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Research Papers

Grapevine pruning strategy affects trunk disease symptoms, wood pathobiome and mycobiome

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Summary. Vine training and pruning are cultural strategies that can be deployed to manage grapevine trunk diseases (GTDs). Forty-year-old commercial vineyards in the Cognac region, France, trained to either Guyot-Arcure (severe pruning) or Guyot-Poussard (minimal pruning), were studied to determine how the two systems affected trunk disease symptomatology. Effects of pruning practices on the pathobiome and mycobiome of asymptomatic grapevines were also assessed, using culture- and amplicon-based Illumina sequencing approaches. The hypothesis examined was that severe pruning of Guyot-Arcure increases trunk diseases incidence and severity, and causes higher pathogen load and microbial diversity, compared to Guyot-Poussard. Numbers of symptomatic and asymptomatic vines for the two training systems were recorded over 3 years, including numbers of vines with esca foliar symptoms, and partially unproductive and dead vines. Six asymptomatic vines from each pruning method were selected, and culturing and sequencing data were obtained from 27 samples per vine. Fungi in the *Phaeomoniellaceae*, *Togniniaceae*, and *Botryosphaeriaceae* were the most frequently identified. The data indicated that severe pruning increased risk of pathogen infections, with *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum* and *Diplodia* sp. being the most commonly identified fungi. Greater numbers of dead or dying vines were recorded in the severely pruned vineyard, indicating that this strategy shortens vine longevity. Results also showed that severe pruning increased endophytic microbial diversity, and that the pruning methods influenced mycobiome community composition. This knowledge will improve recommendations to growers for practical and cost-effective ways to manage GTDs.

Keywords. Botryosphaeria canker, esca, disease management, microbiome.

INTRODUCTION

Grapevines are cultivated for their fresh fruit, dried fruit, wine, and other spirits, producing more than 77 million tons annually and providing US\$ 68 billion production value (Alston *et al.*, 2019; Casolani *et al.*, 2022). Grapevine Trunk Diseases (GTDs) are a significant impediment to grape production worldwide (Bois *et al.*, 2017; Gramaje *et al.*, 2018). GTDs are caused by a complex of taxonomically unrelated fungal pathogens, and vines are often affected by mixed infections of fungi (Bertsch *et al.*, 2013; Gramaje *et al.*, 2018).

Esca, Botryosphaeria canker and Eutypa dieback are among the main GTDs that affect vineyard longevity, yield of productive vines, and quality of the fruit (Kaplan *et al.*, 2016; Gispert *et al.*, 2020; Larach *et al.*, 2020; Dewasme *et al.*, 2022). *Eutypa lata* (*Diatrypaceae*) is the main causal agent of Eutypa dieback, and several taxa in the *Botryosphaeriaceae* cause Botryosphaeria canker. Both diseases have symptoms of brown to black sectorial necroses in grapevine wood, and Eutypa dieback also develop symptoms of shoot and leaf dwarfing (Rolshausen *et al.*, 2006; Úrbez-Torres, 2011; Travadon *et al.*, 2012; Bertsch *et al.*, 2013). The *Ascomycota* fungi *Phaeomoniliella chlamydospora* and *Phaeoacremonium minimum* (formerly *P. aleophilum*), and *Basidiomycota* *Fomitiporia mediterranea* are among the major causal agents of esca. Esca wood symptoms include black spots in trunk cross-sections and streaking in longitudinal sections but can be surrounded by pink to brown wood discolorations, and in older vines, white rot is also common. Foliar symptoms associated with esca include tiger stripe leaf patterns, wilting, and apoplexy, ranging from a few leaves to the entire vine canopies (Mugnai *et al.*, 1999; Surico, 2009; Lecomte *et al.*, 2012; Lecomte *et al.*, 2018).

Among all GTDs, the esca complex is a serious threat to grape production. A survey of European and Mediterranean vineyards by Guerin-Dubrana *et al.* (2019) indicated that esca symptoms were the most common. Wood dieback symptoms caused by Eutypa and Botryosphaeria were less frequently recorded, although incidence of Botryosphaeria canker was reported to be increasing in several countries. In France, the National Grapevine Trunk Disease Survey reported that GTDs incidence increased nationally from 3 to 13% between 2003 and 2013, with esca being the most-widespread, whereas other GTDs such as Eutypa dieback were more region-specific (Fussler *et al.*, 2008; Bruez *et al.*, 2013; Lecomte *et al.*, 2018). Esca foliar symptom expression has been shown to be erratic, linked to different amounts of annual rainfall, particularly in late spring to early summer (Calzarano *et al.*, 2018; Kraus *et al.*, 2019;

Dewasme *et al.*, 2022). Recent studies that have deployed high throughput gene sequencing methods to profile the mycobiome in symptomatic and asymptomatic esca-affected grapevines have also identified the causal agents of Eutypa dieback and Botryosphaeria canker (Bruez *et al.*, 2014; Del Frari *et al.*, 2019). Co-occurrence of these pathogens in the woody tissues could also affect esca symptom expression and may explain inconsistencies of annual observations of foliar disease symptoms.

Grapevine wounds are the main entry points for GTD pathogens, so grapevines are especially susceptible to GTD infections during pruning. Wound susceptibility mainly depends on the time of pruning and the period between pruning and infection events (Munkvold and Marois, 1995). Temperature and rainfall influence wound healing processes, and the period vine susceptibility, as well as the quality and quantity of pathogen inoculum (Eskalen *et al.*, 2007; Martinez-Diz *et al.*, 2020). There are no effective curative methods or treatments for GTDs. These diseases are mainly managed using preventative strategies, among which fungicide applications to protect pruning wounds have been the most effective (Rolshausen *et al.*, 2010). Although removal of infected wood (“curettage”) has been shown to reduce foliar and fruit symptoms of esca (Cholet *et al.*, 2021; Pacetti *et al.*, 2021). Growers often start protecting pruning wounds with the appearance of first GTD symptoms, when it is too late for effective disease control. Pruning wound treatment practices must be used early at vineyard establishment to provide yield benefits (Kaplan *et al.*, 2016; Gispert *et al.*, 2020; Di Marco *et al.*, 2022). The banning of sodium arsenite in Europe, that was long registered for protecting grapevine pruning wounds, left growers with no alternatives, and likely resulted in an increased of GTDs incidence (Bruez *et al.*, 2021). Many growers do not protect pruning wounds, due to costs and lack of immediate visible benefits of fungicide applications because of the long incubation period from pathogen infection to symptoms appearance (Hillis *et al.*, 2017). Implementing alternative disease prevention strategies that are affordable and time efficient would provide large benefits to viticulture industries.

Vine training and pruning have been studied as a strategy to reduce GTDs. Pruning objectives balance vine productivity with fruit quality. Attaining this balance while reducing the number and size of pruning wounds would minimize the point of entry for vascular GTD pathogens. The ‘Vertical Shoot Positioned’ (VSP) system that has been broadly and internationally adopted is an intensive pruning system that is more conducive to GTDs compared to minimal pruning (Gu *et al.*, 2005; Travadon *et al.*, 2016; Kraus *et al.*, 2019; Kraus *et*

al., 2022). There are different types of VSP training systems; the ‘Guyot-Poussard’ system, similar to cordon pruning, trains long mature arms with few large cuts close to main trunks, and can reduce esca (Lafon, 1921). In contrast, the ‘Guyot-Simple’ system, similar to cane-pruned vines, trains one spur on each side of each vine, and creates large cuts close to the trunk head. This system was described to be conducive of GTDs (Lecomte *et al.*, 2018). Following a survey of French vineyards, Lecomte *et al.* (2018) noted that vine training forms with long arms (cordons) decline less rapidly than short arm training forms. However, those observations were not supported with studies in the United States of America (Gu *et al.*, 2005) and Australia (Henderson *et al.*, 2021), that measured less *Eutypa dieback* incidence and severity in cane-pruned vineyards than in cordon-pruned vineyards. Henderson *et al.* (2021) concluded that the spur pruning resulted in wounds that were smaller than those made by cane pruning, but the larger number of cuts made per vine resulted in greater wound area per vine. Thus, limiting the total surface wound area per vine should be taken into account when pruning vines to limit GTDs incidence. While all these comparative studies of pruning strategies have measured disease incidence and severity, only a few have attempted to assess how the strategies affected microbial community diversity and composition of grapevine wood endospheres (Travadon *et al.*, 2016; Kraus *et al.*, 2022).

The research reported here included a comparative analysis of the Guyot-Arcure and Guyot-Poussard pruning strategies that are used in most of the Cognac vineyards of France. The aim was to evaluate, over a 3-year period in a mature (> 40-year-old) commercial vineyard, the effects of vine pruning on trunk disease symptomatology, including foliar and wood symptoms and vine death. In addition, effects of these two pruning practices on vine pathobiome and mycobiome were assessed, using

culture-based and amplicon-based Illumina sequencing approaches. The research hypothesis was that the severe Guyot-Arcure pruning increases disease severity and incidence and provides a gateway for increased pathogen load and microbial diversity compared to Guyot-Poussard pruning. The information generated from this study will provide knowledge for recommendations to growers on practical ways to prevent GTDs.

MATERIALS AND METHODS

Vineyard characteristics.

Two adjacent vineyards with contrasting training systems were selected. They were located near Cognac in the Charente region of southwestern France (Table 1). The vineyards of ‘Ugni Blanc’ on 101-14 (41B) rootstock were planted in 1972 and 1973, and one was trained as ‘Guyot-Arcure’ and one as ‘Guyot-Poussard’ (see Supplemental Figure 1A). The Guyot-Poussard training system consists of horizontal cordons with few small-to-medium-sized pruning wounds primarily concentrated at the top of the cordons, allowing permanent bilateral flow of sap in the lower vasculature of the vine cordons. In contrast, the ‘Guyot-Arcure’ training system consists of V-shaped cordons that are often renewed and restored, a system that requires extensive pruning. The positions of pruning wounds is not controlled, and can disrupt consistent sap flow that can lead to changes in sap routes (Lafon, 1921). The two vineyards were surveyed during 3 years from 2018–2020, and numbers of asymptomatic and symptomatic vines (foliar and wood symptoms) were recorded, as well as the numbers of dead vines. Direct comparisons between the grapevine training systems and incidence of symptomatic *versus* asymptomatic trunk diseases were assessed using Chi-square statistical

Table 1. Vine training systems (Arcure or Poussard) impact on mean grapevine trunk disease incidence and severity in three survey years.

Vineyard training System	Survey year	Percent asymptomatic vines	Mean percent symptomatic vines			
			Vines with esca foliar symptoms ^c	Partially unproductive Vines ^d	Unproductive Vines ^e	Total
Arcure ^a	2018	42.5	1.6	14.7	41.3	57.5
	2019	40.1	1.2	17.8	40.9	59.9
	2020	38.7	2.9	16.2	42.1	61.3
Poussard ^b	2018	70.4	0.7	14.9	14	29.6
	2019	66.9	0.4	18.6	14	33.1
	2020	65.9	1.7	17.9	14.5	34.1

^a 511 total vines; ^b 692 total vines; ^c No other foliar symptoms (e.g., *Eutypa*) recorded; ^d re-trained vines, one arm missing, one dead-arm; ^e dead or missing.

analyses. Twelve asymptomatic vines (six of each training type), were selected for further assessment. Whole vines were uprooted during the dormant season, were numbered, and were then stored in a cold room (4°C) while awaiting processing. All vines were processed (using image analysis, tissue culturing and DNA extraction from woody tissues) within 2 weeks following sampling from the vineyards.

Image analysis of wood decay

All 12 asymptomatic vines from the two pruning methods were cut longitudinally with an upright electric chainsaw. When needed, the trunk and cordons of each vine were sectioned into smaller pieces to ensure that all wood sections were cut in the middle. All the sections were photographed using a NIKON D 3100 digital camera. The proportions (%) of necrotic surface were then evaluated from these photos using the Image J software Fiji version 2.14.0 (Schindelin *et al.*, 2012), by calculating the ratio of the areas of necroses to those of the total cross section areas (Supplementary Figure 2). To do this, each image was cleaned of impurities (e.g., markings made by the saw used to make longitudinal cuts), and the image was scaled to 5 mm. To crop each image from the background, each wood piece was manually traced, and this procedure was replicated three times for accuracy. Thereafter, a threshold for the necrotic regions was created (shown in red in Supplementary Figure 2), and additional tracing of necrosis on the wood was carried out manually as required. A binary image was then created, resulting in black background and white for necrotic tissue, and this was used to calculate proportions (%) necrosis by dividing the necrotic area by the total area of the wood section. The percentage of the necrotic area was calculated for each trunk and both arms separately, then the arms and trunk were averaged. Analysis of the distribution of values was then carried out using nonparametric Kruskal-Wallis tests, with using R software version 2.8.0 (Fox, 2005).

Grapevine processing

For each of the 12 asymptomatic grapevines, a total of 27 samples were collected that consisted of nine samples per cordon and trunk (Supplementary Figure 1B). The lengths of the trunk and the arms were measured, and then sampled at 20%, 50%, and 80% of the length of the cordons or trunk. For each spatial location, wood samples were collected from the top and bottom sec-

tions of the cordon, from approx. 1 cm beneath the bark and from the middle section of the heartwood. Similarly, wood samples were collected from the left and right sides of the trunk from approx. 1 cm beneath the bark, and from the center of the heartwood. All samples were collected with wood chisel treated between each cut with 70% ethanol and heat. For each sample, approximately 2 g of wood was collected for molecular and microbiological studies. Samples for molecular assessments were flash-frozen in liquid nitrogen and stored at -20°C, and samples for microbiological assessments were processed within the same day.

Culture-dependent analyses

Wood samples ($\approx 3 \times 5 \times 2$ mm) from all 27 sampling points on each vine were disinfected (15 sec in 3% calcium hypochlorite), and were then rinsed with twice in sterile water, and dried on sterile filter paper. For each sample, the wood fragments were placed on a malt extract agar (MEA; 20 g L⁻¹ malt extract and 15 g L⁻¹ agar) (five fragments per Petri dish), and the dishes were incubated at room temperature. Fungal development was then observed over a six-week period. Taxonomic classification of resulting fungi was carried out at the family level based on culture and morphological characteristics for *Botryosphaeriaceae* (Phillips *et al.*, 2013), *Diaporthaceae* (van Niekerk *et al.*, 2005), *Diatrypaceae* (Trouillas *et al.*, 2010), *Nectriaceae* (Chaverri *et al.*, 2011; Grafenhan *et al.*, 2011), *Phaeomoniellaceae* (Chen *et al.*, 2022), *Togniniaceae* (Gramaje *et al.*, 2015), or the *Basidiomycota Fomitiporia mediterranea* (Fischer *et al.*, 2005). The identity of *Phaeoacremonium minimum* (*Togniniaceae*) and *Phaeomoniella chlamydospora* (*Phaeomoniellaceae*) was verified by PCR, using primer pairs PaIqR [CGTCATCCAAGATGCCGAATAAAG]-PaIqF [CGGTGGGGTTTTTACGTCTACAG] for *Pm. minimum* and PchQr [CCATTGTAGCTGTTCCAA-GATCAG]-PchQf [CTCTGGTGTGTAAGTTCAATC-GACTC] for *Pa. chlamydospora*, targeting the β -tubulin DNA region (Pouzoulet *et al.*, 2013). Presence or absence of each fungal group was recorded for the 27 data points for each assessed vine. The distributions for the numbers of fungal families recovered from the trunk or cordon samples were analyzed by ANOVA tests or nonparametric Kruskal-Wallis tests using the Rcmdr package of the R software version 2.8.0 (Fox, 2005). The statistical tests were carried out according to the vine training system (Arcure vs. Poussard) and were presented separately for trunk and cordons.

Culture-independent analyses

All 27 frozen wood samples from each vine were individually ground to powder with MM300 grinder (Retsch). DNA was extracted from each of the 324 wood samples (27 samples per vine from 12 grapevines), from 60 mg of wood powder, using the Indvisorb Spin Plant Mini Kit (Eurobio), according to the manufacturer's instructions. The purity of the extracted DNA was evaluated with NanoDrop One (Thermo Fisher Scientific), and was quantified with Qubit 2.0 (Thermo Fisher Scientific). Fungal ribosomal ITS regions were amplified using the forward (AAAACCTTTCAACAACGGATC) and reverse (TYCCTACCTGATCCGAGGTC) GTAA primers designed by Morales-Crus *et al.* (2018). Each 25- μ L PCR reaction mix contained 1 ng of DNA template, Apex 2 \times Taq DNA Polymerase Master Mix solution (Genesee Scientific), and 0.4 μ M of each primer. The PCR program (Veriti thermal cycler, Thermo Fisher Scientific) was as follows: initial denaturation at 95°C for 3 min, followed by 37 cycles each at 95°C for 45 s, 55°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. Following PCR, amplicon sizes and uniqueness were verified using gel electrophoresis. The PCR products were cleaned using 1X Ampure XP magnetic beads (Agencourt, Beckman Coulter). DNA concentration was determined for each purified amplicon using Qubit 2.0 (Thermo Fisher Scientific). For high-throughput sequencing, equimolar amounts of all barcoded amplicons were pooled into a single sample, the total concentration of which was determined. Five hundred nanograms of pooled DNA were then end-repaired, A-tailed and single-index adapter ligated (Kapa LTP library prep kit, Kapa Biosystems). After adapter ligation, the library was completed with two consecutive 1X Ampure XP magnetic beads (Agencourt, Beckman Coulter) cleanups. The size distribution of the library was determined with the Bioanalyzer (Agilent Technologies), and was submitted for sequencing in 250-bp paired-end mode on an Illumina MiSeq (UC Davis, Genome Center DNA Technologies Core). The fungal dataset totalled 173 starting samples of the possible 324 (2 pruning types \times 6 vines \times 27 samples per vine), for downstream computational analyses. The reasons for the missing samples were poor quality or quantity of DNA that was not suitable for Illumina sequencing, or because the PCR yielded no products. The 173 samples included 85 for Arcure pruned vines [Arm20 = 20 samples; Arm50 = 18 samples; Arm80 = 19 samples; Trunk20 = 12 samples; Trunk50 = eight samples; Trunk80 = eight samples], and 88 for Poussard pruned vines [Arm20 = 15 samples; Arm50 = 15 samples; Arm80 = 21 samples; Trunk20 = 12 samples; Trunk50 = 14 samples; Trunk80 = 11 samples].

All sequences were submitted to the National Center for Biotechnology Information Sequence Read Archive, under the bioproject accession number PRJNA1066615.

Computational analyses.

Trimmomatic v 0.39 was used to initially clean the sequencing reads, with a sliding window of 4:19 and a minimum length of 150. The R v4.1.2 software (R Core Team, 2021) was used to carry out perform all computational analyses. Most processing for the reads was done in DADA2 v 1.16.0 (Callahan *et al.*, 2016), including further quality control sequencing filtering, dereplication, chimera identification, merging paired-end reads, and construction of Amplicon Sequence Variant (ASV) tables. Taxonomic identifications were assigned using the UNITE database v 10.5.2021 for fungal taxa. Phyloseq v 1.36.0 (McMurdie and Holmes, 2013) and ggplot2 v3.3.5 packages (Wickham, 2009) were used for much of the graphical and statistical analyses of the data. Unidentified microbes at the kingdom level were removed. Alpha diversity was measured for observed taxa within the communities. Poisson generalized linear modelling with *ad hoc* Tukey tests was used to verify statistical differences among groups. Bar charts were constructed by aggregating taxa at the family and genus levels. Samples were also constructed by tissue compartments, and were transformed to relative abundance. Bray–Curtis dissimilarity was used to calculate the compositional dissimilarities between samples. These dissimilarities were visualized with Non-metric MultiDimensional Scaling (NMDS) plots using the Vegan package v 2.5-7. The Adonis test was used to determine the statistical significance of beta diversity.

RESULTS

The two vineyard blocks were 45 years old in the first year of the survey and showed high incidence of grapevine trunk diseases that increased in each year of the survey (Table 1). The results demonstrate how the two training systems affected GTDs incidence and severity. Arcure-pruned vines displayed greater percentage ($P < 0.0001$) of vines symptomatic for GTD than for Poussard-pruned vines, for all 3 years of the study, mostly for numbers of dead or dying vines. ImageJ analysis of the ratios of the necrotic areas to areas of total vine wood indicated a high percentage (70–80%) of wood decay in the asymptomatic vines, regardless of the training system (Supplementary Table 1). The decay appeared as wood discolourations, ranging from light brown to

dark black in colour with little to no white rot observed in all 12 vines (Supplementary Figures 1 and 2).

The Miseq produced approx. 24 million reads. After trimmomatic (before processing with DADA2) there were 8,672,611 reads across all the 173 starting samples. At the end of DADA2 processing, there were 7,614,009 reads remaining, keeping between 60–98% of reads in each sample through DADA2 processing. No samples had less than 15K reads for analyses. After filtering, a total of 1267 ASVs were recorded from the 173 samples. These results indicated that pruning methods affected microbial diversity richness (Figure 1) and community composition (Figure 2). Alpha diversity plots showed greater observed microbial diversity in trunks of Arcure-pruned vines than in trunks of Poussard-pruned vines [Poisson generalized linear model with Tukey; $P < 0.001$], with trend indicat-

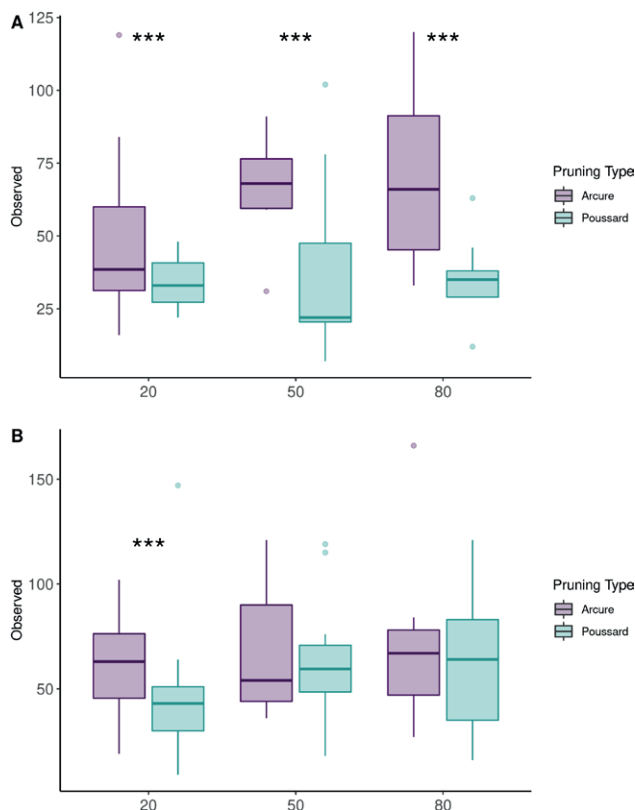


Figure 1. Alpha diversity plots indicating that microbial richness was affected by grapevine pruning practice. Boxplots represent observed diversity at the location on the vine (20%, 50% or 80%) on trunks (A) and arms (B). Statistical significance is indicated for $P < 0.001$ (***), based on Poisson generalized linear models with pairwise Tukey tests. Arcure: Arm20 = 20 samples; Arm50 = 18 samples; Arm80 = 19 samples; Trunk20 = 12 samples; Trunk50 = eight samples; Trunk80 = eight samples. Poussard: Arm20 = 15 samples; Arm50 = 15 samples; Arm80 = 21 samples; Trunk20 = 12 samples; Trunk 50 = 14 samples; Trunk80 = 11 samples.

ing greatest diversity near the heads of the trunks. In vine arms, microbial diversity differences between the two pruning types were only detected in the sections closest to the trunks (arm 20), with Arcure-pruned vines having the greatest taxa richness. Pruning practice type also affected fungal community composition both in vine arms and trunks (Adonis test $P < 0.001$).

Taxa bar plots showed that *Phaeoconiella* (*Phaeoconiellaceae*) and *Phaeoacremonium* (*Togniniaceae*) were the two main pathogen taxa infecting the grapevine trunks and arms, regardless of the pruning method. *Phaeoconiella* and *Phaeoacremonium* represented approx. 60% and 12% in relative abundance, respectively (Figure 3; Supplementary Figure 3). The PCR analyses confirmed that the pathogenic species were *Pa. chlamydospora* and *Pm. minimum*. Sequence data also indicated that the pathogen *Diplodia* (*Botryosphaeriaceae*) was also present in all grapevine sampled compartments, representing approx. 2% relative abundance (Figure 3; Supplementary Figure 3). These three families (*Phaeoconiellaceae*, *Togniniaceae*, *Botryosphaeriaceae*) with known pathogenic fungi represented 81.6% in arms and 77.8% in trunks of all the taxonomic groups in severely-pruned vines, compared with 74.4% in arms and 71% in trunks of minimally-pruned vines (See Supplementary Figure 3A). Of those, *Phaeoconiellaceae* had greater abundance in severely pruned vines than in minimally-pruned vines (See Supplementary Figure 3B).

Microbial isolations from the 324 tissue samplings from the 12 grapevines (27 sample per grapevine) confirmed, to some degree, the sequencing data. Recovery proportions were greatest for fungi in the *Phaeoconiellaceae* (44.8% recoveries), *Botryosphaeriaceae* (42.9%) and *Togniniaceae* (31.5%; Figure 4). The fourth most recovered pathogenic group were *Nectriaceae* (13%), but incidences of other pathogenic groups were low (*Diatrypaceae*, 1.2%; *Diaporthaceae*, 0.6%. *Fomitiporia mediterranea* was only isolated from one trunk sample and one arm sample from Arcure-pruned vines. Severely-pruned vines displayed greater incidence of esca-causing fungi (*Pm. minimum* and *Pa. chlamydospora*) in vine arms and trunks in comparison to the minimally-pruned vines (Figure 4). Similarly, *Botryosphaeriaceae* percent recovery was also greater in the trunks of severely- vs minimally-pruned vines, whereas the opposite was true for the vine arms.

DISCUSSION

This study was designed to gain knowledge on effects of two grapevine pruning practices on incidence

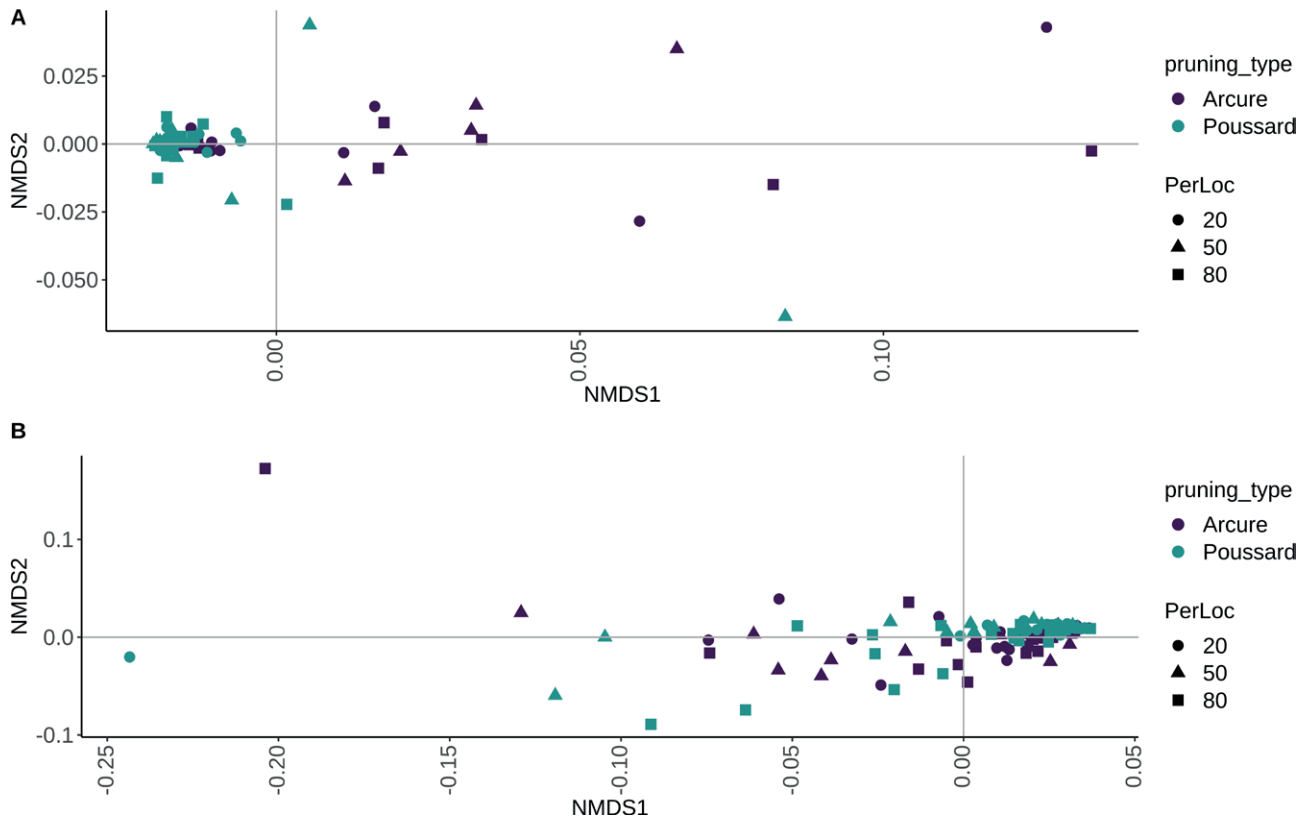


Figure 2. Bray-Curtis beta diversity plots indicating that fungal beta diversity was affected by grapevine pruning practice. Each dot represents the fungal community composition of one vine sample. Points are coloured by each pruning type (Arcure vs. Poussard) and shaped for sampling location on each vine (20%, 50% and 80%) on trunks (A) and arms (B). Statistically significant P and R^2 values were measured by Adonis permutational multivariate analysis of variance for trunks ($P = 0.001$, $R^2 = 0.15$) or arms ($P = 0.001$, $R^2 = 0.0865$). Arcure: Arms = 57 samples; Trunks = 28 samples. Poussard: Arms = 51 samples; Trunks = 37 samples.

and severity of GTDs. In addition, effects were assessed of these pruning strategies on spatial composition and diversity of the mycobiome and pathobiome in asymptomatic grapevines. Incidence of GTD foliar symptoms in the surveyed vineyards was low for all three years of the study, although the assessed vines showed extensive wood decay, regardless of the pruning strategy applied. Leaf stripe symptoms were observed and were indicative of esca, which was confirmed with culture-dependent and independent diagnoses.

Esca has been identified as the major threat to vineyards across Mediterranean climates (Lecomte *et al.*, 2018; Guerin-Dubrana *et al.*, 2019). Community composition analysis from non-symptomatic vines indicated that GTD pathogens dominated the wood mycobiome, supporting previous data (Geiger *et al.*, 2022). *Phaeoconiella chlamydospora* and *Pm. minimum* were the dominant fungi of the wood mycobiome and pathobiome, with, respectively, 60% and 12% of relative abundance. Profiling of the wood microbiome affected by GTDs and esca

using high throughput sequencing showed that *Pa. chlamydospora* is the dominant member in many viticulture areas (Morales-Cruz *et al.*, 2018; Del Frari *et al.*, 2019; Niem *et al.*, 2020; Geiger *et al.*, 2022; Kraus *et al.*, 2022; Vanga *et al.*, 2022). However, *Pm. minimum* was not always the second most prevalent pathogen reported in esca-affected vineyards, as *Fomitiporia mediterranea* was often detected. The GTAA primers that were used in the present study are specific for *Ascomycota* (Morales-Cruz *et al.*, 2018), and will not amplify *Fomitiporia* (*Basidiomycota*). Nonetheless, the presence of white wood rot was not commonly observed in the analysed vines, and *Fomitiporia* isolation was very low. This may explain the low incidence of leaf stripe symptoms observed in the two vineyards. Previous studies (Maher *et al.*, 2012; Pacetti *et al.*, 2021) have shown that incidence leaf stripe symptoms were correlated with presence of white wood rot and abundance of *F. mediterranea*.

Sequencing data indicated that fungi in the *Herpotrichiellaceae* were the third most abundant, and these

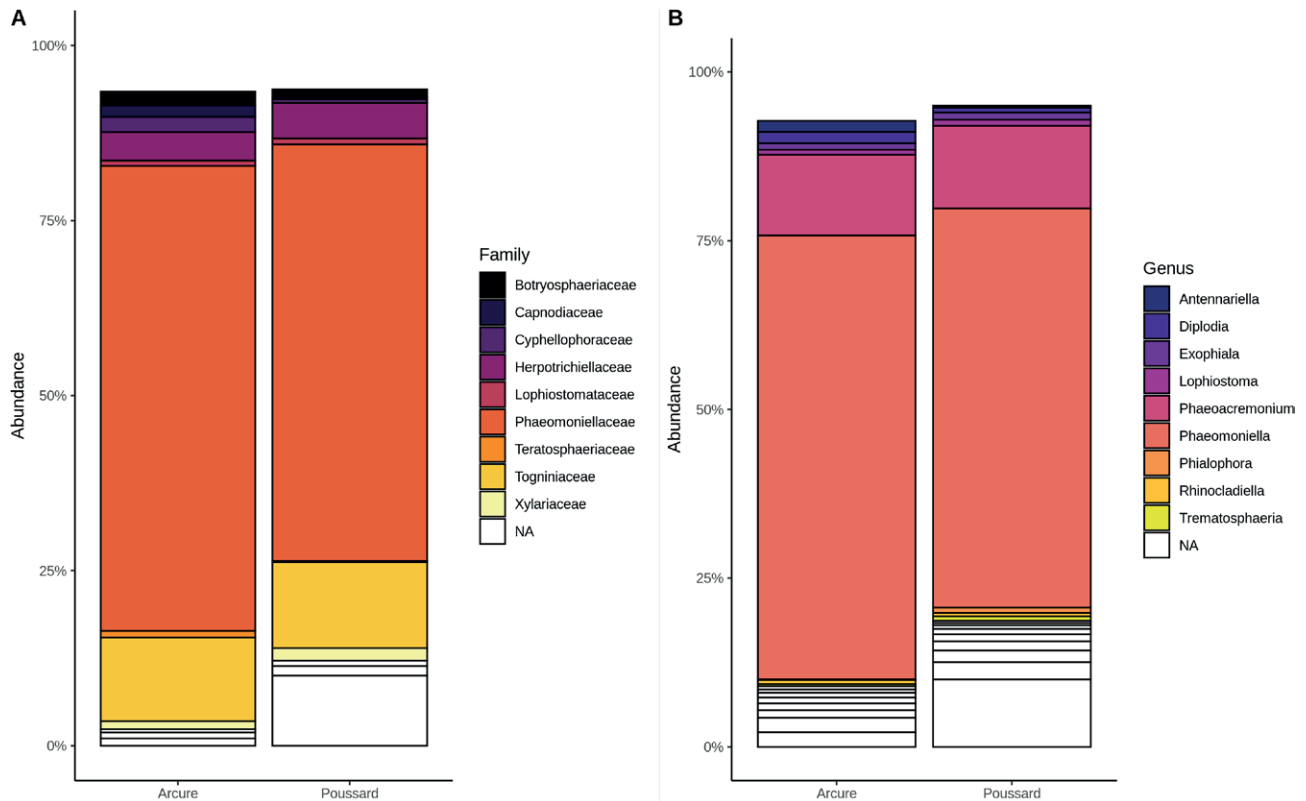


Figure 3. Taxa bar plots showing relative abundance of the top ten fungal families (panel A) and genera (panel B) inhabiting grapevine endospheres for Arcure- (n = 85) and Poussard- (n = 88) trained vines.

fungi have also been reported in other studies (Del Frari *et al.*, 2019; Vanga *et al.*, 2022). However, suspected grapevine pathogens within the *Herpotrichiellaceae* (*Phialophora*; Hawksworth *et al.*, 1976) were not re-isolated from grapevines, possibly due to their slow-growing nature or because other non-pathogenic represented this group. *Botryosphaeriaceae* (*Diplodia*) were the fourth most abundant pathogenic fungi identified, although with disparity between low relative abundance and the high recovery rates from wood samples, because of the rapid growth of these fungi in culture. Fungi within this family cause *Botryosphaeria* canker in a broad host range and have also been associated with esca in several studies (Bruez *et al.*, 2014; Lecomte *et al.*, 2018; Geiger *et al.*, 2022; Kraus *et al.*, 2022). Several other pathogenic fungi in the *Diatrypaceae* (*Eutypa*), *Diaporthaceae* (*Diaporthe*) and *Nectriaceae* (*Fusarium*) were also identified, but at low incidence and abundance, indicating that these fungi played marginal roles in decline of the surveyed vineyards.

Efficient management of GTDs in vineyards is achieved by early adoption of preventative measures (Kaplan *et al.*, 2016; Gispert *et al.*, 2020). Post-prun-

ing fungicide treatment is the most effective practice, mainly because the causal agents are airborne with free water and infect vines through wounds (Rolshausen *et al.*, 2010). Adjusting the timing of pruning during dry weather conditions when pathogen inoculum is low and/or when periods of wound susceptibility are short during warm temperatures, is also recommended (Munkvold and Marois, 1995; Martinez-Diz *et al.*, 2020). However, those strategies are not always practical because the required weather conditions are not always present at pruning, or in are synchronized with the availability of field labour.

Vine training and pruning practices have been investigated for management of GTDs. Evidence suggests that severe pruning with high numbers of cuts and large wound sizes increases GTD incidence and severity (Gu *et al.*, 2005; Lecomte *et al.*, 2018). Henderson *et al.* (2021) proposed that severity of pruning is best defined by the total surface area of pruning cuts per vine, which is affected both by the number and size of wounds per vine. Incidence of esca (number of symptomatic vines) and severity (extent of wood decay) were reduced after commercial vineyards in Germany and France were con-

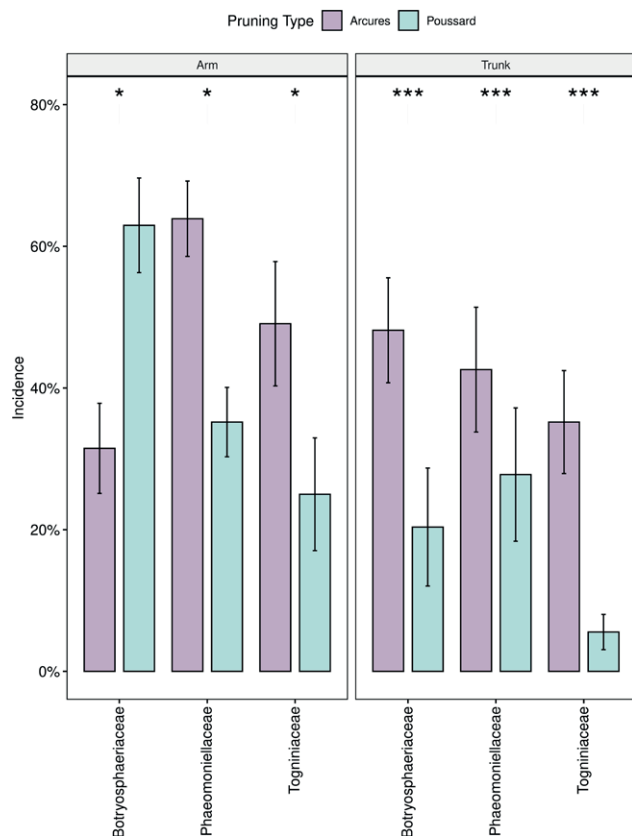


Figure 4. Statistical significant differences in percent recovery for *Phaeomoniellaceae*, *Togniniaceae*, and *Botryosphaeriaceae* fungi between Arcure- and Poussard-pruned grapevines in arms (left panel; six grapevine replicates with two arms per vine and nine data point per arm; $n = 108$), and trunks (right panel; six grapevine replicates with one trunk per vine and nine data points per trunk; $n = 54$). Standard errors are shown on the bar graph and statistical P values are indicated with asterisks (* $P < 0.5$; *** $P < 0.001$).

verted from intensive pruning and training systems (e.g. vertical spur position [VSP]) to minimal pruning (Travadon *et al.*, 2016; Kraus *et al.*, 2019; Kraus *et al.*, 2022). However, improved disease outcomes were only significant when these practices were adopted early in the lives of vineyards (Kraus *et al.*, 2022). Results from the present study were in line with these findings and showed a 45% decrease in vine symptoms and 75% decrease in vine mortality in minimally-pruned grapevines that could be attributed to the reduction of *Pm. minimum* and *Pa. chlamydospora* incidence from vine trunk and arm tissues. *Botryosphaeriaceae* infections were only less present in the vine arms that were severely pruned vines, highlighting the possible contrast in disease etiology for the other two pathogens. However, pruning methods did not affect internal wood decay, with all asymptomatic vines having 70–80% of necrosis in trunks and arms.

Differences in the extent of wood decay between pruning practices could perhaps be better observed in younger vines. The Guyot-Poussard pruning has been shown to minimize the interruption of vine sap flow to foliage, whereas Guyot-Arcure pruning interrupts sap routes causing xylem vessel occlusion and loss of physiological function, thereby stressing vines and supporting escape-pathogen colonization (Lecomte *et al.*, 2018). Together, these results suggest that training methods that decrease wound surface areas per vine, but also pay attention to location of pruning by preserving integrity of continuous sap routes, reduce vine stress. These are factors that minimize the risks of GTDs infections and maximize vineyard lifespans.

The present study has also indicated that the pruning strategy affected fungal community diversity and composition of asymptomatic vines. Two studies, from France (Travadon *et al.*, 2016) and Germany (Kraus *et al.*, 2022), compared minimal pruning with spur pruning, in two cultivars, and both studies yielded inconsistent outcomes on fungal community diversity and composition. It was suggested that fungal abundance and diversity are driven both by cultivar susceptibility to wood-infecting fungi and pruning severity (Travadon *et al.*, 2016). However, in both of these studies, all the vineyards were converted from spur pruning to minimal pruning after several years, which confounded microbial composition analyses. The assemblage of the core endophytic microbiome in perennial wood of grapevines is driven by several factors, including above and below ground wound colonization (Deyett and Rolshausen, 2020; Martinez-Diz *et al.*, 2020). Because pruning methods influence the aboveground endophytic pathobiome it is also likely that it influences the entire host mycobiome, as indicated by the present study. Large-scale sampling from different geographical areas which use contrasting pruning practices will help validate these data.

In conclusion, data from this study support current knowledge that severe pruning increases the risks of GTD pathogen infections and shortens vine longevity and vineyard productivity. Additional comparative studies between intensive and minimal pruning should be carried out over long time periods (years), starting at the vineyard establishment, to increase understanding of the long-term effects of vine training systems on wood endophytic microbiome assembly dynamics and pathobiome profiles. Attention should also be paid to the severity of pruning with respect to wound surface area per vine, and how this affects vine xylem integrity and sap flow routes. This knowledge will improve grower recommendations for practical ways to effectively manage GTDs.

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