Etiology, epidemiology and control of moldy core of apples in Chile

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To Facundo and Víctor with love.

To my parents and brother for their unconditional support.

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CHAPTER I

Introduction

Apple (*Malus domestica* Borkh) is a commercially important crop, being the second most planted fruit crop tree, at present with 35,937 ha, representing 11 % of the total fruit crops in Chile with a total production of approximately 765,000 ton in 2016 (www.odepa.cl). Currently, Chile is the largest apple producer and exporter country in the southern hemisphere and the tenth producer in the world (FAOSTAT 2016).

Several fungal diseases affect apple worldwide, causing foliar and fruit diseases (e.g. apple scab, powdery mildew, rusts), canker and wood diaseases (e.g. European canker, Valsa canker), root rot 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).

Apples are attacked by *Alternaria* species causing leaf and fruit blotch, fruit rot, core rot and moldy core (Gur et al. 2018; Harteveld et al. 2013; Sutton et al. 2014). These apple diseases have been associated with *A. alternata*, *A. arborescens*, *A. longipes*, *A. mali* and *A. tenuissima* (Table 1). *A. mali* is the main species associated to Alternaria blotch, in northern hemisphere and Australia (Gur et al. 2017; Harteveld et al. 2013; Sawamura, 2014).

In Chile, moldy core has been reported (Latorre 1983). However, there is not detailed information on the *Alternaria* species involved. Disease prevalence between 4 and 29% was estimated in 'Oregon Spur' (= 'Red King Oregon') apples at harvest in Chile late in the 1980s (Latorre 1983). Similarly, prevalences varying from 5 to 65% have been reported in susceptible cultivars in Australia, Israel, South Africa, and in the United States (Ellis and Barrat 1983; Serdani et al. 1998).

Moldy core is characterized by a gray to dark olive green cottony mycelium that grows restricted to the carpel without necessarily affecting the fruit flesh (Fig. 1). Moldy core

infection occurs in the orchard, but it can also occur in the packing house (Spotts et al. 1988). It is an important disease in apple fruits with an open sinus from the calyx to the core region (Fig. 1) (McLeod 2014).

Species of *Alternaria* have been frequently associated with moldy core. (Ellis and Barrat 1983; McLeod 2014; Reuveni et al. 2002). However, other genera of filamentous fungi, such as species of *Aspergillus*, *Cladosporium*, *Coniothyrium*, *Epicoccum*, *Phoma* and *Stemphylium* have also been associated with this syndrome; nonetheless, species of *Alternaria* are the most frequently isolated fungi (Ellis and Barrat 1983; Gao et al. 2013; McLeod 2014; Serdani et al. 2002).

At least two small-spored *Alternaria* spp., *A. alternata* and *A. tenuissima*, have been associated with moldy core of apple (McLeod 2014). Nevertheless, recent studies suggest that a complex of small-spored *Alternaria* spp., including *A. alternata*, *A. arborescens*, *A. infectoria* and *A. tenuissima*, may be associated with moldy core of apples (Gao et al. 2013; Ntasiou et al. 2015). A similar situation has been reported for core rot in pomegranate (Luo et al. 2017).

The morphological identification of the small-spored species of the genus *Alternaria* is challenging because of the high diversity and few characteristics that allow their unambiguous identification. At present, molecular and phylogenetic analysis are essential for the identification of species within small-spored *Alternaria* (Lawrence et al. 2016; Woudenberg et al. 2013).

More than 19 genera of fungi had been found to be able to colonize flowers and young fruits. However, *Alternaria* spp. were the most common (over 50%) fungi present as endophytes from early bud develop stages to harvest (Ellis and Barrat 1983; Serdani et al. 1998).

Once, the carpel cavity is colonized, the fungi are protected and hardly have contact with the fungicides applied on the fruit. Moreover, a very favorable environment for the growth

and multiplication of fungi is generated (Ellis and Barrat 1983). Fungicide research conducted to the control of moldy core and core rot of apple are sometime effective (Reuveni et al. 2002), but very often they are infective (Ellis and Barrat 1983; Pinilla et al. 1996; Shtienberg 2012). The difficulties encountered with a spray application to reach and protect carpels can explain, at least in part, these variable results.

Reuveni et al. (2002) through artificial inoculations, with *A. alternata*, revealed that the beginning of bloom and full bloom were the most susceptible developmental stages for infection of moldy core.

In Chile, moldy core remains as a very important apple disease, but the etiology and epidemiology have not been extensively studied, particularly for apple cultivars that are planted at present. Therefore, the hypothesis of this thesis postulates that apple moldy core is associated with filamentous fungi that would infect apples early in the development of the fruit, coexisting in the carpels until harvest.

The general objective of this thesis was to study the etiology, epidemiology and control of moldy core of apples in Chile. The specific objectives were i) to isolate and characterize the fungi associated with the moldy core of apple in Chile (Chapter III), ii) to characterize the *Alternaria* isolates obtained, determine their pathogenicity and virulence (Chapter III), iii) to study the sources of primary inoculum associated with apple trees (Chapter IV), iv) to evaluate the sensitivity of *Alternaria* isolates to fungicides and the effectiveness in the control of moldy core of apples (Chapter V).

Table 1. Diseases caused by *Alternaria* species and their distribution in the world.

Disease	*Alternaria* species	Distribution	References
Leaf and fruit blotch	*A. mali, A. alternata*, *A. arborescens*, *A. longipes, A. tenuissima*	Australia, China, Iran, Israel, Italy, Japan, Russia, Turkey, South Korea, USA, Yugoslavia, Zimbabwe	Gur et al. 2017; Harteveld et al. 2013; Ozgonen and Karaca 2006; Rotondo et al. 2012; Soleimani and Esmailzadeh 2007; Sutton et al. 2014.
Fruit rot	*A. mali*	Israel	Gur et al. 2018.
Core rot	*A. alternata*, *A. arborescens*, *A. tenuissima*	Australia, Canada, Chile, Greece, Israel, Netherlands, New Zealand, South Africa, UK, USA	Latorre 1983; Ntasiou et al. 2015; Sutton et al. 2014.
Moldy core	*A. alternata*, *A. arborescens*, *A. tenuissima*	Australia, Canada, Chile, China, Greece, Israel, Netherlands, New Zealand, South Africa, UK, USA	Gao et al. 2013; Latorre 1983; Ntasiou et al. 2015; Sutton et al. 2014.

Fig. 1. Symptoms of moldy core on 'Red Chief' apple. **A.** Longitudinal view of apple fruit. Arrow shows the open sinus from the calyx to the core region. **B.** Cross view of apple fruit.

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CHAPTER II

Occurrence of Alternaria Blotch Associated with *Alternaria alternata*, *A. arborescens*, *A. infectoria* and *A. tenuissima* on Apples in Chile

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ABSTRACT

Chile is one of the largest apple (*Malus domestica*) producers from the southern hemisphere with more than 37,937 ha planted. Several fungal diseases affect apple in Chile, including Alternaria blotch (AB) which is frequently observed in summer months. AB is characterized by the presence of small (3 to 5 mm in diameter), circular gray to brown necrotic lesions, often with a dark brown to purple margins on leaves of apples. The objective of this study was to identified the species of *Alternaria* associated to Alternaria blotch isolated from apple leaves in Chile. With this purpose, isolations from the margins of the necrotic lesions on the leaves were placed on potato dextrose agar acidified with 0.5 µl/ml of 92% lactic acid (APDA). Isolates were identified morphologically and molecularly for the *Alternaria* major allergen Alt a1 (*Alt a1*), plasma membrane ATPase (*ATPase*) and calmodulin (*CAL*) genes. Prevalences between 0.1 to 4.0 % of AB were estimated in seven apple orchards with cultivas 'Scarlet', 'Red Chief', 'Oregon Spur' and 'Granny Smith' in Chile in 2016. Dark olive to gray with whitish margins colonies (n = 173 isolates) of *Alternaria* species (Simmons, 2007) were recovered. Isolates produced small catenulate muriform conidia on single conidiophores. Consensus sequences were BLASTed showing a 99% similarity with ex-type sequences of *A. alternata*, *A. arborescens*, *A. infectoria* and *A. tenuissima*. Phylogenetic analysis of maximum parsimony was used to confirmed the identification of the *Alternaria* species. The pathogenicity of *Alternaria* spp. was confirmed on detached leaves of 'Red

Chief' apples. Necrotic lesions of 2.6 to 4.4 mm in diameter with or without chlorotic haloes were obtained after 4 days at 20°C. Differences in virulence were significant ($P < 0.001$), with *A. tenuissima* and *A. infectoria* the most and the least virulent species, respectively. The results demonstrated the presence of an *Alternaria* complex associated to AB and to our knowledge, this is the first report of *A. alternata, A. arborescens, A. infectoria* and *A. tenuissima* associated with AB in Chile and the first report of *A. infectoria* worldwide. No evidences of *A. mali*, primarily found in northern hemisphere and Australia, were obtained in this study. Currently AB is considered a minor apple disease in Chile.

Chile is one of the largest apple (*Malus domestica* Borkh) producers from the southern hemisphere with more than 37,937 ha planted. Several fungal diseases affect apple worldwide, including Alternaria blotch which is frequently observed in summer months. AB is characterized by the presence of small (3 to 5 mm in diameter), circular gray to brown necrotic lesions, often with a dark brown to purple margins on leaves of apples (Fig. 1). In cases of severe attack, defoliation of up to 50% can occur in susceptible cultivars (Sawamura, 2014). Symptoms on fruit are uncommon, except in the very susceptible cultivars ('Golden Delicious', 'Starking Delicious', 'Indo', 'Gala', and 'Pink Lady'), commonly limited to small, corky, dark lesions, typically associated with the lenticels (Gur et al. 2017; Sawamura, 2014). However, severe outbreaks have been reported recently in cv. Pink Lady apples in northern Israel, especially on fruit (Gur et al. 2017).

Alternaria mali is the main cited causal agent of Alternaria blotch in northern hemisphere and Australia (Gur et al. 2017; Harteveld et al. 2013; Ozgonen and Karaca 2006; Sawamura, 2014; Soleinami and Esmaizadeh 2007). However, different species of *Alternaria* have been associated to Alternaria blotch in resent studies in Italy and Australia, where *A. alternata*, *A. arborescens*, *A. longipes* and *A. tenuissima* were identified (Harteveld et al. 2013; Rotondo

et al. 2012) and differences in viruences among *Alternaria* isolates were detected (Harteveld et al. 2014).

In Chile, the etiology of Alternaria blotch has not been extensively studied. Therefore, the aim of this study was to identify the species of *Alternaria* associated Alternaria blotch of apples in Chile.

MATERIALS AND METHODS

Sampling locations and fungal isolation. Syntomatics leaves with one or more spots per leaves (Fig. 1) were samples from seven apple orchards located betwen Pirque (33°.40' lat.S) and Los Ángeles (37°33' lat.S) with cultivas 'Scarlet', 'Red Chief', 'Oregon Spur' and 'Granny Smith' in 2016. To determine the prevalence of leaves with symptoms of Alternaria blotch, 16 trees were evaluated per orchard, of which a total of 40 shoots were observed, evaluating ten shoots from each of the cardinal points (north, south, east, west).

Between ten and twenty symptomatic leaves were samples per orchard. Leaves were surface-disinfested (75% ethanol, 30 s), a section taken from the limit between healthy and symptomatic leaf tissue was placed on potato dextrose agar (PDA) acidified with 92% lactic acid at 0.5 ml/liter (APDA) plus 0.1% Igepal CO-630 (Sigma-Aldrich), which was used as a colony growth restrictor (Elfar et al. 2013). The cultures were incubated for 7 to 10 days at room temperature (20-22°C) in the dark prior to the determination of the total number of *Alternaria*-like colonies under a stereoscopic microscope. Fungal colonies were tentatively identified as *Alternaria* by the colony morphology (color, texture) and by the conidiophore and conidial characteristics. Mycelia from *Alternaria*-like colonies of 173 isolates were transferred to APDA, and pure cultures were then obtained by transferring a single hyphal tip to fresh APDA. Isolates were kept on APDA at 5°C until used.

Molecular characterization. A total of nine *Alternaria* isolates representing four morphological groups differentiated on the basis of colony morphology and characteristics

of their conidiophores and conidia were selected for molecular studies (Table 1). Isolates were examined for the *Alternaria* major allergen Alt a1 (*Alt a1*), plasma membrane ATPase (*ATPase*) and calmodulin (*CAL*).

DNA was extracted from the isolates from 7- to 10-day-old mycelia cultured on APDA incubated at 20-22°C. Mycelia were carefully separated from the agar medium with the aid of a sterile scalpel and ground in liquid nitrogen in a porcelain mortar. Genomic DNA was extracted using a DNA extraction kit (Wizard Genomic DNA Purification Kit, Promega, Madison, WI). The DNA yield was estimated by gel electrophoresis and GelRed (Biotium Inc., Alameda, CA) staining and visualized by UV transillumination at 320 nm.

The *Alt a1*, *ATPase* and *CAL* genes were amplified using primer pairs ATPDF1/ATPDR1, CALDF1/CALDR1 (Lawrence et al. 2013) and Alt-for/Alt-rev (Hong et al. 2005) respectively. Polymerase chain reaction (PCR) was conducted in a thermal cycler (Veriti 96-Well, Applied Biosystems, CA) in a final volume of 25 µl containing 5 µl of 10x PCR buffer, 1 µl of 25 mM $MgCl_2$, 0.2 µl of 10 mM dNTPs, 1 µl of a 15 mM solution of each primer, 0.13 µl containing 5 units of *Taq* DNA polymerase (GoTaq Flexi DNA Polymerase, Promega) and 2 µl of template DNA. The amplification protocol included preheating for 3 min at 95°C followed by 35 cycles of denaturation at 95°C for 30 s; annealing for 30 s at 60°C for *ATPase*, at 57°C for *CAL* and at 58°C for *Alt a1*; and extension at 72°C for 90 s, with a final extension for 7 min at 72°C.

The PCR products were separated on 2% agarose gels in 1.0x Tris-acetate-EDTA (TAE) buffer and stained with GelRed, and the DNA fragments were visualized by UV transillumination at 320 nm.

The PCR-amplified products were purified and sequenced from both directions by Macrogen Inc., South Korea. The sequences were edited using Proseq v.2.91 (Filatov 2002) and aligned using ClustalX v.2.1 (Larkin et al. 2007). A BLASTn search analysis was performed against reference sequences in the GenBank (www.ncbi.nlm.nih.gov) database.

A phylogenic analysis was performed for a maximum parsimony (MP) analysis with the aid of MEGA v.7.0 (Kumar et al. 2016). Gaps were treated as missing data. The MP tree was obtained using the Tree-Bisection-Reconnection (TBR) branch-swapping algorithm and 1,000 random sequences additions. Branch stability was estimated with a bootstrap analysis with 1,000 replicates. The alignment included sequences from the nine *Alternaria* isolates obtained from moldy core of apples and eight *Alternaria* isolates obtained from leaf spot of apple in Chile (Table 1). Based on the BLASTn results, *Alternaria* sequences deposited in GenBank were used in phylogenetic analysis. Sequences of *Stemphylium botryosum*, *S. callistephi*, and *S. vesicarium* were included as the outgroup (Table 2). The phylogenetic analysis was performed independently for each gene. The congruence of the evolution between the *ATPase* and *CAL* genes, and between *ATPase*, *CAL* and *Alt a1* was studied using the partition homogeneity tests with the aid of PAUP v.4.0b10 (Swofford 2002).

Morphological characterization. Colony morphology of four *Alternaria* isolates was characterized in 9-cm-diameter plastic Petri dishes containing APDA, potato carrot agar (PCA), containing per 1 L of distilled water 20 g potato, 20 g carrot, and 20 g agar, (Simmons 2007) APDA and PCA plates were incubated for 7 days at 22°C 8 h/ 16 h light/ dark. Light was provided by daylight candles (Philips, TL-D 36W/54-765, Thailand) placed 40 cm above the plates. The color, texture and margins of the colonies on APDA were determined. The color was established with the aid of the Pantone color chart (www.pantone-colours.com). Conidia and conidiophores from three PCA plates per isolate were mounted on colorless 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 and 500 ml distilled water) before being observed under a light microscope. Conidiophore (n = 15 per isolate) length and width, cell numbers and branching were determined. Conidial (n = 50) shape, length, width and number of transepta were measured and compared with published descriptions (Simmons 2007; Pryor and Michailides 2002).

Pathogenicity tests. To stimulate sporulation, isolates were cultivated in 0.05 x PDA (Pryor and Michailides 2002) for 7 days at 22°C with 10 h/14 h of light/dark cycles. Inoculum suspensions were prepared with 10 to 14 day-old cultures on 0.05 x PDA. Dishes were flooded with approximately 20 ml of 0.05% of Tween 80 and scraping the surface of the medium with sterile scalpel. The conidial suspension was filtered through two layers gauze and the final concentration was adjusted to 1×10^6 conidia / ml using a hemacytometer. One Isolate of *A. alternata*, *A. arborescens*, *A. infectoria* and *A. tenuissima* were evaluated for pathogenicity.

Detached leaves of 'Red Chief' apples (n = 4), taken from the newest leaves of the shoots, were surface disinfected in 1% NaOCl for 1 min followed by sterile distilled water for 1 min and air dried. Then, the leaves were wounded with three punctures made with a sterile hypodermic needle in the apical, basal and middle regions of each leaf blade. Leaves were inoculated by delivering 10 µl of conidial suspension per wound site. Leaves were incubated at 25°C in a humid chamber for 7 days before the diameters of the lesions were determined. An equal number of wounded leaves treated with sterile water served as the negative controls. Re-isolations from the necrotic lesions on APDA were attempted, and isolates were identified on the basis of the conidial morphology. This experiment was conducted twice. This experiment was repeated as described above using unwounded leaves.

The experimental treatments were performed in a completely randomized design with four replicates. Means were separated according to the Fisher LSD test ($P < 0.05$). Analysis was performed with the aid of SigmaStat 3.1 (Systat Software Inc.). Necrotic lesion length data were transformed to Ln(x + 1) prior to analysis, but non-transformed data are presented.

RESULTS

Sampling locations and fungal isolation. Symptoms of diseased leaves consisted of the presence of one or more small (3 to 5 mm in diameter), circular gray to brown necrotic lesions per leaves, often with a dark brown to purple margins on leaves of apples (Fig. 1). The prevalence was estimated between 0.1 to 4.0 % of Alternaria blotch in apple leaves of cultivars ‘Scarlet’, ‘Red Chief’, ‘Oregon Spur’ and ‘Granny Smith’. A total of 173 isolates of small-spored *Alternaria* spp. were obtained from diseased leaves.

Molecular characterization. Consensus sequences were BLASTed showing a 99% similarity with ex-type sequences of *A. alternata* (AY563301, JQ811979, JQ646208), *A. arborescens* (AY563303, JQ671880, JQ646214), *A. infectoria* (FJ266502, JQ671804, JQ646138) and *A. tenuissima* (KP275690, JQ811989, JQ646209). Sequences of *Alternaria* spp. used in this study were deposited in GenBank (Table 1).

Phylogenetic analysis of maximum parsimony (MP) was used to confirmed the identification of the *Alternaria* species. Sequences of *ATPase, CAL* and *Alt a1* of the *Alternaria* isolates from apple ranged from 1188 to 1197, 775 to 794 and 483 to 485 nucleotides respectively. In the MP analysis, statistical data for ATPase were as follows: tree length [TL] = 430, consistency index [CI] = 0.837, retention index [RI] = 0.926, and rescaled consistency index [RC] = 0.775; for CAL: [TL] = 346, [CI] = 0.884, [RI] = 0.965, and [RC] = 0.854; for *Alt a1*: [TL] = 127, [CI] = 0.835, [RI] = 0.931, and [RC] = 0.778.

The phylogenetic analysis for the ATPase gene showed that the nine isolates from apples were identified in two small-spored sections. In section *Alternaria*, two isolates were identified as *A. alternata*, clustered with *A. alternata* ex-type (97% bootstrap), four isolates were identified as *A. tenuissima* clustered in the same group with *A. tenuissima* ex-type (82% bootstrap), and two isolates were identified as *A. arborescens*, clustered with the *A. arborescens* ex-type (100% bootstrap). In section *Infectoriae*, one isolate was identified as *A. infectoria* clustered the ex-type by a high bootstrap value of 99% (Fig. 2). The

phylogenetic analysis for the *CAL* gene showed that the nine isolates from apples were identified in two small-spored sections. In sect. *Alternaria*, two isolates were identified as *A. alternata*, clustered with *A. alternata* ex-type (97% bootstrap), four isolates were identified as *A. tenuissima* clustered in the same group with *A. tenuissima* ex-type (82% bootstrap), and two isolates were identified as *A. arborescens*, clustered with the *A. arborescens* ex-type (100% bootstrap). In sect. *Infectoriae*, one isolate was identified as *A. infectoria* clustered the ex-type by a high bootstrap value of 99% (Fig. 2).

In relation with the reference isolates, the MP analysis of *CAL* gene differentiated the *Alternaria* isolates in two small-spored section: sect. *Alternaria* and sect. *Infectoriae*. *A. infectoria* was supported by a high bootstrap value (99% bootstrap); however, one isolate of *A. tenuissima* was grouped in the same cluster with *A. alternata*, and other isolate of *A. tenuissima* was grouped in the same cluster with *A. arborescens*. The MP analysis of Alt a1 gene differentiated the *Alternaria* isolates in two small-spored section: sect. *Alternaria* and sect. *Infectoriae*. *A. infectoria* was supported by a high bootstrap value (97% bootstrap); however, isolate of *A. alternate, A. tenuissima* and *A. arborescens* were grouped in the same cluster.

The partitioned homogeneity test showed significant differences ($P < 0.05$) between the *Alt a1*, *ATPase* and *CAL* genes and between *ATPase* and *CAL*. Therefore, the concatenated analysis of these genes was not performed.

Morphological characterization. All *Alternaria* isolates produced single conidiophores and catenulate muriform, brown to golden brown conidia. Isolates were differentiated based on the colony morphology and characteristics of their conidiophores and conidia. *A. alternata* produced cottony, gray to green (Pantone no. 5777-5763) colony on APDA. Isolate produced conidial chains with numerous secondary chains branching on short conidiophores (56.3 ± 11.9 µm) of 3 to 6 cells. Conidia were ovoid to ellipsoid with 3 to 7 transepta (Table 3).

A. arborescens produced cottony, olive brown (Pantone no. 5815-5825) with prominent concentric growth rings and narrow white margins colonies on APDA. Isolate produced conidial chains of 2 to 7 conidia, with long conidiophores (226.7 ± 94.5 µm) of 7 to 10 cells. Conidia were ovoid to ellipsoid with 1 to 4 transepta (Table 3).

A. tenuissima produced cottony, olive brown (Pantone no. 5815-5825) with slight concentric growth rings and prominent white margins colonies on APDA. The isolate produced conidial chains of 4 to 12 conidia, rarely with a lateral branch, on short conidiophores of 43.1 ± 7.4 of 4 to 5 cells. Conidia were ovoid to obclavate with 3 to 6 transepta and a long beak (Table 3).

A. infectoria produced floccules, gray to yellow-green (Pantone no. 5777-5783) with slight concentric growth rings colonies on APDA. Isolate produced conidial chains with numerous secondary chains branching on relatively short conidiophores (mean 77.3 ± 21.2 µm) of 3 to 6 cells. Conidia were ovoid to ellipsoid with 2 to 5 transepta (Table 3).

Pathogenicity tests. Without exception, all *Alternaria* species were pathogenic in detached apple leaves, developing brown necrotic lesions of 1.2 to 5.7 mm in diameter, with concentric rings and a chlorotic halo, after 5 days at 20°C. Significant differences ($P < 0.001$) in the lesion diameters were found among *Alternaria* spp. Differences in virulence were consistently obtained, with *A. tenuissima* being the most virulent (mean of 4.7 mm), followed by *A. arborescens* (mean of 2.8 mm), *A. alternata* (mean of 2.5 mm) and *A. infectoria*, which was the least virulent isolate (1.2 mm) (Fig. 3). Reisolations from the margins of the necrotic lesions were successful from 100% of the inoculated leaves. The identification of reisolated fungi was confirmed morphologically. No symptoms were observed in wounded but noninoculated controls.

DISCUSSION

In this study it was demonstrated that Altenaria blotch is a disease present in Chile and it is caused by many small-spored *Alternaria* spp., specifically *A. alternata, A. arborescens, A. infectoria* and *A. tenuissima.*

All *Alternaria* spp., isolates were pathogenic inducing small necrotic lesions on detached wounded leaves However, significant ($P < 0.001$) differences in lesion diameter were obtained, suggesting differences in virulence among *Alternaria* isolates, with *A. tenuissima* being the most virulent species, followed by *A. arborescens*, *A. alternata* and *A. infectoria.* Consistently, differences in virulence between *Alternaria* spp. have been detected on other hosts. For example, isolates from *Alternaria* late blight of pistachio and from Alternaria rot of blueberry, *A. infectoria* were less virulent than *A. alternata*, *A. arborescens* and *A. tenuissima* (Pryor and Michailides 2002; Zhu and Xiao 2015).

To our knowledge, this is the first report of these *Alternaria* spp. associated with Alternaria blotch in Chile and the first report of *A. infectoria* worldwide. Previously, *A. alternata, A. arborescens,* and *A. tenuissima* have been reported causing Alternaria blotch on leaves and fruits in Australia and Italy (Harteveld et al. 2013; Rotondo et al. 2012). However, no evidences of *A. mali*, primarily found in northern hemisphere and Australia (Gur et al. 2017; Harteveld et al. 2013; Ozgonen and Karaca 2006; Sawamura, 2014; Soleinami and Esmaizadeh 2007), were obtained in this study. Currently Alternaria blotch is considered a minor apple disease in Chile. These results do not exclude that other plant-pathogenic fungi may be involved in this syndrome.

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Table 1. Source of isolates of *Alternaria* spp. from apples leaves obtained in commercial orchard in Central Chile and GenBank accession numbers for sequences of three genes (*Alternaria* major allergen Alt a1, plasma membrane ATPase, and Calmodulin) of *Altenaria* isolates studied

Species	Locality[x]	Latitude[y]	Apple cultivar	GenBank accession number[z]		
				Alt a1	*ATPase*	*CAL*
Alternaria alternata						
10LS	Pirque, MR	33°40'	Scarlet	MG925165	MG740618	MG925123
12LS[w]	Morza, VII	34°50'	Scarlet	MG925166	MG740619	MG925124
A. arborescens						
13LS[w]	Los Ángeles, VIII	37°33'	Scarlet	MG925167	MG740621	MG925126
14LS	Los Ángeles, VIII	37°33'	Scarlet	MG925168	MG740620	MG925125
A. infectoria						
9LS[w]	Pirque, MR	33°40'	Scarlet	MG925169	MG740622	MG925127
A. tenuissima						
4LS	Morza, VII	34°50'	Scarlet	MG925170	MG740623	MG925128
5LS	Pirque, MR	33°40'	Scarlet	MG925171	MG740625	MG925129
6LS	Colbún, VII	35°43'	Red Chief	MG925172	MG740624	MG925130
11LS[w]	Morza, VII	34°50'	Scarlet	MG925173	MG740626	MG925131

[w] Isolates used for morphological characterization and pathogenicity tests
[x] Administrative Regions of Chile: MR= Metropolitan Region and Regions VI, VII, VIII and IX.
[y] Southern latitude.
[z] Genes: *Alt a1*= *Alternaria* major allergen Alt a1; *ATPase*= plasma membrane ATPase; *CAL*= Calmodulin.

Table 2. Accession numbers for reference sequences of three genes (*Alternaria* major allergen Alt a1, plasma membrane ATPase, and Calmodulin) of *Alternaria* isolates in GenBank used for phylogenetic analysis in this study

		GenBank accession number [z]		
Species	Isolates[x]	*Alt a1*	*ATPase*	*CAL*
Alternaria alternata	EGS 34-016[y]	KP275691	JQ671874	JQ646208
A. alternata	X1191	KJ920991	KJ908234	KJ920956
A. alternata	X1048	KJ920997	KJ908230	KJ920957
A. alternata	BMP0463	na [z]	JQ811981	na
A. arborescens	EGS 39-128 [y]	AY563303	JQ671880	JQ646214
A. arborescens	BMP0462	na	JQ811990	na
A. arborescens	3.J24	KJ921023	KJ908244	KJ920979
A. arborescens	X1013	KJ920987	KJ908247	KJ920949
A. arborescens	X1312	KJ921008	KJ908242	KJ920948
A. argyroxiphii	EGS 35-122 [y]	JQ646434	JQ671926	JQ646260
A. destruens	EGS 46-069 [y]	JQ646402	JQ671873	JQ646207
A. dumosa	EGS 45-007 [y]	na	JQ671877	JQ646211
A. herbiphorbicola	EGS 40-140 [y]	JQ646410	JQ671888	JQ646222
A. infectoria	EGS 27-193 [y]	FJ266502	JQ671804	JQ646138
A. infectoria	3.J14	KJ921017	KJ908219	KJ920977
A. infectoria	X1273	KJ920982	KJ908218	KJ920953
A. limoniasperae	EGS 45-100 [y]	JQ646370	JQ671879	JQ646213
A. rosae	EGS 41-130 [y]	JQ646370	JQ671803	JQ646137
A. parvicaespitosa	X1272	KJ920981	KJ908217	KJ920952
A. tenuissima	EGS 34.015 [y]	KP275690	JQ811989	JQ646209
A. tenuissima	BMP2256	na	JQ811983	na
A. tenuissima	BMP1254	na	JQ811988	na
Stemphylium botryosum	BMP 0360	AY563274	JQ671767	JQ646101
S. callistephi	BMP 0377	AY563276	JQ671769	JQ646103
S. vesicarium	BMP 0374	AY563275	JQ671768	JQ646102

[x] na = sequences not available in GenBank.
BMP: B.M. Pryor, Division of Plant Pathology, Department of Plant Sciences, EGS: E.G. Simmons, Mycological Services, Crawfordsville, IN 47933,
3.J: Pryor and Michailides, 2002.
X : Zhu and Xiao, 2015
[y] ex-type
[z] *Alt a1*= *Alternaria* major allergen Alt a1; *ATPase*= plasma membrane ATPase; *CAL*= Calmodulin.

Table 3. Morphometric characterization of conidiophores and conidia of *Alternaria* spp. isolated from moldy core of apples in Chile

Isolates	Conidiophores[xz]		Conidia[yz]		
	Length, µm	Width, µm	Length, µm	Width, µm	Length/ width
A. alternata					
12LS	56.3 ± 11.9	3.85 ± 0.4	23.4 ± 3.4	10.0 ± 1.3	2.4 ± 0.4
A. arborecens					
13LS	226.7 ± 94.5	3.5 ± 0.5	21.6 ± 3.3	9.2 ± 1.1	2.4 ± 0.4
A. infectoria					
9LS	77.3 ± 21.2	4.6 ± 0.5	30.7 ± 6.4	11.6 ± 2.1	2.7 ± 0.5
A. tenuissima					
11LS	43.1 ± 7.4	4.3 ± 0.4	31.0 ± 5.3	11.9 ± 1.6	2.6 ± 0.5

[x]Means of at least 15 primary conidiophores per isolates ± standard deviation.
[y]Means of at least 50 conidia per isolate ± standard deviation.
[z]Incubated on PCA for 7 days at 22°C 8 h/ 16 h light/ dark.

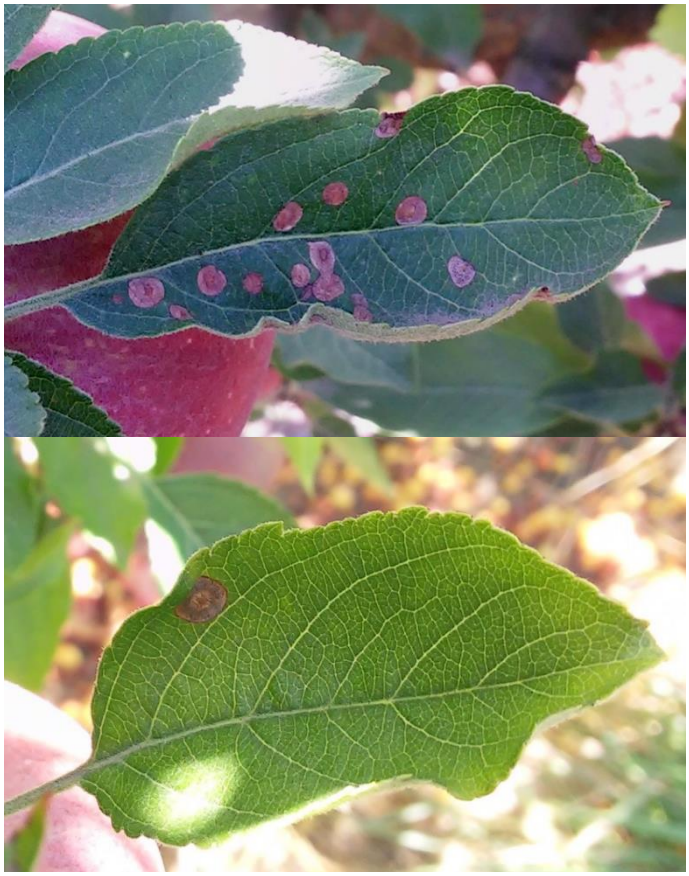

Fig. 1. Naturally infected apple leaves with symptoms of Alternaria blotch.

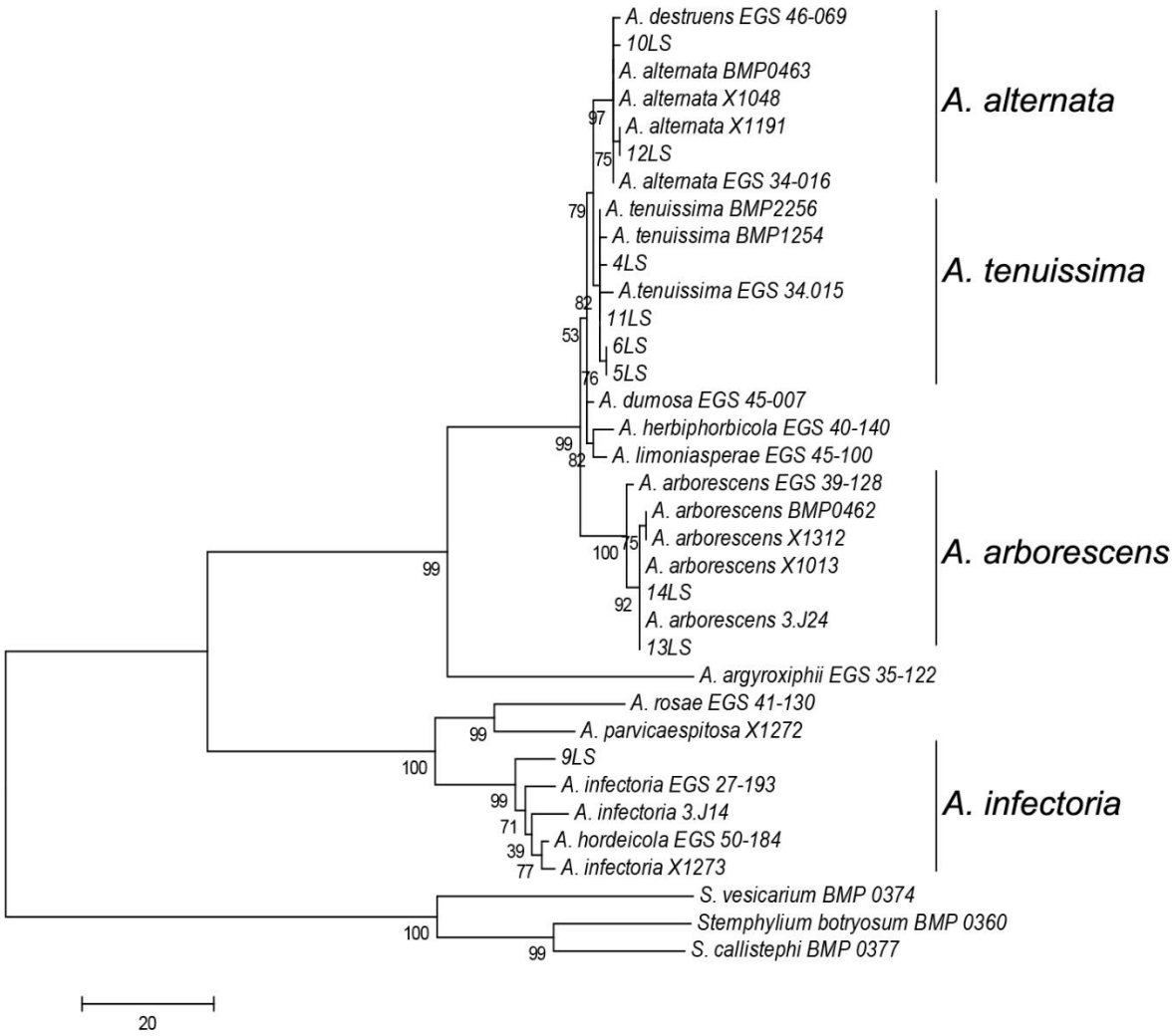

Fig. 4. Phylogenetic tree obtained from maximum parsimony analysis of the plasma membrane ATPase gene from sequences of *Alternaria* species from Chilean apples and from sequences of ex-types in GenBank. The consensus tree inferred from the 81 most parsimonious trees and bootstrap values are shown. The tree was rooted with *Stemphylium botryosum, S. callistephi* and *S. vesicarium.* Tree length = 430, consistency index = 0.837, retention index = 0.926 and rescaled consistency index = 0.775. Numbers and LS are *Alternaria* isolates from Alternaria blotch in Chile; other codes are isolates from GenBank.

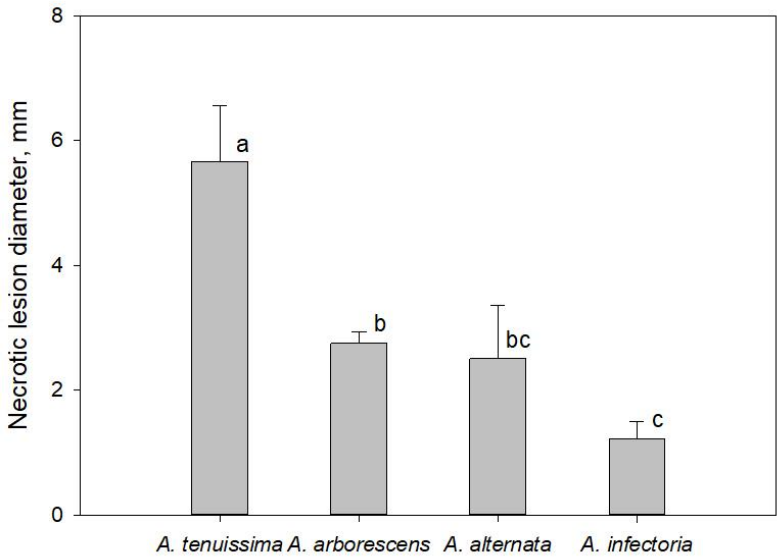

Fig. 3. Pathogenicity of *Alternaria* spp. isolates causing necrotic lesions on detached 'Red Chief' leaves. After 4 days of conidial inoculation at 20°C in humid chambers. Means followed by the same letters are not significantly different according to Fisher LSD test ($P < 0.05$). Vertical bars = standard errors.

CHAPTER III

Identification and Characterization of *Alternaria* species Associated with Moldy Core of Apple in Chile

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Identification and Characterization of *Alternaria* species Associated with Moldy Core of Apple in Chile

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ABSTRACT

Moldy core (MC) of apple is an important disease in Chile with prevalences observed between 4 and 46% in 'Fuji', 'Oregon Spur' 'Red Chief', and 'Scarlet' apples in the 2014-2015 and 2015-2016 growing seasons. However, there is no information on the identity of the causal agents associated with MC in Chile. The analysis of 653 MC fruits revealed the presence of several genera of filamentous fungi. However, species of *Alternaria* (67.7%) were by far the most frequently fungi isolated. A total of 41 *Alternaria* isolates were characterized morphologically and molecularly using *Alternaria* major allergen Alt a1, calmodulin and plasma membrane ATPase gene regions. Six small-spored *Alternaria* spp. were identified namely, in order of importance, *A. tenuissima*, *A. arborescens*, *A. alternata*, and *A. dumosa* in sect *Alternaria*, *A. frumenti*, in sect *Infectoriae*, and *A. kordkuyana in* sect *Pseudoalternaria.* MC symptoms were reproducible and consisted of a light gray to dark olive green mycelium over the carpel and seed of immature and mature fruits, confirming that the isolates of these *Alternaria* spp. were pathogenic. These isolates caused brown necrotic lesions with concentric rings on wounded detached apple leaves. This study demonstrated that at least six *Alternaria* spp. are the cause of MC of apples in Chile. These *Alternaria* spp. were isolated alone or two or more species coexist in the same fruit. This is the first report of *A. tenuissima*, *A. arborescens*, *A. frumenti*, *A. dumosa* and *A. kordkuyana* associated with MC of apples in Chile and the first report of *A. frumenti*, *A. kordkuyana* and *A. dumosa* causing MC of apples worldwide.

Chile is one of the largest apple producers in the southern hemisphere, with more than 37,200 ha planted and a total production of approximately 765,000 tons in 2016 (www.odepa.cl). Moldy core and core rot are considered two important diseases in apples with an open sinus from the calyx to the core region (McLeod 2014). Core rot usually follows a moldy core condition. This study primarily focuses on moldy core, which considerably affects apple production in Chile. A disease prevalence between 4 and 29% was estimated in 'Oregon Spur' (= 'Red King Oregon') apples at harvest in Chile late in the 1980s (Latorre 1986; Morales 1986). Recently, a moldy core prevalence between 4 and 46% was determined at harvest in new apple cultivars ('Fuji', 'Red Chief', 'Scarlet') planted in Chile. Similarly, prevalences varying from 5 to 12% have been reported in susceptible cultivars in Australia, Israel and South Africa (Kupferman 1992; Reuveni 2006; Serdani et al. 1998). A moldy core prevalence exceeding 40% was reported in Israel in 2003 (Reuveni 2006), and 38 to 65% of fruits with moldy core were estimated in Ohio in 1980 and 1981 (Ellis and Barrat 1983)

Moldy core infection occurs in the orchard, but it can also occur in the packing house (Spotts et al. 1988). It is characterized by fungal mycelial growth that is restricted to the carpel without necessarily affecting the fruit flesh. Infected fruits ripen earlier, are often smaller and slightly deformed and fall prematurely compared with healthy fruits (Latorre 1983; McLeod 2014; Niem et al. 2007). Because of the importance of moldy core, apple packing houses commonly estimate disease prevalence at the reception of each apple lot in Chile. Only those lots with no evidence of moldy core are kept in controlled atmosphere chambers.

The open sinus condition that characterizes most 'Red Delicious' apples, unlike 'Granny Smith' apples, can explain the relatively high susceptibility to moldy core exhibited by this cultivar (Pinilla et al. 1996; Serdani et al. 1998). However, other factors such as mesoderm

pH and locule sensitivity to colonization can affect apple susceptibility to moldy core in open sinus apples (Niem et al. 2007).

Species of *Alternaria* have been frequently associated with moldy core. (Ellis and Barrat 1983; McLeod 2014; Reuveni 2002). However, other genera of filamentous fungi, such as species of *Aspergillus*, *Cladosporium*, *Coniothyrium*, *Epicoccum*, *Phoma* and *Stemphylium* have also been associated with this syndrome; nonetheless, species of *Alternaria* are the most frequently isolated fungi (Ellis and Barrat 1983; Gao et al. 2013; McLeod 2014; Serdani et al. 2002).

At least two small-spored *Alternaria* spp., *A. alternata* and *A. tenuissima*, have been associated with moldy core of apple (McLeod 2014). Nevertheless, recent studies suggest that a complex of small-spored *Alternaria* spp., including *A. alternata*, *A. arborescens*, *A. infectoria* and *A. tenuissima*, may be associated with moldy core of apples (Gao et al. 2013; Ntsiou et al. 2015). A similar situation has been reported for core rot in pomegranate (Luo et al. 2017; Michailides et al. 2008).

The identification of the small-spored species of the genus *Alternaria* is challenging based on taxonomically characters due to a high diversity and few characteristics that allow their unambiguous identification (Andrew et al. 2009). At present, molecular and phylogenetic analysis are essential for recognizing species within small-spored *Alternaria*. Different loci have been used, such as nuclear rDNA, internal transcribed spaces (ITS) regions, mitochondrial small subunit (mtSSU), and many protein-coding genes (Andrew et al. 2009; Hong et al. 2005; Lawrence et al. 2013; Lawrence et al. 2016; Pryor and Gilbertson 2000; Woudenberg et al. 2013; 2015). A multilocus study was carried out by Lawrence et al. (2013), who showed that the most informative protein-coding genes to identify *Alternaria* spp., in order of importance, are plasma membrane ATPase, calmodulin, *Alternaria* major allergen Alt a1, glyceraldehyde-3-phosphate dehydrogenase and actin. However, plasma membrane ATPase and calmodulin have been suggested as the most appropriate markers

for molecular identification within small-spored *Alternaria.* Other genes, such as beta-tubulin and translation elongation factor 1-alpha (*TEF*), were the least informative genes for the identification of *Alternaria* spp. (Lawrence et al. 2013, 2016). Furthermore, the histone 3 gene has been used to separate *A. alternata* from *A. tenuissima* (Kang et al. 2002; Sun and Huang 2017).

In Chile, the etiology of moldy core remains to be determined, particularly for cultivars that are planted at present. Therefore, the objective of this work was to characterize and study the role of *Alternaria* species associated with moldy core of apples in Chile.

MATERIALS AND METHODS

Sampling locations and fungal isolation. A total of 2,000 apparently healthy fruits of apple cultivars 'Fuji', 'Red Chief', 'Oregon Spur' and 'Scarlet', with open sinus fruits, were sampled randomly from three commercial orchards located in Graneros (34°.40' lat.S), Río Claro (35°15' lat.S) and Colbún (35°43' lat.S) in 2015. Eight additional orchards, between Pirque (33°.40' lat.S), and Traiguén (38°.34' lat.S), were surveyed in 2016 (Fig. 1). In addition, apparently healthy fruits (n=200) of 'Granny Smith' apples, with a closed sinus, were sampled in one orchard in Graneros. Fruits were sampled 2-3 weeks before harvest, transported to the laboratory, stored at 5°C and evaluated within two weeks. The apples were washed under running tap water and surface disinfected (75% ethanol, 5 min) before sectioning each fruit longitudinally. The carpels and flesh were examined and classified as healthy or diseased (Gao et al. 2013). The later fruits were classified as (i) brown core, with brown necrotic lesions of the carpel walls and absence of mycelium growth; (ii) moldy core (Fig. 2a), with carpels colonized by gray to dark olive green mycelium; (iii) dry rot, with dry and corky brown rot of the flesh, in addition to the presence of mycelium in the carpels; or (iv) wet rot, with wet and soft brown rot of the flesh, in addition to mycelium growth in the carpels.

Isolations were attempted from small pieces (2-5 mm in length) of mesocarp (flesh), carpel walls and seeds on potato dextrose agar (PDA) acidified with 92% lactic acid at 0.5 ml/liter (APDA) plus 0.1% Igepal CO-630 (Sigma-Aldrich), which was used as a colony growth restrictor (Elfar et al. 2013). The cultures were incubated for 7 to 10 days at room temperature (20-22°C) in the dark prior to the determination of the total number of *Alternaria*-like colonies under a stereoscopic microscope. Fungal colonies were tentatively identified as *Alternaria* by the colony morphology (color, texture) and by the conidiophore and conidial characteristics. Colonies of other filamentous fungi were counted and identified and to genus level on the basis of colony morphology (color, texture) and conidiophore and conidial characteristics.

Mycelia from *Alternaria*-like colonies of 330 isolates were transferred to APDA, and pure cultures were then obtained by transferring a single hyphal tip to fresh APDA. Isolates were kept on APDA at 5°C until used.

Molecular characterization. A total of 33 *Alternaria* isolates from moldy core and eight isolates from leaf spot of apple (Elfar et al. 2018), representing each of six morphological groups differentiated on the basis of colony morphology and characteristics of their conidiophores and conidia were selected for molecular studies (Table 1). Fourteen of these isolates were initially examined molecularly by the internal transcribed spacer (ITS) and histone 3 gene (*HIS3*) sequence analysis. However, these analyses were not very informative. Therefore, the 41 isolates were examined for the *Alternaria* major allergen Alt a1 (*Alt a1*), plasma membrane ATPase (*ATPase*), calmodulin (*CAL*).

DNA was extracted from the isolates from 7- to 10-day-old mycelia cultured on APDA incubated at 20-22°C. Mycelia were carefully separated from the agar medium with the aid of a sterile scalpel and ground in liquid nitrogen in a porcelain mortar. Genomic DNA was extracted using a DNA extraction kit (Wizard Genomic DNA Purification Kit, Promega,

Madison, WI). The DNA yield was estimated by gel electrophoresis and GelRed (Biotium Inc., Alameda, CA) staining and visualized by UV transillumination at 320 nm.

The ITS, *Alt a1*, *ATPase*, *CAL* and *HIS3* genes were amplified using primer pairs ITS5/ITS4 (White et al. 1990), ATPDF1/ATPDR1, CALDF1/CALDR1 (Lawrence et al. 2013) and Alt-for/Alt-rev (Hong et al. 2005) H3-1a and H3-1b (Glass and Donaldson 1995), respectively. Polymerase chain reaction (PCR) was conducted in a thermal cycler (Veriti 96-Well, Applied Biosystems, CA) in a final volume of 25 µl containing 5 µl of 10x PCR buffer, 1 µl of 25 mM $MgCl_2$, 0.2 µl of 10 mM dNTPs, 1 µl of a 15 mM solution of each primer, 0.13 µl containing 5 units of *Taq* DNA polymerase (GoTaq Flexi DNA Polymerase, Promega) and 2 µl of template DNA. The amplification protocol included preheating for 3 min at 95°C followed by 35 cycles of denaturation at 95°C for 30 s; annealing for 30 s at 59°C for the ITS, at 60°C for *ATPase*, at 57°C for *CAL*, at 58°C for *Alt a1* and at 64.5 for *HIS3*; and extension at 72°C for 90 s, with a final extension for 7 min at 72°C.

The PCR products were separated on 2% agarose gels in 1.0x Tris-acetate-EDTA (TAE) buffer and stained with GelRed, and the DNA fragments were visualized by UV transillumination at 320 nm.

The PCR-amplified products were purified and sequenced from both directions by Macrogen Inc., South Korea. The sequences were edited using Proseq v.2.91 (Filatov 2002) and aligned using ClustalX v.2.1 (Larkin et al. 2007). A BLASTn search analysis was performed against reference sequences in the GenBank (www.ncbi.nlm.nih.gov) database.

A phylogenic analysis was performed for a maximum parsimony (MP) analysis with the aid of MEGA v.7.0 (Kumar et al. 2016). Gaps were treated as missing data. The MP tree was obtained using the Tree-Bisection-Reconnection (TBR) branch-swapping algorithm and 1,000 random sequences additions. Branch stability was estimated with a bootstrap analysis with 1,000 replicates. The alignment included sequences from the 33 *Alternaria* isolates obtained from moldy core of apples and eight *Alternaria* isolates obtained from leaf spot of

apple in Chile (Table 1). Based on the BLASTn results, *Alternaria* sequences deposited in GenBank for mainly *Alternaria* spp. included in sections: *Alternaria*, *Infectoriae* and *Pseudoalternaria*, including ex-types, were used in phylogenetic analysis. Sequences of *Stemphylium botryosum*, *S. callistephi*, and *S. vesicarium* were included as the outgroup (Table 2). The phylogenetic analysis was performed independently for each gene. The congruence of the evolution between the *ATPase* and *CAL* genes, and between *ATPase*, *CAL* and *Alt a1* was studied using the partition homogeneity tests with the aid of PAUP v.4.0b10 (Swofford 2002).

Morphological characterization. Colony morphology of 19 *Alternaria* isolates from apple was characterized in 9-cm-diameter plastic Petri dishes containing APDA, potato carrot agar (PCA), containing per 1 L of distilled water 20 g potato, 20 g carrot, and 20 g agar, (Simmons 2007) and dichloran rose Bengal yeast extract sucrose agar (DRYES), containing per 1 L of distilled water 2 mg dichloran, 25 mg rose Bengal, 20 g yeast extract and 150 g sucrose, (Frisvad 1983). APDA and PCA plates were incubated for 7 days at 22°C 8 h/ 16 h light/ dark. Light was provided by daylight candles (Philips, TL-D 36W/54-765, Thailand) placed 40 cm above the plates. The DRYES plates were incubated for 7 days at 22°C either under daylight or darkness conditions. The color, texture and margins of the colonies and the development of pigments on APDA were determined. The color of the colonies on DRYES was determined. The color was established with the aid of the Pantone color chart (www.pantone-colours.com). Conidia and conidiophores from three PCA plates per isolate were mounted on colorless 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 and 500 ml distilled water) before being observed under a light microscope. Conidiophore (n = 15 per isolate) length and width, cell numbers and branching were determined. Conidial (n = 50) shape, length, width, number of transepta and beak length were measured and compared

with published descriptions (Gannibal and Lawrence 2016; Simmons 2007; Poursafar et al. 2018; Pryor and Michailides 2002).

Vegetative compatibility tests. The vegetative compatibility within *A. alternata* (n = 3 isolates), *A. arborescens* (n = 3 isolates), *A. dumosa* (n = 1 isolate), *A. frumenti* (n = 2 isolates), *A. kordkuyana* (n = 1 isolate), and *A. tenuissima* (n = 3 isolates) were conducted based on van der Waals et al. (2004). Two isolates were paired on APDA on 90-mm Petri dishes. Two mycelial plugs (4 <mm in diameter) were cut from actively growing APDA cultures and placed 2 cm apart. Reactions were scored after 7 days of incubation in the dark at 20°C. All isolates were paired with themselves to ensure self-compatibility. Pairings were repeated to confirm the results. Pairings were examined macroscopically for the presence of an antagonism zone in the region of mycelial contact (van der Waals et al. 2004).

Effect of temperature on mycelial growth. The effect of temperature on the mycelial growth of *A. alternata* (n = 5 isolates), *A. arborescens* (n = 5 isolates), *A. dumosa* (n = 1 isolate), *A. frumenti* (n = 2 isolates), *A. kordkuyana* (n = 1 isolate), and *A. tenuissima* (n = 5 isolates) was studied. For this purpose, a 4-mm-diameter mycelial plug was placed in the center of each Petri dish (9 cm) containing 15 ml of APDA and incubated in thermal chambers (Velp Scientifica, Milan, Italy) adjusted to 0 to 40°C (±1°C) in 5°C intervals. Temperatures were checked with a HOBO PRO temperature sensor (Onset Computer Corp., MA) with two terminals, one was outside the Petri dish and the second one placed inside the Petri dish on the agar medium surface. Petri dishes were incubated for at least 12 h at the desired temperature before use. The growth of two perpendicular radials mycelial was determined after 2, 4 and 6 days of incubation. Isolates showing no growth after 6 days at 0 and/or 40°C were reincubated at 22°C for 5 days to determine their viability. This experiment was conducted twice.

Pathogenicity tests. To stimulate sporulation, isolates were cultivated in 0.05 x PDA (Pryor and Michailides 2002) for 7 days at 22°C with 10 h/14 h of light/dark cycles. Inoculum

suspensions were prepared with 10- to 14-day-old cultures on 0.05 x PDA. Dishes were flooded with approximately 20 ml of 0.05% of Tween 80 and scraping the surface of the medium with sterile scalpel. The conidial suspension was filtered through two layers gauze and the final concentration was adjusted to 1×10^6 conidia / ml using a hemacytometer. Isolates of *A. alternata* (n = 5 isolates), *A. arborescens* (n = 5 isolates), *A. dumosa* (n = 1 isolate), *A. frumenti* (n = 2 isolates), *A. kordkuyana* (n = 1 isolate), and *A. tenuissima* (n = 5 isolates) were evaluated for pathogenicity.

Apple fruit. Immature fruits (mean equatorial diameter of 31.6 mm) of 'Red Chief' apples (n = 4) were surface disinfected in 75% ethanol for 5 min and air-dried before sectioning transversally. One half of each fruit were inoculated with 10 µl of conidial suspensions that were placed in each of the five carpels and then covered with the other half of the fruit to avoid rapid dehydration. Fruits were incubated at 20°C in a humid chamber for 10 days prior to determination of the proportion of carpels with visible mycelial growth. An equal number of fruits treated with sterile water served as the controls. Re-isolations from carpels were performed on APDA. This experiment was repeated as described above using mature fruits (mean total soluble solids 13.1%), except that the fruits were inoculated with 30 µl of conidial suspensions placed in each of the five carpels.

Immature fruits and mature fruits of 'Red Chief' apples (n=4) were surface disinfected in 75% ethanol for 5 min and air-dried. Each fruit was inoculated with 10 µl of conidial suspension that was deposited on top of four punctures made with the aid of a sterile hypodermic needle. Fruits were incubated in humid chambers at 20°C for 10 days prior to determine the necrotic lesions. An equal number of fruits, injured but not inoculated, treated with sterile solution were left as negative control.

Apple seeds. Seeds from immature and mature 'Red Chief' apples were surface disinfected with 75% ethanol for 30 s. Five seeds were aseptically placed in humid chambers (n = 4), inoculated with 10 µl of conidial suspension per seed and incubated at 20°C for 7

days before examining each seed for the presence of mycelial growth, sporulation and necrotic lesions. An equal number of seeds treated with sterile water served as the negative controls. Re-isolations from surface mycelial growth and necrotic lesions were attempted on APDA.

Apple leaves. Detached leaves of 'Red Chief' apples (n = 4), taken from the newest leaves of the shoots, were surface disinfected in 1% NaOCl for 1 min followed by sterile distilled water for 1 min and air dried. Then, the leaves were wounded with three punctures made with a sterile hypodermic needle in the apical, basal and middle regions of each leaf blade. Leaves were inoculated by delivering 15 µl of conidial suspension per wound site. Leaves were incubated at 20°C in a humid chamber for 7 days before the diameters of the lesions were determined. An equal number of wounded leaves treated with sterile water served as the negative controls. Re-isolations from the necrotic lesions on APDA were attempted, and isolates were identified on the basis of the conidial morphology. This experiment was conducted twice. This experiment was repeated as described above using unwounded leaves.

Design and statistical analysis. All experimental treatments were performed in a completely randomized design with four replicates. Means were separated according to the Fisher LSD test ($P < 0.05$). All analyses were performed with the aid of SigmaStat 3.1 (Systat Software Inc.). Data percentages were arcsine$\sqrt{(x/100)}$ transformed, and necrotic lesion length data were transformed to Ln(x + 1) prior to analysis, but non-transformed data are presented. The mean daily radial growth rates were estimated by linear regression analysis between y = mycelial growth and x = time.

RESULTS

Sampling locations and fungal isolation. Of 2200 fruit sampled, 653 (29.7%) were diseased fruits, characterized by the presence of a gray to dark olive green cottony mycelium

(moldy core). In addition, 158 (7.2%) fruits showed a brown core, 31 (1.4%) fruit with dry rot and 4 (0.2%) fruits showed wet rot. A moldy core was most prevalent, varying from 16 to 46% in 'Oregon Spur', 'Red Chief', and 'Scarlet' apples and between 4 and 13% in 'Fuji' apples. The brown core prevalence varied from 0.1 to 69%. A low prevalence of core rot was determined in 'Oregon Spur', 'Red Chief' and 'Scarlet' apples (0.3 to 3.0%). 'Granny Smith' apples were symptomless (Table 3).

Species of *Alternaria* were the most frequently and consistently isolated fungi, with 56.2% of the apple samples in 2014-2015 and 79.2% in 2015-2016, followed in order of species by frequency *Cladosporium*, *Penicillium*, *Stemphylium*, *Epicoccum*, *Botrytis* and *Fusarium* (Table 3). In total, 330 isolates of *Alternaria* spp. were obtained from moldy core apples (Table 3).

Molecular characterization. Sequences of *ATPase* of the *Alternaria* isolates from apple ranged from 1188 to 1197 bp. Maximum parsimony analysis produced a consensus tree (Fig. 4) inferred from the two most parsimonious trees (tree length [TL] = 567, consistency index [CI] = 0.747, retention index [RI] = 0.953, and rescaled consistency index [RC] = 0.713). The phylogenetic tree obtained with *ATPase* sequences delimited six main clades (groups A to F) (Fig. 4). Clade I (group A), with 97% bootstrap support, contains sequences of isolates of *A. alternata* from Chilean apples together with the *A. alternata* ex-type (JQ671874) and *A. destruens* ex-type (JQ671873). Clade II (group B), with 88% bootstrap support, contains isolates of *A. tenuissima*, including the sequence of the *A. tenuissima* ex-type (JQ811989). Clade III (group F), with 70% bootstrap support, groups isolates *A. dumosa* together with the *A. dumosa* ex-type (JQ671877). Clade IV (group C), with 100% bootstrap support, contains isolates of *A. arborescens*, including the *A. arborescens* ex-type (JQ671880). Clade V (group D), with 91% bootstrap support, group isolates from moldy core of Chilean apples together with *ATPase* sequences of *A. frumenti* (JQ671823) in sect. *Infectoriae*. Clade VI (group E), with 99% bootstrap support, contains one isolate from

Chilean apples that was grouped together with *A. kordkuyana* ex-type (MF033860) in sect. *Pseudoalternaria* (Fig. 4).

Sequences of *CAL* of the *Alternaria* spp. from apple ranged from 775 to 794 nucleotides. Maximum parsimony analysis produced one parsimonious trees (tree length [TL] = 355, consistency index [CI] = 0.785, retention index [RI] = 0.977, and rescaled consistency index [RC] = 0.768). The phylogenetic tree obtained with *CAL* sequences delimited four main clades. Clade I, with 82% bootstrap support, contains isolates of the *A. tenuissima*, *A. destruens* ex-type (JQ646207) and *A. limoniasperae* ex-type (JQ646213). Clade II, with 79% bootstrap groups isolates *A. dumosa* together with the *A. dumosa* ex-type (JQ646211). Clade III, with 99% bootstrap support, contains sequences of isolates of *A. arborescens*, including the sequences of the *A. arborescens* ex-type (AY563303), *A. cerealis* (JQ646217) and *A. angustiovoidea* (JQ646203). Clade IV, with 96% bootstrap support, groups isolates *A. alternata* and *A. tenuissima* together with the ex-types (JQ646208, JQ646209). Clade V, with 97% bootstrap support, contains isolate from Chilean apples including the *A. parvicaespitosa* ex-type (KJ920952) of sect. *Pseudoalternaria.* DNA sequence of *A. kordkuyana* ex-type for *CAL* gene is not available. Clade VII, with 100% bootstrap support, contains sequences of isolates from Chilean apples including the ex-type of *A. infectoria*, *A. frumenti* and other *Alternaria* spp. of sect. *Infertoriae*.

Sequences of *Alt a1* of the *Alternaria* spp. from apple ranged from 483 to 485 nucleotides. Maximum parsimony analysis yielded four most parsimonious trees (tree length [TL] = 200, consistency index [CI] = 0.700, retention index [RI] = 0.940, and rescaled consistency index [RC] = 0.658). The phylogenetic tree obtained with *Alt a1* sequences delimited five main clades. Clade I, with 100% bootstrap support, contains sequences of isolates of *A. alternata* and *A. tenuissima* from Chilean apples that were grouped together with *Alt a1* sequences of the *A. alternata* and *A. tenuissima* ex-types (KP275691, KP275690). Clade II, with 100% bootstrap support, contains isolates of *A. arborescens* and *A. frumenti*, including sequences

of the *A. arborescens* ex-type (AY563303). Clade III, with 100% bootstrap support, groups isolates *A. alternata*, *A.dumosa* and *A. tenuissima* together with the *A. destruens* and *A. herbiphorbicola* ex-types. Clade IV, with 70% bootstrap support, contains the ex-type of *A. infectoria* and other *Alternaria* spp. of sect. *Infertoriae*, but none of the Chilean isolates were grouped in Cluster IV. Clade V, with 98% bootstrap support, contains one isolates from Chilean apples including the ex-type *A. rosae* and *A. parvicaespitosa* of sect. *Pseudoalternaria.* DNA sequence of *A. kordkuyana* ex-type for *Alt a1* gene is not available.

The partitioned homogeneity test showed significant differences ($P < 0.05$) between the *Alt a1*, *ATPase* and *CAL* genes and between *ATPase* and *CAL*. Therefore, the concatenated analysis of these genes was not performed.

The maximum parsimony analysis of the rDNA ITS sequence was not informative to clearly delimit the small-spored species of *Alternaria* associated with moldy core of apple in the present study. Similarly, the phylogenetic analysis of the partial coding sequence of the *HIS* gene was not useful to distinguish isolates of *Alternaria* spp. from moldy core of apple.

Morphological characterization. All *Alternaria* isolates produced single conidiophores and catenulate muriform, brown to golden brown conidia. However, six groups (A, B, C, D, E, F) of isolates were differentiated based on the colony morphology and characteristics of their conidiophores and conidia. Isolates in Group A were identified as *A. alternata*, in sect. *Alternaria* and produced cottony, gray to green (Pantone no. 5777-5763) colonies on APDA and gray-green colonies on DRYES medium. Isolates produced conidial chains with numerous secondary chains branching on short conidiophores (mean 49.8 ± 11.5 × 4.0 ± 0.5 µm) of 3 – 6 cells. Conidia were ovoid to ellipsoid (mean 28.3 ± 4.4 × 11.7 ± 1.6 µm) with 3 – 7 transepta and 0 – 1 longisepta (Fig. 3, Table 4).

Isolates in Group B were identified as *A. tenuissima* in sect. *Alternaria* and produced cottony, olive brown (Pantone no. 5815-5825) with slight concentric growth rings and prominent white margins colonies on APDA and gray-green to yellow-green colonies on

DRYES medium. The isolates produced conidial chains of 4 – 12 conidia, rarely with a lateral branch, on conidiophores of 41.1 ± 9.7 × 4.2 ± 0.5 µm of 4 – 5 cells. Conidia were ovoid to obclavate with a narrow tapered upper half (mean 34.7 ± 5.9 × 11 ± 1.3 µm) with 3 – 6 transepta with the majority of the conidia exhibiting a dark transverse median septa, with 0 – 2 longisepta and a long beak (Fig. 3, Table 4).

Isolates in Group C were identified as *A. arborescens* in sect. *Alternaria* and produced cottony, olive brown (Pantone no. 5815-5825) with prominent concentric growth rings and narrow white margins colonies on APDA and dark yellow-green colonies on DRYES medium. Isolates produced conidial chains of 2 – 7 conidia, with very distinctive long conidiophores (mean 225.4 ± 80 × 3.8 ± 0.5 µm) of 7 – 10 cells and extended secondary conidiophores. Sporulation were concentrated near the apex of secondary, tertiary and even quaternary conidiophores. Conidia were ovoid to ellipsoid (mean 23.2 ± 4.2 × 10.1 ± 1.3) with 1 – 4 transepta and no longisepta or rarely with 1 longisepta (Fig. 3, Table 4).

Isolates in Group D were identified as *A. frumenti* in sect. *Infectoriae* and produced floccules, gray to yellow-green (Pantone no. 5777-5783) colonies with slight concentric growth rings on APDA and white colonies on DRYES medium. Primary conidiophores were relatively short (mean 63.8 ± 16.4 × 4.2 ± 0.7 µm) of 3 – 6 cells. Secondary conidiophores were branched and geniculated. Conidia were ovoid to ellipsoid (mean 33.7 ± 6 × 10.2 ± 1.6 µm) with 2 – 5 transepta and 0 – 1 longisepta in 0 – 3 transverse segment, produced on secondary and tertiary conidiophores (Fig. 3, Table 4).

Isolate in Group E was identified as *A. kordkuyana* in sect*. Pseudoalternaria.* This isolate produced gray to dark-green (Pantone no. 5763-5743) colonies with slight concentric growth rings on APDA and white colonies on DRYES medium. Isolates produced short conidial chains of 1 – 6 conidia with lateral chains branching on short conidiophores (mean 56.1 ± 10.8 × 3.8 ± 0.5 µm) of 4 – 6 cells. Conidia were ovoid to elliptical (mean 24.7 ± 6.8 × 9.7 ± 2.2 µm) with 1 – 4 transepta and 0 – 1 longisepta (Fig. 3, Table 4).

Isolate in Group F was identified as *A. dumosa* in sect. *Alternaria* produced cottony, light brown-green (Pantone no. 5803-5783) colonies with slight concentric growth rings on APDA and gray-green colonies on DRYES medium. Isolates produced conidial chains with numerous secondary chains branching on short conidiophores (mean 60.2 ± 19.9 × 3.9 ± 0.4 µm) of 4 – 5 cells. Conidia were ovoid (mean 18.3 ± 3 × 8.9 ± 1.6 µm) with 1 – 4 transepta and 0 – 1 longisepta (Fig. 3, Table 4).

Vegetative compatibility tests. Of 169 pairings studied, 150 pairings were consistently compatible, developing a clear non-antagonistic line at the contact zone. One pairing was incompatible and 18 pairings were uncertain (Table 5).

Effect of temperature on mycelial growth. The radial mycelial growth of the *Alternaria* isolates was significantly ($P < 0.001$) affected by temperature and isolate with a significant ($P < 0.001$) temperature × isolate interaction. All *Alternaria* isolates were able to grow between 5 and 35°C (Fig. 5). However, some isolates showed very slight growth (<1 mm/6 days) at 0°C. All isolates regained normal growth at 22°C. Optimal radial mycelial growth was obtained at 25°C. No growth occurred at 40°C in 8 days. Only some isolates of *A. arborescens* (isolate 38MC), *A. dumosa* (isolate 28MC), and *A. tenuissima* (isolate 33 MC) were able to regain growth after 5 days at 22°C. The differences in the mycelial growth rate among the *Alternaria* isolates were significant ($P < 0.001$). The highest growth rate was obtained for *A. kordkuyana* (12.0 mm/day), followed by *A. dumosa* (9.8 mm/day), *A. frumenti* (8.9 mm/day), *A. alternata* (8.1 mm/day), *A. tenuissima* (7.6 mm/day), and *A. arborescens* (7.2 mm/day) (Fig. 6).

Pathogenicity tests. All *Alternaria* spp. isolates developed moldy core characterized by the presence of a gray to dark olive green mycelium with or without sporulation restricted to the carpels (Fig. 2b) in immature and mature apple fruits (Table 6). Reisolations were accomplished for 100% of the inoculated fruits. Non-inoculated fruits remained asymtomatic.

All *Alternaria* isolates affected 100% of the inoculated seeds, developing a superficial gray to dark olive green mycelium and rotted 100% of the immature seeds. Isolates of *A. arborescens*, *A. tenuissima*, *A. kordkuyana* and one isolate of *A. alternata* sporulated on the seed after 5 days at 20°C.

Independently of the *Alternaria* spp., none of the isolates were able to cause necrotic lesions on the epidermis and flesh of immature and mature fruits after conidial inoculations, except for a very superficial oxidation and minimal but not measurable necrosis.

Independently of the *Alternaria* isolates, seeds from mature fruits were superficially colonized, developing a gray to dark olive green mycelium and abundant dark sporulation (Fig. 2c). However, the seeds were not rotted, comprising only the testa without evidence of internal seed invasion after 10 days at 20°C. Significant differences ($P < 0.001$) in the percentage of seed colonization were obtained among isolates and *Alternaria* spp.

Without exception, all *Alternaria* isolates were pathogenic in detached apple leaves, developing brown necrotic lesions of 0.4 to 5.7 mm in diameter, with concentric rings and a chlorotic halo (Fig. 2d), after 5 days at 20°C. Significant differences ($P < 0.001$) in the lesion diameters were found among isolates and *Alternaria* spp. Differences in virulence were consistently obtained, with isolates of *A. tenuissima* being the most virulent (mean of 4.3 mm), followed by isolates of *A. alternata* (mean of 2.9 mm), *A. arborescens* (mean of 2.5 mm), *A. dumosa* (2.1 mm), *A. frumenti* (mean of 2.0 mm), and *A. kordkuyana*, which was the least virulent isolate (0.4 mm) (Table 6). Reisolations from the margins of the necrotic lesions were successful from 100% of the inoculated leaves. The identification of reisolated fungi was confirmed morphologically. Unwounded but inoculated leaves remained asymtomatic.

DISCUSSION

The analysis of 653 fruits showing moldy core revealed the presence of several genera of filamentous fungi, however, species of *Alternaria* (67.7%) were by far the most commonly and consistently isolated causal agents. Moldy core was only found on apple cultivars having open sinus fruits but it was absent in 'Granny Smith', a cultivar with close sinus fruits.

Based on the results of this study, at least six species of small-spored *Alternaria* were found, namely, in order of importance, *A. tenuissima*, *A. arborescens*, *A. alternata*, *A. dumosa* in sect *Alternaria*, *A. frumenti*, in sect. *Infectoriae*, and *A. kordkuyana* in sect. *Pseudoalternaria* These species were identified morphologically and molecularly according to Gannibal and Lawrence (2016), Lawrence et al. (2013), Poursafar et al. (2018), Pryor and Michailides (2002), Simmons (2007), and Zhu and Xiao (2015). The identification of *A. kordkuyana* should be further study using additional gene sequences. A concatenated analysis using DNA sequences of *ATPase*, *CAL* and *Alt a1* genes would be advisable. However, DNA sequences of *CAL* and *Alt a1* genes are not available for *A. kordkuyana* ex-type.

These *Alternaria* spp. were isolated alone or two or more species coexist in the same fruit. To some extent, the vegetative compatibility exhibited by most species and isolates explain the coexistence of more than one species on the same fruit carpel. Complex of small-spored *Alternaria* spp. have been previously identified causing diseases in different fruit crops, such as core rot of apples (Serdani et al. 2002), Alternaria leaf blotch and fruit spot (Harteveld et al. 2013; Rotondo et al. 2012), heart rot of pomegranates (Luo et al. 2017; Michaelides et al. 2008), Alternaria fruit rot of blueberry (Zhu and Xiao 2015), and Alternaria late blight of pistachio (Pryor and Michailides 2002).

All *Alternaria* isolates obtained from moldy core apples were small-spored *Alternaria* with single conidiophores, catenulate and muriform conidia (Simmons 2007). There is a general agreement that the identification of *Alternaria* spp. is taxonomically difficult, particularly the

small-spored *Alternaria*, because they share and have overlapping morphological characteristics (Andrew et al. 2009, Serdani et al 2002). Colonies of isolates in sect. *Alternaria* varied between gray-green to yellow-green. Isolates of *A. frumenti* and *A. kordkuyana* as expected for *Alternaria* isolates of sect. *Infectoriae* and *Pseudoalternaria* developed white and distinctive colonies on DRYES medium (Andersen et al. 2002; Lawrence et al. 2014; Poursafar et al. 2018).

In contrast to previous works (Kang et al. 2002; Sun and Huang 2017; Zheng et al. 2015), the phylogenetic analysis of the partial coding sequence of *HIS* gene was inadequate to distinguish isolates of *Alternaria* spp. from moldy core of apple in this study. Similarly, to previous studies (Lawrence et al. 2013, 2016; Luo et al. 2017; Zheng et al. 2015), the maximum parsimony analysis of the rDNA ITS sequence allowed to clearly delimit the small-spored species of *Alternaria* within sections *Alternaria*, *Infectoriae* and *Pseudoalternaria*. However, it was not informative enough to segregate the species within each section.

Phylogenetic analysis using the *ATPase*, *CAL* and *Alt a1* genes has been proposed to differentiate species of *Alternaria* in previous works (Lawrence et al. 2013; Zhu and Xiao 2015). In the present study, the *Alt a1* gene provides sufficient information to clearly distinguish *Alternaria* isolates in sect. *Pseudoalternaria* from sect. *Alternaria* and sect. *Infectoriae*. The *CAL* gene allowed the differentiation of *A. arborescens*, *A. dumosa*, isolates of *Alternaria* sect. *Infectoriae* and sect. *Pseudoalternaria*., placing them in four separate clusters, but the information provided by this gene was not sufficient to distinguish *A. tenuissima* and *A. alternata* as separate species. These last two species were clearly separated using the *ATPase* gene. However, in agreement with Zhu and Xiao (2015), *A. destruens* was clustered into the same group with *A. alternata* isolates from apples and the reference isolates of *A. alternata*, but *A. alternata* and *A. destruens* could be differentiated by their sporulation patterns, which widely differ.

In Greece, Ntasiou et al. (2015) determined the presence of *A. tenuissima* and *A. arborescens* as the cause of core rot of apple. Gao et al. (2013) reported an association of *A. alternata*, *A. arborescens*, *A. infectoria* and *A. tenuissima* with moldy core in 'Fuji' apples in China. Similarly, Serdani et al. (2002), studying the *Alternaria* spp. associated with dry core rot of apples in South Africa, demonstrated the presence of *A. arborescens*, *A. infectoria* and *A. tenuissima.* However, isolates of *A. infectoria* from China and South Africa were not pathogenic. In contrast to Gao et al. (2013) and Serdani et al. (2002), isolates of *Alternaria frumenti* in sect. *Infectoriae* from Chile were pathogenic on apple fruits and leaves. Additionally, in this study, two other pathogenic *Alternaria* spp., *A. dumosa* and *A. kordkuyana* were found.

Independently of the *Alternaria* spp., all isolates were pathogenic, colonizing the carpels and rotting seed from immature fruits, but the seeds from mature fruit were only surface-colonized without affecting the seeds internally. All isolates induced small necrotic lesions on detached wounded leaves, but were unable to infect non-wounded leaves, suggesting that leaf injuries are needed for infection under field conditions. However, significant ($P < 0.001$) differences in lesion diameter were obtained, suggesting differences in virulence among *Alternaria* isolates, with *A. tenuissima* being the most virulent species, followed by *A. alternata*, *A. arborescens*, *A. dumosa*, *A. frumenti* and *A. kordkuyana* Independently of the virulence on leaf assays and the lack of pathogenicity on immature and mature fruits, all *Alternaria* spp. showed a high capacity to colonize the carpels of both immature and mature fruits, which is the main concern regarding moldy core of apples. Furthermore, differences in virulence between *Alternaria* spp. have been detected on other hosts. For example, on blueberry and pistachio, isolates of *A. infectoria* were less virulent than *A. alternata*, *A. arborescens* and *A. tenuissima* (Pryor and Michailides 2002; Zhu and Xiao 2015).

The *Alternaria* isolates that were not able to produce fruit rot lesions in this study as it has been reported previously for some isolates of *A. alternata*, *A. arborescens* and *A. tenuissima*

(Harteveld et al. 2014; Rotondo et al. 2012). These differences in the pathogenic capacities suggests the presence of different pathotypes. Nevertheless, differences in the susceptibility of apple cultivars used in the pathogenicity tests can also explain these results. Large differences in susceptibility have been reported; being 'Golden Delicious' very susceptible, 'Top Red' moderately resistant and 'Jonathan' resistant (Gur et al. 2017). Besides, the inoculated tissue should be considered, because it has been shown that the mesoderm close to the skin is highly resistant to *Alternaria* rot increasing in susceptibility towards the carpels (Gur et al. 2017). Interestingly, no evidences of *A. mali* were obtained in this study, which has been associated mainly with fruit rot of apples (Harteveld et al. 2013; Gur et al. 2017; Rotondo et al. 2012).

All the reference isolates obtained from necrotic leaf spot of apples were equally able to colonize carpels internally, developing a cottony gray to olive green mycelium that characterized apple moldy core. Therefore, there was no evidence for apple tissue specificity among isolates of *Alternaria* spp. attacking apples. Similar to Kang et al. (2002), our results suggested that moldy core resulted from opportunistic and favorable conditions rather than true specific pathological abilities exhibited by the isolates of *Alternaria* spp.

Moldy core prevalences between 4 and 46% were estimated in this study, reinforcing the economic importance of moldy core in the Chilean apple industry. As has been reported in other apple-producing countries (Ellis and Barrat 1983, Kupferman 1992; Miller 1959; Reuveni 2006; Serdani et al. 1998), moldy core has been associated with the open-sinus condition of Delicious cultivars. In this study, 40 and 53% of 'Oregon Spur' and 'Red Chief' apples with an open sinus were very susceptible, showing the highest moldy core prevalence (16 to 46%). In the present study, 'Fuji' (Red Delicious x Ralls Janet) had a high proportion of open sinus fruits at harvest (42%). However, a relatively low (<13%) proportion of moldy core was determined. This finding can be explained by factors other than the sinus condition of the fruits (Shtienberg 2012). Differences in the mesoderm and locule sensitivity

of each apple cultivar to colonization by moldy core fungi could also explain the substantial variation in prevalence obtained among open sinus apple cultivars in this study (Niem et al. 2007).

The apparent resistance of 'Granny Smith' apples to moldy core appeared to be associated with the closed sinus condition characterizing 'Granny Smith' fruits (Serdani et al. 1998).

In summary, this study demonstrates that moldy core of apple is caused by many small-spored *Alternaria* spp. The most frequently isolated species was *A. tenuissima*, which was virulent on apple leaf assays and showed a high capacity to colonize apple carpels. However, *A. tenuissima* and the other *Alternaria* spp. show no capacity to cause lesion on the epidermis and flesh of apple fruits. This species coexisted with one or more *Alternaria* spp. This is the first report of *A. arborescens*, *A. dumosa*, *A. frumenti*, *A. tenuissima* and *A. kordkuyana* associated with moldy core of apples in Chile and the first report of *A. dumosa*, *A. frumenti*, and *A. kordkuyana* causing moldy core of apples worldwide. The results of this study do not exclude the possibility that other species may be involved in the *Alternaria* complex associated with moldy core of apples. The presence of different species of *Alternaria* may have implications to consider when establishing control strategies, because *Alternaria* spp. may have different sensitivities to fungicides. More information on moldy core epidemiology and sensitivity to fungicides of *Alternaria* spp. is needed in order to implement successful control strategies.

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Table 1. Source of isolates of *Alternaria* spp. from apples obtained in commercial orchard in Central Chile and GenBank accession numbers for sequences of three genes (*Alternaria* major allergen Alt a1, plasma membrane ATPase, and Calmodulin) of *Altenaria* isolates studied

Species	Symptom	Locality[x]	Latitude[y]	Apple cultivar	GenBank accession number[z]		
					Alt a1	*ATPase*	*CAL*
Alternaria alternata							
7MC[w]	Brown core	Río Claro, VII	35°15'	Red Chief	MG925174	MG740627	MG925132
10LS	Leaf spot	Pirque, MR	33°40'	Scarlet	MG925165	MG740618	MG925123
12LS[w]	Leaf spot	Morza, VII	34°50'	Scarlet	MG925166	MG740619	MG925124
16MC[w]	Moldy core	Pirque, MR	33°40'	Scarlet	MG925175	MG740628	MG925133
17MC	Moldy core	Graneros, VI	34° 4'	Oregon Spur	MG925176	MG740629	MG925134
30MC[w]	Moldy core	Graneros, VI	34° 4'	Oregon Spur	MG925177	MG740630	MG925135
36MC[w]	Moldy core	Los Niches, VII	35° 3'	Scarlet	MG925178	MG740631	MG925136
A. arborescens							
8MC[w]	Brown core	Colbún, VII	35°43'	Red Chief	MG925179	MG740632	MG925137
13LS[w]	Leaf spot	Los Ángeles, VIII	37°33'	Scarlet	MG925167	MG740621	MG925126
14LS	Leaf spot	Los Ángeles, VIII	37°33'	Scarlet	MG925168	MG740620	MG925125
19MC[w]	Moldy core	Pirque, MR	33°40'	Scarlet	MG925180	MG740633	MG925138
23MC	Moldy core	Longaví, VII	36°12'	Scarlet	MG925181	MG740637	MG925142
31MC	Moldy core	Los Ángeles, VIII	37°33'	Scarlet	MG925182	MG740634	MG925139
35MC	Moldy core	Río Claro, VII	35°15'	Red Chief	MG925183	MG740636	MG925141
38MC[w]	Moldy core	Traiguén, IX	38°21'	Fuji	MG925184	MG740638	MG925143
39MC	Moldy core	Morza, VII	34°50'	Red Chief	MG925185	MG740639	MG925144
40MC[w]	Moldy core	Graneros, VI	34° 4'	Oregon Spur	MG925186	MG740635	MG925140
A. dumosa							
28MC[w]	Moldy core	Morza, VII	34°50'	Red Chief	MG925187	MG740640	MG925145
A. frumenti							
2MC[w]	Brown core	Colbún, VII	35°43'	Red Chief	MG925188	MG740641	MG925146
37MC[w]	Moldy core	Traiguén, IX	38°21'	Red Chief	MG925189	MG740642	MG925147
A. kordkuyana							
34MC[w]	Moldy core	Longaví, VII	36°12'	Scarlet	MG925190	MG740643	MG925148
A. tenuissima							
1MC	Brown core	Río Claro, VII	35°15'	Red Chief	MG925191	MG740644	MG925149
3MC	Dry rot	Graneros, VI	34° 4'	Oregon Spur	MG925192	MG740645	MG925150
4LS	Leaf spot	Morza, VII	34°50'	Scarlet	MG925170	MG740623	MG925128
5LS	Leaf spot	Pirque, MR	33°40'	Scarlet	MG925171	MG740625	MG925129
6LS	Leaf spot	Colbún, VII	35°43'	Red Chief	MG925172	MG740624	MG925130
11LS[w]	Leaf spot	Morza, VII	34°50'	Scarlet	MG925173	MG740626	MG925131
15MC[w]	Brown core	Río Claro, VII	35°15'	Red Chief	MG925193	MG740646	MG925151
18MC[w]	Moldy core	Longaví, VII	36°12'	Scarlet	MG925194	MG740647	MG925152
20MC	Moldy core	Longaví, VII	36°0'	Scarlet	MG925195	MG740648	MG925153
21MC	Moldy core	Morza, VII	34°50'	Scarlet	MG925196	MG740649	MG925154
22MC	Moldy core	Río Claro, VII	35°15'	Red Chief	MG925197	MG740650	MG925155
24MC	Moldy core	Los Niches, VII	35° 3'	Red Chief	MG925198	MG740651	MG925156
25MC	Moldy core	Río Claro, VII	35°15'	Red Chief	MG925199	MG740652	MG925157
26MC	Moldy core	Longaví, VII	36°12'	Red Chief	MG925200	MG740653	MG925158
27MC	Moldy core	Longaví, VII	36°12'	Red Chief	MG925201	MG740654	MG925159
29MC	Moldy core	Longaví, VII	36°12'	Scarlet	MG925202	MG740655	MG925160
32MC	Moldy core	Río Claro, VII	35°15'	Red Chief	MG925203	MG740656	MG925161
33MC[w]	Moldy core	Los Ángeles, VIII	37°33'	Scarlet	MG925204	MG740657	MG925162
41MC	Moldy core	Graneros, VI	34° 4'	Oregon Spur	MG925205	MG740658	MG925163
42MC[w]	Moldy core	Graneros, VI	34° 4'	Oregon Spur	MG925206	MG740659	MG925164

[w] Isolates used for morphological characterization, effect of temperature on mycelial growth test and pathogenicity tests
[x] Administrative Regions of Chile: MR= Metropolitan Region and Regions VI, VII, VIII and IX.
[y] Southern latitude.
[z] Genes: *Alt a1*= *Alternaria* major allergen Alt a1; *ATPase*= plasma membrane ATPase; *CAL*= Calmodulin.

Table 2. Accession numbers for reference sequences of three genes (*Alternaria* major allergen Alt a1, plasma membrane ATPase, and Calmodulin) of *Alternaria* isolates in GenBank used for phylogenetic analysis in this study

		GenBank accession number [z]		
Species	Isolates[x]	*Alt a1*	*ATPase*	*CAL*
Alternaria alternata	EGS 34-016[y]	KP275691	JQ671874	JQ646208
A. alternata	X1191	KJ920991	KJ908234	KJ920956
A. alternata	X1048	KJ920997	KJ908230	KJ920957
A. alternata	BMP0463	na [z]	JQ811981	na
A. angustiovoidea	EGS 36-172[y]	JQ646398	JQ671869	JQ646203
A. arborescens	EGS 39-128[y]	AY563303	JQ671880	JQ646214
A. arborescens	BMP0462	na	JQ811990	na
A. arborescens	3.J24	KJ921023	KJ908244	KJ920979
A. arborescens	X1013	KJ920987	KJ908247	KJ920949
A. arborescens	X1312	KJ921008	KJ908242	KJ920948
A. argyroxiphii	EGS 35-122[y]	JQ646434	JQ671926	JQ646260
A. arrhenatheri	56492-12FD[y]	na	JQ693603	na
A. californica	EGS 52-082[y]	JQ646373	JQ671813	JQ646147
A. cerialis	EGS 43-072[y]	JQ646405	JQ671883	JQ646217
A. conjuncta	EGS 37-139[y]	AY563281	JQ671824	JQ646158
A. daucicaulis	EGS 36-1947[y]	na	JQ671822	JQ646156
A. destruens	EGS 46-069[y]	JQ646402	JQ671873	JQ646207
A. dumosa	EGS 45-007[y]	na	JQ671877	JQ646211
A. ethzedia	EGS 37-143[y]	AY563284	JQ671805	JQ646139
A. frumenti	EGS 44-001[y]	JQ646378	JQ671823	JQ646157
A. graminicola	EGS 41-139[y]	na	JQ671819	JQ646153
A. herbiphorbicola	EGS 40-140[y]	JQ646410	JQ671888	JQ646222
A. hordeiaustralica	EGS 44-200[y]	na	JQ671811	JQ646145
A. hordeicola	EGS 50-184[y]	JQ646372	JQ671812	JQ646146
A. humuli	EGS 47-140[y]	na	JQ671821	JQ646155
A. incomplexa	EGS 17-103[y]	JQ646374	JQ671815	JQ646149
A. infectoria	EGS 27-193[y]	FJ266502	JQ671804	JQ646138
A. infectoria	3.J14	KJ921017	KJ908219	KJ920977
A. infectoria	X1273	KJ920982	KJ908218	KJ920953
A. intercepta	EGS 49-137[y]	JQ646380	JQ671826	JQ646160
A. kordkuyana	KQ-20[y]	na	MF033860	na
A. limoniasperae	EGS 45-100[y]	JQ646370	JQ671879	JQ646213
A. malorum	EGS 52.153[y]	JQ646369	JQ671800	JQ646134
A. merytae	EGS 46-153[y]	na	JQ671820	JQ646154
A. metachromatica	EGS 38-132[y]	AY563285	JQ671809	JQ646143
A. novae-zelandiae	EGS 48-092[y]	JQ646379	JQ671825	JQ646159

A. oregonensis	EGS 29-194 [y]	AY563283	JQ671827	JQ646161
A. parvicaespitosa	X1272 [y]	KJ920981	KJ908217	KJ920952
A. rosae	EGS 41-130 [y]	JQ646370	JQ671803	JQ646137
A. tenuissima	EGS 34.015 [y]	KP275690	JQ811989	JQ646209
A. tenuissima	BMP2256	na	JQ811983	na
A. tenuissima	BMP1254	na	JQ811988	na
A. triticimaculans	EGS 41-050 [y]	na	JQ671806	JQ646140
A. triticina	EGS 17-061 [y]	JQ646371	JQ671808	JQ646142
A. ventricosa	EGS 52-075 [y]	JQ646377	JQ671818	JQ646152
A. viburni	EGS 49-147 [y]	JQ646375	JQ671816	JQ646150
Stemphylium botryosum	BMP 0360	AY563274	JQ671767	JQ646101
S. callistephi	BMP 0377	AY563276	JQ671769	JQ646103
S. vesicarium	BMP 0374	AY563275	JQ671768	JQ646102

[x] na = sequences not available in GenBank.
BMP: B.M. Pryor, Division of Plant Pathology, Department of Plant Sciences, EGS: E.G. Simmons, Mycological Services, Crawfordsville, IN 47933, 3.J: Pryor and Michailides, 2002, X: Zhu and Xiao, 2015
[y] ex-type
[z] *Alt a1*= *Alternaria* major allergen Alt a1; *ATP*= plasma membrane ATPase; *CAL*= Calmodulin.

Table 3. Frequency of isolation of *Alternaria* spp. and other filamentous fungi obtained from apples fruits (n = 2,200) collected and harvest in commercial orchards in Central Valley of Chile

	Prevalence[y], %				Isolates[z], %							
Cultivars	MC	BC	DR	WR	Alt	Bot	Cla	Epi	Fus	Pen	Ste	Others
2014-2015 growing season												
Open sinus cultivars:												
Fuji (n=200)	4.0	4.5	0.5	0.0	6.4	0.0	0.5	0.0	0.0	0.0	1.1	1.1
Oregon Spur (n=100)	16.0	69.0	3.0	1.0	23.0	0.0	7,0	4.8	0.5	8.0	0.5	3.7
Red Chief (n=200)	26.5	19.0	1.0	0.0	26.8	0.0	8.0	0.0	0.0	1.1	1.1	6.4
Total,%					56.2	0.0	15.5	4.8	0.5	9.1	2.7	11.2
Close sinus cultivars:												
Granny Smith (n=100)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2015-2016 growing season												
Open sinus cultivars:												
Fuji (n=100)	13.0	7.0	0.0	0.0	3.5	0.0	0.3	0.0	0.0	0.0	0.0	0.0
Oregon Spur (n=100)	46.0	24.0	2.0	1.0	16.0	0.3	2.4	0.0	0.0	3.1	3.1	4.9
Red Chief (n=700)	38.9	0.1	0.9	0.3	30.9	0.3	0.0	0.0	0.3	0.3	0.0	2.1
Scarlett (n=600)	40.8	1.7	2.8	0.0	28.8	0.0	0.3	0.0	0.0	0.7	0.7	1.4
Total,%					79.2	0.7	3.1	0.0	0.3	4.2	3.8	8.4
Close sinus cultivars:												
Granny Smith (n=100)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[y] Percentages based on total number of fruits per apple cultivar. MC= moldy core, BC= brown core, DR= dry rot, WR= wet rot.

[z] Percentages were calculated from the total fungal isolates, n=187 and n=287 obtained respectively in 2014-2015 and 2015-2016 growing season. Alt= *Alternaria* spp., Bot= *Botrytis* spp., Cla= *Cladosporium* spp., Epi= *Epicoccum* spp., Fus= *Fusarium* spp., Pen= *Penicillium* spp. and Ste= *Stemphylium* spp.

Table 4. Morphometric characterization of conidiophores and conidia of *Alternaria* spp. isolated from moldy core of apples in Chile

Isolates	Colony[w]	Conidiophores[xz]		Conidia[yz]		
	Growth rate, mm/day	Length, µm	Width, µm	Length, µm	Width, µm	Length/ width
A. alternata (Group A)						
7MC	7.3 ± 0.3	36.4 ± 7.5	4.3 ± 0.5	32.3 ± 5.2	13.1 ± 1.7	1.8 ± 0.3
12LS	8.5 ± 0.4	56.3 ± 11.9	3.9 ± 0.4	23.4 ± 3.4	10.0 ± 1.3	2.4 ± 0.4
16MC	8.3 ± 0.2	54.2 ± 13.1	3.9 ± 0.5	35.4 ± 6.1	13.3 ± 2.1	2.7 ± 0.5
30MC	9.0 ± 0.1	57.9 ± 14.9	4.2 ± 0.5	26.2 ± 4.2	11.6 ± 1.6	2.3 ± 0.4
36MC	7.5 ± 0.2	44.3 ± 10.3	3.9 ± 0.4	24.3 ± 3.2	10.7 ± 1.2	2.3 ± 0.3
A. arborecens (Group C)						
8MC	6.3 ± 0.3	239.0 ± 104.3	4.1 ± 0.5	29.1 ± 5.8	10.6 ± 1.6	2.8 ± 0.6
13LS	6.7 ± 0.3	226.7 ± 94.5	3.5 ± 0.5	21.6 ± 3.3	9.2 ± 1.1	2.4 ± 0.4
19MC	8.1 ± 0.2	208.9 ± 73.6	3.8 ± 0.6	22.2 ± 3.6	11.1 ± 1.2	2.1 ± 0.3
38MC	7.6 ± 0.3	236.5 ± 62.9	3.5 ± 0.5	19.9 ± 4.1	9.3 ± 1.2	2.2 ± 0.5
40MC	7.3 ± 0.3	216.1 ± 64.6	4.0 ± 0.3	23.4 ± 4.2	10.4 ± 1.6	2.3 ± 0.4
A. dumosa (Group F)						
28MC	9.8 ± 0.2	60.2 ± 13.9	3.9 ± 0.4	18.3 ± 3.0	8.9 ± 1.6	2.1 ± 0.4
A. frumenti (Group D)						
2MC	9.0 ± 0.3	56.6 ± 18.6	4.5 ± 0.8	28.8 ± 4.8	9.3 ± 1.0	2.2 ± 0.6
37MC	8.9 ± 0.1	70.9 ± 14.1	3.9 ± 0.6	38.6 ± 7.1	11.0 ± 2.1	3.6 ± 0.9
A. kordkuyana (Group E)						
34MC	12.0 ± 0.2	56.1 ± 10.8	3.8 ± 0.5	24.7 ± 6.8	9.7 ± 2.2	2.6 ± 0.5
A. tenuissima (Group B)						
11LS	7.9 ± 0.3	43.1 ± 7.4	4.3 ± 0.4	31.0 ± 5.3	11.9 ± 1.6	2.6 ± 0.5
15MC	6.0 ± 0.3	39.3 ± 7.9	3.9 ± 0.5	35.6 ± 5.7	10.7 ± 1.3	2.4 ± 0.5
18MC	6.6 ± 0.2	41.6 ± 11.2	4.3 ± 0.5	36.5 ± 5.9	11.5 ± 1.4	3.2 ± 0.7
33MC	8.2 ± 0.2	49.1 ± 10.9	4.4 ± 0.7	31.2 ± 6.7	9.1 ± 1.0	2.3 ± 0.6
42MC	8.6 ± 0.3	32.2 ± 11.1	4.0 ± 0.5	39.0 ± 6.1	11.8 ±1.1	3.3 ± 0.6

[w]Means of four plates ± standard error. Incubated on APDA for 6 days at 25°C in the dark.
[x]Means of at least 15 primary conidiophores per isolates ± standard deviation.
[y]Means of at least 50 conidia per isolate ± standard deviation.
[z]Incubated on PCA for 7 days at 22°C 8 h/ 16 h light/ dark.

Table 5. Vegetative compatibility tests among isolates of *Alternaria* spp. obtained from moldy core of apples: compatible (light grey), uncertain (dark grey) and: incompatible (black) *Alternaria* isolates

7 MC	12 LS	16 MC	8 MC	13 LS	38 MC	28 MC	2 MC	37 MC	34 MC	11 LS	15 MC	18 MC	Isolates	Species
													7 MC	*A. alternata*
													12LS	
													16 MC	
													8 MC	*A. arborescens*
													13 LS	
													38 MC	
													28 MC	*A. dumosa*
													2 MC	*A. frumenti*
													37 MC	
													34 MC	*A. kordkuyana*
													11 LS	*A. tenuissima*
													15 MC	
													18 MC	

Table 6. Pathogenicity of *Alternaria* spp. isolates on fruit and leaves of 'Red Chief' apples, producing colonization of carpels and seeds and causing necrotic lesions on leaves

	Immature fruit, [xy]			Mature fruit, %[xy]			Leaves[xz]
	Carpel moldy, %	Seed rot, %	Dry rot, mm	Carpel moldy, %	Seeds moldy, %	Dry rot, mm	Necrotic lesions, mm
A. alternata							
7MC	85 a-d	100	0.0	100 ns	90 b	0.0	4.7 a-c
12LS	65 d	100	0.0	70	95 ab	0.0	2.5 c-g
16MC	80 a-d	100	0.0	80	90 b	0.0	1.3 g
30MC	95 ab	100	0.0	90	100 a	0.0	4.0 a-d
36MC	75 cd	100	0.0	80	100 a	0.0	2.0 d-g
Mean	80	100	0.0	84	95	0.0	2.9
A. arborescens							
8MC	80 b-d	100	0.0	100	100 a	0.0	2.9 b-f
13LS	85 a-d	100	0.0	90	100 a	0.0	2.8 b-f
19MC	85 a-d	100	0.0	90	100 a	0.0	3.0 a-e
38MC	95 ab	100	0.0	95	100 a	0.0	1.9 d-g
40MC	90 a-c	100	0.0	90	100 a	0.0	2.1 e-g
Mean	87	100	0.0	93	100	0.0	2.5
A. dumosa							
28MC	100 a	100	0.0	95	100 a	0.0	2.1 e-g
A. frumenti							
2MC	80 a-d	100	0.0	85	45 c	0.0	2.2 c-g
37MC	90 a-c	100	0.0	80	50 c	0.0	1.5 fg
Mean	95	100	0.0	83	48	0.0	2.0
A. kordkuyana							
34MC	95 ab	100	0.0	95	90 b	0.0	0.4 h
A. tenuissima							
11LS	95 ab	100	0.0	95	100 a	0.0	5.7 a
15MC	100 a	100	0.0	95	100 a	0.0	3.4 a-e
18MC	90 a-c	100	0.0	85	100 a	0.0	4.9 ab
33MC	95 ab	100	0.0	90	100 a	0.0	5.3 ab
42MC	95 ab	100	0.0	90	100 a	0.0	2.3 d-g
Mean	95	100	0.0	91	100	0.0	4.3

[x] Non-inoculated controls remained symptomless. These data were excluded from the statistical analysis. Means of four replicates followed by the same letter in each column did not differ significantly separated according to the Fisher LSD test ($P < 0.05$). ns = not significant at $P = 0.05$.

[y] Five carpels per fruit (n = 4) and five seeds per replicate (n = 4) were inoculated with a conidial suspension (10^6 conidia/ml) incubated in humid chambers at 20°C for five days for immature fruit and for 10 days for mature fruit. Data percentages were arcsine$\sqrt{(x/100)}$ transformed but non-transformed data are presented.

[z] Leaves were inoculated with conidial suspension (10^6 conidia/ml), and lesion diameters were determined after 4 days at 20°C in humid chambers. Data were transformed to Ln(x + 1) prior to analysis, but non-transformed data are presented.

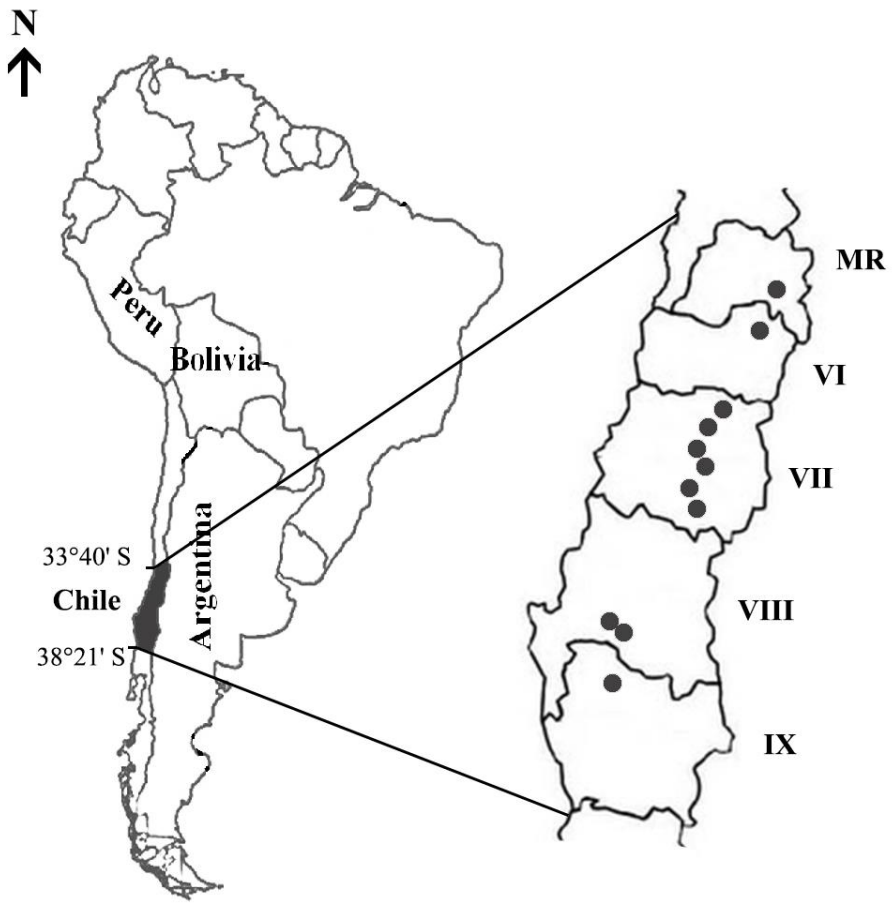


Fig. 1. Map of Chile showing the geographic distribution of apple orchards. Administrative Regions of Chile: RM= Metropolitan Region and Regions VI, VII, VIII and IX.

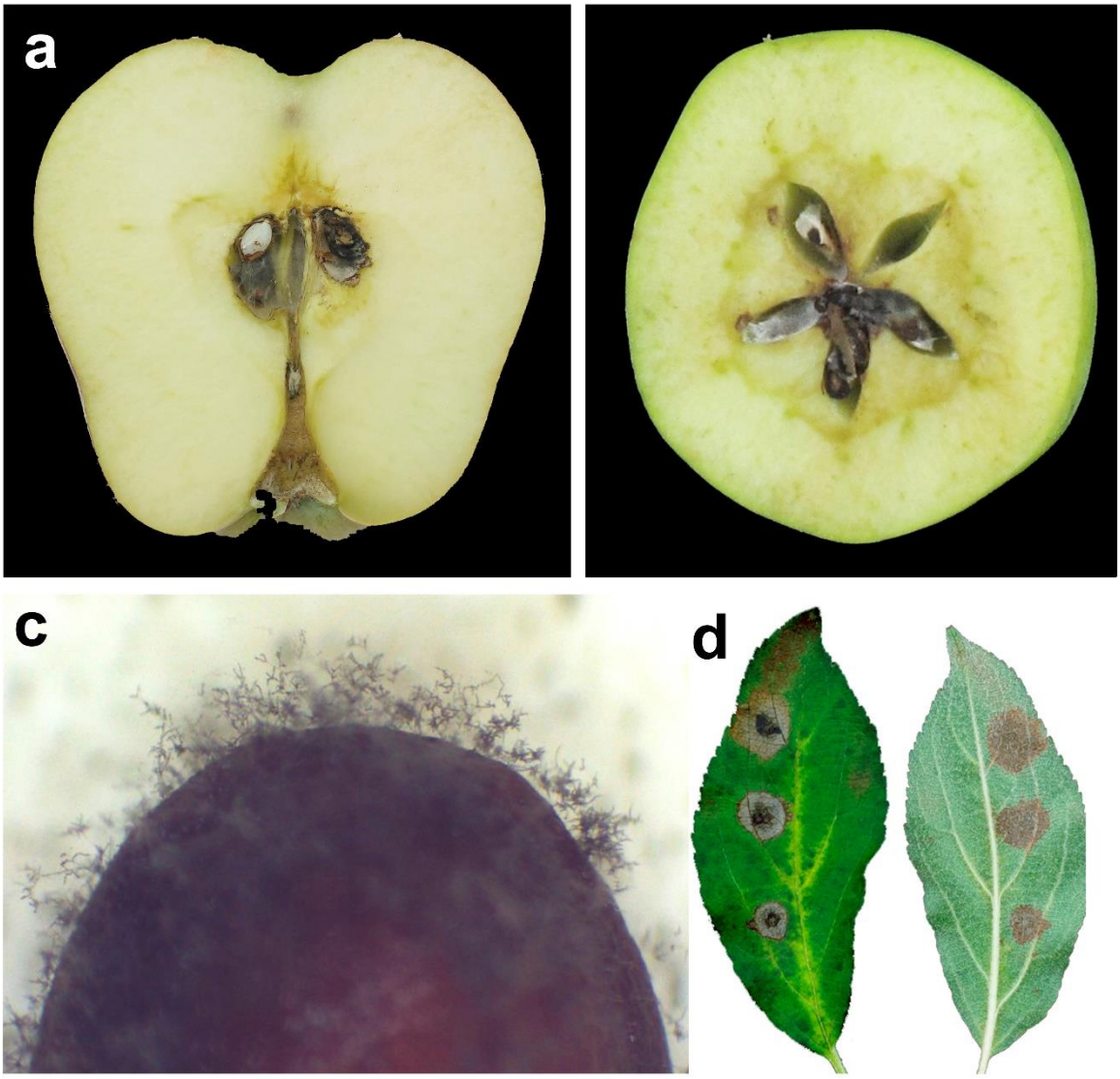


Fig. 2. Symptoms and pathogenicity of moldy core of 'Red Chief' apples caused by *Alternaria tenuissima*. **a**, naturally infected fruit. **b**, artificially inoculated immature fruit. **c**, colonization and sporulation of *A. tenuissima* on the apple seed surface. **d**, necrotic lesions on apple leaves inoculated with conidia of *A. tenuissima*.

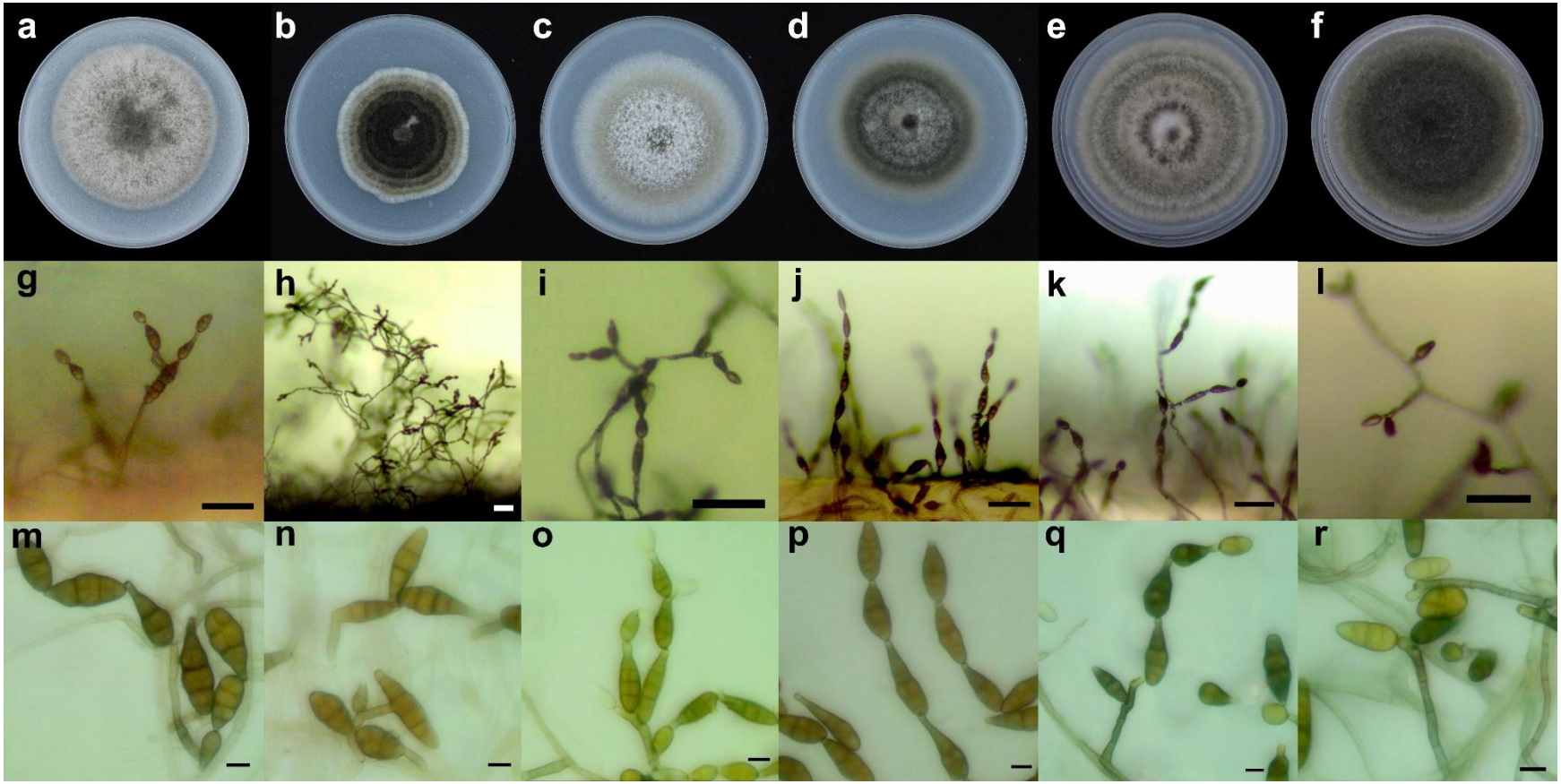
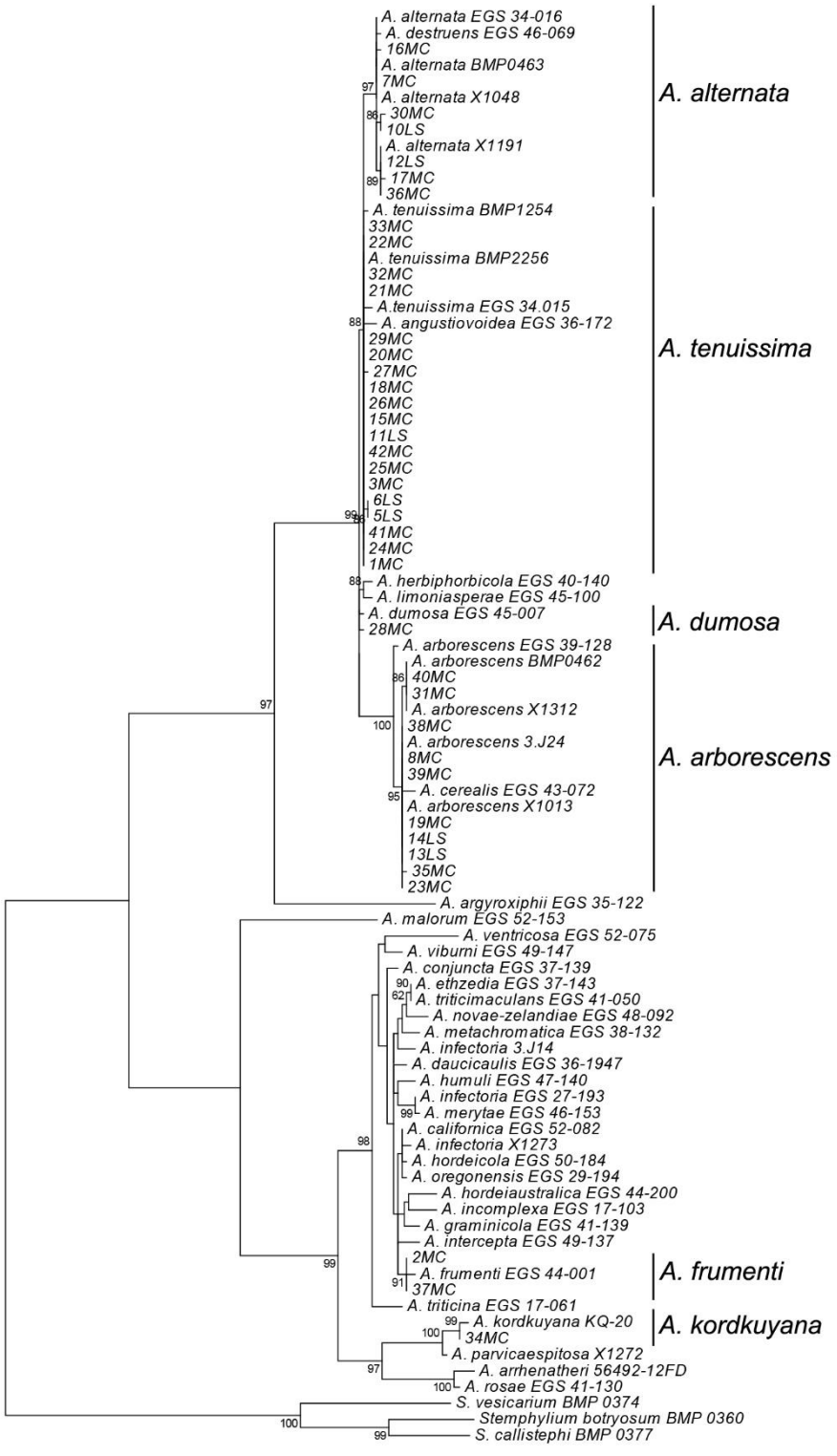


Fig. 3. Morphological characteristics of species of *Alternaria* isolated from apple moldy core. **a** to **f**, Colony morphology on acidified potato dextrose agar plates incubated for 7 days at 22°C 8 h/ 16 h light/ dark. **g** to **r**, sporulation pattern on potato carrot agar plates incubated for 7 days at 22°C 8 h/ 16 h light/ dark. **a**, **g**, and **m**, *A. alternata*. **b**, **h**, and **n**, *A. arborescens*. **c**, **i**, and **o**, *A. frumenti*. **d**, **j** and **p**, *A. tenuissima*. **e**, **k** and **q**, *A. dumosa*. **f**, **l** and **r**, *A. kordkuyana*. Scale bars: g-l = 50 µm, m-r = 10 µm.



1

2 **Fig. 4.** Phylogenetic tree obtained from maximum parsimony analysis of the plasma

3 membrane ATPase gene from sequences of *Alternaria* species from Chilean apples and

from sequences of ex-types in GenBank. The consensus tree inferred from the two most parsimonious trees and bootstrap values are shown. The tree was rooted with *Stemphylium botryosum, S. callistephi* and *S. vesicarium.* Tree length = 567, consistency index = 0.747, retention index = 0.953 and rescaled consistency index = 0.713. Numbers and MC or LS are *Alternaria* isolates from apples in Chile; other codes are isolates from GenBank.

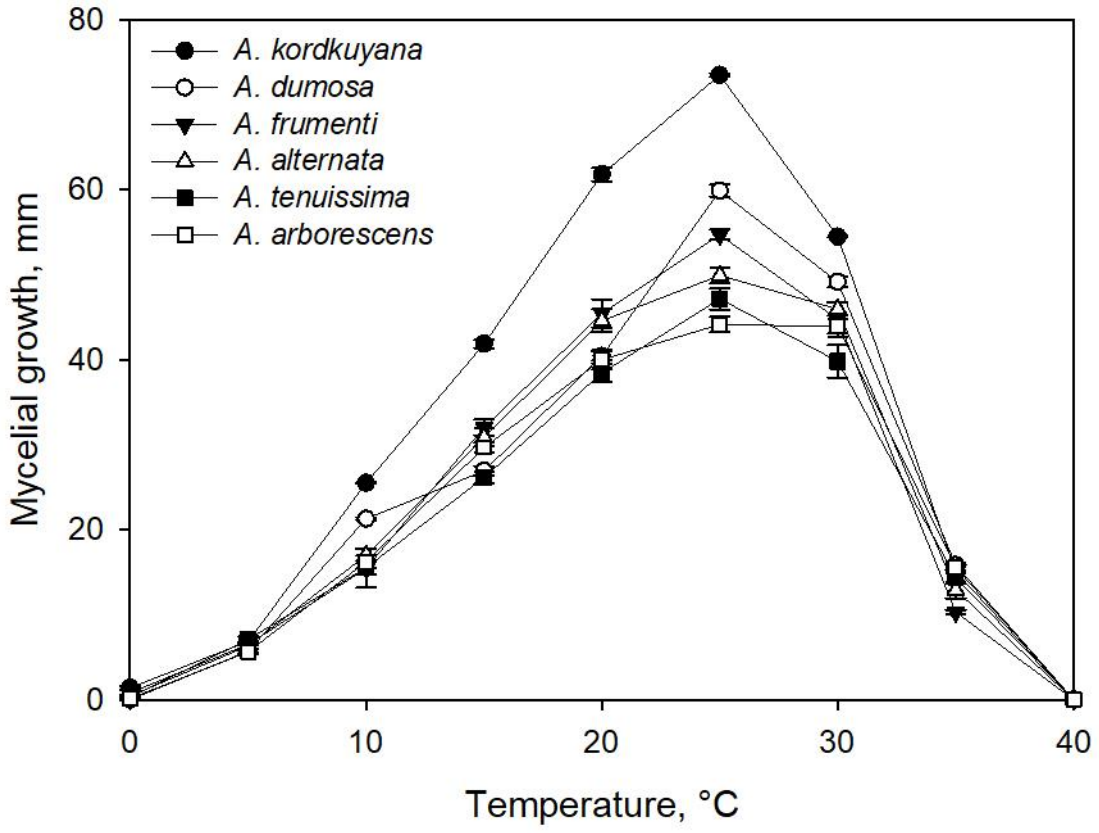


Fig. 5. Effect of temperature on the mean radial mycelial growth of isolates of *Alternaria* spp. determined after 6 days of incubation on acidified potato dextrose in the dark. Vertical bars = standard errors.

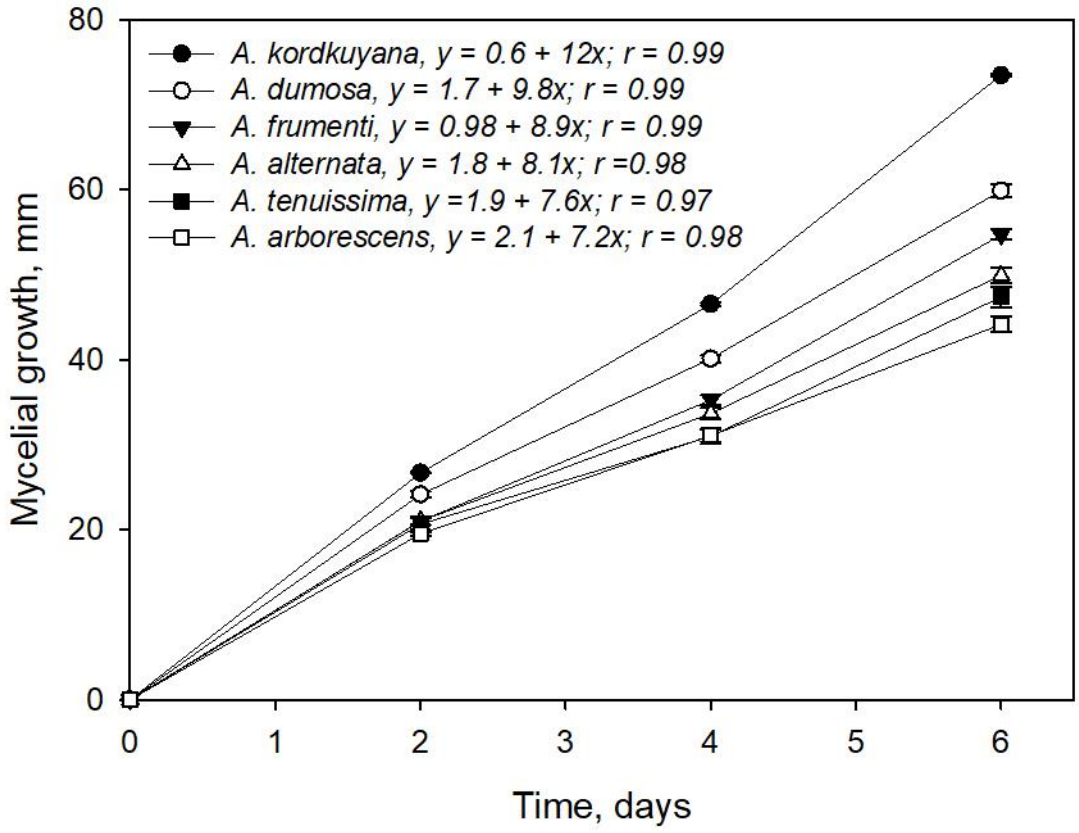


Fig. 6. Mycelial growth rate of isolates *Alternaria* spp. estimated on acidified potato dextrose in the dark at 25°C. Vertical bars = standard errors.

CHAPTER IV

Alternaria spp. on apparently healthy apples as a potential inoculum source for moldy core development and the effect of resistant and susceptible apple cultivars

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ABSTRACT

Apple (*Malus domestica* Borkh) is a major fruit crop in Chile. Among apple diseases, moldy core (MC) in the fruit have a high prevalence (16 to 46%) in susceptible cultivars. Apparently healthy flowers and fruit were sampled at six growth stages, from the pink bud to the mature fruit, from the susceptible 'Oregon Spur' and from the resistant 'Granny Smith' apples during two growing seasons. At least seven fungal genera were detected colonizing flowers and the fruit of both cultivars. Small-spored *Alternaria* spp. were the primary species identified throughout all the growing stages. Independently of the growth stages, sepals, stamens and carpels of flowers were similarly colonized by *Alternaria* spp. in the susceptible and resistant apple cultivars. However, in fruits of 4-6 cm in diameter, big differences in the frequency of *Alternaria* spp. in fruit carpels were observed between susceptible and resistant apples, with 55% and 1%, respectively. At least five *Alternaria* spp. were identified at the species level using plasma membrane ATPase, namely, *A. alternata*, *A. arborescens*, *A. limoniasperae*, *A. tenuissima* in sect *Alternaria*, *A. kordkuyana* in sect. *Pseudoalternaria* and *Alternaria* sp. in sect. *Infectoriae*. All *Alternaria* isolates, from sepals, stamens and carpels, from apparently healthy flowers and fruits were pathogenic, producing MC symptoms in apples. These results suggest that apparently healthy flowers and fruits may act as an important inoculum source of *Alternaria* spp. for MC infections and also suggest that control strategies

against MC using fungicide applications should be carried at flowering, from pink bud onwards.

Apple (*Malus domestica* Borkh) is commercially important crop, being the second most planted fruit crop tree, at present with 35,937 ha, representing 11% of the total fruit crops in Chile.

Among apple diseases, moldy core disease is an economically important disease that occurs primary in open sinus apple cultivars such us 'Oregon Spur',' Red Chief' and 'Scarlet'. The symptoms are characterized by the presence of a gray to dark olive green cottony mycelium that grows in the carpels and over the apple seeds. Prevalences from 16 to 46% were estimated at harvest in the 2015-2017 growing seasons in Chile. Moldy core is more difficult to find in close sinus cultivars such as 'Granny Smith' and 'Golden Delicious' (Elfar et al. 2018b; Niem et al. 2007; Serdani et al. 1998).

At least six small-spored *Alternaria* spp. have been associated with moldy core in Chile, namely, *A. alternata*, *A. arborescens*, *A. dumosa*, *A. frumenti*, *A. kordkuyana* and *A. tenuissima*, with the latter species being the most frequently isolated species (Elfar, et al. 2018b).

Serdani et al. (1998) studied the endophytic fungi associated with the core rot of 'Top Red' apples in South Africa, identifying more than 19 fungal taxa. However, *Alternaria* spp. were the most common (over 50%) fungi present as endophytes at bud development stages early in the season. Similarly, Ellis and Barrat (1983) found 14 genera of fungi associated with the core region of Delicious apples, with *Alternaria* spp. being the most commonly isolated fungi. *Alternaria* spp. colonized flowers and young fruits and were detected in flowers briefly after bloom.

The aims of this research were (i) to identify the species of *Alternaria* and other filamentous fungi associated with apparently healthy flowers and fruits of apples, (ii) to study

89 the ability of *Alternaria* spp. to colonize carpels in apple fruits, and (iii) to study the population
90 dynamics of species of *Alternaria* in the flowers and fruits of susceptible and resistant apples.
91
92 **MATERIALS AND METHODS**
93 **Sampling location and fungal isolation.** Flowers and fruits were randomly obtained
94 from the moldy core susceptible ('Oregon Spur') and resistant ('Granny Smith') apple
95 cultivars in the 2015-2016 and 2016-2017 growing seasons. Samplings were conducted
96 from mid-September (spring) to mid-February (summer), in a commercial apple orchard in
97 Graneros (34°4'S, 70°43'W). Flower samples (n=100) were collected randomly at the pink
98 bud, full bloom, and petal fall growing stages. Fruit samplings were started with fruits of 1 to
99 2 cm in diameter (16-23 days after full bloom, DAFB) until maturity (11% total soluble solids,
100 TSS, 132 DAFB). Flowers and fruits were sampled from at least four trees per row in four
101 rows. Samples were transported to the laboratory in an ice chest and were immediately
102 processed.
103 Flowers and fruits were surface disinfected in 0.25% NaOCl for 1.5 min and then rinsed
104 in sterile distilled water for 1 min before sectioning them longitudinally and separating 100
105 stamens, 100 sepals (fresh stamens and fresh sepals were collected at the flower stages,
106 and senescent stamens and senescent sepals attached to the calyx end were collected at
107 fruit stages) and 100 carpels, which were placed on potato dextrose agar (PDA) acidified
108 with 92% lactic acid at 0.5 ml l^{-1} (APDA) plus a colony growth restrictor and 0.1% Igepal CO-
109 630 (Sigma-Aldrich) (Elfar et al., 2013). The plates were incubated at 20°C for 10 days prior
110 to determination of the total number of *Alternaria* spp. and other filamentous fungal colonies
111 under a stereoscopic microscope.
112 Fungal colonies were tentatively identified by their colony (color, texture) and conidial
113 morphology and compared with genera descriptions (MycoBank Database,
114 www.mycobank.org). A total of 252 *Alternaria* colonies, characterized the presence of gray

115 to dark olive green colonies and single or chains of ovoids to obclavate dark conidia, were
116 sub-cultured on APDA. Pure cultures were obtained by a single hyphal tip transfer to fresh
117 APDA. Cultures were kept in Eppendorf tubes at 5°C in 10% sterile glycerol.

118 **Identification of *Alternaria* isolates.** A total of 27 *Alternaria* isolates obtained from
119 flowers and fruit samples were identified at the species level using plasma membrane
120 ATPase (*ATPase*). Based on previous results, the *ATPase* gene was highly informative and
121 allowed discrimination among small-spored *Alternaria* species associated with the moldy
122 core of apples (Elfar et al., 2018b). DNA was extracted from 7 to 10 day-old cultures on
123 APDA incubated at 20-22°C. Mycelia were carefully separated from agar medium with the
124 aid of a sterile scalpel and ground in liquid nitrogen in a porcelain mortar. The genomic DNA
125 was extracted using a DNA extraction kit (Wizard Genomic DNA Purification Kit, Promega,
126 Madison, WI). The DNA yield was estimated by gel electrophoresis and GelRed (Biotium
127 Inc., CA) staining and visualized by UV transillumination at ʎ = 320 nm.

128 The *ATPase* gene was amplified using the primer pair ATPDF1/ATPDR1 (Lawrence et
129 al. 2013). A polymerase chain reaction (PCR) was conducted in a thermal cycler (Veriti 96-
130 Well, Applied Biosystems, CA) in a final volume of 25 µl containing 5 µl of 10x PCR buffer,
131 1 µl of 25 mM $MgCl_2$, 0.2 µl of 10 mM dNTPs, 1 µl of a 15 mM solution of each primer, 0.13
132 µl containing 5 units of *Taq* DNA polymerase (GoTaq Flexi DNA Polymerase, Promega) and
133 2 µl of template DNA. The amplification protocol included preheating for 3 min at 95°C,
134 followed by 35 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 60°C for plasma
135 membrane ATPase and extension at 72°C for 60 s, with a final extension for 7 min at 72°C.

136 The PCR products were separated on 2% agarose gels in 1.0x Tris-acetate-EDTA (TAE)
137 buffer and stained with GelRed, and the DNA fragments were visualized by UV
138 transillumination at ʎ = 320 nm.

139 The PCR-amplified products were purified and sequenced from both directions by
140 Macrogen Inc., South Korea. The sequences were edited using Proseq v.2.91 (Filatov,

2002) and aligned using ClustalX v.2.1 (Larkin et al., 2007). A BLAST search analysis was performed against reference sequences in the GenBank (www.ncbi.nlm.nih.gov) database.

A phylogenic analysis was performed for a maximum parsimony (MP) analysis with the aid of MEGA v.7.0 (Kumar et al., 2016). Gaps were treated as missing data. The MP tree was obtained using the Tree-Bisection-Reconnection (TBR) branch-swapping algorithm and 1,000 random sequence additions. Branch stability was estimated with a bootstrap analysis of 1,000 replicates. The alignment included sequences from the 27 *Alternaria* isolates obtained from apples and eight *Alternaria* isolates obtained from leaf spot of apple in Chile (Table 1). Based on the BlASTn results, *Alternaria* sequences, including ex-types in sections of *Alternaria*, *Infectoriae* and *Pseudoalternaria,* were selected for phylogenetic analyses. Sequences of *Stemphylium botryosum*, *S. callistephi* and *S. vesicarium* were included as the outgroup (Table 2).

Pathogenicity tests. Inoculum suspensions were prepared with 10 to 14 day-old cultures on 0.05 x PDA. Dishes were flooded with approximately 20 ml of 0.05% Tween 80, and the surface of the medium was scraped with a sterile scalpel. The resulted suspension was filtered through two layers of gauze, and the final concentration was adjusted to 1×10^6 conidia/ml using a haemocytometer. Isolates of *A. alternata* (n = 3 isolates), *A. arborescens* (n = 3 isolates), *A. limoniasperae* (n = 1 isolate), *A. kordkuyana* (n = 2 isolates), *A. tenuissima* (n = 3 isolates) and *Alternaria* sp. (n = 1 isolate) were evaluated for pathogenicity.

Mature fruits (13.2% TSS, code 6 of starch index 1–9) of 'Royal Gala' apples (n = 5) were surface disinfected in 75% ethanol for 5 min and air-dried before sectioning longitudinally. One-half of each fruit was inoculated with 60 µl of conidial suspensions placed in the carpels and covered with the other half of the fruit to avoid rapid dehydration. Fruits were incubated at 20°C in a humid chamber for 10 days prior to determining the proportion of affected moldy core carpels. An equal number of fruits treated with sterile water served as the controls. Re-

166 isolations were performed on APDA. This experiment was repeated on mature (12.9% TSS,
167 code 6 of starch index 1–9) 'Fuji' apples.

168 **Design and statistical analysis.** The effect of growth stages and apple cultivars on the
169 isolation frequency of *Alternaria* spp. from carpels was studied following a 2 × 6 (apple
170 cultivars × growth stages) factorial design with four replicates of 25 flowers or 25 fruits as
171 experimental units. The pathogenicity was performed according to a 2 × 13 (apple cultivar ×
172 isolates) factorial design, with five replicates and one fruit as the experimental unit. Data
173 were subjected to a two-way analysis of variance, and the means were separated with
174 Tukey's test (P = 0.05) with the aid of SigmaStat 3.1 software (Systat Software Inc., San
175 José, CA).

176

177 **RESULTS**

178 **Sampling location and fungal isolation.** Colony morphology, conidiophore and conidial
179 characteristics were used to identify seven fungal genera, namely, *Alternaria*, *Botrytis*,
180 *Cladosporium*, *Epicoccum*, *Fusarium*, *Penicillium*, and *Stemphylium*. These species were
181 observed in the flowers and fruit of 'Granny Smith' and 'Oregon Spur' apple cultivars (Table
182 3). However, small-spored *Alternaria* spp. were the most frequently and consistently isolated
183 fungi in both apple cultivars.

184 Colonies of *Alternaria* were light gray to dark olive green colonies and single or chains of
185 ovoids to obclavate muriform dark conidia. *Botrytis* colonies were white to gray with
186 abundant erect light brown conidiophores branched at the apical region with oval hyaline
187 non-septated conidia. *Cladosporium* colonies were olive green with a velvet appearance,
188 abundant 0-3 septated, lemon shaped, and conidia with a prominent scar. *Epicoccum*
189 colonies were yellow to orange to red, woolly with dark sporodochia and solitary, muriform,
190 brown, globular conidia. *Fusarium* colonies were white to yellow or pink, with masses of
191 macro and micro conidia in sporodochia. *Penicillium* colonies were white with green to blue

192 sporulation on penicillated conidiophores with spherical to oval non-septate hyaline conidia.
193 *Stemphylium* colonies were dark olive green with dark, muriform, solitary conidia ellipsoidal
194 to obclavate.

195 At the flower stages, the frequency of isolation of *Alternaria* spp. was relatively similar
196 between apple cultivars, varying from 12 to 27% and 8 to 23% in fresh stamens, 27.5 to
197 45% and 30.0 to 47% in alive sepals and 38.5 to 44.5% and 36.5 to 44.0% in carpels of
198 resistant and susceptible apple cultivars respectively (Table 3).

199 At the fruit stages, the frequency of isolation of *Alternaria* spp. was relatively similar
200 between apple cultivars in senescent stamens and senescent sepals that remained attached
201 to the fruits, varying from 23.5 to 43.5% and 14 to 26% in senescent stamens, and 28.5 to
202 44.5% and 31.0 to 39.5% in senescent sepals (Table 3). However, big differences in the
203 frequency of isolation of *Alternaria* spp. in the carpels of immature fruits of 4-5 cm in diameter
204 and mature fruits were obtained between 'Oregon Spur' and 'Granny Smith' (Fig. 1). A
205 significant effect of cultivars ($P < 0.001$) and growth stages ($P < 0.001$) was obtained in the
206 isolation frequency of *Alternaria* spp. from apple carpels in the 2015-2016 and 2016-2017
207 growing seasons. The interaction between cultivars and growth stages was also significant
208 ($P < 0.001$) (Table 4). Interestingly, carpel isolations significantly ($P \leq 0.05$) varied from 46
209 to 56% in susceptible 'Oregon Spur' apples and to 1 to 3% in resistant 'Granny Smith' apples
210 (Table 4).

211 **Molecular identification of *Alternaria* isolates.** DNA sequences of *ATPase* of the
212 *Alternaria* isolates from apples ranged from 1188 to 1203 bp. Maximum parsimony analysis
213 produced a consensus tree (Fig. 2), which was inferred from the 23 most parsimonious trees
214 (tree length [TL] = 514, consistency index [CI] = 0.802, retention index [RI] = 0.969, and
215 rescaled consistency index [RC] = 0.776). The phylogenetic analysis for the ATPase gene
216 showed that the 27 isolates from apples were identified in three small-spored sections. In
217 section *Alternaria*, seven isolates were identified as *A. alternata*, clustered with *A. alternata*

ex-type (72% bootstrap); eight isolates were identified as *A. tenuissima* clustered in the same group with *A. tenuissima* ex-type (80% bootstrap); one isolate was identified as *A. limoniasperae*, clustered with the *A. limoniasperae* ex-type (53% bootstrap); and eight isolates were identified as *A. arborescens*, clustered with the *A. arborescens* ex-type (99% bootstrap). In section *Pseudoalternaria*, two isolates were *A. kordkuyana* clustered with the ex-type by a high bootstrap value of 99%. One unidentified isolate was clustered in section *Infectoriae* (Fig. 2).

Pathogenicity test. In mature 'Royal Gala' and 'Fuji' apples, all *Alternaria* isolates developed a moldy core, characterized by the development of light gray to dark olive green cottony mycelium with or without sporulation restricted to the carpels. The majority of the isolates produced a brown discoloration of the carpels walls, but none of the isolates were able to penetrate the carpel and rot the mesocarp. The effect of *Alternaria* isolates was statistically significant ($P < 0.001$), but a non-significant ($P = 0.238$) effect was obtained for apple cultivars on the percentage of the moldy core among the isolates. The interaction cultivar × isolate was non-significant ($P = 0.992$). For each *Alternaria* spp., differences in virulence were dependent on the isolate. The most virulent isolate was *A. alternata* isolate C.FL10. (Table 5). Re-isolations were accomplished for 100% of the inoculated fruits. Non-inoculated fruits remained symptomless.

DISCUSSION

After analysing 2400 apparently healthy flowers and fruits of 'Granny Smith' and 'Oregon Spur' apples in two growing seasons, the mycoflora associated with the flowers and fruits were predominantly composed of species of *Alternaria*. At least five *Alternaria* spp. were identified, namely, *A. alternata*, *A. arborescens*, *A. limoniasperae*, *A. tenuissima* in sect *Alternaria*, *A. kordkuyana* in sect. *Pseudoalternaria* and *Alternaria* sp. in sect. *Infectoriae*. These, results showed that *Alternaria* spp. were well established in apple orchards colonizing apparently healthy apple flowers and fruits. Apparently, the environmental

244 conditions, particularly temperature and humidity, that prevailed in the orchard allowed
245 colonization and establishment of *Alternaria* spp. in the apple orchard.

246 In this and previous studies, it was demonstrated that the same species of *Alternaria*
247 were the most prevalent fungi associated with apple moldy core in Chile (Elfar et al. 2018).
248 Similarly, *Alternaria* spp., has been reported as the most frequent fungi associated with
249 moldy core in Ohio (Ellis and Barrat 1983), core rot in South Africa (Serdani et al. 1998),
250 fruit and leaf spot in Australia (Harteveld et al. 2014) and leaf spot in Chile (Elfar et al.
251 2018a). Interestingly, no evidence for the presence of *A. mali* was found in this and in
252 previous studies in Chile (Elfar et al. 2018a, b). At present, *A. mali* has only been isolated in
253 the northern hemisphere and Australia (Harteveld et al. 2013, Sawamura, 2014). Other
254 filamentous fungi such as *Botrytis*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Penicillium*, and
255 *Stemphilium* were also identified. However, the frequency of isolation of these fungi was
256 below the frequency of isolation of *Alternaria* spp (Ellis and Barrat 1983, Elfar et al., 2018,
257 Gao et al 2013).

258 Independent of the growth stages, fresh sepals, fresh stamens and carpels of flowers
259 were similarly colonized by *Alternaria* spp. in the susceptible ‘Oregon Spur’ and in the
260 resistant ‘Granny Smith’ apples. Similar results were obtained previously in the susceptible
261 ‘Red Delicious’ and in the resistant ‘Golden Delicious’ apples at flowering stages (Niem et
262 al. 2007).

263 In agreement with Niem et al. (2007), similar frequencies of *Alternaria* spp. in the carpels
264 were detected in fruits of 1-2 cm in diameter between susceptible and resistant cultivars.
265 However, in the fruits of 4-6 cm in diameter, big differences in the frequency of *Alternaria*
266 spp. in fruit carpels were observed between susceptible ‘Oregon Spur’ and resistant ‘Granny
267 Smith’ at 55% and 1%, respectively. At the time of sampling, the calycine tubes were well
268 developed in both the fruits of 4-6 cm in diameter and mature fruits in apple cultivars.
269 However, in the susceptible ‘Oregon Spur’ apples, the calycine tube remained open and

closed in the resistant ‘Granny Smith’ apples, preventing the invasion and colonization of the carpels by the superficial *Alternaria* inoculum.

Furthermore, previous studies conducted in resistant ‘Golden Delicious’ apples demonstrated that carpel sections near the calyx end had high levels of colonization by *A. alternata*, and the carpel section distant from the calix end was not colonized by *A. alternata* (Niem et al. 2007). In contrast, ‘Red Delicious’ apples, which are considered susceptible carpel sections independent of the distance from the calyx end, were profusely colonized by *A. alternata*. This suggests that carpel samples of very small fruits (1-2 cm in diameter) may be contaminated with external *Alternaria* inoculum, explaining the lack of differences between susceptible and resistant apples obtained in this study in 1-2 cm diameter fruit.

Alternaria spp. isolates from sepals, stamens and carpels from apparently healthy flowers and fruits were pathogenic and produced moldy core in apples, suggesting that apparently healthy flowers and fruits may act as an important inoculum source of *Alternaria* spp. for moldy core infections. Important differences in virulence were associated with specific isolates in each *Alternaria* spp. However, the most virulent isolate was found among *A. alternata* species and the less virulent isolates were among *A. tenuissima* species.

In conclusion, this study demonstrated that many small-spored species of *Alternaria* were well distributed on flowers early in the season, colonizing fresh sepals, fresh stamens and carpels. The senescent tissues, sepals and stamens that remain attached to the calyx end on the apple fruit can also serve as potential inoculum sources for the moldy core. These results also suggest that control strategies against moldy core using fungicide applications should be carried out at flowering, from the pink bud onwards. In addition, *A. limoniasperae* was found for the first time on apples in the world.

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340

341 **Table 1.** Source of isolates of *Alternaria* spp. from apples obtained in a commercial
342 orchard in Central Chile and GenBank accession numbers for sequences of plasma
343 membrane ATPase of *Alternaria* isolates studied

Species	Sample source	Flower/fruit part	Gen Bank no
Alternaria alternata			
Se.FL5	Flower	Sepal	MJ226516
C.FL8	Flower	Carpel	MJ226517
C.FL10 [z]	Flower	Carpel	MJ226518
S.FL12 [z]	Flower	Stamen	MJ226519
Se.FR16 [z]	Fruit	Sepal	MJ226520
C.FR52	Fruit	Carpel	MJ226521
C.FR53	Fruit	Carpel	MJ226522
A. arborescens			
C.FL7	Flower	Carpel	MJ226523
C.FL11[z]	Flower	Carpel	MJ226524
S.FL27[z]	Flower	Stamen	MJ226525
S.FR31	Fruit	Stamen	MJ226526
Se.FR37 [z]	Fruit	Sepal	MJ226527
S.FR40	Fruit	Stamen	MJ226528
Se.FL44	Flower	Sepal	MJ226529
C.FR54	Fruit	Carpel	MJ226530
A. limoniasperae			
C.FR13 [z]	Fruit	Carpel	MJ226531
A. kordkuyana			
S.FL43 [z]	Flower	Stamen	MJ226532
C.FL47 [z]	Flower	Carpel	MJ226533
Alternaria sp.			
C.FR14 [z]	Fruit	Carpel	MJ226534
A. tenuissima			
Se.FL1	Flower	Sepal	MJ226535
S.FL2	Flower	Stamen	MJ226536
C.FL6 [z]	Flower	Carpel	MJ226537
Se.FL9 [z]	Flower	Sepal	MJ226538
S.FR15 [z]	Fruit	Stamen	MJ226539
C.FR55	Fruit	Carpel	MJ226540
C.FR56	Fruit	Carpel	MJ226541
C.FR57	Fruit	Carpel	MJ226542

344 [z] Isolates used for morphological characterization and pathogenicity tests

345

346 **Table 2.** Accession numbers for reference sequences of plasma
347 membrane ATPase of *Alternaria* isolates in GenBank used for
348 phylogenetic analysis in this study

Species	Isolates[z]	GenBank no
Alternaria alternata	EGS 34-016[y]	JQ671874
A. alternata	X1191	KJ908234
A. alternata	X1048	KJ908230
A. alternata	BMP0463	JQ811981
A. alternata	16MC	MG740628
A. alternata	17MC	MG740629
A. alternata	30MC	MG740630
A. alternata	36MC	MG740631
A. alternata	10LS	MG740618
A. angustiovoidea	EGS 36-172[y]	JQ671869
A. arborescens	EGS 39-128[y]	JQ671880
A. arborescens	BMP0462	JQ811990
A. arborescens	3.J24	KJ908244
A. arborescens	X1312	KJ908242
A. arborescens	8MC	MG740632
A. arborescens	31MC	MG740634
A. arborescens	35MC	MG740636
A. arborescens	40MC	MG740635
A. arborescens	14LS	MG740620
A. arrhenatheri	56492-12FD	JQ693603
A. californica	EGS 52-082	JQ671813
A. cerialis	EGS 43-072	JQ671883
A. conjuncta	EGS 37-139	JQ671824
A. daucicaulis	EGS 36-1947	JQ671822
A. destruens	EGS 46-069[y]	JQ671873
A. dumosa	EGS 45-007[y]	JQ671877
A. dumosa	28MC	MG740640
A. ethzedia	EGS 37-143[y]	JQ671805
A. frumenti	EGS 44-001[y]	JQ671823
A. frumenti	2MC	MG740641
A. frumenti	37MC	MG740642
A. graminicola	EGS 41-139[y]	JQ671819
A. herbiphorbicola	EGS 40-140[y]	JQ671888
A. hordeiaustralica	EGS 44-200[y]	JQ671811
A. hordeicola	EGS 50-184[y]	JQ671812
A. humuli	EGS 47-140[y]	JQ671821

A. incomplexa	EGS 17-103[y]	JQ671815
A. infectoria	EGS 27-193[y]	JQ671804
A. infectoria	3.J14	KJ908219
A. infectoria	X1273	KJ908218
A. infectoria	9LS	MG740622
A. intercepta	EGS 49-137[y]	JQ671826
A. kordkuyana	KQ-20[y]	MF033860
A. kordkuyana	34MC	MG740643
A. limoniasperae	EGS 45-100[y]	JQ671879
A. merytae	EGS 46-153[y]	JQ671820
A. metachromatica	EGS 38-132[y]	JQ671809
A. novae-zelandiae	EGS 48-092[y]	JQ671825
A. oregonensis	EGS 29-194[y]	JQ671827
A. parvicaespitosa	X1272[y]	KJ908217
A. rosae	EGS 41-130[y]	JQ671803
A. tenuissima	EGS 34.015[y]	JQ811989
A. tenuissima	BMP2256	JQ811983
A. tenuissima	BMP1254	JQ811988
A. tenuissima	11LS	MG740626
A. tenuissima	15MC	MG740646
A. tenuissima	18MC	MG740647
A. tenuissima	27MC	MG740654
A. tenuissima	42MC	MG740659
A. triticimaculans	EGS 41-050[y]	JQ671806
A. triticina	EGS 17-061[y]	JQ671808
A. ventricosa	EGS 52-075[y]	JQ671818
A. viburni	EGS 49-147[y]	JQ671816
Stemphylium botryosum	BMP 0360	JQ671767
S. callistephi	BMP 0377	JQ671769
S. vesicarium	BMP 0374	JQ671768

[y] ex-type

BMP: B.M. Pryor, Division of Plant Pathology, Department of Plant Sciences, EGS: E.G. Simmons, Mycological Services, Crawfordsville, IN 47933, 3.J: Pryor and Michailides, 2002, X: Zhu and Xiao, 2015, MC: Elfar et al. 2018b, LS: Elfar et al. 2018a.

Table 3. Frequency of isolation of *Alternaria* spp. and other filamentous fungi isolated from flowers of apples collected at the pink bud, full bloom and petal fall, and immature and mature fruit stages, in a commercial orchard in the Central Valley of Chile

	Isolate frequency[x], %													
	Alt	Cla	Epi	Pen	Bot	Ste	Others	Alt	Cla	Epi	Pen	Bot	Ste	Others
Flower stages	Oregon Spur							Granny Smith						
Pink bud														
Fresh stamens[z]	12.0	1.0	0.0	2.5	0.0	0.0	0.5	8.0	2.5	0.5	7.0	0.5	0.0	1.0
Fresh sepals[z]	27.5	1.5	16.5	3.5	2.5	0.0	3.0	30.0	5.0	19.0	6.0	1.5	0.0	3.5
Carpels	44.5	1.0	18.5	0.5	6.0	1.0	5.0	41.0	2.5	23.0	1.0	6.0	1.0	7.5
Mean	28,0	1,2	11,7	2,2	2,8	0,3	2,8	26,3	3,3	14,2	4,7	2,7	0,3	4,0
Full bloom														
Fresh stamens[z]	14.5	14.0	2.0	7.5	4.5	0.0	0.5	15.5	7.5	7.5	5.0	4.5	0.5	2.0
Fresh sepals[z]	45.0	12.5	12.5	3.0	2.5	1.0	7.0	47.0	6.5	16.0	1.0	1.0	1.5	9.0
Carpels	44.0	8.0	12.5	1.5	7.5	2.0	9.5	44.0	3.5	17.0	2.5	7.5	0.5	11.0
Mean	34,5	11,5	9,0	4,0	4,8	1,0	5,7	35,5	5,8	13,5	2,8	4,3	0,8	7,3
Petal fall														
Fresh stamens[z]	27.0	12.5	5.5	3.0	8.5	0.5	16.0	23.0	10.0	12.0	1.5	4.0	0.0	21.0
Fresh sepals[z]	37.0	7.0	13.0	2.5	10.0	0.5	14.0	38.5	9.0	13.5	1.5	6.5	0.0	18.0
Carpels	38.5	5.5	10.0	2.5	14.5	0.5	19.5	36.5	5.0	15.0	1.5	13.0	0.0	17.5
Mean	38,5	5,5	10	2,5	14,5	0,5	19,5	36,5	5	15	1,5	13	0	17,5
Immature fruits (<10% TSS[y])														
Fruit 1-2 cm														
Senescent stamens[z]	23.5	5.5	5.5	2.0	4.0	1.0	12.5	14.0	3.5	7.5	0.0	5.0	1.0	14.0
Senescent sepals[z]	28.5	12.5	5.0	3.0	5.0	0.0	14.0	35.0	7.0	11.0	1.0	9.5	4.0	20.5
Carpels	39.5	9.0	10.5	3.0	8.0	1.5	15.5	48.5	6.0	12.0	1.0	5.0	1.5	16.0
Mean	30,5	9,0	7,0	2,7	5,7	0,8	14,0	32,5	5,5	10,2	0,7	6,5	2,2	16,8
Fruit 4-5 cm														
Senescent stamens[z]	24.0	13.0	6.0	4.5	0.5	0.0	12.0	26.0	7.5	7.5	2.0	4.5	0.5	2.5
Senescent sepals[z]	35.0	15.5	4.0	3.5	0.5	2.5	13.5	39.5	12.0	9.0	0.0	4.5	0.5	2.5
Carpels	55.5	11.0	2.0	4.5	1.0	3.5	12.5	1.0	16.0	0.5	5.0	1.5	0.0	1.0
Mean	38,2	13,2	4,0	4,2	0,7	2,0	12,7	22,2	11,8	5,7	2,3	3,5	0,3	2,0
Mature fruit (>10% TSS[y])														
Senescent stamens[z]	43.5	10.5	12.5	20.0	1.5	1.0	5.0	25.0	13.0	6.5	10.0	2.5	0.0	2.5
Senescent sepals[z]	44.5	6.5	7.5	21.5	2.0	0.0	9.0	31.0	13.0	9.0	8.5	1.5	0.0	1.5
Carpels	49.0	9.0	2.0	14.0	0.0	6.0	15.5	2.0	34.0	0.0	9.5	0.0	0.0	1.5
Mean	45,7	8,7	7,3	18,5	1,2	2,3	9,8	19,3	20,0	5,2	9,3	1,3	0,0	1,8

[x] Percentages are the mean of the two growing seasons' data, each with four replicates of 25 flowers or fruits. Alt= *Alternaria* spp., Bot= *Botrytis* spp., Cla= *Cladosporium* spp., Epi= *Epicoccum* spp., Fus= *Fusarium* spp., Pen= *Penicillium* spp., and Ste= *Stemphylium* spp.

[y] TSS= Total soluble solids.

[z] Fresh stamens and fresh sepals were collected at the flower stages, and senescent stamens and senescent sepals attached to the calyx end were collected at the fruit stages.

Table 4. The effect of the growth stages and apple cultivars on the isolation frequency of *Alternaria* spp. from carpels

Growth stages[y]	Frequency of *Alternaria* isolation, %[x]					
	Cultivars, 2015-2016			Cultivars, 2016-2017		
	Oregon Spur	Granny Smith	Mean	Oregon Spur	Granny Smith	Mean
PB	46 ab	40 a	43.0	43 abc	42 a	42.5
FB	59 a	47 a	53.0	29 c	41 a	35.0
PF	45 ab	50 a	47.5	32 bc	23 b	27.5
F_{1-2}	40 b	58 a	49.0	39 abc	39 a	39.0
F_{4-5}	55 ab	1 b	28.0	56 a	1 c	28.5
FM	46 ab	3 b	24.5	52 ab	1 c	26.5
Mean	48.5	33.2		41.8	24.5	
Analysis of variance						
	Df	*P*	SED[z]	df	*P*	SED[z]
Cultivars (C)	1	<0.001	0.052	1	<0.001	0.028
Growth stage (GS)	5	<0.001	0.030	5	<0.001	0.049
C × GS interaction	5	<0.001	0.074	5	<0.001	0.070

[x] 'Oregon Spur' and 'Granny Smith' are considered susceptible and resistant apple cultivars, respectively. Percentages are the mean of four replicates of 25 flowers or fruits. Means followed by the same letter in each column did not differ significantly according to Tukey's pairwise multiple comparison test ($P = 0.05$).
[y] PB= pink bud, FB= full bloom, PF= petals fall, F_{1-2}= fruits of 1-2 cm in diameter, F_{4-5}= fruits of 4-5 cm in diameter, FM= mature fruits.
[z] SED = standard error of the difference (standard error of the mean × √2). Data were arcsine √(x/100) transformed before the analysis, but non-transformed data are presented.

Table 5. Pathogenicity of *Alternaria* isolates studied on fruits of 'Gala' and 'Fuji' apples, by the percentage of moldy core developed after controlled inoculation

	Moldy core[x], %		
Alternaria isolates	'Royal Gala'	'Fuji'	Mean
A. alternata			
C.FL10	92.0	92.0	92 a
S.FL12	40.0	32.0	36 cd
Se.FR16	52.0	52.0	52 a-d
Mean	61.3	58.6	
A. arborescens			
C.FL11	74.0	68.0	71 abc
S.FL27	62.0	44.0	53 a-d
Se.FR37	50.0	34.0	42 cd
Mean	62.0	48.7	
A. limoniasperae			
C.FR13	92.0	82.0	87 ab
A. kordkuyana			
S.FL43	36.0	30.0	33 cd
C.FL47	50.0	50.0	50 bcd
Mean	43.0	40.0	
A. tenuissima			
C.FL6	42.0	58.0	50 bcd
Se.FL9	22.0	18.0	20 d
S.FR15	36.0	22.0	29 d
Mean	33.3	31.7	
Alternaria sp.			
C.FR14	28.0	22.0	25 d
Analysis of variance			
	df	*P*	SED
Cultivar (C)	1	0.238	0.047
Isolate (I)	12	<0.001	0.119
C ×I interaction	12	0.992	0.168

[x] Non-inoculated controls remained symptomless. These data were excluded from the statistical analysis. Means of five replicates followed by the same letter in each column did not differ significantly according to Tukey's test (P = 0.05). SED = standard error of the difference (standard error of the mean × $\sqrt{2}$)

Fruit carpels of five fruits were inoculated with 60 µl of conidial suspension (10^6 conidia/ml) per fruit. Incubated in humid chambers at 20°C for 10 days.

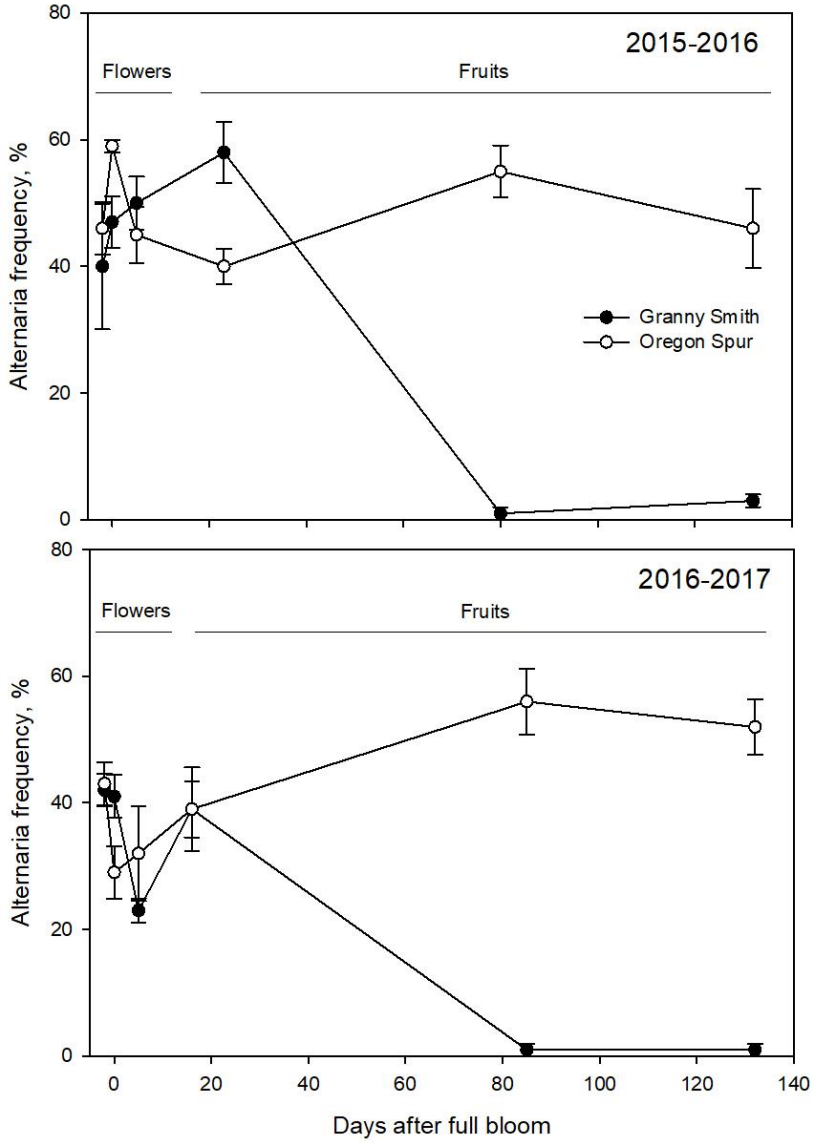

Fig. 1. *Alternaria* frequency in carpels of flowers and fruits of 'Oregon Spur' and 'Granny Smith' apples, collected between the pink bud and mature fruit.

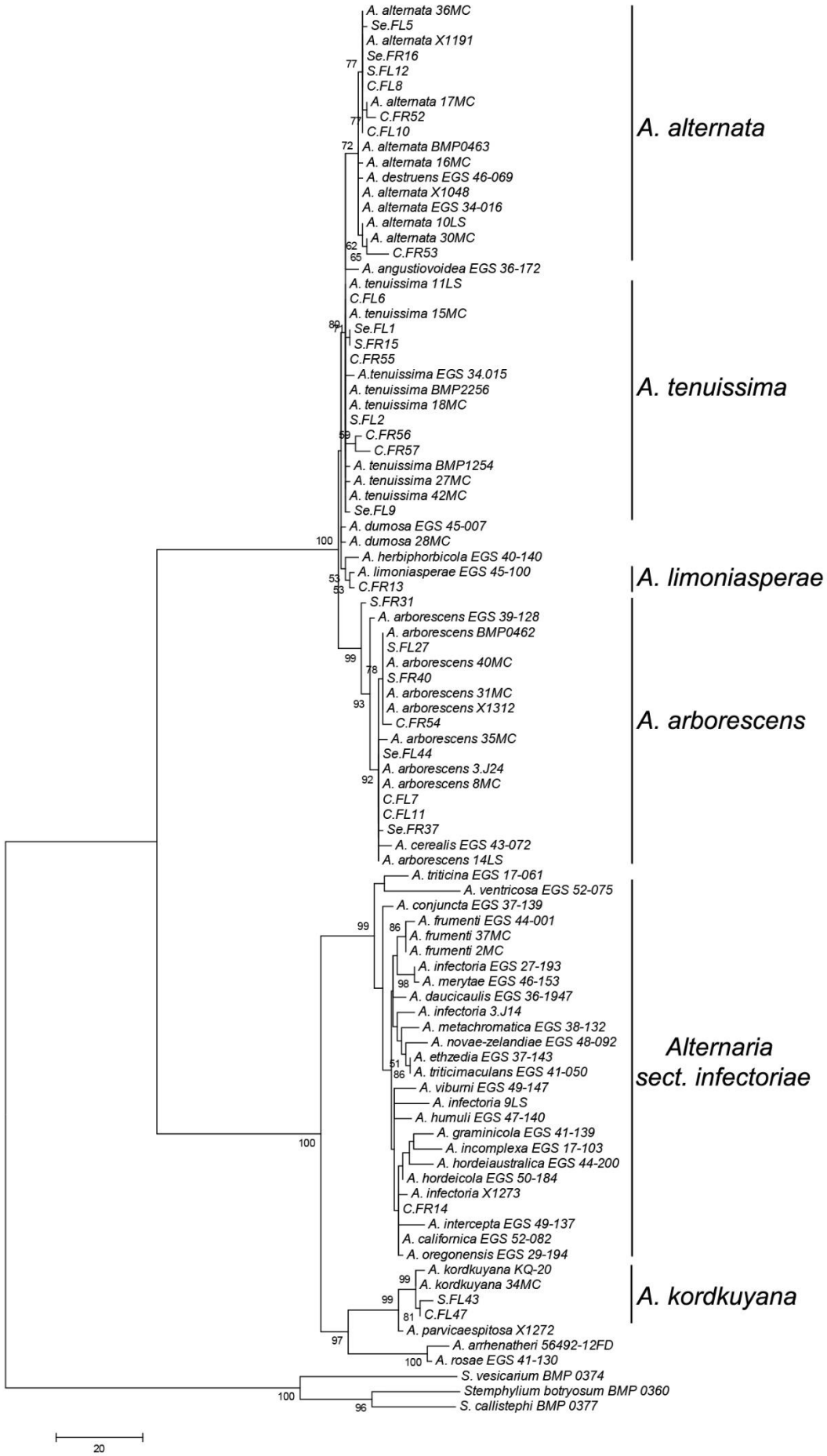
A. alternata 36MC
Se.FL5
A. alternata X1191
Se.FR16
S.FL12
C.FL8
A. alternata 17MC
C.FR52
C.FL10
A. alternata BMP0463
A. alternata 16MC
A. destruens EGS 46-069
A. alternata X1048
A. alternata EGS 34-016
A. alternata 10LS
A. alternata 30MC
C.FR53
A. alternata
A. angustiovoidea EGS 36-172
A. tenuissima 11LS
C.FL6
A. tenuissima 15MC
Se.FL1
S.FR15
C.FR55
A.tenuissima EGS 34.015
A. tenuissima BMP2256
A. tenuissima 18MC
S.FL2
C.FR56
C.FR57
A. tenuissima BMP1254
A. tenuissima 27MC
A. tenuissima 42MC
Se.FL9
A. tenuissima
A. dumosa EGS 45-007
A. dumosa 28MC
A. herbiphorbicola EGS 40-140
A. limoniasperae EGS 45-100
C.FR13
A. limoniasperae
S.FR31
A. arborescens EGS 39-128
A. arborescens BMP0462
S.FL27
A. arborescens 40MC
S.FR40
A. arborescens 31MC
A. arborescens X1312
C.FR54
A. arborescens 35MC
Se.FL44
A. arborescens 3.J24
A. arborescens 8MC
C.FL7
C.FL11
Se.FR37
A. cerealis EGS 43-072
A. arborescens 14LS
A. arborescens
A. triticina EGS 17-061
A. ventricosa EGS 52-075
A. conjuncta EGS 37-139
A. frumenti EGS 44-001
A. frumenti 37MC
A. frumenti 2MC
A. infectoria EGS 27-193
A. merytae EGS 46-153
A. daucicaulis EGS 36-1947
A. infectoria 3.J14
A. metachromatica EGS 38-132
A. novae-zelandiae EGS 48-092
A. ethzedia EGS 37-143
A. triticimaculans EGS 41-050
A. viburni EGS 49-147
A. infectoria 9LS
A. humuli EGS 47-140
A. graminicola EGS 41-139
A. incomplexa EGS 17-103
A. hordeiaustralica EGS 44-200
A. hordeicola EGS 50-184
A. infectoria X1273
C.FR14
A. intercepta EGS 49-137
A. californica EGS 52-082
A. oregonensis EGS 29-194
Alternaria sect. infectoriae
A. kordkuyana KQ-20
A. kordkuyana 34MC
S.FL43
C.FL47
A. parvicaespitosa X1272
A. kordkuyana
A. arrhenatheri 56492-12FD
A. rosae EGS 41-130
S. vesicarium BMP 0374
Stemphylium botryosum BMP 0360
S. callistephi BMP 0377
20

Fig. 2. Phylogenetic tree obtained from maximum parsimony analysis of the plasma membrane ATPase gene from sequences of *Alternaria* species from Chilean apples and from sequences of ex-types in GenBank. The consensus tree inferred from the 23 most parsimonious trees and bootstrap values are shown. The tree was rooted with *Stemphylium botryosum, S. callistephi* and *S. vesicarium.* Tree length = 514, consistency index = 0.802, retention index = 0.969 and rescaled consistency index = 0.776.

CHAPTER V

Sensitivity of *Alternaria* spp. to fungicides with diverse modes of action and control of moldy core of apples

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Sensitivity of *Alternaria* spp. to fungicides with diverse modes of action and control of moldy core of apples

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ABSTRACT

Moldy core is frequently found affecting apples in Chile. Five fungicides (difenoconazole, fludioxonil, penthiopyrad, pyrimethanil, and mancozeb) with different modes of action were evaluated in vitro for their efficacy in reducing mycelium growth and suppressing conidial germination. These same fungicides were tested in vivo against moldy core in detached apples inoculated with *A. alternata*, *A. arborescens* or *A. tenuissima*. All fungicides inhibit the mycelial growth and the germination of conidia. However, *Alternaria* isolates were very sensitive to fludioxonil, with EC_{50} values of 0.09 to 0.72 $\mu g \cdot ml^{-1}$ and 0.5 to 2.4 $\mu g \cdot ml^{-1}$, respectively for mycelium growth and conidial germination. Independently of the EC_{50} values, the five fungicides reduced moldy core on apple carpels from 41.7 to 95.0%, ~~obtained for~~ in the untreated controls, to 0.0 to 21.7%. Regardless of the *Alternaria* isolate, fludioxonil achieved a 100% control of moldy core. Therefore, fludioxonil represent an alternative against moldy core of apples in Chile. However, additional studies are necessary to demonstrate its effectiveness under field conditions.

Apple (*Malus domestica* Borkh) is a crop with economic importance in Chile, being the second most planted fruit tree in the country, with more than 37,500 ha. Currently, Chile is the largest producer and exporter of apples in the southern hemisphere and the tenth apple producing country in the world (FAOSTAT 2016).

Several fungal diseases affect apple in Chile, among which Alternaria leaf blotch (Elfar et al. 2018a) and moldy core are frequently found affecting apples. Moldy core is characterized by the presence of a gray to dark olive green cottony mycelium that grows in the carpels and over the apple seeds. It is considered a major disease, especially in cultivars with fruit sinus remaining open until harvest, such as 'Oregon Spur', 'Red Chief', and 'Scarlet' (Elfar et al. 2018b). Moldy core in Chile is mainly associated with A*. tenuissima*, *A. arborescens* and *A. tenuissima* (Elfar et al., 2018b)

Once, the carpel cavity is colonized, the fungi are protected and hardly have contact with the fungicides applied on the fruit. Moreover, a very favorable environment for the growth and multiplication of fungi is generated (Ellis and Barrat 1983). Research conducted with fungicides applications to the control of moldy core and core rot of apple in some cases are effective and in others are ineffective.

Reuveni et al. (2002), through field trials with fungicide applications and inoculations with *A. alternata*, determined that beginning of bloom and full bloom were the most susceptible growth stages for infection. Four applications of polyoxine B, difenoconazole or azoystrobin from the beginning of bloom to fruit set, reduced the incidence of moldy core by 50-70%. Subsequently, Reuveni (2006) obtained an effective reduction of moldy core in apple fruits, making three applications from the beginning of bloom to petal fall of bromuconazole or a mixture of pyraclostrobin (6.7%) + boscalid (26.7%), decreasing the incidence of the disease by 50 to 70% and 45 to 80% respectively, compared to the untreated control (Reuveni, 2006.)

Ellis and Barrat (1983), studied successive applications of benomyl, captan, mancozeb and dodine applied from green tips up to two weeks before harvest in Delicious apple trees in Ohio. However, the fungicide treatments had no effect on the control of the colonization of *Alternaria* in the carpel cavity of fruits, being unable to find a treatment that provides satisfactory control of moldy core. Shtienberg (2012), neither obtained an effective control

of moldy core in ‘Red Delicious’ apple trees in Israel, by three applications at pink-cluster stage, 60% bloom and full bloom of bromuconazole + captan. In contrast, Reuveni and Prusky (2007) had a significant reduction in core rot and moldy core by spraying bromuconazole + captan or difenoconazole + captan in ‘Top Red’ and ‘Oregon Spur’ apple in Israel.

Similarly, spray applications of mancozeb or hexaconazole at full bloom on ‘Atwood’ and ‘Richared Delicious’ apples in Chile were ineffective against ~~control of~~ moldy core, evaluated at harvest and after four months of conventional cold storage or in controlled atmosphere (Pinilla et al., 1996).

The aim of the present work was to study the efficacy of fungicides with diverse modes of action on the three main *Alternaria* species causing moldy core in Chile, namely *A. alternata*, *A. arborescens* and *A. tenuissima*. This was studied was conducted in vitro and in vivo on detached apples.

MATERIALS AND METHODS

Fungicides. The efficacy of the following fungicides with diverse modes of action were studied: penthiopyrad; difenoconazole, fludioxonil, pyrimethanil, and mancozeb. Fungicides were obtained as commercial formulations (Table 1). For the in vitro tests, an aqueous suspension of each fungicide was prepared and added aseptically to molten sterile agar media after autoclaving and cool down to approximately 50°C.

Isolates. Isolates of *Alternaria alterata* (30MC and 36MC), *A. arborescens* (8MC and 19MC) and *A. tenuissima* (15MC and 33MC) originally obtained from apples with moldy core were used (Elfar et al. 2018b). The isolates were maintained on potato dextrose agar (PDA) acidified with 92% lactic acid at 0.5 ml/liter (APDA) at 5°C prior to testing. To stimulate sporulation, isolates were cultivated in 0.05 x PDA at 22°C with 10 h/14 h of light/dark cycles (Pryor and Michailides 2002).

Mycelial growth and conidial germination tests. The effective concentration required to obtain a 50% (EC_{50}) inhibition of the mycelial growth was studied on APDA for all fungicides with the exception of pyrimethanil, which was tested on glucose gelatin agar (GGA) containing per 1 L of distilled water 4 g glucose, 4 g gelatin, 1.77 g K_2HPO_4, 0.36 g $MgSO_47H_2O$, and 20 g agar. The fungicides penthiopyrad; difenoconazole, and pyrimethanil were tested at 0, 0.1, 0.5, 1.0, 5.0, and 10.0 $\mu g \cdot ml^{-1}$; fludioxonil at 0, 0.05, 0.1, 0.5, 1.0, and 5.0 $\mu g \cdot ml^{-1}$; and macozeb at 0, 1.0, 5.0, 10.0, 50.0, and 100 $\mu g \cdot ml^{-1}$. Plates were seeded with either a mycelial disc (4 mm in diameter), taken from the margin of an actively growing colony of 7-day-old cultures on APDA, or with100 µl conidial suspension (1×10^5 conidia / ml). Three plates of each fungicide concentration were used for each isolate. Colony diameter was measured after 6 days and conidial germination after 20 h at 20°C in darkness.

Control of moldy core in detached apples. Mature fruits (13 % total soluble solids, code 6 of starch index 1–9) of 'Royal Gala' apples were surface disinfected in 1% NaOCl for 1 min and then in 75% ethanol for 5 min and air-dried before sectioning longitudinally. The two halves of each fruit were sprayed with the fungicides (400µl.) studied at the concentration indicated in Table 1. After 24 h one half of each fruit was inoculated with 60 µl of conidial suspensions that were placed in the carpels and then covered with the other half of the fruit to avoid rapid dehydration. Fruits were incubated at 20°C in a humid chamber for 10 days prior to determinate the proportion of moldy core carpels. An equal number of fruits treated with sterile water served as the controls. Re-isolations were performed on APDA. The results were expressed as % fungicide efficacy relative to the untreated controls.

Experimental design and statistical analysis. The EC_{50} values for mycelial growth and conidial germination were estimated with a linear regression analysis where X = the log fungicide concentration and Y = Probit % of the control. For control of moldy core in detached apples the fungicide treatments were randomly distributed in a complete randomized design with a 5 × 6 (fungicide × isolates) factorial structure, with six replicates of one apple each.

Data were subjected to a two-way analysis of variance and mean were separated according with Tukey's test (P = 0.05) with the aid of SigmaStat 3.1 software (Systat Software Inc., San José, CA).

RESULTS

Mycelial growth and conidial germination tests. The EC_{50} values for mycelial growth and conidial germination were estimated from the respective regression equations with R^2 values of from 0.80 to 0.99 for mycelial growth and R^2 values from 0.85 to 0.99 for conidial germination.

Independently of the *Alternaria* species, the mycelium of all *Alternaria* isolates was highly sensitive to fludioxonil with EC_{50} values varying from 0.09 to 0.72 $\mu g \cdot ml^{-1}$. In the same way, isolates were sensitive to pyrimethanil, with EC_{50} values of 0.65 to 3.80 $\mu g \cdot ml^{-1}$. Differences in the sensitive to difenoconazole and penthiopyrad were detected between isolates, EC_{50} values of 1.17 to 2.07 and 1.38 to 4.02 $\mu g \cdot ml^{-1}$ were obtained for difenoconazole and penthiopyrad, respectively. However, *A. alternata* isolate 36MC and *A. tenuissima* isolate 33MC had values >10 $\mu g \cdot ml^{-1}$ for difenoconazole and penthiopyrad. High values of EC_{50} for mancozeb were obtained with all *Alternaria* isolates (>42.8 to >100 $\mu g \cdot ml^{-1}$) (Table 2).

Regardless of the *Alternaria* isolates, the germination of conidia was also highly affected by fludioxonil and pyrimethanil, with EC_{50} of 0.5 to 2.4 and 0.14 to 1.19 $\mu g \cdot ml^{-1}$ respectively. Differences in the sensitive to difenoconazole were detected between isolates, EC_{50} values of 1.30 to 4.90 $\mu g \cdot ml^{-1}$ were obtained. However, *A. alternata* isolate 30MC, *A. arborescens* isolate 8MC, and *A. tenuissima* isolate15MC had values >10 $\mu g \cdot ml^{-1}$ for difenoconazole. For mancozeb, EC_{50} values of 4.29 to 7.44 $\mu g \cdot ml^{-1}$ were obtained for conidial germination. Differences in the conidia sensitivity to penthiopyrad were detected between the species of *Alternaria*. Isolates of *A. arborescens* were highly sensitive with EC_{50} values of 1.02 to 2.39 $\mu g \cdot ml^{-1}$, in contrast *A. tenuissima* isolates had values >10 $\mu g \cdot ml^{-1}$ for penthiopyrad. In the

case of *A. alternata* isolate 36MC was highly sensitive with 01.07 $\mu g \cdot ml^{-1}$ EC_{50} and isolate 30MC had >10 $\mu g \cdot ml^{-1}$ EC_{50} value for penthiopyrad (Table 2).

Control of moldy core in detached apples. A significant effect of fungicides ($P < 0.001$) and *Alternaria* isolates ($P < 0.001$) were obtained in the percentage of moldy core developed on mature 'Royal Gala' apples. The interaction between fungicides and *Alternaria* isolates was also significant ($P < 0.001$) (Table 3). The five fungicides applied were effective in reducing the development of moldy core on apple carpels from 41.7 to 95.0%, obtained for the untreated controls, to 0.0 to 21.7%. Fludioxonil achieved a 100% inhibition of mycelial growth of all the *Alternaria* isolates (Table 3).

DISCUSSION

In this study five fungicides with different modes of action were evaluated in vitro for their efficacy in reducing mycelium growth and suppressing conidial germination and in vivo for the control of moldy core in detached apples caused by *A. alternata*, *A. arborescens* and *A. tenuissima* which are the main *Alternaria* species associated with apple moldy core in Chile (Elfar et al. 2018).

Considering that the fungicides evaluated had the capacity of inhibit the mycelial growth and the germination of conidia, independently of the EC_{50} values obtained, all the fungicides, at the rates used, were able to reduce the percentage of moldy core on carpels by *A. alternata*, *A. arborescens*, and *A. tenuissima*. Therefore, the fungicide difenoconazole, fludioxonil, penthiopyrad, pyrimethanil, and mancozeb represent an alternative to control of moldy core of apples in Chile. However, fludioxonil, a phenylpyrrole fungicide, was the most effective with better control levels of moldy core in in vivo inoculations of detached apples, and lower EC_{50} values to inhibit the mycelial growth and the germination of conidia than the other fungicides tested. Thus, additional studies are necessary to demonstrate its effectiveness under field conditions.

Similarly, a study on ‘Bosc’ pear showed that fludioxonil was highlighted in the inhibition of mycelial growth and conidia germination of *A. alternata* in vitro tests. In vivo tests, fludioxonil provided >90% of control on postharvest decay of artificially inoculated pear fruits (Lutz et al. 2017). Field application of a mix of fludioxonil + cyprodinil was very effective for controlling Alternaria late blight in pistachio orchards (Avenot and Michailides 2015).

Previous results showed that *Alternaria* spp. are well established in apple orchards colonizing apparently healthy apple flowers and fruits from early in the growing stage of pink bud (Niem et al. 2007). Additionally, Reuveni et al. (2002) through artificial inoculations, with *A. alternata*, revealed that the beginning of bloom and full bloom were the most susceptible developmental stages for infection of moldy core.

In previous studies, foliar sprays of benomyl, captan, mancozeb and dodine were unsuccessful in the control of moldy core of apples (Ellis and Barrat 1983), probably due to the low efficacy in the control of *Alternaria* spp. or insufficient doses. Similarly, an application of mancozeb was ineffective against moldy core on ‘Atwood’ and ‘Richared Delicious’ apple, probably due that number of applications was insufficient or were applied too late in bloom stage. However, the present study showed that mancozeb was able to control moldy core developed on detached apples thought in vivo inoculations. Thus, additional studies are necessary to clarify effectiveness of mancozeb against moldy core under field conditions.

Considering that each of the tested fungicides have different modes of action represent an alternative to be incorporated in a disease management program to maximize the moldy core control and to minimize the risk of development of resistance of *Alternaria* spp.

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Table 1. Fungicides used in the in vitro studies for mycelium growth and spore germination and in in vivo studies for apples inoculation

Common name	Group name [y]	Mode of action [y]	Commercial name	Dose a.i. g / 100L[z]
Difenoconazole	DMI	Sterol biosynthesis in membranes	Dominio 250 EC	5
Fludioxonil	Phenylpyrroles	Signal transduction	Starter 230 SC	46
Penthiopyrad	SDHI	Respiration	Fontelis 20 SC	30
Pyrimethanil	Anilinopyrimidines	Amino acids and protein synthesis	Bonnus 400 SC	40
Mancozeb	Dithiocarbamates	Chemicals with multi-site activity	Manzate 200 WP	192

[y] Based on FRAC (Fungicide Resistance Action Committee) 2018. SDHI = succinatedehydrogenase inhibitors, DMI = demethylation inhibitor.
[z] a.i = active ingredient.

Table 2. Sensitivity of *Alternaria* isolates obtained from moldy core of apples to fungicides with different modes of action

	Median effective concentration EC_{50}[z], µg·ml^{-1}									
	Mycelium					Conidia				
Isolate	Dif	Flu	Man	Pen	Pyr	Dif	Flu	Man	Pen	Pyr
A. alternata										
30MC	1.17	0.09	42.8	1.38	1.21	>10	1.09	5.19	>10	0.25
36MC	>10	0.72	>100	>10	2.46	1.30	0.79	4.80	1.07	0.24
A. arborescens										
8MC	1.98	<0.05	84.9	1.42	3.80	>10	2.40	5.23	1.02	0.14
19MC	1.18	0.23	50.6	3.90	2.15	2.74	0.64	4.29	2.39	0.17
A. tenuissima										
15MC	2.07	0.26	74.0	4.02	0.65	>10	1.38	7.44	>10	1.19
33MC	>10	0.34	>100	>10	1.65	4.90	0.50	5.15	>10	0.65

[z] Dif= difenoconazole, Flu= fludioxonil, Pen= penthiopyrad, Pyr= pyrimethanil, Man= mancozeb. Effective concentrations were determined in vitro using APDA, except for pyrimethanil, which were tested on glucose gelatin agar (GGA) medium.

Table 3. Effect of fungicides with different modes of action in the control of moldy core caused by isolates of *Alternaria* on 'Royal Gala' apples

	Moldy core[y], %						
	A. alternata		*A. arborescens*		*A. tenuissima*		
Fungicide	30MC	36MC	8MC	19MC	15MC	33MC	Mean
Difenoconazole	5.0 b	6.7 ab	15.0 b	3.3 b	21.7 b	0.0 b	8.6
Fludioxonil	0.0 b	0.0 b	0.0 b	0.0 b	0.0 d	0.0 b	0.0
Penthiopyrad	6.7 b	3.3 b	8.3 b	8.3 b	6.7 cd	3.3 b	6.1
Pyrimethanil	5.0 b	0.0 b	8.3 b	3.3 b	16.7 bc	8.3 b	6.9
Mancozeb	6.7 b	6.7 ab	5.0 b	3.3 b	13.3 bcd	6.7 b	6.9
Untreated control	71.7 a	30.0 a	81.7 a	41.7 a	95.0 a	80.0 a	66.7

Analysis of variance

	df	*F*	*P*	SED[z]
Fungicide (F)	5	122.7	<0.001	0.032
Isolate (I)	5	8.2	<0.001	0.032
F × I interaction	25	3.3	<0.001	0.078

[y] Fruit carpels of six fruits were inoculated with 60 µl of conidial suspension (10^6 conidia/ml) per fruit at 24 h after the fungicide application. Fruits were incubated in humid chambers at 20°C for 10 days.

[z] SED = standard error of the difference (standard error of the mean × √2).

CHAPTER VI

Final conclusions

Small-spored *Alternaria* spp. were well established in apple orchards colonizing apparently healthy apple flowers and fruits, causing Alternaria leaf blotch and moldy core in Chile. Moldy core of apple was caused by many small-spored *Alternaria* spp. namely *A. alternata*, *A. arborescens*, *A. dumosa*, *A. frumenti*, *A. tenuissima* and *A. kordkuyana*. These species coexisted with one or more *Alternaria* spp. *A. tenuissima* was the most frequently isolated species, it was pathogenic on apple leaf assays and showed a high capacity to colonize apple carpels. However, these *Alternaria* spp. were unable to infect the epidermis and flesh of apple fruits. *Alternaria* spp. were well distributed on flowers early in the season, colonizing fresh sepals, fresh stamens and carpels. The senescent tissues, sepals and stamens, that remain attached to the calyx end on the apple fruits can also serve as potential inoculum sources for the moldy core. Fludioxonil provided 100% control, representing an alternative to control of moldy core of apples.

ANNEXES

SCIENTIFIC PRESENTATIONS

2017 The American Phytopathological Society Annual Meeting, San Antonio, Texas.

AM-Toxin and virulence in *Alternaria tenuissima* isolated from apple moldy core

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Moldy core (MC) is an important disease affecting 'Red Chief', 'Red King Oregon' and 'Scarlet' apples, characterized by fruits with open sinus until harvest. *Alternaria tenuissima* and *Alternaria* spp. have been associated to MC in Chile. The presence of AM-toxin gen and virulence in six isolates of *A. tenuissima* were studied. Isolates of *A. alternata*, *A. arborescens*, and *A. infectoria*, previously isolated from MC of apple, were included as reference isolates. Pure cultures were obtained by single hypal tip transfer to PDA, and the presence of AM-toxin gen was examined with the specific primers LINF1/ LINR and AM-f/AM-r. The presence of AM-toxin gen was determined in four out of six *A. tenuissima* isolates, and in two *A. infectoria* isolates. No evidence of the presence of AM-toxin gen were found in isolates of *A. alternata* and *A. arborescens*. Virulence was study on detached wounded leaves (n=4) of 'Red Chief' apples, inoculated with 15 µl of conidial suspensions (10^6 conidia/ml) and incubated for 7 days at 25°C. All isolates of *Alternaria* studied induced grey brown necrotic lesions, but differences in virulence were obtained. Based on our results, AM-toxin producing isolates of *A. tenuissima* were more virulent than AM-toxin producing isolates of *A. infectoria* and non AM-toxin producing isolates of *A. alternata* and *A. arborescens*.

350-P

AM-toxin and virulence in *Alternaria tenuissima* isolated from apple moldy core

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INTRODUCTION

Moldy core (MC) of apple (*Malus* x *domestica*) (Fig.1) is an important disease frequently occurs affecting Red Delicious apples and its spots, characterized by fruit with open sinus until harvest. *Alternaria tenuissima* (Fig. 1) is the most frequent species causing MC in Chile. The objective this research was to study the presence of AM-toxin gene and virulence in isolates of *A. tenuissima.*

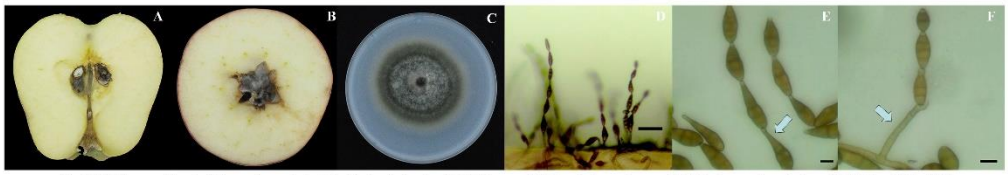

Fig. 1. Moldy core of apple (*Alternaria tenuissima*). A. Seed colonization. B. Moldy core. C. A.tenuissima colony on PCA. D. Long unbranched conidial chains. E. Conidial beak (arrow). F. Short unbrunched coniophore (arrow).

METHODOLOGY

Isolates. *A. tenuissima* (n=6) were obtained on APDA from MC of apples collected at harvest. Isolates of *A. alternata* (n=2), *A. arborescens* (n=3) and *A. infectoria* (n=2) were included in this study. All isolates were identified previously (3,4) .

AM-toxin gene detection. Polymerase chain reaction (PCR) was performed using AM Toxin-specific primers LinF1/ LinR (2) and AM-forward/AM-reverse (1).

Pathogenicity and virulence tests. Detached wounded leaves (n=4, per isolate) of 'Red Chief' apples were inoculated with 15 µl of 10^6 conidia/ml suspension and incubated for 5 days at 25°C in humid chambers.

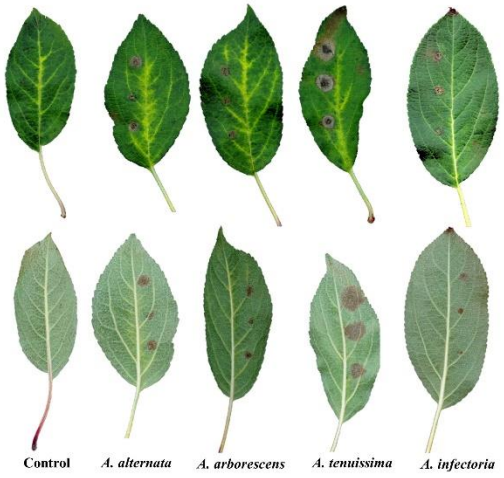

Fig. 2. Necrotic lesions on adaxial and abaxial leaves surface obtained on 'Red Chief' apples inoculated after 5 days at 25°C. Control =non-inoculated.

RESULTS

Evidences for the presence of AM-toxin gene were detected in four *A. tenuissima* isolates, and in two *A. infectoria* isolates. No evidences for the presence of AM-toxin gene were found in isolates of *A. alternata* and *A. arborescens* (Table 1.).

All *Alternaria* isolates induced grey brown necrotic lesions (Fig. 2). *A. tenuissima* was the most virulent and *A. infectoria* the less virulent species (Fig. 3).

Table 1. Detection of AM-toxin gene

Alternaria species	Primer	
	LinF1/ LinR	AM-f/ AM-r
A. tenuissima	+	+
A. infectoria	+	+
A. alternata	-	-
A. arborescens	-	-

+ = AM-toxin gene present,
- – AM-toxin gene absent

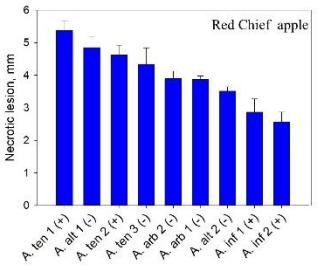

Fig. 3. Pathogenicity test on apple leaves. *Alternaria tenuissima (A.ten), A. alternata (A. alt), A. arborescens (A. arb)* and *A. infectoria (A. inf).* Five days at 25°C. (+) = AM-toxin gene present, (-)= AM-toxin gene absent. Means of four replicates, significant at $P< 0.001$. Bars = standard error.

DISCUSION

Evidences for the present of AM-toxin gene were obtained in isolates of *A. tenuissima* which were more virulent than other *Alternaria* species. Further studies are needed to demonstrate the production of AM-toxin and it correlation with virulence

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Alternaria species causing moldy core of apple in Chile

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Moldy core (MC) of apple is an important disease in Chile, reducing yields and quality. Infected fruits ripe and drop prematurely, and show a slight deformation, a black fungal mycelia colonize the carpels and eventually a core rot occur. Disease prevalence of 12 to 60% were estimated in 'Fuji', 'Red Chief', 'Red King Oregon' and 'Scarlet' apples in 2015–2016. The etiology of MC was studied. Apple samples (n=100) were collected from ten orchards in Central Chile two weeks before harvest. A total of 319 of *Alternaria* isolates were obtained on PDA, and selected small-spore *Alternaria* isolates (n=14) were examined molecularly with the primer of plasma membrane ATPase, calmodulin and Alternaria major allergen sequence analysis. Based on a concatenated phylogenetic analysis, *A. alternata*, *A. arborescens*, *A. infectoria* and *A. tenuissima* were identified. All *Alternaria* spp. were pathogenic in inoculated mature apples and seeds and detached leaves of 'Red Chief' apples. A gray to dark olive green mycelium colonized the carpel cavity after 10 days at 25°C. Inoculated seeds were superficially colonized. Leaf symptoms consisted on round grayish brown necrotic lesions after 7 days at 25°C. *Alternaria* spp. were re-isolated from inoculated fruits, seeds and leaves. In conclusion, MC of apple was associated to an *Alternaria* complex, being *A. alternata*, *A. arborescens*, *A. infectoria* and *A. tenuissima* identified in this study.

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Alternaria species Causing Moldy Core of Apple in Chile

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INTRODUCTION

Moldy core (MC) is an important disease of apples, previously associated with *Alternaria alternata* in Chile. Infected fruit ripe and drop prematurely. A gray mold colonizes the carpels (Fig.1), reducing the storage of apples. In this study, *Alternaria* isolates were characterized and the relative importance of the *Alternaria* species was determined.

Fig. 1. Moldy core of apple.

METHODOLOGY

Isolates. A total of 319 of *Alternaria* isolates were obtained on APDA.

Morphology. Colony, conidiophore and conidia morphology of 14 selected isolates were determined on APDA and PCA (3).

Molecular characterization. ITS1- 5.8S-ITS2 (4), plasma membrane ATPase (Atp), calmodulin (Cal) (2) and *Alternaria* major allergen genes (Alt a1) (1) from 14 isolates were amplified and sequenced. Phylogenetic analyses were performed.

Pathogenicity tests. Carpels of mature fruits, detached wounded leaves and seeds of 'Red Chief' apples were inoculated with 10^6 conidial/ml.

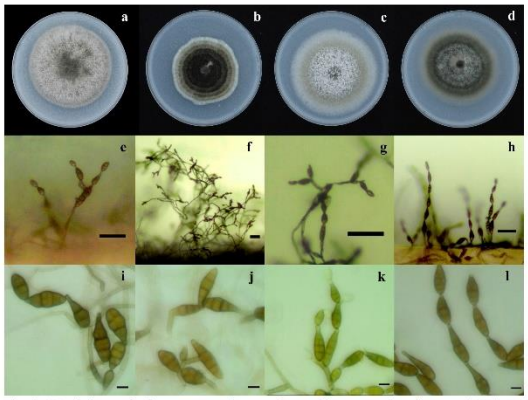

Fig. 2. Morphology of *Alternaria* spp. from apples moldy core. a-d, colony on APDA; e-l, sporogenesis pattern on PCA after for 7 days at 22°C 8 h/ 16 h light/ dark; a,e,i, *A. alternata*; b, f, j, *A. tenuissima*; c, g, k, *A. arborescens*; d, h, l, *A. infectoria*. Scale bar of e-h = 50 µm; i-l= 10 µm.

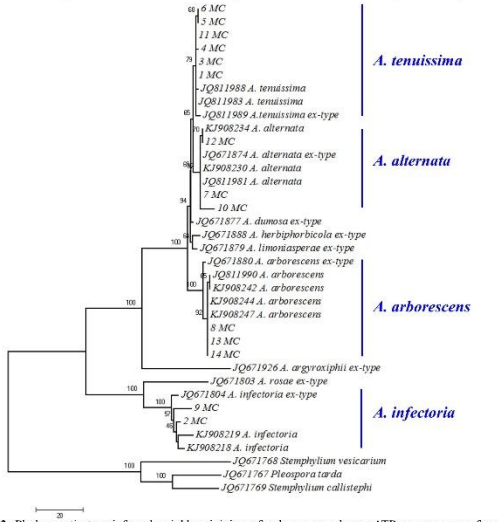

Fig. 3. Phylogenetic tree inferred neighbor joining of plasma membrane ATPase sequences from *Alternaria* isolates from Chile (MC). Bootstrap support values from 1,000 replicates.

RESULTS

MC prevalence of 12-60% were estimated in 'Fuji' and Red Delicious apple orchards in 2015-2016. MC was absent in Granny Smith.

Alternaria alternata, A. arborescens, A. infectoria and *A. tenuissima* were identified (Fig. 2).

The phylogenetic analyses using Cal gene differentiated *A. arborcens* and *A. infectoria*. Atp gene distinguished *A. alternata* from *A. tenuissima* isolates (Fig.3). The ITS and Alt a1 gene sequences were not informative enough to delimited the *Alternaria* species.

All isolates were pathogenic in mature carpel fruits and leaves, and were able to superficially colonize seeds. Seed viability was not affected.

CONCLUSION

Alternaria tenuissima was the most frequently identified species, following by *A. arborecens, A. alternata* and *A. infectoria.*

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Situación del corazón mohoso de la manzana en Chile

Moldy core situation of apple in Chile

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Chile es uno de los mayores productores y exportadores de manzana del hemisferio sur, con 765.000 ton exportadas en 2016. El corazón mohoso (CM) de la manzana es una enfermedad importante en Chile que reduce la calidad de la fruta, debido a la presencia de micelio en los carpelos. En manzanas con tubo calicinal abierto, se estimaron prevalencias de 3-56% de CM al iniciar la cosecha en 2015 y 2016. Contrariamente, no hubo evidencias de CM en manzanas 'Granny Smith' con tubo calicinal cerrado. Este trabajo tuvo por objetivo establecer la etiología de CM en muestras de manzanas de los cultivares 'Fuji', 'Red Chief', 'Red King Oregon' y 'Scarlet'. Muestras de frutos (n=100) de diez huertos entre Santiago y Temuco, fueron colocados en placas de APD acidificado con 0,5 ml/L de ácido láctico 92% (APDA). De un total de 650 frutos enfermos se aisló mayoritariamente especies de *Alternaria* de conidia pequeña y secundariamente especies de los géneros *Botrytis, Cladosporium, Epicoccum, Fusarium, Penicillium, Stemphylium, Ulocladium.* Los aislados de *Alternaria* (n=14) fueron examinados morfológicamente en APDA y agar papa zanahoria y molecularmente analizando los genes *Alternaria major allergen* Alt a1 (Alt-for/Alt-rev,), ATPasa de membrana plasmática (ATPDF1/ATPDR1) y Calmodulina (CALDF1/CALDR1). En función de estos resultados se identificó a *A. alternata*, *A. arborescens*, *A. infectoria* y *A. tenuissima*. Aislados (n=2) de cada una de estas especies resultaron patogénicos en frutos y en hojas de manzano 'Red Chief'. Estos resultados demuestran de CM de la manzana se debe a un complejo de especies de *Alternaria.*