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Distribution of three grapevine trunk pathogens in Chilean vineyards, determined using molecular detection from asymptomatic woody pruning material

Felipe GAÍNZA-CORTÉS[1], Rosa ROA-ROCO[1], Paulina ARRAÑO-SALINAS[1], Pablo RODRÍGUEZ-HERRERA[1], Mauricio A. LOLAS[2], José Carlos CARIS-MALDONADO[1], Patricia SILVA-FLORES[3,4,*], Álvaro GONZÁLEZ[1]

[1] *Viña Concha y Toro S.A, Center for Research and Innovation, Fundo Pocoa s/n, Km10 Ruta K-650, Región del Maule, Pencahue, Chile*
[2] *Universidad de Talca, Faculty of Agricultural Sciences, Fruit Pathology Laboratory, 3460000, Talca, Chile*
[3] *Centro de Investigación de Estudios Avanzados del Maule (CIEAM), Vicerrectoría de Investigación y Postgrado, Universidad Católica del Maule, Avenida San Miguel 3605, Talca, Chile*
[4] *Centro del Secano, Facultad de Ciencias Agrarias y Forestales, Universidad Católica del Maule, Avda. San Miguel 3605 Talca, Chile*
*Corresponding author. E-mail: psilva@ucm.cl

Summary. Grapevine Trunk Diseases (GTDs) cause important economic losses in vineyards. Diagnosis of pathogens causing GTDs is usually achieved using culture methods from symptomatic plants, but these methods may not be definitive. This study aimed to detect three fungi associated with GTDs in Chile (*Diplodia seriata*, *Phaeomoniella chlamydospora* and *Eutypa lata*) using qPCR methods, after extracting DNA from asymptomatic woody pruning material taken from 912 grapevine plants and 346 rootstocks. Pathogen incidence and distribution across different regions, vineyards, grape and wine cultivars, plant ages and rootstocks were determined. Forty percent of assessed grapevine plants and 42% of rootstocks were positive for the assayed GTD pathogens. The fungus with the greatest incidence was *D. seriata* (36% of plants and 30% of rootstocks), followed by *E. lata* (6% of plants and 20% of rootstocks) and *P. chlamydospora* (4% of plants and 5% of rootstocks). Positive relationships were detected between the presence of *D. seriata* and *P. chlamydospora* and grapevine age. However, the three fungi were randomly distributed across graprevine plants and rootstocks since none of the assessed factors had statistically significant influence in their distribution. This study is the first to assess incidence and distribution of *E. lata* in Chile. The qPCR-based method described could be used for detecting fungi in asymptomatic nursery plants, and be used to avoid distribution of infected plants into new vineyard plantings.

Keywords. qPCR, early detection, *Vitis vinifera*, trunk pathogens, wood fungi.

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INTRODUCTION

Grapevine Trunk Diseases (GTDs) causes major economic problems in vineyards, decreasing their longevity, yields and quality of wine produced. International cost for the replacement of dead grapevine plants resulting from GTDs is estimated to be over 1.5 billion dollars each year (Hofstetter *et al.,* 2012). Accordingly, much research has been carried out to understand these important diseases in many countries (Mondello *et al.,* 2018; Grozić *et al.,* 2019; Songy *et al.,* 2019). The currently accepted GTDs are Esca, Botryosphaeria dieback and canker, Eutypa dieback and Phomopsis dieback (Bertsch *et al.,* 2013; Fontaine *et al.,* 2016; Gramaje *et al.,* 2018). The status of these diseases in Chilean wine production is scarce but they are likely to be detrimental to the wine industry (Besoain, 2018).

Chile is the world's sixth most important wine producer and the fourth most important wine exporter. Grapevines are grown in several valleys between the northern Coquimbo region (29°54'S, 71°15'W), and the southern Biobío region (36°46'22"S, 73°03'47"W). A total of 137,000 ha of cultivated wine grapes are grown, with the Maule (53,687 ha) and O´Higgins (45,782 ha) regions being the main wine producers (SAG, 2018).

Several fungi have been internationally associated with GTDs, and have been identified and described in Chile during the last decade. These include, the Ascomycete species of the *Botryosphaeriaceae*, *Phaeoacremonium* spp., *Diatrypaceae* spp., *Phaeomoniella chlamydospora*, and some Basidiomycetes such as *Inocutis* spp. (Auger *et al.,* 2004; Díaz *et al.,* 2009; 2011). In addition, Díaz *et al.* (2013) in a survey including 67 vineyards from the Antofagasta region (27°18'S) to the Biobío region (37°42'S), and using traditional culture methods, as well as molecular identification techniques, found a total of 12 fungal species. These fungi were present at varying frequencies, all were pathogenic, coexisting in plants in most cases, and inducing dark brown streaking in grapevine wood tissues of different ages. The most frequently isolated species were *P. chlamydospora* (85% incidence), *Diplodia seriata* (56%), and *Inocutis* sp. (47%). These results were partially confirmed in a survey from 2016 to 2018, where vineyards from the Valparaiso (33°02'S) to Maule (35°25'S) region were assessed. This focused on GTD symptomatic plants from vineyards of different ages. The pathogens most frequently isolated were *Inocutis* sp. (49% of isolates), *Botryosphaeriaceae* (*i.e. D. seriata* and *N. parvum,* 23%) and *P. chlamydospora* (18%) (Unpublished data). *Eutypa lata* was also reported from Chile, using molecular and morphological analyses (Lolas *et al.,* 2020). This species is one of the most internationally common GTD pathogen (Siebert, 2001; Travadon *et al.,* 2012). However, and due to recent findings, distribution of *E. lata* is not known within the Chilean wine production regions.

Diagnosis of GTD pathogens from grapevine plants has traditionally been carried out using culture isolation methods, morphological analyses, and molecular markers based on the Internal Transcribed Spacer (ITS) region and/or the beta-tubulin gene (Rolshausen *et al.,* 2013; Luque *et al.,* 2014; Úrbez-Torres *et al.,* 2015; Gramaje *et al.,* 2018). However, these methods can lead to inconsistent results, and identification of pathogen species can be complicated due the plasticity of morphological characters (Rolshausen *et al.,* 2004; Pavlic *et al.,* 2009). Based on the increasing availability of fungal genomic sequences from public repositories (Blanco-Ulate *et al.,* 2013a,b; Morales-Cruz *et al.,* 2015), and on the PCR specificity and sensibility, strategies using quantitative real-time PCR (qPCR) have been shown to be more suitable for detecting and quantifying the DNA of most of the main fungi associated to GTDs (Pouzoulet *et al.,* 2013, 2017; Reis *et al.,* 2019).

Most of the GTD diagnoses described above have been conducted on symptomatic plants. In nursery propagation processes, the sensitivity of the PCR-based diagnostic tool becomes relevant (compared with traditional culture methods) where mother plants and the propagation material (mostly asymptomatic) are important sources of inoculum for fungal trunk pathogens (Aroca and Raposo, 2007; Aroca *et al.,* 2010).

Factors affecting the diversity of fungi associated with GTDs are poorly understood (Del Frari *et al.,* 2019). Grape cultivar has been related to the fungi causing these diseases (Travadon *et al.,* 2016), but this has not been studied in Chilean vineyards. Molecular tools can provide accurate identification of GTD pathogens.

The aim of the present study was to determine the incidence of two of the most important fungi associated with GTDs in Chile (*D. seriata* and *P. chlamydospora*), and the recently reported *E. lata*, using qPCR-based methods and extracting DNA directly from asymptomatic woody pruning material. This was to develop understanding of the distribution patterns of the fungi associated with GTDs in this country.

MATERIALS AND METHODS

Sampling

Eight vineyards (hereafter designated V0 to V7), in the main wine grapevine production valleys of Chile, were surveyed from 2016 to 2019. The vineyards were

between 5 to 23 years old and were in several regions of Chile, including Coquimbo (29°54'S), Valparaíso (33°02'S), O´Higgins (34°10'S) and Maule (35°25'S), with mean annual rainfall ranging from 1 to 1,000 mm, mostly during winter months. Collected samples consisted of at least 5–8 pruned 1-year-old canes per plant, taken from asymptomatic woody pruning material (no visually evident wood symptoms). Sixteen *V. vinifera* cultivars were sampled, including: 'Cabernet franc' (CF), 'Cabernet Sauvignon' (CS), 'Carménère' (CA), 'Chardonnay' (CH), 'Gewürztraminer' (GT), 'Malbec' (MA), 'Marselan' (Ma), 'Merlot' (ME), 'Pedro Jimenez' (PJ), 'Petit Syrah' (PS), 'Petit Verdot' (PV), 'Pinot noir' (PN), 'Riesling' (RI), 'Sauvignon blanc' (SB), 'Syrah' (SY), and 'Viognier' (VI). Five rootstocks located in V6 from Maule region were also sampled, including: 101-14 MGt (*V. riparia* Michx × *V. rupestris* Scheele), 110 Richter (*V. berlandieri* Planch × *V. rupestris*), 1103 Paulsen (*V. berlandieri* × *V. rupestris*), 3309 Couderc (*V. riparia* × *V. rupestris*), and SO4 (*V. berlandieri* × *V. riparia*). The samples were transported at 4°C to a laboratory within 24 hours of collection and were subsequently stored at -80°C until processed.

Incidence of the three main GTD pathogens considered for this study (*D. seriata*, *E. lata* and *P. chlamydospora*) was determined as the percentage of the positively infected samples in relation to the total number of plants samples per region, vineyard, grape cultivar or wine cultivar (red or white), depending on the particular case. For the rootstock samples, pathogen incidence was determined as the percentage of positively infected plants in relation to the total number of plants sampled per rootstock type.

Sequence alignment and primer design

Partial sequences of the *P. chlamydospora* beta-tubulin gene were retrieved from the GenBank database at the NCBI website (https://www.ncbi.nlm.nih.gov/genbank/). To evaluate sequence variability between international and Chilean isolates, *P. chlamydospora* beta-tubulin sequences were aligned using Clustal Omega from the European Molecular Biology Laboratory, EMBL-EBI. The international sequences used were *P. chlamydospora* strain CBS 229.95 (accession No. AF253968), STE-U 3066 (AF253969), Y83-8-1 (EU078329), Y121-20-1 (EU078333), Pch-2 (GQ903724), Pch-3 (GQ903725), UCD2548MO (HQ288313), UCD-2586MO (HQ288316), PARC143 (KF764664), PARC326 (KF764667), and IBVD01 (KP213108). Chilean sequences used were *P. chlamydospora* isolate Pach-525 (JX679869), Pach-394 (JX679870), Pach-304 (JX679871), Pach-300 (JX679873), Pach-297 (JX679874), Pach-132 (JX679877), Pach-128 (JX679878), Pach-102 (JX679880), Pach-59 (JX679882), Pach-36 (JX679885), Pach-7 (JX679888), and Pach-6 (JX679889). Primers and TaqMan probe designs were carried out using the GenScript Real-time PCR (TaqMan) Primer Design tool (https://www.genscript.com/tools/real-time-pcr-taqman-primer-design-tool). To detect *D. seriata* and *E. lata*, the SYBR Green primer sets described by Pouzoulet *et al.* (2017) were used.

All primer conditions have been standardized for efficiency between 90 to 110%, R^2 ≥0.995. Specificity of primers has been validated by sequencing amplicons obtained and using BLASTN (NCBI), and in all cases the sequenced amplicons matched the expected amplicons. For *D. seriata*, the sequencing results coincided with *D. seriata* beta-tubulin annotated sequences from New Zealand (accession nº MN245025), United States of America (MN318125), Spain (MK522087) and Chile (MG952722), among other beta-tubulin sequences from *D. seriata*. For *E. lata*, amplicon sequences aligned with Australian sequences (MN433695), and others annotated in the United States of America (MN329778, HM164739, HM164762). In the case of *P. chlamydospora*, sequencing results aligned with beta tubulin sequences from Brazil (MN747132, MK903808), Spain (MG7458109), and Canada (KF764664), among other sequences from *P. chlamydospora*. In addition, the sequence from this fungus had 100% similarity with the Chilean *P. chlamydospora* beta-tubulin sequences annotated in GenBank used for primer design (JX679869, JX679870, JX679871, JX679873, JX679874, JX679877, JX679878, JX679880, JX679882, JX679885, JX679888, JX679889).

Total DNA purification

Frozen wood samples from all the pruned 1-year-old canes sampled per plant were each ground transversally at the center of the cane using a drill and liquid nitrogen, without grinding bark. Approx. 100 mg of wood chip representative of the sample was used to isolate total DNA using the DNeasy Plant Mini Kit (Qiagen, Germany). DNA quality and quantity were determined using an Infinite M200Pro NanoQuant spectrophotometer (Tecan) and a Qubit 3.0 fluorometer (Thermo Fisher Scientific Inc.).

Quantitative real-time PCR (qPCR) analyses

To assess DNA quality and absence of inhibitors, all samples were first amplified using Actin primers with the TaqMan probe (Bruisson *et al.*, 2017). The TaqMan system was also used to detect *P. chlamydospora*. In both

cases, the reactions were each prepared in a final volume of 10 µL, containing 5 µL of TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific), 2 µL of DNA sample, and primers were each used at a final concentration of 600 nM and probe at 200 nM. The amplification profile used was 50ºC for 2 min, 95ºC for 20 sec, followed by 40 cycles each of 95ºC for 1 sec, 56ºC for 5 sec and 60ºC for 15 sec.

To detect *D. seriata* and *E. lata,* the SYBR green system was used. Reactions were performed in a final volume of 10 µL, containing 5 µL of PowerUp™ SYBR© Green Master Mix (Thermo Fisher Scientific), 2 µL of DNA sample, and primers were each used at a final concentration of 500 nM. The amplification profile used was 50°C for 2 min, 95°C for 2 min, followed by 40 cycles each of 95°C for 1 sec and 60°C for 30 sec. The melting curve was at 95°C for 15 sec and then from 60°C to 95°C (increasing 1.5°C sec^{-1}). All reactions were run in the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). Primers and probe sequences are outlined in Table 1.

Statistical analyses

In order to understand the distribution patterns of the fungi associated with GTDs and the factors that might be explaining these patterns, the presence/absence data matrix of *P. chlamydospora*, *D. seriata* and *E. lata* was used. Samples were grouped by region, vineyard, grape cultivar, wine cultivar or age, constituting 25 different samples. Incidence was calculated for each sample. With both data sets (presence/absence and incidence), several analyses were performed using R 3.5.1 (R Development Core Team, 2020).

Firstly, whether age of the grape cultivar affected GTDs presence/absence was analyzed. For this the ggpubr package (Kassambara, 2020) was used, using the *stat_compare_means* function, in order to perform mean comparisons with a Kruskal-Wallis test.

Secondly, and to understand the distribution of the GTD pathogens across samples, a heatmap was built using the function *heatmap.2* of the gplots package (Warnes *et al.,* 2020). This made it possible to visualize how similar the samples were in terms of the fungal species of interest. Possible correlations between the incidence of GTD pathogens and the age of plants was then assessed. For the regressions the ggpubr package (Kassambara, 2020) was used for these regressions, using the *stat_regline_equation* function. To understand which factors affected the GTD pathogen composition in the samples and considering the unbalanced design, ANOSIM tests were performed to determine whether the region, vineyard, grape cultivar, wine cultivar (red or white) or vine age had effects on pathogen composition. Analyses were carried out with the incidence table, using the *anosim* function of the *vegan* package (Oksanen *et al.,* 2019), considering a Jaccard distance matrix with 999 permutations. The samples with absence of all the three species of fungi were removed in order to conduct the analysis.

For rootstocks, the presence/absence data matrix was used to calculate the incidence of the GTD pathogens. Since five rootstocks were sampled, the analysis considered five different samples, and a heatmap was constructed as described above. The matrix of pathogen incidence was used to test whether the type of rootstock had an influence of pathogen composition, through an ANOSIM.

RESULTS

Samples from a total of 912 plants, from four regions of Chile (Table 2), and from eight vineyards (Table 3) containing a total of 16 grape cultivars (Table 4), were

Table 1. Oligonucleotides used on this study.

Target	Detection system	Primer sequence (5'->3')	Reference
Phaeomoniella chlamydospora	TaqMan	PchF: GCTGACGACGTCCAGGGTAA PchR: TCAAGGCCGTGTTCACCAGA PchProbe: CCTCGACGACTACAGACGCGCCA	This study
Diplodia seriata	SYBR Green	DseCQF: CTCTGCAATCGCTGACCCTTG DseCQR: ACGTGTTTGTCTAACTAGTAGAGAGTACC	Pouzoulet *et al.,* 2017
Eutypa lata	SYBR Green	ElQF:GCCAGCTAATAAAACAATTGCTTACCT ElQR: AGATAACCTCGTGTGATTGTGTGATT	Pouzoulet *et al.,* 2017
Actin	TaqMan	ActF: GTATTGTGCTGGATTCTGGTGAT ActR: GCAAGGTCAAGACGAAGGATAG ActProbe: CACTGTGCCAATTTATGAAGGTTATGCACTTC	Bruisson *et al.,* 2017

Table 2. Incidence of GTD pathogens by region in Chile. The regions are ordered from north to south of the country. No. = number, GTD- = plants with no GTD fungi, GTD+ = plants with GTD fungi. *D_s = Diplodia seriata*, *E_l = Eutypa lata* and *P_c = Phaeomoniella chlamydospora*. The number below each GTD fungus corresponds to the number of positive plants infected by that fungus.

Region	No. Total Plants	No. Plants GTD-	% Plants GTD-	No. Plants GTD+	% Plants GTD+	*D_s*	*E_l*	*P_c*
Coquimbo	7	7	100	0	0	0	0	0
Valparaíso	50	37	74.0	13	26.0	13	3	0
O`Higgins	316	121	38.0	195	62.0	175	20	18
Maule	539	382	71.0	157	29.0	137	32	18
No. Total	912	547		370		325	55	36
%			60.0		40.6	35.6	6.0	3.9

Table 3. Incidence of GTDs and GTD pathogens in seven Chilean vineyards. The vineyards are ordered from north to south of Chile. V = Vineyard, C = Coquimbo, V = Valparaíso, O = O´Higgins and M = Maule, n = number, GTD- = plants with no GTD fungi, GTD+ = plants with presence of GTD fungi. *D_s = Diplodia seriata*, *E_l = Eutypa lata* and *P_c = Phaeomoniella chlamydospora*. The numbers below each GTD fungus are the number of positive plants for that fungus.

Vineyard (Region)	No. Total Plants	No. Plants GTD-	% Plants GTD-	No. Plants GTD+	% Plants GTD+	*D_s*	*E_l*	*P_c*
V0 (C)	7	7	100	0	0	0	0	0
V1 (V)	50	37	74.0	13	26.0	13	3	0
V2 (O)	59	54	91.5	5	8.5	2	1	2
V3 (O)	257	67	26.1	190	73.9	173	19	16
V4 (O)	93	78	83.9	15	16.1	13	1	1
V5 (M)	24	21	87.5	3	12.5	2	1	0
V6 (M)	334	203	60.8	131	39.2	116	29	15
V7 (M)	88	80	90.9	8	9.1	6	1	2
No. Total	912	547		365		325	55	36
%			60.0		40.0	35.6	6.0	3.9

analyzed in this study. The samples included white and red cultivars (Table 5), and the sampled plants were of age between 5 and 23 years.

The data analyzed for GTD pathogen incidence in the different regions indicated that in the Valparaíso and Maule regions, the proportions of plants that were negative for these fungi were greater than the proportions of those infected. The samples from the O`Higgins region, on the other hand, had greater incidence of GTD pathogens in relation to plants with no pathogenic fungal species. The most frequently detected fungus was *D. seriata*, and samples from the O'Higgins and Maule regions were infected by *D. seriata*, *P. chlamydospora* and *E. lata* (Table 2). None of these pathogens were found in Coquimbo region, and *P. chlamydospora* was not detected in samples from Valparaíso.

Samples from two vineyards (V3 and V6) had high numbers of GTD pathogens. Vineyard V3 was in a coastal area, while vineyard V6 was located towards the Central Valley of Chile.

Incidence of GTD pathogens per grape cultivar showed the presence of at least one GTD fungus in most of the cultivars examined (Table 4). High incidence was detected in several cultivars, and *D. seriata* was always the dominant pathogen in these cultivars.

White wine cultivars had higher incidence of GTD pathogens than red wine cultivars (Table 5). However, the proportion of healthy plants was higher for both cases.

The age of the vines had a significant effect on the presence/absence of *D. seriata* (Figure 1a) and *P. chlamydospora* (Figure 1c). For both pathogens, that the older plants had more infections with these fungi than young plants.

The regressions between age of plants and the incidence of GTD pathogens showed positive but not statistically significant relationships for the three fungal species: *D. seriata* (R = 0.3, *P* = 0.19), *E. lata* (R = 0.12, *P* = 0.71) and *P. chlamydospora* (R = 0.5, *P* = 0.21).

Diplodia seriata had the greatest incidence across most samples, followed by *E. lata* (Figure 2). *Phaeomo-*

Table 4. Incidence of GTDs by grape cultivar. No. = number, GTD- = plants with no GTD fungi, GTD+ = plants with GTD fungi. *D_s* = *Diplodia seriata*, *E_l* = *Eutypa lata* and *P_c* = *Phaeomoniella chlamydospora*. The numbers below each fungus are the number of positive plants for that fungus.

Grape cultivar (vine age: years)	No. Total Plants	No. Plants GTD-	% Plants GTD-	No. Plants GTD+	% Plants GTD+	*D_s*	*E_l*	*P_c*
Cabernet franc (8 –11)	29	25	86.2	4	13.8	4	0	0
Cabernet Sauvignon (10–13)	157	99	63.1	58	36.9	51	20	9
Carménère (7–3)	84	69	82.1	15	17.9	11	1	4
Chardonnay (11–15)	90	48	53.3	42	46.7	40	5	1
Gewürztraminer (11)	10	9	90.0	1	10.0	1	0	0
Malbec (11)	10	10	100	0	0	0	0	0
Marselan (11)	9	8	88.9	1	11.1	1	0	0
Merlot (5–11)	141	120	85.1	21	14.9	18	2	1
Pedro Jimenez (7–11)	7	7	100	0	0	0	0	0
Petit Syrah (11)	2	2	100	0	0	0	0	0
Petit Verdot (11)	18	1	5.6	17	94.4	17	0	0
Pinot noir (14)	98	6	6.1	92	93.9	91	7	0
Riesling (11)	5	4	80.0	1	20.0	1	0	0
Sauvignon blanc (11–14)	225	117	52.0	108	48.0	85	20	21
Syrah (13)	6	3	50.0	3	50.0	3	0	0
Viognier (6)	21	19	90.5	2	9.5	2	0	0
No. Total	912	547		365		325	55	36
%			60.0		40.0	35.6	6.0	3.9

niella chlamydospora had the least incidence across the 25 samples studied. For the Coquimbo region, none of the seven analyzed plants from the cultivar 'Pedro Jimenez' were positive for GTD pathogens, in contrast with the other three regions. In addition, in the O`Higgins region and specifically in V2, the 20 plants of 'Cabernet franc' analyzed were also negative for all three GTD pathogens investigated. In the Maule region and specifically in V6, 'Malbec' (ten plants) and 'Petit Syrah' (two plants) cultivars were negative for all three GTD pathogens (Figure 2).

The analysis of GTD pathogen composition in relation to region, vineyard, grape cultivar, wine cultivar and vine age factors showed that none of these factors had statistically significant effects upon the composition of GTD pathogens detected in the sampled grapevine plants. However, region had an almost significant effect ($P = 0.06$) on pathogen composition, explaining 20% of the variance between regions.

Assessments of GTD pathogen incidence in rootstock plants, showed that *D. seriata* and *E. lata* were the most frequently detected species in all five sampled rootstocks (Table 5). None of the rootstocks were free of GTD fungi and almost half of the rootstocks were infected with *D. seriata* and *E. lata* (Table 6).

The heatmap (Figure 3) showed that rootstocks 101-14 and 3309 had similar incidences of GTD patho-

Table 5. Incidence of GTD pathogens by wine cultivar type. No. = number, GTD- = plants with no GTD fungi, GTD+ = plants with GTD fungi. *D_s* = *Diplodia seriata*, *E_l* = *Eutypa lata* and *P_c* = *Phaeomoniella chlamydospora*. The numbers below each fungus are the number of positive plants for that fungus.

Wine cultivar type	No. Total Plants	No. Plants GTD-	% Plants GTD-	No. Plants GTD+	% Plants GTD+	*D_s*	*E_l*	*P_c*
Red	554	343	62.0	211	38.0	196	30	14
White	358	204	57.0	154	43.0	129	25	22
No. Total	912	547		365		325	55	36
%			60		40.0	35.6	6.0	3.9

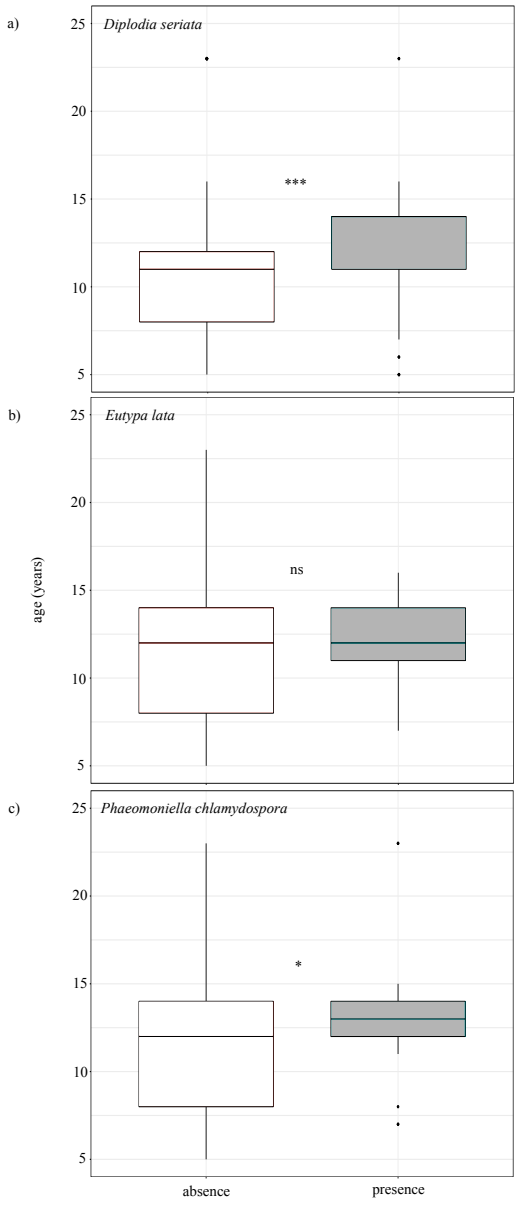

Figure 1. Effect of age of plants on the presence and absence of a) *Diplodia seriata* (*** $P < 0.001$) b) *Eutypa lata* (not significantly different) and c) *Phaeomoniella chlamydospora* (*$P < 0.05$).

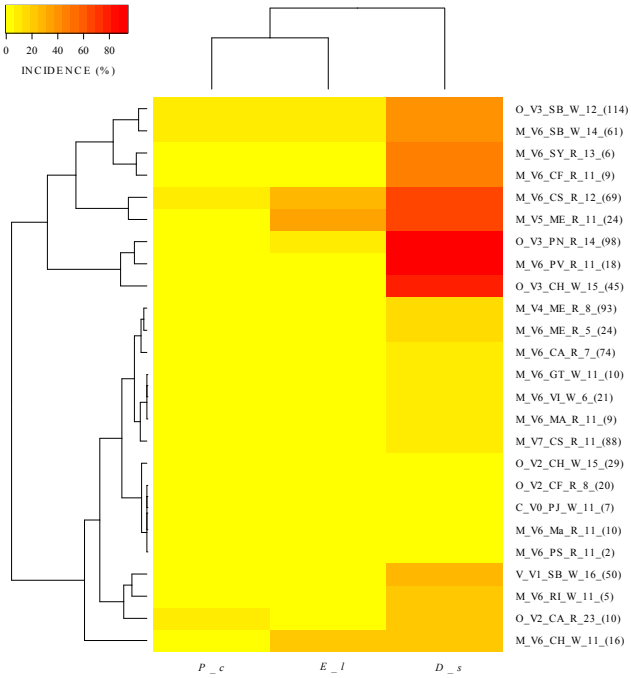

Figure 2. Heatmap with GTDs fungal distribution in the 25 samples of this study. M: Maule region, O: O`Higgins region, V: Valparaíso region, C: Coquimbo region, V0-V7: vineyards 0 to 7, CF: 'Cabernet franc', CS: 'Cabernet Sauvignon', CA: 'Carménère', CH: 'Chardonnay', GT: 'Gewürztraminer', MA: 'Malbec', Ma: 'Marselan', ME: 'Merlot', PJ: 'Pedro Jimenez', PS: 'Petit Syrah', PV: 'Petit Verdot', PN: 'Pinot noir', RI: 'Riesling', SB: 'Sauvignon blanc', SY: 'Syrah', VI: 'Viognier', W: white wine cultivar, R: red wine cultivar, numbers in the fifth position of each sample name indicates age in years. Next to each sample in brackets the number of plants per sample is depicted. *P_c*: *Phaeomoniella chlamydospora*, *E_l*: *Eutypa lata* and *D_s*: *Diplodia seriata*.

gens, with the pathogens occurring simultaneously and *D. seriata* and *E. lata* occurring more frequently. In the other three rootstocks, only *E. lata* and *D. seriata* were detected (Figure 3). The pathogen composition analysis revealed that the type of rootstock had a statistically significant effect ($P < 0.01$)on GTDs fungal composition.

Table 6. Incidence of GTD pathogens in grapevine rootstock plants. NO. = number, GTD- = plants with no GTD fungi, GTD+ = plants with GTD fungi. *D_s* = *Diplodia seriata*, *E_l* = *Eutypa lata* and *P_c* = *Phaeomoniella chlamydospora*. The number below each GTD fungus corresponds to the number of positive plants infected by that fungus.

Rootstock (age in years)	No. Total Plants	No. Plants GTD-	% Plants GTD-	No. Plants GTD+	% Plants GTD+	*D_s*	*E_l*	*P_c*
101-14 (20)	80	39	49.0	41	51.3	27	27	10
1103P (20)	77	53	69.0	24	31.0	15	13	2
110-R (20)	46	21	46.0	25	54.0	21	9	0
3309 (20)	49	24	49.0	25	51.0	16	15	5
SO4 (20)	94	63	67.0	31	33.0	25	6	2
No. Total	346	200		146		104	70	19
%			58.0		42.0	30.0	20.0	5.0

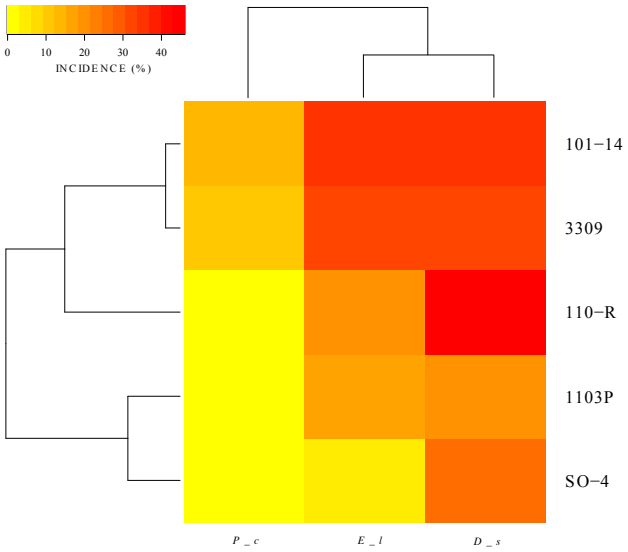

Figure 3. Heatmap with GTDs fungal distribution in the five rootstocks considered on this study. *P_c: Phaeomoniella chlamydospora, E_l: Eutypa lata* and *D_s: Diplodia seriata.*

DISCUSSION

Part of the aim of this study was to detect two of the most frequently occurring fungi (*P. chlamydospora* and *D. seriata*) associated with GTDs in Chile, and the globally important pathogen *E. lata* recently reported for Chile. In this research, the three pathogens were detected using qPCR-based methods and obtaining DNA material directly from asymptomatic grapevine woody pruning material. Using these methods is an advance compared with traditional methods of pathogen detection (Diaz *et al.,* 2013). Traditional methods have involved isolation and morphological identification of pathogens, and have been widely used due to simplicity and low cost. However, they require considerable time investment and have several disadvantages. They lack specificity, sometimes giving inaccurate results (false negatives) (Pouzoulet *et al.,* 2017), and are particularly complex since the fungi involved do not form key reproductive structures that allow identification from *in vitro* cultures (*e.g. E. lata*) (Rolshausen *et al.,* 2004; Pavlic *et al.,* 2009). Mycological experience is also essential to achieve accurate pathogen identification (Fischer and Dott, 2002). Use of qPCR for the detection of fungal pathogen has recently been demonstrated for grapevine pruning wounds (Pouzoulet *et al.,* 2017) and for apple trees (Gringbers *et al.,* 2020). Pouzoulet *et al.* (2017) demonstrated this for *E. lata* and *D. seriata,* and the present study has confirmed their results with Chilean material, and has added the possibility of detection of *P. chlamydospora* in grapevines through the design of a specific probe and primers based on international and Chilean isolates.

The present study has demonstrated of high sensitivity and specificity through direct diagnosis using qPCR, which efficiently differentiated several GTD-causing fungi from complex samples of woody pruning material. This study has confirmed that asymptomatic grapevine mother plants and propagation material can also be sources of fungal trunk pathogens as has been previously suggested (Aroca and Raposo, 2007; Aroca *et al.,* 2010). Consequently, sensitive PCR-based diagnostic tools become relevant in early grapevine propagation stages to assist prevention of pathogen dissemination. Another advantage of this approach is that it can be rapidly applied, and the results are quickly obtained relative to classical *in vitro* culturing methods. These methods are useful for rapid and accurate decisions in vineyards, and for early detection of pathogens in nursery plants, to assist high quality and production standards of for young plants that are free of GTD pathogens.

The present study also determined the relative incidence of the three fungal pathogens in the main grape-

vine growing regions of Chile. In the samples examined, incidence of *D. seriata* was 36%, *E. lata* was 6% and *P. chlamydospora* was 4%. This was found in analyses of samples from 912 plants, from four regions of Chile, eight vineyards, 16 grapes cultivars of white and red grape varieties, with plants from 5 to 23 years old. These results contrast with those of Diaz *et al.* (2013), who reported incidence of *D. seriata* at 56% and *P. chlamydospora* at 85%. However, Diaz *et al.* (2013) examined only GTD symptomatic plants. This difference suggests the presence of biotic or abiotic filtering in the environment, that could favour one pathogen species over another once symptoms appear (Crowther *et al.,* 2014). It is widely acknowledged that once the fungi sporulate, airborne spores are transported after rain (Úrbez-Torres *et al.,* 2010), and natural or artificial wounds are sites of host infection, followed by develop and reproduction (Bertsch *et al.,* 2013). However, even when different fungal species spores attach to the same wound, other factors, such as intrinsic species traits, may determine infection success of one species over another (Crowther *et al.,* 2014).

The incidence of *E. lata* infections was only 6% in asymptomatic plants. Future research should also consider symptomatic plants, to establish accurate incidence of this GTD pathogen. However, GTD pathogens were only detected only in some grape cultivars ('Sauvignon blanc', 'Pinot noir', 'Merlot', 'Chardonnay', 'Carménerè' and 'Cabernet Sauvignon'), and it has been reported that their occurrence can be related to warm temperatures and high humidity (Ramírez and Pineda, 2003).

In contrast for rootstocks, incidence for *D. seriata* was 30%, *E. lata* 20%, and *P. chlamydospora*, 5%. The only rootstock for which particular GTD pathogens were not detected was 110-R, where *P. chlamydospora* was not found. This suggest that rootstocks are important reservoirs for GTD pathogens. This raises the possibility that during grafting processes, nursery treatments (disinfection), biotic approaches (cultivar choice) or abiotic filtering (climatic conditions) could inhibit pathogen at least for *E. lata* (Crowther *et al.,* 2014).

The current study also considered to provide understanding of distribution patterns of the fungi associated with GTDs, and the factors that explain those patterns. A relationship was detected between grapevine age and the presence of GTD pathogens. This occurred for *D. seriata* and *P. chlamydospora*, but not for *E. lata*. Older plants had greater presence of the two GTD fungi than young plants, which was probably related to greater exposure to pruning and more exposed wounds susceptible to infections from pathogen inoculum.

In contrast with previous studies (Travadon *et al.,* 2016), the present research did not indicate effects of region, vineyard, grape and wine cultivar or plant age on GTD pathogen composition, factors which have not been previously considered in Chilean viticulture. Similarly, different rootstocks did not influence fungal composition. In most cases, random distributions of the GTD fungi occurred for all the plants investigated. This strongly suggests that these fungi were opportunists, and the first to arrive at susceptible host sites will be the successful colonizer. This study has demonstrated that GTD pathogens can be detected in asymptomatic plants, and in in grapevine nurseries. Avoiding transmission of these pathogens into productive vineyards is likely to be a prominent option for managing GTDs.

CONCLUSIONS

This study reports a rapid, accurate and reliable method for detecting three GTD fungi (*D. seriata, E. lata* and *P. chlamydospora*) in asymptomatic grapevine plants, and random distribution of the GTD pathogens in relation to environmental conditions. This knowledge supports adoption of appropriate disease management practices for detecting the fungi in asymptomatic nursery plants, which will reduce transmission of these pathogens into vineyard through young planting material.

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LITERATURE CITED

Aroca Á., Gramaje D., Armengol J., García-Jiménez J., Raposo R., 2010. Evaluation of the grapevine nursery propagation process as a source of *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* and occurrence of trunk disease pathogens in rootstock mother vines in Spain. *European Journal of Plant Pathology* 126: 165–174. https://doi.org/10.1007/s10658-009-9530-3

Aroca A., Raposo R., 2007. PCR-based strategy to detect and identify species of *Phaeoacremonium* causing grapevine diseases. *Applied and Environmental Microbiology* 73: 2911–2918. https://doi.org/10.1128/AEM.02176-06

Auger J., Esterio M., Pérez I., Gubler W.D., Eskalen A., 2004. First Report of *Phaeomoniella chlamydos-*

pora on *Vitis vinifera* and French American Hybrids in Chile. *Plant Disease* 88: 1285–1285. https://doi.org/10.1094/PDIS.2004.88.11.1285C

Bertsch C., Ramírez-Suero M., Magnin-Robert M., Larignon P., Chong J., … Fontaine F., 2013. Grapevine trunk diseases: complex and still poorly understood. *Plant Pathology* 62: 243–265. https://doi.org/10.1111/j.1365-3059.2012.02674.x

Besoain X., 2018. Grapevine Trunk Diseases (GTDs): Impact on Table Grapes and Wine Vineyards in Chile. In: *Grapes and Wines - Advances in Production, Processing, Analysis and Valorization* (A.M. Jordão, ed.), InTechOpen, 43–58. https://doi.org/10.5772/intechopen.72607

Blanco-Ulate B., Rolshausen P., Cantu D., 2013a. Draft Genome Sequence of the Ascomycete *Phaeoacremonium aleophilum* Strain UCR-PA7, a Causal Agent of the Esca Disease Complex in Grapevines. *Genome Announcements* 1: e00390-13. doi:10.1128/genomeA.00390-13

Blanco-Ulate B., Rolshausen P., Cantu D., 2013b. Draft Genome Sequence of *Neofusicoccum parvum* Isolate UCR-NP2, a Fungal Vascular Pathogen Associated with Grapevine Cankers. *Genome Announcements* 1: e00339-13. doi:10.1128/genomeA.00339-13

Bruisson S., Lebel S., Walter B., Prevotat L., Seddas S., Schellenbaum P., 2017. Comparative detection of a large population of grapevine viruses by TaqMan® RT-qPCR and ELISA. *Journal of Virological Methods* 240: 73–77. https://doi.org/10.1016/j.jviromet.2016.12.003

Crowther T.W., Maynard D.S., Crowther T.R., Peccia J., Smith J.R., Bradford M., 2014. Untangling the fungal niche: The trait-based approach. *Frontiers in Microbiology* 5: 1–12. https://doi.org/10.3389/fmicb.2014.00579

Díaz G.A., Esterio M., Auger J., 2009. Effects of *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* on grapevine rootstocks. *Ciencia e Investigación Agraria* 36: 381–390. http://dx.doi.org/10.4067/S0718-16202009000300005

Díaz G.A., Prehn D., Besoain X., Chávez E.R., Latorre B.A., 2011. *Neofusicoccum parvum* associated with grapevine trunk diseases in Chile. *Plant Disease* 95: 1032–1032. https://doi.org/10.1094/PDIS-03-11-0260

Díaz G.A., Auger J., Besoain X., Bordeu E., Latorre B.A., 2013. Prevalencia y patogenicidad de hongos asociados con enfermedades de la madera en viñedos chilenos. *Ciencia e Investigacion Agraria* 40: 327–339. http://dx.doi.org/10.4067/S0718-16202013000200008

Fischer G., Dott W., 2002. Quality assurance and good laboratory practice in the mycological laboratory – compilation of basic techniques for the identification of fungi. *International Journal of Hygiene and Environmental Health* 205: 433–442. https://doi.org/10.1078/1438-4639-00190

Fontaine F., Pinto C., Vallet J., Clément C., Gomes A.C., Spagnolo A., 2016. The effects of grapevine trunk diseases (GTDs) on vine physiology. *European Journal of Plant Pathology* 144: 707–721. https://doi.org/10.1007/s10658-015-0770-0

Del Frari G., Gobbi A., Aggerbeck M.R., Oliveira H., Hansen L.H., Ferreira R.B., 2019. Characterization of the wood mycobiome of *Vitis vinifera* in a vineyard affected by Esca. Spatial distribution of fungal communities and their putative relation with leaf symptoms. *Frontiers in Plant Science* 10: 910. https://doi.org/10.3389/fpls.2019.00910

Gramaje D., Úrbez-Torres J., Sosnowski M., 2018. Managing grapevine trunk diseases with respect to etiology and epidemiology: Current strategies and future prospects. *Plant Disease* 102: 12–39. https://doi.org/10.1094/PDIS-04-17-0512-FE

Grinbergs D., Chilian J., Carrasco-Fernández J., France A., Moya-Elizondo E., Gerding M., 2020. A PCR-based method for the rapid detection of *Chondrostereum purpureum* in apple. *Plant Disease* 104: 702–707. https://doi.org/10.1094/PDIS-10-19-2086-RE

Grozić K., Bubola M., Poljuha D., 2019. Symptoms and management of grapevine trunk diseases. *Journal of Central European Agriculture* 20: 876–890. https://doi.org/10.5513/JCEA01/20.3.1991

Hofstetter V., Buyck B., Croll D., Viret O., Couloux A., Gindro K., 2012. What if esca disease of grapevine were not a fungal disease? *Fungal Diversity* 54: 51–67. https://doi.org/10.1007/s13225-012-0171-z

Kassambara A, 2020. Ggpubr: "ggplot2" Based Publications Ready Plot v0.4.0. https://rpkgs.datanovia.com/ggpubr/

Lolas M.A., Castro A., Polanco R., Gaínza-Cortés F., Ferrada E., … Díaz G.A., 2020. First report of *Eutypa lata* causing dieback of grapevines (*Vitis vinifera*) in Chile. *Plant Disease* 104: 7, 2024. https://doi.org/10.1094/PDIS-12-19-2531-PDN

Luque J., Elena G., Garcia-Figueres F., Reyes J., Barrios G., Legorburu F.J., 2014. Natural infections of pruning wounds by fungal trunk pathogens in mature grapevines in Catalonia (Northeast Spain). *Australian Journal of Grape and Wine Research* 20: 134–143. https://doi.org/10.1111/ajgw.12046

Mondello V., Songy A., Battiston E., Pinto C., Coppin C., … Fontaine F., 2018. Grapevine trunk diseases: A review of fifteen years of trials for their control with chemicals and biocontrol agents. *Plant Disease*

102: 1189–1217. https://doi.org/10.1094/PDIS-08-17-1181-FE

Morales-Cruz A., Amrine K.C., Blanco-Ulate B., Lawrence D.P., Travadon R., … Cantu D., 2015. Distinctive expansion of gene families associated with plant cell wall degradation, secondary metabolism, and nutrient uptake in the genomes of grapevine trunk pathogens. *BMC Genomics* 16: 469. https://doi.org/10.1186/s12864-015-1624-z

Oksanen J., Blanchet F.G., Friendly M., Kindt R., Legendre P., … Wagner H., 2019. vegan: Community Ecology Package, ver.2.5-6. https://github.com/vegandevs/vegan

Pavlic D., Slippers B., Coutinho T.A., Wingfield M.J., 2009. Molecular and phenotypic characterization of three phylogenetic species discovered within the *Neofusicoccum parvum/N. ribis* complex. *Mycologia* 101: 636–647. https://doi.org/10.3852/08-193

Pouzoulet J., Mailhac N., Couderc C., Besson X., Daydé J., … Jacques A., 2013. A method to detect and quantify *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* DNA in grapevine-wood samples. *Applied Microbiology and Biotechnology* 97: 10163–10175. https://doi.org/10.1007/s00253-013-5299-6

Pouzoulet J., Rolshausen P.E., Schiavon M., Bol S., Travadon R., … Jacques A., 2017. A Method to Detect and Quantify *Eutypa lata* and *Diplodia seriata* - Complex DNA in Grapevine Pruning Wounds. *Plant Disease* 101: 1470–1480. https://doi.org/10.1094/PDIS-03-17-0362-RE

R Core Team, 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/

Ramírez E., Pineda J., 2003. Distribución de *Eutypa lata* y *Lasiodiploidia theobromae* en las zonas vitícolas de Venezuela. *Revista de la Facultad de Agronomía*, *Universidad del Zulia* 20: 43–51.

Reis P., Pierron R., Larignon P., Lecomte P., Abou-Mansour E., … Fontaine F., 2019. *Vitis* methods to understand and develop strategies for diagnosis and sustainable control of grapevine trunk diseases. *Phytopathology* 109: 916–931. https://doi.org/10.1094/PHYTO-09-18-0349-RVW

Rolshausen P.E., Baumgartner K., Travadon R., Fujiyoshi P., Pouzoulet J., Wilcox W.F., 2013. Identification of *Eutypa* spp. causing *Eutypa* dieback of grapevine in Eastern North America. *Plant Disease* 98: 483–491. https://doi.org/10.1094/PDIS-08-13-0883-RE

Rolshausen P.E., Trouillas F., Gubler W.D., 2004. Identification of *Eutypa lata* by PCR-RFLP. *Plant Disease* 88: 925–929. https://doi.org/10.1094/PDIS.2004.88.9.925

SAG, 2018. Catrasto viticola nacional. http://www.sag.cl/content/catastro-viticola-nacional-2018.

Songy A., Fernandez O., Clément C., Larignon P., Fontaine F., 2019. Grapevine trunk diseases under thermal and water stresses. *Planta* 249: 1655–1679. https://doi.org/10.1007/s00425-019-03111-8

Siebert J.B., 2001. *Eutypa*: the economic toll on vineyards. *Wines Vines* 50–56.

Travadon R., Baumgartner K., Rolshausen P.E., Gubler W.D., Sosnowski M.R., … P´eros J.P., 2012. Genetic structure of the fungal grapevine pathogen *Eutypa lata* from four continents. *Plant Pathology*. 61: 85-95. https://doi.org/10.1111/j.1365-3059.2011.02496.x

Travadon R., Lecomte P., Diarra B., Lawrence D.P., Renault D., … Baumgartner K., 2016. Grapevine pruning systems and cultivars influence the diversity of wood-colonizing fungi. *Fungal Ecology* 24: 82–93. https://doi.org/10.1016/j.funeco.2016.09.003

Úrbez-Torres J.R., Battany M., Bettiga L.J., Gispert C., McGourty G., … Gubler W.D., 2010. Botryosphaeriaceae species spore-trapping studies in California vineyards. *Plant Disease* 94: 717–724. http://dx.doi.org/10.1094/PDIS-94-6-0717

Úrbez-Torres J.R., Haag P., Bowen P., Lowery T., O'Gorman D.T., 2015. Development of a DNA Macroarray for the Detection and Identification of Fungal Pathogens Causing Decline of Young Grapevines. *Phytopathology* 105: 1373–1388. https://doi.org/10.1094/PHYTO-03-15-0069-R

Warnes G.R., Bolker B., Bonebakker L., Gentleman R., Huber W., … Galili T., 2020. gplots: Various R Programming Tools for Plotting Data v3.0.4. https://github.com/talgalili/gplots