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RESEARCH PAPER

Prevalence and pathogenicity of fungi associated with grapevine trunk diseases in Chilean vineyards

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Abstract

G. A., Díaz, J., Auger, X., Besoain, E., Bordeu, and B. A. Latorre, 2013. Prevalence and pathogenicity of fungi associated with grapevine trunk diseases in Chilean vineyards. Cien. Inv. Agr. 40(2): 327-339. Trunk diseases in grapevine (*Vitis vinifera*) have been identified as a major problem in the wine and table grape industries around the world, reducing the productivity, quality and longevity of vineyards. The present study examined 694 wood samples from the cordons and trunks of vines with trunk disease symptoms in 67 Chilean vineyards located between Copiapó (27°18' S) and Los Angeles (37°42' S). A total of 1,363 fungal isolates were obtained from diseased cordons and trunks with dark brown streaking, yellowish soft-spongy cankers and brown hard V-shaped cankers. Using molecular identification, a total of 12 fungal genera were identified in Chile at varying frequencies: *Phaeomoniella chlamydospora* (85%); Botryosphaeriaceae (56%) including *Diplodia mutila*, *D. seriata*, *Neofusicoccum parvum* and *Spencermartinsia viticola*; *Inocutis* sp. (47%); *Diatrypaceae* (*Cryptovalsa ampelina* and *Eutypella leprosa*) (4.8%); *Seimatosporium botan* (1.7%); *Phomopsis viticola* (0.4%); *Cylindrocarpon* sp. (0.4%); and *Phaeoacremonium aleophilum* (0.2%). All species were pathogenic, inducing dark brown streaking on various aged grapevine wood tissue. In conclusion, several fungal species are associated with grapevine trunk diseases in the Chilean vineyards being *Pa. chlamydospora*, *D. seriata* and Inocutis sp. the most frequent isolated species. These are pathogens that can be found alone or they can coexist in the same plant. This is the first report of Pho. viticola associated with trunk diseases in Chile.

Key words: Botryosphaeria canker, Esca, fungal trunk pathogens, *Vitis vinifera*, wood decay.

Introduction

Grapevine (*Vitis vinifera* L.) is an important crop in Chile, with currently over 182,000 ha planted. In the past two decades, the importance of grapevine trunk diseases in Chile has been recognized. It has recently been estimated that 22% of vineyards are affected, reducing productivity, quality and the productive lifespan of Chilean vineyards (Díaz *et al.*, 2011a; Morales *et al.*, 2012). Although symptoms can be observed in the trunks and cordons of young (<7-year-old) grapevines, the prevalence and severity considerably increases as

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the vines get older (Bertsch *et al*., 2013; Morales *et al*, 2012; Úrbez-Torres, 2011).

Esca, Petri disease, black foot disease, Botryosphaeria dieback and Eutypa dieback are the predominant trunk diseases that affect vineyards worldwide (Bertsch *et al*., 2013; Halleen *et al*., 2006; Úrbez-Torres, 2011). However, only Botryosphaeria dieback, black dead arm and basal canker, and wood necrosis and dieback associated with diatrypaceous fungi and *Seimatosporium botan* have been reported among trunk diseases in Chile (Auger *et al*., 2004a; Auger *et al*., 2004b; Besoain *et al*., 2013; Díaz *et al*., 2011a,b, 2012; Latorre *et al*., 1986; Morales *et al*., 2012). In addition, chlorotic leaf roll similar to Eutypa dieback has been recognized in Chile (Auger *et al*., 2005).

Foliar symptoms include malformation, chlorosis and partial necrosis of the leaves associated with short internodes, reduced growth and a general decline. Wood symptoms are characterized by the presence of dark brown to black spots in cross-sections of cordons and trunks, which are visible as dark brown wood streaking in longitudinal sections. In addition, brown hard V-shaped cankers and/or yellow soft cankers in the wood can also be observed (Cortesi *et al*., 2000; Bertsch *et al*., 2013, Mugnai *et al*., 1999; Úrbez-Torres *et al*., 2012; White *et al*., 2011a). The dark brown wood streaking associated to Esca-like symptoms is the most prevalent internal symptom in young and adult grapevines (Bertsch *et al*., 2013, Mugnai *et al*., 1999).

Several fungi are associated with trunk diseases throughout the world, including species of Ascomycetes, such as Botryosphaeriaceae spp., Diatrypaceae spp., *Phaeoacremonium (Pm.)* spp., *Phaeomoniella (Pa.) chlamydospora,* and Basidiomycetes, such as *Fomitiporia (F.) punctata, F. mediterranea* and *Inocutis* spp. (Armengol *et al*., 2001; Cortesi *et al*., 2000; Fisher, 2006; Martin and Cobos, 2007; Rolshausen *et al.,* 2006; Taylor *et al*., 2005; Úrbez-Torres *et al*., 2012; White *et al*., 2011b).

In Chile, *Botryosphaeria dothidea*, *Cryptovalsa (C.) ampelina*, *Diplodia (D.) seriata*, *D. mutila*, *Eutypella (Eu.) leprosa*, *Neofusicoccum (N.) parvum, Pm. aleophilum*, *Pa. chlamydospora, Seimatosporium (Se.) botan* and *Spencermartinsia (S.) viticola* have been reported as associated with trunk diseases (Auger *et al*., 2004a; Auger *et al*., 2004b; Besoain *et al*., 2013; Díaz *et al*., 2009, 2011a,b, 2012; Latorre *et al*., 1986; Morales *et al*., 2012). Interestingly, *Eutypa lata*, which has been isolated from other grape-growing areas around the world, has not been reported in Chile.

Considering the importance of the grapevine industry in Chile, this study was conducted to identify the fungi associated with declining grapevines and to determine their relative importance and pathogenicity as fungal trunk pathogens in *V. vinifera* in Chile.

Materials and methods

Sampling

Sixty-seven vineyards in the main wine and table grapevine production valleys of Chile were surveyed from 2009 to 2012. Vineyards from 4 to 35 years old were sampled from Copiapó (27°18’ S) to Los Angeles (37°42’ S), with a mean annual rainfall ranging from 1 to 1,000 mm, most of which fell during the winter months. The samples consisted of at least 8-12 trunk and/or cordons per vineyard taken from diseased grapevines of wine and table grape cultivars with decline symptoms, including leaf malformation, chlorotic and necrotic leaves, short internodes, dead spurs and dieback.

Fungal isolation

The bark of each sample was removed prior to surface disinfection with 96% ethanol for 15 s and immediately flamed for 15 s. The outer tissues were removed aseptically in a laminar flow chamber. Small wood pieces (3-5 mm) were taken

from the margins between healthy and discolored tissues and placed in Petri dishes containing 2% potato dextrose agar acidified with 0.5 ml/liter of 92% lactic acid (APDA) plus 0.005% tetracycline, 0.01% streptomycin and 0.1% Igepal CO-630 (Sigma-Aldrich, MO, USA) (APDAm). The plates were incubated for 14 to 21 days at 20 °C until fungal colonies were observed.

Morphological and molecular identification

Fungi were identified to the genus level on the basis of the culture characteristics and on the morphology of the asexual structures, including conidia, conidiophores and the presence or absence of fruiting bodies (Crous and Gams, 2000; Crous *et al*., 1996; Gottlieb *et al*., 2002; Hatakeyama and Harada, 2004; Mostert *et al*., 2006; Trouillas *et al*., 2010; Úrbez-Torres *et al*., 2006). One or two isolates of each fungal taxon were further identified to the species level by molecular analysis of the internal transcribed spacer (ITS1-5.8S-ITS2) region of the nuclear ribosomal DNA (rDNA). The rDNA was extracted from mycelium using a DNA extraction kit (Axygen Biosciences, CA, USA). The ITS region was amplified using ITS4 and ITS5 primers (White *et al*., 1990). Polymerase chain reactions (PCR) were conducted as previously reported (Úrbez-Torres *et al*., 2006) in a Maxygene Gradient thermal cycler (Axygen Biosciences, Union City, CA, USA). Each PCR reaction mixture contained 2.5 µL of 10x PCR buffer, 1.0 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 0.5 µL of 0.5 mM of each primer, 0.2 µL of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) at 5 units·µL^{-1}, and 1 µL of template DNA for a final volume of 25 µL. A negative control was included in each PCR reaction. The PCR products were separated on a 2% agarose gel in 1.0x Tris-acetate-EDTA (TAE) buffer and observed by UV illumination after staining with GelRedTM (Biotium Inc., Hayward, CA, USA). The PCR products were purified and sequenced in both directions by Macrogen (Macrogen Inc., Geumchen-gu, South Korea). The fungal sequences were manually- edited, assembled and aligned using Bioeditor version 7.1.3.0 (Tom Hall, Isis Pharmaceutical Inc., Carlsbad, CA, USA). Alignment gaps were treated as missing data. The sequences of each fungus were compared using the GenBank database and basic local alignment search tools (Blast) for nucleotide analyses. The identification was confirmed with >98% similarity to the fungal species deposited in GenBank (Table 1).

Pathogenicity tests

The pathogenicity of one isolate from each identified species was tested in four independent experiments conducted on *V. vinifera*. The following isolates were used: *C. ampelina* (Cry-18-2009), *D. mutila* (Dm-25-2010), *D. seriata* (Ds-7-2009), *Eu. leprosa* (Eu-86-2010), *N. parvum* (Neo-105-2010), *Pm. aleophilum* (Pal-357-2010), *Pa. chlamydospora* (Pa.ch-3-2009), *Phomopsis (Pho.) viticola* (Pho-

Table 1. Isolates from GenBank used for nucleotide basic local alignment search tool (Blastn).

Fungal species	Isolate	Hosts	GenBank Nº ITS	Country of origin
Cryptovalsa ampelina	CA014	*Vitis vinifera*	GQ293913	USA
Cylindrocarpon sp.	CY144	*Vitis vinifera*	AM419074	Portugal
Diplodia mutila	STE-U 5038	*Vitis vinifera*	AY343484	South Africa
Diplodia seriata	UCD46Fr	*Vitis vinifera*	DQ008326	USA
Eutypella leprosa	CBS 276.87	*Tilia* sp.	AJ302463	Switzerland
Inocutis jamaicensis	1500	*Diostea juncea*	AY072033	Argentina
Neofusicoccum parvum	B02-07	*Vaccinium corymbosum*	EU833984	Chile
Phaeoacremonium aleophilum	CBS 246.91	*Vitis vinifera*	AF017651	France
Phaeomoniella chlamydospora	CBS 229.95	*Vitis vinifera*	AF197973	Italy
Phomopsis viticola	UCD2236MO	*Vitis eastivalis*	HQ288245	USA
Seimatosporium botan	-	*Peonia suffruticosa*	HM067840	China
Spencermartinsia viticola	CBS 117006	*Vitis vinifera*	AY905555	Spain

221-2010), *Se. botan* (Sei-302-2010), *S. viticola* (Spe-578-2011), *Cylindrocarpon* sp. (Cyl-476-2011) and *Inocutis* sp. (Bas-91-2010).

Axenic grapevine plantlets. Eight 30-day-old 'Carménère' plantlets that were 40 mm in height and six leaves, micropropagated on semisolid medium (Gregori and Tizio, 1997), were inoculated with a 2-mm-diameter mycelial plug from a 7- to 21-day-old APDA culture of each fungal isolate. The inoculum was placed upside down in a small injury (2 mm diameter) at the stem base that was made with a sterile scalpel. After inoculation, the inoculated site was left open. The plantlets were incubated under 200 $\mu mol \cdot m^2 s^{-1}$ of white fluorescent light for a 16-h photoperiod at 20°C in a plant growth room. An equal number of plantlets that were inoculated with sterile agar were used as controls. The number of leaves, along with shoot and root lengths were assessed at 28 days post-inoculation. In an attempt to fulfill Koch's postulates, small pieces of colonized tissue taken from the margins of lesions were placed on APDAm.

Rooted grapevines. Five 'Carménère' vines (2 years old) were inoculated, during winter dormancy using a 5-mm-diameter mycelial plug from a 7- to 21-day-old APDA culture of each fungal isolate per vine, in a wound made with a 5-mm-diameter cork-borer into the trunk and sealed with Parafilm to avoid dehydration. An equal number of vines that were inoculated with sterile agar plugs were left as controls. All vines were maintained in a mixture of peat (70%) and perlite (30%) in 3.5 L containers in a lath house. The internal length of dark brown streaking was measured 15 mo post-inoculation. Re-isolations from the margins of dark brown streaking were performed by placing nine small wood pieces (2x2 mm) on APDAm per inoculated vine.

Grapevine shoots. Five green shoots per 8-year-old vine from the 'Cabernet Sauvignon' vineyard located in Alto Jahuel, Metropolitan Region, were aseptically wounded with a cork-borer (3-mm-diameter) at the third internode and inoculated with mycelial plugs from a 7- to 21-day-old APDA culture of each fungal isolate placed side down and wrapped with Parafilm to avoid dehydration in November (spring). One vine was selected per isolate. An equal number of shoots inoculated with sterile agar plugs were used as controls. Shoots were removed from the vines 60-days post-inoculation and taken to the laboratory for assessment by measuring the extent of the dark brown streaking from the inoculation site. Re-isolations were performed from small pieces of the dark brown streaking that were placed on APDAm.

Detached grapevine shoots. Five 'Carménère' shoots (15-cm in length) obtained from a 12-year-old vineyard were disinfected superficially with 75% ethanol for 30 s and air dried in a laminar flow chamber. Wounds (3x3 mm) were made with a cork-borer in the middle of the shoot and inoculated immediately with a 3x3-mm mycelium plug from a 7- to 21-day-old APDA culture of each fungal isolate per shoot. The control treatment was a sterile mycelium plug. The inoculated wounds were sealed with Parafilm to avoid dehydration. All shoots were placed in a humid chamber (>85% relative humidity) (35x27x13 cm) at 25°C. The dark brown streaking was measured at 14 days post-inoculation. Fungi were re-isolated from the margins of the dark brown streaking on the tissues and placed on APDAm.

Design and statistical analysis

Pathogenicity studies were performed using a completely randomized design with eight replicates for plantlet tests and five replicates for rooted grapevines, attached shoots and detached shoots. The results were subjected to an analysis of variance, and the means were separated according to a pairwise multiple comparison Tukey's test ($P<0.05$) using SigmaStat 3.1 (Systat Software Inc. San José, CA, USA). All experiments were repeated, except for the tests conducted with the rooted grapevines.

Results

Sampling and fungal isolation

Cordons and trunks from young (≤10 years old) grapevines mainly presented brown streaking that appeared as necrotic spots in cross-sections or dark brown streaking in longitudinal sections. Brown, hard, V-shaped cankers were sporadically observed in young grapevines. In addition to the dark brown streaking, yellowish soft, spongy cankers (Figure 1C) and/or a brown, hard, V-shaped cankers were commonly observed in the cross-sections of cordons and the trunks of mature (≥ 11 years old) grapevines (Figure 1). Leaf malformation, chlorotic and necrotic leaves, short internodes, death of spurs and dieback were commonly associated with the cordon and/or trunk symptoms (Figure 2).

A total of 1,363 fungal isolates were obtained from 694 wood samples of diseased cordons and/or trunks. A total of 12 genera were identified on the basis of their cultural and morphological characteristics. These genera included *Cryptovalsa*, *Cylindrocarpon*, *Phomopsis (Diaporthe)*, *Diplodia*, *Dothiorella*, *Eutypella*, *Inocutis*, *Neofusicoccum*, *Phaeoacremonium*, *Phaeomoniella*, *Seimatosporium* and *Spencermartinsia*.

The analysis and comparison of the sequences of the ITS region of the rDNA with known sequences deposited in GenBank (Table 1) led to the identification of *Pa. chlamydospora, Inocutis* sp.; the Botryosphaeriaceae *D. seriata*, *D. mutila, N. parvum* and *S. viticola*; and the Diatrypaceae *C. ampelina* and *Eu. leprosa*. In addition, *Se. botan, Pho. viticola, Cylindrocarpon* sp. and *Pm. aleophilum* were also identified. The sequences of the Chilean isolates were deposited in GenBank (Table 2).

Overall, 85% of samples contained *Pa. chlamydospora*, followed by species of Botryosphaeriaceae (56%) and *Inocutis* sp. (47%) (Table 3). Other fungi were isolated only occasionally from diseased grapevine samples, including 4.8% Diatrypaceae, 1.7% *Se. botan,* 0.4% *Pho. viticola,* 0.4% *Cylindrocarpon* sp. and 0.2% *Pm. aleophilum*.

Of the total isolates from samples affected by dark brown streaking (n=657), *Pa. chlamydospora* was isolated from 85%, including young and mature grapevine (Figure 1). Species of Botryosphaeriaceae, including *D. mutila*, *D. seriata*, *N. parvum* and *S. viticola*, were isolated

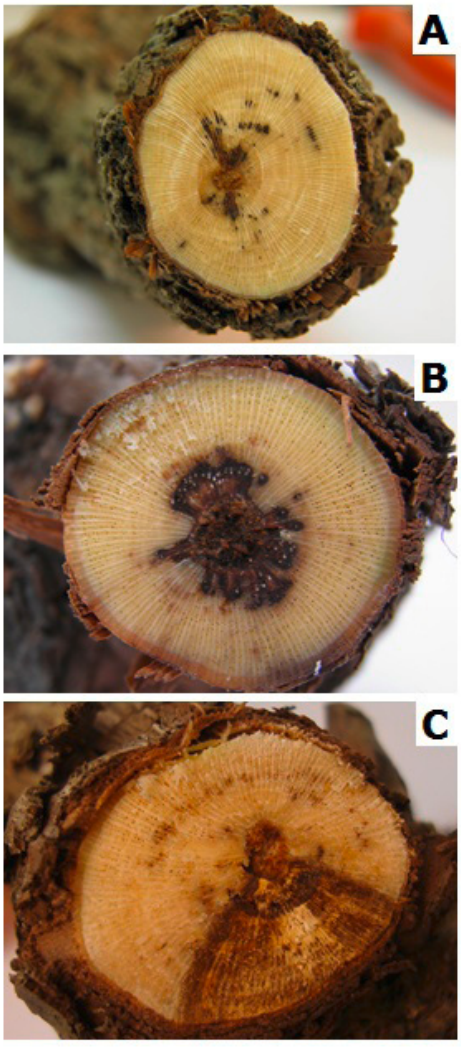

Figure 1. Cross-sections of diseased cordons or trunks with internal symptoms obtained from Chilean vineyards. A, Cordon of 8-year-old 'Carménère' vine with black spotting. B, Trunk of 15-year-old 'Carménère' vine with central black spotting. C, Cordon of 25-year-old 'Chardonnay' vine with canker with brown spotting and yellowish, soft and spongy rotten wood.

Table 2. Fungal species obtained from *Vitis vinifera* with trunk diseases in Chile that were identified by Blast nucleotide analysis..

Species	Isolate[1]	Host cultivars	Origin	GenBank[2] Nº	IMI[3] Nº
Cryptovalsa ampelina	Cry-17	Pinot Noir	Casablanca	HQ694976	398781
Cryptovalsa ampelina	Cry-18	Cabernet Sauvignon	Alto Jahuel	HQ694977	-
Cylindrocarpon sp.	Cyl-476	Cabernet Sauvignon	Nancagua	-	-
Diplodia mutila	Dm-20	Carménère	Alto Jahuel	-	-
Diplodia mutila	Dm-5	Cabernet Sauvignon	Alto Jahuel	-	-
Diplodia seriata	Ds-7	Cabernet Sauvignon	Alto Jahuel	-	-
Diplodia seriata	Ds-80	Pinot Noir	Casablanca	-	-
Eutypella leprosa	Eu-86	Pinot Noir	Casablanca	HQ694974	398667
Eutypella leprosa	Eu-121	Chardonnay	Casablanca	HQ694975	-
Inocutis sp.	Bas-91	Cabernet Sauvignon	Alto Jahuel	-	399078
Inocutis sp.	Bas-184	Cabernet Sauvignon	Nancagua	-	-
Neofusicoccum parvum	Neo-104	Syrah	Lolol	JF273631	-
Neofusicoccum parvum	Neo-105	Cabernet Sauvignon	Lolol	JF273632	-
Phaeoacremonium aleophilum	Pal-357	Cabernet Sauvignon	Nancagua	-	-
Phaeomoniella chlamydospora	Pach-3	Cabernet Sauvignon	Alto Jahuel	JF968607	397989
Phaeomoniella chlamydospora	Pach-55	Pinot Noir	Mulchén	JF968608	-
Phomopsis viticola	Pho-210	Carménère	Almahue	-	-
Phomopsis viticola	Pho-221	Cabernet Sauvignon	Almahue	-	-
Seimatosporium botan	Sei-302	Pedro Jimenez	Ovalle	JN088482	-
Seimatosporium botan	Sei-316	Cabernet Sauvignon	Cauquenes	JN088483	-
Spencermartinsia viticola	Spe-578	Carménére	Apalta	-	-
Spencermartinsia viticola	Spe-643	Cabernet Sauvignon	Alhué	-	-

[1]Isolate= isolate code, specie-sample no.-collected year.
[2]GenBank= isolates deposited in GenBank as accession number by ITS gen.
[3]IMI= isolates deposited in CABI as IMI number.

Table 3. Frequency of fungal genera obtained from *Vitis vinifera* in the main grapevine production regions of Chile.

Region[1]	Total samples[2] no.	Positive sample, %	Pa[3]	Bot[4]	Ino[5]	Dia[6]	Sei[7]	Pho[8]	Cyl[9]	Pal[10]
			Positive samples, no.							
Copiapó	16	93.8	0	14	6	0	0	0	0	0
Limarí	68	100.0	58	12	9	0	5	0	0	2
Aconcagua	40	97.5	32	18	13	0	0	0	0	0
Casablanca	40	100	35	59	35	8	0	0	0	0
San Antonio	26	76.9	19	2	0	0	0	0	0	0
Maipo	182	100.0	168	88	85	12	0	3	2	0
Cachapoal	36	100.0	23	35	25	5	0	0	0	0
Colchagua	160	98.1	141	109	91	9	0	0	1	0
Curicó	20	95.0	12	15	14	0	7	0	0	0
Maule	40	100.0	35	28	35	0	0	0	0	0
Itata	22	100.0	21	7	12	0	0	0	0	0
Bío-Bío	44	95.5	36	36	3	0	0	0	0	0
Total	694	97.9	590	391	328	34	12	3	3	2
%	-	-	85.0	56.3	47.3	4.8	1.7	0.4	0.4	0.2

[1]The regions were sorted by geographical location from north to south of Chile. [2]Samples=Number cordons and/or trunks obtained from grapevine with trunk diseases. [3]Pa=*Phaeomoniella chlamydospora*. [4]Bot=Botryosphaeriaceae species; *Diplodia seriata*, *D. mutila*, *Neofusicoccum parvum* and *Spencermartinsia viticola*. [5]Ino=*Inocutis* sp. [6]Dia=Diatrypaceae species; *Cryptovalsa ampelina* and *Eutypella leprosa*. [7]Sei=*Seimatosporium botan*. [8]Pho=*Phomopsis viticola*. [9]Cyl=*Cylindrocarpon* sp. [10]Pal=*Phaeoacremonium aleophilum*.

from 91% of the samples exhibiting hard brown V-shaped cankers (n=392). *Diplodia seriata* alone was isolated from 68% of the samples with a V-shaped canker. *Inocutis* sp. was observed in 96% of the samples with yellowish soft-spongy cankers (n=314) (Figures 1 and 3).

The Diatrypaceae species *C. ampelina* and *Eu. leprosa* were isolated from 1.8 and 2.9% of the samples with dark brown streaking, respectively. *Seimatosporium botan* was identified on 2.4% of samples with hard brown V-shaped cankers. *Phaeoacremonium aleophilum, Pho. viticola* and *Cylindrocarpon* sp. were isolated from 0.3-0.5% of the samples with dark brown streaking (Figure 3).

According to the distribution by valley, where the annual rainfall increases from 1 to 1,300 mm and annual temperature decreases from 18 to 11 °C, *Pa. chlamydospora* was located from the north to the south of Chile and was found in all valleys except in Copiapó. Species of Botryosphaeriaceae were found in all valleys sampled, *D. seriata* was found in all valleys, and *D. mutila*, *N. parvum* and *S. viticola* only were obtained between the Casablanca and Curico Valleys (data not shown). The basidiomycete *Inocutis* sp. was isolated in all valleys, except in San Antonio (Table 3).

Pathogenicity test

All fungal species were pathogenic on axenic grapevine plantlets. Independent of the pathogen, the foliar symptoms consisted of shortened internodes and chlorosis and necrosis of the leaves

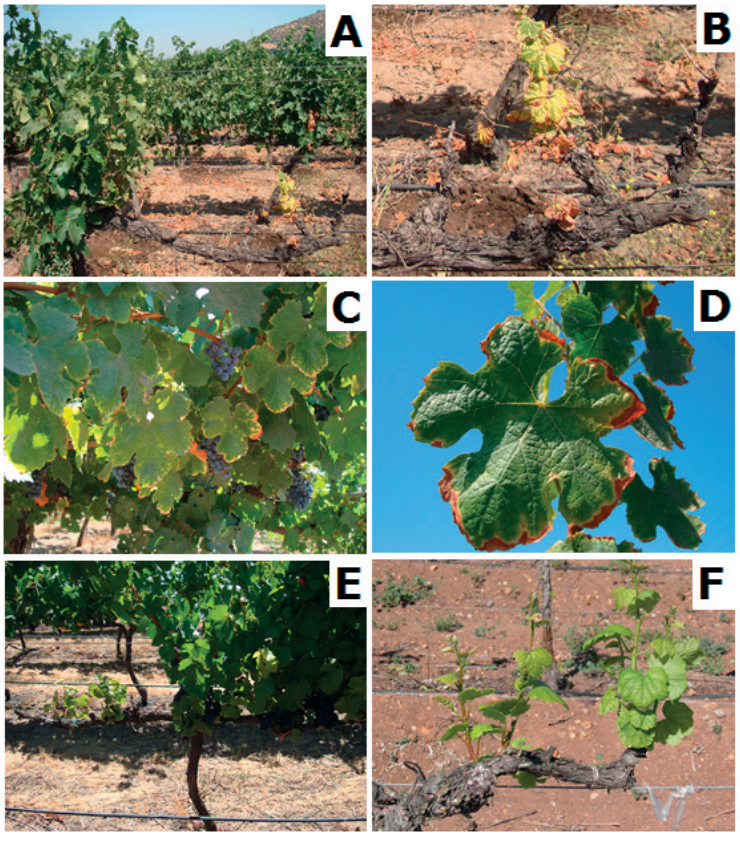

Figure 2. Decline symptoms observed in grapevines with trunk diseases in Chile. **A-B,** 'Cabernet Sauvignon' vine with diseased cordon, dead spurs, full chlorotic leaves, short internodes and stunted shoots. **C-D,** Shoots on 'Cabernet Sauvignon' vine with deformed necrotic leaves with marginal chlorosis. **E,** 'Pinot Noir' vine with cordon dieback. **F,** 'Chardonnay' vine cordon with cupped leaves.

at 28 days post-inoculation. The non-inoculated grapevine plantlets remained healthy. The length of the roots, shoots and leaf numbers decreased significantly (P≤0.05) in inoculated grapevine plantlets (Table 4). Re-isolation was 100% successful from inoculated grapevine plantlets, and pathogens were not re-isolated from non-inoculated controls.

All two-year-old grapevines inoculated with all fungal species developed brown streaking in the trunk. The length of the brown streaking varied among pathogens from 41.4 mm for *S. botan* to 109.9 mm for *N. parvum* after 15 months of incubation in lath-house conditions. Control treatments produced a slight discoloration (mean of 10.1 mm) that was significantly different from the inoculated grapevines (Table 4). The pathogens were successfully re-isolated from more than 80% of the inoculated grapevines. No pathogens were isolated from non-inoculated controls.

Independent of the pathogens, significant (P≤0.05) brown streaking was obtained in inoculated shoots in the bioassay in the laboratory as well in inoculated shoots in the vineyard. In the laboratory, after 14 days at 25 °C, inoculated shoots developed vascular discoloration that varied from 11.3 to 79.6 mm for *Inocutis* sp. and *N. parvum*, respectively. Field-inoculated shoots developed brown streaking that varied from 15.5 for *Pho. viticola* to 81.1 mm for *N. parvum* after 60 days with mean air temperature of 18.7 °C (Table 4). Re-isolations were successful in 60 to 100% of the inoculated shoots in the laboratory and in 89 to 100% of the field-inoculated shoots. Control treatments developed a slight discoloration in the detached shoots (5.9 mm) and in field shoot (6.8 mm), but no pathogens were isolated from these shoots.

Discussion

Trunk diseases were observed in vineyards located from the Copiapó valley in the north to the Bío-Bío valley in the south, which covers a distance of approximately 1,500 km in Chile. In the Copiapó valley, which is characterized by a desert climate, *D. seriata* and *Inocutis* sp. were

Table 4. Pathogenicity of fungal species associated with grapevine trunk diseases in Chile on axenic grapevine plantlets, 2-year-old grapevine trunks and attached and detached shoots from mature grapevines in the field.

	Axenic plantlets				Shoots	
					Detached	Attached
Fungi	Roots[1] mm	Shoots[1] mm	Leaves[1] no.	Trunks BS[2], mm	BS[2], mm	
Cryptovalsa ampelina	58.5 bc	61.9 bc	7 ab	57.1 bcd	14.1 b	18.6 b
Cylindrocarpon sp.	65.8 cd	69.2 cd	12 d	nd	nd	nd
Diplodia mutila	48.9 ab	49.6 ab	7 ab	77.7 de	39.9 ef	63.9 e
Diplodia seriata	62.6 bc	58.9 c	11 cd	72.1 cde	53.5 g	64.3 e
Eutypella leprosa	58.8 bc	63.2 c	7 ab	51.4 b	33.2 de	33.1 c
Inocutis sp.	74.2 de	73.9 cd	11 cd	53.1 bc	11.3 ab	19.1 b
Neofusicoccum parvum	41.5 a	38.4 a	6 a	109.9 f	79.6 i	81.1 f
Phaeoacremonium aleophilum	64.3 cd	68.5 cd	10 bcd	nd	24.7 c	nd
Phaeomoniella chlamydospora	63.4 bc	65.7 c	10 bcd	88.0 e	43.8 f	45.0 d
Phomopsis viticola	80.4 e	78.6 d	10 bcd	nd	12.1 ab	15.5 b
Seimatosporium botan	62.7 cd	62.1 bc	8 abc	41.4 b	29.8 cd	37.1 c
Spencermartinsia viticola	42.1 a	42.8 a	6 a	89.9 ef	71.9 h	60.2 e
Control	122.4 f	124.9 e	15 e	10.1 a	5.9 a	6.8 a

[1]Mean length of roots, shoots and leaves of axenic 'Carménère' grapevine plantlets. [2]BS=Mean length of brown streaking. Means followed by the same letters in each column did not differ significantly according to Tukey's pairwise multiple comparison test (P=0.05). nd = not determined.

found, but no evidence of *Pa. chlamydospora* was obtained. However, *Pa. chlamydospora* was the most frequently isolated species in the valleys that are characterized by rather humid conditions, whereas in central Chile (Aconcagua to Colchagua valleys) with a cool Mediterranean climate, a great diversity of fungi were found associated with trunk diseases of grapevines. Vineyards in San Antonio Valley are relatively young, and this may explain the low isolation frequency of fungal trunk pathogens obtained in this study. *Phaeomoniella chlamydospora*, Botryosphaeriaceae spp. and *Inocutis* sp. were also detected in the valleys located in south-central Chile between Curicó and Bío-Bío, which is associated with a Mediterranean to humid temperate climate.

The present study reports 12 different fungal taxa associated with trunk diseases in Chilean vineyards. These results are in agreement with those reported in Argentina (Gatica *et al.*, 2000), France (Larignon and Dubos, 1997), Italy (Mugnai *et al.*, 1999), the USA (Úrbez-Torres *et al.*, 2012), Spain (Armengol *et al.*, 2001; Martin and Cobos, 2007) and South Africa (White *et al.*, 2011b) where trunk diseases are associated with up to 16 different fungal species. However, the relative prevalence of each taxon varied by country.

On the basis of these results, the most frequently isolated species was *Pa. chlamydospora*, followed by species of Botryosphaeriaceae and *Inocutis* sp. Similar results were reported in Italy and Spain, where *Pa. chlamydospora* was the most frequent species associated with grapevine trunk diseases (Martin and Cobos, 2007; Mugnai *et al.*, 1999). However, species of the families Diatrypaceae and Botryosphaeriaceae have been reported as the main fungal pathogens associated with grapevine trunk diseases in Australia and California (Pitt *et al.*, 2010; Trouillas *et al.*, 2010; Trouillas *et al.*, 2011; Úrbez-Torres *et al.*, 2006).

Similar to other grapevine-producing regions of the world (Martin and Cobos, 2007; Mugnai *et al.*, 1999; Úrbez-Torres *et al.*, 2012), *Pa. chlamydospora* has been associated with dark brown streaking that appears in very young grapevines,

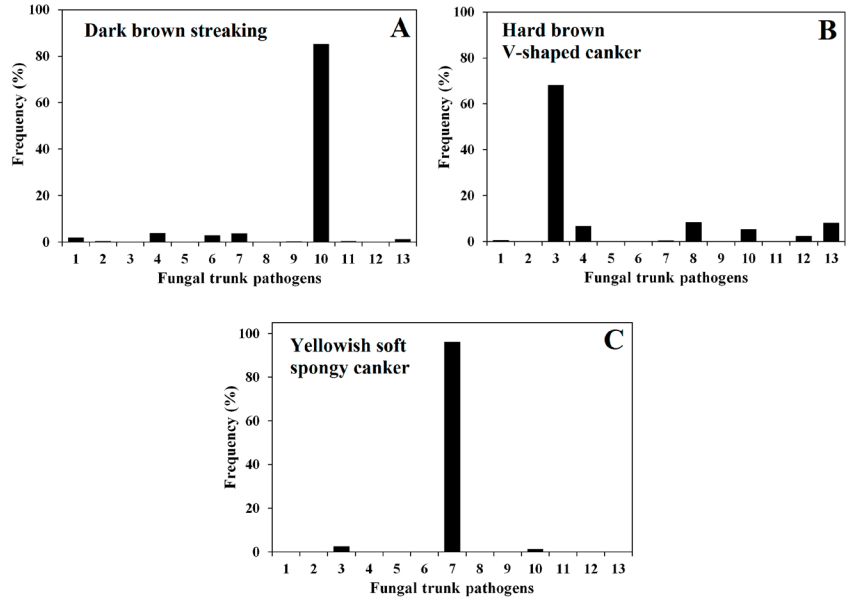

Figure 3. Frequency of fungal species obtained from samples according to wood canker type. A, Frequency according to presence of dark brown streaking canker (n=657 isolates). B, Frequency according to presence of hard brown V-shaped canker (n=392 isolates). C, Frequency according to presence of yellowish soft-spongy canker (n=314 isolates). 1, *Cryptovalsa ampelina*; 2, *Cylindrocarpon* sp.; 3, *Diplodia seriata*; 4, *Diplodia mutila*; 5, *Eutypa lata*; 6, *Eutypella leprosa*; 7, *Inocutis* sp.; 8, *Neofusicoccum parvum*; 9, *Phaeoacremonium aleophilum*; 10, *Phaeomoniella chlamydospora*; 11, *Phomopsis viticola*; 12, *Seimatosporium botan*; 13, *Spencermartinsia viticola*.

even at nurseries. The Botryosphaeriaceae species were mainly isolated from hard brown V-shaped cankers that developed primarily in relatively older (>7-year-old) plants. Several other studies have also associated Botryosphaeriaceae spp. with V-shaped cankers (Auger *et al*., 2004a; Morales *et al*., 2012; Úrbez-Torres *et al*., 2012; White *et al*., 2011a).

On the basis of morphology and ITS analysis, it was impossible to identify the species of the Chilean isolates of *Inocutis*. These *Inocutis* isolates were only obtained from yellowish soft-spongy cankers that were observed in grapevines over 10 years old. Other studies have demonstrated that basidiomycete fungi attack relatively old grapevine wood (Cortesi *et al*., 2001). *Fomitiporella vitis*, a basidiomycete fungus previously associated to chlorotic leaf roll in Chile (Auger *et al*., 2005), and other species of *Fomitiporella* were absent in this study, but these results do not exclude that these pathogens may be present in Chilean vineyards.

On the other hand, Diatrypaceae species, including *C. ampelina and Eu. leprosa*, were mainly obtained at a relatively low frequency from brown wood streaking. Previously, *C. ampelina* isolates have been obtained from hard brown cankers and brown vascular streaking (Díaz *et al*., 2011b). *Seimatosporium botan* was isolated from hard brown cankers (Díaz *et al*., 2012). For the first time, *Pho. viticola* was isolated from the dead arms of grapevines in Chilean vineyards. Previously, *Pho. viticola* was reported only as a foliar and fruit pathogen of grapevines in Chile (Acuña, 2010). However, the relative importance of these pathogens varies considerably from other vineyards worldwide (Armengol *et al*., 2001, Laringnon and Dubos, 1997; Pitt *et al*., 2010; Rolshausen *et al*., 2006; Trouillas *et al*., 2010; White *et al*., 2011b).

Several studies have reported that *Phaeoacremonium* spp. is an important fungi associated with the trunk diseases of grapevines (Armengol *et al*., 2001; Bertsch *et al*., 2013; Mugnai *et al*., 1999; Mostert *et al*., 2006). However, *Pm. aleophilum* was occasionally isolated in northern Chile in this study.

Regardless of the fungal species, all fungal isolates were pathogenic when they were tested in axenic 'Carménère' grapevine plantlets, but the symptoms varied among pathogen species from leaf chlorosis (*e.g., Inocutis* sp.) and reddening (*e.g*., *Pa. chlamydospora*) to a rapid death (*e.g*., Botryosphaeriaceae spp.) of the entire plantlets. These results were similar to those obtained by Gatica *et al*. (2000) working with 'Cabernet Sauvignon', 'Malbec' and 'Tempranillo' grapevines plantlets inoculated with *I. jamaicensis* (=*Phellinus* sp.), *Pa. chlamydospora* and Botryosphaeriaceae sp. The inoculated trunks of young grapevines displayed brown streaking lesions that varied in length from 41 mm (*Se. botan*) to 109 mm (*N. parvum*). Foliar symptoms, chlorosis and/or reddish leaves were only produced by *Pa. chlamydospora*, *Inocutis* sp. and Diatrypaceae spp., and no foliar symptoms were observed when grapevine plantlets were inoculated with Botryosphaeriaceae spp. and *Se. botan* (Úrbez-Torres, 2011). Pathogenicity was also demonstrated on inoculated shoots in bioassays as well in inoculated shoots in the vineyards, but only the induction of brown streaking was observed. In agreement with previous studies, the Botryosphaeriaceae species were the most virulent pathogens isolated in this study (Úrbez-Torres *et al*., 2012).

Samples of grapevines presenting chlorotic leaf roll, a grapevine disease previously described in Chile (Auger *et al.,* 2005), were analyzed in this study. However, the symptoms of chlorotic leaf roll were not induced in artificially inoculated plants in this study. Based on our results, it is very possible that several fungal trunk pathogens can be associated with chlorotic leaf roll, including *Inocutis* sp., *Fomitiporella* spp., *Pa. chlamydospora*, *Pm. aleophilum* and Diatrypaceae species. However, further research is needed to clarify the role of the pathogens identified in this study in the etiology of chlorotic leaf roll. In addition, *E. lata*, a pathogen inducing symptoms similar

to those described for chlorotic leaf roll (Bertsch *et al.,* 2013; Pitt *et al.,* 2010) was not identified in this study. To our knowledge, *E. lata* is absent in Argentina where "hoja de malvón," a disease similar to chlorotic leaf roll, has been described on grapevines (Gatica *et al*., 2000).

In conclusion, several fungal species are associated with grapevine trunk diseases in the Chilean vineyards, with *Pa. chlamydospora*, *D. seriata* and *Inocutis* sp. being the most frequent isolated species. These pathogens can be found alone, or they can coexist in the same plant. The tiger stripe foliar symptoms that were previously described for Esca disease were not observed in this study (Mugnai *et al*., 1999; White *et al*., 2011b). Interestingly, Eutypa dieback (*E. lata*), which is commonly found in other grape-growing areas of the world, was not identified in this study. Accepting that the climatic conditions in central and southern Chile are not a limiting factor for *E. lata,* and considering that the long-distance dissemination of *E. lata* only occurs in association with infected plant materials, it is possible that this pathogen has not yet been introduced into the country.

Resumen

G.A., Díaz, J., Auger, X., Besoain, E., Bordeu y B.A. Latorre. 2013. Prevalencia y patogenicidad de hongos asociados con enfermedades de la madera en viñedos Chilenos. Cien. Inv. Agr. 40(2): 327-339. Las enfermedades de la madera de la vid (*Vitis vinifera*) se han identificado como un importante problema sanitario de la industria vitícola en el mundo, reduciendo la productividad, calidad y longevidad de los viñedos. En el presente estudio se examinó 694 muestras de brazos y troncos de vides con cancrosis en la madera recolectada en 67 viñedos entre Copiapó (27° 18' S) y Los Ángeles (37° 42' S), Chile. Se obtuvieron 1.363 aislados fúngicos desde brazos y troncos enfermos, caracterizados por presentar estrías necróticas vasculares, cancros blandos, esponjosos, blanco-cremosos y cancros firmes en consistencia, pardos, a menudo en forma de V e cortes transversales. Por análisis molecular de la región ITS del ADNr, se identificaron 12 géneros de hongos fitopatógenos los correspondieron a: *Phaeomoniella chlamydospora* (85%), especies de Botryosphaeriaceae (56%) (*Diplodia mutila*, *D. seriata*, *Neofusicoccum parvum* y *Spencermartinsia viticola*), *Inocutis* sp. (47%), *Diatrypaceae spp.* (*Cryptovalsa ampelina* y *Eutypella leprosa*) (4,8%), *Seimatosporium botan* (1,7%), *Phomopsis viticola* (0,4%), *Cylindrocarpon* sp. (0,4%) y *Phaeoacremonium aleophilum* (0,2%). Estas especies fueron patogénicas, induciendo estrías necróticas vasculares, pardo oscuras, a negras en tejidos semi-lignificados y lignificados de vid. En conclusión, varias especies de hongos fitopatógenos están asociados a cancrosis de la madera en viñedos siendo *Pa. chlamydospora*, *D. seriata* e *Inocutis* sp. las especies mas frecuentemente encontradas. Estos hongos fitopatógenos pueden encontrarse solos o se pueden encontrar coexistiendo en la misma planta. Este es el primer reporte de *Pho. viticola* asociado con enfermedades de la madera en Chile.

Palabras clave: Esca, hongos de la madera, pudrición de la madera.

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