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Microbiota of grapevine woody tissues with or without esca-foliar symptoms in northeast Spain

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Summary. Grapevine Trunk Diseases (GTDs), particularly esca, are major concerns in Spain. This study characterized the fungal and bacterial communities in a 30-year-old 'Cabernet Sauvignon' vineyard in Catalonia, Spain. Six symptomatic vines expressing esca-foliar symptoms and six additional asymptomatic plants were surveyed. Non-necrotic and necrotic tissues were sampled from rootstocks, trunks and cordons of the selected vines. Fungal isolations and molecular techniques were used in combination to investigate the endophytic microflora and to characterize the fungal and bacterial diversity of the vines. Pathogenic fungi involved in GTDs were identified, and the associations were assessed between all the isolated fungal taxa and the health status of vines, host parts and tissue types. *Phaeomoniella chlamydospora* and *Diplodia seriata* were found in non-necrotic tissues, whereas *Fomitiporia mediterranea* was mainly found in soft-rot tissues, and *Neofusicoccum parvum* and *Eutypa* cf. *lata* in hard necrotic tissues. Several *Alternaria* and *Trichoderma* species were isolated, mainly from non-necrotic tissues. The single strand conformation polymorphism fingerprinting method showed differences in bacterial and fungal communities between the different types of tissue in healthy and diseased vines. However, no differences were detected in these microbial communities between non-necrotic tissues from asymptomatic and symptomatic vines. Two frequently-isolated species of *Paraconiothyrium* were tested for pathogenicity, but their pathogenic roles on grapevines was unclear.

Key words: endophyte, grapevine trunk diseases, *Paraconiothyrium*, SSCP, *Vitis vinifera*.

Introduction

Spain is the country with the largest area of production given over to grapevine (*Vitis vinifera* L.), with 920,180 ha grown in 2016 that yielded 5.9 million of tons, and making this country the second in world wine production (FAO, 2017). In the last decades, reports on the incidence of grapevine trunk diseases (GTDs) in Spain have increased, involving rootstock vines (Aroca *et al.*, 2006; Aroca and Raposo, 2009; Aroca *et al.*, 2010), young vineyards (Armengol

et al., 2002; Giménez-Jaime *et al.*, 2006; Moreno-Sanz *et al.*, 2013) and mature vines (Úrbez-Torres *et al.*, 2006; Sánchez-Torres *et al.*, 2008; Gramaje *et al.*, 2009; Luque *et al.*, 2009; 2012). There are no known grapevine varieties that are resistant to these diseases (Surico *et al.*, 2006; Wagschal *et al.*, 2008; Larignon *et al.*, 2009).

Esca, Botryosphaeria and Eutypa dieback are the most destructive GTDs caused by fungi in mature vineyards, and are responsible for important crop losses in most grape-growing countries, including Spain (Armengol *et al.*, 2001). The principal fungal pathogens involved in esca are *Fomitiporia mediterranea* M. Fisch. (Fischer, 2002), *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous

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& W. Gams, and *Phaeoacremonium minimum* (Tul. & C. Tul.) Gramaje, L. Mostert & Crous (Crous *et al.*, 1996). Esca is a Complex where symptoms and their expression are highly variable (Mugnai *et al.*, 1999; Surico *et al.*, 2006). Most recognised foliar symptoms are interveinal chlorosis or discolourations that coalesce in large necrotic areas (Surico *et al.*, 2006; Gubler et al., 2015). This is the most common disease in the studied area, so the vines sampled in our study were characterised by the expression or not of esca-foliar symptoms.

For many years, sodium arsenite was the only apparently efficient chemical for control of this disease (Fussler *et al.*, 2008; Larignon *et al.*, 2008; Mondello *et al.*, 2018). However, this compound was banned in Europe at the beginning of the 21st century due to its environmental impacts and harmful effects on humans (Trouba *et al.*, 2002). In Spain, use of the chemical was forbidden in 2003. The widely reported increase of esca worldwide in the last decades has been associated with the restrictions on sodium arsenite, and with different factors related with cultural practices. These include insufficient protection of pruning wounds, which can remain susceptible to fungal infections up to 12 weeks after pruning (Elena and Luque, 2016), and a reduced sanitary care of rootstocks and vine propagation material (Gramaje and Armengol, 2011). Several surveys of young vineyards carried out in Spain have reported trunk pathogens occurring in all the main wine grape production areas (Armengol *et al.*, 2001; Aroca *et al.*, 2006; Giménez-Jaime *et al.*, 2006; Luque *et al.*, 2009; Aroca *et al.*, 2010; Gramaje *et al.*, 2011; Agustí- Brisarch *et al.*, 2013). This suggests that newly produced plants were infected during propagation in nurseries, and that the planting material used in the propagation process may be infected with these pathogens (Gramaje *et al.*, 2018).

The fungal pathogens involved in esca are responsible of wood necrosis. However, their role in the disease process is still a matter of debate (Hofstetter *et al.*, 2012). The involvement of microorganisms other than fungi in esca has been recently speculated. Two preliminary studies (Bruez *et al.*, 2014; 2015) showed that pathogenic fungi involved in GTDs and also pathogenic bacteria colonize the wood both of healthy and esca-diseased grapevines. Bacteria can also be found in such tissues and they may interact with these fungal colonizers. However, research into bacteria having roles in GTD development needs further assessment. The role of bacteria as helpers of the necrosis

development has also been highlighted by Haidar *et al.* (2016).

In Spain, studies of diseases and pathogenic mycoflora associated with grapevine rootstocks, both from young and mature vines, have been widely performed, as cited above. However, no systematic studies have been carried out to elucidate the microbiological communities of grapevines affected by trunk diseases, which could provide increased understanding of these complex diseases. For this reason, the present study aimed to characterize the microflora of mature Spanish 'Cabernet Sauvignon' vines with or without esca-foliar symptoms. Two approaches were used: i) determining the fungal diversity from fungal isolations conducted with non-necrotic, necrotic and white-rot tissues of trunks and rootstocks, and ii) determining the genetic structure of both types of fungal and bacterial communities present in the different tissues by using a fingerprinting method. In addition, pathogenicity tests were carried out with two species of *Paraconiothyrium* Verkley, which were very frequently isolated from the surveyed vines.

Materials and methods

Fungal and bacterial communities of diseased and healthy grapevine wood

Vineyard

Plant material was obtained from a 30-year-old commercial 'Cabernet Sauvignon' vineyard located in El Pla del Penedès (Catalonia, NE Spain; UTM 31 T 392200, 4586700). The vines were grafted onto 140 Ruggeri rootstocks, and maintained as bilateral cordons and spur-pruned. The vineyard was monitored for the presence of foliar GTDs symptoms from 2008 to 2013 (Luque, *unpublished data*).

Plant material and sampling

Six plants which had expressed chronic esca-foliar symptoms at least twice over the previous 5 years (2010 to 2014) were chosen as the group of symptomatic plants. An additional group of six plants which did not express symptoms over the same period was selected as the asymptomatic group. The sampling method was vine-destructive, and the plants with or without esca-foliar symptoms in July/August 2014 were uprooted in August 2014. The trunks and rootstocks were cut longitudinally to verify the status of the wood (necrotic or healthy). Fifteen non-necrotic

wood fragments (approx. 5 × 0.5 cm) were collected from upper, central or basal areas of the trunk and from the rootstock of each plant. When symptomatic plants showed areas of necrotic wood or white rot, 15 additional fragments were also collected from these necrotic tissues.

Fungal isolation and identification

Wood fragments were collected with a sterile scalpel and were surface sterilised in 5% sodium hypochlorite for 30 s, rinsed in sterile distilled water three times for 10 s, and dried on sterile filter paper in a flow cabinet. The tissue fragments were then placed on 2% Malt Extract Agar (Difco™; Becton Dickinson and Company) in Petri plates (three fragments per plate) and incubated at 25°C under combined near-UV and white fluorescent light (respectively, Philips TL-D 18W BLB and Sylvania Standard F18W/33-640-TS cool white sources), with a 12/12 h diurnal light/dark period. Fungal development was monitored during 3 weeks. Whenever possible, identification of the fungi was based on morphological and cultural characters, and with microscope examination of fruiting structures and conidia. When morphological identification was not possible or dubious, sequencing of the Internal Transcribed Spacer (ITS) regions ITS1 and ITS2 of the rDNA was performed, following protocols described elsewhere (Alves *et al.*, 2004; Luque *et al.*, 2012). The sequences obtained were matched with deposited reference sequences in GenBank, using BLAST to confirm tentative identifications.

Analysis by single strand conformation polymorphism (SSCP) of fungal and bacterial communities colonizing the grapevine wood

Each wood sample was ground in liquid nitrogen with a one-ball Dangoumau type mill and then kept at -80°C prior to DNA extraction. DNA was extracted from 60 mg aliquots of woody tissues using the Indvisorb Spin Plant mini Kit (Invitek Inc.) in accordance with manufacturer's instructions (Bruez *et al.*, 2014). The DNA extracts were then quantified with a Nanodrop (ND-1000, Thermoscientific, Labtech International Ltd) and adjusted at 10 ng µL^{-1}. DNA was extracted in duplicate for each of the collected wood samples.

The pair of primers ML1 and ML2 (White *et al.*, 1990), targeting the mitochondrial large subunit rDNA gene, was used in the SSCP analyses. For bacteria, the 799f and 1115r primers pair was used, tar-

geting the V5–V6 region of the bacterial 16S rRNA gene (Redford *et al.*, 2010). The DNA was amplified by PCR in a Mastercycler Gradient Thermocycler (Eppendorf) in a reaction mixture (30 µL final volume) consisting of 2 µL of DNA template (10 ng µL^{-1}), 3 µL of 10× reaction buffer (Eurogentec), 1 µL of MgCl$_2$ (50 mM), 0.6 µL of dNTP (10 mM), 0.6 µL of each primer (10 µM) of fungal primers or bacterial primers, 3 µL of BSA (10 µg µL^{-1}) (New England BioLabs), 0.1 µL of SilverStar DNA polymerase (Eurogentec) and 19.1 µL of sterile distilled water.

The fungal PCR cycling parameters were 95°C for 2 min, followed by 35 cycles at 95°C for 1 min, 56°C for 1 min, 72°C for 1 min, and final extension at 72°C for 10 min. The PCR products (about 250 bp) were visualized using 2% TBE agarose gel electrophoresis prior to SSCP analysis. The bacterial PCR cycling conditions were: enzyme activation at 95°C for 2 min; followed by 25 cycles of denaturation at 95°C for 45 s; hybridization at 54°C for 30 s; extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR products were visualized using 2% TBE agarose gel electrophoresis prior to SSCP analysis.

The SSCP analyses were performed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) equipped with four 36-cm long capillaries. One microliter of each PCR product was mixed with 18.8 µL formamide Hi-Di (Applied Biosystems) and 0.2 µL of the standard internal DNA molecular weight marker Genescan 400 HD ROX (Applied Biosystems). The sample mixture was denatured at 95°C for 5 min, immediately cooled on ice, and then loaded into the instrument. The non-denaturing polymer consisted of 5.6% POP conformational analysis polymer (Applied Biosystems), 10% glycerol, EDTA buffer 10× (Applied Biosystems), and water. The migration time was set to 2,000 s, the voltage to 15 kV, and the temperature was 32°C. Samples were co-migrated with the fluorescent size standard (Applied Biosystems) to allow comparison of migration profiles between samples. Patterns were then aligned with the package StatFingerprints of R version 2.0, and were gathered into a single numerical database before statistical analyses.

Statistical analyses

The association of the type of plant (asymptomatic or symptomatic), plant part (rootstock, basal trunk, medium trunk or upper trunk) and the tissue symptom (non-necrotic or necrotic/white rot) with fungal community was determined by Correspondence

Analysis (CA) using the package ca (Nenadić and Greenacre, 2007) on R (version 3.0.1). The analysis was based on the relative abundance of the species found in the samples. Type of plant, plant part and type of tissue were used as classifying variables. The proximity of a species score to a classification variable centroid indicated that the species had the greatest relative abundance in that specific plant type, part or tissue. The characterization of wood-colonizing fungal and bacterial communities was analyzed using Principal Component Analysis (PCA). Each record was calculated by analyzing 250 variables corresponding to the SSCP profile scans of each sample. PCA was performed in R using the princomp function within the Rcmdr interface. Pearson's correlation coefficients were used in the analyses. Variables with a cos2 ≥ 0.5 on one of the first two principal components were estimated as sufficiently well represented by the principal plan generated by the PCA.

Pathogenicity of *Paraconiothyrium brasiliense* and *Paraconiothyrium variabile*

Paraconiothyrium brasiliense Verkley and *P. variabile* Riccioni, Damm, Verkley & Crous were frequently found in symptomatic and asymptomatic plants, so isolates (four of *Paraconiothyrium brasiliense* and two of *P. variabile*) were tested for pathogenicity to grapevines. Two different tests were conducted using i) a detached 'Tempranillo' cane assay, and ii) 'Tempranillo' vines in an experimental vineyard.

Detached canes assay

Dormant canes were used, each with three internodes and uniform thickness. A 4 mm diam. hole was drilled in the mid internode. A mycelium plug from an actively growing colony on PDA (Difco™; Becton Dickinson and Company) was placed into the hole and the wound was wrapped with Parafilm® (Bemis). Control canes were each treated with a sterile PDA plug instead of the mycelial inoculum. The ends of the canes were sealed with paraffin to prevent excessive water loss from canes. Twenty canes per isolate were used as replicates. Inoculated canes were placed in a plastic bag and incubated at 25°C in darkness for 1 month. The bark of the canes was then removed with a sterile scalpel, and the necrotic lesions developed in the vascular tissues were measured. The total necrosis extension to both sides from each inoculation site was calculated and used in the

statistical analyses. Inoculated fungi were reisolated as follows: two 1-cm fragments of each debarked cane was cut 1 cm away at both sides from the inoculation site. One additional 1-cm fragment was obtained from each end the necrotic lesion. Wood pieces were surface-sterilized in 70% ethanol for 4 min, and were then plated onto PDA amended with streptomycin sulphate (50 mg L^{-1}). Plates were incubated at 25°C until isolates of *P. brasiliense* and *P. variabile* were identified from colony morphology, growth rate and colour characteristics.

Vine assay

The experiment was conducted in a 4-year-old 'Tempranillo' vineyard located at IRTA facilities in Cabrils, Barcelona, Spain. The vines were grafted onto Richter 110 rootstocks, and grown as spur-pruned bilateral cordons. In February 2015, dormant canes from the current season were each pruned leaving four nodes and then inoculations were performed in the internode between the second and the third node, as described for the detached cane assay (above). Control vines were each treated with a sterile PDA plug instead of the mycelial inoculum. Twenty canes were used per treatment. Inoculated canes were removed from the vines in January 2016, and measurements of necrosis length and reisolations were made, as described above.

Statistical analyses

All statistical analyses were performed using R (https://www.r-project.org/). Mean necrosis lengths and percentage of reisolations were calculated per each isolate. Data of necrosis lengths were subjected to analysis of variance (ANOVA) whereas data on reisolations were analysed by non-parametric tests (Kruskal-Wallis and pairwise Mann-Whitney U tests), as it was not possible to fit linear models to these data. Prior to ANOVA, data on necrosis lengths were checked for normality and transformed if necessary. Residuals were checked to meet ANOVA assumptions. Tukey HSD test was used to detect differences among the means at $P = 0.05$.

Results

Fungal and bacterial communities

Status of trunk wood

Examination of longitudinal stem sections of the sampled vines showed that non-necrotic tissues

predominated in the trunks and rootstocks of non-symptomatic and symptomatic plants. However, two symptomatic plants (R6P20 and R9P15) showed high proportions of necrotic tissues along the whole trunks, and one plant (R6P20) also showed white rot tissues along the rootstock (Table 1). These necrotic tissues were also sampled. The appearance of normal, living functional tissues was yellowish to bright creamy in colour and with a humid touch, regardless of the health status of the vine.

Identification and distribution the fungi isolated from the wood tissues

Successful isolations (total of 479 from 825 attempts (58.1%) were obtained during the experiment, which yielded 550 fungal isolates (Table 2): 316 were from the symptomatic vines and 234 from the asymptomatic vines; 124 were from roots, 143 from the basal trunks, 143 from the medium trunks, and 140 from the upper trunks; 135 isolates were from necrotic or white rot tissues, and 415 were from non-necrotic tissues. Details of isolations of specific taxa from each type of plant, plant part or tissue type are presented in Table 2. Pre-

viously known species associated to grapevine trunk diseases were isolated 223 times (Table 2; Figure 1). *Diplodia seriata* De Not. was isolated from both types of vines, but only from non-necrotic tissues. *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips was only isolated from symptomatic vines: 71 isolations were from trunk necrotic tissues, six isolations were from white rot rootstock tissues, and five isolations were from the non-necrotic tissues. *Eutypa* cf. *lata* was found 12 times in the necrotic tissues of the upper trunks of symptomatic plants. *Fomitiporia mediterranea* was only isolated from white rot (12 isolations) or necrotic tissues (20 isolations) of symptomatic grapevines. *Phaeomoniella chlamydospora* was found 23 times in the asymptomatic vines and 19 times in the symptomatic ones; 15 times in the non-necrotic rootstock tissues, four times from non-necrotic trunk tissues. *Phaeoacremonium minimum* was mainly isolated from the asymptomatic vines (26 isolations). Additionally, this fungus was isolated from non-necrotic tissues of the symptomatic vines (12 isolations) and once from the white rot tissues. *Phomopsis* sp. was found three times in the necrotic upper trunk tissues of a symptomatic grapevine. The other fungal species

Table 1. Status of the grapevine wood tissues sampled from symptomatic and asymptomatic 'Cabernet Sauvignon' grapevines.

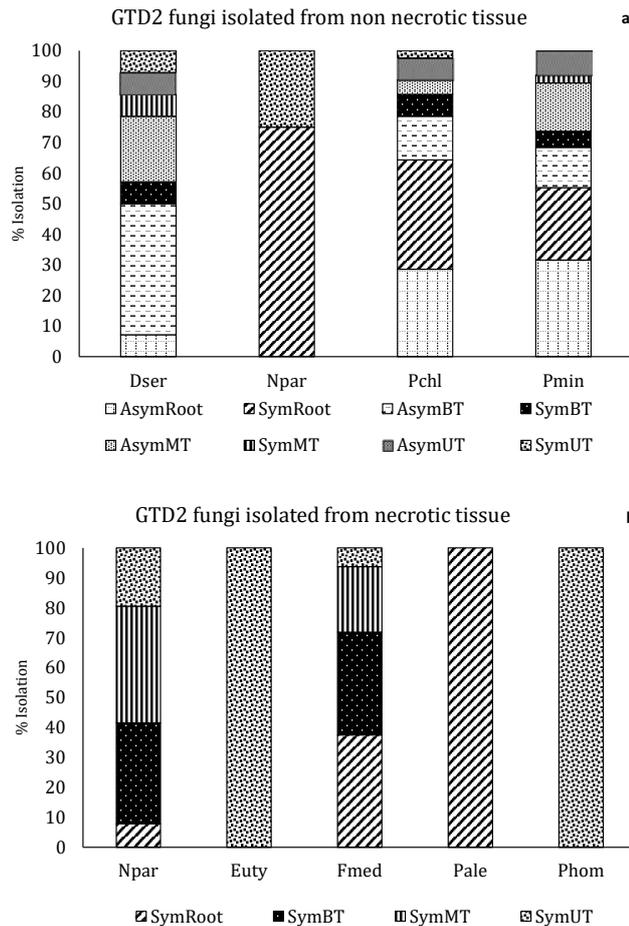| Plant code	Status	Necrotic tissue			Non-necrotic tissue		
		White rot	Necrotic tissue	Rootstock	Basal trunk	Medium trunk	Upper trunk
R6P20	Symptomatic	x	x				
R12P7	Symptomatic			x	x	x	x
R9P15	Symptomatic		x	x			
R19P15	Symptomatic			x	x	x	x
R13P13	Symptomatic			x	x	x	x
R12P25	Symptomatic			x	x	x	x
R19P28	Asymptomatic			x	x	x	x
R19P26	Asymptomatic			x	x	x	x
R11P9	Asymptomatic			x	x	x	x
R18P28	Asymptomatic			x	x	x	x
R11P11	Asymptomatic			x	x	x	x
R13P39	Asymptomatic			x	x	x	x

Table 2. Fungal taxa isolated from non-necrotic, necrotic or white rot affected tissues, and from different plant parts (roots, or basal, medium or upper trunks) of symptomatic (Sym) or asymptomatic (Asym) 'Cabernet Sauvignon' grapevines.

Species	Division	Order	Rootstock Asymp	Rootstock Symp	Basal Trunk Asymp	Basal Trunk Symp	Medium Trunk Asymp	Medium Trunk Symp	Upper Trunk Asymp	Upper Trunk Symp	Necrotic Basal Trunk Symp	Necrotic Medium Trunk Symp	Necrotic Upper Trunk Symp	White rot Upper Trunk Symp	White rot Rootstock Symp	Total
Acremonium sp.	Ascomycota	Hypocreales	0	0	0	0	0	0	0	1	0	0	0	0	0	1
Alternaria sp.	Ascomycota	Pleosporales	1	1	1	0	1	0	0	1	0	0	0	0	0	5
Arthrinium sp.	Ascomycota	Xylariales	0	0	1	1	0	0	0	0	0	0	0	0	0	2
Aspergillus cf. *flavus*	Ascomycota	Eurotiales	1	1	0	0	0	0	0	0	0	0	0	0	0	2
Aspergillus cf. *niger*	Ascomycota	Eurotiales	0	0	0	1	1	0	0	0	0	0	0	0	0	2
Aureobasidium pullulans	Ascomycota	Dothideales	1	0	1	0	1	1	5	2	0	0	0	0	0	11
Beauveria bassiana	Ascomycota	Hypocreales	1	3	0	11	1	1	0	4	0	0	0	0	0	21
Chaetomium erectum	Ascomycota	Sordariales	0	0	0	0	1	0	0	0	0	0	0	0	0	1
Chaetomium cf. *globosum*	Ascomycota	Sordariales	1	0	0	0	0	0	0	0	0	0	0	0	0	1
Chaetomium sp.	Ascomycota	Sordariales	0	0	1	0	0	0	0	0	0	0	0	0	0	1
Cladosporium sp.	Ascomycota	Capnodiales	5	1	6	1	2	2	4	5	0	0	0	0	0	26
Dendrothyrium variisporum	Ascomycota	Pleosporales	0	0	1	0	0	0	1	0	0	0	0	0	0	2
Diplodia seriata	Ascomycota	Botryosphaeriales	1	0	6	1	3	1	1	1	0	0	0	0	0	14
Eutypa sp.	Ascomycota	Xylariales	0	0	0	0	0	0	0	0	0	0	12	0	0	12
Fomitiporia mediterranea	Basidiomycota	Hymenochaetales	0	0	0	0	0	0	0	0	11	7	2	0	12	32
Gliomastix murorum	Ascomycota	Hypocreales	0	0	1	0	0	0	0	0	0	0	0	0	0	1
Lophiostoma sp.	Ascomycota	Pleosporales	0	2	0	0	0	0	0	1	0	0	0	0	0	3
Mollisia sp.	Ascomycota	Helotiales	1	0	0	0	0	0	0	0	0	0	0	0	0	1
Macrophomina phaseolina	Ascomycota	Botryosphaeriales	0	0	0	0	0	1	1	0	0	0	0	0	0	2
Neofusicoccum parvum	Ascomycota	Botryosphaeriales	0	3	0	0	0	0	0	1	26	30	15	0	6	81
Penicillium sp.	Ascomycota	Eurotiales	4	11	6	8	1	6	3	0	0	0	8	0	0	47
Paraconiothyrium brasiliense	Ascomycota	Pleosporales	2	1	5	10	24	8	10	2	0	0	0	0	2	64
Paraconiothyrium variabile	Ascomycota	Pleosporales	2	4	4	5	4	3	3	7	0	0	0	0	0	32

(Continued)

Table 2. (Continued).

Species	Division	Order	Non-necrotic tissues								Necrotic tissues		White rot		Total
			Rootstock		Basal Trunk		Medium Trunk		Upper Trunk		Basal Trunk	Medium Trunk	Upper Trunk	Rootstock	
			Asymp	Symp	Asymp	Symp	Asymp	Symp	Asymp	Symp	Symp	Symp	Symp	Symp	
Phaeomoniella chlamydospora	Ascomycota	Phaeomoniellales	12	15	6	3	2	0	3	1	0	0	0	0	42
Phaeoacremonium minimum	Ascomycota	Diaporthales	12	9	5	2	6	1	3	0	0	0	0	1	39
Phomopsis sp.	Ascomycota	Diaporthales	0	0	0	0	0	0	0	0	0	0	3	0	3
Pyrenochaeta sp.	Ascomycota	Pleosporales	0	0	1	0	0	0	2	4	0	0	0	0	7
Seimatosporium sp.	Ascomycota	Amphisphaeriales	0	0	1	0	0	1	1	0	0	0	0	0	3
Stachybotrys sp.	Ascomycota	Hypocreales	0	0	1	0	0	0	0	0	0	0	0	0	1
Trichoderma cf. *viride*	Ascomycota	Hypocreales	2	0	0	0	0	0	0	0	0	0	0	0	2
Xylogone sp.	Ascomycota	Helotiales	3	1	1	0	3	0	0	0	0	0	0	0	8
Unknown			1	1	12	3	13	18	24	9	37	37	40	21	81
All			50	53	60	46	63	43	61	39	37	37	40	21	550

GTD2 fungi isolated from non necrotic tissue   **a**

GTD2 fungi isolated from necrotic tissue   **b**

Figure 1. Isolation percentages of grapevine trunk pathogens from non-necrotic host tissues (a) and necrotic tissues (b) of 30-year-old 'Cabernet Sauvignon' grapevines in NE Spain. Data are from asymptomatic (Asym) and symptomactic (Sym) plants, and from different plant parts (roots, basal trunks (BT), medium trunks (MT) and upper trunks (UT)). Values are expressed as percentages to the total number of reisolations of fungal species. There is no data for *Eutypa* cf. *lata* (Euty), *F. mediterranea* (Fmed) and *Phomopsis* sp. (Phom) in a, and for *D. seriata* (Dser) and *P. chlamydospora* (Pchl) in b. *N. parvum* (Npar) and *P. minimum* (Pmin) are represented in both figures.

were always found in non-necrotic tissues, except for *P. brasiliense,* which was isolated twice from white rot tissue (Table 2).

The output obtained from the CA (Figures 2a and 2b) gave a total inertia of 2.62, and the two first dimensions accounted for 49% of the total inertia (respectively, 34 and 15%). The principal co-ordinates

for the first two dimensions (k = 1 and k = 2) were also obtained for each fungus. These co-ordinates are shown in two complementary plots with the same axis co-ordinates; the first with pathogenic fungi known to be associated to grapevine trunk diseases, and the second with the rest of the fungal species. The different fungi typically associated with grapevine trunk diseases were related with specific tissues, plant parts and the two plant symptom expression types (Figure 2a). *Diplodia seriata, P. chlamydospora* and *P. minimum* were related to the non-necrotic tissues of asymptomatic and symptomatic plants and *F. mediterranea, N. parvum, Eutypa* cf. *lata* and *Phomopsis* sp. with the necrotic or white rot tissues of symptomatic plants. *Diplodia seriata* was located close to non-necrotic tissues of trunks and *P. chlamydospora* and *P. minimum* close to non-necrotic tissues of rootstocks. *Neofusicoccum parvum, Eutypa* cf. *lata* and *Phomopsis* sp. were associated with necrotic tissue of trunks and *F. mediterranea* was also related to white rot tissues in rootstocks. The other isolated fungi were associated with non-necrotic tissues from both types of vines (Figure 2b); none of these fungi were located close to necrotic tissues.

Fingerprinting of microflora

The SSCP profiles of the fungal and bacterial microflora from the symptomatic and asymptomatic vines did not show differences between these groups of vines (data not shown), but they did depend on plant parts and tissues (Figures 3 and 4). Each point in Figure 3 represents the fungal or bacterial communities present in the different parts of the symptomatic and asymptomatic vines. The first two principal components, Dim 1 and Dim 2, accounted for 54% of the data variance for the fungal community (Figure 3a), and 55% of the variance for bacteria (Figure 3b). The results showed that differences between cordon and rootstock parts, according to the first component, were only observed for bacterial communities. Comparison of the fungi inhabiting the different parts of the vines, either in necrotic or non-necrotic and white rot tissues, are shown represented in Figure 4a, and for bacterial communities in Figure 4b. The first two principal components, Dim 1 and Dim 2, accounted for 57% of the data variance for the fungi (Figure 4a), and 69% for the bacteria (Figure 4b). In both cases, the clusters representing the samples from non-necrotic tissues differed from those from necrotic and white-rot affected tissues.
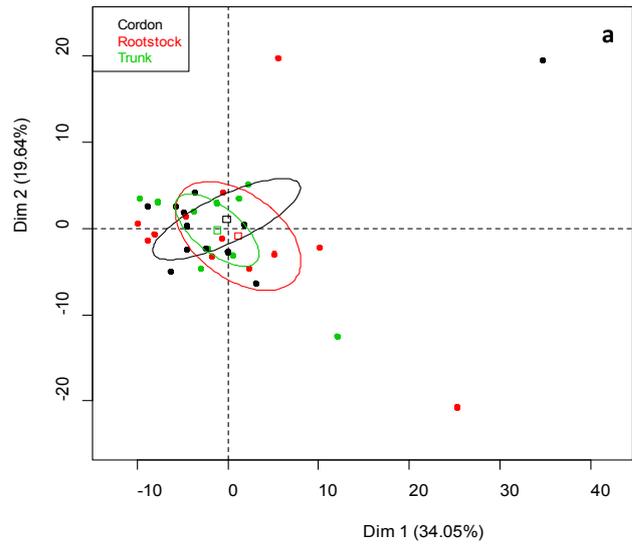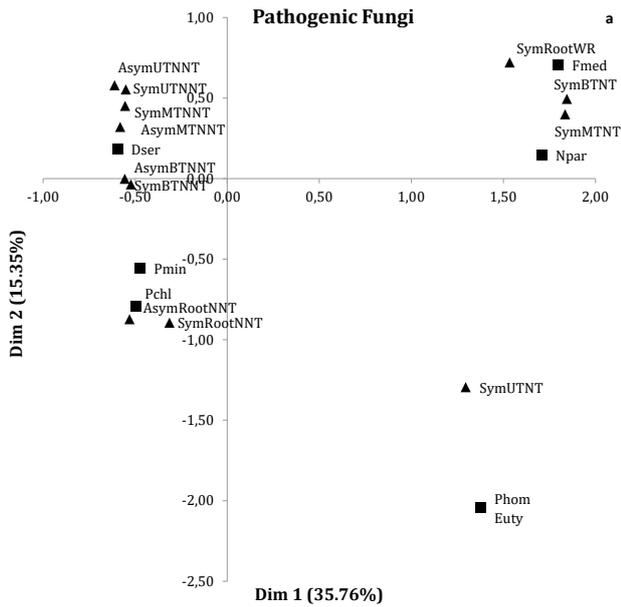
Figure 2. Plots resulting from the correspondence analyses on the fungi isolated from 30-year-old asymptomatic and symptomatic 'Cabernet Sauvignon' grapevines located in a vineyard in NE Spain. Results are shown in two complementary views of the same bidimensional space; (a) fungi known to be associated with grapevine trunk diseases (squares), and (b) other fungal taxa (circles). Legends to individually separated types of vines (Sym, symptomatic; Asym, asymptomatic), plant parts (Root; BT, basal trunk; MT, medium trunk; UT, upper trunk) or tissue types (NNT, non-necrotic tissue; NT, necrotic tissue; WR, white rot) are shown in triangles. Abbreviation of fungal species associated to grapevine trunk diseases are: Dser, *Diplodia seriata*; Npar, *Neofusicoccum parvum*; Euty, *Eutypa* cf. *lata*; Fmed, *Fomitiporia mediterranea*; Pchl, *Phaeomoniella chlamydospora*; Pmin, *Phaeoacremonium minimum*; Phom, *Phomopsis* sp. Codes for other isolated taxa isolated are: 1, *Paraconiothyrium brasiliense*; 2, *Paraconiothyrium variabile*; 3, *Acremonium* sp.; 4, *Alternaria* sp.; 5, *Arthrinium* sp.; 6, *Aspergillus* cf. *flavus*; 7, *Aspergillus* cf. *niger*; 8, *Aureobasidium pullulans*; 9, *Beauveria bassiana*; 10, *Chaetomium erectum*; 11, *Chaetomium* cf. g*lobosum*; 12, *Chaetomium* sp.; 13, *Cladosporium* sp.; 14, *Dendrothyrium variisporum*; 15, *Gliomastix murorum*; 16, *Lophiostoma* sp. 1; 17, *Lophiostoma* sp. 2; 18, *Mollisia* sp.; 19, *Macrophomina phaseolina*; 20, *Penicillium* sp. 1; 21, *Penicillium* sp. 2; 22, *Penicillium* sp. 3; 23, *Penicillium* sp. 4; 24, *Pyrenochaeta* sp. 1; 25, *Pyrenochaeta* sp. 2; 26, *Seimatosporium* sp.; 27, *Stachybotrys* sp.; 28, *Trichoderma* cf. *viride*; 29, *Xylogone* sp.; 30, Unknown_A; 31, Unknown_B; 32, Unknown_C; 33, Unknown_D; 34, Unknown_E; 35, Unknown_F; 36, Unknown_G; 37, Unknown_H; 38, Unknown_I; 39, Unknown_J.
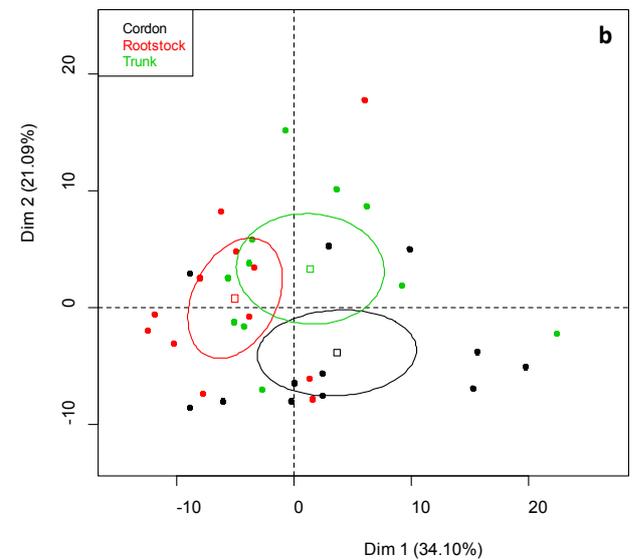
Figure 3. Principal component analyses (PCA) of fungal (a) and bacterial (b) communities colonizing the non-necrotic tissue of cordons, trunks or rootstocks of esca-foliar symptomatic or asymptomatic 'Cabernet Sauvignon' grapevines, based on SSCP profiles. The variation (%) explained by each PCA axis is given in parentheses. PCA eigenvalues indicate that the first two components, Dim 1 and Dim 2, account for a percentage of the total bacterial variability. Squares correspond to individual SSCPs and ellipses to the 95% confidence intervals calculated for each community.
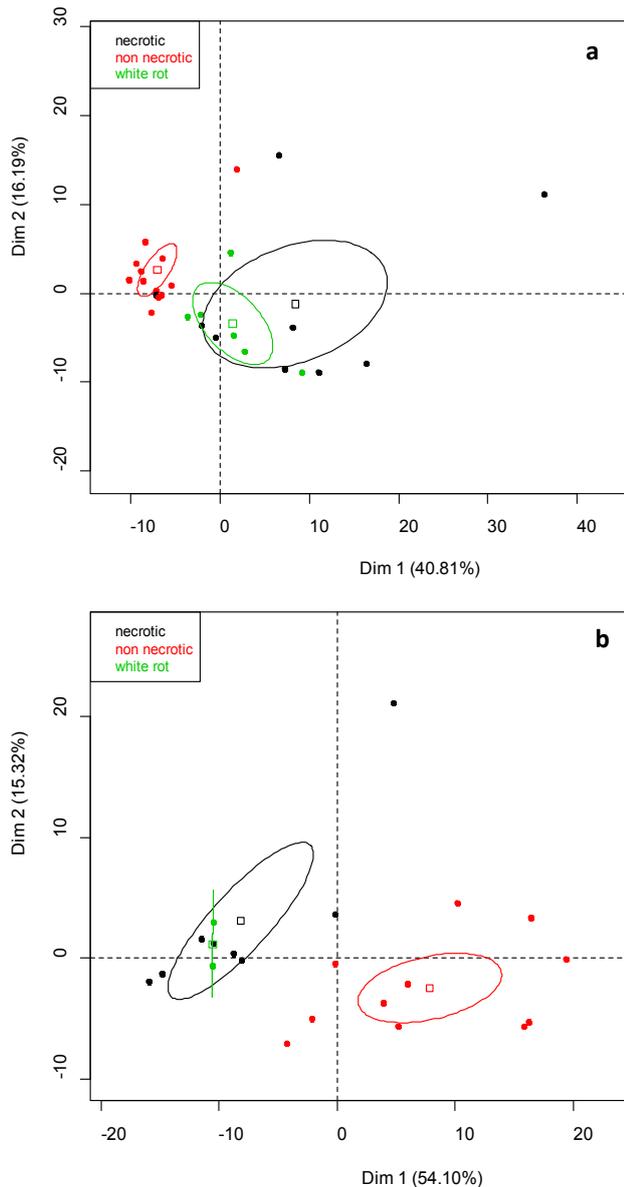
Figure 4. Principal component analysis (PCA) of fungal (a) and bacterial (b) communities colonizing the non-necrotic and necrotic tissues of cordons or trunks of esca-foliar symptomatic or asymptomatic 30-year-old grapevines, based on SSCP profiles. The variation (%) explained by each PCA axis is given in parentheses. PCA eigenvalues indicate that the first two components, Dim 1 and Dim 2, account for a percentage of the total bacterial variability. Squares correspond to individual SSCPs and ellipses to the 95% confidence intervals calculated for each community.

Pathogenicity of *Paraconiothyrium brasiliense* and *P. variabile* to grapevine tissues

In the detached cane and standing vine pathogenicity assays, necrotic lesions were observed in the wood of the inoculated canes, with the lesions extending from the inoculation sites. In the detached cane assay, statistical analysis showed significant differences ($P < 0.001$) in mean necrosis lengths among the isolates, although the mean lesion lengths were never greater than 0.3 cm. All isolates caused necrotic lesions that were longer than the controls (Table 3), with mean values ranging from 0.15 cm (Pbra 4) to 0.23 cm (Pbra 1 and Pbra 2). Percentages of reisolation were not statistically different between isolates ($P = 0.4116$). Isolates of *P. brasiliense* were recovered from more than 70% of the inoculated canes, except for isolate Pbra2 that was reisolated only from 54% of the inoculated canes, whereas isolates of *P. variabile* were recovered from 61% of the inoculated canes (Table 3). In the vine assay, only isolates Pbra 2, Pbra 3 and Pbra 4 produced necrotic lesions significantly longer than the controls ($P < 0.0001$; Table 3). However, mean necrosis lengths from isolates ranged from 1.88 cm (Pbra 1) to 3.48 cm (Pbra 2) 11 months after inoculation. No significant differences were detected for proportions of reisolations among isolates ($P = 0.2444$). Isolates of both inoculated species were recovered at greater than 90% from the inoculated canes (Table 3).

Discussion

This study aimed to characterize the microbial communities occurring in a 30-year-old 'Cabernet-Sauvignon' vineyard affected by GTDs. The fungal and bacterial microbiota of different types of host tissues were compared, and the results showed that typical microbiota differentially colonized the host tissue types. The CA and PCA analyses of fungal isolation data, and the SSCP-PCR band patterns, indicated that fungal communities of non-necrotic tissues in trunks and rootstocks of asymptomatic and symptomatic plants were similar. The PCA analysis of the non-necrotic tissues from both types of grapevines showed that fungal communities from different parts of the plants overlapped. In the CA analysis, the centroids corresponding to each plant part with non-necrotic tissues of asymptomatic and symptomatic plants were very similarly distributed. Moreover, 17

Table 3. Mean necrosis lengths (± standard errors) and mean reisolation proportions for isolates of *Paraconiothyrium brasiliense* (Pbra) and *P. Variabile* (Pvar) recorded in the pathogenicity tests on 'Tempranillo' detached canes and standing vines. Statistically significant differences (*P*<0.05) between means are shown in different letters.

Species and isolate	Detached cane assay		Vine assay	
	Necrosis length (cm)	% reisolation	Necrosis length (cm)	% reisolation
Control non- inoculated	0.09±0.02 a	0 a	2.08±0.35 a	0 a
Pbra 1	0.23±0.02 c	82.50 b	1.88±0.13 a	100 b
Pbra 2	0.23±0.01 c	53.75 b	3.48±0.40 b	93.75 b
Pbra 3	0.18±0.03 bc	70 b	3.04±0.33 b	100 b
Pbra 4	0.15±0.02 b	70 b	3.25±0.25 b	90 b
Pvar 1	0.16±0.02 b	61.25 b	2.04±0.25 a	98.67 b
Pvar 2	0.16±0.02 b	61.25 b	2.12±0.30 a	100 b

of the 26 species isolated from non-necrotic tissues were equally found in both types of plants.

These results are similar to those reported by Bruez *et al.* (2014), who did not find differences between the endophytic mycoflora in non-necrotic tissues of 10-year-old symptomatic and asymptomatic 'Cabernet Sauvignon' grapevines. Endophytic fungi are frequently found in healthy tissues of grapevines, as reported previously. González and Tello (2011) described the composition of the endophytic fungal communities within tissues of several *V. vinifera* cultivars from central Spain, and found Ascomycota to be the most prevalent endophyte group. Similarly, Ascomycota were most frequently isolated in the present study, and specifically fungi in the Pleosporales, with up to six different species from this order. Casieri *et al.* (2009) studied the fungal communities occurring in the wood of five *V. vinifera* cultivars grown in Switzerland, and they also found that the fungal community was mainly dominated by Ascomycota, with numerous isolates belonging to Hypocreales, Sordariales and Diaporthales. Casieri *et al.* (2009) also reported that fungal genera and species isolated from different plant parts were quite similar, suggesting that they contributed equally to the composition of the fungal communities. Bruez *et al.* (2014) determined Hypocreales and Botryosphaeriales as the most abundant orders in non-necrotic tissues of symptomatic and asymptomatic vines. However, these results are not in accordance with those obtained in the present study, probably due to the different fungal taxa that were

isolated in each country from the non-necrotic tissues of different plant parts. In addition, these results are based on microbiology techniques, and approaches, such as metagenomics, could give more information about the endophytic grapevine microflora.

Despite the similarities in the fungal microbiota found in non-necrotic tissues of symptomatic and asymptomatic vines, clear differences in microbial communities were observed between necrotic and non-necrotic tissues of different plant parts. *Eutypa* cf. *lata* and *N. parvum*, causal agents, respectively, of Eutypa dieback (Carter, 1991) and Botryosphaeria dieback (Úrbez-Torres, 2011), were associated with necrotic tissues of the trunks of symptomatic plants. *Phomopsis* sp. was also isolated from necrotic tissues of symptomatic plants. *Phomopsis* Sacc. & Roum. is a cosmopolitan genus that contains plant pathogens, such as *Phomopsis viticola* (Sacc.) Sacc., and endophytes described from a wide range of annual and perennial hosts (Uecker, 1988). *Diplodia seriata*, usually associated with Botryosphaeria dieback (Úrbez-Torres, 2011), was only isolated from non-necrotic tissues of asymptomatic and symptomatic plants. The pathogenicity of *D. seriata* on grapevines has been a matter of discussion. Luque *et al.* (2014) reported high incidence of *D. seriata* in naturally infected grapevines in NE Spain, although Elena *et al.* (2015) considered this fungus as a weak pathogen, suggesting an important role in grapevine decline. Recently, Reis *et al.* (2016) reproduced typical esca-foliar symptoms on cv. Tempranillo inoculated with *D. seriata*. *Phaeomon-*

iella chlamydospora and *P. minimum* were mainly found in non-necrotic tissues (*P. minimum* was only isolated once from white rot tissues). The absence of symptoms produced by these two pathogens in the sampled vines could be due to: i) latent or early infections, which had not developed visible symptoms, or ii) the co-existence with other endophytic microorganisms, and particularly those that protect the plants. Bruez *et al.* (2016) suggested that *Trichoderma* spp. and *Bionectria ochroleuca* (Schwein). Schroers & Samuels, isolated from non-necrotic outer wood tissues, could interact with the wood-decaying fungi in the same tissues to mask or inhibit pathogen activity. The Basidiomycete *F. mediterranea* (Hymenochaetales), was identified in the white-rot and hard necrotic basal trunk tissues, causing wood decay (Surico, 2009; Bertsch *et al.*, 2013; Cloete *et al.*, 2015).

Beauveria bassiana (Bals.-Criv.) Vuill., *Cladosporium* sp. and *Penicillium* spp. were the most frequently isolated fungi from non-necrotic tissues in the present study. Only one species of *Cladosporium* (*C. herbarum* (Pers.) Link) has been known to be antagonistic to *E. lata* (Munkvold and Marois, 1993). Further research is necessary to assess interactions between some of these microorganisms and the grapevine trunk pathogens, to evaluate their potential roles as pathogen antagonists.

Paraconiothyrium brasiliense and *P. variabile* were also frequently isolated from non-necrotic tissues of asymptomatic and symptomatic plants. *Paraconiothyrium variabile* has been isolated from necrotic wood from different host plants such as *Laurus nobilis* L. (Göre and Bucak, 2007), *Actinidia chinensis* Planch. and *A. deliciosa* (A. Chev.) A. Chev. (Riccioni *et al.*, 2007), and *Prunus persica* L. (Damm *et al.*, 2008). This indicates that this fungus may also have a broad host range, including several distantly related hosts. In the pathogenicity tests performed in the present study, *P. brasiliense* and *P. variabile* were frequently reisolated from inoculated host tissues, and they produced necrotic lesions that were longer than the control treatments in the detached cane assay. However, only three isolates of *P. brasiliense* caused lesions that were significantly longer than controls in the standing vine assay. Moreover, the mean lengths of necroses never exceeded 0.25 cm in the detached cane assay or 3.5 cm in vine assay. This indicates that virulence of these fungi on grapevines is not great as that of other GTD pathogens (Rolshausen *et al.*, 2008; Aroca and Raposo, 2009; Úrbez-Torres and Gubler, 2009). Other *Paraconiothyrium* species have been associated with wood rot

of birch and pine (*P. minitans* (W.A. Campb.) Verkley; see Nilsson, 1973) and core rot of apples (*P. sporulosum* (W. Gams & Domsch) Verkley; see Michailides *et al.*, 1994). Tsuda *et al.* (2003) described some secondary metabolites of a *Paraconiothyrium* isolate with antagonistic and antifungal characteristics. Due to the previous results and those from the present study, further research is required to more accurately assess the pathogenicity of these fungi to grapevines.

The bacterial communities characterized in this study showed differences between non-necrotic and the necrotic tissues, including those affected by the white rot. The PCA showed differences between the rootstock, trunk and cordon tissues, so we suggest that there is a diversification of communities, especially in different grapevine parts. These results do not agree with those of Bruez *et al.* (2015). They did not detect differences between the specific host parts, or between the necrotic tissue and white rot affected tissues. Our study was carried out with 30-year-old vines while Bruez *et al.* (2015) examined 10-year-old vines.

The study reported here showed that a complex and diverse microflora colonized the different woody grapevine tissues in a 30-year-old 'Cabernet-Sauvignon' vineyard in NE Spain. Some fungal species typically associated with GTDs, such as *D. seriata*, *P. chlamydospora* and *P. minimum*, were isolated from non-necrotic tissues of symptomatic plants, whereas other potentially antagonistic species were also isolated from the same types of tissues. Differences in bacterial communities were also observed between the different types of tissues in the asymptomatic and symptomatic grapevines. Two species of *Paraconiothyrium*, frequently found in non-necrotic tissues of symptomatic vines, were tested for their pathogenicity. However, results of the pathogenicity tests did not confirm clear pathogenic roles of these fungi on grapevine. Further research is required to determine relationships between microbial communities in grapevines with or without symptoms of GTDs.

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