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Deciphering the Multifaceted Determinants of Esca Incidence Across a Vineyards Network

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Presented by

VINCIANE MONOD

Professor Daniel Croll, Université de Neuchâtel (thesis director)

Dr Katia Gindro, Agroscope (supervisor)

Professor Joop EM Vermeer, Université de Neuchâtel (internal expert)

Dr Chloé Delmas, INRAE Bordeaux (external expert)

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unine
Université de Neuchâtel
Faculté des sciences

Rue Emile-Argand 11
CH-2000 Neuchâtel
doctorat.sciences@unine.ch

IMPRIMATUR POUR THESE DE DOCTORAT

La Faculté des sciences de l'Université de Neuchâtel autorise l'impression de la présente thèse soutenue par

Madame Vinciane MONOD

Titre :

"Deciphering the Multifaceted Determinants of Esca Incidence Across a Vineyards Network"

sur le rapport des membres du jury composé comme suit :

- Prof. Daniel Croll , directeur de thèse, Université de Neuchâtel, Suisse
- Prof. Joop EM Vermeer, Université de Neuchâtel, Suisse
- Dr Chloé Delmas, INRAE, Bordeaux, F

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Summary

In complex diseases like tree decline, where multiple factors are involved and difficult to isolate, understanding the relationships between biotic and abiotic factors is a challenge. Ensuring the ongoing health of perennial crops for sustained profitability remains a significant concern due to the multitude of threats and associated economic losses. From the late 1980s onwards, established vineyards experienced a decline in yields and an increase in plant mortality attributed to the emergence of fungal vascular diseases influenced by pedoclimatic factors. Esca disease is among the most devastating vascular disease in Europe threatening grapevine. Symptoms are expressed at two levels: i) in the wood by different levels of necrosis and the presence of brown longitudinal bands or cankers; ii) in the leaves by leaf discoloration with a "tiger stripe" pattern. This thesis aims to improve our understanding of the multifactorial nature of esca dieback through the analysis of the plant mycobiome offering insights into potential microbial contributions to the onset and progression of dieback symptoms and through the evaluation of pedo-climatic factors linked with esca incidence. To achieve these objectives, we worked on a network of vineyards all planted with the same grape variety (Gamaret) with plants of the same age to reduce confounding factors, including the variability in host susceptibility already highlighted. We aimed to test for compositional differences in trunk inhabiting fungal communities in vineyards affected to varying degrees by esca. The analysis of the vine trunk mycobiome revealed a remarkably diverse fungal community with weak differentiation at the vineyard of regional level. We found overrepresentation of several taxa in asymptomatic plants; however, no taxa were overrepresented in symptomatic plants. Key taxa typically implicated in esca were also not showing any significant association with plant health status. In parallel, we collected epidemiological and physiological data once a year for four consecutive years in the network of studied vineyard. We compared epidemiological data on the incidence of esca with climatic data obtained from weather stations, as well as certain physiological indicators (berry and wood weights, δ 13C, analyses of the chemical composition of leaves and musts) to compare them with the measured incidence of esca. We identified a positive correlation between soil water retention capacity and the amount of precipitation in early summer and the incidence of esca.

Keywords: esca, grapevine, mycobiome, dieback, perennial plants, pedo-climatic factors.

Résumé

Dans le cas de maladies complexes comme le dépérissement des arbres, où de multiples facteurs sont impliqués et difficiles à isoler, la compréhension des relations entre les facteurs biotiques et abiotiques est un défi. Garantir la santé des cultures pérennes pour une rentabilité durable est une préoccupation majeure en raison de la multitude des menaces et des pertes économiques qui en découlent. À partir de la fin des années 1980, les vignobles existants ont connu une baisse des rendements et une augmentation de la mortalité des plantes attribuée à l'émergence de maladies vasculaires fongiques influencées par des facteurs pédoclimatiques. La maladie de l'esca est l'une des maladies vasculaires les plus dévastatrices en Europe menaçant la vigne. Cette thèse vise à améliorer notre compréhension de la nature multifactorielle du dépérissement de l'esca. Cela grâce à l'analyse des communautés fongiques de la plante qui permet d'évaluer la contribution des facteurs microbiens avec l'apparition et la progression des symptômes de dépérissement ainsi que par l'évaluation de facteurs pédoclimatiques liés à l'incidence de l'esca. Pour atteindre ces objectifs, nous avons travaillé sur un réseau de vignobles planté avec le même cépage (Gamaret) avec des plantes du même âge pour réduire les facteurs confondants, notamment l'incidence de la variabilité de la sensibilité de l'hôte déjà observée. Nous avons analysé les différences dans la composition des communautés fongiques des troncs de vignes affectés à des degrés divers par l'esca. L'analyse du mycobiome du tronc de vigne a révélé une communauté fongique remarquablement diversifiée avec une faible différenciation au niveau du vignoble ou de la région. Nous avons constaté une surreprésentation de plusieurs taxons dans les plantes asymptomatiques ; cependant, aucun taxon n'était surreprésenté dans les plantes symptomatiques. Les taxons clés typiquement impliqués dans l'esca ne présentaient pas non plus d'association significative avec l'état de santé des plantes. Parallèlement, nous avons collecté des données épidémiologiques et physiologiques une fois par an pendant quatre années consécutives dans le réseau des vignobles étudiés. Nous avons comparé les données épidémiologiques sur l'incidence de l'esca avec les données climatiques obtenues à partir des stations météorologiques, ainsi que certains indicateurs physiologiques (poids des baies et du bois, δ 13C, analyses de la composition chimique des feuilles et des moûts) pour les comparer avec l'incidence mesurée de l'esca. Nous avons identifié une corrélation positive entre la capacité de rétention de l'eau du sol et la quantité de précipitations au début de l'été et l'incidence de l'esca.

Mots-clés : esca, vigne, communautés fongiques, dépérissement, plantes pérennes, facteurs pédoclimatiques.

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Scientific output

Talks and Posters

InoVino Bi-Annual Viticultural Meeting, Savigny, February 2023 (talk)

Agroscope Plant Protection Annual Meeting, Nyon, Switzerland, December 2020 (poster)

Annual PhD Students Meeting, Neuchâtel, Switzerland, March 2019 (talk)

Publications

Monod V, Hofstetter V, Zufferey V, Viret O, Gindro K, Croll D. 2022. Quantifying Trade-Offs in the Choice of Ribosomal Barcoding Markers for Fungal Amplicon Sequencing: a Case Study on the Grapevine Trunk Mycobiome. Microbiology Spectrum 02513-22

Unpublished manuscripts

Monod V, Zufferey V, Wilhelm M, Viret O, Gindro K, Croll D, Hofstetter V. 2023. A systemic approach allows to identify the pedoclimatic conditions most critical in the susceptibility of a grapevine cultivar to esca dieback. bioRxiv doi.org/10.1101/2023.05.23.541976

Introduction

Decline of perennial plants

Perennial woody plants are characterized as organisms that have a lifespan of more than two years, with the feature of retaining persistent aboveground dormant parts and woody material during the non-growing season (Bettenfeld et al. 2020). The role of woody plants in ecosystem functioning is multifaceted and crucial. They contribute to the cycling of water and CO_2, provide valuable goods and services, support biodiversity, and play a vital part in carbon sequestration, making them key actors in addressing environmental challenges and maintaining ecological balance (Jackson et al. 2002; Costanza et al. 1997). Trees experience significant decline or death affecting many species (Anderson et al. 2004) with diverse symptoms on branches and foliage (twig drying, wood rotting, leaves yellowing) or dieback of the entire tree (Bettenfeld et al. 2020). In most trees decline, symptoms may express for several seasons not always consecutively leading usually to the plant death (Yadeta and J Thomma 2013). It is a widespread issue affecting forests, woodlands, and perennial crops worldwide (Santini et al. 2013; Wingfield et al. 2010). Tree decline are complex and multi-factorial diseases with biotic and abiotic components (Vieites et al. 2009; Tiew et al. 2020). Determining the combination of factors leading to decline is challenging (Bettenfeld et al. 2020). Among tree declines, vascular wilts are among the most destructive (Yadeta and J Thomma 2013).

Abiotic factors

Tree decline may be linked to various abiotic factors. Climate change is considered one of the primary drivers of tree decline. Rising temperature, changes in precipitation patterns, and extreme weather events like droughts, extreme temperature both hot and cold can stress trees, weaken their immune systems, and make them more vulnerable to diseases, pests, and other stressors (Denman et al. 2018; Camarero et al. 2015). These stresses limit carbon assimilation during the active period and cause damage to the growing tissues (Rozas and Sampedro 2013; Claverie et al. 2020). Attention has primarily focused on the impact of drought identified as a predominant cause of tree decline (Anderegg et al. 2016). Several studies brought also attention to the potential role of soil water saturation caused by higher rainfall frequency and intensity or high irrigation contributing to tree decline (i.e., in oak decline in wet Atlantic forest, (Rozas and García-González 2012); in esca disease of grapevine, (Marchi et al. 2006; Guérin-Dubrana et al. 2013); in Dutch elm disease, (Solla and Gil 2002); in olive tree affected by *Verticillium dahliae*, (Jiménez-Díaz et al. 2012)). Excessive water levels have been found to influence the size and morphology of xylem vessels, which are responsive to environmental signals (Pouzoulet et al.

2014). The characteristics of the xylem vessels seems to impact the susceptibility of perennial plants to vascular pathogens (in grapevine esca disease, (Pouzoulet et al. 2017); in Dutch elm disease, (Solla and Gil 2002). Smaller Xylem vessels being negatively correlated with disease incidence (Pouzoulet et al. 2014; Solla and Gil 2002). Soil degradation may also be involved with poor soil quality, erosion, compaction, or contamination that can negatively impact tree health and contribute to diebacks (Thomas and Büttner 1998; Gaertig et al. 2002; Helama et al. 2009). Even a slight deficiency in one nutrient can make trees more susceptible to deficiencies in other resources, and this can predispose them to the negative impacts of additional stresses imposed on them (Niinemets 2010). Perennial crops require specific nutrients for healthy growth and productivity. Nutritional deficiencies or imbalances, such as insufficient nitrogen, phosphorus, potassium, or micronutrients, can weaken the plants and make them more susceptible to diseases, pests, and diebacks (Ghorbani et al. 2008; Huber, Römheld, and Weinmann 2012). Improper pruning or training techniques in perennial crops can also contribute to diebacks. Pruning wounds create entry points for pathogens, that can lead to infections and dieback (Amponsah et al. 2012; Philippe E. Rolshausen et al. 2010; Claverie et al. 2020). The speed and severity of symptom development are also influenced by factors such as plant age and fitness, and pathogen virulence (Yadeta and J Thomma 2013).

Biotic factors

A wide range of organisms such as insects, nematodes, viruses, bacteria, fungi, and oomycetes contribute to tree decline (Yadeta and J Thomma 2013; Boyd et al. 2013; Cazorla and Mercado-Blanco 2016). These organisms can infect different parts of the plant, such as leaves, stems, flowers, fruits, trunk, roots or the vascular system (Yadeta and J Thomma 2013), causing a range of symptoms such as cankers, wilting, dieback, and decay. Fungal and bacterial pathogens are common biotic factors involved in tree decline. They can invade tree tissues, disrupt the vascular system, and produce toxins that affect tree health (Pouzoulet et al. 2017). *Armillaria*, *Phytophthora ramorum*, *Ophiostoma ulmi* and *O. novo-ulmi* (Gladieux et al. 2011) or Botryosphaeriacea species (Berraf-Tebbal et al. 2020) are well known fungal pathogens responsible for significant decline of wild or cultivated trees. Bacterial pathogens include species like *Xylella fastidiosa* threatening many perennial and annual plants (Hopkins 1989) or *Pseudomonas syringae* known to cause diseases such as canker in fruit trees (Kennelly et al. 2007). In addition to pathogens, insect pests can contribute to tree decline by introducing or spreading fungal or bacterial pathogens. These pathogens can then colonize the tree's vascular system, impeding water and nutrient transport and leading to decline symptoms (Yadeta and J Thomma 2013). The interactions between different organisms can exacerbate tree decline. For

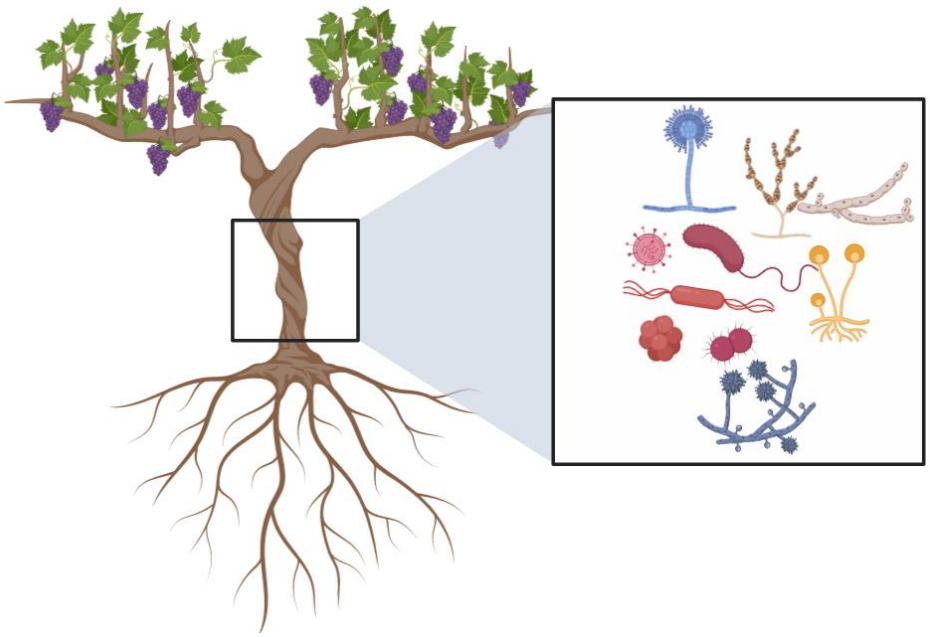
instance, some insects act as vectors, transmitting pathogens from tree to tree, thereby facilitating the spread of diseases. In some cases, multiple fungal species can interact, forming complex disease (Lamichhane and Venturi 2015)that collectively contribute to tree decline. Some organisms are considered as latent opportunistic pathogens and cause symptoms only when their host suffer from abiotic stress as describe in the above section (Pearce 1996; Slippers and Wingfield 2007).

In human pathology, it is increasingly recognized that infectious agents do not act alone, but interact with other commensals or pathogenic organisms (Singer 2010). However, in plant pathology, the notion of monospecies/monostrain infection has been more prevalent. Plant disease epidemics have traditionally been associated with a single pathogen from a clonal group (Lamichhane and Venturi 2015). Our knowledge of synergies between pathogens is relatively limited, but it seems that a more severe severity linked to the presence of several pathogens occurs more frequently than previously thought. In some situations, the "one pathogen causes one disease" model is unsuitable for proving causality, and needs to be adapted to take account of polymicrobial infections (Denman et al. 2018). Understanding these synergistic interactions in plants is crucial for gaining insights into microbial pathogenesis, evolution, and the development of effective disease control strategies (Lamichhane and Venturi 2015). One field of research aims to improve our understanding of the multifactorial nature of tree decline and the analysis of the plant microbiome, which can play a significant role in these complex interactions. Microbiome analysis holds promise in unravelling the intricate relationship between tree decline and the microbial communities inhabiting trees, offering insights into potential microbial contributions to the onset and progression of dieback symptoms (Denman et al. 2018).

Microbiome influence on tree decline

Microorganisms form complex co-associations with plants influencing plant health and productivity (Trivedi et al. 2020). The holobiont concept posits that plants are comprised of the host organism and its associated microbiota, including inherited endosymbionts and various microorganisms (Margulis and Fester 1991; Rohwer et al. 2002). This holistic entity undergoes coevolutionary selection to maintain overall stability over ecological and evolutionary timescales (Trivedi et al. 2020). The microbiome refers to the community of microorganisms, including bacteria, fungi, and other microbes, that inhabit various parts of a plant, such as the roots, leaves and bark and contributes to the plant holobiont's fitness, with the sum of their genetic information termed the microbiome (Mishra, Bhattacharjee, and Sharma 2021). The plant-microbiota relationship has evolved over millions of years, resulting in highly specialized

ecosystems that support essential ecological functions such as fitness, structure, growth, and health (Heckman et al. 2001). Microbiome assembly is influenced by selective forces such as dispersal, species interactions, and adaptation to different plant compartments (Morella et al. 2020). The plant holobiont's microbiota is distributed across distinct habitats, including the soil (root microbiota), rhizosphere, phylosphere, and endosphere, each with specific functions and structures. Microbial communities become more plant-specific and less diverse as the plant matures (Morella et al. 2020). A plant gets its microbiota either through vertical transmission (from seeds or vegetative propagules) and/or horizontal transmission from the environment (mainly from phyllosphere or rhizosphere) (Mishra, Bhattacharjee, and Sharma 2021). The microbiota, by providing additional genes to the host (Turner, James, and Poole 2013), plays a crucial role in the plant's dynamic adaptation to biotic and abiotic environmental conditions, enhancing its ability to cope with various types of stresses. Microbial communities can promote plant growth by simulating water and nutrient intake, increase health trough antibiosis against pathogens and pests or induce plant resistance against biotic and abiotic stresses (Rodriguez et al. 2009; Hyde et al. 2019; Trivedi et al. 2020; Rolli et al. 2015). This is particularly important for plants due to their immobility and sessile lifestyle (Vandenkoornhuyse et al. 2015). The microbiota also plays a role in plant health and disease resistance by directly affecting plant pathogens through competition, antimicrobial activity, and the selection of members capable of producing enzymes and specialized metabolites (Hansen et al. 2020). Indirectly, the microbiota



The 'Plant Microbiome' encompasses a wide array of microbial communities residing both externally on the plant's surface and internally within its tissues. Created with BioRender.com

acts as a secondary immune system for the host by priming and modulating plant defenses and inducing systemic resistance (Pieterse et al. 2014). However, the definition of a healthy microbiota remains unknown and is not specific to the presence of a particular genus or distribution of a community (Begum et al. 2022).

Boundaries between the symbiotic associations are not yet well defined and depending upon the environmental conditions, the relationship between plants and their microbiome (i.e., commensalism, mutualism or parasitism) may change (Mishra, Bhattacharjee, and Sharma 2021). For instance, different strains from *Pantoea ananatis* isolated from healthy maize seeds exhibited to has either promoting properties, be weakly pathogenic or have no observed effect on the plant likely reliable to the protein secretion systems and effector protein (Sheibani-Tezerji et al. 2015). Environmental factors and host genotype may also influence the lifestyle of a unique microbe from endophyte to pathogen as observed with *Fusarium verticillioides* in maize (Bacon, Glenn, and Yates 2008). Some endophytes have a latent temporary state and turn symptomatic when the plant encounters stress conditions as drought, humidity or nutrient starvation (Mishra et al 2021). Defining a taxon strictly within a singular category (i.e., endophyte or pathogen) poses a challenge. An alternative method for studying endophytism involves analyzing genomic data to identify specific genes that can differentiate endophytic strains from pathogenic strains within closely related lineages. In a study conducted by Lòpez-Fernàndez et al. (2015), this approach was employed to compare the genomes of seven bacterial endophytes isolated from grapevine with those of related pathogenic strains from the same genera. The comparison revealed a significant overlap of virulence-related genes in both endophytes and pathogens, indicating a shared core pangenome with consistent conservation across different genera, irrespective of their endophytic or pathogenic lifestyles. This suggests that the structural organization of endophyte genomes reflects the preservation of properties associated with various behaviors, including pathogenicity (Pacifico et al. 2019).

The mycobiome, focusing on plant-associated fungal communities, has been less studied than the bacterial microbiota (Fitzpatrick et al. 2020; Pagano et al. 2017). It comprises various functional groups, including saprotrophic, pathogenic, epiphytic, endophytic, and mycorrhizal fungi (Porras-Alfaro and Bayman 2011). Fungal endophytes, residing asymptomatically within plant tissues, play essential roles in plant fitness, growth, and development (Anal et al. 2020). However, characterizing their diversity remains challenging (Harrison and Griffin 2020). Endophytic colonization represents a finely balanced interaction between fungi and host plants, leading to mutualistic or commensal associations (Schulz and Boyle 2005). The symbiotic continuum suggests that fungi may transition between latent, active pathogenicity, and

mutualism, influenced by environmental factors and host conditions (Schulz and Boyle 2005). The distinction between endophyte and pathogen is subtle, with both producing similar virulence factors, and the balance depends on physiological and genetic factors (Schulz and Boyle 2006; Kogel, Franken, and Hückelhoven 2006).

Plant health is therefore not a binary concept of being either healthy or diseased, but rather exists along a continuum. In complex diseases like tree decline, where multiple factors are involved and difficult to isolate, understanding the relationships between microbiome communities and the health status of the host plant is crucial. The presence of endophytes, which reside within plants without causing harm, challenges our traditional understanding of infection processes leading to plant diseases (Mishra, Bhattacharjee, and Sharma 2021). Further research is needed to determine what disruptions or imbalances in the plant microbiome are considered detrimental to plant health (Romani 2011; Begum et al. 2022) and what can define boundaries between endophytes and pathogens (Lòpez-Fernàndez et al. 2015).

Amplicon sequencing and microbiome analysis

Availability of methodologies to comprehensively analyze microbial populations in diseased plant tissues has been limited until recently. The evolution of sequencing and multiplexing technology has expanded our ability to characterize microbiome diversity in many environments (Zhou et al. 2015; Trivedi et al. 2020). DNA sequencing has transformed microbiome diversity assessments, with the potential to improve data collection and annotation of both taxonomic and ecological traits (Zanne et al. 2020).

To characterize a microbiome of environmental samples, High Throughput Sequencing (HTS) has become the gold standard tool. It has enabled the sequencing of taxonomically informative loci (i.e., barcoding) to reproducibly determine community structures and species diversity (Nilsson et al. 2019), overcoming the bias of culture-dependent approaches (i.e., creating bias in recovered diversity and neglecting true diversity) (O'Brien et al. 2005). HTS produces a large amount of data in a timely and cost-effective manner (Cappellato et al. 2021).

However, obtaining an unbiased picture of the microbiome diversity within a plant through DNA sequencing using next-generation sequencing techniques is still challenging (Zhou et al. 2015). DNA extraction methods may introduce biases during the sample preparation process, leading to uneven representation of microbial taxa (Krsek and Wellington 1999; McOrist, Jackson, and Bird 2002). PCR amplification of DNA fragments can also introduce biases, favoring the amplification of certain taxa over others (Krehenwinkel et al. 2017). Additionally, sequencing errors and limitations in the reference databases used for taxonomic assignment can

affect the accuracy of microbial identification. Accurate species delineation and taxonomic assignment is still a challenge too (Tedersoo et al. 2010; Bazzicalupo, Bálint, and Schmitt 2013; Lindahl et al. 2013). Taxonomic assignment of many taxa and even large lineages are sometimes missing from our barcode databases, making fungal name assignment particularly challenging (Zanne et al. 2020). Errors can also cascade into misidentified sequences deposits (Hofstetter et al. 2019). Significant efforts are needed to deposit and curate sequence data of type material in public databases (Abarenkov et al. 2010; Nagy et al. 2011; Osmundson et al. 2013; Nilsson et al. 2014; Schoch et al. 2014).

Analyzing the results of sequencing datasets from next-generation sequencing (NGS) poses challenges due to several factors. The data generated by NGS are highly specific and often contain a significant number of null values (Cappellato et al. 2021). Additionally, the abundance information (count data) obtained through sequencing does not directly represent absolute abundance, but rather reflect the relative proportions of individuals within a specific taxonomic group, influenced by the sequencing depth (Gloor et al. 2017). Furthermore, the sequencing depth, which refers to the amount of sequencing performed on each sample, can vary greatly between samples (Cappellato et al. 2021). These challenges need to be carefully considered when interpreting and comparing NGS data in microbiome studies.

Moreover, identifying biologically informative features differentiating health status or a gradient between an asymptomatic and a symptomatic plant is challenging with any genomic dataset. Typically, the high-dimensional data and the number of potential biomarkers is often much higher than the number of samples. Strong inter-subject variability characterized many microbial communities (Segata et al. 2011) which increases the difficulty of analyzing it. Environmental microbiome communities are characterized by the presence of a long tail of rare organisms (Pedrós-Alió 2006; Gobet, Quince, and Ramette 2010) and determining the function or impact of rare organisms is a challenge. It has recently been proposed that rare organisms have a genuine function for the host health balance (Jousset et al. 2017). Understanding and quantifying the proportion of inactive and active rare microbes is crucial for predicting the potential effects on ecosystem functioning resulting from the loss of these rare species (Jousset et al. 2017).

Furthermore, the importance of microorganisms to the host cannot be determined solely by measures of the overall relative abundance of their genetic material in the microbiome. The production of metabolites and small molecules by fungi or bacteria may have a greater impact

on the microbiota and the host than the mere presence or abundance of certain species (Begum et al. 2022).

Grapevine as a major crop

Grapevine (*Vitis vinifera* L.), distributed and planted worldwide, has an important cultural heritage and economical importance (This, Lacombe, and Thomas 2006). Domesticated and cultivated for century, the *Vitis* species has retained the most interest and has become among the major fruit crops in the world in terms of area cultivated and economic value (Myles et al. 2011). *Vitis vinifera* is part of the Vitaceae family, which includes about 60 wild *Vitis* species spread in Europe, North America and Asia under Mediterranean and temperate climatic conditions (Terral et al. 2010; Torregrosa et al. 2015). Other species such as *V. rupestris*, *V. riparia* or *V. berlandieri* are used for breeding rootstock for their resistance properties against some grapevine threats such as Oidium, mildews or the aphid *Phylloxera* (Terral et al. 2010). Grapevine was originally found growing along riverbanks and in alluvial and colluvial deciduous and semi-deciduous forest (Arnold, Gillet, and Gobat 1998). Wild populations are nowadays in progressive decline due to anthropogenic pressure on their natural habitat and introduction of non-endemic pathogens against which wild type has no inherited defence (Arnold et al. 2005). The conservation of wild forms close to cultivated plants is essential to maintain genetic variability and combat genetic erosion. The genetic characteristics of wild grapevines may prove essential in this case (Arnold, Gillet, and Gobat 1998).

Surfaces dedicated to cultivated vines was 7.3 million hectares in 2021 worldwide (OIV 2022). In Europe Mediterranean basin, viticulture is a very old practice and landscape occupied by vineyards are cultural and economic patrimonies (Greinert, Kostecki, and Vystavna 2019). In some regions of wine-producing countries such as Spain, France and Italy, vines occupy over 20% of agricultural land (EC 2019 European Commission). As perennial agricultural systems, vineyards shape the appearance of entire landscapes, generating unique ecosystems (Daniel et al. 2012; Cazorla and Mercado-Blanco 2016). Each vine productive region developed or adopted cultivars selected for their organoleptic properties, their agronomical characteristics such as yield or fruit characterization, adaptation to soil and climate specificities or resistance to various abiotic and biotic factors. Breeding development efforts in viticulture aim to improve resilience to abiotic and biotic stressors (Alleweldt and Possingham 1988; De Lorenzis et al. 2022). Through targeted breeding programs, researchers focus on identifying traits that confer drought tolerance, heat resistance, cold hardiness, and adaptability to specific soil conditions. These breeding efforts specially target traits that can confer resistance to pests and diseases, more specifically fungal disease that are the most devastating (B. M. Fischer et al. 2004). In

viticulture, similar to agriculture in a broader sense, the evolving natural and structural conditions present inherent difficulties for perennial production. To maintain or increase production while resources become scarcer, high-quality seeds and planting materials with suitable genetic potential are crucial. These enable resource-efficient, environmentally friendly production and meet consumer demands for quality and safety. Plant breeding aims to provide the viticulture sector with diverse varieties adapted to local conditions, resilient to changing natural factors, and meeting market requirements. Like many other fruit crops, grapevine is mostly grown as clonal lineage (Ramos-Madrigal et al. 2019). Clonal selection arose to preserve the genetic profile and preserve the selected traits (Roach et al. 2018).

Gamaret cultivar

The Gamaret cultivar, developed in 1970 by the Federal Agronomic Research Station of Agroscope in Switzerland, resulted from the crossing of Gamay and Reichensteiner grape varieties. This cultivar quickly gained popularity among winegrowers and consumers due to its desirable taste characteristics and resistance to grey mold. It was cultivated to a significant extent mainly in the regions along Lake Geneva, especially in the Canton of Vaud and Geneva, and has since expanded to the northern areas of Canton of Vaud and Canton of Valais. One of the notable advantages of Gamaret is its resistance to grey mold, which allows the grapes to remain intact on the vine even during the later stages of ripening. Harvesting Gamaret grapes two to three weeks after the normal harvest enhances the quality of the resulting wines, particularly in terms of tannin content. Several factors contribute to the success of this cultivar, such as its suitability for use in late vineyards with a limited range of grape varieties, its adaptability to diverse soil and climatic conditions, its resistance to *Botrytis* in humid climates and its ability to adapt to different consumer tastes and preferences. (https://www.academievin.org/). Gamaret is planted in a variety of soil and climate conditions, due to its good adaptability. However, the cultivar exhibits varying occurrences of grapevine trunk diseases, particularly esca, as evidenced by observable leaf symptoms. Grapevine trunk diseases are one of the main causes of loss of productivity in vineyards today. We decided to study Gamaret because of its variable susceptibility to decline, making it a valuable model.

Grapevine Trunk Disease and Esca

Grapevine faces diverse biotic and abiotic stresses across its lifespan of 30 years or more (Songy et al. 2019; Suzuki et al. 2014). Ensuring the ongoing health of plants in vineyards for sustained profitability remains a significant concern within the industry due to the multitude of threats and associated economic losses. From the late 1980, established vineyards experienced reduced yields and increased plant mortality (C. Bertsch et al. 2013; Christophe Bertsch et al. 2009).

These issues were attributed to the emergence of fungal vascular diseases influenced by soil and climate factors (Christophe Bertsch et al. 2009; Bortolami, Farolfi, et al. 2021; Pastore, Frioni, and Diago 2022; Songy et al. 2019).

Esca symptoms with on the left wilting of the grape bunch and on the right foliar symptoms.
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Among the emerging fungal diseases of grapevine, grapevine trunk disease (GTD) is responsible for the death of young and mature grapevines worldwide (Claverie et al. 2020; Mondello et al. 2018). The establishment of new vineyards in the 1990s, coupled with the discontinuation of certain chemicals (such as sodium arsenate) that were effective against GTD, along with the widespread adoption of poor management and pruning practices, is believed to have contributed to the rise in disease incidence (Azevedo-Nogueira et al. 2022; Claverie et al. 2020). These diseases are a worrying threat to the viability and sustainability of viticulture (C. Bertsch et al. 2013; Kenfaoui et al. 2022). Symptoms are expressed at two levels: i) in the wood by different levels of necrosis and the presence of brown longitudinal bands or cankers; ii) in the leaves by leaf discoloration (Fontaine et al. 2016; Cobos et al. 2022). Leaf symptoms are mainly visible during the summer months. Symptoms can be chronic for a few years or very acute with the apoplectic form (Azevedo-Nogueira et al. 2022) eventually causing the vineyard to shrink. The manifestation of foliar symptoms primarily occurs in vineyards that have reached a mature age of 7 to 12 years or older (Bortolami, Gambetta, et al. 2021; Liu et al. 2017). Among the GTDs, the most destructive diseases of adult plants are esca, Botryosphaeria dieback (Úrbez-Torres and Gubler 2011), Eutypa dieback (Kuntzmann et al. 2010; P. E. Rolshausen et al. 2008; Trouillas and Gubler 2010) and, to a lesser extent, Phomopsis dieback (Úrbez-Torres et al. 2013). Several of these diseases can affect grapevine plants simultaneously, due to their longevity and the multiple opportunities for fungal pathogens to infect the wood (Bruez et al. 2014; Del Frari et al. 2019; Gramaje, Úrbez-Torres, and Sosnowski 2018; Hofstetter et al. 2012). GTD-infected plants may express foliar symptoms for several years and not always consecutively on individual plants

(Calzarano et al. 2018; Songy et al. 2019). They usually die within a few years after the first disease symptoms being expressed (Guérin-Dubrana et al. 2013; Kenfaoui et al. 2022).

Grapevine trunk disease represents a complex disease in which the presence of symptoms cannot be attributed to a single organism, necessitating the consideration of both biotic and abiotic factors (Claverie et al. 2020). Due to the significant economic importance of grapevine cultivation, extensive research has been conducted on the plant and associated diseases, making it an ideal model for investigating the factors contributing to complex perennial crop decline. I will further focus on esca disease because it is the most prevalent GTD disease in Europe (Bortolami, Gambetta, et al. 2021; Claverie et al. 2020). The next section presents the main factors thought to be involved in esca etiology.

Biotic factors related to esca disease

Esca is characterized as being caused by a range of taxonomically unrelated fungi that colonize the xylem (Hrycan et al. 2020; C. Bertsch et al. 2013) mainly through pruning wounds (Philippe E. Rolshausen et al. 2010). Esca is associated with several fungi, including *Phaeomoniella chlamydospora*, *Phaeoacremonium spp.*, *Fomitiporia mediterranea* and *Botryosphaeriaceae* (e.g. *Diplodia seriata* or *Neofusicoccum parvum*) species because these taxa have been frequently isolated from diseased plants (Claverie et al. 2020; Luque et al. 2009). These fungi live exclusively in wood and have never been isolated from leaves despite the presence of leaf symptoms (C. Bertsch et al. 2013; Claverie et al. 2020). All fungal species associated with esca tested to date produce phytotoxic compounds (Andolfi et al. 2011; Martos et al. 2018; Masi et al. 2018). The hypothesis is that foliar symptoms result from translocation of these phytotoxic compounds from the trunk to the leaves via sap flow (Bortolami, Gambetta, et al. 2021). Nevertheless, esca leaf symptoms have rarely been reproduced under controlled conditions (Reis et al. 2016; Claverie et al. 2020) and the etiology and role of the fungi considered as responsible remain poorly understood (C. Bertsch et al. 2013; Claverie et al. 2020; Mondello et al. 2018). Recent studies have shown that the composition of the fungal community isolated from symptomatic or asymptomatic plants from a single vineyard or comparable vineyards cannot be distinguished (Del Frari et al. 2019; Bruez et al. 2014; Bruez et al. 2016; Hofstetter et al. 2012). Because symptoms of esca vary from year to year and esca fungi have been found in asymptomatic tissues, it has been suggested that some of these fungi might function as latent pathogens (Hrycan et al. 2020). What cause the shift of the wood inhabiting fungi from endophyte to pathogen is still a challenge and is thought to be related to abiotic factors influencing the microbiome composition and its dynamic. The interactions between hosts and pathogens can involve various dialogues. These dialogues can occur between vines and fungi,

different fungal species and strains, as well as between fungi and associated microflora, including fungi and bacteria. Furthermore, the overall dynamics of these interactions are likely to be influenced by environmental conditions (Claverie et al. 2020).

Abiotic factors related to esca disease

Soil and climate conditions are believed to play a role in the development of grapevine trunk diseases, particularly esca (Dubos et al. 2002; Marchi et al. 2006). Soils with high water-holding capacity, such as deep clay soils, and climatic variations during the summer have been identified as factors that can increase the risk of sap flow disruption (Surico, Mugnai, and Marchi 2006). Symptoms seem to be enhanced by conditions of humidity (Andreini et al. 2009). The impact of water accessibility in relation to symptoms presence was tested in a controlled study, in which drought conditions were found to prevent the expression of esca symptoms in grapevines (Bortolami, Gambetta, et al. 2021).

Annual changes in symptom expression may be related to differences in the size of new vessels developed under various water regime and vigor conditions (Pouzoulet et al. 2014). Vulnerability to esca disease is also influenced by the plant genotype (Andreini et al. 2009). Grape genotypes have different xylem vessel diameter size ranges, which are key determinant of disease resistance (Pouzoulet et al. 2017). In addition to plant genotype, environmental conditions also influence xylem architecture. During the development of new shoots, the amount of water available to the plant will influence the size of xylem vessels (i.e. a high water supply during development will favour larger vessels) (observed in *Ulmus*, (Solla and Gil 2002)). Xylem morphology influence the compartmentalization achieved by vessel occlusion (tylosis formation) to impair pathogens movement within plant tissues. Larger vessels reported to be more sensitive to vascular pathogens with slower occlusion and hence pathogen restriction (Pouzoulet et al. 2014, 2017). Vines showing even mild foliar symptoms experience hydraulic failure due to blockages in their vascular system, leading to decreased water transport and stomatal conductance in petioles and shoots compared to healthy plants (Ouadi et al. 2019; Bortolami, Farolfi, et al. 2021; Bortolami et al. 2019). Leaves are more vulnerable to tyloses formation compared to perennial organs (Bortolami, Farolfi, et al. 2021). Vulnerability of leaves could also be related to the accumulation of toxins and/or elicitors transported from the perennial part of the plant to the leaves where they accumulate and create higher occlusion density (Bortolami, Farolfi, et al. 2021). Some phytotoxins as Eutypine produced by *Eutypa lata* or some exopolysaccharides produced by Botryosphaeriaceae species have been identified (Bertsch et al 2013). However, the exact role of toxin and/or elicitor produced or triggered by esca-associated fungi in the expression of leaf symptoms is still under investigation.

Disease mitigation

The occurrence of the disease may take place prior to the visible appearance of symptoms. By the time symptoms manifest, they often indicate an intermediate to advanced stage of the disease. This complicates early mitigation and disease control. Therefore, a top priority is to enhance our understanding of the causes and triggers that initiate the disease, as well as the environmental conditions that promote its development. Antagonistic microorganisms have been explored as potential novel approaches to controlling pathogens, with some trials showing promising results in terms of reducing or even eliminating symptoms associated with the disease (Niem et al. 2020). However, the mere detection of metabolites or toxins is insufficient to identify the specific causal pathogen, as the metabolic profile obtained often represents the complex interactions between plants, pathogens, and endophytic microorganisms. It may not accurately reflect the profile of the pathogen itself (Amponsah et al. 2012; Azevedo-Nogueira, Martins-Lopes, and Gomes 2020). Additionally, certain secondary metabolites are shared among different fungal species (such as tyrosol, isosclerone, or 4-hydroxybenzaldehyde) (Andolfi et al. 2011; Masi et al. 2018; Azevedo-Nogueira et al. 2022). In some cases, co-infections can exacerbate or expedite the onset of the disease (for example, when *Diplodia seriata* is co-inoculated with *Seimatosporum vitifusiforme*) (Azevedo-Nogueira et al. 2022). Consequently, more in-depth research is clearly necessary to unravel the causal mechanisms and develop effective strategies for their control.

Objectives

Tree decline, particularly in the case of esca of grapevine, represents complex diseases characterized by intricate biotic and abiotic interactions, making it challenging to decipher. Numerous studies have been carried out in different regions, countries, or continents, often on different grape varieties with different susceptibilities to the disease, with plants of different ages, grown and pruned in different ways. The disparity of these studies has made it difficult to generalize the results obtained on the role of pedoclimatic and biotic factors on the variability of esca incidence. To overcome these disparities, we used a single vineyard network comprising 21 plots. What sets this network apart is its distinctive nature: all the vine plants were planted in 2003, ensuring they share the same age; all plants are from the same cultivar that underwent cloning in the same nursery stock, guaranteeing genetic uniformity (variability in cultivar susceptibility has been demonstrated). This setup allowed for the minimization of confounding factors and provided a valuable model to study the disease and understand the factors contributing to the incidence of esca. Due to the multi-factorial components of esca disease; we used a systemic approach focusing on fungal communities inhabiting the vine trunk and pedo-climatic factors. The first aspect we wanted to investigate is the degree to which fungal community composition is a contributing factor in the outreach of disease symptoms of esca. The second aspect we wanted to investigate is the role of abiotic factors in the incidence of esca disease within the vineyards network.

To analyze the fungal community, we first needed to establish a reliable method for characterizing the inhabiting fungal communities of grapevine plants. The evolution of sequencing technology and multiplexing has rapidly expanded our ability to characterize fungal diversity in the environment. However, obtaining an unbiased assessment of the fungal community using ribosomal markers remains challenging. In my **first chapter**, we examined the implications of barcoding strategies by amplifying and sequencing two ribosomal DNA fragments. We analyzed the performance of the full internal transcribed spacer (ITS) and a longer fragment including ITS and a part of the 28S ribosomal subunit replicated on 60 grapevine trunk core samples. Using identical handling, amplification, and sequencing procedures, we obtained higher sequencing depths for the shorter ITS amplicon. Despite the more limited access to polymorphism, the overall diversity in amplified sequence variants was higher for the shorter ITS amplicon. We detected no meaningful bias in the phylogenetic composition due to the amplicon choice across analyzed samples. Despite the increased resolution of the longer ITS-28S amplicon, the higher and more consistent yields of the shorter amplicon produced a clearer resolution of the fungal community of grapevine stem samples.

My first chapter provided the methodological basis for my **second chapter**, in which we extended the sampling efforts to 21 vineyards (described above), carefully selected for their different levels of esca incidence. On each vineyard, we sequenced the trunk fungal communities of 10 asymptomatic and 5 symptomatic plants. Despite extensive investigations into fungal communities of grapevines, a definitive answer to vine esca disease remains elusive, highlighting the intricate nature of the disease. By conducting mycobiome community composition analysis, we showed that the fungal communities of asymptomatic and symptomatic plants exhibit substantial similarities. The presence of symptoms does not result in a significant overall shift in the composition of the fungal community. In our observations, we did not detect any significant increase or decrease in overall diversity when comparing asymptomatic and symptomatic plants, nor did we observe the appearance or disappearance of specific taxa. Furthermore, we examined the proportion of typically related species of esca in both diseased and asymptomatic plants and found no notable disparities. These findings raise and reaffirm questions regarding the roles of these species in the presence and development of esca. They also raise questions about the transition of these species within the plant, potentially from commensal to pathogenic behavior. Notably, our experimental design did not include testing the specific conditions for this transition.

For my **third chapter** we investigated the abiotic factors related to various esca incidence on the same vineyards network. To identify the environmental factors influencing the expression of esca, we conducted a four-year experiment. This study involved collecting epidemiological and physiological data annually for four consecutive years on the vineyards of the network. To complement this data, we obtained long-term climatic data from weather stations for the same plots. Additionally, we assessed the soil water holding capacity of each plot. Through principal component and regression analyses, combining epidemiological, biotic, and pedo-climatic data, we identified a positive correlation between soil water retention capacity and plant mortality resulting from esca. Furthermore, these analyses revealed that leaf disease symptoms and apoplexy occurred more frequently during periods of cold, wet weather followed by hot, dry weather, or when there were abrupt shifts from the long-term climatic conditions. We found that the impact of soil water holding capacity on disease expression was less pronounced during warm and dry climate conditions, both at the regional and year-specific levels. The results of this chapter provide crucial insights into the significant environmental factors influencing the expression of esca. These findings can guide recommendations to winegrowers regarding the specific cultivar studied. Moreover, the study can serve as a model for identifying environmental factors involved in complex and multifactorial diseases like tree and perennial crop disease.

In the context of climate change, deciphering the mechanisms involved in perennial plant decline is of crucial importance. For future crop management, as well as for woody perennials in natural ecosystems because they are and will be threatened by emerging diseases and climatic hazards. Understanding the complex mechanisms and multidimensional interactions between host, environment and pathobiome that contribute to the development of complex tree diseases is a challenging task. However, meeting this challenge is crucial, as it represents a major gap in our current knowledge.

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Chapter 1: Quantifying Trade-Offs in the Choice of Ribosomal Barcoding Markers for Fungal Amplicon Sequencing: A Case Study on the Grapevine Trunk Mycobiome

Vinciane Monod,[a,b] Valérie Hofstetter,[b] Vivian Zufferey,[b] Olivier Viret,[c] Katia Gindro,[b] Daniel Croll[a]

[a]Laboratory of Evolutionary Genetics, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland

[b]Agroscope, Plant Protection, Mycology, Nyon, Switzerland

[c]Direction générale de l'agriculture, de la viticulture et des affaires vétérinaires (DGAV), Département de l'Economie, de l'innovation et du sports (DEIS), Morges, Switzerland

Abstract

The evolution of sequencing technology and multiplexing has rapidly expanded our ability to characterize fungal diversity in the environment. However, obtaining an unbiased assessment of the fungal community using ribosomal markers remains challenging. Longer amplicons were shown to improve taxonomic resolution and resolve ambiguities by reducing the risk of spurious operational taxonomic units. We examined the implications of barcoding strategies by amplifying and sequencing two ribosomal DNA fragments. We analyzed the performance of the full internal transcribed spacer (ITS) and a longer fragment including also a part of the 28S ribosomal subunit replicated on 60 grapevine trunk core samples. Grapevine trunks harbor highly diverse fungal communities with implications for disease development. Using identical handling, amplification, and sequencing procedures, we obtained higher sequencing depths for the shorter ITS amplicon. Despite the more limited access to polymorphism, the overall diversity in amplified sequence variants was higher for the shorter ITS amplicon. We detected no meaningful bias in the phylogenetic composition due to the amplicon choice across analyzed samples. Despite the increased resolution of the longer ITS-28S amplicon, the higher and more consistent yields of the shorter amplicons produced a clearer resolution of the fungal community of grapevine stem samples. Our study highlights that the choice of ribosomal amplicons should be carefully evaluated and adjusted according to specific goals.

Importance

Surveying fungal communities is key to our understanding of ecological functions of diverse habitats. Fungal communities can inform about the resilience of agricultural ecosystems, risks to human health, and impacts of pathogens. Community compositions are typically analyzed using ribosomal DNA sequences. Due to technical limitations, most fungal community surveys were based on amplifying a short but highly variable fragment. Advances in sequencing technology enabled the use of longer fragments that can address some limitations of species identification. In this study, we examined the implications of choosing either a short or long ribosomal sequence fragment by replicating the analyses on 60 grapevine wood core samples. Using highly accurate long-read sequencing, we found that the shorter fragment produced substantially higher yields. The shorter fragment also revealed more sequence and species diversity. Our study highlights that the choice of ribosomal amplicons should be carefully evaluated and adjusted according to specific goals.

Introduction

Fungi occur in nearly all environments, are highly diverse, and can form tight associations with other organisms as pathogens or mutualists (1). The mycobiome associated with plants has important implications for agricultural ecosystems (2). Vascular diseases affecting plant stems, including xylem and phloem, are often difficult to diagnose or the causal agent is not yet known (3–6). Surveys of fungal communities (i.e., the mycobiome) have become key tools to understand how environmental and temporal factors influence species compositions associated with diseases (7, 8). The evolution of sequencing technology and multiplexing has rapidly expanded our ability to characterize fungal diversity in many environments (9). However, the implementation of molecular tools to establish unbiased mycobiome surveys remains challenging (9). Early impediments of surveying fungal diversity included the need to culture species, which creates significant biases in the estimation of community compositions (10, 11). Next-generation sequencing (NGS) technology has enabled the sequencing of taxonomically informative loci (i.e., barcoding) to reproducibly determine community structures and species diversity (1). Second-generation sequencing techniques (i.e., short-read sequencing) can generate deep-coverage amplicon data sets (12). Read length constraints limit amplicons to ca. 550 bp using an overlapping paired-end design (13).

Fungal nuclear ribosomal internal transcribed spacers 1 and 2 (ITS1 and ITS2), in addition to the 5.8S subunit, constitute the prevalent barcoding locus for fungi (14). With a typical amplicon length of 400 to 600 bp, the locus is compatible with second-generation sequencing read length

limitations (15). However, several studies have highlighted potential shortcomings of relying on short barcoding markers (16, 17). Targeting longer amplicons has been made possible by third-generation sequencing technology. Longer amplicons were shown to improve taxonomic resolution (18) and resolve ambiguities by reducing the risk of spurious operational taxonomic units (OTUs) (19–21). An important factor in establishing long-read amplicon sequencing is error correction approaches such as PacBio circular consensus sequencing (CCS). CCS drastically reduces base-calling errors through multiple sequencing passes on the same molecules (19). Importantly, CCS provides a per-base accuracy comparable to that of short-read sequencing (22, 23). However, the choice of the locus, challenges in amplifying longer fragments, and downstream analyses remain important considerations.

Comparisons between long and short amplicon studies of fungal communities show that long amplicons have typically lower taxonomic coverage in sequence databases (24). However, longer reads can improve taxonomic resolution (25, 26). This raises the question of whether targeting a longer amplicon (i.e., full ITS or full ITS plus flanking rRNA subunit regions) can provide sufficient sequence read depth per sample to accurately capture relevant differences in species richness and community composition (27). Use of the full ITS region combines the benefits of capturing both ITS1 and ITS2 subregions (20). Targeting the full 5.8S rRNA gene provides improvements for fungal identification, notably the precision of genus-level identification because of a much lower substitution rate than for ITS1 or ITS2 (1).

The higher taxonomic resolution given by the full ITS can be used for strain-level identifications (28, 29). Tedersoo et al. (26) have shown that the identification rate was 33% higher at genus rank when using full-length ITS sequences than when using either ITS1 or ITS2. Longer sequences that combine the ITS with a portion of the small (18S) or large (28S) nuclear ribosomal subunit can also facilitate taxonomic assignments at the family or order level (17). In addition, more than 50% of taxonomically unassigned fungal ITS sequences, i.e., sequences corresponding to species belonging to underrepresented or not yet represented groups in sequence databases, can be identified at least at the divisional level by adding flanking ribosomal DNA (rDNA) regions (26). Assessing the impact of using one or more ribosomal DNA regions for fungal identification should inform decisions about the design of fungal community and barcode studies.

As a model to assess fungal barcoding amplicon suitability, we focused on the complex grapevine trunk mycobiome. The grapevine trunk is inhabited by various fungal species from different taxonomic and functional groups (30). Determining fungal diversity is of high interest

because various dieback diseases are thought to be caused by fungal pathogens, constituting severe threats to vineyards worldwide with substantial economic consequences (31). Grapevine is subject to a complex set of interacting pathogenic or commensal microorganisms (2). Despite significant efforts over the past 3 decades, the causal agents among the grapevine trunk microbiome and the outbreak dynamics are poorly understood (30, 32). Expansive characterizations of the fungal diversity present in grapevine trunks may help to identify one or more fungal species strongly associated with disease outbreaks. To date, the grapevine mycobiome has been analyzed largely based on culture-dependent approaches. Up to 159 OTUs were described using Sanger sequencing (31) and up to 259 OTUs with second-generation NGS technology (30). Third generation long-fragment sequencing has not been used to describe the grapevine mycobiome to our knowledge.

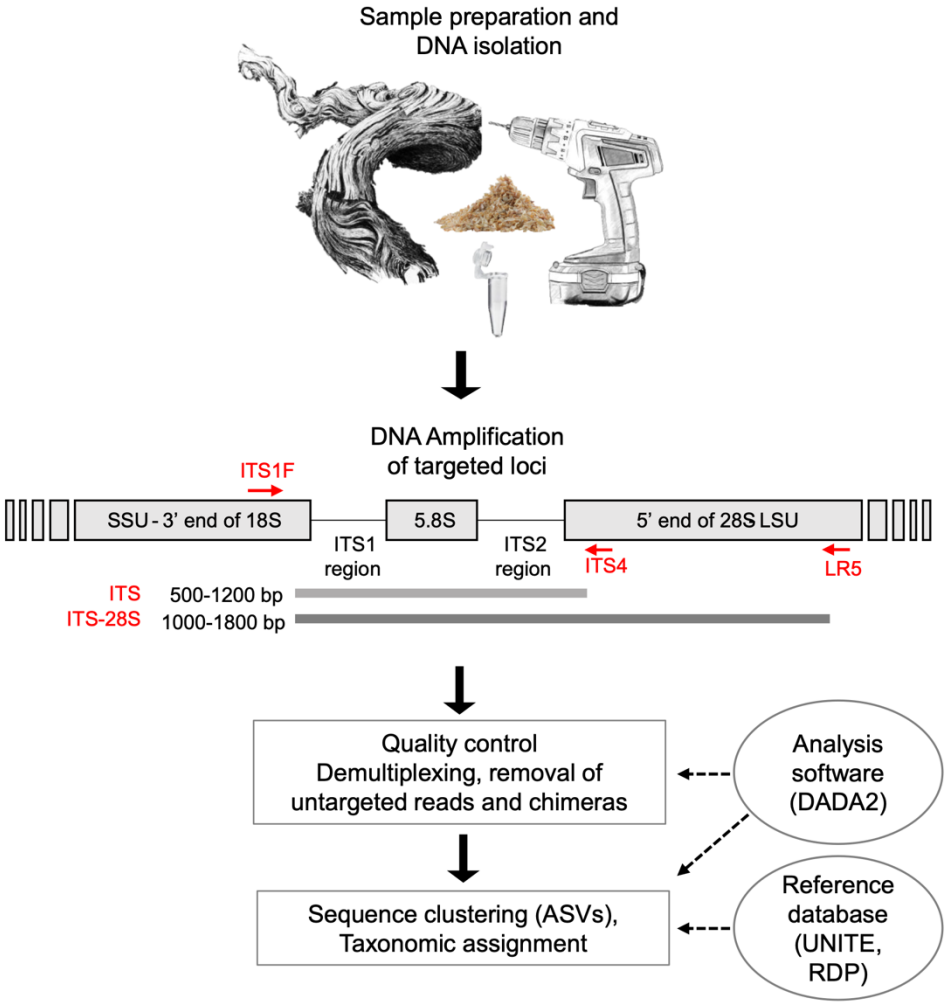

Figure 1: Trunk sample collection and barcoding loci. Sampling method used to extract wood cores from the grafting point of vine plants (n = 60) of a single vineyard before proceeding to DNA extraction. Genomic regions targeted for the amplification: internal transcribed spacer (ITS) and ITS - large subunit (LSU/28S) using the ITS1F – ITS4 and ITS1F – LR5 primer pairs, respectively. Bioinformatics workflow to filter and trim sequences following the DADA2 method. Inference of amplicon sequence variants (ASVs) from sequencing data and taxonomic assignments of each ASVs.

In this study, we examined the implications of barcoding strategies in the context of the grapevine trunk mycobiome using third-generation sequencing technology. We amplified and sequenced two fragments to analyze their performance in characterizing the grapevine trunk mycobiome: the full ITS (14) and a longer fragment composed of the full ITS and a part of the large subunit (LSU) (ITS-28S) to assess the impact on taxonomic resolution and phylogenetic coverage. We analyzed whether the length of the target amplicon influences the sequencing depth and whether the sequencing depth correlates with the detected diversity. Finally, we examined if the amplicon choice influenced the detected fungal diversity.

Results

Amplicon sequencing and read recovery. A total of 60 grapevine plants located in a single plot were sampled at the grafting point using wood cores (Fig. 1). The prevalence of grapevine trunk diseases (GTD) among the 60 randomly selected plants was 14% in the sampling year. DNA extracted from the 60 wood samples could be successfully amplified in 59 and 50 samples with the primer pairs ITS1F-ITS4 (ITS) and ITS1F-LR5 (ITS-28S), respectively. One ITS and 10 ITS-28S amplicon samples were not sequenced because of a too-low PCR product yield after amplification. A total of 382,672 PacBio CCS reads were successfully demultiplexed using the 8-bp barcode with 100% identity, generating 682 to 15,663 reads per sample for the ITS (>4,156 reads for 75% of the samples). For the ITS-28S, 227,549 raw reads were demultiplexed, generating 707 to 22,288 reads per samples (>1,876 reads for 75% of the samples). Sequencing depths were highly variable among samples for both loci (Fig. 2A). A total of 90% of the reads were successfully demultiplexed for the ITS and 89% for the ITS-28S. In proportion, the ITS locus accounted for 63% of the total number of reads. The number of reads per sample between the two amplicons was not correlated (Fig. 2A). This shows that the equimolar pooling of the PCR products was successful in balancing sequencing yields. Sequence lengths were comparable to the expected PCR amplicon sizes. The average sequence lengths were 629 bp for the ITS and 1,526 bp for the ITS-28S (Fig. 2B).

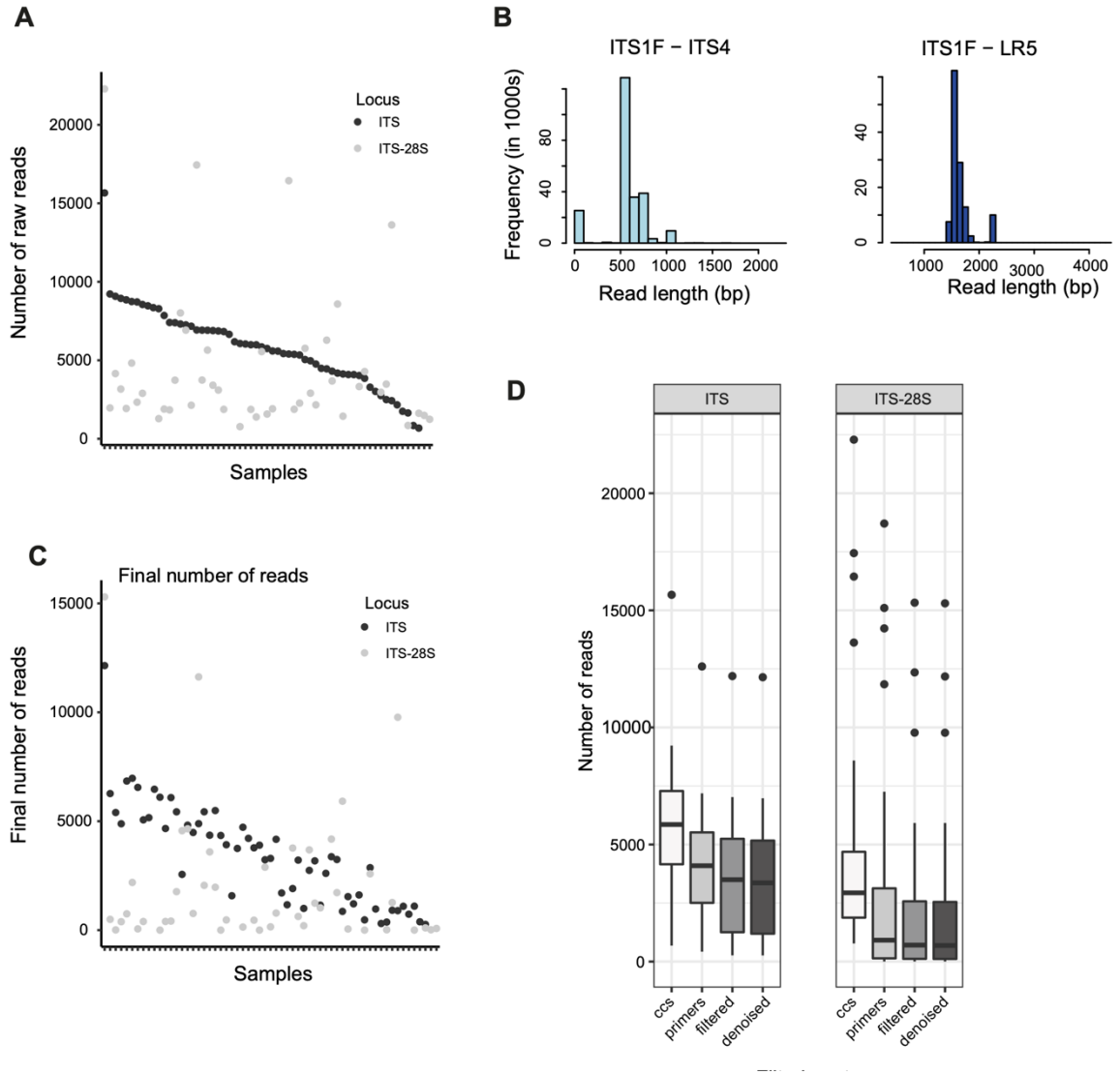

Figure 2 Circular consensus sequencing (CCS) analyses of two ribosomal amplicons. **A**) Number of raw CCS reads obtained for each of the 109 samples for the internal transcribed spacer (ITS) and ITS - large subunit (28S) amplicons amplified using the ITS1F – ITS4 and ITS1F – LR5 primer pairs, respectively. Samples are ranked by raw read counts of the ITS amplicon. **B**) Distribution of raw read lengths for each of the two amplicons for all samples combined. **C**) Number of final read numbers for the two amplicons after all filtering steps. Samples are ranked by raw read counts of the ITS amplicon. **D**) Impact of individual filtering steps (dereplication, primer detection, filtering for amplicon length, denoising according to error detection and presence of chimeras) on the read counts per sample for each of the two amplicons.

Dereplication according to detected flanking primer sequences is a critical step to ensure high-quality sequences, but this step can also discard a substantial number of sequences. For the ITS, 29% of reads were discarded at this step; 38% were discarded for the ITS-28S. Three samples from the ITS-28S saw no reads passing the dereplication, as no matching primer sequences were detected. Read filtering for amplicon length showed disparities between the ITS and ITS-28S data sets, with 70% of reads retained for the ITS and 61% retained for the ITS-28S (Fig. 2D). Error detection and denoising retained most of the reads for both loci ($98%). Chimeras were identified in ;2% of the ITS sequence reads but in ,1% for ITS-28S sequence reads. At the end of the quality filtering procedure, 58% of the reads were retained for the ITS and 45% for the ITS-

28S. The final sequencing depth was 1,190 reads or more for 75% of the samples for the ITS, but it was only 112 reads or more for 75% of the samples for the ITS-28S. Hence, the ITS amplicon produced significantly more high-quality reads after the filtering steps. The two target amplicons differed in total sequencing yield and proportion of reads kept after the quality filtering steps (Fig. 2C and D). Successfully identifying both primer sequences was a critical step for read retention during the ITS-28S filtering process. The ITS-28S amplicons showed alsomore variability among samples in retained read proportions than the ITS (Fig. 3A to C). Samples with more reads showed a weak tendency to have a higher proportion of kept reads (R = 0.57 [Fig. 3B]). This tendency was stronger for ITS-28S amplicons (R = 0.66). To better understand the shifts between the two data sets, we tested multiple filter parameter values for their impact on read retention. However, more relaxed primer matching parameters did not meaningfully increase retained reads. This is true for up to six allowed mismatches for primer detection. Beyond six allowed mismatches, a higher proportion of reads were kept as expected (Fig. 3D). In summary, the difference in retained reads between amplicon data sets is largely unrelated to the primer matching stringency.

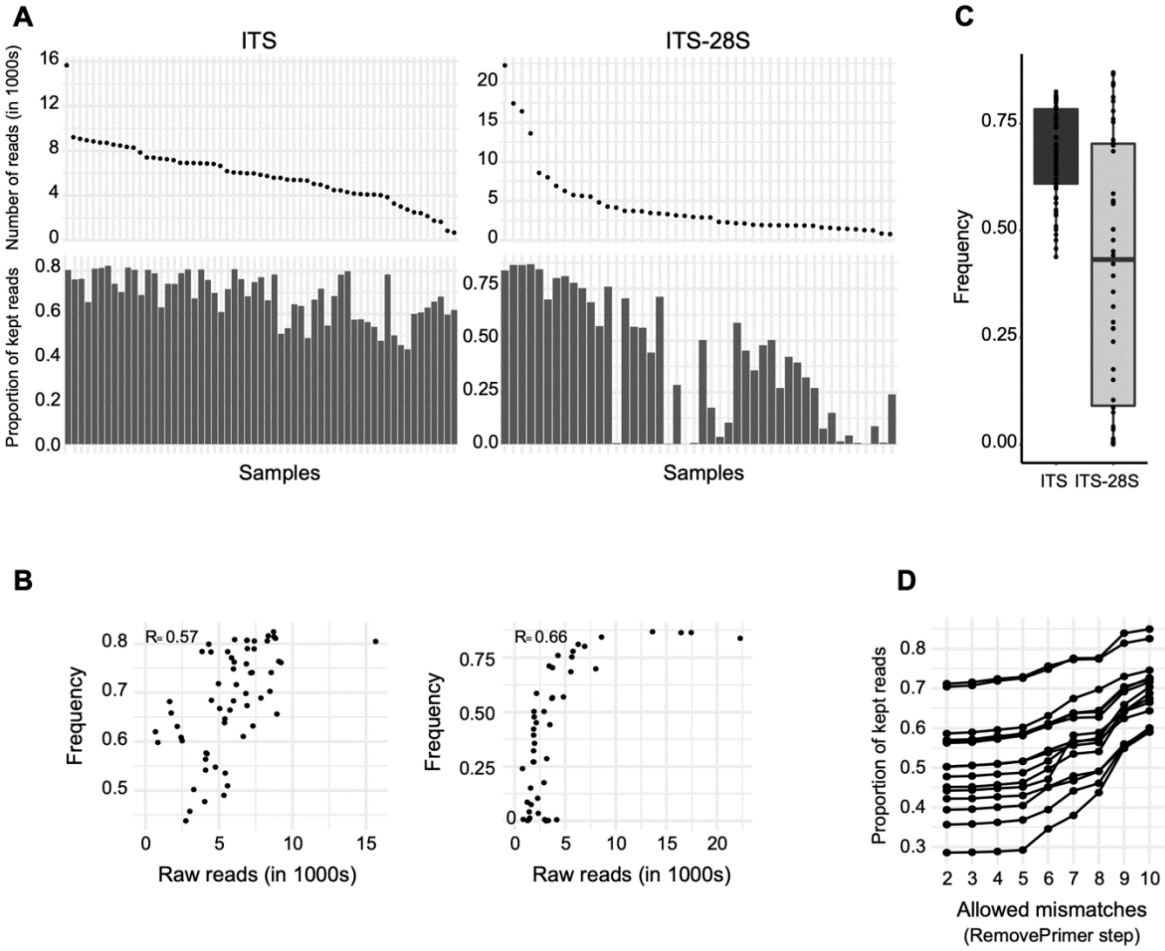

Figure 3: Impact of read filtering steps. **A**) Samples ranked by raw read counts and proportion of kept reads by sample at the primer trimming step for ITS and ITS-28S. **B**) Relation between raw read counts and proportion of kept reads at the primer trimming step for ITS and ITS-28S. **C**) Proportion of retained reads at the primer trimming step for both markers. **D**) Proportion of kept reads according to the number of allowed mismatches in primers detection for ITS-28S.

Comparison of sequence variant recovery for the two barcoding loci. The number of inferred amplified sequence variants (ASVs) was highly variable among samples for both loci (Fig. 4A). We found only a moderate correlation between the number of reads and the ASVs inferred per sample (R = 0.39 for the ITS and R = 0.53 for the ITS-28S [Fig. 4B]). For the ITS, we found a strong density peak of 500 reads by unique ASVs with a normal distribution.

For the ITS-28S data set, the distribution of reads by ASVs was more variable, with a flatter curve going from 50 to 1,000 reads by unique ASVs (Fig. 4C). Overall, 933 ASVs were detected including both data sets. The ITS data set comprises 888 ASVs among 59 samples; the ITS-28S data set comprises 175 ASVs among 46 samples. The artificially cut ITS-28S fragment still covered 164 ASVs for the ITS subset and 140 for the 28S subset (Fig. 4D). The subset creation for the long fragment provides a direct comparison of the represented sequences in the ITS and ITS-28S amplicon data sets. In a direct comparison, we found 888 ASVs with the ITS and 164 ASVs with the ITS-28S amplicon subset to the ITS, of which 119 (12.7%) were shared among the two amplicon data sets (Fig. 5A). A total of 45 ASVs were detected only by the ITS-28S subset to the ITS amplicon and 769 ASVs were detected only by the ITS amplicon. Among the shared ASVs, the proportions occupied by individual ASVs in the two data sets were very similar. Overall, 83% of the shared ASVs differed by less than 1% in relative proportion between the two amplicon sets (Fig. 5B). The most differentiated ASVs in terms of relative proportion were ASVs assigned to Fomitiporia punctata (8% more abundant in the ITS-28S subset to ITS) and Bacidina neosquamulosa (4% more present in the ITS). Hence, the long fragment revealed only a minor degree of additional ASVs not already captured by the ITS amplicon.

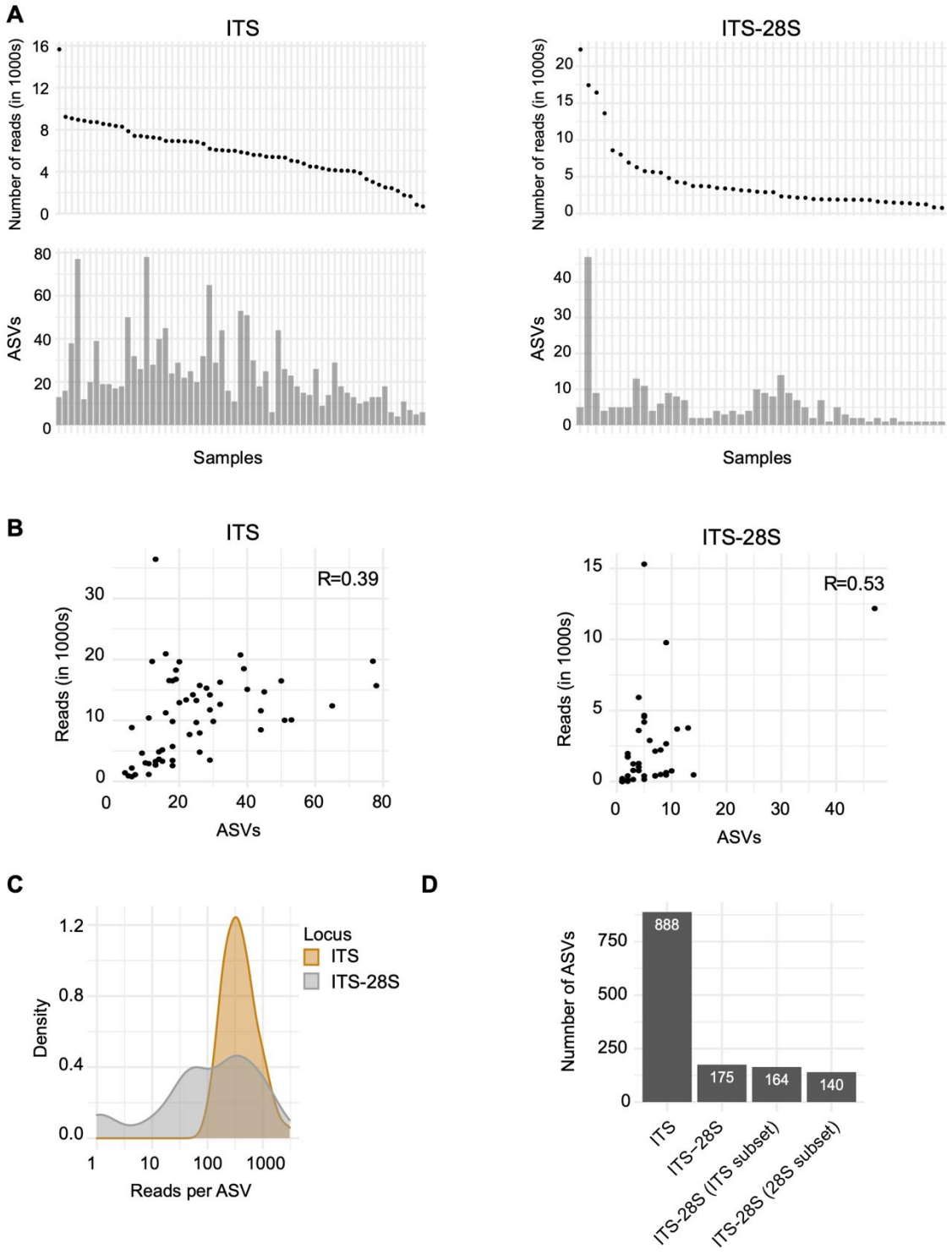

Figure 4 Amplified sequence variant (ASV) diversity. **A**) Samples ranked by raw read counts and inferred ASVs by sample for ITS and ITS-28S. **B**) Relation between raw read counts and ASVs detected for ITS and ITS-28S. **C**) Distribution of reads by ASVs for ITS and ITS-28S. **D**) ASVs detected by markers.

Community composition of the grapevine mycobiome. To better understand the consequences of targeting either the ITS or the ITS-28S on the detected fungal community composition, we examined the diversity present in both data sets across several taxonomic ranks. First, we created a subset of the ITS-28S amplicon consisting of a more conserved LSU portion. This subset of the ITS-28S typically provided only genus-level resolution using the RDP database. For the ITS portion of the ITS-28S amplicon, as well as the ITS amplicon, $60% of ASVs were assigned at the species level (Fig. 5C). Next, we analyzed the relative abundance of reads assigned to each phylum. Ascomycota represented the highest proportion of the reads (77%) in the ITS data set and slightly less in the ITS-28S ITS subset (67%) and 28S subset (65%). Basidiomycota proportions showed opposite patterns, with the ITS data set showing 22%, the ITS-28S ITS subset showing 33%, and the 28S subset showing 33% (Fig. 5E). For classes represented by more than 2% of the reads, we identified Eurotiomycetes and Agaricomycetes as the most abundant in all data sets. Dothideomycetes, Sordariomycetes, and Lecanoromycetes were represented by >2% of the reads only in the ITS data set. Similarly, Exobasidiomycetes were only found in the ITS-28S 28S subset at 2% (Fig. 5E). The most represented genera according to the ITS amplicon were *Phaeomoniella* (40%), *Fomitiporia* (9%), and *Bacidina* (4.6%). For the ITS-28S ITS subset, the genera were similarly *Phaeomoniella* (46%), *Fomitiporia* (15%), and *Mollisia* (7%). For the ITS-28S 28S subset, we found *Xenocylindrosporium* (25%), *Fomitiporia* (16%), and *Mollisia* (8%) (Fig. 5E).

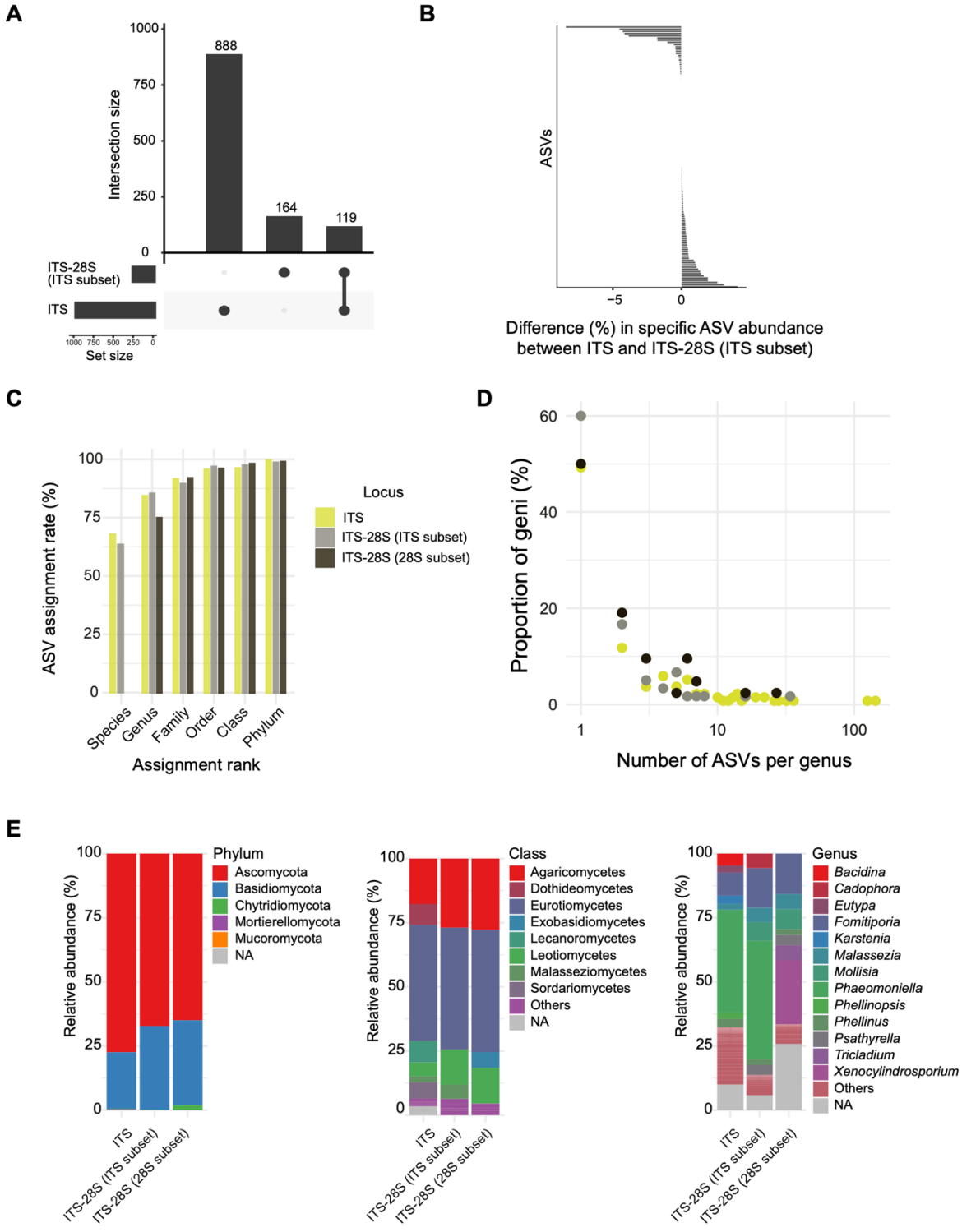

Figure 5 Taxonomical diversity among grapevine trunk samples. **A**) Intersecting sets of ASVs from ITS and ITS-28S ITS subset dataset. **B**) Proportional divergence of the shared ASVs of ITS and ITS-28S ITS subset. **C**) Proportion of assigned reads at several taxonomical ranks. **D**) Proportion of geni by number of ASVs for ITS, ITS-28S ITS subset and ITS-28S 28S subset. **E**) Relative abundance of several taxonomical ranks (phylum, class, genus) for ITS, ITS-28S ITS subset and ITS-28S 28S subset.

At the species level, the diversity detected for the ITS-28S ITS subset was 45 ASVs. These ASVs correspond to 17 taxa with around half (n = 8) of the species or genera unable to be detected with the ITS marker (see Table S1 in the supplemental material). The taxa detected only by the ITS subset of the ITS-28S amplicon were *Mollisia* (8,301 sequences), *Pseudoophiobolus rosae* (49 sequences), *Meyerozyma guilliermondii* (19 sequences), *Coniochaeta coluteae* (9 sequences), *Bipolaris drechsleri* (6 sequences), *Seimatosporium pistaciae* (6 sequences), *Wojnowiciella cissampeli* (3 sequences), and the Ceratobasidiaceae family (2 sequences). For the 769 ASVs detected only based on the ITS amplicon, 103 species corresponded to species uniquely detected by the ITS amplicon. The most abundant species in the data set typically included multiple distinct ASVs matching to the same species. A total of 50% (ITS and ITS-28S 28S subset) and 60% (ITS-28S ITS subset) of the detected genera were represented by unique ASVs. Around 18% (ITS and ITS-28S ITS subset) and 16% (ITS-28S 28S subset) of the genera were represented by three distinct ASVs (Fig. 5D).

The species represented by the highest number of ASVs was *Phaeomoniella chlamydospora* for both data sets. This species was represented by 304 (ITS) and 75 (ITS-28S ITS subsets) different ASVs, highlighting significant intraspecific variation. *P. chlamydospora* was also the most abundant species in the ITS and ITS-28S ITS subset data sets. This species, which is typically associated with grapevine trunk disease, was identified on 88% (ITS) and 97% (ITS-28S ITS subset) of the sampled plant. *P. chlamydospora* was not detected in the ITS-28S 28S subset. As this was surprising, we independently analyzed assignments of some ASVs using BLAST searches inNCBI GenBank. Some ASVs classified as belonging to the *Xenocylindrosporium* genus on the basis of matches in the RDP database rather belong to the *Phaeomoniella* genus based on GenBank matches. Uncertainty about taxonomic assignments using RDP is consistent with concerns about representativeness issues of long ribosomal fragment databases.

OTU-based clustering and contrast to ASV analyses. We used the same error-corrected sequences as for the ASV-based analyses to obtain operational taxonomic units (OTUs) prior to taxonomic assignments. Using the commonly used threshold of 97% sequence identity, we obtained 559 OTUs for the ITS fragment (versus 888 ASVs), 102 OTUs for the ITS-28S subset to the ITS (versus 164 ASVs), and 70 OTUs for the LSU-28S subset to the LSU (versus 140 ASVs). We examined the proportion of genera assigned to each OTU for each marker set. Overall, the proportions of assigned genera differed very little, with typically ,1% changes in the assigned categories, including the proportion of unassigned sequences (Table S2). Using the 99% threshold for OTU assignments, we observed a spike in the proportion of OTUs without assigned genera compared to other OTU thresholds and ASV-based assignments.

Discussion

We used PacBio long-read technology to amplify and sequence two fungal barcoding amplicons (ITS and ITS-28S) in parallel from DNA extracted from a set of vine wood samples. Using identical handling, amplification, and sequencing procedures, we obtained higher sequencing depth and higher ASV diversity for the shorter amplicon (i.e., ITS). We found no meaningful bias in the phylogenetic representation of the samples according to the selected amplicon. Despite the increased resolution of the long ITS-28S amplicon, the higher and more consistent yields of the shorter amplicons produced a clearer resolution of the fungal community of grapevine stems.

Recovery of fungal barcoding sequences. The PacBio sequencing libraries prepared in parallel for the two different ribosomal amplicons differed in yield, with ;1.5 times more sequences obtained for the ITS. Similarly, quality filtering retained a higher proportion of sequencing reads from the shorter amplicon. The higher yield and quality were consistent with findings by Tedersoo et al. (26). Differences in the number of sequences recovered for the two amplicons could be due to less efficient PCR amplification, e.g., due to competition among primed amplicons during the adaptor ligation step (26). Another possible explanation is that LR5 might be less efficient than ITS4 to amplify fungal diversity (33). Longer amplicons can also show reduced yields due to template positioning in sequencing wells (24, 26). Difficulties in properly detecting primer sequences on the long fragment could be due to a deterioration of sequencing quality. However, circular consensus sequencing should produce homogeneous quality scores for the entire template. Technological progress with the Sequel I system of PacBio and library preparation overall likely benefited read retention during quality filtering. Both of our amplicons showed higher retention (58% for ITS and 45% for ITS-28S) than obtained by Tedersoo et al. (26) (28% of retained reads with a Sequel I system for a 400-to 700-bp amplicon and 24% with the RSII system for a 1,250- to 1,700-bp amplicon). With further progress, sequencing of longer fragments should become even more efficient.

Contrasting the recovered sequence diversity. Targeting either a shorter amplicon with higher sequencing depth or a longer amplicon with reduced depth creates a tradeoff that needs to be resolved. Both longer fragments and higher depth have the potential to improve the resolution of taxa present in a sample. We detected more ASVs using the ITS data set, but we found no clear relationship between the sequencing depth and detected diversity as expected. As our study covered environmental samples, the sequence diversity is most likely highly heterogeneous. Hence, sequencing depth versus sequence diversity correlations among samples are only of limited use. Indeed, we detected samples of intermediate sequencing depth but with

some of the highest numbers of recovered ASVs. Above some threshold, increasing sequencing depth is not expected to yield more recovered diversity (27). Purahong et al. (21) showed also that sequencing depth alone is a poor performance metric to evaluate the representation of the fungal community. Kennedy et al. (27) suggested a threshold of 100 reads per sample, beyond which additional reads are unlikely to substantially shift community assessment of environmental samples. In our study, we obtained >1,190 reads for the ITS and >112 reads for the ITS-28S amplicons for 75% of the samples. Hence, regardless of the selected amplicon, fungal communities should be reasonably well assessed in our study.

A basic argument for preferring longer amplicon sequences is the ability to detect a larger number of sequence variants present in the data sets (i.e., ASVs). Interestingly, our study recovered only a few ASVs using the longer ITS-28S that were not detected by the shorter amplicon (45 ASVs). It is likely that we have somewhat underestimated ASV diversity based on the shorter amplicon due to the chosen length cutoffs. Hence, using ITS is more beneficial to assess the fungal diversity present in the analyzed grapevine trunk samples than targeting a longer amplicon making use of the highly accurate PacBio consensus reads. Some studies have even reported that increasing target amplicon lengths has negative effects on the assessment of microbial richness and community composition (34, 35). Our own comparative analyses have not revealed such detrimental effects. ASVs recovered with the ITS and ITS-28S amplicons showed very similar relative abundances.

Recovery of taxonomic diversity. We compared how well amplicons could be assigned to different taxonomic levels depending on the marker targeted. Taxonomic identification for the ITS data set was similar to that for the ITS sequence extracted from the longer amplicon as well as for the entire ITS-28S amplicon (>75% of the ASVs assigned to the genus level for the three amplicon data sets). A somewhat lower proportion of the 28S sequences extracted from the ITS-28S amplicon were assigned, which is most likely explained by the poor taxon coverage of the 28S subunit, compared to ITS, in sequence databases (;15 times more ITS sequences available in UNITE). The generally high taxonomic assignment rates are consistent with the general growth of amplicon databases and the high accuracy of consensus sequences compared to the case with previous studies relying on short-read sequencing (i.e., Illumina). Furthermore, improvements in the analysis pipelines (i.e., DADA2) reduced erroneous chimera sequences and increased the accuracy of amplicon data sets. Our parallel diversity analyses based on two ribosomal amplicons revealed highly diverse fungal communities across grapevine trunks sampled across a vineyard. Our findings are consistent with previous analyses by Del Frari et al. (30) and Travadon et al. (36). We found high consistency in the identified taxa (8 out of 14 genera

identified by Del Frari [30]). The consistently recovered taxa include *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp., and *Fomitiporia mediterranea*, fungal species commonly associated with esca disease of grapevine (37). This is consistent with expectations for diversity of fungi sampled at the grafting point. Our implementation of PacBio amplicon sequencing opens up opportunities for high-resolution profiling across large sets of samples covering space and time in mature vineyards. The high precision of the recovered sequences will allow the monitoring of fungal strains associated with GTD and more generally of wood endophyte fungal species.

Limitations in our comparison lie in the lack of mock community analyses. Comparing the resolutions of barcoding markers on reference DNA mixtures but also more realistic mock analyses of wood core extracts will further substantiate our performance assessments of ribosomal profiling. Additionally, a truly comparable reference database for taxonomic assignments would be needed. Excising the ITS portion of the longer amplicon allowed for a direct comparison using the same database, but a full comparison between the shorter and longer amplicon would require a fully equivalent ITS-28S sequence database. Our analyses match findings by Brown et al. (38) and Porras-Alfaro et al. (39) similarly comparing ITS and 28S amplicon diversity. It is evident that progress in exploiting longer amplicons to their full potential will require more comprehensive databases commensurate with the progress of sequencing technology.

Material and Methods

Sample collection. Grapevine trunk sampling was conducted in La Côte vineyards in Echichens, Switzerland (46°32907.0340N, 6°30903.8070E, WGS 84, 475 m above sea). Samples were collected from 60 different plants in a single vineyard plot of 150 m by 40 m. Grapevine plants were all of the variety Gamaret (a cross between Gamay and Reichensteiner) grafted onto 3309C rootstock (Vitis riparia Vitis rupestris). All the plants came from the same nursery (Dutruy in Founex, Switzerland) and were planted in 2003. Plants affected by GTD had been replaced continuously and were not considered in our sampling. A plant was considered diseased if one or more shoots showed leaf necrosis up to wilt or dieback symptoms. These are typically symptoms classified as grapevine trunk disease (4, 40, 41). Grapevine wood was sampled at the grafting point (where fungal diversity was previously shown to be highest) using a non-destructive method. A 0.5-cm2 piece of bark was removed with a surface-sterilized (80% ethyl alcohol [EtOH]) scalpel. The sampling was then performed with a power drill with a surface-sterilized drill bit (Ø 3.5 mm) by running the drill gently where the bark was removed to collect the coiled wood (;60 mg) in an Eppendorf tube held underneath with sterilized tweezers.

In the Eppendorf tubes, kept in an ice box during the sampling process, two 5-mm iron beads had previously been deposited to ease the next step of the protocol. As soon as possible, the Eppendorf tubes containing the coiled wood were stored at 280°C (Fig. 1).

DNA extraction from wood samples. Eppendorf tubes (safe lock) containing two 5-mm iron beads and wood samples were taken out of the 280°C freezer and put in liquid nitrogen. The Eppendorf tubes were then placed two times for 1 min at 30 Hz in a TissueLyser (Qiagen Inc., Germantown, MD, USA) to disrupt wood tissues. Between and after these two steps of tissue disruption, tubes were placed in liquid nitrogen for 1 min. After the tubes were placed on ice to let them gently thaw, 1 mL of cetyltrimethylammonium bromide (CTAB) was poured into each tube. The samples were then centrifuged for 1 min at 15,000 rounds/min and the supernatant was transferred to a new tube. The fungal DNA extraction was then performed with phenol-chloroform as described by Hofstetter et al. (42). The DNA quality was checked with an electrophoresis gel, and the extracted products were stored at 280°C.

Amplification of fungal ribosomal DNA. Two loci were targeted for amplification: ITS using primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) and a longer amplicon including ITS and a portion of the 28S subunit using primers ITS1F and LR5 (TCCTGAGGGAAACTTCG) (Fig. 1). We followed the PacBio procedure using barcoded universal primers for multiplexing amplicons, which includes two PCR steps (see https://www.pacb.com). The first PCR program was 30 s of denaturation at 98°C and then 30 cycles of 15 s at 98°C, 15 s at 55°C, and 1 min 30 s at 72°C, followed by a final elongation step for 7 min at 72°C. The second PCR program was 30 s of denaturation at 98°C and then 20 cycles of 15 s at 98°C, 15 s at 64°C, and 1 min 20 s at 72°C, followed by a final elongation step for 7 min at 72°C. We performed purificationbetween the two PCRs to reduce contaminants or carryover of primer dimers using 96-well PCR purification plates (Qiagen Inc., Germantown, MD, USA). The final libraries were quantified with a Qubit fluorometer (Thermo Fisher, Foster City, CA, USA), and then all samples were pooled equimolarly. The pooled samples were then purified with 1 AMPure XP beads (Beckman Coulter Inc., Indianapolis, IN, USA) as per the manufacturer's instructions. Amplicons were prepared for SMRT sequencing at the Functional Genomics Center in Zürich (FGCZ), Switzerland. Sequencing was performed on the PacBio Sequel II platform.

Demultiplexing and trimming. The raw reads were demultiplexed using lima (https://lima.how/). After obtaining fastq files, reads were processed for quality filtering with the DADA2 package for R (43) (Fig. 1) (https://github.com/benjjneb/dada2). The DADA2 processing steps include the following: (i) dereplication with primer detection (reads without

primer sequences are discarded); (ii) filtering for amplicon length (between 500 and 1,000 bp for the ITS and 1,000 to 2,000 bp for the longer fragment); (iii) error detection, in which error rates are learned by alternating between sample inference and error rate estimation until convergence (a feature table of observed transitions for each type and quality scores are produced); (iv) denoising to reduce sequencing errors based on error models; and (v) checking for chimeras, in which each sequence is evaluated against a set of putative parental sequences drawn from the sequence collection. Several tests were performed with removeBimeraDenovo by increasing the minFoldParentOverAbundance parameter from 4 to 8. We chose to continue with a minFoldParentOverAbundance of 8 to retain a maximum of reads.

Analyses of amplicon sequence variants and taxonomic assignments. We used the DADA2 algorithm to infer amplicon sequence variants (ASVs) from the filtered reads (Fig. 1). ASVs represent reads with 100% similarity accounting for sequencing errors by appropriately modeling PacBio CCS sequencing errors (18). Taxonomic assignments were performed with the function AssignTaxonomy of the DADA2 pipeline, which classifies sequences based on reference training data sets. The databases used for assignments were UNITE for the ITS (44, 45) and the Ribosomal Database Project (46) (RDP LSU training set). To compare sequence diversities directly between the short and long amplicon sets, ITS-28S fragments were cut in two subset fragments corresponding to the ITS and the 28S subunit (beginning of LSU), respectively. This was performed using the removePrimers step in the DADA2 pipeline. Instead of the ITS1F-LR5 primer pair as described above, we used ITS1F-ITS4 for the ITS and rcITS4-LR5 for the 28S subunit. The sequence subset creation produced the two subsets, ITS-28S subset to ITS and ITS-28S subset to 28S. OTU clustering was performed with the R package DECIPHER using three identity levels (95, 97, and 99% identity) in R (v4.1.2) (47, 48).

Data availability

All PacBio sequencing data are available from the NCBI Sequence Read Archive (SRA) under BioProject PRJNA847708.

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Chapter 2: Endophytic Fungal Community Analyses in Replicated Grapevine Stands Reveal no Drivers of Dieback

Authors: Monod V.[1,2], Hofstetter V.[2], Viret O.[4], Zufferey V.[3], Gindro K.[2], Croll D.[1]*

[1] Laboratory of Evolutionary Genetics, Institute of Biology, University of Neuchâtel, CH-2000 Neuchâtel, Switzerland

[2] Agroscope, Plant Protection, Mycology, 1260 Nyon, Switzerland

[3] Agroscope, Viticulture, Avenue de Rochettaz 21, 1009 Pully, Switzerland

[4] Direction générale de l'agriculture, de la viticulture et des affaires vétérinaires (DGAV), Département de l'Economie, de l'innovation et du sports (DEIS), CH-1110 Morges, Switzerland

*Corresponding author: Daniel Croll, Laboratory of Evolutionary Genetics, Institute of Biology, University of Neuchâtel, CH-2000 Neuchâtel, Switzerland; e-mail: daniel.croll@unine.ch

Abstract

Tree decline are complex and multi-factorial diseases with biotic and abiotic components. Microbiome assembly effects on perennial plant health and decline rates are challenging to assess due to the large number of coexisting microbes including endophytic fungi. Grapevine wood dieback, esca included, is considered the main threat to sustainable grapevine cultivation worldwide but is caused by yet unresolved factors. We sampled asymptomatic and esca symptomatic plants on a network of vineyards in two different years to perform amplicon sequencing analyses of the fungal communities of grapevine trunks. Variability in the recovered diversity is not associated with the health status of the sampled plant. Key taxa typically implicated in esca did not show any significant association with plant health status.

Introduction

Tree decline is the deterioration of tree health observed increasingly in forests and perennial crops constituting a global concern (Denman et al. 2018; Santini et al. 2013; Cohen et al. 2016). Environmental warming is a key factor in increasing tree decline likelihood and favors the spread of plant diseases (Millar and Stephenson 2015; Singh et al. 2023). Tree decline are complex and multi-factorial diseases with biotic and abiotic components (Vieites et al. 2009; Tiew et al. 2020). Determining the combination of factors leading to decline is challenging (Bettenfeld et al. 2020). Vascular wilts are among the most destructive tree declines (Yadeta and J Thomma 2013). Complex biotic interactions, including polymicrobial and insect activity, influence the onset of dieback and increase severity (Denman et al. 2018). Even though most plant diseases are thought to be caused by discrete pathogen species, there is growing evidence that complex plant diseases can arise from synergistic interactions among multiple microorganisms (Lamichhane and Venturi 2015; Denman et al. 2018). Given the complexity of the microbial communities associated with perennial plants, investigating links between microbiome composition and disease status is essential.

Microbiome assembly effects on perennial plant health and decline rates are challenging to assess due to the large number of coexisting microbes including endophytic fungi (Martins et al. 2021). Members of fungal communities interact with each other and with their hosts to cause a wide range of beneficial or pathogenic effects (Compant et al. 2019). The microbiota, by providing additional ecological functions to the host (Turner, James, and Poole 2013), plays a crucial role in plant adaptation to biotic and abiotic environmental conditions potentially enhancing plant health and stress resistance (Pacifico et al. 2019; Stewart et al. 2021). Microbial communities can promote plant growth by simulating water and nutrient intake, increase health trough antibiosis against pathogens and pests (Rodriguez et al. 2009; Hyde et al. 2019; Trivedi et al. 2020; Rolli et al. 2015; Pacifico et al. 2019). The spectrum of symbiotic associations and their consequences are not well defined and depend upon environmental conditions and can transition between commensalism, mutualism or parasitism (Mishra, Bhattacharjee, and Sharma 2021). For instance, different strains of *Pantoea ananatis* bacteria isolated from healthy maize seeds exhibited either growth-promoting properties, weakly pathogenic or had neutral effects on the plant. Such variation in plant-microbe relationships is likely governed by protein secretion systems and effector proteins (Sheibani-Tezerji et al. 2015; Stewart et al. 2021). Environmental factors and host genotype may also influence the lifestyle of fungi transitioning from endophyte to pathogen as observed in *Fusarium verticillioides* on maize (Bacon, Glenn, and Yates 2008). Some endophytes display a latent state and turn symptomatic when the plant

encounters stress conditions such as drought, humidity, or nutrient starvation (Mishra, Bhattacharjee, and Sharma 2021). Fungal endophytes include a diverse group of species with some known to cause plant disease as pathogens but are also present on asymptomatic plants (Trivedi et al. 2020; Sieber 2007). How endophytes transition from commensalism or mutualist interactions to pathogens remains poorly understood (Hardoim et al. 2015; Douanla-Meli, Langer, and Mouafo 2013).

The host microbiome can undergo substantial changes in community structure in presence of pathogenic species and the progression of diseases (Douanla-Meli, Langer, and Mouafo 2013; Mina et al. 2020). Olive orchards suffering from anthracnose show lower endophyte diversity under higher disease incidence (Martins et al. 2021). In Acute Oak decline, the dominant bacterial species was stimulated by a co-invading beetle with additional effects likely caused by other micro-organisms associated with the host or the beetle (Doonan et al. 2020). Synergism among different pathogens can increase disease severity in various tree species including apple, chestnut, hazelnut and grapevine (Lamichhane and Venturi 2015). The impact on tree health resulting from microbial interactions with sequential or cumulative effects can also be modulated by abiotic factors. The deterioration of trees frequently includes abiotic predisposing elements, such as soil microclimate attributes that interact with microbial or insect-induced harm (Doonan et al. 2020). Rising temperature, changes in precipitation patterns, and extreme weather events such as droughts, extreme temperatures can induce stress in trees, weaken their immune systems, and make them more vulnerable to pests and other stressors (Denman et al. 2018; Camarero et al. 2015). Decline diseases where abiotic and biotic interaction contribute likely to disease development need to be addressed with an integrated system approach (Denman et al. 2018).

Grapevine wood dieback is considered the main threat to sustainable grapevine cultivation worldwide but is caused by yet unresolved factors (Cobos et al. 2022). A range of wood-colonizing fungal pathogens were suggested to contribute to disease progression (P. Larignon and Dubos 1997; Mugnai, Graniti, and Surico 1999; Philippe Larignon et al. 2009; C. Bertsch et al. 2013) in addition to changes in climatic and soil conditions (Marchi et al. 2006; Surico, Mugnai, and Marchi 2006). The main form of dieback is identified as grapevine trunk disease (GTD) with significant impacts on yield and reduced fruit quality leading to high plant replacement rates and economic losses (Gramaje, Úrbez-Torres, and Sosnowski 2018; C. Bertsch et al. 2013). GTD is classified into several disease types including the most damaging esca (Bortolami, Gambetta, et al. 2021). Similar plant declines were also observed on many other woody species including lemon, olive, apple, pomegranate trees without clear associations of

potential pathogens and the onset of symptoms (Markakis et al. 2017). Esca includes trunk necrosis development in mature vine as well as foliar symptoms and/or symptoms on the shoots with grape wilting. The expression of foliar symptoms can be discontinuous, but plants usually die within a few years after the expression of the first symptoms (Bruez et al. 2013; Kenfaoui et al. 2022). The discontinuous expression of the disease suggests complex interactions with potential pathogenic species and environmental conditions (Andolfi et al. 2011). Esca disease is thought to be associated with the activity of three distantly related fungi: *Phaeoacremonium* spp., *Phaeomoniella chlamydospora*, and *Fomitiporia mediterranea*, considered as the most serious of the vine pathogens and the main agents of the vascular disease (Andolfi et al. 2011; Brown, Lawrence, and Baumgartner 2020; C. Bertsch et al. 2013). Members of the Botryosphaeriaceae family are also considered to play a role in the disease complex (Gramaje, Úrbez-Torres, and Sosnowski 2018; C. Bertsch et al. 2013). These fungi have consistently been isolated from symptomatic grapevines, displaying a close association with esca symptoms such as foliar necrosis and wood discoloration (Bruno and Sparapano 2006; Mugnai, Graniti, and Surico 1999). However, fungal species isolated from symptomatic plants often occur both on symptomatic and asymptomatic plants suggesting that the disease is not solely triggered by the presence of specific species (Hofstetter et al. 2012; Del Frari et al. 2019; E. Bruez et al. 2016). Shared occurrence of fungal species in both symptomatic and asymptomatic plants suggests a potential endophytic phase (Gramaje, Úrbez-Torres, and Sosnowski 2018). The exact mechanisms and interactions between these fungi and the grapevine host remain poorly understood (C. Bertsch et al. 2013). Whether the association of fungal species with symptom development of esca is based on causal relationships remains unknown (C. Bertsch et al. 2013; M. Fischer and Peighami-Ashnaei 2019). The major limitation of the system is that disease symptoms cannot be reproduced in controlled infections (Reis et al. 2016).

A systematic investigation of esca symptom development using standardized grapevine genotypes planted in diverse environments showed that soil water retention capacity is likely a factor favoring symptom development (Monod et al. 2023). Further studies have shown that water availability can influence symptom expression (Sosnowski et al. 2007; Marchi et al. 2006) and symptom development was likely favored by stronger amplitudes in wet/dry climate transitions (Monod et al. 2023). High-throughput amplicon sequencing techniques can generate high-resolution assessments of fungal diversity within grapevine trunks (Monod et al. 2022; Pacifico et al. 2019; Dissanayake et al. 2018). By examining changes in microbiome composition as a function of symptom development in various environments, helps pinpoint species potentially involved in the disease.

Here, we aimed to identify the most likely drivers of grapevine esca disease using a replicated set of 21 vineyards planted simultaneously with a single susceptible cultivar (*i.e.* Gamaret). We sampled asymptomatic and symptomatic plants on each vineyard in two different years to perform amplicon sequencing analyses of the fungal communities of grapevine trunks. We analyzed mycobiome composition partition within and across vineyards to quantify the community stability in absence of the disease. Using repeated assessment of asymptomatic and symptomatic plants detected in vineyards, we tested for potential association of particular taxa with disease symptom expression.

Results

Replicated assessment of the trunk mycobiome across vineyards

We analyzed 21 vineyard plots planted simultaneously in 2003 with the cultivar Gamaret in Western Switzerland (Figure 1A). All plants originate from a single nursery to standardize both age and genetic makeup. The set of replicated Gamaret plots was tracked based on physiological indicators (yield, must and leaf chemical composition), meteorological and climatic recordings, soil analyses as well as the incidence of esca (Monod et al. 2023). The mortality rate was highly variable between studied sites, ranging from 0-47% in 2017 (Figure 1C, see Monod et al 2023 for details). To determine whether the grapevine trunk wood fungal community could explain the prevalence of esca, we sampled vine plants in the plot network in 2019 and 2021 (Figure 1B). We randomly selected 10 asymptomatic and 5 symptomatic plants showing either foliar symptoms or apoplexy (Figure 1B, 1D). To enhance the likelihood of obtaining a representative mycobiome, we sample 10 asymptomatic plants per vineyards. However, due to the low incidence in specific plots, only five symptomatic plants per vineyard were sampled. We used an optimized protocol to obtain wood cores at the grafting point for each plant (Monod et al. 2022; Hofstetter et al. 2012). To barcode the endophytic fungal community present in the wood cores, we amplified the ITS with primer pairs ITS1F-ITS4. Previous work on the mycobiome of grapevine trunks showed that utilizing a shorter ITS fragment offered the better trade-off between depth of coverage and taxonomic resolution compared to the analysis of longer fragments including also segments of the 28S ribosomal gene sequence (Monod et al. 2022).

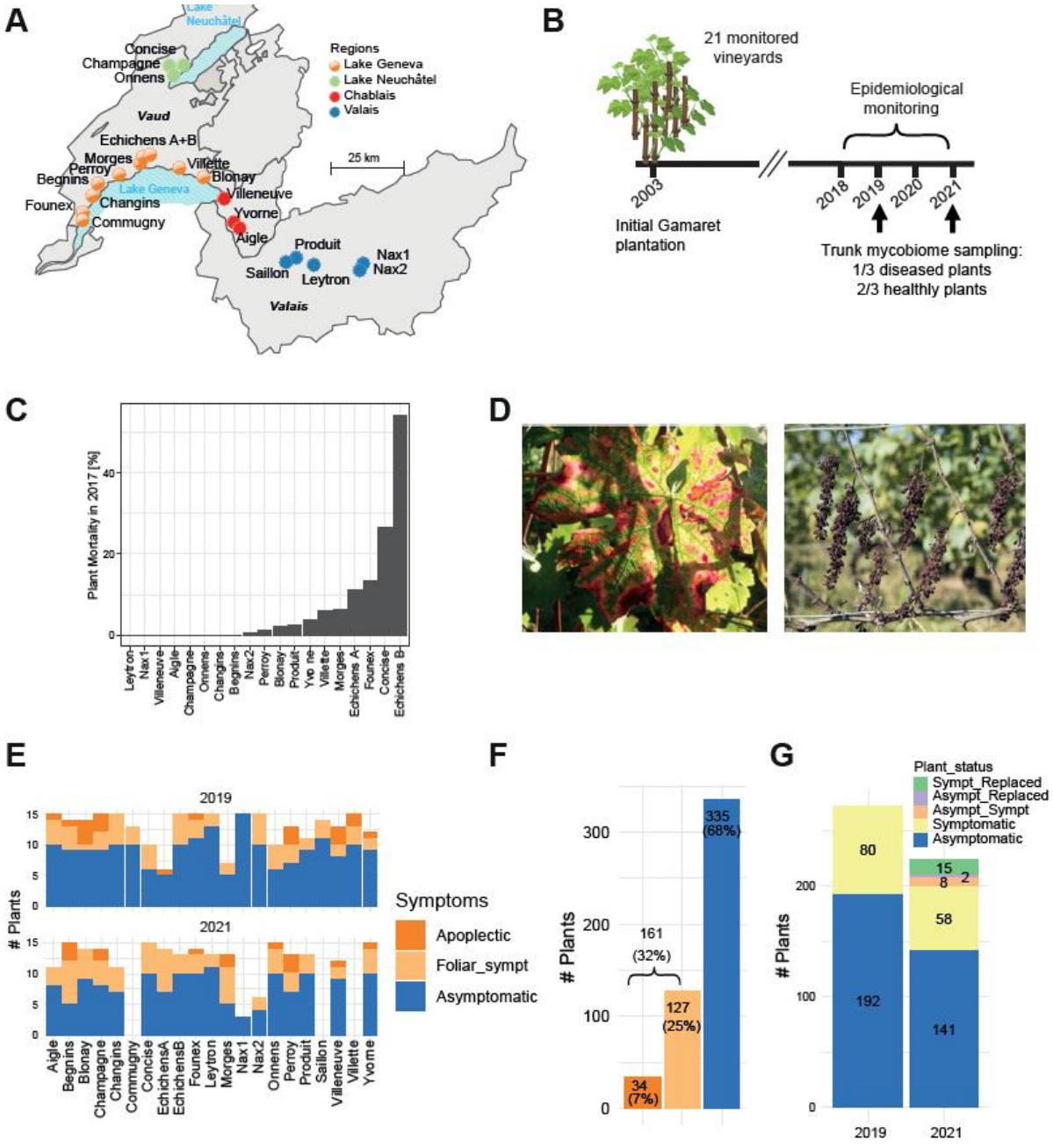

Figure 1 Collection of vine trunk samples to survey the mycobiome community composition. **A** Location of the studied vineyards (n=21) in Western Switzerland, coloured according to the main viticultural regions. **B** History of the studied vineyards with planting in 2003, followed by the monitoring of esca symptoms (2018-2021) and the sampling seasons (2019 and 2021). **C** Mortality rates attributed to esca in the studied vineyards (status 2017). **D** Esca symptoms: typical foliar symptoms ("tiger-stripes") and apoplectic symptoms with wilting of the whole plant. **E** Number of samples successfully sequenced according to categories. **F** Proportion of plants sampled by category (asymptomatic plants n=335, symptomatic plants with leaf symptoms n=127, and symptomatic plants with apoplexy n=34). **G** Overview of sequenced plants by symptom category and sampling year: Asymptomatic plants in 2019 still asymptomatic in 2021 - Asymptomatic; Symptomatic plants in 2019 still symptomatic in 2021 - Symptomatic; Asymptomatic plants in 2019 became symptomatic in 2021: Asympt_Sympt; asymptomatic plants in 2019 that were replaced in 2021: Asympt_Replaced; symptomatic plants in 2019 that were replaced in 2021: Sympt_Replaced).

We successfully amplified 496 samples over all sites. Samples with low PCR yield were excluded as well as samples from sites where the vineyard was uprooted during the sampling period (see Methods for details). We generated PacBio circular consensus sequencing (CCS) data for 192 asymptomatic and 80 symptomatic plants for the 2019 sampling period (Figure 1G). In 2021, 141 plants (out of 192) had remained asymptomatic and 58 (out of 80) had remained symptomatic. We also found plants asymptomatic in 2019 turn symptomatic (n=8) (Figure 1G). Furthermore, two asymptomatic and 15 symptomatic plants from 2019 were uprooted by the plot owners and we selected new plants at random in 2021 as a replacement. The composition of the sampled plants, including proportions of asymptomatic and symptomatic individuals varies among plots in particular for esca symptom categories (Figure 1E). Overall, we sequenced 335 asymptomatic plants (68%) and 161 symptomatic plants (32%) (Figure 1F).

We analyzed 3,390,060 CCS reads after quality filtering steps with a mean of 6,834 reads per sample. The reads clustered into 4,129 distinct amplicon sequence variants (ASVs), assigned to 697 species based on matches in the UNITE fungal ribosomal DNA database (Kõljalg et al. 2019). The median number of reads per ASV was 25 (Figure 2A). Most AVSs were rare with 30% of the ASVs having 10 reads or less. We obtained a median of 121 ASVs per sample (Figure 2C). The number of detected ASVs per sample was correlated with the number of reads (r=0.47, p<0.05; Figure 2B). Overall, the recovered endophytic community was composed of Ascomycota (87.8%), Basidiomycota *(6.82%),* Chytridiomycota (3.29%) and others (2.1%). Among the 41 identified classes, the most abundant classes were Eurotiomycetes (34.4%), Dothideomycetes (30.3%), Sordariomycetes (12.2%), Lecanoromycetes (4.45%) and Leotiomycetes.(4.21%). Among the 497 detected genera, we found a dominance of *Phaeomoniella* (27.6%), *Aspergillus* (5.55%), *Phaeoacremonium* (4.50%), *Pseudopithomyces* (4.01%), *Angustimassarina* (3.90%) (Figure 2D, E, F).

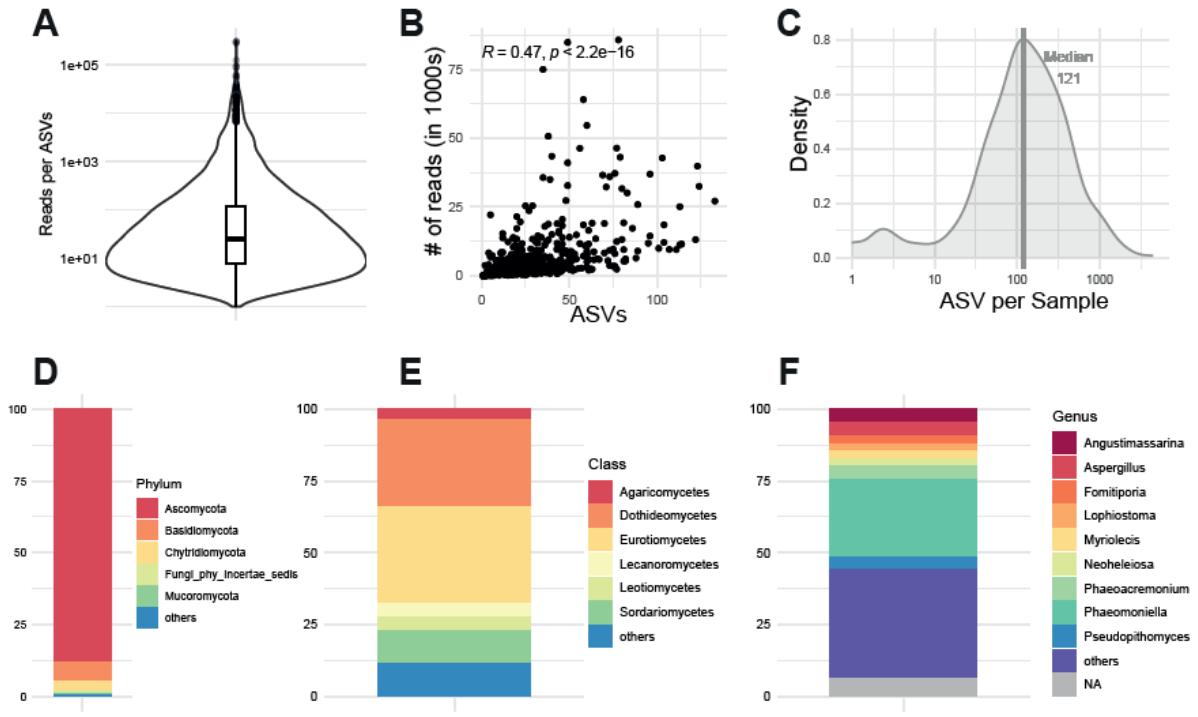

Figure 2 Amplified sequence variants (ASV) recovery and diversity. **A** Recovered reads per ASV (median= 25 reads). **B** Relationship between the number of raw reads and the ASVs detected per sample. **C** Distribution of ASVs per sample (median=121 ASVs). **D** Proportion of phyla represented by the amplicon sequences ("others": phyla with <0.5%). **E** Proportion of classes in the total sequences ("others": classes with <2%). **F** Proportion of genus in the total sequences ("others": comprises classes with <2%).

Fungal microbiome structure among healthy and symptomatic plants

Plants are typically associated with diverse microbiomes independent of their health status. To assess the fungal microbiome structure of asymptomatic plants, we analyzed the 502 fungal species detected in asymptomatic grapevine trunks. The diversity of the recovered mycobiome varied between the two sampling years with 1-133 ASVs recovered per plant and a total of 3124 ASVs. The total diversity between the two sampling years was comparable with 351 species recovered in 2019 (1751 ASVs; *n* =192 samples) and 374 species in 2021 (2057 ASVs; n=143 samples. The mycobiome was only weakly shared between regions across Western Switzerland with 158 (3.8%) out of 4129 ASVs found in all regions (Figure 3A). If we consider the proportions of reads associated with each ASVs, fungal communities are more similar with 64% of reads assigned to the same taxa across geographical regions. Differences in mycobiome composition between regions were mostly due to rare ASVs. *P. chlamydospora* was the most abundant species in all regions. A principal coordinate analysis (pCoA) of the mycobiome revealed substantial overlaps among regions and vineyards, yet fungal communities differ significantly among the regions

(PERMANOVA, R^2=0.016, p=0.001) and among vineyards (PERMANOVA, R2= 0.096, P= 0.001). The pCoA highlights the substantial mycobiome variability among plants, vineyards and regions and the challenge to test for consistent species occurrences across fungal communities (Figure 3B).

Symptomatic plants did not differ significantly from asymptomatic plants in recovered species or ASV diversity with 502 species (3124 ASVs) detected among asymptomatic plants (n=335) and 418 species (1999 ASVs) detected among symptomatic plants (n=161) (ANOVA, p>0.05). Comparisons of Chao1 diversity among different plant health status categories revealed significant differences among plants remaining healthy (*i.e.* asymptomatic) or keep showing symptoms between the sampling years (Wilcoxon p=1.6e^{-5} for asymptomatic plants; p=0.061 for symptomatic plants; Figure 3C). Variability in the recovered diversity is not associated with the health status of the sampled plant. The fungal diversity recovered for the same plant varied across the two time points. However, differences in diversity for plants turning from asymptomatic to symptomatic across sampling years were not significant (Chao1 diversity index; Wilcoxon p>0.5; Figure 3C). However, this assessment is based on a comparatively low number of observations (n = 8). Asymptomatic and symptomatic plants shared overall 24% of ASVs (Figure 3D). If we consider the proportions of reads associated with each ASVs, fungal communities are more similar with 89% of reads assigned to the same taxa between health status. Differences in fungal community composition across samples of different health status were largely due to rare taxa. The pCoA revealed no obvious clustering between plants remaining asymptomatic, persistant in a symptomatic stage or turning symptomatic over the sampling period (Figure 3E). Community composition was nevertheless significantly different between symptomatic and asymptomatic plants (PERMANOVA, R^2=0.003, p=0.014). Fungal community composition was significantly different between asymptomatic plants compared to plants suffering from apoplexy (PERMANOVA, R^2=0.00465, p=0.005). Fungal communities of plants presenting either foliar symptoms or apoplexy were not significatively different (PERMANOVA, p>0.05).

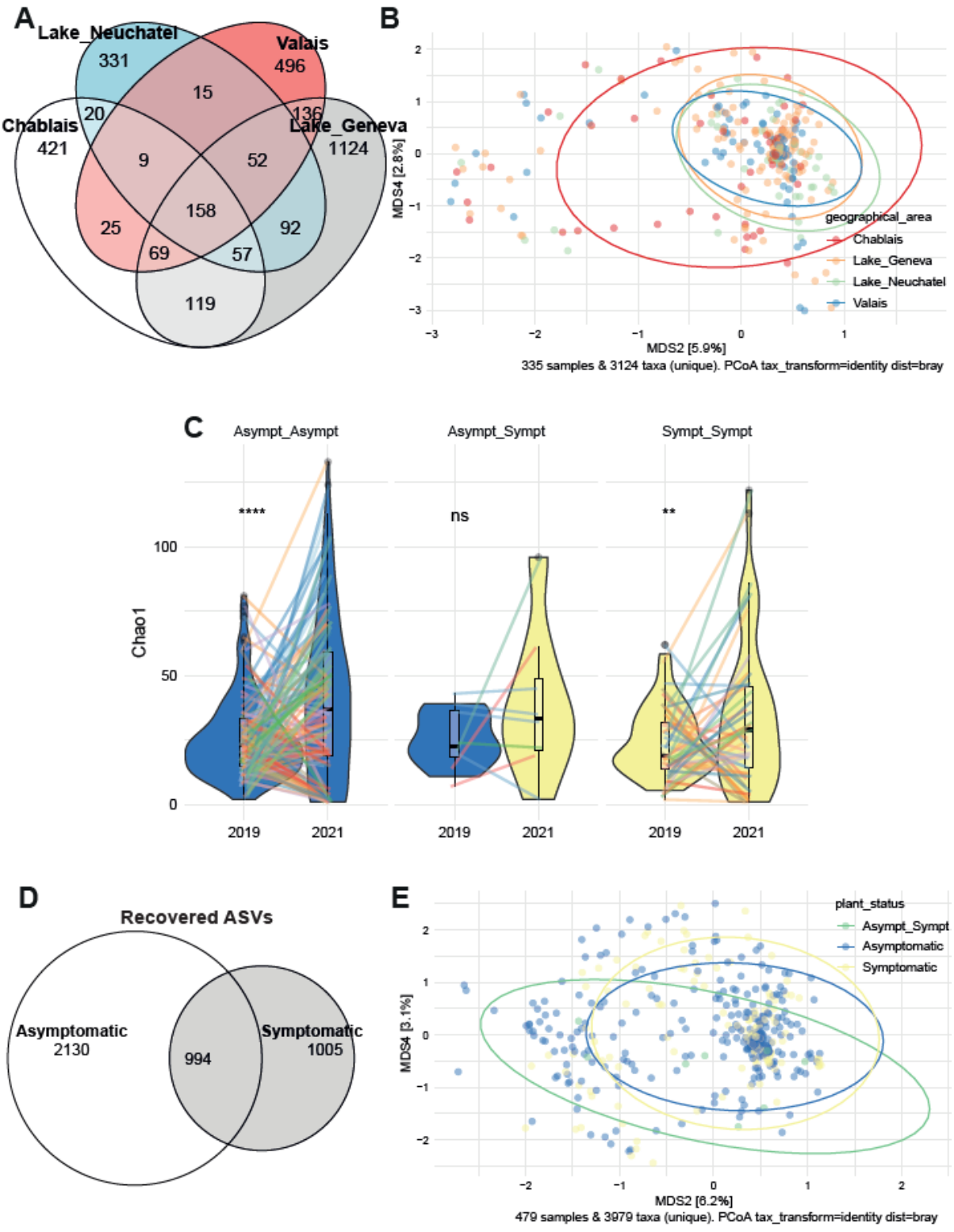

Figure 3 Diversity of asymptomatic or symptomatic plant mycobiomes. **A** Proportion of ASVs shared among asymptomatic plants per geographic regions. **B** Principal coordinate analysis (PCoA, no transformation, Bray-Curtis distance on ASV diversity, *n* = 3124) of mycobiome diversity of asymptomatic plants across geographic regions. Each point represents the mycobiome composition at the ASV level of the trunk of once sampled vine plants. **C** Violin plots displaying the α-diversity on esca asymptomatic and symptomatic sampled plants (Chao1 index) taking into account their epidemiological history (Asympt_Asympt: plants that remained asymptomatic during the two years of sampling; Asympt_Sympt: plants that changed from asymptomatic in 2019 to

symptomatic in 2021; Sympt_Sympt: plants recorded as symptomatic during the two years of sampling) with individual samples linked and colour-marked by vineyard. **D** Proportion of ASVs shared between asymptomatic and symptomatic sampled plants **E** Principal coordinate analysis (PCoA, no transformation, Bray-Curtis distance on ASVs diversity, n= 4169) of the mycobiome diversity of the samples.

Taxonomic profiles highlight taxa linked to asymptomatic plants

We assessed evidence of significantly overrepresented taxa in either asymptomatic and symptomatic plants using discriminant analyses (DA). DA indices were constructed for a total of 496 plant samples. We used three different approaches to assess evidence for taxa enrichment in symptomatic versus asymptomatic plant trunk mycobiome. First, linear discriminant analysis effect size (LEfSe) analyses identified the *Neosetophoma* genus, a species of the same genus (*N. shoemaker*) and the related Phaeosphaeriaceae family and the class of Tremellomycetes as enriched in asymptomatic plants (Figure 4A). Second, an analysis of composition of microbiomes (ANCOM) identified six enriched genera including two enriched in asymptomatic plants (*Neosetophoma* and *Filobasidium*) and three enriched in symptomatic plants (*Tausonia*, *Verrucoccum* and *Mortierella*). ANCOM also identified the Asycomycota phylum as enriched in symptomatic samples (Figure 4B). ANCOM-BC identified seven genera (*Neosetophoma*, *Calloriaceae*, *Naganishia*, *Curvibasidium*, *Trichoderma*, *Cyphellophora* and *Lophiostoma*) and an order (Pleosporales) as having reduced abundance (negative LFC) in symptomatic compared to asymptomatic plants (Figure 4C). Hence, the ANCOM method was the only one to identify enriched taxa in symptomatic plants. The *Neosetophoma* genus was supported by evidence and consensus between methods for association with asymptomatic plants. Proportionally, *Neosetophoma* genus represents 0.5% of the reads of asymptomatic plants compared to 0.03% in symptomatic plants (Sup. Figure 1A). Upon examining the distribution of the *Neosetophoma* genus across various geographical regions, a notably higher occurrence was observed in the Valais region (Sup. Figure 1B). Valais is the region that exhibits the lowest incidence of esca impact (Sup. Fig., (Monod et al. 2023). When we examined the presence of the *Neosetophoma* genus alongside the recorded mortality rates in each vineyard, we did not detect any correlation though (R=0.05 p=0.518) (Sup. Figure1).

Fungal ecological characteristics in the composition between symptomatic and asymptomatic plants revealed no shift

To determine potential differences in the ecological roles of fungal communities in symptomatic and asymptomatic plant samples, we classified each identified genus into functional groups using the FungalTrait database (Põlme et al. 2020). After considering predicted guilds and trophic modes, our analysis revealed no significant differentiation between asymptomatic and

symptomatic plants (Figure 4D, E). It should be noted that many genera are classified as pathogenic. This is potentially a bias of the database, as pathogenic genera are more studied and described than others.

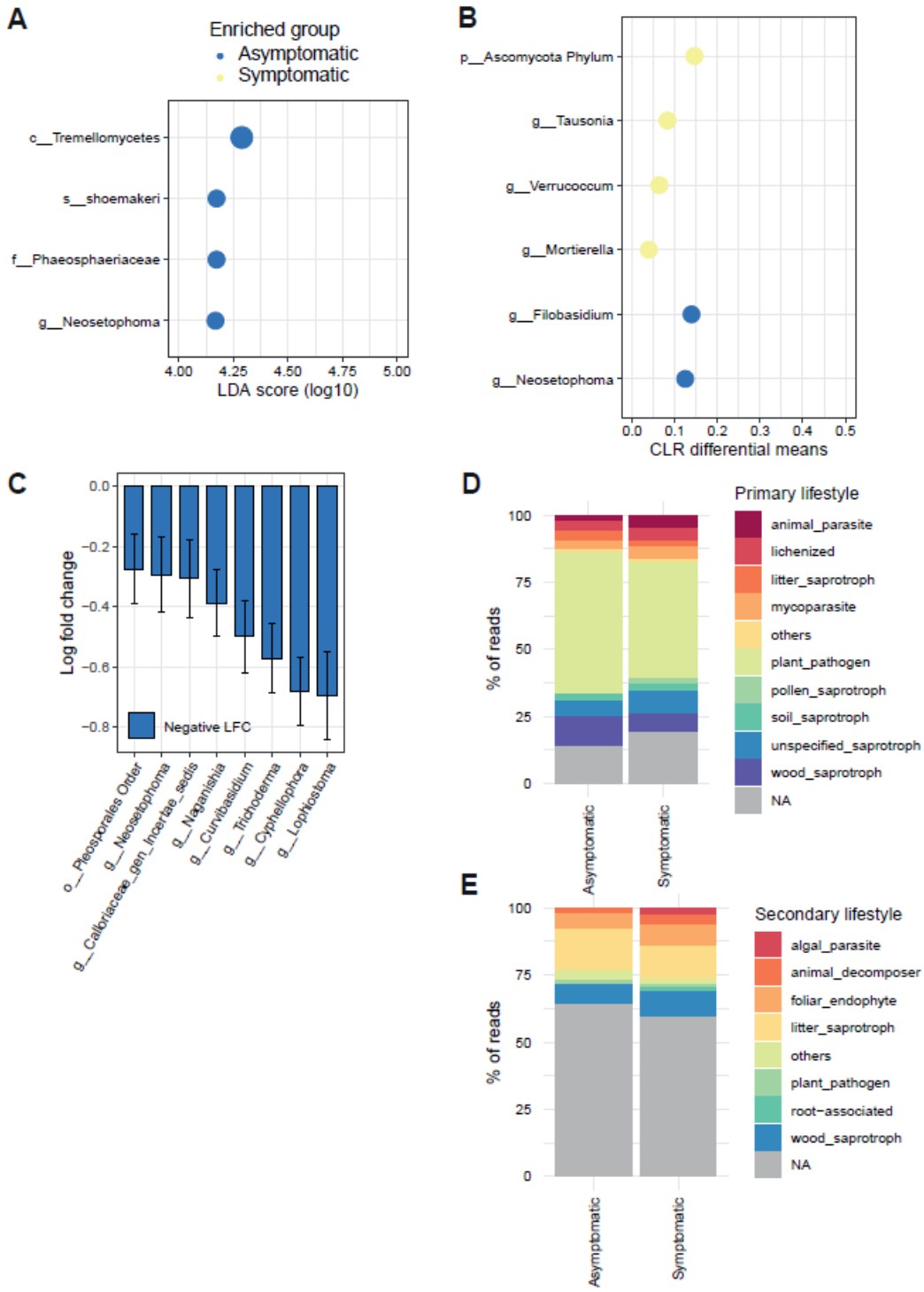

Figure 4 Identification of differentially abundant taxa in the mycobiome of asymptomatic and symptomatic plants. **A** Linear discriminant analysis (LEfSe) was used to identify overabundant taxa in asymptomatic and symptomatic plants (CLR normalization, p-value < 0.05). Enriched taxa in the asymptomatic group are shown in blue, enriched taxa in the symptomatic group in yellow. The list of discriminating features according to the classes (asymptomatic and symptomatic) is ordered by the magnitude of the effect with which they differentiate the classes. **B** Microbiome composition analysis (ANCOM) identified compositional differences (p-value < 0.05) in the mycobiome communities of asymptomatic and symptomatic sampled plants. Enriched taxa in the asymptomatic group are shown in blue, enriched taxa in the symptomatic group in yellow. **C** Analysis of microbiome composition represented by effect size (log fold change) and 95% confidence interval bars (two-

sided; Bonferroni adjusted) derived from the ANCOM-BC model. **D** Traits based approach with proportion of primary lifestyle between asymptomatic and symptomatic sampled plants. **E** Traits based approach with proportion of secondary lifestyle between asymptomatic and symptomatic sampled plants.

Genera previously associated with esca

We retrieved a set of taxa commonly described to be associate with grapevine trunk diseases (Andolfi et al. 2011; C. Bertsch et al. 2013; Brown, Lawrence, and Baumgartner 2020). We focused on presence and relative abundance of the genera *Phaeomoniella*, *Phaeoacremonium* and *Fomitiporia*, as well as the Botryosphaeriaceae family. We retrieved ASVs assigned to each taxonomic unit across vineyards and plant health status. We found no evidence that these taxonomic units were enriched in symptomatic plants (Figure 5). The proportions of *Phaeomoniella* (mean of 34.1% in asymptomatic; 33.1% in symptomatic plants), *Phaeoacremonium* (mean of 14.7% in asymptomatic; 11.7% in symptomatic plants) and *Fomitiporia* (mean of 16.5% in asymptomatic; 11.6% in symptomatic plants) in both symptomatic and asymptomatic plants were comparable (Figure 5 A, C, E). Relative abundance of the focal taxa varied across vineyards ranging for *Phaeomoniella* from 100% to 0.02% in asymptomatic and symptomatic plants, for *Phaeoacremonium* from 100% to 0.02% in asymptomatic plants and from 100% to 0.06% in symptomatic plants and for *Fomitiporia* from 95.6% to 0.03% in asymptomatic plants and 77,5% to 0.02% in symptomatic plants (Figure 5 B, D, F).

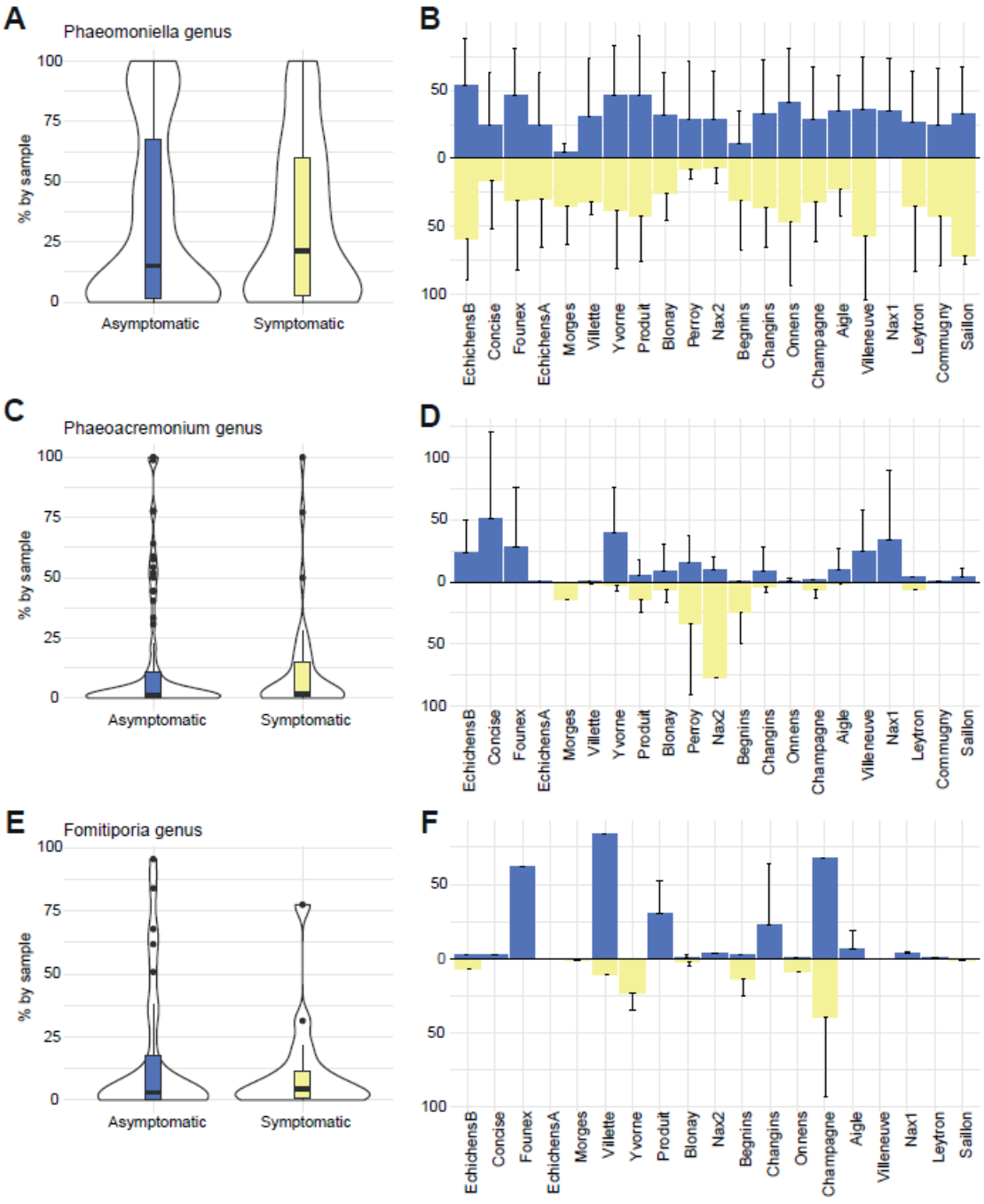

Figure 5 Fungal taxa commonly associated with esca per genus. **A** *Phaeomoniella* genus with symptomatic and asymptomatic taxa. **B** The mean (standard deviation in black) of the *Phaeomoniella* genus proportion by vineyard for asymptomatic (blue) and symptomatic (yellow) sampled plants. **C** Proportion of *Phaeoacremonium* genus between asymptomatic and symptomatic plants. **D** The proportion of the *Phaeoacremonium* genus varies between vineyards in asymptomatic (blue) and symptomatic (yellow) sampled plants. **E** Proportion of the *Fomitiporia* genus between asymptomatic and symptomatic sampled plants. **F** The proportion of the *Fomitiporia* genus across vineyards and asymptomatic (blue) and symptomatic (yellow) sampled plants.

Discussion

Esca disease is likely caused by a multitude of fungal species and is facilitated by environmental conditions. Here, we aimed to test for compositional differences in trunk inhabiting fungal communities in vineyards affected to varying degrees by esca. The analysis of the vine trunk mycobiome revealed a remarkably diverse fungal community with weak differentiation at the vineyard of regional level. We found overrepresentation of several taxa in asymptomatic plants, however no taxa was overrepresented in symptomatic plants. Key taxa typically implicated in esca were also not showing any significant association with plant health status.

Extensive mycobiome variability across sampling scales

Fungal diversity among sampled plants showed high among plant heterogeneity confirming analyses across many wild or cultivated plant species (Pozo et al. 2021; Vandenkoornhuyse et al. 2015). The grapevine trunk mycobiome was largely constituted by rare taxa consistent with many host-associated mycobiome studies (Del Frari et al. 2019; Travadon et al. 2013; Lundberg et al. 2012; Segata et al. 2011; Gobet, Quince, and Ramette 2010; Vaz et al. 2018). Variation in sample diversity may be attributed to factors such as sampling bias, disparities between plant tissues containing both living and deceased material, intra-vineyard diversity, or differences in pedoclimatic conditions (Bettenfeld et al. 2022; Vandenkoornhuyse et al. 2015; Pacifico et al. 2019). We observed dissimilarities in fungal composition across geographic areas consistent with findings of previous studies on grapevine microbiome composition (Bekris et al. 2021) and other woody plants (Proença et al. 2017). These observations suggest that fungal endophytes colonize the tissues of the hosts by a potential horizontal transfer of diversity from the surrounding environment via soil- or airborne spores (Vaz et al. 2018; Saikkonen et al. 2004; Rana et al. 2019).

Alpha diversity declined with increasing disease symptom severity and such a decline was suggested in other systems to play a protective role mediated by the retained members of the fungal community (Koskella et al. 2017). Similar findings were obtained from acute oak decline (Doonan et al. 2020), from fungal root pathogens (Mendes et al. 2011) or bumble bees (Koch and Schmid-Hempel 2011). The opposite was also observed though in pine wilt disease (Proença et al. 2017) or ash dieback (Griffiths et al. 2020) where higher diversity of the microbiome was observed along with symptom severity. Higher microbiome diversity is thought to stem from the pathogen suppressing plant resistance mechanisms and, thereby, facilitating the colonization by other microorganisms (Proença et al. 2017). Plants affected by esca showed neither a decrease or increase in alpha diversity in our study. No significant differences in alpha diversity of declined and healthy tree were found in key tree species (holm oak, cork oak, chestnut and pyrenean oak) of the mediterranean forest (Diez-Hermano et al. 2022). While richness in diversity

remained unchanged, alterations in community composition could still have occurred depending on the health status of the plant. However, the interpretation of the functional role of the fungal community can be challenging because fungal species can shift from diverse types of symbiosis (Sieber 2007; Romeralo et al. 2022).

Grapevine fungal community structures are shaped by rare taxa

Despite no overall diversity effects, we detected a broad range of alterations in the community composition between asymptomatic and symptomatic plants. Yet, plant health was not a strong enough factor to reveal distinct community effects using beta dissimilarity analyses. Across geography and esca health status, we observed high inter-sample variability. Such variability in the host-associated mycobiome creates significant challenges to pinpoint cryptic species underpinning diseases. Yet, many environmental microbiome communities are typically characterized by the presence of a long tail of rare taxa (Pedrós-Alió 2006; Gobet, Quince, and Ramette 2010). Overcoming statistical limitations in associating rare taxa with disease development would require either substantially expanding the sampling effort or reducing environmental noise.

No differentiated fungal community associated with symptomatic plants

We tested for enrichment in particular taxonomic groups on symptomatic plants using three distinct approaches and found no robustly associated taxon. This aligns with earlier research on fungal trunk communities of esca revealing no direct association between particular taxa and symptomatic esca plants (Del Frari et al. 2019; Hofstetter et al. 2012; Bruez et al. 2014). Our study expands on previous works by substantially increasing the number of samples highlighting that even sampling strategies with hundreds of datapoints remain underpowered. Previous research conducted on the same vineyards has linked the incidence of esca symptoms to pedo-climatic factors (Monod et al. 2023) suggesting that soil water retention capacity is a key factor for disease development. Retention capacity is influenced by the amount of precipitation and various soil characteristics. Whether such soil properties are causal or only showing correlated responses to a yet unknown factor remains unknown. If soil properties are at the origin of the disease, a number of fungal taxa may in turn sporadically associate with particular soil types without playing a relevant role in the disease. Furthermore, any association of endophyte taxa may be similarly due to correlations of soil characteristics and fungal diversity. Furthermore, endophytes may exit the latent asymptomatic state after the plant encountered stress conditions such as drought, humidity, or nutrient starvation (Mishra, Bhattacharjee, and Sharma 2021). An interesting observation was the notable enrichment of the *Neosetophoma* genus in asymptomatic plants underpinned by a consensus of all differential abundance methods. The

prevalence of this genus is most remarkable in the Valais region, which also exhibits the lowest esca incidence. Additionally, it's noteworthy that the *Neosetophoma* genus is absent from numerous studied vineyards. Hence, the strong relationship between the presence of the *Neosetophoma* genus and the absence of esca symptoms should be interpreted with caution. It is conceivable that endophytes residing in woody plants play a defensive role for the host plant by generating a range of protective mycotoxins and enzymes (Pacifico et al. 2019; Stewart et al. 2021). Lack of associated taxa with trunk disease does not negate causal interactions of fungal taxa with the disease but rather suggests that the complexity of biotic drivers is too high.

Plant health or disease should not be viewed as a binary concept but rather an expression of symptoms along a continuum. In complex diseases like tree decline, multiple factors are likely involved and resolving causal relationships between taxa and health is challenging. The presence of endophytes residing within plants without causing harm, challenges our traditional understanding of plant infection processes and how causal taxa should be identified (Mishra, Bhattacharjee, and Sharma 2021). The ecological relevance of rare species is increasingly recognized with key functions in host-associated microbiomes (Jousset et al. 2017). Yet, determining perturbations caused by rare species is challenging as most study systems are underpowered (Säterberg et al. 2019). Further research under more controlled conditions is needed to determine what disruption or imbalance in the plant microbiome is considered detrimental to plant health (Romani 2011; Begum et al. 2022) and what meaningful boundaries can be drawn between endophytes and pathogens (Lòpez-Fernàndez et al. 2015).

Methods

Sample collection

Wood samples were collected in August 2019 and 2021 from vineyards located in four viticultural regions in western Switzerland. A total of 21 vineyards planted with the Gamaret variety were sampled. Gamaret originates from a cross between Gamay and Reichensteiner varieties grafted onto 3309C rootstock (*V. riparia* X *V. rupestris*). All plants originate from the same nursery (Les frères Dutruy SA; Founex, Switzerland) and were planted in 2003. The 21 vineyards had been under similar viticultural management. In each vineyard, five symptomatic plants displaying the typical foliar esca symptoms including leaf discoloration, tiger-stripe pattern and plant wilting (Mugnai, Graniti, and Surico 1999) were collected alongside ten asymptomatic plants in 2019 and 2021. Plants were 16 and 18 years old when samples were collected in 2019 and 2021, respectively. Some vineyards were uprooted (*i.e.,* Villette and Saillon vineyards) and some replacements were managed inconsistently (*i.e.,* Commugny), hence three vineyards were

excluded for the second sampling year. Each vine plant selected was sampled at the grafting point using a non-destructive method (Hofstetter et al 2012). A 0.5 cm^2 piece of bark was removed with a surface-sterilized scalpel (80% ethyl alcohol). Next, sampling was performed using a power drill with a surface-sterilized drill bit (Ø 3.5 mm) at the spot where the bark was removed. Coiled wood (~60 mg) extracted by the power drill was collected in Eppendorf tubes held underneath using sterilized tweezers. Eppendorf tubes containing the coiled wood were stored at -80°C.

DNA extraction from wood samples

Eppendorf tubes containing wood samples and two 5-mm iron beads were placed in liquid nitrogen. Material was ruptured two times for 1 min at 30 Hz in a TissueLyser (Qiagen Inc., Germantown, MD, USA). Between and after these two steps of tissue disruption, tubes were placed in liquid nitrogen for 1 min. The tubes were placed on ice for slow thawing and 1 mL of cetyltrimethylammonium bromide (CTAB) was added to each tube. The samples were then centrifuged for 1 min at 15,000 rpm and the supernatant was transferred to a new tube. Fungal DNA was extracted using a Qiacube robot with the DNeasy Plant Pro Kit 69206 (Qiagen).

Amplification of fungal ribosomal DNA

The ITS was targeted for amplification using primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) (Gardes and Bruns 1993). We followed the PacBio procedure using barcoded universal primers for multiplexing amplicons, which includes two PCR steps (see https://www.pacb.com). The first PCR program was 30 s of denaturation at 98°C and then 30 cycles of 15 s at 98°C, 15 s at 55°C, and 1 min 30 s at 72°C, followed by a final elongation step for 7 min at 72°C. The second PCR program was 30 s of denaturation at 98°C and then 20 cycles of 15 s at 98°C, 15 s at 64°C, and 1 min 20 s at 72°C, followed by a final elongation step for 7 min at 72°C (Pacific Biosciences, 2019). The final libraries were quantified with a Qubit fluorometer (Thermo Fisher, Foster City, CA, USA), and then all samples were pooled equimolarly. Amplicons were purified and prepared for SMRT sequencing at the Functional Genomics Center in Zürich (FGCZ), Switzerland. Sequencing was performed on the PacBio Sequel II platform.

Demultiplexing and analyses of amplicon sequence variants

Raw reads were processed with the DADA2 package in R (Callahan Github, https://github.com/benjjneb/dada2). We quality-trimmed, filtered reads and inferred amplicon sequence variants (ASVs) with DADA2. For chimera detection we observed a too light detection made by DADA2 algorithm (isBimeraDenovo) and consequently we used QIIME2 uchime-

denovo function. Taxonomic assignments were performed with the function *AssignTaxonomy()* of the DADA2 pipeline, which classifies sequences based on the reference training data sets and based on the UNITE general FASTA release database (2023-07-25, (Abarenkov et al. 2023). We calculated Chao1 indices to assess the richness and diversity of the trunk mycobiome using the *vegan* R package (Oksanen et al 2022). Differences in taxonomic diversity were tested using a Wilcoxon test ($p < 0.05$).

Analyses and characterization of taxa associated with esca

We assessed dissimilarity distances to visualize beta diversity and quantified differences in the overall ASV composition based on a principal coordinates analysis (PCoA) with Bray-Curtis distances (Bray and Curtis 1957). Beta diversity dissimilarities in fungal communities were assessed at the sample level, health status and geographic region. Differences between groups in taxonomic composition were tested using three distinct methods. Linear discriminant analysis effect size (LEfSe) (Segata et al. 2011) is based on Kruskal-Wallis rank sum tests to identify taxa with significant differential abundance (alpha = 0.05) between groups using one-against-all comparisons. The analyses are followed by a linear discriminant analysis (LDA) to estimate the effect size of each differentially abundant feature (LDA >2). ANCOM2 based on Aitchison's methodology uses relative abundances to infer absolute abundances (Mandal et al 2015). Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) was used with an adjustment for sampling fraction by adding a sample-specific offset term in a linear regression model (Lin and Peddada 2020). This offset term corrects for biases and the log-transformed linear regression framework addresses microbiome data compositionality (Lin and Peddada 2020). Niche characteristics and traits shared by identified genera were analyzed using the FungalTraits database (Põlme et al. 2020). Figure panels were generated using the R package *ggplot2* v3.3.3 (Wickham n.d.).

Data availability

All PacBio sequencing data are available from the NCBI Sequence Read Archive

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Author contributions

Monod, Zufferey, Viret, Gindro, Hofstetter and Croll conceived the ideas and designed methodology; Monod collected the data; Monod and Croll analyzed the data; Monod and Croll led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication and ensured that questions related to the accuracy or integrity of any part of their work are appropriately investigated and resolved.

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Supplementary Figure

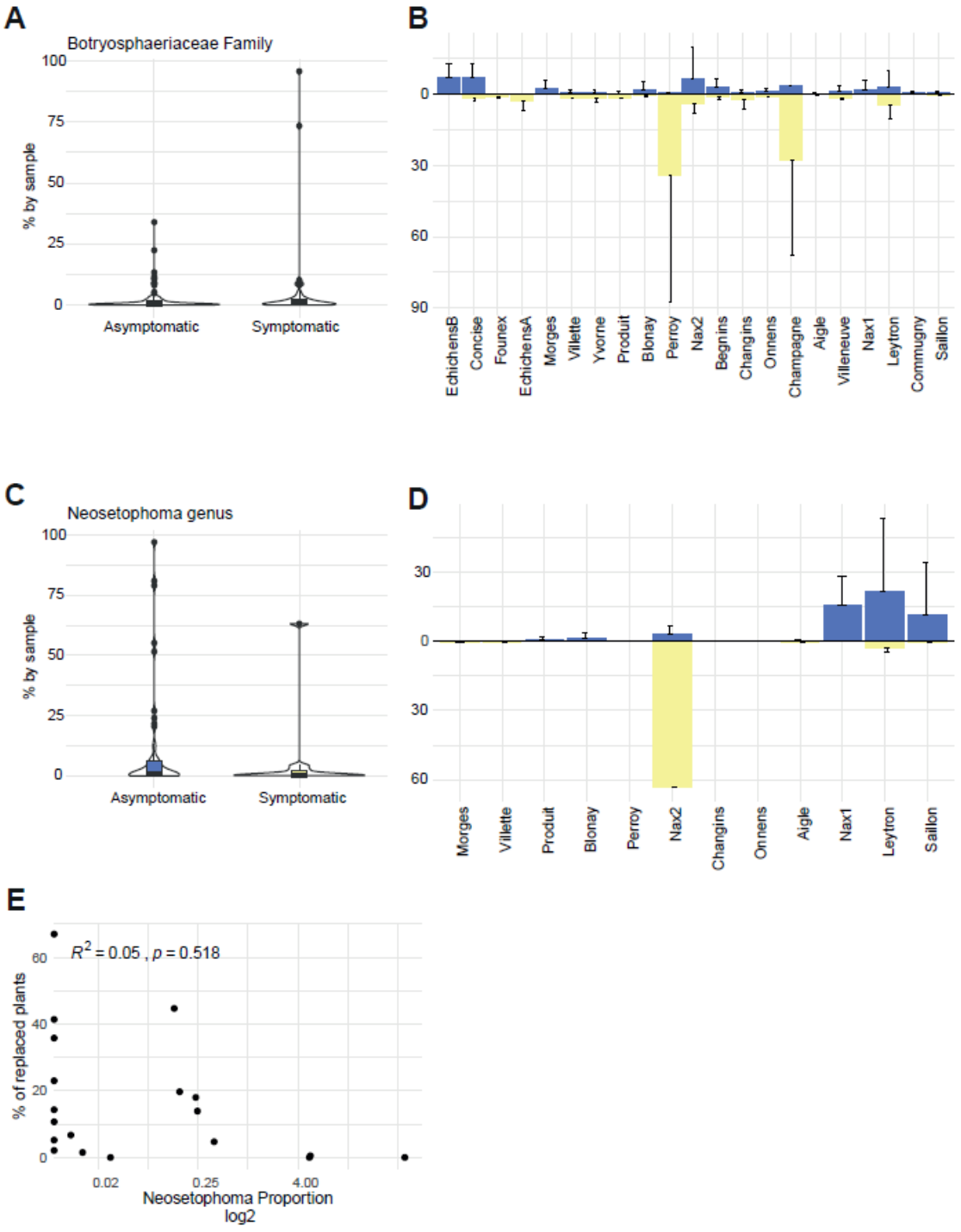

Figure 6 A Botryosphaeriaceae family with symptomatic and asymptomatic taxa. **B** The mean (standard deviation in black) of the Botryosphaeriaceae family proportion by vineyard for asymptomatic (blue) and symptomatic (yellow) sampled plants. **C** Proportion of *Neosetophoma* genus between asymptomatic and symptomatic plants. D The proportion of the *Neosetophoma* genus varies between vineyards in asymptomatic (blue) and symptomatic (yellow) sampled plants. **E** Relationship between the proportion of *Neosetophoma* genus by plot and the proportion of replaced plants.

Chapter 3: A systemic approach allows to identify the pedoclimatic conditions most critical in the susceptibility of a grapevine cultivar to esca

Vinciane Monod[1,2], Vivian Zufferey[3], Matthieu Wilhelm[2], Olivier Viret[4], Katia Gindro[2], Daniel Croll[1], Valérie Hofstetter[2*]

[1] Laboratory of Evolutionary Genetics, Institute of Biology, University of Neuchâtel, CH-2000 Neuchâtel, Switzerland

[2] Agroscope, Plant Protection, Mycology, 1260 Nyon, Switzerland

[3] Agroscope, Viticulture, Avenue de Rochettaz 21, 1009 Pully, Switzerland

[4] Direction générale de l'agriculture, de la viticulture et des affaires vétérinaires (DGAV), Département de l'Economie, de l'innovation et du sports (DEIS), CH-1110 Morges, Switzerland

*Corresponding author: Valérie Hofstetter, Agroscope, Plant Protection, Mycology, 1260 Nyon, Switzerland; e-mail: valerie.hofstetter@agroscope.admin.ch

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Abstract

Esca is the most destructive grapevine trunk disease in the world. The disease is complex and remains poorly understood. As some vine cultivars show highly variable incidence to esca, we designed a four-year experiment to identify which environmental factors influence the expression of the disease. We collected epidemiological and physiological data once a year for four consecutive years in 19 vineyard plots located in four wine-growing regions of Western Switzerland. We compared these data with climatic data obtained from weather stations for these same plots for four years and over the long term. We also estimated the soil water holding capacity of each plot. Confounding factors were minimal because all vineyards were planted in 2003 with the same cultivar and all plants grafted in the same nursery with genetically homogeneous grafting material. Principal component and regression analyses of combined

epidemiological, biotic and pedoclimatic data identified a positive correlation between soil water retention capacity and plant mortality due to esca. These analyses also showed that leaf disease symptoms and apoplexy are more frequent when cold, wet periods are followed - or alternate with - hot, dry periods, and that apoplexy occurs more frequently when weather conditions change abruptly (cold, wet May followed by a hot June) and deviate significantly from long-term climatic conditions. Regression analyses show that the soil water holding capacity impacts less the disease expression when the climate is warm and dry, both at the regional and at year-specific levels. Having identified the most important environmental factors towards expression of esca, this study allows recommendations to be given to the winegrowers for the cultivar studied but can also be used as a model to identify the environmental factors that influence the expression of fungal diseases in other grapevine cultivars, other grapevine trunk diseases and even in other woody plants.

Introduction

Grapevine plants, with a lifespan of 30 years or more, are subject to a variety of biotic and abiotic stresses (Songy et al., 2019; Suzuki et al., 2014). Since the early 1980s, mature vineyards have begun to show increased yield and plant losses (Bertsch et al., 2013; Larignon et Dubos 1997; Mugnai et al 1999). This situation is attributed to the emergence of fungal diseases, linked to soil and climate factors (Bertsch et al., 2009; Bortolami et al., 2021a; Pastore et al., 2022; Songy et al., 2019). The cost of these diseases is high, involving numerous chemicals and/or biological fungicide treatments and replacement of dead plants (De Moura et al., 2012).

Among the emerging fungal diseases of grapevine, a complex called grapevine trunk disease (GTD) is primarily responsible for the death of young and mature grapevines worldwide (Claverie et al., 2020; Mondello et al., 2018). A very large number of new vineyards were planted during the 1990s, probably as a result of the spread of GTD in all countries where grapevines are grown (Gramaje & Armengol, 2011). The majority of these plants reached the age of disease expression at the same time, 7-10 years after their plantation, making the problem more noticeable (Gramaje et al., 2018). In addition, the generalisation of mechanical pruning has increased the number of pruning wounds, the main entry point for the fungi associated with GTD (Makatini et al., 2014). These diseases pose a real threat to the viability and sustainability of viticulture (Bertsch et al., 2013; Kenfaoui et al., 2022).

The adult plants' most destructive GTD worldwide are esca, Botryosphaeria dieback (Úrbez-Torres & Gubler, 2011), Eutypa dieback (Kuntzmann et al., 2010; Rolshausen et al., 2008;

Trouillas & Gubler, 2010) and, to a lesser extent, Phomopsis dieback (Úrbez-Torres et al., 2013). All these diseases generate necroses in the grapevine trunk as well as foliar and/or shoot symptoms. Grapevine plants often suffer from more than a single GTD, due to their longevity and the multiple opportunities for fungal pathogens to infect wood mostly via pruning wounds (Bruez et al., 2014; Del Frari et al., 2019; Gramaje et al., 2018; Hofstetter et al., 2012). GTD infected plants may express foliar symptoms for several years and not always consecutively, but they usually die within a few years after the first disease symptoms are expressed (Bruez et al., 2013; Kenfaoui et al., 2022). All GTD-associated fungal species tested to date produce phytotoxic compounds (Andolfi et al., 2011; Martos et al., 2018; Masi et al., 2018). However, because these fungi live exclusively in wood, they have never been isolated from leaves (Bertsch et al., 2013; Bortolami et al., 2019), foliar symptoms are thought to result from the translocation of these phytotoxic compounds from the trunk to the leaves via sap flow (Bortolami et al., 2021b). To date, GTD foliar symptoms have rarely been reproduced under controlled conditions (Reis et al., 2016). The etiology of GTD pathogenic fungi remains poorly understood (Bertsch et al., 2013; Claverie et al., 2020; Mondello et al., 2018).

Soil and climate factors have also been suspected since long to influence GTD expression, particularly esca (Dubos et al., 2002). Environmental constraints seem to play a key role as they impact grapevine physiological behaviour by influencing host-microbiome interactions (Delmas, 2021), trigger the transition from a fungal endophyte state to a pathogenic state (Porras-Alfaro & Bayman, 2011; Saikkonen et al., 2003), and/or accelerate the translocation of metabolic compounds throughout the plant via sap flow (Claverie et al., 2020). Soils with high water holding capacity (deep, clayey soils) and climatic variations during the summer are factors reported to increase the risk of sap flow disruption (Surico et al., 2006). Vines with even mild foliar symptoms already suffer from hydraulic failure, a decrease in conductance in petioles and shoots, compared to healthy plants (Ouadi et al., 2019). Other important factors influencing disease expression appear to be linked to the plant physiology including the plant water status during growth periods, vigour (leaf area), carbon/nitrogen ratio in plant tissues (Berger et al., 2007) and functioning of the vascular system (Andreini et al., 2009; Edwards, Pascoe, et al., 2007). Two main hypotheses have been put forward to explain conductance failure: gas embolism (Canny, 1997) and/or occlusions (Sun et al., 2007), the latter either related or not with fungal pathogens. A recent study has shown that occlusions by tyloses and gels, probably induced remotely by phytotoxins produced by esca-associated fungi, lead to hydraulic failure in veins of esca-symptomatic leaves (Bortolami et al., 2019). However, the role of esca associated fungi in the expression of foliar symptoms remains to be proven. Another study recently tested

the effect of drought on grapevines under controlled conditions (Bortolami et al., 2021b) and found that drought prevents the expression of esca symptoms.

Numerous studies have attempted to understand the impact of soil type, particularly its ability to retain water, and/or climate on esca disease expression (Bortolami et al., 2021a; Calzarano et al., 2018; Dubos et al., 2002; Fischer & Peighami-Ashnaei, 2019; Guérin-Dubrana et al., 2013; Marchi et al., 2006; Surico et al., 2000). However, such studies have been conducted in different regions, countries, or continents, often on different grape varieties with different disease susceptibility (Andreini et al., 2014), with plants of different ages, grown and pruned in different ways (Gramaje et al., 2018). The disparity of these studies has made it difficult to generalize the results obtained and to precisely identify the role of the pedoclimatic and biotic factors responsible for the variability in the incidence of esca observed in different regions and for different grape varieties (Bertsch et al., 2009). Therefore, the impact of individual and/or combined pedoclimatic on the expression of esca is not yet clearly established. While differences in esca susceptibility between cultivars are relatively well documented (Bruez et al., 2013; Chacón-Vozmediano et al., 2021; Marchi, 2001; Serra et al., 2021), variation in esca susceptibility of a single grape variety across multiple viticultural regions has rarely been tracked in long-term epidemiological studies (Dewasme et al., 2022; Guérin-Dubrana et al., 2013) and never, to our knowledge, by combining epidemiological data not only with climatic conditions but also with soil characteristics and plant physiological data.

To determine the impact of soil, climate and biotic factors on esca expression, we reduced the effects of confounding factors by using a network of vineyards and studied for esca expression since planting in 2003. In this network, 19 plots of an esca susceptible cultivar (Gamaret) were selected, with all plants grafted with homogeneous scion and rootstock material in the same nursery and managed under uniform cultural practices. Such a network of vineyards can serve as a model to study the relationship between esca expression and soil and climatic conditions. Since climatic demand and soil characteristics are varying within this network, this creates optimal conditions to study the influence of pedoclimatic factors on the prevalence of esca. This experimental design allowed us i) to examine the variability of esca incidence in vineyards located in different geographical areas, this independently of plant age, cultivar and vineyard cultural practices; ii) to explore the relationship (independence or correlation) between esca expression, pedoclimatic factors and vine physiological factors in different vine-growing regions iii) to identify which factor(s) could be good candidate(s) to predict esca expression.

Material and Methods

Study site, esca epidemiology, and plant material

The 19 Gamaret plots studied are spread over a perimeter of about 3700 ha of cultivated vineyards in Western Switzerland (Fig. 1). The plots monitored are located in four wine-growing regions with plots located at the shore of the Lake of Geneva (9 plots), of the Lake of Neuchâtel (3 plots), in Chablais (3 plots), all at an altitude of 350 m., and in Valais (4 plots) located in a large alpine valley between 500 m and 700 m altitude.

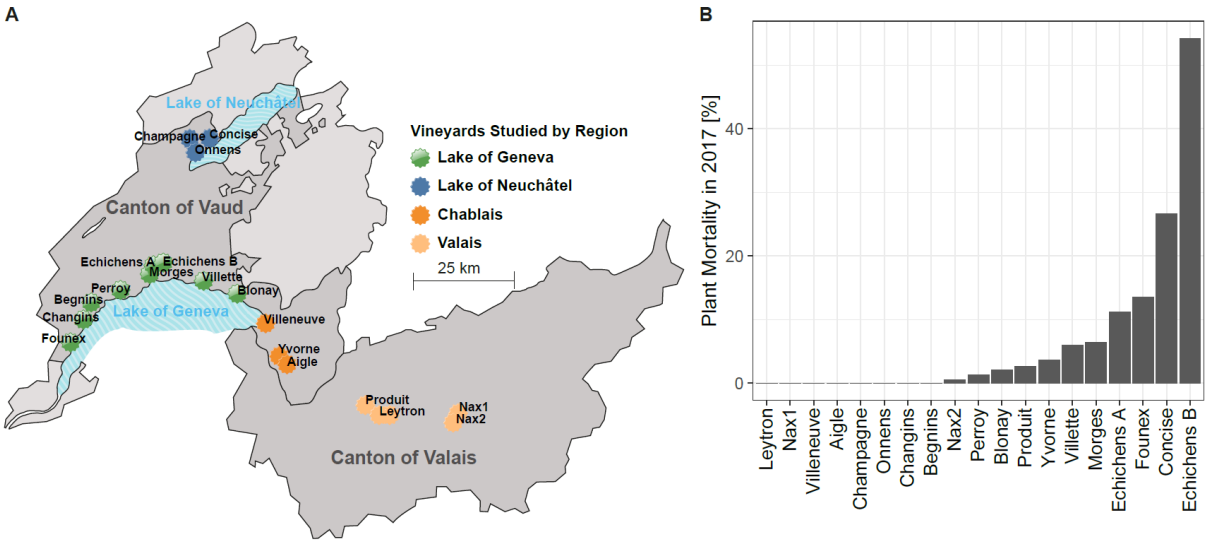

Figure 1 A Map of the Gamaret vineyards monitored across western Switzerland. **B** Cumulative replacement rate [% of plants] from vineyard plantation in 2003 through 2017 across the study plot network.

These plots were all planted in 2003 with *Vitis vinifera* L. cv Gamaret, a Swiss red variety developed from a cross between Gamay x Reichensteiner. This vine variety is well established in Switzerland with circa 500 ha (representing 3% of the Swiss vineyard area). Vine plants were all grafted onto 3309C rootstock (*V. riparia x V. rupestris*) with the same vegetal/genetic material coming from the same nursery stock. All vines were trained in the single Guyot system (vertical shoot-positioned foliage) with an average planting density of 7500 ± 500 vines ha^{-1}. The soil management included a natural grassing between the rows for all the plots implanted in Vaud (15 plots, Fig. 1. A). On the Valais network (4 plots), chemical weed control was carried out.

The vineyard plots (Fig. 1B) were monitored for GTD leaf symptoms and mortality since their planting (2003) until the beginning of this study (2018). Foliar symptoms (Fig. 2B) in the studied vineyard plots were typical of esca. Cumulated plant mortality data gathered before the experiment (2003-2017) appeared highly variable (Fig. 1B), ranging from 0% to 48 % depending

on the vineyard plots. Consequently, Gamaret cultivar seemed to be a good candidate to study the influence of abiotic factors on the incidence of esca.

For four consecutive experimental years (2018-2021), when esca symptoms were most visible in late August or early September, about 300 vines in each of the studied vineyard plots were examined for the presence of esca symptoms (5700 plants on the whole network). For the epidemiological monitoring, we identified five categories of symptoms (Fig. 2): apparently healthy plants; foliar symptoms taking in account incidence and severity weak foliar symptoms: one symptomatic shoot, medium foliar symptoms: more than a single symptomatic shoot, strong foliar symptoms: whole plant shoots symptomatic), apoplectic symptoms; replacement of the plant after apoplexy. The proportion of affected plants per plot, from year to year, was inferred based on the number of plants originally planted in 2003 and still present in 2021.

Figure 2 Epidemiological data recorded according to visual symptoms **A**. Asymptomatic leaves; **B**. Leaves expressing esca symptoms; **C**. Apoplectic plant.

Climatic data

Meteorological data were collected from the Data portal for teaching and research (IDAweb; https://www.meteoswiss.admin.ch/services-and-publications/service/weather-and-climate-products/data-portal-for-teaching-and-research.html) of the Federal Office of Meteorology and Climatology MeteoSwiss. Climatic data were collected from meteorologic stations for locations representative of each of the four wine-growing regions under study (Fig. 1.A): Lake of Geneva (Pully), Lake of Neuchâtel (Method), Chablais (Aigle) and Valais (Sion). We collected weather data for the four years of the experiment (2018-2021) as well as for previous years (1990-2017) to derive a climate norm for comparison. Meteorological norms (min, max, quartiles and median) were then computed for these four stations.

In order to increase the accuracy of meteorological data, meteorological stations from Agrometeo (http://www.agrometeo.ch) were chosen according to their proximity to the network plots (Table SM 1). This network consists in automatic meteorological stations. Those stations

were thus not suitable for the computations of climatic norms but were used for the measurements during the experiment (2018-2021). We gathered from Agrometeo precipitations, temperatures, and evapotranspiration measurements for 11 stations. Secondary variables were computed from the raw data. From the precipitation data we created nine secondary variables (Table SM 2): monthly precipitation; number of days per month with rainfall; number of days per month with >= 0.1mm, >0.3mm, >10mm, >100mm; absolute deviation from monthly median sum; deviation of the number of days per month with precipitation from the monthly average; number of days per month superior and inferior to the median. From the temperature data we created four secondary variables: number of days per month above and below the median; number of days per month with >25°C; number of unusually hot days (maximum temperature ≥ 30 °C) per month, deviation from average (1991-2020 norm). We consider only meteorological data from April to September, period most likely to influence the incidence of esca foliar symptoms and plant mortality.

Soil types and soil water holding capacity (SWHC)

Around 80% of the vineyard plots studied are alpine moraines. Moraines can be classified into three types (Letessier & Fermond, 2004): bottom moraines with few stones (< 30% coarse elements), stony moraines (30-60% coarse elements), and gravely moraines (> 60% large elements and stones).

For most of the plots (15), a hole of 1.5 m deep x 1 m wide was dug between two rows of vines. For four plots, the hole was dug to a depth of 2 m because the roots were deeper and bedrock not reached. The SWHC was calculated according to Letessier et Fermond (2004). SWHC was assessed through a combination of direct and indirect estimation methods. Indirectly, the influence of soil texture classes, including sand, silt, and clay, on water holding capacity was considered. This approach recognises the general characteristics associated with each soil texture: sandy soils, characterized by larger particles, tend to drain quickly and hold less water; silt soils, with medium-sized particles, exhibit moderate water retention; and clay soils, composed of smaller particles, can retain more water, although potentially draining less efficiently. The texture of each soil was estimated by a pedologist on each site (Letessier et Fermond 2004). Additionally, the SWHC is also conditional to the organic matter content. The presence of organic matter acts as a water-absorbing sponge, thereby enhancing the soil's capacity to retain water. Consequently, soils with elevated organic matter content generally demonstrated a higher water holding capacity. This index was also estimated by Letessier et Fermond (2004). A cultural coefficient was applied for each of the soil textures (Baize & Jabiol, 2011).

Physiological and agronomical monitoring of the vineyard plots

To determine the vine water status, two approaches were used: the measurement of predawn leaf water potential (Ψ_{PD}) and analysis of the carbon isotope composition *($\delta^{13}C$)* in berries at harvest. Predawn leaf water potentials (Ψ_{PD}) were measured using a pressure chamber (Scholander et al., 1965) between 2 and 5 a.m., in complete darkness, on eight mature, undamaged, and non-senescent leaves centrally placed in the foliage on each location when evapotranspiration was at the minimum. The mean values of eight leaves per plot were used as predawn water potential variable. The level of water stress of each plot was assigned according to Van Leeuwen et al. (2009). Predawn water potentials were measured at the veraison (BBCH 83-85) stage once a year in August. The stable carbon isotope ($\delta^{13}C$) composition of the must sugars (200 berries sample by plot) was determined at harvest at the Stable Isotopes Laboratory of the University of Lausanne by elemental analysis-isotope ratio mass spectrometry (EA-IRMS) using a Carlo Erba 1108 elemental analyzer connected to a Thermo Fischer Scientific (Bremen, Germany) DeltaV mass spectrometer. The stable isotope composition was reported as $\delta^{13}C$ values per mille (‰), with deviations of the isotope ratio relative to known standards as follows: $\delta = [(R_{sample} - R_{standard})/R_{standard}] \times 1000$, where R is the ratio of heavy to light isotopes ($^{13}C/^{12}C$). The $R_{standard}$ value for $\delta^{13}C$ in Vienna Pee Dee Belemnite limestone is 0.0112372 (Deléens et al., 1994).

At veraison, a foliar analysis was performed to determine the levels of leaf nitrogen, potassium, phosphorous, calcium and magnesium. The samples consisted of 30 leaves gathered in the cluster zone. Leaves with petioles were washed, over-dried, ground and analyzed by Sol Conseils (Gland, Switzerland). Leaf chlorophyll index was measured using an N-tester device (Yara, Nanterre, France) on adult leaves situated at the middle of shoots. In early winter, the total weight of the pruned vine shoots was recorded (30 shoots per vineyard). The second or last shoot of the fruiting cane was chosen for the measurement. Thirty shoots per plot (one per plant) were sampled and cut to a length of 1.0 meter and individual shoot weights determined (g/linear meter).

At harvest, 200 berries per vineyard were randomly selected and weighted. After weighing the berries, the juices extracted from the individual berries were analyzed by the Oenology Laboratory of Agroscope to establish their sugar, pH, malic and tartaric acid contents, and their assimilable nitrogen content Using WinScan® infrared spectroscopy (FOSS NIRSystems, USA).

Statistical analyses

We used principal component analyses (PCA) to see if meteorological and physiological parameters could have an influence on annual esca incidence and test if these variables could be used to predict disease incidence. These analyses also feature collinearity between meteorological and physiological variables. Three variables reporting for esca incidence were created: the number of apoplectic plants over the four years of the experiment, the sum of all types of foliar symptoms, and the overall symptoms.

We then used two distinct methods to assess the strength of the relationships between our variables. We first have considered Pearson's correlation coefficient and a recently developed correlation-coefficient index ξ —correlation coefficient (Chatterjee, 2019) that considers any kind of functional relationship. In particular, it has the property of being equal to zero if and only if there is independence. It has been implemented in R in the package xicor (Holmes & Chatterjee, 2021). To screen the explanatory variables that might influence the esca incidence, we fixed some threshold and considered the variables whose correlation with the variables of incidence was higher (in absolute value for the correlation).

Results

Esca Epidemiological data across the four years experiment

Looking at the percentage of plant apoplexy and foliar symptoms in the different regions from 2018 to 2021 (Fig. 3) the plots located on the shores of Lake of Geneva, and to a lesser extent the plots located on the shores of Lake of Neuchâtel, expressed more esca symptoms of both types than the other two regions studied, consistently over the years under study.

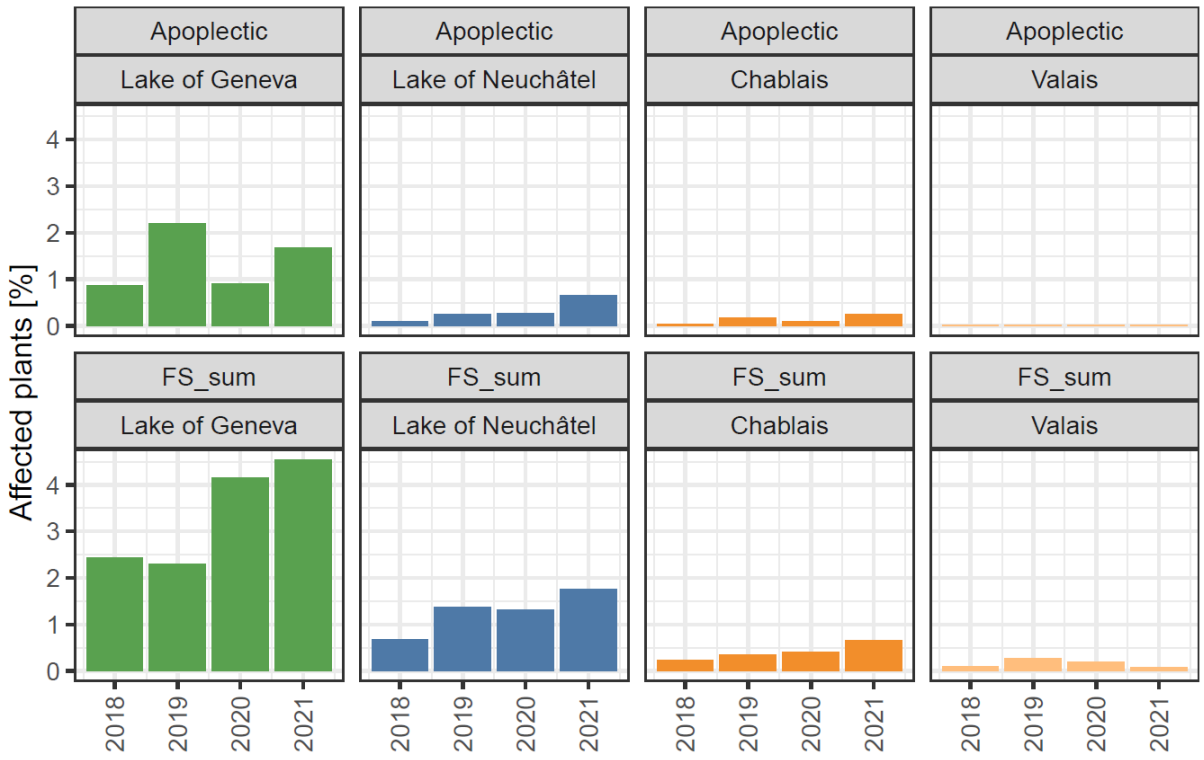

Figure 3 Sum of plants [%] affected by apoplectic symptoms and by sum of foliar symptoms (FS_sum) for the four monitored vintages (2018-2021) by wine-growing region.

In Valais, annual symptoms (apoplexy and foliar symptoms) were close to zero, while in Chablais they were slightly higher but always inferior to 1%. However, apoplexy and foliar symptoms showed a different evolution during the four years of experimentation. The years 2019 and 2021 were the worst for apoplexy in three of the regions (Lake of Geneva, Lake of Neuchâtel and Chablais; Fig. 3), as were the leaf symptoms of esca, but only in Lake of Neuchâtel. In 2020 and 2021, more than 4% of the vines in the Lake of Geneva plots expressed foliar symptoms, compared to just over 2% in 2018 and 2019. Foliar symptoms were generally slightly increasing from year to year in all regions except Valais. Of the four regions studied, Lake of Geneva is clearly the most susceptible to esca, while Valais and Chablais seem much less susceptible.

A closer look at the epidemiological data taking into account individual plots instead of wine-growing regions, indicates that esca also varies greatly between plots in each region (Fig. 4). At Lake of Geneva, Morges appears to be the worst for cumulative symptoms of the disease, ranging from 21% in 2018 and increasing over the years to 56% in 2021. Esca has also higher incidence in Begnins (17-40%), Echichens A (18-27%), and Villette, ranging from 7-18%, than in the other plots of the region considered. However, the incidence of cumulative symptoms did not exceed 10% in Blonay during the four-year experiment. In the Lake of Neuchâtel region, the

cumulative incidence of esca also varied. While Onnens expressed the lowest percentage of esca symptoms over the years 2018-2020 (4-11%), Champagne showed slightly more symptoms over the same period (8-12%) and Concise was the most affected plot (10-25%). However, in 2021, the situation was reversed, with Onnens and Champagne both expressing more than 20% of cumulative esca symptoms while Concise expressed less than half (10%) of symptoms than the other two plots. In Chablais, the plot expressing the highest rate of cumulative symptoms of the disease from 2018 to 2021 was Yvorne (with a peak of 14% in 2018), while the plots in Villeneuve and Aigle expressed 1-2% of symptoms in the first two years and a little more thereafter but still less than 5% except in Villeneuve in 2021 with 9% of affected plants. In Valais, Produit was the plot with the most symptoms, but less than 7%, except for the plot in Nax 2 (5% vs. 4% in Produit) in 2020. In Leytron and Nax 1, the incidence of esca was almost zero in all four years. Overall, these results indicate that esca symptoms incidence varies between years and between plots whatever the region considered.

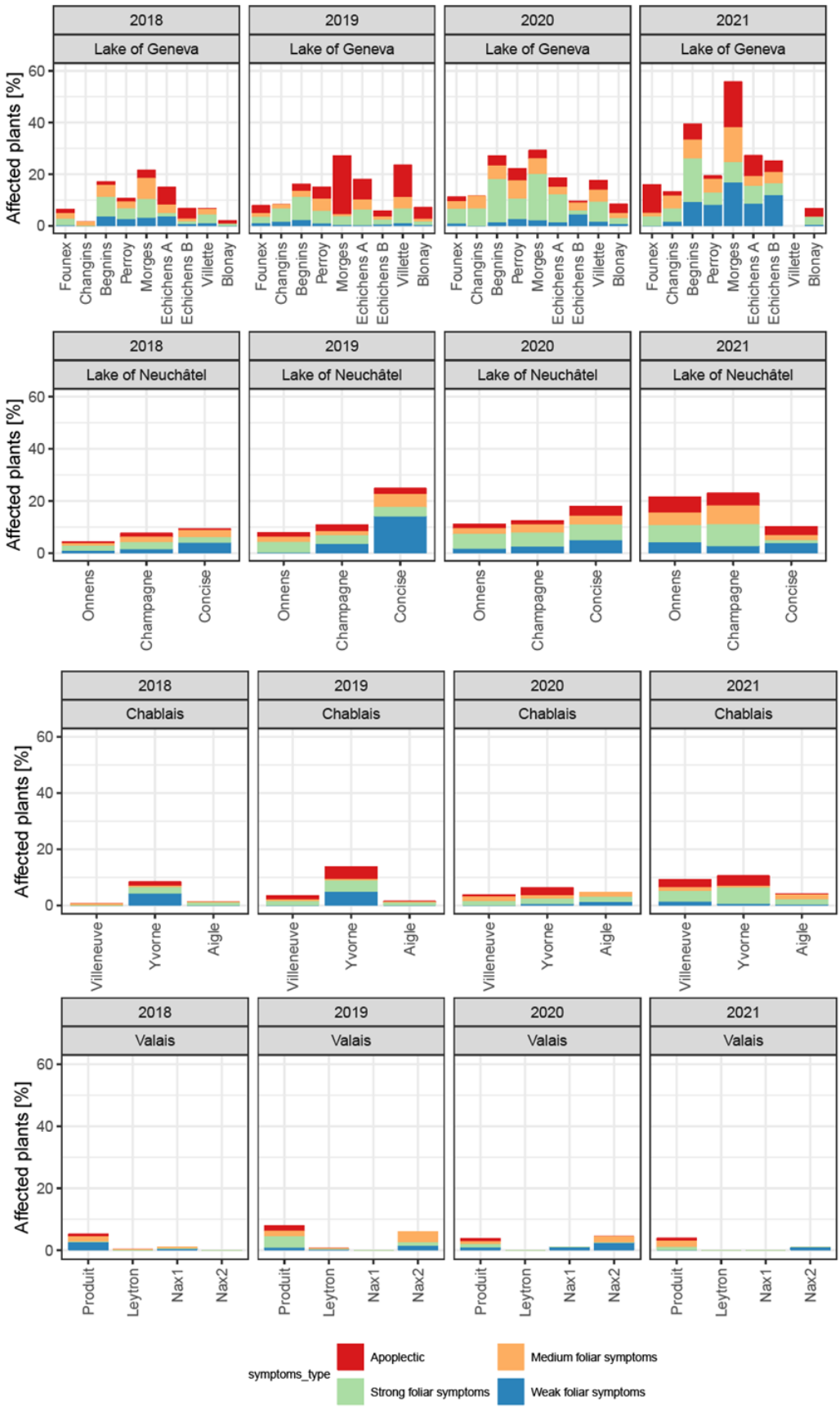

Figure 4 Annual rate of esca symptomatic plants by vineyard plots and by region for weak, medium, strong foliar symptoms and apoplectic plants (2018-2020)

Climatic characterization of the survey period

The four-year survey period was marked by unusual precipitation regimes (Fig. 5). While 2018 was drier than the long-term norm, except in Lake of Neuchâtel region from May to July (Fig. 1A), 2019 and 2020 were characterized by an alternance of drier or wetter months than the long-term norm during the six months of grapevine vegetative period considered.

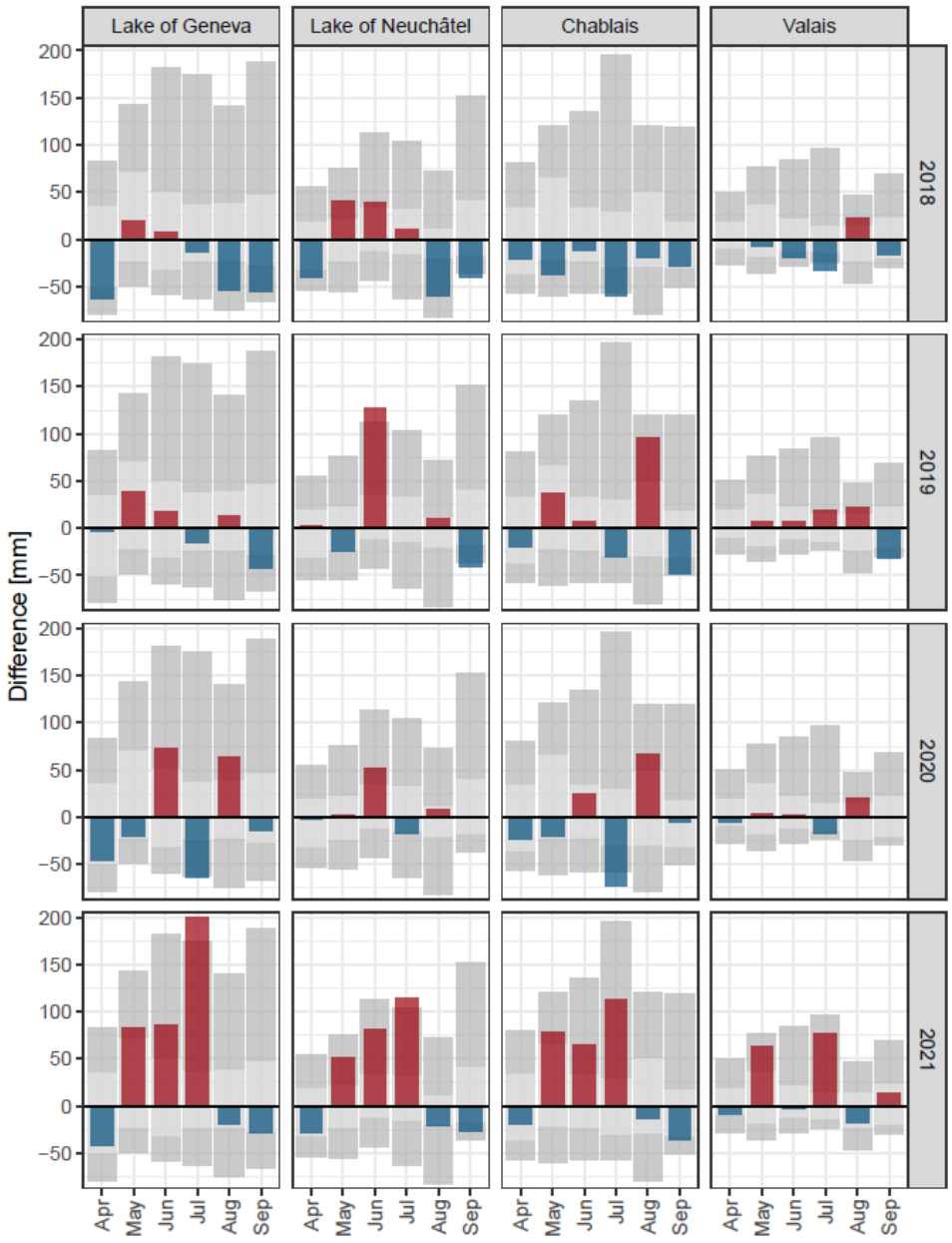

Figure 5 Positive (red) or negative (blue) differences to the long-term median (1990-2017) in monthly spring and summer precipitations (mm) in four wine-growing regions of Western Switzerland from 2018 to 2021. The background rectangles are the minimum and maxim um (light grey) and the 25% and 75% percentile (dark grey) of the same data over the period 1990-2017.

Year 2021 encompassed an overall very wet period from May to July, preceded and followed by months drier than the long-term norm, this except for Valais where June was very close to the norm while very rainy in all the other regions. Precipitation differences to the long-term norms were observed in all four wine-growing regions (Fig. 5), however more pronounced in Lake of Geneva, Lake of Neuchâtel and Chablais than in Valais, where except in 2021 in May and July, the precipitation regime remained closer to the norm than in the three other regions.

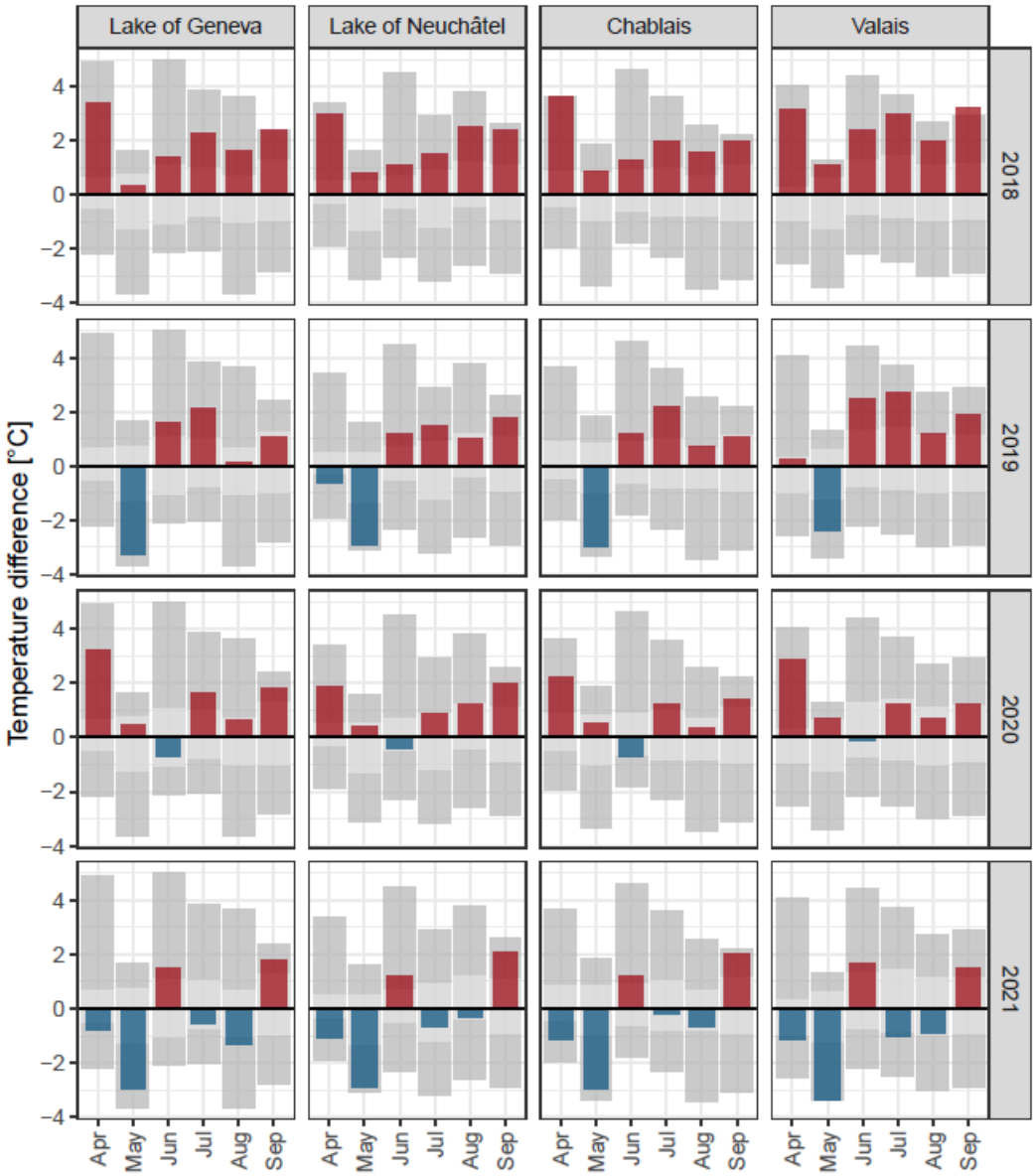

Figure 6 Positive (red) or negative (blue) differences to the long-term median (1990-2017) in monthly spring and summer temperatures (oC) in four wine-growing regions of Western Switzerland from 2018 to 2021. The background rectangles are the minimum and maximum (light grey) and the 25% and 75% percentile (dark grey) of the same data over the period 1990-2017.

Temperatures (Fig. 6) were overall higher than the long-term norm during the first three years of the survey period (2018-2020) in the four wine-growing regions, except for May in 2019, which was particularly cold everywhere, and June which was a bit colder than usually in all regions except in Valais. Year 2021 was very peculiar regarding temperatures and highly similar in the four wine-growing regions. This year was characterized by an alternance of cold and hot months in all regions. While April and particularly May were colder than the long-term temperature norm, June appeared hotter than the norm, July and August cooler and September hotter. The month of May appeared the most singular month in 2019 and 2021, with very cold temperature compared to the long-term norm in all four regions. Also, during the four years of the survey period, temperatures were more homogenous across regions (Fig. 6) than the precipitations (Fig. 5) and Valais appeared not different than the other three wine-growing regions regarding temperature fluctuations.

Soil types and soil water holding capacity (SWHC)

The sites studied were roughly grouped by category of SWHC (Table 1).

Table 1 Experimental sites in four wine-growing regions (Switzerland), with their soil type managements (% soil cover cropping) and their soil water holding capacity (SWHC).

Sites	**Region**	**Soil types**	**% Cover cropping**	**SWHC** (mm)	**SWHC category**
Founex	Lake of Geneva	Gravelly moraines	70	155	Medium
Changins	Lake of Geneva	Bottom moraines	70	110	Medium
Begnins	Lake of Geneva	Bottom moraines	70	130	Medium
Perroy	Lake of Geneva	Bottom moraines	70	155	High
Morges	Lake of Geneva	Bottom moraines	70	240	High
Echichens A	Lake of Geneva	Bottom moraines	70	230	High
Echichens B	Lake of Geneva	Bottom moraines	70	230	High
Villette	Lake of Geneva	Marly sandstones	70	250	High
Blonay	Lake of Geneva	Marly sandstones	70	200	High
Onnens	Lake of Neuchâtel	Jurassic sandy stones	70	120	Medium
Champagne	Lake of Neuchâtel	Jurassic sandy stones	60	95	Low
Concise	Lake of Neuchâtel	Jurassic sandy stones	70	180	High
Villeneuve	Chablais	Gravelly moraines	70	90	Low
Yvorne	Chablais	Gravelly moraines	50	100	Low
Aigle	Chablais	Gravelly moraines	50	80	Low
Produit	Valais	Clay schists	0	160	High
Leytron	Valais	Stony moraines	0	100	Low
Nax1	Valais	Stony moraines	0	90	Low
Nax2	Valais	Colluvial/stony moraines	0	140	Medium

The SWHC was calculated on each plot by taking into account the amount of stones, texture, root colonization and rooting depth (Letessier & Fermond, 2004). The SWHC corresponds to the maximum amount of water in a soil that the vine can extract (Baize & Jabiol, 2011). A quarter of the plots have a SWHC ≤ 100 mm (low), another quarter of the plots have a SWHC between 100 and 150 mm (medium), and half of the plots a SWHC > 150 mm (high).

Physiological and agronomical monitoring of the vineyard plot

Comparing the different record year, none of the plant physiological indices (Ψ_{PD}, $\delta^{13}C$, assimilable nitrogen, chlorophyll content of the leaves, weight of the pruning and of the berries) remained stable across the four years experiment (Fig. 7). Apart from water potential (Ψ_{PD}), which increased in 2020 for plots with an average SWHC, remained stable for plots with a high SWHC and decreased for plots with a low SWHC (Fig. 7A), all the other abiotic factors (Fig. 7B-F) followed the same pattern depending on the vintage and independently of the SWHC. Grapevine plants grown on soils having a low SWHC exhibit lower pre-dawn water potential and pruning weight but a higher rate of assimilable nitrogen and chlorophyll than vineyards planted in soils with medium and high SWHC. For these latter plots, assimilable nitrogen in harvested berries (Fig. 7C) was generally low in 2018 and 2021 (with average values between 60 and 80 mg N/litre), tendency less pronounced for plots with low SWHC, and reflecting a marked nitrogen deficiency in grapes during the first and last year of the experiment. Pruning weight (Fig. 7E) was particularly low in 2020 but only for plots having a low SWHC. Berries weight was high in 2019, this independently of SWHC (Fig. 7F). Consequently, the abiotic factors associated with the vineyard plots seem to vary more from one vintage to another than according to the SWHC category.

By grouping the plots by region (Fig. SM1), the physiological variables seem to be more influenced by the regional climates, particularly for the predawn leaf water potential (Fig. S1A), and the nitrogen content (Fig. S1C-D). The Valais plots are atypical according to these indicators, highlighting a regional particularism. The Chablais appears, as the Valais, characterized by a high predawn leaf water potential and a high nitrogen content.

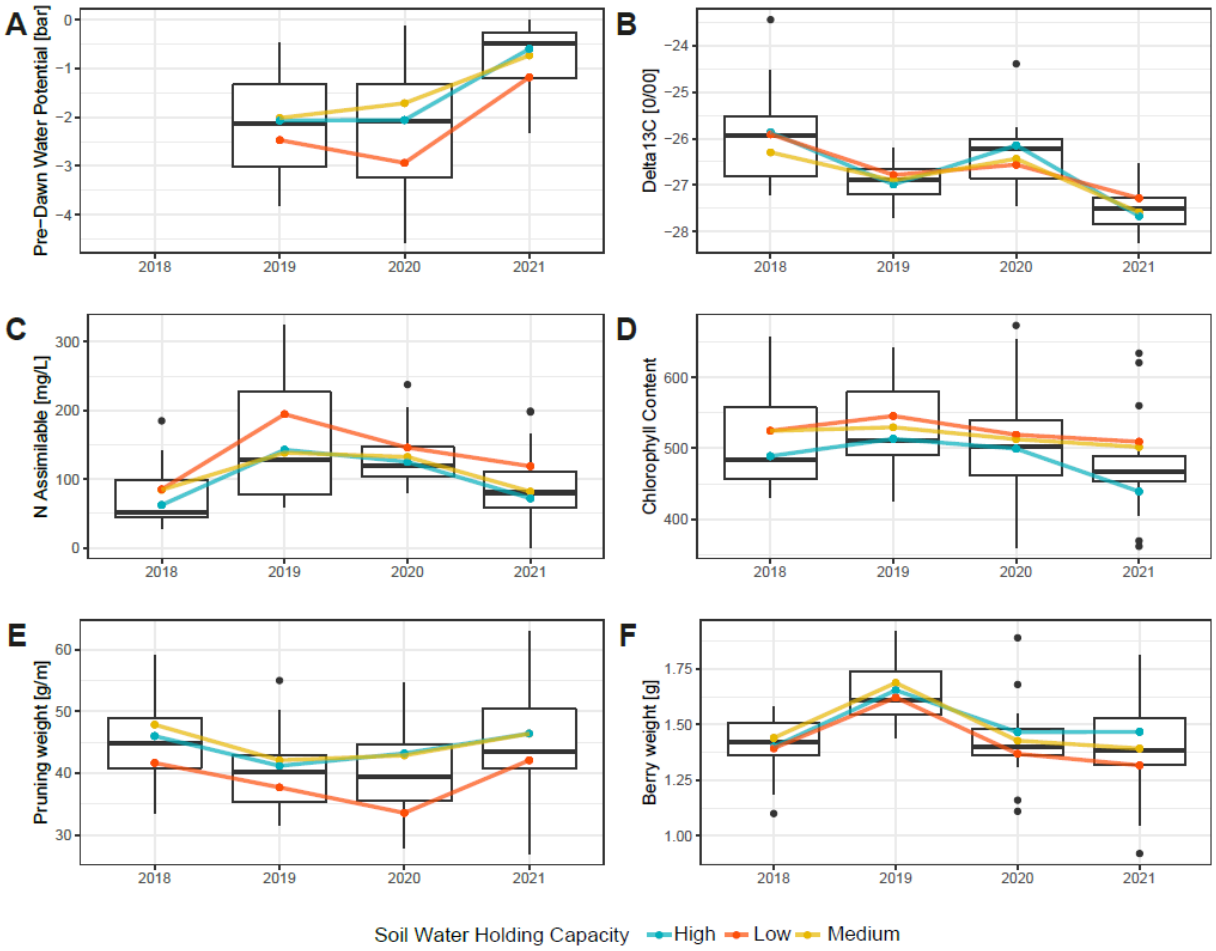

Figure 7 Mean values of measured physiological indexes of plots categorized by SWHC (<100 low [orange], >100-150 medium [yellow], > 150 high [blue]) across four consecutive years (2018-2021) **A** Pre-dawn leaf water potential (ΨPD), **B** δ13C, **C** Assimilable nitrogen, **D** Chlorophyll content index (N-tester), **E** Pruning weight, **F** Berries weight.

Examining abiotic variables alongside disease incidence

Collinearity between measured abiotic parameters and incidence of the disease with main pattern of variation (Fig. 8) were summarized with a principal component analysis (PCA). The first PCA axis explains between 43.8% and 54.8% of the variance depending on vintages and is mainly driven by nitrogen indices, esca incidence indices, cover cropping and SWHC. The second PCA axis explains between 12.9 to 24.9% of the variance and is mainly driven by wood and berries weight and, however to a lower rate, by water stress indices (Delta13C and Base_pot) depending on the vintages.

According to PCA results, SWHC is correlated with the annual incidence esca (arrows point in the same direction). SWHC arrow is closer to plant mortality in 2019 and 2021 and with the sum of foliar symptoms in 2018 and 2020. The variables corresponding to cover cropping, berry weight, and wood weight also point, more than the other abiotic factors, in the same direction as

the esca incidence indices. This is more pronounced in 2019 and 2021. Plots located in Valais (Leytron, Nax1, Nax2 and Produit) are clearly discriminated by PCA for the four observed years and are more correlated with nitrogen and water stress indices. These indices were well correlated, especially in 2019 and 2021. These plots are also the least affected by esca (according to annual symptom incidence; Fig. 3). Plots most affected by esca are usually characterized by high SWHC and cover cropping indices. Vigor indices (wood and berry weight) tend to be negatively correlated with high nitrogen indices (more strongly detected in 2019, 2020 and 2021). Cover cropping is always negatively correlated with nitrogen indices.

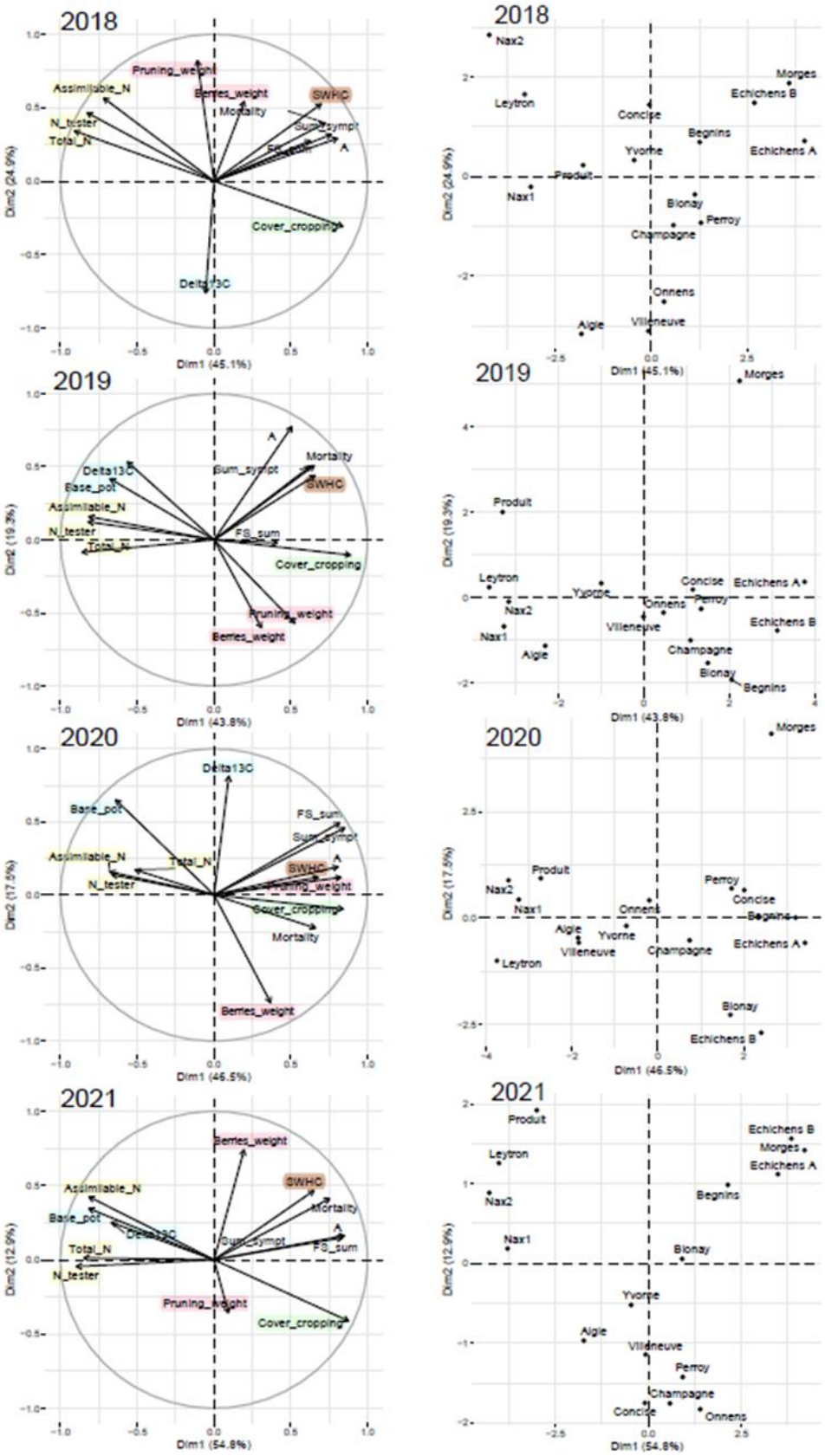

Figure 8 Principal Component Analysis (biplot PCA) based on abiotic and epidemiological variables by vintages 2018-2021 (left) taking in account the 19 plots (right). Color code account for nitrogen content (yellow), vigor (pink), water stress index (blue), cover cropping (green), soil water holding capacity (brown) and epidemiological esca variables (no color) with apoplexy (A), sum of foliar symptoms (FS_sum), all symptoms (A+FS_sum) and mortality.

As SWHC appeared well correlated with plant mortality, the relationship between the total mortality rate since the vines were planted (2003) and the SWHC was tested for the 19 studied vineyard plots. Linear regression (Fig. 9) inferred a positive correlation (R^2 = 0.6 [P <0.001]) between plant mortality and SWHC. However, while such a correlation is clearly inferred for most of the plots (13 out of 19), SWHC does not explain the plant mortality for six plots, especially for Echichens B, Concise and Villette. However, for the latter, this is probably due to the absence of epidemiological data in 2021, as this vineyard was uprooted in 2020. Nevertheless, the correlation between plant mortality and SWHC suggests that soil water storage capacity is one of the main factors explaining the incidence of esca mortality for the Gamaret cultivar.

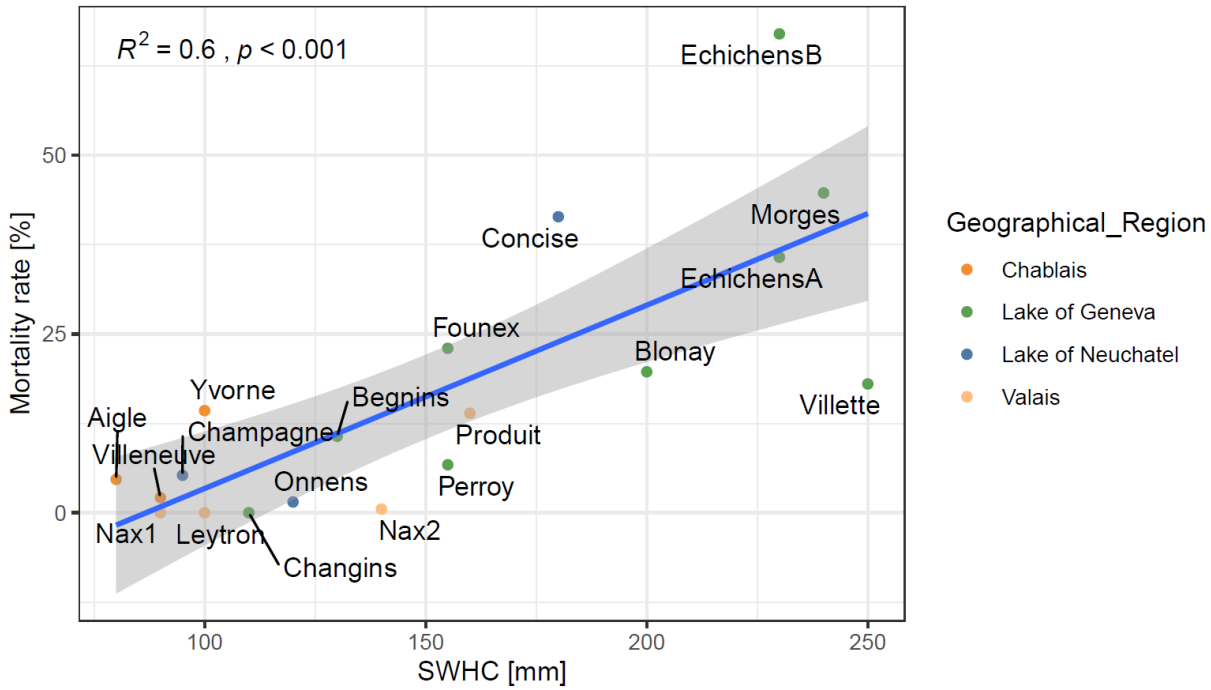

Figure 9 Linear regression between total mortality rate by vineyard plot (from 2003-2021) and soil water holding capacity (SWHC) index for each plot based on Pearson's correlation coefficient.

According to our correlation test among 70 variables (Table SM 2) with our esca incidence indexes, the variables most correlated are variables accounting for monthly precipitation regimes in May and June, SWHC, cover cropping and chlorophyll content for Pearson's correlation coefficient (PCC) (Fig. 10). The highest PCC obtained (0.58) describe a positive relation between the sum of symptoms and the rainfall in June (Fig. 10A). PCC also underscore a positive correlation for the precipitation regime observed in June and May between the amount of rainfall and the number of days with rain >10mm in May and June and the difference of precipitation compared to the norm (1991-2017) in June and our esca incidence indices (Fig. 10A). SWHC is also positively correlated with all the esca incidence indices but more strongly with apoplexy. Chlorophyll content was negatively correlated with our esca incidence. This negative relationship between nitrogen and esca incidence was also underscored by the PCA (Fig. 8). Variable most linked with esca incidence indices according to ξ-correlation (XCC) are the rainfall in June and to a lesser extent in July with only apoplexy. Cover cropping and SWHC are also retained as not independent from our esca incidence indices (Fig. 10B).

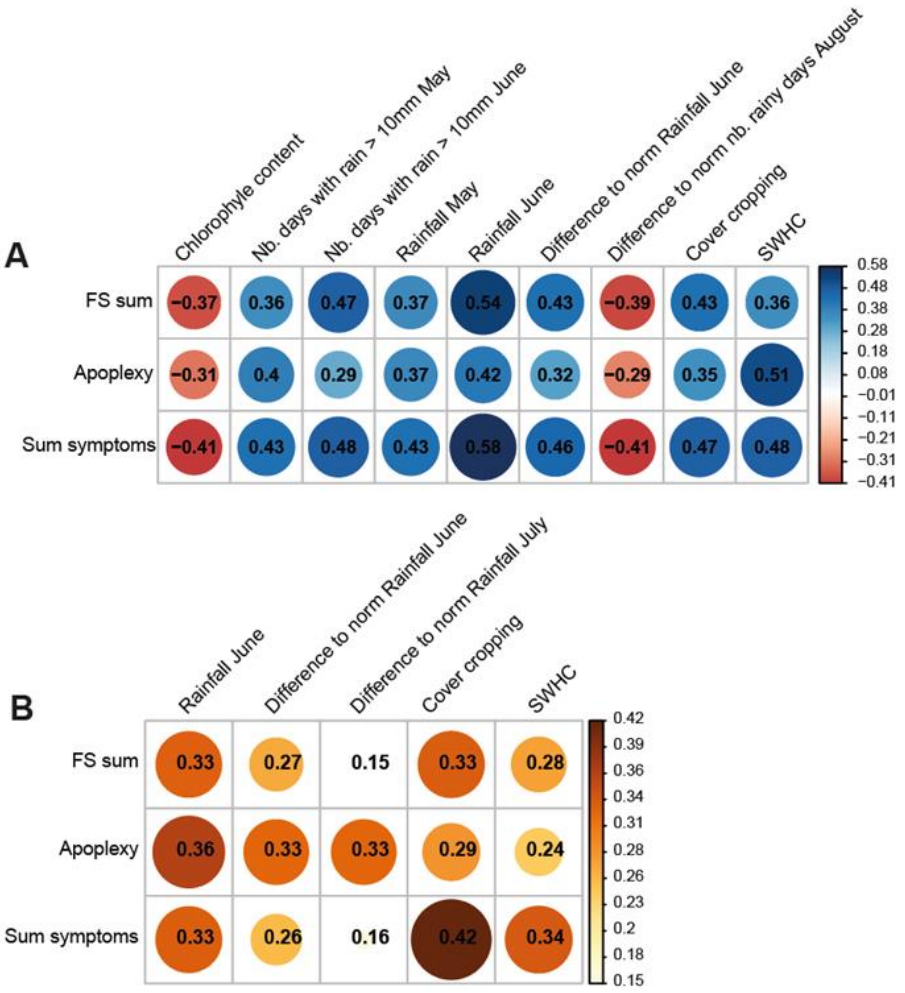

Figure 10 Correlation coefficient matrix with variables most correlated with the esca incidence indices: A (plant with apoplexy), FS_sum (plant with foliar symptoms), Sum_sympt (all plants affected by esca; A+FS_sum). Size of the dot reflects the strength of the correlation. **A** Variables with the highest correlation rate with the esca incidence indices according to the Pearson correlation coefficient (red corresponds to a negative relationship and blue to a positive relationship). **B** Variables most correlated with the esca incidence indices according to Xicor correlation coefficient.

Discussion

In monitoring prior to this study (2003-2017), Gamaret showed a large variability in susceptibility to esca between plots in the study network. Such a wide range of susceptibility was rarely observed for other grape varieties and already suggested an impact of soil-climate factors on disease expression. By relating the incidence of esca to regional climatic conditions, our results show that in a region like Valais, with a drier climate and with June and July hotter compared to other wine-growing regions in Western Switzerland, the incidence of the disease is almost null. Also, the year 2018 was characterized by an abnormally dry and hot vegetation period compared to the other three years. This vintage was also the one in which esca symptom expression was the lowest in the four viticultural regions. Grapevines are often grown at the limit of water stress, providing less water than the plant can transpire (Castellarin et al., 2007; Chaves et al., 2010). This irrigation strategy aims to improve grape quality and reduce water consumption but was reported to increase the sensitivity of plants to heat waves (Edwards et al. 2011). The present study suggests that heat and water stress may not be as deleterious to viticulture as generally assumed (Fernandez et al., 2023; Fischer & Peighami-Ashnaei, 2019; Songy et al., 2019). Our results agree with those of Bortolami et al. (2021b) who showed that water-stressed grapevines do not express esca symptoms, while watered plants express them. Our results run counter to the idea that heat combined with drought exacerbates plant damage (Pandey et al., 2015). As Bortolami et al. (2021) pointed out, the susceptibility of a given grape cultivar to esca does not seem to be altered in the long term by drought, an assumption verified in this study for Valais. Gamaret shows a susceptibility to the disease that is primarily regional, but that varies from one vintage to another.

Over the four years that these vineyards were monitored, temperatures in each region for a given vintage were comparable, with the same monthly deviations from the long-term norm. The differences in esca incidence observed between wine-growing regions thus appear to be due to annual precipitation more than to temperature patterns. Previous studies mentioned that cool, rainy summers favored the expression of esca foliar symptoms, whereas hot and dry summers favored apoplexy (Surico et al., 2000; Marchi et al. 2006). We did not observe this trend. The year 2018 was particularly hot and dry during the summer and was the year with the lowest number of apoplectic events and foliar symptom rate. The year 2021 does not fit this trend either, as it was the year with the most rainfall and the highest number of foliar symptoms and apoplectic events in three of the four viticultural regions monitored. Apoplectic events were most numerous in 2019 and 2021 in three of the wine regions. Both years were characterized by a particularly rainy and cold May, followed by a hot (2019) or rainy and hot (2021) summer. These

observations are rather in agreement with Calzarano et al. (2018) who showed that temperature and precipitation from May to July, or even only in July (in Italy), are key factors for the expression of esca. In our four years of monitoring, the month that has an impact on plant apoplexy is May, if it is cold and rainy, followed by a hot summer (2019) or alternating periods during which temperatures vary when the summer is particularly rainy (2021).

The Gamaret plants studied here were grafted onto rootstock 3309C reported to have low drought tolerance (Verslype et al., 2023). According to our results, this cultivar appears to be less sensitive to drought than expected, particularly in 2018, the driest year of the monitoring, and more generally in the Valais region. Kondouras et al. (2006) reported that rootstock 3309C may allow scions to maintain higher stomatal conductance and water uptake under water deficit conditions. In addition, de Souza et al. (2022) observed that plants grafted to this rootstock had the slowest increase in water deficit throughout the growing season which is thought to be due its larger and deeper root system. In the *Catalogue des vignes cultivées en France* (http://plantgrape.plantnet-project.org), rootstock 3309C is described as particularly sensitive to water stress when it occurs suddenly during the growing season and as having a poor adaptation to excess water. This could also explain why apoplexy cases were more frequent in 2019 and 2021, years characterized by a sudden change in climatic conditions (between May and June in 2019) or by a particularly rainy summer accompanied by alternating cool and hot periods (2021). These observations agree with previous studies (Surico et al., 2000) that suggested that apoplexy was related to alternating dry, hot periods and wet, cool periods, conditions leading to high leaf and canopy production and high evapotranspiration that could lead to disruption of sap flow due to gas embolism. Bortolami et al. (2021) found that hydraulic failure and occlusion of vessels by tyloses and gels induced at a distance by esca are not mutually exclusive and that occlusions lead to hydraulic misfunction in the veins of esca symptomatic leaves.

The variability of esca expression observed between wine-growing regions was also observed between plots within the same wine-growing region. For example, the cumulative expression of esca symptoms in the Lake Geneva region varied from 21-56% in Morges during the four years of monitoring, whereas in Blonay it was less than 10%. This intra-regional variability was also observed in the Chablais region, with the plot in Yvorne showing more symptoms than the plots in Aigle or Villeneuve during the four years of monitoring. In Valais, the plot in Produit expressed more esca symptoms than the other two plots in this region during the four years of monitoring. The fact that the expression of esca is variable between plots in the same wine-growing region, and thus subject to similar climatic conditions, suggests that rainfall and temperature only partially explain the incidence of the disease. These results confirm the idea,

put forward by several authors (Marchi et al., 2006; Mugnai et al., 1999; Surico et al., 2000), that other abiotic factors than climate are involved in esca expression.

The abiotic factor, apart from climate, most often suggested to have an impact on esca expression is the soil water holding capacity (Calvo-Garrido et al., 2021; Calzarano et al., 2018; Graniti et al., 2000; Lecomte et al., 2009; Sosnowski et al., 2011; Van Niekerk et al., 2011). Grouping the plots into three categories based on their SWHC (Table 1) and testing its relationship with some physiological and agronomic factors, results show that abiotic factors vary more with vintage than according to plot SWHC category. Although water stress was moderate during the four years of the experiment, the physiological behavior of Gamaret seems to be more sensitive to sudden changes in climatic conditions than to the accessibility of water, probably in relation with the root capacity of rootstock 3309C to access water deep in the soil during dry periods (de Souza et al., 2022). Soil management seems to play a role in the accumulation of nitrogen in the grapes. The Valais plots, the only ones in the network that are not weeded, have higher levels of available nitrogen in the grapes than the plots in the other three wine regions, regardless of the vintage. Cover cropping is mainly grass in these three wine regions. These results are consistent with Abad et al. (2021) who found that cover crops generally do not compete with grapevines for nitrogen, except for grasses. Also, foliar symptoms are less expressed in Valais than in the other wine regions considered in this study, which contradicts the hypothesis that high nitrogen levels increase the severity of foliar symptoms (Lemmens et al., 2004). It also contradicts the idea that dense cultures as vineyards (Gramaje et al., 2018), well supplied with nitrogen, may favor pathogen load (Liu et al., 2017). As suggested by Sun et al. (2020), while nitrogen appears to affect physical defenses and the production of antimicrobial phytoalexins, it also appears to stimulate the production of enzymes and proteins related to systemic resistance. These authors also suggest that, although nitrogen is known to play an important role in plant defense, its impact on pathogen virulence and plant resistance remains understudied.

To determine which abiotic factors could explain the incidence and severity of esca symptoms, the potential correlation of these factors with the epidemiological data was tested. PCA results suggest that esca symptoms are, among all tested factors and across all vintages, most correlated with SWHC, especially variable accounting for plant mortality in 2019 and 2021. Water deficit related to soils with low SWHC has often been suggested as a source of stress for grapevines (Lanari et al., 2015; Lovisolo et al., 2010) and the impact of drought on esca expression often studied (Edwards, Pascoe, et al., 2007; Edwards, Salib, et al., 2007; Fischer & Kassemeyer, 2012; Luque et al., 2009; Ramegowda & Senthil-Kumar, 2015; Surico et al., 2000, 2006). These studies

suggested that in the presence of esca pathogens, drought favors the development of disease symptoms. However, Bortolami et al. (2021b) found that water stress inhibits esca symptoms expression. This last hypothesis seems to be verified in our study, especially in Valais, which has drier summers than the other regions and where the expression of esca is the lowest. But it is also possible that Valais vineyards are less infected by esca fungal pathogens than the other wine regions considered. A study of the fungal community associated with the studied vineyards will allow to answer that question.

The cost of plant replacement is high and is one of the major concerns of winemakers (Bertsch et al., 2013; Gramaje et al., 2018; Hofstetter et al., 2012). Having identified SWHC as one of the main factors related with plant mortality, we performed a linear regression analysis to establish the relationship between plant mortality rate per plot and SWHC. Regression analyses identified SWHC as clearly correlated with esca plant mortality. This trend is observed at regional and at the intraregional level. In Valais, the impact of SWHC on plant mortality appears much less pronounced than in the other wine-growing regions. SWHC is less impacting plant mortality when regional climatic conditions are hot and dry. At intraregional level, Produit, the only plot in Valais with a high SWHC has the highest mortality rate in this wine region. The same tendency is observed for Concise (Lake Neuchâtel) and for the three plots with an average SWHC compared to the other plots located at the edge of Lake Geneva, which have a high SWHC. The fact that the three plots of the Chablais have all a low SWHC may also explain the low rate of esca symptoms and mortality expressed in that region.

Although the correlation coefficient of the regression analysis remains relatively low to assess strong relationships, the influence of water availability on esca prevalence, a hypothesis already advanced by several studies (Andreini et al., 2014; Marchi et al., 2006; Surico et al., 2000), is strongly suggested here to be the limiting factor for plant mortality. Regression analyses allowed us to sort out among 70 abiotic variables that water availability in June given by the amount of rainfall, the number of days where it rained more than 10 mm and the difference of precipitation compared to the average (1990-2017), were the factors the most correlated with esca incidence. The precipitation regime in June would likely be the one that, among the variables considered, influences the most the prevalence of esca for a given year. As precipitation regime in May and August were also retained for specific monitored years, we can consider that the precipitation regime during the spring and early summer where the vegetation growth is the greatest (Keller, 2020) will be determinant for esca prevalence. These results, confirm those of Calzarano et al. (2018) who also showed that climatic conditions from May to July, particularly July in Italy, are driving esca prevalence. Although the role of water is increasingly established, the variability in

the incidence of esca within a plot, with some plants being affected earlier than others, is not yet clear. The precise role of water availability or restriction in the expression of esca symptoms has yet to be clarified.

Fischer and Peighami-Ashnaei (2019) suggested that environmental factors were driving the prevalence of esca, primarily due to the time lag (often several years) between pathogenic fungal infection of esca and symptom expression (Di Marco & Osti, 2008). This hypothesis, already suggested by previous studies (Marchi et al., 2006; Mugnai et al., 1999; Surico et al., 2000) seems to be verified in our four-year vineyard network monitoring. Both climatic and soil factors (SWHC) seem to have a strong impact on disease expression. Climate seems to be responsible for the differential expression of leaf symptoms of esca and apoplexy, both at the regional and intra-regional level. Apoplexy also appears to be expressed more than foliar symptoms under specific climatic conditions, when cold and hot periods, deviating from the long-term norm, alternate between May and July. This study identified for the first time the water retention capacity of the soil as a factor limiting plant mortality, a result that remains to be confirmed by studies on other grape varieties susceptible to esca.

Although our four years of vineyard monitoring were characterized by particular climatic conditions compared to the long-term norm, esca symptoms and plant mortality remained low, except for some of the plots of the Lake of Geneva region, where cumulative symptoms reached up to 60% (Morges) and mortality up to 58% (EchichensB). This systemic approach allowed the identification of the combination of factors responsible for the expression of the different types of symptoms of esca in Gamaret. In terms of recommendations to winegrowers, an esca-susceptible cultivar such as Gamaret seems to be better adapted to soils with low water retention capacity and to dry and hot climatic conditions, with the rootstock 3309 providing the scions with a lower sensitivity to drought. Adherence to these recommendations will allow for at least partial control of esca for a susceptible grape variety such as Gamaret, and probably for other susceptible grape varieties, as no plant protection product has proven effective against this disease since the ban on sodium arsenite (Songy et al., 2019).

Climate change and temperature elevation are known to increase the number and severity of fungal infections (Fischer & Kassemeyer, 2012; Nnadi & Carter, 2021). Esca, and more generally all GTD, are considered emerging fungal diseases related to climate change and to human activity (Bertsch et al., 2009; Gramaje et al., 2018). Symptoms similar to those of esca have been observed and described since Antiquity (Mugnai et al., 1999), but this disease has only become a concern in recent decades. There are several reasons why a disease like esca may emerge (Garrett

et al., 2021). First, pathogen populations may have changed and/or new species introduced. Since the late nineteenth century, most grapevine plants are grafted with European scions and American rootstocks, the latter being resistant to phylloxera (Carton et al., 2007). Young plants are traded worldwide and are a source of newly introduced species everywhere vines are cultivated (Gramaje et al., 2018). For example, *Eutypa lata* (Pers.) Tul. & C. Tul., species responsible for Eutypa dieback, is suggested to have been introduced several times in North America by importation of infected material (Rolshausen et al., 2014; Travadon et al., 2012). Second, host populations may have change. New grapevine cultivars are constantly produced, most of them selected for their resistance to diseases like downy and powdery mildews, respectively *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni. and *Erysiphe necator* Schwein., but not to GTD. Third, cultural practices may have changed which is the case in viticulture with the introduction of mechanical pruning which has been reported to increase the number of wounds and being less respectful of sap flow than manual pruning (Gramaje et al., 2018; Lecomte et al., 2018). Pruning wounds are the main route for the entrance for GTD associated fungi (Rosace et al., 2023). Consequently, other reasons than climate change may explain esca emergence.

Also according to Garret et al. (2021), the arguments for attributing a significant role to climate change in disease emergence are the ubiquitous presence of the pathogen(s), the absence of changes in pathogen and host populations that might alter resistance dynamics, the absence of changes in cultural practices, peaks in disease expression that should correspond to the climatic demand of the pathogen (but this assessment is only possible when the climatic demand of the pathogen is well known), and long-term monitoring of the disease pattern to derive a convincing trend in the relationship between climatic conditions and disease incidence. Having used a systemic approach, our study fits most of the criteria to attribute an impact to climate change on esca expression. Esca disease, based on foliar symptoms, is present in all the studied vineyard plots. As we used plants grafted in a unique nursery, with genetically homogenous material all planted the same year, a change in host populations is to be discarded but cannot be excluded for fungal pathogen populations. Cultural practices were the same for all the plots, except for cover cropping in Valais. The four years of intense monitoring of the vineyard network have inferred a correlation between climatic conditions and disease expression rate.

The systemic approach used allowed to identify the pedoclimatic factors impacting disease expression and can serve as a model to identify the environmental factors most impacting other esca susceptible grapevine cultivars to esca and other GTD associated diseases. Such approach can also be useful to identify the abiotic factors accounting for fungal diseases of other

cultivated woody plants and for successful reforestation for which climate and soil characteristics have been shown to be important (Baird & Pope, 2022; Hermoso et al., 2021).

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Conflict of interest

We have no conflicts of interest to disclose.

Author's Contributions

Monod, Zufferey, Viret, Gindro, Croll and Hofstetter conceived the ideas and designed methodology; Monod, Zufferey, and Wilhelm collected the data; Monod, Zufferey; Wilhelm, Viret, Gindro and Hofstetter analyzed the data; Monod, Zufferey and Hofstetter led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication and ensured that questions related to the accuracy or integrity of any part of their work are appropriately investigated and resolved.

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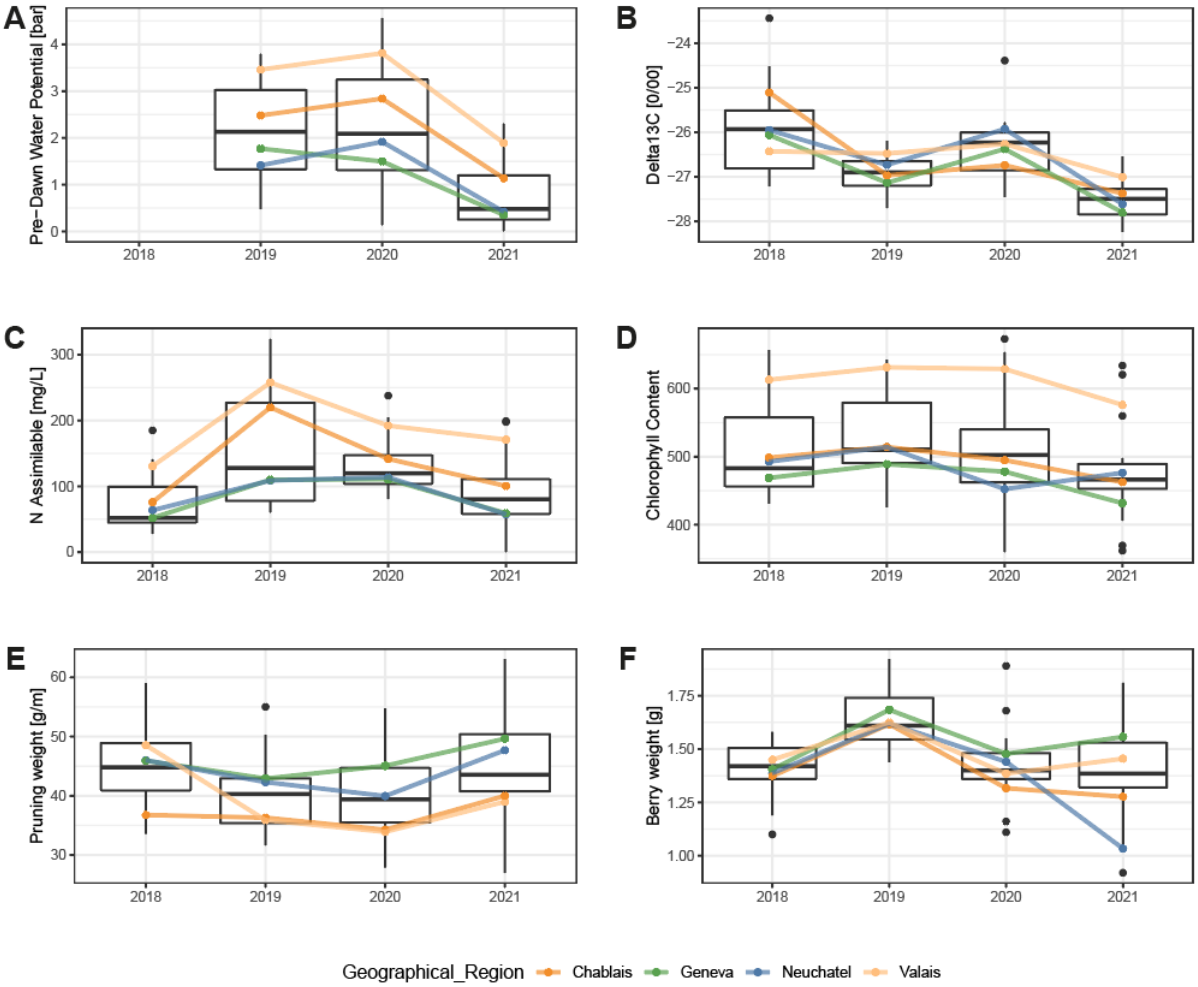
Supplementary material

Supp. Table 1 Meteorological station selected for the 19 study sites.

Agrometeo stations	**Plot represented by the station**
Leytron	Leytron, Produit
Sion	Nax1, Nax2
Champagne	Champagne, Concise, Onnens
Marcelin	Morges, Echichens A, Echichens B
Yvorne	Yvorne, Aigle
Villeneuve	Villeneuve
Blonay	Blonay
Begnins	Begnins
Commugny	Founex
Changins	Changins
Bourg-en-Lavaux	Villette
Perroy	Perroy

Supp. Table 2 Biotic and abiotic variables considered to establish Pearson and Xicor correlation coefficients with esca epidemiological monitoring data on the entire plot network.

Variables name	Variable Description	Variables name	Variable Description
Total_acidity Brix K Mg P N_Assim N_tester N_tot Delta13C Berries_weight Pruning_weight SWHC Cover_cropping	see Material and Methods	EPT_Apr EPT_Aug EPT_Jul EPT_Jun EPT_Mar EPT_May	Evapotranspiration per month
		NbDay01_Apr NbDay01_Aug NbDay01_Jul NbDay01_Jun NbDay01_Mar NbDay01_May	Nb days with rain >1mm per month
ColderDays_Apr ColderDays_Aug ColderDays_Jul ColderDays_Jun ColderDays_Mar ColderDays_May	Days colder than the norm (1990-2017) per month	NbDay03_Apr NbDay03_Aug NbDay03_Jul NbDay03_Jun NbDay03_Mar NbDay03_May	Nb days with rain >3mm per month
		NbDay10_Apr NbDay10_Aug NbDay10_Jul NbDay10_Jun NbDay10_Mar NbDay10_May	Nb days with rain >10mm per month
diffRainfallNorm_Apr diffRainfallNorm_Aug diffRainfallNorm_Jul diffRainfallNorm_Jun diffRainfallNorm_Mar diffRainfallNorm_May	Difference to norm (1991-2017) amount of rainfall per month		
		Rainfall_Apr Rainfall_Aug Rainfall_Jul Rainfall_Jun Rainfall_Mar Rainfall_May	Precipitations [mm] per month
diffRainyDaysNorm_Apr diffRainyDaysNorm_Aug diffRainyDaysNorm_Jul diffRainyDaysNorm_Jun diffRainyDaysNorm_Mar diffRainyDaysNorm_May	Difference to norm (1991-2017) nb. rainy days per month		
		SummerDays_Aug SummerDays_Jul SummerDays_Jun	Nb days with >25°C per month
WarmerDays_Apr WarmerDays_Aug WarmerDays_Jul WarmerDays_Jun WarmerDays_Mar WarmerDays_May	Difference to norm temperaure per month		



Supp. Figure 1 Mean values of measured physiological indexes of plots categorized by geographical region across four consecutive years (2018-2021) **A** pre-dawn leaf water potential (ΨPD), **B** δ13C, **C** Assimilable nitrogen, **D** Chlorophyll content index (N-tester), **E** Pruning weight, **F** Berries weight.

General Discussion

In perennial plants, which are exposed to a multitude of threats throughout their extended lifecycles, tree decline is a recurrent phenomenon resulting from a complex interplay of various factors (Koskella et al., 2017; Denman et al., 2018). The imperative need to maintain yield and the associated management practices accentuate this susceptibility in perennial crops. Disentangling the complex web of causal factors contributing to tree decline is a challenge. This is why we investigated biotic and abiotic factors linked to the presence and prevalence of esca, a dieback threatening vineyards worldwide (Bertsch et al., 2013; Gramaje, Úrbez-Torres and Sosnowski, 2018).

This doctoral thesis was dedicated to exploring the intricate facets of esca from multiple perspectives. Esca is the result of complex interaction of factors. Esca incidence is variable from one vineyard to another, influenced by climate, soil types, grape varieties or management practices (Gramaje, Úrbez-Torres and Sosnowski, 2018; Claverie et al., 2020). Esca involves multiple fungal pathogens (repeatedly isolated from plants showing symptoms) and their interactions with each other and with the grapevine host are not well understood (Graniti, Surico and Mugnai, 2000). Our objective was to scrutinize potential biotic and abiotic factors associated with the outbreak of the disease. To characterize the biotic factors, we developed a method to properly describe the trunk inhabiting fungal community in my first chapter. In our study, we assess the impact on both yield and quality of the recovered sequences when targeting the ITS region alone compared to a longer ribosomal fragment encompassing the ITS region and a portion of the 28S. Our findings indicated superior outcomes (sequencing depth and recovered diversity) when focusing on the ITS fragment. We subsequently applied this method in the second chapter, to investigate the fungal communities inhabiting grapevine trunks in plants displaying varying symptom severity across a network of 21 vineyards. Fungi have long been implicated in esca due to their consistent presence in affected plants. However, our investigation revealed no discernible disparities in the fungal communities retrieved from asymptomatic and symptomatic plants. The mere presence of specific species traditionally linked with esca does not suffice to explain the presence of symptoms. Moreover, the relative abundance of these species did not vary between asymptomatic and symptomatic plant samples. Thus, the precise role of these species and the trunk fungal communities more generally remains a question that warrants further exploration. In the third chapter, we delved into the connection between esca prevalence and a range of abiotic factors in the same network of vineyards displaying varying degrees of disease impact. Notably, we identified correlations between the presence of esca and specific climatic and pedological factors, which are associated

with the plant's water accessibility. We observed a greater incidence of esca (foliar symptoms and apoplexy) in vineyards with important Soil Water Holding Capacity (SWHC) and we observed a higher incidence of esca during rainy year, particularly when rain occurred in late spring. In the course of our research, we acquired insights into the variability of the susceptibility of a unique vine variety to esca disease and we were able to link this disease susceptibility to pedo-climatic factors. We also observed in the analysis of the myocobiome of asymptomatic and symptomatic plant that the fungal composition of the host plant is very diverse but does not differ according to the health status of the sampled plant. This work reaffirms the complex interaction between grapevines, their associated fungal communities, the variability of esca prevalence and the abiotic conditions conducive to disease development. Above all, our results open new avenues of exploration in this field (Figure 1).

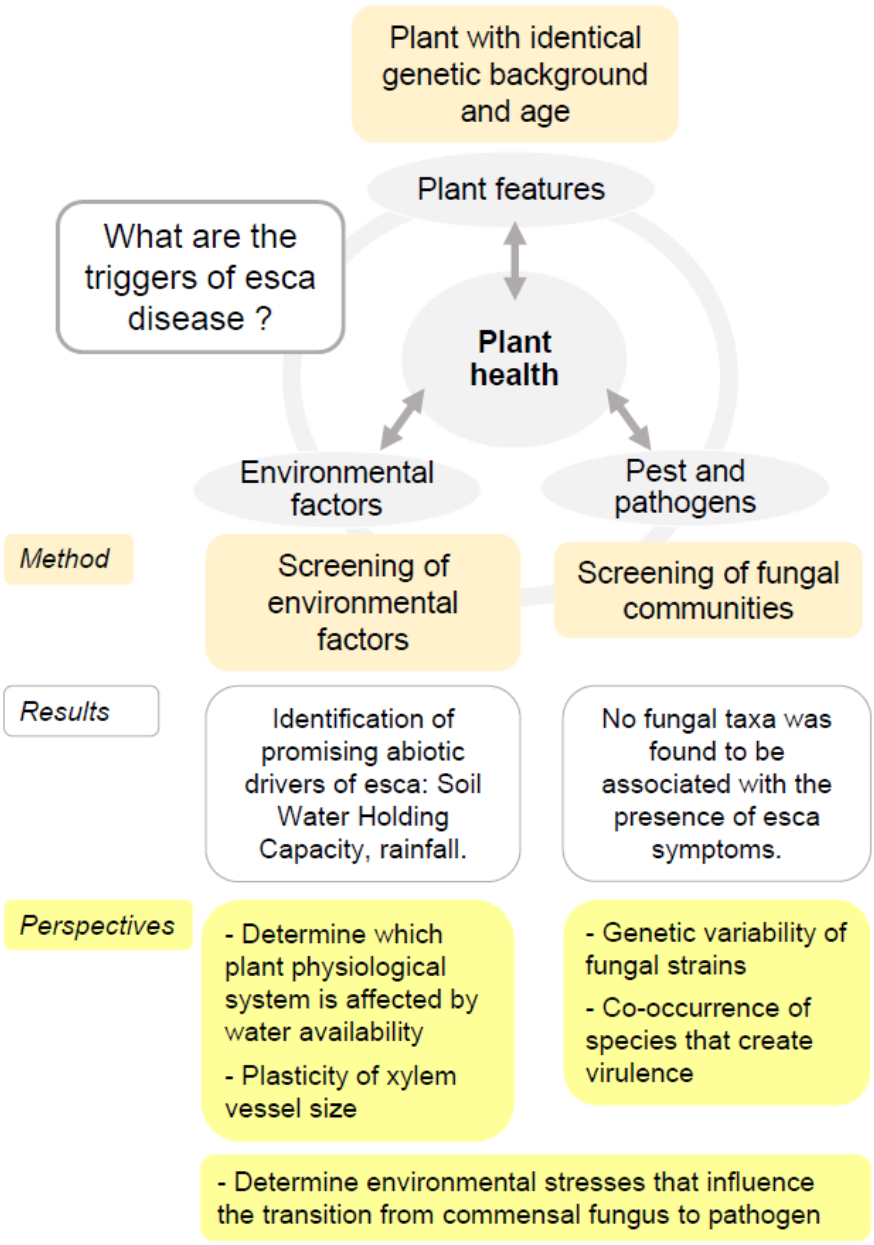


Figure 1 Schematic representation of the themes addressed, with: the general problematic in which the research question lies, the various decline factors investigated, the main results obtained and the perspectives.

The study of the mycobiome in the context of esca disease poses several challenges. We aimed to test for compositional differences in trunk inhabiting fungal communities in vineyards affected to varying degrees by esca. The analysis of the vine trunk mycobiome revealed a remarkably diverse fungal community with weak differentiation at the vineyard or regional level. We found overrepresentation of several taxa in asymptomatic plants; however no taxa were overrepresented in symptomatic plants. Key taxa typically implicated in esca were also not showing any significant association with plant health status. In complex diseases like tree decline, multiple factors are likely involved and resolving causal relationships between the presence of taxa and health is challenging. The presence of endophytes residing within plants without causing harm, challenges our traditional understanding of plant infection processes

and how causal taxa should be identified (Mishra, Bhattacharjee and Sharma, 2021). The spectrum of symbiotic associations and their consequences are not well defined and depend upon environmental conditions and can transition between commensalism, mutualism or pathogenicity (Mishra, Bhattacharjee and Sharma, 2021). Some fungi associated with plants can promote health or cause disease depending on the specific strain or their location (Selosse, Baudoin and Vandenkoornhuyse, 2004; Schulz and Boyle, 2005; Busby, Ridout and Newcombe, 2016). Microbial community assembly is influenced by cooperative and competitive interactions among the myriad microbial members that perform functions for plant health as a whole (Gao et al., 2021). Microbiome composition is also described as a mechanism for creating novel traits that enhance the plant's ability in different environmental conditions (Trivedi et al., 2020). This illustrates the difficulty to isolate species and link the presence of these species to an observed condition (i.e. symptomatic or asymptomatic). Several hypotheses may explain why we were not able to link the presence of fungal species with the presence of symptoms. The ability of an endophyte to either grow asymptomatically within its host or induce disease depends not only on its adaptation to the host but also on the variable virulence of the endophyte, the response of the host's defence mechanisms, and the prevailing environmental conditions both biotic and abiotic (Schulz and Boyle, 2005; Delaye, García-Guzmán and Heil, 2013; Busby, Ridout and Newcombe, 2016).

Species conventionally linked with esca are prevalent in the mycobiome that we recovered, with some, such as *Phaeomoniella chlamydospora*, ranking among the most abundant. The substantial abundance of the species link to esca implies their adaptation to the trunk environment and suggests that their interaction with the plant is not, at least exclusively, pathogenic. If these species typically associated with esca were responsible for symptoms, it would imply a transition from mutualistic to pathogenic relationship at a certain stage. If the relationship was purely pathogenic, the mere presence of these species would induce symptoms, which is not the case in many asymptomatic plants housing the species. Similar abundance of esca related species between symptomatic and asymptomatic plants were observed in other studies as well (Hofstetter et al., 2012; Bruez et al., 2014; Del Frari et al., 2019) and these species are also known to be widely distributed in many grape-growing areas worldwide (Bertsch et al., 2013). Taxa typically linked with esca were often hosted by the vine plant in our study suggesting a certain adaptation to the host. Several endophytes have been considered as latent pathogens but later act as pathogens under changed environmental conditions (Delaye, García-Guzmán and Heil, 2013). Esca outbreak can be the result of certain species functioning as opportunistic pathogen on the host plant. Shift from commensal to pathogenic species can be considered as

an unbalanced symbiosis (Kogel, Franken and Hückelhoven, 2006). Suboptimal environmental conditions may stress the host plant and weaken its defence status, creating favourable conditions for the disease development (Schulz and Boyle, 2005). Both the predispositions of the host and the prevailing environmental conditions exert influence over the equilibrium between the host and the endophyte (Schulz and Boyle, 2005). Endophytes modification is context dependent because environmental factors (temperature, pH, humidity) are pivotal in determining the prevalence and activity of fungal species, influencing both their growth and the production of mycotoxins (Busby, Ridout and Newcombe, 2016; Magan and Medina, 2016; Giorni, Bertuzzi and Battilani, 2019). Ecological function of an endophyte (neutrality, pathogenicity, pathogen antagonism or pathogen facilitation) can be modified by environmental factors such as soil properties, nutrients status and climatic conditions or host related factors such as physiological status (Romeralo et al., 2022). Abiotic condition can also influence indirectly the relation between the host and the endophytes. In maize, for example, no difference in fungal growth was observed according to shift in temperature and rainfall but the production of mycotoxin was influenced by the climatic condition, triggering the presence of the disease (Giorni, Bertuzzi and Battilani, 2019). In the case of esca disease, one prominent hypothesis states that toxins are transported from pathogen niche in the trunk to leaves through the transpiration stream (Claverie et al., 2020) and that this translocation can be influenced by abiotic factors like drought. Drought will reduce fungal toxic activity and/or enhance plant defence and/or decrease water transpiration which will influence the symptoms expression (Bortolami et al., 2021). In our vineyards network, we identified that sites with an excess of water and rainfall fluctuations were more affected by esca compared to plot characterized by dryer conditions. In another study, vine plant kept in a state of water deficit do not express esca symptoms over a two-year experiment (Bortolami et al., 2021). The perception of water deficit or excess by plants, driven by soil water availability, is primarily governed by local soil and hydrological conditions. These hydrological conditions, in turn, are significantly influenced by topography according to specific soil and climate characteristics (Esteban et al., 2021). An excess of water could lead to an imbalance in the host plant, thereby creating favourable conditions for a shift in the fungal community. Isolating environmental stressful conditions for the host while holding other factors constant is nevertheless difficult to achieve (Busby, Ridout and Newcombe, 2016).

In our experimental design, we focused on field-collected sample with a certain degree of standardization (i.e. age and genetic background of the host plant, sampling location on the grafting point and time for sampling). However, our vineyards were located in diverse soil and

climate area and the variation of environmental conditions was not controlled. We measured several physiological indicators on each vineyard, but we obtained a mean by vineyard and not a plant-by-plant measurements. Moreover, we did not control any parameter to directly test its incidence on the microbiome or the health status of the plant. To delve deeper into the analysis, we could have adopted a machine learning approach to pinpoint the critical abiotic factors that exert an influence on microbial communities. However, the variability of the recovered mycobiome composed of many rare taxa did not allow to reach a sufficient power to discriminate the microbiome according to the plant location or health status. Indeed, a shortcoming of our approach was that the sampling effort was probably not sufficient to discriminate mycobiome among health status or location. Bullington et al (2021) warned about the under-sampling issues which may obscure underlying patterns in microbial distributions and compromise the estimated diversity and degree to which sites, conditions or individual host truly differ. Subsamples from the same plant (milkweed) recovered completely different sets of species (more pronounced for foliar fungal endophytes compared to arbuscular mycorrhizal fungi), illustrating the undersampling bias that implies a lower power and accuracy to characterize the subtler aspects of plant microbiome interactions (Bullington, Lekberg and Larkin, 2021). Based on these patterns and our results with large variability among sample, more individual plants and a greater sampling effort within individuals are likely needed to better characterize trunk fungal communities and reach a sufficient power that enable the discrimination of health status by the mycobiome analysis.

Another avenue to explore is the genetic variability of species related to esca may revealed some mutation responsible for the pathogenicity. The changes could be very discrete and difficult to detect (Kogel, Franken and Hückelhoven, 2006). The idea would be to detect potential markers for mutualism or pathogenicity looking at intra-species diversity. However, to detect markers of mutualism or pathogenicity the experimental design would have to be adapted. Sequencing of the fungal genome of many organisms would be required to provides detailed genetic information, allowing to explore the genetic basis of specific traits, including those related to mutualism or pathogenicity through Single Nucleotide Polymorphisms (SNPs), insertion/deletion (indels), gene expression or epigenetic modifications. This could be achievable only if we have specific target species to focus on. Sequencing the entire genome of an entire fungal community would be impractical. To effectively pursue this direction, it is essential to gain a better understanding of which species are genuinely involved.

Esca disease may also be the result of multispecies interaction. The co-occurrence of species should be investigated to ascertain whether the presence of a species alone does not harm the

host plant, but when that species is present alongside others, their interaction becomes detrimental to the plant. Numerous fungal species have the capacity to coexist and exert mutual influences on each other with regard to growth and mycotoxin production (Busby, Ridout and Newcombe, 2016; Giorni, Bertuzzi and Battilani, 2019). Exploring the relationships between species could aid in unravelling certain aspects of disease occurrence. This underscores the complexity of intermicrobial interactions, which can play a role in both disease mitigation and facilitation. In our analysis, species commonly associated with esca appear to exhibit a broad distribution with similar relative abundance in plant showing symptoms and in asymptomatic plant. The symptoms might be the results of the co-occurrence of multiple species. Co-occurrence of species may help to identify taxa that have a key position in microbial network (Romeralo et al., 2022), explain virulence (Hassani, Durán and Hacquard, 2018) or joint production of phytotoxic metabolites (Giorni, Bertuzzi and Battilani, 2019). To explore potential microbial connections and assess community stability based on network structure, co-occurrence network analysis has gained prominence (Gao et al., 2021). However, our understanding of potential interactions within complex microbiomes associated with plants and their response to pathogen intrusion remains limited (Gao et al., 2021). A better understanding of intermicrobial interactions within the plant microbiota will enable us to define more clearly the functional importance of microbial networks for the overall fitness of the microbiome and its host (Hassani, Durán and Hacquard, 2018).

Another aspect we could focus on is the morphology of xylem vessels in grapevine plants and how this influences their susceptibility to esca. This factor plays a crucial role in the host plant's ability to repel fungal threats. The host's capacity to contain infections and colonization of vascular pathogen is influenced by the morphological characteristics of its vascular system (Pouzoulet et al., 2020). Water availability impacts the morphology of the xylem vessel due to plant plasticity and adaptation to its direct environment (Solla and Gil, 2002). When wider vessels experience a loss of hydraulic function due to pathogen-induced occlusion or embolism, the reduction in hydraulic conductance is more pronounced compared to narrower vessels (Pouzoulet et al., 2020). Exploring the hypothesis of plasticity in xylem vessel morphology could provide valuable insights into the variability of esca symptom incidence within the same grapevine variety planted in different locations characterized by specific pedoclimatic properties. In our network of vineyards, it would be interesting to study whether differences in xylem vessel size are indeed linked to site-specific location and pedoclimatic conditions, and whether a link with the incidence of esca can be established through xylem morphology.

Outlook

This thesis underlines the intricates relationship between vine trunk fungal inhabiting communities and abiotic factors regarding esca incidence. Our work focused on a unique network of vineyards with a single grape variety (Gamaret) and plants of the same age, ensuring homogeneity of plant material, to analyse the incidence of esca, something that has never been done before. Many high-quality studies have been carried out, but heterogeneity in terms of plant cultivar, plant age or location has made the outcomes difficult to generalize. We assessed of the variability of the susceptibility of a unique vine variety regarding its location and pedo-climatic regimes. In parallel, our analysis of the fungal communities of the vine trunk revealed an important variability across sampled plant independently of their health status.

Identifying microbiomes that are considered 'healthy' and distinguishing them from those associated with a diseased state is proving to be challenging in the context of esca disease. This challenge arises in part due to significant unexplained variation in microbiome composition and diversity among asymptomatic individuals. Increasing the number of diseased plants in highly affected vineyards might have reduced the variability among samples, potentially facilitating the identification of patterns that distinguish between asymptomatic and symptomatic plants. Incorporate co-evolution or co-dynamic of several fungal species might also help decipher the dialogue within the trunk inhabiting mycobiome. Our results do not rule out the influence of trunk-inhabiting fungi in triggering esca symptoms. However, our results do point to sophisticated relationships between the presence of one or possibly several species and the environmental conditions leading to the emergence of symptoms and dieback. Given the complex influences and interactions of the microbiome with plant health, there is certainly still much to be discovered about fungal communities and their link to the onset of esca. However, the difficulty of isolating species or controlling certain conditions to study the dynamics of the microbiome in living plants remains a challenge, given the many factors involved.

By focusing on a single vine variety, we have shown that susceptibility to esca disease varies from one vineyard to another, independently of the susceptibility of the host itself. The environmental factors involved in, or responsible for, the variability in susceptibility of a single grapevine variety should be studied in greater depth. An experimental design with controlled conditions will certainly be needed to test specific hypotheses, particularly the impact of water availability (quantity, seasonality, influence on xylem architecture, mycotoxin production linked to water availability) on the incidence of esca.

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