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Romain Darriaut

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By **Romain DARRIAUT**

**Role of the microbiome in the rhizosphere
functioning of different grapevine rootstocks and
influence on scion growth**

Under the supervision of :
Mme **Virginie LAUVERGEAT**, Maître de conférences, Université de Bordeaux

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Jury members:

| | | |
|-----------------------------|---|------------|
| Mme Alia DELLAGI | Professeure, AgroParisTech | President |
| M. Aziz AZIZ | Full professor, Université de Reims Champagne-Ardenne | Rapporteur |
| M. David GRAMAJE | Chargé de recherche, Instituto de Ciencias de la Vid y del Vino | Rapporteur |
| Mme Angela SESSITSCH | Professeure, Austrian Institute of Technology | Reviewer |
| Mme Nathalie OLLAT | Ingénieure de recherche, INRAE Bordeaux | Guest |

Rôle du microbiome dans le fonctionnement de la rhizosphère de différents porte-greffes de Vigne et influence sur la croissance du greffon

Chez la Vigne, le génotype du porte-greffe détermine le développement du greffon et la résistance du plant face aux contraintes biotiques et abiotiques. Le dépérissement du vignoble est un phénomène complexe, qui se traduit par une baisse subie de la productivité du cep et éventuellement de sa mort. La principale stratégie pour pallier ce manque de production est le remplacement des pieds non productifs ou morts par de nouveaux jeunes plants. Ce processus est causé par de nombreux facteurs parfois liés à la qualité du sol viticole et à son microbiote. En effet, l'interaction entre les racines et le microbiote du sol joue un rôle essentiel dans le contrôle des phytopathogènes telluriques mais aussi dans l'acquisition des ressources minérales nécessaires à la croissance du cep. La rhizosphère, qui est la portion de sol proche des racines, est au cœur de ses interactions. L'objectif de ces travaux vise à mieux comprendre le rôle du microbiome du sol et des racines dans un contexte de dépérissement du vignoble. Les sols d'inter-rang de quatre parcelles en dépérissement, qui n'était pas dû à des symptômes de carences minérales ou de maladies, ont révélé un dérèglement de la diversité microbienne et de l'activité enzymatique. Une analyse plus poussée sur les racines et la rhizosphère des plants en déclin d'une de ces parcelles a mis en évidence une forte présence de microorganismes potentiellement pathogènes mais aussi bénéfiques. Dans un second temps, une expérimentation en serre sur de jeunes plants de vignes plantées sur du sol de parcelle viticole présentant des signes de dépérissement, a montré que le porte-greffe modifiait la composition microbienne des racines et de la rhizosphère. Une caractérisation fonctionnelle *in vitro* de rhizobactéries de ces plants a permis d'identifier des isolats modifiant les systèmes aériens et racinaires de plantule de vignes. Ces résultats contribuent à la compréhension des interactions entre la vigne et le microbiote du sol, mettant en évidence le rôle de la dimension génétique du porte-greffe.

Mots clés : Diversité microbienne, Qualité du sol, *Vitis vinifera*, Dépérissement du vignoble, PGPR

Role of the microbiome in the rhizosphere functioning of different grapevine rootstocks and influence on graft growth

In the Vine, the genotype of the rootstock determines the development of the scion and the resistance of the plant to biotic and abiotic pressures. Vine decline is a complex phenomenon, which results in a sudden or progressive decrease in productivity of the vine and eventually its death. The main strategy to overcome this lack of production is the replacement of non-productive or dead plants by new young plants. This process of decline is caused by many factors sometimes related to the quality of the vineyard soil and its microbiota. Indeed, the interaction between the roots and the soil microbiota plays an essential role in the control of telluric phytopathogens but also in the acquisition of mineral resources required for the vine growth. The rhizosphere, which is the portion of soil close to the roots, is at the core of these interactions. The objective of this work is to better understand the role of the soil and root microbiome in a context of vineyard decline. The inter-row soils of four vineyard plots in decline, which was not due to symptoms of mineral deficiencies or diseases, revealed a disruption of microbial diversity and enzymatic activity. Further analysis of the roots and rhizosphere of declining plants in one of these plots revealed a high presence of potentially pathogenic but also beneficial microorganisms. In a second step, a greenhouse experiment on young grapevines planted on the soil of a vineyard plot exhibiting signs of decline, showed that the rootstock modified the microbial composition of the roots and rhizosphere. An *in vitro* functional characterization of rhizobacteria from these plants allowed the identification of isolates modifying the aerial and root systems of vine plantlets. These results contribute to the understanding of vine-soil interactions, highlighting the role of the genetic dimension of the rootstock.

Keywords: Microbial diversity, Soil quality, *Vitis vinifera*, Vineyard decline, PGPR

Résumé substantiel

La vigne est cultivée dans divers environnements à travers le monde et cette adaptation est en partie dû au système greffé, combinant les qualités organoleptiques des baies de vignes européennes (*Vitis vinifera*) et la tolérance à divers ravageurs des vignes américaines (*Vitis* spp.). Le choix du porte-greffe varie selon les conditions pédoclimatiques et s'inscrit dans la dimension géographique et culturelle de terroir. Outre l'influence du porte-greffe sur la vigueur du greffon et autres propriétés agronomiques, celui-ci est en contact direct avec le sol, dont la qualité va impacter l'état de santé du plant.

Une bonne qualité biologique des sols est donc primordiale pour la pérennité des potentiels agronomiques de nos terroirs. En effet, les sols fournissent des services écosystémiques indispensables au bon développement de la vigne tels que les cycles de régulation des nutriments ainsi que leur approvisionnement dans la plante par leur assimilation au niveau des racines. Ces services sont régulés en parties par les micro-organismes tels que les bactéries et champignons, qui sont les plus représentatifs, via des interactions microscopiques extrêmement complexes et nombreuses. Le microbiote chez la vigne, qui se définit par l'ensemble des micro-organismes, est en très grande partie originaire du sol et influence positivement ou négativement l'adaptation du plant dans son environnement. Le sol est un réservoir microbien contenant une pléthore de microbes, et sont considérés comme pathogènes, neutres ou bénéfiques vis-à-vis de la plante.

Une fois à proximité des racines, ces micro-organismes peuvent rester à la surface ou pénétrer les racines, en jouant un rôle protecteur ou infectieux. Parmi ces micro-organismes endophytes se trouvent des champignons symbiotiques, dont les fameux champignons mycorhiziens à arbuscule (CMA). Ceux-ci ont souvent montré leur efficacité pour réduire les effets de stress vis-à-vis du plant de vigne et des plants mycorhizés sont mêmes proposés aux viticulteurs par les pépiniéristes. Le microbiote du sol est donc un ensemble d'interactions qui est déterminant pour l'état de santé du cep, et cette dynamique peut également aller dans l'autre sens, c'est-à-dire que le microbiote du sol peut être influencé par le génotype du porte-greffe et permettre à certaines espèces de micro-organismes de proliférer, voire de devenir majoritaires par rapport à d'autres. L'impact du génotype du porte-greffe sur la composition microbienne de la rhizosphère et de l'endosphère des racines est donc un sujet d'intérêt dans la compréhension de la sélection des microorganismes associés à la vigne.

La vigne est sujette à de nombreux stress biotiques et abiotiques pouvant mener à un phénomène de dépérissement. Le dépérissement du vignoble peut se définir comme étant une baisse

pluriannuelle de production et/ou de qualité du cep souvent accompagnée de sa mort prématurée, brutale ou progressive dues à de nombreux facteurs. La principale solution pour pallier le manque de production demeure la complantation, qui consiste à remplacer le plant de vigne improductif ou mort par un nouveau jeune plant. Le dépérissement du vignoble est une problématique majeure dont le phénomène grandissant inquiète les viticulteurs à travers le monde. En France, les interprofessionnelles de santé avec le soutien de FranceAgriMer et du ministère de l'Agriculture ont mis en place le Plan National du Dépérissement du Vignoble (PNDV). Ces travaux de thèse s'inscrivent dans ce plan, au sein du projet Vitirhizobiome qui vise à comprendre l'implication microbiome du sol proche des racines, appelé rhizosphère, ainsi que des racines sur le fonctionnement du jeune plant de vigne dans un contexte de dépérissement du vignoble. On pourrait supposer que des vignobles présentant des signes de dépérissement seraient caractérisés par un dérèglement de l'équilibre microbiotique du sol. Par exemple, les microorganismes bénéfiques seraient moins présents et actifs dans un sol dépérissant que dans un sol non dépérissant.

Le manuscrit est constitué de cinq parties. La première présente le contexte, un état de l'art lié aux interactions triparties sol × microbes × porte-greffe de vigne et se termine par les objectifs et plan des travaux de thèse. La seconde partie du manuscrit met en évidence des indicateurs biologiques du sol d'inter-rang de quatre parcelles viticoles sujettes à dépérissement, mais également la composition du microbiome bactérien et fongique des sols présentant des vignes dépérissantes. En troisième partie, une approche holistique met en lumière la dérégulation de la qualité de la baie ainsi que du microbiome de la rhizosphère et des racines dans une des quatre parcelles viticoles. L'analyse a été faite également dans les horizons profonds d'une fosse pédologique. La quatrième partie est basée sur une expérimentation en serre visant à étudier l'effet de deux portes greffes et de deux sols différents sur la composition microbienne des racines. La cinquième partie cherche à observer l'effet de l'addition de microorganismes potentiellement bénéfiques sur le développement de plants en serre. Et en fin de manuscrit, des éléments de discussion sont apportés par rapport aux résultats généraux obtenus, ouvrant de nouvelles perspectives de recherche.

Chapitre II - Dérégulation des communautés microbiennes dans du sol d'inter-rang de parcelles viticoles sujettes à dépérissement

L'objectif de cette première partie consiste à caractériser les profils microbiologiques des sols de deux zones issues d'une même parcelle viticole, dont l'une présente des signes de dépérissement des vignes et l'autre présentant des vignes saines n'ayant pas de problème de croissance. Pour ce faire, quatre parcelles issues du Haut-Médoc et de Graves ont été

sélectionnées. Les travaux de ce chapitre sont en partie exploratoire car le dépérissement des vignobles étudiés n'a pas été identifié comme étant causé par une maladie ou une carence minérale, mais par une faible vigueur, un déclin des vieux plants et une mauvaise reprise des jeunes plants. Ce phénomène a été observé dans certaines parcelles dans des zones délimitées, que l'on a nommées S (pour symptomatique) tandis que les zones alentours nommées AS (pour asymptomatique) ne présentaient pas ces signes de dépérissement. Des analyses physico-chimiques, microbiologiques et enzymatiques ont été réalisées sur des prélèvements de sols d'inter-rangs en automne et au printemps.

Les résultats des analyses physico-chimiques montrent qu'aucune des zones S ou AS ne présentait de carence ou d'excès en teneur minérale qui pourrait expliquer ce dépérissement. La granulométrie s'est montrée relativement similaire dans les zones S et AS, quelle que soit la parcelle.

Les activités de l'arylamidase, de la β -glucosidase et de la phosphatase étaient significativement supérieures dans les sols AS par rapport aux S. Ces enzymes sont impliquées respectivement dans les cycles de l'azote, du glucose et du phosphate. Les premières analyses microbiologiques basées sur la culture de bactéries et champignons sur milieux gélosés ont révélé, globalement, une densité plus faible de bactéries et plus forte de champignons dans les sols S. Ces observations ont pu être également confirmées par PCR quantitative (q-PCR) sur l'ADN d'inter-rang, montrant que les gènes 16S archées et bactériens étaient significativement plus abondants, avec également une quantité de gène 18S fongique moindre, dans les sols AS que S. L'activité microbienne, mesurée par un système d'oxydation de sources carbonées appelé Biolog EcoPlate™, était cependant plus importante dans les zones S que AS.

Dans un second temps, les séquençages Illumina MiSeq sur du 16S, ITS et 18S basés sur les bactéries, les champignons, et les CAM, respectivement, ont permis d'identifier certaines des communautés microbiennes présentes dans ces sols d'inter-rang. Les indices de l'alpha-diversité que sont la richesse et la diversité ont montré une dérégulation des microbiomes dans les S par rapport aux sols AS. Il a notamment été observé un enrichissement des champignons potentiellement pathogènes et de microorganismes potentiellement bénéfiques au développement de la vigne dans les sols S par rapport aux sols AS.

Cette partie II des travaux a permis de confirmer que les sols d'inter-rang des zones dépérissantes présentaient des profils microbiologiques différents des zones saines au sein des quatre parcelles.

Chapitre III - Approche holistique dans un vignoble dépérissant, avec une attention particulière aux microorganismes des racines et de la rhizosphère

Comme mentionné précédemment, le microbiome tellurique peut donner un aperçu de la qualité du sol dans les vignobles. Les micro-organismes endophytes de la vigne proviennent principalement du sol environnant et traversent la rhizosphère pour atteindre le cortex racinaire et les tissus vasculaires. Certains de ces endophytes sont pathogènes et d'autres sont bénéfiques pour la plante, agissant comme des promoteurs de croissance en régulant l'absorption des nutriments ou les réponses hormonales, ou en stimulant les voies de défense. Les objectifs de ce chapitre étaient de caractériser l'effet du déclin sur la vigne, et d'explorer la taxonomie et la fonctionnalité des microorganismes associés à sa rhizosphère et à son endosphère. De plus, les horizons profonds des zones symptomatiques et asymptomatiques précédemment décrites ont été explorés pour leur impact potentiel sur la croissance de la vigne.

Un vignoble parmi les quatre présentés au chapitre 2 a été sélectionné pour une analyse *in situ* plus approfondie. Le phénotypage des plantes, ainsi que les échantillonnages de la rhizosphère, du sol brut et des racines ont été réalisés un an après ceux effectués dans le chapitre 2, et ont également comparé la zone symptomatique à la zone asymptomatique. Tout d'abord, le sol brut, correspondant au sol d'inter-rang, a été examiné une fois de plus pour confirmer le dérèglement microbien. Ensuite, la teneur en minéraux des feuilles et la composition du moût a été déterminé. Les profils microbiens ont été dressés, sur la base de méthodes dépendantes de la culture, de mesures Eco-Plates, de q-PCR et de séquençage d'amplicons 16S, ITS et 18S. De plus, une fosse dans chaque zone a été générée pour visualiser les horizons les plus profonds et explorer leurs paramètres physico-chimiques et microbiens.

Les effets du déclin ont été observés sur la composition des feuilles et du moût, avec un contenu minéral réduit dans les échantillons symptomatiques par rapport aux échantillons asymptomatiques. Conformément aux résultats du chapitre 2, la diversité fonctionnelle mesurée par les Eco-Plates était plus importante dans la zone en déclin, tant dans le sol brut que dans la rhizosphère. En outre, les compartiments du sol d'inter-rang, des racines et de la rhizosphère présentaient une dysrégulation taxonomique dans les communautés bactériennes et fongiques, mettant en évidence la perturbation microbienne liée au déclin observé. De façon similaire à la deuxième partie du chapitre 2, un enrichissement en bactéries potentiellement bénéfiques, ainsi qu'en champignons pathogènes, a été constaté dans les échantillons symptomatiques. Les horizons profonds des zones asymptomatiques et symptomatiques ont présenté une diminution des activités enzymatiques, du nombre de copies de gènes et du niveau des populations cultivables de microbes, à l'exception de l'activité phosphatase qui est devenue plus importante dans les horizons profonds symptomatiques. En raison d'un séquençage tardif et d'une mise à

jour dans les bases de données MaarJAM et Unite lors de la rédaction du manuscrit, les affiliations des taxons pour le 18S des *Glomeromycota* n'ont pas été présentées dans ce travail. Le séquençage à base d'amplicons s'est démocratisé et est largement utilisé dans les études écologiques pour révéler la structure des communautés microbiennes. Cependant, une des limites de cette méthodologie est le manque d'informations concernant les microbes actifs. Par conséquent, la combinaison des méthodes indépendantes et dépendantes des cultivables peut refléter un profil microbien plus complet et plus pertinent dans les environnements étudiés que la méthode à base unique. Cette approche holistique a permis de mieux comprendre le déséquilibre microbien qui se produit dans le vignoble. Cependant, un seul génotype de porte-greffe était présent dans cette étude, ce qui est un facteur déterminant dans l'association de la vigne avec son microbiote. Le prochain chapitre est lié à ce sujet du génotype du porte-greffe, et sera étudié dans une expérience en serre.

Chapitre IV - Influence du génotype du porte-greffe sur le microbiome souterrain des jeunes vignes

Comme de nombreuses cultures pérennes, les vignes improductives et mourantes sont remplacées par de jeunes plants qui ont besoin d'au moins deux ans pour devenir performants et productifs. Comme nous l'avons vu précédemment, la dimension génétique du porte-greffe exerce une influence sur l'assimilation des minéraux mais aussi sur les microorganismes associés à la vigne. Dans ce contexte, l'impact du génotype du porte-greffe sur la diversité et la fonctionnalité microbienne du sol et des racines est une question pertinente.

Ce chapitre vise à explorer l'influence du génotype du porte-greffe sur le microbiome souterrain de jeunes vignes, empotées avec des sols symptomatiques ou asymptomatiques. Ces sols ont été excavés du vignoble étudié dans le chapitre précédent et utilisés comme substrat pour une expérience en serre. Le dispositif expérimental a duré 4,5 mois, a été répété deux fois, et était basé sur des Cabernet Sauvignon d'un an greffés sur Riparia Gloire de Montpellier (RGM) ou 1103 Paulsen (1103P), qui induisent respectivement une faible et une forte vigueur du greffon. Pour comparer l'influence du génotype du porte-greffe sur les microbes, des approches dépendantes et indépendantes de la culture des microorganismes ont été utilisées dans les compartiments des racines, de la rhizosphère et du sol brut. Toutes les méthodes précédentes, y compris les Eco-Plates, la q-PCR, le niveau de microbes cultivables, le séquençage des amplicons et la colonisation mycorhizienne des racines, ont été évaluées. En outre, les isolats rhizobactériens ont été identifiés par MALDI-TOF-MS, et les genres bactériens prédominants ont été visualisés par microscopie DOPE-FISH pour confirmer leur présence dans l'endosphère des racines.

Bien que cette expérience ait été menée en serre, les résultats obtenus ont mis en évidence le génotype du porte-greffe et le sol comme moteurs des communautés bactériennes et fongiques dans les jeunes vignes.

La contribution microbienne initiale du sol du vignoble et des plantes de la pépinière au microbiome de la serre a été explorée, ce qui a permis de détecter certains genres de champignons pathogènes. Aucun des symptômes affiliés causés par ces taxons n'a été observé dans le vignoble ou dans la serre, suggérant l'hypothèse d'une régulation naturelle par d'autres microorganismes présents dans les racines et le sol. Exclusivement pendant la première année de l'expérience en serre, la combinaison CS×1103P cultivée dans des sols symptomatiques a présenté une biomasse aérienne et souterraine significativement plus faible que la combinaison CS×1103P cultivée dans un sol asymptomatique.

Étant donné les rôles potentiels de protection contre les pathogènes fongiques et de promotion de la croissance, on peut se demander si l'ajout de micro-organismes bénéfiques sélectionnés pourrait favoriser la croissance de la vigne. Cette question est abordée dans le chapitre suivant.

Chapitre V - Isolement et caractérisation d'isolats bactériens potentiellement bénéfiques pour la croissance de la vigne

Les sols abritent une pléthore de micro-organismes qui sont essentiels à la croissance et à la santé de la plante. Dans de nombreuses études, l'isolement de rhizobactéries bénéfiques pour la vigne a été effectué principalement pour le contrôle des pathogènes. En plus de ces bactéries bénéfiques, le potentiel des CAM pour favoriser la croissance a également été bien étudié en viticulture et est même proposé par les pépinières aux viticulteurs. Cependant, très peu d'études ont combiné les effets promoteurs de croissance de ces deux types de microorganismes dans la vigne. De plus, l'ajout de microorganismes peut créer ou appauvrir des niches écologiques, créant ainsi un déséquilibre microbien potentiel, ce qui est une question très peu étudiée.

Les effets de l'ajout de micro-organismes bénéfiques sur les racines de la vigne et les compartiments du sol ont donc été étudiés dans une expérience en serre mais ne seront pas présentés dans le manuscrit en raison de la consistance des données et du manque de temps pour présenter une analyse décente. Cependant, les résultats préliminaires consistant en la caractérisation des rhizobactéries potentiellement bénéfiques seront présentés. Certains résultats de la serre seront brièvement discutés. Les rhizobactéries isolées dans le chapitre précédent ont été testées pour des traits bénéfiques à la croissance du plant en utilisant des tests biochimiques et biologiques. Les huit isolats les plus pertinents ont d'abord été inoculés sur des graines germées de *Lepidium sativum*. Cette plante a été choisie en raison de sa croissance rapide et de la facilité des mesures phénotypiques. Ces huit isolats ont ensuite été inoculés sur

des plantules du cv. 1103P (*Vitis rupestris* × *Vitis berlandieri*), puisque ce porte-greffe était le plus sensible aux paramètres de croissance dans le chapitre précédent. Pour réaliser l'expérience en serre, l'inoculum ayant le plus grand effet positif sur les paramètres de croissance a été sélectionné et testé en serre sur la combinaison CS×1103P empotée dans le sol symptomatique étudié dans les chapitres précédents. Certains des CS×1103P ont été inoculés par la pépinière avec des champignons mycorhiziens commerciaux afin d'étudier le potentiel des champignons bénéfiques proposés aux viticulteurs. En outre, la moitié de ces plantes mycorhizées ont été inoculées avec les rhizobactéries isolées pour tester si leurs effets combinés sur la croissance étaient synergiques ou inhibiteurs.

Les résultats ont montré des effets bénéfiques des rhizobactéries isolées sur la croissance de *L. sativum* et *V. rupestris* × *Vitis berlandieri* cv 1103P. Certains isolats à haute capacité fonctionnelle étaient spécifiques au porte-greffe 1103P. L'utilisation de consortiums composés de deux souches a induit un effet plus important que l'inoculation d'un seul isolat. L'utilisation du mélange rhizobactérien le plus efficace couplé aux CAM a montré une augmentation de la biomasse racinaire alors qu'une réduction du diamètre des branches a été observée dans les plantes mycorhizées.

Chapitre VI : Discussion générale

Dans ce chapitre est mentionnée divers concepts de recherche envisageable suite aux résultats obtenus. Dans un premier temps, le concept de « cry-for-help » est mentionné, soulignant l'intérêt d'étudier les microorganismes présents dans les milieux stressés. Dans un second temps, des méthodes de culture sont mentionnées, afin de cultiver de façon optimales les microorganismes présents dans le sol ou les plantes. La notion d'ingénierie du microbiome est évoquée par le biais de transplant du microbiome d'un sol sain sur un microbiome dérégulé. Il est ensuite discuté du lien possible entre la composition microbienne de l'endosphère et du phénotype qui varie selon le génotype du porte-greffe×greffon. Enfin, l'intérêt de ces travaux de thèse dans le milieu professionnel de la viticulture est discuté.

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From now on, I allow myself to switch to French for the rest of the acknowledgements.

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Abbreviation list

1103P = 1103 Paulsen

ACC = 1-aminocyclopropane-1-carboxylic acid

ACCd = 1-aminocyclopropane-1-carboxylate deaminase

AMF = Arbuscular Mycorrhizal Fungi

ANOVA = Analysis of variance

ArMV = Arabic Mosaic Virus

AS = Asymptomatic

ASV = Amplicon Sequence Variants

AUC = Area under curve

AWCD = Average well color development

BCA = Biological Control Agent

CAS = Chrome-azurol S

CEC = Cation exchange capacity

CFU = Colony forming unit

CLPP = Community level physiological pattern

CS = Cabernet-Sauvignon

cv. = Cultivar

DGGE = Denaturing gradient gel electrophoresis

DOPE-FISH = Double labeling of oligonucleotide probes - Fluorescence in situ hybridization

EPS = Exopolysaccharides

FDR = False discovery rate

GFLV = Grapevine Fan Leaf Virus

GLRaV = Grapevine leafroll-associated viruses

GTD = Grapevine trunk disease

IAA = Indole-3-acetic acid

ISR = Induced Systemic Resistance

ITS = Internal transcribed spacers

LDA = Linear discriminant analysis

LEfSe = Linear discriminant analysis effect size

LSU = Large Subunit Region

MALDI-TOF-MS = Matrix assisted laser desorption ionization - Time of flight – Mass spectrometry

MAMP = Microbe-Associated Molecular Patterns
NGS = Next-generation sequencing
NMDS = Non-metric multidimensional scaling
N-NH₃ = Ammoniacal nitrogen
NOPA = Alpha-amino nitrogen
OTU = Operational taxonomic unit
PCR = Polymerase chain reaction
PDA = Potato dextrose agar
PERMANOVA = Permutational multivariate analysis of variance
PGP = Plant Growth Promoting
PGPB = Plant Growth-Promoting Bacteria
PGPM = Plant Growth-Promoting Microbe
PGPR = Plant Growth-Promoting Rhizobacteria
PMD = Potential metabolic diversity
PMF = Peptide mass fingerprint
PNDV = Plan National Dépérissement du Vignoble
PR = Pathogenesis-related
q-PCR = Quantitative-PCR
R2A = Reasoner's 2A agar
RGM = Riparia Gloire de Montpellier
S = Symptomatic
SOM = Soil organic matter
spp. = Species *pluralis*
SSU = Small Subunit
V. spp. = Vitis spp.
VOC = Volatile organic compounds

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General introduction

Since the dawn of agriculture, grapevine (*Vitis* spp.) has been cultivated worldwide mainly for its berries harboring oenological properties of interest. Wine aromas and structure are dependent on the human practices, climate, and undoubtedly soil interactions. Due to increasing environmental pressures, partly originated from climate change, viticulture must adapt to keep wine typicity while maintaining healthy vineyard, against so-called grapevine decline. Interestingly, some grapevine declines occur without any apparent pathological cause or apparent mineral deficiency. To counteract this decrease in production, some winegrowers have no choice but to replace dying or dead vines with new young plants.

Grapevine is a grafted plant composed of a rootstock, which is at the interface between the soil and the scion, which produces berries. Indeed, grafting in viticulture has been widely used since the emergence of pests and diseases from the XIXth century, such as phylloxera aphid that almost destroyed European vineyards. Since then, the choice of the rootstock is made according to the soil and climatic conditions and its intrinsic agronomic properties and pest tolerance, which strongly influence the composition of the berries partly because of its direct contact with the soil.

Rootstock uptakes essential nutrients for grapevine growth, and directly interacts with soil microorganisms. Indeed, soil is considered as a reservoir of microbes for plants and some of them pass through the roots to migrate to aerial compartments while others stay close to the roots. The rhizosphere compartment, which is the tight portion of soil proximal to the roots, is a particular hot spot for microorganisms' activities. The grapevine associated microbiome is known to modulate the health of the plant, with either beneficial or pathogenic effects, and may reflect the fitness of the host, especially in young vines. Viticulture is quite greedy in pesticides, and some alternatives to counteract the pathogens emergence or mineral deficiencies exist, based on microbial preparation.

Since rootstock acts as a selector of soil microorganisms, grapevine decline might be associated to unbalanced soil microbiome. But to what extent is this microbiome dysregulated? Is there any keystone taxa depletion or at the opposite the emergence of pathogenic and opportunistic microorganisms?

Very little is known about the link between the soil microbiome and vine health. In addition, no research has been conducted on the microbiome of vineyard soils showing unexplained signs

of decline. This work aims to understand the interaction between rootstock and microbiome of belowground compartments under unexplained decline context in vineyards. It also investigates the role of soil and root microbiome for grapevine development using two rootstocks from different genetic background in greenhouse.

Chapter I

Context and literature

1. A plant intimately linked with the human practice

1.1. Viticulture history in a glimpse

The vine is a woody perennial plant, including both wild and cultivated species belonging to the *Vitis* genus found all around the world (Robinson *et al.*, 2013). The cultivated grapevine, also known as *Vitis vinifera* var. *sativa* which derived from the wild form *Vitis vinifera* subsp. *Sylvestris* (Terral *et al.*, 2010), is a crop, famous for its oenological hallmarks and for its specific products such as table grapes, juice, brandies, or even jams.



Figure 1 : Grapevine as a powerful symbol in old (A-B) Egyptian (Tomb of the Vineyards: around 1439-1413 B.C), (C) Roman (Sarcophagus in Rome: around 140-150 A.D.), and (D) Hellenistic (Sarcophagus: around the 2nd-3rd centuries A.D.) civilizations. Reproduced from (Savo *et al.*, 2016).

The grapevine domestication happened more than 6,000 years ago during the Neolithic period, and probably occurred somewhere around the Caspian and Black seas, where the most ancient traces of grape culture have been identified (McGovern *et al.*, 2017; Miller, 2008). This plant took part in the transition to urbanization and travelled with human beings to other parts of the world (Fuller and Stevens, 2019). Grapevine had a great influence in the Eurasian culture among the most important civilizations (**Figure 1**) (Savo *et al.*, 2016). In Europe, viticulture could have started due to Phoenician influence in Southern Italy during the 2nd millennium B.C.

or in Southern Spain at the beginning of the 1st millennium B.C. and is considered as the crop with the greatest diffusion back in those times (Pérez-Jordà *et al.*, 2021).

In Europe, viticulture practices were transmitted from the Phoenician and the Greeks to the Roman Empire, which upgraded the plantations and winemaking processes until the empire decline and fall. Based on these legacies, contemporary grapevine varieties started to be recorded in the Middle-Age in Europe (Ramos-Madrigal *et al.*, 2019). After millennia of selective breeding, over 10,000 varieties of grapevines are known to be cultivated nowadays, with a broad range of flavors and aromas (Robinson *et al.*, 2013; This *et al.*, 2006).

Some wild varieties originated from North America have co-evolved endemically with grapevine pathogens, resulting in tolerant American *Vitis* and sensitive European varieties to invasive and devastating pathogens such as phylloxera, powdery and downy mildews. The introduction of powdery mildew caused by the fungal agent *Erysiphe necator* was first recorded in western Europe in 1845, followed by the sap-sucking aphid phylloxera in 1863 and rapidly became worldwide grapevine diseases (Töpfer *et al.*, 2011). Fontaine *et al.* (2021) tracked down the pathogen presence of downy mildew caused by *Plasmopara viticola* across the world through its first record in France in 1878, highlighting its spreading aspect (**Figure 2**).

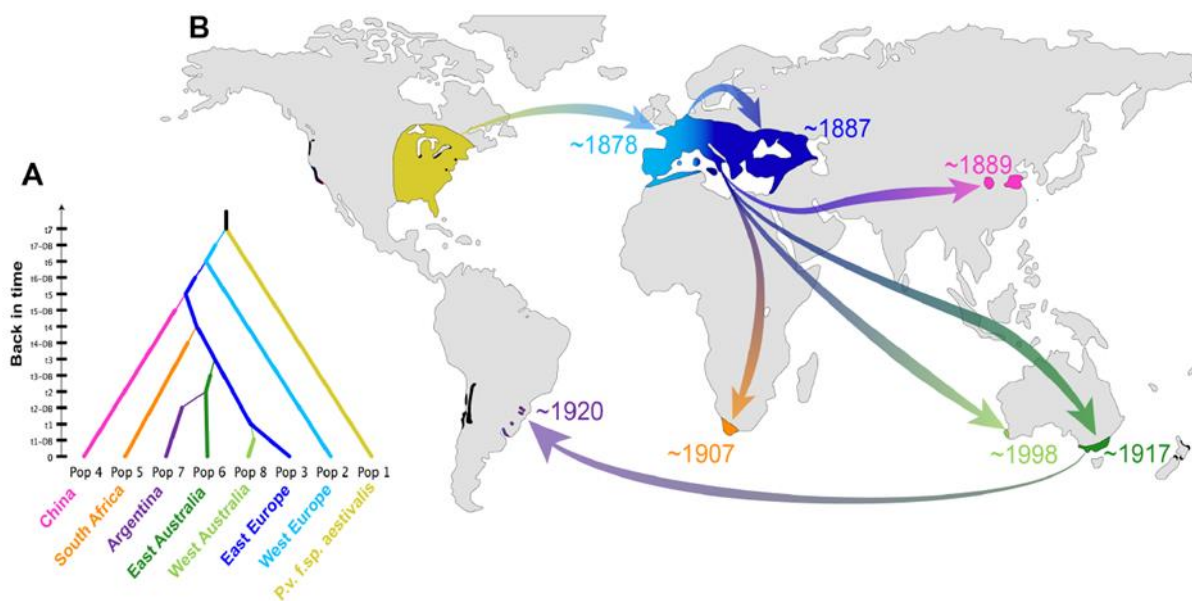


Figure 2 : Worldwide invasion history of downy mildew with (A) the population divergence scenario coupled to (B) its geographic representation, from Fontaine *et al.* (2021).

These historical events afflicted the old-world viticulture, and resulted in a drastic change of growing practices by spreading and democratizing, even nowadays, the grafting process of the

usually own-rooted *Vitis vinifera* grapevines onto American *Vitis* hybrids partially resistant to phylloxera aphid (Tello *et al.*, 2019; This *et al.*, 2006).

1.2. Bordeaux region in the terroir concept

1.2.1. Terroir as a multifactorial effect on grape composition

Terroir is a French word that has been applied to wine regions for a long time. This term can be defined as a spatial and temporal interactive ecosystem that influences the grape flavors and winemaking process in a specific area, including climate, soil and vine genotypes (Deloire *et al.*, 2005; Van Leeuwen and Seguin, 2006). Grapes aromas are also impacted by cultural management by winegrowers, and their complex interactions are understudied (Alem *et al.*, 2019). The final contribution of the winemakers remains the winemaking techniques to transform the compounds of the berries in order to reveal their aromas in their own quality wines. Terroir effect on aromas expression is mainly driven by air temperature, radiation, vine nitrogen and water status factors that are impacted by soil water holding, reference evapotranspiration, and rainfall (Figure 3).

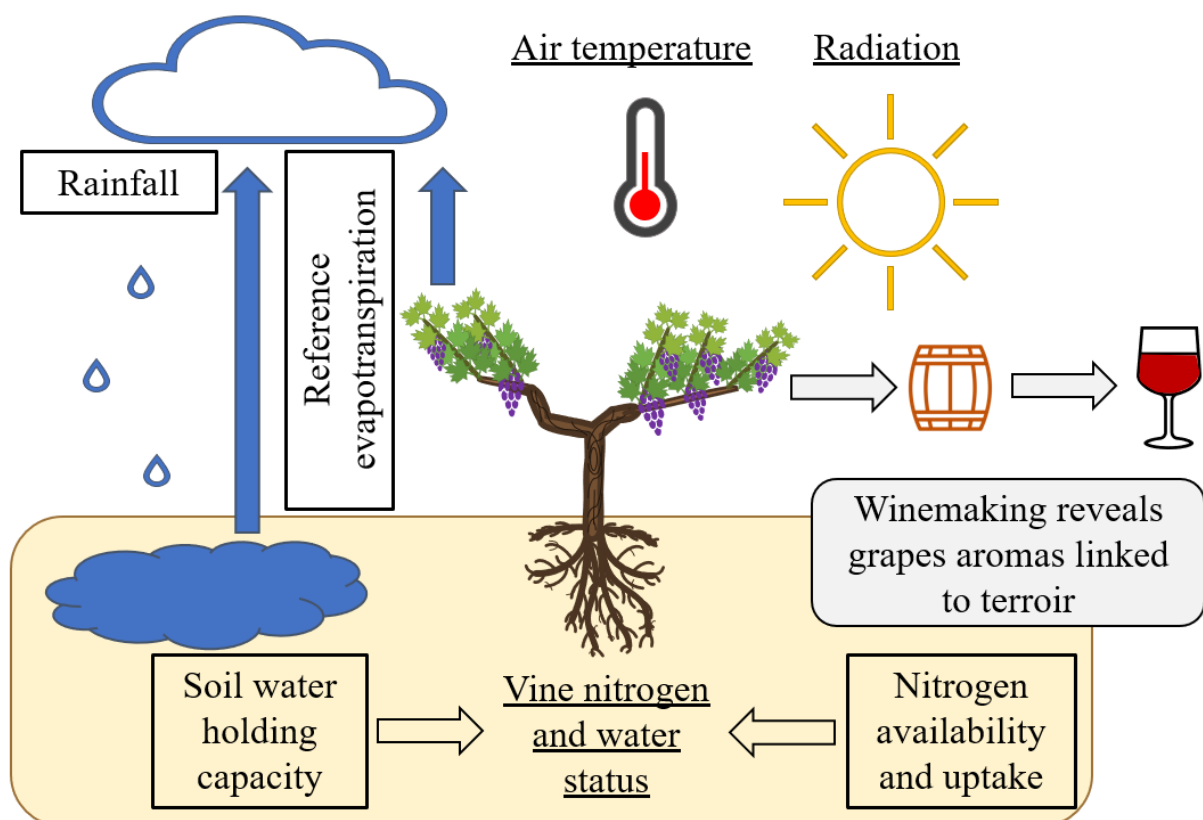


Figure 3 : Overview of the factors (framed) impacting the terroir effects (underlined), which modify aromas in grapes and wines. Adapted and modified from Leeuwen *et al.* (2020).

The aromatic profiles of the berries are partly determined by the choice of the genotype variety (Anderson and Aryal, 2013). For instance, Cabernet Sauvignon is poor in sugar and rich in tannin content, while Merlot displays less tannin content with higher sugar rate, making interesting associations between these common cultivars in Bordeaux (Gergaud and Ginsburgh, 2008).

1.2.2. Bordeaux, a place to grow

Vitis vinifera is the most valuable crop with around 7.3 million of hectares worldwide specialized in the production of 260 million hectoliters of wine in 2020 with a value of 29.6 billion euros. In France, the areas supporting vineyards represent around 797,000 of hectares with a production of 46.6 million hectoliters of wine and are globally divided in 16 wine terroirs with their own traditions, varieties, and histories (OIV, 2021).

Among those wine regions, we can distinguish Bordeaux area, which flourished since the Middle-Ages thanks to a prosperous merchandising of its quality wines through the seas. Wines from Bordeaux region were already famous in the 17th century, and its specific vineyard soil was thought to be the reason of this quality and fame (van Leeuwen and Seguin, 2006). Lately in the second half of the XIXth century, in this France area, the notion of a "wine château" was born. This notion inaugurated the concept of property producing wines of quality with a specific terroir (Roudié, 2000). In parallel, the first Bordeaux Wine Official Classification dated back from 1855 and was requested by Emperor Napoleon III in order to export a classification system for France's best Bordeaux wines abroad. Wines were categorized based on château's reputation and their prevailing trading prices (Thomson and Mutkoski, 2011).

The Bordeaux region is composed of six geographical entities, also known as Appellations. These Appellations enjoy a mild oceanic climate composed of soft winters and relatively wet springs with cool nights and hot summer days that are ideals for vine blossoming (Baciocco *et al.*, 2014; Bois *et al.*, 2018). Bordeaux vineyards are mainly located on clay-limestone, gravelly, and sandy soils which filter rapidly and favor the grape maturation with these calorific soil properties. Regarding the vines genotypes within the Bordeaux region, the most common cultivars are black Merlot, Cabernet Sauvignon, and Cabernet Franc for the red wines while Sémillon and Muscadelle are the most common cultivars for the white wines (Bélis-Bergouignan, 2011).

1.3. The grapevine as a product of domestication and adaptation

1.3.1. The parental origin of the current European rootstock

The genus *Vitis* L. is one of the 16 genera in the *Vitaceae* family and encompasses 79 species (The Plant List, 2021). Like many other crops, grapevine is mostly grown as clonal lineages and the most valuable varieties are selected and maintained through *in vitro* propagation, softwood cuttings, field grafting, and mostly bench grafting (Waite *et al.*, 2015). Through target-enriched genome-wide sequencing, an archaeological sample dating from the 12th century was matching perfectly with the actual cultivated variety Savagnin Blanc, suggesting at least a 900 years of uninterrupted propagation (Ramos-Madrugal *et al.*, 2019).

Grafting is a common and ancient method for plant propagation and adaptation applied to several crops such as apples, citrus, coffee, avocado or olives (Warschefsky *et al.*, 2016). The rootstock, making up the trunk and root system, is at the interface between the soil and the scion for water supply, nutrient uptake, and influences the scion development and thus the fruit formation. Due to its proximity with the soil and influence on berry composition, the rootstock is chosen regarding the pedoclimatic conditions (Corso and Bonghi, 2014; Tramontini *et al.*, 2013). Rootstocks may have different genetic backgrounds because of breeding histories. In viticulture, many of the current rootstocks used were obtained to adapt to the diseases from the XIXth century (*e.g.*, phylloxera, powdery, and downy mildews), and most of them have been used for at least a hundred years (**Figure 4**).

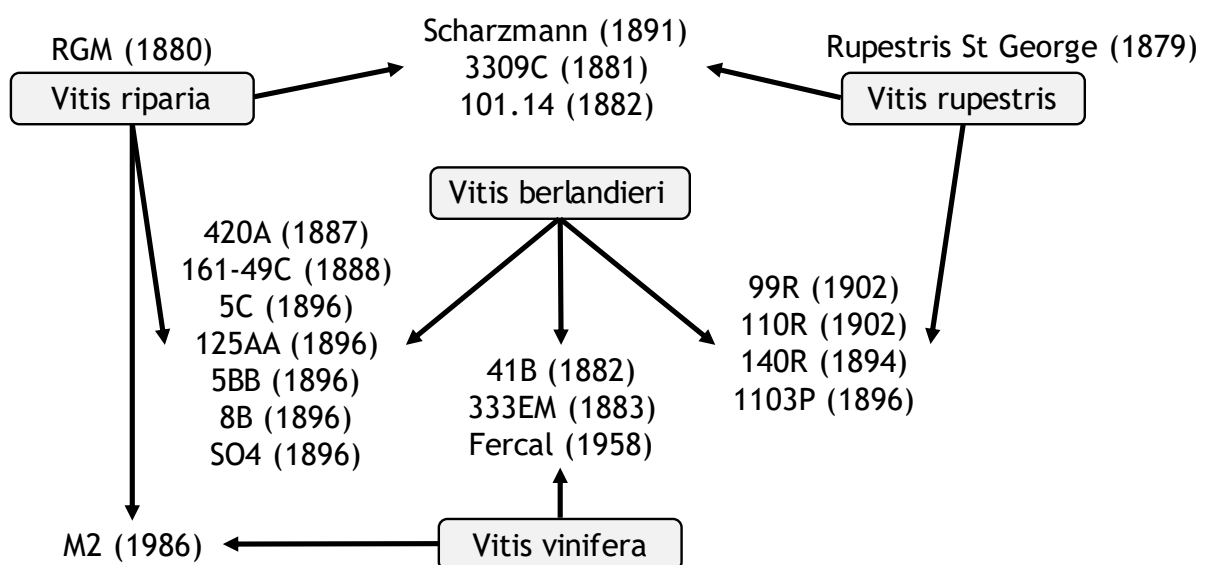


Figure 4: Common rootstocks, their parentage, and year of release according to Pl@ntGrape (<https://plantgrape.plantnet-project.org>), modified and adapted from Corso and Bonghi (2014).

Initially, the first generation of rootstocks used to counteract these afflictions were pure American species of *V. riparia* and *V. rupestris*, namely Riparia Gloire de Montpellier and Rupestris St George, respectively. However, it appeared that these rootstocks, having a good response when grafted with *V. vinifera*, were unsuitable for most of the European soils known to be calcareous. This led to the introduction of *V. berlandieri*, highly tolerant to limestone due to its calcareous-site origin but with poor rooting ability (Schmid *et al.*, 2009). These historical events explain the parental basis of most of the common rootstocks used in today's viticulture, which are the pure and hybrid forms of *V. riparia*, *V. rupestris*, and *V. berlandieri*. Riaz *et al.* (2019) highlighted the lack of diversity in the rootstock genetic landscape and pointed out the limited knowledge regarding the genetic ascendance as some pedigree information of commercialized rootstock was erroneous. However, the rootstock properties and their capacity to adapt to their environment have been well investigated.

1.3.2. Cultivars are selected for their agronomic traits

Extensive studies have been performed to unravel the rootstock genotype capacity to display agronomic features (Ibacache *et al.*, 2019). The most sought-after agronomic characteristics depend on the topology of the vineyard and the expectations of the winegrowers. The rootstock is known to modify the vegetative growth of the scion, which induces a change in the composition of the berries (Clingleffer *et al.*, 2019; Jin *et al.*, 2016). Consequently, the winegrowers must carefully select the appropriate rootstock × scion combination to fulfill their yield and quality criteria.

The most common traits of selection, due to the historical background, are the resistance to diseases and pests (*e.g.*, phylloxera aphid, nematodes, crown gall, phytophthora) and the tolerance to soil constraints (*e.g.*, drought, salinity, limestone, acidity) and texture (*i.e.*, clay, sand, silt) (Reynolds and Wardle, 2001). For instance, Riparia Gloire de Montpellier (*Vitis riparia*) presents poor tolerance to drought and low tolerance to limestone while 140 Ruggeri (*V. berlandieri* × *V. rupestris*) displays high and medium tolerances to drought and limestone, respectively (**Table 1**).

Certain physiological traits (*e.g.*, scion vigor, rooting ability, bench grafting, ripening time) are the primary selection attributes for grape flavors, as these characteristics impact the vegetative cycle, as well as berry size and content (Shaffer, 2004; Zombardo *et al.*, 2020). However, those attributes depend on the rootstock × scion combination used and are therefore difficult to generalize for each variety (Tandonnet *et al.*, 2010). Rootstocks attributes are also famous for their capacity to affect canopy expansion (Soar *et al.*, 2006), pruning weight which is a relevant

indicator of the vegetative grapevine growth (Dias *et al.*, 2017), bud fertility (Al-Obeed *et al.*, 2010), and of course, yield (Bascañán-Godoy *et al.*, 2017). Different balances between the root system and canopy development are observed depending on the rootstock and scion genotypes which impact the uptake of the aerial and belowground resources, hence affecting the wine typicity (Comas *et al.*, 2000; Gaiotti *et al.*, 2016; Grechi *et al.*, 2007).

Some of the most concerning issues in viticulture are related to the climate change and its main problematic lies in the adaptation of grapevine confronted to these environmental constraints while preserving the wine typicity. Once again, the rootstock selection might be the solution to this equation through the help of genetic, genomic, and breeding approaches (Delrot *et al.*, 2020). Breeding programs of cultivars aim also to gather multiple traits of resistance for a sustainable viticulture (Schneider *et al.*, 2019). In France, viticulture is one of the most greedy crop in pesticides and other synthetic protection products in addition to its relatively small cultivable surface (Butault *et al.*, 2011).

Table 1 : Rootstock comparison for traits of agronomic relevance, adapted and modified from Ibacache *et al.* (2020). Not all rootstocks are represented. Scale: Excellent (++); high (+); intermediate (\pm); low (-); P, poor (--).

| Rootstock | Parent species | Resistance | | Tolerance | | Influenced traits | |
|----------------------|---|------------|----------|-----------|-------|-------------------|---------|
| | | Phylloxera | Nematode | Drought | Lime | Scion Vigor | Rooting |
| Riparia Gloire | <i>V. riparia</i> | ++ | -- | -- | - | - | \pm |
| Rupestris St. George | <i>V. rupestris</i> | + | -- | -- | -- | + | \pm |
| Rupestris du Lot | <i>V. rupestris</i> | + | \pm | \pm | -- | + | \pm |
| 420A | <i>V. berlandieri</i> x <i>V. riparia</i> | + | -- | -- | \pm | -- | - |
| 5BB | <i>V. berlandieri</i> x <i>V. riparia</i> | + | -- | - | \pm | -- | -- |
| SO4 | <i>V. berlandieri</i> x <i>V. riparia</i> | + | -- | - | -- | \pm | -- |
| 8B | <i>V. berlandieri</i> x <i>V. riparia</i> | + | -- | -- | -- | \pm | - |
| 5C | <i>V. berlandieri</i> x <i>V. riparia</i> | + | + | - | -- | + | -- |
| 161-49 C | <i>V. berlandieri</i> x <i>V. riparia</i> | ++ | -- | - | \pm | \pm | - |
| 99 R | <i>V. berlandieri</i> x <i>V. rupestris</i> | + | -- | \pm | -- | + | -- |
| 110 Richter | <i>V. berlandieri</i> x <i>V. rupestris</i> | + | -- | ++ | -- | \pm | - |
| 1103 Paulsen | <i>V. berlandieri</i> x <i>V. rupestris</i> | + | -- | \pm | -- | \pm | \pm |
| 140 Ruggeri | <i>V. berlandieri</i> x <i>V. rupestris</i> | + | - | + | \pm | + | -- |

As a matter of fact, cultivar breeding remains an efficient strategy to counteract diseases or pests and to increase resistance to phytopathogens, but is a complex and time-consuming investigation that can last for decades (Cadle-Davidson, 2008; Töpfer *et al.*, 2011). Moreover, several other factors, that could be qualified as dieback-related, afflict the viticulture that rootstock or cultivar breeding cannot solve currently on its own.

1.4. Decline and dieback are linked to environmental and unpredictable stresses but also triggered by human practices

1.4.1. Viticulture whistleblowers

Decline and dieback are both terms used to describe general symptoms of diseases among perennial crops. “Decline” refers to general loss of vigor and quality of the plants with necrotic cankers, “dieback” clearly means the death of branches and sometimes the plant itself. These phenomena are therefore linked to the loss in productivity and fruit quality, especially in viticulture (Bettenfeld *et al.*, 2020).

In France, around 19% of the surface area of graft nurseries has been lost since the last 10 years due to dieback, and this phenomenon led to annual yield losses of about 4.6 hl/ha since 2014. In the 70’s, the French wine production was estimated at 67.5 million of hl while in 2013 the production was close to 44.9 million of hl, meaning that in 40 years, the annual French production has decreased by one third (Rapport mission FAM-CNIV-BIPE, 2016). In response to these alarming facts, the French interprofessional winegrowers with the help of Agriculture Ministry initiated in 2016 the French National Plan against Vine Decline (Plan National Dépérissement du Vignoble; PNDV) with the aim to increase knowledge and solutions towards current grapevine decline and dieback problematics. Many complex factors and interactions lead to the decline and dieback of vineyards. These can be distinguished between biotic and/or abiotic processes.

1.4.2. Biotic factors causing decline and/or dieback issues

Many grapevine diseases and pests are referenced worldwide with well-described symptoms (**Table 2**), and among the most investigated ones are the Grapevine Trunk Diseases (GTDs), which are believed to afflict viticulture since its beginning (Mugnai *et al.*, 1999). The term GTDs encompasses many diseases caused by fungal pathogens in mature and established vineyards (mainly Esca complex, black dead arm, and Eutypiose) or young ones (mostly Petri disease and Black foot). Around 133 fungal species within 34 genera have been found to cause

GTDs. The most famous symptom is the apoplectic form of the vine with chlorotic foliage, but each disease expresses distinct symptoms all depicted in Gramaje *et al.* (2018).

Intensive research has also been made for airborne fungi, namely downy mildew, powdery mildew, black rot, and dead arm, as well as soilborne fungi like the root rot and Black foot diseases. These aerial and telluric diseases account for the largest number of fungicide treatments in vineyard, especially due to the mildew that can spread easily through the sensitive European vineyards supporting *V. vinifera* (Pertot *et al.*, 2017). The bacterial pathogens transmitted by pests, such as grapevine yellows caused by different phytoplasmas (Bois noir, Flavescence dorée) are also well-documented.

Other pathogenic microorganisms are also especially involved in berry degradation such as gray and acid mold diseases and provoke drastic loss on sensitive varieties. Interestingly, under particular conditions, *Botrytis cinerea* can be referred as noble rot which allow the production of highly aromatic and sweet wines, referred as botrytized wines (Magyar, 2011). Apart this beneficial aspect of the disease for some winemakers, the winegrowers seek to control this pathogen.

The most encountered viruses in France are the ones associated to Court-noué (*e.g.*, Grapevine Fan Leaf Virus = GFLV, Arabic Mosaic Virus = ArMV) and leafroll diseases (Grapevine leafroll-associated viruses = GLRaV) which express symptoms of leaves malformation, branches shortening, and vine mortality.

Table 2 : Non-exhaustive list of biotic factors leading or affecting the decline in vineyards by interfering with the vine mortality and/or the berry degradation. Strong effects on vine decline were notated with “++”, while relatively low effect with “+”, and no effect with “-”.

| Biotic factor | Causal agents | Vine mortality | Berry degradation | Reference |
|----------------------------|---|----------------|-------------------|--|
| Esca complex | Many fungal pathogens: <i>Phaeoconiella chlamydospora</i> , <i>Fomitiporia mediterranea</i> ... | ++ | ++ | (Mondello <i>et al.</i> , 2018a; Rooney-Latham <i>et al.</i> , 2005) |
| Black dead arm | <i>Botryosphaeriaceae</i> | ++ | ++ | (Reis <i>et al.</i> , 2019) |
| Eutypiose | <i>Eutypa lata</i> | ++ | ++ | (Živković <i>et al.</i> , 2019) |
| Downy mildew | <i>Plasmopara viticola</i> | + | ++ | (Kennelly <i>et al.</i> , 2007) |
| Powdery mildew | <i>Erysiphe necator</i> | + | ++ | (Guilpart <i>et al.</i> , 2017; Thind <i>et al.</i> , 2006) |
| Black rot | <i>Guignardia bidwellii</i> | - | + | (Ullrich <i>et al.</i> , 2009) |
| Dead arm/Excoriose dieback | <i>Phomopsis viticola</i> | ++ | + | (Úrbez-Torres <i>et al.</i> , 2013) |
| Gray mold | <i>Botrytis cinerea</i> | - | ++ | (Steel <i>et al.</i> , 2013; Vatsa-Portugal <i>et al.</i> , 2017) |

| | | | | |
|---------------------------|---|----|----|--|
| Root rot | <i>Armillaria mellea</i> , <i>Rosellinia necatrix</i> , <i>Roesleria subterranea</i> | ++ | + | (Aguín <i>et al.</i> , 2006; Freire Cruz and Carvalho Pires, 2014; Neuhauser <i>et al.</i> , 2011) |
| Black foot | <i>Ilyonectria liriodendri</i> and <i>Dactylonectria macrodidyma</i> | ++ | + | (Probst <i>et al.</i> , 2019) |
| Petri disease | Many fungal pathogens: <i>P. chlamydospora</i> , <i>Phaeoacremonium</i> spp... | ++ | + | (Pilar Martínez-Diz <i>et al.</i> , 2021) |
| Bacterial blight | <i>Xylophilus Ampelinus</i> | ++ | ++ | (Bisztray <i>et al.</i> , 2012; Szegedi and Civerolo, 2011) |
| Flavescence dorée | Phytoplasma <i>Candidatus vitis</i> transmitted by leafhopper <i>Scaphoideus titanus</i> | ++ | ++ | (Oliveira <i>et al.</i> , 2020; Quiroga <i>et al.</i> , 2017) |
| Bois noir | Phytoplasma <i>Candidatus solani</i> transmitted by planthopper <i>Hyalesthes obsoletus</i> | ++ | ++ | (Hren <i>et al.</i> , 2009; Padovan <i>et al.</i> , 1996) |
| Crown gall | <i>Agrobacterium vitis</i> | ++ | ++ | (Diana and Dejeu, 2011; Filo <i>et al.</i> , 2013) |
| Pierce disease | <i>Xylella fastidiosa</i> | ++ | + | (Hopkins and Purcell, 2002; Wallis and Chen, 2012) |
| Court-noué | GFLV, ArMV both transmitted by nematodes <i>X. index</i> and <i>X. Diversicaudatum</i> , respectively | ++ | ++ | (Digiario <i>et al.</i> , 2017) |
| Leafroll disease | GLRaV, transmitted by mealybugs | + | ++ | (Angelini <i>et al.</i> , 2017; Porotikova <i>et al.</i> , 2019) |
| Grapevine Fleck Virus | Maculavirus | + | ++ | (Martelli, 2017) |
| Other viruses | RSP, KSG, GCB, GVN, GVM | + | + | (Mannini and Digiario, 2017; Meng and Rowhani, 2017) |
| Grill leafhopper | <i>Empoasca vitis</i> | - | + | (Román <i>et al.</i> , 2021) |
| Phylloxera | <i>Daktulosphaira vitifoliae</i> | ++ | ++ | (Forneck <i>et al.</i> , 2017) |
| <i>Drosophila suzukii</i> | <i>Drosophila suzukii</i> | - | ++ | (Tonina <i>et al.</i> , 2020) |
| Grape berry moths | <i>Eudemis</i> , <i>Cochylis</i> , and <i>Eulia</i> | - | ++ | (Kreiter, 2018) |
| Grape rust mites | Many species: erinose, acariosis, yellow, or red mites | - | ++ | (Schreiner <i>et al.</i> , 2014) |

1.4.3. Abiotic factors causing decline and/or dieback issues

Grapevine crops are often subject to suboptimal growing parameters, referred as abiotic constraints, which are mainly related to climate change. It has been observed that phenological stages were advanced, compared to historical data, due to the rise of temperature, resulting sometimes in altered grape composition and thus reduced wine quality (van Leeuwen and Darriet, 2016). Moreover, 75% of the French vineyard surface is planted with grape varieties

considered as sensitive, but other European vineyards are also concerned (FAM-CNIV-BIPE, 2016).

However, many agricultural practices are also linked to the vineyard predisposition to decline or dieback (**Table 3**).

Table 3 : Overview of some abiotic factors leading or affecting the decline in vineyards.

| Factors | Link with decline | Reference |
|--|---|--|
| Genetic anomalies (Variegation, fasciation) | Discoloration of organs including berries (= variegation). Flattening and grouping of stems, petioles, and pedicels (=fasciation). | (Martin <i>et al.</i> , 2021) |
| Physiopathology (Coulure, millerandage) | Abnormal post-flowering fall of fertilized ovaries and young berries (= coulure). Reduced development of some berries (= millerandage). | (Ibáñez <i>et al.</i> , 2019; Tello <i>et al.</i> , 2021) |
| Fallow practice | Recommended to reduce soil borne pathogens. | (Liu <i>et al.</i> , 2021) |
| Vineyard selection (Soil agronomic histories and topology) | Determine in part the berries yield, their quality, and the vines longevity. | (Chrobak <i>et al.</i> , 2020) |
| Rootstock × scion combination | Determine a balanced vigor and quality. Some combinations are incompatibles and more sensitive to decline. | (Marín <i>et al.</i> , 2021) |
| Earthwork/hedges/shutters, planting density, orientation of the rows | Reduce the risk of contamination by pathogens and can thus increase vines longevity. | (Kobus Hunter <i>et al.</i> , 2020; Petrov <i>et al.</i> , 2017) |
| Drain | Water drain reduces the possibility of root asphyxia in case of temporary waterlogging, which can lead to a decrease in yields and grape quality. | (Dambros <i>et al.</i> , 2016) |
| Canopy management | Improves quantity and quality by better exposure of the leaves to the sun. | (France <i>et al.</i> , 2018) |
| Management of missing vines | Different replacement strategies of the missing vines to regain the vineyard productivity. | (Sisterson and Stenger, 2013; Waite <i>et al.</i> , 2018) |
| Tillage | Soil compaction or decompaction have an impact on the root system of the vine and therefore its yield. | (Myburgh, 2013; Ostandie <i>et al.</i> , 2021) |
| Cover crop | Controls susceptibility to certain diseases but can also a habitat for pests and pathogens if not managed properly. | (Linares Torres <i>et al.</i> , 2018; Richards <i>et al.</i> , 2020) |
| Soil amendment | An adapted amendment to the soil conditions improves the yield. | (Gaiotti <i>et al.</i> , 2016; Pereg <i>et al.</i> , 2018) |
| Irrigation | Influence on the wines typicity during the different phenological stages and impact vine adaptation to hydric stresses. | (Chaves <i>et al.</i> , 2010, 2007) |
| Climate change | Influence on the phenological stages of the vine. | (Mosedale <i>et al.</i> , 2016, 2015) |
| Frost | Decreased yield, or even harvest destruction. | (Molitor <i>et al.</i> , 2014) |
| Water stress | Decreased yield and reduced quality of the grapes. | (Lovisolo <i>et al.</i> , 2010; Pagay <i>et al.</i> , 2016) |
| Salinity | Burning symptoms of the edge of the leaves with necroses, and leaves fall which can lead to yield losses and even to death. | (Aragüés <i>et al.</i> , 2015; X. Zhang <i>et al.</i> , 2002) |

Vineyard settlement is the first step in preventing from decline with a soil prospection to unravel the suitability of the soil to support vineyard. The establishment of the vineyard on natural

slopes, or on man-made terraces system, is known to optimize solar radiation upon vines even if the risk of soil erosion is higher due to runoff (Corti *et al.*, 2011; Lazcano *et al.*, 2020). One of the first criteria for site selection is soil topography, which provides insight into the roots capacity to explore the soil. Furthermore, analysis on the soil composition gives guidance to select the most relevant rootstock × scion combination in line with the production objectives. In addition, the choice of biological material must be exempted of pathogens or genetic anomalies, which is mainly affected by nurseries management. Prior to planting, fallowing is counseled when the vineyard site is replanted with grapevines or other fruit trees, as it can suppress the soilborne pathogens and restore the soil microbial and nutrient pool.

Subsequently to vine plantation, it usually takes three years to obtain the first harvest, and ten more years are needed before reaching the full vineyard potential. To maintain the vines health and prevent the apparition of decline symptoms, an adequate management of the canopy, amendment, and cover crop should be operated. Altogether, defining the agricultural practices is consequently primordial to a reliable settlement in order to optimize the yields, the grapes quality, and to ensure soil of good quality, which is the matrix of a perennial viticulture (Lazcano *et al.*, 2020; White, 2015).

2. The soil as a complex ecosystem

2.1. Soil quality is a pillar to plant fitness

Soils have been observed since the dawn of agriculture and their agronomic properties were already classified back in the old civilizations. The first Chinese soil classification based on soil fertility, color, and texture is 4,000 years old, while Theophrastus, one of the Aristotle students, wrote the first recorded manual of land management with agronomic soil properties (Brevik and Hartemink, 2010; Krupenikov, 1993).

Soil erosion is a well-known problematic and has been reduced through millenniums with specific agricultural practices such as invention of the ard plough by the Babylonians around 6,000 to 4,000 B.C., or the construction of bench terraces by the Phoenicians around 1,200 to 800 B.C. (Brevik and Hartemink, 2010). Nowadays, Borrelli *et al.* (2017) estimated an annual average potential soil erosion amount of 36 billion tons driven by spatial changes of land use such as water and wind erosion.

Sometimes referred as the skin of Earth, soil is one of the most interactive matrix on Earth and provide many ecosystemic services, including nutrient regulation for plant, and defense against plant pathogens (Faucon *et al.*, 2017). Soil health, or soil quality, corresponds to the soil's capacity to maintain its ecosystemic functions towards plants, animals, and humans, and is estimated with a large panel of physical, chemical, and biological indicators (Stewart *et al.*, 2018). These indicators assess the primordial features of soil quality that are mainly based on underground biodiversity, nutrient availability, soil structure, and water regulation (**Figure 5**).

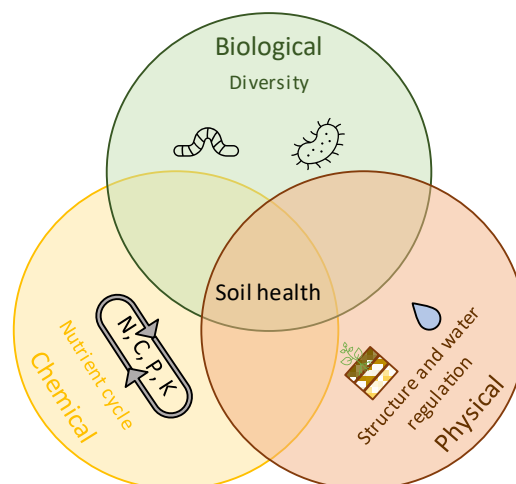


Figure 5 : The triptych of soil health indicators represented by biological, chemical, and physical factors.

Many scientists support the idea that defining all the ideal quality indicators for each type of soil is impossible due to its complexity and its site-specificity factors related to climate (Bünemann *et al.*, 2018; Karlen *et al.*, 2008). However, many universal techniques are available to assess these indicators and can give an overview of the soil biochemical properties and provide a diagnosis of soil capacity to support crop system.

2.1.1. Physical indicators as the framework to grapevine development

Physical indicators of soil health are mostly related to soil structure, tilth, surface covering and comprise horizon texture, rooting depth, morphological features, aggregate stability, penetration resistance, and bulk density (Arshad *et al.*, 2015). Some others are water related such as water content, infiltration, hydraulic conductivity, or field water storage capacity (Lowery *et al.*, 2015).

Texture is the main soil physical property and permits its classification according to the USDA triangle method based on the fractions of clay, sand, and silt (**Figure 6.A**). Agronomists usually investigate the subsoil layers, namely horizons, with a vertical pit (**Figure 6.B**) in order to create a so-called “agronomic profile” (Peigné *et al.*, 2013). This method allows deciphering the layered horizons that have different roles in the biological and geochemical processes. These horizons usually describe the sharpness of boundaries, the soil color and mottling, the percentage and distribution of the stones, the presence of carbonate or any other salts deposits. Samples of each horizon are also collected to additional laboratory analysis. These layers are often visible to the naked eye (**Figure 6.B**), corresponding to different composition from the topsoil, which is enriched with organic matter from the plant litter decomposition, to the below horizons until the parent material.

The surface of topsoil has an impact on wine production since it can manage heat transfer and storage properties of the soil. For instance, stony soils reverberate heat if they are whitish as the *galets* in Chateauneuf-du-Pape (France) or the *codols* in Monsant (Spain), at the opposite of dark colored soils heating rapidly and promoting vines ripening as in Franconia district (Germany) (Maltman, 2008). Grapevine has the peculiarity to grow on large types of conditions that harbor different soil physical characteristics such as hyper arid Chilean Entisol (Verdugo-Vásquez *et al.*, 2021), semi-arid Spanish Xeric Haplocalcids (Marín-Martínez *et al.*, 2021), or even tropical Brazilian Ultisol (Silva *et al.*, 2018).

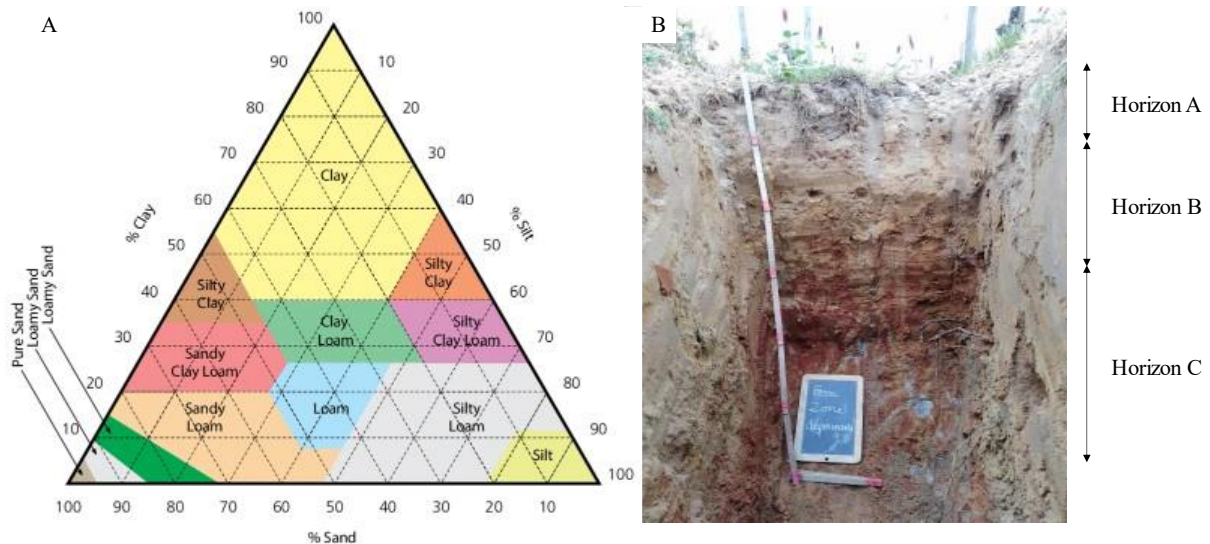


Figure 6 : (A) USDA classification of the soil texture, from (Durner, 2017) and (B) example of a pit, used to generate an agronomic profile (personal picture). Horizon A is called the topsoil and differs in activity compared to deeper horizons B and C which can be distinguished by naked eye, and by their texture.

The upper horizon is also a region of intense biological activity and is rich in soil organic matter (SOM). SOM displays crucial biological and physicochemical properties that reside in its capacity to store carbon and nutrients for plant development, and its action as a cementing agent for soil stability, making this complex a centerpiece component that promotes soil aggregation (Sarker *et al.*, 2018), soil biological diversity (Bending *et al.*, 2002), and nutrient availability (Tiessen *et al.*, 1994). However, land degradation in vineyards is worldwide and often associated with loss of SOM because of management practices resulting in topsoil compaction and reduced water infiltration capacity that aggravate soil erosion (Ferreira *et al.*, 2020). These soil compaction and incapacity of water infiltration drastically inhibit root propagation beneath soil, affecting the aboveground compartments and berries quality (Lipiec and Hatano, 2003).

2.1.2. Chemical indicators reflect the soil capacity to provide nutrient to grapevine

Soil physical parameters are closely related to chemical ones, which are linked to plant nutrition and soil toxicity. Indeed, the availability of trace elements (Cu, Zn, Mn, Fe, Al, B, S, Mo) and nutrients (N, P, K, Ca, Mg, Na, NO₃-N, extractable NH₄) for their absorption by the vine depends mainly on the so-called pH (**Figure 7.A**), which is the hydrogen-ion activity measured in a soil suspended in a solution, and the cation exchange capacity (CEC), representing the soil's buffer capacity to hold positively charged nutrients (**Figure 7.B**). The pH modulates the solubility of soil minerals and depends mainly on the parent material, soil leaching and SOM content.

The pH is often linked to the CEC which modulates the exchange of hydrogen ions between the plant and the host for the absorption of nutrients by the roots. A higher CEC corresponds to a higher clay content, while a lower CEC is related to a high sand fraction in the soil (Arias *et al.*, 2005). High CEC can lead to symptoms of toxicity when the threshold for nutrient uptake by the vine is exceeded or favored over other nutrients (Oliver *et al.*, 2013; Schoenholtza *et al.*, 2000). Soil toxicity can also be related to trace element, which refers to the elements that have, once present in a certain quantity in soil, negative impact on plant and microorganisms development (Acosta-Martínez and Tabatabai, 2001).

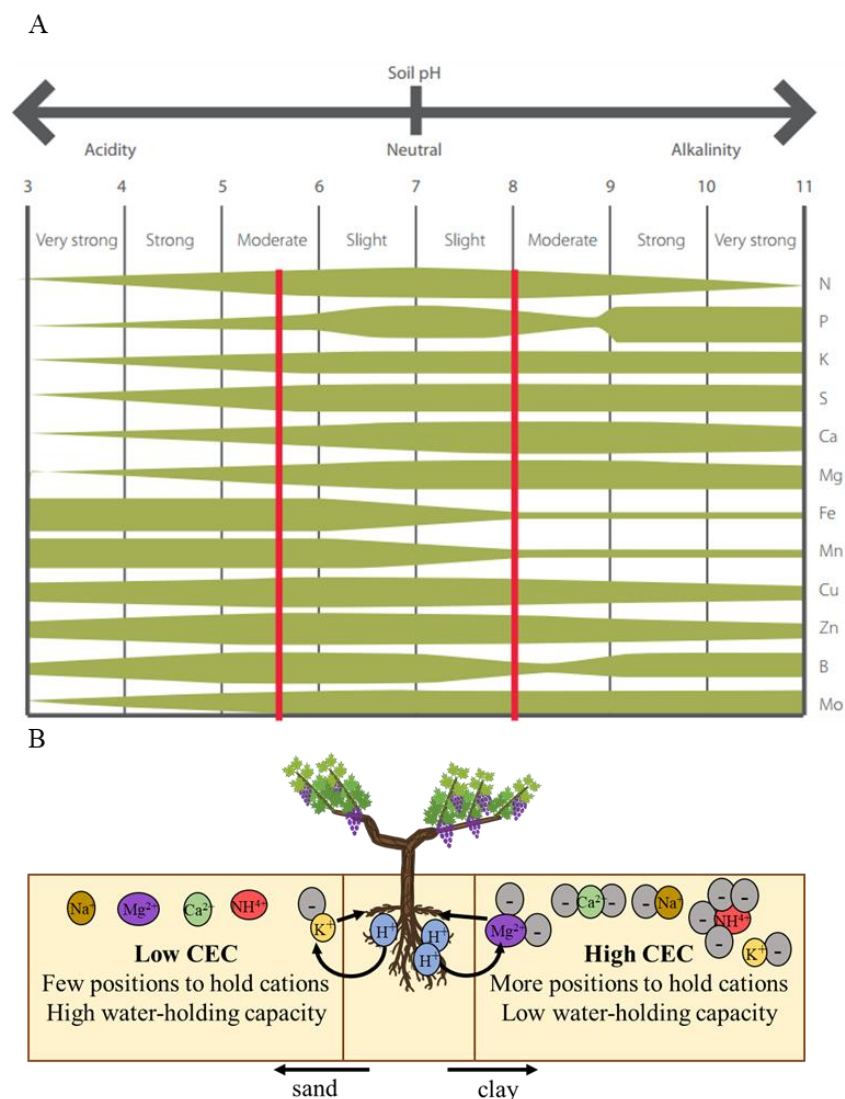


Figure 7 : (A) The effect of soil pH on the availability of nutrients to grapevines originated from Proffitt and Campbell-Clause (2012). In red is represented the optimal range for nutrient uptake by grapevine, i.e., between 5.5 and 8. (B) The effects of CEC on plant cationic nutrient uptake, where more hydrogen ions are required for positively charged nutrients.

In regard to soil toxicity, vineyards have a long history with chemical treatments due to grapevine sensitivity to a great number of pathogens. Heavy metals contamination in soils can be of natural origin or due to human practices, especially in viticulture. Their presence in soil can be persistent over the long term and most of them bioaccumulates because they do not decompose through biological processes (Abioye, 2011). Conventional vineyards are mainly treated with Cu- and Zn-based fungicides such as the famous Bordeaux mixture ($\text{CuSO}_4 + \text{Ca}(\text{OH})_2$) used as foliar application over the last century as high as 50 kg per hectare per year. In 2018, the European Union decreased its soil application from 6 to 4 kg of Cu per hectare per year over seven years. This intensive use of copper-based fungicides ended up with concentrations of Cu in soils exceeding the thresholds authorized by European legislation in a large majority of vineyards (Droz *et al.*, 2021; Komárek *et al.*, 2010). For instance, uncontaminated soils display concentration of Cu ranging from 2 to 40 mg per kg of soil, while 15% of European vineyards exceed 100 mg of Cu per kg of topsoil (Ballabio *et al.*, 2018).

2.1.3. Enzymatic activities as relevant biological indicators of soil health

The biological indicators to assess the soil quality are globally distinguished into the assessment of microfauna, macrofauna, and microbes. While the soil physicochemical parameters provide the framework to plant growth, the biotic parameters modulate this framework and adapt to environmental conditions. One of the complexity and beauty of the soil kingdom is that inversely, the abiotic parameters trigger the biogeochemical processes of the microbial communities and therefore shape the soil microorganisms' diversity (Falkowski *et al.*, 2008; Schimel *et al.*, 2007).

The microbiological indicators of soil are mainly based on microbial biomass, and microbial enzyme activities. Enzymes furnish relevant insights in soil ecosystem activity since they can be produced by living microorganisms or delivered as free enzymes by dead microbes. Microbial enzyme activities reflect metabolic factors and may serve as early indicators of soil quality improvement or degradation in agroecosystems (Alkorta *et al.*, 2003). These soil metabolic activities drive the plant decomposition, carbon, and nitrogen cycling, nutrient availability, and soil productivity. Soil enzymology discipline has been extensively studied since the late XIXth century, and no major breakthrough has been made in the last thirty years (Nannipieri *et al.*, 2018). Several enzymes have been used as standardized biological indicators involved in SOM dynamics and soil biogeochemical cycling (**Table 4**).

Phosphorus is a macronutrient needed for biological sustain of all organisms and its limited resource in soil makes it primordial for terrestrial ecosystems (Filippelli, 2009). This nutrient

is available in two forms, inorganic and organic. Inorganic phosphorus is required for all forms of life, and only inorganic orthophosphate anion PO_4^{3-} is used by plants. Phosphomonoesterases, distinguished between acid and alkaline phosphatases, play key roles in the release and solubilization of phosphorus through the hydrolysis of ester and anhydrides of phosphoric acid from organic compounds (Eivazi and Tabatabai, 1977).

Table 4 : Common enzymes used as biological indicators of soil quality.

| Hydrolase name | Role | Substrate used | Product measured | Reference |
|--------------------------------------|-----------------------------|--|---------------------|---------------------------------------|
| Acid and alkaline phosphatases | Organic P mineralization | ρ -Nitrophenyl-phosphate | ρ -nitrophenol | (Acosta-Martínez and Tabatabai, 2015) |
| β -glucosidase | Cellulose degradation | ρ -Nitrophenyl- β -D-glucopyranoside | ρ -nitrophenol | (Eivazi and Tabatabai, 1988) |
| N-acetyl- β -D-glucosaminidase | Chitin degradation | ρ -Nitrophenyl-N-acetyl- β -D-glucosaminide | ρ -nitrophenol | (Parham and Deng, 2000) |
| Arylsulfatase | Mineralization of organic S | ρ -Nitrophenyl sulfate | ρ -nitrophenol | (Tabatabai and Bremner, 1970) |
| Arylamidase | Nitrogen mineralization | L-Leucine β -naphthylamine | 2-naphthylamine | (Acosta-Martínez and Tabatabai, 2001) |

Regarding the carbon cycling, β -glucosidase takes part in the degradation of SOM and plant residues. This hydrolase catalyzes the degradation of cellulose into simple sugars, reflecting the decomposition of plant residues in soil (Stott *et al.*, 2010).

Nitrogen is primordial for most agricultural crops but only trace quantities are available in its mineral form, while its organic form is a major component of SOM and may account for greater than 95% of the total N in most topsoils (Acosta-Martínez, 2000). Arylamidase activity in soils strikes as being a key enzyme involved in N mineralization since this hydrolase releases some amino acids from SOM that are also substrates for other catalyzers taking part in N cycling (Acosta-Martínez and Tabatabai, 2001).

Altogether, these soil enzymes highlight the ability of microorganisms to metabolize nutrients for plant development, but other specific tools exist for monitoring the microbial belowground communities that reflect soil biodiversity and health.

2.2. Cultivable approaches to study the microbial communities

2.2.1. Medium-based techniques to quantify active microorganisms

The use of selective culture media to study the specific composition of microbial communities is subject to debate since only a small fraction of the microorganisms can be cultivable (Ritz, 2007). Although these methods provide results that are not very representative of the communities, they are still used to study the function and ecological contribution of species or population groups in soil (Armalytè *et al.*, 2019; Moreno *et al.*, 2021; Siebers *et al.*, 2018).

The *in vitro* multiplication of microorganisms is operated by the spreading of an environmental sample on a culture medium and is usually used for clonal isolation or for the estimation of the level of populations of cultivable microorganisms. The quantification of microorganisms, especially bacteria and fungi, from culture media is based on the principle that microorganism, after incubation and multiplication, generates a macroscopically detectable colony, namely Colony Forming Units (CFUs) (**Figure 8**). To reduce variability due to moisture between soil samples, CFUs are enumerated from fresh soil but are then usually expressed from dry soil.

Microorganisms need basic nutrients and a source of energy to turn on the cellular machinery. Some microbes use carbon (*e.g.*, sugars, starches, carbohydrates, organic acids), nitrogen (*e.g.*, urea, ammonia), or even light as source of energy (Burgin *et al.*, 2011; Christie-Oleza *et al.*, 2017). Even though a single medium cannot reproduce all the environmental conditions required for all the indigenous microorganisms' growth, there are some generic medium composition and specific conditions available to standardize the cultivation of microorganisms (Davis *et al.*, 2005; Pham and Kim, 2016). Glucose in its dextrose form is usually used as carbon source while nitrogen sources include peptone, yeast extract, or amino acids.

The most common media used for bacterial cultivation from soil are nutrient broth, Luria Bertani, tryptic soy broth, mineral salts medium, and Reasoner's 2A agar (R2A) which vary in carbon and nitrogen source composition as well as trace elements concentration. In regard to fungi, the usual media found for their appropriate growth are potato dextrose agar (PDA), corn meal agar, potato carrot agar which vary in their carbohydrate composition. Therefore, these culture media allow the quantification and isolation of microorganisms present in the soil, and can also be used to further identify those isolates.

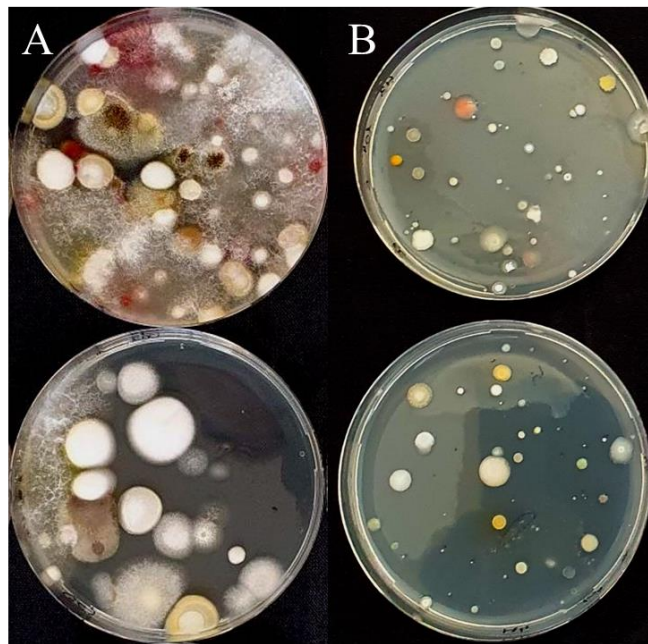


Figure 8 : Plates filled with (A) Potato Dextrose Agar (PDA) generating fungal CFUs and (B) Reasoner's 2A Agar (R2A) medium generating bacterial CFUs spread with soil suspension, from personal pictures.

2.2.2. MALDI-TOF coupled to MS permits fast species identification of isolates

One of the most widely used technique to analyze biomolecules is the matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) coupled to mass spectrometry (MS). This methodology, based on peptide mass fingerprint (PMF) profile, enables fast and reliable identification of fresh isolates which can be bacteria, yeasts, and sometimes filamentous fungi (Singhal *et al.*, 2015). Some microorganisms can be identified directly by MS, namely direct cell profiling, while for some others, whole complete cell extraction or semi extraction steps are required for optimal identification.

The process of semi-extraction method is depicted in **Figure 9**, and starts with the sampling of fresh microorganisms since it is known that the content of ribosomal proteins changes under nutrient deficiencies and long-time storage on agar plates (Pavlovic *et al.*, 2013). The isolates are loaded on stainless steel MALDI plate (**Figure 9.B**) and are mixed first with formic acid to extract proteins that are subsequently entrapped and crystallized within an organic compound called matrix (**Figure 9.C**). Once samples are prepared, they are charged into the MALDI-TOF-MS complex (**Figure 9.D**) and are subjected to short laser pulse (**Figure 9.E**) in order to prepare the extracted proteins for desorption (**Figure 9.F**) and ionization (**Figure 9.G**). These steps generate singly protonated ions that are accelerated (Figure 9.H) and separated according to the ratio mass to charge of ionized (m/z^+) proteins and peptides (**Figure 9.I**) which are measured by MS and TOF analytical technique. The mass ranges between 2 and 20 kDa, which reflects

essentially ribosomal proteins, and m/z ratio measurement is determined with the time taken for traveling the length of the flight tube until the TOF detector (**Figure 9.J**). Finally specific spectra, namely PMFs, are generated and compared to database containing PMF of known microbial isolates (**Figure 9.K**), enabling the identification of tested isolates if the PMF is recognized.

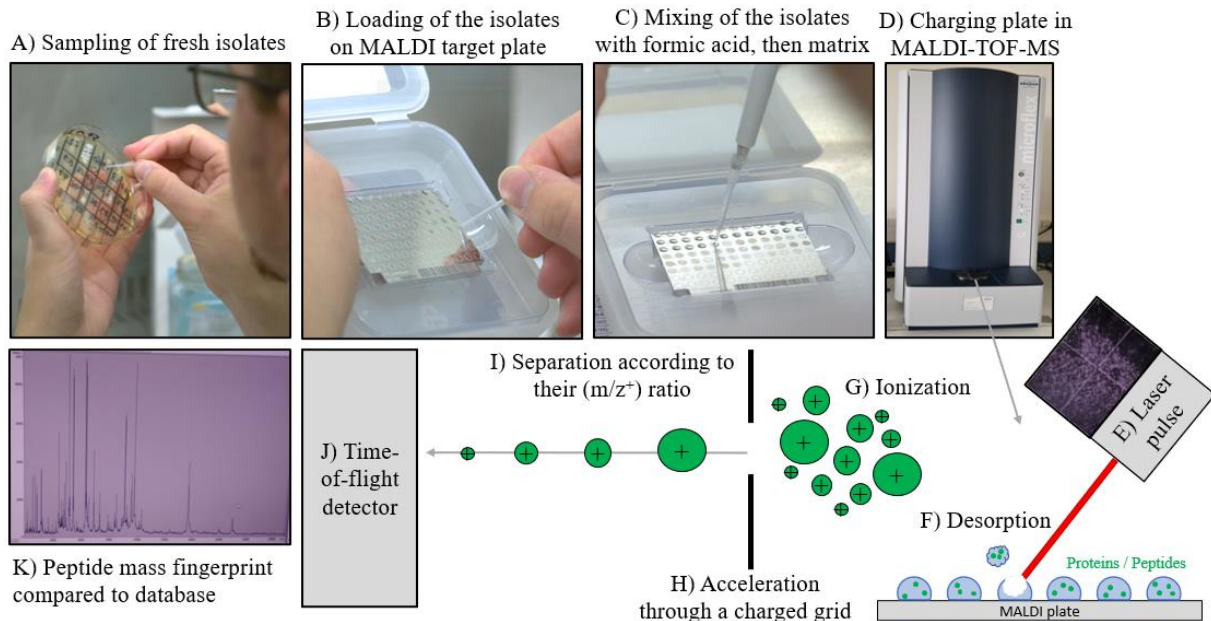


Figure 9 : Schematic process of the MALDI-TOF-MS workflow, from the sampling of fresh isolates to the peptide mass fingerprint acquisition, illustrated with personal pictures.

This recognition relies on the PMF provided in the used database, and its matching is rated with a score value ranging from 0 to 1.7 for unrecognized PMF, 1.7 to 2.0 for genus identification, and above 2.0 for species recognition (Nagy *et al.*, 2012). MALDI-TOF coupled to MS has a large scope of application and is often operated in routine in agri-food and medical industries for pathogen detection in clinical laboratories and quality checking for food safety (Singhal *et al.*, 2015). This technique is also used to detect yeast and specific bacteria in wine and grape must with high reliability (Gutiérrez *et al.*, 2017; Kačániová *et al.*, 2020), as well as bacteria isolated from vineyard soil (Chong *et al.*, 2016; Martins *et al.*, 2020; Oyuela Aguilar *et al.*, 2021). MALDI-TOF-MS is therefore an interesting technique to combine with plating method for the identification of isolates.

2.2.3. Biochemical assays and cultivable-based methods to isolate effective microbial consortium with functional traits

The study of soil microbial community can be carried out from a point of view of taxonomic diversity but also from a perspective of metabolic potential. The correlation between these two

approaches has been the subject of numerous research works in various types of soil (Çakmakçi *et al.*, 2010; Goyal *et al.*, 2021; Jeanbille *et al.*, 2016). Therefore, a large panel of medium is used in the isolation of microorganisms having contrasted metabolic processes through selection with biochemical assays. This property is the pillar element of screening and characterization of microbial consortium having beneficial effects on plant health, also known as plant-growth promoting (PGP) traits (**Table 5**). These PGP traits have been usually attributed to microorganisms inhabiting the rhizosphere, which is the tight portion of soil close to the roots. More information related to rhizosphere are discussed lately in part 3, entitled “Interaction between grapevine rootstock and soil microorganisms”.

Ethylene is a plant hormone that is expressed under stress and diverse mechanisms of regulation to reduce plant growth. Its direct precursor is 1-aminocyclopropane-1-carboxylic acid (ACC) which can be degraded by ACC deaminase, thus reducing ethylene production in the plant (Orozco-Mosqueda *et al.*, 2020). ACC deaminase is produced by certain soil microorganisms such as bacteria, fungi, or yeast, underscoring the ability of soil microorganisms to promote plant growth. Another primordial phytohormone is the auxin indole-3-acetic acid (IAA), produced by plants for several regulatory parameters such as root development, cell division and elongation, apical dormancy or differentiation of vascular tissues (Aloni *et al.*, 2006). IAA can also be produced by microorganisms based primarily on L-tryptophan precursor (Naveed *et al.*, 2015).

Unlike ethylene and IAA, ammonia (NH₃) directly promotes plant growth as it acts as a macronutrient and a source of nitrogen that is widely used as fertilizer in agricultural crops (Ojha and Dhiman, 2010). Other nutrient necessary for plant development (*e.g.*, nitrogen and phosphorus) can be increased by telluric microorganisms. Indeed, specific species of bacteria belonging mainly to *Pseudomonas*, *Bacillus*, *Rhizobium*, or even *Burkholderia* convert insoluble phosphates (*e.g.*, tricalcium phosphate, dicalcium phosphate, hydroxyl phosphate) to an assimilable form for the plant (Hayat *et al.*, 2010), while some *Rhizobia* form mutualistic symbiosis with roots to fixate atmospheric N₂ into usable nitrogen form (Zaidi *et al.*, 2015). In regard to iron uptake by plants, the microbial siderophore convert insoluble iron to its soluble form which is a growth promoter for plant but also a molecule for pathogen control (Sayyed *et al.*, 2013).

Table 5 : List of common PGP traits in microbial species characterized using culture-dependent methods.

| PGP trait | Media used (Condition) | Reference |
|--------------------------|--|--------------------------------|
| ACC deaminase | DF Minimal salts pH 7.2 (ACC as nitrogen source) | (Penrose and Glick, 2003) |
| IAA synthesis | Unspecific medium (Salkowski reagent as pH and colored indicator) | (Gordon and Weber, 1951) |
| Ammonia production | Peptone water (Nessler's reagent as pH and colored indicator) | (Cappuccino and Sherman, 1992) |
| Nitrogen fixation | NfB pH 6.8 (bromothymol blue as pH and colored indicator) | (Döbereiner, 1989) |
| Phosphate solubilization | Pikovskaya medium pH 7 (bromophenol blue as pH and colored indicator) | (Pikovskaya, 1948) |
| Siderophore production | Chrome-azurol S medium (FeCl ₃ ·6H ₂ O as iron source and chrome azurol as pH and colored indicator) | (Schwyn and Neilands, 1987) |

Plant growth-promoting rhizobacteria (PGPR) have been isolated from the rhizosphere of numerous crops such as maize (Agbodjato *et al.*, 2015), tomato (Guerrieri *et al.*, 2020) and of course grapevine (Funes Pinter *et al.*, 2017; Salomon *et al.*, 2014). The beneficial role of PGPR has been well discussed in the literature and is known to improve crop productivity, berry quality, plant health, and undoubtedly soil quality (Kumari *et al.*, 2019). Therefore, the functional diversity of a soil reflects its capacity to perform bio-geochemical process essential for its quality and thus the health of crop.

2.2.4. Eco-Plates from Biolog™ system to study metabolic activities of microbial communities

Enzymatic activity measurement is a common method to study soil functional diversity through screening of individual microorganisms with PGP traits. Analysis of metabolic diversity of a community can also be achieved by examining the catabolic behavior with respect to several substrates. Degradation data of these substrates can be gathered to form a metabolic profile. Carbon, nitrogen, and organic compounds being a key factor in several microbial ecosystems, their consumption as substrates appear to be suitable for the determination of the metabolic versatility of an environmental sample. The metabolic fingerprints generated by this method, named community level physiological pattern (CLPP) (Lehman *et al.*, 1997), has been used to study the quality of vineyard soil (Lagomarsino *et al.*, 2012; Viti *et al.*, 2008).

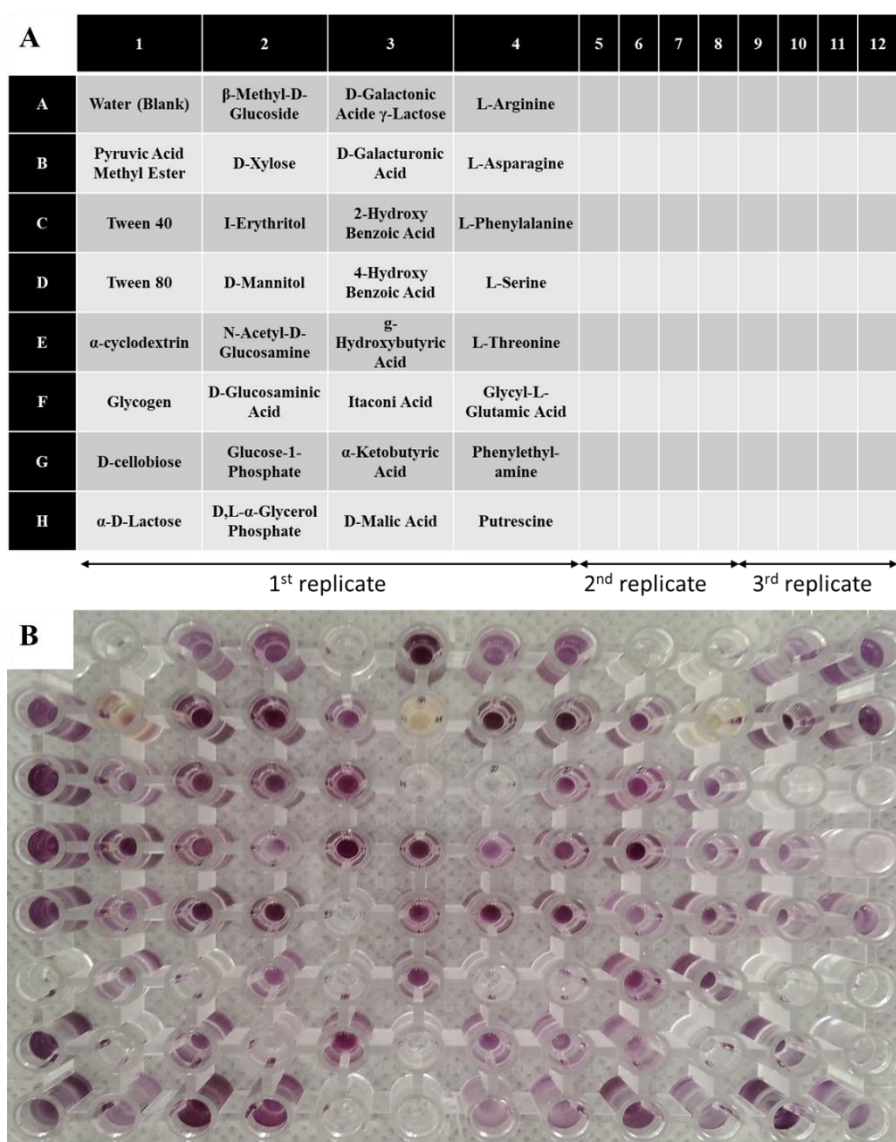


Figure 10 : (A) Carbon source pattern of Eco-Plates from Biolog™ system and (B) Eco-Plates after 96h of incubation with soil suspension, from personal picture.

An interesting, low cost, and fast-response approach to use is the Eco-Plates from Biolog™ system which are 96-well plates containing one blank and 31 sources of carbohydrates, amino acids, carboxylic acids, amines, amides, and polymers in three replicates (**Figure 10.A**). These several substrate sources permit microbial communities to be characterized, subsequently to the inoculation of soil suspension, according to their CLPP determined by colorimetric redox reactions based on tetrazolium violet indicator. The more important the substrate consumption is, the higher the color intensity is (**Figure 10.B**). Kinetics of substrate consumption can also be made to visualize the microbial capacity to degrade components through time.

Eco-Plates were used in vineyards to assess soil quality (Capó-Bauçà *et al.*, 2019; Jacometti *et al.*, 2007), or the effect of some chemicals on the functioning of vineyard soil (Guo *et al.*, 2015;

Xie *et al.*, 2009). This approach is therefore interesting to use for the assessment of soil functionalities inherent to microbial populations.

2.3. Molecular approaches to unravel the microbial composition

2.3.1. Molecular markers for microbial identification and quantification

Profiling of microbial communities using culture-independent methods has been used for decades with several approaches such as phospholipid fatty acid biomarkers (Willers *et al.*, 2015), amplified ribosomal DNA restriction analysis (ARDRA) (Wu *et al.*, 2006), terminal restriction fragment length polymorphism (T-RFLP), or denaturing gradient gel electrophoresis (DGGE) (Siqueira *et al.*, 2017). Studying microbial communities is also performed with multi-omics approaches which are relatively recent technologies based on molecular techniques coupled to computational tools (*e.g.*, metagenomics, metaproteomics, metatranscriptomics) (Gutleben *et al.*, 2018). Culture-independent methodologies do not stop to get meliorated with for instance the emergence of integrative tools such as ChocoPhiAn 3 for taxonomic, functional, strain-level, and phylogenetic profiling (**Figure 11**). Despite the technology and time differences between these methods, they share certain characteristics, such as the use of molecular markers for microbial profiling.

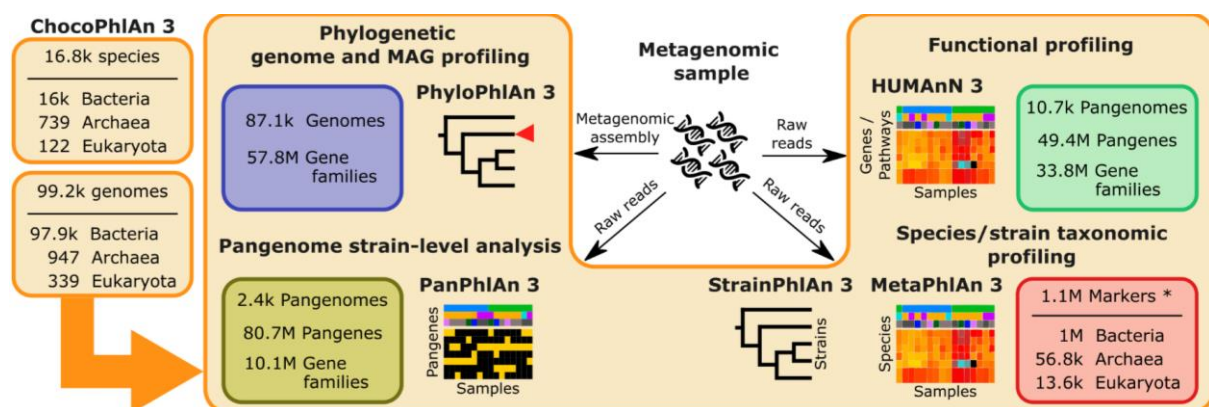


Figure 11 : ChocoPhiAn 3 principle using metagenomic sample to draw up a microbial profile of an environmental sample including phylogenetic genome, strain-level pangenome, taxonomic profiling and functional diversity, from Beghini *et al.* (2021).

Among different possible molecular markers, rDNA encoding ribosomal RNAs has proven to be the molecular target of choice for ecological studies (Rastogi and Sani, 2011). These sequences are ubiquitous to all organisms, with a conserved function associated to rRNA coding, composing the structure responsible for protein synthesis, namely the ribosomes. These genes have the advantage of being made up of both highly conserved fragments suitable for annealing sites for the corresponding polymerase chain reaction (PCR) primers, which target

specific microbial communities (*e.g.*, fungi, bacteria, archaea), and variable domains allowing the distinction of species within the community used for phylogenetic differentiation (Liu *et al.*, 2012; Rastogi and Sani, 2011). Ribosomal DNA genes are grouped as an operon, which may be present in multiple copies also known as tandem repeats (**Figure 12**).

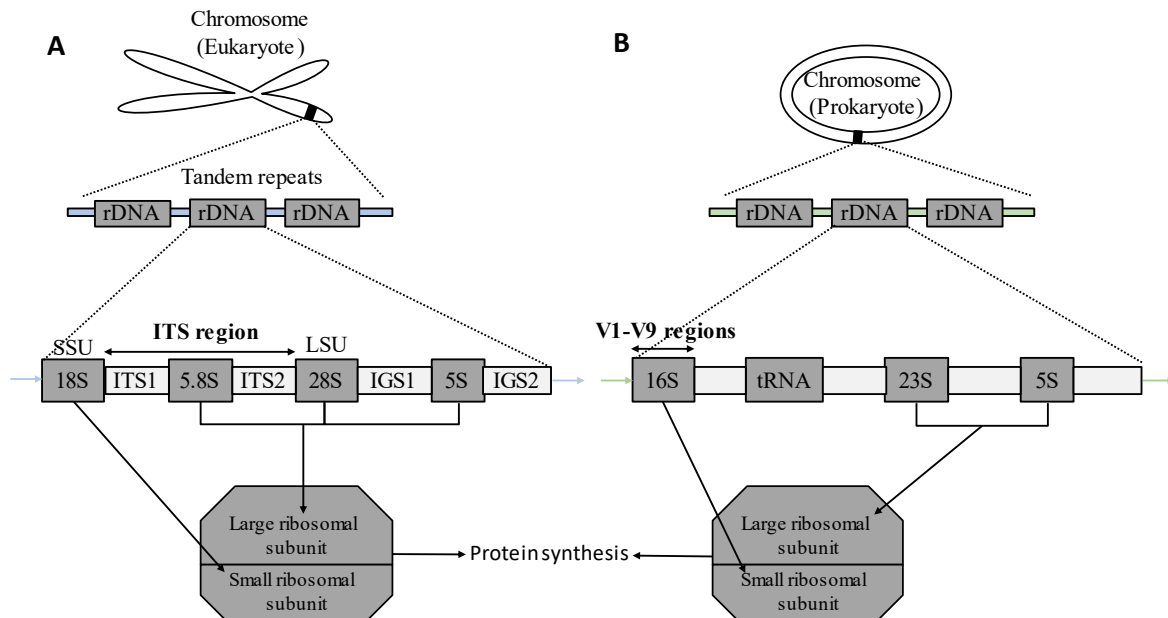


Figure 12 : Schematic representation of rDNA operon with the variable regions of (A) eukaryotic and (B) prokaryotic organisms, modified and adapted from Lavrinienko *et al.* (2021).

These copies are usually identical or very close due to strong evolutionary pressures (Nelson *et al.*, 2019). Nevertheless, variations have been observed between the different copies within the same strain, constituting a limiting factor for the use of these genes as molecular markers since this heterogeneity leads to an overestimation of the number of species (Lavrinienko *et al.*, 2021). In prokaryotic organisms, the rDNA operon consists of three genes (*i.e.*, 5S, 16S, and 23S), while eukaryotic organisms present rDNA operon constituted of four genes (*i.e.*, 5S, 5.8S, 18S, and 28S), classified according to their coefficient of sedimentation expressed in Svedberg (S) ranging from 8 to 12 kb. These genes are separated by two types of regions: internal transcribed spacers (ITS) and intergenic spacers.

In prokaryote's domain (*i.e.*, bacteria and archaea), the 16S rRNA is the most common gene marker while in eukaryote (*e.g.*, fungi) the internal transcribed regions (ITS-1 et ITS-2) are preferred over the 5.8S and 26S rRNA for molecular analysis. All of the nine hypervariable regions (V1–V9) from 16S rRNA gene have been targeted for the estimation of bacterial diversity in soil vineyard (Burns *et al.*, 2015a; Campisano *et al.*, 2014; Gupta *et al.*, 2019; Wang *et al.*, 2021; Wright *et al.*, 2021). The neighboring conserved parts, where the rate of evolution

is almost zero, are identical in all bacterial communities and are referred as hybridization sites for universal primers.

The ubiquity of ribosomal genes and the possibility that unwanted DNA can be amplified simultaneously with the one under consideration may provide bias to communities' analysis. This is the case for plastid and mitochondrial DNA, which have similarities with bacteria in 16S rRNA genes, that are therefore also the target for hybridization of certain universal primers (Song and Xie, 2020). To avoid this problem and reduce impairs in microbiome studies, it is necessary to choose regions that are specific to the community of interest or in the case where its presence is unavoidable, to remove afterwards this unwanted DNA from analysis. An interesting methodology is the use of peptide nucleic acids clamps, which bind to specific DNA and therefore block its amplification (Fitzpatrick *et al.*, 2018).

2.3.2. Quantitative-PCR (q-PCR) permits the quantification of microbial genes of interest

The principle of real-time quantitative PCR is based on the detection and quantification, during amplification, of a fluorescent emitter which is proportional to the quantity of amplicons formed. It is thus possible to follow the amplification process cycle by cycle, "in real time". To quantify the target gene, either relative or absolute methodologies can be used. Relative based q-PCR relies on the comparison of a sequence to the tested one, resulting in ratios as output, and is preferably used to observe the effect of a treatment on microbial communities. Whereas absolute quantification, resulting in gene copy numbers as output, is based on calibration curve with a standard having a known concentration supplemented with a no template control, usually water (**Figure 13.A**), making it more suitable for microbial communities quantification (Smith and Osborn, 2009; Taylor *et al.*, 2019).

The kinetics of a real-time PCR reaction, as for classical PCR, can be divided into three phases: initiation phase, exponential growth, and plateau phase (**Figure 13.A**). The intensity of the emitted signal is measured at each stage of elongation, until the plateau phase where the fluorescence becomes constant. In real-time PCR, the initial phase corresponds to the background, ending up when the number of amplicons formed exceeds the fluorescence threshold value. The cycle to which this latter value corresponds is called cycle threshold (Ct). Denaturation melting curve, which is performed after q-PCR cycling, is supposed to give rise to a single distinct peak in the plot of the negative derivative of fluorescence versus temperature. This single peak indicates that the generated amplicons are specific to primers and prove the specificity of the PCR (**Figure 13.B**). The principle and basis of quantification is the direct

linear correlation between the amount of the target sequence at the beginning and the Ct obtained (**Figure 13.C**).

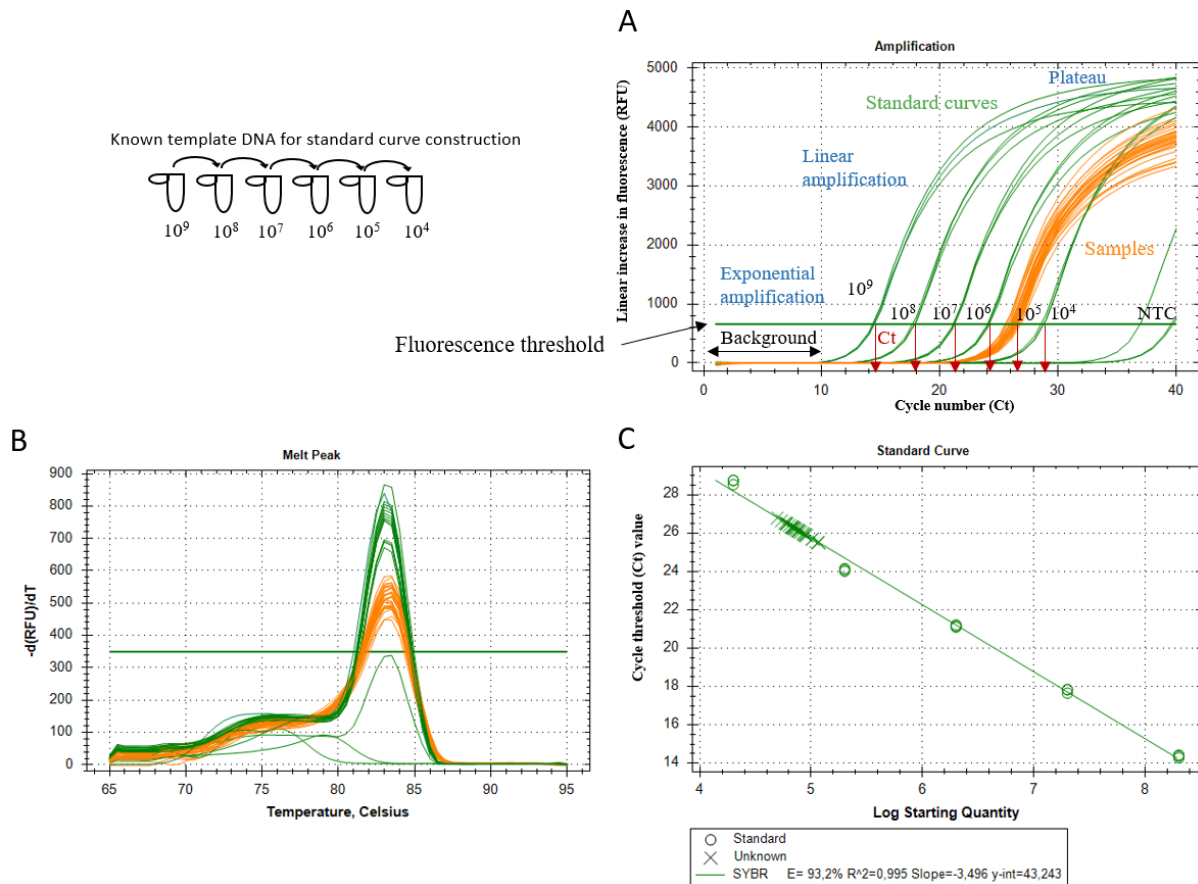


Figure 13 : Construction of standard curves (green) from known concentrations of template DNA for q-PCR amplifications of unknown soil samples (orange). (A) Log plot of the increase in fluorescence vs. cycle number of DNA standards ranging from 10^4 to 10^9 of 16S rRNA gene amplicons. (B) Melt curve analysis. (C) Simple linear regression of the Ct values versus log of the initial rRNA gene number associated to the descriptors shown in the right box. From personal pictures.

Fluorescent markers used in q-PCR are distinguished into non-specific markers that intercalate into the double-stranded DNA (e.g., SYBR® Green), and specific markers consisting of modified DNA oligonucleotides that bind exclusively to the DNA sequence between the two primers (e.g., Taqman®) (Cao and Shockey, 2012). Although SYBR® Green-based technology has its drawbacks, including primer mismatches that can generate non-specific amplicons which overestimate quantification, it is one of the most frequently used intercalating agents because it binds to double-stranded DNA molecule, without any specificity or inhibition effect on the reaction. On the other hand, probes such as Taqman® are tagged at their 5' end by a reporter, while their 3' end is tagged by a quencher that inhibits reporter emission when they are proximal. The reporter is then separated from the quencher, which emits a signal proportional to the number of hydrolyzed probes (Smith and Osborn, 2009). Although, fluorescent probes offer higher specificity than DNA intercalators, the risk of false negatives is

more important due to the presence of mutations in the region recognized by the probe preventing hybridization.

Entomopathogenic nematodes (Blanco-Pérez *et al.*, 2020) and phylloxera aphids (Giblot-Ducray *et al.*, 2016) in vineyards have been quantified using real-time PCR. Regarding soil microorganisms, q-PCR has been widely used to detect and quantify several microbial taxa (Fierer *et al.*, 2005). Beside taxonomic communities, other functional markers such as genes involved in nutrient cycling can be targeted by q-PCR. For instance, genes taking part in nitrogen cycling (*e.g.*, *nifH*, *nirK*, *nirS*, *nosZ*, *amoA-B*, *amoA-arch*) were quantified in vineyards (Pereg *et al.*, 2018; Tatti *et al.*, 2013). This molecular technique is also used in vineyards to detect some specific species such as the pathogen *P. chlamydospora* (Quince *et al.*, 2017; Saccà *et al.*, 2018) through nested PCR approach. This molecular procedure is used when DNA of interest is known to be present at relatively low concentrations and consists in performing q-PCR on amplicons obtained from a first PCR. Even though q-PCR remains an interesting and sensitive tool to quantify microorganisms and global taxa, it does not have the potential to depict large diversity in microbial communities.

2.3.3. Amplicons sequencing technology to unravel microbial diversity

Until the advent of omics approaches, molecular analysis of microbial communities was based either on individual isolate identification or on cloning and sequencing of PCR-amplified ribosomal RNA genes from environmental samples. To carry out microbial characterizations, those tools used first-generation sequencing technology, which was initially developed by Sanger *et al.* (1977). The term "next-generation sequencing" (NGS) refers to second- and third-generation sequencing technologies that are not based on Sanger sequencing, and that have made a breakthrough in the last decade in characterizing complex microbial environments such as soil or plant tissue (Nkongolo and Narendrula-Kotha, 2020).

Microbial community analysis using high throughput sequencing can be distinguished between shotgun or amplicons-based sequencing approaches. The first aims at sequencing short fragments from the extracted total DNA, while the second aims at sequencing multiple copies of amplicons (*e.g.*, 16S, ITS, 18S) (**Figure 14**). Amplicon-based sequencing is often considered as metagenomics, albeit strictly speaking it is not, since only a few specific genes are sequenced at a time. Both have their advantages and limitations to study microbial communities, but amplicons sequencing is sometimes more attractive because of its lower cost and reduced time for analysis (Brumfield *et al.*, 2020).

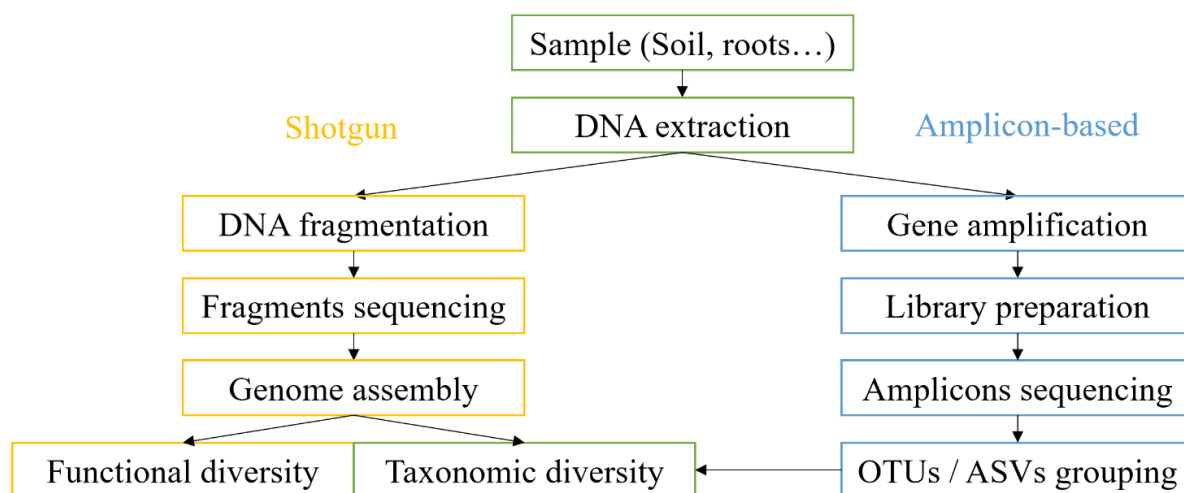


Figure 14 : Schematic workflow of shotgun and amplicon-based approaches.

Several reference databases are available for gene marker such as 16S or ITS (*e.g.*, RDP for Ribosomal Database Project, SILVA Ribosomal RNA Gene Database Project, or Greengenes), and for whole-genome databases (*e.g.*, RefSeq for Reference Sequence, GenBank, or PATRIC for Pathosystems Resource Integration Center). An argument for amplicons-based approach is the availability of more complete databases for environmental samples such as soil (Quince *et al.*, 2017). The use of primers to amplify specific microbial genes and avoid host DNA or other unwanted organisms is also an advantage of this methodology. Both methods provide insights in microbial taxonomic diversity, but shotgun sequencing provides functional profile informations, whereas amplicon-based sequencing can only predict the functional traits of the identified microbiome. Some tools are available to predict this functional diversity based on taxonomic tables such as PICRUSt (Douglas *et al.*, 2018) for 16S rRNA or FUNguild (Nguyen *et al.*, 2016) for ITS or any other fungal markers.

Obviously, these NGS-based approaches rely on computational bioinformatic and require multiple steps of data processing. For instance, Galaxy interface proposes FROGS pipeline to treat 16S rRNA sequencing and propose interactive pipeline to build. Common methodology used to process data obtained from amplicons-based sequencing is depicted in **Figure 15**. Processes used are initiated by a demultiplexing step, if required, followed by an assembly of R1 and R2 into contigs which are overlapping of merged reads. Contigs are submitted to other filtering step until their aggregation into clusters which are groups of sequences based on shared similarity adjusted with a threshold. Chimeric operational taxonomic unit (OTUs) are removed before filtering of OTUs with for instance removal of chloroplast-like sequences compared to databases. Filtered OTUs are therefore blasted with available databases before providing taxonomic and abundance tables needed for diversity and other taxonomic analysis.

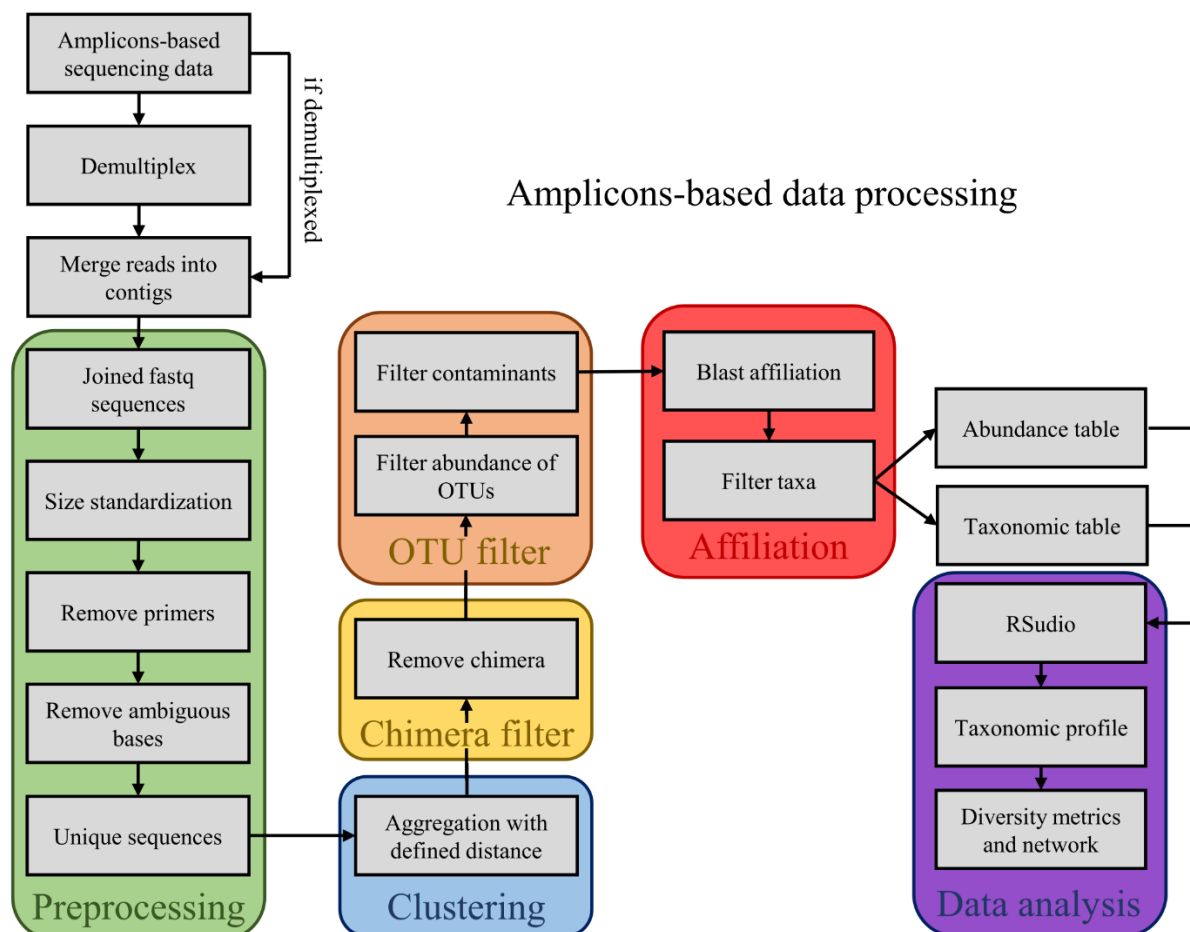


Figure 15 : Common methodology to treat sequences obtained from amplicons-based sequencing, using OTUs clustering, inspired from FROGS pipeline in Galaxy instance.

2.3.4. *Fluorescent in situ hybridization as a tool to visualize cultivable and uncultivable microbial taxa*

Specific identification of individual microbial cells in their natural habitats (*e.g.*, roots, soil) is possible using fluorescence *in situ* hybridization (FISH). This method combines microscopy with fluorescent probes targeting sequences of interest, and allows the detection of specific taxa ranging from phylum (Crocetti *et al.*, 2006) to species level (Posada *et al.*, 2016). FISH methodology involves several steps, including fixation and permeabilization of cells in samples, followed by hybridization of specific probes, washing of samples to remove unbound probes, and detection of labeled microorganisms by epifluorescence microscopy (**Figure 16**).

FISH probes are usually between 15 to 30 nucleotides long and contain a fluorescent dye (*e.g.*, fluorescein, carbocyanine such as Cy3 or Cy5) labelled at the 5' -end, and are often designed to target 16S and 23S rRNA molecules from microorganisms within samples (Amann *et al.*, 2001). Low signal intensity, and target inaccessibility are commonly encountered problems in FISH methodology. Double-labeling-of-oligonucleotide-probes (DOPE) -FISH is a derived

from FISH, with a second identical fluorochrome at 3'-end, which increase the fluorescent signal rRNA intensity (Suyal *et al.*, 2019).

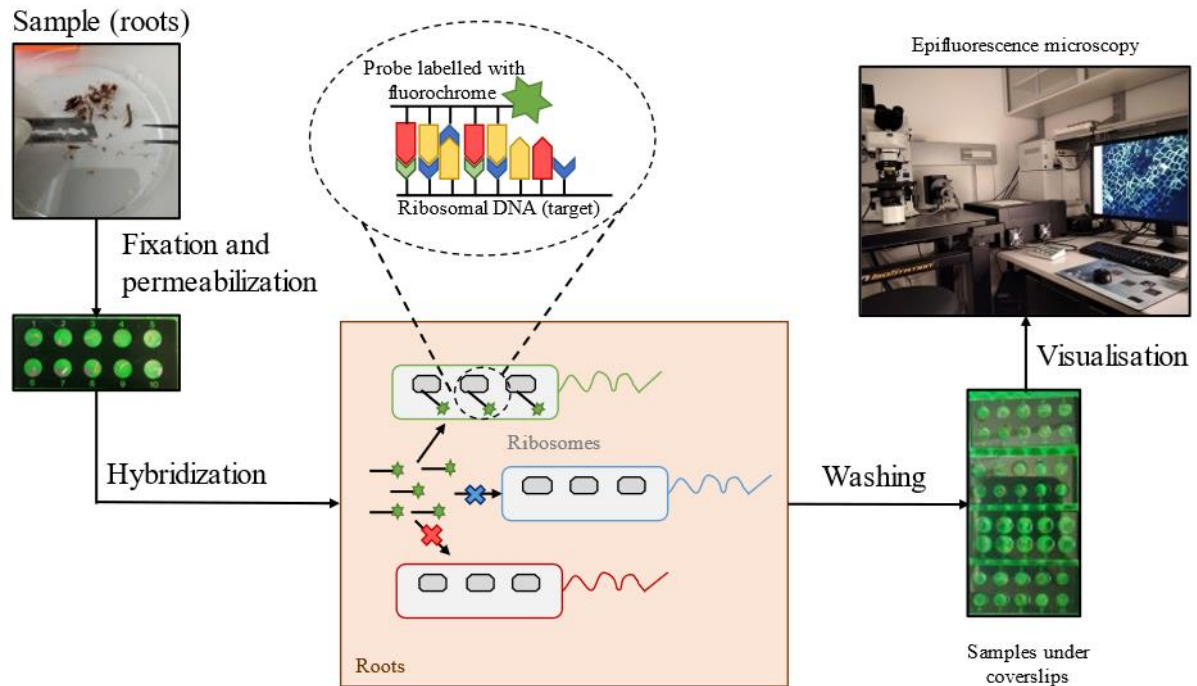


Figure 16 : Methodology of FISH from root samples to microscopy visualization, provided from personal pictures.

FISH can be performed on soil microorganisms (Rogers *et al.*, 2007) but are more often used to investigate microbes associated to plants, such as grapevine (Compant *et al.*, 2011), and more specifically roots (Compant *et al.*, 2013b). This microscopy-based method is therefore relevant to visualize specific microbial endophytes within their natural environment (Hardoim *et al.*, 2015a).

2.3.5. The importance of combining both culture-dependent and -independent approaches

Soil diversity has been extensively studied using traditional microbiological methods including plating methods on different agar media, traditional microscopy, and biochemical assays. Nevertheless, these techniques are often time-consuming and biased since only microorganisms capable of growing on the culture media and under specific conditions can be identified. It is generally known that about 0.1 to 1% of the diversity of microorganisms is detected on an agar plate supplemented with a medium (Amann *et al.*, 1995). In addition, these approaches may over-represent opportunistic species that would grow rapidly on the environments used.

On the other hand, the democratization of DNA-based molecular techniques such as PCR and the identification of stable molecular marker genes such as rRNA genes, have improved the

identification of microbial species with deeper resolution, and greater reliability. Molecular tools based on independent culture methods have the advantage of accessing many species present in a community, including microorganisms that cannot be cultured yet (Suyal *et al.*, 2019). Even though these tools are sometimes less time-consuming than culture-dependent techniques while providing insights in the uncultivable part of microbial communities, they display limitations and can lead to bias since DNA from unviable microorganisms might be targeted. Indeed, this accession to total DNA of microorganisms may belong to dormant microbes as well as microorganisms that do not functionally contribute to soil.

Culture-dependent methods, despite all the biases associated with them, are still important for ecological studies. They allow the isolation of species for collection, and their subsequent analysis provide information on the functional potential of a soil (Greening *et al.*, 2019). In addition, culture methods continue to be improved and allow the isolation of species that were not previously cultivable (Pham and Kim, 2012). The application of these two methodologies must therefore be done in synergy, so that the complementary information generated can contribute to a better understanding of the microbial soil and the grapevine.

3. Interaction between grapevine rootstock and soil microorganisms

This part was the subject of a literature review entitled “**Grapevine rootstock and soil microbiome interactions: Keys for a resilient viticulture**”. This review was accepted in Horticulture Research on January 17, 2022.

Grapevine rootstock and soil microbiome interactions: Keys for a resilient viticulture

Horticulture Research, 9, <https://doi.org/10.1093/hr/uhac019>

Running title: Rootstock microbiome in vineyards

Romain Darriaut¹, Vincent Lailheugue¹, Isabelle Masneuf-Pomarède^{2,3}, Elisa Marguerit¹, Guilherme Martins^{2,3}, Stéphane Compant⁴, Patricia Ballestra², Steven Upton³, Nathalie Ollat¹, Virginie Lauvergeat^{1*}

¹ EGFV, Univ. Bordeaux, Bordeaux Sciences Agro, INRAE, ISVV, F-33882, Villenave d'Ornon, France

² Université de Bordeaux, INRAE, Bordeaux INP, UR Œnologie EA 4577, USC 1366, ISVV, 33140 Villenave d'Ornon, France

³ Bordeaux Sciences Agro, 33170 Gradignan, France

⁴ AIT Austrian Institute of Technology, Center for Health and Bioresources, Bioresources Unit, Konrad Lorenz Straße 24, Tulln, A-3430, Austria

romain.darriaut@inrae.fr; vincent.lailheugue@u-bordeaux.fr; isabelle.masneuf@agro-bordeaux.fr; elisa.marguerit@agro-bordeaux.fr; guilherme.martins@agro-bordeaux.fr; steven.upton@agro-bordeaux.fr; patricia.ballestra@u-bordeaux.fr; nathalie.ollat@inrae.fr; stephane.compant@ait.ac.at

*corresponding author: virginie.lauvergeat@inrae.fr

Abstract

Soil microbiota has increasingly been shown to play an integral role in viticulture resilience. The emergence of new metagenomic and culturomic technologies has led to significant advances in the study of microbial biodiversity. In the agricultural sector, soil and plant microbiomes have been found to significantly improve resistance to environmental stressors and diseases, as well as influencing crop yields and fruit quality thus improving sustainability under shifting environments. Grapevines are usually cultivated as a scion grafted on rootstocks, which are selected according to pedoclimatic conditions and cultural practices, known as terroir. The rootstock connects the surrounding soil to the vine's aerial part and impacts scion growth and berry quality. Understanding rootstock and soil microbiome dynamics is a relevant and important field of study, which may be critical to improve viticulture sustainability and resilience. This review aims to highlight the relationship between grapevine roots and telluric microbiota diversity and activity. In addition, this review explores the concept of core microbiome regarding potential applications of soil microbiome engineering with the goal of enhancing grapevine adaptation to biotic and abiotic stress.

Keywords: Environmental stress, grapevine rootstock, microbiome engineering, microorganisms' interactions, plant growth-promoting rhizobacteria, rhizosphere, soil diversity, sustainable viticulture, terroir, vine health

Introduction

Omics technologies have deepened our knowledge and understanding of telluric and ecosystemic processes; these developments underscore the importance of soil microbiome to plant health. The microbiome has recently been redefined as the microbiota and its theater of activity which combine microbial structural elements such as proteins, peptides, lipids, nucleic acids, polysaccharides, and microbial metabolites as signaling molecules, toxins, (in)organic molecules, and the environmental conditions (Berg *et al.*, 2020). Currently, the primary methods used to explore the taxonomic and functional soil microbiome diversity utilize plating methods and computed metagenomics which respectively rely on media composition and high-throughput sequencing (Sarhan *et al.*, 2019). Through the use of these techniques, it has been suggested that plant-associated microorganisms are recruited from the soil microbiota, thus serving as the microorganisms' reservoir of rich microbial diversity (Hardoim *et al.*, 2015b). In viticulture, the soil microbiome is now considered as a terroir component that could influence grape berry composition (White, 2020). Studying the microbiome in vineyards, especially fungi and bacteria, is an emerging field of science as it holds the potential to improve grapevine

adaptation to climate change and prevention of pathogenic infection. Thus, the study of vineyard microorganisms holds tremendous potential for improving vine resilience and helping vineyards better face increasing environmental stress.

The composition of the soil microbiota, and therefore its related biological activity, is dependent on many factors (*e.g.*, physicochemical characteristics of the soil, plant species and cultivars, climatic conditions, cultural practices ...) (Compant *et al.*, 2019; Fierer, 2017). Regardless of the microbiota already present in the soil, the main drivers of the composition of the microbial community associated with the root system (epiphytic and endophytic) are the primary and secondary metabolites exudated by the roots (Pascale *et al.*, 2020). The composition of the exudates vary depending on environmental factors, as well as plant species and cultivars (Ghatak *et al.*, 2021; Herz *et al.*, 2018), which collectively shape the root microbiome.

Cultivated grapevines are typically grafted plants composed of a scion (*Vitis vinifera* L.), which produces grape berries, and a rootstock (*Vitis* spp., tolerant to phylloxera aphids), which is selected considering pedoclimatic conditions. Grafting is a practice widely used to improve resistance to environmental stresses, yield and quality of the harvested product (Williams *et al.*, 2021). The rootstock works as an interface between the soil and the grapevine-associated microbiota, hence modulating the plant holobiont. The scion cultivar is another factor in this complex rootstock × scion × soil interaction, which may influence the root-associated microbiome. The rootstock's capacity to interact with soil microorganisms differs between genotypes due to their intense breeding and genetic background histories (Marín *et al.*, 2021). Rootstocks display contrasting root system in terms of root architecture, as well as synthesis and exudation of metabolites. Some of these compounds are signaling molecules, which shape and attract soil microorganisms. It is therefore essential to understand the role of the rootstock in these interactions that could be further utilized to isolate and promote biofertilizers and bioprotectors. Moreover, the use of rootstocks appears to be an appropriate strategy to conserve wine quality produced by the scion while simultaneously conferring resistance to biotic and abiotic constraints (Ollat *et al.*, 2016). This review serves to update and expand upon the role of soil microbiome and rootstock dynamics in improving grapevine resilience.

3.1. Close to the roots, a dynamic spot for molecular exchange

3.1.1. The soil acts as a microbial reservoir for the plant

The grapevine microbiome has been investigated in every compartment using culture-dependent and independent techniques. Independent of soil type and cultivar genotype, the prokaryotic microbiome of *V. vinifera* is mainly composed of *Proteobacteria*, followed by *Firmicutes*, *Actinobacteria*, *Acidobacteria*, and *Bacteroidetes* (**Table S1**). The grapevine's eukaryotic microbiome consists of *Ascomycota* and *Basidiomycota* on both the above and below-ground parts of the vine (**Table S2**) while the *Glomeromycota* division is established in the vine roots. Wei *et al.* (2018) found in their multi-compartment study that *Proteobacteria* and *Firmicutes* are more common to berries, leaves, and grape must, whereas *Bacteroidetes* and *Actinobacteria* adapt better to soil. The authors found that even in the phyllosphere, which is the target of several air-borne pathogens, the relative abundance of bacterial genus and class depends on the plant organs.

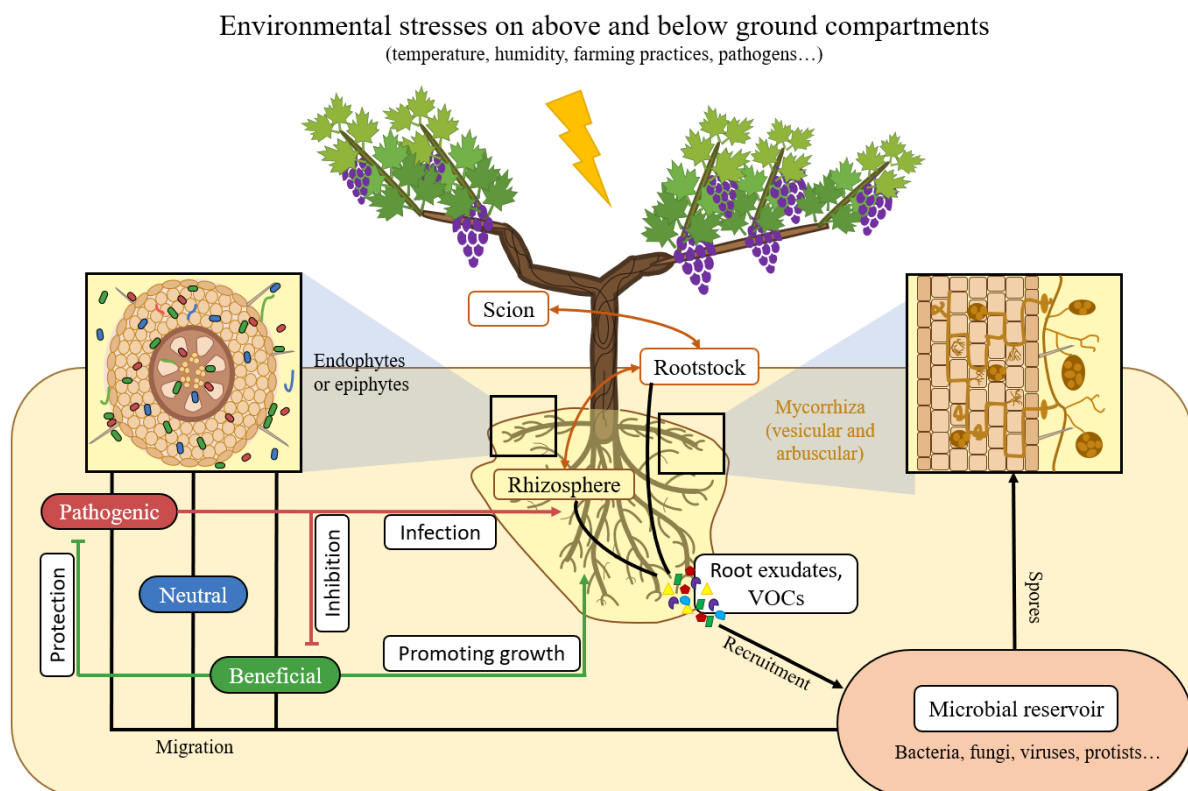


Figure 17 : Schematic representation of the vine-soil interactions. Environmental stresses afflict both below and above ground compartments of vine. Scion and rootstock communicate through long distance signaling compounds. These signaling pathways modulate the root exudates composition (e.g., VOCs, Volatile Organic Compounds) into the soil microbial reservoir. Microorganisms are therefore chemoattracted and present pathogenic, neutral or beneficial functions towards the vine. They can be either epiphytic and/or endophytic (box on the left), such as mycorrhizal fungi (box on the right).

The rhizosphere, defined as the tight area of soil enveloping the plant roots, hosts a tremendous number of microorganisms, which interact directly or indirectly with the plant. This soil compartment supports a complex microbiome and is considered as one of the most dynamic ecosystems on Earth. Part of the rhizosphere microbiome, also known as rhizomicrobiome, has been shown to provide the host plant with better capacities to adapt to environmental stresses, potentially playing an integral role in plant health (Qu *et al.*, 2020). Soil microflora is mainly composed of bacteria, archaea, fungi, protists, and viruses, which have either beneficial, neutral, or pathogenic relationships with the plant (**Figure 17**). Pathogenic microorganisms participate in the root infection processes whereas beneficial microbiota promote the plant's growth and defense mechanisms (Compant *et al.*, 2019).

The relative abundance of bacterial and fungal rhizomicrobiome varies with scion/rootstock combination features, soil type, climatic conditions, soil depth, and cultural practices (Marasco *et al.*, 2018; Mezzasalma *et al.*, 2018; Nerva *et al.*, 2021b; Samad *et al.*, 2017; Zarraonaindia *et al.*, 2015). Among fungi, the most encountered taxa in the vineyard soil are principally from the *Ascomycota* and *Basidiomycota* phyla (**Table 6**). With regard to bacteria, the most abundant genera found in the grapevine rhizosphere belong to *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Acidobacteria* phyla.

Table 6 : Examples of the main bacterial and fungal taxa found in the rhizomicrobiome of grafted and ungrafted grapevine, with their relative abundances and associated sequencing target region.

| Major bacterial taxa (% of relative abundance), and the associated target region | Major fungal taxa (% of relative abundance), and the associated target region | Studied scion/rootstock combination | Reference |
|---|---|--|---------------------------------------|
| | Root / surrounding soil (ITS1): <i>Ascomycota</i> , <i>Mortierellomycota</i> , <i>Basidiomycota</i> . Relative abundances not provided | Pinot noir cv. (<i>V. vinifera</i>). Presence or absence of rootstock not provided. | (Liu and Howell, 2021) |
| Rhizosphere (16S V4-V5): <i>Acidobacteriota</i> (35%), <i>Proteobacteria</i> (22%), <i>Latescibacteriota</i> (15%), <i>Methylomirabilota</i> (6%), <i>Gemmatimonadota</i> (4%) | | Ungrafted 1103P, 140 Ru, 161-49 C, and Kober 5BB cv. | (Dries <i>et al.</i> , 2021) |
| Rhizosphere (16S V3-V4): <i>Proteobacteria</i> (~45%), <i>Bacteroidetes</i> (~15%), <i>Firmicutes</i> (~9%), <i>Actinobacteria</i> (~7%), <i>Acidobacteria</i> (~6%) | Rhizosphere (ITS1): <i>Ascomycota</i> (~47%), <i>Basidiomycota</i> (~15%), <i>Mortierellomycota</i> (~10%) | Ungrafted Malbec (<i>V. vinifera</i>) and Cabernet Sauvignon cv. | (Oyuela Aguilar <i>et al.</i> , 2020) |

| | | | |
|---|---|---|-------------------------------------|
| Rhizosphere (16S V4): <i>Proteobacteria</i> (~70%), <i>Actinobacteria</i> (~18%), <i>Bacteroidetes</i> (~8%), <i>Firmicutes</i> (~5%) | Rhizosphere (ITS1): <i>Ascomycota</i> (~50%), <i>Basidiomycota</i> (~45%) | Syrah cv. (<i>V. vinifera</i>) grafted on 1103P | (Deyett and Rolshausen, 2020) |
| Rhizosphere (16S V4): <i>Proteobacteria</i> (27%), <i>Actinobacteria</i> (21%), <i>Acidobacteria</i> (15%), <i>Bacteroidetes</i> (6%) | Rhizosphere (ITS2): <i>Ascomycota</i> (67%), <i>Basidiomycota</i> (16%), <i>Zygomycota</i> (12%) | Tempranillo (<i>V. vinifera</i>) cv. grafted on 110R, 140 Ru, 1103P (all above are <i>V. berlandieri</i> × <i>V. rupestris</i>), 41 B (<i>V. vinifera</i> × <i>V. berlandieri</i>), and 161-49 C (<i>V. riparia</i> × <i>V. berlandieri</i>) | (Berlanas <i>et al.</i> , 2019) |
| | Rhizosphere (ITS2): <i>Ascomycota</i> (61%), <i>Basidiomycota</i> (21%) | Tempranillo cv. grafted on 110R | (Martínez-Diz <i>et al.</i> , 2019) |
| Root and Rhizosphere (16S V3- V4): <i>Proteobacteria</i> (53%), <i>Actinobacteria</i> (24%), <i>Bacteroidetes</i> (5%), <i>Chloroflexi</i> (4%), <i>Acidobacteria</i> (4%) | | Barbera cv., ungrafted (<i>V. vinifera</i>) and grafted on SO4, 420A, 161-49C and 157-11C (all are <i>V. riparia</i> × <i>V. berlandieri</i>) | (Marasco <i>et al.</i> , 2018) |
| Rhizosphere (16S V1-V4): <i>Actinobacteria</i> (52%), <i>Proteobacteria</i> (36%), <i>Gemmatimonadetes</i> (2%), <i>Bacteroidetes</i> (~2%) | | Pinot noir cv. Presence or absence of rootstock not provided. | (Novello <i>et al.</i> , 2017) |
| Rhizosphere (16S V5-V7): <i>Actinobacteria</i> (47%), <i>Proteobacteria</i> (22%), <i>Bacteroidetes</i> (13%) | | Zweigelt cv. clone GU4 (<i>V. vinifera</i>) grafted on Kober 5BB (<i>Vitis berlandieri</i> × <i>Vitis riparia</i>) | (Samad <i>et al.</i> , 2017) |

These phyla are keystone taxa that perform a broad range of functions in the soil ecosystem (Banerjee *et al.*, 2018). Zarraonaindia *et al.* (2015) and Marasco *et al.* (2018) showed an enrichment of the rhizosphere compared to bulk soil for main phyla such as *Gammaproteobacteria*, *Betaproteobacteria*, and *Actinobacteria*. This increase in bacterial richness might be promoted, through the use of flagella, by chemoattractants (*e.g.*, sugars, amino acids, organic acids, vitamins, phytohormones, flavonoids, terpenes) (Musilova *et al.*, 2016). Indeed, genes involved in bacterial chemotaxis and motility as well as flagella association, are more present in microbial communities found in root-associated environments, in comparison to bulk soil (Trivedi *et al.*, 2020). Root microbial communities in grapevines were also investigated using 16S/ITS rRNA amplicon sequencing, shotgun metagenomics, and cultivable approaches (Pacífico *et al.*, 2019). It appears that bacterial diversity is lower in the root compartment than in the rhizosphere, and the majority of root-associated bacterial taxa matched the bacteria found in the soil (Marasco *et al.*, 2018; Zarraonaindia *et al.*, 2015), which

also occurs with fungal diversity (Martínez-Diz *et al.*, 2019; Zahid *et al.*, 2021), highlighting soil microbial reservoir capacity.

3.1.2. Soil and rhizosphere: a microbial source of inoculum of grape berry microbiota

Must and wine microorganisms belong mainly to the microbial consortia of grape berries (Ramírez *et al.*, 2020). Many studies support that the main source of these microorganisms is the vineyard soil (Belda *et al.*, 2017; Zarraonaindia *et al.*, 2015), even though the atmospheric microbiome also influences the composition of fungal and bacteria communities associated with leaves, flowers, and fruits (Abdelfattah *et al.*, 2019). The root endophytes can shape the microbial community of aboveground organs by changing endophytic microbial loads in grapes (Zarraonaindia *et al.*, 2015). A significant input of soil microorganisms to grapes through epiphytic migration during harvest was also suggested (Martins *et al.*, 2013). Contrary to the bacterial component, studies on vineyard soil contribution to the yeast community of grapes are scarce. A hypothetical endophytic way of colonization was proposed for the fermentative yeast *S. cerevisiae* to be transported from the soil via roots and stems to the surface of the grape berry (Mandl *et al.*, 2015) as shown for bacteria (Compant *et al.*, 2011). As for bacteria, vineyard soil appears to be a permanent natural reservoir of non-*Saccharomyces* yeasts via possible contamination of grapes with edaphic microorganisms due to deposit of dust from vineyard soil (Ramírez *et al.*, 2020). Microbial communities on grapes could have the potential to influence grape composition and thus the organoleptic properties of the wine, contributing to a regional terroir. Zarraonaindia *et al.* (2015) showed that the aboveground bacterial community was significantly influenced by soil edaphic factors such as total carbon, moisture, and soil temperature, which would ultimately impact the quality of grapes due to changes in nutrient availability for the plant. Weather and soil properties influence soil and must microbial diversity that will indirectly impact wine aroma profiles (Griggs *et al.*, 2021). The contribution of the soil microbial component on the berry and the final wine composition should be evaluated in light of other factors including pedoclimatic, human parameters, rootstock and scion genotypes that define the concept of terroir.

3.1.3. The impact of telluric microbiota on grape berry composition

In agriculture, plant probiotic bacteria significantly impact crop quality and fruit composition by increasing vitamins, flavonoids, and antioxidants content, among other benefits (Jiménez-Gómez *et al.*, 2017). For example, the addition of a Plant-Growth Promoting Bacterium (PGPB)

K. radicincitans modifies amino acid, sugar, and volatile composition of ripened tomato fruits, thus contributing to a more pleasant-tasting fruit (Berger *et al.*, 2017). Aoki *et al.* (2017) investigated the activation in grape berries of the gene expression of stilbene synthase, a key enzyme in resveratrol synthesis, by a *Bacillus cereus* strain. Native microorganisms can exert an accumulation of volatile compounds in grape berries that could be activated by phytopathogens in the case of volatile precursors of volatile thiols (3MH) responsible for grapefruit aroma in white wines (Otoguro and Suzuki, 2018). The production of aroma by grape-associated microorganisms could also directly impact grape berry composition (Verginer *et al.*, 2010).

Grape berry endophytic and epiphytic microorganisms are known to activate metabolic pathways leading to an increase in phenolic compounds or other aroma compounds biosynthesis, as reviewed in Otoguro and Suzuki (2018). Even if the endophytic berry microbial community is largely derived from the soil, very few studies evaluate the impact of telluric microbiota on berry composition and are mainly focused on arbuscular mycorrhizal fungi (AMF).

By using Biolog™ EcoPlates technology, Ji *et al.* (2019) showed a correlation between metabolic activities and functional diversity of rhizosphere microbial communities and physicochemical indices of grape berry quality. Association of grapevine with AMF facilitates the synthesis of plant secondary metabolites such as resveratrol, flavonol or anthocyanin, which improve berry quality and plant tolerance to environmental stresses (Torres *et al.*, 2018). Wine produced from a vineyard with cv. Sangiovese had better oxidative stability and a significantly higher level of bioactive compounds such as gallic acid, resveratrol, caffeic acid and, quercetin, when treated with a consortium of *Glomus* species plus soil bacteria, fungi and, yeast to a lesser extent, compared to the wine produced by control vines (Gabriele *et al.*, 2016). The protective role of AMF against warming effects on berries on three clones of Tempranillo was shown to improve their antioxidant properties and anthocyanin content (Torres *et al.*, 2016). The inoculation of eight ancient grapevine varieties with a mixture of five AMF species reduced the berry mass and increased the soluble sugars and anthocyanin contents for most of the cultivars (Antolín *et al.*, 2020). The intensity of these variations on berries was different among the cultivars, suggesting a genotype dependent effect. These studies do not take into account the effect of the rootstock genotype as almost all were performed with ungrafted cultivars. Therefore, the functional potential of the rootstocks to impact the soil microbiota effect on fruit physiology, susceptibility to pathogen and grape berry quality remains to be explored.

3.1.4. Root-associated and rhizosphere microbiomes are regulated by grapevine genotype and possess useful plant growth-promoting features

Plant species and genotypes play determinant roles in selecting the telluric microorganisms that will surround the host. As most cultivated grapevines are chimeric plants composed by *Vitis vinifera* cultivars grafted on American *Vitis* species and hybrids, it is essential to consider the effect of the scion/rootstock combination. To date, only one study analyzed the bacterial community structure in the rhizosphere of 4 cultivars × 4 rootstocks combinations (Vink *et al.*, 2021). Authors showed that the diversity of rhizosphere bacteria is impacted first by the cultivar followed by rootstock genotypes, but the effect was dependent on the diversity index used. The distinct genetic component and capacity to produce photosynthate components of the cultivars might alter the exudate composition and could explain this difference in bacterial diversity. Bacterial microbiomes in the rhizosphere of five different rootstocks grafted with the same Barbera cv. were significantly different in terms of richness, diversity, and community networking, within the same vineyard (Marasco *et al.*, 2018). Biget *et al.* (2021) demonstrated through their multi-site analysis within a vineyard that vine age was one of the main drivers of bacterial and fungal root endophytes, even though the genetic background of rootstock was not investigated. Considering this, Berlanas *et al.* (2019) highlighted that rootstock genotype had a greater impact than millesimal or sampling date on bacterial and fungal microbiome structure in the rhizosphere exclusively in mature vineyards. Predominant amounts of *Proteobacteria* and *Actinobacteria* were found in all samples of rhizosphere, but bacterial genera varied depending on the rootstocks. With regard to fungi, the *Ascomycota* and *Basidiomycota* phyla varied greatly among rootstocks. Specific genera were affiliated to distinct rootstock genotypes, such as *Geopyxis* for the 110R rootstock, or *Clonostachys* for 1103P and 140 Ru rootstocks. Regarding functional screening of indigenous isolates, Samad *et al.* (2017) and Marasco *et al.* (2018) confirmed the significant enrichment of *Proteobacteria* in grapevine root tissues (Kober 5BB rootstock, and ungrafted/grafted Barbera cv. on 402A, 157-11C, SO4, 161-49C, respectively), while *Actinobacteria* and *Bacteroidetes* remained at relatively constant levels in both rhizosphere and root compartments. Conversely, *Gemmatimonadetes* and *Firmicutes* were less abundant in roots than the surrounding soils. In both studies, Plant-Growth Promoting (PGP) activities of strains belonging to the *Enterobacteriaceae* and *Pseudomonadaceae* families were tested for production of hydrogen cyanide, ACC deaminase (ACCd), siderophores, indole acetic acid (IAA), and for phosphate solubilization. It has been shown by Marasco *et al.* (2018) that PGP functional genes were conserved in both the rhizosphere and root endosphere despite selecting different bacterial communities, and therefore that the

frequencies of these PGP traits were not dependent on the rootstock genotype. For Syrah cv. grafted on 1103P rootstock, Deyett and Rolshausen (2020) observed a different enrichment composed mainly of *Rhizobium*, *Devosia*, *Streptomyces*, and *Pseudomonas* genera in the rhizosphere. This study also revealed that fungal and bacterial richness in roots accounted for 64% of the amplicon sequence variants (ASVs) found in the rhizosphere and soil compartments. *Streptomyces* and *Pseudomonas* genera are often associated with PGP activities but also inhibit the colonization of pathogens in grapevine woods (Niem *et al.*, 2020). Using a disruptive approach based on metaproteomic, Bona *et al.* (2019) confirmed that the high biochemical activity (*i.e.*, phosphorus metabolic processes and regulation of nitrogen compounds) in the rhizosphere of ungrafted *Vitis vinifera* cv. Pinot noir was largely attributed to bacteria belonging to the *Proteobacteria* phylum. To another extent, D'Amico *et al.* (2018), observed a depletion and sometimes a total absence of potassium (K) solubilizing bacterial members from the *Micrococcaceae*, *Comamonadaceae*, *Cytophagaceae*, *Sphingomonadaceae*, *Rhizobiaceae*, *Xanthomonadaceae*, and *Microbacteriaceae* in the rhizosphere and roots of 1103P rootstock, whereas they were detected in 5BB rootstock with the same Lambrusco cultivar. This dysregulation of the functional microbiome was linked to the problem of K absorption observed in the studied *V. berlandieri* × *V. rupestris* rootstocks. Except for AMF, more studies have been focused on the bacterial communities of grapevine roots and rhizosphere compared to studies of fungal communities. Given the importance of rhizosphere functions, it is relevant and crucial to examine the link between rootstock agronomic features and rhizosphere microbiome traits.

3.1.5. Case of the famous symbiont, the arbuscular mycorrhizal fungi

AMF symbioses are endomycorrhizal associations with obligate biotrophic fungi belonging to the *Glomeromycota* division. This is the most frequently encountered mycorrhizal form encompassing grapevines as approximately 80 % of terrestrial plants are able to associate with AMF (Likar and Regvar, 2017; Popescu, 2016; Trouvelot *et al.*, 2015). AMF symbioses are mainly induced in soil where P availability is low, and play a key role in providing P and N to plant root cells, which can be attributed to increased soil exploration surface due to extra-radicular hyphae proliferation (Lanfranco *et al.*, 2018). In return, fungi receive photosynthetically fixed carbon assimilated from plant cells. AMF do not only affect plant growth traits, water and nutrient uptake, but also protect their host from pathogens. Since the first description of two AMF species by Tulasne et Tulasne in 1845, more than 260 Glomeromycotan species have been discovered (Öpik and Davison, 2016). The most common species identified using culture-dependent approaches are included in the *Glomeraceae* order

such as *G. intraradices* or *G. mosseae*. New technologies based on molecular approaches provided deeper insights about AMF diversity in vineyards by sequencing ribosomal Internal Transcribed Spacers (ITS) or their small subunit (SSU) rRNA fragments (Schreiner, 2020; Van Geel *et al.*, 2017). Drain *et al.* (2019) proposed a standardized protocol to study AMF communities from root samples of vines. The authors amplified the D2 domain from the Large Subunit Region (LSU) and revealed the predominance of the *Rhizophagus* and *Glomus* genera coupled to eight other genera from the *Glomeromycota* division. However, a clear picture of how AMF diversity colonizes grapevine roots in different parts of the world is incomplete, especially since the classification of AMFs remains controversial and molecular techniques for their identification have not been standardized (Kryukov *et al.*, 2020).

Although it is assumed that sustainable practices enhance the spore abundance and diversity of AMF (Radić *et al.*, 2014), they are influenced by several factors including edaphic parameters and grapevine genotype. Moukarzel *et al.* (2021) demonstrated a significant difference in the AMF community associated with nine rootstocks grafted or not with Pinot noir cv. using denaturing gradient gel electrophoresis (DGGE) and trap cultures. Nerva *et al.* (2021a) identified the influence of the rootstock genotype in activating distinct defense pathways by young cuttings, grafted on either 1103P or SO4 rootstock, when treated with *R. irregulare* and *F. mossea*. While studies of citrus have shown scions to be more influential to the AMF community structure than on rootstock (Song *et al.*, 2015), the role that scion genotype could play in AMF diversity in grapevines has yet to be explored. The selection of rootstock and scion genotype are important in determining grapevine capacity to form mycorrhizal associations that could enhance host mineral uptake and increase grapevine sustainability.

3.2. Microbiome engineering, a tool to promote plant health

3.2.1. The concept of compositional and functional core microbiome

The concept of core microbiome relies on operational taxonomic units (OTUs), and to some extent on ASVs, shared between different individuals of the same species, as was first proposed in humans (Turnbaugh *et al.*, 2007). Despite its complexity, the concept of core microbiome is gaining support and several definitions have been made with regard to either microbiome's functionality, temporal stability, taxonomy, plant-adapted, or ecology (Risely, 2020). Most of the time, core microbiome is referred to as the compositional core based on taxonomy or functional core. Indeed, this core concept is not only considered as the microorganism's diversity, but also as the core interactions that are used to maintain an individuals' health, and

on a larger scale the ecosystem. Crops and plants in general, are associated with distinct soil microbiomes which are influenced, independent of temporal factors, by biotic and abiotic components (Thakur and Geisen, 2019).

Swift *et al.* (2021) suggested, subsequently to a multi-compartment analysis submitted to irrigation stress, that the core microbiome is quite conserved in the different analyzed rootstocks (cv. Chambourcin grafted on 1103P, 3309C, and SO4). The different irrigations lead to microbial changes in aerial compartments such as different amounts of *Acetobacterales* and *Saccharomycetes* in berries which could affect wine quality. Carbone *et al.* (2021) recently pointed out this shift in fungal communities under three distinct irrigation regimes (25%, 50%, or 100% of field capacity) with 22.3% of fungal OTUs shared in roots among those conditions, while 66.8% and 55.6% OTUs were found to be common in rhizosphere, and bulk soil compartments, respectively. Despite neglecting the role of rootstock, Liu and Howell (2021) unveiled the fungal core microbiome in Merlot cv. which displays 32.75% of shared OTUs between roots and soil, fluctuating in abundance across the season. This supports the idea that the grapevine core microbiome relies on the composition of microbial soil reservoir, which is recruited differently according to the rootstock.

Core functions such as biogeochemical processes in the soil appear to be related to taxonomically distinct patterns but with similar metabolic functions, hence confirming that the theater of microbiota activity can be distinguished into taxonomy and functioning that interact with the terroir (Griggs *et al.*, 2021). Terroir is a broad concept that can be described as the components driving the aromas and wine typicity within a defined geographical region with specific soil topology, and viticultural practices including cultivar variety (Van Leeuwen *et al.*, 2018). As discussed previously, different rootstocks are able to be associated with different microbial communities sharing similar functional traits (D'Amico *et al.*, 2018; Marasco *et al.*, 2018). Functional redundancy is indeed the idea that more than one taxon can exert the same function within a microbial community (Louca *et al.*, 2018). Unravelling the core species recruited through rootstocks could be a powerful tool in determining microbiome responses to environmental constraints. Therefore, microbiome functioning must be understood in order to predict plant health in response to various stresses, even though microbiome-plant partnerships are complex belowground-based interactions linked with the soil.

3.2.2. Microbial diversity as a biological marker for grapevine fitness

Many biotic and abiotic stresses occur in vineyards and can lead to plant decline or dieback if not managed properly. Grapevine dieback afflict viticulture worldwide and can be defined as a

pluriannual decrease in vine productivity linked to its sudden premature or gradual death due to environmental causes and/or agronomic practices (Riou *et al.*, 2016). Despite evidence of negative impact on microbial communities in young replanted vines due to long-term monoculture and intense replanting management, replacing the dead vines with young vines remains sometimes the only solution to palliate this problematic dieback (Liu *et al.*, 2021; Westphal *et al.*, 2002). Grapevines are a perennial plant which require significant time-consuming cultivation; at least three years are needed for the new plant to harbor productive grapes (Sanmartin *et al.*, 2017). To this end, accelerating the growth of young cuttings with plant growth-promoting rhizobacteria (PGPR) or AMF may be an interesting approach to compensate for the lack of productivity during the beginning of replantation, but this approach has not been widely studied in vineyards (Rolli *et al.*, 2017). However, this strategy may increase the incidence and severity of grapevine trunk diseases (GTDs) symptoms due to the predisposition of GTD to affect such vineyards managed using training and pruning techniques which promote vine growth (Hrycan *et al.*, 2020). On that account, microbiome engineering which is an actual trend which encompasses crops and numerous cultivars (Orozco-Mosqueda *et al.*, 2018), appears to be a promising strategy against environmental stressors. Microbiome engineering often refers to a set of tools which strengthen the soil microbiome and hence the plant-associated microbiome through nutrient uptake and pathogen control (**Figure 18**). Among these tools, agricultural practices (*e.g.*, cover crop, irrigation, tillage), soil amendment, and plant material choice (*i.e.*, grafted rootstock or not) can interfere with microbial diversity which is considered as a key biomarker in plant protection and growth strategies (Berg *et al.*, 2017). The greatest microbial diversity was found in organic vineyards compared to conventional ones (Vega-Avila *et al.*, 2015) but with a lower soil microbial biomass (Ostandie *et al.*, 2021). This difference in diversity may be related to the abundance of organic matter which are a rich source of exogenous microbial inoculants which can colonize the vines. A meta-analysis made by Karimi *et al.* (2020) highlighted the effect of viticultural practices on soil microbiological diversity and showed that tillage, absence of cover crop, and mineral fertilization all contributed significantly to reductions in soil biodiversity. Microbiome inoculation is another interesting tool that directly modify the soil and/or rootstock microbiome functionalities and compositions.

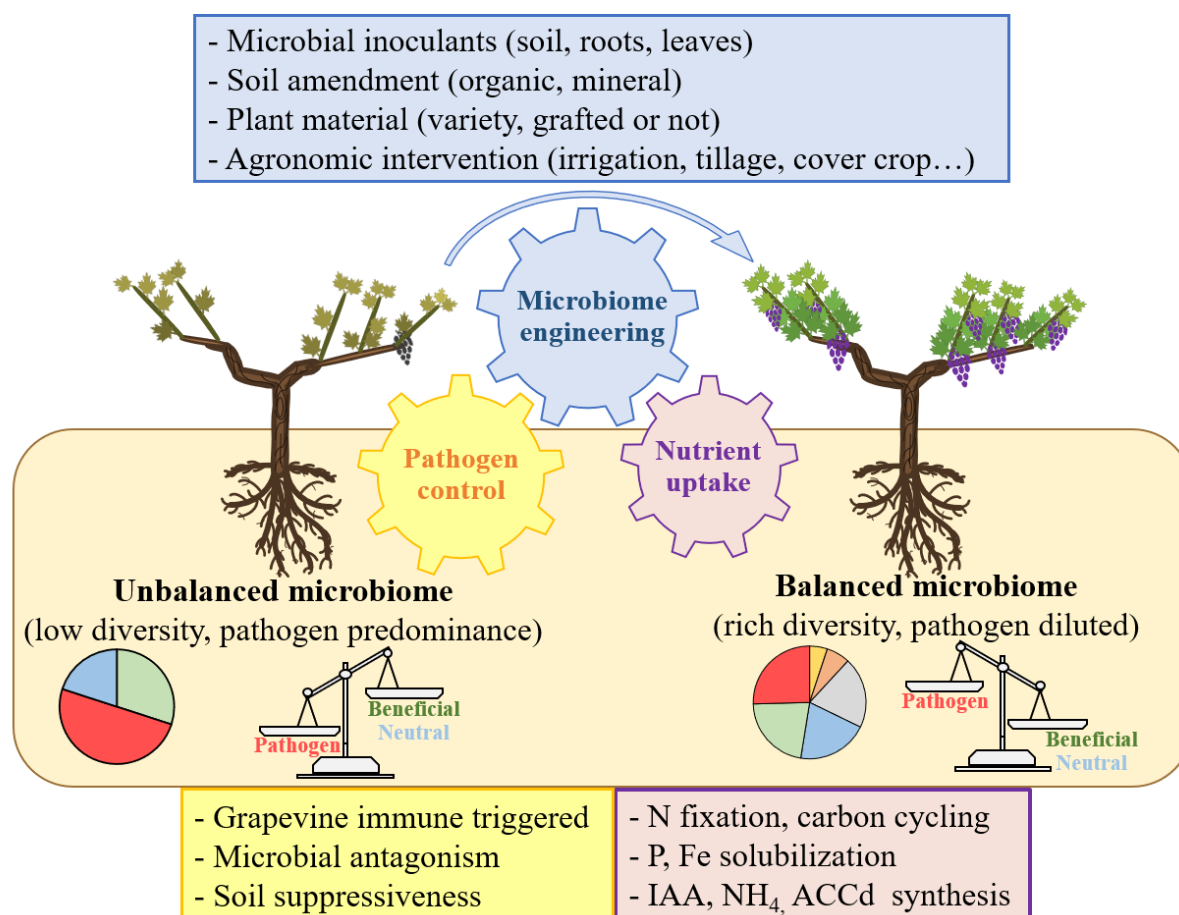


Figure 18 : Schematic representation of grapevine health affected by soil microbiome services, pathogen control (yellow box) and nutrient uptake (purple box), which are enhanced by microbiome engineering (blue box). Unbalanced microbiome comes along with a low microbial diversity with predisposition to pathogen predominance, while high microbial diversity is found in balanced microbiome and inhibits the pathogen capacity to afflict grapevine.

3.2.3. Biological control agents (BCAs) as limited but efficient disease management strategies

Nurseries have proposed to winegrowers the possibility of inoculating rootstocks with specific microorganisms such as AMF prior to planting, in an effort to improve grapevine resilience to abiotic and biotic stresses. Biological control provides tools for disease management which are partly based on soil microbial properties that promote plant health and fruit quality. This strategy called biocontrol, has been exploited recently as an alternative to synthetic or chemical pesticides (Ilaria Pertot *et al.*, 2017). The most common BCAs in viticulture are used in spray application and are partly efficient, compared to the synthetic solutions, against powdery, downy mildew or gray mold, caused by *Erysiphe necator*, *Plasmopara viticola*, and *Botrytis cinerea* respectively (Dagostin *et al.*, 2011). Currently, commercial microbial fungicides sprayed on the grapevine aerial part can be derived from bacteria, yeast, and multicellular fungi (Table 7). Those listed microorganisms are present in a variety of habitats worldwide, and can

naturally be found in vineyard soils (Andreazza *et al.*, 2011; Angeli *et al.*, 2009; Nally *et al.*, 2012; Salunkhe *et al.*, 2013), hence comforting the vineyard soil studies for BCA screening.

Table 7 : Non-exhaustive list of common biological control products used in the wine-growing industry to apply on the grapevine's foliar part.

| Microorganism as active ingredient | Target pathogen | Tradename (manufacturer) | Mode of action | Reference |
|------------------------------------|---|------------------------------------|--|--|
| <i>Bacillus subtilis</i> | <i>Botrytis cinerea</i> | Rhapsody® Serenade Max® (Bayer) | Antimicrobial, eliciting plant defense | (Thomidis <i>et al.</i> , 2016) |
| <i>B. pumilus</i> | <i>Uncinula necator</i> | Sonata® (Bayer) | Antimicrobial, antibiosis | (Serrano <i>et al.</i> , 2013) |
| <i>Streptomyces griseoviridis</i> | <i>Botrytis cinerea</i> , <i>Fusarium</i> , <i>Alternaria</i> | Mycostop® (Verdera) | Competition | (Lahdenperä <i>et al.</i> , 1991) |
| <i>Ampelomyces quisqualis</i> | <i>Uncinula necator</i> | AQ10® (Ecogen) | Competition, antibiosis | (Hofstein <i>et al.</i> , 1996) |
| <i>Trichoderma harzianum</i> | <i>Botrytis cinerea</i> | Trichodex® (Makhteshim-Agan) | Competition | (O'neill <i>et al.</i> , 1996) |
| <i>T. atroviride</i> | <i>Phaeoacremonium minimum</i> , <i>Phaeoconiella chlamydospora</i> , <i>Botrytis cinerea</i> | Vintec® (Belchim Crop Protection) | Antibiosis | (Pertot <i>et al.</i> , 2017; Pertot <i>et al.</i> , 2016) |
| <i>Saccharomyces cerevisiae</i> | <i>Botrytis cinerea</i> | Julietta® (Agrauxine) | Antibiosis | (São-José <i>et al.</i> , 2017) |
| <i>Metschnikowia fructicola</i> | <i>Botrytis cinerea</i> | Noli® (Koppert Biological Systems) | Antimicrobial, eliciting plant defense | (Sipiczki, 2006) |
| <i>Aureobasidium pullulans</i> | <i>Botrytis cinerea</i> | Botector® (Nufarm) | Competition | (Calvo-Garrido <i>et al.</i> , 2019) |

Usually, spray applications are applied on the aerial part of the vine, targeting the leaves and berries where the first symptoms of the disease occur. However, the vine architecture and dense foliage may reduce the efficiency of the product, allowing the pathogen to sporulate on the untreated part of the crop. One solution to counteract the pathogen growth in viticulture is to leverage the microbe-associated molecular patterns (MAMPs) from beneficial microbes through belowground host-specific receptors, which prime grapevine immune response (Héloir *et al.*, 2019). This strategy is referred to as induced systemic resistance (ISR) and can benefit both the aboveground parts of the plant and the roots via BCAs when applied to the soil or grapevine root system (**Table 8**). ISR leads to the production of phytoalexins and/or pathogenesis-related (PR) proteins in the distal parts. Phytoalexins are low weight

metabolites synthesized after microbial recognition and signaling in plant cells acting as defense compounds. In grapevines, these molecules (**Table 8**) are mainly stilbenes and encompasses *trans*-resveratrol, *trans*- ϵ -viniferin, and its derivative *trans*-piceid (Jeandet *et al.*, 2014). Moreover, it has been shown that the BCA oomycete *Pythium oligandrum* inoculated at the root level can modulate the transcriptome of the grapevine but also of the *Phaeomoniella chlamydospora* virulence factors, a GTD ascomycota fungus, even when the two microorganisms are not in direct contact (Yacoub *et al.*, 2020). Among the GTDs, black-foot and Petri diseases are the most common and are present in nurseries and young vineyards. Their symptoms in fields include overall reduced growth, dysregulation in the budbreak and sprouting, with chlorotic leaves and necrosis on the rootstock (Gramaje and Armengol, 2011). *Trichoderma* spp., *Bacillus*, and *Pseudomonas*-based commercialized products as well as two potential BCAs (*i.e.*, *P. oligandrum* Po 37, *Streptomyces* sp. E1 and R4) reduced the Black-foot and Petri diseases by dipping the roots before planting under field conditions (Pilar Martínez-Diz *et al.*, 2021). Stempien *et al.* (2020) unveiled the grapevine defense activation triggered by *Trichoderma atroviride* (T-77 and USPP T1) drenching and its colonization on rootstock cultivars 110R, US 8-7, 1103P. It appeared that the level of expression of genes such as *VvSTS* and *VvChit4c* encoding proteins involved in stilbene synthesis and chitinase, respectively, was dependent on the rootstock genotype and *Trichoderma* strain used. Recently Jaarsveld *et al.* (2021) showed the higher colonization capacity by six *Trichoderma* products on graftlings (Sauvignon blanc cv. Grafted onto Ramsey) basal ends compared to middle or root tip part, even though *Trichoderma* spp. treatments were not sufficient to prevent fungal infections. Clear evidence of the biocontrol effects was observed *in vitro*, in greenhouse and in field (**Table 8**).

Table 8 : List of inocula used for their biological control properties on grapevine and applied on the soil or root system.

| Target pathogen (Disease) | Inoculum identification (Origin) | Observations | Plant material (Type of application) | Reference |
|----------------------------------|--|--|---|------------------------------------|
| <i>B. cinerea</i> (Gray mold) | <i>Bacillus subtilis</i> PTA-271, (Grapevine rhizosphere) | Systemic resistance. Accumulation of stilbenic phytoalexins, <i>trans</i> - resveratrol and ϵ -viniferin in leaves and berries. | Field, 15 years-old cv. Chardonnay-41B (Soil drenching) | (Aziz <i>et al.</i> , 2016) |
| | <i>P. fluorescens</i> PTA-CT2, and (Grapevine stem) | | | |
| | <i>Pantoea agglomerans</i> PTA- AF2 (Grapevine leaf) | | | |
| | <i>Pantoea agglomerans</i> Pa-AF2, (Grapevine leaf) | Local and systemic resistance. | <i>In vitro</i> , 4 weeks-old cv. Chardonnay | (Verhagen <i>et al.</i> , 2011) |
| | <i>Acinetobacter lwoffii</i> Al-113, (Grapevine roots) | Early oxidative burst and stilbenic phytoalexins | (Root dipping) | |
| | <i>B. subtilis</i> Bs271, and (Grapevine rhizosphere) | (<i>trans</i> -resveratrol and <i>trans</i> - ϵ -viniferin) accumulation in leaves. | | |
| | <i>P. fluorescens</i> PfCT2 (Grapevine stem) | | | |

| | | | | |
|---|--|--|--|--|
| | <i>Bacillus subtilis</i> PTA-271, <i>A. lwoffii</i> PTA-113, <i>P. agglomerans</i> PTA-AF1 and PTA-AF2, and <i>P. fluorescens</i> PTA-268 and PTA-CT2 (All isolated from grapevine rhizosphere) | Systemic resistance. Accumulation of chitinase and β -1,3-glucanase in leaves and berries. | Field, 10 years-old cv. Chardonnay-41B (Soil drenching) | (Magnin-Robert <i>et al.</i> , 2007) |
| | <i>Burkholderia</i> sp. BE17 and BE24 | Systemic resistance. H ₂ O ₂ accumulation and upregulations of <i>PR5</i> and <i>PR10</i> in leaves. | <i>In vitro</i> , 4 weeks-old cv. Chardonnay (Root dipping) | (Esmaeel <i>et al.</i> , 2020) |
| | <i>Paraburkholderia phytofirmans</i> PsJN | Systemic resistance. H ₂ O ₂ accumulation and upregulations of <i>PR1</i> , <i>PR2</i> , <i>PR5</i> , <i>WRKY</i> , and <i>JAZ</i> in leaves. | <i>In vitro</i> , 4 weeks-old cv. Chardonnay (Root dipping) | (Miotto-Vilanova <i>et al.</i> , 2016) |
| <i>P. viticola</i> (Downy mildew) and <i>B. cinerea</i> (Gray mold) | <i>Pseudomonas fluorescens</i> PTA-CT2 (Grapevine rhizosphere) | Systemic resistance. <i>P. viticola</i> : Stilbenes accumulation. Upregulations of <i>PR1</i> , <i>PR2</i> , <i>GST</i> , <i>ACO</i> , and <i>HSR</i> . <i>B. cinerea</i> : Stilbenes and resveratrol accumulation. Upregulations of <i>ACO</i> , <i>PR1</i> , <i>GST</i> genes and <i>HSR</i> downregulation. | Greenhouse, 2 years-old cv. Pinot noir-5BB and Solaris30-5BB (Soil drenching) | (Lakkis <i>et al.</i> , 2019) |
| <i>E. necator</i> (Powdery mildew) | <i>T. harzianum</i> 5R (Citrus rhizosphere, <i>T. viride</i> F-01812 (sugarcane soil), and F-01951 (forest soil), and <i>T. asperellum</i> F-01769 (soil)) | Systemic resistance. Increase in total phenol contents, chitinase, and β -1,3-glucanase in leaves. | Field, 8 years-old cv. Centennial Seedless (Soil drenching) | (Sawant <i>et al.</i> , 2020) |
| <i>Phaeoconiella chlamydospora</i> (Esca) | <i>Pythium oligandrum</i> Oth-2, Oth-3, Sto-1, Oth-4, Sto-7, and Sto-11 (Grapevine rhizosphere) | Systemic resistance. Oligandrin synthesis <i>in vitro</i> . <i>PR10</i> , <i>Glu</i> , <i>Gst</i> , and <i>Lox</i> upregulations. | Greenhouse, 4 months-old cv. Cabernet Sauvignon (Collar inoculation) | (Yacoub <i>et al.</i> , 2016) |
| <i>Neofusicoccum parvum</i> (Botryosphaeria dieback) | <i>Bacillus subtilis</i> PTA-271 (Grapevine rhizosphere), and <i>Trichoderma atroviride</i> SC1 (Hazelnut wood) | Decrease of salicylic acid (SA)-dependent defenses compared to symptomatic non plants. <i>LOX9</i> , <i>PR2</i> , <i>PAL</i> , and <i>STS</i> upregulation in leaves. | Culture chamber, 1 year-old cv. Chardonnay and Tempranillo. Soil drenching (<i>B. subtilis</i> PTA-271) and wound painting (<i>T. atroviride</i> SC1). | (Leal <i>et al.</i> , 2021) |
| <i>Agrobacterium tumefaciens</i> (Crown gall) | <i>Pseudomonas kilonensis</i> Sn48, (Grapevine roots) and <i>P. agglomerans</i> Sa14 (Wild-grapevine stem) | Systemic resistance. Stilbenic phytoalexins (<i>trans</i> -resveratrol, <i>trans</i> -piceid, and <i>ϵ</i> -viniferin) global accumulation in leaves, roots, and stems. <i>PR1</i> , <i>PR2</i> , and <i>PR4</i> genes upregulation in leaves. | Greenhouse, 4 weeks-old cv. Chardonnay (Root dipping) | (Asghari <i>et al.</i> , 2020) |

These findings suggest that preventive application by soil drenching or root inoculation could be a promising strategy for disease management since the molecular mechanisms underlying the biocontrol effects of the inoculum are deciphered.

3.2.4. Microbiome can enhance abiotic stress tolerance

By mitigating abiotic stresses, microbiome × rootstock interactions could be a relevant way to contribute to adaptation in the global climate change context. Up to now, the mechanisms developed by the plants to recruit their microbiomes in response to specific abiotic stresses remain poorly understood.

The root microbiome can enhance water deficit tolerance by acting in hormone regulation or by increasing plant antioxidant activity (de la Fuente Cantó *et al.*, 2020). To this end, trends in microorganisms' biomass, diversity, and activity under water deficit conditions have been explored (Caddell *et al.*, 2019; de la Fuente Cantó *et al.*, 2020). Exopolysaccharides (EPS) allow beneficial microbes to efficiently colonize the rhizosphere by increasing the percentage of stable soil aggregates and thus by increasing water and nutrient uptake (Caddell *et al.*, 2019). It was also demonstrated that microorganisms from more fluctuating environments have a higher functional acclimatization (Hawkes and Keitt, 2015). In addition, plants benefit from their associated microbiome to tolerate water deficit, especially when the microbiome has been previously exposed to water deficit with the host plant in years before (Zolla *et al.*, 2013). In grapevines, few studies have been made on the microbiome impact on abiotic stress (Pacífico *et al.*, 2019). However, all the microorganisms tested were originated from root endosphere compartment and some of them vary in their effect depending on the rootstock genotype (**Table 9**). This comforts the hypothesis that microbiota from resistant rootstock in stressed environment might be an interesting strategy to investigate.

In addition, several microorganisms isolated from grapevine roots were studied for their capacity to synthesize protective molecules that might alleviate abiotic stresses. Carotenoids, known for their antioxidant activities and as precursors of abscisic acid, were produced by *Microbacterium imperial* Rz19M10, *Kocuria erythromyxa* Rt5M10, and *Terribacillus saccharophilus* Rt17M10 (Salomon *et al.*, 2016) but also by *B. licheniformis* Rt4M10 (Cohen *et al.*, 2018). The metabolism of abscisic acid could be modulated in the advantage of inoculated grapevines with arbuscular mycorrhizal symbiosis (Torres *et al.*, 2018). Among the protective molecules, the melatonin allows to counteract the negative effects of abiotic stresses and it has been shown that inoculated grapevines with *Bacillus amyloliquefaciens* SB-9 (Jiao *et al.*, 2016) or with *Pseudomonas fluorescens* RG11 (Ma *et al.*, 2017) accumulate more melatonin.

Additionally to bacterial endophytes, water deficit stress can be alleviated by the presence of AMF thanks to their external mycelium that increase water use efficiency even though there is no current evidence of direct water transfer to the plant (Trouvelot *et al.*, 2015).

Table 9 : List of inocula used for their beneficial effect on grapevine submitted to abiotic stress and applied on the soil or root system.

| Abiotic stress (Factor to counter) | Inoculum identification (Origin) | Observations | Plant material (Type of application) | Reference |
|------------------------------------|---|--|--|-------------------------------------|
| Arsenic | <i>Bacillus licheniformis</i> Rt4M10, <i>Micrococcus luteus</i> Rz2M10 and <i>P. fluorescens</i> Rt6M10 (Grapevine root endosphere and rhizosphere) | Reduction of arsenic toxicity indicators with enhanced ascorbate peroxidase activity (<i>B. licheniformis</i>) and increased peroxidase activity (<i>M. luteus</i> and <i>P. fluorescens</i>) | Greenhouse, 2 years-old cv. Malbec (Leaf sprayed and stem-based inoculation) | (Funes Pinter <i>et al.</i> , 2018) |
| Drought | <i>Acinetobacter</i> and 2 <i>Pseudomonas</i> spp. (Grapevine root endosphere) | Higher tolerance to water deficit by maintaining photosynthetic activity and growth which was rootstock dependent. Positive effect on evapotranspiration and stomatal conductance. | Greenhouse, 1 year-old cv. SO4, 420A, 5BB (Roots dipping) Field, 1 year-old cv. Barbera (Roots dipping) | (Rolli <i>et al.</i> , 2015) |
| Drought | <i>Glomus mosseae</i> (not specified) | Higher tolerance to water deficit by maintaining photosynthetic activity and growth which was rootstock dependent. Positive effect on evapotranspiration and stomatal conductance. Increase of phosphorus content in leaves. | Greenhouse, 1 year-old cv. Cabernet-Sauvignon grafted on 110R, 41B, 1103P, 5BB, 44–53 Malegue, 140R and 101–14MGt (Soil inoculation) | (Nikolaou <i>et al.</i> , 2003) |

Besides the issues surrounding water deficit, the problem of soil salinization impacts a large percentage of irrigated vineyards worldwide (Aragüés *et al.*, 2015). AMF are known to improve growth related traits in saline conditions. Khalil (2013) demonstrated on three rootstocks genotypes (1103P, Harmony, and Dogridge) that AMF addition contributes to increase plant height, stem diameter, leaf area, total leaf number, and total dry weight even if the effects were not significant. The total carbohydrates, leaf free proline content, and total leaf chlorophyll content were higher in inoculated seedlings than in uninoculated ones, suggesting a higher osmoprotection coupled to a photosynthesis maintenance. Moreover, mycorrhizal inoculation tends to decrease the Na and Cl concentrations while increasing P and K leaves content. A

relevant choice of rootstock with mycorrhizal inoculation could be one way to avoid salinity problems in a vineyard.

The complexity of the interactions between the plant, the microbiome, and the surrounding environment is an issue that must be overcome to understand the beneficial associations between plants and microbes. It appears more relevant to isolate plant growth-promoting microbe (PGPM) that can promote tolerance to a specific abiotic stress from environments in which this stress occurs (Rodriguez *et al.*, 2008). It could be outstanding to study the plasticity of the PGPM to rootstock × scion × interactions at the field level, hence the importance of including the microbiome in grapevine breeding programs (Gómez-Bellot *et al.*, 2015). As suggested for the tree species, association of rootstocks with different beneficial microbiota could be a relevant way to share the benefits of the microbiota from one individual to another to get a “microbial complementarity” (Bettenfeld *et al.*, 2020).

3.2.5. Are soil microbial inoculum a safe and relevant process to increase grapevine resilience?

The establishment and persistence of the BCAs in the soil and root compartments remain one of the most important concerns in microbial inoculant preparation (Verbruggen *et al.*, 2013). Although the transfer of inoculation to different climatic regions can be a success, the effect may not be the same depending on pedoclimatic features (Chibeba *et al.*, 2018). Aside from these technical aspects, the BCAs legislation among EU, USA, and worldwide markets are quite different but remain important for their biosafety which are based on molecular identification coupled to pathogenicity, toxicological, and 37°C-growth tests (Velivelli *et al.*, 2014). While the biosafety issue has always been evaluated for human healthcare and plant health, the mass application of PGPM in the environment is never considered during the BCAs development. What if the PGPM application provokes soil or plant microbiome dysbiosis and lately its degradation (Keswani *et al.*, 2019)? What if a BCA turns out to become pathogenic, due to horizontal gene transfer from other surrounding microbes or because of the evolution or speciation?

In grapevine wood tissues, Haidar *et al.* (2021) unveiled the synergistic effect of some bacterial strains with the basidiomycete *Fomitiporia mediterranea* involved in esca complex, to degrade wood components. The interesting part is the capacity of some of these bacterial strains to inhibit the pathogen growth *in vitro*, while having cellulose and xylan degradation properties. In grapevines, colonization process by inoculating beneficial endophytes such as *Paraburkholderia phytofirmans* strain PsJN or strains of *Enterobacter ludwigii* and *Pantoea*

vagans have been studied in young plants (Compant *et al.*, 2005; Lòpez-Fernàndez *et al.*, 2016), and among the PGPR inoculated on grapevine roots, they are mainly composed from *Pseudomonas*, *Bacillus*, *Pantoea*, and *Burkholderia* genera (**Table 8**). However, depicting the PGPR inoculation impact on the soil microbiome remains a challenge and should combine both culture-dependent and independent approaches. Indeed, exogenous microorganisms might affect soil quality negatively by modifying soil capacity to process bio-geochemical cycles and hence, its potential to promote vine growth.

Soil exhibits the natural ability to suppress disease through its microbiome composition which is enhanced by agricultural processes that positively influence microbial diversity (Cook, 2014; Richards *et al.*, 2020). For instance, Nerva *et al.* (2019) investigated the microbial profile of both Esca-symptomatic and asymptomatic soils which suggested that higher proportions of *Curvularia*, *Coprinopsis*, *Bacillus*, and *Streptomyces* genera could suppress disease symptoms. These studies further support the idea that bulk soils are a major source of inoculum for pathogens. Microbial transplant is now assumed in medical research as a solution to modulate the human microbiota coupled to therapeutic effects (Smits *et al.*, 2013). While not conducted in a vineyard, Siegel-Hertz *et al.* (2018) used soil transplants from suppressive soil to show inhibiting effects on Fusarium wilt conducive soils. Exclusive bacterial and fungal genera were found in Fusarium wilt-suppressive soils compared to conducive soils which suggest that microbiome transplant could be an efficient and promising way to promote microbiome diversity. This strategy within a vineyard could counteract the microbiome dysbiosis and the problematic effect of the inoculum survival since the soils possess quite similar abiotic features. Biocontrol is assumed to be less efficient in disease management compared to chemical and synthetic products. One biotechnology-based tool that must be mentioned for increasing the microorganisms' efficiency in pathogen control is the protoplast fusion technique, which is mainly studied for genetic transformation and somatic hybridization. This approach is quite difficult in grapevines and has recently been used for whole grapevine generation from protoplasts (Bertini *et al.*, 2019). Protoplast fusion technique is also used in PGP and biocontrol bacteria to merge distinct traits. For instance, Gaziea *et al.* (2020) attempted to merge, the biocontrol ability of *Bacillus thuringiensis* I977 against *Meloidogyne* spp. and the PGP capacity of *Pseudomonas aeruginosa* in grapevine seedlings and successfully controlled the root-knot nematode while promoting the plant growth. While this approach has not been tested on the field, it has already been considered against root-knot nematodes (Abdel-Salam *et al.*, 2018) and remains an interesting solution for BCA or biofertilizer products. *Trichoderma* spp., which are one of the most famous BCAs worldwide, have also been subjected to capacity enhancement

for soil-borne disease suppressiveness (Lakhani and Vakharia, 2016). Strains engineered via protoplast fusion are not affiliated to genetically modified organisms' regulations since this technique is a form of natural homologous recombination (Zhang *et al.*, 2002), hence giving the possibility for BCAs to have more positive impacts on grapevine health.

3.3. Conclusions and future prospects

Altogether, these findings demonstrate that the grapevine is able, via rootstock and scion genotypes, to select distinct but potentially beneficial microorganisms close to the roots. Although there is no consensus regarding the choice of hypervariable regions to amplify and sequence (**Table 6**), it is still possible to make comparable taxonomic descriptions between studies at the phyla level. However, it may be quite difficult to compare at the genera or species level since bias, in addition to “universal primers” choice, can occur until data processing (Pollock *et al.*, 2018). The rhizosphere and root-associated microbiome, which are a balance between stress and fitness, would be relevant biological indicators of plant health status. The rhizosphere could be considered as an extended root phenotype, presented by Dawkins (1982), which is a trait that may also reflect the agronomic properties of the rootstock as well as its health status. To this end, soil microbial diversity could explain many dysbiosis and symbiosis observed in the grapevine organs since most of them are recruited from the surrounding soil. Until now, no research of soil virome in vineyards has been done even though it is known that the viruses are playing important roles in ecological processes and microorganism evolution (Pratama and van Elsas, 2018), whereas the grapevine associated virome has been well investigated in leaf and trunk tissues (Martelli, 2017).

Given increasing environmental constraints, improving viticulture sustainability is currently a major challenge. One important area of study to improve sustainability includes better understanding soil microbiome functionalities and its effects on the grapevine metabolism and agronomic responses. Based on the current literature, the soil microbiome could offer new engineering solutions to palliate intensive phytosanitary use and climate change issues. To this end, molecular and microbial dialogues between the scion and the soil through the rootstock must be considered. The core microbiome of the grape should be preserved as it represents a sensitive balance for the plant protection, growth, nutrition, and health.

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Conflict of interests

The authors declare no competing interests.

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Supplemental information

Supplementary information accompanies the manuscript on the Horticulture Revised Research website <http://www.nature.com/hortres>

Table S1 : Examples of the bacterial diversity among the different grapevine compartments.

| Compartments | Approaches used and main phyla detected | Main microorganisms detected | Scion/Rootstock combinations | References |
|---|--|---|--|---------------------------------|
| Berry surface, leaves | MiSeq on 16S rRNA (V3-V4 regions) | Leaves: <i>Alphaproteobacteria</i> , <i>Saprospirae</i> , <i>Cytophagia</i> , <i>Actinobacteria</i> | Dolcetto, Sangiovese cv. grafted on different rootstocks (not specified) | (Vitulo <i>et al.</i> , 2019) |
| | Leaves: <i>Proteobacteria</i> , <i>Bacteroidetes</i> , <i>Actinobacteria</i> | Berry surface: <i>Alpha/Beta/Gamma-Proteobacteria</i> , <i>Bacilli</i> , <i>Actinobacteria</i> | | |
| | Berry surface: <i>Proteobacteria</i> , <i>Firmicutes</i> , <i>Actinobacteria</i> | | | |
| Berry surface, grape must, leaves, wine, soil | MiSeq on 16S rRNA (V4 region) | Berry surface: <i>Pseudomonas</i> , <i>Acinetobacter</i> , <i>Kaistobacter</i> , <i>Sphingomonas</i> | Cabernet Sauvignon, not specified if grafted or not | (Wei <i>et al.</i> , 2018) |
| | Berry surface and must, leaves, wine: <i>Proteobacteria</i> , <i>Firmicutes</i> | Grape must: <i>Oenococcus</i> , <i>Pseudomonas</i> | | |
| | Soil: <i>Proteobacteria</i> , <i>Firmicutes</i> , <i>Bacteroidetes</i> , <i>Actinobacteria</i> | Leaves: <i>Pseudomonas</i> , <i>Acinetobacter</i> , <i>Kaistobacter</i> , <i>Sphingomonas</i> , <i>Oenococcus</i> | | |
| | | Wine: <i>Oenococcus</i> | | |
| | | Soil: <i>Kaistobacter</i> , <i>Arthrobacter</i> , <i>Skermanella</i> , <i>Sphingomonas</i> | | |
| Soil, roots, graft union, cane | MiSeq on 16S rRNA (V4 region) | Soil: <i>Nitrososphaera</i> , <i>Flavobacterium</i> , <i>Agrobacterium</i> | Cabernet Dorsa grafted onto SO4 | (Faist <i>et al.</i> , 2016) |
| | Soil: <i>Proteobacteria</i> , <i>Acidobacteria</i> , <i>Actinobacteria</i> , <i>Bacteroidetes</i> , <i>Firmicutes</i> , <i>Crenarchaeota</i> , <i>Planctomycetes</i> , <i>Verrucomicrobia</i> , <i>Chloroflexi</i> | Roots: <i>Methylobacterium</i> , <i>Pseudomonas</i> , <i>Steroidobacter</i> , <i>Erwinia</i> , <i>Sediminibacterium</i> , <i>Bradyrhizobium</i> | | |
| | Root: <i>Proteobacteria</i> , <i>Actinobacteria</i> , <i>Bacteroidetes</i> | Graft union: <i>Pseudomonas</i> , <i>Agrobacterium</i> , <i>Erwinia</i> , <i>Sodalis</i> | | |
| | Graft union: <i>Proteobacteria</i> , <i>Actinobacteria</i> | Cane: <i>Pseudomonas</i> | | |
| | Cane: <i>Proteobacteria</i> | | | |
| Berry surface | Ion Torrent on the 16S rRNA (V4 region) | <i>Bacillus</i> , <i>Erwinia</i> , <i>Acinetobacter</i> , <i>Oenococcus</i> | Ungrafted Carignan and Grenache cultivars | (Portillo <i>et al.</i> , 2016) |

| | | | | |
|---|---|---|--|--------------------------------------|
| Bulk soil, rhizosphere, roots, leaves, grape surface, must, flowers | <p>MiSeq on 16S rRNA (V4 region)</p> <p>Soil: <i>Proteobacteria</i>, <i>Acidobacteria</i>, <i>Bacteroidetes</i>, <i>Verrucomicrobia</i>, <i>Planctomycetes</i></p> <p>Roots: <i>Proteobacteria</i>, <i>Acidobacteria</i>, <i>Bacteroidetes</i>, <i>Verrucomicrobia</i>, <i>Actinobacteria</i></p> <p>Leaves, grapes, flowers: <i>Proteobacteria</i></p> | <p>Roots: <i>Xanthomonadales</i> (<i>Steroidobacter</i>), <i>Cytophagaceae</i>, <i>Chitinophagaceae</i>, <i>Rhizobiales</i>, <i>Actinomycetales</i></p> <p>Leaves, grape: <i>Sphingomonas</i>, <i>Pseudomonas</i>, <i>Methylobacterium</i></p> <p>Flowers: <i>Pseudomonas</i> and <i>Erwinia</i> spp.</p> | Merlot cv. grafted onto 3309C | (Zarraonaindia <i>et al.</i> , 2015) |
| Grape must, wine | <p>MiSeq on 16S rRNA (V4 region)</p> <p>Grape must: <i>Firmicutes</i>, <i>Proteobacteria</i></p> <p>Wine: <i>Firmicutes</i></p> | <p>Grape must: <i>Leuconostocaceae</i>, <i>Enterobacteriaceae</i>, <i>Bacillaceae</i>, <i>Pseudomonas</i>, <i>Sphingomonas</i></p> <p>Wine: <i>Leuconostocaceae</i></p> <p>Grape must: <i>Botryotinia fuckeliana</i>, <i>Cladosporium</i>, <i>S. cerevisiae</i></p> <p>Wine: <i>Cladosporium</i>, <i>Botryotinia fuckeliana</i>, <i>S. cerevisiae</i></p> | Cabernet Sauvignon, Chardonnay, Zinfandel cv., not specified if grafted or not | (Bokulich <i>et al.</i> , 2014) |
| Leaves | <p>Pyrosequencing 16S rRNA (V5-V9 regions)</p> <p><i>Proteobacteria</i>, <i>Actinobacteria</i></p> <p>Pyrosequencing 16S rRNA (V6 region)</p> <p><i>Proteobacteria</i>, <i>Actinobacteria</i></p> | <p><i>Pseudomonas</i>, <i>Frigoribacterium</i>, <i>Sphingomonas</i>, <i>Erwinia</i>, <i>Acetobacter</i>, <i>Curtobacterium</i></p> <p><i>Enterobacteriaceae</i>, <i>Pseudomonadaceae</i>, <i>Moraxellaceae</i>, <i>Comamonadaceae</i>, <i>Streptococcaceae</i>, <i>Actinobacteria</i></p> | Pinot gris cv., not specified if grafted or not | (Perazzolli <i>et al.</i> , 2014) |
| | | | Tempranillo cv., not specified if grafted or not | (Pinto <i>et al.</i> , 2014) |

Table S2 : Examples of the fungal diversity among the different grapevine compartments.

| Compartments | Approaches used and main phyla detected | Main microorganisms detected | Scion/Rootstock combinations | References |
|---|---|--|--|-------------------------------------|
| Rootstock xylem | MiSeq on ITS2 <i>Ascomycota</i> | <i>Cladosporiaceae, Dothioraceae, Nectriaceae, Pleosporaceae, Ploettnerulaceae, Trichocomaceae</i> | Ungrafted 110R and 41B | (Gramaje <i>et al.</i> , 2021) |
| Roots, rhizosphere | MiSeq on ITS2, <i>Ascomycota, Basidiomycota</i> | <i>Nectriaceae, Ceratobasidiaceae, Mortierellaceae</i> | Ungrafted SO4 | (Carbone <i>et al.</i> , 2021) |
| Grapes, flowers, leaves, roots | MiSeq on ITS1 <i>Ascomycota, Basidiomycota</i> | <i>Aureobasidium, Cladosporium, Epicoccum, Mortierella, Cryptococcus, Debaryomyces, Saccharomyces, Mycosphaerella, Lophiostoma, Alternaria, and Penicillium</i> | Pinot Noir cv., not specified if grafted or not | (Liu and Howell, 2021) |
| Roots, bulk soil, rhizosphere | MiSeq on ITS2 <i>Ascomycota, Basidiomycota</i> | <i>Mortierellaceae, Nectriaceae, Pleosporaceae</i> | Tempranillo cv. grafted onto 110R | (Martínez-Diz <i>et al.</i> , 2019) |
| Branches | Plating method <i>Ascomycota, Basidiomycota</i> | <i>Alternaria spp., Aureobasidium pullulans, Diplodia seriata, Cladosporium spp., Epicoccum nigrum</i> | Riesling cv., not specified if grafted or not | (Kraus <i>et al.</i> , 2019) |
| Berry surface | MiSeq on ITS <i>Ascomycota, Basidiomycota</i> | <i>Pleosporaceae, Cladosporiaceae, Sporidiobolales, Aureobasidiaceae</i> | Table grape Crimson Seedless cv., not specified if grafted or not | (Carmichael <i>et al.</i> , 2019) |
| Total berry, flower, leaves, air | Plating method and MiSeq on ITS1 / ITS4 <i>Ascomycota, Basidiomycota</i> | Plating: <i>Alternaria, Cladosporium, Aspergillus, Botryosphaeria</i> MiSeq: <i>Alternaria, Cladosporium, Pleosporaceae, Lasiodiplodia, Phoma, Botrytis, Aspergillus, Penicillium</i> | Midnight beauty cv. | (Dissanayake <i>et al.</i> , 2018) |
| Berry surface, grape must, leaves, soil | MiSeq on ITS All compartments: <i>Ascomycota, Basidiomycota</i> | Berry surface: <i>Aureobasidium, Pleosporaceae, Dothideale, Cryptococcus</i> Grape must: <i>Aureobasidium, Erysiphe, Aspergillus, Cryptococcus</i> Leaves: <i>Aureobasidium, Pleosporaceae, Dothideales, Erysiphe, Alternaria, Cryptococcus</i> Soil: <i>Ascomycota, Sordariales, Tetracladium, Dothideales, Pleosporales</i> | Cabernet Sauvignon cv., not specified if grafted or not | (Wei <i>et al.</i> , 2018) |
| Wood: crown area and grafting area from rootstock | Plating Method and MiSeq on ITS2 from cDNA | MiSeq: <i>Erysiphaceae, Lasiosphaeriaceae, Mycosphaerellaceae, Nectriaceae, Pleosporaceae</i> Plating: <i>Bionectriaceae, Nectriaceae, Microascaceae, Trichocomaceae</i> | Garnacha Tintorera cv. grafted onto rootstock 110R, and Sauvignon Blanc cv. grafted onto SO4 | (Eichmeier <i>et al.</i> , 2018) |

| | | | | |
|------------|--|--|---|--------------------------------------|
| Grape must | MiSeq on ITS <i>Ascomycota</i> , <i>Saccharomycotina</i> | <i>Cladosporium</i> spp., <i>Botrytis cinerea</i> , <i>Penicillium</i> spp., <i>Davidiella tassiana</i> , <i>Aureobasidium pullulans</i> , <i>S.</i> <i>cerevisiae</i> , <i>Hanseniaspora uvarum</i> , <i>Candida zemplinina</i> | Cabernet Sauvignon, Chardonnay, Zinfandel cv., not specified if grafted or not | (Bokulich <i>et al.</i> , 2014) |
| Leaves | Pyrosequencing on ITS <i>Ascomycota</i> , <i>Basidiomycota</i> | <i>Cryptococcus</i> , <i>Dioszegia</i> , <i>Sebacina</i> | Pinot gris cv., not specified if grafted or not | (Perazzolli <i>et al.</i> , 2014) |
| | Pyrosequencing on ITS2 and D2 <i>Ascomycota</i> , <i>Basidiomycota</i> | <i>Rhizopus</i> , <i>Mucor</i> , <i>Zoophthora</i> , <i>Pandora</i> , <i>Aureobasidium</i> , <i>Sporormiella</i> , <i>Alternaria</i> | Tempranillo cv., not specified if grafted or not | (Pinto <i>et al.</i> , 2014) |

Objectives and thesis plan

As just reviewed, grapevine is confronted to several environmental stresses that induces decline. Microorganisms associated with grapevines originate largely from the soil interface and are taken up by the rootstock through the rhizosphere and root compartments. The genetic dimension of the rootstock is a consistent feature of the grapevine-associated microbiome selection since different compounds are exudated from the roots to attract distinct endophytic microbes. The structure of endophytic microbial communities is considered as a relevant biological indicator of grapevine fitness because of their negative or beneficial impacts on host plant. To another extent, soil microbial quality might also be determinant for the development and health of the vine.

In France, the National Vineyard Decline Plan (PNDV) was set up in 2016 by the Ministry of Agriculture to understand the causes and mechanisms leading to vineyard decline. This thesis work is part of the PNDV and is included in the Vitirhizobiome project which aims to comprehend the role and functioning of the rhizosphere and root microbiome in the development of grapevine in a decline context. The decline context is definite and at the same time exploratory since Vitirhizobiome seeks to investigate unexplained grapevine declines. Herein, the term "unexplained" refers to the lack of identified reasons such as symptoms related to fungal, viral or bacterial pathological causes, or even mineral deficiencies, or excess nutrients.

The main working hypothesis is that soil microbial quality is a biological indicator of vine health and is linked to its growth and development. In this context of decline, one can assume that the microbial communities of symptomatic vineyards could be dysregulated. But to what extent? Is there an overall imbalance between beneficial and pathogenic microbes, or is there a specific depletion of certain keystone taxa?

In addition, since the rootstock acts as a selector of grapevine-associated microorganisms, there are questions related to its interactions with soil microbes that raise interest. For instance, what is the importance of the rootstock in the selection process of the microbes, and to what degree do soil microbes influence the grapevine development? Considering that soil communities are in dysbiosis, is it possible to stimulate soil and root communities to promote vine growth?

This work does not pretend to predict vine health by the initial microbial composition of the soil, but rather to link microbial structures and activities to observed phenotypes.

The strategy adopted in this work to investigate the role of soil microbiome on grapevine development is schematized in **Figure 19**. The first objective is to understand how are related the bulk soil microbiome and the grapevine health in four Bordeaux vineyards subjected to

unexplained decline (Chapter 2). Then, the second objective is to know whether this unexplained decline, with a focus on a single vineyard, is affecting the root and rhizosphere microbiome (Chapter 3).

The third objective is to see if the decline is reproducible under controlled conditions by transplanting vineyard soil into pots filled with young grapevines having different genetic backgrounds (Chapter 4). More precisely, this experiment aims to perceive the rootstock genotype and soil microbiome interactions, and additionally to isolate beneficial rhizobacteria. Finally, the last questioning is about the impact of addition of previously isolated (*i.e.*, PGPR) and commercially available (*i.e.*, AMF) beneficial microorganisms on soil microbiome and young grapevine (Chapter 5).

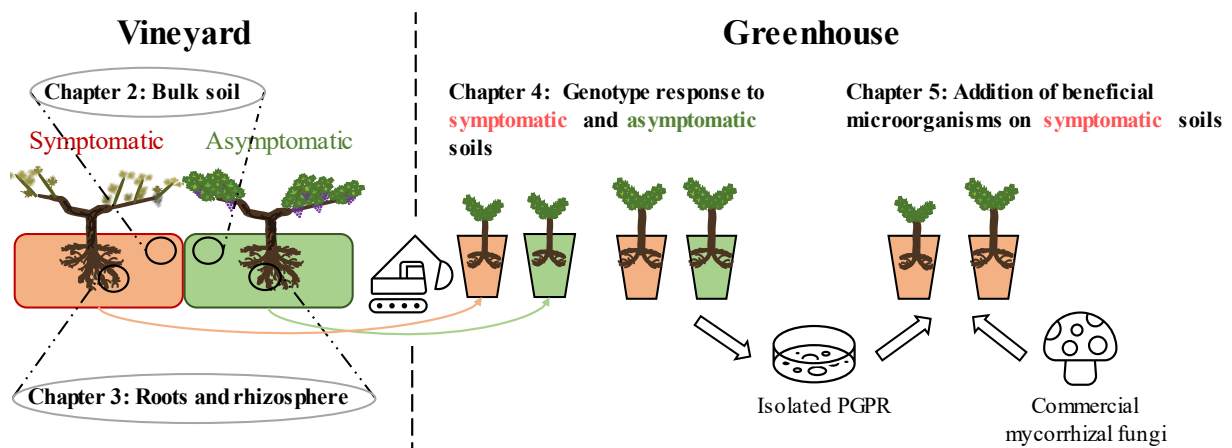


Figure 19: Schematic overview of the adopted strategy to investigate the role of soil microbiome on grapevine development.

Chapter II

Dysregulation of the microbial communities in bulk soil from declining vineyards

Preface

Grapevine decline is due to plurifactorial causes linked to environmental issues or agricultural practices and are sometimes not clearly related to pathological incidences or mineral deficiencies. Vineyard soil composition and functionalities may therefore explain the degradation of grapevine fitness.

The purpose of Chapter 2 was to find some quality indicators of the bulk soil from the inter-row of unexplained vineyards decline. Through a multisite study in four vineyards located in two terroirs from Bordeaux area (*i.e.*, Haut-Médoc and Graves appellations), diverse parameters of soil quality were assessed. Comparisons were performed between declining (symptomatic) and non-declining zones (asymptomatic) for each vineyard. In addition, samples were collected during autumn and spring to add a temporal dimension for these quality assessments in diverse locations.

At first, physicochemical and microbial parameters were investigated through cultivable dependent approach, Eco-Plates measurements, and q-PCR methods, on the bulk soils from the inter-row of these four vineyards subjected to unexplained decline.

Results obtained were the subject of the research article entitled “**Grapevine decline is associated with difference in soil microbial composition and activity**”. This article was published in OenoOne on July 20, 2021 and constitutes the first part of this chapter.

Dressing a profile of the bacterial and fungal structures present in these symptomatic soils is the subject of the second part of this chapter. The objectives were to compare the microbiome composition from the same samples investigated during the first part. The spatial (*i.e.*, terroir: four vineyards from two distinct terroir) and temporal (*i.e.*, season: during autumn and spring periods) dimensions were added to the soil status factor (*i.e.*, symptomatic and asymptomatic) of the inter-rows. To this end, libraries were prepared for amplicons-sequencing based on ITS region, as well as 16S and 18S genes, for the identification of fungi, bacteria, and *Glomeromycota* divisions, respectively.

This second part was the subject of a research article in preparation entitled “**Bacterial and fungal soil microbiomes in vineyard subjected to decline**” that will soon be submitted to Soil biology and biochemistry.

1. Grapevine decline is associated with difference in soil microbial composition and activity

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Romain Darriaut¹, Guilherme Martins^{2,4}, Coralie Dewasme¹, Séverine Mary³, Guillaume Darrieutort³, Patricia Ballestra², Elisa Marguerit¹, Philippe Vivin¹, Nathalie Ollat¹, Isabelle Masneuf-Pomarède^{2,4}, Virginie Lauvergeat^{1*}

¹ EGFV, Univ. Bordeaux, Bordeaux Sciences Agro, INRAE, ISVV, F-33882, Villenave d'Ornon, France

² Université de Bordeaux, ISVV, Unité de recherche Œnologie EA 4577, USC 1366 INRA, Bordeaux INP, 33140 Villenave d'Ornon, France

³ Université de Bordeaux, Vitinnov, Bordeaux Sciences Agro, ISVV, 1 cours du Général de Gaulle, 33170 Gradignan, France

⁴ Bordeaux Sciences Agro, 1 cours du Général de Gaulle, 33170 Gradignan, France

*corresponding author: virginie.lauvergeat@inrae.fr

Abstract

Grapevine decline is a top concern in viticulture worldwide and is often associated with many biotic and abiotic factors. Grape trunk diseases and viruses are some of the most frequently identified causes of vine dieback. However, a decline is sometimes observed when no mineral deficiency or excess, or pathogenic causes can be identified. Soil enzymatic and microbial activities are relevant bio-indicators since they are known to influence vine health. Grapevine associated microbiota, linked to vine fitness, is known to be influenced by soil microbiota coming from the microbial pool inhabiting the vineyard. This work describes the microbial diversity and activity of four different vineyard plots of Bordeaux region, selected due to the presence of localized declining areas unexplained yet by disease symptoms. Soils were sampled in declining areas and in areas within the same plot showing no decline symptoms, during autumn and spring periods. Significant differences in enzymatic activities, microbial biomass and activity were found among soils even if those soils presented quite similar physicochemical characteristics that could not explain these observed declines. The results of enzymatic assays distinguished patterns in autumn and spring periods with an overall greater enzymatic activity in soils from non-declining areas. This work suggests that soils displaying decline symptoms present a dysbiosis in functionality and diversity which is linked to vine health.

Keywords: Enzymatic activities, Grapevine decline, Microbial diversity, Terroir, Vineyard soil

Introduction

Aside from its economic value, viticulture in France plays a significant historical and cultural role due to differing agricultural practices depending on pedoclimatic conditions and geographical traditions. Certain French wine production areas, and more globally terroirs such as Val de Loire and Saint-Emilion, are even considered “World heritage UNESCO” sites (Rochard, 2016). Obviously, terroir, which is defined as a region linked to a specific ecosystem with a distinct quality of grapes and therefore wines, is shaped by several factors. It is well-known that core parameters of terroir such as climate, soil, plant material, and human practices influence vineyard productivity, and berry quality (Van Leeuwen *et al.*, 2010; Rotaru *et al.*, 2010). Facing the increased challenges posed by climate change, viticulture needs new tools to adapt to these environmental constraints that perpetuate the issue of vineyard decline observed for several decades (Marín *et al.*, 2020; Mosedale *et al.*, 2015; Reineke and Thiéry, 2016).

Vineyard decline, defined as a vine multiannual yield diminution or its premature, brutal or progressive death, is afflicting viticulture worldwide (Riou *et al.*, 2016). It is a high concern in French viticulture with more than 4.6 hl/ha yield loss estimated in 2014, encompassing around 10 % of grapevine plantation at the national scale (BIPE, 2015). The causes of this decline are complex. Grapevine decline is often linked to disease symptoms that can be due to bacteria (Hopkins and Purcell, 2002), fungi and oomycetes (Mondello *et al.*, 2018), viruses (Maree *et al.*, 2013), pests (Reineke and Thiéry, 2016), and even genetic susceptibility of the rootstock (Renault-Spilmont, 2007).

Currently, the best understood pathologies associated to grapevine decline remain the grapevine trunk diseases (GTD) which include *Eutypa* dieback, Esca disease complex, and *Botryosphaeria* dieback which are the most predominant ones caused by specific fungi with well-documented rot symptoms (Bertsch *et al.*, 2013). Besides GTDs, viruses such as Grapevine Fanleaf Virus (GFV) and Arabis Mosaic Virus (ArMV) are also known to cause specific symptoms affecting mainly the scion (Martelli, 2017). This type of decline is known to be linked with the soil microbiological status. For instance, Nerva *et al.* (2019) recently showed a link between bulk microbiome composition in vineyard soil and Esca severity by comparing symptomatic and asymptomatic vines, suggesting that bulk soil is the source of GTD inoculum. However, in many cases, no pathologic causes can be identified in declining vineyards. These declines could be caused by numerous abiotic factors such as climate (water stress, light exposure, heat stress ...), viticultural practices, soil quality, and/or the use of pesticides.

Physicochemical and biological parameters interact in a delicate balance that may easily flip into vineyard decline. It is assumed that environmental abiotic factors such as climate (Sosnowski *et al.*, 2007) and soil features (Lecomte *et al.*, 2011) are, most of the time, linked to pathogen predisposition to provoke decline issues. At the microscopic scale, soil microbiota have a broad range of interactions with host-plant, from pathogenic to commensal or beneficial effects that can be observed at a macroscopic scale (Newton *et al.*, 2010). Moreover, soil inhabiting microbes shape grapevine associated microbiota (Martins *et al.*, 2013) and are fully considered as determinant factors for wine quality (Zarraonaindia *et al.*, 2015). These micro-organism dynamics play key roles in host plant health and productivity through several direct and indirect processes with for instance plant immune response triggering (Chisholm *et al.*, 2006), carbon (Schimel and Schaeffer, 2012), nitrogen (Mooshammer *et al.*, 2014), and phosphorus (Richardson and Simpson, 2011) cycling. Soil microbiota composition in terms of genetic diversity is currently under investigation by the international grapevine scientific community since metagenomics-based tools are more affordable. A strong effort is being made to describe the microbial soil community depending on geographic location, soil physicochemical composition, and other parameters such as cultural practices (Berlanas *et al.*, 2019; Burns *et al.*, 2015b; Canfora *et al.*, 2018; Coller *et al.*, 2019).

Notwithstanding, to our knowledge, only Bacci *et al.* (2018) investigated the relationship between soil microbiome as a biological indicator and a plant health status, the common reed *Phragmites australis*, when subjected to decline without any known causes. Several hypotheses can be offered to explain unknown decline, but the quality and microbiological balance of the soil may be a coherent biological indicator. One can hypothesize that soil displaying decline features could either having a global downsize of its microbial diversity and activity or either having a dysbiosis specific of its beneficial microorganisms such as Plant-Growth Promoting Rhizobacteria (PGPR) or Arbuscular Mycorrhiza Fungi (AMF) which can be reduced or even absent. In grapevine decline problematics, some studies have focused on the restauration of soil microbial diversity and pathogens suppressiveness by adding cover crops which stimulates beneficial microorganisms activity (Richards *et al.*, 2020; Vukicevich *et al.*, 2018). Nevertheless, the profiles of symptomatic and asymptomatic soil featuring unexplained grapevine decline have yet to be studied within a same plot.

In this context, this work aims to investigate soils displaying decline features with symptoms that were not associated to explainable pathologic causes. To this end, four vineyards from two different terroirs of Bordeaux were chosen in order to dig out the physicochemical,

microbiological, and enzymatic differences in bulk soil profile between a declining area and a non-declining one within a same vineyard during autumn and spring.

Materials and methods

Studied sites

The Bordeaux wine region is in southwestern France, 20 to 150 km from the Atlantic Ocean coasts, between 44.5° and 45.5°N. The predominant climate is sub-humid temperate with cool nights and low risk of extreme temperatures (Tonietto and Carbonneau, 2004). Four plots, namely 1, 2, 3, and 4, were selected in two different Appellations with distinct terroir, namely Médoc (north of Bordeaux) and Graves (east of Bordeaux). Each of these plots presented an area displaying decline notable features (*e.g.* higher percentage of dying plants, smaller vigour of the scions, loss of yield, smaller berries...), compared to the rest of the plot where grapevines showed a “normal” growth and yield. The declining areas were annotated with S (for Symptomatic), whereas other areas in the same plot presenting “normal” vines were annotated AS (for ASymptomatic). Each S and AS areas was delimited with four rows and 20 plants per row. The two major viruses, Arabis Mosaic Virus (ArMV) and Grapevine Fan Leaf Virus (GFLV) (Boscia *et al.*, 1997) were assessed by the ELISA method. DAS-ELISA was carried out with crude plant extracts from leaves samples. GFLV and ArMV were detected using the reagents provided by Bioreba AG (Reinach, Switzerland). Substrate hydrolysis was recorded at 405 nm with a Dynex MRX II microplate reader. Vigour of the vine was measured by weighting the winter-pruned wood of 28 plants spread on four rows within the AS and S areas for each plot. The four plots were all located on sandy soils, and according to the World Reference Base for Soil Resources vineyards in Graves (Villenave d’Ornon) are on superior Pleistocene and Holocene sediments whereas Médoc’s (Saint Julien) plots are located on inferior Pleistocene sediments (WRBSR, 2015). GPS coordinates, ages of vines, and combinations of rootstocks with scions are presented in **Table 10**.

Soil sampling

Bulk soils (10-30 cm of depth) from inter-row vineyards were sampled at those eight different sites. Sampling was performed in November 2018 and in April 2019 using an auger (10 cm × 25 cm) for three subsamples with 1 meter of distance between each that were afterwards pooled. For physicochemical analysis, three aliquot portions were made from this pool whereas five aliquot portions were made for the enzymatic, molecular, and microbiological analysis.

Regarding Eco-Plates® assays, upper layer soils (5-10 cm) were sampled during the two periods (autumn and spring) and used at their fresh state 24 hours after their sampling.

Table 10 : Characteristics of the 4 studied plots and the GPS coordinates of the soils with symptomatic (S) and asymptomatic (AS) vines. CS stands for Cabernet-Sauvignon and RGM for Riparia Gloire Montpellier.

| Terroir Plot | Graves | | | | Médoc | | | |
|------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| | 1 | | 2 | | 3 | | 4 | |
| Plantation year | 2011 | | 2008 | | 1990 | | 1963 | |
| Scion/rootstock combinations | CS/RGM | | CS/RGM | | Merlot/3309C | | CS/Kober 5BB | |
| Inter-row distance (m) | 1.1 | | 1.1 | | 1.5 | | 1.3 | |
| Inter-plant distance (m) | 0.90 | | 0.90 | | 1 | | 1 | |
| Vine status | S | AS | S | AS | S | AS | S | AS |
| GPS coordinates | 44°45'14.0"N 0°33'31.4"W | 44°45'14.1"N 0°33'32.0"W | 44°45'13.0"N 0°33'24.8"W | 44°45'14.3"N 0°33'25.4"W | 45°09'25.5"N 0°48'19.7"W | 45°09'25.4"N 0°48'18.8"W | 45°09'31.3"N 0°46'19.1"W | 45°09'29.9"N 0°46'16.9"W |

Physicochemical analysis of soils

Five hundred grams from the three subsamples described above were dried at 40°C for 72 hours, sieved at 2 mm, homogenized, and sent to INRAe LAS (62000, Arras, France) to perform physicochemical analysis encompassing granulometry, pH, nutrients, and major trace elements contents listed in **Table 11**. According to Proffitt and Campbell-Clause (2012), the physicochemical parameters measured were not affiliated to mineral deficiencies that could explain the decline observed. At the texture level, the studied soils were all considered as “sand” soils which was defined by the USDA classification. Regarding the pH, all the studied soils were considered as moderate to slightly acid but are among the same rank within plots between S and AS soils.

DNA extraction

Subsequently to sampling, 5 grams of soil sampled from the 5 subsamples described above during autumn and spring were lyophilized for 48 hours using Christ Alpha® 1-4 (Bioblock Scientific) and stored at -80°C prior to DNA extraction. DNA was extracted from 250 mg of the lyophilized soils using the DNeasy PowerSoil Pro kit (Qiagen) using the manufacturer recommendations with an additional C5 washing step. DNA samples were quantified on Qubit® 3.0 fluorometer (Thermo Fisher Scientific) using Qubit™ dsDNA HS Assay kit, and their quality was checked with a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific). DNA was then stored at -20°C until further use.

Table 11 : Physicochemical characteristics of the inter-row soils from the 4 studied plots with (S) and without (AS) decline symptoms. Data shown are the values obtained after pooling 3 subsamples.

| Terroir | Graves | | | | Médoc | | | |
|---|--------|------|------|------|-------|------|------|------|
| | 1 | | 2 | | 3 | | 4 | |
| Plot | S | AS | S | AS | S | AS | S | AS |
| Vine status | S | AS | S | AS | S | AS | S | AS |
| Basic soil properties | | | | | | | | |
| Sand (%) | 84 | 82 | 89 | 88 | 90 | 88 | 95 | 87 |
| Silt (%) | 10 | 10 | 7 | 7 | 5 | 7 | 2 | 7 |
| Clay (%) | 7 | 8 | 4 | 5 | 5 | 5 | 3 | 6 |
| pH (water) | 6.65 | 6.86 | 5.79 | 5.75 | 6.05 | 6.78 | 7.09 | 6.2 |
| pH (KCl) | 5.93 | 6.01 | 4.64 | 4.62 | 5.07 | 5.96 | 6.37 | 5.13 |
| Organic carbon (%) | 0.41 | 0.56 | 0.24 | 0.53 | 0.43 | 0.46 | 0.23 | 1.39 |
| Total nitrogen (%) | 0.03 | 0.05 | 0.02 | 0.04 | 0.03 | 0.03 | 0.01 | 0.08 |
| Organic matter (%) | 0.71 | 0.97 | 0.41 | 0.91 | 0.73 | 0.79 | 0.39 | 2.40 |
| C/N | 11.8 | 12.2 | 14.1 | 12 | 15.2 | 13.1 | 16.4 | 16.9 |
| Micro/macronutrients | | | | | | | | |
| Phosphorus (mg.kg ⁻¹) | 35 | 30 | 17 | 65 | 38 | 13 | 45 | 81 |
| CEC (cmol ⁺ .kg ⁻¹) | 3.1 | 3.9 | 1.3 | 2.5 | 2.5 | 3.3 | 1.8 | 5.5 |
| Ca (cmol ⁺ .kg ⁻¹) | 2.8 | 3.5 | 0.5 | 1.3 | 1.7 | 2.9 | 1.4 | 3.4 |
| Mg (cmol ⁺ .kg ⁻¹) | 0.4 | 0.4 | 0.5 | 0.2 | 0.2 | 0.3 | 0.3 | 0.7 |
| K (cmol ⁺ .kg ⁻¹) | 3.8 | 2.6 | 2.9 | 4.5 | 4.7 | 3.3 | 5.3 | 3.1 |
| Na (cmol ⁺ .kg ⁻¹) | 0.05 | 0.04 | 0.02 | 0.03 | 0.02 | 0.03 | 0.01 | 0.03 |
| NO ₃ ⁻ (mg.kg ⁻¹) | 9.8 | 5.2 | 0.6 | 5.8 | 2.7 | 2.1 | 0.7 | 6.9 |
| NH ₃ -N (mg.kg ⁻¹) | 2.2 | 2.7 | 1.7 | 1.9 | 2 | 1.8 | 1.5 | 2.3 |
| Trace elements | | | | | | | | |
| Cu (mg.kg ⁻¹) | 18.5 | 22.3 | 3.24 | 5.38 | 53 | 56.8 | 19.9 | 37.8 |
| Fe (mg.kg ⁻¹) | 117 | 137 | 65 | 195 | 142 | 96 | 27 | 133 |
| Mn (mg.kg ⁻¹) | 23.4 | 10.2 | 3.3 | 6.6 | 5.3 | 6.1 | 3.4 | 3.7 |
| Zn (mg.kg ⁻¹) | 3.6 | 5.6 | 2.6 | 1.6 | 4.7 | 2.8 | 5 | 6.6 |

Quantitative PCR amplification of bacterial and archaeal 16S and fungal 18S rRNA genes

Analyses of qPCR were performed on the DNA extracted from the soil samples using three primers pairs to quantify bacterial and archaeal 16S rRNA genes as well as the fungal 18S rRNA genes (**Table S3**).

Bacterial and archaeal 16S qPCR reactions were monitored in 20 µL mixture consisting in 10 µL of GoTaq® qPCR Master Mix (Promega), 1 and 0.6 µL of each primer (10 µM) for bacterial and archaeal quantification, respectively, and 1 ng of extracted DNA. Cycling conditions were starting with an initial denaturation at 95°C for 10' followed by 40 cycles of denaturation at 95°C for 15", annealing temperature for 30" at 60°C and elongation at 60°C for 30". Melt curves were obtained at 65°C by increasing 0.5°C / 5" until 95°C. Fungal 18S qPCR reactions were performed in the same conditions except that the annealing temperature was at 50°C. Each sample was quantified in three replicates in Hard-Shell® 96-Well PCR plates sealed with

Microseal® « B » film (Bio-Rad) using the CFX96™ Real-Time PCR Detection System and the CFX™ Manager software, version 3.1 (Bio-Rad laboratories, France). The software algorithm calculates the efficiency (E) and threshold cycle (CT) based on the kinetics within each reaction. The efficiencies of the qPCR were 85 % to 99 % ($R^2 > 0.99$). The initial template concentration N (gene copy numbers per qPCR reaction volume) was then calculated with the following equation: $N = (1 + E)^{CT}$.

Standard curves and absolute quantification of target genes

This qPCR approach based on universal bacterial, archaeal, and fungal subunit rRNA genes amplification were followed by absolute abundance quantification using standard curves. To draw those curves, PCR were performed in a T100™ Thermal Cycler (Bio-Rad) on 1 ng of DNA extracted from plot 1 in 20 μ L mixture consisted of 10 μ L of GoTaq® qPCR Master Mix (Promega), 1 and 0.6 μ L of each primer (10 μ M) for bacterial and archaeal quantification, respectively. Similar cycling conditions were initial denaturation at 95°C for 10' followed by 30 cycles of denaturation at 95°C for 30", annealing temperature for 30" at 50°C and 60°C, for 16S and 18S genes respectively, and elongation at 72°C for 30", finished by a final elongation step at 72°C for 3'. Obtained amplicons were then sub-cloned using the pGEM®-T easy vector system (Promega) and sequenced to confirm the identity of the amplified fragments. Calibration curves (log gene copy number per reaction volume versus log N) were obtained using serial dilutions of standard from 2×10^8 to 2×10^3 copies of pGEM-T vector containing the corresponding sequence. The numbers of copies of the qPCR standards were calculated by assuming average molecular masses of 660 Da for 1 bp of double-stranded DNA.

$$\text{Copies per nanogram} = \frac{n \times mw}{Na \times 10^{-9}}$$

where n is the length of the standard in base pairs,

mw is the molecular weight per bp or nucleotide,

and Na is the Avogadro constant (6.02×10^{23} molecules per mol).

Enzymatic assays

As explained above, following enzymatic assays were done with fresh, homogenized, and sieved soil sampled from each site, coming from five subsamples. One gram of soil for each site was dried and weighted for the final calculation of enzymatic activities.

Alkaline phosphatase

Colorimetric estimation of the *p*-nitrophenol released by soil phosphatase activity when incubated with basic buffered sodium *p*-nitrophenyl phosphate solution and toluene was used to determine alkaline phosphatase as described by Tabatabai (1994), excepted the filtration step which was replaced by an 8000 g centrifugation. Assays were performed with 1 gram of homogenized and sieved (2mm) fresh soil.

β -glucosidase

Herein, the procedures are similar to those of phosphodiesterase activity (see above) and are based on colorimetric estimation of the *p*-nitrophenol released by soil β -glucosidase activity when incubated, as described by Tabatabai (1994) with a centrifugation step at 8000 g replacing the filtration.

Arylamidase

Arylamidase activity was detected using Acosta-Martínez and Tabatabai (2000) colorimetric assay, based on 2-naphtylamine released from 1 g of sieved (<2 mm) fresh soil when incubated with L-Leucine β -naphthylamine.

Potential metabolic diversity (PMD) of cultivable bacteria

PMD, represented by functional richness (R) and global metabolic activity (AWCD) were assessed with Biolog Eco-Plates™ system (Biolog Inc., CA) using Calbrix *et al.* (2005) preparation. Those plates are containing 96 wells filled with 31 different carbon sources, plus a control well. Briefly, fresh soil from the 10 cm of the upper layer from 3 biological replicates were pooled, sieved at 2 mm, and homogenized prior to suspend 5 grams of fresh soil into 50 mL of 0.85 % NaCl. Suspensions were shaken for 10 minutes at 300 rpm and rested for 10 minutes under ambient temperature. Supernatants were diluted with ultrapure sterile water 1:100 and the 31 Eco-Plate wells were filled with 120 μ L of this diluted supernatant, incubated at 20°C in the dark and subsequently, their absorbance at 590 nm were measured every 24 hours for 4 days. Each Eco-Plate was subdivided into three replicates for each tested soil, and the absorbance value of each carbon source was corrected by subtracting the absorbance value of the well containing only water. Negative values were set to zero. Global microbial metabolic activity in each replicate was expressed as the Average Well Colour Development (AWCD). Microbial richness functionality R were calculated as the number of utilized substrates (> higher AWCD mean among the tested soils at 96h) and Shannon evenness index (SEI) were calculated

according to Zak *et al.* (1994) (**Table S4**). Area Under AWCD Curve (AUC), which gives better insights for curve dynamics, was calculated with trapezoidal method for each soil using “caTools” packaging.

Cultivable bacteria and fungi colonies quantification

Quantification of cultivable bacterial population from the eight soils was done on R2A medium (0.5 % yeast extract, 0.5 % proteose peptone, 0.5 % casamino acids, 0.5 % glucose, 0.5 % soluble starch, 0.3% sodium pyruvate, 0.3 % H₂KO₄P, 0.05 % MgCl₂, pH 7) amended with 25 mg/L of nystatin to inhibit yeasts and fungi growth. Sterile Petri dishes filled with R₂A medium were plated with the same soil suspensions used above for PMD which were tenfold serial diluted. They were then incubated at 25°C and Unities Forming Colonies (UFC) were numerated 4 days after plating. Additionally, cultivable fungal population were quantified on Potato Dextrose Agar (BioKar) amended with 500 µg mL⁻¹ of gentamicin and 50 µg mL⁻¹ of chloramphenicol to inhibit bacterial growth. Incubation was done at 25°C, and UFC were numerated 7 days after plating.

Statistical analysis

All statistical analyses were performed using the R software version 4.0.2 (R Core Team, 2020) and RStudio version 1.3.1056. Histograms and principal component analysis (PCA) were made using ggplot2, ggthemes, and FactoMineR packages.

Normality and homogeneity of variances were checked by the Shapiro-Wilk and the Leven tests, respectively (Zar, 1999).

ANalysis Of VAriance (ANOVA) with soil status factor (AS or S) was performed for enzymatic activities, microbial biomass, and genes quantities. When significant effects were detected, multiple comparisons of means were done with pairwise t-tests ($\alpha = 0.05$). Residuals were prior checked for their independency, normality and variance homogeneity with the Durbin Watson, Shapiro-Wilk, and Bartlett tests, respectively. When assumptions for parametric tests were not respected, a multiple pairwise comparison with Wilcoxon test was performed after a Kruskal-Wallis test using the multcomp packaging. Bonferroni correction was applied for pairwise comparison. Two-way ANOVA with seasonal (autumn or spring) and terroir factors were performed on molecular biomasses.

Results

Declining areas display higher mortality rate and weaker vigour of plants, which are not associated to the presence of viruses

To quantify the decline empirically observed by the winegrowers within the S area in each plot, the percentages of missing vines and young plants, which were recently planted to replace dead plants, as well as the pruning weight of the old vines were assessed in symptomatic and asymptomatic areas (**Table 12**).

Table 12 : Characterization of the observed decline in the 4 studied plots by comparing the areas with symptomatic (S) and asymptomatic (AS) vines. Means \pm SE (n = 28) are represented. Missing vines correspond to dead plants that were not replaced, and young plants are grafted plants that have been recently planted (less than 5 years) to replace the dead ones. For pruning means, asterisks represent significant differences between S and AS soils with $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

| Terroir | Graves | | | | Médoc | | | |
|---------------------------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| | 1 | | 2 | | 3 | | 4 | |
| Plot | S | AS | S | AS | S | AS | S | AS |
| Decline features | S | AS | S | AS | S | AS | S | AS |
| Missing vines (%) | 0 | 0 | 0 | 0 | 35 | 2 | 0 | 5 |
| Young plants (%) | 65 | 1 | 57 | 1 | 13 | 5 | 38 | 14 |
| GFLV/ArMV (%) ^a | 0 | 0 | 0 | 0 | 0 | 12.5 | 12.5 | 100 |
| Number of pruned woods per vine | 7.6 \pm 0.4 | 9.6 \pm 0.4 | 8.0 \pm 0.3 | 9.7 \pm 0.5 | 4.4 \pm 0.4 | 7.9 \pm 0.4 | 6.6 \pm 0.3 | 10 \pm 0.5 |
| | | ** | | * | | *** | | *** |
| Pruning weight per vine (g) | 197 \pm 98 | 361 \pm 120 | 139 \pm 69 | 307 \pm 185 | 82 \pm 46 | 183 \pm 84 | 104 \pm 99 | 289 \pm 113 |
| | | *** | | ** | | ** | | *** |
| Vigour ^b | 25 \pm 9 | 38 \pm 10 | 14 \pm 6 | 33 \pm 20 | 19 \pm 9 | 23 \pm 8 | 15 \pm 11. | 29 \pm 8 |
| | | *** | | *** | | * | | *** |

a The presence of GFLV and/or ArMV viruses has been tested using ELISA tests in eight plants within each area. Data are presented as the percentage of positive samples.

b Vigour was calculated as the pruning weight divided by the number of pruned woods.

Although the mortality of the vines in each plot is higher in S areas compared to AS ones with a higher number of missing plants and/or higher number of young plants.

Significant differences were detected among the soils regarding the number of pruned woods (ANOVA: $F(7,216) = 19.21$, $P < 0.001$) and the average pruning weight (Kruskal-Wallis: $\chi^2 = 116.35$, $ddl = 7$, $P < 0.001$) per vine, and lower levels were observed for vines growing in S

areas than those growing in AS areas, for all the studied plots. Even if no visual disease symptoms could be associated with the observed decline when comparing the two areas within each plot, the presence of the main viruses responsible of “court noué” was checked. GLFV and ArMV were detected in the AS area of plot 3 and in the S and AS areas of plot 4. Thus, the presence of the viruses appeared to be not correlated with the observed decline of vines.

Soils from declining areas contain less bacteria and archaea DNA than well growing areas

Molecular analyses revealed that the quantity of total DNA extracted per g of dry soil was significantly higher (Kruskal-Wallis: $\chi^2 = 37.49$, $ddl = 7$, $P < 0.001$) in each AS area compared to its corresponding S area in all plots, whatever the seasonal period (**Figure 20; Figure S1**). Molecular biomass of bacteria (Kruskal-Wallis: $\chi^2 = 34.016$, $ddl = 7$, $P < 0.001$) and archaea (Kruskal-Wallis: $\chi^2 = 37.496$, $ddl = 7$, $P < 0.001$) followed the same pattern except for bacterial 16S detection in plot 3 where no significant difference has been detected ($P = 0.931$). Interestingly, no significant difference was detected for fungal 18S gene between S and AS areas in spring samples, except for plot 4 (Kruskal-Wallis: $\chi^2 = 34.943$, $ddl = 7$, $P < 0.001$), while higher signals were detected in S soils compared to AS ones within three plots during autumn. It appeared that higher quantity of DNA was extracted during spring compared to autumn with an increase in S of 5, 85, 70, and 426 % as well as in AS of 24, 258, 137, and 63 % for the plots 1, 2, 3, and 4, respectively. To this extent, more bacterial and archaeal 16S genes were found in spring samples compared to autumn ones, especially for the plots 1 and 2 located in Graves. For instance, in these plots, in AS soils it took a rise of 1000 and 258 % in 16S archaeal genes, and 470 and 259 % in 16S bacterial genes. Seasonal effect was not significant on the number of 18S gene copies (ANOVA: $F(1,76) = 0.23$, $P = 0.63$), which always remain lower than the number of bacterial, and even the archaeal, 16S genes.

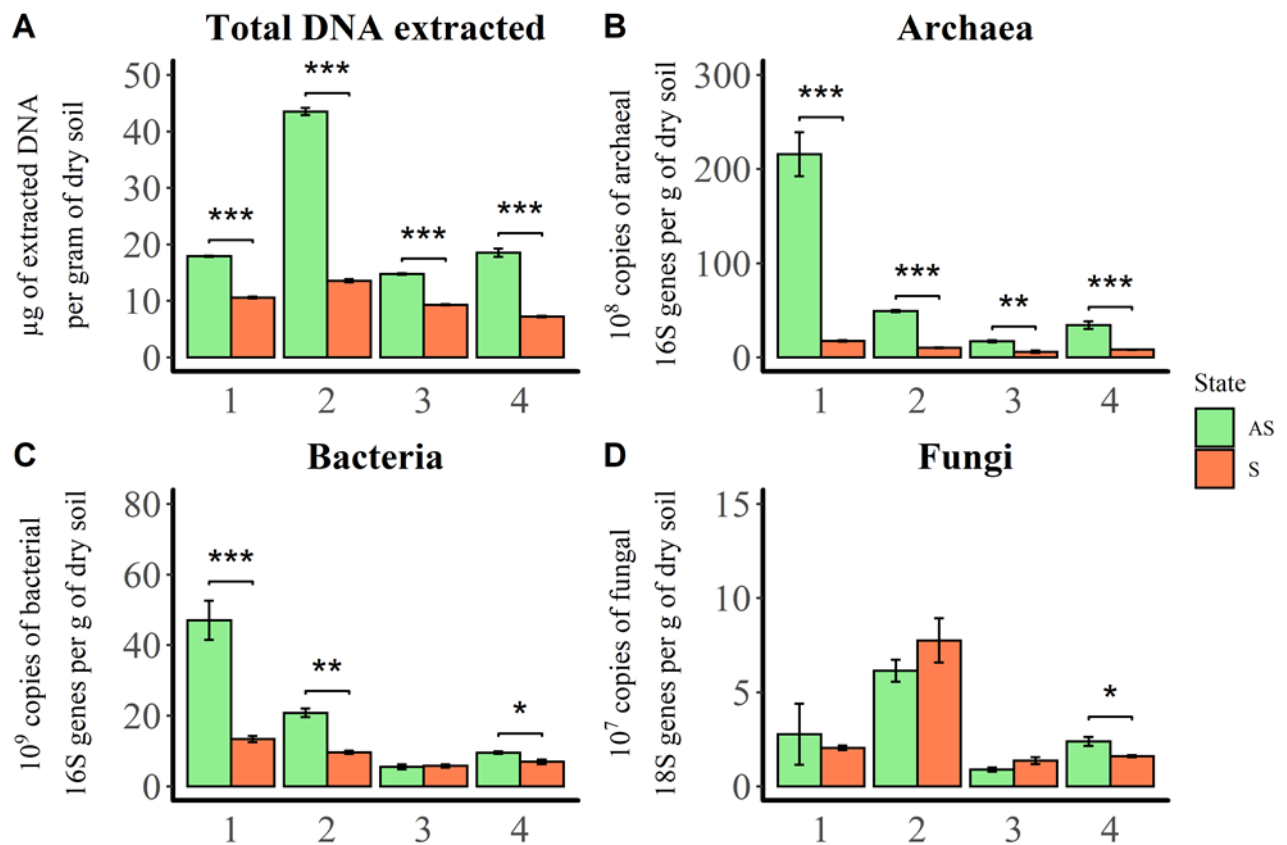


Figure 20 : Quantifications of (A) total DNA, (B) archaeal 16S rRNA genes, (C) bacterial 16S rRNA genes, and (D) fungal 18S rRNA genes in asymptomatic (AS = green) and symptomatic (S = orange) soils, among plots 1, 2, 3, and 4 during spring. Bars represent means \pm SE (n = 5). Significant differences were detected through pairwise t or Wilcoxon tests after an ANOVA or Kruskal test, where $\alpha = 0.05$, corrected with Bonferroni method. Asterisks represent significant differences between S and AS soils with $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

Asymptomatic soils displayed more cultivable bacteria and fewer fungi, with a lower microbial activity, compared to symptomatic soils

Differences in molecular biomass found between S and AS soils concerning the bacterial and fungal level were confirmed with cultivable approaches. Significant differences were detected among the soils regarding the level of bacterial cultivable populations (ANOVA: $F(7,16) = 33.28$, $P < 0.001$), and a higher level was observed in AS soils compared to S soils, excepting the plot 4 where no significant difference was detected ($P = 0.100$) (**Table 13**). Unlike the fungi (Kruskal-Wallis: $\chi^2 = 22.273$, $ddl = 7$, $P = 0.002$), the level of cultivable population was significantly higher in S soils compared to AS soils. Cultivable population of bacteria and fungi were also assessed during autumn which corroborate, as with the spring measurement, with a higher and lower population levels of bacteria and fungi, respectively, in AS compared to S

soils (**Table S5**). A seasonal effect was observed with higher number of bacteria and fungi found during spring compared to samplings made in autumn.

Table 13 : Cultivable population levels of bacteria and fungi, and Eco-Plates measurements (Area Under Curve (AUC), Shannon’s evenness (E) and richness (R) functionality at 96 hours post-incubation) within the 4 studied plots with (S) and without (AS) decline symptoms during spring. Means \pm SE are presented with (n = 5) for bacterial and fungal counts, whereas (n = 3) for Eco-Plates measurements. Asterisks represent significant differences between S and AS soils with $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)).

| Terroir | Graves | | | | Médoc | | | |
|--------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| | Plot | 1 | 2 | 3 | 4 | | | |
| Status | S | AS | S | AS | S | AS | S | AS |
| Bacterial | | | | | | | | |
| counts (log (CFU / g of soil)) | 7.6 \pm 0.03 | 7.9 \pm 0.03 | 7.6 \pm 0.04 | 7.8 \pm 0.04 | 7.5 \pm 0.03 | 7.7 \pm 0.03 | 7.7 \pm 0.01 | 7.7 \pm 0.03 |
| | | ** | | ** | | ** | | ns |
| Fungal counts | | | | | | | | |
| (log (CFU / g of soil)) | 7.3 \pm 0.04 | 6.1 \pm 0.04 | 7.2 \pm 0.02 | 6.9 \pm 0.03 | 6.5 \pm 0.04 | 6.2 \pm 0.15 | 7.5 \pm 0.06 | 7.0 \pm 0.15 |
| | | *** | | *** | | *** | | *** |
| AUC | | | | | | | | |
| | 7.6 \pm 0.19 | 6.4 \pm 0.13 | 9.0 \pm 0.26 | 8.0 \pm 0.13 | 8.4 \pm 0.21 | 8.3 \pm 0.07 | 8.5 \pm 0.37 | 7.9 \pm 0.3 |
| | | *** | | *** | | ns | | *** |
| E | | | | | | | | |
| | 0.995 \pm 0.002 | 0.991 \pm 0.000 | 0.998 \pm 0.001 | 0.991 \pm 0.004 | 0.986 \pm 0.001 | 0.992 \pm 0.001 | 0.993 \pm 0.001 | 0.999 \pm 0.001 |
| | | ns | | ns | | ns | | ns |
| R | | | | | | | | |
| | 23.3 \pm 0.6 | 13.3 \pm 2.1 | 27.7 \pm 0.6 | 19.7 \pm 0.6 | 20.7 \pm 1.1 | 22 \pm 1 | 27.3 \pm 0.6 | 25 |
| | | *** | | *** | | ns | | * |

Microbial activities during spring sampling represented by AWCD from Biolog Eco-plates™ system were significantly (Kruskal-Wallis: $\chi^2 = 37.496$, $ddl = 7$, $P < 0.001$) more important in S soils compared to AS soils at the end point (96 hours after incubation), excepting the plot 3 where no significant difference was detected ($P = 0.799$) (**Figure 21**).

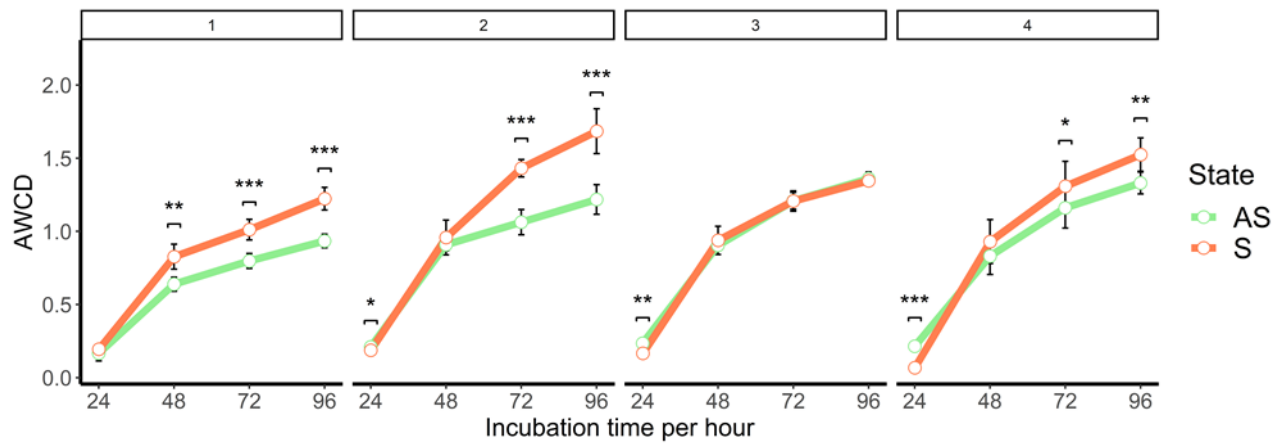


Figure 21 : Eco-Plates™ measurements displaying microbial activities represented by Average Well Colour Development (AWCD) of metabolized substrates in Eco-Plates based on 96-h incubation ($n = 3$) in symptomatic ($S = \text{red}$) and in asymptomatic ($AS = \text{green}$) soils of decline among for plots 1, 2, 3, and 4 during spring. Points on the curves represent means \pm SE ($n = 3$). Asterisks represent significant differences between S and AS soils with $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

The AUC was neither significantly different for the plot 3 ($P = 0.8$) (**Table 13**). Shannon’s evenness was not significantly different among the soils (Kruskal-Wallis: $\chi^2 = 20.44$, $ddl = 7$, $P = 0.0569$), however the richness functionality R was significantly more important in S soils compared to AS soils (ANOVA: $F(3,8) = 82.83$, $P < 0.001$), excepting the plot 3 where no significant difference was detected ($P = 0.12$). Interestingly, the microbial activity measured by Eco-Plates™ were inverted during the autumn season, with significantly more important values in AWCD (ANOVA: $F(3,8) = 164.4$, $P < 0.001$), and richness R (ANOVA: $F(3,8) = 82.83$, $P < 0.001$) for the AS soils compared to S ones (**Figure S2; Table S5**).

Table 14 : Ratios between 16S bacterial and 18S fungal genes, and between cultivable bacterial and fungal CFUs from the soils within the 4 studied plots with (S) and without (AS) decline symptoms during spring. Means \pm SE are represented ($n = 5$). Asterisks represent significant differences between S and AS soils with $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

| Terroir | Graves | | | | Médoc | | | |
|-----------------------|--------------|----------------|--------------|--------------|----------------|---------------|---------------|--------------|
| | 1 | | 2 | | 3 | | 4 | |
| Plots | | | | | | | | |
| Soil status | S | AS | S | AS | S | AS | S | AS |
| (B / F) molecular | 657 \pm 52 | 1754 \pm 100 | 135 \pm 45 | 345 \pm 46 | 661 \pm 110 | 473 \pm 106 | 440 \pm 114 | 413 \pm 79 |
| | *** | | *** | | * | | ns | |
| (B / F) cultivable | 108 \pm 17 | 1911 \pm 44 | 121 \pm 14 | 436 \pm 12 | 1009 \pm 295 | 756 \pm 42 | 72 \pm 10 | 265 \pm 98 |
| | *** | | *** | | * | | ** | |

The molecular (ANOVA: $F(7, 32) = 11.02, P < 0.001$) and cultivable (ANOVA: $F(7, 16) = 94.58, P < 0.001$) (B / F) ratios were significantly higher in AS soils compared to the S ones for plots 1, 2, and 4, excepted for the plot 3 where the B / F ratio was significantly lower in the AS soil (Table 14).

An overall higher enzymatic activity was detected in asymptomatic soils

Soils within the 4 studied plots showed significant differences in enzymatic analysis (Figure 22). Significantly higher activity in AS soils compared to S soils was observed in arylamidase, β -glucosidase, and alkaline phosphatase during spring period, except in plot 4 and plot 3 for the arylamidase ($P = 0.967$) and β -glucosidase ($P = 0.339$), respectively. The enzymatic activity was also recorded during autumn but only for plots 1 and 2. Alkaline phosphatase activity increased in spring compared to the samples made in autumn among the soils 1 AS, 1 S, 2 AS, and 2 AS with an uprise of 357, 608, 564, and 504 %, respectively (Figure S3). Unlike the β -glucosidase where the activity was more important during autumn than spring with an increase of 84, 73, 41, and 6 % for plots 1, 2, 3, and 4, respectively.

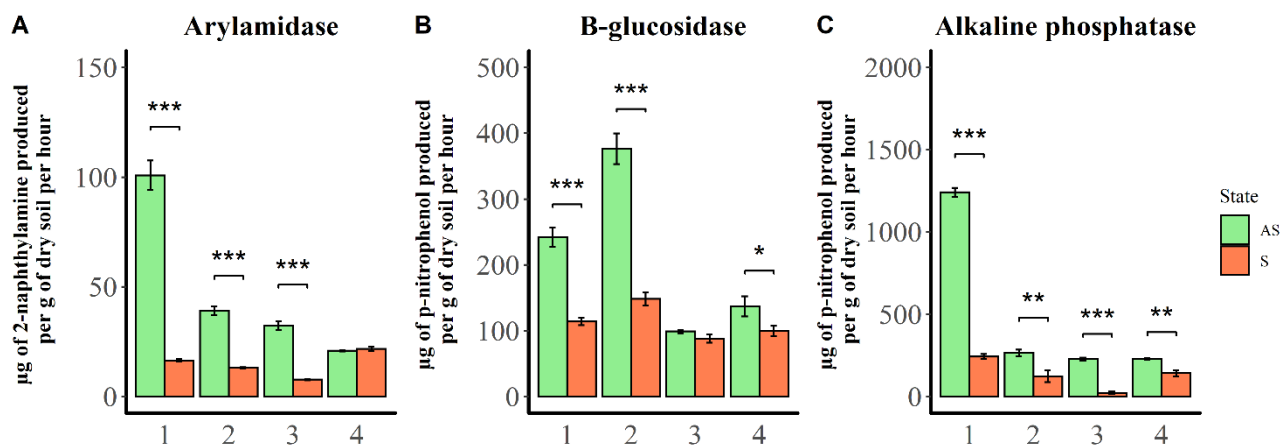


Figure 22 : Enzymatic activities in asymptomatic (AS = green) and in symptomatic (S = orange) soils among plots 1, 2, 3, and 4. Soils were assessed for the activity of (A) arylamidase I, (B) β -glucosidase, and (C) alkaline phosphatase during spring. Bars represent means \pm SE (n = 5). Significance differences corrected with Bonferroni method were detected through pairwise t or Wilcoxon tests after an ANOVA or Kruskal test. To facilitate the graph reading, usual letters were replaced with annotations: $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

Linking microbial profiles and enzymes activities

To visualize the similarities and differences between the profiles of the 8 studied soils, a PCA was performed considering all the enzymatic, molecular, and microbial values (Figure 23).

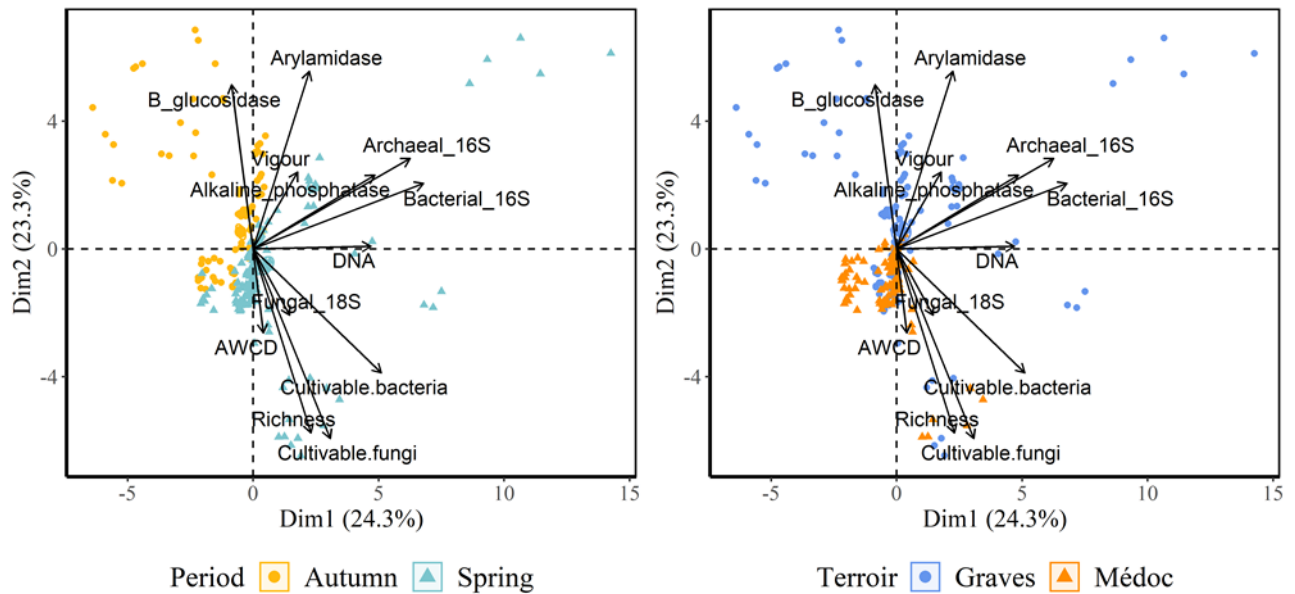


Figure 23 : Ordination biplot analysis of PCA for enzymatic (arylamidase, B-glucosidase, acid, and alkaline phosphatases), molecular (total DNA, archaeal and bacterial 16S and fungal 18S), and microbial (Eco-Plates™ measurements represented by AWCD and richness, cultivable bacteria and fungi) variables among the 4 plots displaying (A) season and (B) terroir. The size of the arrows indicates the contribution strength of the variables. Standard error ellipses show 95 % confidence areas.

The first two dimensions (Dim) accounted for 47.6 % of the variance. Dim1 axis accounted for 24 % of total variance and was positively correlated with vigour of the vines, DNA, fungal 18S genes, bacterial and archaeal 16S genes, arylamidase and alkaline phosphatase activities, cultivable fungi, and bacteria, with Eco-Plates™ measurements while β -glucosidase activity were negatively correlated. Dim2 axis accounted for 23 % of total variance and was correlated with vigour of the vines, DNA, bacterial and archaeal 16S genes, alkaline phosphatase, arylamidase, and β -glucosidase activities while cultivable bacteria and fungi, Eco-Plates™ measurements and fungal 18S genes were negatively correlated. In other extend, the Graves and autumn samples were mainly found on the positive side of Dim2 whereas Médoc and spring were mainly found on its negative side.

To have an overview over the variables that could explain the unexplained dieback, PCAs were performed vineyard plot per vineyard plot considering all the enzymatic, molecular, and microbial values (**Figure 24**).

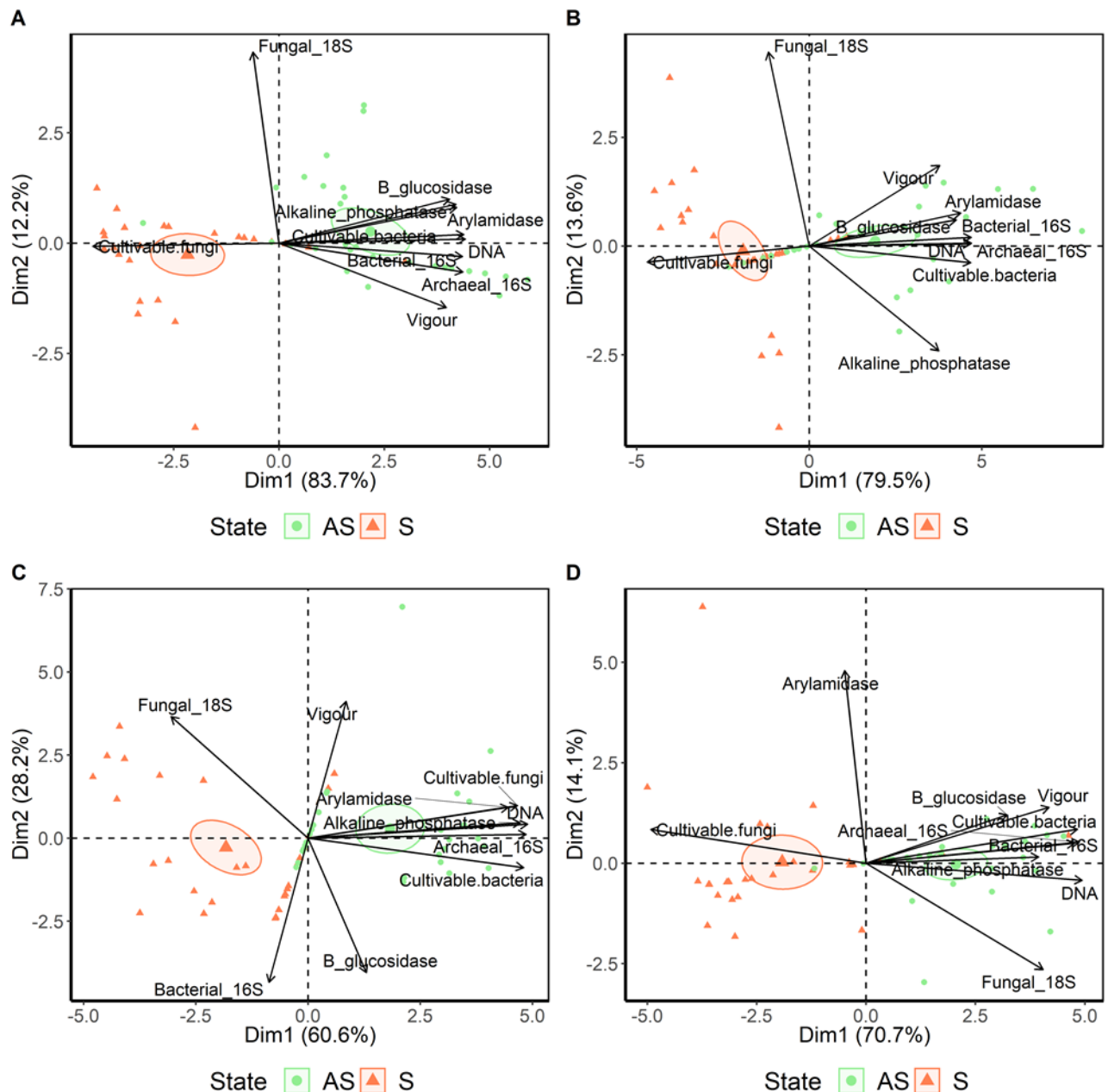


Figure 24 : Ordination biplot analysis of PCA for enzymatic, molecular, and microbial variables among the plots (A) 1, (B) 2, (C) 3, and (D) 4 displaying symptomatic (orange) and asymptomatic (green) features during spring. The size of the arrows indicates the contribution strength of the variables. Standard error ellipses show 95 % confidence areas.

The first two dimensions (Dim) accounted for 95%, 93%, 68%, and 84 % of the variance in plot 1, 2, 3, and 4, respectively. Asymptomatic feature is generically explained by enzymatic activities recorded for arylamidase, β -glucosidase, and phosphatase, by the level of cultivable bacteria, and by the total DNA extracted coupled with the number of 16S copies of archaeal and bacterial genes. On the other hand, symptomatic features are explained by the number of cultivable fungi for plots 1, 2 and 4, and by the number of 18S fungal genes for plots 2 and 3.

Eco-Plates™ variables were not presented in these PCAs since they did not have a clear pattern that could explain the soil status during spring.

Discussion

The decline of vineyards, which has been accelerating over the past few decades, is increasingly worrying stakeholders in the wine industry. Among the main factors that can play a role in these declines, global warming can influence the progression of certain diseases by altering the functioning and microbiome diversity (Dubey *et al.*, 2018). It is well-known that soil microorganisms provide many ecosystem services, such as solubilizing and mineralizing insoluble soil phosphorus or increasing nitrogen available for plants. To better understand the causes of vine decline unexplained by disease symptoms, we investigated the physicochemical, enzymatic, and microbial profiles with declining areas and compared them with those of asymptomatic areas within the same vineyard, in four plots from two Bordeaux's appellations.

Soil abiotic parameters may not explain the observed decline

Altogether, the physicochemical, enzymatic, and microbial components determine the soil ecosystemic processes. These processes are correlated with soil functions which influence vine growth and grape quality (Riches *et al.*, 2013; Van Leeuwen *et al.*, 2018). Within these plots, soil with decline features (S) was compared with soil sampled in an area with well-growing and asymptomatic vines (AS). In the eight investigated soils, none of the physicochemical parameters measured in **Table 11** could explain the decline observed in S soils compared to corresponding AS soils. Indeed, no symptoms could be associated with a lack or excess of trace elements as described in Proffitt and Campbell-Clause (2012) and their toxicity thresholds usually observed in vineyards were above the levels observed in the top 30 cm of the sampled soils. For instance, copper salts also known as Bordeaux mixture ($\text{Ca}(\text{OH})_2 + \text{CuSO}_4$) have been traditionally used intensively in vineyards since the 19th century to prevent damages provoked by mildew pathogens, which lead to extreme concentrations in top-soils exceeding 500 mg.kg^{-1} of Cu (Brun *et al.*, 2001). Herein the highest value of Cu (56.8 mg.kg^{-1}) was found in AS soil from plot 3 and is not considered to have a potential negative effect on vine growth. Although, it is well known that soil physicochemical parameters are important drivers of the microbial communities (Plassart *et al.*, 2019). Dequiedt *et al.*, (2011) hierarchized these factors with fine texture and CEC as the top drivers, followed by organic C and N contents, and by soil pH. In our study, the AS and S soils among the four plots had a similar fine soil structure. CEC and total C and N contents differed between AS and S soils for only half of the plots (*Table 2*). This

might explain the differences observed in 16S gene copies for archaea and bacteria found between AS and S soils for these plots, but not for plot 1 and 3. However, only the upper topsoils were analysed with a unique measurement. To unravel the belowground interactions with the physicochemical features of the soil and the vine roots, deeper analysis should be made.

Enzymatic and microbial profiles are relevant biological indicators of the observed decline

Dequiedt *et al.* (2011) observed, through a French survey, that vineyard soils displayed the lowest microbial biomass compared to other land uses. In our study, we obtained higher means in both autumn (**Figure S1.A**) and spring (**Figure 20.A**) periods (1.5 and 3 times more, respectively) compared to Dequiedt *et al.* (2011), which may be due to differences in the DNA extraction methods used.

We observed significant differences between S and AS soils in the four vineyard plots, suggesting that the soils with declining vines have a reduced amount of microbial biomass compared to the soils with non-declining vines. The archaeal and bacterial amount of specific 16S genes during autumn (**Figure S1.B-C**) and spring periods (**Figure 20.B-C**) follow the same trend as total DNA. This is less clear for 18S fungal gene quantity which is significantly more abundant in S soils compared to AS soils during the autumn period for at least three plots (**Figure S1.D**) but does not seem to follow this trend during the spring period (**Figure 20.D**). One explanation of these dissimilarities in the number of 16S gene copies between S and AS soils is that bacterial communities are more sensitive than fungal communities to alteration of nutrient availability (Liang *et al.*, 2019).

Alkaline phosphatase in soils is known to be produced exclusively by microorganisms and not by plants (Dick *et al.*, 1983). Its activity has been reported to be linked with the level of bacteria under P limiting conditions (Fraser *et al.*, 2015). In our study, alkaline phosphatase activity was highly positively correlated with archaeal and 16S genes and seems to highlight the difference in soil quality between S and AS soils. Highly positive correlations were also found for arylamidase, with archaeal and bacterial 16S genes, which is considered as a key indicator of soil quality and are primary products of microorganisms (Dodor *et al.*, 2002). To a lesser extent, the β -glucosidase activity is more correlated with the fungal 18S gene than with the archaeal or bacterial 16S genes. Level of cultivable fungi appeared to be also correlated with the Eco-Plates measurements such as amino acids, carbohydrates, carboxylic acids, and polymers consumptions. Copies of 18S genes and the level of cultivable fungi are correlated, and seem to have a strong impact on soil enzymatic activity. Miguéns *et al.* (2007) deciphered the critical

level of degradation of vineyard soils and our β -glucosidase and phosphatase activities values were in the same range as in their study which suggests that vineyards display soils with poor enzymatic activities. Among the fungi, the AMF or AM fungal spores are known to be highly influenced by soil conditions and reveal the status of the soil (José *et al.*, 2021; Mahmoudi *et al.*, 2021). High-throughput sequencing using 18S or ITS specific primers is commonly used for the description of AMF diversity in vineyards (Berruti, *et al.*, 2017), and might be a promising perspective to evaluate the health status in vineyard soil. In our case, PCAs highlighted, vineyard plot by vineyard plot, that the observed variables could explain the differences between symptomatic and asymptomatic soils for spring samples. Symptomatic features in vineyards were generically explained by the number of cultivable fungi and the number of 18S fungal genes whereas the asymptomatic feature was explained by enzymatic activities, by the level of cultivable bacteria, and by the number of 16S copies of archaeal and bacterial genes. These results suggest a dysbiosis in the microbial communities coupled and maybe linked to a dysregulation of the ecosystemic processes.

Eco-Plates™ are quite controversial in their interpretation because, like the level of population of cultivable microorganisms, it may be biased because of fast growing microorganisms which alter the substrate consumption within the wells (Verschuere *et al.*, 1997). In vineyard soils, this technology has been used to investigate cover crop (Capó-Bauçà *et al.*, 2019) and chemical input (Aballay *et al.*, 2017) effects on the physiological profiles of telluric microorganisms. In our case, this system remains interesting to use since we compare similar textures of soil during different seasons. It appeared that the levels of AWCD, AUC, and R measured were significantly higher in S soils compared to AS soils during the spring season whereas this pattern seems inverted during autumn with higher microbial activities in AS soils compared to the S ones. It is hard to explain this inverted tendency, though one of the explanations would be the soil amendment made between the autumn and spring periods that are levelling up the enzymatic and microbial activities in a more important way in the S soils compared to the AS soils due to the higher level of fungi. Indeed, it has been suggested that organic matter derived from fungal metabolic processes may be more chemically resistant, and thus increasing the stable carbon storage (Liu *et al.*, 2011; Martin and Haider, 1979). This tool remains interesting to compare vineyard physiological profiles, even though the Eco-Plates from Biolog system has yet to be proven as a deep-analysis soil quality indicator since fast growing microorganisms alter the substrate consumption.

Microbial enzymatic activities and molecular biomasses evolve with the season and depend on the terroir

The tendency for higher enzymatic activities, in AS soils compared to S soils, observed during the autumn period (**Figure S3**) was similar to the observations made during the spring period. Although, phosphatase activities were quite reduced during the autumn compared to the spring period which corroborate the results found in Zuccarini *et al.* (2020), suggesting that warming increases some enzymatic production in soils, but not for β -glucosidase in our case. The higher level of β -glucosidase during autumn might be due to leaf-fall, pruned woods, and cover crops degradation that are known to produce organic matter (McGourty and Reganold, 2005). This change in enzymatic activities comes up with an alteration in cultivable level of microbial populations. As far as organic matter is concerned, the amount found in vineyards is considered to be poor compared to other woody perennial crops (Midwood *et al.*, 2020). The potential of organic matter, provided by cover crops, is known to increase microbial activity and therefore the soil quality (Belmonte *et al.*, 2018; Steenwerth and Belina, 2008; Winter *et al.*, 2018). Herein we observed an increase in both bacterial and fungal CFU per gram of dry soil (**Table 13; Table S5**), that is quite different with Corneo *et al.* (2013) results since they had shown no such seasonal effect on cultivable bacteria and fungi over 2 years of samplings. Measurements made with the Eco-Plates highlighted the microbial dynamics through specific substrates consumption which seems to be more important during spring than autumn (**Figure 21; Table S5**). It may not be surprising since higher level of bacteria and fungi in cultivable and molecular approaches have been found during spring sampling. Indeed, Hernandez and Menéndez (2019) showed a change in fungal diversity with seasonal fluctuation. Bacterial/fungal (B / F) gene and cultivable ratios can be used as indicators of soil quality of vineyards but are hardly comparable between studies since different methods to measure biomass were applied (Zehetner *et al.*, 2015; Holland *et al.*, 2013). Herein, (B / F) ratios based on copies number of 16S and 18S genes, indicate that bacteria are more abundant than fungi in all soils and globally more abundant in spring than in autumn (**Table 14; Table S6**). In the Graves plots, the (B / F) ratio is lower in S soils compared to AS soils in both periods of sampling. Interestingly, the (B / F) ratios based on cultivable approach are 3460, 45764, 7881, and 4782 % higher in spring compared to autumn samples for 1 S, 1 AS, 2 S, and 2 AS soils, respectively. These observations clearly show an increase in level of cultivable bacteria during spring, suggesting that the richness in bacteria is lowered during autumn whereas cultivable fungi are more stable with the season. This effect was noticed in Pietikäinen *et al.* (2005), with a different approach, that fungi are more adapted

to low temperature compared to bacteria, which could drastically affect the (*B / F*) ratios that we obtained.

However, the season is assumed to cause less effect on the soil microbiota than the localization (Corneo *et al.*, 2013; Siles and Margesin, 2016). Our study demonstrated that both season and terroir strongly impact the variables observed among the soils. The 18S fungal gene was neither significantly impacted by the terroir (ANOVA: $F(1,76) = 3.645$, $P = 0.32$) and the period of sampling (ANOVA: $F(1,76) = 0.84$, $P = 0.63$), underscoring the idea that fungi were more adapted to low temperature, whereas archaea and bacteria were significantly impacted by both the season and the geographical location.

Conclusion

Investigations among four vineyards in the Bordeaux French region that were subjected to unexplained decline revealed a dysbiosis in their microbial diversity and enzymatic activities. The level of cultivable bacteria coupled to the number of 16S bacterial gene copies were significantly more important in the asymptomatic soils compared to symptomatic ones, while the level of cultivable fungi was higher in the soils subjected to decline. Enzymes involved in N, C, and P cycling were significantly more present in the asymptomatic soils, suggesting a decrease in the ecosystemic processes in the area experiencing decline. The dysregulation of the ecosystemic processes coupled to the microbial dysbiosis observed in studied vineyards in decline is linked to the soil status and therefore the grapevine fitness.

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Supplemental information

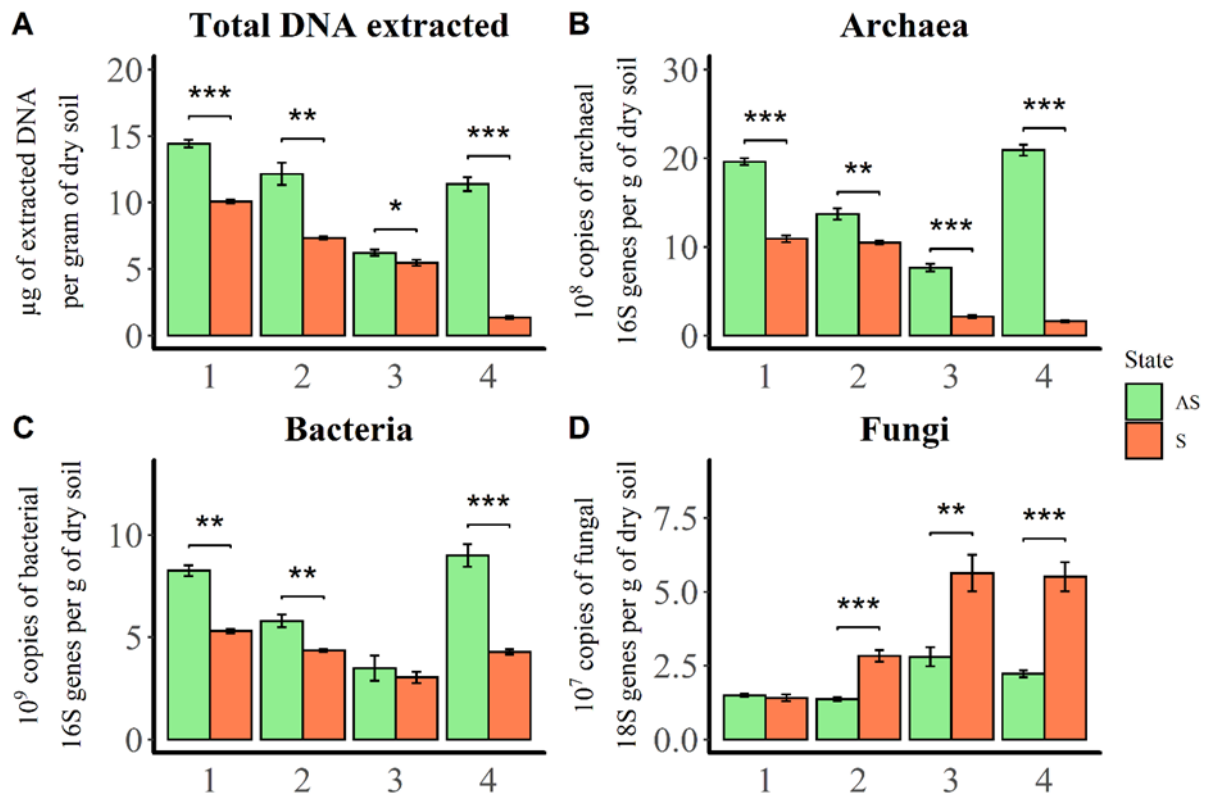


Figure S1 : Quantifications of (A) crude extracted DNA, (B) archaeal 16 rRNA genes, (C) bacterial 16S rRNA genes, and (D) fungal 18S rRNA genes in asymptomatic (AS = green) and in symptomatic (S = orange) soils among plots 1, 2, 3, and 4 during autumn. Bars represent means \pm SE (n = 5). Significance differences corrected with Bonferroni method were detected through pairwise t or Wilcoxon tests after ANOVA or Kruskal test. To facilitate the graph reading, usual letters were replaced with P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***).

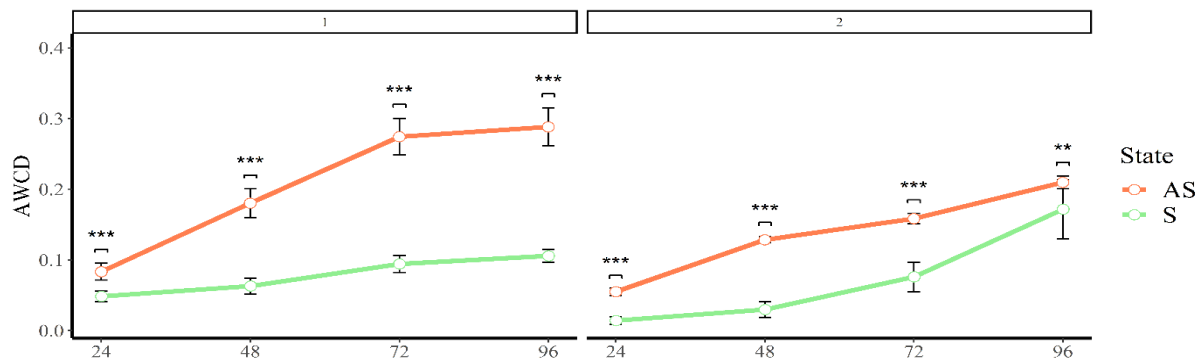


Figure S2 : Eco-Plates™ measurements representing microbial activities represented by Average Well Colour Development of metabolized substrates based on 96h incubation (n = 3) in symptomatic (S = orange) and in asymptomatic (AS = green) soils of decline among for plots 1 and 2 during autumn. Points on the curves represent means \pm SE (n = 3). Asterisks represent significant differences between S and AS soils with P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***).

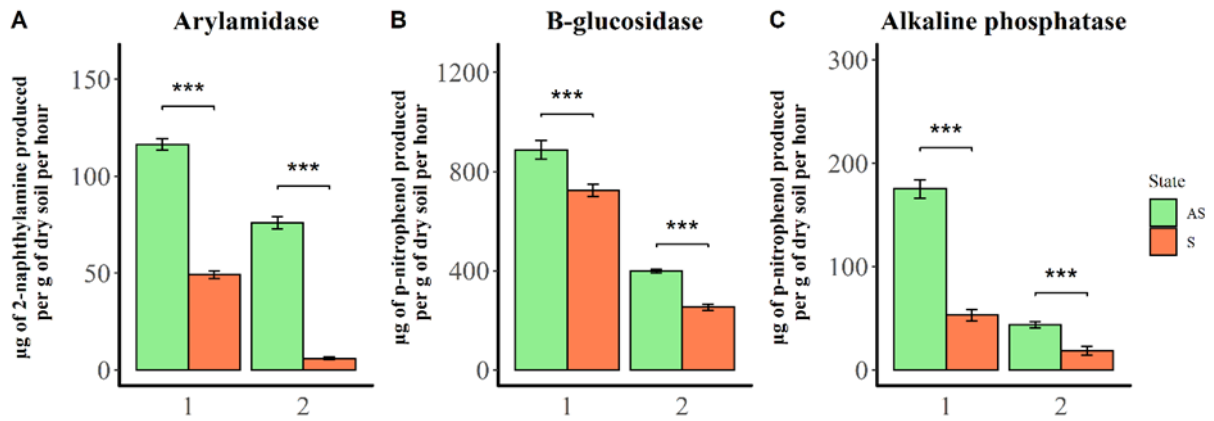


Figure S3 : Enzymatic activities in asymptomatic (AS = green) and in symptomatic (S = orange) soils among plots 1 and 2. Soils were assayed for the activity of (A) arylamidase in μg of 2-naphthylamine per g dwt h^{-1} of soil, (B) β -glucosidase, (C) alkaline phosphatase in μg of p-nitrophenol produced per g of dry soil per hour during autumn. Bars represent means \pm SE ($n=5$). Significant differences corrected with Bonferroni method were detected through pairwise t or Wilcoxon tests after an ANOVA or Kruskal test. To facilitate the graph reading, usual letters were replaced with $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)).

Table S3 : Primers for 16S and 18S rRNA amplification.

| Primer | Primer sequence (5' → 3') | Target and size of the amplicon | Reference |
|------------------|----------------------------------|--|---|
| 515R | CCTACGGGAGGCAGCAG | Bacterial 16S rRNA gene 174 bases | (López-Gutiérrez <i>et al.</i> , 2004) |
| 341F | ATTACCGCGGCTGCTGGCA | | |
| Arch967F | ATTGGCGGGGAGCAC | Archaeal 16S rRNA gene 140 bases | (Cadillo-Quiroz <i>et al.</i> , 2006) |
| Arch1060R | GGCCATGCACCWCCTCTC | | |
| FR1 | AICCATTC AATCGGTAIT | Fungal 18S rRNA gene 340 bases | (Vainio and Hantula, 2000) |
| FF390 | CGATAACGAACGAGACCT | | |

Table S4 : Formulae for Biolog index calculations.

| Index | Definition | Formulae | Notes |
|----------|-------------------------------|--------------------------------|--|
| AWCD | Sum of the corrected OD value | $\sum_{i=1}^n \frac{OD_i}{31}$ | OD _i = absorbance of the response well |
| <i>H</i> | Richness diversity | $-\sum p_i(\ln p_i)$ | p _i = ratio between OD _i and OD _i sum |
| <i>E</i> | Evenness calculated from H | $H / \ln S$ | S = Number of wells |

Table S5 : Bacterial and fungal numbers of isolates from the soils within the plots 1 and 2 with (S) and without (AS) dieback symptoms during autumn period. Means \pm SE are presented (n = 5). Asterisks represent significant differences between S and AS soils with P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***).

| Terroir Plots State | Graves | | | |
|---|-------------------|-------------------|-------------------|-------------------|
| | 1 | | 2 | |
| | S | AS | S | AS |
| Bacterial counts (log (CFU / g of soil)) | 7.14 \pm 0.03 | 7.42 \pm 0.03 | 6.78 \pm 0.04 | 7.44 \pm 0.03 |
| | | * | | *** |
| Fungal counts (log (CFU / g of soil)) | 6.67 \pm 0.05 | 6.51 \pm 0.08 | 6.60 \pm 0.06 | 6.39 \pm 0.09 |
| | | * | | ** |
| AUC | 2.49 \pm 0.03 | 3.36 \pm 0.06 | 2.33 \pm 0.04 | 2.91 \pm 0.01 |
| | | *** | | *** |
| E | 0.915 \pm 0.008 | 0.927 \pm 0.013 | 0.878 \pm 0.003 | 0.890 \pm 0.004 |
| | | ns | | * |
| R | 13 \pm 2 | 22 \pm 1.73 | 14 | 17 \pm 1 |
| | | *** | | *** |

Table S6 : Ratios between 16S bacterial and 18S fungal genes and also between cultivable bacterial and fungal CFUs from the soils within the 4 studied plots with (S) and without (AS) dieback symptoms during autumn period. Means \pm SE are represented (n = 5). Asterisks represent significant differences between S and AS soils with P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***).

| Terroir | Graves | | | | Médoc | | | |
|-----------------------|-----------------|-----------------|-----------------|-----------------|------------|--------------|-------------|--------------|
| | 1 | | 2 | | 3 | | 4 | |
| Plot | | | | | | | | |
| Soil | S | AS | S | AS | S | AS | S | AS |
| (B / F) molecular | 384 \pm 63 | 551 \pm 37 | 158 \pm 30 | 425 \pm 40 | 54 \pm 4 | 122 \pm 17 | 80 \pm 18 | 411 \pm 88 |
| | | * | | *** | | *** | | *** |
| (B / F) cultivable | 3.03 \pm 0.48 | 4.17 \pm 0.89 | 1.52 \pm 0.17 | 8.93 \pm 1.60 | nd | nd | nd | nd |
| | | * | | *** | | | | |

Table S7 : Statistical results testing the effects of season (autumn and spring), terroir (Médoc and Graves) on enzymatic (arylamidase, B-glucosidase, acid, and alkaline phosphatases), molecular (total DNA, archaeal and bacterial 16S and fungal 18S), and microbial (Eco-Plates® measurements, and cultivable bacteria and fungi) variables. Asterisks represent significant differences between S and AS soils with $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***). Annotations with “nd” were due to the absence of samplings and data for Médoc terroir during autumn.

| Parameter | Terroir | | Period | | Terroir*Period | |
|----------------------|----------|----------|----------|----------|----------------|----------|
| | <i>F</i> | <i>P</i> | <i>F</i> | <i>P</i> | <i>F</i> | <i>P</i> |
| Arylamidase | 12.98 | *** | 10.50 | ** | nd | nd |
| β-glucosidase | 13.38 | *** | 122.13 | *** | nd | nd |
| Alkaline phosphatase | 38.57 | *** | 61.60 | *** | nd | nd |
| DNA | 17.20 | *** | 25.08 | *** | 1.48 | 0.23 |
| Archaeal 16S | 9.53 | ** | 11.23 | ** | 6.42 | * |
| Bacterial 16S | 20.59 | *** | 25.92 | *** | 16.08 | *** |
| Fungal 18S | 1.00 | ns | 0.23 | ns | 39.83 | *** |
| Cultivable bacteria | 0.02 | ns | 61.06 | ns | nd | nd |
| Cultivable fungi | 0.04 | ns | 49.83 | ns | nd | nd |
| AWCD | 1.04 | ns | 83.37 | *** | nd | nd |
| SEI | 0.01 | ns | 69.23 | *** | nd | nd |
| Richness | 2.57 | ns | 37.43 | *** | nd | nd |
| Amines | 0.68 | ns | 139.73 | *** | nd | nd |
| Amino acids | 0.58 | ns | 104.29 | *** | nd | nd |
| Carbohydrates | 0.83 | ns | 197.63 | *** | nd | nd |
| Carboxylic acids | 5.33 | * | 289.77 | *** | nd | nd |
| Phenolic compounds | 1.05 | ns | 59.96 | *** | nd | nd |
| Polymers | 5.22 | * | 134.33 | *** | nd | nd |

2. Bacterial and fungal microbiomes in vineyard soils subjected to unexplained decline

Romain Darriaut¹, Joseph Tran¹, Coralie Dewasme¹, Guilherme Martins^{2,3}, Nathalie Ollat¹, Isabelle Masneuf-Pomarède^{2,3}, Virginie Lauvergeat^{1*}

¹ EGFV, Univ. Bordeaux, Bordeaux Sciences Agro, INRAE, ISVV, F-33882, Villenave d'Ornon, France

² Université de Bordeaux, INRAE, Bordeaux INP, UMR Œnologie 1366, ISVV, 33140 Villenave d'Ornon, France

³ Bordeaux Sciences Agro, 1 cours du Général de Gaulle, 33170 Gradignan, France

*corresponding author: virginie.lauvergeat@inrae.fr

Abstract

Grapevine declines inducing a progressive yield decrease and an early mortality can be caused by diverse biotic and abiotic factors, sometimes combined. However, some declines are not affiliated to known pathological symptoms or mineral deficiency, and remain unexplained. In such vineyards, soil microbial community should be explored as it may reflect soil health and therefore influences the plant fitness. Indeed, grapevine rhizospheric and endospheric associated microbiota are mainly originated from vineyard soil and play key roles in plant nutrient uptake, health, and development. In this study, the microbial composition of asymptomatic (AS) and symptomatic (S) bulk soils microbiomes from four Bordeaux vineyards presenting unexplained grapevine decline were investigated using high-throughput sequencing based on 16S rRNA and 18S rRNA genes, as well as ITS, during spring and autumn periods. Vineyard geographical location was the strongest driver of microbial communities, while season was mostly driving bacterial community. Symptomatic soils presented enriched bacterial taxa that were potentially beneficial for grapevine. In addition, fungal diversity and richness, including *Glomeromycota* division, was more important in symptomatic soils. Fungal genera associated to grapevine diseases were detected across the different conditions, with higher abundances in symptomatic soils. These findings highlighted the soil from vineyard subjected to unexplained decline as a potential source for fungal pathogens but also for potentially beneficial microorganisms.

Keywords: grapevine decline, metabarcoding, taxa enrichment, terroir, season

Introduction

Important microbial dynamics take place in the soil and drastically influence the health status of the surrounding plants through direct or indirect processes (Trivedi *et al.*, 2020; Wei *et al.*, 2019). Environmental factors such as salinity, drought, agricultural practices, or crop system shape the soil microbial communities that influence these dynamics (Delgado-Baquerizo *et al.*, 2018; Hariharan *et al.*, 2017; Schimel, 2018; Zhang *et al.*, 2019). The dialogues between plant × microbes are therefore modulated by different stressors and can be distinguished into beneficial, commensal, and negative interactions. Undoubtedly, besides these environmental factors, the temporal and spatial variations highly impact the soil microbiome composition (Nuccio *et al.*, 2020).

Pathogen infection can modulate the microbiome diversity and change the host responses and functionalities, resulting in a microbiome dysregulation. Microbiome dysbiosis describes a disequilibrium in the microbiome composition, a microbial profile, that deviates from a healthy and asymptomatic one, which therefore catalyzes the pathogen predisposition to impact the host immune system (Berg *et al.*, 2020). This dysbiosis term is usually used in medical fields, where it is assumed that the high or low relative abundance of a taxon is a marker that contributes to the diagnosis and treatment of the disease (Levy *et al.*, 2017). Parallely, the gut microbiome could be compared to the rhizosphere microbiome since these specific niches are essential to the nutrition, immunity, and pathogen resistance of the individuals (Berendsen *et al.*, 2012). This microbiome dysregulation was observed in plants such as in apple rootstock as well as its surrounding bulk soil when subjected to apple replant disease (Balbín-Suárez *et al.* 2021).

Usually in biological control problematics, researchers investigate the capacity of specific strain to display defensive trait against plant pathogens. However, it has been suggested that higher the microbiome diversity is, better the chance is to generate functional features beneficial for the plants like resistance to phytopathogens or plant-growth promoting traits (Hu *et al.*, 2020; Saleem *et al.*, 2019; Wei *et al.*, 2015). According to this, one can say that the biological soil's quality would be considered as the soil ability to display a broad range of diversity and quantity of microorganisms involved in ecosystemic processes (Loreau and de Mazancourt, 2013). Some keystone taxa are known to trigger those processes, particularly in litter degradation, nitrogen fixation, soil remediation, or plant nutrient solubilization. Therefore, the absence of one of those taxa would negatively impact the soil quality and hence the plant health. Moreover, the keystone taxa removal might alter the soil microbiome stability and increase the soil dysbiosis with new depletions of microbial communities (Herren and McMahan, 2018).

The identification and characterization of the soil microbial communities are usually done with the DNA metabarcoding technology (Francioli *et al.*, 2021). Amplicons-based sequencing is one of the preferred tools in microbial ecology due to decreasing cost of high throughput sequencing, improvement of computational methods, and expansion of DNA sequences databases. This methodology was largely applied in every grapevine compartments such as berry (Zhang *et al.*, 2019), leaves (Wei *et al.*, 2018), bark (Vitulo *et al.*, 2019), rhizosphere (Berlanas *et al.*, 2019), and roots (Carbone *et al.*, 2021). The most targeted regions are the 16S rRNA gene for bacteria and ITS or 18S rRNA gene for fungal communities. Besides soil bacteria and fungi, one of the most studied microorganisms in vineyards, are the root-associated *Glomeromycota* fungi, famous for their mycorrhizal association with the roots contributing to grapevine health, which harbor their beneficial effects based on nutritional support and competition with phytopathogens (Popescu, 2016; Scandellari, 2017; Trouvelot *et al.*, 2015). To investigate grapevine health, most of the microbiome research has been made on the grapevine trunk diseases (GTD) such as esca complex, *Botryosphaeria* dieback, Petri disease, which are widespread diseases causing grapevine decline with easily recognizable foliar symptoms (Gramaje *et al.*, 2018). These works usually focus on rhizosphere (Saccà *et al.*, 2019) and wood (Fotios *et al.*, 2021) interfaces, however, relatively few research have been made on the bulk soil in vineyard in relation to grapevine health (Geiger *et al.*, 2021; Nerva *et al.*, 2019). And no research has been made on the bulk soil microbiome from vineyards afflicted to decline unrelated to mineral deficiencies or pathogen infection, and remain unexplained.

Previous analysis made in Darriaut *et al.* (2021) demonstrated the dysregulation of microbial and functional profiles in four vineyards in the Bordeaux region of France afflicted by unexplained grapevine decline. The symptomatic soils, harbored grapevines with low vigor and high mortality rate, were compared to soils located within the same vineyards that supported well-growing, asymptomatic vines. Physicochemical parameters and virus presence did not explain these observed declines. These vineyards were investigated during autumn and spring periods, and revealed higher enzymatic activities, level of cultivable bacteria, and 16S gene copies, as well as lower level of cultivable fungi and 18S gene copies in asymptomatic bulk soils compared to symptomatic ones. The aim of this present study was to compare the microbial communities' compositions between the soils displaying asymptomatic and declining vines located in the same vineyards. We hypothesized that the symptomatic soils were either subjected to microbial dysbiosis regarding some keystone taxa implied in soil ecosystemic processes, or the dysregulation of the richness and diversities of bacterial and fungal communities. Subsequently to preliminary analysis made in Darriaut *et al.* (2021), the

objectives of this work were to investigate the bacterial and fungal communities associated with the bulk soil from inter-row of asymptomatic and symptomatic areas within four vineyards, during spring and autumn season, presenting vines subjected to unexplained decline unrelated to mineral deficiencies or pathogen infection. The analysis was coupled with a focus on the *Glomeromycota* division because of their biological indicators of viticultural soil health (Popescu, 2016; Trouvelot *et al.*, 2015). Attention was paid to microbial richness and diversities coupled to the community structure subjected to soil status, season, and terroir factors.

Material & Methods

Study sites and sampling

Samplings were the ones performed in Darriaut *et al.* (2021). Briefly, four plots from two Bordeaux region appellations proper to their terroir, namely Graves and Haut-Médoc, were investigated during autumn and spring periods. Small and located area displaying unexplained and symptomatic (S) decline features were sampled in these vineyards, as well as bulk soil from healthy well-growing and asymptomatic (AS) vines (**Figure 25**).

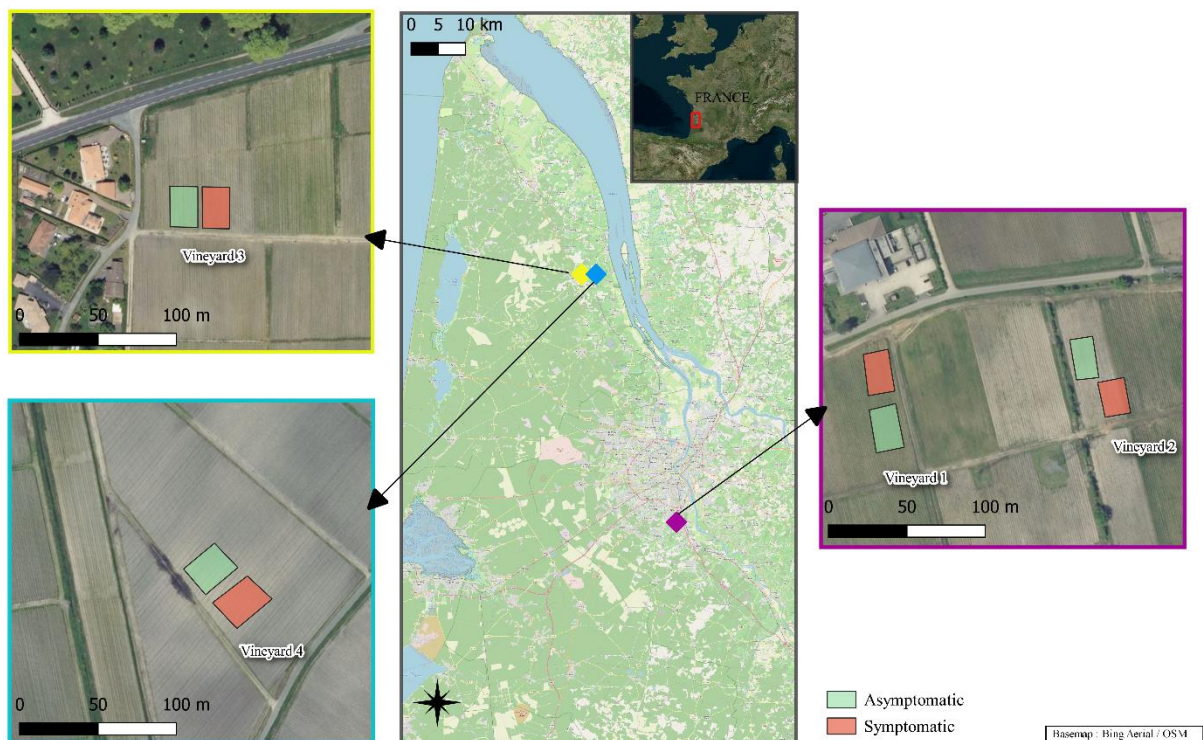


Figure 25 : Proximity of the studied vineyards, located in the Bordeaux region, from the terroirs of Graves (Vineyard 1 and 2) and Haut-Médoc (Vineyard 3 and 4).

The samplings were performed in triplicates and accounted for a total of 48 samples. The upper surface of inter-rows to approximately 30 cm deep, was collected with an auger (10 cm × 25

cm), sieved (< 2 mm), lyophilized for 48 hours using Christ Alpha® 1-4 (Bioblock Scientific) and stored at –80 °C prior to DNA extraction.

DNA extraction, library preparation, and sequencing

Total DNA was extracted from 250 mg of the lyophilized soils using the DNeasy PowerSoil Pro kit (Qiagen) according to the manufacturer recommendations with an additional C5 washing step. Quantification of the extracted DNA samples were performed on a Qubit® 3.0 fluorometer (Thermo Fisher Scientific) using the Qubit™ dsDNA HS Assay Kit, while the quality checking was done with a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific). DNA was then stored at –20 °C until further use.

The DNA samples were randomized across plates and amplified using the primers listed in **Table 15**, specific to either the bacterial and archaeal 16S rRNA gene, the fungal ITS1 region, or the fungal SSU which is used for *Glomeromycota* identification.

Table 15 : Primers used for 16S rRNA, ITS, and 18S rRNA amplifications. Specific overhang Illumina adapters are in italic and underlined.

| Primer | Primer sequence (5' to 3') | Target and size of the amplicon | Reference |
|-----------------|--|---|-----------------------------------|
| 341F | <u><i>TCGTCGGCAGCGTCAGATGTGTATAAG</i></u> <u><i>AGACAGCCTACGGGNGGCWGCAG</i></u> | Bacterial 16SrRNA gene V3-V4 regions (464 bp) | (Klindworth <i>et al.</i> , 2013) |
| 785R | <u><i>GTCTCGTGGGCTCGGAGATGTGTATAA</i></u> <u><i>GAGACAGGACTACHVGGGTATCTAATCC</i></u> | | |
| ITS1F | <u><i>TCGTCGGCAGCGTCAGATGTGTATAAG</i></u> <u><i>AGACAGCTTGGTCATTTAGAGGAAGTAA</i></u> | Fungal ITS1 region (highly variable) | (Gardes and Bruns, 1993) |
| ITS2 | <u><i>GTCTCGTGGGCTCGGAGATGTGTATAA</i></u> <u><i>GAGACAGGCTGCGTTCTTCATCGATGC</i></u> | | |
| AMV4.5 Nf AMDGr | <u><i>TCGTCGGCAGCGTCAGATGTGTATAAG</i></u> <u><i>AGACAGAAGCTCGTAGTTGAATTTTCG</i></u> <u><i>GTCTCGTGGGCTCGGAGATGTGTATAA</i></u> <u><i>GAGACAGCCCAACTATCCCTATTAATCAT</i></u> | Fungal 18SrRNA (350bp) | (Suzuki <i>et al.</i> , 2020) |

All the primers included the specific overhang Illumina adapters used for the amplicon library construction. Each 25 µl reaction contained 12.5 µl of 5X GoTaq® Reaction Buffers (Promega, France), 8 µl of Nuclease-free water, 1 µl of each primer (10 µM), 2.5 µl of DNA template (5 ng/µl), and 0.625 u of GoTaq® G2 DNA Polymerase (Promega, France). PCR amplifications were performed in triplicate for each condition. The cycling conditions are listed in **Table S8**. Further steps were processed at the PGTB sequencing facility (Genome Transcriptome Facility

of Bordeaux, Pierroton, France) using first a Nano V2 with 2×250 nucleotides paired reads to calibrate the homogeneity of the 3 genes target, subsequently followed by a V2 with 2×250 nucleotide paired reads protocol. The PCR products were purified with platform-specific SPRI magnetic beads (1X ratio) and quantified using Quant-iT™ dsDNA Assay kit (ThermoFisher, France). MID and Illumina sequencing adapters were added. Libraries were pooled in equimolar amounts using a Hamilton Microlab STAR robot and sequenced on an Illumina MiSeq platform using the MiSeq Reagent Kit v2 (2×250 bp). Obtained sequences were demultiplexed with index search at the PGTB facility.

Bioinformatics methods

Sequences quality obtained were preliminarily checked with FastQC v.0.11.8 (Andrews, 2010). Sequences were quality filtered, trimmed, denoised, and clustered into Operational Taxonomy Units (OTUs) using FROGS pipeline from Galaxy instance (Escudié *et al.*, 2018). Briefly, raw forward and reverse reads for each sample were assembled into paired-ended reads with a minimum overlapping of 50 nucleotides and 0.1 mismatch using the VSEARCH tool (Rognes *et al.*, 2016). Primers were removed using Cutadapt (Martin, 2011), chimeras were detected and removed with UCHIME (Edgar *et al.*, 2011), clustering was performed using SWARM (Mahé *et al.*, 2014) within FROGS pipeline. Minimum proportion of sequences abundance to keep OTUs was set to $5e-05$, and singletons suppression was done with phiX contaminant databank. Taxonomic assignments of 16S rRNA, 18S rRNA gene OTUs and ITS-based OTUs were performed against silva138.1 (16S pintail100) (Quast *et al.*, 2012), MaarJAM (2019) (Öpik *et al.*, 2010), Unite8.2 (Nilsson *et al.*, 2019), respectively, using RDPClassifier from Galaxy.

All analysis and graphs were performed on R (4.1.2) using RStudio (2021.9.1.372). Figures were generated with *ggplot2* (3.3.5) and *ggthemes* (4.2.4) packages and arranged using *ggpubr* (0.4.0) (Arnold, 2021; Kassambara, 2020; Wickham and Chang, 2008). Dataset were gathered and analyzed through *phyloseq* package (1.38.0) (McMurdie and Holmes, 2013). Taxa related to mitochondrial and chloroplast OTUs were removed. Venn diagrams were generated to demonstrate the distinct and shared OTUs within soils through soil status, season, and terroir factors. Bacterial and fungal OTUs shared between Soil \times Terroir \times Season conditions were visualized using *UpSetR* (1.4.0) (Conway *et al.*, 2017). Richness and α -diversity metrics, represented by Chao1, Shannon's diversity, and Bray-Curtis dissimilarity, respectively, were calculated through *phyloseq* using "estimate_richness" function. To test for significant differences between the means of alpha diversity metrics by conditions, pairwise comparisons were used, based on either t or wilcoxon test, subsequently to homogeneity and normalization

verifications using Levene and Shapiro tests. Nonmetric multidimensional scaling (NMDS) was used to ordinate samples in two-dimensional space based on Bray-Curtis distance using ordinate function from *phyloseq* with “NMDS” method. Linear models and permutational multivariate analysis of variance (PERMANOVA), for richness and diversities metrics, were demonstrated using the formula: variable ~ Soil status × Season + Terroir. Type-II analysis of variances (ANOVAs) were performed using *car* (3.0-12) on Chao1 and Shannon’s diversity metrics while PERMANOVAs were assessed on Bray-Curtis dissimilarity using *vegan* package (2.5-7) with 999 permutations, and tests of multivariate homogeneity of group dispersions were checked using “betadisper” function within the same package. Vector fitting to ordinations using “envfit” function from *vegan* was used to identify the environmental factors that best predicted bacterial and fungal community structures. Functions “ggeffectsiz”, “ggdiffbox”, and ‘ggdiffclade’ from *MicrobiotaProcess* (1.2.2) were used to discriminate significantly different taxa across conditions (Xu and Yu, 2021). This process was set with Kruskal ($\alpha = 0.05$) test based on linear discriminant analysis (LDA) effect size (LEfSe) and Wilcox ($\alpha = 0.05$), corrected with False Discovery Rate (FDR).

Results

Taxonomic distribution across the different conditions

A total of 4,649,863 16S, 4,191,712 ITS, and 3,844,836 18S raw sequences were generated. Subsequently to chimera removal, paired-end sequences of 16S rRNA gene, ITS, and 18S rRNA gene were clustered into 2684, 810, and 244 operational taxonomic units (OTUs), respectively.

Shared OTUs between bacterial communities across the soil status, season, and terroir factors were 96.36%, 96.22%, and 92.71%, respectively (**Figure 26.A**). Fungal OTUs from ITS sequencing were more shared regarding season (89.01%) and soil status factors (88.89%) than terroir (75.80%) (**Figure 26.B**). *Glomeromycota* division displayed more distinct OTUs across factors than fungal ITS or bacterial 16S, with 63.11% shared OTUs across symptomatic and asymptomatic soils, 55.74% regarding season, and 63.11% between Graves and Haut-Médoc terroirs (**Figure 26.C**). Co-occurrence of OTUs revealed 1423 bacterial OTUs, 206 fungal OTUS, and 14 OTUs associated to *Glomeromycota*, shared across the eight Soil × Terroir × Season conditions (**Figure S4**).

Regardless of the soil status, terroir, and season, *Actinobacteriota* (33%), *Proteobacteria* (15%), *Chloforexi* (14%), *Acidobacteria* (13%), *Firmicutes* (14%), *Verrucomicrobiota* (3%)

were the most abundant bacterial phyla accounting for 92% of total bacterial communities (**Figure 26.D**) while “Others” group was composed of *Gemmatimonadota*, *Myxococcota*, *Methylomirabilota*, *Nitrospirota*, *Bacteroidota*, *Desulfobacterota*, *Latescibacterota*, *RCP2-54*, *MBNT15*, *Entotheonellaeota*, *GAL15*, *Halobacterota*, *Cyanobacteria*, *Patescibacteria*, *Fibrobacterota*, and *Bdellovibrionota*.

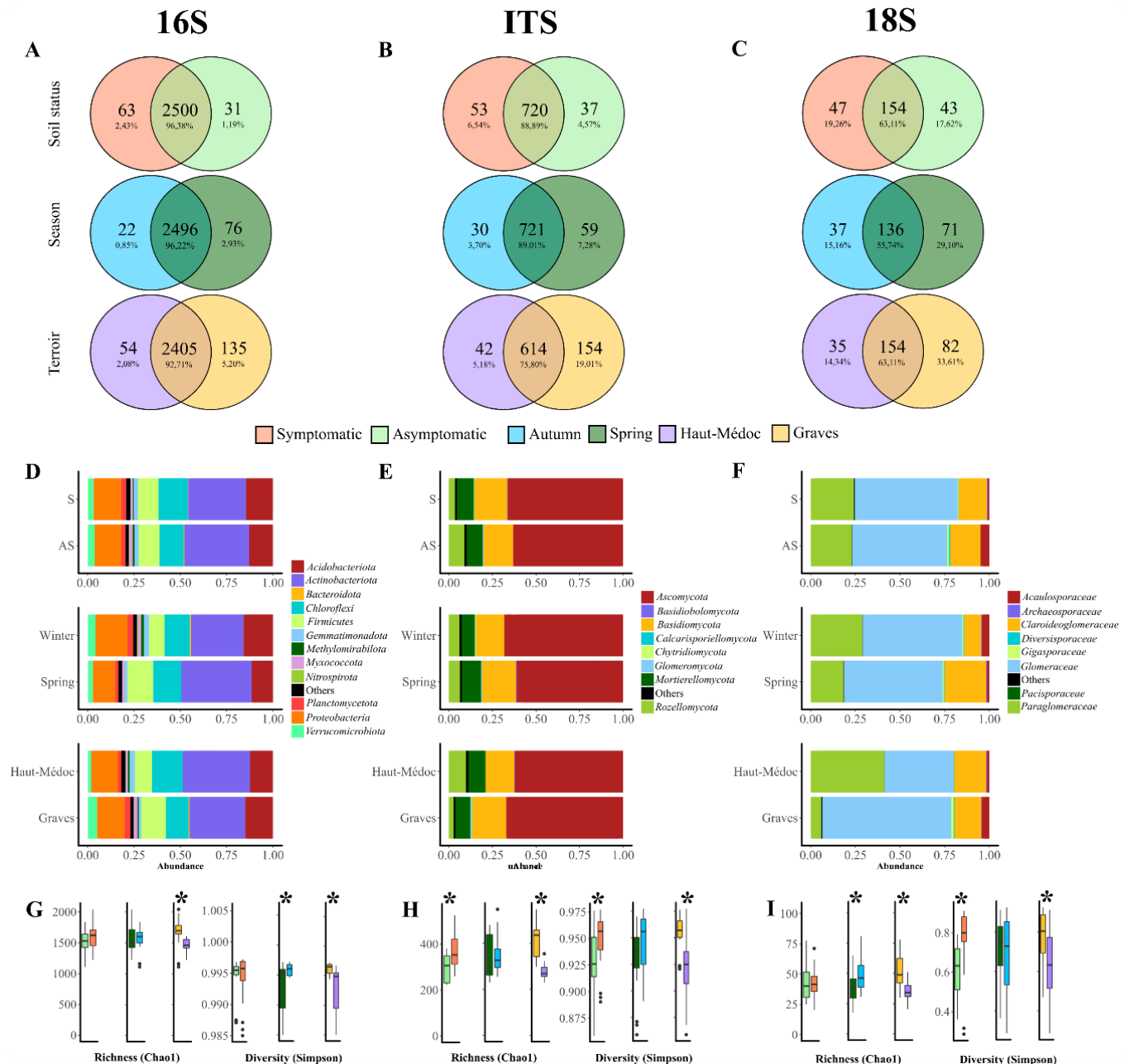


Figure 26 : Taxonomic distribution across the different conditions. Shared and unique OTUs related to soil status, season, and terroir factors among (A) bacterial, (B) fungal, and (C) Glomeromycota communities. Relative abundances of (D) bacterial, (E) fungal phyla, and (F) Glomeromycota identified families. The phyla individually represented less than 1% of the total communities were grouped in “Others”. Richness and diversity, represented by Chao1 and Simpson’s index diversity, of (G) bacterial, (H) fungal, and (I) *Glomeromycota* taxa. Asterisks are presenting significant differences ($P < 0.05$) between conditions.

In regard to ITS sequencing, *Ascomycota* (58%), *Basidiomycota* (16%), *Mortierellomycota* (8%), and *Rozellomycota* (6%) were the predominant phyla, while unaffiliated fungal OTUs accounted for 9% (**Figure 26.E**) and “Others” group was constituted of *Calcarisporiellomycota*, *Chytridiomycota*, *Glomeromycota*, *Basidiobolomycota*, *Kickxellomycota*, *Monoblepharomycota*, *Blastocladiomycota*, *Zoopagomycota*, *Olpidiomycota*, and *Entorrhizomycota*. Regarding the 18S rRNA sequencing, 31% of OTUs were unaffiliated. *Glomus* (55%), *Paraglomus* (24%), *Claroideoglomus* (16%), and *Acaulospora* (3%) were the predominant identified genera while less abundant ones were belonging to *Scutellospora*, *Diversispora*, *Archaeospora*, *Gigaspora*, *Pacispora*, and *Ambispora* (**Figure 26.F**). Taxonomy of the microbial communities of each vineyard is depicted in **Figure S5**.

Several fungi affiliated to grapevine diseases (*i.e.*, grey mold, Petri disease, black foot, grapevine canker) listed in **Table S9** were detected across the conditions. Among them, *Phaeoacremonium* (0.43%, 0.10%), *Ilyonectria* (8.17%, 0.61%), *Neonectria* (12.32%, 0.05%), *Cadophora* (30.72%, 28.90%), *Botrytis* (1.78%, 0.02%), *Curvularia* (46.58%, 70.32%) were identified in both asymptomatic and symptomatic soils, respectively (**Figure 27**).

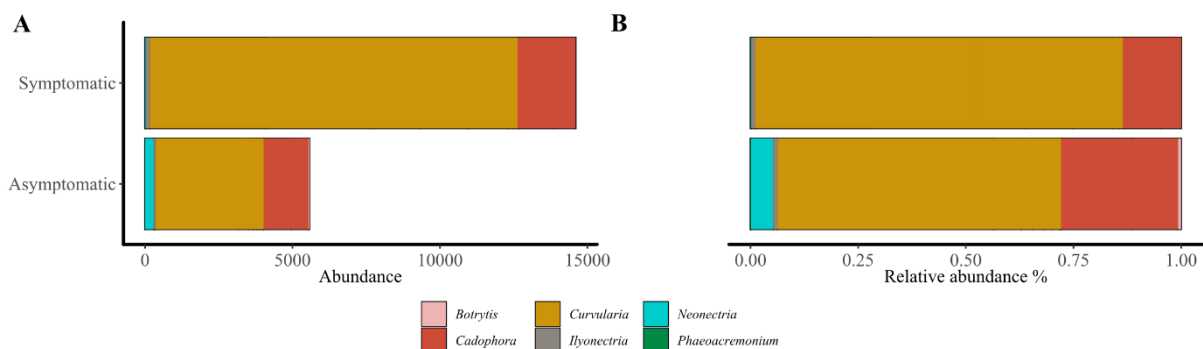


Figure 27 : Fungal genera associated to grapevine diseases across the symptomatic and asymptomatic conditions represented by their abundance (**A**) and relative abundance (**B**).

Richness differed between terroir and season while diversity was rather affected by soil status

Similar bacterial richness, represented by Chao1 metric, was found during spring and autumn seasons, as well as among symptomatic and asymptomatic soils, but significant different Chao1 richness were found between Graves and Haut-Médoc appellations (**Figure 26.G**). In regard to ITS-sequenced fungal community, richness was significantly influenced by soil status and terroir, accounting for 4.4% and 78.2% of the observed variance, respectively (**Table 16**). Symptomatic soils displayed higher ITS-based richness compared to asymptomatic ones, as well as more important richness in Graves compared to Haut-Médoc (**Figure 26.H**). Regarding

Glomeromycota division, Chao1 metric was influenced by both terroir and season, explaining 4.4% and 7.1% of the total variance (**Table 16**), respectively, with lower richness in Haut-Médoc and during autumn compared to Graves appellation and spring period (**Figure 26.I**).

Shannon's index diversity was influenced by terroir for bacterial, and both ITS and 18S-sequenced fungal communities with higher diversity in Graves compared to Haut-Médoc (**Figure 26.G, H, and I**). Bacterial and ITS-sequenced fungal diversities were also influenced by season with less important diversity, respectively, in autumn in comparison to spring period (**Figure 26G and H**). In addition, decline features of the soil were also driving Simpson's index diversities of both ITS-sequenced fungal and 18S-sequenced fungal communities with, respectively, significantly higher diversity in symptomatic soil compared to asymptomatic ones (**Figure 26H and I**). In regard to bacterial diversity, season and terroir factors explained 16.7% and 22.6% of the total variance observed, while 11.4% and 31.7% of observed variance were explained by soil and terroir, respectively for ITS-based fungal OTUs (**Table 16**).

Table 16 : Factors effects related to soil composition (S, AS), season (autumn, spring), and terroir (Graves and Haut-Médoc) on richness (Chao1), diversity (Simpson), and β -diversity (Bray-Curtis) related to bacterial, fungal, and Glomeromycotan communities in the sampled bulk soils. Significances were assessed through a Type II ANOVA for richness and α -diversity, while PERMANOVA was used for β -diversity.

| | | Richness (Chao1) | | α -diversity (Simpson) | | β -diversity (Bray-Curtis) | | |
|----------------------|----------------------|------------------|----------------|-------------------------------|----------------|----------------------------------|-----------------------|--------------|
| | | <i>F</i> (1,43) | <i>P</i> | <i>F</i> (1,43) | <i>P</i> | <i>F</i> (1,43) | <i>R</i> ² | <i>P</i> |
| Bacteria | Soil | 2.407 | 0.128 | 0.314 | 0.578 | 4.611 | 0.074 | 0.001 |
| | Season | 0.0001 | 0.994 | 16.742 | < 0.001 | 4.628 | 0.074 | 0.001 |
| | Terroir | 14.306 | < 0.001 | 22.636 | < 0.001 | 8.346 | 0.134 | 0.001 |
| | Soil \times Season | 0.002 | 0.965 | 0.441 | 0.510 | 2.730 | 0.027 | 0.003 |
| | Residuals | 83.2849 | | 59.867 | | 79.685 | | |
| Fungi | Soil | 4.402 | 0.042 | 11.454 | 0.001 | 3.909 | 0.063 | 0.001 |
| | Season | 0.261 | 0.612 | 5.650 | 0.022 | 3.543 | 0.057 | 0.001 |
| | Terroir | 78.245 | < 0.001 | 31.663 | < 0.001 | 10.223 | 0.164 | 0.001 |
| | Soil \times Season | 0.096 | 0.758 | 6.664 | 0.013 | 1.646 | 0.075 | 0.075 |
| | Residuals | 16.996 | | 44.569 | | 80.679 | | |
| <i>Glomeromycota</i> | Soil | 0.278 | 0.601 | 7.157 | 0.010 | 4.690 | 0.073 | 0.001 |
| | Season | 7.134 | 0.044 | 0.245 | 0.623 | 3.600 | 0.056 | 0.001 |
| | Terroir | 4.418 | < 0.001 | 8.695 | 0.005 | 9.021 | 0.141 | 0.001 |
| | Soil \times Season | 0.004 | 0.950 | 1.075 | 0.305 | 3.614 | 0.056 | 0.001 |
| | Residuals | 88.166 | | 82.828 | | 20.925 | | |

Taxa enrichment is influenced by soil status, season, and terroir

The LEfSe was conducted to report significantly enriched taxa and to get an overview of microbial community changes between soil, sampling time or vineyard location. The results presented in **Figure 28** showed enrichment related to each of the three environmental factors. For a better visualization, please consider observing the high quality figure in the **Annex**.

Enrichments in bacterial communities were more important due to season and terroir than soil status factor, while fungi enrichments were mainly caused by terroir and soil factors.

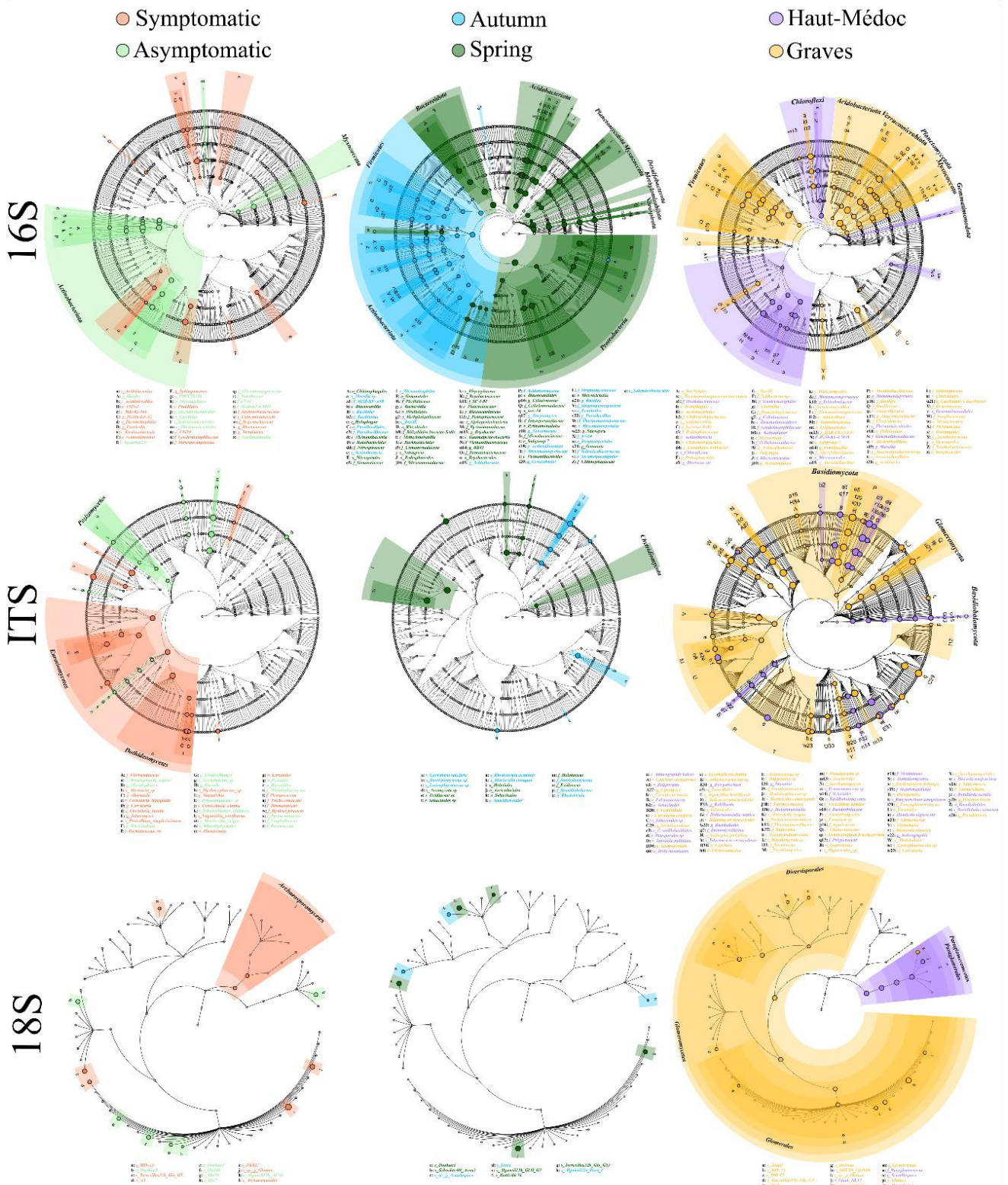


Figure 28 : Circular cladograms reporting LEfSe analysis related to identified OTUs from bacterial, fungal, and Glomeromycota communities according to phylogenetic features around the circle. The

center of the circle represents the kingdom while the outer circle presents the OTUs at species level. The color of the points and sectors indicate the factor in which the related OTUs are enriched.

Asymptomatic soils were, regardless of season or vineyard location, significantly enriched in 18 taxa, mainly from *Actinobacteriota* (i.e., *Acidimicrobiia*, *MB-A2-108*, *Thermoleophilia* classes) and *Myxococcota* (i.e., *bacteriap25* class) phyla while symptomatic soils were rather enriched in 16 taxa belonging partly to *Ktedonobacteria* and *Acidobacteriae* classes, as well as *Blastococcus*, *Terrabacter*, *Gemmatimonas*, *Sphingomonas*, and *Fonticella* genera.

During autumn, 30 taxa were increased mainly taking part in *Actinobacteriota*, *Firmicutes* phyla while 56 taxa were enriched during spring, belonging to *Proteobacteriota*, *Acidobacteriota*, *Planctomycetota*, *Methylomirabiota*, *Bacteroidota*, *Nitrospirota*, *Myxococcota*, *Desulfobacterota*. In regard to terroir, 22 taxa largely belonging to *Chloroflexi* and *Gemmatimonadota* were enriched in Haut-Médoc while 58 taxa were more abundant in Graves from *Firmicutes*, *Acidobacteriota*, *Verrucomicrobiota*, *Planctomycetota*, and *Myxococcota* phyla.

Regarding fungi sequenced-based on ITS, 21 taxa were significantly more abundant in symptomatic soils, with most taxa from *Trichocomaceae*, *Filobasidiaceae*, *Pleosporaceae*, *Hyaloscyphaceae*, and *Dermateaceae* families while asymptomatic soils were enriched in 17 taxa which globally belong to *Pyronemataceae*, *Russalaceae*, *Lyophyllaceae*, and *Minutisphaeraceae* families. Few taxonomic enrichments were found in fungal communities due to season with 9 taxa enriched during autumn (e.g., *Sporidiobolales* family) and also 9 taxa enriched during spring (e.g., *Helotiales*, *Sebacinales*, and *Auriculariales* orders). On the other hand, the factor inducing the most important effect towards a microbial community was the terroir component on the ITS-sequenced fungal community with 59 taxa more abundant in Graves appellation (e.g., *Tremellales*, *Thelebolales*, *Eurotiales*, *Pleosporales*, and *Glomerales* orders) and 34 enriched taxa in Haut-Médoc (e.g., *Holtermanniales* and *Cystofilobasidiales* orders).

In *Glomeromycota* division, the taxa enrichment was more influenced by terroir with 17 taxa (e.g., species from *Glomerales*, and *Diversiporales* orders) in Graves and 5 in Haut-Médoc (exclusively from *Paraglomus* genus), while 7 taxa were more abundant in symptomatic and 6 were significantly more present in asymptomatic soils. Sampling time was the less influential factor on *Glomeromycota* division with 3 enriched taxa in autumn and 5 during spring period.

Microbial community structure is modified by soil status, season, and terroir

To explore whether differences in microbiome structure and composition were correlated with decline features, sampling time, or vineyard location, the β -diversity using Bray-Curtis distance was computed. NMDS analysis identified similar groupings of microbial communities based on soil status depending on season or terroir (**Figure 29**).

Each of the three factors displayed significant correlations with the first two dimensions of the NMDS analysis. Even though, clustering was more pronounced regarding the terroir effect, compared to the season, statistical differences were confirmed through PERMANOVA and revealed that the overall microbial community across both bacterial and fungal samples, differed from decline features, sampling time, and vineyard location (**Table 16**).

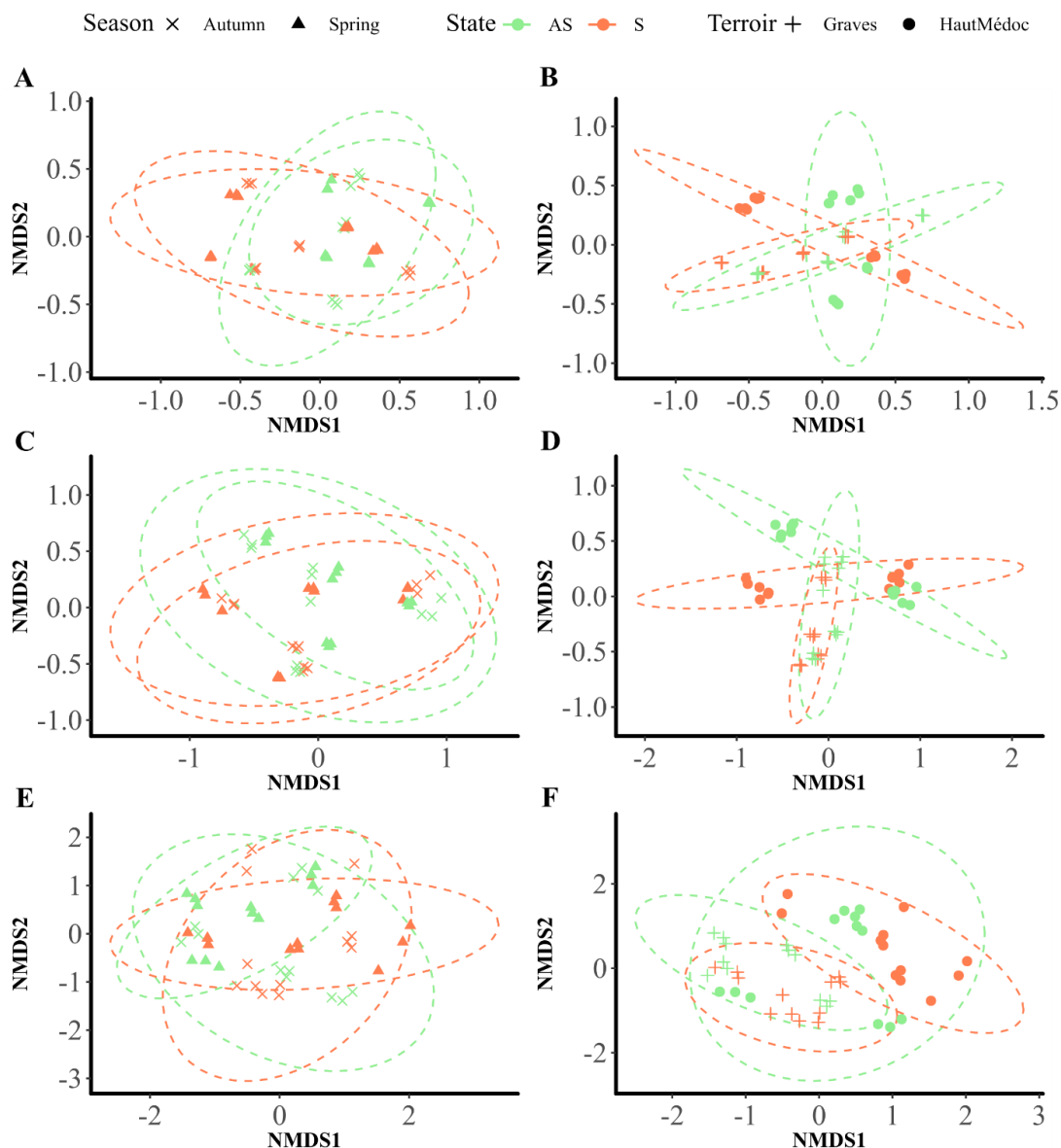


Figure 29 : Non-metric multidimensional scaling (NMDS) plot ordination of (A, B) bacterial, (C, D) fungal, and (E, F) Glomeromycota communities among the Graves (cross) and Haut-Médoc (circle) appellations across symptomatic S (orange) and asymptomatic AS (light green) soils during spring

(triangle) and autumn (cross) seasons. Ordinations were based on Bray-Curtis distance method, and dashed lines represent 95% confidence ellipses.

The beta-dispersion analysis showed insignificant comparisons for soil status factor across each microbial community (**Table S11**). These results indicated that the significant effects related to symptomatic or asymptomatic features observed above were likely not an artifact of dispersion and do reflect biological differences. On other hand significant effects were observed for bacteria related to season, as well as terroir factor regarding the fungal and *Glomeromycota* communities (**Table S11**). In parallel, the strongest relationship between factors and bacterial community was with soil status ($R^2 = 0.087$) while ITS-fungal and *Glomeromycota* communities were rather related to terroir component ($R^2 = 0.125$ and $R^2 = 0.246$, respectively).

Discussion

An essential goal in microbial ecology is to understand the influence of factors that modulate the microbial communities. The bulk soil is considered as the microbial reservoir where the plant uptake its associated microbiome through the chemoattraction property of root exudates. A balanced grapevine-associated microbiome is primordial for the vine development while preserving berry quality, and therefore its surrounding soil should propose microorganisms meeting these criteria.

Microbial diversity metrics in bulk soil as a biological indicator of grapevine decline?

Vineyard decline is related to a large panel of environmental factors and can be alleviated by several human practices if managed properly. For instance, cover crop are used to counteract vine decline by conferring an increased microbial diversity which acts as line of defense against soil pathogen (Richards *et al.*, 2020). In the studied vineyards, cover cropping was not used in symptomatic and asymptomatic soils, and the results obtained suggest that microbial richness is influenced rather by vineyard location and season than by soil status. It may not be surprising since main drivers of microbial communities in soils from vineyards are edaphic (*e.g.*, soil physicochemical parameters) and environmental factors (*e.g.*, temperature or moisture) (Burns *et al.*, 2015b; Coller *et al.*, 2019). Two terroirs were explored in this study, within Graves (*i.e.*, vineyards 1 and 2) and Haut-Médoc appellations (*i.e.*, vineyards 3 and 4). Both were located in Bordeaux region managed with conventional practices, presenting different physicochemical parameters depicted in Darriaut *et al.* (2021).

Notwithstanding the terroir and season impacts on microbial communities, no significantly different bacterial richness and diversity were found between symptomatic and asymptomatic soils. On a previous study made on vineyard affected to esca complex, no significant differences in fungal and bacterial richness and diversity were found between symptomatic and asymptomatic bulk soils (Nerva *et al.*, 2019). In addition, similar bacterial diversity was found in rhizosphere of symptomatic and asymptomatic grapevine affected to esca complex (Saccà *et al.*, 2019). Herein, symptomatic soils presented significantly higher fungal richness and diversity compared to asymptomatic ones, which did not corroborate to Nerva *et al.* (2019) or with previous results related to lower level of cultivable fungi in symptomatic soils (Darriaut *et al.*, 2021).

Moreover, the *Glomeromycota* division, affiliated to arbuscular mycorrhizal fungi (AMF), presented as well significantly higher diversity in symptomatic soils compared to asymptomatic ones (**Table S10**). To our knowledge, only Bezerra *et al.* (2021) investigated the richness of AMF in soil with decline and dieback features by comparing them to asymptomatic soils within the same vineyards. It appeared that the location of the vineyard had more influence on the AMF diversity and richness than the soil status.

Usually, degraded soils present less fungal diversity or richness than normal soil since soil biodiversity has a positive correlation with the soil ecosystemic processes, while loss of fungal diversity impairs several functions such as nutrient cycling, organic matter decomposition, and plant defense system (Wagg *et al.*, 2019, 2014). In our case, the soils supporting grapevine decline were presenting similar bacterial richness and diversities while surprisingly at the opposite the fungal communities were displaying higher diversity metrics in soils with grapevine in decline.

Detected fungal pathogens were more abundant in symptomatic soils compared to asymptomatic ones

Among these genera, *Cadophora luteo-olivacea*, *Ilyonectria destructans*, *Neonectria lugdunensis*, *Curvularia spicifera*, *Curvularia lunata*, *Curvularia inaequalis*, and *Curvularia portulacae* species were detected with some of them clearly identified as grapevine pathogens (Bahmani *et al.*, 2021; Gramaje *et al.*, 2021, 2018; Lade *et al.*, 2022). Interestingly, the abundances affiliated to these fungi genera were rather affected by soil ($F(1, 284) = 7.21$, $P = 0.008$) and terroir ($F(1, 284) = 7.31$, $P = 0.007$) than season ($F(1, 284) = 0.15$, $P = 0.694$) and were higher in symptomatic soils compared to asymptomatic ones. The abundance of the genera *Phaeoacremonium* and *Phaeoconiella* was higher in soils associated to symptomatic vines

affected by esca, compared to asymptomatic ones (Nerva *et al.*, 2019), while in our case only *Phaeoacremonium* genus was detected and in a similar abundance between both bulk soils. These findings were not correlated to grapevine diseases, comforting the idea that the observed declines were not affiliated to pathogen invasion from the bulk soil. However, it supports the postulate that soil is one of the inoculum sources of fungal pathogens of grapevine that are even present in healthy and asymptomatic bulk soils (Giménez-Jaime *et al.*, 2006; Gramaje and Armengol, 2011; Nerva *et al.*, 2019).

Symptomatic soil harbors high amount of potentially beneficial bacteria to grapevine

The greater abundance of potential fungal pathogens in symptomatic soils was accompanied by an enrichment of several bacterial taxa compared to asymptomatic conditions, regardless of season or vineyard location. Among these enriched taxa were included the bacterial genera *Blastococcus*, *Terrabacter*, *Sphingomonas*, *Gemmatimonas*, and *Fonticella*. These genera were related to pathogen control or nutrient regulation, and were not affiliated to known plant diseases. For instance, *Blastococcus* is involved in both nitrogen metabolism (Cobo-Díaz *et al.*, 2015) and pathogen inhibition such as *Fusarium oxysporum* (Zhao *et al.*, 2019). *Terrabacter* genus is also a potential keystone taxa involved in pathogen suppression responsible of wheat decline (Chng *et al.*, 2015; Wei *et al.*, 2019). *Terrabacter*, on the other hand, has been negatively correlated with bacterial wilt disease index, and was related to the restoration of declining soil properties by increasing soil nutrients (Qi *et al.*, 2020). *Gemmatimonas* has also been reported to be linked with *Fusarium* wilt suppressiveness (Ou *et al.*, 2019), as well as organic matter degradation and conversion into soil nutrient. *Sphingomonas* genus displayed high nitrogenase activity and plant-promoting growth capacity towards maize and wheat plants (Xu *et al.*, 2018), and is known to harbor biocontrol properties on powdery mildew and *Fusarium* blight (Innerebner *et al.*, 2011; Wachowska *et al.*, 2013).

In addition, higher *Glomeromycota* diversity was found in symptomatic conditions compared to asymptomatic ones (**Table 16; Table S10**). Even though Landi *et al.* (2021) did not investigate diversity, the authors found greater intensity of mycorrhizal fungi colonization, as well as greater abundance of *Glomeromycota*, in the rhizosphere and roots of esca-affected grapevine compared to asymptomatic ones.

The « cry-for-help » concept is a plant adaptative response to biotic (Rolfe *et al.*, 2019) or abiotic stresses (Rolli *et al.*, 2021), and make sense in this decline context since there was no evidence of edaphic factors or mineral deficiencies explaining the growth issue of grapevine

among the four vineyards (Darriaut *et al.*, 2021). Wei *et al.* (2019) demonstrated the determinant function of soil microbiome composition in plant health through the presence of rare taxa and pathogen-suppressing genus. It would be outstanding to study the potential beneficial effects of isolates from declining areas. Indeed, stressful environments may provide microorganisms that can alleviate diverse stress and furnish interesting growth-promoting traits to plants or relevant metabolic activities for soil nutrient cycling (Ashry *et al.*, 2021).

Conclusion

The present investigation carried out for the first time on the microbiome from bulk soil of vineyards affected by unknown decline during spring and autumn period among two different Bordeaux terroirs. The differences between symptomatic and asymptomatic soils from the surrounded grapevine of both bacterial and fungal divisions were pointed out. Despite the presence of fungal genera associated to grapevine diseases, soils in symptomatic area were even enriched in potentially beneficial bacterial genera, with also higher diversity in fungal communities, including the *Glomeromycota* phylum. These findings suggest the soil as one of the sources of fungal pathogen but also of beneficial microorganisms for the grapevine, rising interest in the isolation of bacteria in stressful environments.

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2. Bacterial and fungal microbiomes in vineyard soils subjected to unexplained decline

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Supplemental information

Table S8 : Cycling conditions used for the first PCR before indexing.

| Primer's pair | Initial denaturation | Number of cycles | Denaturation | Annealing | Extension | Final extension |
|--------------------|----------------------|------------------|--------------|--------------|--------------|-----------------|
| 341F/785R | 5 min at 95°C | 25 | 30 s at 95°C | 30 s at 55°C | 30 s at 72°C | 5 min at 95°C |
| ITS1F/ITS2 | 5 min at 95°C | 30 | 30 s at 95°C | 30 s at 55°C | 45 s at 72°C | 5 min at 95°C |
| AMV4.5Nf/ AMDGr | 5 min at 95°C | 30 | 30 s at 95°C | 45 s at 55°C | 45 s at 72°C | 5 min at 95°C |

Table S9 : List of fungal genera associated to grapevine diseases.

| | Associated disease | References |
|----------------------------|----------------------------------|---|
| <i>Anthostoma</i> | Eutypa | (Gramaje <i>et al.</i> , 2018; Perazzolli <i>et al.</i> , 2019) |
| <i>Botrytis</i> | Grey mould | (González-Fernández <i>et al.</i> , 2020) |
| <i>Botryosphaeria</i> | <i>Botryosphaeria</i> dieback | (Mondello <i>et al.</i> , 2015) |
| <i>Cadophora</i> | Petri disease | (Gramaje <i>et al.</i> , 2021) |
| <i>Campylocarpon</i> | Black foot | (Gramaje <i>et al.</i> , 2018) |
| <i>Cryptosphaeria</i> | Canker, Eutypa | (Trouillas <i>et al.</i> , 2010) |
| <i>Cryptovalsa</i> | Canker, Eutypa | (Niem <i>et al.</i> , 2020; Trouillas <i>et al.</i> , 2010) |
| <i>Cytospora</i> | Canker, Eutypa | (Lawrence <i>et al.</i> , 2017) |
| <i>Curvularia</i> | Canker | (Bahmani <i>et al.</i> , 2021) |
| <i>Cylindrocladiella</i> | Black foot | (Gramaje <i>et al.</i> , 2018) |
| <i>Dactylonectria</i> | Black foot | (Gramaje <i>et al.</i> , 2018) |
| <i>Diaporthe</i> | <i>Phomopsis dieback</i> | (Yan <i>et al.</i> , 2013) |
| <i>Diatrype</i> | Eutypa dieback | (Trouillas <i>et al.</i> , 2010) |
| <i>Diatrypella</i> | Eutypa dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Diplodia</i> | <i>Botryosphaeria</i> dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Dothiorella</i> | dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Eutypa</i> | Eutypa dieback | (Cardot <i>et al.</i> , 2019) |
| <i>Eutypella</i> | Eutypa dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Fomitiporia</i> | Esca | (Gramaje <i>et al.</i> , 2018) |
| <i>Fomitiporella</i> | Esca | (Gramaje <i>et al.</i> , 2018) |
| <i>Fusicoccum</i> | Phomopsis dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Ilyonectria</i> | Black foot | (Lade <i>et al.</i> , 2022) |
| <i>Inocutis</i> | Esca | (Gramaje <i>et al.</i> , 2018) |
| <i>Inonotus</i> | Esca | (Gramaje <i>et al.</i> , 2018) |
| <i>Lasiodiplodia</i> | <i>Botryosphaeria</i> dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Neofusicoccum</i> | dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Neonectria</i> | Black foot | (Gramaje <i>et al.</i> , 2018) |
| <i>Neoscytalidium</i> | <i>Botryosphaeria</i> dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Phaeobotryosphaeria</i> | <i>Botryosphaeria</i> dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Phaeoacremonium</i> | Petri disease | (Lade <i>et al.</i> , 2022) |
| <i>Phaeomoniella</i> | Petri disease | (Lade <i>et al.</i> , 2022) |
| <i>Phellinus</i> | Esca | (Gramaje <i>et al.</i> , 2018) |
| <i>Pleurostoma</i> | Petri disease | (Gramaje <i>et al.</i> , 2018) |
| <i>Spencermartinsia</i> | <i>Botryosphaeria</i> dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Stereum</i> | Esca | (Gramaje <i>et al.</i> , 2018) |
| <i>Thelonectria</i> | Black foot | (Gramaje <i>et al.</i> , 2018) |

Table S10 : Reports of alpha diversities metrics represented by Chao1 and Simpson for each of the conditions among the bacterial and fungal (ITS and 18S) communities. Different letters indicate significant differences among the communities (pairwise test, $P < 0.05$).

| Community | Terroir | Season | Soil | Richness (Chao1) | Diversity (Simpson) |
|-----------|------------|--------|------|------------------|---------------------|
| Bacteria | Haut-Médoc | Spring | S | 1535 ± 30 abc | 0.9949 ± 0.0009 a |
| | | | AS | 1553 ± 24 abc | 0.9960 ± 0.0003 a |
| | | Autumn | S | 1345 ± 48 d | 0.9882 ± 0.0010 c |
| | | | AS | 1405 ± 33 cd | 0.9915 ± 0.0019 b |
| | Graves | Spring | S | 1672 ± 9 b | 0.9969 ± 0.0001 a |
| | | | AS | 1483 ± 153 acd | 0.9957 ± 0.0005 a |
| | | Autumn | S | 1858 ± 54 e | 0.9967 ± 0.0001 a |
| | | | AS | 1636 ± 30 ab | 0.9952 ± 0.0020 a |
| Fungi ITS | Haut-Médoc | Spring | S | 300 ± 12 b | 0.9359 ± 0.0185 a |
| | | | AS | 285 ± 13 b | 0.9224 ± 0.0041 a |
| | | Autumn | S | 269 ± 14 b | 0.9382 ± 0.0035 ad |
| | | | AS | 258 ± 2 b | 0.8918 ± 0.0118 c |
| | Graves | Spring | S | 425 ± 37 ac | 0.9613 ± 0.0016 bd |
| | | | AS | 386 ± 29 a | 0.9651 ± 0.0049 b |
| | | Autumn | S | 450 ± 8 c | 0.9615 ± 0.0035 bd |
| | | | AS | 388 ± 28 a | 0.9360 ± 0.0061 a |
| Fungi 18S | Haut-Médoc | Spring | S | 37 ± 1 cd | 0.5920 ± 0.0967 bc |
| | | | AS | 36 ± 4 cd | 0.5953 ± 0.0269 bc |
| | | Autumn | S | 39 ± 4 cd | 0.8550 ± 0.0278 a |
| | | | AS | 30 ± 1 d | 0.4928 ± 0.0426 c |
| | Graves | Spring | S | 61 ± 10 b | 0.8609 ± 0.0194 a |
| | | | AS | 58 ± 6 ab | 0.7091 ± 0.0961 ab |
| | | Autumn | S | 44 ± 3 acd | 0.7367 ± 0.0358 ab |
| | | | AS | 48 ± 6 abc | 0.7626 ± 0.0406 a |

Table S11 : Tests of multivariate homogeneity of group dispersions and vector fitting analysis based on the relationship between microbial communities and factors.

| | | Dispersion | | Vector fitting analysis | |
|-------------|---------|------------|--------------|-------------------------|--------------|
| | | F(1,46) | <i>P</i> | R ² | <i>P</i> |
| Bacteria | Soil | 0.753 | 0.380 | 0.087 | 0.023 |
| | Terroir | 0.076 | 0.780 | 0.041 | 0.142 |
| | Season | 6.120 | 0.021 | 0.022 | 0.358 |
| Fungi (ITS) | Soil | 0.001 | 0.968 | 0.048 | 0.135 |
| | Terroir | 210.04 | 0.001 | 0.125 | 0.004 |
| | Season | 0.621 | 0.431 | 0.006 | 0.750 |
| Fungi (18S) | Soil | 0.255 | 0.630 | 0.055 | 0.076 |
| | Terroir | 17.879 | 0.001 | 0.246 | 0.001 |
| | Season | 0.419 | 0.350 | 0.034 | 0.204 |

The R² values represent the proportion of variances explained by the ordination. P-values represent the significance of correlations based on a post hoc permutations test (n = 999).

Figure S4 : Co-occurrence of OTUs with the absence or presence of (A) bacterial, (B) fungal phyla, and (C) Glomeromycota divisions across soil status (AS for asymptomatic, S for symptomatic), terroir (Graves and Haut-Médoc appellations) and season (autumn and spring).

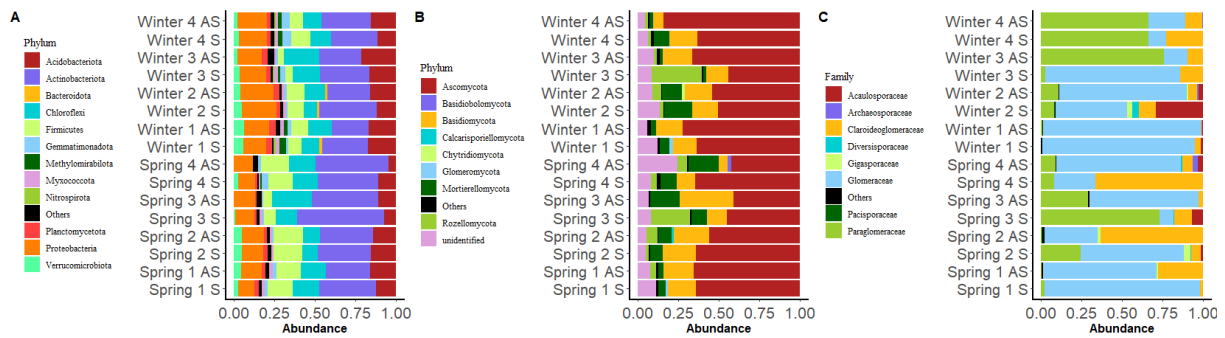


Figure S5 : Relative abundances of (A) bacterial, (B) fungal phyla, and (C) Glomeromycota identified families across the 16 conditions (n = 3) among the asymptomatic AS and symptomatic S areas, during autumn and spring seasons, and among the four vineyards (1, 2, 3, 4). The phyla individually representing less than 1% of the total communities were grouped in "Others".

Brief discussion

Symptomatic soils showed a depletion of bacteria, as well as lower enzymatic activities compared to asymptomatic soils suggesting a microbial dysbiosis. Soil physicochemical characteristics from the inter-row did not present excess or lack of nutrients and trace-elements. Considering this, bacterial and fungal communities' structure in these symptomatic areas could be correlated with the decline observed and unbalanced enzymatic activities. As reviewed, bulk soil from vineyard is known to display various microorganisms that would potentially interact with grapevine through rhizosphere and roots compartments. For instance, soil is the main source of various pathogenic fungi responsible of GTDs or beneficial AMF from *Glomeromycota* division.

The bulk soils of the studied declining vineyards with no explained causes displayed different microbial communities. The structure of bacterial and fungal communities was highly impacted by the spatial and temporal factors. Interestingly higher abundances of potentially beneficial bacteria, as well as pathogenic fungi, were found in the symptomatic areas.

This Chapter highlighted the functional and taxonomic microbial dysregulation in vineyards subjected to decline without any apparent pathogenic symptoms. These findings comfort the idea that the microbes from the bulk soil can be used as quality indicator of soil health, which is determinant for the future health of the plant. However, further analysis is required on the declining grapevine and its surrounding, as well as deep soil horizons, which is the purpose of the following chapter.

Chapter III

Holistic approach in a declining vineyard with a focus on the root and rhizosphere microorganisms

Preface

As previously mentioned, the bulk soil microbiome can provide insight into soil quality in vineyards. Endophytic microorganisms in grapevines originate mainly from the surrounding soil and pass through the rhizosphere to reach the root cortex and vascular tissue. Some of these endophytes are pathogenic and others are beneficial to the plant, acting as growth promoters by regulating nutrient uptake or hormonal responses, or by stimulating defense pathways. The objectives of this chapter were to characterize the effect of the decline on the grapevine, and to explore the taxonomy and functionality of the microorganisms associated with its rhizosphere and endosphere. In addition, deep horizons of previously described symptomatic and asymptomatic areas were explored for their potential impact on vine growth.

One vineyard from the four presented in chapter 2 was selected for further *in situ* analysis. The plant phenotyping, as well as the rhizosphere, bulk soil, and root samplings were performed one year after those made in chapter 2, and likewise compared the symptomatic area with the asymptomatic area. At first, the bulk soil was investigated once again to confirm bulk soil microbial dysregulation. Then, the mineral content of the leaves and the composition of the must were determined. Microbial profiles were dressed, based on cultivable-dependent methods, Eco-Plates measurements, q-PCR, and amplicons-based sequencing. In addition, one pit in each area was generated to visualize the deepest horizons and explore their physicochemical and microbial parameters.

The sequencing data were obtained very recently (early February 2022), and the Galaxy platform, which is the instance used for sequence preprocessing, has updated its version. This update does not currently allow the taxonomic affiliation of the Glomeromycota division using the MaarJAM database and will be corrected by the time of the thesis defense. The preliminary results are presented in the following draft of a research article entitled “**Grapevine decline is affiliated to soil and root microbiome dysbiosis**”. This article is planned to be submitted to BMC Plant Biology subsequently to further analysis and co-authors corrections.

Grapevine decline is affiliated to soil and root microbiome dysbiosis

Romain Darriaut¹, Guilherme Martins^{2,3}, Elisa Marguerit¹, Isabelle Masneuf-Pomarède^{2,3}, Virginie Lauvergeat^{1*}

¹ EGFV, Univ. Bordeaux, Bordeaux Sciences Agro, INRAE, ISVV, F-33882, Villenave d'Ornon, France

² Université de Bordeaux, INRAE, Bordeaux INP, UR Œnologie EA 4577, USC 1366, ISVV, 33140 Villenave d'Ornon, France

³ Bordeaux Sciences Agro, 1 cours du Général de Gaulle, 33170 Gradignan, France

*corresponding author: virginie.lauvergeat@inrae.fr

Abstract

Vineyard decline is characterized by a decrease of plant growth and berry yield that can lead to vine death. Some of these declines are not explainable by pathogen infection or unbalance of soil nutrients. A previous analysis in four vineyards showed a disruption in the taxonomy and functionality of the soil microbiome in symptomatic (S) declining areas compared to healthy ones (AS, asymptomatic). As soil is a microbial reservoir in which grapevine uptakes its associated microbiome through roots compartment, its diversity is considered as interesting biological indicator of plant health. The objective of this work was to pursue the analysis in one of the previously studied vineyards by exploring the bacterial and fungal microbiome of the root endosphere, rhizosphere, and bulk soils of vines grown in the S and AS zones. Declining vines had poor berry quality and lower petiole mineral content than those in AS zones. Profiles of the deeper soil horizons could not explain this observed decline, but the bulk soil from zone S showed a dysregulation of enzyme and microbial activities. Bacterial richness and fungal diversity were lower in symptomatic soils and roots, respectively, highlighting the root functionality of acting as a selective barrier for microbes. However, higher microbial activities, as well as enrichment of potentially beneficial bacterial and fungal pathogens were found in the S soils. These results could not explain the observed decline but revealed stressed vines with active microbial profiles in the belowground compartments, highlighting the interest of exploring the functional microbiota of plants under stressed conditions.

Keywords: vineyard decline, microbial dysregulation, root endosphere, *Vitis vinifera* microbiota

Introduction

Plant decline is defined as the decrease of the plant physiological processes leading to the loss of vigor and sometimes its death. In perennial crops, this decline refers to an economic loss related to productivity downsizing, and is mostly affiliated to pathogenic causes (Chuche *et al.*, 2018; Cox *et al.*, 2005). Pathogens predisposition to invade crops is facilitated with climate change phenomenon and undoubtedly with the soil resilience loss (Chakraborty *et al.*, 2008; Cienciala *et al.*, 2017). The abiotic uncontrolled stresses such as temperature variations, salinity, and water deficiencies disturb the ecosystemic processes and establish optimal growth conditions for opportunistic and sometimes pathogenic microorganisms (Fraser and Brown, 2017; Marçais and Bréda, 2006; Pandey and Senthil-Kumar, 2019).

Among the telluric microorganisms, fungi and bacteria are the most dominant ones, and lots of descriptive analyses have been made in diverse habitats and under different crops (Karimi *et al.*, 2020b). In addition, soil microorganisms trigger all the ecosystemic processes such as nutrient cycling, litter decomposition, soil remediation, or phytopathogen biocontrol (Fierer, 2017). To this end, a good equilibrium between keystone taxa has to be settled for optimal soil biogeochemical services (Banerjee *et al.*, 2018). Agricultural practices, besides climatic conditions, are one of the most important drivers for microbial communities and can therefore easily promote the dysregulation of those services, ending up in decline features (Banerjee *et al.*, 2019; Lin *et al.*, 2019). For instance, the incidence of grapevine trunk diseases (GTDs), which are the most studied fungal pathogens related to grapevine decline, are mainly influenced by soil management practices and edaphic parameters (Gramaje *et al.*, 2018). Vineyards are assumed to display worldwide one of the poorest soils in term of biodiversity across the agricultural systems due to the intensive uses of chemical treatments (Karimi *et al.*, 2019).

Soil microbiome shapes the plant-associated microbiome and has a strong influence on its fitness (Wei *et al.*, 2019). There is evidence in the impact of taxonomic and functional diversity on plant health and productivity (Chen *et al.*, 2020; Trivedi *et al.*, 2020). Bacterial and fungal microbiome in vineyard soil have been well investigated using 16S rRNA and ITS sequencing, respectively, on bulk, rhizosphere, and roots compartments (Berlanas *et al.*, 2019; D'Amico *et al.*, 2018; Marasco *et al.*, 2018; Zarraonaindia and Gilbert, 2015), but few studies showed a direct correlation with grapevine health. Saccà *et al.* (2019) unraveled the rhizosphere microbiome of grapevine infected by Esca complex which displays specific and well-documented symptoms, but few research has been made on unexplained grapevine decline.

A first study on four French vineyards with areas of local decline (low vigor and high mortality) whose causes were not clearly identified focused on the analysis of the composition and microbial functioning of the inter-row soil (Darriaut *et al.*, 2021 ; Darriaut *et al.*, in preparation). Bulk soils in the symptomatic (S) area of all the studied plots showed a lower presence and activity of bacteria and fungi and a higher abundance of putative pathogenic fungi and PGPB bacteria than in the asymptomatic (AS) area.

To go further and investigate how does the soil microbiome influence the rhizosphere and root microbial composition, as well as the plant responses, a deeper and holistic analysis of both S and AS areas located within a same vineyard plot has been done by several approaches ranging from yield estimation and berry components analysis to soil and studies of roots microbial communities using cultivable and amplicons-based sequencing.

Material and methods

Studied site

The studied vineyard has been depicted in Darriaut *et al.*, 2021 (plot number 2). This plot is located in Graves terroir, GPS coordinates 44°45'13.0"N 0°33'24.8"W. It has been established in 2008 with *Vitis vinifera* Cabernet Sauvignon scion grafted on *V. riparia* Gloire de Montpellier rootstock (CS/RGM) vines. Two areas have been defined showing significant differences in vigour and number of missing vines. The symptomatic area (S) showed a lower vigour and higher number of recently replanted vines than the so-called asymptomatic area (AS). Vines were revealed negative in ELISA tests for grapevine fan leaf virus (GFLV) and arabis mosaic virus (ArMV) in S and AS soils. These two areas are only 20 m apart in the same plot.

Plant phenotyping

Young, recently replanted vines in both areas were not considered in this study, only mature (>10 years old) grapevines were phenotyped and sampled. All the primary and secondary bunches of grapes of 28 vines spread on four rows for both AS and S areas were collected and weighted. The weight of a bunch of grapes was calculated as the weight of the bunches divided by the number of bunches for each vine.

Approximately 10 berries of the same size were collected per vine from all over the bunches by hand for a total of 100 berries in triplicate for each of the S and AS areas. The sampling was done in bags with vertical filter (BagFilter® 400 ml, Interscience). These 100 berries were weighted, and the average mass of a berry was calculated. The berries juice was extracted using

a crusher (BagMixer® 400 W, Interscience), collected in 50 ml tubes, and then centrifuged for 10 minutes at 20089 g. The juice components were then analyzed using a WineScan™ Auto based on Fourier Transform Infrared Spectroscopy (FTIR; FOSS Analytical, Hillerød) (Destrac *et al.*, 2015; Suter *et al.*, 2021). Sugar content represented by total soluble solids (°Bx) was measured with a digital refractometer.

Vine water status was determined from the juice extracted from the berries by measuring the $\delta^{13}\text{C}$, which refers to the ratio of carbon isotopes $^{13}\text{C}/^{12}\text{C}$. The $\delta^{13}\text{C}$ (in ‰) varies between -20 ‰, considered as severe water stress, and -28 ‰, considered as no water stress (van Leeuwen *et al.*, 2001).

For S and AS soils, approximately 60 leaves per area were randomly sampled, dried at 40°C for 48h. Limbs were then separated from the petioles, and both were sent for Mg, Ca, K, C, P, and N contents estimations to Auréa Agrosociences (Blanquefort, France).

Soil and roots sampling

Three bulk soil samples (10 to 30 cm depth), spaced one meter apart, were collected in each area using an auger (10 cm x 25 cm).

Whitish juvenile roots, with rhizosphere soil attached to them, were collected from 5 old grapevine plants (> 3 years) in each S and AS areas. Rhizosphere was separated from roots using centrifugation at 2,000 g subsequently to 10s of vortex and repeated twice. At this stage, roots were separated from the rhizosphere and subdivided into two groups with one for the estimation of mycorrhizal colonization, and the other for microbial endosphere analysis by amplicon sequencing. Regarding the latter group, roots were surface sterilized by adding 3% of sodium hypochlorite for 1 minute, followed by 3% H_2O_2 for 1 minute. The roots were washed three times with sterile water and stored at -80°C until subsequent DNA extraction.

Rhizosphere and bulk soil collected were also subdivided into two groups with one for cultivable analysis and the other for quantitative-PCR (q-PCR) and amplicon sequencing. The latter group was lyophilized for 48 hours using Christ Alpha® 1-4 (Bioblock Scientific) and stored at -80°C prior to DNA extraction.

Physicochemical parameters from bulk soil

Five hundred grams of each bulk soil sample were dried at 40°C for 72 hours, sieved (< 2 mm), and sent to INRAe LAS (62000, Arras) for physicochemical measurements including basic soil properties (*i.e.*, grain size, pH, CEC, C and N contents), micro and macronutrients (organic matter, P, Ca, Mg, K, Na, NO_3^- , $\text{NH}_3\text{-N}$), and trace elements (Cu, Fe, Mn, Zn).

Potential metabolic diversity (PMD) and quantification of microorganisms in rhizosphere and bulk soil, coupled to mycorrhizal root colonization

PMD and quantification of cultivable bacteria and fungi from fresh rhizosphere and bulk soils were assessed using the methods described in Darriaut *et al.* (2021). Briefly, PMD was evaluated using Biolog Eco-Plates™ system (Biolog Inc., CA), by measuring the consumptions of thirty-one different substrates (*i.e.*, amines, amino acids, carbohydrates, carboxylic acids, phenolic compounds, and polymers) by present microorganisms, every 24 hours for 4 days.

In parallel to PMD evaluation, soils dilutions were plated on R2A medium amended with 25 mg.l⁻¹ of nystatin for the quantification of cultivable bacterial population, while the fungal populations were quantified on PDA medium supplemented with 500 mg.l⁻¹ of gentamicin and 50 mg.l⁻¹ of chloramphenicol.

From the subgroup of fresh sampled roots that were not surface sterilized, thirty subsamples of fresh roots were used for the estimation of their colonization by mycorrhizal fungi. Those roots were stained by the ink-KOH-H₂O₂ method modified from (Phillips and Hayman, 1970). Briefly, fresh roots were rinsed in sterile water and incubated in 10% KOH for 30 minutes at 95°C. Immediately after the incubation, to the KOH-roots mixture was incorporated 3% of H₂O₂. The solution was discarded, and the roots were rinsed twice with sterile water and stained, in 5% India ink (Super Black™) solution with 8% acetic acid, by incubating at 90°C for 5 minutes. Roots were destained at ambient temperature with 8% acetic acid for 15 minutes before washed with sterile water. Stained roots were then placed on glass slides with pure glycerol and observed with a light microscope LEICA DM750 equipped with a LEICA ICC50 W camera. Subsequently, arbuscular mycorrhizal colonization was estimated with Trouvelot *et al.* (1986) method and MycoCalc program (www2.dijon.inra.fr/mychintec/MycoCalc-prg/download.html).

Enzymatic assays

Following enzymatic assays were done as described in Darriaut *et al.* (2021), with fresh, homogenized, and sieved bulk soil sampled from each site, coming from five subsamples. One gram of soil for each site was dried for the final calculus of enzymatic activities.

DNA extraction

DNA was extracted from 250 mg of the lyophilized soils using the DNeasy PowerSoil Pro kit (Qiagen) using the manufacturer recommendations with an additional C5 washing step. Soil

DNA extraction was initiated with a FastPrep device set on power 4 m.s⁻¹ for 30 seconds and performed twice with a vortex step between each run.

Regarding the root samples, 100 mg of powder were obtained by freezing in liquid nitrogen and by grinding using bead beating in steel containers using Retsch MM400. DNA was extracted from the powder using DNeasy® Plant Mini kit, following the manufacturer's instructions.

DNA samples were quantified on Qubit® 3.0 fluorometer (Thermo Fisher Scientific) using Qubit™ dsDNA HS Assay kit, and their quality was checked with a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific). DNA was then stored at -20°C until further use.

Quantitative-PCR (q-PCR) amplification of bacterial, archaeal, and fungal genes

Quantitative PCR analyses were performed on the DNA extracted from the soil samples using three primers pairs to quantify bacterial and archaeal 16S rRNA genes as well as the fungal and mycorrhizal 18S rRNA genes (**Table S12**), according to Darriaut *et al.* (2021). These q-PCR were based on absolute quantification using standard curves obtained from amplicons subcloned into pGEM®-T easy vector system (Promega). The efficiencies of the q-PCR were 80% to 99% ($R^2 > 0.99$).

16S rRNA gene and ITS sequencing pre-processing

The DNA samples were randomized across plates and amplified using the universal primers listed in (**Table S12**), specific to either the bacterial and archaeal 16S rRNA gene, or the fungal ITS1 region. All the primers included the specific overhang Illumina adapters used for the amplicon library construction. Each 25 µl reaction contained 12.5 µl of 5X GoTaq® Reaction Buffers (Promega, France), 8 µl of Nuclease-free water, 1 µl of each primer (10 µM), 2.5 µl of DNA template (5 ng/µl), and 0.625 u of GoTaq® G2 DNA Polymerase (Promega, France). PCR amplifications were performed in triplicate for each condition. The cycling conditions of 16S rRNA gene and ITS amplifications were initiated with a denaturation at 95°C for 5 min, followed by 25 and 30 cycles, respectively, with denaturation at 95°C for 30s, annealing step at 55°C for 30s, followed by an extension step at 72°C for 30s and 45s, respectively. Further steps were processed at the PGTB sequencing facility (Genome Transcriptome Facility of Bordeaux, Pierroton, France) using a V2 with 2 × 250 nucleotide paired reads protocol. The PCR products were purified with platform specific SPRI magnetic beads (1X ratio) and quantified using Quant-iT™ dsDNA Assay kit (ThermoFisher, France). MID and Illumina sequencing adapters were added. Libraries were pooled in equimolar amounts using a Hamilton

Microlab STAR robot and sequenced on an Illumina MiSeq platform using the MiSeq Reagent Kit v2 (2×250 bp). Obtained sequences were demultiplexed with index search at the PGTB facility.

Profiles of deep soil horizons

Pedological profiles were performed in both S and AS areas using a mini excavator, distinguishing three horizons in S area and four horizons in AS area (**Figure 30**). Samples for each horizon was done in triplicate and pooled for analysis, consisting in physicochemical parameters assessment, enzymatic assays, DNA extraction, cultivable bacteria and fungi coupled to Eco-Plates measurements and q-PCR listed above.

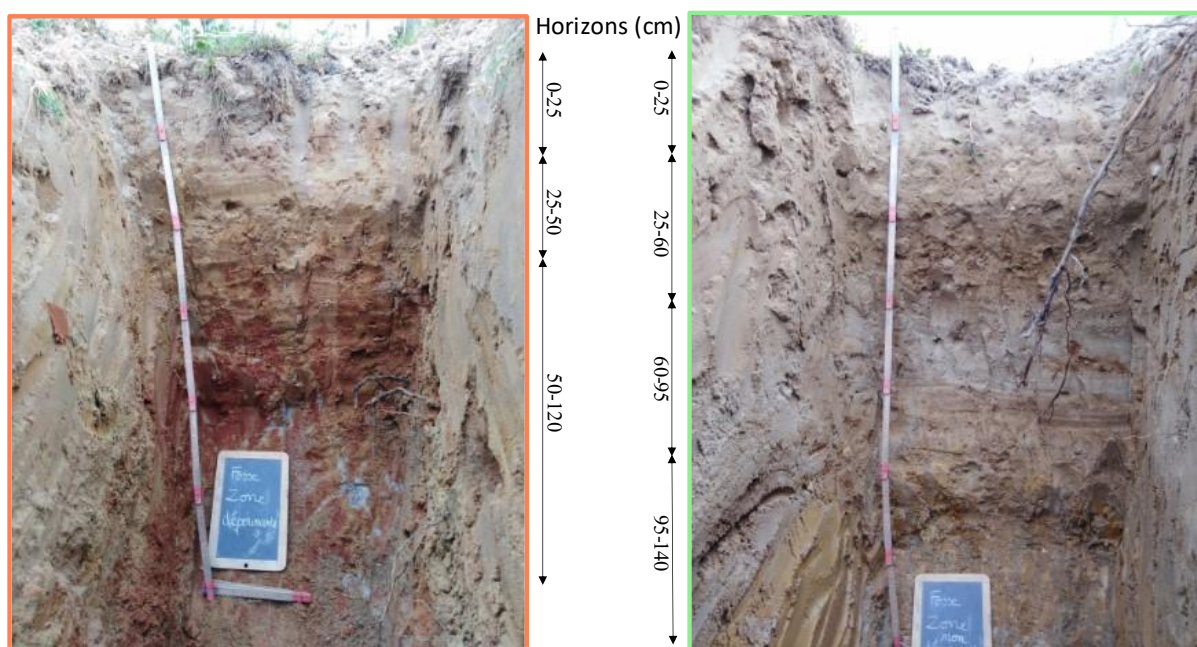


Figure 30 : Soil profiles generated in symptomatic (orange) and asymptomatic (green) areas across the different horizons.

Bioinformatics analysis and statistics

All analysis and graphs were performed on R (R-4.1.2) using RStudio (2021.9.1.372). Figures were generated with *ggplot2* (3.3.5) and *ggthemes* (4.2.4) packages and arranged using *ggpubr* (version 0.4.0) (Arnold, 2021; Kassambara, 2020; Wickham and Chang, 2008). Two-way Analysis of Variance (ANOVA) with soil status (AS or S) and depth of horizons in the pit (four horizons in AS and three horizons in S areas) factors were performed on enzymatic activities, cultivable, q-PCR, and Eco-Plates measurements. Residuals were checked for their independency, normality, and variance homogeneity with the Durbin Watson, Shapiro-Wilk, and Bartlett tests, respectively. When assumptions for parametric tests were not respected, a

multiple pairwise comparison using Wilcoxon test was performed subsequently to Kruskal–Wallis test using the *multcomp* (1.4-18) package (Hothorn *et al.*, 2008). Principal Component Analysis (PCA) was performed using *FactoMineR* (2.4) and *missMDA* (1.18) (Josse & Husson, 2016; Le *et al.*, 2008). Area under curve (AUC) of average color well development (AWCD) which gives better insights for curve dynamics, was calculated with the trapezoidal method for each soil using “caTools” (1.18.2) packaging.

Regarding the amplicons sequencing, sequences quality obtained were preliminarily checked with FastQC v.0.11.8 (Andrews, 2010). Sequences were quality filtered, trimmed, denoised, and clustered into Operational Taxonomy Units (OTUs) using FROGS pipeline from Galaxy instance (Escudié *et al.*, 2018). Briefly, raw forward and reverse reads for each sample were assembled into paired-ended reads with a minimum overlapping of 50 nucleotides and 0.1 mismatch using the VSEARCH tool (Rognes *et al.*, 2016). Primers were removed using Cutadapt (Martin, 2011), chimeras were detected and removed with UCHIME (Edgar *et al.*, 2011), clustering was performed using SWARM (Mahé *et al.*, 2014) within FROGS pipeline. Minimum proportion of sequences abundance to keep OTUs was set to 5e-05, and singletons suppression was done with phiX contaminant databank. Taxonomic assignments of 16S rRNA and ITS-based OTUs were performed against silva138.1 (16S pintail100) (Quast *et al.*, 2012) and Unite8.2 (Nilsson *et al.*, 2019), respectively, using RDPClassifier from Galaxy.

Dataset were gathered and analyzed through *phyloseq* package (1.38.0) (McMurdie and Holmes, 2013). Taxa related to mitochondrial and chloroplast OTUs were removed. Shared OTUs were visualized with Venn diagrams that were generated with *VennDiagram* (1.7.1) (Chen, 2021).

Richness and α -diversity metrics, represented by Chao1, Shannon’s diversity, and Bray-Curtis dissimilarity, respectively, were calculated through *phyloseq* using “estimate_richness” function. In order to test for significant differences between the means of alpha diversity metrics by conditions, pairwise comparisons were used, based on either t or Wilcoxon test, subsequently to homogeneity and normalization verifications using Levene and Shapiro tests. Nonmetric multidimensional scaling (NMDS) was used to ordinate samples in two-dimensional space based on Bray-Curtis distance using ordinate function from *phyloseq* with “NMDS” method. Linear models and PERMANOVA, for richness and diversities metrics, were demonstrated using the formula: variable ~ Soil status \times Compartment. Type-II Analysis Of Variances (ANOVAs) were performed using *car* (3.0-12) on Chao1 and Shannon’s diversity metrics while PERMANOVAs were assessed on Bray-Curtis dissimilarity using *vegan* package (2.5-7) with 999 permutations. Functions “ggeffects” and “ggdiffbox” from *MicrobiotaProcess* (1.2.2)

were used to discriminate significantly different taxa across conditions (Xu and Yu, 2021). This process was set with Kruskal ($\alpha = 0.05$) test based on linear discriminant analysis (LDA) effect size (LEfSe) and Wilcox ($\alpha = 0.05$), corrected with False Discovery Rate (FDR).

Results

The observed decline was associated with the decrease in vine vigor and the difference in must composition

The biplot analysis of petiole (**Figure 31.A**) and must (**Figure 31.C**) composition revealed distinct profiles across symptomatic S and asymptomatic AS areas, with dimensions accounting for 83.6% and 94.5% of total variance, respectively. Regarding limb composition, AS profile was overlapping S profile (**Figure 31.B**), with dimensions accounting for 97.4%. In the biplot PCA of petiole, AS samples were found in the positive side of Dim1, which correlated with Phosphorus (P), magnesium (Mg), calcium (Ca), potassium (K), and sodium (Na) while nitrogen (N) was found in the negative side of Dim1 where all the S samples were represented. Significantly lower amount of P, Ca, and Mg and higher content of N was found in petioles from S area, whereas no significant differences were found in limbs (**Table S13**).

In the must composition, alpha-amino N (NOPA), assimilable N, ammoniacal N (N-NH₃), total acidity, malic acid, tartaric acid, $\delta^{13}\text{C}$ were correlated with the positive side of Dim1 where the asymptomatic samples were found while K, pH, volatile acidity, reducing sugars, brix content, and degree in correlation with negative side of Dim1, harboring all the symptomatic samples (**Figure 31.C**). Similarly, significantly less titratable acidity, malic acid, tartaric acid, and content in assimilable N, NOPA, N-NH₃ and significantly higher must pH, volatile acidity, and potassium in must composition from S area compared to the AS one (**Table S13**). The vine water status represented here by $\delta^{13}\text{C}$ was not reflecting severe water stresses in both symptomatic and asymptomatic areas.

During harvest time in autumn, the yield, represented here by the total biomass of primary and secondary bunches divided by the number of bunches per vine, was significantly higher in AS area compared to S area, as well as the average mass of the berries (**Figure 31.D**).

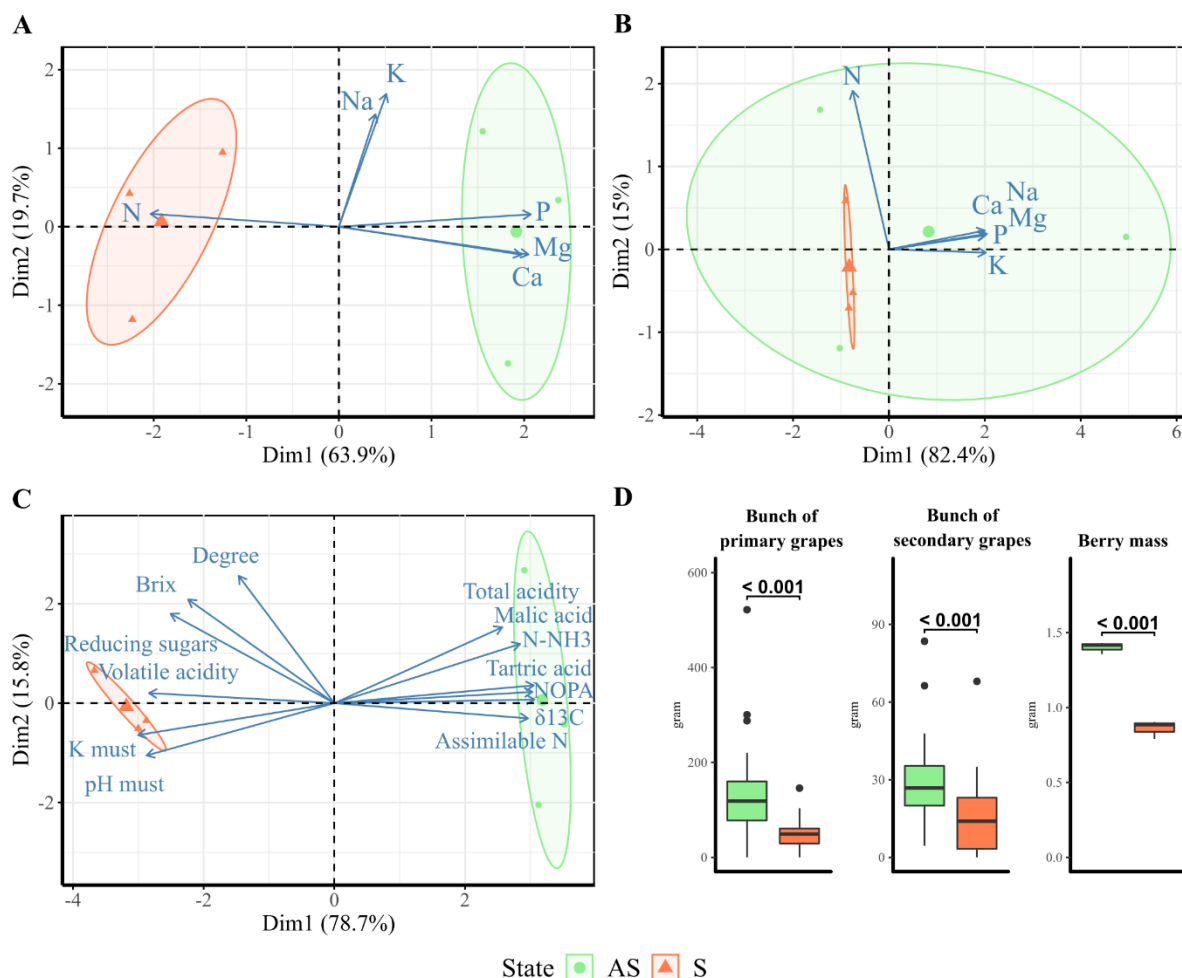


Figure 31 : Ordination biplot PCA for (A) petiole, (B) limb, and (C) must compositions across symptomatic (orange, S) and asymptomatic (green, AS) areas. Standard error ellipses show 95 % confidence areas. (D) Yield is presented as the mass of primary and secondary bunches per plant (n = 28) and the berry mass (n = 100). P values were calculated using t tests or Wilcoxon tests depending of the parametric assumption.

Bulk soils presented quite similar physicochemical properties but contrasted enzymatic and microbial profiles

The biplot PCA for physicochemical parameters revealed an overlap of confidence interval of the S and AS profiles, with dimensions accounting for 87.2% of total variance (**Figure 32.A**). Nitrogen, calcium, NO_3^- , and manganese content were significantly more important whereas inversely, carbon / nitrogen ratio was significantly less important in AS compared to S bulk soil (**Table S14**).

Regarding the Eco-Plates measurements, the biplot analysis of PCA, with Dim1 and Dim 2 accounting for 86.8% of total variance, revealed a smaller overlap with distinct S and AS profiles (**Figure 32.B**). Level of cultivable bacterial and fungal populations were significantly higher in AS compared to S bulk soil (**Table S15**), while no significant differences were found in Eco-Plates measurements except for AUC that was significantly more important in S than

AS area. However, enzymatic activities involved in nitrogen, carbon, and phosphorus cycling (*i.e.*, arylamidase, β -glucosidase, and alkaline phosphatase, respectively) were significantly more important in AS compared to S bulk soil (**Figure 32.C**). Similarly, total molecular biomass, and copies of fungal 18S genes, as well as bacterial and archaeal 16S genes, were significantly higher in AS compared to S bulk soils (**Figure 32.D**).

Regarding the amplicons sequencing, a total of 1,094,170 16S and 1,723,461 ITS raw sequences were generated. Subsequently to chimera removal, paired-end sequences of 16S rRNA gene and ITS were clustered into 1,566 and 961 operational taxonomic units (OTUs), respectively. Around 62% and 53% of the bacterial and fungal OTUs, respectively, were shared between S and AS conditions (**Figure 32.E**). Richness, represented here by the Chao1 metrics, was similar for fungal communities but significantly higher in AS compared to S bulk soil for the 16S OTUs (**Figure 32.F**). On other hand, diversity represented here by Simpson's index was similar for bacterial communities while the asymptomatic bulk soil presented higher fungal diversity than the symptomatic one.

Actinobacteriota (29%), *Proteobacteria* (22%), *Acidobacteria* (15%), *Firmicutes* (11%), *Chloforexi* (9%), *Verrucomicrobiota* (5%), *Plancomycetota* (3%), *Gemmatimonodata* (2%), *Bacteroidota* (1%), *Myxococcota* (1%), *Nitrospirota* (1%), were the most abundant bacterial phyla of bacterial communities (**Figure 32.G**) while "Others" group was composed of *Dependentiae*, *Desulfobacterota*, *GAL 15*, *Latescibacterota*, *Methylomirabilota*, *RCP2-25*, *WPS-2* phyla. Regarding the ITS-based sequences, *Ascomycota* (65%), *Basidiomycota* (17%), *Mortierellomycota* (14%), *Rozellomycota* (5%), and *Chytridiomycota* (1%) were the predominant phyla, while "Others" phyla were belonging to *Blastocladiomycota*, *Calcarisporiellomycota*, *Entorrhizomycota*, *Glomeromycota*, *Kickxellomycota*, *Olpidiomycota*, and *Zoopagomycota*.

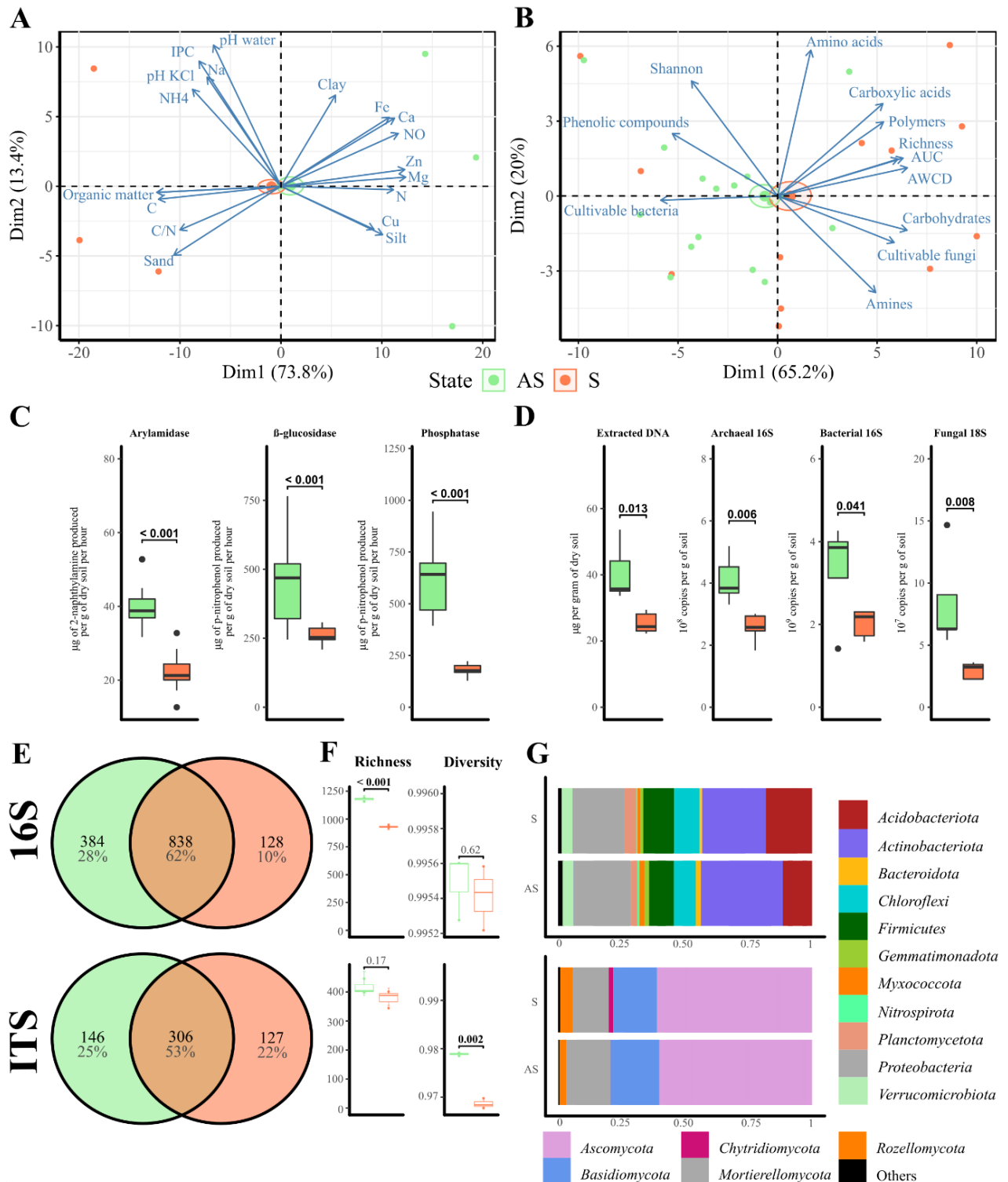


Figure 32 : Bulk soil profile in symptomatic (orange, S) and asymptomatic (green, AS) areas. Biplot PCA of (A) physicochemical parameters (n = 3), and (B) Eco-Plates measurements represented by Shannon's index, AWCD, AUC, functional richness, and family compounds consumed (i.e., amines, amino acids, carbohydrates, carboxylic acids, phenolic compounds, and polymers) coupled to bacterial and fungal level of cultivable populations. Standard error ellipses show 95 % confidence areas. (C) Enzymatic activities represented by arylamidase, β -glucosidase, and phosphatase alkaline, and (D) q-PCR measurements for archaeal and bacterial 16S and 18S genes (n = 5). (E) Shared OTUs represented by Venn diagram, and (F) α -diversity metrics (richness = Chao1, diversity = Simpson), as well as (G) relative abundance of phyla. Significant differences were calculated with t or Wilcoxon tests, depending on the normality hypothesis.

Rhizosphere and root associated microorganisms

The biplot PCA from rhizosphere samples, accounting for 97.3% of total variance, revealed overlapping profiles between S and AS areas, with Eco-Plates measurements explaining the symptomatic features (**Figure 33.A**). Interestingly, the global AWCD represented by AUC, was significantly more important in S rhizosphere than AS, specifically with significantly more carbohydrates, carboxylic acids, phenolic compounds, and polymers consumed after 96 hours of Eco-Plates incubation (**Table S15**). Similarly, significantly higher functional richness and Shannon evenness index was found in S rhizosphere, while no significant difference in level of cultivable bacterial and fungal populations was detected. Regarding the q-PCR assays, only bacterial 16S genes was significantly more important in AS rhizosphere compared to S one (**Figure 33.B**). In roots, no difference was found between S and AS areas regarding the mycorrhizal frequency or intensity (**Figure S6**).

In regard to amplicons-based sequencing, 11.2% of the total bacterial OTUs were shared between the four conditions (*i.e.*, symptomatic and asymptomatic root, and rhizosphere) (**Figure 33.C**). In the rhizosphere, 62% of the assigned bacterial sequences were common to S and AS, while only 0.2% were shared between S and AS in the root samples. Higher amount of OTUs were exclusive to AS rhizosphere (12.4%) compared to the symptomatic condition (3.7%), while only 0.3% and 0.4% were unique to AS and S root samples, respectively. Regarding the ITS sequences, 10.7% were common to rhizosphere and root endosphere, regardless of soil status. Between S and AS areas, 36.6% and 2.4% were shared in the rhizosphere and root samples, respectively.

Both bacterial and fungal richness were similar between AS and S conditions in root endosphere samples, while significantly higher richness was found in asymptomatic area in the rhizosphere compartment (**Figure 33.D**). The diversity was not significantly different between the S and AS conditions across the root and rhizosphere samples.

The observed phyla in roots and rhizosphere were the same that were observed in bulk soil (**Figure 33.E**; **Figure 32.G**).

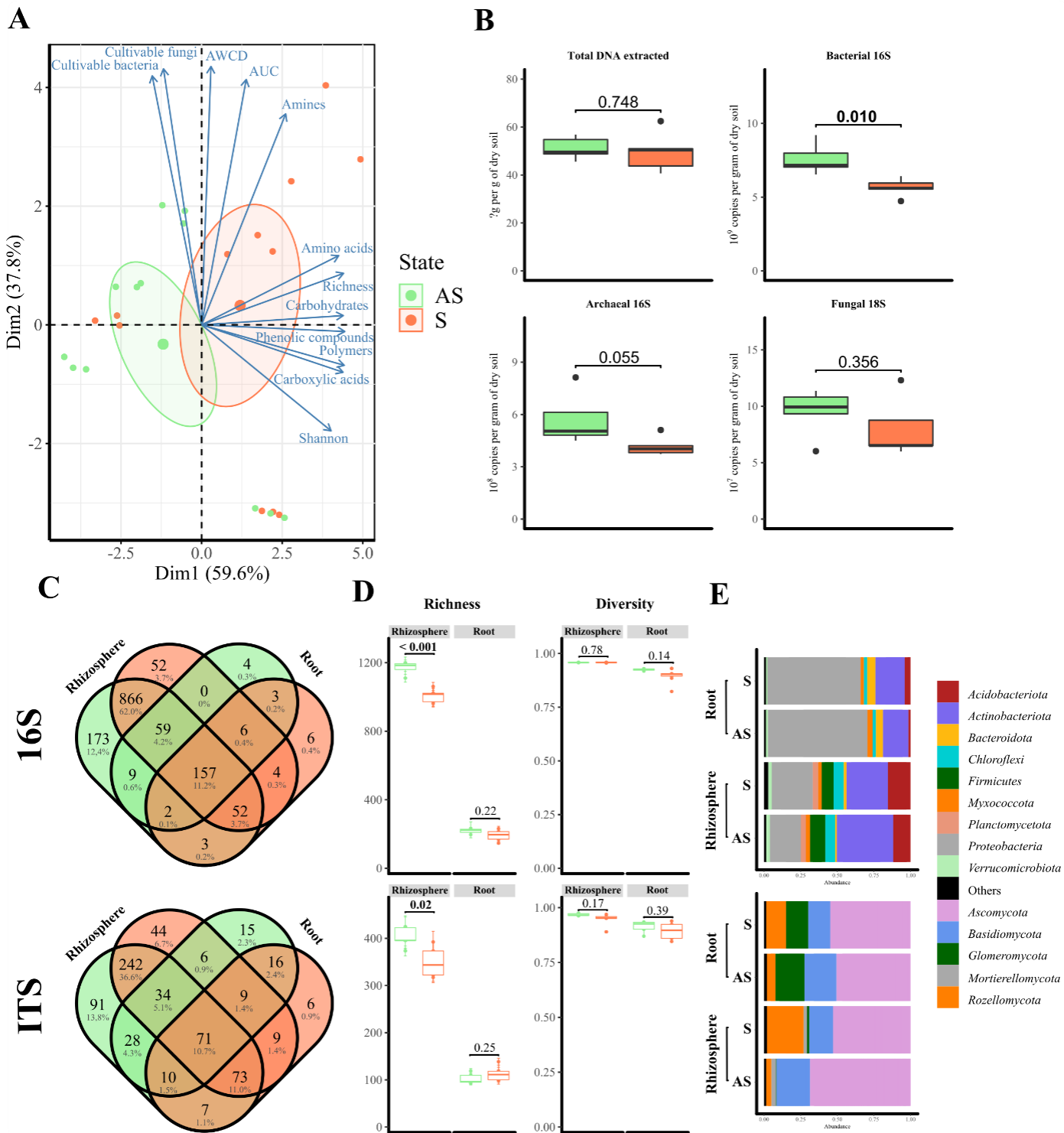


Figure 33 : Rhizosphere profile and composition of the root endosphere microbiome in symptomatic (orange, S) and asymptomatic (green, AS) areas. Ordination biplot analysis of PCA for (A) Eco-Plates measurements ($n = 3$) represented by Shannon's index, AWCD, AUC, functional richness, and family compounds consumed coupled to bacterial and fungal level of cultivable populations ($n = 5$). (B) Total DNA extracted with associated q-PCR measurements for archaeal and bacterial 16S and 18S genes ($n = 5$). (C) Shared OTUs represented by Venn diagram for 16S and ITS sequencing. (D) α -diversity of both root and rhizosphere associated to (E) their phyla.

The effect of compartmentalization on microbial communities

Higher number of 16S and 18S genes copies were found in rhizosphere compared to bulk soil (**Figure S8.A**), with the same trend observed for the level of cultivable bacteria and fungi, as well as Eco-Plates metrics (**Figure S8.B**).

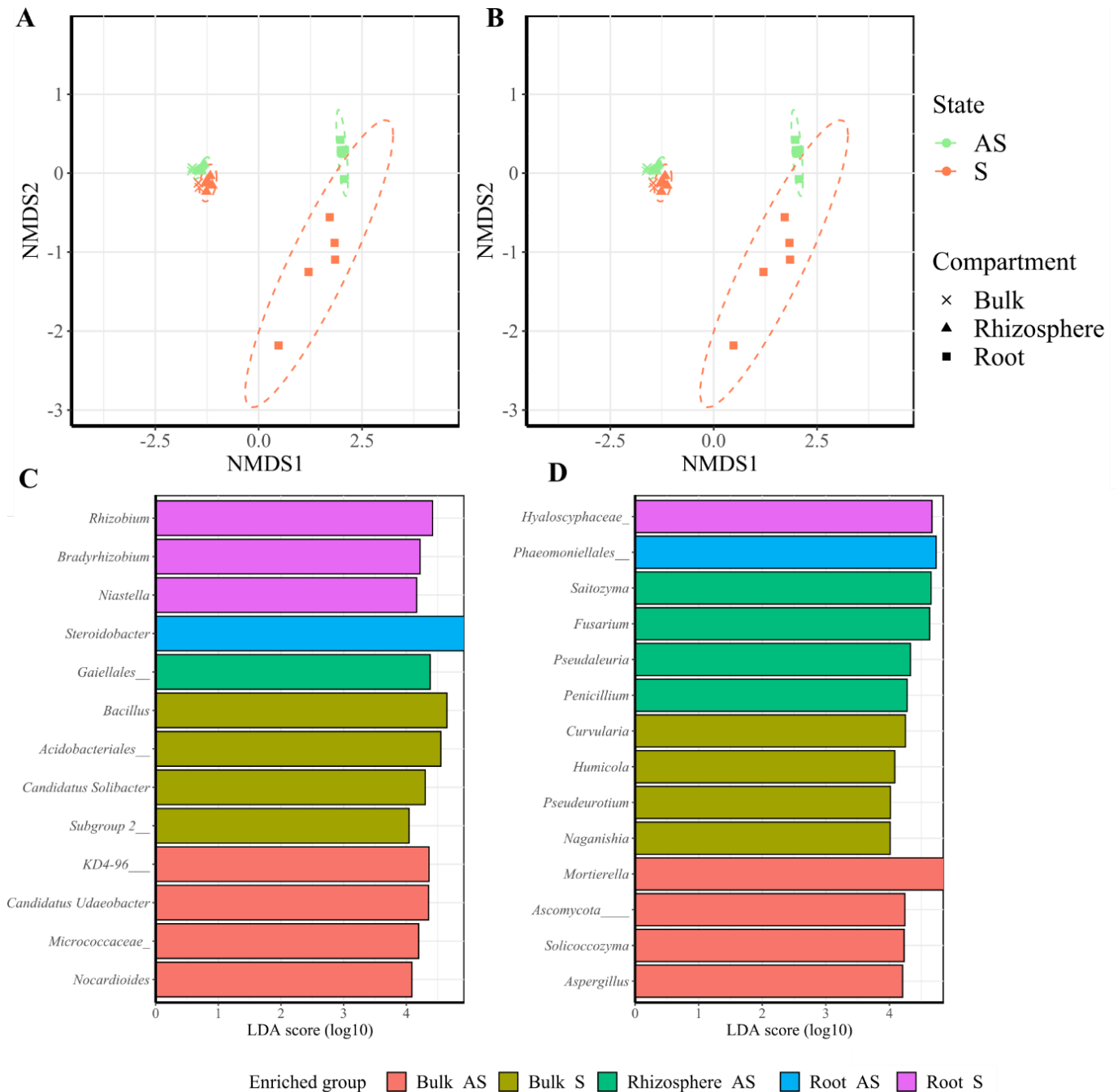


Figure 34: Compartmentalization effect on microbial communities. NMDS plot ordination of (A) bacterial, and (B) fungal communities among the bulk (cross), rhizosphere (triangle), and root endosphere (square) across symptomatic S (orange) and asymptomatic AS (light green) soils. Dashed lines represent 95% confidence ellipses. LEfSe analysis (LDA > 4) of enriched genera among the soil status × compartment conditions.

In regard to amplicons sequencing, compartmentalization and soil status had significant effect on both α - and β - diversities (**Table S18**). Soil bacterial and fungal richness, as well as diversity, were significantly higher than that of root endosphere (**Table S19**). In addition, NMDS based on Bray-Curtis dissimilarities demonstrated that the compartment was the main factor of difference in β -diversity (**Table S18, Figure 34.A.B**).

The LEfSe ($P < 0.05$, FDR, LDA > 4) revealed 9 enriched bacterial genera in bulk and rhizosphere soils mainly belonging to *Actinobacteriota* (*i.e.*, unidentified genera from *Gaiellales*, *Micrococcaceae*, *Nocardioides*) and *Acidobacteriota* (*i.e.*, unidentified genera from *Acidobacteriales*, *Candidatus solibacter*) phyla, while 4 enriched bacterial genera were found in root endosphere which belonged to the *Proteobacteria* (*i.e.*, *Rhizobium*, *Bradyrhizobium*, and *Steroidobacter*) and *Bacteroidota* (*i.e.*, *Niastella*) phyla (**Figure 34.C**). Twelve fungal genera were enriched in soil compartments principally pertaining to the *Ascomycota* (*i.e.*, *Fusarium*, *Pseudaleuria*, *Penicillium*, *Curvularia*, *Humicola*, *Pseudeurotium*, and *Aspergillus*) and *Basidiomycota* (*i.e.*, *Saitozyma*, *Naganishia*, and *Solicoccozyma*) phyla. No taxa were enriched in the rhizosphere from S area.

Moreover, fungal pathogens associated to grapevine diseases were detected in each compartment but with different abundance (**Figure S9**). *Curvularia* was more abundant in rhizosphere and bulk soil, while *Cadophora* and *Botrytis* were more abundant in root endosphere. Among these genera, *Botrytis caroliniana*, *Curvularia lunata*, *Curvularia spicifera*, *Curvularia portulacae*, *Curvularia inaequalis*, *Diaporthe columnaris*, *Ilyonectria destructans*, *Cadophora luteo-olivacea*, *Diplodia intermedia*, *Neonectria lugdunensis*, and *Phaeomoniella chlamydospora* were detected.

The deep horizons show different characteristics between the S and AS areas

A pit was dug in both S and AS area, and visually, there was no justified cessation of growth explained by the observations on these horizons in S area. There was the presence of brown spots, but this could not explain the observed decline (**Figure 30**). Each of the perceived horizons displayed distinct physicochemical features, and were classified as sand, except for latter depth that were considered as loamy sand and sandy clay loam in S and AS pit, respectively (**Table S16**). Horizon from 95-140 cm depth in AS soil displayed more CEC, C, N, P, organic matter, Ca, Mg, K, Na, $\text{NH}_3\text{-N}$, and Fe than the horizon above from 60-95 cm depth. Similarly in S pit, more important CEC, C, N, organic matter, Ca, K, Na, $\text{NH}_3\text{-N}$, and Fe content were found in the deep horizon from 50-120 cm depth compared to the above horizon from 25-50 cm depth.

The different horizons displayed definite profile from the measurements made through the biplot analysis of PCA, accounting for 84.8% of total variance (**Figure S7**). The biplot PCA also revealed distinct profile for both S and AS (**Figure 35.A**), with, surprisingly, symptomatic feature explained by alkaline phosphatase. Indeed, the deeper the horizon in the S pit, the higher the phosphatase activity, whereas regarding the other enzymatic activities, arylamidase and β -glucosidase drastically drop off after the second horizon of both areas (**Figure 35.B**). Similar trend was found for q-PCR measurements (**Figure 35.C**) and level of cultivable bacterial and fungal populations (**Figure 35.D**), with significantly more important copies of genes, DNA, and cultivable microbial counts in AS area compared to S one in the first horizon (**Table S17**).

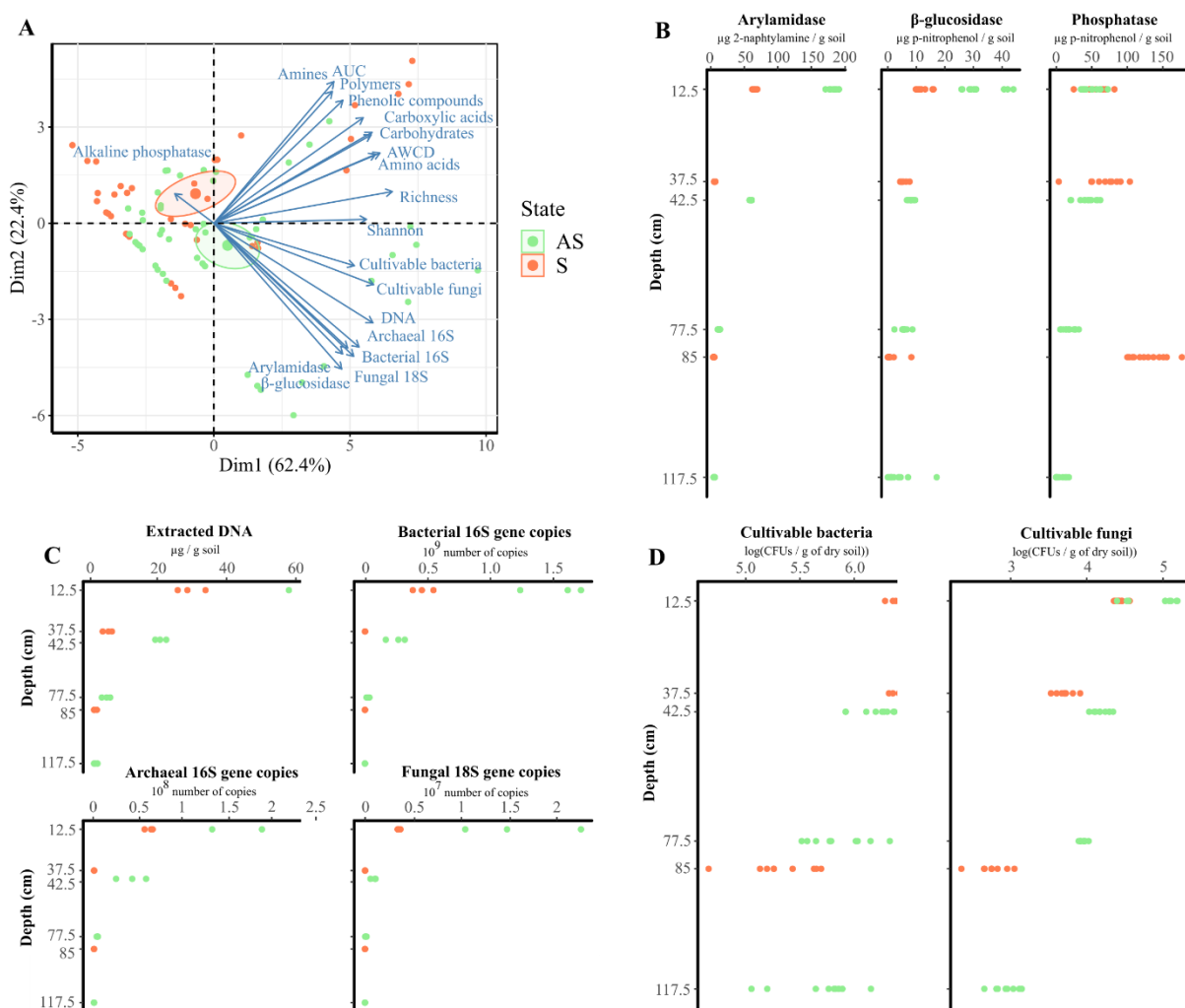


Figure 35 : Horizons profiles (0-25 = 12.5, 25-50 = 37.5, 25-60 = 42.5, 60-95 = 77.5, 50-120 = 85, and 95-140 = 117.5 cm depth) across pits made in symptomatic (orange, S) and asymptomatic (green, AS) areas. (A) Ordination biplot analysis of PCA for Eco-Plates measurements (n = 3) represented by Shannon's index, AWCD, AUC, functional richness, and family compounds consumed (i.e., amines, amino acids, carbohydrates, carboxylic acids, phenolic compounds, and polymers) coupled to microbial (i.e., bacteria and fungi) level of cultivable populations, enzymatic measurements (i.e., arylamidase, β -glucosidase, alkaline phosphatase), and q-PCR measurements (i.e., archaeal and bacterial 16S, and 18S genes). The size of the arrows indicates the contribution strength of the variables. Standard error ellipses

show 95 % confidence areas. **(B)** Enzymatic activities (n = 10), **(C)** q-PCR measurements (n = 3), and **(D)** microbial level of cultivable populations (n = 8).

Discussion

This work investigated the microbial, enzymatic, and physicochemical components within a Bordeaux vineyard presenting unexplained decline with no symptoms of mineral deficiencies or pathogen infection.

Pedological profile could not explain the observed decline

Agronomists usually employ a field method to generate what is called a crop profile to study the composition and structure of deep horizons of soils supporting cultivated plants (Peigné *et al.*, 2013). This method is also used in vineyards to estimate soil erosion and grapevine fertility (Mondini *et al.*, 2018; Rodrigo-Comino *et al.*, 2018). In our case, all values from the measurements made were falling in the deepest horizons compared to the topsoil surfaces, except for alkaline phosphatase in the symptomatic zone. On the contrary, the phosphatase activity was increasing in deeper horizons and was even significantly higher compared to the topsoil of the asymptomatic zone. Phosphatase catalyzes the release of inorganic phosphorus from organic-bound phosphorus playing a crucial role in the P cycling. However, this activity was not correlated with P content which was the lowest observed, below 2 mg.g⁻¹. Tarafdar *et al.* (1989) reported an increase of acid phosphatase activity in deeper horizons (45-60 cm) of arid soil profiles in crop and tree lands and suggested the root exudates causing this increase of enzymatic activity in deep horizons. It has been suggested for acid phosphatase that plants increase their activity under stress (Miller *et al.*, 2001; Tadano and Sakai, 1991). In our case, symptomatic grapevines may increase the alkaline phosphatase enzymatic activity through root exudates as a “cry-for-help” strategy (Rolli *et al.*, 2021).

As the wine quality is linked to the quality of berries, the must composition is one of the most concerning components for the winegrowers. Must composition is known to correlate with water deficit and soil composition (Brillante *et al.*, 2018), and its microbiota, mainly composed of yeast, has been studied for its effects on the wine making process (Martins *et al.*, 2012; Morgan *et al.*, 2017). Sugars, nitrogen content (assimilable N, NOPA, ammoniacal) as well as acidity in must composition are considered as quality indicator of grapes (Cagnasso *et al.*, 2008; Downey *et al.*, 2006). Here we found less malic and tartaric acids, as well as titratable acidity, in symptomatic samples compared to asymptomatic ones, showing a potential incidence in wine quality. Similarly, NOPA which is an essential compound in yeast nutrition and therefore of

primary importance in must fermentation (Vilanova *et al.*, 2007), was lower in the symptomatic area. In addition, inadequate assimilable nitrogen content in must composition might cause problems in yeast functioning and therefore wine fermentation, which can be alleviated by nitrogen addition (Paolini *et al.*, 2016). Essential macronutrients (*i.e.*, Ca²⁺, P, Mg) with fundamental physiological roles in plant development and structure (Gilliam *et al.*, 2011; Rustioni *et al.*, 2018) were lower in leaves content of symptomatic samples, confirming the declining context.

The physicochemical values across the different horizons were ranging in the nutritional range according to Proffitt and Campbell-Clause (2012). The soil physicochemical parameters could not explain the observed decline and were not related to symptoms of nutrient deficiency or excess. The higher level of clay in deep horizons compared to topsoil explains the greater CEC found which correlated with higher amount of nutrient known to be available in their cationic form (Ca, Na, K). Nutrient content in bulk soil was sometimes twice as high in the AS zone as in the S zone, which could explain the lower calcium, phosphorus, and magnesium content, but not nitrogen, which was higher in the symptomatic leaves. Nitrogen is essential for the synthesis of metabolites regulating the plant development (Xu *et al.*, 2012). Perchlik and Tegeeder (2018) demonstrated that enhanced allocation of nitrogen from roots to leaves could increase the photosynthetic rate. A hypothesis would be that declining grapevines mobilized nitrogen in leaves to optimize their growth under stressed environments.

Microbial structures were more influenced by compartmentalization than by soil status

Soil serves as the primary reservoir of microbiota within the grapevine (Marasco *et al.*, 2018; Swift *et al.*, 2021; Zarraonaindia *et al.*, 2015), as such, the root endosphere shares a fraction of the microbial diversity present in its surrounding soil. Here, only 0.9% of bacterial OTUs and 5.6% of fungal OTUs were found solely in the root endosphere, confirming that the soil supplies the root grapevine microbiome. Regardless of the compartment and soil status, the major bacterial phyla belonged to *Proteobacteria*, *Actinobacteriota*, *Acidobacteriota*, and *Firmicutes*, while the predominant fungal phyla were *Ascomycota* and *Basidiomycota*. These findings were consistent with the previous work made in grapevine belowground compartments (Berlanas *et al.*, 2019; Marasco *et al.*, 2018; Wei *et al.*, 2018; Zarraonaindia *et al.*, 2015).

The structure of fungal and bacterial communities from root endosphere were distinct from the ones found in soil. In agreement with previous research in grapevine, reduced bacterial and fungal richness as well as diversity were found in the root endosphere compared to soil samples

(Carbone *et al.*, 2021; D'Amico *et al.*, 2018; Marasco *et al.*, 2018; Swift *et al.*, 2021). The fungal root microbiome was influenced by soil status, revealing its sensitivity to soil composition. In the rhizosphere and bulk soil, the α -diversity metrics were lower in symptomatic condition compared to the asymptomatic one, suggesting a dysregulation of the microbiome surrounding the declining grapevines. It has been proposed that higher diversity provided greater ecosystemic processes such as organic matter degradation (Maron *et al.*, 2018). On other hand, the bacterial and fungal α -diversity metrics of root endosphere microbiome was not significantly different between the conditions.

Latent fungi associated to grapevine diseases cohabit with potentially beneficial bacteria

These differences in microbiome structure might be determinant in disease development, as the presence of dysregulated root endosphere microbiome might ease the pathogen colonization (Balbín-Suárez *et al.*, 2021). Even if symptoms of grapevine diseases were not observed in the vineyard, some fungal pathogens were detected in the different compartment, with higher abundances in symptomatic conditions. As observed in several studies, healthy plant may harbor potential latent endophytic fungal pathogens (Manzotti *et al.*, 2020; Martínez-Diz *et al.*, 2019; Yang *et al.*, 2018). These latent pathogens may induce disease incidence under environmental constrains or during plant growth (Sosnowski *et al.*, 2021). Indeed, black foot and Petri diseases occur in vineyards supporting young grapevines (< 5 years) while Esca complex, *Botryosphaeria* and *Eutypa* diebacks are usually observed in mature vineyards (Gramaje *et al.*, 2018; Hrycan *et al.*, 2020). Herein, the vineyard presented at least 10 years old grapevines, suggesting that the soil composition was rather the source of these detected fungi, comforted with the higher abundance found in symptomatic samples.

In addition to the presence of these fungi, enriched bacterial genera were detected in symptomatic conditions belonging to *Rhizobium*, *Bradyrhizobium*, *Bacillus*, *Candidatus solibacter*, and *Niastella*. Using a metaproteome approach on the grapevine rhizosphere, Bona *et al.* (2019) demonstrated the involvement in protein and nitrogen metabolism of several bacterial genera including *Bacillus* and *Bradyrhizobium*, here, enriched in the symptomatic rhizosphere and root endosphere, respectively. The plant growth-promoting properties of *Rhizobium* and *Bradyrhizobium*, which are common genera found in grapevine roots, are related to siderophores and phytohormones production, as well as nitrogen fixation and phosphate solubilization (Wright *et al.*, 2021). The *Niastella* genus, enriched in symptomatic root samples, is known to produce indole acetic acid (IAA), which is the most common phytohormone with

growth-promoting ability (Visioli *et al.*, 2018). Interestingly, *Candidatus solibacter* genus was predominant in soil contaminated by acid mine drainage or mudflat with long-term rice cultivation, and were correlated with sulfur metabolism (Wang *et al.*, 2018), and carbohydrate degradation (Zhang *et al.*, 2019b).

Eco-plates measurements revealed greater degradation of amines, amino acids, carbohydrates, carboxylic acids, phenolic compounds, and polymers, in the symptomatic rhizosphere and bulk soils compared to asymptomatic samples. A decrease in many soil biodiversity parameters with lower soil organic matter content was reported in a multisite study (Rutgers *et al.*, 2016), which is not consistent with our findings, as no significant difference in organic matter was found between S and AS areas in topsoils. Core functions such as biogeochemical processes in the soil might be related to distinct taxonomic composition but with similar metabolic functions (Griggs *et al.*, 2021). Even though Eco-Plates are not deep-informative analysis, here were reported higher functional diversity while lower microbial richness, as well as reduced fungal diversity in bulk soil. Further analysis should be made to identify the taxa responsible of this high microbial activity.

All these findings were in accordance with the previous results from bulk soil samples described in Darriaut *et al.* (2021), confirming the microbial activity promotion and microbiome dysregulation in symptomatic soils.

Conclusion

This work demonstrated the microbiome dysbiosis from root endosphere, rhizosphere and bulk soils of a vineyard subjected to unexplained decline. The deep soil horizons could not explain the declining symptoms affiliated to low vigour and poor quality of the berries. The symptomatic samples were presenting enriched taxa of potentially beneficial bacteria, that might be explained by the presence of latent fungal genera associated to grapevine diseases. The functional diversity was also higher in the rhizosphere and bulk soil samples, suggesting that stressed vineyards are harboring pathogens and beneficial microorganisms.

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Supplemental information

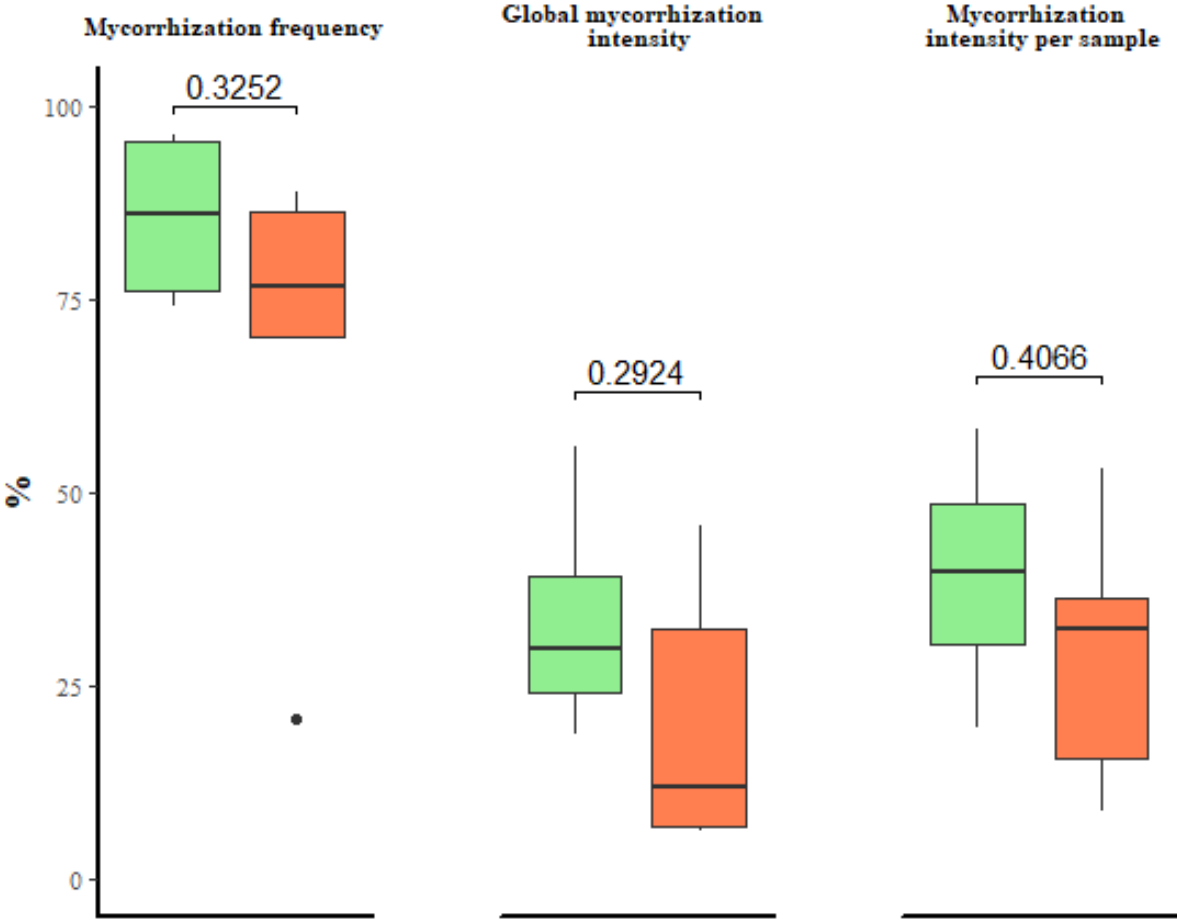


Figure S6 : Frequency and intensity of the colonization of grapevine roots by AMF (n = 5) from the studied plot with (S) and without (AS) decline symptoms.

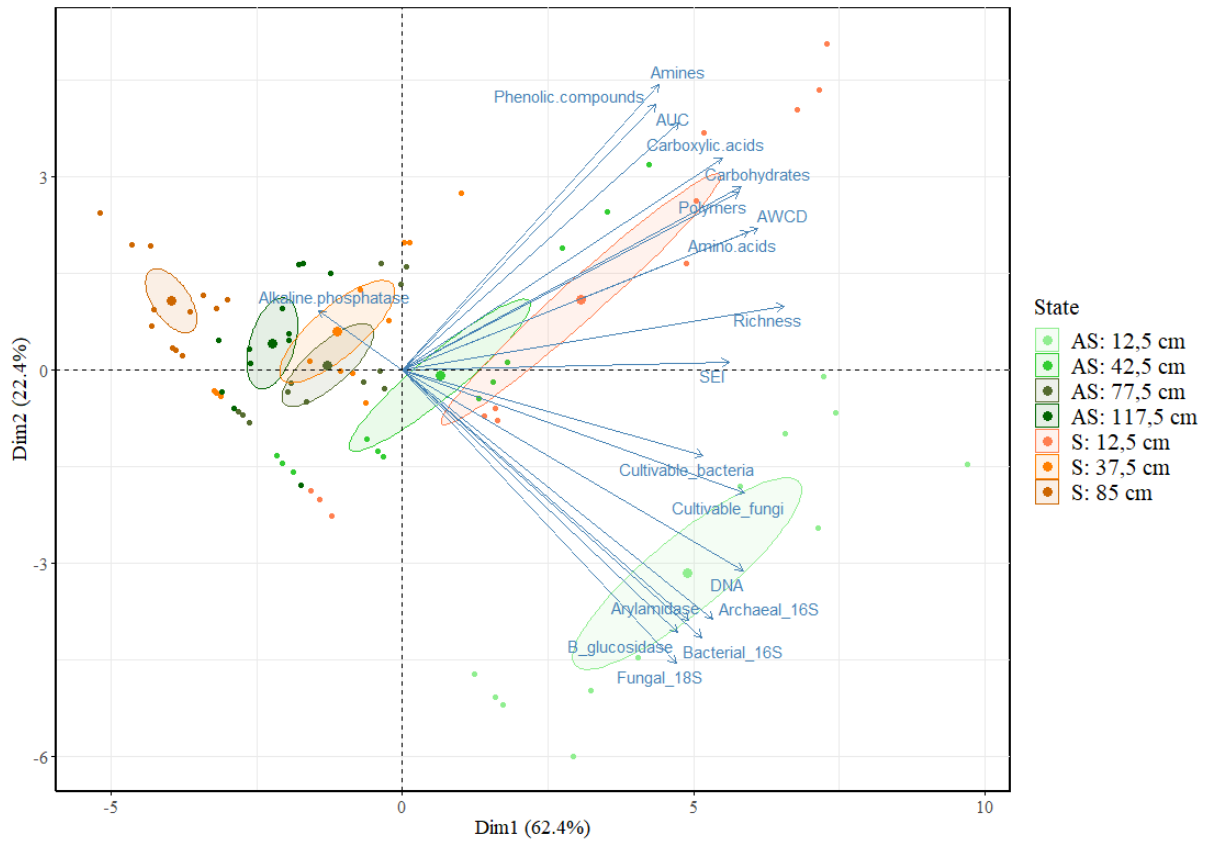


Figure S7 : Ordination biplot PCA across horizons profiles (12.5, 37.5, 42.5, 77.5, 85, and 117.5 cm depth) within pits made in symptomatic (orange, S) and asymptomatic (green, AS) areas. Analysis of PCA was made on Eco-Plates measurements ($n = 3$) represented by Shannon's index, AWCD, AUC, functional richness, and family compounds consumed (*i.e.*, amines, amino acids, carbohydrates, carboxylic acids, phenolic compounds, and polymers) coupled to microbial (*i.e.*, bacteria and fungi) level of cultivable populations, enzymatic assays (*i.e.*, arylamidase, β -glucosidase, alkaline phosphatase), and q-PCR measurements (*i.e.*, archaeal and bacterial 16S, and 18S genes).

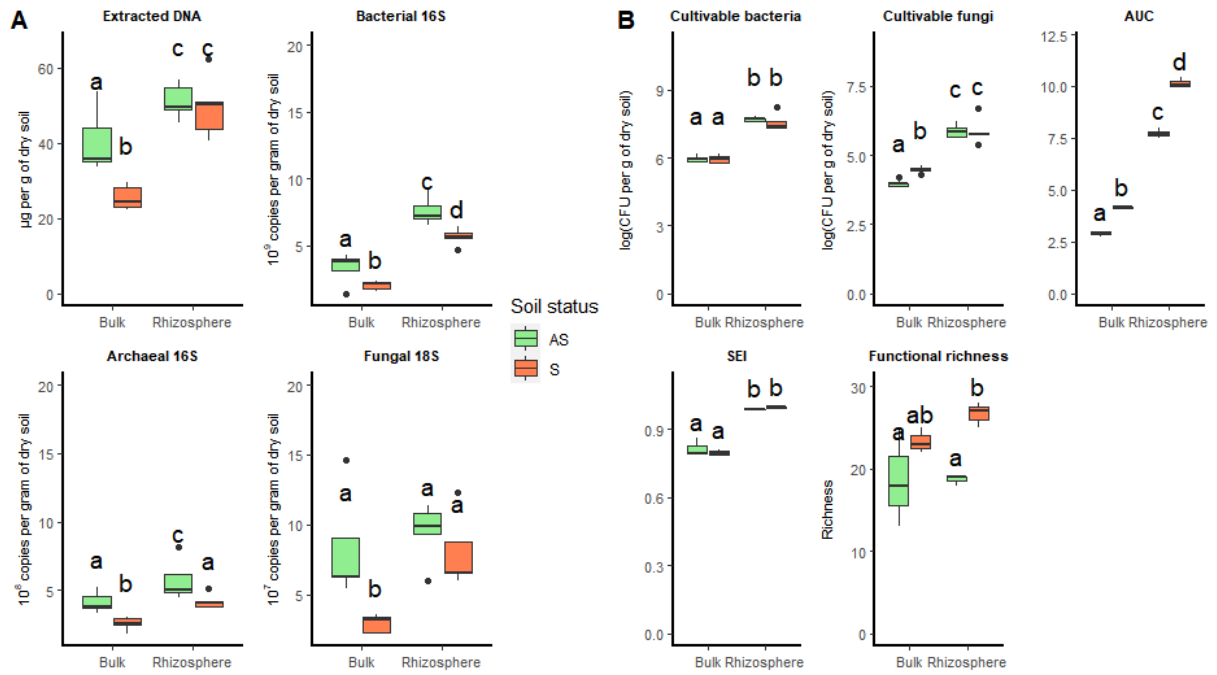


Figure S8 : Comparison of microbial profiles between the rhizosphere and bulk soils. (A) Comparison between DNA extracted and q-PCR measurements (*i.e.*, number of copies of fungal 18S, bacterial and archaeal 16S genes), as well as level of cultivable microbes and Eco-Plates measurements (SEI stands for Shannon’s evenness index). Different letters indicate different groups obtained subsequently to pairwise comparisons.

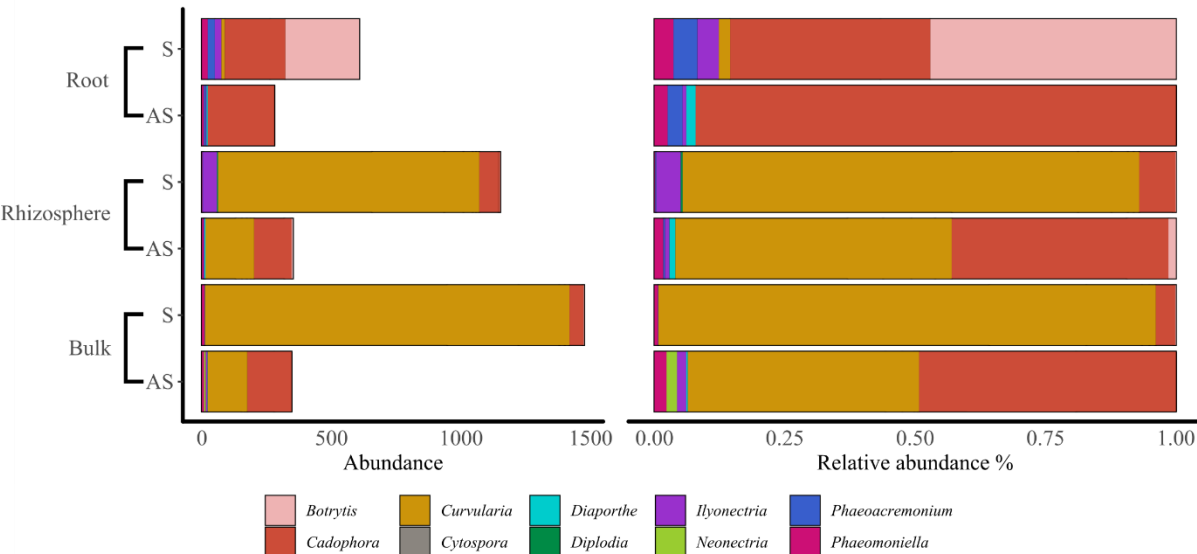


Figure S9 : Fungal genera associated to grapevine diseases across the symptomatic and asymptomatic conditions represented by their abundance and relative abundance.

Table S12 : Primers for 16S and 18S rRNA amplification for q-PCR, as well as for 16S rRNA gene and ITS sequencing. Specific overhang Illumina adapters are in italic and underlined.

| Primer | Primer sequence (5' → 3') | Target and size of the amplicon | Reference |
|------------------|--|---|--|
| 515R | CCTACGGGAGGCAGCAG | Bacterial 16S rRNA gene 174 bp | (López-Gutiérrez <i>et al.</i> , 2004) |
| 341F | ATTACCGCGGCTGCTGGCA | | |
| Arch1060R | GGCCATGCACCWCCTCTC | Archaeal 16S rRNA gene 140 bp | (Cadillo-Quiroz <i>et al.</i> , 2006) |
| Arch967F | ATTGGCGGGGGAGCAC | | |
| FR1 | AICCATTCAATCGGTAIT | Fungal 18S rRNA gene 340 bp | (Vainio and Hantula, 2000) |
| FF390 | CGATAACGAACGAGACCT | | |
| 341F | <u><i>TCGTCGGCAGCGTCAGATGTGT</i></u> <u><i>ATAAGAGACAGCCTACGGGNG</i></u> GCWGCAG | Bacterial 16SrRNA gene V3-V4 regions (464 bp) | (Klindworth <i>et al.</i> , 2013) |
| 785R | <u><i>GTCTCGTGGGCTCGGAGATGTG</i></u> <u><i>TATAAGAGACAGGACTACHVGG</i></u> GTATCTAATCC | | |
| ITS1F | <u><i>TCGTCGGCAGCGTCAGATGTGT</i></u> <u><i>ATAAGAGACAGCCTTGGTCATTT</i></u> AGAGGAAGTAA | Fungal ITS1 region (highly variable) | (Gardes and Bruns, 1993) |
| ITS2 | <u><i>GTCTCGTGGGCTCGGAGATGTG</i></u> <u><i>TATAAGAGACAGGCTGCGTTCT</i></u> TCATCGATGC | | (White <i>et al.</i> , 1990) |

Table S13 : Vineyard decline assessment for the symptomatic (S) and asymptomatic (AS) soils. It includes yield by plant (n = 28), must composition, leaves content, and vine water status (n = 3). Numbers represent means \pm SE, letters.

| | S | AS | P |
|--|-------------------|-------------------|---------------|
| Yield estimation per plant | | | |
| Primary and secondary grapes number | 7.8 \pm 1.3 | 15.25 \pm 2.2 | 0.0160 |
| Primary and secondary grapes mass (g) | 394 \pm 110 | 1867 \pm 378.1 | 0.0009 |
| Grapes bunch (g) | 42.17 \pm 6.8 | 104.10 \pm 16.7 | 0.0003 |
| Berry mass (g) | 0.86 \pm 0.03 | 1.40 \pm 0.02 | 0.0005 |
| Must composition | | | |
| Total soluble solids ($^{\circ}$ Brix) | 23.6 \pm 0.1 | 23.4 \pm 0.1 | 0.44 |
| Must pH | 3.61 \pm 0.01 | 3.53 \pm 0.01 | 0.0222 |
| Degree | 13.53 \pm 0.03 | 13.3 \pm 0.11 | 0.1733 |
| Reducing sugars (g.L ⁻¹) | 228 \pm 0.4 | 224 \pm 1.6 | 0.1108 |
| Total acidity (g.L ⁻¹) | 3.46 \pm 0.03 | 3.69 \pm 0.07 | 0.0655 |
| Malic acid (g.L ⁻¹) | 1.13 \pm 0.02 | 1.48 \pm 0.06 | 0.0253 |
| Tartaric acid (g.L ⁻¹) | 5.73 \pm 0.06 | 6.27 \pm 0.03 | 0.0060 |
| Volatile acidity | 0.09 \pm 0.01 | 0.06 | 0.0434 |
| Assimilable nitrogen (mg.L ⁻¹) | 168.33 \pm 2.9 | 248 \pm 1.53 | 0.0001 |
| Alpha-amino nitrogen (NOPA) (mg.L ⁻¹) | 99 \pm 3.60 | 119.67 \pm 0.88 | 0.0238 |
| Ammoniacal nitrogen (N-NH ₃) (mg.L ⁻¹) | 31.67 \pm 1.33 | 68.67 \pm 1.20 | 0.0001 |
| K (mg.L ⁻¹) | 1955.3 \pm 5.24 | 1759 \pm 18.50 | 0.0056 |
| Petiole content (g.kg⁻¹ dry matter) | | | |
| K | 9.24 \pm 0.86 | 9.66 \pm 1.29 | 0.7802 |
| Na | 0.44 \pm 0.03 | 0.46 \pm 0.04 | 0.7051 |
| P | 0.36 \pm 0.02 | 0.52 \pm 0.02 | 0.0053 |
| Ca | 15,25 \pm 1.95 | 31.51 \pm 0.92 | 0.0058 |
| Mg | 4.11 \pm 0.57 | 10.4 \pm 0.61 | 0.0017 |
| N | 4.96 \pm 0.04 | 4.47 \pm 0.09 | 0.0208 |
| Limb content (g.kg⁻¹ dry matter) | | | |
| K | 8.6 \pm 0.15 | 10.7 \pm 5.23 | 0.7802 |
| Na | 0.26 \pm 0.01 | 0.32 \pm 0.14 | 0.7 |
| P | 1.28 \pm 0.08 | 1.95 \pm 0.90 | 0.534 |
| Ca | 22.66 \pm 0.50 | 54.75 \pm 23.02 | 0.1 |
| Mg | 2.56 \pm 0.12 | 5.83 \pm 2.48 | 0.1 |
| N | 22.61 \pm 1.45 | 22.97 \pm 3.27 | 0.9273 |
| Vine water status | | | |
| δ C13 | -26.73 \pm 0.07 | -25.87 \pm 0.10 | 0.9273 |

Table S14 : Physicochemical characteristics of the inter-row soils with (S) and without (AS) decline symptoms. Numbers represents means \pm SE (n = 3). In bold are indicated significant differences based on either student t or Mann-Whitney tests.

| | S | AS | P |
|---|-------------------|-------------------|---------------|
| Basic soil properties | | | |
| Clay (%) | 5.13 \pm 0.18 | 5.73 \pm 0.57 | 0.4054 |
| Silt (%) | 6.3 \pm 0.23 | 7 \pm 0.25 | 0.1103 |
| Sand (%) | 88.57 \pm 0.24 | 87.27 \pm 0.50 | 0.1036 |
| pH (H ₂ O) | 6.49 \pm 0.11 | 6.34 \pm 0.03 | 0.3758 |
| pH (KCl) | 5.55 \pm 0.03 | 5.48 \pm 0.03 | 0.1962 |
| IPC | 0.35 \pm 0.01 | 0.31 \pm 0.01 | 0.0664 |
| CEC (cmol ⁺ .kg ⁻¹) | 1.72 \pm 0.11 | 2.57 \pm 0.07 | 0.0011 |
| C (g.kg ⁻¹) | 5.7 \pm 0.01 | 5.46 \pm 0.02 | 0.0722 |
| N (g.kg ⁻¹) | 0.4 \pm 0.01 | 0.45 \pm 0.02 | 0.0008 |
| C / N | 14.12 \pm 0.12 | 12.22 \pm 0.11 | 0.0004 |
| Micro/macronutrients | | | |
| P (g.kg ⁻¹) | 0.037 \pm 0.001 | 0.045 \pm 0.001 | 0.0722 |
| Organic matter (g.kg ⁻¹) | 9.86 \pm 0.01 | 9.45 \pm 0.02 | 0.0722 |
| Ca (g.kg ⁻¹) | 0.40 \pm 0.01 | 0.63 \pm 0.05 | 0.0349 |
| Mg (g.kg ⁻¹) | 0.02 \pm 0.01 | 0.06 \pm 0.01 | 0.1 |
| K (g.kg ⁻¹) | 0.04 \pm 0.01 | 0.06 \pm 0.01 | 0.1 |
| Na (g.kg ⁻¹) | 0.030 \pm 0.001 | 0.028 \pm 0.001 | 0.3435 |
| NO ₃ ⁻ (mg.kg ⁻¹) | 3.15 \pm 0.23 | 5.15 \pm 0.20 | 0.0028 |
| NH ₃ -N (mg.kg ⁻¹) | 2.81 \pm 0.01 | 2.51 \pm 0.16 | 0.2034 |
| Trace elements | | | |
| Cu (mg.kg ⁻¹) | 7.64 \pm 0.49 | 9.21 \pm 0.11 | 0.0787 |
| Fe (mg.kg ⁻¹) | 169 \pm 2.65 | 179 \pm 0.58 | 0.0575 |
| Mn (mg.kg ⁻¹) | 5.83 \pm 0.34 | 15.13 \pm 0.07 | 0.0009 |
| Zn (mg.kg ⁻¹) | 2.17 \pm 0.14 | 2.42 \pm 0.07 | 0.2073 |

Table S15 : Cultivable population levels of bacteria and fungi, and Eco-Plates measurements (AUC, Shannon's index, family compounds consumed, and functional richness at 96 hours post-incubation) within the symptomatic (S) and asymptomatic (AS) rhizosphere and bulk soils. Means \pm SE are presented with (n = 5) for bacterial and fungal counts whereas (n = 3) for Eco-Plates measurements. Letter ^a represents variables in log (CFUs / g of dry soil)), while ^b represents variables calculated based on AWCD values.

| | | S | AS | P | |
|----------------------------------|----------------------------------|----------------------------------|-------------------|-----------------|--------|
| Bulk | Cultivable bacteria ^a | 6.85 \pm 0.04 | 7.10 \pm 0.02 | < 0.0001 | |
| | Cultivable fungi ^a | 5.47 \pm 0.03 | 5.88 \pm 0.09 | 0.0057 | |
| | AUC ^b | 4.14 \pm 0.06 | 2.89 \pm 0.08 | 0.0003 | |
| | Shannon's index ^b | 0.815 \pm 0.023 | 0.798 \pm 0.008 | 0.5343 | |
| | Functional richness ^b | 23.33 \pm 0.88 | 18.67 \pm 3.48 | 0.3106 | |
| | Amines ^b | 0.74 \pm 0.74 | 0.40 \pm 0.21 | 0.6996 | |
| | Amino acids ^b | 3.55 \pm 1.45 | 2.76 \pm 0.79 | 0.4 | |
| | Carbohydrates ^b | 8.74 \pm 1.77 | 3.88 \pm 0.71 | 0.0953 | |
| | Carboxylic acids ^b | 4.21 \pm 0.54 | 2.89 \pm 0.83 | 0.2633 | |
| | Phenolic compounds ^b | 0.008 \pm 0.01 | 0.52 \pm 0.31 | 0.3537 | |
| | Polymers ^b | 3.54 \pm 0.17 | 1.58 \pm 0.17 | 0.1191 | |
| | Rhizosphere | Cultivable bacteria ^a | 7.57 \pm 0.18 | 7.69 \pm 0.05 | 0.5531 |
| | | Cultivable fungi ^a | 5.89 \pm 0.22 | 5.87 \pm 0.12 | 0.9517 |
| AUC ^b | | 10.16 \pm 0.13 | 7.73 \pm 0.14 | < 0.001 | |
| Shannon's index ^b | | 0.996 \pm 0.002 | 0.989 \pm 0.001 | 0.0456 | |
| Functional richness ^b | | 26.67 \pm 0.88 | 18.67 \pm 0.33 | 0.006 | |
| Amines ^b | | 3.36 \pm 0.28 | 2.63 \pm 0.01 | 0.1 | |
| Amino acids ^b | | 11.81 \pm 1.09 | 7.44 \pm 0.17 | 0.0538 | |
| Carbohydrates ^b | | 18.52 \pm 0.11 | 15.75 \pm 0.2 | 0.0012 | |
| Carboxylic acids ^b | | 12.21 \pm 0.14 | 9.36 \pm 0.23 | 0.0011 | |
| Phenolic compounds ^b | | 2.87 \pm 0.18 | 1.73 \pm 0.13 | 0.0086 | |
| Polymers ^b | 7.77 \pm 0.37 | 5.14 \pm 0.17 | 0.0085 | | |

Table S16 : Physicochemical characteristics of the different depth soils from the studied plot with (S) and without (AS) decline symptoms. Data shown are the values obtained after pooling 3 subsamples.

| Depth | S | | | AS | | | |
|---|-------|--------|------------|-------|-------|-------|-----------------|
| | 0-25 | 25-50 | 50-120 | 0-25 | 25-60 | 60-95 | 95-140 |
| Soil classification | Sand | Sand | Loamy sand | Sand | Sand | Sand | Sandy clay loam |
| Basic soil properties | | | | | | | |
| Clay (%) | 3.7 | 3.7 | 19.2 | 5.7 | 6.8 | 3.3 | 23.8 |
| Fine silt (%) | 3.6 | 3.8 | 2.3 | 3.9 | 4.6 | 3.6 | 17.5 |
| Coarse silt (%) | 3 | 3.1 | 2.1 | 3.8 | 3.1 | 3.1 | 7.3 |
| Fine sand (%) | 12.1 | 21.5 | 9.6 | 13 | 11 | 11.2 | 6.9 |
| Coarse sand (%) | 77.6 | 67.9 | 66.8 | 73.6 | 74.5 | 78.8 | 44.5 |
| pH (H ₂ O) | 5.82 | 6.31 | 5.58 | 5.44 | 5.16 | 5.56 | 6.49 |
| pH (KCl) | 4.84 | 5.01 | 4.58 | 4.33 | 4.05 | 4.51 | 5.28 |
| CEC (cmol ⁺ .kg ⁻¹) | 1.01 | 0.96 | 4.14 | 2.38 | 2.30 | 0.87 | 4.78 |
| C (g.kg ⁻¹) | 1.57 | 0.45 | 0.79 | 4.85 | 1.75 | 0.46 | 1.33 |
| N (g.kg ⁻¹) | 0.27 | 0.22 | 0.24 | 0.53 | 0.31 | 0.19 | 0.37 |
| C / N | 5.81 | 2.07 | 3.32 | 9.09 | 5.64 | 2.42 | 3.63 |
| Micro/macronutrients | | | | | | | |
| P (g.kg ⁻¹) | 0.005 | <0.002 | <0.002 | 0.018 | 0.005 | 0.002 | 0.003 |
| Organic matter (g.kg ⁻¹) | 7.71 | 0.77 | 1.37 | 8.39 | 3.02 | 0.8 | 2.3 |
| Ca (g.kg ⁻¹) | 0.10 | 0.10 | 0.52 | 0.17 | 0.20 | 0.06 | 0.84 |
| Mg (g.kg ⁻¹) | 0.007 | 0.011 | 0.08 | 0.02 | 0.016 | 0.005 | 0.052 |
| K (g.kg ⁻¹) | 0.02 | 0.01 | 0.03 | 0.06 | 0.02 | 0.01 | 0.04 |
| Na (g.kg ⁻¹) | 0.005 | 0.003 | 0.016 | 0.014 | 0.003 | 0.002 | 0.02 |
| NO ₃ ⁻ (mg.kg ⁻¹) | 0.3 | <0.15 | <0.15 | 1.51 | 0.26 | <0.15 | <0.15 |
| NH ₃ -N (mg.kg ⁻¹) | 1.14 | 0.67 | 1.12 | 1.67 | 2.17 | 0.74 | 0.81 |
| Trace elements | | | | | | | |
| Cu (mg.kg ⁻¹) | 4.66 | 0.58 | 0.5 | 6.16 | 5.03 | 1.30 | <0.5 |
| Fe (mg.kg ⁻¹) | 61.91 | 20.92 | 25.05 | 109.4 | 87.24 | 13.52 | 29.22 |
| Mn (mg.kg ⁻¹) | 21.18 | 6.16 | <0.5 | 13.65 | 50.86 | 6.74 | 2.68 |
| Zn (mg.kg ⁻¹) | 0.60 | 0.53 | 0.23 | 1.61 | 0.95 | 0.50 | 0.35 |

Table S17 : Measurements made at different depth across the pits in both S and AS areas. Numbers represent means \pm SE, and letters indicate different groups obtained for each variable after pairwise comparisons with Bonferroni correction.

| | Depth (cm) | S | Depth (cm) | AS |
|---|------------|-------------------------|------------|-------------------------|
| Bacterial counts log(CFUs / g of dry soil) | 0-25 | 6.47 \pm 0.03 ad | 0-25 | 6.54 \pm 0.02 a |
| | 25-50 | 6.45 \pm 0.02 d | 25-60 | 6.19 \pm 0.06 b |
| | 50-120 | 5.32 \pm 0.11 e | 60-95 | 5.87 \pm 0.09 c |
| | | | 95-140 | 5.69 \pm 0.11 c |
| Fungal counts log(CFUs / g of dry soil) | 0-25 | 4.43 \pm 0.02 e | 0-25 | 4.90 \pm 0.10 a |
| | 25-50 | 3.71 \pm 0.04 f | 25-60 | 4.20 \pm 0.03 b |
| | 50-120 | 2.75 \pm 0.07 d | 60-95 | 3.95 \pm 0.01 c |
| | | | 95-140 | 2.94 \pm 0.05 d |
| Arylamidase activity (μ g 2-naphthylamine g ⁻¹ dry soil h ⁻¹) | 0-25 | 65.26 \pm 0.57 e | 0-25 | 183.20 \pm 1.72 a |
| | 25-50 | 5.77 \pm 0.23 d | 25-60 | 59.87 \pm 0.39 b |
| | 50-120 | 4.23 \pm 0.23 f | 60-95 | 12.18 \pm 0.35 c |
| | | | 95-140 | 5.13 \pm 0.23 d |
| β -glucosidase activity (μ g p-nitrophenol g ⁻¹ dry soil h ⁻¹) | 0-25 | 12.61 \pm 0.73 d | 0-25 | 32.16 \pm 1.81 a |
| | 25-50 | 5.20 \pm 0.27 c | 25-60 | 8.15 \pm 0.25 b |
| | 50-120 | 1.20 \pm 0.66 d | 60-95 | 5.66 \pm 0.41 c |
| | | | 95-140 | 3.64 \pm 1.36 cd |
| Phosphatase activity (μ g p-nitrophenol g ⁻¹ dry soil h ⁻¹) | 0-25 | 53.42 \pm 4.35 a | 0-25 | 50.66 \pm 3.28 a |
| | 25-50 | 65.94 \pm 7.52 a | 25-60 | 43.23 \pm 3.85 a |
| | 50-120 | 129.57 \pm 6.94 d | 60-95 | 17.72 \pm 2.43 b |
| | | | 95-140 | 5.03 \pm 1.92 c |
| Total DNA extracted (μ g.g ⁻¹ of dry soil) | 0-25 | 29.23 \pm 2.36 d | 0-25 | 61.44 \pm 1.85 a |
| | 25-50 | 5.29 \pm 0.78 c | 25-60 | 20.61 \pm 0.93 b |
| | 50-120 | 1.63 \pm 0.26 c | 60-95 | 4.86 \pm 0.70 c |
| | | | 95-140 | 1.75 \pm 0.30 c |
| Archaeal 16S (10 ⁸ number of copies) | 0-25 | 0.62 \pm 0.03 b | 0-25 | 1.88 \pm 0.32 a |
| | 25-50 | 0.0009 \pm 0.0007 c | 25-60 | 0.42 \pm 0.09 b |
| | 50-120 | 0.0002 \pm 0.00005 c | 60-95 | 0.038 \pm 0.004 c |
| | | | 95-140 | 0.00018 \pm 0.00005 c |
| Bacterial 16S (10 ⁹ number of copies) | 0-25 | 0.46 \pm 0.01 d | 0-25 | 1.51 \pm 0.15 a |
| | 25-50 | 0.0007 \pm 0.00002 c | 25-60 | 0.25 \pm 0.04 b |
| | 50-120 | 0.0003 \pm 0.00009 c | 60-95 | 0.024 \pm 0.007 c |
| | | | 95-140 | 0.00021 \pm 0.00002 c |
| Fungal 18S (10 ⁷ number of copies) | 0-25 | 0.352 \pm 0.009 d | 0-25 | 1.58 \pm 0.35 a |
| | 25-50 | 0.0012 \pm 0.0006 c | 25-60 | 0.09 \pm 0.02 b |
| | 50-120 | 0.00027 \pm 0.00018 c | 60-95 | 0.008 \pm 0.004 c |
| | | | 95-140 | 0.09 00002 c |

Table S18 : Factors effects related to compartment (bulk, rhizosphere, root endosphere) and soil composition (S, AS) on richness, diversity, and β -diversity related to bacterial and fungal communities. Significances were assessed through a Type II ANOVA for richness and diversity, while PERMANOVA (n=999) was used for distance dissimilarities.

| Community | Factor | Richness (Chao1) | | Diversity (Simpson) | | β -diversity (Bray-Curtis) | | |
|-----------|-------------|---------------------|----------------|------------------------|----------------|-------------------------------------|-------|--------------|
| | | F | P | F | P | R ² | F | P |
| 16S | Compartment | 2472.77 | < 0.001 | 17.17 | < 0.001 | 0.55 | 22.91 | 0.001 |
| | State | 98.96 | < 0.001 | 3.53 | 0.075 | 0.09 | 7.85 | 0.001 |
| ITS | Compartment | 484.47 | < 0.001 | 23.82 | < 0.001 | 0.45 | 14.47 | 0.001 |
| | State | 7.46 | 0.013 | 2.87 | 0.106 | 0.11 | 7.06 | 0.001 |

Table S19 : Reports of α -diversities metrics represented by Chao1 and Simpson for each of the conditions among the bacterial and fungal communities. Different letters indicate significant differences among the communities (pairwise test, $P < 0.05$).

| Community | Compartment | State | Richness (Chao1) | Diversity (Simpson) |
|-----------|-------------|-------|---------------------|------------------------|
| Bacteria | Bulk | AS | 1178 \pm 10 a | 0.99 \pm 0.01 a |
| | | S | 931 \pm 9 b | 0.99 \pm 0.01 a |
| | Rhizosphere | AS | 1174 \pm 20 a | 0.99 \pm 0.01 a |
| | | S | 1022 \pm 18 ab | 0.99 \pm 0.01 a |
| | Root | AS | 189 \pm 7 c | 0.96 \pm 0.01 b |
| | | S | 160 \pm 15 c | 0.93 \pm 0.01 b |
| Fungi | Bulk | AS | 416 \pm 15 a | 0.98 \pm 0.01 a |
| | | S | 378 \pm 17 b | 0.97 \pm 0.01 a |
| | Rhizosphere | AS | 408 \pm 10 a | 0.97 \pm 0.01 a |
| | | S | 355 \pm 15 b | 0.94 \pm 0.01 ab |
| | Root | AS | 91 \pm 5 c | 0.91 \pm 0.01 bc |
| | | S | 102 \pm 7 c | 0.89 \pm 0.01 c |

Brief discussion

The effects of the decline were observed on the leaves and must composition, with reduced mineral content in symptomatic samples compared to the asymptomatic ones. Consistent with Chapter 2 results, the functional diversity measured by Eco-Plates was more important in the declining area in both bulk and rhizosphere components. In addition, the bulk, root, and rhizosphere compartments exhibited taxonomic dysregulation in bacterial and fungal communities, highlighting the microbial disruption linked to the observed decline. In accordance to the second part of chapter 2, enrichment of potentially beneficial bacteria, as well as fungal pathogen, were found in the symptomatic samples. Deep horizons from both asymptomatic and symptomatic areas presented decreasing enzymatic activities, number of gene copies, and level of cultivable populations of microbes, with the exception of phosphatase activity that got more important in deep symptomatic horizon. Due to late sequencing and update in MaarJAM and Unite databases, taxa affiliations for *Glomeromycota* were not presented in this work. These results will be processed and incorporated before the article submission.

Amplicons-based sequencing has been democratized and is widely used in ecological studies to reveal the structure of microbial communities. However, one limitation of this methodology is the lack of information regarding active microbes. Therefore, the combination of the cultivable-dependent and independent methods can reflect a more comprehensive and relevant microbial profile in the environments studied than single-based method. This holistic approach was able to provide insights in the microbial unbalance occurring in the vineyard. However, only one rootstock genotype was present in this study, which is a determinant factor in the association of grapevine with its microbiota. The next chapter is related to this topic of rootstock genotype, and will be studied in a greenhouse experiment.

Chapter IV

Influence of the rootstock genotype on belowground microbiome of young vines

Preface

Like many perennial crops, unproductive and dying vines are replaced by young plants which require at least three years to become fruitful and productive. As reviewed previously, the genetic dimension of the rootstock has an influence on the mineral uptake but also on the microorganisms associated to the grapevine. In this context, the impact of rootstock genotype on soil and root microbial diversity and functionality is an insightful question.

The present chapter aims to explore the influence of the rootstock genotype on belowground microbiome of young vines, potted with either symptomatic or asymptomatic soils. These soils were excavated from the vineyard studied in the previous chapter and used as soil substrate for a greenhouse experiment. The experimental design lasted for 4.5 months, was repeated twice, and was based on 1 year-old Cabernet Sauvignon grafted on either Riparia Gloire de Montpellier or 1103 Paulsen, which induce low and high vigour to the scion, respectively.

To compare the influence of the rootstock genotype on microbes, cultivable dependent and independent approaches were used in root, rhizosphere, and bulk compartments. All the previous methods including, Eco-Plates, q-PCR, level of cultivable microbes, amplicons sequencing, and mycorrhizal root colonization were assessed. In addition, rhizobacterial isolates were identified with MALDI-TOF-MS, and the predominant bacterial genera were visualized with DOPE-FISH microscopy to confirm their presence in root endosphere.

This chapter part was the subject of a research article entitled “**Soil composition and rootstock genotype drive the root associated microbial communities in young grapevines**” that will be submitted to Journal of Experimental Botany subsequently to co-authors corrections.

Soil composition and rootstock genotype drive the root associated microbial communities in young grapevines

Romain Darriaut¹, Livio Antonielli², Guilherme Martins^{3,4}, Patricia Ballestra³, Philippe Vivin¹, Elisa Marguerit¹, Birgit Mitter², Isabelle Masneuf-Pomarède^{3,4}, Stéphane Compant², Virginie Lauvergeat^{1*}

¹ EGFV, Univ. Bordeaux, Bordeaux Sciences Agro, INRAE, ISVV, F-33882, Villenave d'Ornon, France

² Bioresources Unit, Center for Health & Bioresources, AIT Austrian Institute of Technology GmbH, Tulln, Austria

³ Université de Bordeaux, INRAE, Bordeaux INP, UR Œnologie EA 4577, USC 1366, ISVV, 33140 Villenave d'Ornon, France

⁴ Bordeaux Sciences Agro, 1 cours du Général de Gaulle, 33170 Gradignan, France

*corresponding author: virginie.lauvergeat@inrae.fr

Abstract

The young plant, whose health and growth are largely influenced by microorganisms, draws its associated microbiome from the soil. In grapevine, which is often a grafted plant, the genetic dimension of the rootstock is another factor to consider in its belowground associations with microorganisms. In this greenhouse study, the impact of the soil and the rootstock genotype on the microbial community in roots, rhizosphere and bulk soil were investigated using culture-dependent analysis, as well as amplicon sequencing of the bacterial 16S rRNA gene and the fungal ITS. *Vitis vinifera* L. cv. Cabernet Sauvignon plants grafted on two different rootstocks, namely Riparia Gloire de Montpellier (RGM) and 1103 Paulsen (1103P) were grown in two different soils, which originated from the same vineyard partially subjected to unexplained decline (*i.e.*, asymptomatic AS, and symptomatic S areas). The observed decline in vineyard was not related to a pathological cause or mineral disturbance, but to low growth and high mortality of the plants. Soils used for greenhouse experiment possessed similar physicochemical properties and different microbial compositions. After five months in greenhouse, cultivable microorganisms in rhizosphere compartment and mycorrhizal colonization were rather influenced by the soil status than the rootstock genotype. Fungal diversity and richness of sequenced amplicons were largely influenced by the soil status and the rootstock genotype while bacterial richness was only slightly affected by the genetic dimension of the rootstock. Compartmentalization affected both bacterial and fungal communities, but differently according to the genotype, underscoring the rootstock influence on microorganism selection. In addition, predominant root associated bacteria were visualized using fluorescence *in situ* hybridization (FISH) revealing their niches inside root tissues.

Interestingly, some fungal genera associated to grapevine pathogens were detected in vineyard soils and in each greenhouse conditions. Higher prevalence was found in roots, and especially in roots before plantation (*i.e.*, originated from the nursery). The decline observed in the vineyard was not replicated in the greenhouse, and the fungal pathogens detected did not express symptoms on the hosts. These results emphasized the importance of rootstock genotype and soil composition in shaping the microbiome of young vines.

Keywords: grapevine microbiome, rootstock selection, root endosphere, rhizosphere, mycorrhiza colonization

Introduction

The microbiome, which is defined as the community of microorganisms and their theatre of activity (Berg *et al.*, 2020), is a biological indicator of plant health and productivity (Trivedi *et al.*, 2020). Its composition depends on the niche location such as the phyllosphere, rhizosphere, or plant endosphere (Rossmann *et al.*, 2017; Vandenkoornhuysen *et al.*, 2015). The plant microbiota is mainly originated from the surrounding soil, which acts as a reservoir, passing through the rhizosphere and roots compartments. The rhizosphere compartment, where most of the biogeochemical and nutrient cycling occur, is considered as a hot spot for the microbial activity, while the plant endosphere also contains microorganisms which intimately interact with plant host (Reinhold-Hurek *et al.*, 2015). Plant endophytes have received special attention since the functions of plant-associated microbiome can be either mutualistic or pathogenic to the host, which is determinant for crop health and productivity (Compant *et al.*, 2021). There are many factors, both biotic and abiotic, that can influence the composition of soil microbial communities, and therefore plant endosphere.

Abiotic parameters such as soil physicochemical characteristics (Hartman and Tringe, 2019), environmental conditions (Dubey *et al.*, 2019), or even agricultural practices (Ke *et al.*, 2021) could alter the microorganism compositions within soil and plants. Biotic factors such as pathogen invasion (Byers *et al.*, 2020), as well as the application of plant growth-promoting rhizobacteria (Zhang *et al.*, 2019a) or mycorrhizal fungi (Zhou *et al.*, 2020) might also alter telluric and plant endosphere microbial communities, in addition to plant age or plant genotype (Wagner *et al.*, 2016).

Keystone microbial taxa, which are the ecosystem engineers having a large influence in the communities of microorganisms, are therefore primordial to maintain the health of the plant host (Banerjee *et al.*, 2018). The depletion or downsize of these taxa in soil could cause a

microbial dysbiosis responsible of the decline and could predict the plant health (Wei *et al.*, 2019). This dysbiosis term is mainly used in medical fields and are used as markers in pathological conditions. Recently in tomato, it has been shown that the abundances of *Actinobacteria* and *Firmicutes* taxa were lower in the rhizosphere of diseased plants than in healthy ones (Lee *et al.*, 2021).

Vineyard decline is a process observed in viticulture which lead to a decrease in vine productivity and sometimes to plant death (Riou *et al.*, 2016). This phenomenon is often associated with several individual or combined stresses, including biotic and abiotic factors. Most of the work on the microbiome related to grapevine decline refers to biotic stressors such as grapevine trunk diseases (GTDs), which present distinct and well-documented symptoms (Gramaje *et al.*, 2018). Additionally, grapevine growth disturbance might be provoked by nutrient deficiencies or other abiotic stresses (Ollat *et al.*, 2016). However, winegrowers are sometimes confronted with grapevine decline that is not associated to pathological causes or mineral imbalance, but might be related to soil biological dysfunction (Darriaut *et al.*, 2021).

Plant roots through exudates synthesis shape the rhizomicrobiome by selective enrichment of microbiota from the bulk soil. The biochemical composition of root exudates varies with the host age and genotype, and consequently drives the wellness and microbial communities of the plant (Vives-Peris *et al.*, 2020). Cultivated grapevine, among other crops, is usually a grafted plant composed of a scion and a rootstock. The rootstock is at the interface between the soil and provide nutrient to the scion, while the latter is the aerial part producing the berries. The rootstock modifies scion phenotype while conferring specific rooting ability, resistance features, and other agronomic properties (Gautier *et al.*, 2019). In viticulture, rootstocks have different genetic background due to intensive breeding and selections of agronomic traits (Riaz *et al.*, 2019). These genotype-specific traits correspond to different physiological features with distinct root exudates that determine the microbiota in the rhizosphere and root compartments (Berlanas *et al.*, 2019; Dries *et al.*, 2021; Marasco *et al.*, 2018; Zarraonaindia *et al.*, 2015).

As with many perennial crops, unproductive, dying, or dead vines are usually replaced by new young plants. The vines take at least three years to become productive, and in the meantime form their associated microbiota through the rootstock. This period is crucial for the development and health of the future grapevine, especially in a soil subjected to unexplained decline. Given the above, there is a lack of knowledge in the selection process of the microbiota of young vines. The impact of the rootstock genotype is neither well understood in term of the microbiota uptake in roots. To our knowledge, no research has been carried out on the roots and

soil microbiome of young, grafted vines plants grown with substrate taken from decline vineyards.

The aim of this work was to observe the evolution of belowground microbiome from two genetically different rootstocks grown with soil substrate taken from vineyards subjected to unexplained decline. To this end, cultivable and molecular based approaches are used on the bulk soil, rhizosphere, and root compartments on young grapevine plants.

Material & Methods

Plant material

One year-old grapevines were obtained from the Pépinière Guillaume nursery (70700, Charcenne, France) by grafting clone 169 of *V. vinifera* L. cv. Cabernet Sauvignon (CS) with rootstocks known for their low (RGM: Riparia Gloire de Montpellier) or high (1103P: 1103 Paulsen) vigour-conferred. The two scion × rootstock combinations (*i.e.*, CS×RGM and CS×1103P) were obtained in traditional bare root plants without any microbial addition. Before planting, a few roots of CS×RGM and CS×1103P were sampled for 16S rRNA gene and ITS amplicon sequencing.

Experimental design

For this experimental design, two soils originating from the same vineyard were sampled. The first soil supported well-growing and asymptomatic (AS) vines while the second sampled soil was in an area where unexplained decline was observed with symptomatic vines (S). Previous analysis performed in Darriaut *et al.* (2021) (*i.e.*, vineyard n°2) revealed relatively similar physicochemical features with higher enzymatic activities and different microbial profiles between S and AS soils.

Soil samples from the 0-30 cm depth horizon were collected in mid-April 2019 with a mini-excavator and sieved (mesh size < 3 cm) to remove large roots and gravels. Seventy plants from each combination were randomly divided and planted in 7.5 L pots (diameter 26 cm, height 21 cm) filled with either S or AS soils, supported by a geotextile membrane and amended with sterilized gravels. These 35 pots per condition, namely S-1103P, S-RGM, AS-1103P, AS-RGM, were placed in a greenhouse under ambient light and temperature. The plants were watered twice a week with 60 ml per pot per watering without adding nutrient solution. The shoots were tied with thread to stakes in order to let the plants grow on a fence in an upright

position. This experimental design was repeated in the next growing season in 2020, with freshly excavated soil and new plants with similar scion × rootstock combinations.

Plant and pot substrate sampling

Plants were harvested in early September, after 4.5 months of greenhouse experience. Measurements of the following parameters were done right after the sampling: fresh biomass of aerial plant parts including leaves and shoots, fresh biomass of trunk and roots, diameters and lengths of shoots and trunk. To measure leaf greenness of the plants, chlorophyll index of the top fourth and third leaves were estimated using a portable chlorophyll meter (SPAD-502, Konica Minolta Sensing, Inc., Japan).

In parallel to plant sampling for phenotypic measurements, roots, rhizosphere, and bulk soil were individually collected.

Large and small soil aggregates were removed from the roots by manual shaking. The roots with few portions of soil left, considered here as the rhizosphere compartment, were placed in tubes containing sterile 0.85% NaCl solution and vortexed prior to 5,000 g centrifugation for 10 minutes to detach the rhizosphere from the roots. The root and rhizosphere samples were separated, and both divided into two subgroups, as depicted in **Figure S10**. The first root subgroup was surface sterilized with 3% hypochlorite sodium for 1 minute subsequently to 3% H₂O₂ for 1 minute and rinsed thrice using sterile water. This subgroup was stored at -80°C prior to DNA extraction for the 16S rRNA gene and ITS amplicon sequencing of root endosphere. The second subgroup of roots was used at fresh state for staining to observe mycorrhizal colonization.

Rhizosphere samples obtained after centrifugation and detachment from the roots were also separated into two subgroups. The first subgroup of rhizosphere samples was lyophilized for 48 hours using Christ Alpha® 1-4 (Bioblock Scientific) and stored at -80°C prior to DNA extraction. The second subgroup was used for the potential metabolic diversity (PMD), the isolates quantification with plating method, as well as the isolates identification through MALDI-TOF-MS.

DNA extraction

DNA was isolated using FastDNA Spin kit for soil (MP Biomedicals) following manufacturer's instructions except that bead beating step on FastPrep device and aspiration of liquid samples were performed twice. Bead beating power on FastPrep device was set on power 5 for 30 seconds for soils and to power 6 for 40 seconds for root samples. DNA was isolated from 500

mg of soils and 200 mg of root powders prepared by freezing in liquid nitrogen and pulverized by bead beating in steel containers on a Retsch mill. DNAs were eluted from DNA binding matrix into 100 µl of sterile H₂O. For q-PCR measurements, DNA was isolated using DNeasy PowerSoil Pro Kit (Qiagen) on 250 mg of rhizosphere soil following manufacturer's instructions except that bead beating step was performed thrice at 4 m.s⁻¹ for 30s and that an additional washing step with C5 solutions was performed.

Potential metabolic diversity coupled to quantification of microorganisms, and mycorrhizal root colonization

PMD, quantification of cultivable bacteria and fungi, and quantitative PCR of bacterial 16S, archaeal 16S, and fungal 18S rRNA genes were demonstrated according to Darriaut *et al.* (2021). Briefly, PMD is evaluated by measuring 31 different substrates consumption every 24 h for 4 days within the Biolog Eco-Plates™ system (Biolog Inc., CA, USA). These substrates are related to 6 compounds families (*i.e.*, amines, amino acids, carbohydrates, carboxylic acids, phenolic compounds, and polymers).

In parallel to PMD assay, the quantification of cultivable bacterial population was investigated on R2A medium amended with 25 mg/L of nystatin to inhibit yeast and fungal growth, while the fungal populations were quantified on PDA medium supplemented with 500 µg ml⁻¹ of gentamicin and 50 µg ml⁻¹ of chloramphenicol to inhibit yeast and bacterial growth.

From the DNA extracted in the rhizosphere among the four conditions, quantitative PCR analyses based on absolute quantification were performed on the DNA extracted using three primers pairs to quantify bacterial and archaeal 16S rRNA genes as well as the fungal 18S rRNA genes, as detailed in Darriaut *et al.* (2021). The efficiencies of the q-PCR were 80% to 99% ($R^2 > 0.99$).

In addition, from the second subgroup of prepared fresh roots, thirty subsamples were collected for an estimation of mycorrhizal root colonization during the second year of sampling. Those roots were stained by the ink-KOH-H₂O₂ method modified from Phillips & Hayman (1970). Briefly, fresh roots were rinsed in sterile water and incubated in 10% KOH for 30 min at 95°C. Immediately after the incubation, to the KOH-roots mixture was incorporated 3% H₂O₂. The solution was discarded, and the roots were rinsed thrice with sterile water and stained, in 5% India ink (Super Black™) solution with 8% acetic acid, by incubating at 90°C for 5 min. Roots were destained at ambient temperature with 8% acetic acid for 15 min before washed with sterile water. Stained roots were then placed on glass slides with pure glycerol and observed with a light microscope LEICA DM750 equipped with a LEICA ICC50 W camera.

Subsequently, arbuscular mycorrhizal colonization was estimated with Trouvelot *et al.* (1986) method and MycoCalc program (www2.dijon.inra.fr/mychintec/MycoCalc-prg/download.html).

Identification of bacterial isolates through MALDI-TOF-MS

From the R2A plates, 100 isolates were randomly selected for each of the four conditions (i.e., AS-1103P, AS-RGM, S-1103P, S-RGM) of each year (i.e., 2019 and 2020) and grown individually on new R2A plates, accounting for a total of 800 isolates. The 100 isolates were collected across three biological replicates from the same dilution. Single fresh isolates were smeared on MSP96 target polished steel BC plate and overlaid with 1 µl of 70% formic acid. Once dried at room temperature, samples were overlaid with MALDI matrix (1 µl, 10 mg/ml of α -cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid) for crystallization. Once dried, the target plate was submitted to MALDI-TOF MS analysis using Microflex MALDI-TOF (Bruker Daltonik GmbH, Leipzig, Germany) bench-top mass spectrometer scanned with laser wavelength of 337 nm and acceleration voltage of 20 kV. The analysis was performed using Flex Control, MTB Compass, and MALDI-Biotyper™ software (Bruker Daltonics, Germany) by comparing the mass profile of the isolates to mass profiles in the Biotyper database. Bacterial test standard was added to every plate in order to calibrate the mass spectral data performed by the MALDI-TOF-MS. Mass profiles matching were obtained as score values and ranged from 0 to 3 as indicated by the manufacturer. Score values above 2.2 corresponded to highly probable species identification, the ones between 1.8 and 2 displayed identifications at the genus level, while score values below 1.8 were not considered as trustful identifications.

Amplicon libraries preparation and sequencing

All PCR amplifications were carried out by KAPA HiFi HotStart PCR Kit (Roche) mixture containing template DNA, 1x KAPA HiFi buffer with magnesium, 300 µM dNTPs, 0.25 units of KAPA HiFi polymerase, and specified concentration of primers. PCR amplification of each sample was repeated three times and amplicons pooled together for further indexing according to cycling conditions listed in (Table S20).

Amplification of 16S rRNA gene was performed on 1:10 of diluted extracted DNA using 300nm of primers 799f/1175r, designed to amplify V5-V7 bacterial regions with the exclusion of chloroplast DNA (Chelius and Triplett, 2001). PCR bands were excised and separated from plant mitochondrial amplicons for further indexing.

Two internal transcribed spacer region (ITS) libraries were created to sequence ITS1 and ITS2 based on primers listed in **Table S21**. PCR amplification for the first ITS library which target ITS1 region was performed on 1:10 of diluted extracted DNA using 500 nm of primer 5.8S-Fun_NeXTf coupled to reverse primers (ITS5_Mix = ITS4-Fun_NeXTr + ITS43S-Fun_NeXTr, adapted from Taylor *et al.* (2016)). Second ITS library, targeting ITS2, was created by applying nested PCR approach. First PCR amplification was performed on 1:10 of diluted extracted DNA using 300 nm of primers ITS1F/TW13 (Klaubauf *et al.*, 2010), designed to amplify fungal ITS and part of fungal large subunit (LSU). This was followed by the second amplification using 450 nm of primers mixes containing forward (ITS3_Mix = ITS31_NeXTf + ITS32_NeXTf + ITS33_NeXTf + ITS34_NeXTf + ITS35_NeXTf) and reverse primers (ITS4_Mix = ITS4_NeXTr + ITS43S_NeXTr) on 3 µl of the first PCR amplicon (Tedersoo *et al.*, 2014).

Indexing-PCR of 16S rDNA and ITS DNA amplicons was performed using Illumina Nextera XT indexing primers (forward S502-S503, S505-S508, S510-S511 and reverse N701-N707, N710-N712, N714-N715) under following conditions: 1 µl of 16S rRNA gene or ITS PCR amplicons (each derived from three pooled independent PCR amplifications), 1x KAPA HiFi buffer with 2 mM MgCl₂, 300 µM dNTPs, 300 nM of each forward and reverse indexing primer, 0.25 unit of KAPA HiFi polymerase and H₂O up to 50 µl. Amplification was performed making initial denaturation step at 95°C for 3 minutes, 12 cycles including denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and elongation at 72°C for 30 sec, and final elongation at 72°C for 5 minutes.

Intensity of bands was measured and compared using Image Lab 6.1 software (BioRad). Amplicons were then mixed in equimolar amounts to create pooled libraries. Libraries were cleaned first by extraction with Phenol-Chloroform-Isoamyl (24:24:1) and Chloroform-Isoamyl (24:1) followed by the spin filtration using Amicon Ultracel 30K centrifugal filters (Millipore UFC503096) applying 2 x 500 µl ddH₂O and finally using AmPure XP magnetic beads (Beckman Coulter) according to the manufacturer instruction. Two libraries, based on either soil or root samples were created, and sequenced separately. For sequencing 6 pM library was spiked with 8% PhiX and sequencing was performed on the MiSeq System (Illumina) using Illumina MiSeq® Reagent Kit v3 (600 cycle) (MS-102-3003).

16S rRNA gene and ITS sequencing pre-processing

MiSeq sequences were filtered with Bowtie 2 v.2.3.4.3 (Langmead and Salzberg, 2012) to remove PhiX control reads, if still present, and sequence quality was preliminarily checked with

FastQC v.0.11.8 (Andrews, 2010). Primers were removed using Cutadapt v.1.18 (Martin, 2011). Sequences were quality filtered, trimmed, denoised and amplicon sequence variants (ASVs) were generated with DADA2 v1.20.0 (Callahan *et al.*, 2016). Denoised forward and reverse ASV sequences were merged, and chimeras were removed. Filtered ASVs were checked using Metaxa2 v2.2.3 (Bengtsson-Palme *et al.*, 2016) and ITSx v1.1.3 (Bengtsson-Palme *et al.*, 2013) for targeting the presence of V5-V7 16S rRNA and ITS2 region, in archaeal and bacterial sequences and fungal sequences, respectively. Taxonomic assignment of 16S rRNA gene ASVs and ITS based ASVs was performed using the RDP classifier of DADA2 against the SILVA v138 database (Quast *et al.*, 2012) and UNITE 8.2 database (Nilsson *et al.*, 2019), respectively. After taxonomic classification, ASVs classified as other than archaea, bacteria or fungi were removed.

Bioinformatics analysis and statistics

All analysis and graphs were performed on R (R-4.1.2) using RStudio (2021.9.1.372). Figures were generated with *ggplot2* (3.3.5) and *ggthemes* (4.2.4) packages and arranged using *ggpubr* (version 0.4.0) (Arnold, 2021; Kassambara, 2020; Wickham and Chang, 2008). Two-way Analysis of Variance (ANOVA) with soil status (AS or S) and rootstock genotype (RGM or 1103P) factors were performed on cultivable, q-PCR and Eco-Plates measurements. Residuals were checked for their independency, normality, and variance homogeneity with the Durbin Watson, Shapiro-Wilk, and Bartlett tests, respectively. When assumptions for parametric tests were not respected, a multiple pairwise comparison using Wilcoxon test was performed subsequently to Kruskal–Wallis test using the *multcomp* (1.4-18) package (Hothorn *et al.*, 2008). Principal Component Analysis was performed using *FactoMineR* (2.4) and *missMDA* (1.18) while Venn diagrams were generated using *VennDiagram* (1.7.1) (Chen, 2021; Josse & Husson, 2016; Le *et al.*, 2008). Area under curve (AUC) of average color well development (AWCD) which gives better insights for curve dynamics, was calculated with the trapezoidal method for each soil using “caTools” (1.18.2) packaging.

Regarding the amplicons-based sequencing data, low abundant ASVs with a maximum relative abundance below 0.1% per sample were discarded using “filter.OTU” function from *RAM* package (1.2.1.7) (Chen *et al.*, 2018). The diversity was estimated using the Simpson’s diversity index while richness metric was counted based on observed ASVs, among every samples. Richness (observed ASVs) and diversity (Simpson’s index) values were calculated employing the *rtk* (0.2.6.1) package, averaging the results obtained after 999 rarefactions (Saary *et al.*, 2017). Richness and diversity metrics were compared between compartments, rootstock

genotype, sampling year, and soil status by means of pairwise comparisons from *RVAideMemoire* (0.9-81) (Hervé, 2021). Prior to any beta-diversity calculation, differences in sequencing depth were addressed applying the median of ratios method implemented in the DESeq2 Bioconductor package (Love *et al.*, 2021). The differences between microbial communities were investigated using Bray-Curtis dissimilarity distance. Multivariate analysis of bacterial and fungal communities was performed based on constrained multidimensional scaling using Constrained Analysis of Principal Components (CAP) from *vegan* (Oksanen *et al.*, 2020). The significance of rootstock genotype, compartment, and soil status factors for each sampling year used as constraint in the CAP was assessed via the permutation test from *vegan*. Bray-Curtis dissimilarity distances were also investigated using permutational multivariate analysis of variance (PERMANOVA) based on “adonis” function from *vegan* package (Anderson, 2001). Dissimilarities in the relative abundance of bacterial and fungal communities were visualized by network analysis of Bray-Curtis distances using the “make_network” and “plot_network” functions from the *phyloseq* package.

MicrobiomeMarker (version 1.1.1) was used for “limma_voom” function to discriminate microbial above family taxa between vineyard soil used for greenhouse experiment (Cao, 2020).

Visualization of bacterial endophytic taxa

DOPE-FISH microscopy was carried out to visualize bacterial taxa within surface sterilized root samples, from the first subgroup described above, of the four conditions during the first year of sampling. Fixation was carried out overnight at 4°C, in a paraformaldehyde solution (4% w/v in PBS pH 7.2) and rinsed three times with PBS. Samples were then treated with a lysozyme solution (1 mg ml⁻¹ in PBS) for 10 min at 37°C, followed by dehydration in an ethanol series (25%, 50%, 75%, and 99.9%; 15 min each step). DOPE-FISH was performed after cutting samples into small pieces, and then using probes from Eurofins (Germany) labelled at both 5' and 3' positions, summarized in (Table S22). A mixEUB (equivalent mixture of EUB338, EUB338II, EUB338III coupled with a Cy3 fluorochrome), a Chit probe specific to *Chitinophaga*, a Rhizo4 a probe specific to *Rhizobium* (16S), a Pseu22 probe specific to *Pseudomonas* from C3, C4, C5 clusters (16S), and a Pce probe specific to *Burkholderia* (23S), all coupled to Cy5 fluorochrome, were used. A NONEUB probe, coupled with Cy3 and Cy5, was also used independently as a negative control. Hybridization was performed at 46°C for all the probes except for the Pce which was done at 40°C, during 2 h 30 min, with 20 µl hybridization solution applied to each plant sample, placed on slides in a 50 ml moist chamber

(also housing a piece of tissue imbibed with 5 ml of hybridization buffer). Each hybridization solution contained 20 mM Tris-HCl pH 8.0, 0.01% w/v SDS, 0.9 M NaCl, formamide at the concentration adapted for each probe: 15 ng μl^{-1} for a general probe, and 10 ng μl^{-1} for a specific probe. Post-hybridization was performed in 20 μl at 48°C for 30 min with a post-FISH pre-warmed solution containing 20 mM Tris-HCl pH 8.0, 0.01% (w/v) SDS, 5 mM EDTA pH 8.0 and NaCl at a concentration corresponding to the formamide concentration used. Samples were rinsed with distilled water before being air-dried in the dark.

The samples were then observed under a confocal microscope (Olympus Fluoview FV1000 with multiline laser FV5-LAMAR-2 HeNe(G) and laser FV10-LAHEG230-2). X, Y, Z pictures were taken at 405, 488, 633 nm and with 20X objectives. Pictures were analyzed on Imaris software. Pictures were cropped and whole pictures were sharpened. The light/contrast balance was also improved to better observe the image details, as seen when samples are observed in the dark under the microscope. Images shown in this publication represent the average of colonization.

Results

Initial matrix soil used for greenhouse experiment displayed different microbial communities

A total of 22,030,894 bacterial 16S rRNA gene sequences and 24,753,799 fungal ITS sequences were generated from 108 samples covering 36 sample types from each of three replicates which encompass two different years of sampling of bulk soil, rhizosphere, and roots from greenhouse plus bulk soil from vineyard and roots of young plants before planting. Subsequently to quality filtering, denoising, merging, chimera, and contaminant removing, 8,553,704 bacterial 16S rRNA gene sequences and 14,764,550 fungal ITS sequence remained and generated 31,096 bacterial and 7,994 fungal Amplicon Sequences Variants (ASVs). ASVs having less than 0.1% sequencing depth were removed.

Concerning the initial matrix soil originated from vineyard used for greenhouse experiment, 915 bacterial ASVs (**Figure 36.A**) and 584 fungal ASVs (**Figure 36.B**) were shared between AS and S soils, regardless of sampling year. ASVs specific to AS condition were accounted for 522 in bacterial kingdom and 142 in fungal kingdom, while 118 bacterial and 222 fungal ASVs were specific to S condition.

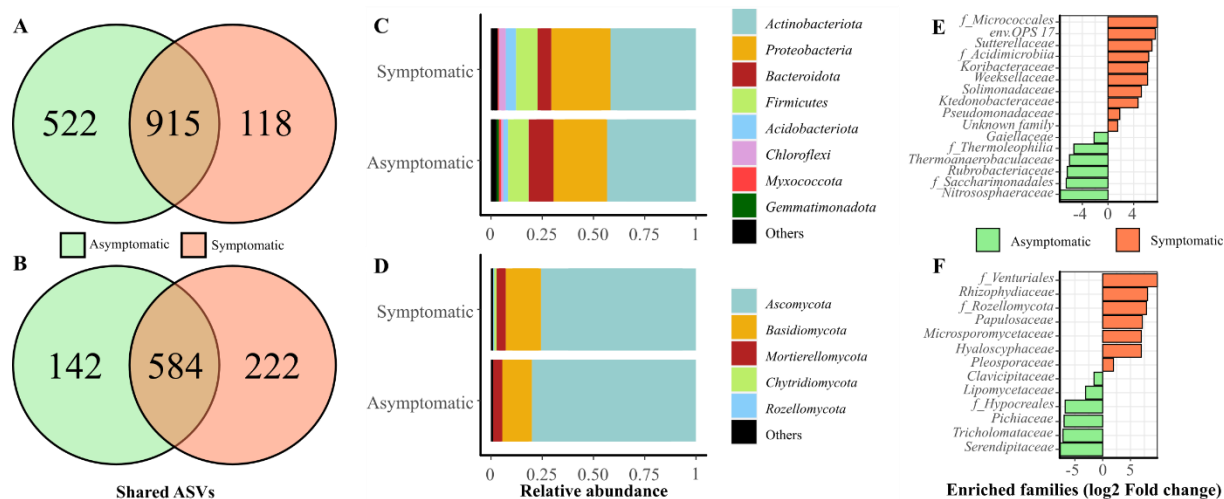


Figure 36 : Microbial comparison between vineyard symptomatic S and asymptomatic AS soils used for greenhouse experiment. Venn diagrams encompassing shared and distinct ASVs for (A) bacterial, and (B) fungal communities. Abundance at phylum level for S and AS among (C) bacterial and (D) fungal communities, where phyla individually representing less than 1% of the total communities were grouped in “Others”. Enriched (E) bacterial and (F) fungal class, order, and families using Limma-Voom differential analysis ($P < 0.001$; FDR).

The predominant bacterial phyla are represented in **Figure 36.C**. Bacterial phyla representing less than 1% abundance in AS and S soils, respectively, belong to *Desulfobacterota* (0.80%, 0.75%), *Nitrospirota* (0.47%, 0.27%), *Crenarchaeota* (0.38%, 0.22%), *RCP2-54* (0.12%, 0.33%) *Verrucomicrobiota* (0.11%, 0.23%), *Bdellovibrionota* (0.12%, 0.15%), *Patescibacteria* (0.12%, 0%), *Fibrobacterota* (0%, 0.03%). The predominant fungal phyla are represented in **Figure 36.D** with phyla representing “Others” group in AS and S soils, respectively, belonging to *Mucoromycota* (0.05%, 0.15%) and *Glomeromycota* (0.02%, 0%). Dominant bacterial classes for both sampling years in AS and S soils were, respectively, *Actinobacteria* (31.70%, 34.99%), *Gammaproteobacteria* (14.96%, 20.62%), *Alphaproteobacteria* (11.61%, 8.76%), *Bacilli* (9.25%, 9.49%), *Bacteroidia* (12.25%, 6.45%), *Thermoleophilia* (8.85, 5.76%), and *Acidobacteriae* (2.11%, 5.16%). In regard to fungi, most represented classes were *Sordariomycetes* (35.24%, 36.64%), *Dothideomycetes* (19.19%, 19.22%), *Tremellomycetes* (13.68%, 15.87%), *Leotiomycetes* (15.08%, 10.45%), *Eurotiomycetes* (6.77%, 6.62%), and *Mortierellomycetes* (4.47%, 4.77%).

Limma-Voom differential analysis was performed to get a better overview on the differences occurring across the bacterial (**Figure 36.E**) and fungal (**Figure 36.F**) communities between the two soils. This analysis detected ten enriched bacterial groups in S soil (1.56 to 7.82 log₂ fold change), mainly composed of *Proteobacteria* (*Sutterellaceae*, *Solimonadaceae*, *Pseudomonadaceae*) and *Bacteroidota* (*env.OPS 17*, *Weeksellaceae*), while AS soil was enriched with six groups (-2.17 to -7.57 log₂ fold change) with a majority of *Actinobacteria*

(*f_Thermophilia*, *Gailllellaceae*, *Rubrobacteriaceae*). Regarding fungi, seven enriched families were found in S soil (1.91 to 9.86 log₂ fold change), accounting from a majority of *Ascomycota* phylum (*Papulosaceae*, *f_Venturiales*, *Hyaloscyphaceae*, *Pleosporaceae*), while six enriched families were detected in AS soil (-1.50 to -7.76 log₂ fold change) mainly belonging to *Ascomycota* phylum (*f_Hypocreales*, *Clavicipitaceae*, *Lipomycetaceae*, *Pichiaceae*).

Table 17 : Factors effects related to soil composition (S, AS), rootstock genotype (RGM, 1103P), and sampling year (Year 1, Year 2) on richness, diversity (Simpson), and β -diversity (Bray-Curtis) based on bacterial and fungal communities among the bulk, rhizosphere, and roots compartments in the greenhouse disposal.

| | | | Richness | | α -diversity | | β -diversity | | |
|----------|-------------|-------------|----------|------------------|---------------------|------------------|--------------------|----------------|--------------|
| | | | F | P | F | P | F | R ² | P |
| Bacteria | Vineyard | Soil | 1.147 | 0.312 | 4.553 | 0.041 | 34.203 | 0.490 | 0.001 |
| | | Year | 13.841 | 0.005 | 0.308 | 0.592 | 21.993 | 0.315 | 0.001 |
| | Bulk | Soil(S) | 0.860 | 0.364 | 0.551 | 0.467 | 9.693 | 0.238 | 0.001 |
| | | Genotype(G) | 0.043 | 0.837 | 1.374 | 0.256 | 1.210 | 0.029 | 0.259 |
| | | Year | 0.253 | 0.620 | 2.406 | 0.137 | 9.151 | 0.225 | 0.001 |
| | S × G | S × G | 5.031 | 0.037 | 0.560 | 0.463 | 1.595 | 0.039 | 0.129 |
| | | Soil | 1.045 | 0.319 | 2.961 | 0.101 | 14.437 | 0.308 | 0.001 |
| | | Genotype | 0.021 | 0.885 | 0.042 | 0.841 | 1.118 | 0.024 | 0.273 |
| | Year | Year | 4.629 | 0.045 | 10.011 | 0.005 | 11.460 | 0.245 | 0.001 |
| | | S × G | 0.399 | 0.535 | 0.001 | 0.977 | 0.798 | 0.017 | 0.521 |
| | | Soil | 1.648 | 0.215 | 0.001 | 0.996 | 2.632 | 0.085 | 0.001 |
| | Root | Genotype | 6.866 | 0.017 | 1.577 | 0.224 | 2.132 | 0.069 | 0.003 |
| | | Year | 0.808 | 0.380 | 1.440 | 0.245 | 5.813 | 0.189 | 0.001 |
| | | S × G | 0.086 | 0.773 | 2.610 | 0.123 | 1.232 | 0.040 | 0.192 |
| Soil | | 0.797 | 0.395 | 8.273 | 0.018 | 16.974 | 0.351 | 0.001 | |
| Fungi | Vineyard | Year | 144.183 | <0.001 | 62.261 | <0.001 | 16.887 | 0.349 | 0.001 |
| | | Soil | 1.328 | 0.263 | 0.684 | 0.419 | 10.648 | 0.226 | 0.001 |
| | Bulk | Genotype | 0.922 | 0.350 | 2.561 | 0.126 | 0.947 | 0.020 | 0.403 |
| | | Year | 14.951 | 0.001 | 15.109 | <0.001 | 15.623 | 0.331 | 0.001 |
| | | S × G | 2.047 | 0.169 | 1.082 | 0.311 | 0.908 | 0.019 | 0.442 |
| | Rhizosphere | Soil | 0.222 | 0.643 | 0.104 | 0.751 | 11.822 | 0.254 | 0.001 |
| | | Genotype | 0.015 | 0.904 | 0.014 | 0.907 | 1.580 | 0.033 | 0.132 |
| | | Year | 5.280 | 0.033 | 7.127 | 0.015 | 12.901 | 0.278 | 0.001 |
| | S × G | S × G | 1.372 | 0.256 | 0.058 | 0.812 | 1.172 | 0.025 | 0.291 |
| | | Soil | 3.804 | 0.066 | 15.161 | <0.001 | 1.868 | 0.065 | 0.010 |
| | | Genotype | 5.058 | 0.037 | 6.518 | 0.019 | 2.557 | 0.089 | 0.001 |
| | Year | Year | 130.83 | <0.001 | 6.401 | 0.020 | 4.279 | 0.149 | 0.001 |
| | | S × G | 0.396 | 0.537 | 0.771 | 0.391 | 1.083 | 0.038 | 0.306 |

Significances were assessed through a Type II ANOVA for richness and α -diversity while PERMANOVA was used for β -diversity with F(1,19) for bulk, rhizosphere, and root compartments, while F(1,9) for vineyard soils. P values below 0.05 are represented in bold.

The richness of bacterial and fungal communities (*i.e.*, observed ASVs), as well as fungal diversity (*i.e.*, Simpson's index), were significantly impacted by the sampling year, while bacterial diversity was significantly influenced by soil status (**Table 17**).

The two soils S and AS described were used as matrix soil for the greenhouse experiment with two scion × rootstock combinations, namely CS×RGM and CS×1103P.

Soil composition and rootstock genotype influenced the microbial communities

Regarding amplicons-based sequencing of 16S rRNA gene and ITS in the greenhouse experiment, bacterial communities were largely composed of *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (**Figure 37.A**) while *Ascomycota* and *Basidiomycota* were predominant in the fungal division (**Figure 37.B**). Sampling year was significantly driving α - and β -diversity in most of the compartment for both bacterial and fungal communities (**Table 17**). The highest numbers of bacterial and fungal ASVs were observed in rhizosphere from S-1103P and AS-1103P conditions, respectively, while the lowest were found in the roots from S-RGM and roots from 1103P rootstock before planting (*i.e.*, roots from nursery), respectively (**Table S23**). Interestingly, genetic background of the rootstock significantly affected both bacterial and fungal richness, as well as α - and β -diversity exclusively in roots. The soil status significantly impacted the β -diversity of both bacterial and fungal communities in every compartment, while only the fungal α -diversity in roots was driven by the soil composition. This segregation between AS and S soils was more pronounced in the bulk and rhizosphere compartments than in the roots in each year for both microbial communities (**Table 17**).

Furthermore, CAP based on the two rootstock genotypes, two soils, and three compartments displayed for both rootstocks that bulk and rhizosphere clustered together, distinctly to the roots, and were grouped depending on the soil status for both bacterial (**Figure 37.C**) and fungal communities (**Figure 37.D**).

From this greenhouse dataset, PERMANOVA revealed that the most influencing factor on both bacterial and fungal communities was the compartment (*i.e.*, rhizosphere, bulk soil, and roots) while soil status and sampling year had similar effects (**Table 18**).

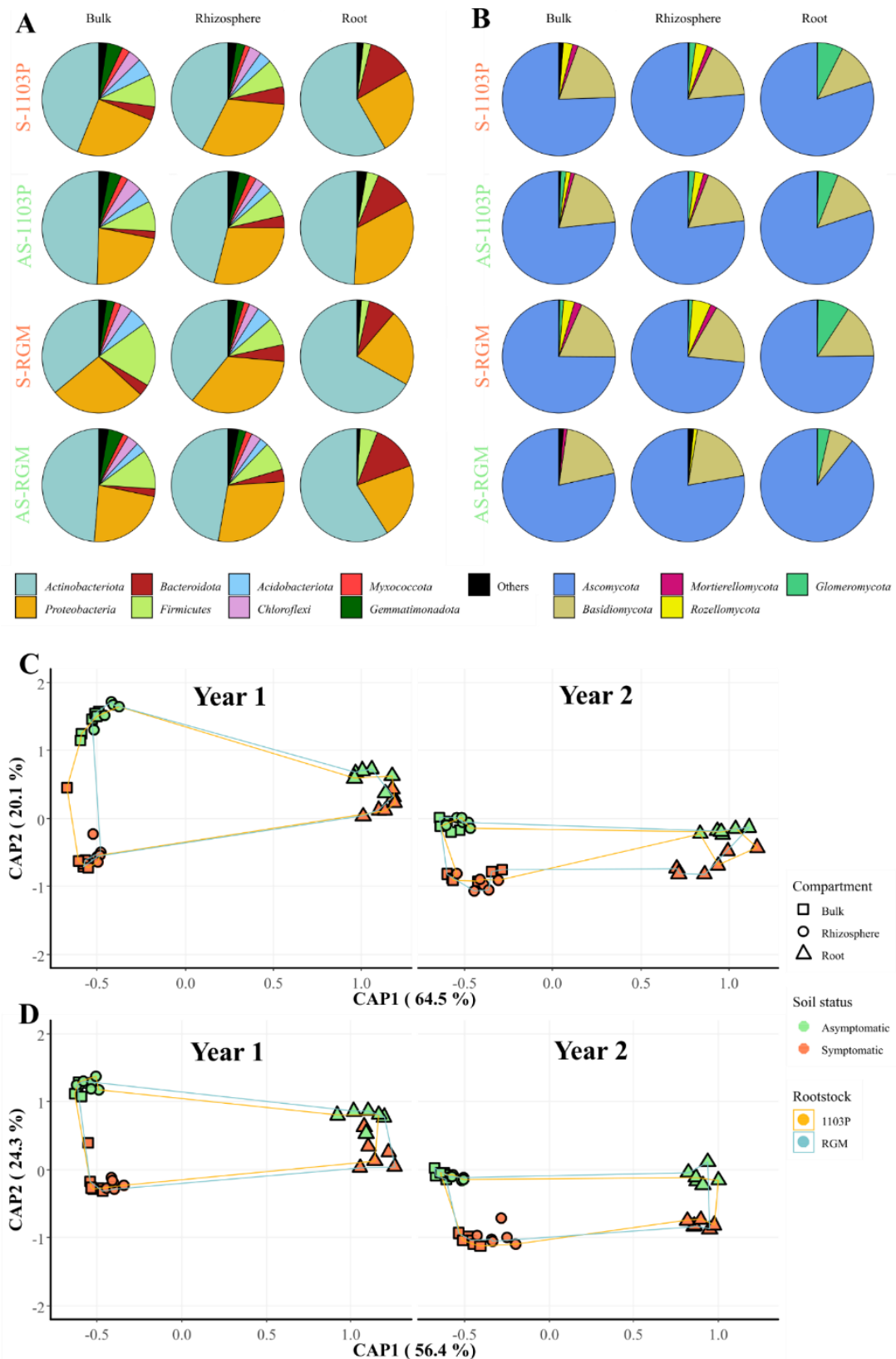


Figure 37 : Bacterial and fungal communities across the conditions. Relative abundances at the phylum level among the four conditions (S-1103P, AS-1103P, S-RGM, AS-RGM) in the bulk, rhizosphere, and root compartments for (A) bacterial and (B) fungal communities. Phyla accounting for less than 1% of the total abundance in communities were grouped in “Others”. Constrained analysis of principal coordinates (CAP) of samples by compartment related to P and RGM rootstocks grown in Symptomatic and Asymptomatic soils in greenhouse, repeated twice (Year 1 and Year 2) for (C) bacterial and (D) fungal communities.

Table 18 : Experimental factors predicting β -diversity of bacterial and fungal communities in the dataset from greenhouse experiment, based on Bray-Curtis distance.

| | | Richness (obs) | | α -diversity (Simpson) | | β -diversity | |
|----------|-------------|----------------|------------------|-------------------------------|------------------|--------------------|--------------|
| | | F | P | F | P | F | P |
| Bacteria | Soil | 0.454 | 0.503 | 0.002 | 0.967 | 11.65 | 0.001 |
| | Compartment | 120.31 | <0.001 | 55.512 | <0.001 | 24.25 | 0.001 |
| | Rootstock | 1.441 | 0.234 | 1.793 | 0.185 | 1.44 | 0.144 |
| | Year | 0.423 | 0.517 | 1.420 | 0.238 | 10.72 | 0.001 |
| Fungi | Soil | 2.996 | 0.088 | 8.892 | 0.004 | 11.60 | 0.001 |
| | Compartment | 224.196 | <0.001 | 28.604 | <0.001 | 17.13 | 0.001 |
| | Rootstock | 1.717 | 0.194 | 5.259 | 0.025 | 1.77 | 0.068 |
| | Year | 44.178 | <0.001 | 24.148 | <0.001 | 11.78 | 0.001 |

Significances were assessed through a Type II ANOVA for richness and α -diversity while PERMANOVA (permutations = 999) was calculated by terms from “capscale” function using Soil + Compartment + Rootstock + Year model.

Concerning the cultivable methods, 200 isolates per condition (*i.e.*, AS-1103P, S-1103P, AS-RGM, and S-RGM) were analyzed on MALDI-TOF-MS. Among the 800 isolates, 401 (50%) of the mass profiles matched to the Biotyper database with a score values > 1.8 . Asymptomatic soils had lower identification with 169 isolates (21%) compared to the 230 isolates (28%) from the S soils. Score values above 1.7 matched for 36 genera while the score values above 2.0 matched for 83 species. The isolated bacteria were predominantly members of the genus *Bacillus* (16.6%), followed by *Pseudomonas* (5.25%), *Arthrobacter* (4%), and *Burkholderia* (3.4%) while the less frequently detected genera were categorized in the “Others” group (**Figure 38.A**). These last isolates belonged to the genera *Ralstonia*, *Buttiauxella*, *Variovorax*, *Paenarthrobacter*, *Rhizobium*, *Streptomyces*, *Flavobacterium*, *Peanibacillus*, *Dyella*, *Serratia*, *Caballeronia*, *Brevibacillus*, *Microbacterium*, *Sphingomonas*, *Falsibacillus*, *Staphylococcus*, *Acinetobacter*, *Amicolaptosis*, *Aquicola*, *Brachybacterium*, *Cupriavidus*, *Gordonia*, *Herbaspirillum*, *Leifsonia*, *Rhodococcus*, and *Sinomonas*. Distinct genera were specific to each condition (**Figure 38.B**), with 1 genus being common to the S soils (*Rhizobium*), 3 genera common to the AS soils (*Peanibacillus*, *Brevibacillus*, *Buttiauxella*), but interestingly no identified genera were exclusively specific to the RGM, or 1103P rootstock. The Simpson’s index generated from these isolates was higher among the conditions in AS soils compared to the S soils, and lower in the RGM compared to the 1103P rootstock (**Figure 38.C**).

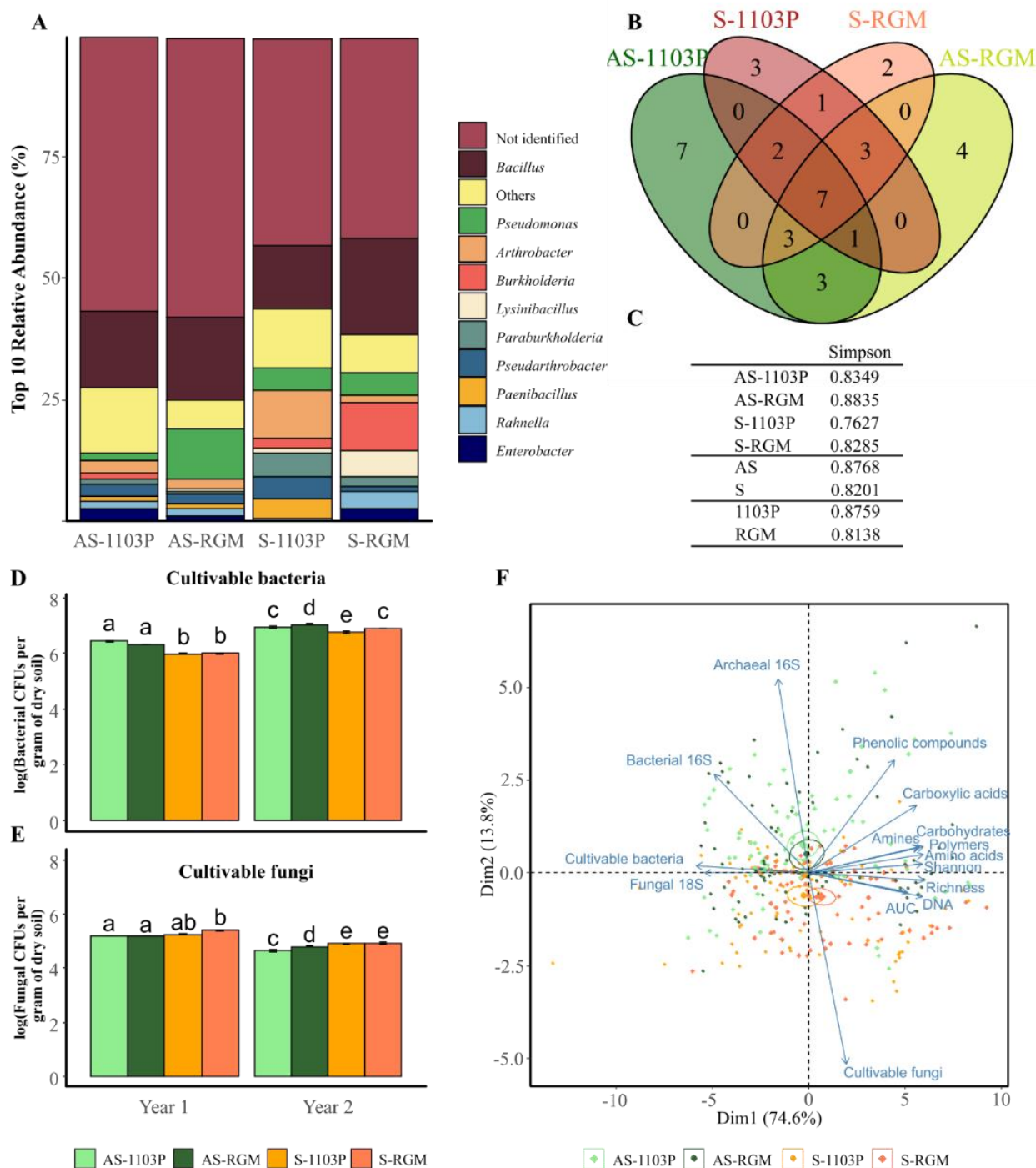


Figure 38 : Rhizosphere microbial profile using cultivable-based approaches and q-PCR measurements. Diversity of cultivable bacteria isolated from rhizosphere and identified among the 4 conditions through MALDI-TOF-MS, demonstrated by (A) the relative abundance of the top 10 taxa at the genus level, (B) the Venn diagram illustrating the overlap of the genera, and (C) the associated Simpson's diversity index. Histograms representing the level of populations of cultivable (D) bacteria and (E) fungi for both sampling years. Bars represent means \pm SE (n = 3). (F) Ordination biplot of principal component analysis (PCA) for level of cultivable microorganisms, Eco-Plates measurements (AUC, Shannon's evenness index, functional richness, and the families of consumed substrates), the total DNA extracted from the rhizosphere and the amplicons (fungal 18S, archaeal and bacterial 16S genes).

Impact of time on microbial community structure in bulk soil

This study was carried out twice with plants from similar scion × rootstock combinations, as well as freshly excavated soil substrate from the same vineyard. Regardless the sampling year, initial substrate soils S and AS from vineyard used for the greenhouse experiment displayed different microbial profiles (**Figure 36; Table 17**).

Nevertheless, time spent in greenhouse had some effects on the microbial diversity and richness of the soil between the soil right after sampling and after the greenhouse experiment (**Figure S11.A**). The bacterial richness was more affected by the time spent in greenhouse while its diversity was globally similar. At the opposite, the richness of fungal communities was less impacted than the diversity by the time spent in greenhouse.

In addition, CAP based on the bulk soil from the greenhouse experiment and bulk soil collected in the vineyard demonstrated segregation in bacterial (**Figure S11.B**) and fungal communities (**Figure S11.C**), mainly due to soil status for both sampling years.

Specific genera were enriched in both investigated vineyard and greenhouse bulk soils, regardless of sampling year (**Figure S11.B**), with higher number of bacterial and fungal taxa enriched in vineyards (36 and 24, respectively) compared to greenhouse (7 and 13, respectively).

Rootstock genotype had more influence than soil status on grapevine growth

Rootstocks included in this study have different genetic backgrounds and are especially bred to provide distinct agronomic traits (Ibacache *et al.* 2020) (**Table S24**). During these four and a half months of growth repeated twice, some additional sampling points were performed to better evaluate the growth performance on the different symptomatic and asymptomatic soils substrate. Some significant differences were found in growth between RGM and 1103P rootstocks related to aerial and root parameters (**Figure S12; Table S25**). It appeared that significantly higher growth in both root and aerial systems were observed, exclusively for CS×1103P combination, in asymptomatic condition compared to the symptomatic one at the end of the greenhouse experiment during the first year.

Vineyard and nursery microbiome contributions to root associated microbiome in the greenhouse experiment

Network analysis of Bray-Curtis distances clustered the root compartment from greenhouse and root from nursery, and separated them from the other cluster consisting in bulk, rhizosphere, and vineyard soil for bacterial communities (**Figure 39.A**).

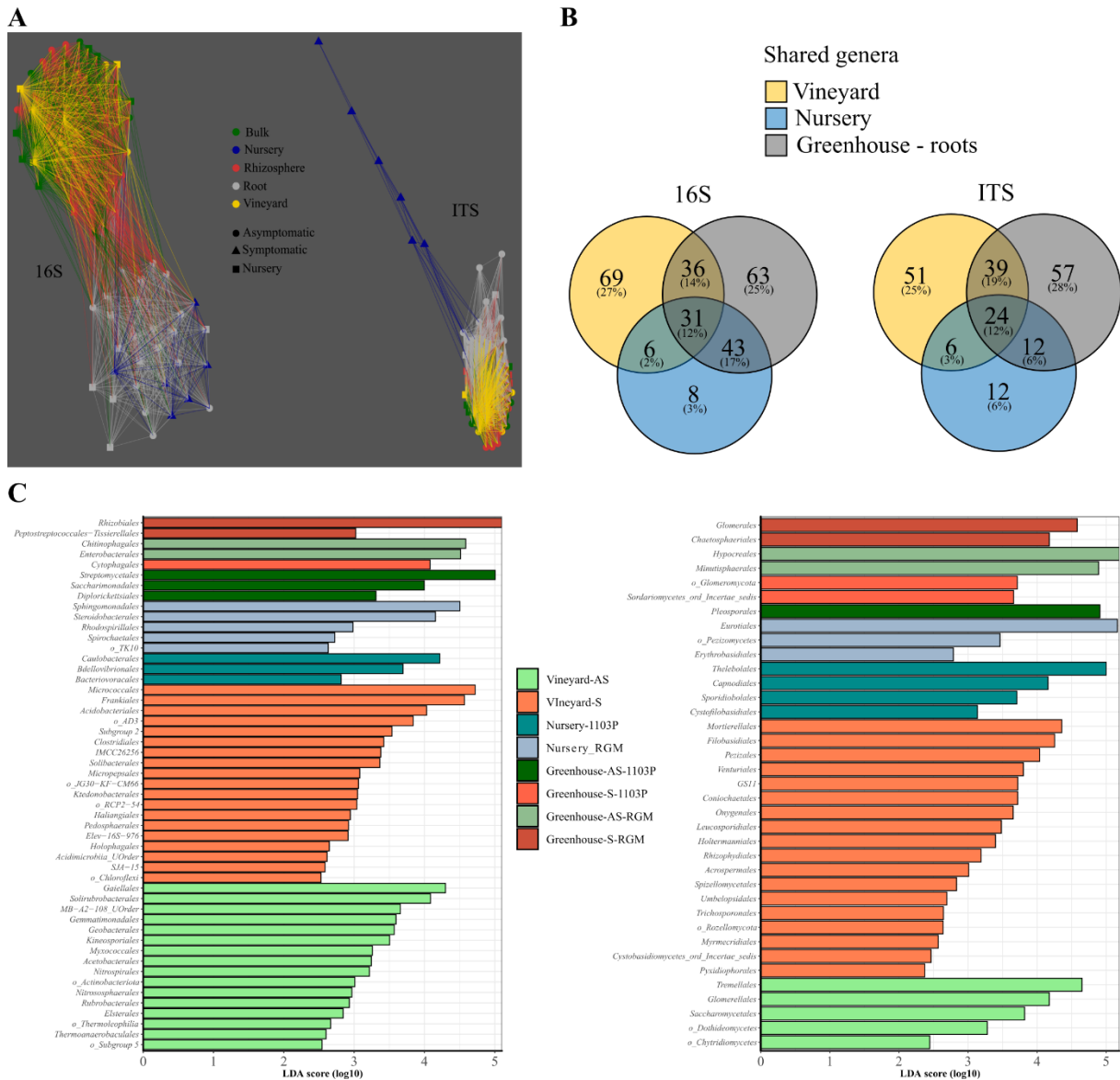


Figure 39 : Vineyard and nursery microbiome contributions to root associated microbiome from greenhouse disposal. **(A)** Network analysis of bacterial (16S) and fungal (ITS) taxa, in terms of relative abundances, in vineyard, nursery, and greenhouse compartments (i.e., bulk, rhizosphere, root), using Bray–Curtis distances less than 0.95. **(B)** Venn diagram presenting the shared bacterial and fungal genera between vineyard, nursery, and root compartment from the greenhouse disposal. **(C)** LEfSe displaying the enriched orders in vineyard (symptomatic S and asymptomatic AS), nursery (1103P and RGM rootstocks), and root compartment from greenhouse experiment in each condition (AS-1103P, S-1103P, AS-RGM, S-RGM).

Moreover, 3% and 6% of the common bacterial (i.e., *Nordella*, *Paenisporosarcina*, *Allokutzneria*, *Salinispira*, *Phaselicystis*, *Peredibacter*, *FFCH7168*, *SWB02*) and fungal (i.e.,

mainly *Ascomycota* from *Ramularia Debaryomyces*, *Neosetophoma*, *Botrytis*, *Vermiconia*, *Microdochium*, *Zymoseptoria*) genera, respectively were distinct to nursery samples and were not detected in root samples from greenhouse (**Figure 39.B**). Enriched taxa were largely found in vineyard soils, accounting for 35 bacterial and 23 fungal orders, while only 8 and 7, respectively, were found in nursery (**Figure 39.C**).

In addition, some fungal genera associated to known grapevine diseases listed in **Table S26**, were detected across the samples belonging to *Botrytis*, *Cadophora*, *Curvularia*, *Diaporthe*, *Diplodia*, *Ilyonectria*, *Phaeoacremonium*, and *Phaeomoniella* (**Figure 40**).

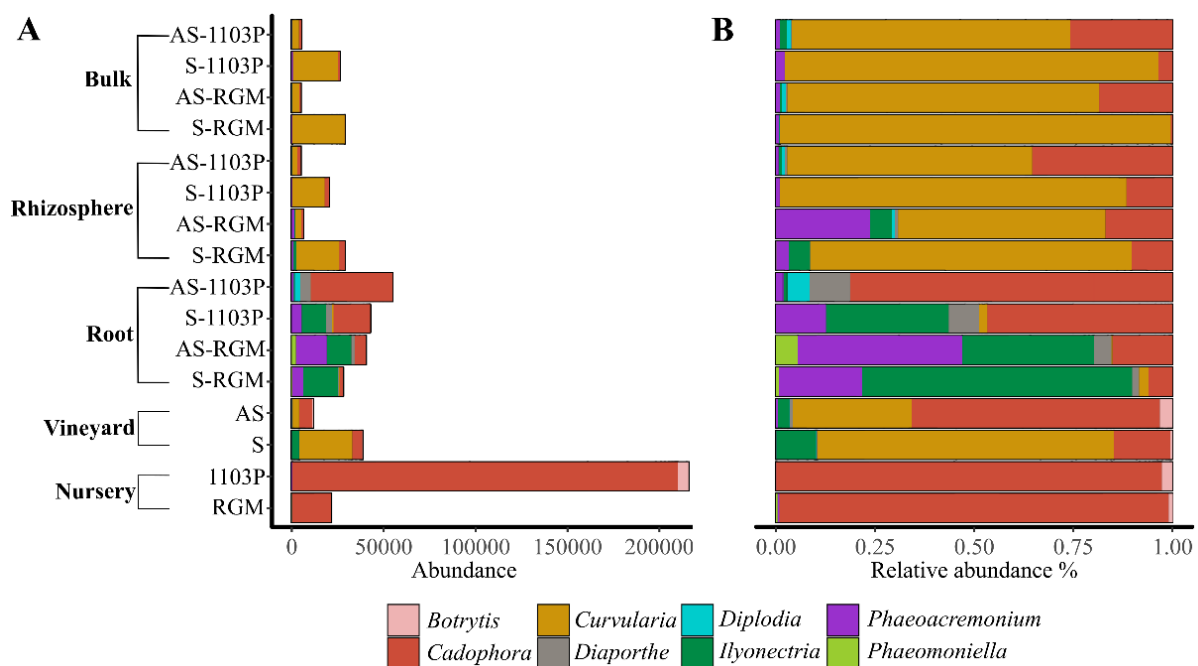


Figure 40 : Fungal genera associated to grapevine diseases in the greenhouse experiment (*i.e.*, bulk, rhizosphere, root), vineyard, and the root before planting (*i.e.*, nursery) related to symptomatic (S) or asymptomatic (AS) soils using 1103P or RGM rootstocks.

Regarding these fungal pathogens, significant differences were observed among the different conditions (**Table S27**), with significantly contrasted richness and Simpson's index in RGM roots between S and AS modalities. Overall, they revealed higher abundances in symptomatic initial soil compared to the asymptomatic condition, as well as initially more important abundances in 1103P from nursery compared to RGM rootstock ($F(2, 712) = 14.092, P < 0.001$) (**Figure 40.A**).

Microbial activities and level of cultivable populations in the rhizosphere compartment differed according to the soil composition

The biplot PCA for q-PCR, level of cultivable microbes, and q-PCR measurements in the rhizosphere revealed two overlaps of confidence between the S-1103P and S-RGM conditions,

as well between AS-1103P and AS-RGM (**Figure 38.F**). Dimensions (Dim1 and Dim2) accounted for 88.4% of total variance. Symptomatic samples were mostly found in the negative side of Dim2, which was correlated with the level of population of cultivable fungi, the total extracted DNA, the general activities, and richness measured in Eco-Plates (*i.e.*, AUC, richness, respectively). On other hand, asymptomatic samples were mainly found in the positive side of Dim2, which correlated with all the other measurements including the level of population of cultivable bacteria, the number of bacterial and archaeal 16S genes, and all the rest of Eco-Plates measurements (*i.e.*, Shannon diversity, amino acids, polymers, carbohydrates, amines, carboxylic acids, and phenolic compounds).

Table 19: Effects of the rootstock genotype (1103P or RGM), the rhizosphere status (S or AS) and the sampling year (Year1 and Year2) on the microbial level of cultivable populations, the Biolog system parameters, the microbial DNA, and the q-PCR measurements. Significances were assessed through a Type II ANOVA.

| | Rootstock | | Soil | | Year | | |
|---------------------|---------------------|--------------|--------------|------------------|------------------|------------------|------------------|
| | F | P | F | P | F | P | |
| Cultivable bacteria | 11.68 | 0.001 | 43.61 | <0.001 | 403.93 | <0.001 | |
| Cultivable fungi | 8.629 | 0.004 | 49.603 | <0.001 | 164.001 | <0.001 | |
| Biolog™ System | Area Under Curve | 12.33 | 0.002 | 48.59 | <0.001 | 781.71 | <0.001 |
| | Shannon's evenness | 0.178 | 0.678 | 0.030 | 0.865 | 201.371 | <0.001 |
| | Functional richness | 0.024 | 0.880 | 8.489 | 0.009 | 158.114 | <0.001 |
| | Amines | 0.554 | 0.466 | 1.330 | 0.263 | 98.976 | <0.001 |
| | Amino Acids | 1.529 | 0.231 | 15.655 | <0.001 | 273.105 | <0.001 |
| | Carbohydrates | 0.015 | 0.903 | 14.349 | <0.001 | 563.578 | <0.001 |
| | Carboxylic acids | 0.022 | 0.884 | 9.353 | 0.006 | 122.215 | <0.001 |
| | Phenolic compounds | 1.689 | 0.209 | 2.157 | 0.158 | 137.548 | <0.001 |
| | Polymers | 1.043 | 0.320 | 3.951 | 0.061 | 73.700 | <0.001 |
| | q-PCR | DNA | 0.189 | 0.666 | 1.295 | 0.260 | 179.021 |
| Bacterial 16S | | 2.056 | 0.157 | 21.367 | <0.001 | 122.098 | <0.001 |
| Archaeal 16S | | 0.375 | 0.543 | 41.456 | <0.001 | 39.956 | <0.001 |
| Fungal 18S | | 0.239 | 0.627 | 1.016 | 0.318 | 86.702 | <0.001 |

Results from the q-PCR analysis revealed significantly higher level of archaeal and bacterial amplicons in AS rhizosphere compared to S rhizosphere for both rootstocks during the two years of sampling, while no differences were detected for fungal amplicons (**Data not shown**). Cultivable bacteria associated with the rhizosphere soil of the RGM and 1103P rootstocks grown in S and AS soils during both years of sampling ranged from 10^5 to 10^8 CFUs/g (**Figure 38.D**) while cultivable fungi ranged from 10^4 to 10^7 CFUs/g (**Figure 38.E**). Plating methods revealed that the level of cultivable bacteria was significantly different among the four conditions at the final point of sampling, during the first year ($F(3, 8) = 113.1$, $P < 0.001$), and the second year ($F(3, 8) = 15.67$, $P < 0.001$) (**Figure 38.D**) with a higher level observed in the

AS soil compared to the S one for both rootstocks. In parallel, significant differences were also observed at the final point of sampling for the level of cultivable fungi during the first year ($F(3, 8) = 34.97, P < 0.001$), and the second year ($\chi^2 = 27.578, \text{ddl} = 3, P < 0.001$) but with a lower level observed in the AS soil compared to the S one for both rootstock (**Figure 38.E**).

Besides the year of sampling, the status of the rhizosphere represented by either symptomatic or asymptomatic soils had a higher effect on the abundance of microorganisms as observed by cultivation and q-PCR than the rootstock genotype (**Table 19**).

Microbes of both soils showed the same activities in Eco-Plates measurements, but with different intensities (**Table S28**). The microbial activities represented by the AUC from the Biolog Eco-Plates™ technology were significantly more important for S soils compared to AS ones for both rootstock during the first year ($F(3, 8) = 25.25, P < 0.001$), and the second year ($F(3, 8) = 34.1, P < 0.001$).

Visualization of endophytic microorganisms associated to roots

Based on ITS sequencing, the phylum *Glomeromycota* was globally enriched in roots (6.38%) compared to bulk (0.98%) and rhizosphere (1.30%) compartments across the four conditions (**Figure 37.B**), with higher amount in S (RGM: 8.98%, P: 7.31%) compared to AS (RGM: 3.50%, P: 5.68%) roots for RGM and 1103P rootstocks, independently of sampling year. The mycorrhizal colonization of the four conditions was investigated at the final point of sampling and only during the second year of sampling using staining and microscopy methods (**Figure S13**). Mycorrhizal colonization observed in microscope was consistent with the ITS-based sequencing analysis on Year 2 since higher intensity was found in S conditions (**Table S29**). The mycorrhizal frequency in roots was not significantly different among the conditions ($\chi^2 = 5.9862, \text{ddl} = 3, P = 0.1123$). However, the global intensity of the mycorrhizal colonization ($F(3,16) = 5.313, P = 0.001$) and within the samples were significantly different ($F(3,16) = 5.976, P = 0.006$) with AS-RGM condition having more colonization over S-RGM while no significant difference was observed for the 1103P rootstock.

Regarding the bacterial communities in roots, top bacterial genera were belonging to *Streptomyces*, *Stenotrophomonas*, *Pseudomonas*, *Cutibacterium*, *Chitinophaga*, *Novosphingobium*, *Burkholderia* (*Caballeronia-Paraburkholderia*), and *Rhizobium* (*Allorhizobium-Neorhizobium-Pararhizobium*) (**Figure S14**).

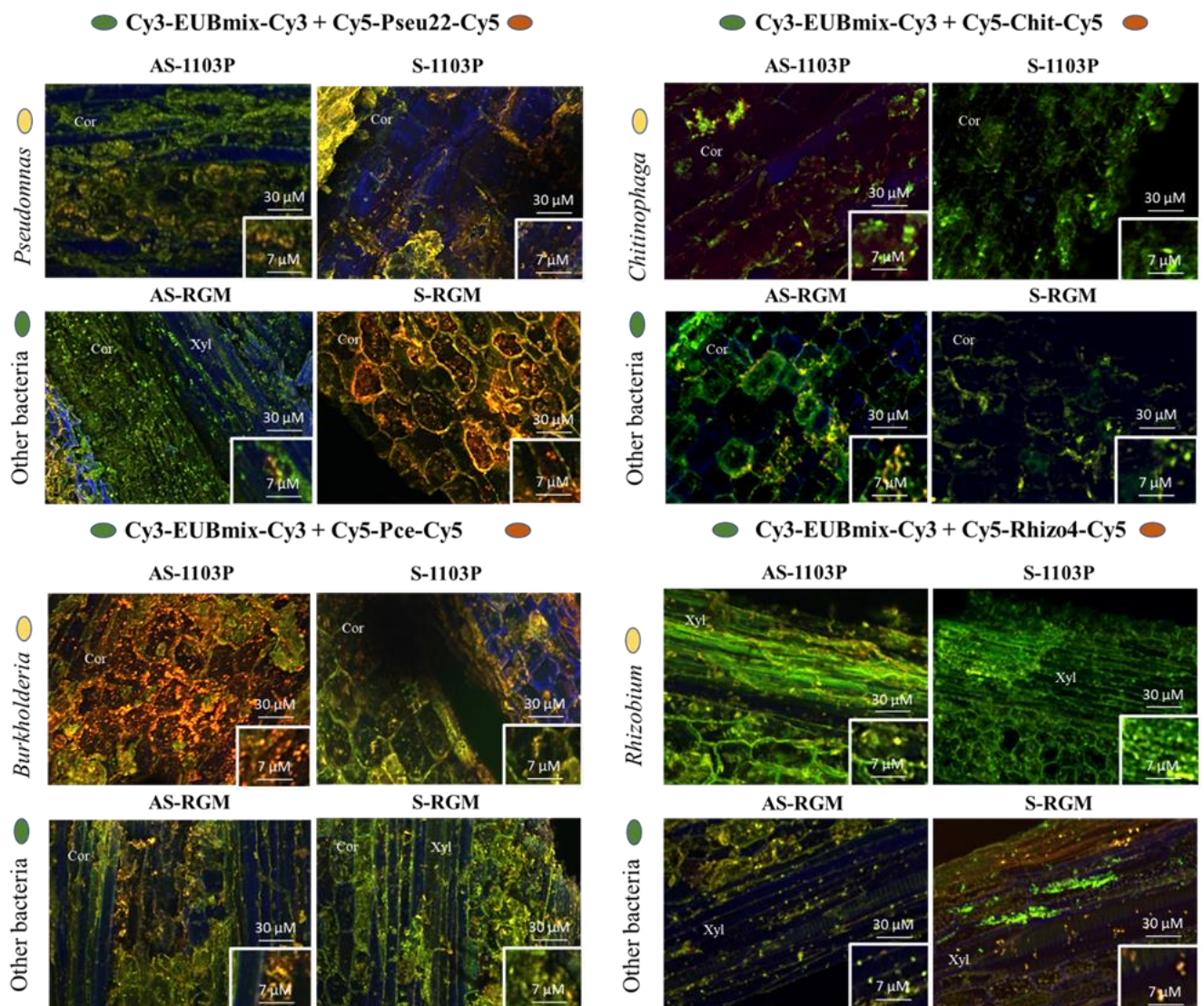


Figure 41 : Microphotographies of the root colonization by *Chitinophaga*, *Rhizobium*, *Burkholderia*, and *Pseudomonas* genera using DOPE-FISH microscopy across the different conditions within roots sampled during the first year of greenhouse experiment. Cor = cortex, Xyl = xylem.

Four of these genera were targeted for DOPE-FISH microscopy and were all visualized in root endosphere compartment of both RGM and 1103P rootstocks in either cortex or xylem zones (**Figure 41**). Naturally autofluorescent microbes were slightly detected in root endosphere using negative NONEUB probe, confirming the specificity of the probes used to target microorganisms (**Figure S15**).

Discussion

In this study, we characterized the bacterial and fungal communities in the roots, rhizosphere, and bulk soil of vine plants – Cabernet Sauvignon grafted onto RGM and 1103P rootstocks, respectively. These young vines were grown in two different soils. Those soils were from the same vineyard presenting unexplained decline features with an area with symptomatic (S) and asymptomatic (AS) vines. The two soils were chosen due to their quite similar physicochemical features and different microbial composition, described in Darriaut *et al.* (2021) as vineyard n°2.

Fungal and bacterial communities in bulk soil were affected by the time spent in greenhouse

The bulk soil is assumed to be the compartment least influenced by the rootstock due to its remote proximity to the roots. However, time spent in greenhouse modified the initial microbial diversity and richness found in vineyard bulk soil.

Soil microorganisms have complex interrelationships within natural soils, and their transposition into greenhouse experiment is known to simplify the co-occurrence network (Zhou *et al.*, 2020). In our case, the Simpson's index was rather impacted than richness for fungal communities, suggesting that their sensitivity to environment change is greater than for bacterial communities. Moreover, higher number of bacterial and fungal taxa were enriched in the bulk from the vineyard compared to the one from the greenhouse.

A reduced diversity or altered structure in pot experiment is expected compared to soils in their natural system (Berg *et al.*, 2016), which was partly true regarding the Simpson's index of both bacterial (Symptomatic soils during year 2) and fungal (Asymptomatic soils during year 1) communities (**Figure S11.A**). Pots in greenhouse for long-term cultivation are usually supplied with nutrient solution or are potted with plant substrate, which drastically diminish the microbial richness and diversity (Granzow *et al.*, 2017; Zachow *et al.*, 2014). Indeed, the interaction and cooperation among soil microbial individuals is supposed to be less important and less competitive when high level of soil nutrients are present (Wang *et al.*, 2017). Herein, the pots in greenhouse were daily watered without any nutrient addition, which rather affected the fungal diversity. Kaisermann *et al.*, (2015) demonstrated through small fluctuations of soil water content that fungal communities were largely affected, at the opposite of bacterial communities that were less sensitive to these small environmental constraints.

However, the initial differences in the composition of microbiomes associated with symptomatic and asymptomatic vineyard soils persisted during the greenhouse experiment and was differently affected.

The composition of the root endosphere microbiome is more sensitive to rootstock genotype and soil status than that of the rhizosphere and bulk soil compartments

Even though microbial richness and diversity were similar in bulk and rhizosphere compartments, the soil diversity metrics were significantly more important compared to roots (**Table S30**). Indeed, plant compartments provide specific microbial niches leading to distinct microbiome associations and functionalities (Rossmann *et al.*, 2017). The microbial diversity is usually lower with a higher degree of specialization proximal to the roots (Bonito *et al.*, 2014).

Despite the lack of consensus in the choice of primers for amplicon sequencing, the bacterial communities found in our samples through V5-V7 sequencing were consistent to previous findings with a predominant relative richness of *Actinobacteria*, *Proteobacteria*, *Bacteroidota*, *Firmicutes*, *Acidobacteria*, *Chloroflexi*, *Myxococcota*, and *Gemmatimonadota* (Berlanas *et al.*, 2019; Deyett & Rolshausen, 2020; Dries *et al.*, 2021; Samad *et al.*, 2017; Swift *et al.*, 2021).

The structure of root bacterial communities was distinct to the ones found in the rhizosphere and bulk soil, as reported in Swift *et al.* (2021). Overall, bacterial α -diversity was only significantly driven by the compartment factor, confirming the roots as a selective barrier for large panel of bacterial taxa. The most represented bacterial genera (*i.e.*, *Pseudomonas*, *Chitinophaga*, *Rhizobium*, and *Burkholderia*) among the four conditions were visualized using DOPE-FISH microscopy and revealed their presence in cortical cell layers or xylem vessels in roots for each condition.

Although this methodology has been described as lacking information in some cases due to high hypervariability, identification of fungal communities by amplicon sequencing is generally based on a region of the ITS (Kiss, 2012; Vu *et al.*, 2019). Here, two libraries were used based on both fungal ITS1 and ITS2 regions, the latter being created with two primers mixes by a nested PCR approach. Indeed, the fungal ITS2 barcoding region was found to recover more DNA sequences for fungal analysis than LSU, SSU, or even ITS1 (Schoch *et al.*, 2012), and remains the favorite molecular marker to study fungal communities (Tedersoo *et al.*, 2014).

The detected fungal communities were dominantly composed of *Ascomycota* and *Basidiomycota* in bulk soil (76.39%, 18.87%), rhizosphere (76.14%, 17.81%), and root

endosphere (81.16%, 12.26%), respectively, as reported in previous studies (Berlanas *et al.*, 2019; Deyett & Rolshausen, 2020; Martínez-Diz *et al.*, 2019; Swift *et al.*, 2021; Zahid *et al.*, 2021). While fungal richness was significantly influenced by sampling year and compartment, the fungal diversity was mostly affected by compartmentalization in addition to soil status, rootstock genotype, and sampling year. Fungal communities are known to be distinct in diversity, compositions, and functionalities in the different grapevine-associated compartments (Carbone *et al.*, 2021; Swift *et al.*, 2021). In our case, the dissimilarities were predominant in the root-associated microbiome, as it was demonstrated for bacterial communities.

The root-associated microbiota and microbiome are influenced by rootstock genotype and are rather influenced by vineyard soil microbiome than root initial microbiome from nursery

Among the fungal root-associated communities subsist the ones forming symbiotic associations, namely the arbuscular mycorrhizal fungi (AMF) that are well-studied microorganisms, especially in the grapevine model (Holland *et al.*, 2016; Popescu, 2016; Trouvelot *et al.*, 2015). Their capacity to form associations with rootstocks are known to be influenced by their genetic background (Karagiannidis *et al.*, 2002; Moukarzel *et al.*, 2021) and soil composition (Schreiner and Mihara, 2009). Our results demonstrated that soil had more influence than rootstock genotype on mycorrhizal intensity, which correlated with the observations of the ITS-based sequencing on roots. In addition, no affiliation was found in vineyard with *Glomeromycota* in S soil while 0.02% of total fungal phyla was belonging to *Glomeromycota* in AS soil. However, AMF were colonizing the roots in each of the symptomatic conditions, suggesting either the presence of indigenous fungi from *Glomeromycota* division in the young vines obtained from nursery, or the proliferation of this undetected taxa during the greenhouse experiment. Before planting, roots from young 1103P and RGM rootstocks were only composed of *Ascomycota* (74.68%, 89.42%), *Basidiomycota* (22.43%, 9.37%), and *Mortierellomycota* (2.89%, 1.21%), respectively, suggesting the likelihood of the second hypothesis (**Figure S16**).

Network analysis of Bray-Curtis distances distinguished root from soil samples. This network is consistent with the results from Zarraonaindia *et al.* (2015) and Marasco *et al.* (2018) which found distinct clusters and connections from the soil × root samples. However, a different pattern was found for the fungal communities with a clustering of the greenhouse and vineyard samples, probably due to the strong segregation of nursery samples. This separation suggests

that the initial nursery fungal microbiome was diluted in the vineyard soil with time spent in the greenhouse.

Modulation of the root-associated microbiome is known to be based on several plant molecules that would suppress some potential diseases (Pascale *et al.*, 2020), and in our case *Botrytis*, responsible of grey mold which is a serious grapevine disease was controlled in roots. Several mechanisms were exposed to control *Botrytis* in grapevine which were mediated by endophytic bacteria, such as *Streptomyces*, *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Burkholderia*, *Erwinia*, *Pantoea agglomerans*, or *Micromonospora* (Compant *et al.*, 2013a). Some of these potentially antagonistic genera (*i.e.*, *Pseudomonas*, *Streptomyces*, *Acinetobacter*, *Burkholderia*) were found in rhizosphere samples among the four conditions (*i.e.*, AS-1103P, S-1103P, AS-RGM, S-RGM) using MALDI-TOF-MS, as well as in root samples using DOPE-FISH microscopy (*i.e.*, *Burkholderia*, *Pseudomonas*).

Other fungal pathogens were detected (*i.e.*, *Cadophora*, *Curvularia*, *Diaporthe*, *Diplodia*, *Ilyonectria*, *Phaeoacremonium*, and *Phaeoconiella*). Among *Cadophora* genus, species were identified as *C. luteo-olivacea* (Navarrete *et al.*, 2011), *C. malorum* (Travadon *et al.*, 2015), and *C. melinii* (Gramaje *et al.*, 2011) that are associated to Petri disease. It appeared that the detected phytopathogens were more present in roots, especially in roots from nursery, than soil compartments, which may not be surprising since these genera are affiliated to GTDs (Lade *et al.*, 2022). Detected fungal pathogens did not cause any symptoms in vineyard or in greenhouse. However, these findings support the idea that soil and nursery are the source of GTDs inoculum (Gramaje and Armengol, 2011; Nerva *et al.*, 2019). But the prevalence of these fungal pathogens decreased during the greenhouse experiment, suggesting a role of soil microbial diversity in the control of pathogens.

Differences in root and soil associated microorganisms between symptomatic and asymptomatic conditions have few impacts on plant growth

Rootstocks included in this study have different genetic backgrounds and are especially bred to provide distinct agronomic traits (Ibacache *et al.* 2020). Soil status had significant effect on growth aerial and root parameters only for CS×1103P combination at the final sampling point during the first year. Soil physicochemical properties are known to affect grapevine development (Conradie *et al.*, 1986; Echenique *et al.*, 2005), however, no work has demonstrated the impact of the natural soil microbiome, independent of soils infected with phytopathogens, in promoting grapevine growth while having similar physicochemical characteristics. Besides these contrasted phenotypic properties, our results suggested that the

rootstock genotype, in addition to soil composition, could be considered as a driver of the level of bacterial and fungal cultivable populations from the rhizosphere compartment. In addition, MALDI-TOF-MS revealed different bacterial diversity depending on the rootstock and soil status.

It has already been demonstrated that the choice of rootstock genotype in grapevine influences the fungal and bacterial communities associated to the root endosphere, rhizosphere, and bulk soil (Berlanas *et al.*, 2019; D'Amico *et al.*, 2018; Marasco *et al.*, 2018). Furthermore, the bacterial communities associated to the aboveground compartments, such as phyllosphere and carposphere, are assumed to be mostly originated from the soil through the roots where they are recruited with conserved plant-growth promoting traits (Marasco *et al.*, 2018; Samad *et al.*, 2017). These attraction and selection processes are ruled by the different signaling compounds, primary (*e.g.*, carbohydrates, organic acids, and amino acids), and secondary (*e.g.*, glucosinolates, and flavonoids) metabolites exudated by the rootstock towards soil (Sasse *et al.*, 2018; Vives-Peris *et al.* 2020). Marastoni *et al.* (2019) unveiled the different root exudates composition of distinct grapevine rootstocks, and root exudates have also been investigated in copper toxicity (Marastoni *et al.*, 2019) and iron deficiency (Marastoni *et al.*, 2020) conditions but no studies have been made on the grapevine exudates impact on microbial communities. Potential metabolic diversity, based on potential root exudates consumption such as amines, amino-acids, carbohydrates, carboxylic acids, phenolic compounds, and polymers, revealed distinct profiles with higher activities in S soils compared to AS ones for both rootstock combinations. These findings suggested higher effect from the soil status than rootstock genotype on the microbial functional diversity, while no significant effect by both rootstock or soil factors was observed on the taxonomic diversity or richness in the rhizosphere compartment for bacterial community. In fact, the soil status was more important than rootstock genotype in driving the fungal diversity, besides sampling year and compartment effects. In this study, fungal communities were therefore more impacted by the soil status than the bacteria, suggesting their determinant role in the future of young grapevine health.

Conclusion

The decline observed in the vineyard could not be emulated in the greenhouse. However, soils used for the controlled experiment altered the roots microbiome and the rhizosphere profiles among the two rootstocks used. This finding highlighted the importance of soil microbial composition and rootstock genotype to shape the root microbiome in young grapevine plant, as well as the sensitivity of fungal communities under greenhouse experiment. In addition, the

grapevine obtained from nursery were colonized by GTDs associated fungi that got suppressed in the greenhouse experiment, which underscore the importance of the soil microbial diversity as a natural disease suppressor. This study provides new insights in the bacterial and fungal communities of roots and soil interfaces, which confirm the importance of the rootstock and soil composition on the associated belowground compartment of the young grapevines.

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Supplemental information

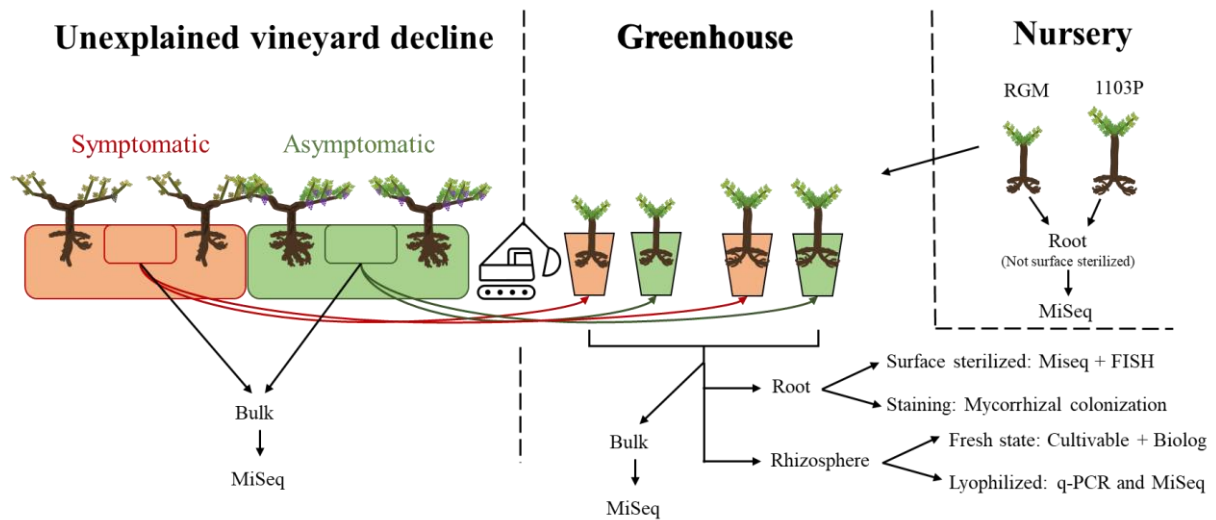


Figure S10 : Schematic overview of the experimental design carried out in this study from the vineyard soil excavation to the greenhouse disposal. Sampling strategy to separate root and rhizosphere subgroups is also depicted.

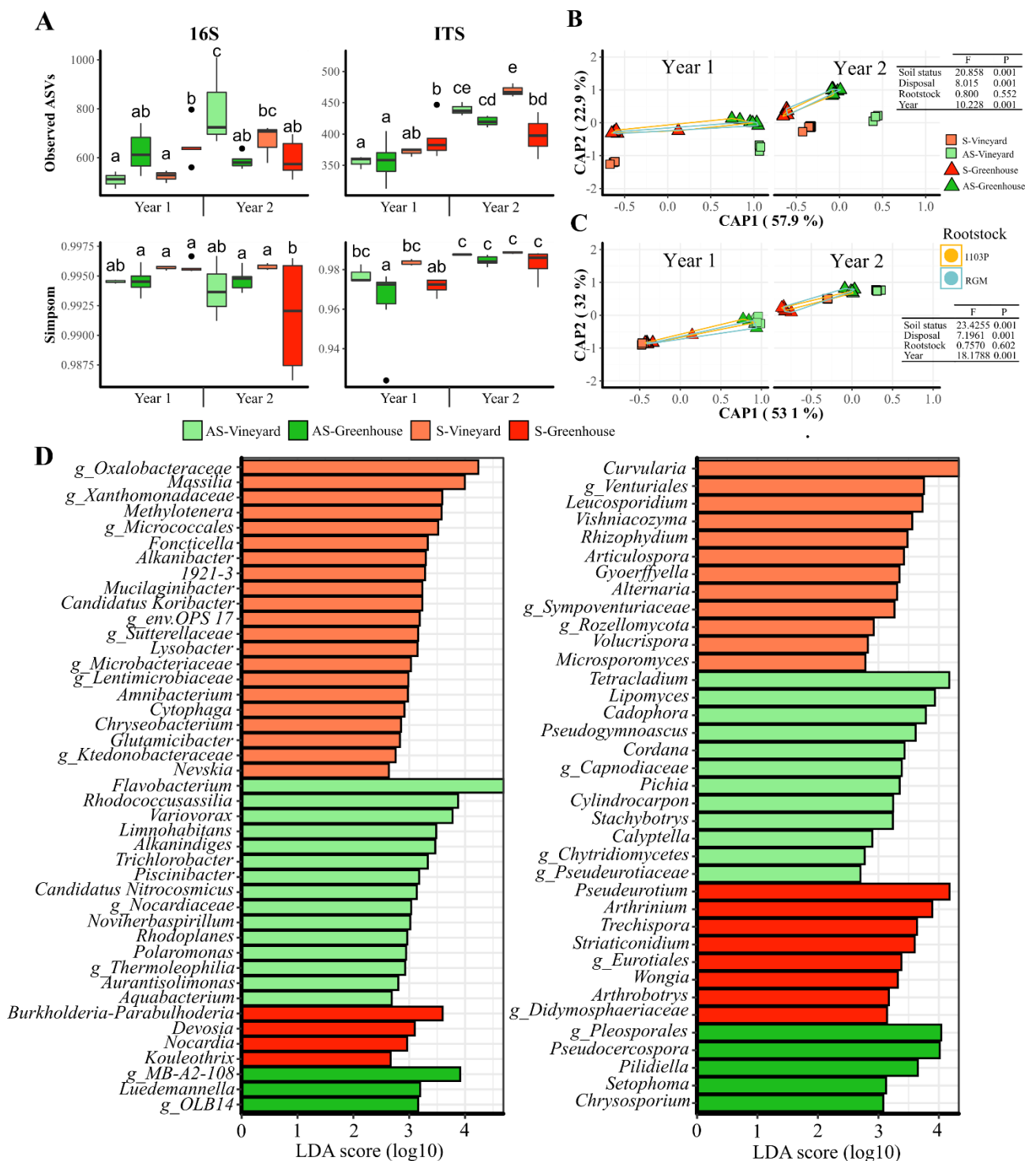


Figure S11: (A) Diversity metrics (*i.e.*, observed ASVs and Simpson’s diversity index) of bulk symptomatic and asymptomatic soils from vineyard and greenhouse across the two sampling years for bacterial and fungal communities. CAP of bulk symptomatic and asymptomatic soils from vineyard and greenhouse disposal, related to 1103P and RGM rootstocks, repeated twice (Year 1 and Year 2) for (B) bacterial and (C) fungal communities. Tables represent PERMANOVA by terms from “capscale” function (permutations = 9,999). (D) Linear discriminant analysis Effect Size based on LDA (LEfSe) for bacterial and fungal genera on bulk and vineyard samples regardless of sampling year and rootstock genotype.

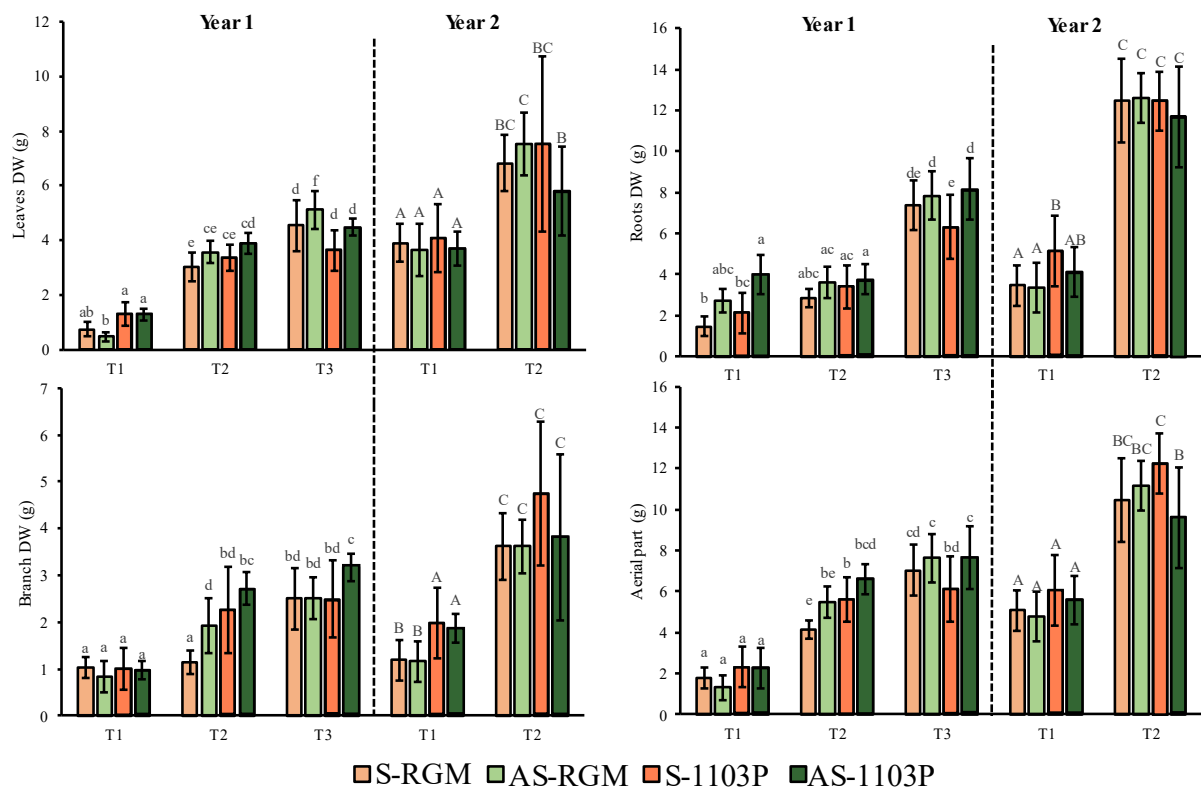


Figure S12 : Phenotypic measurements of aerial and root systems (DW = dry weight) at different sampling points during year 1 and year 2. Microbiome analysis were performed at the final sampling point (Year 1: T3 and Year 2: T2). Letters indicate significantly different means ($P < 0.05$).

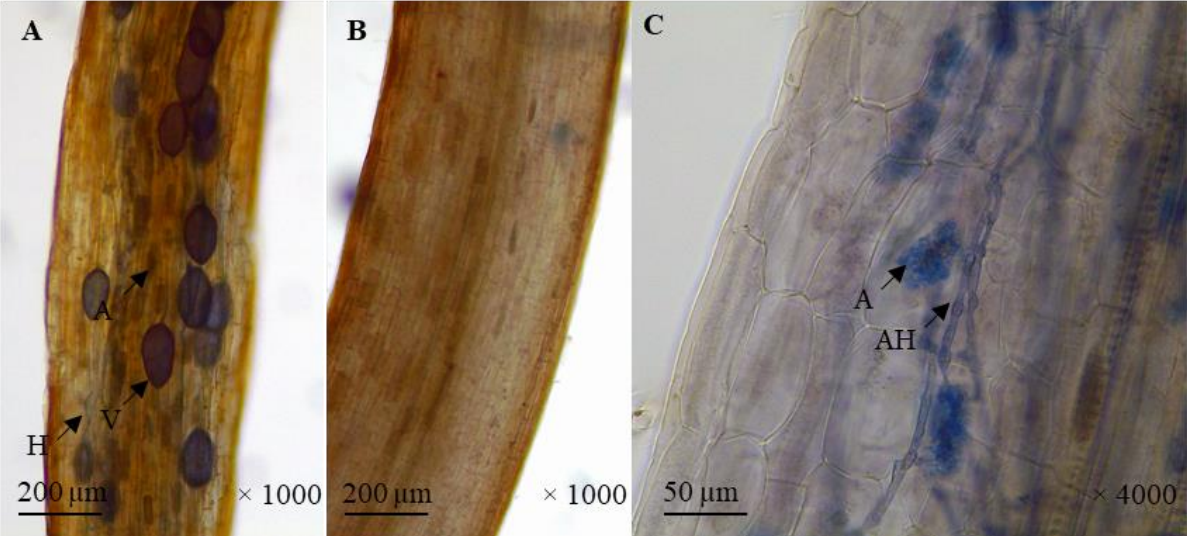


Figure S13 : Photomicrographs of (A) structural mycorrhization, (B) absence of mycorrhization, and (C) AMF within young grapevine roots. H = Hyphae, V = Vesicle, A = Arbuscule, AH = Aseptate hyphae.

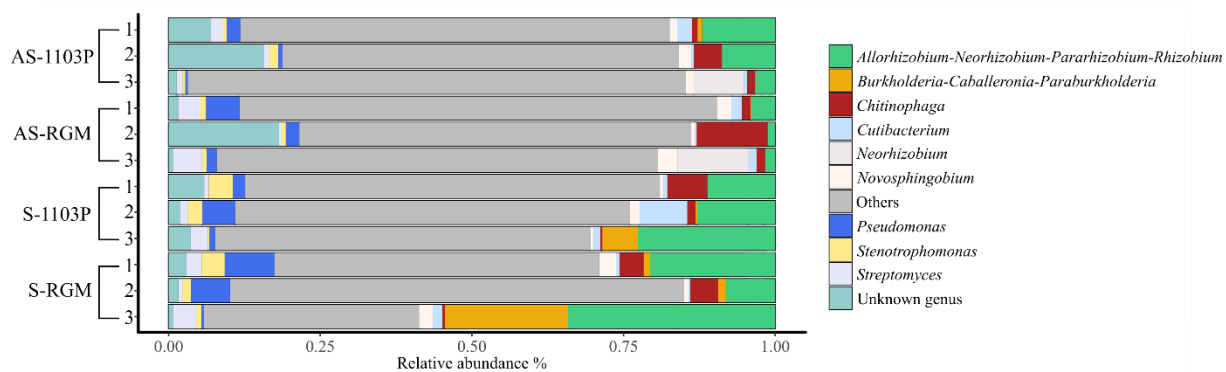


Figure S14 : Relative abundance of the 10 most represented bacterial genera in roots during the first year of sampling for the three replicates. The less represented genera were grouped in “Others”.

● **Cy3-NONEUB-Cy3 + Cy5-NONEUB-Cy5** ●

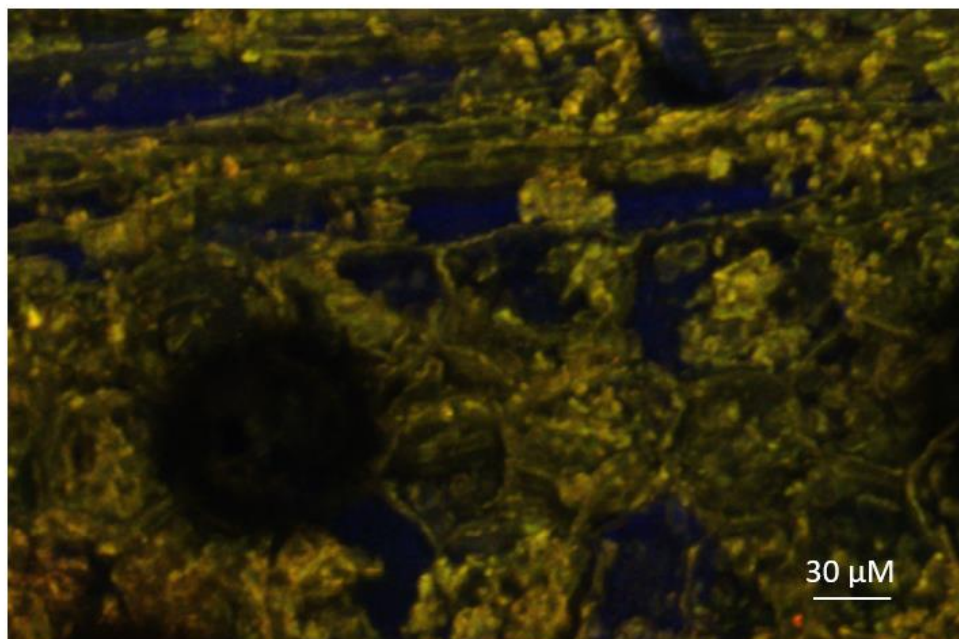


Figure S15 : Detection of naturally autofluorescent microbes in root endosphere using negative NONEUB probe.

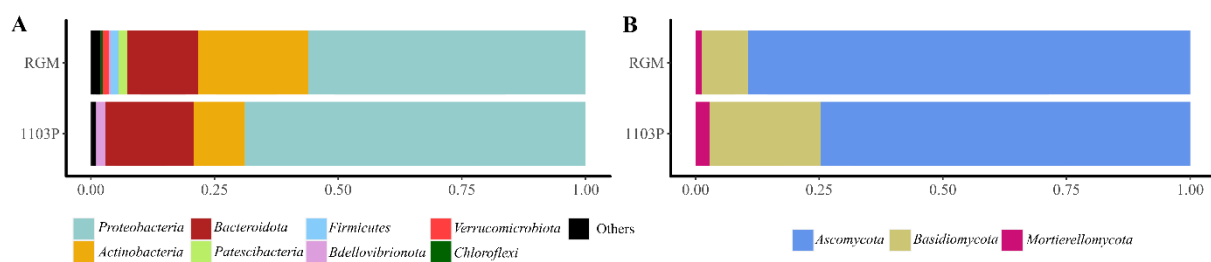


Figure S16 : Relative abundance of phyla in roots from RGM and 1103P rootstocks before plantation, where the less represented phyla were grouped in “Others”.

Table S20 : Conditions cycling for amplicon preparation of 16S and ITS libraries.

| Primers | Initial denaturation | Number of cycles | Denaturation | Annealing | Extension | Final extension |
|--------------------------------|----------------------|------------------|--------------|--------------|--------------|-----------------|
| 799f and 1175r | 5 min at 95°C | 32 | 30 s at 95°C | 30 s at 55°C | 45 s at 72°C | 5 min at 72°C |
| ITS1F and TW13 | 5 min at 95°C | 30 | 30 s at 95°C | 30 s at 50°C | 90 s at 72°C | 5 min at 72°C |
| ITS3_Mix and ITS4_Mix | 3 min at 95°C | 30 | 30 s at 95°C | 30 s at 60°C | 45 s at 72°C | 5 min at 72°C |
| 5.8S-Fun_NeXTf and ITS5_Mix | 3 min at 95°C | 42 | 35 s at 95°C | 30 s at 53°C | 42 s at 72°C | 5 min at 72°C |

Table S21 : Primers used for 16S rRNA, and nested-based PCR for ITS amplifications. Specific overhang Illumina adapters are underlined.

| Primer | Primer sequence (5' → 3') |
|------------------|---|
| 799f | <u>TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGA</u> ACMGGATTAGATACCCKG |
| 1175r | <u>GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG</u> ACGTCRTCCCCDCCTTCCTC |
| ITS1F | CTTGGTCATTTAGAGGAAGTAA |
| TW13 | GGTCCGTGTTTCAAGACG |
| ITS31_NeXTf | <u>TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG</u> CATCGATGAAGAACGCAG |
| ITS32_NeXTf | <u>TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG</u> CAACGATGAAGAACGCAG |
| ITS33_NeXTf | <u>TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG</u> CACCGATGAAGAACGCAG |
| ITS34_NeXTf | <u>TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG</u> CATCGATGAAGAACGTAG |
| ITS35_NeXTf | <u>TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG</u> CATCGATGAAGAACGTGG |
| ITS4_NeXTr | <u>GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG</u> TCCTCCGCTTATTGATATGC |
| ITS43S_NeXTr | <u>GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG</u> TCCTSSSCTTATTGATATGC' |
| 5.8S-Fun_NeXTf | <u>TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG</u> GGAACCTTTYRRC AAYGGATCWCT |
| ITS4-Fun_NeXTr | <u>GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG</u> AAAGCCTCCGCTTATTGATATGCTTAART |
| ITS43S-Fun_NeXTr | <u>GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG</u> AAAGCCTSSSCTTATTGATATGCTTAART |

Table S22 : Probes used for DOPE-FISH, with the modification and target associated.

| Oligoname | Sequence (5' -> 3') | Modification | Target |
|------------------|-------------------------------|---------------------|---------------------------|
| EUB338 | GCTGCCTCCCGTAGGAGT | 5'-CY3 3'-CY3 | |
| EUB338II | GCAGCCACCCGTAGGTGT | 5'-CY3 3'-CY3 | All bacteria |
| EUB338III | GCTGCCACCCGTAGGTGT | 5'-CY3 3'-CY3 | |
| NONEUB | ACTCCTACGGGAGGCAGC | 5'-CY5 3'-CY5 | Non-negative control |
| NONEUB | ACTCCTACGGGAGGCAGC | 5'-CY3 3'-CY3 | |
| Chit | GGAAGGTTGCGTACGTGT | 5'-CY5 3'-CY5 | <i>Chitinophaga</i> |
| Rhizo4 | GCGTTATTCCGTAGTCAAGG | 5'-CY5 3'-CY5 | <i>Rhizobium</i> (16S) |
| Pseu4 | CACCAGGTACAAGTACCCGT | 5'-CY5 3'-CY5 | <i>Pseudomonas</i> (16S) |
| Pce | CCCATCGCATCTAACAAT | 5'-CY5 3'-CY5 | <i>Burkholderia</i> (23S) |

Table S23 : Diversity metrics of bacterial and fungal communities for each condition, regardless of sampling year.

| | | Rootstock | Soil | Observed ASVs | Simpson | |
|----------|-------------|-----------|-------------|----------------|----------------|---------------|
| Bacteria | Bulk | 1103P | AS | 783 ± 55 a | 0.99 ± 0.01 a | |
| | | | S | 709 ± 61 a | 0.99 ± 0.01 a | |
| | Rhizosphere | 1103P | RGM | AS | 681 ± 23 a | 0.99 ± 0.01 a |
| | | | S | 774 ± 21 a | 0.99 ± 0.01 a | |
| | | RGM | AS | 672 ± 37 a | 0.99 ± 0.01 a | |
| | | | S | 792 ± 50 a | 1.00 ± 0.01 a | |
| | | RGM | AS | 691 ± 49 a | 1.00 ± 0.01 a | |
| | | | S | 772 ± 15 a | 1.00 ± 0.01 a | |
| | Roots | 1103P | AS | 400 ± 34 bc | 0.95 ± 0.01 cd | |
| | | | S | 360 ± 55 bc | 0.97 ± 0.01 be | |
| | | RGM | AS | 329 ± 19 bc | 0.96 ± 0.01 ce | |
| | | | S | 296 ± 24 c | 0.94 ± 0.01 d | |
| | Vineyard | | AS | 788 ± 113 a | 0.99 ± 0.01 a | |
| | | | S | 666 ± 45 a | 1 ± 0.01 a | |
| Nursery | 1103P | | 427 ± 14 bc | 0.98 ± 0.01 ab | | |
| | RGM | | 493 ± 40 c | 0.98 ± 0.01 ab | | |
| Fungi | Bulk | 1103P | AS | 476 ± 13 a | 0.98 ± 0.01 ab | |
| | | | S | 465 ± 22 a | 0.98 ± 0.01 ab | |
| | | RGM | AS | 425 ± 20 a | 0.97 ± 0.01 ab | |
| | | | S | 469 ± 13 a | 0.98 ± 0.01 ab | |
| | Rhizosphere | 1103P | AS | 477 ± 16 a | 0.98 ± 0.01 ab | |
| | | | S | 468 ± 24 a | 0.98 ± 0.01 ab | |
| | | RGM | AS | 454 ± 24 a | 0.98 ± 0.01 ab | |
| | | | S | 489 ± 12 a | 0.98 ± 0.01 ab | |
| | Roots | 1103P | AS | 205 ± 41 b | 0.95 ± 0.01 f | |
| | | | S | 218 ± 47 b | 0.97 ± 0.01 ac | |
| | | RGM | AS | 171 ± 29 b | 0.94 ± 0.01 e | |
| | | | S | 203 ± 33 b | 0.96 ± 0.01 cf | |
| | Vineyard | | AS | 453 ± 21 a | 0.98 ± 0.01 ab | |
| | | | S | 484 ± 23 a | 0.99 ± 0.01 b | |
| Nursery | 1103P | 1103P | 132 ± 6 b | 0.92 ± 0.01 de | | |
| | RGM | RGM | 141 ± 11 b | 0.91 ± 0.01 d | | |

Means ± SE are presented (n = 6) and different letters indicate the groups are significantly different (pairwise comparison, p < 0.05).

Table S24 : Genetic parentage, some agronomic, and tolerance traits related to the rootstocks RGM and 1103P, according to (Ibacache et al., 2020).

| | Genetic parentage | Vigor induced | Rooting ability | Tolerance | | |
|-------|---|---------------|-----------------|-----------|---------|------|
| | | | | Lime | Drought | Salt |
| RGM | <i>V. riparia</i> | Low | Moderate | Low | Poor | Low |
| 1103P | <i>V. berlandieri</i> × <i>V. rupestris</i> | Moderate/High | Moderate | Poor | Medium | High |

Table S25 : Raw phenotype data from phenotype sampling points (T1, T2, T3) and both sampling years. Letters in bold indicate significant differences (pairwise $P < 0.05$) among the 4 conditions (AS-1103P, S-1103P, AS-RGM, S-RGM).

| | | | Stem length | Stem diameter | Chlorophyll | Trunk | dry | Length (cm) | Diameter |
|--------|----|----------|----------------|---------------|----------------|----------------|---------------|--------------|----------|
| | | | (cm) | (cm) | leaves content | weigh (g) | | | |
| Year 1 | T1 | AS-1103P | 49.8 ± 8.1 b | 1 ± 0.2 a | 20.6 ± 1.6 c | 19.7 ± 3.5 ab | 30.7 ± 1.7 a | 1.1 ± 0.1 a | |
| | | S-1103P | 40.8 ± 16.3 ab | 1 ± 0.5 a | 17.6 ± 1.4 a | 20.4 ± 2.3 a | 30.2 ± 0.4 a | 1 ± 0.1 ab | |
| | | AS-RGM | 24 ± 7.4 c | 0.7 ± 0.1 a | 25.9 ± 1.9 b | 14.7 ± 4.1 b | 32.5 ± 1.1 b | 0.8 ± 0.1 bc | |
| | | S-RGM | 29.2 ± 6.9 ac | 0.4 ± 0.1 b | 24.3 ± 1.7 b | 16.9 ± 5.6 ab | 32.5 ± 1.8 b | 0.8 ± 0.1 c | |
| | T2 | AS-1103P | 67.1 ± 9.7 b | 0.7 ± 0.2 a | 22.9 ± 3.7 a | 20.5 ± 5 a | 30.1 ± 0.5 b | 1.2 ± 0.2 a | |
| | | S-1103P | 61.6 ± 7.3 ab | 0.9 ± 0.2 a | 23 ± 6.7 a | 17 ± 5.1 ab | 31.2 ± 1.8 ab | 1 ± 0.1 a | |
| | | AS-RGM | 57.6 ± 11 ab | 0.8 ± 0.1 a | 25.8 ± 4.3 a | 12.4 ± 2.6 bc | 32.4 ± 1.4 a | 0.9 ± 0.1 a | |
| | | S-RGM | 50.4 ± 6.4 a | 0.7 ± 0.1 a | 23.7 ± 0.6 a | 11.2 ± 2.7 c | 32.7 ± 1.5 a | 0.9 ± 0.1 a | |
| | T3 | AS-1103P | 68.9 ± 7.5 c | 0.9 ± 0.1 a | 28.1 ± 1.2 c | 20.8 ± 3.6 a | 32.3 ± 1 ab | 1.1 ± 0.1 a | |
| | | S-1103P | 55.2 ± 13.8 ab | 0.9 ± 0.2 a | 21.4 ± 2 a | 23.6 ± 6.5 a | 31.7 ± 2.2 a | 1.2 ± 0.1 a | |
| | | AS-RGM | 61.7 ± 8.6 bc | 0.9 ± 0.2 a | 27.9 ± 1.9 c | 15.1 ± 4 b | 34 ± 2 c | 0.9 ± 0.1 b | |
| | | S-RGM | 50.3 ± 8 a | 0.8 ± 0.2 a | 25.8 ± 2 b | 16.6 ± 4.6 b | 33.4 ± 1.4 bc | 0.9 ± 0.1 b | |
| Year 2 | T1 | AS-1103P | 56.6 ± 9.1 a | 1.3 ± 0.2 c | 23.1 ± 0.1 c | 25.9 ± 7.2 a | 34.6 ± 1.8 a | 1.3 ± 0.2 a | |
| | | S-1103P | 54.9 ± 16.6 a | 1.1 ± 0.2 a | 23 ± 0.1 a | 27.6 ± 6.9 a | 35 ± 1.3 a | 1.3 ± 0.2 a | |
| | | AS-RGM | 42.7 ± 13.1 b | 0.9 ± 0.2 b | 23.2 ± 0.1 d | 19.1 ± 4 b | 32.9 ± 1.1 b | 1.1 ± 0.1 b | |
| | | S-RGM | 47.2 ± 13.3 ab | 1 ± 0.2 ab | 24.3 ± 0.5 b | 18.5 ± 2.5 b | 32.3 ± 1.9 b | 1.1 ± 0.1 b | |
| | T2 | AS-1103P | 78.6 ± 14.9 a | 0.9 ± 0.2 ab | 20.9 ± 3.1 a | 29.7 ± 10.1 ab | 33.5 ± 2 a | 1.4 ± 0.2 a | |
| | | S-1103P | 78.5 ± 17.1 a | 1 ± 0.1 a | 21.9 ± 2.5 a | 32 ± 8 a | 33.1 ± 1 a | 1.4 ± 0.2 a | |
| | | AS-RGM | 71.3 ± 13.9 a | 0.8 ± 0.1 b | 22 ± 3.7 a | 24.9 ± 3.3 b | 31.4 ± 3.1 b | 1.2 ± 0.1 b | |
| | | S-RGM | 69.5 ± 13 a | 0.8 ± 0.1 b | 23 ± 3 a | 22.6 ± 2.4 b | 29.7 ± 0.8 b | 1.2 ± 0.1 ab | |

Table S26 : List of the fungal genera, associated to grapevine diseases.

| | Associated disease | References |
|----------------------------|-------------------------------|---|
| <i>Anthostoma</i> | Eutypa | (Gramaje <i>et al.</i> , 2018; Perazzolli <i>et al.</i> , 2019) |
| <i>Botrytis</i> | Grey mould | (González-Fernández <i>et al.</i> , 2020) |
| <i>Botryosphaeria</i> | <i>Botryosphaeria</i> dieback | (Mondello <i>et al.</i> , 2015) |
| <i>Cadophora</i> | Petri disease | (Gramaje <i>et al.</i> , 2021) |
| <i>Campylocarpon</i> | Black foot | (Gramaje <i>et al.</i> , 2018) |
| <i>Cryptosphaeria</i> | Canker, Eutypa | (Trouillas <i>et al.</i> , 2010) |
| <i>Cryptovalsa</i> | Canker, Eutypa | (Niem <i>et al.</i> , 2020; Trouillas <i>et al.</i> , 2010) |
| <i>Cytospora</i> | Canker, Eutypa | (Lawrence <i>et al.</i> , 2017) |
| <i>Curvularia</i> | Canker | (Bahmani <i>et al.</i> , 2021) |
| <i>Cylindrocladiella</i> | Black foot | (Gramaje <i>et al.</i> , 2018) |
| <i>Dactylonectria</i> | Black foot | (Gramaje <i>et al.</i> , 2018) |
| <i>Diaporthe</i> | <i>Phomopsis dieback</i> | (Yan <i>et al.</i> , 2013) |
| <i>Diatrype</i> | Eutypa dieback | (Trouillas <i>et al.</i> , 2010) |
| <i>Diatrypella</i> | Eutypa dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Diplodia</i> | <i>Botryosphaeria</i> dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Dothiorella</i> | <i>Botryosphaeria</i> dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Eutypa</i> | Eutypa dieback | (Cardot <i>et al.</i> , 2019) |
| <i>Eutypella</i> | Eutypa dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Fomitiporia</i> | Esca | (Gramaje <i>et al.</i> , 2018) |
| <i>Fomitiporella</i> | Esca | (Gramaje <i>et al.</i> , 2018) |
| <i>Fusicoccum</i> | <i>Phomopsis dieback</i> | (Gramaje <i>et al.</i> , 2018) |
| <i>Ilyonectria</i> | Black foot | (Lade <i>et al.</i> , 2022) |
| <i>Inocutis</i> | Esca | (Gramaje <i>et al.</i> , 2018) |
| <i>Inonotus</i> | Esca | (Gramaje <i>et al.</i> , 2018) |
| <i>Lasiodiplodia</i> | <i>Botryosphaeria</i> dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Neofusicoccum</i> | <i>Botryosphaeria</i> dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Neonectria</i> | Black foot | (Gramaje <i>et al.</i> , 2018) |
| <i>Neoscytalidium</i> | <i>Botryosphaeria</i> dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Phaeobotryosphaeria</i> | <i>Botryosphaeria</i> dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Phaeoacremonium</i> | Petri disease | (Lade <i>et al.</i> , 2022) |
| <i>Phaeomoniella</i> | Petri disease | (Lade <i>et al.</i> , 2022) |
| <i>Phellinus</i> | Esca | (Gramaje <i>et al.</i> , 2018) |
| <i>Pleurostoma</i> | Petri disease | (Gramaje <i>et al.</i> , 2018) |
| <i>Spencermartinsia</i> | <i>Botryosphaeria</i> dieback | (Gramaje <i>et al.</i> , 2018) |
| <i>Stereum</i> | Esca | (Gramaje <i>et al.</i> , 2018) |
| <i>Thelonectria</i> | Black foot | (Gramaje <i>et al.</i> , 2018) |

Table S27 : Diversity metrics for fungal pathogens detected across the conditions.

| | Rootstock | Soil | Observed ASVs | Simpson |
|-------------|-----------|------|-------------------|-------------------|
| Bulk | 1103P | AS | 10.83 ± 0.98 abcd | 0.80 ± 0.03 ab |
| | | S | 9.50 ± 0.67 abce | 0.69 ± 0.02 cdef |
| | RGM | AS | 9.33 ± 1.11 abce | 0.79 ± 0.02 abcd |
| | | S | 9.66 ± 0.67 abc | 0.70 ± 0.01 acde |
| Rhizosphere | 1103P | AS | 10.17 ± 0.48 abc | 0.84 ± 0.01 b |
| | | S | 10.33 ± 0.49 abcd | 0.73 ± 0.02 abcde |
| | RGM | AS | 9.50 ± 1.61 abce | 0.79 ± 0.03 abc |
| | | S | 13.67 ± 1.08 d | 0.77 ± 0.01 abcde |
| Roots | 1103P | AS | 8.83 ± 1.78 abcef | 0.68 ± 0.03 def |
| | | S | 7.50 ± 1.12 cef | 0.58 ± 0.06 fg |
| | RGM | AS | 6.17 ± 1.74 ef | 0.51 ± 0.10 g |
| | | S | 5.83 ± 0.48 f | 0.67 ± 0.03 ef |
| Vineyard | | AS | 11.33 ± 1.61 abd | 0.81 ± 0.02 ab |
| | | S | 13.67 ± 1.69 d | 0.78 ± 0.04 abcd |
| Nursery | 1103P | | 13.00 ± 1.00 ad | 0.68 ± 0.05 acdef |
| | RGM | | 7.33 ± 2.40 bcef | 0.52 ± 0.08 g |

Means ± SE are presented (n = 6) and different letters indicate the groups are significantly different (pairwise comparison, $P < 0.05$).

Table S28 : Rhizosphere Eco-Plates measurements (AUC, Shannon's evenness (E) and richness (R) functionality at 96 hours post-incubation) for the two studied rootstocks RGM and P with (S) and without (AS) microbial dysbiosis symptoms for the two sampling years.

| | Year 1 | | | | Year 2 | | | |
|-----|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| | RGM | | 1103P | | RGM | | 1103P | |
| | S | AS | S | AS | S | AS | S | AS |
| AUC | 10.6 ± 0.4 b | 9.5 ± 0.2 a | 12 ± 0.5 c | 9.6 ± 0.4 a | 3.1 ± 0.3 b | 3.8 ± 0.1 c | 2.9 ± 0.1 b | 2.6 ± 0.1 a |
| E | 0.97 ± 0.01 b | 0.97 ± 0.01 a | 0.97 ± 0.01 b | 0.98 ± 0.01 b | 0.98 ± 0.02 b | 0.88 ± 0.01 a | 0.90 ± 0.03 a | 0.87 ± 0.01 a |
| R | 25 ± 1 c | 19 ± 2.6 a | 22.3 ± 3.2 ac | 19.3 ± 3.1 a | 9.7 ± 0.5 b | 7.7 ± 0.2 a | 7 ± 1.7 a | 6.7 ± 0.6 a |

Means ± SE are presented (n = 3) and different letters indicate the groups are significantly different (pairwise comparison, $P < 0.05$).

Table S29 : Colonization of grapevine plants by AMF using microscopy-staining method, and relative abundance of Glomeromycota using ITS-sequencing for RGM and P rootstocks in asymptomatic (AS) and symptomatic (S) soils at the final sampling point during the second year.

| | RGM | | P | |
|--|----------------|---------------|----------------|---------------|
| | S | AS | S | AS |
| Frequency (%) | 96.7 ± 4.7 a | 96 ± 7.2 a | 88 ± 12.2 a | 87.3 ± 10.9 a |
| Global intensity (%) | 52.5 ± 13.2 b | 34.8 ± 12.5 a | 39.7 ± 10.7 ab | 25.5 ± 5.7 a |
| Samples intensity (%) | 54 ± 11.7 b | 36.1 ± 12.2 a | 44.8 ± 8.1 ab | 29.2 ± 5.9 a |
| Relative abundance <i>Glomeromycota</i> (%) | 16.69 ± 4.79 b | 2.35 ± 0.45 a | 11.26 ± 3.70 a | 7.09 ± 2.38 a |

Means ± SE are presented with n = 10 for microscope approach while n = 3 for sequencing approach. Means with different letters are significantly different (pairwise comparison, $P < 0.05$).

Table S30 : Diversity metrics of bacterial and fungal communities in the global dataset.

| | | Obs | Simpson |
|----------|-------------|------------|----------------|
| Bacteria | Bulk | 736 ± 22 a | 0.99 ± 0.01 a |
| | Rhizosphere | 732 ± 22 a | 0.98 ± 0.01 a |
| | Root | 346 ± 18 c | 0.96 ± 0.01 c |
| | Vineyard | 727 ± 61 a | 0.99 ± 0.01 a |
| | Nursery | 460 ± 24 b | 0.98 ± 0.01 b |
| Fungi | Bulk | 459 ± 9 a | 0.98 ± 0.01 a |
| | Rhizosphere | 472 ± 10 a | 0.98 ± 0.01 a |
| | Root | 199 ± 18 c | 0.95 ± 0.01 c |
| | Vineyard | 469 ± 16 a | 0.98 ± 0.014 a |
| | Nursery | 136 ± 6 b | 0.92 ± 0.01 b |

Means ± SE are presented (n = 6). Means with different letters are significantly different (pairwise comparison, $P < 0.05$).

Brief discussion

Although this experiment was conducted in a greenhouse, the results obtained highlighted rootstock genotype and soil as drivers of bacterial and fungal communities in young vines.

Not surprisingly, the bulk soil from the vineyard differed from that at the end of the greenhouse experiment. A more meaningful comparison of the effect of the greenhouse on this soil could have been completed by sampling the vineyard soil at the same time as the final greenhouse sampling.

However, the initial microbial contribution of the vineyard soil and nursery plants to the greenhouse microbiome was explored, resulting in the detection of some pathogenic fungal genera. None of the affiliated symptoms caused by these taxa were observed in the vineyard or in the greenhouse, suggesting the hypothesis of natural regulation by other microorganisms present in the roots and soil. In addition, and exclusively during the first year of the greenhouse experiment, the CS×1103P combination grown in symptomatic soils exhibited significantly lower aboveground and belowground biomass than the CS×1103P combination grown in asymptomatic soil.

Given the potential protective roles against fungal pathogens and growth promotion of growth, it is questionable whether the addition of selected beneficial microorganisms could promote vine growth. This question is addressed in the next chapter.

Chapter V

Isolation and characterization of bacterial isolates potentially beneficial for grapevine growth

Preface

Soils harbor a plethora of microorganisms that are critical to the growth and health of the plant. Isolation of PGPR in grapevine has been performed mainly for pathogen control in many studies. In addition to these beneficial bacteria, the potential of AMF in promoting growth has also been well studied in viticulture and is even offered by nurseries to winemakers. However, very few studies have combined the growth-promoting effects of these two types of microorganisms in grapevine. Furthermore, the addition of microorganisms can create or deplete ecological niches creating a potential microbial imbalance, which is a very understudied issue.

The effects of the addition of beneficial microorganisms on grapevine roots and soil compartments were studied in a greenhouse experiment but will not be presented due to the consistence of the data and lack of time to present a decent analysis. However, preliminary results consisting in the characterization of the potentially beneficial rhizobacteria will be presented. Some results of the greenhouse will be briefly discussed, but the core will be presented during the thesis defense. Rhizobacteria isolated in the previous chapter were tested for PGP traits using biochemical and biological assays. The eight most relevant isolates were first inoculated onto *Lepidium sativum* sprouted seeds. This plant was chosen because of its rapid growth and ease of phenotypic measurements. These eight isolates were then inoculated onto cv. 1103P (*Vitis rupestris* × *Vitis berlandieri*) seedlings, since this rootstock was the most sensitive to growth parameters in the previous chapter.

To conduct the greenhouse experiment, the inoculum with the greatest positive effect on growth parameters was selected and tested in the greenhouse onto the CS×1103P combination potted in the symptomatic soil studied in the previous chapters. Some of the CS×1103P were inoculated by the nursery with commercial mycorrhizal fungi to study the potential of the beneficial fungi proposed to grape growers. In addition, half of these mycorrhized plants were inoculated with the isolated rhizobacteria to test whether their combined effects on growth were synergistic or inhibitory. This chapter part presents few results from of a research article entitled **“The addition of isolated plant growth-promoting rhizobacteria and commercial arbuscular mycorrhizal fungi alters the microbiome associated with the roots of young vines”**, planned to be submitted to *Frontiers in Microbiology* subsequently to further analysis and co-authors corrections.

The addition of isolated plant growth-promoting rhizobacteria and commercial arbuscular mycorrhizal fungi alters the microbiome associated with the roots of young vines

Romain Darriaut¹, Jules Wastin², Joseph Tran¹, Philippe Vivin¹, Elisa Marguerit¹, Isabelle Masneuf-Pomarède^{3,4}, Virginie Lauvergeat^{1*}

¹ EGFV, Univ. Bordeaux, Bordeaux Sciences Agro, INRAE, ISVV, F-33882, Villenave d'Ornon, France

² Université Libre de Bruxelles, Brussels, Belgium

³ Université de Bordeaux, INRAE, Bordeaux INP, UMR Œnologie EA 4577, USC 1366, ISVV, 33140 Villenave d'Ornon, France

⁴ Bordeaux Sciences Agro, 1 cours du Général de Gaulle, 33170 Gradignan, France

*corresponding author: virginie.lauvergeat@inrae.fr

Abstract

Arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) are considered highly-efficient agents for conferring development to perennial crops by improving nutrition and pathogen control. In viticulture, combining AMF and PGPR is an understudied association, especially regarding the impact on soil and root microbes. The objective of this study was to evaluate the impact of single inoculation or in combination of commercial AMF (*Rhizoglyphus irregularis* strain BEG7 and *Funneliformis mosseae*) and PGPR (*Pseudomonas veronii* and *Pseudomonas brassicacearum*) on *Vitis vinifera* belowground microbiome. First, 200 rhizobacterial isolates were screened for PGP traits using plating and colorimetric biochemical assays. The most promising isolates were tested for their growth-promoting capacity onto *L. sativum* and *Vitis rupestris* × *Vitis berlandieri* plantlets cv. 1103P in single, dual, or triple mix combination. The most effective combination was inoculated alone or in combination with commercial AMF in greenhouse on one year old grafted vine potted with soil from a declining vineyard. The microbial profile, which is not presented here, was dressed up using MALDI-TOF-MS identification, quantitative-PCR, and 16S rRNA gene, as well as ITS, sequencing.

Keywords: *Vitis vinifera* microbiota, grapevine endosphere, rhizosphere, *Lepidium sativum*, grapevine plantlets

Introduction

Cultivated grapevine, also known as *Vitis vinifera* L., is a perennial, often grafted, and valuable crop able to grow worldwide. This plant is confronted to several abiotic and biotic stressors that reduce the productivity and quality of grapes. Among abiotic factors, drought and salinity are becoming increasingly important under the pressure of climate change (Bernardo *et al.*, 2018). Regarding biotic stresses, grapevine trunk diseases (GTDs), pests and viruses pose a major threat to viticulture because few solutions are effective in countering these incidences (Claverie *et al.*, 2020; Mannini and Digiario, 2017; Tello *et al.*, 2019). These incidences adversely impact grapevines growth and development, thus affecting vineyard yield and quality. Most of the time, dead or too unproductive vines are replaced by new young ones which will take at least two years, after establishment, to become profitable (Sanmartin *et al.*, 2017).

In the meantime, the young vines are confronted to environmental constraints that might be determinant for their health and development. Indeed grapevines, and plants in general, draw the large part of their associated microbiota from the soil through chemoattractants exudated from the roots (Vives-Peris *et al.*, 2020). Telluric pathogenic microorganisms, such species from *Botryosphaeriaceae* family (Mondello *et al.*, 2020), or even from *Phaeoacremonium* genus (Aigoun-Mouhous *et al.*, 2021) are among those attracted colonizing microbes and infect young and mature grapevines through root system.

In addition, grapevine plants from nurseries have been shown to contain fungal pathogens leading to the decline of young vines. The infection process usually occurred during the cutting and grafting preparation which produce a great number of wounds which facilitate the colonization of fungal pathogens (Gramaje and Armengol, 2011). Indeed, bad quality material has sometimes been provided by nurseries due to lack of quality criteria and assessment for grapevine propagations (Waite *et al.*, 2015). Besides these well-known GTDs incidences originated from nurseries, some defaults affecting vigour and longevity of the young vines were also reported (Waite *et al.*, 2013). Altogether, these low-quality vines do not last long and need to be replaced in a short time after their plantation.

No current solutions have been established to fully control soilborne pathogens infecting grapevine roots, but some biological control strategies have been developed. Rhizosphere, defined as the tight portion of soil close to the roots, is a particular hot spot for microbe × plant interactions (de la Fuente Cantó *et al.*, 2020). Plant growth-promoting rhizobacteria can confer better plant development through direct nutrient transfer or by controlling phytopathogen (Sayyed and Arora, 2019). In grapevine, rhizobacteria have been isolated and tested for their

capacity to reduce GTD incidence (Álvarez-Pérez *et al.*, 2017; Haidar *et al.*, 2021a; Wu *et al.*, 2020), and even to improve grafting efficiency (Sabir, 2013). The PGP activities in grapevine have been studied *in vitro* (Sabir *et al.*, 2012), in greenhouse (Funes Pinter *et al.*, 2018), and even in field condition (Rolli *et al.*, 2017). Interestingly, the capacity of grapevine rootstock to possess rhizobacteria harboring PGP traits seemed to be a core function independent to vineyard location (Marasco *et al.*, 2013) or rootstock genotype (Marasco *et al.*, 2018).

Another interesting microorganism which can promote grapevine growth while providing resistance to pathogens is the arbuscular mycorrhizal fungi. This fungal symbiont can impart features that improve plant growth by supplying soil nutrient to roots and by controlling soilborne pathogens (M. Chen *et al.*, 2018). In this mutualistic symbiosis, the fungi provide soil nutrients such as nitrogen (N) or phosphorus (P) thanks to their external mycelium in exchange of carbon from plant photosynthates released from the roots. It has been estimated, for *Picea abies* trees, that 40% of fine root carbon were allocated to mycorrhizal structure (Gorzela *et al.*, 2020). In viticulture, AMF have been greatly studied for these powerful and beneficial nutrition traits (Likar and Regvar, 2017; Massa *et al.*, 2020; Schreiner, 2020). In addition to nutrient uptake and pathogens inhibition, AMF are known to modify berry content, making these microorganisms quite relevant for wine production (Antolín *et al.*, 2020; Torres *et al.*, 2018a). Nowadays, it is a current practice in nurseries to provide material plant already mycorrhized to winegrowers.

The combination of both AMF and PGPR seems a relevant strategy for pathogens control and plant growth while conferring good quality fruits (Noceto *et al.*, 2021). This methodology has been applied in strawberry (Lowe *et al.*, 2012), or even apple trees (Przybyłko *et al.*, 2021), but very few studies have been investigated the potential synergistic effect of PGPR and AMF in grapevine development (Nerva *et al.*, 2022; Velásquez *et al.*, 2020).

The objectives of this work were first to characterize growth effects of previously isolated rhizobacteria from symptomatic and asymptomatic soils from Chapter 4. Assessments of growth promotion were performed using biochemical tests and inoculations *in vitro* on *Lepidium sativum* sprouted seeds (*i.e.*, fast growing plant) and *Vitis rupestris* × *Vitis berlandieri* plantlets. Afterwards, a single inoculation of the most efficient PGPR combination was performed on mycorrhized and non-mycorrhized young grapevines in greenhouse to observe their effect on the grapevine development as well as the rhizosphere and root endosphere microbiome.

Material & Methods

Screening for *in vitro* PGPR activities

From the rhizobacteria isolated previously in Chapter 4, two hundred isolates were randomly selected (*i.e.*, fifty from each condition: S-RGM, AS-RGM, S-1103P, AS-1103P) and tested for the PGP activities listed below.

Indole-3-acetic-acid (IAA) production

The production of IAA was determined using the Salkowski reaction adapted from Gordon & Weber (1951). The bacterial isolates were grown in LB medium supplemented with 100 $\mu\text{g}\cdot\text{ml}^{-1}$ l-tryptophan, acting as a precursor for IAA synthesis, for 48 hours at 28°C under 200 rpm continuous shaking. Bacterial suspensions were centrifuged at 8000 g for 10 min at 4°C. One ml of the supernatant was then mixed with 4 ml of Salkowski reagent (1.0 ml 0.5 M FeCl_3 in 50 ml of 35% HClO_4), followed by measuring the color changes using a spectrophotometer at 530 nm. Calibration curve for estimating auxin concentration was made with standards ranging from 10 to 100 $\mu\text{g}\cdot\text{ml}^{-1}$ of IAA.

Capacity to produce 1-aminocyclopropane-1-carboxylate deaminase (ACCd)

The presence of ACCd activity was determined using ACC as sole source of nitrogen adjusted from Penrose & Glick (2003), which estimate the amount of α -ketobutyrate. Cells initially grown in R2A medium were inoculated at $\text{OD}_{600} = 0.1$ in DF medium supplemented with 3 mM of ACC and incubated at 30°C for 48 hours. Subsequently to an 8000g centrifugation, pellets were respectively washed with 0.1 M Tris-HCl (pH 7.6) and resuspended in 600 μl of 0.1 M Tris-HCl (pH 8.5) amended with 30 μl of toluene, and finally vortexed for 30 s. The toluenized cells (200 μl) were gently mixed with 0.5 M ACC and incubated at 30°C for 15 minutes. The reaction was stopped by adding 1 ml of 0.56 M HCl and vortexed, followed by a 5-minute centrifugation at 16000 g. The supernatant (1 ml) was mixed with 800 μl of 0.56 M HCl and 300 μl of 2,4 dinitrophenylhydrazine (0.2% in 2M HCl), and finally incubated at 30°C for 30 minutes. Colorimetric reactions occurred with the addition of 2 ml of 2 N NaOH and were measured at 540 nm. Calibration curve for estimating ACCd concentration was made with standards ranging from 0.1 to 1 $\mu\text{g}\cdot\text{ml}^{-1}$ of α -ketobutyrate.

Ammonia production

The production of ammonia for each rhizobacteria was assessed using the Nesslerization reaction described by Cappuccino & Sherman (1992). Each rhizobacterial isolate was grown in peptone water for 72 hours at 28°C at 200 rpm. Culture supernatant (200 ml) was mixed with 1 ml of Nessler's reagent which was supplemented with 7.3 ml of ammonia-free water. The development of brown to yellow color indicating the ammonia production was spectrophotometrically monitored at 450 nm. The concentration of ammonia produced was estimated using a standard curve based on ammonium sulphate ranging from 0.1 to 1 µmol per ml.

Siderophore synthesis

The synthesis of siderophores was determined using the plating method based on Chrome-azuroil S (CAS) medium adjusted from Schwyn & Neilands (1987). The CAS assay solution consisted in 6 ml of 10 mM HDTMA solution diluted upto 100 ml with distilled water and a mixture of 1.5 ml iron (III) solution (1 mM FeCl₃·6H₂O in 100 mM HCl) supplemented to 7.5 ml of 2 mM aqueous CAS solution which was added under stirring. Anhydrous piperazine (4.307 g) was dissolved in 30 mL of water, and 6.25 ml of HCl (37%) was carefully added to it. This buffer solution (pH 5.6) was adjusted to 100 ml and the CAS shuttle solution was obtained by adding 4 mM of 5-sulfosalicylic acid to the above solution. Bacterial isolates were plated on CAS agar and incubated for 72 hours at 28°C. Siderophore production was therefore assessed by measuring the distance between the colony and its surrounding halo.

Phosphate solubilization

The ability of the rhizobacteria to solubilize phosphate was determined using the Pikovskaya medium adjusted from Pikovskaya (1948). Each bacterial isolate was plated on Pikovskaya agar (1% glucose, 0.5% Ca₃(PO₄)₂, 0.05% (NH₄)SO₄, 0.02% NaCl, 0.01% MgSO₄·7H₂O, 0.02% KCl, 0.0002% MnSO₄·7H₂O, 0.0002% FeSO₄·7H₂O, 0.05% yeast extract, 1.5% agar) supplemented with bromophenol blue to assess phosphate solubilization capacity. Plates were incubated for 72 hours at 28°C. Phosphate solubilization was assessed by measuring the distance between the colony and the edge of its surrounding halo.

Nitrogen fixation

The capacity of the isolates to fix nitrogen was assessed with the NfB solid medium adjusted from Döbereiner (1989). Each bacterial isolate was plated on pH 6.8 NfB (0.05% D-malic acid,

0.05% K_2HPO_4 , 0.02% $MgSO_4$, 0.01% $NaCl$, 1.5% agar) complemented with 2 ml of bromothymol blue (0.5% in 0.2M KOH), 1 ml of vitamin solution (per 100 ml: 10 mg biotin, 20 mg pyridoxine-HCl), and 2 ml of micronutrient solution (per liter: 40 mg $CuSO_4 \cdot 5H_2O$, 120 mg $ZnSO_4 \cdot 7H_2O$, 1.4g H_3BO_3 , 1g $Na_2MoO_4 \cdot 2H_2O$, 1.5g $MnSO_4 \cdot H_2O$). Plates were incubated for 72 hours at 28°C. Nitrogen fixation was assessed by measuring the distance between the colony and the edge of its surrounding halo.

Identification of the most promising strains

The isolates having the most efficient PGP activities were sent for 16S rRNA gene sequencing to confirm their identities. To do so, extracted DNA was used as template for PCR amplification specific primers for the 16S gene, namely 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1063R (5'-ACGGGCGGTGTGTRC-3'). Obtained amplicons were sequenced using Sanger technology (Plateforme Génomique Fonctionnelle, Université Victor Segalen, Bordeaux 2, France), and their sequences were aligned and compared using GenBank database, using the NCBI BLAST tool (Basic Local Alignment Search Tools BLAST). The identification was considered valid when the identity was at least 99%.

In vitro evaluation of growth promotion on *Lepidium sativum*

Seeds of *Lepidium sativum* were surface sterilized by an immersion in 2.5% sodium hypochlorite for 1 minute followed by an immersion in 3% H_2O_2 for 1 minute. Seeds were then rinsed three times with sterile distilled water. Sterilization was checked by macerating some seeds in sterile 0.86% $NaCl$, 100 μ l of the macerate was plated on R2A medium. Seeds were then plated on water agar and incubated for 24 hours at 25°C. Fifteen pre-germinated seeds with uniform radicles length (1.5-2 mm) were plated on new water agar dishes. The eight selected PGPR were inoculated in single, dual, or triple combinations (*i.e.*, 92 unique combinations in total), with 100 μ L of solution at a final concentration of 10^9 CFUs.ml⁻¹. The control was considered as a treatment with water only. Plates containing the inoculated pre-germinated sterilized seeds were then incubated for 72 hours at 25°C. To assess the capacity of PGPR to promote *L. sativum* growth, the length, as well as the fresh weight, of stem and roots were measured.

In vitro evaluation of growth promotion on 1103P plantlets

Vitis rupestris × *Vitis berlandieri* plantlets cv. 1103P were propagated *in vitro* on McCown Woody Plant Medium (Duchefa) supplemented with 3% sucrose, 0.27 μ M 1-naphthalene acetic

acid, and 0.75% agar, in a growth chamber at 25°C / 20°C and subjected to a photoperiod of 16 h light/8 h dark with a light intensity of 145 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. After six weeks of growth, fifteen plantlets were transplanted into plant pots filled with McCown Woody Plant Medium with 0.5% agar without any other supplement. The eight selected PGPR were inoculated in single, or dual combinations (i.e., 36 unique combinations), with 300 μL of solution at a final concentration of $10^9 \text{ CFUs.ml}^{-1}$, on the basal part and root extremities of the plantlets. The control was considered as a treatment with water only. Plant pots containing the inoculated plantlets were then put back to growth chamber for 4 weeks. To assess the capacity of PGPR to promote plantlets growth, the length, as well as the fresh weight, of stem and roots were measured. In addition, the number of primary roots and secondary roots were counted, and the petiole length were measured.

Greenhouse experimental design

The same symptomatic soil from the inter-rowed analyzed in Chapters 3 and 4, was used during this greenhouse experiment, and was performed in parallel to Chapter 4 in 2020. The soil from the upper surface to approximately 30 cm deep was collected with a mini excavator, sieved (< 3 cm) to remove roots and gravels. Twenty plants from each treatment (i.e., untreated, mycorrhized, bacterized, and mycorrhized + bacterized) were put in 7.5 L pots (diameter 26 cm, height 21 cm) filled with S soil, supported with geotextile membrane, and amended with sterilized gravels. Those 35 pots per conditions, namely Untreated (control plant), Bac (inoculated with rhizobacteria), Myc (commercially inoculated with mycorrhizal fungi), Myc+Bac (inoculated with both rhizobacteria and mycorrhizal fungi), were placed in greenhouse at middle of April 2020 under ambient light and temperature. The plants were watered twice a week with 60 ml per pots with no nutrient supply. The shoots were tied with thread to stakes in order to let the plants grow on a fence in an upright position. Plants were harvested and phenotyped twice; after 2 and 4.5 months of greenhouse experience. Measurements of the following parameters were done right after the samples harvesting: aerial fresh biomass including leaves and shoots, fresh biomass of trunk and roots, diameters and lengths of shoots and trunk. To measure foliar surface of the plants, main vein length was measured and estimated using $y = 0.5576x^{2.3184}$ equation from Marguerit (2010).

Bioinformatic analysis

All analysis and graphs were performed on R (R-4.1.2) using RStudio (2021.9.1.372). Figures were generated with *ggplot2* (3.3.5) and *ggthemes* (4.2.4) packages and arranged using *ggpubr*

(version 0.4.0) (Arnold, 2021; Kassambara, 2020; Wickham and Chang, 2008). Analysis of Variance (ANOVA) with the combination of isolates used was performed. Residuals were checked for their independency, normality, and variance homogeneity with the Durbin Watson, Shapiro-Wilk, and Bartlett tests, respectively. When assumptions for parametric tests were not respected, a multiple pairwise comparison using Wilcoxon test was performed subsequently to Kruskal–Wallis test using the *multcomp* (1.4-18) package (Hothorn *et al.*, 2008). Principal Component Analysis was performed using *FactoMineR* (2.4) and *missMDA* (1.18) (Josse and Husson, 2016; Le *et al.*, 2008). Hierarchical cluster analysis (HCA) (Gentle *et al.*, 1991; Ruppert, 2004) was applied to visualize the dissimilarities between isolates capacity to promote growth on *L. sativum* and 1103P plantlets. Dissimilarities relied on the same observations that were presented in the PCA. HCA was performed using “hclust” function from *fastcluster* (1.2.3) package based on Euclidean distance metric and Ward’s group linkage. The results were presented as a circular dendrogram, where the smaller the linkage distances in the dendrogram, the more similar the effect of isolate combination.

Results

Diversity and functional characteristics of isolates

From the 800 rhizobacteria isolated in the four conditions from the Chapter 4 experiment (*i.e.*, AS-1103P, S-1103P, AS-RGM, S-RGM), 200 were randomly selected and tested for biochemical tests related to PGP traits. The rhizobacterial isolates belonged to 17 genera with *Bacillus* (24%), *Pseudomonas* (11%), *Rahnella* (7%), and *Streptomyces* (2%) were present in all conditions, while 35% were not identified through MALDI-TOF-MS (**Figure 42.A**).

Paenibacillus (2%), *Buttiauxella* (4%), *Brevibacillus* (1%) were specific to asymptomatic soils while *Rhizobium* (2%) was exclusively found in symptomatic conditions. *Enterobacter* (4%) was found only in 1103P samples, while no genera of the selected isolates were exclusively obtained from RGM samples. Other isolates were defined as *Amicolaptosis* (1%), *Burkholderia* (2%), *Cupriavidus* (1%), *Dyella* (1%), *Lysinibacillus* (2%), *Ralstonia* (2%), *Serratia* (2%), and *Staphylococcus* (1%). All the genera tested for ammonia production, siderophore synthesis, phosphate solubilization, nitrogen fixation, or IAA production, were at least presenting one PGP trait (**Figure 42.B**). *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, and *Rhizobium* were the genera identified with functional ability to contribute to plant growth promotion in each PGP trait. ACC deaminase was the least common PGP trait (15%) found in the isolates tested, while the most common was siderophore production (55.5%), followed by nitrogen

fixation (55%), ammonia production (54.5%), IAA synthesis (49%), and phosphate solubilization (48%) (**Figure 42.C**). Regarding the functional potential of each condition, S-1103P was inhabiting the greatest number of PGP capacity, accounting for 156 traits, followed by AS-1103P (145), S-RGM (132), and AS-RGM (121).

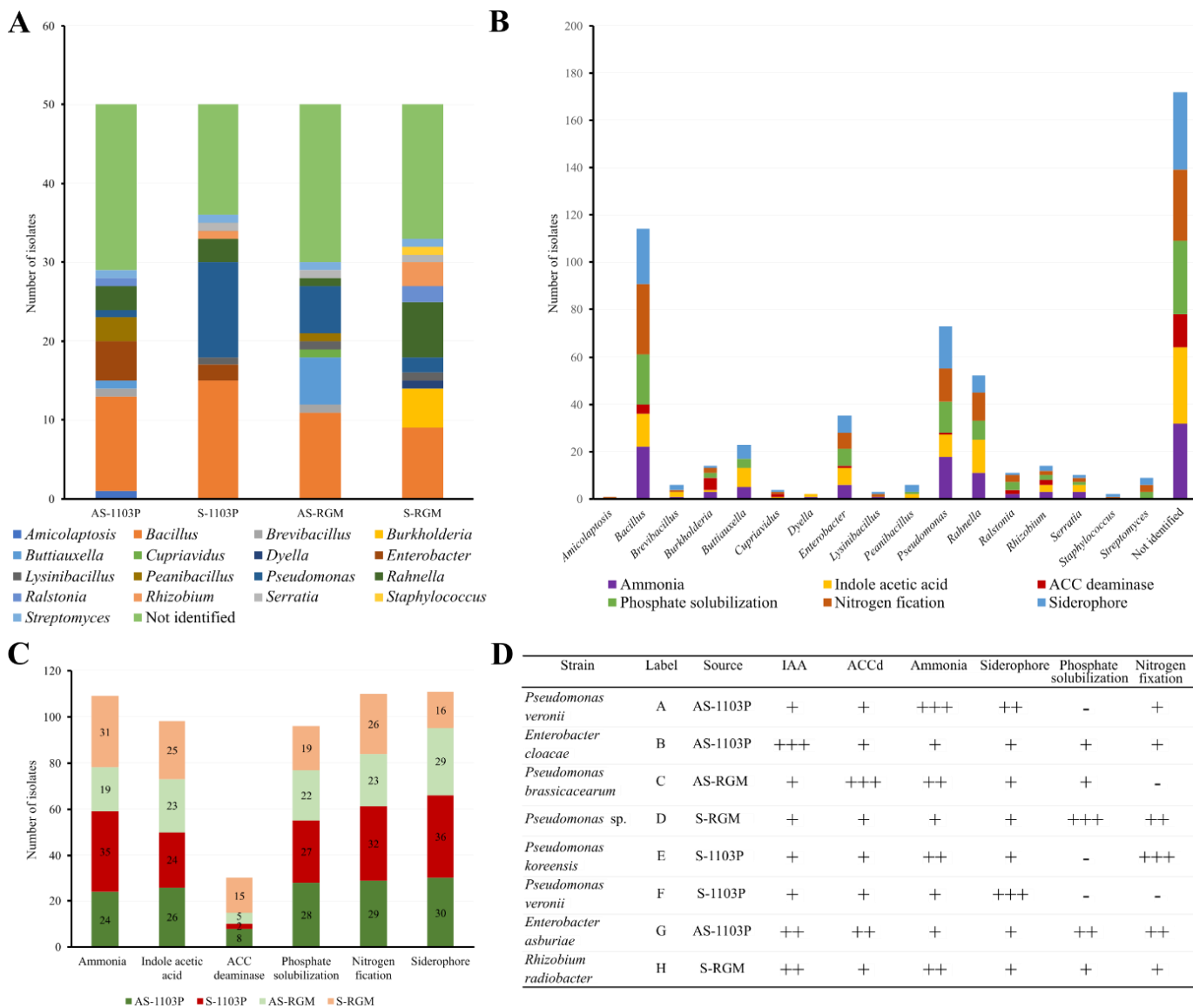


Figure 42 : (A) Bacterial isolates identified at the genus level using MALDI-TOF-MS across the asymptomatic (AS-) and symptomatic (S-) conditions from the rhizosphere of RGM and 1103P rootstocks of the previous study in Chapter 4. (B) Functional capacity of the identified genera to possess PGP traits. (C) Distribution of the isolates having PGP traits. (D) Summary of the most promising isolates tested for *in vitro* growth promotion on *Lepidium sativum* and *Vitis vinifera* L. plantlets with (+) as an effective isolate and (-) as a non-effective isolate in the corresponding PGP function.

After this biochemical screening, the most efficient isolate for each of the PGP traits, as well as two isolates efficient for all traits tested were selected (**Figure 42.D**). Their 16S rRNA sequencing confirmed MALDI-TOF-MS identification as two *Pseudomonas veronii* (labelled as A and F isolates), one *Enterobacter cloacae* (isolate B), *Pseudomonas brassicacearum* (isolate C), *Pseudomonas* sp. (isolate D), *Enterobacter asburiae* (isolate G), and *Rhizobium*

radiobacter (isolate H). Their origins came from each of the conditions (*i.e.*, AS-1103P, S-1103P, AS-RGM, and S-RGM).

Growth promotion effects on *Lepidium sativum* and *Vitis vinifera* plantlets

The best performing selected isolates were inoculated onto *Lepidium sativum* seeds and *Vitis rupestris* × *Vitis berlandieri* cv. 1103P. Phenotypic traits related to their growth were measured and compared to the control corresponding to the water treatment. The inoculates were done in single combination, double combination (*i.e.*, isolate X × isolate Y) or triple combination (*i.e.*, isolate X × isolate Y × isolate Z), with the latter exclusively performed in *L. sativum*.

For *Lepidium sativum*, among the 92 combinations tested, only 2% were inhibiting stem mass, and 4% were inhibiting root length while 24% were inhibiting root mass, and 39% were inhibiting stem length (**Table S31**). Regarding 1103P plantlets, among the 36 combinations tested, 39% were inhibiting leaves and stem mass, while 53% were reducing stem length and 70% were reducing petiole length (**Table S32**). In regard to root system in grapevine plantlets, 78% were promoting the mass of root, with 70% of the tested combinations promoting the length of secondary roots and 39% increasing the length of primary roots.

To compare phenotypic traits between *L. sativum* and *V. vinifera*, only simple and dual combinations effects on total weight and length compared to water treatment were presented in **Figure 43.A**. Significant groups were detected across the different inoculates. The significantly best performing single or double combinations in terms of total weight promotion of *L. sativum* were A×C, B×D, C, C×H, C×G, F, B×F, and A×H. Regarding the promotion of root and stem lengths, A×C, B×F, A×H, and C×H combinations were the most efficient ones. Similarly, significant groups were distinguished in the growth traits of 1103P plantlets. The combinations C×G, D×F, B×E, A×G, F×H, and A×C were inducing a significantly greater weight gain compared to water treatment, while only A×C and A×B combinations were significantly different from water control in terms of total length promotion.

Biplot PCA was used to visualize the inoculates effects on the phenotypic traits measured for *L. sativum*, and 1103P plantlets, distinctly (**Figure 43.B**). The first two dimensions (Dim1 and Dim2) accounted for 88.4%, and 72.3% of the total variance in *L. sativum* and *Vitis rupestris* × *Vitis berlandieri* PCA, respectively. For the PCA of *L. sativum* samples, Dim1 was positively correlated with all the traits measured (*i.e.*, mass of the roots and stem, as well as length of root and stem). Dim2 was positively correlated with mass of both root and stem while negatively correlation with length of both root and length was found with Dim2. Interestingly, the combinations comprising single or dual isolates in *L. sativum* samples were found on the

positive side of Dim2, whereas the combinations composed of triple isolates were found on the negative side of Dim2. Regarding the PCA of 1103P plantlet samples, Dim1 was positively correlated to root weight, petiole length, as well as secondary root length and number, while aerial weight and stem length were negatively correlated to stem length and weight of the aerial part. On other hand, Dim1 was positively correlated to each of the measured variables except the length of the primary root and the number of the secondary roots. As with the *L. sativum* PCA, combinations including the double isolates were predominantly on the positive side of Dim2 while the single-based inoculates were on its negative side. The HCA dendrogram depicts the combinations of tested inoculates in *L. sativum* seeds and 1103P plantlets, and identified seven and five clusters, respectively, based on the phenotypic similarities of the samples (**Figure 43.C**). It appeared that the combinations with the greatest positive effects on the development of *L. sativum* was A×C, while A×C and A×B were the ones for *V. vinifera*. Considering this, the A×C consortium, composed of *Pseudomonas veronii* and *Pseudomonas brassicacearum* was used, as the Bac treatment, in the greenhouse experiment on young vines cv. 1103P potted with the symptomatic soil in Chapter 4.

Effects of the microbial addition on the phenotype of greenhouse plants

Here were only presented the phenotypic measurements made in the greenhouse experiment. The first sampling point, namely T1, did not present any growth differences from the aerial or root system across the four conditions (**Table 20**). However, during the second sampling point T2, significantly higher branch diameter was observed in untreated vines and vines treated with rhizobacteria compared to the mycorrhized plants ($\chi^2 = 21.05$, $P < 0.001$). In addition, mycorrhized plants inoculated with the rhizobacteria displayed significantly greater dry root biomass compared to Bac and Myc conditions ($F(3, 36)=2.27$, $P = 0.047$).

Table 20 : Phenotype measurements on aerial and root system of grapevine grown in greenhouse experiment (n = 10). Different letters indicate significant different group ($p < 0.05$).

| | | Leaves number | Foliar surface (cm ²) | Aerial biomass (g) | Branch diameter (cm) | Dry root biomass (g) | Dry trunk biomass (g) |
|----|-----------|------------------|--------------------------------------|-----------------------|-------------------------|----------------------------|-----------------------------|
| T1 | Untreated | 22.7 ± 4 a | 623 ± 137 a | 6.1 ± 2 a | 1.1 ± 0.2 a | 5.1 ± 1.8 a | 27.6 ± 6.9 a |
| | Myc | 22.9 ± 5.9 a | 743 ± 198 a | 6.8 ± 1.8 a | 1.2 ± 0.2 a | 4.8 ± 1.1 a | 30.3 ± 6.5 a |
| | Myc+Bac | 24.8 ± 4.3 a | 699 ± 114 a | 6.6 ± 1.4 a | 1.1 ± 0.2 a | 4 ± 0.8 a | 25.6 ± 6.3 a |
| | Bac | 25.1 ± 5.3 a | 747 ± 132 a | 6.4 ± 1.4 a | 1.1 ± 0.2 a | 3.9 ± 1.1 a | 23.3 ± 6.3 a |
| T2 | Untreated | 27.3 ± 3.9 a | 434 ± 80 a | 12.2 ± 4 a | 3.2 ± 0.2 a | 11.3 ± 2.5 ab | 32 ± 8 a |
| | Myc | 29.1 ± 4.4 a | 441 ± 79 a | 12.3 ± 2.4 a | 1.5 ± 0.4 b | 10.9 ± 2.5 a | 36.4 ± 9.4 a |
| | Myc+Bac | 28 ± 4.7 a | 444 ± 159 a | 11.9 ± 3.5 a | 1.3 ± 0.3 b | 14.2 ± 5.1 b | 35.5 ± 12.3 a |
| | Bac | 27.4 ± 5.9 a | 585 ± 554 a | 10.8 ± 2.2 a | 3.6 ± 0.5 a | 11.2 ± 1.9 a | 31.1 ± 9.3 a |

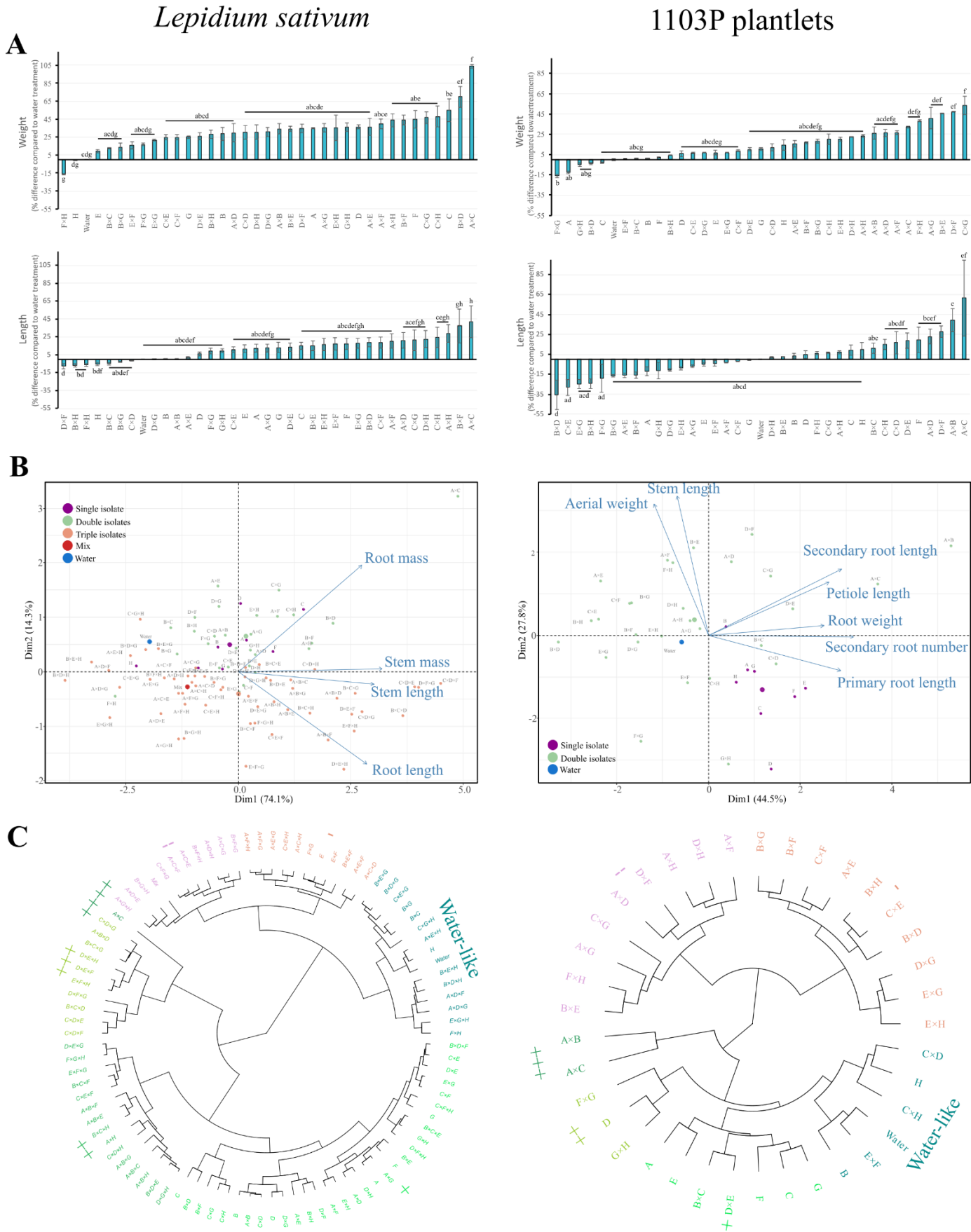


Figure 43 : Growth promotion effects of potentially beneficial isolates on *L. sativum* sprouted seeds (n = 15) and *V. vinifera* plantlets cv. 1103P (n = 15). (A) HCA and (B) biplot PCA of several growth traits from aerial and root systems. HCA clusters were colored according to the efficiency of isolates in growth

influence with (+) as promoting, (-) as inhibitive growth compared to water-treated conditions. PCA individuals were colored according to their inoculation type (single, dual, triple, water, or mix; the latter combination standing for the mix of the eight isolates). (C) Influence of single and dual combinations of isolates compared to water treatment.

Discussion

Characterization of the PGP traits in isolated rhizobacteria

Among 125 rhizobacteria isolated from *V. vinifera* L. cv. Zweigelt grafted onto Kober 5BB, Samad *et al.* (2017) identified a majority of *Pseudomonas* and *Arthrobacter* using R2A medium, whereas no *Arthrobacter* was characterized in our samples. This could be due to the lack of identification of isolated rhizobacteria by MALDI-TOF-MS. This technology is a relatively reliable and fast recognition technique, but the lack of identification is rather dependent on in-house databases (Kopcakova *et al.*, 2014). Here, the genera of the eight isolates tested correspond to the 16S rRNA sequencing results. To continue the analysis and combine taxonomic and functional diversity, the use of 16S rRNA on the remaining isolates should be done.

Although many isolates failed to be identified, the predominant genera characterized were *Pseudomonas*, *Bacillus*, *Rahnella*, and *Streptomyces*, present in the four conditions studied in the chapter 4 (*i.e.*, AS-1103P, S-1103P, AS-RGM, and S-RGM). In grapevine, PGP activities of strains belonging to the *Enterobacteriaceae* and *Pseudomonadaceae* families were tested for the production of hydrogen cyanide, ACCd, siderophores, IAA, and for P solubilization (Marasco *et al.*, 2018; Oyuela Aguilar *et al.*, 2021; Samad *et al.*, 2017). Bona *et al.* (2019) investigated the rhizosphere of *V. vinifera* cv. Pinot Noir using a metaproteome approach. They showed that bacteria belonging to *Streptomyces*, *Bacillus*, *Bradyrhizobium*, *Burkholderia*, and *Pseudomonas* were the genera with highly active protein expression, mainly involved in P and N metabolism. Herein, *Bacillus* and *Pseudomonas* were involved in all the PGP traits tested, with less activity in ACCd. This activity, which principle is to lower the plant ethylene levels (Glick *et al.*, 2007), was the least common functional property. It is concordant with Samad *et al.* (2017), who found this primarily in root endosphere. In addition, *Streptomyces* and *Pseudomonas* are known to exhibit PGP activities but also to inhibit the growth of fungal pathogens in grapevine wood (Niem *et al.*, 2020), rising interest in further antifungal activities of our samples.

With respect to the functional potential of each condition, the 1103P rootstock appears to harbor the greatest number of PGP capabilities, while, regardless of rootstock genotype, the S

conditions show greater functional diversity. Marasco *et al.* (2018) showed that PGP functional genes were conserved in both the rhizosphere and root endosphere despite the selection of different bacterial communities, and thus the frequencies of these PGP traits did not depend on rootstock genotype. Some of the detected genera were exclusively present in 1103P, such as *Enterobacter*, which exhibited high PGP characteristics, with two strains among the eight isolates with the most effective functional capabilities. Functional redundancy is the idea that several taxa can perform the same function within a microbial community (Louca *et al.*, 2018). Exploring rootstock-recruited isolates could be a powerful tool for determining microbiome responses to environmental stresses.

Effects on plants phenotype

Few studies have found the ability of PGPRs to promote grapevine yield (Aziz *et al.*, 2016; Rolli *et al.*, 2017), as most PGPR-related research has focused on pathogen control. Deployment of consortia may promote plant growth more effectively than individual strains (Finkel *et al.*, 2017). Here, the effects of single and double inoculation were distinguished in the biplot analysis, with better growth promotion abilities on *V. vinifera* plantlets when inoculated with a mixture of two isolates. In this preliminary study, only effects on plant growth were presented, with significant *in vitro* and greenhouse effects on aerial and root systems. Interestingly in the greenhouse, mycorrhized plants showed reduced branch diameters compared to non-mycorrhized plants, while greater root biomass was observed in mycorrhized plants inoculated with rhizobacteria. These results suggest a preferential allocation of carbon in roots over branches. However, the rest of the results obtained regarding the impact of the addition of these beneficial microorganisms on the root endosphere and the rhizosphere microbiome still need to be addressed.

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Supplemental information

Table S31 : Percentage of growth promotion or inhibition on *L. sativum* on stem and root measured parameters compared to water treatment.

| Combination | Inocula | Stem mass | Root mass | Stem length | Root length |
|-------------|---------|-----------|-----------|-------------|-------------|
| A | Single | 37 | 31 | 7 | 22 |
| B | Single | 28 | 30 | -14 | 28 |
| C | Single | 47 | 76 | 2 | 40 |
| D | Single | 31 | 51 | 0 | 18 |
| E | Single | 9 | 11 | 6 | 21 |
| F | Single | 49 | 32 | 11 | 29 |
| G | Single | 33 | 4 | 11 | 18 |
| H | Single | 5 | -16 | -10 | 3 |
| A×B | Double | 34 | 33 | -11 | 24 |
| A×C | Double | 77 | 124 | 39 | 47 |
| A×D | Double | 30 | 29 | 18 | 27 |
| A×E | Double | 37 | 36 | 4 | -3 |
| A×F | Double | 32 | 58 | 16 | 28 |
| A×G | Double | 34 | 39 | 6 | 25 |
| A×H | Double | 38 | 58 | 21 | 45 |
| B×C | Double | 10 | 20 | -12 | 9 |
| B×D | Double | 70 | 69 | 7 | 42 |
| B×E | Double | 40 | 19 | 9 | 27 |
| B×F | Double | 44 | 44 | 41 | 30 |
| B×G | Double | 15 | 10 | -13 | 16 |
| B×H | Double | 31 | 21 | -17 | 13 |
| C×D | Double | 30 | 31 | -14 | 23 |
| C×E | Double | 26 | 21 | 1 | 30 |
| C×F | Double | 28 | 15 | 11 | 35 |
| C×G | Double | 44 | 52 | 28 | 9 |
| C×H | Double | 46 | 52 | 26 | 22 |
| D×E | Double | 24 | 31 | 7 | 26 |
| D×F | Double | 42 | 15 | -12 | 0 |
| D×G | Double | 26 | 45 | -8 | 16 |
| D×H | Double | 36 | 16 | 20 | 27 |
| E×F | Double | 17 | 13 | 14 | 23 |
| E×G | Double | 24 | 15 | 8 | 38 |
| E×H | Double | 31 | 47 | 15 | 19 |
| F×G | Double | 14 | 24 | 4 | 18 |
| F×H | Double | -14 | -21 | -18 | 16 |
| G×H | Double | 35 | 38 | -3 | 34 |
| Water | Water | 48 | 33 | 10 | 46 |
| A×B×C | Triple | 65 | 31 | 28 | 52 |
| A×B×D | Triple | 40 | 23 | 15 | 53 |
| A×B×E | Triple | 62 | 20 | 15 | 68 |
| A×B×F | Triple | 63 | 23 | 6 | 43 |

| | | | | | |
|-------|--------|-----|-----|-----|----|
| A×B×G | Triple | 43 | 15 | 15 | 42 |
| A×B×H | Triple | 72 | 55 | 40 | 77 |
| B×C×D | Triple | 38 | 34 | 0 | 38 |
| B×C×E | Triple | 33 | 2 | 8 | 46 |
| B×C×F | Triple | 69 | 45 | 24 | 60 |
| B×C×G | Triple | 50 | 27 | 17 | 58 |
| B×C×H | Triple | 39 | 25 | 13 | 39 |
| B×D×E | Triple | 71 | 63 | 60 | 66 |
| C×D×E | Triple | 74 | 75 | 63 | 72 |
| C×D×F | Triple | 78 | 31 | 30 | 59 |
| C×D×G | Triple | 54 | 39 | 25 | 42 |
| C×D×H | Triple | 37 | 2 | 19 | 50 |
| C×E×F | Triple | 8 | -8 | -20 | 18 |
| C×E×G | Triple | 57 | 35 | 37 | 62 |
| D×E×F | Triple | 43 | 14 | -3 | 47 |
| D×E×G | Triple | 51 | 15 | 34 | 77 |
| D×E×H | Triple | 98 | 64 | 25 | 71 |
| D×F×G | Triple | 32 | 24 | 0 | 38 |
| D×F×H | Triple | 42 | 23 | 10 | 37 |
| D×G×H | Triple | 31 | -14 | 5 | 58 |
| E×F×G | Triple | 60 | 29 | 35 | 67 |
| E×F×H | Triple | -3 | -35 | -28 | 19 |
| E×G×H | Triple | 42 | 5 | -1 | 49 |
| F×G×H | Triple | 24 | -2 | 21 | 21 |
| A×C×D | Triple | 17 | -12 | -4 | 20 |
| A×C×E | Triple | 21 | -3 | -3 | 17 |
| A×C×F | Triple | 28 | 6 | -12 | 21 |
| A×C×G | Triple | 16 | -8 | 13 | 17 |
| A×C×H | Triple | 15 | -25 | -12 | 23 |
| A×D×E | Triple | 1 | -25 | -21 | 0 |
| A×D×F | Triple | 13 | -24 | -37 | 12 |
| A×D×G | Triple | 24 | 1 | -11 | 25 |
| A×D×H | Triple | 26 | 6 | 25 | 31 |
| A×E×F | Triple | 7 | -1 | -4 | 20 |
| A×E×G | Triple | 6 | -19 | -6 | -6 |
| A×E×H | Triple | 11 | -8 | 1 | 22 |
| A×F×G | Triple | 8 | -9 | 3 | 27 |
| A×F×H | Triple | 14 | -26 | -7 | 34 |
| A×G×H | Triple | 32 | 23 | 7 | 31 |
| B×D×F | Triple | 10 | 2 | -23 | 9 |
| B×D×G | Triple | -10 | -38 | -38 | -2 |
| B×D×H | Triple | 0 | 18 | 6 | 31 |
| B×E×F | Triple | 15 | 4 | -19 | 10 |
| B×E×G | Triple | 2 | -22 | -39 | 0 |
| B×E×H | Triple | 25 | 14 | -12 | 35 |
| B×F×G | Triple | 25 | -13 | -17 | 13 |

| | | | | | |
|-------|--------|----|-----|-----|----|
| B×F×H | Triple | 21 | -19 | -20 | 42 |
| B×G×H | Triple | 16 | -9 | 12 | 27 |
| C×E×H | Triple | 28 | -2 | -5 | 18 |
| C×F×G | Triple | 32 | 3 | 1 | 29 |
| C×F×H | Triple | 21 | 8 | -33 | -1 |
| C×G×H | Triple | 8 | 17 | -25 | 40 |
| Mix | Mix | 37 | 31 | 7 | 22 |

Table S32 : Percentage of growth promotion or inhibition on *V. vinifera* L. plantlets on aerial and root measured parameters compared to water treatment.

| | Leaves and stem mass | Root mass | Stem length | Total petiole length | Secondary root length | Number of secondary roots | Primary root length |
|-----|----------------------|-----------|-------------|----------------------|-----------------------|---------------------------|---------------------|
| A | -22 | -2 | -9 | 6 | 46 | 188 | -27 |
| B | -3 | 7 | 4 | 9 | -5 | 94 | 5 |
| C | -26 | 22 | -15 | -18 | 69 | 150 | 13 |
| D | -33 | 49 | -27 | -14 | 12 | 88 | 26 |
| E | -20 | 36 | -17 | 6 | 59 | 212 | -10 |
| F | -24 | 31 | -13 | 0 | 63 | 97 | 33 |
| G | -4 | 26 | -12 | -9 | 63 | 153 | -7 |
| H | -19 | 51 | -4 | -6 | -18 | 6 | 25 |
| A×B | 0 | 54 | 8 | 23 | 228 | 365 | 20 |
| B×C | -14 | 18 | -2 | 0 | 75 | 106 | 6 |
| C×D | -17 | 44 | 0 | -9 | 28 | 141 | 26 |
| D×E | -7 | 29 | 3 | 3 | 117 | 124 | 8 |
| E×F | -10 | 11 | -7 | -5 | -31 | 24 | 4 |
| F×G | -24 | -7 | -16 | -19 | -52 | 29 | -13 |
| G×H | -35 | 29 | -23 | -15 | -30 | 128 | 2 |
| A×C | 24 | 41 | 19 | 20 | 135 | 171 | 75 |
| B×D | 18 | -24 | 0 | -21 | -55 | -29 | -48 |
| C×E | 20 | -6 | 3 | -12 | -37 | -17 | -44 |
| D×F | 52 | 43 | 10 | 1 | 82 | 83 | 6 |
| E×G | 13 | 2 | -4 | -27 | -26 | 26 | -38 |
| F×H | 41 | 36 | 8 | -20 | 68 | 51 | -34 |
| A×D | 44 | 10 | 1 | 3 | 102 | 93 | -12 |
| B×E | 51 | 41 | 8 | -5 | 45 | 47 | -28 |
| D×G | 17 | -3 | -5 | -22 | 24 | 61 | -37 |
| E×H | 16 | 24 | -3 | -25 | 33 | 83 | -39 |
| A×E | 37 | -4 | 8 | -10 | -12 | -51 | -36 |
| C×F | 22 | -3 | 6 | -16 | 23 | -18 | -22 |
| B×F | 17 | 16 | 0 | -18 | -21 | 11 | -24 |
| C×G | 40 | 66 | 5 | -8 | 31 | 226 | -9 |
| D×H | 27 | 19 | -1 | -1 | 16 | -19 | -4 |
| A×F | 38 | 16 | 8 | 2 | 29 | -2 | -32 |
| B×G | 33 | 5 | 1 | -12 | 3 | 22 | -41 |
| C×H | 3 | 36 | -9 | -23 | 30 | 19 | 22 |
| A×G | 19 | 61 | -6 | -17 | 41 | 40 | -38 |
| B×H | 15 | -6 | 2 | -16 | -8 | -42 | -53 |
| A×H | 23 | 24 | -5 | -11 | 60 | 11 | -18 |

Brief discussion

In this chapter, the beneficial effects of isolated rhizobacteria on growth of *L. sativum* and *V. vinifera* cv 1103P was shown. Some isolates with high functional capacity were specific to 1103P rootstock. The use of consortia consisting of two strains was inducing a greater effect than inoculate of single-based isolate. The use of the most efficient rhizobacterial mix coupled to AMF displayed an increased root biomass while a reduced branch diameter was observed in mycorrhized plants.

However, further analysis is required to observe the effects on grapevine, and its associated belowground microbiome structure.

Chapter VI

General discussion

This thesis is part of the Vitirrhizobiome project, which aims to shed light on the functioning of the soil and roots of grapevines facing decline with unidentified cause. In this exploratory context, the strategy adopted sought to understand the importance of the bulk, rhizosphere, and root microbiomes in vineyards, as well as in young, grafted grapevine plants potted with soil from symptomatic vineyard.

The main working hypothesis was based on the microbial dysbiosis of these dysfunctional soils, with either the depletion of important microorganisms carrying ecological processes essential to soil quality, or the overall reduction in diversity and abundance of soil microbes.

To explore this hypothesis, some biological indicators were evaluated, in the first part of Chapter 2, on bulk soils from vineyard supporting declining vines with no apparent pathological causes or nutrient dysregulation. Results indicated unbalanced microbial profiles, as well as reduced enzymatic activities, suggesting a dysbiosis of the microbial communities functioning and composition. In the second part of Chapter 2, high throughput sequencing of bacterial and fungal amplicons confirmed the microbial disequilibrium but with different results than expected. Compared to the asymptomatic soils, the fungal richness and diversity were higher in soils subjected to decline. In addition, genera of potentially beneficial bacteria, as well as pathogenic fungal genera, were more abundant in symptomatic soils.

1. Investigating stressed soils for beneficial isolates

Even though this part of the study was not based on isolates, sequencing of 16S rRNA gene revealed higher abundances of *Blastococcus*, *Terrabacter*, *Sphingomonas*, *Gemmatimonas*, and *Fonticella* genera. These genera are affiliated to potentially beneficial traits involved in soil nutrient regulation and pathogen control. Enrichment of pathogen-controlling bacteria was explained by a greater abundance of potentially fungal pathogens. The cry-for-help hypothesis was proposed to understand this predominance of beneficial and pathogenic taxa.

However, it is worth mentioning that the synergy between plant and soil rules most of the telluric geobiochemical processes. The synergetic co-evolution between soil and plant could explain the dynamics taking place in the belowground compartment (Gouda *et al.*, 2018). Certain microorganisms are considered as beneficial by favoring plant development, but in return obtain reduced carbon from root exudates and an ecological niche such as host endosphere or soil rhizosphere (Lyu *et al.*, 2021). As a result of this coevolution, the high diversity and metabolic richness of microbial communities are common in the rhizosphere of perennial plants such as grapevine (Marasco *et al.*, 2018). Rhizodeposition is the process of

carbon released from the plants which vary between 10% to 40% of produced photosynthates (Hennion *et al.*, 2019). This carbon flux is an attractive nutrient source for bacteria and fungi, especially the ones forming mycorrhizal association.

In addition to signaling compounds exudated from roots, stress or other environmental stimuli modulate the biochemical pathways of microorganisms. For instance, the composition and production of EPS or anti-oxidative enzymes in cyanobacteria under salt stress was modified (Ozturk and Aslim, 2010; Verma *et al.*, 2019). Herein, the metabolic diversity measured by Eco-plates technology was more important in the symptomatic bulk (Chapter 2) and rhizosphere (Chapter 3) soils compared to asymptomatic ones. Therefore, one hypothesis would be that the grapevine under decline produces compounds stimulating the microbial communities in the surrounding soil, and that the presence of high abundance of fungi potentially associated to grapevine diseases created a niche for beneficial bacteria.

Ethylene is one of plant hormones that coordinate stress signaling in the host and is produced under various environmental stimulus (Khan *et al.*, 2017). Among the different PGP traits, ACC deaminase is known to alleviate the ethylene-negative effects on plant development (Olanrewaju *et al.*, 2017). Strains possessing the highest efficiency in ACC deaminase have been isolated from some nutrient-poor and alkaline areas (Leontidou *et al.*, 2020). Similarly, the best siderophore producers have been isolated in rhizosphere of tolerant cultivar under iron stress (de Souza *et al.*, 2015). In our case, the best candidates in phosphate solubilization, nitrogen fixation, siderophore, and IAA synthesis, identified as *Pseudomonas* sp., *Pseudomonas koreensis*, *Pseudomonas veronii*, and *Enterobacter cloacae*, respectively, were all isolated in symptomatic soils (Chapter 5).

Studying isolates in extreme or stressed soils could be an interesting goal to pursue. Similarly, the endosphere of stressed or diseased plant roots may harbor highly active and beneficial microbes. This strategy could have promising prospects for agricultural management and disease suppression. To this end, some tools improvements are required to better characterize the beneficial isolates for plants.

2. Culturomics: when the old comes back in fashion

Although the study of microbial communities is old, it has long been restricted to the use of imaging and microscopy techniques that simply allow the observation of morphological characteristics. In this context, only microorganisms that could be grown *in vitro* could be

studied. Thus, before the development of molecular biology technologies, only a low-resolution study of a small fraction of the cultivable microbes was possible.

Core functional microbiome is an interesting aspect to look over. To do so, certain culture-independent methods are used to unravel the metabolic diversity of microorganisms. For instance, metaproteomic analyses have provided insights into the molecular phenotypes of microbial communities from the rhizosphere of grapevine (Bona *et al.*, 2019). Another strategy, namely the metabolomic profiling, is able to depict the metabolites taking part in the microorganisms \times rhizosphere chemical interactions (Mhlongo *et al.*, 2018). In addition, shotgun metagenomics has also proven to be an effective methodology to link functional and taxonomic diversity in vineyards (Castañeda and Barbosa, 2017). Even though, these methodologies are not democratized yet in grapevine research, shotgun metagenomics tends to be more affordable, expanding the opportunities to study the functionality of microbial communities (Azevedo-Silva *et al.*, 2021).

But as a major limitation of these techniques are well named, they are not based on collecting isolates. Looking for microorganisms capable of triggering priming defense or nutrient uptake in plants requires their cultivation prior to their functional characterization. Moreover, database of genomic references must be supplied with characterized isolates, making the isolation process an essential step in the investigation of microbial communities. Although this scientific discipline has been studied since the dawn of molecular approaches, it is suggested that the genomic diversity of cultured bacteria has not yet been fully explored (Trivedi *et al.*, 2021).

Therefore, culturomics approaches can provide valuable instruments for the isolation, discovery, and biochemical characterization of new microbial taxa, essential to better comprehend the functioning of plant and soil microbes. Different protocols, namely high-throughput culturing, could be developed such as the use of several sample dilutions, pH, temperature, atmospheres, or incubation time, and especially the composition of the media to improve the cultivability of the microbiota. The composition of the medium acts as a first filter of active microbes that could be screened in function of their metabolic profiles.

For instance, some specialized saprophytic microorganisms were isolated using contaminants as sole carbon sources (Chicca *et al.*, 2022). By changing condition cultures (*e.g.*, broth culture, detection of microcolonies, specific media composition...), and using MALDI-TOF coupled to MS for microorganisms identification, Lagier *et al.* (2016) cultured human gut microorganisms with sequences previously unassigned. The culturomics of the plant microbes lags significantly behind the human microorganisms, and should be inspired by its advances. The development of an in-house MALDI-TOF MS database specific to grapevine rhizosphere bacteria and fungi

could be a valuable tool to identify, at the species level, the microbiota that can be cultivated in the implementation of plant and soil culturomics.

The recovery of rare or non-culturable taxa in plant and soil habitat can be improved by certain methodologies. Indeed, the nutrient excess in artificial media contributes to select fast-growing microbes, a simulated environment such as the use of the diffusion chamber can alleviate this issue (Bollmann *et al.*, 2007). This is based on the incubation of a diluted sample in contact with the natural habitat of the target microorganisms through a membrane with pores of a few μm . As with diffusion chamber, the use of soil substrate membrane system is quite interesting to imitate natural conditions (Pudasaini *et al.*, 2017). Moreover, the ichip, which acts as a diffusion chamber but is directly placed in the natural habitat, has been shown to increase the recovery of microbial taxa from 5 to 300 fold (Berdy *et al.*, 2017). In addition, novel plant-based culture media are getting increased attention in plant culturomics (Sarhan *et al.*, 2019), and rhizosphere samples (Mourad *et al.*, 2018).

Microorganisms \times plant interactions have been explored using holistic ecological studies, however grapevine microbiome research have yet to benefit from all these available tools. Although the mechanisms involved in vineyard ecology need to be better understood, one understudied strategy deserves greater attention.

3. Soil microbiome transplant as part of microbiome engineering

A secondary objective of this work was to find out if the addition of potentially beneficial microorganisms would impact the microbiome functioning. The rhizobacteria inoculated had positive effects in controlled environment on *L. sativum* sprouted seeds and *V. vinifera* plantlets and had small effects in root growth of grapevine in greenhouse (Chapter 5). This inoculation is part of the microbiome engineering concept since it modifies the microbiome functioning and taxonomic diversity. Another relevant result to mention was the high abundance of fungal genera associated to grapevine diseases in roots from plants obtained from the nursery. After potting plants and few months in the greenhouse, the abundance and richness of the pathogenic fungi were significantly reduced. The natural protective role of soil was mentioned, which is another microbiome engineering concept to explore.

Disease-suppressive soils are possible explanations for protecting plants from root infections by soilborne pathogens. This interesting microbiological phenomenon can be distinguished into two types, namely the general suppressiveness of soils and the specific one, that are reviewed in Gómez Expósito *et al.* (2017). The latter is promoted by certain microorganisms that interfere

with soil pathogens, while the general repression is attributed to global biotic and abiotic soil parameters that control the pathogens. It has been proposed that the suppressive effects of some soil could be transferred to conducive (*i.e.*, soil presenting soil pathogens) ones by soil transplantation (Klein *et al.*, 2013). By mixing small amounts (1-10%) of the suppressive soil with the conducive soil, the growth of soil pathogens can be inhibited (Raaijmakers and Mazzola, 2016; van der Voort *et al.*, 2016). Since the rhizosphere microbiome can be a biomarker of plant health, this microbiome transplantation can be related to the medical field one with fecal transfer, which is a well-known therapy for severe diseases (Antushevich, 2020; Smits *et al.*, 2013; Suskind *et al.*, 2015).

4. Plant phenotype and microbial composition

As stated previously, grapevine is a grafted perennial crop. The genetic dimension of this hybrid form brings supplemental questioning regarding the contribution of the scion into the microbiome association. Up to now, very few studies have focused on this aspect, opening new perspectives related to grapevine \times soil \times microbiome interactions. However, some questions still remain opened; for instance, does the scion have a strong impact on the selection of microbes? Do some scions attract some endophytic microorganisms favoring microorganisms beneficial for vine health or even impacting grapes composition and finally wine quality? Most of the potential answers relies on the signaling between the scion and rootstock, and it would be outstanding to explore the scion \times rootstock combination effects in this kind of contexts.

Variations of the root and rhizosphere microbial communities depending on the rootstock may be due, in addition to exudated chemoattractants, to differences in root development, morphology and architecture (Chapter 4). The root system is responsible for the acquisition of microbes, as well as water and mineral resources for the whole plant. The uptake depends on the rootstock genotype which differs in terms of biomass allocation and selection of microorganisms. Herein, CS \times 1103P combination showed higher vigour, compared to CS \times RGM. The balance between the root system and the aerial part is therefore an important parameter for the mineral supply of the plant. These results suggest that 1103P has a stronger ability to provide mineral nutrition to its aerial part, which is consistent with previous results based on these rootstocks (Cochetel *et al.*, 2018; Gautier *et al.*, 2021). But what about the microbial endosphere from the aerial part; does it have a role in this development? For instance, it has been found that vines subjected to practices inducing fast growth (*e.g.*, pruning) were

more subjected to Esca symptoms (Hrycan *et al.*, 2020). Some endophytes might be linked with the nutrient allocation as it was proposed by Henning *et al.* (2019) for *Populus deltoides* tree. In the present work, inoculations of some mixing of isolates (*e.g.*, A×B isolates) presented increased number of secondary roots. In addition, the samples treated with both rhizobacteria (*i.e.*, A×C isolates), and mycorrhizal fungi increased the root biomass. The hallmarks of a root system are related to its ability to explore the soil and acquire nutrients (Hodge *et al.*, 2009). However, depending on the availability of nutrients in the soil, the root system is more or less adapted to acquire them. This depends mainly on the capacity of the rootstock to develop ramifications in the soil. Mixing of some beneficial isolates need further investigations to better evaluate the impact in the root architecture system. As with the aerial compartment discussed above, root endophytes of rootstock with dense and highly rooting ability might also provide interesting PGP traits.

5. Future prospects

Here, most of the results were based on taxonomic identification and some were conducted on microbial functionalities which were Eco-Plates measurements and PGP isolations. Omic-based technologies have opened new opportunities to study microbial processes in soil and plants. As noted in chapter 1, shotgun sequencing allows taxonomic diversity to be associated with functional diversity (Quince *et al.*, 2017). As the use of metagenomics and metabarcoding becomes more widespread, other resources are available to explore plant-microbe interactions. The advent of omic-based technologies is intended to detect and characterize genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) (Sharma *et al.*, 2020). Transcriptomics and metabolomics are also used to assess soil quality (Chen *et al.*, 2014; Withers *et al.*, 2020), which encourage the integration of omics methodologies to evaluate the functional characteristics of soil and plant microbiome.

6. Usefulness of this thesis work, and application to winemaking professionals

The work performed had the potential to explore theoretical and applicable issues. In theory, the main problematic was to investigate the dysregulated microbiome in declining vineyards. To prevent decline in vineyards, winegrowers usually prospect the soil physicochemical parameters to ensure the presence of good nutrient balance. This work encourages the use of microbiological indicators in soil prior to establish a vineyard, such as the detection of fungal

pathogens, the diversity metrics, and the enzymatic activities. In chapter 4, certain fungal pathogens were detected so even if it sounds evident, the choice of healthy cultivar is recommended. Standards of the hygiene in nursery, as well as the treatments subsequently to cuttings, greatly influence the quality and health of grapevines (Gramaje and Armengol, 2011; Waite *et al.*, 2018). However, the protective effects of hot-water treatments against phytoplasmas and some fungal GTDs do not last long, and further treatments are required when the vineyard is established (Bruez *et al.*, 2017).

The microbial composition of the soil reflects the quality of the soil and is correlated with the overall health of the vine. This modification linked to the diversity of the microbial composition seems to be closely related to the observed decline. Thus, cultural practices known to stimulate microbial diversity should be adopted so that soils can provide the ecosystem services essential to the proper development of the vineyard. Moderate addition of organic matter to the soil can stimulate microbial activity, so organic amendments should be favored over mineral fertilization (Belmonte *et al.*, 2018b; Di Giacinto *et al.*, 2020). Similarly, tillage operations should be minimized to preserve the soil aggregate structure that provides the microhabitat for microbial communities. It is also recognized that maintaining a vegetative cover is an agronomic option that promotes biological life in the soil. And finally, it would be preferable to moderate the use of pesticides and in particular copper, whose negative impact on the microbial population is known (Mackie *et al.*, 2013).

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General conclusion

This work has shown the interest of promoting the use of biological indicators to detect microbial disturbances occurring in vineyard soils and conducive to grapevine decline. Microbiome dysregulation might be determinant for the future of grapevine health, but symptomatic plants harbored highly active microorganisms with potentially beneficial bacterial. The combination of several approaches to explore the diversity of the belowground microbiota and its functioning should continue to provide insights into vineyard management.

Annex

Publications

Darriaut, R., Martins, G., Dewasme, C., Mary, S., Darrieutort, G., Ballestra, P., Marguerit E., Vivin P., Ollat, N., Masneuf-Pomarède, I., Lauvergeat, V. (2021). Grapevine decline is associated with difference in soil microbial composition and activity. *OENO One*, 55(3), 67-84.

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Oral communications

Darriaut, R., Wastin, J., Martins, G. E., Ballestra, P., Dewasme, C., Mary, S., Darrieutort, G., Marguerit, E., Vivin, P., Ollat, N., Masneuf-Pomarède, I., Compant, S., Lauvergeat, V. (2019). Contrasting soil microbial profiles in healthy and declined vineyards. In *MiCrope 2019 International Symposium*. Vienna, Austria

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Poster communication

Darriaut, R., Vivin, P., Schmutz, M., Martins, G., Ballestra, P., Masneuf-Pomarède, I., Marguerit, E., Ollat, N., Guenser, J., Darrieutort, G., Dewasme, C., Lauvergeat, V., (2020) Vitirhizobiome : Influence of the rhizosphere microbiota on the scion growth. In *Séminaire du PNDV : Recherche et transfert, quelles synergies?*. Beaune, France.

Scientific popularization

Dewasme Laveau, C., Derycke M., Janoueix, A., Mary, S., Darriaut, R., G., Darrietort, Martins, G., Ballestra, P., Schmutz, M., Marguerit, E., Vivin, P., Ollat, N., Masneuf-Pomarède, I., Lauvergeat, V. (2020). Quels rôles pour le microbiote du sol dans la lutte contre le dépérissement du vignoble et l'aide à la complantation de jeunes vignes greffées? *Revue des Œnologues*, S177, 24-27.

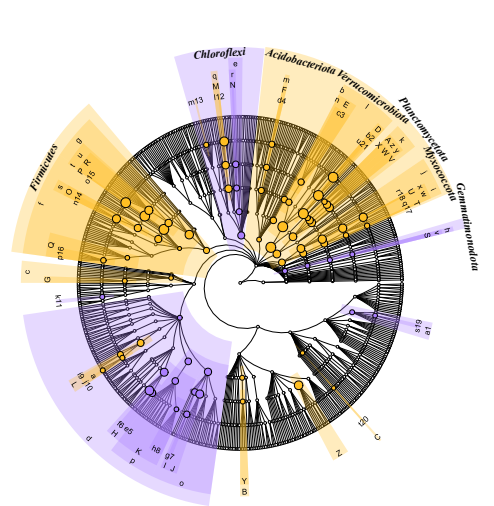
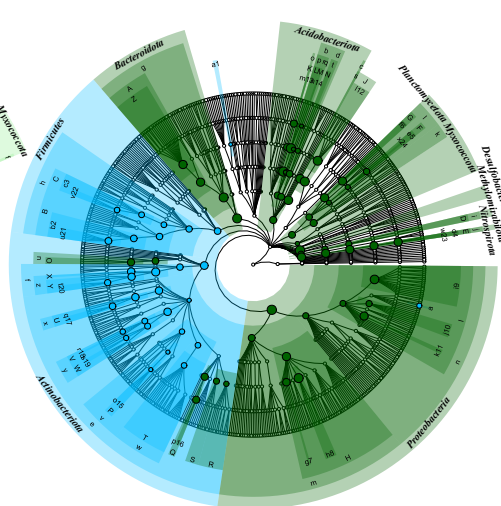
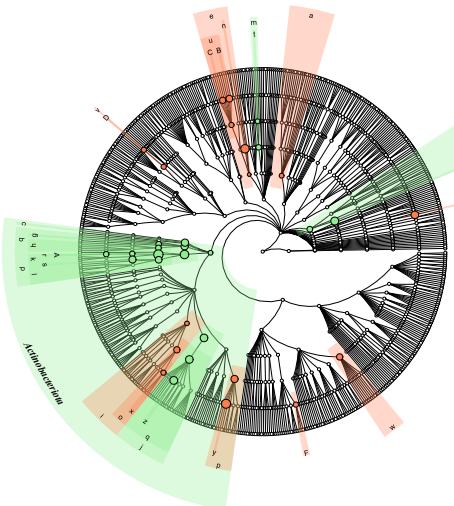
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● Symptomatic
● Asymptomatic

● Autumn
● Spring

● Haut-Médoc
● Graves

16S

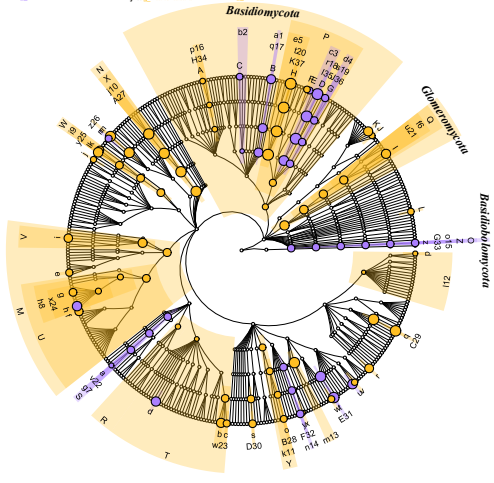
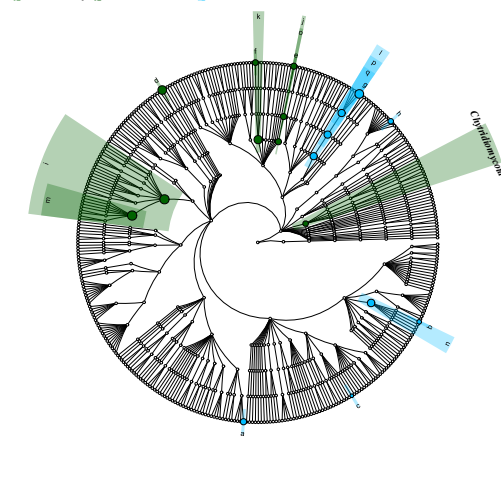
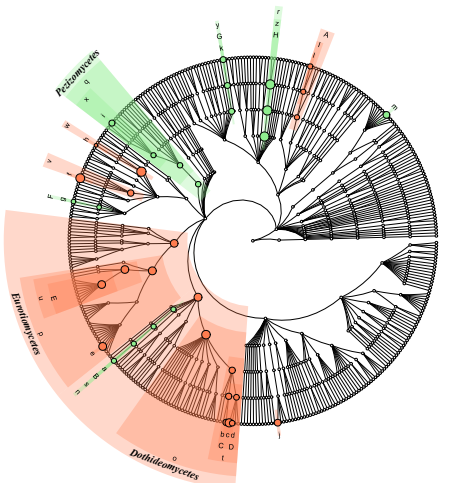


- A: *Aschbachia*
- B: *Aschbachia*
- C: *Aschbachia*
- D: *Aschbachia*
- E: *Aschbachia*
- F: *Aschbachia*
- G: *Aschbachia*
- H: *Aschbachia*
- I: *Aschbachia*
- J: *Aschbachia*
- K: *Aschbachia*
- L: *Aschbachia*
- M: *Aschbachia*
- N: *Aschbachia*
- O: *Aschbachia*
- P: *Aschbachia*
- Q: *Aschbachia*
- R: *Aschbachia*
- S: *Aschbachia*
- T: *Aschbachia*
- U: *Aschbachia*
- V: *Aschbachia*
- W: *Aschbachia*
- X: *Aschbachia*
- Y: *Aschbachia*
- Z: *Aschbachia*

- A: *Chilodactylus*
- B: *Chilodactylus*
- C: *Chilodactylus*
- D: *Chilodactylus*
- E: *Chilodactylus*
- F: *Chilodactylus*
- G: *Chilodactylus*
- H: *Chilodactylus*
- I: *Chilodactylus*
- J: *Chilodactylus*
- K: *Chilodactylus*
- L: *Chilodactylus*
- M: *Chilodactylus*
- N: *Chilodactylus*
- O: *Chilodactylus*
- P: *Chilodactylus*
- Q: *Chilodactylus*
- R: *Chilodactylus*
- S: *Chilodactylus*
- T: *Chilodactylus*
- U: *Chilodactylus*
- V: *Chilodactylus*
- W: *Chilodactylus*
- X: *Chilodactylus*
- Y: *Chilodactylus*
- Z: *Chilodactylus*

- A: *Prothothallo*
- B: *Prothothallo*
- C: *Prothothallo*
- D: *Prothothallo*
- E: *Prothothallo*
- F: *Prothothallo*
- G: *Prothothallo*
- H: *Prothothallo*
- I: *Prothothallo*
- J: *Prothothallo*
- K: *Prothothallo*
- L: *Prothothallo*
- M: *Prothothallo*
- N: *Prothothallo*
- O: *Prothothallo*
- P: *Prothothallo*
- Q: *Prothothallo*
- R: *Prothothallo*
- S: *Prothothallo*
- T: *Prothothallo*
- U: *Prothothallo*
- V: *Prothothallo*
- W: *Prothothallo*
- X: *Prothothallo*
- Y: *Prothothallo*
- Z: *Prothothallo*

ITS

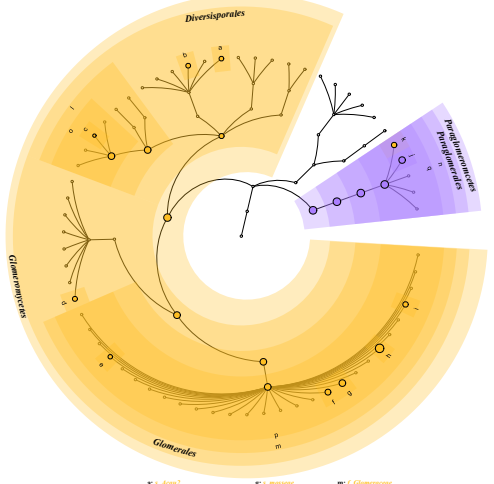
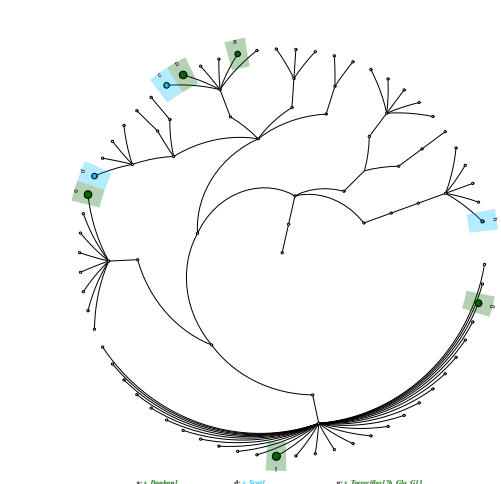
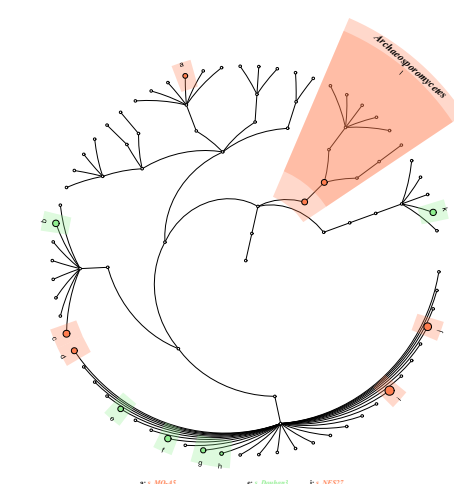


- A: *Filobolus*
- B: *Filobolus*
- C: *Filobolus*
- D: *Filobolus*
- E: *Filobolus*
- F: *Filobolus*
- G: *Filobolus*
- H: *Filobolus*
- I: *Filobolus*
- J: *Filobolus*
- K: *Filobolus*
- L: *Filobolus*
- M: *Filobolus*
- N: *Filobolus*
- O: *Filobolus*
- P: *Filobolus*
- Q: *Filobolus*
- R: *Filobolus*
- S: *Filobolus*
- T: *Filobolus*
- U: *Filobolus*
- V: *Filobolus*
- W: *Filobolus*
- X: *Filobolus*
- Y: *Filobolus*
- Z: *Filobolus*

- A: *Carolinia sp.*
- B: *Carolinia sp.*
- C: *Carolinia sp.*
- D: *Carolinia sp.*
- E: *Carolinia sp.*
- F: *Carolinia sp.*
- G: *Carolinia sp.*
- H: *Carolinia sp.*
- I: *Carolinia sp.*
- J: *Carolinia sp.*
- K: *Carolinia sp.*
- L: *Carolinia sp.*
- M: *Carolinia sp.*
- N: *Carolinia sp.*
- O: *Carolinia sp.*
- P: *Carolinia sp.*
- Q: *Carolinia sp.*
- R: *Carolinia sp.*
- S: *Carolinia sp.*
- T: *Carolinia sp.*
- U: *Carolinia sp.*
- V: *Carolinia sp.*
- W: *Carolinia sp.*
- X: *Carolinia sp.*
- Y: *Carolinia sp.*
- Z: *Carolinia sp.*

- A: *Stropharia*
- B: *Stropharia*
- C: *Stropharia*
- D: *Stropharia*
- E: *Stropharia*
- F: *Stropharia*
- G: *Stropharia*
- H: *Stropharia*
- I: *Stropharia*
- J: *Stropharia*
- K: *Stropharia*
- L: *Stropharia*
- M: *Stropharia*
- N: *Stropharia*
- O: *Stropharia*
- P: *Stropharia*
- Q: *Stropharia*
- R: *Stropharia*
- S: *Stropharia*
- T: *Stropharia*
- U: *Stropharia*
- V: *Stropharia*
- W: *Stropharia*
- X: *Stropharia*
- Y: *Stropharia*
- Z: *Stropharia*

18S



- A: *18S-47*
- B: *18S-47*
- C: *18S-47*
- D: *18S-47*
- E: *18S-47*
- F: *18S-47*
- G: *18S-47*
- H: *18S-47*
- I: *18S-47*
- J: *18S-47*
- K: *18S-47*
- L: *18S-47*
- M: *18S-47*
- N: *18S-47*
- O: *18S-47*
- P: *18S-47*
- Q: *18S-47*
- R: *18S-47*
- S: *18S-47*
- T: *18S-47*
- U: *18S-47*
- V: *18S-47*
- W: *18S-47*
- X: *18S-47*
- Y: *18S-47*
- Z: *18S-47*

- A: *18S-1*
- B: *18S-1*
- C: *18S-1*
- D: *18S-1*
- E: *18S-1*
- F: *18S-1*
- G: *18S-1*
- H: *18S-1*
- I: *18S-1*
- J: *18S-1*
- K: *18S-1*
- L: *18S-1*
- M: *18S-1*
- N: *18S-1*
- O: *18S-1*
- P: *18S-1*
- Q: *18S-1*
- R: *18S-1*
- S: *18S-1*
- T: *18S-1*
- U: *18S-1*
- V: *18S-1*
- W: *18S-1*
- X: *18S-1*
- Y: *18S-1*
- Z: *18S-1*

- A: *18S-1*
- B: *18S-1*
- C: *18S-1*
- D: *18S-1*
- E: *18S-1*
- F: *18S-1*
- G: *18S-1*
- H: *18S-1*
- I: *18S-1*
- J: *18S-1*
- K: *18S-1*
- L: *18S-1*
- M: *18S-1*
- N: *18S-1*
- O: *18S-1*
- P: *18S-1*
- Q: *18S-1*
- R: *18S-1*
- S: *18S-1*
- T: *18S-1*
- U: *18S-1*
- V: *18S-1*
- W: *18S-1*
- X: *18S-1*
- Y: *18S-1*
- Z: *18S-1*