Various fungal communities colonise the functional wood tissues of old grapevines externally free from grapevine trunk disease symptoms

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Abstract

Background and Aims: The objective of this study was to analyse the various fungal communities that colonise functional wood tissues of old vines that did not express symptoms of grapevine trunk diseases (i.e. Esca and Eutypa dieback) in the year of sampling. Plants of the cultivar Baco Blanc a grapevine hybrid of Folle Blanche and Noah used to produce Armagnac in France were sampled.
Methods and Results: Forty-two and 58-year-old vines, planted in the same vineyard, were uprooted, cut longitudinally and their functional wood tissues sampled. Culture-dependent and single-strand conformational polymorphism methods were used to compare the fungal communities colonising these wood tissues. It was shown that the fungal communities were significantly different depending on the age of the grapevines. A total of 421 fungal strains were isolated and identified by internal transcribed spacer region sequencing.
Conclusions: Many grapevine trunk diseases fungal pathogens, particularly the causal agents of Esca (42-year-old vines) and Eutypa dieback (58-year-old vines), as well as numerous potentially plant-beneficial mycoparasites (e.g. *Trichoderma* spp.), were isolated from the functional wood tissues of old grapevines.
Significance of the Study: The lack of foliar symptoms among older grapevines may reflect an 'equilibrium' among trunk fungal pathogens, mycoparasites and saprobes in the functional wood tissues of trunks.

Keywords: *fingerprinting method, fungal community, grapevine trunk disease, grapevine wood tissue, pathogenic fungi, potentially beneficial fungi*

Introduction

As was the case for earlier vine-health crises (powdery and downy mildews, phylloxera) at the end of 19th century, viticulture is now confronted with equally vast upheavals brought about by global climate change, the major phenomenon of grapevine trunk disease (GTDs), that is Esca, Eutypa and Botryosphaeriaceae diebacks, and strong consumer demand for an environmental-friendly viticulture. Although GTDs, mainly Esca, have been recorded since antiquity, they were known to affect only old vines, without inducing serious damage to vineyards. Now, within the span of a mere two decades, they have become a subject of major concern for the wine industry (Scheck et al. 1998, Mugnai et al. 1999, Armengol et al. 2001, Rumbos and Rumbou 2001, Edwards and Pascoe 2004, Gimenez-Jaime et al. 2006, Úrbez-Torres et al. 2006, 2009, Rego et al. 2009, Bertsch et al. 2013). Vineyards throughout the world are being attacked by GTDs and, in France, all the wine-producing regions are affected. Grapevine trunk diseases are on the increase and, currently, approximately 13% of French vineyards are unproductive, essentially because of these diseases. Understandably, this situation has engendered great apprehension in the viticulture sector.

Esca is generally thought to result from the pathogenic activity of fungal species, including *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* and *Fomitiporia mediterranea*. *Eutypa lata* and *Stereum hirsutum*, however, may also be involved in this disease (Fischer and Kassemeyer 2003, White et al. 2011). Botryosphaeriaceae species are mostly associated with Botryosphaeriaceae diebacks (Larignon et al. 2009). These fungi deconstruct the wood, causing various types of wood necrosis: central necrosis, black punctate necrosis, sectorial necrosis and a typical necrosis called white-rot associated with Esca. The chronic form, with typical foliar symptoms, i.e. 'tiger stripes', expresses itself consecutively, or not, over a few years. This form generally ends with the death of the grapevine after a few years (Guerin-Dubrana et al. 2013). Another form, apoplexy, is more severe, resulting in plant death within a few days (Larignon and Dubos 1997).

One of the key enigmas relating to these fungi is that foliar symptoms of Esca are often lacking, despite the occurrence of wood necroses following vine inoculation (Laveau et al. 2009). Koch's postulate is thus not always validated, and the possible role of other microorganisms in the process of wood degradation remains a matter of debate. The fungi implicated in Esca and Botryosphaeriaceae diebacks may also be present in the wood without causing symptoms.

Trunk diseases are rare in young vineyards. The canopy symptoms (shoot dieback, dead spurs, discoloured and altered

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leaves) do not become apparent until a vineyard is 8–10 years old but, by the time they are 15–20 years old, vineyards reach a peak level of disease incidence (Munkvold et al. 1993). Interestingly, the foliar symptoms of Esca and Eutypa dieback (Bertsch et al. 2013), GTDs with characteristic foliar symptoms, are relatively rare among older vines (Bruez et al. 2013). Our objective was to examine the wood-colonising fungi of vines planted prior to 1990, which marked the beginning of the grapevine trunk disease epidemic in France. Our approach was to isolate and compare, in a vineyard with 42-and 58-year-old vines, the wood-colonising fungi of epidemic-surviving vines, which did not show foliar symptoms of either Esca or Eutypa dieback. We sampled the functional non-necrotic wood, the key tissue for plant growth and development. Wood-colonising fungi were isolated in culture, and identified, using a combination of culture dependent, and sequencing of the nuclear ribosomal (r)DNA internal transcribed spacer (ITS) region. Communities of wood-colonising fungi were additionally defined by a DNA-mitochondrial large subunit fingerprinting method, single-stranded conformational polymorphisms (SSCPs).

Materials and methods

Vineyard site

The experiment was conducted in a vineyard in the Armagnac wine-producing region, located in the southwest of France (Parleboscq, 200 km south from Bordeaux, France). The vineyard was established in tawny sand, a type of Bas-Armagnac soil, with two separate plots being planted in 1953 (58-year old, the date of sampling was taken into account) and in 1969 (42-year old) with ungrafted Baco Blanc, a hybrid of Folle Blanche (*Vitis vinifera* L.) and Noah, a natural hybrid of *V. labruscana* and *V. riparia* Michx. In France, Baco Blanc is the only hybrid authorised for the production of Armagnac. Baco Blanc is sensitive to phylloxera, flavescence dorée, mites and downy mildew on leaves. It is relatively tolerant to *Botrytis cinerea* and powdery mildew. Its tolerance to trunk diseases is unknown. In the 5 years prior to the study, average yield was 80 to 90 hL/ha. No external foliar symptoms of Esca or Eutypa dieback were observed in the year of sampling.

Sampling of wood tissues of grapevines

In order to study the wood-colonising fungi, seven 42-year-old vines and seven 58-year-old vines were uprooted, and their trunks (approximately 80 cm in length x 10 cm in diameter) were cut longitudinally to reveal areas of necrotic and non-necrotic wood. The non-necrotic wood in the outer part (functional tissues) was sampled from the upper, central and basal locations of the trunk. Samples consisted of 15 fragments (each fragment approximately 0.5 cm^3) were cut from a trunk section of each of the 14 vines with a flame-sterilised scalpel. The wood fragments were surface sterilised in 5% calcium hypochlorite for 30 s, rinsed in sterile distilled water for 10 s and dried on sterile filter paper. They were then used either for the culture-based identification of the fungal communities or for the DNA-fingerprinting study (SSCP).

Fungal community structure based on DNA fingerprinting

From each trunk section sampled, 10 g of non-necrotic wood was taken for SSCPs. Samples were ground in liquid nitrogen with a one-ball mill of Dangoumau type, and stored at −80°C; DNA was extracted from 60 mg aliquots of ground wood using the Indvisorb Spin Plant mini Kit (Eurobio, Courtabouef, Les Ulis, France) in accordance with the manufacturer's instructions, and quantified with a Nanodrop (ND-1000, Thermoscientific, Labtech Software, Tampa, FL, USA) and standardised to a concentration of 10 ng/μL.

The mitochondrial large subunit rDNA gene was amplified, using PCR primers ML1 and ML2 (White et al. 1990) for SSCP. Polymerase chain reaction was carried out in an Epgradient Mastercycler (Eppendorf, Hamburg, Germany) in a 25 μL reaction volume consisting of 1 μL of DNA template (10 ng/μL), 0.2 mmol/L dNTP, 1 ng/μL of bovine serum albumin (New England BioLabs, Ipswich, MA, USA),0.2 μmol/L primer, Pfu Turbo buffer 1X, and 0.05 U of Pfu Turbo DNA polymerase (Stratagene/Agilent Technologies, Santa Clara, CA, USA). Cycling parameters were 95°C for 2 min, followed by 30 cycles at 95°C for 1 min, 56°C for 1 min, 72°C for 1 min, with a final extension at 72°C for 10 min; PCR products (~250 bp) were confirmed by 2% TBE agarose gel electrophoresis prior to SSCP analysis.

The SSCP was analysed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA) equipped with four 36-cm capillaries. The PCR product (1 μL) was mixed with 18.8 μL of formamide Hi-Di (Applied Biosystems) and 0.2 μL of standard internal DNA molecular mass marker (Genescan 400 HD ROX, Applied Biosystems). The sample mixture was denatured at 95°C for 5 min, and immediately cooled on ice before loading. The non-denaturing polymer consisted of 5.6% POP Conformational Analysis Polymer (Applied Biosystems), 10% glycerol, EDTA buffer 10X (Applied Biosystems) and water. Migration time was 33 min, at 15 kV and 32°C. All 56 samples co-migrated with the fluorescent size standard (GeneScan-400 ROX), which allowed profiles to be obtained.

These profiles were aligned using the StatFingerprints package (Michelland et al. 2009) in R v2.14.2 (R Core Team 2012), resulting in 250 total variables (i.e. 250 peaks). Genetic differentiation among samples was then compared through principal component analysis (PCA), based on Pearson's correlation coefficient, with cos2 ≥0.5, and also on one of the first three principal components (Dim1, Dim2 or Dim 3), which were estimated as sufficiently well represented by the principal plane generated by this PCA.

Isolation and identification of fungi by culture-dependent methods

Isolation of the fungi. Surface-sterilised wood fragments were plated on malt extract agar (three fragments per plate) and incubated in the dark at 25°C for 1 month. Colonies that emerged during that time were subcultured, and the location from which the wood was sampled was noted. For isolates that sporulated, taxonomic identification was initially based on colony morphology, and later identification of representative isolates confirmed by sequencing of the internal transcribed spacer of the rDNA region. For isolates that did not sporulate, ITS sequencing of representative isolates was the sole method of identification.

DNA extraction. Fungal genomic DNA was isolated from fresh mycelium scraped from the surface of a 14-day culture. Mycelium was freeze-dried overnight (Alpha 1-4 LO plus, Fisher Scientific, Illkirch-Graffenstaden, France) and then ground with a Tissue Lyser II (Retsch, Qiagen, KJ Venlo, the Netherlands) for 1 min at a frequency of 29.9/s. After incubation in 400 μL of cetyltrimethylammonium bromide (1X) at 65°C for 1 h, 400 μL of chloroform: isoamyl alcohol (24:1, v/v) was added, and samples were centrifuged for 30 min at 1638 *g*. The aqueous phase was transferred to a new tube, and 200 μL of isopropanol was added. Samples were then kept overnight at

−20°C for DNA precipitation. After centrifugation for 20 min at 1638 *g*, the supernatant was discarded, and 500 µL of 70% ethanol was added in order to wash the pellets. Once the ethanol was discarded, the pellets were air dried and re-suspended in 50 µL sterile distilled water.

Identification of the isolates. The ITS region was sequenced with primers ITS1f and ITS4 (White et al. 1990, Chanderbali et al. 2001). For species-level identification, sequences were subjected to a bulk BLASTn search against the International Nucleotide Sequence Databases, as implemented in the PlutoF workbench of the UNITE database (Abarenkov et al. 2010). The DNA of the sterile fungi and of some fungi involved in the GTDs or potentially biocontrol agents were sequenced and analysed with PlutoF. To determine the species names, the proportion of sequence BLAST was set at 99%.

Statistical analysis

The species diversity the of wood-colonising fungi was compared for 42- and 58-year-old vines: Shannon's diversity, H' (Shannon and Weaver 1963, Magurran 2004); Simpson's diversity, D (Lyons and Hutcheson 1979); species evenness, J (Gotelli and Colwell 2001); species richness (total number of species observed); and relative abundance (proportion of wood fragments per location from which a species was isolated). All calculations were made using Agricolaea in R v.2.14.2 software. The data for the seven 42-year-old plants and the seven 58-year-old plants were then pooled to calculate the species accumulation curves, which were used to estimate the number of species in a given sample. The estimators of the total number of species and of the species without the singletons were calculated (Colwell 2005). The species accumulation was plotted in order to study the relationships between the number of fungal taxa isolated, and the number of plants per age. This accumulation was used to calculate both the species accumulation curves (Sobs) and the Mao-Tau estimator, itself used to compare the species richness with a 95% confidence interval.

Fungal community structure based on species-level identification

The effect of both vine age (42 or 58-year-old) and trunk location (upper, central or basal) on fungal community structure was determined by canonical correspondence analyses (CCAs) with the R package ade4TkGUI software (Dray and Dufour 2007). Analyses were based on the relative abundance of the ten most common species found in samples. The unanalysed species were those that were present in fewer than four of the 42 samples. In the first CCA, vine age was treated as an independent variable, species relative abundance as dependent variables, and trunk section as the co-variable. Centroids for the independent variables (vine age or trunk section) and species scores for the fungi are presented in a biplot. Proximity of a species score to the centroid of an independent variable signifies that the species has the highest relative abundance in vines of that age or in that trunk section.

Results

Status of the trunk wood

Trunks of the seven 58-year-old vines were hollow at the centre and had a high proportion of necrotic wood (Figure 1). Four of these vines were characterised by the presence of white rot, which is associated with the wood-rotting Basidiomycete fungi that sometimes colonise vines with Esca (Bertsch et al. 2013). None of the 42-year-old vines had white rot, but they all had other types of necrosis (Figure 1). In plants of both 58 and 42-year-old vines, functional tissues in the outer part looked yellow or bright brown.

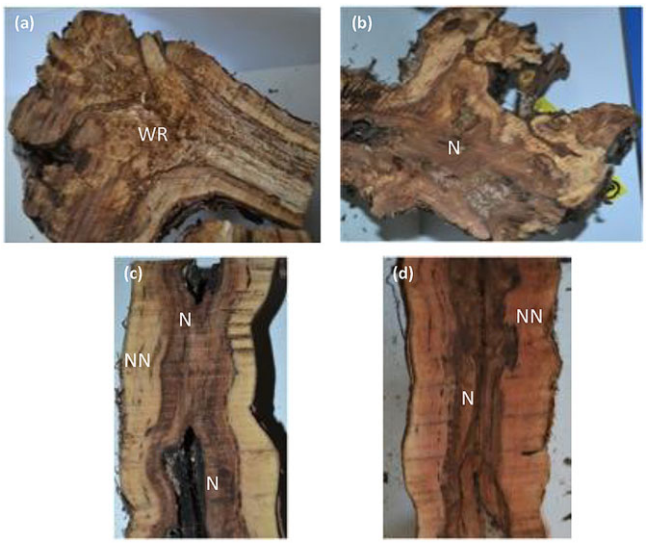

Figure 1. (a,b) Longitudinal cordon sections and (c,d) longitudinal trunk sections of (b,d) 42-year-old and (a,c) 58-year-old Baco Blanc grapevines. N: necrotic tissue, NN: non-necrotic tissue, WR: white rot.

Fungal community structure based on DNA fingerprinting

From the profiles obtained by SSCP analyses, PCA eingenvalues indicated that the first two principal components, Dim 1 and Dim 2, accounted for 62.89% of total data variance (Figure 2). The bunch representing the wood-colonising fungi of 42 year-old plants is clearly separated from that of the 58-year-old vines by Dim 1, negative versus positive coordinates, respectively.

For each 42-year-old vine, the SSCP profiles of the fungal microflora from the trunk (upper, central, basal) and cordon were compared and analysed in order to obtain a PCA (Figure 3). The first two principal components, Dim 1 and Dim 2, accounted for 60% of the data variance. Dim 1 and Dim 2, negative versus positive coordinates, respectively, did not separate the clusters representing the different grapevine parts. Similar trends (not shown) were obtained with the 58-year-old grapevines.

Fungal community structure based on species-level identification

We identified 421 fungal taxa: 218 taxa from the 58-year-old vines and 203 from the 42-year-old vines. Of the 421 taxa, 103 were identified at species level (61 isolates from 58-year-old vines, 42 isolates from 42-year-old vines), and 318 were identified at genus level (Table 1).

Ascomycota, representing 11 orders, was the predominant fungal division (83% of 421 taxa), followed by Basidiomycota, with three orders (13%), and one order of Zygomycota (4%) (Figure 4). The most common order was Hypocreales (Ascomycota), which included isolates from each vine. The other common orders were Xylariales (Ascomycota), Eurotiales (Ascomycota) and Mucorales (Zygomycota). Isolates of Saccharomycetales (Ascomycota) were isolated from only one 42-year-old vine, and Botryosphaeriales (Ascomycota) from three 58-year-old vines (Figure 4). Basidiomycota were identified in vines of both ages, with the highest number of isolates coming from the 42-year-old vines.

More genera were isolated from the 58-year-old vines than from the 42-year-old vines (40 vs 30, respectively). In the 58-year-old vines, four taxa accounted for 50% of all isolates:

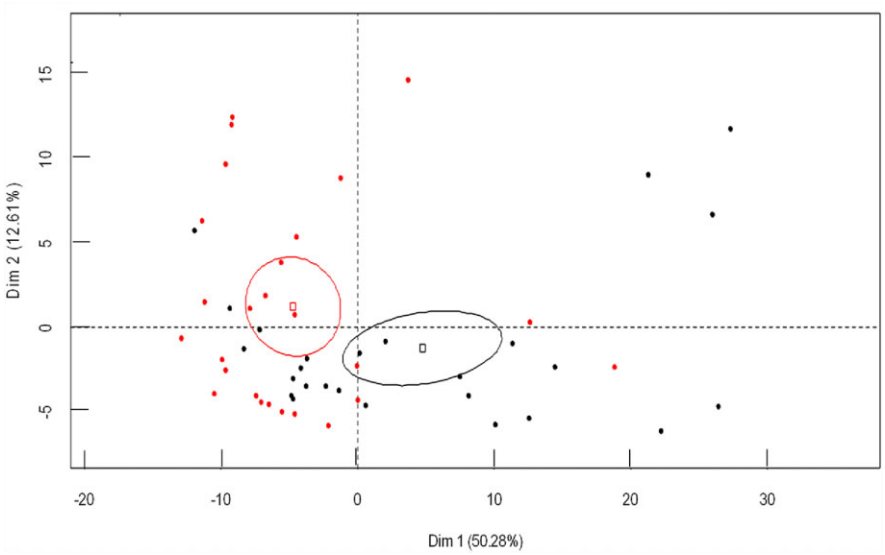

Figure 2. Principal component analysis (PCA) of the fungal microflora colonising the cordon, upper, central and basal parts of trunks of 42-year-old (●) and 58-year-old (●) Baco Blanc grapevines based on single-stranded conformational polymorphism (SSCP) profiles. The variation (%) explained by each PCA axis is given in brackets. Individual SSCPs (,□), and ellipses to the 95% confidence intervals calculated for each community are indicated.

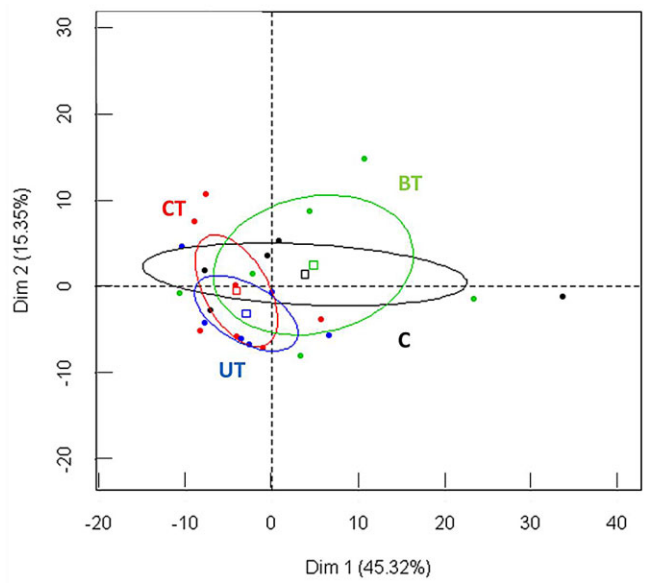

Figure 3. Distribution of the fungal communities is based on the principal plane represented by principal component analysis (PCA) of the fungal microflora colonising the trunk [upper (UT) (●), central (CT) (●), basal (BT) (●) parts] and the cordon (C) (●) of the 42-year-old Baco Blanc grapevines. The variation (%) explained by each PCA axis is given in brackets. Individual single-stranded conformational polymorphisms (, , ,□) and ellipses to the 95% confidence intervals calculated for each community are indicated.

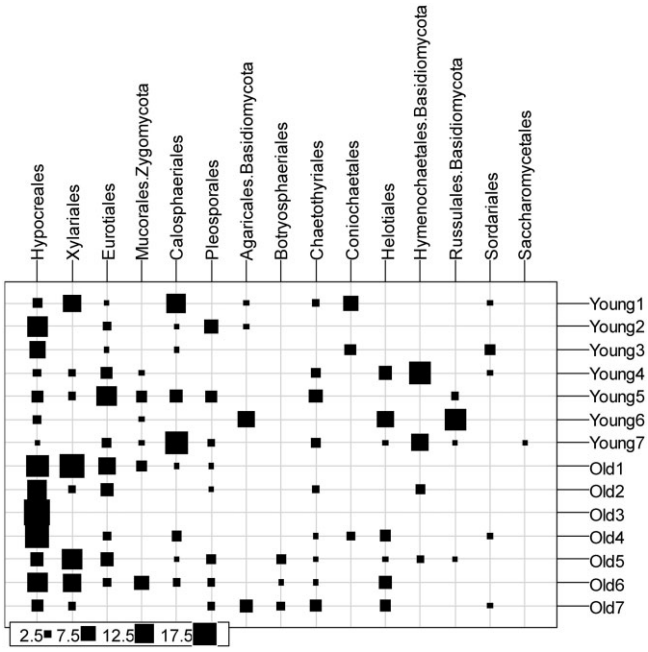

Figure 4. Distribution of the fungi orders per Baco Blanc grapevines aged 42 years (seven plants coded Young1 to Young7) or 58 years (seven plants coded Old1 to Old7). A square represents the number of isolates per plant and the size of the square depends of the number of isolates per plant.

trunk pathogen *Eutypa lata*, putative mycoparasite *Trichoderma harzianum*, an unidentified *Trichoderma* species and the saprobe *Penicillium* sp. (Figure 5). In the 42-year-old vines, 50% of all isolates represented five taxa: Esca pathogens such as *P. aleophilum* and *P. chlamydospora*, white rot fungi such as *F. mediterranea*, *S. hirsutum* and saprobe such as *T. harzianum* and *Penicillium* sp. In the 58-year-old vines, there was a significantly greater frequency of three taxa: Eutypa dieback pathogen *E. lata*, putative mycoparasite *Trichoderma* sp. and *Bionectria ochroleuca*. In the 42-year-old vines, there was a significantly greater frequency of five taxa: Esca pathogen *P. aleophilum*, saprobic white rot fungi *F. mediterranea* and *S. hirsutum*, and saprobes *Coniochaeta velutina* and *Scytalidium cuboideum*. Although the 58- and 42-year-old plants shared many similar species, the number of strains of *Trichoderma* sp. (*P* value = 0.007) and of *E. lata* (*P* value = 0.025) differed.

Species richness was higher for the 58-year-old vines than for the 42-year-old vines (12 vs 10, respectively), but this difference was not significant (Kolmogorov–Smirnov test, $P = 0.9$; Figure 6). Neither the Shannon ($P = 0.2$) nor the Simpson diversity indices ($P = 0.2$) were significantly different. Species evenness was, likewise, not significantly different ($P = 0.3$).

When all the fungi were considered (continuous lines), the curves of species accumulation were not asymptotic, but when the plural and double fungal taxa were considered (broken lines), the asymptotes were obtained (Figure 7).

The ten most abundant species isolated from the 42-year-old and/or 58-year-old plants were analysed according to age and vine parts (Figure 8). Some of these ten species, as revealed by CCA, were specific to the fungal community of the 42-year-old vines, with *S. hirsutum*, *F. mediterranea* and *P. aleophilum* being the most common fungi. In the 58-year-old vines, however,

Table 1. Number of endophytic fungal species isolated from trunk of 42- and 58-year-old Baco Blanc grapevines.

Order	Family	Genus	Species	58-year old	42-year old
Xylariales	Diatrypaceae	*Eutypa*	*lata*	34	12
Hypocreales	Hypocreaceae	*Trichoderma*	sp.	31	9
Hypocreales	Hypocreaceae	*Trichoderma*	*harzianum*	29	19
Eurotiales	Trichodermaceae	*Penicillium*	sp.	17	19
Hypocreales	Bionectriaceae	*Bionectria*	*ochroleuca*	11	2
Mucorales	Cunninghamellaceae	*Gongronella*	*butleri*	10	6
Chaetothyriales	Herpotrichiellaceae	*Phaeomoniella*	*chlamydospora*	9	13
Helotiales	Vibrisseaceae	*Phialocephala*	*dimorphospora*	8	0
Hymenochaetales	Hymenochaetaceae	*Fomitiporia*	*mediterranea*	5	23
Agaricales	Psathyrellaceae	*Psytharella*	*candolleana*	5	10
Helotiales	?	*Cadophora*	*hiberna*	5	3
Hypocreales	Nectriaceae	*Fusarium*	sp.	5	0
Xylariales	Amphisphaeriaceae	*Pestalotiopsis*	*disseminata*	5	0
Calosphaeriales	Calosphaeriaceae	*Phaeoacremonium*	*aleophilum*	4	31
Calosphaeriales	Calosphaeriaceae	*Phaeoacremonium*	*mortoniaceae*	3	3
Hypocreales	Hypocreaceae	*Trichoderma*	*hamatum*	3	1
Botryosphaeriales	Botryosphaeriaceae	*Botryosphaeria*	sp.	3	0
Botryosphaeriales	Botryosphaeriaceae	*Diplodia*	*seriata*	3	0
Hypocreales	Hypocreaceae	*Trichoderma*	*atroviride*	3	0
Coniochaetales	Coniochaetaceae	*Coniochaeta*	*velutina*	2	11
Eurotiales	Trichodermaceae	*Penicillium*	*citreonigrum*	2	3
Xylariales	Amphisphaeriaceae	*Truncatella*	*angustata*	2	1
Eurotiales	Trichodermaceae	*Penicillium*	*pancosmium*	2	0
Russulales	Stereaceae	*Stereum*	*hirsutum*	1	17
Helotiales	?	*Scytalidium*	*cuboideum*	1	9
Pleosporales	Montagnulaceae	*Paraconiothyrium*	sp.	1	6
Sordariales	Lasiosphaeriaceae	*Arthrinium*	sp.	1	3
Pleosporales	Sporormiaceae	*Preussia*	sp.	1	2
Pleosporales	Pleosporaceae	*Pithomyces*	*valparadisiacus*	1	2
Pleosporales	Didymellaceae	*Epicoccum*	*nigrum*	1	1
Pleosporales	Leptosphaeriaceae	*Leptosphaeria*	sp.	1	1
Eurotiales	Trichodermaceae	*Penicillium*	*expansum*	1	1
Pleosporales	Pleosporaceae	*Alternaria*	*alternata*	1	0
Pleosporales	Pleosporaceae	*Alternaria*	sp.	1	0
Sordariales	Chaetomiaceae	*Chaetomium*	*coarctatum*	1	0
Hypocreales	Nectriaceae	*Fusarium*	*oxysporum*	1	0
Pleosporales	Pleosporaceae	*Lewia*	*infectoria*	1	0
Pleosporales	Lophiostomataceae	*Lophiostoma*	*corticola*	1	0
Eurotiales	Trichodermaceae	*Penicillium*	*commune*	1	0
Xylariales	Amphisphaeriaceae	*Pestalotiopsis*	*mangiferae*	1	0
Xylariales	Amphisphaeriaceae	*Seiridium*	*ceratosporum*	1	0
Sordariales	Chaetomiaceae	*Chaetomium*	*globosum*	0	3
Helotiales	Dermateaceae	*Cryptosporiopsis*	sp.	0	2
Hypocreales	Hypocreaceae	*Trichoderma*	*virens*	0	1
Mucorales	Mucoraceae	*Mucor*	*circinelloides*	0	1
Saccharomycetales	Saccharomycetaceae	*Saccharomyces*	*guilliermondii*	0	1

Trichoderma sp. and *E. lata* were more common. Axis 1 of the CCA biplot significantly accounted for these differences between the 42 and 58-year-old plants.

Discussion

In the context of the current epidemic outbreak of GTDs in France (Bruez et al. 2013), we studied the endophytic fungal microflora colonising the wood of 42- and 58-year-old grapevines that did not express foliar GTDs symptoms during the year of sampling. In the trunk and cordons of the sampled grapevines, wood necroses were significant, with the wood tissues being completely black and damaged in the inner part; functional tissues in the outer part looked yellow or bright brown. In spite of the significant wood degradation, however, the vines did produce berries that were subsequently used to produce brandy (i.e. Armagnac). We therefore considered that those plants could be used to study the fungal microflora colonising the functional tissues of old grapevines. They could also be used to determine whether plant-pathogenic or plant-beneficial fungi colonise these structures, which are vital for grapevine growth and life.

As shown by SSCP, the global fungal microflora of 42 or 58-year-old plants were different. This finding is in line with Arnold et al. (2001) who reported that, for tropical fungal endophytes, the age and pathogenic status of the plant may exert some influence. In addition, other factors, such as the

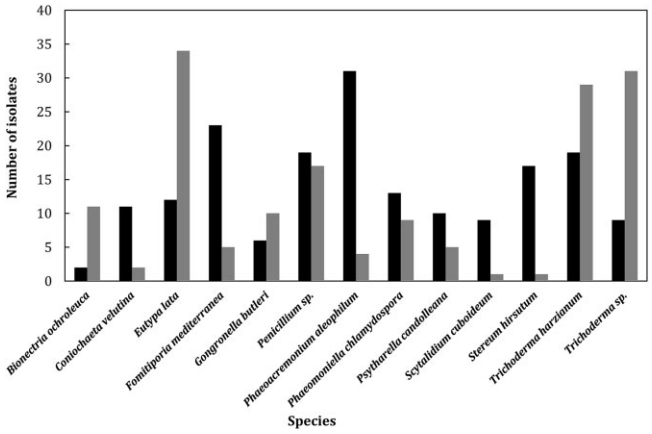

Figure 5. Distribution of the number of isolates from cultured fungi representing the 13 principal genera and species distinguished by DNA-internal transcribed spacer sequencing of Baco Blanc grapevines aged 42 ( ) and 58 ( ) years.

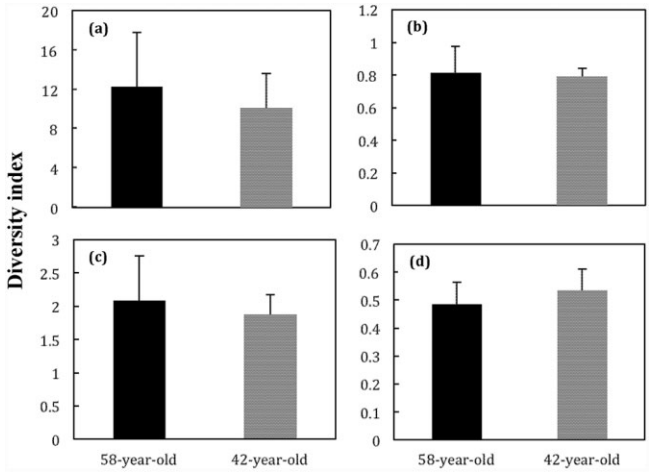

Figure 6. Diversity indexes with the confidence limits: (a) Richness; (b) Simpson; (c) Shannon; and (d) Evenness, for the 42- and 58-year-old Baco Blanc grapevines. Data on ordinate axis synthesise average values of seven plants per age.

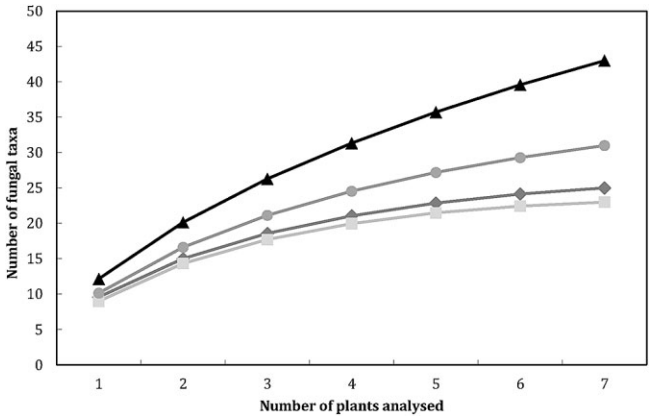

Figure 7. Species accumulation curves showing the relationship between the number of plants analysed and the number of fungal taxa isolated in Baco Blanc aged 42 years (●),42 years without singletons ( ), 58 years (▲) and 58 years without singletons (◆).

sampling locality (Arnold et al. 2001, Higgins et al. 2007) and the climate (Arnold and Lutzoni 2007) may have an influence on the composition of the endophytic fungal microflora. In our experiment, as the grapevines were planted in one specific vineyard site, with the same type of soil, and as those grapevines were submitted to the same climate, our results suggest that the fungal microflora colonising the grapevine trunk are also age dependent. Within plants of the same age class, SSCP also revealed that the fungal communities colonising the cordon and the various parts of the trunk could not be differentiated, thus indicating that the same mycoflora colonise these outer wood tissues. This result agrees with that of Bruez et al. (2014) in their study of the mycoflora colonising the various healthy wood tissues of younger, 10-year-old, Esca-foliar symptomatic or asymptomatic grapevines.

The results of culturable strain identification using DNA–ITS sequencing showed that at least 60% of the wood chips were colonised by fungi for both 42- and 58-year-old grapevines, and 421 fungal strains were isolated and identified by DNA–ITS sequencing. Forty-six taxa were obtained from 630 chips of wood. The most abundant fungal division was Ascomycota, followed by Basidiomycota and Zygomycota. This distribution pattern of Ascomycota is consistent with other studies on the endophytic communities of woody tissues or shoots, and on the leaves of different plant hosts (Bills 1996, Frohlich et al. 2000, Rungjindamai et al. 2008), including grapevines (Hofstetter et al. 2012, Pancher et al. 2012, Bruez et al. 2014). Previous research showed that the Basidiomycota were the most abundant fungi isolated from wood decay tissue (Pouska et al. 2010). As we mainly sampled the non-necrotic wood tissues, the proportion of Ascomycota versus Basidiomycota was as expected.

Out of the 46 fungi species identified, 41 were isolated from 42-year-old plants and 30 species from 58-year-old plants; 25 were common to the two ages of grapevine. Twelve singleton species were isolated in both types of plant. Casieri et al. (2009) isolated endophytic fungi from healthy 1-year-old vines, 66 operational taxonomic units were determined, and Hofstetter et al. (2012) isolated, in a Swiss vineyard, 158 different species from 15- to 30-year-old grapevine wood. The particularly low number of species we isolated in the old Baco Blanc plants could be related to their age. The species accumulation curves indicated that, without the singleton, the sampling appeared to be representative of the isolation of species from the wood tissues. Few fungal species were abundant, and CCA results indicated that some species are specifically associated with a particular grapevine type.

In 42-year-old plants, the most numerous pathogenic fungi were: *Phaeoacremonium aleophilum*, followed by *F. mediterranea*, *S. hirsutum*, *P. chlamydospora* and *E. lata*. All of these fungi are involved in Esca, and are able to cause wood necroses in grapevines, as reported by Bertsch et al. (2013) and Laveau et al. (2009). In the 58-year-old plants, the same fungi were isolated, but the most abundant pathogenic fungus was *E. lata*, which is associated with Eutypa dieback (Bertsch et al. 2013).

Another fungus, a wood-decay fungus, *Psathyrella candolleana*, also relatively abundant in the wood of grapevines (ten strains in 42-year-old plants and five strains in 58-year-old ones), selectively decomposes carbohydrates in the wood (Fukasawa et al. 2005).

As previously shown, all the plant pathogens were isolated in grapevines, at different proportions. Two different pathogenic profiles were obtained: one that was associated with Esca in the 42-year-old plants and the other with Eutypa dieback in the 58-year-old plants. In all these grapevines other fungi, with potentially antagonistic activities, were isolated but, depending on plant age, at different proportions. If pathogens or wood-decaying fungi colonise the relatively preserved outer wood tissues, potentially plant-protective fungi, such as *Trichoderma*

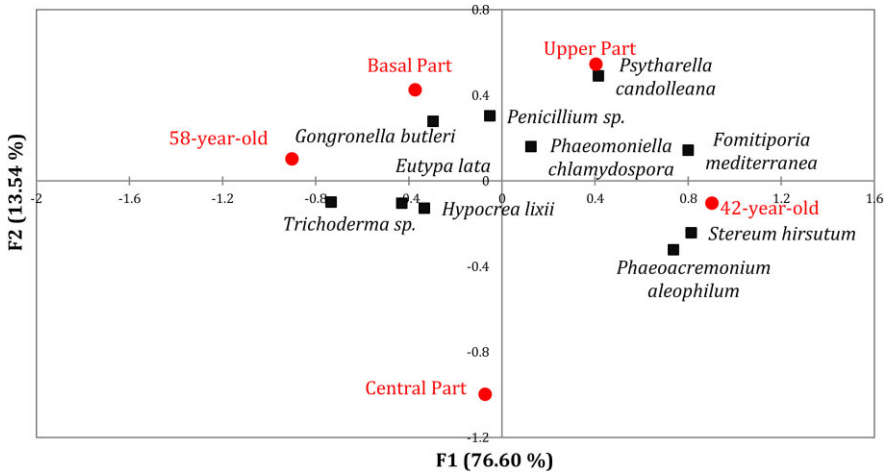

Figure 8. Biplot of canonical correspondence analysis of the effect of vine age and trunk parts on the ten most abundant species isolated from the 42-year-old and/or 58-year-old Baco Blanc grapevines.

spp. and *B. ochroleuca*, inhabit the same tissues. In the 58-year-old plants, *Trichoderma* sp., *T. harzianum* and *T. atroviride* were the most abundant fungi, with their numbers exceeding those of *E. lata*. The same pattern of fungi was found in the 42-year-old plants, with *Trichoderma* spp. being predominant.

Trichoderma spp. are known to be opportunistic, avirulent plant symbionts (Harman et al. 2004), and the antagonistic activity of some *Trichoderma* spp. strains against pathogenic fungi has been extensively described (Perveen and Bokhari 2012, Schwarze et al. 2012). Generally, this activity consists of mycoparasitism and antibiosis (Lu et al. 2004, Brunner et al. 2005). So, in the present case, it can be assumed that *Trichoderma* spp., as well as other mycoparasites such as *B. ochroleuca*, could interact in the wood with other fungi, such as Esca-pathogenic fungi and *E. lata*.

Trichoderma harzianum is a fungus which can reduce the recovery of *E. lata* in the trunk of grapevines in the field. In vitro, it produces metabolites able to decrease the development of *E. lata* (John et al. 2005). Fourie et al. (2001) showed that, in the nursery, *T. harzianum* strains that were mixed together produced a change in the microflora associated with the roots. In fact, the mixture reduced the incidence of *Cylindrocarpon* spp., *Phaeoacremonium* spp. and *Phaeomoniella* spp. By colonising the roots, *Trichoderma* spp. also prevented the development of pathogens in the roots by antagonist competition. In the field, a *Trichoderma* mixture (isolates of *T. harzianum* specie) was effective in reducing the necrosis produced by *P. chlamydospora* on the rootstock (Di Marco and Osti 2007).

In the present experiment, the colonisation of the wood trunk by potentially antagonistic fungi, e.g. *Trichoderma* spp. and *B. ochroleuca*, at various proportions, suggests that the interactions that occur between fungi differ for the global mycoflora in the 42- and 58-year-old plants. It can be hypothesised that competition between non-pathogenic and pathogenic fungi result in the inhibition of the development of some fungi. For instance, if *F. mediterranea* is hampered in its development, this may explain why we did not observe white rot symptoms in the functional woody tissue. Further research, aimed at studying these interactions in grapevine trunk, needs to be undertaken in order to determine how the microbial communities can evolve over time. For instance, the properties of *Trichoderma* spp. colonising the grapevine wood tissues, for example direct mode of action against pathogens through competition, or indirect mode of action via plant induction of resistance, have to be determined. Recently, Bruez et al. (2014) reported that shifts occurred in the fungal communities colonising the healthy wood tissues of grapevines over a period of 1 year. They indicated that these shifts could be associated with plant physiology. During spring, the plant produces leaves and grapes, and numerous molecules are transported into the vessels. This source of food probably has an influence on the fungal communities and can, at least in part, explain the shifts observed in the microflora. In contrast, in winter, when the plant is in a latent state, there are either no, or only a limited number of, vascular exchanges within the tissues. Low temperature in winter and high in summer may also shape, at least in part, the fungal communities. So, in the present study, in addition to the interactions between fungi, one may assume that the various mycoflora of Baco Blanc grapevines may also result from the influence of abiotic factors, such as the climate and the presence of nutrients moving in the xylem tissues.

In conclusion, our investigation showed that fungal communities, which are associated with Esca or Eutypa-dieback diseases, colonise the two differently aged plants, however, GTD-foliar symptoms were not observed. It can be hypothesised that, within the relatively preserved outer wood tissues, the microbial balance between the potentially plant-beneficial and plant-pathogenic fungi may prevent the pathogens from developing extensively and, therefore the grapevine from becoming diseased. Consequently, studying this fragile equilibrium and how it shapes the mycoflora should open up major new opportunities for plant protection.

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References

Abarenkov, K., Nilsson, R.H., Larsson, K.H., Alexander, I.J., Eberhardt, U., Erland, S., Hoiland, K., Kjoller, R., Larsson, E., Pennanen, T., Sen, R., Taylor, A.F.S., Tedersoo, L., Ursing, B.M., Vralstad, T., Liimatainen, K., Peintner, U. and Koljalg, U. (2010) The UNITE database for molecular identification of fungi – recent updates and future perspectives. New Phytologist **186**, 281–285.

Armengol, J., Vicent, A., Torne, L., Garcia-Figueres, F. and Garcia-Jimenez, J. (2001) Fungi associated with esca and grapevine declines in Spain: a three-year survey. Phytopathologia Mediterranea **40**, S325–S329.

Arnold, A.E. and Lutzoni, F. (2007) Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? Ecology **88**, 541–549.

Arnold, A.E., Maynard, Z. and Gilbert, G.S. (2001) Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. Mycological Research **105**, 1502–1507.

Bertsch, C., Ramirez-Suero, M., Magnin-Robert, M., Larignon, P., Chong, J., Abou-Mansour, E., Spagnolo, A., Clément, C. and Fontaine, F. (2013) Grapevine trunk diseases: complex and still poorly understood. Plant Pathology **62**, 243–265.

Bills, G.F. (1996) Isolation and analysis of endophytic fungal communities from woody plants. Redlin, S.C. and Carris, L.M., eds. Endophytic fungi in grasses and woody plants: systematics, ecology, and evolution (American Phytopathological Society: St Paul, MN, USA) pp. 31–65.

Bruez, E., Lecomte, P., Grosman, J., Doublet, B., Bertsch, C., Fontaine, F., Ugaglia, A., Teisedre, P.-L., Da Costa, J.-P., Guerin-Dubrana, L. and Rey, P. (2013) Overview of grapevine trunk diseases in France in the 2000s. Phytopathologia Mediterranea **52**, 262–275.

Bruez, E., Vallance, J., Gerbore, J., Lecomte, P., Da Costa, J.-P., Guerin-Dubrana, L. and Rey, P. (2014) Analyses of the temporal dynamics of fungal communities colonizing the healthy wood tissues of Esca leaf-symptomatic and asymptomatic vines. PLoS ONE **9** (5), e95928.

Brunner, K., Zeilinger, S., Ciliento, R., Woo, S.L., Lorito, M., Kubicek, C.P. and Mach, R.L. (2005) Improvement of the fungal biocontrol agent *Trichoderma atroviride* to enhance both antagonism and induction of plant systemic disease resistance. Applied and Environmental Microbiology **71**, 3959–3965.

Casieri, L., Hofstetter, V., Viret, O. and Gindro, K. (2009) Fungal communities living in the wood of different cultivars of young *Vitis vinifera* plants. Phytopathologia Mediterranea **48**, 73–83.

Chanderbali, A.S., van der Werff, H. and Renner, S.S. (2001) Phylogeny and historical biogeography of Lauraceae: evidence from the chloroplast and nuclear genomes. Annals of the Missouri Botanical Garden **88**, 104–134.

Colwell, R.K. (2005) EstimateS: statistical estimation of species richness and shared species from samples, version 8. http://viceroy.eeb.uconn.edu/estimates/.

Di Marco, S. and Osti, F. (2007) Applications of *Trichoderma* to prevent *Phaeomoniella chlamydospora* infections in organic nurseries. Phytopathologia Mediterranea **46**, 73–83.

Dray, S. and Dufour, A.B. (2007) The ade4 package: implementing the duality diagram for ecologists. Journal of Statistical Software **22** (4), 1–20.

Edwards, J. and Pascoe, I.G. (2004) Occurrence of *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* associated with Petri disease and esca in Australian grapevines. Australasian Plant Pathology **33**, 273–279.

Fischer, M. and Kassemeyer, H.-H. (2003) Fungi associated with Esca disease of grapevine in Germany. Vitis **42**, 109–116.

Fourie, P.H., Halleen, F., van der Vyver, J. and Schreuder, W. (2001) Effect of *Trichoderma* treatments on the occurrence of decline pathogens in the roots and rootstocks of nursery grapevines. Phytopathologia Mediterranea **40**, S473–S478.

Frohlich, J., Hyde, K.D. and Petrini, O. (2000) Endophytic fungi associated with palms. Mycological Research **104**, 1202–1212.

Fukasawa, Y., Osono, T. and Takeda, H. (2005) Decomposition of Japanese beech wood by diverse fungi isolated from a cool temperate deciduous forest. Mycoscience **46**, 97–101.

Gimenez-Jaime, A., Aroca, A., Raposo, R., Garcia-Jimenez, J. and Armengol, J. (2006) Occurrence of fungal pathogens associated with grapevine nurseries and the decline of young vines in Spain. Journal of Phytopathology **154**, 598–602.

Gotelli, N.J. and Colwell, R.K. (2001) Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. Ecology Letters **4**, 379–391.

Guerin-Dubrana, L., Labenne, A., Labrousse, J.C., Bastien, S., Rey, P. and Gegout-Petit, A. (2013) Statistical analysis of grapevine mortality associated with esca or eutypa dieback foliar expression. Phytopathologia Mediterranea **52**, 276–288.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I. and Lorito, M. (2004) Trichoderma species – opportunistic, avirulent plant symbionts. Nature Reviews. Microbiology **2**, 43–56.

Higgins, K.L., Arnold, A.E., Miadlikowska, J., Sarvate, S.D. and Lutzoni, F. (2007) Phylogenetic relationships, host affinity, and geographic structure of boreal and arctic endophytes from three major plant lineages. Molecular Phylogenetics and Evolution **42**, 543–555.

Hofstetter, V., Buyck, B., Croll, D., Viret, O., Couloux, A. and Gindro, K. (2012) What if esca disease of grapevine were not a fungal disease? Fungal Diversity **54**, 51–67.

John, S., Wicks, T.J., Hunt, J.S., Lorimer, M.F., Oakey, H. and Scott, E.S. (2005) Protection of grapevine pruning wounds from infection by *Eutypa lata* using *Trichoderma harzianum* and *Fusarium lateritium*. Australasian Plant Pathology **34**, 569–575.

Larignon, P. and Dubos, B. (1997) Fungi associated with esca disease in grapevine. European Journal of Plant Pathology **103**, 147–157.

Larignon, P., Coarer, M., Girardon, K., Berud, F. and Jacquet, O. (2009) Propagation of pioneer fungi associated with esca disease by vegetative material in French grapevine nurseries. Phytopathologia Mediterranea **48**, 177.

Laveau, C., Letouze, A., Louvet, G., Bastien, S. and Guerin-Dubrana, L. (2009) Differential aggressiveness of fungi implicated in esca and associated diseases of grapevine in France. Phytopathologia Mediterranea **48**, 32–46.

Lu, Z.X., Tombolini, R., Woo, S., Zeilinger, S., Lorito, M. and Jansson, J.K. (2004) In vivo study of *Trichoderma*-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. Applied and Environmental Microbiology **70**, 3073–3081.

Lyons, N.I. and Hutcheson, K. (1979) Distributional properties of Simpson's index of diversity. Communication in Statistics – Theory and Methods **8**, 569–574.

Magurran, A.E. (2004) Measuring biological diversity (Blackwell Science: Oxford, England).

Michelland, R.J., Dejean, S., Combes, S., Fortun-Lamothe, L. and Cauquil, L. (2009) StatFingerprints: a friendly graphical interface program for processing and analysis of microbial fingerprint profiles. Molecular Ecology Resources **9**, 1359–1363.

Mugnai, L., Graniti, A. and Surico, G. (1999) Esca (Black measles) and brown wood-streaking: two old and elusive diseases of grapevines. Plant Disease **83**, 404–418.

Munkvold, G.P., Duthie, J.A. and Marois, J.J. (1993) Spatial patterns of grapevines with Eutypa dieback in vineyards with or without perithecia. Phytopathology **83**, 1440–1448.

Pancher, M., Ceol, M., Corneo, P.E., Longa, C.M.O., Yousaf, S., Pertot, I. and Campisano, A. (2012) Fungal endophytic communities in grapevines (Vitis vinifera L.) respond to crop management. Applied and Environmental Microbiology **78**, 4308–4317.

Perveen, K. and Bokhari, N.A. (2012) Antagonistic activity of *Trichoderma harzianum* and *Trichoderma viride* isolated from soil of date palm field against *Fusarium oxysporum*. African Journal of Microbiology Research **6**, 3348–3353.

Pouska, V., Svoboda, M. and Lepsova, A. (2010) The diversity of wood-decaying fungi in relation to changing site conditions in an old-growth mountain spruce forest, Central Europe. European Journal of Forest Research **129**, 219–231.

R Core Team (2012) R: a language and environment for statistical computing (R Foundation for Statistical Computing: Vienna, Austria). http://www.R-project.org/.

Rego, C., Vaz, A., Nascimento, T., Cabral, A. and Oliveira, H. (2009) Diseases incited by Botryosphaeriaceae fungi in Portuguese vineyards. Phytopathologia Mediterranea **48**, 181.

Rumbos, I. and Rumbou, A. (2001) Fungi associated with esca and young grapevine decline in Greece. Phytopathologia Mediterranea **40**, S330–S335.

Rungjindamai, N., Pinruan, U., Choeyklin, R., Hattori, T. and Jones, E.B.G. (2008) Molecular characterization of basidiomycetous endophytes isolated from leaves, rachis and petioles of the oil palm, *Elaeis guineensis*, in Thailand. Fungal Diversity **33**, 139–161.

Scheck, H.J., Vasquez, S.J., Gubler, W.D. and Fogle, D. (1998) First report of three *Phaeoacremonium* spp. causing young grapevine decline in California. Plant Disease **82**, 590.

Schwarze, F., Jauss, F., Spencer, C., Hallam, C. and Schubert, M. (2012) Evaluation of an antagonistic *Trichoderma* strain for reducing the rate of wood decomposition by the white rot fungus *Phellinus noxius*. Biological Control **61**, 160–168.

Shannon, C.E. and Weaver, W. (1963) The mathematical theory of communication (University of Illinois Press: Champaign, IL, USA).

Úrbez-Torres, J.R., Leavitt, G.M., Voegel, T.M. and Gubler, W.D. (2006) Identification and distribution of Botryosphaeria spp. associated with grapevine cankers in California. Plant Disease **90**, 1490–1503.

Úrbez-Torres, J.R., Adams, P., Kamas, J. and Gubler, W.D. (2009) Identification, incidence, and pathogenicity of fungal species associated with grapevine dieback in Texas. American Journal of Enology and Viticulture **60**, 497–507.

White, C.-L., Halleen, F., Fischer, M. and Mostert, L. (2011) Characterisation of the fungi associated with esca diseased grapevines in South Africa. Phytopathologia Mediterranea **50**, 204–223.

White, T., Birns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Innis, D.G.M., Sninsky, J. and White, T., eds. PCR protocols: a guide to methods and applications (Academic Press: San Diego, CA, USA) pp. 315–322.

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