



Année 2013

Thèse n°2027

THÈSE

pour le

DOCTORAT DE L'UNIVERSITÉ BORDEAUX 2

Mention : **Sciences, Technologie, Santé**

Option : **Biologie végétale**

Etude comparative des communautés fongiques et bactériennes colonisant le bois de ceps de vigne ayant exprimé ou non des symptômes d'esca

Présentée et soutenue publiquement le
25 janvier 2013

Par Emilie Bruez

Membres du Jury

Josep ARMENGOL, Professeur à l'Université Polytechnique de Valence (Espagne)
Georges BARBIER, Professeur à l'Université de Bretagne Occidentale

Rapporteur
Rapporteur

Anne-Françoise ADAM-BLONDON, Directrice de recherche INRA, Evry
Kendra BAUMGARTNER, Chercheur à l'Université de Californie (Etats-Unis)
Lucia GUERIN-DUBRANA, Maître de Conférences à Bordeaux Sciences Agro
Laura MUGNAI, Assistant-Professeur à l'Université de Florence (Italie)

Présidente du jury
Examinatrice
Examinatrice
Examinatrice

Pr Patrice REY, Professeur à Bordeaux Sciences Agro

Directeur de thèse

Remerciements

Je tiens à remercier mon directeur de thèse, le Pr Patrice Rey. Il m'a été d'un très grand soutien durant ces trois années de thèse. Il a été très patient avec moi à des moments où j'avais besoin de ses conseils. Jusqu'au bout j'ai été soutenue et pour cela un très grand merci chef !

Je porte une attention particulière à mes rapporteurs, Mrs Josep Armengol et Georges Barbier qui ont accepté de lire ma thèse et d'en juger le contenu, à la présidente de mon jury, Mme Anne-Françoise Adam-Blondon, ainsi qu'aux autres membres de mon jury : Kendra Baumgartner et Laura Mugnai.

Je remercie Lucia Guerin-Dubrana pour sa gentillesse et pour son aide lorsque j'en avais besoin (surtout à la fin).

Je voudrais remercier Angélique Gautier, Valérie Laval et Marc-Henri Lebrun de l'UR BIOGER de l'INRA de Paris Grignon ainsi que Nicolas Lapalu de l'INRA de Versailles, qui m'ont grandement aidée pour le pyroséquençage (préparation des échantillons, analyses des résultats).

Merci Jess pour son soutien de tous les instants, pour son aide, pour ses corrections. Je suis arrivée en ne sachant pas grand-chose à la biologie moléculaire et aujourd'hui je sors avec « spé bio mol ! ». Tu m'as beaucoup appris. Mais bon comme tu le sais « tu n'es pas bête toi ! ».

Je voudrais remercier particulièrement Christophe Bertsch sans qui je n'aurais jamais connu les maladies du bois ! Cette sortie dans les vignes alsaciennes en troisième année de licence a été une révélation ! Merci à l'équipe de l'UHA de Colmar (Julie, Sibylle, Flore,...)

Merci aux personnes du laboratoire de l'UMR SAVE. J'ai passé trois années sympathiques avec de bons moments gravés en ma mémoire.

Je dis un grand merci à ceux qui m'ont supporté pendant ces trois ans : mes acolytes et amis Jonathan et Mélanie. Quand on écrit les remerciements d'une thèse, cela annonce la fin de trois ans, trois ans de soutien mutuel entre trois thésards dans le même bureau. Merci pour votre aide, votre soutien et votre amitié. Ces trois ans seront des souvenirs du vendredi « chocolatines », de sapins de Noël, de coups de gueules contre les expés qui ne marchent pas pour X raisons, des moments de tristesse, des moments de joie... Bref, entre thésards on se comprend...

Merci à tous mes amis qui m'ont de près comme de loin suivi dans cette aventure de la thèse, dans les bons moments comme dans les mauvais : Amandine L, Amandine D, Marie (et Seb), Gégé (et Sylvain), Valérie, Amélie, Marion (et FX), Anne-Sophie, Julien H, Florine (et Julien), Amandine LB, Lionel(s), Willwill, les copains du bad (Angela, Thierry, MatMat, ...), les copains de l'harmonie de Gradignan (Jessica, Fred, Florian, ...), les copains que je n'ai pas cités parce que j'ai oublié mais de manière non volontaire...

Merci à tous ceux qui ont cru en moi et qui ont toujours été là pour moi.

Un très grand merci à ma famille ainsi que ma belle-famille qui m'ont soutenue à leur façon durant ces trois ans.

Merci ma petite sœur qui a subi quelque fois mes plaintes au téléphone et qui a toujours été là pour moi malgré les distances. Un grand merci à mes parents sans qui je ne serais pas arrivée là où j'en suis. Leur soutien moral a été très important pour moi et ils ont toujours été là quand j'avais besoin et ils le seront toujours, j'en suis sûre... J'espère que vous êtes fiers de votre fille qui, d'un BTS est allée jusqu'à la thèse !

Je tiens à te remercier, Arnaud, pour la très grande patience dont tu as su faire preuve. Durant trois années nous avons vécu beaucoup de choses. Cela n'a pas toujours été facile, mais nous nous sommes bien débrouillés. Tu as toujours été là pour moi. Merci d'avoir été si patient et particulièrement à la fin.

Ca y est, la fin est proche !

Résumé : L'esca est une maladie de dépérissement du bois de la vigne conduisant à la mort des ceps. Actuellement le vignoble mondial est atteint, et au niveau français, cette maladie ne cesse de progresser. Ainsi, 8% des ceps dans le Jura et 4,5% dans la région de Bordeaux manifestent des symptômes d'esca, selon les parcelles des chiffres beaucoup plus élevés sont obtenus, certains cépages sont aussi beaucoup plus sensibles que d'autres. Plusieurs champignons seraient impliqués dans l'esca mais leur rôle ainsi que la détermination de la microflore responsable de cette maladie est encore sujette à interrogation.

Dans ce contexte, l'objectif de cette thèse a été de caractériser et de comparer les microflore fongiques et bactériennes colonisant le bois de ceps de vigne ayant exprimé ou non des symptômes foliaires d'esca.

Dans un premier temps, nous avons prélevé des ceps (cultivar Cabernet Sauvignon) relativement jeunes (10 ans d'âge) car ils présentaient l'intérêt d'être peu dégradés au niveau du bois du tronc, les symptômes foliaires étant associés à la présence d'amadou (une nécrose typique de l'esca) uniquement dans les bras. Une grande diversité dans les communautés fongiques (674 OTUs) et bactériennes (222 OTUs) colonisant le bois a été observée. Cette diversité est plus importante dans le bois sain de la vigne que dans celui partiellement ou totalement nécrosé. Les techniques utilisées, *i.e.* isolement/séquençage de souches, empreinte moléculaire (Single Strand Conformation Polymorphism, SSCP) et pyroséquençage 454, ont montré que les communautés bactériennes ou fongiques étaient différentes dans les tissus dégradés comparés à ceux qui ne l'étaient pas. Des changements de microflore en fonction du temps (expérimentation durant 1 année) ont aussi été observés. D'une façon générale, les espèces de champignons impliquées dans l'esca sont déjà présentes dans le bois apparemment sain de ceps esca-foliaires symptomatiques mais aussi des asymptomatiques. Il n'a pas été possible de différencier ces 2 types de microflore au niveau du bois sain des plants, cette différenciation se faisant au niveau des nécroses, qui sont plus abondantes dans les ceps esca-symptomatiques. Pour la première fois nous avons montré que des communautés bactériennes spécifiques étaient associées à l'esca, leurs aptitudes trophiques étant différentes selon les tissus où elles étaient prélevées. Les espèces isolées suggèrent que certaines pourraient avoir un rôle dans la protection du végétal, d'autres dans la dégradation des structures du bois, *e.g.* de la lignine, préparant ainsi le terrain aux champignons dégradateurs des tissus ligneux, déjà présents à l'intérieur des ceps.

Nous avons aussi étudiés des ceps plus âgés (cultivar Baco blanc), de 42 et 58 ans, qui avaient un rendement acceptable et n'avaient pas manifesté de symptômes d'esca ou eutypiose (une autre maladie du bois) l'année du prélèvement. Au niveau des tissus fonctionnels du bois, les communautés fongiques étaient caractéristiques de plants atteints par l'eutypiose (ceps de 42 ans) ou de l'esca (ceux de 58 ans). La non expression par les ceps de ces 2 maladies pourrait cependant être associée à la forte présence de champignons mycoparasites et protecteur du végétal, comme *Trichoderma* spp., dans ces tissus fonctionnels. Les interactions au sein des communautés fongiques créant un équilibre où le pathogène ne se développerait pas de façon extensive. Les caractéristiques du Baco blanc, un hybride, moins sensible à certaines maladies de la vigne, pourrait aussi expliquer ce résultat. Ainsi la présence d'une microflore bénéfique naturellement présente dans le bois des ceps associée à des plants ayant une tolérance à ces maladies pourrait ouvrir de nouvelles perspectives pour lutter l'esca, voire l'eutypiose, pour lesquelles aucun moyen de protection n'existe aujourd'hui.

Mots-clés : Vigne, esca, microflore fongique, microflore bactérienne, empreinte moléculaire, pyroséquençage

Abstract: Esca is a Grapevine Trunk Disease (GTD) that induces a decline in grapevine vigour that generally leads up with the death of the plants. Nowadays, vineyards worldwide are attacked by esca and, in France this disease increases steadily. In the Jura, 8% of the grapevines are esca-foliar symptomatic and approximately 4.5% in the Bordeaux region. However, some vineyards are more severely attacked by esca, and certain cultivars are more susceptible than others. Although several pathogenic fungi are associated with esca, their individual roles and their interaction with other microorganisms for the esca have still to be determined.

In this context, the objective of the present PhD study is to characterize and compare the bacterial and fungal microflora that colonize the wood tissues of esca-foliar symptomatic and asymptomatic grapevines.

First, we sampled young (10 year-old) grapevines (Cabernet Sauvignon cultivar) because they had only few necroses in the trunk and white-rot (also called amadou) was only present in the cordons of symptomatic plants. Great diversity in the fungal (674 OTUs) and bacterial (222 OTUs) communities was observed. This diversity was higher in the apparently healthy wood than in the partially or totally necrotic wood tissues. The methods used isolation/sequencing of microbial strains, a molecular fingerprinting method (Single Strand Conformation Polymorphism, SSCP) and 454 pyrosequencing showed that the fungal and bacterial communities of the necrotic and healthy wood tissues were different. Changes in the microflora over time (over a one-year period) have been observed. Fungal species involved in esca are already present in the apparently healthy wood of esca-foliar symptomatic plants but also in the asymptomatic ones. It was not possible to differentiate these 2 microflora. Only microflora from the necroses differed from those of the healthy wood with these necroses being more developed in the esca-foliar symptomatic grapevines. For the first time, we were able to determine that specific bacterial communities are associated with esca. Depending on the wood tissues, different types of bacteria were isolated, with different trophic behaviour. Two roles could be assigned to the species isolated from the various wood tissues: (i) a positive role, due to the biocontrol potential that many species have; (ii) a negative one, by predisposing the wood of grapevines to fungal attacks.

We also studied, old (42 and 58 year-old) grapevines of the cultivar, Baco blanc, that produced regular harvests. The plants had no expressed foliar symptoms of esca or eutypa dieback during the sampling year. Many plant pathogens colonized the functional wood tissues, but in 58 year-old plants they were associated with esca, and in 42 year-old plants, with eutypa dieback. The absence of GTDs expression could be linked to the numerous plant protectant mycoparasites, such as *Trichoderma* spp., that colonized the functional wood tissues. Interactions between species within the fungal communities may create a balance that is unfavourable to the development of the pathogens. The use of Baco blanc, a hybrid less susceptible to certain grapevine diseases could also explain this result. So, because no means of protection are currently available, the combination of beneficial microflora within the grapevine wood tissues with plants that are tolerant to esca, or even eutypa dieback, could be helpful to control those diseases.

Key words: grapevine, esca, fungal microflora, bacterial microflora, molecular fingerprinting method, pyrosequencing

Sommaire

| | |
|---|-----|
| Introduction générale | 1 |
| Publication n°1..... | 27 |
| Overview of grapevine trunk diseases in France in the early 2000s | |
| | |
| Chapitre 1 | 61 |
| Caractérisation de la microflore fongique des tissus du bois non colorés de ceps ayant exprimé ou non des symptômes foliaires d'esca | |
| Publication n°2..... | 69 |
| The complex relationship of fungi with esca. Analyses of the fungal communities that colonize the wood tissues of leaf-symptomatic and asymptomatic vines | |
| Publication n°3..... | 107 |
| Old <i>Vitis vinifera</i> : when pathogenic fungal patterns of grapevine trunk diseases are not always associated with foliar disease expression or plant decline | |
| | |
| Chapitre 2 | 137 |
| Mise en évidence de communautés bactériennes spécifiques associées aux bois de ceps de vigne ayant exprimé ou non des symptômes foliaires d'esca | |
| Publication n°4..... | 141 |
| Specific bacterial microflora are associated with grapevine asymptomatic and esca-symptomatic wood tissues | |
| | |
| Chapitre 3 | 185 |
| Etude par pyroséquençage de la microflore fongique et bactérienne colonisant le bois de ceps de vigne | |
| | |
| Conclusions générales et Perspectives | 217 |
| | |
| Références | 225 |

Introduction générale

Préambule

Le sujet de cette thèse concerne l'étude d'une pathologie de dépérissement de la vigne nommée l'esca dont l'étiologie est relativement complexe puisqu'il est admis qu'elle est due à plusieurs facteurs biotiques (champignons pathogènes), abiotiques, associés aux pratiques culturales.

Certains agents fongiques comme *Phaemoniella chlamydospora*, *Phaeoacremonium aleophilum*, *Fomitiporia mediterranea* et *Botryosphaeria* spp ont été décrits dans la littérature comme étant impliqués dans cette maladie (Edwards and Pascoe, 2001; Fischer and Kassemeyer, 2003; Gimenez-Jaime *et al.*, 2006; Halleen *et al.*, 2003; Larignon and Dubos, 1997; Rumbos and Rumbou, 2001; Retief *et al.*, 2006 ; Scheck *et al.*, 1998; Serra *et al.*, 2000). Néanmoins, de nombreuses interrogations sur leur rôle existent puisque la reproduction des symptômes, en particulier foliaires, n'est que partiellement obtenue. Le quatrième point du postulat de Koch n'est donc pas totalement reproduit. L'hypothèse que d'autres micro-organismes soient impliqués dans ce phénomène de dégradation du bois de la vigne est posée par plusieurs scientifiques, dont ceux de notre groupe au sein de l'UMR Santé et Agroécologie du Vignoble. En outre, la connaissance des micro-organismes impliqués ou associés à l'esca n'est que partielle, puisque les techniques d'isolement couramment utilisées (méthodes culturales) ne permettent de mettre en évidence qu'une partie de cette microflore.

Dans ce contexte, il nous a paru intéressant d'étudier la diversité microbienne à l'intérieur des ceps, en particulier en comparant les communautés colonisant le bois de plants exprimant des symptômes foliaires d'Esca à ceux n'en exprimant pas. Il s'agira d'identifier avec précision les communautés fongiques colonisant le bois des ceps, leurs compositions, leurs évolutions et d'identifier les interactions potentielles existant entre elles. Nous nous sommes aussi intéressés à la microflore bactérienne, plusieurs publications rapportant leur rôle dans la dégradation des structures du bois dont la lignine. Les approches pasteurienne et moléculaires, ces dernières permettant souvent d'acquérir une vision plus exhaustive de la microflore, seront utilisées afin d'obtenir selon les besoins, une représentation globale ou spécifique des communautés de micro-organismes colonisatrices du bois de la vigne. Les objectifs du travail de thèse seront décrits à la fin du chapitre introductif présentant la culture étudiée, la vigne et les maladies du bois et leurs extensions en France durant la dernière décennie (publication n°1).

I. La vigne (*Vitis vinifera*)

1.1) Présentation générale

La vigne, plante grimpante pérenne, appartient au genre *Vitis*. Elle fait partie des angiospermes dicotylédones de la classe des *Magnoliopsida*, de l'ordre des *Rhamnales* (Chadefaud and Emberger, 1960). La famille des *Vitacées*, comprend 14 genres différents (Ren *et al.*, 2011) dont le genre *Vitis*, qui est le seul utilisé, de part le monde, pour produire du raisin. Ce genre se divise en deux sous-groupes : *Muscadina* qui possèdent $2n=40$ chromosomes et *Euvitis* à $2n=38$ chromosomes (Levadoux, 1956). Les hybrides issus de croisement entre ces deux groupes sont rarement fertiles du fait de la différence du nombre de chromosomes. Ces sous-groupes ont été constitués en fonction de l'anatomie et de la morphologie des plants (Planchon, 1887). Le sous-groupe *Muscadinia* ne comprend que trois espèces diploïdes (Keller, 2010) dont la plus connue est *Muscadinia rotundifolia*. Quant au sous-genre *Euvitis*, il se compose d'une soixantaine d'espèces diploïdes différentes se rassemblant dans 3 groupes génétiquement et géographiquement distincts :

- (i) Les vignes américaines comprennent une vingtaine d'espèces possédant des gènes de résistance aux principales maladies de la vigne (mildiou, oïdium, phylloxéra). Ces vignes sont très fréquemment utilisées comme porte-greffe. *Vitis riparia*, *Vitis labrusca*, *Vitis rupestris*, *Vitis rotundifolia* sont les espèces plus connues ($2n=38$ chromosomes). *Vitis rotundifolia* appartient au sous-genre *Muscadinia* ($2n=40$ chromosomes).
- (ii) Les vignes asiatiques comprennent une dizaine d'espèces peu résistantes aux différentes maladies. L'espèce la plus connue est *Vitis amurensis* ; elle est utilisée dans les pays de l'ex-URSS pour des croisements variétaux en raison de sa résistance au mildiou et au froid (Levadoux, 1956).
- (iii) Les vignes européennes ne comprennent qu'une seule espèce : *Vitis vinifera*. Celle-ci comprend un archétype de la vigne sauvage, *Vitis vinifera sylvestris* (Grasso, 1984) et un autre, *Vitis vinifera sativa* qui est la vigne cultivée (Rego *et al.*, 2000; Rego *et al.*, 2005). La vigne sauvage a été quasiment éliminée suite aux attaques de mildiou et de phylloxéra, aux déforestations des forêts vierges et aux réaménagements territoriaux.

A l'origine cultivée dans le bassin méditerranéen, la vigne s'est étendue sur tous les continents. Le fort potentiel vinicole de *Vitis vinifera*, la sélection de cépages spécifiques et l'adaptation de pratiques culturales ont permis l'expansion de cette espèce sur tous les continents. Plus de 10 000 variétés ou cultivars de *Vitis* seraient aujourd'hui existants dans le monde. En France, plus de 200 cultivars sont plantés, douze cépages seulement couvrent 70% du vignoble et sept sont majoritaires : le Cabernet-Sauvignon, le Cabernet-Franc, le Merlot, le Syrah, le Pinot noir, le Sauvignon et l'Ugni blanc. Le choix d'un cépage s'effectue en fonction de sa capacité à produire du raisin, des qualités aromatiques et gustatives des baies.

Différents types de raisin existent : le raisin de table, le raisin de cuve ou le raisin destiné à produire des raisins secs (Reynier, 2003).

1.2) Le développement de la vigne

La vigne est une plante pérenne qui effectue chaque année un cycle composé de deux phases : le cycle végétatif qui permet la production de l'appareil végétatif et le cycle productif qui amène à la formation des fleurs puis des fruits.

Après la récolte des baies qui s'effectue en automne, la plante se met en repos végétatif jusqu'au mois de février de l'année d'après. Après avoir été en état de dormance, elle développe des bourgeons qui vont produire des feuilles ainsi que des fleurs. Il s'agit de l'étape de débourrement qui est caractérisée par le gonflement du bourgeon latent mais aussi du rejet de la bourre. Une fois l'éclatement du bourgeon, les feuilles apparaissent et se développent. Le cycle végétatif se poursuit avec l'allongement des rameaux ainsi que le développement d'autres feuilles. La plante fait alors des réserves principalement sous forme d'amidon et de lignine dans les sarments afin de passer la future période de latence (en hiver).

Environ un an avant la floraison se produit l'initiation florale dans les bourgeons latents. La différenciation des fleurs se réalise au printemps, lorsque le plant reprend son activité. Une fois l'étape de débourrement finie, les inflorescences se développent. La floraison qui a lieu entre mai et juin, aboutit à la libération du pollen. La vigne étant une plante hermaphrodite (fleurs mâles et femelles à la fois), la fécondation peut être autogame (de la plante elle-même) ou allogame (pollen provenant d'une autre plante) (Galet, 2000). Une fois la fécondation terminée, les ovaires vont se gonfler. Cette étape, la nouaison, conduit à la formation des grappes de raisin. Les baies vont alors se développer pour atteindre leur taille définitive au mois d'août. Elles vont ensuite passer du stade véraison (phase de développement des baies vertes) puis à l'étape de maturation qui se caractérise par leur coloration. Une fois que les baies ont atteint leur pleine maturité, elles peuvent être cueillies pour la vendange. Cette dernière étape se déroule de mi-septembre à début octobre, selon le cépage et la maturité voulue. Si les baies n'étaient pas vendangées, il y aurait une sur-maturation qui entrainerait un flétrissement de celles-ci.

1.3) La filière viti-vinicole dans le monde et en France

Le vignoble mondial représente 7,585 millions d'hectares et une production, hors jus et moût, de 260 millions d'hectolitres qui se répartit à 58,4% en Europe, 21,1% en Asie, 12,8% en Amérique, 5% en Asie et 2,7% en Océanie. La production mondiale du raisin de table est de 677,9 millions de quintaux et celle du vin est de 269 millions d'hectolitres (OIV, 2009).

La France est le premier producteur mondial avec 44,75 millions d'hectolitres, devant l'Italie (42,58 d'hectolitres) et l'Espagne (35,14 millions d'hectolitres) (<http://www.planetoscope.com>). L'Europe est ainsi le premier producteur mondial de vin avec 66,5% de la production devant les Etats-Unis (19%) (Communiqué de presse OIV 2012).

Dans le Bordelais, plus de 115 000 ha sont consacrés à la culture de la vigne pour produire 60 vins AOC (25% des AOC de France). Le vignoble bordelais représente 11,3% de la production de vin en France (<http://www.gironde.chambagri.fr>). De nombreux cépages sont cultivés mais les principaux sont le Merlot, le Cabernet-Sauvignon et le Cabernet-Franc pour les vins rouges ; le Sémillon et le Sauvignon pour les vins blancs.

1.4) Trois maladies épidémiques majeures de la vigne

L'évolution des pratiques culturales vers des modes production intensifs sur d'importantes surfaces parfois uniformes conduit à fragiliser la vigne à différents parasites et ravageurs. En outre, l'espèce *Vitis vinifera*, majoritairement cultivée, présente une forte sensibilité à de nombreux parasites. De nombreux virus et deux phytoplasmes sont capables d'infecter la vigne. La protection de la vigne contre ces agents redoutables est basée sur des mesures prophylactiques et des pratiques culturales. La sélection sanitaire accompagnant la certification des variétés commercialisées permet d'éviter l'introduction de plants contaminés dans le vignoble. Les pratiques culturales et les traitements contre les insectes vecteurs participent à la protection du vignoble.

Le mildiou, l'oïdium et la pourriture grise représentent trois maladies aériennes à développement épidémique fréquemment observées en France. L'impact de ces maladies sur la quantité et la qualité de la récolte peut être très important. D'autres maladies, telles que le black rot, sont présentes mais moins fréquentes ou conduisent à des dégâts moins importants.

1.4.1) Eléments de biologie

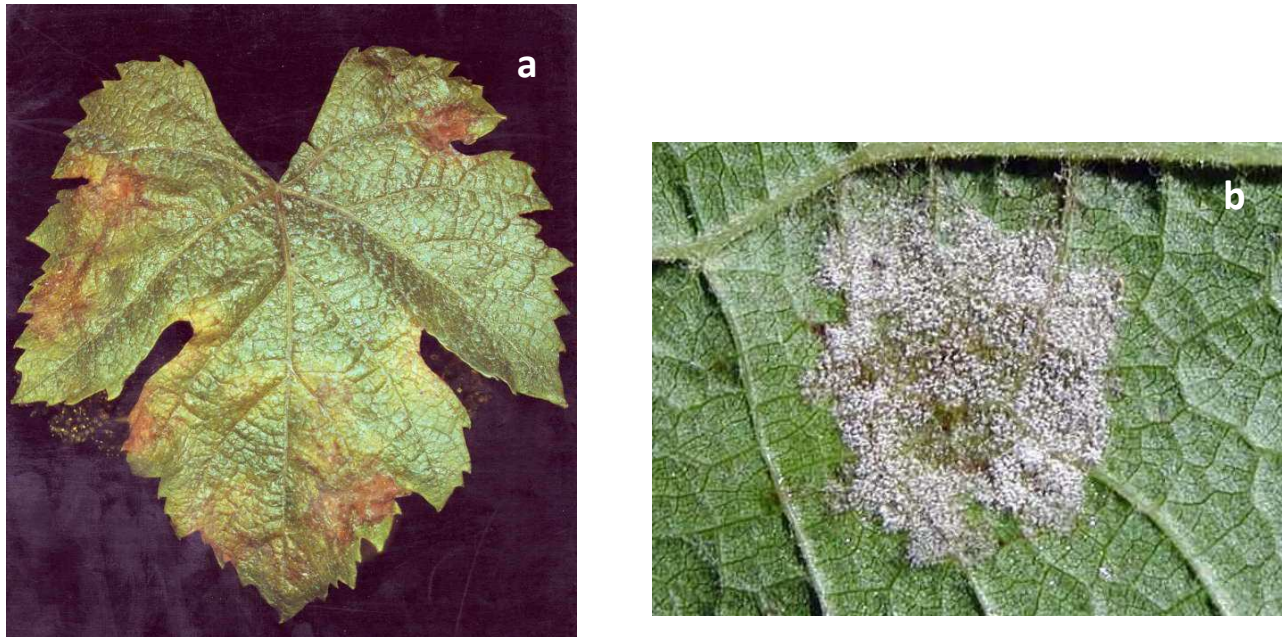
Le mildiou de la vigne est provoqué par un oomycète parasite obligatoire du nom de *Plasmopara viticola*. Le mildiou, originaire d'Amérique du Nord, a été introduit en Europe au 19^{ième} siècle et a été observé pour la première fois en France en 1878.

Ce pathogène touche principalement les feuilles et les grappes. Sur les feuilles, les symptômes se manifestent sur la face supérieure par des plages décolorées, jaunes et d'aspect huileux communément appelées « tâches d'huile » et sur la face inférieure, par un feutrage blanchâtre qui correspond aux fructifications de l'oomycète. Sur les grappes, les symptômes apparaissent lors du stade de floraison avec, dans un premier temps, un brunissement et une déformation des rafles puis, un recouvrement des futures baies par un duvet blanc. Les sarments de vigne peuvent également être infectés par l'oomycète, lorsque celui-ci a déjà envahi les feuilles et les baies.

Les conditions favorables à l'établissement de cette maladie sont des températures comprises entre 15 et 20°C avec des précipitations d'au moins 10 mm. Les oospores produites par reproduction sexuée sont transportées par les gouttes de pluie sur les feuilles et les organes verts et germent dans un film d'eau à leur surface.

Le parasite attaque tous les organes herbacés et si les conditions climatiques sont favorables, l'épidémie peut être très rapide et peut anéantir totalement la récolte. Delière *et al.* (2010) ont

mis en évidence un affaiblissement des réserves en cas de fortes attaques, qui a pour conséquence de diminuer la vigueur de la vigne l'année suivante.



(www.chateaudesmauves.fr)

Figure 1. Mildiou colonisant la face supérieure (Fig. 1a) et la face inférieure d'une feuille de vigne (Fig 1.b).

L'oïdium est une maladie causée par un pathogène appelé *Erysiphe necator*. C'est un champignon ascomycète qui appartient à l'ordre des *Erysiphales* et à la famille des *Erysiphacées*. Il est un parasite obligatoire, c'est-à-dire qu'il a besoin d'un hôte pour se développer. Il a été introduit involontairement en France vers 1845.

Comme le mildiou, ce champignon peut s'attaquer à tous les organes verts de la vigne. Après la véraison complète, il ne colonise pas les baies et l'aoûtement, il ne se développe plus sur le bois. La maladie se manifeste principalement par des tâches blanc-grisâtre et poudreuses à la surface des feuilles avec une crispation des bords du limbe. Quant aux baies, les grains sont d'abord recouverts d'une poussière grise d'aspect cendré avant de finir par éclater sous l'effet de la pression des cellules en développement. Cet éclatement crée alors une porte béante pour la pourriture grise. Ce champignon infecte aussi les jeunes rameaux et les sarments.

Le développement optimal de l'oïdium se situe entre 25 et 30°C avec une humidité relative de 70-80%. Contrairement au mildiou, la présence d'eau libre gêne la germination des conidies.

En plus de diminuer la récolte, l'oïdium peut avoir un impact qualitatif sur la maturation des raisins (Calonnec *et al.*, 2004).



Figure 2. Aspect poussiéreux sur les feuilles dues aux attaques de l'oïdium.

Botrytis est une maladie cryptogamique causée par un champignon ascomycète appelé *Botrytis cinerea*. Il appartient à la classe des *Leotiomycetes*, à l'ordre des *Helotiales* et la famille des *Sclerotiniaceae*. *Botrytis cinerea*, forme anamorphe (asexuée) de *Botryotinia fuckeliana* (forme téléomorphe), est un champignon capable de coloniser les tissus végétaux sains (parasitisme), déjà infectés (opportunisme) ainsi que les tissus morts (saprophytisme). Sur feuille, les symptômes apparaissent sous forme de taches brunes avec un feutrage grisâtre sur la face inférieure (fructifications du champignon) qui ont tendance à s'accroître et à envahir tout le limbe. Les grappes peuvent être touchées avant la floraison et se dessécher. Elles sont surtout sensibles au stade de la véraison où il y a développement d'une coloration brune des baies des cépages blancs et l'apparition d'un épais feutrage gris. Les conidies sont disséminées par le vent et pénètrent dans les organes herbacés de façon directe ou par le biais de blessures. C'est pourquoi l'éclatement des baies dû au mildiou favorise les infections par *Botrytis cinerea*.

Cette maladie entraîne non seulement des pertes de rendement pouvant aller jusqu'à 40% (Viniflor, données 2006) mais elle altère également les qualités organoleptiques des vins (Darriet *et al.*, 2001). Néanmoins, *Botrytis cinerea* est également responsable de la « pourriture noble » nécessaire à l'obtention de certains vins liquoreux.



Figure 3. Symptômes sur baies caractéristiques de *Botrytis cinerea*.

1.4.2) Protection raisonnée

Ces trois maladies nécessitent la mise en œuvre d'une lutte chimique efficace, seule méthode pour l'instant disponible. La lutte chimique est raisonnée de façon à limiter l'apparition de population de parasites résistantes à certaines matières actives composant les fongicides. Il est fortement recommandé de limiter le nombre d'applications annuelles de fongicides d'une même famille chimique et d'alterner les matières actives. La gestion raisonnée passe également par l'utilisation de modèles de prévision des risques et par des observations au vignoble des premières attaques. Pour l'oïdium, Les traitements de floraison sont obligatoires, en raison de la forte réceptivité des jeunes baies.

Des recherches sont menées à l'Unité Mixte de Recherche, l'UMR SAVE, pour déterminer le rôle de l'architecture du couvert végétal sur le développement des épidémies d'oïdium. Son influence peut être directe sur le développement des parasites foliaires en modifiant la physiologie des tissus (Burie *et al.*, 2011) ou indirecte via la modification du micro-climat. Outre les modèles de prévision des risques, des outils d'aide à la décision sont développés. Par exemple, le processus MILDIUM, développé à l'UMR SAVE, comprend des règles de décision de traitements vis-à-vis de l'oïdium et du mildiou basées sur des traitements dits « obligatoires » (- 2 pour le mildiou et 2 pour l'oïdium) et complétées par des traitements facultatifs selon la pression de l'inoculum et d'autres indicateurs, tels que les données de prévision météorologiques (Delière *et al.*, 2010).

La protection de la vigne vis-à-vis de la pourriture grise comprend la maîtrise de la vigueur (choix de porte-greffe peu vigoureux), la maîtrise de la fertilisation azotée et l'enherbement de l'inter-rang. La protection chimique en utilisant des fongicides anti-botrytis comprend en général un à deux traitements positionnés à une ou deux des quatre périodes clés du cycle de développement de la vigne: fin floraison/chute des capuchons floraux (stade A), fermeture de la grappe (stade B), début véraison (stade C), trois semaines avant récolte (stade D).

Les méthodes de lutte moins conventionnelles, telles que l'utilisation de biopesticides et de Stimulateurs Naturels des Défenses des plantes (SDN) sont en cours de développement. Par exemple, Stifénia® est un SDN contre l'oïdium de la vigne et Milstop® est un biopesticide utilisé aussi contre l'oïdium de la vigne.

Des programmes de sélection variétale sont en cours en France et à l'étranger afin de proposer des nouveaux cultivars résistants. Il existe actuellement des hybrides résistants, qui ne sont cependant pas répandus en France en raison de leur faible niveau qualitatif. Le programme de sélection à l'Institut National de Recherche Agronomique (INRA) est orienté sur la recherche de résistance durable (utilisation de résistance polygénique).

Si le mildiou, l'oïdium et la pourriture grise représentent les trois principales maladies cryptogamiques affectant les vignobles à travers le monde, les maladies du bois causées par des agents fongiques deviennent des facteurs limitant de la production de raisins. Les viticulteurs sont actuellement confrontés à deux problèmes majeurs concernant ces maladies du bois: l'absence de méthodes de lutte et une méconnaissance profonde des différents facteurs biotiques et abiotiques impliqués notamment en ce qui concerne l'esca de la vigne. Le paragraphe suivant présente les caractères généraux des maladies du bois de la vigne.

II. Les maladies du bois de la vigne

Les maladies du bois sont définies comme étant des maladies causées ou associant des champignons pathogènes des parties aériennes, ligneuses et pérennes de la vigne : tronc et bras ou cordons. En cela, elles se distinguent des maladies racinaires, foliaires et des organes fructifères. Elles regroupent plusieurs maladies, certaines affectant les jeunes vignes d'autres les vignes plus âgées. Ainsi les maladies nommées : Pied noir, maladie de Petri, Verticilliose et Fusariose touchent les jeunes plantes (1-5 ans) en pépinière ou au vignoble. Les dépérissements à Botryosphaeriaceae, l'esca et l'eutypiose sont généralement décrits sur les vignes plus âgées. Ces dernières sont responsables de dépérissements importants dans de nombreux vignobles de par le monde, diminuant la longévité d'une plantation de vigne et affectant la qualité des raisins produits sur vignes malades (Lorrain *et al.*, 2012). Selon la maladie considérée, les méthodes de lutte préconisées peuvent varier, cependant aux vues des caractéristiques de ces pathosystèmes, les méthodes directes n'ont souvent qu'un effet partiel, les méthodes prophylactiques restant parfois les seules préconisées. Dans cette partie seront développées les connaissances actuelles concernant la microflore fongique pathogène associée aux maladies du bois affectant les plantes jeunes et les plantes âgées et les principales méthodes de lutte. La situation sanitaire française concernant l'esca et l'eutypiose, à partir de l'analyse des données de l'observatoire national sera présentée dans une seconde partie sous la forme d'une publication scientifique.

2.1) La maladie de Petri

Cette maladie, associée à deux champignons Ascomycètes *Phaeomoniella chlamydospora* (*Pch*) et *Phaeoacremonium aleophilum* (*Pal*), affecte les cepes de vigne âgés de 1 à 10 ans (Del Rio Conesa, 2002; Fourie and Halleen, 2002) dans la plupart des vignobles européens et mondiaux (Ferreira *et al.*, 1994; Mugnai *et al.*, 1999; Pascoe and Cottral, 2000). Les symptômes externes se caractérisent par un retard de développement des organes aériens ; la végétation est affaiblie et les entre-nœuds des rameaux sont plus courts. Des chloroses et des nécroses sont présentes sur les feuilles (Scheck *et al.*, 1998; Fourie and Halleen, 2002). Cette maladie doit son nom au phytopathologiste qui l'a décrite en 1912 en observant notamment les symptômes internes caractérisés par des nécroses et des ponctuations noires dans la moelle des cepes atteints.

2.2) La maladie du pied noir

Cette maladie est associée aux champignons du genre *Cylindrocarpon* spp. L'espèce *Cylindrocarpon destructans* est celle qui a été découverte en premier en 1961 dans le vignoble français (Maluta and Larignon, 1991) avant d'être également isolée en Sicile (Grasso, 1984), au Portugal (Rego *et al.*, 2000; Rego *et al.*, 2005) et en Pennsylvanie (Gugino and Travis, 2003). D'autres espèces de *Cylindrocarpon* spp responsables de cette pathologie ont également été isolées au Chili, en Grèce, en Espagne, en Afrique du Sud (Fourie et Halleen, 2002) et en Australie (Whitelaw-Weckert *et al.*, 2007). Cette maladie affecte les

jeunes ceps âgés de 2 à 10 ans. Les symptômes extérieurs sont caractérisés par un raccourcissement des entre-nœuds et une production de petites feuilles. A l'intérieur des ceps peut se développer une grosse et grande nécrose du xylème, de couleur brun foncé allant vers le noir. Le nombre de radicules diminuant, cela peut amener à une perte de vigueur du cep et donc à la mort de celui-ci.

Les maladies de Petri et du Pied noir sont souvent difficiles à déterminer car les champignons impliqués agissent simultanément, un même cep pouvant donc être atteint par ces deux pathologies à la fois.

2.3) L'Eutypiose

L'Eutypiose est une des maladies de dépérissement les plus importantes causée par le champignon ascomycète *Eutypa lata* (Carter, 1991). Elle est présente dans quasiment tous les vignobles du monde et entraîne des pertes économiques conséquentes liées à la diminution de la production de raisin par les plantes. En Californie, le coût annuel des pertes économiques liées à l'eutypiose a été évalué à 260 millions de Dollars américains (<http://www.sardi.sa.gov.au>).

E. lata a été isolé et identifié sur 88 espèces végétales réparties en 28 familles de plantes ligneuses pérennes dicotylédones présentes sur les 5 continents (Berraf and Peros, 2005; Cortesi and Milgroom, 2001; Hua *et al.*, 2007 ; Wenneker *et al.*, 2006). Les principales espèces d'arbres attaquées par ce pathogène sont l'abricotier (Carter and Moller, 1974), le pistachier (Rumbos, 1986), l'olivier (Rumbos, 1993), le citronnier (Kouyeas, 1978), le pommier (Glawe *et al.*, 1983) et la vigne (Boubals, 1986). *E. lata* a été isolé pour la première fois en 1900 dans un prunier écossais sous sa forme anamorphe, *Libertella blepharis*. Il a ensuite été déterminé en 1957 sous sa forme téléomorphe par Carter dans le sud de l'Australie.

Le cycle biologique d'*E. lata* est présenté sur la figure 4. Seule la forme sexuée est pathogène. Le champignon forme des périthèces sur les bois morts dans lesquels vont se former les ascospores (forme sexuée du champignon) qui seront disséminées par la pluie (Paillassa *et al.*, 1992) et par le vent sur de grandes distances (Ramos *et al.*, 1975; Petzoldt *et al.*, 1983). Les plaies de taille et les blessures constituent une porte d'entrée pour le mycélium qui va coloniser les vaisseaux du xylème des bras et du tronc et provoquer l'apparition d'une nécrose brune sectorielle. Extérieurement, les symptômes se caractérisent par la formation d'internœuds courts, l'apparition de chloroses et le repli des feuilles (Fig. 5). Lorsque le bras est coupé, une nécrose en forme de V est visible (Fig.6). Des métabolites toxiques tels que l'eutypinol, l'eulatinol et le méthyleutypinol produits par le champignon sont responsables de l'apparition de ces symptômes.

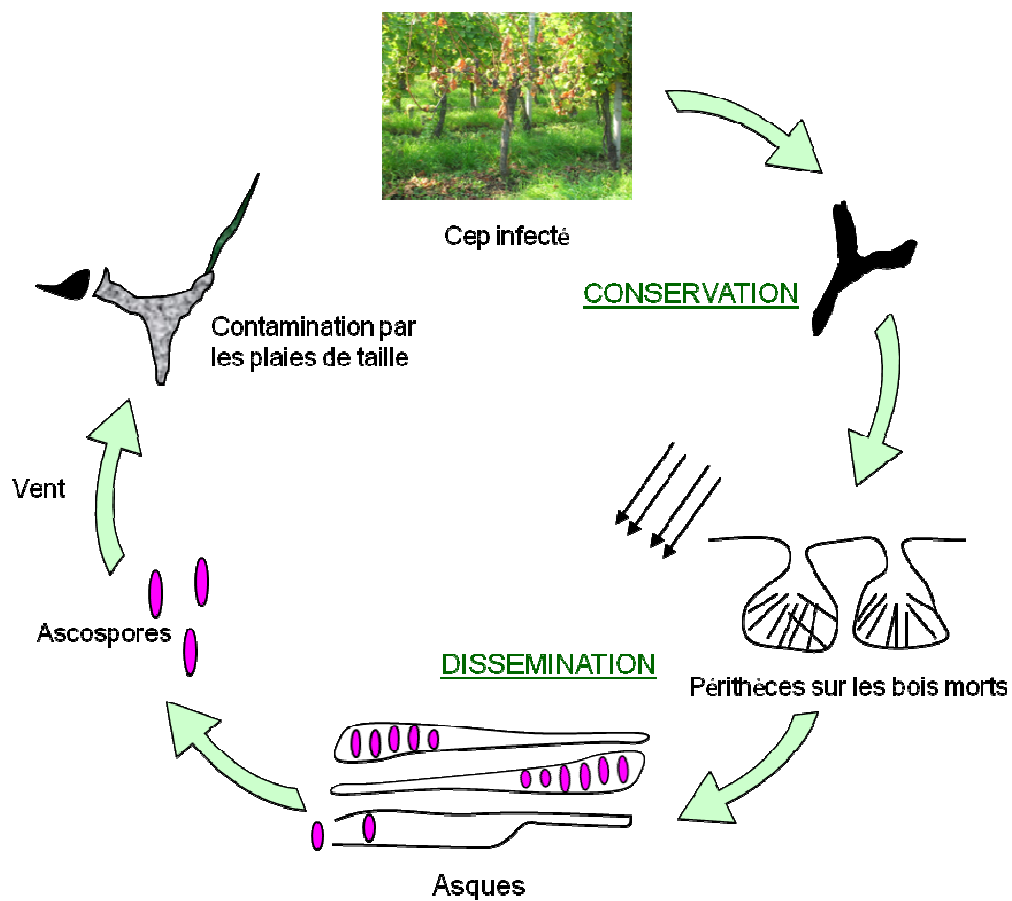


Figure 4. Cycle biologique d'*Eutypa lata*.



(source : Jose Urbez-Torres)

Figure 5. Raccourcissement de rameaux dû à *Eutypa lata*.



(Source : Jose Urbez-Torres)

Figure 6. V-Shape caractéristique de la maladie dans le bois de vigne.

2.4) L'esca

Connue des grecs et des romains, L'esca est la plus ancienne des maladies décrites sur la vigne. Rapportée sous le nom de folletage, ou d'apoplexie, l'esca doit son nom à la présence de pourriture blanche ou amadou (esca en langue d'Oc signifie amadou) (Viala, 1926). Il s'agit d'une maladie à étiologie très complexe dont les causes exactes restent encore à élucider. En effet, le Postulat de Koch n'est que partiellement vérifié : seule la formation des nécroses du bois est reproductible, non les symptômes foliaires. Appelée aussi Black Measles, l'Esca touche normalement les ceps de vigne âgés de 15 à 25 ans, mais des études ont récemment montré que les jeunes plantes pouvaient également être atteintes (Romanazzi *et al.*, 2009). En Europe, trois principaux champignons pathogènes, isolés des nécroses du bois, sont associés à l'esca : deux ascomycètes, *Phaeoacremonium aleophilum* et *Phaeomoniella chlamydospora* et un basidiomycète *Fomitiporia mediterranea*. De nombreux autres champignons dont certains pathogènes sont fréquemment isolés des nécroses du bois de plantes atteintes d'esca. C'est le cas d'*Eutypa lata*, agent responsable de l'Eutypiose, de différentes espèces de *Phaeoacremonium* sp. ou de nombreuses espèces de *Botryosphaeriaceae* (Larignon and Dubos, 1997).

Cette maladie se présente sous deux types de formes : la forme lente et la forme apoplectique (Fig. 8 et 9). Les symptômes foliaires sont caractéristiques de la forme lente même s'ils peuvent être présents chez la forme apoplectique (Arnaud et Arnaud, 1931; Chiarappa, 1959; Larignon and Dubos, 1997; Viala, 1926). La forme lente se caractérise par des colorations foliaires spécifiques : tâches internervaires jaunâtres sur cépages blancs et bordées de rouges sur cépages noirs les nervures restant vertes. Ces tâches évoluent progressivement vers un brunissement et un dessèchement. Les symptômes foliaires de la forme lente peuvent être visibles une année sur un cep et disparaître l'année suivante (Maher *et al.*, 2012). La forme apoplectique est caractérisée par un dessèchement rapide des organes

aériens, rameaux, feuilles et grappes d'une partie ou de la totalité du cep de vigne. Ce symptôme se manifeste généralement lorsque les étés sont chauds, entraînant la mort des ceps en quelques jours seulement sans symptômes annonciateurs.

Sur les baies, les symptômes observés diffèrent selon les pays. En France, les grappes se développent mais les baies flétrissent ou leur maturation est retardée. En Italie (Graniti, 1960; Grasso, 1969) et en Californie (Chiarappa, 1959), les symptômes sur baies sont décrits avec l'apparition de tâches violettes foncées ou gonflement des baies conduisant à leur éclatement.

Dans le bois, le développement de champignons associés à l'Esca conduit à la formation de deux types de nécroses : (i) une nécrose centrale de couleur claire, entourée d'une zone brune et dure, causée par *Phaeoacremonium aleophilum* et *Phaeomoniella chlamydospora* ; (ii) une nécrose sectorielle claire et tendre à partir de laquelle sont isolés *Eutypa lata* et *Fomitiporia mediterranea* (Larignon and Dubos, 1997). Ce dernier est responsable de la formation d'amadou ou pourriture blanche, caractérisée par du bois clair, dégradé et mou présent généralement au centre des nécroses.

Les symptômes foliaires peuvent être considérés comme des symptômes « conséquences » et seraient causés par des toxines émises par les champignons présents dans le bois et véhiculées jusqu'aux organes herbacés par les vaisseaux conducteurs (Luini *et al.*, 2010). La relation entre la sévérité des symptômes foliaires et la sévérité des nécroses internes n'est pas toujours vérifiée (Maher *et al.*, 2012). Les symptômes foliaires de la forme lente de l'esca ne sont pas toujours accompagnés d'amadou dans les bras.

Pour la forme apoplectique, Maher *et al.* (2012) ont montré que les ceps exprimant la forme apoplectique contenaient au niveau des bras et du tronc un volume de bois fonctionnel plus altéré par rapport aux ceps exprimant la forme lente ou asymptomatiques.

En Europe, les principaux champignons associés à l'esca sont *Phaeomoniella chlamydospora* (Crous and Gams, 2000), *Phaeoacremonium aleophilum* (Edwards and Pascoe, 2004) et *Fomitiporia mediterranea* (Tabacchi *et al.*, 2000; Bruno and Sparapano, 2006).

- *Phaeomoniella chlamydospora* (Pch)

Ce champignon ascomycète, présent dans la majorité des vignobles mondiaux, appartient à l'ordre des *Chaetothyriales* et à la famille des *Herpotrichiaceae*. Sa forme sexuée n'est pas connue. Il est également impliqué dans la maladie de Petri. Des études ont montré que la variabilité génétique des populations de ce champignon était faible (Péros *et al.*, 2000; Tegli *et al.*, 2000; Comont *et al.*, 2010).

Lorsqu'il se développe dans la plante hôte, il produit différents types d'enzymes dont des polygalacturonases, des polyméthylgalacturodases (Marchi *et al.*, 2001), des β -glucosidases et des endo- β 1,4 glucanases (Mugnai *et al.*, 1999). Différents types de phytotoxines émises par Pch ont été caractérisées (Tabacchi *et al.*, 2000) ainsi que des polysaccharides extracellulaires (Sparapano *et al.*, 2000).

Pch, champignon à dissémination aérienne est capable de pénétrer dans les ceps par les plaies de taille lors des périodes douces et pluvieuses de la saison hivernale (Larignon *et al.*, 2000). Sa dissémination peut également avoir lieu pendant la période végétative de la vigne (Fig. 7). Les pycnides, contenant des pycniospores, constituent la source d'inoculum de *Pch*. Elles sont localisées sur les plaies de taille âgées et sous l'écorce, dans les zones où les vaisseaux sont exposés aux blessures (Eskalen and Gubler, 2001). Plusieurs hypothèses ont été émises quant au mode de propagation de ce champignon dans les tissus ligneux : (i) le mycélium issu d'une pycniospore colonise progressivement les différents tissus présents à partir d'une blessure ; (ii) les pycniospores sont véhiculées par la sève des racines jusqu'aux rameaux (Bertelli *et al.*, 1998; Feliciano and Gubler, 2001) et (iii) *Pch* existe à l'état endophytique dans les tissus des rameaux ou des sarments (Aroca *et al.*, 2008). Il a d'ailleurs été montré que des plants en pépinière étaient colonisés par ce champignon (Bertelli *et al.*, 1998 ; Gimenez-Jaime *et al.*, 2006). Au cours des étapes de fabrication des plants, il a été montré que le taux d'infection des tissus ligneux par *Pch* augmentait, notamment pendant la phase de réhydratation avant greffage (Whiteman *et al.*, 2004). Bien que certaines études aient porté sur la détection et la viabilité de *Pch* dans le sol (Damm and Fourie, 2005; Gubler *et al.*, 2004; Retief *et al.*, 2006; Whiteman *et al.*, 2005), il n'existe à l'heure actuelle, aucune preuve d'une relation entre la présence du pathogène dans le sol et sa présence dans les plantes.

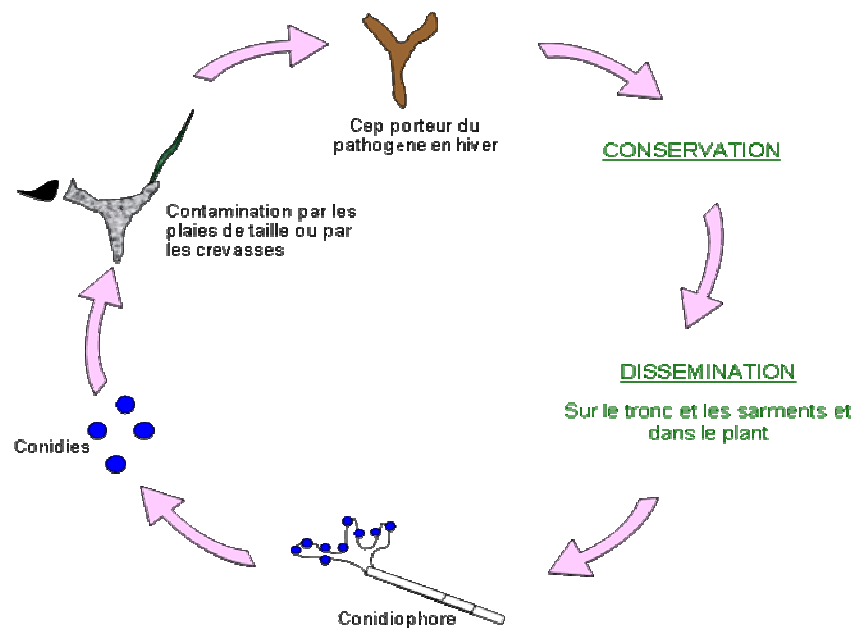


Figure 7. Cycle biologique de *Phaeoacremonium aleophilum*.

- *Phaeoacremonium aleophilum* (Pal)

Si *Phaeoacremonium aleophilum* (Pal) est la principale espèce de ce genre associée à l'esca, d'autres espèces peuvent être isolées de nécroses du bois de ceps atteints d'esca (Moster *et al.*, 2006; Gramaje *et al.*, 2009). Pal, champignon ascomycète, appartient à l'ordre

des *Calosphaeriales* et la famille des *Chaelosphaeriaceae* (Mostert *et al.*, 2003). Il a été isolé dans de nombreux vignobles en France (Larignon and Dubos, 1997), en Espagne (Armengol *et al.*, 2001), en Grèce (Rumbos and Rumbou, 2001), en Italie (Surico *et al.*, 2000), en Algérie (Berraf and Peros, 2005) et aux Etats-Unis (Scheck *et al.*, 1998). Cet agent pathogène est, comme *Pch*, également impliqué dans la maladie de Petri. Sa forme téléomorphe, *Togninia minima*, a été découverte par croisement de souches *in vitro* (Mostert *et al.*, 2003, Pascoe *et al.*, 2004) avant d'être observée *in natura* en Californie (Rooney-Latham *et al.*, 2005). *Pal* est aussi lié à des dépérissements survenant sur kiwi (Di Marco *et al.*, 2000).

Ce champignon se développe à l'intérieur des parois secondaires du bois et provoque la formation de cavités. Il produit des enzymes telles que des xylanases, des β -glucosidases et des endo- β 1,4 glucanases (Mugnai *et al.*, 1999) et pourrait être à l'origine de la production de certaines molécules de naphthalenones (Evidente *et al.*, 2000 ; Abou-Mansour *et al.*, 2004). Malgré son implication dans les maladies du bois, les déterminants de son pouvoir pathogène n'ont pas encore été clairement identifiés.

Le cycle biologique de cet ascomycète est peu connu . *Pal* est un champignon à dissémination aérienne qui contamine les plaies de taille dues à l'effeuillage, au rognage ou à l'éclaircissage durant la période végétative (Larignon *et al.*, 2000). Les conidies se situent sur le tronc et les bras excoriés, dans les crevasses et les craquelures des ceps (Rooney-Latham *et al.*, 2005). Ce champignon est présent dans la partie des rameaux de porte-greffe à proximité de la tête de saul où il a été isolé (Larignon *et al.*, 2009). Il est aussi détecté par l'outil PCR à la surface de matériel de propagation : greffon et porte greffe (Larignon *et al.*, 2009). Les jeunes plants greffés transplantés au champ après greffage peuvent être contaminés (Larignon *et al.*, 2006).

- *Fomitiporia mediterranea* (Fm)

Ce basidiomycète de l'ordre des *Hyménochaetales* longtemps désigné sous le nom de *Phellinus punctatus* (Walla, 1984) et de *Fomitiporia punctata* (Cortesi *et al.*, 2000) est présent dans les ceps atteints d'esca en Europe (Mugnai *et al.*, 1999; Fischer, 2006). Dans les autres régions du monde, d'autres espèces de basidiomycètes sont présentes. Chez la vigne, ce mycète est responsable de la formation d'amadou ; bois dégradé, très clair et mou. Il est également présent chez d'autres espèces ligneuses telles que le kiwi (Di Marco *et al.*, 2004), le noisetier (Pilotti *et al.*, 2010) et le platane (Pilotti *et al.*, 2005).

Fm est considéré comme un colonisateur secondaire qui dégrade complètement les parois cellulaires. Il produit les mêmes enzymes que *Pch* et *Pal* mais également des enzymes lignolytiques comme la laccase, la peroxydase et la phenoloxydase (Mugnai *et al.*, 1999; Bruno and Sparapano, 2006).

Le cycle biologique est peu connu. *Fm* se conserve sous la forme de carpophores (ou basidiomes) dans les parties attaquées de la plante malade. Les basidiospores issues de la reproduction sexuée assurent majoritairement la dissémination par voie aérienne. Ce champignon a été détecté au niveau des plaies de taille, par outil PCR. Il n'est jamais isolé à partir des bois ou des plants à la pépinière.



Figure 8. Photos représentant la forme dite lente (8. a) et la forme apoplectique (8. b).



Figure 9. Symptômes caractéristiques d'une feuille atteinte par la maladie.

2.4.2) Dépérissement à *Botryosphaeria*

Les *Botryosphaeria* spp représentent un groupe très divers de champignons ascomycètes capables d'affecter de nombreuses plantes ligneuses dont la vigne, les eucalyptus (Smith *et al.*, 1996) et les oliviers (Moral *et al.*, 2008), par exemple. Dans la revue de Slippers and Wingfield (2007), les auteurs montrent la diversité des espèces par leur affinité avec leur hôte, leur degré de pathogénie ou encore par leur comportement endophytique : certaines espèces sont capables de se développer dans la plante sans causer de dommage apparent. Sur vigne, il existe de nombreuses études associant ce groupe d'ascomycètes à des dépérissements.

Les *Botryosphaeria* spp. produisent des composés phénoliques toxiques (Djoukeng *et al.*, 2009) : la mélléine, la (3R,4R) cis-4-hydroxymélléine, (3R)-7-hydroxymélléine, et la (3R,4R)-cis-4,7-dihydroxymélléine. D'après Djoukeng *et al.* (2009) la mélléine, bien que produite également par des champignons du genre *Aspergillus* (Cole and Kirskey, 1971; Sasaki *et al.*, 1970), peut donc être utilisée comme un marqueur de la présence des *Botryosphaeria* spp dans le cadre des maladies du bois.

Les symptômes observés sont très divers selon le type d'espèces présentes et la nature de l'organe atteint. Mortalité de bourgeons, dépérissement de rameaux, mort d'un cordon ou de la totalité du cep, nécroses de baies, nécrose sectorielle du bois, ponctuations noires au niveau du xylème en coupe transversale, plaies infectées, chlorose foliaire ou échec au greffage illustrent les symptômes très variés (Voir Fig. 8 et Fig. 9). En France, la maladie appelée Black Dead Arm (BDA), associée à différentes espèces de champignons du genre *Botryosphaeria*, a été décrite en 2001 (Larignon *et al.*, 2001). Cette maladie a été décrite pour la première fois par Lehoczky en 1974 en Hongrie, puis par la suite en Italie par Cristinzio en 1978. Cette maladie est aussi décrite au Chili (Auger *et al.*, 2004), Liban (Choueiri *et al.*, 2006), en Espagne (Luque *et al.*, 2009). Elle est facilement confondue avec l'esca du fait des symptômes foliaires similaires (Lecomte *et al.*, 2012). En effet, le BDA présente des digitations internervaires sans liseré jaune seulement durant les premiers stades d'expression des symptômes. Comme pour l'esca, le BDA présente une forme lente caractérisée par l'apparition de tâches, rouge vineux sur les cépages noirs et jaune-orange sur les cépages blancs, en bordure et à l'intérieur des feuilles. A la fin, ne subsiste plus qu'une petite bande verte (entourée d'un liseré rouge vineux pour les cépages noirs) le long des nervures. De plus, un autre symptôme n'est visible qu'après écorçage du cep ; il s'agit d'une bande brune située en périphérie du tronc et large de plusieurs centimètres. Lors de la coupe transversale du tronc, une partie jaune orangée où les vaisseaux conducteurs sont obstrués ou une nécrose sectorielle brune à noire selon l'état d'avancement de la maladie, peuvent être observées. La forme sévère atteint la totalité du cep et provoque un dessèchement complet des feuilles qui tombent en commençant par la partie apicale du rameau. Les fruits des ceps atteints peuvent également être détruits (Larignon *et al.*, 2001). Cette forme sévère conduit cependant rarement à la mort des ceps qui peuvent apparaître comme sains l'année suivante. Ces symptômes sont associés à la présence d'une bande brune tangentielle sous l'écorce qui peut atteindre la base du greffon . Cette bande brune peut parfois correspondre à une nécrose sectorielle dans le tronc ou le cordon en coupe transversale. Il a ensuite été isolé dans le vignoble californien (Urbez-Torres *et al.*, 2006a), mexicain (Urbez-Torres *et al.*, 2006b) et chilien (Auger *et al.*, 2004).

Plus d'une vingtaine d'espèces de Botryosphaeriaceae sont répertoriées comme pathogènes sur *Vitis vinifera*, isolées de différents organes et pour nombre d'entre elles testées pour leur pouvoir pathogène (Van Niekerk *et al.*, 2006). Aujourd'hui, les espèces de *Botryosphaeriaceae* sont très souvent désignées par le nom de leur forme anamorphe puisque les formes téléomorphes sont rares (Denman *et al.*, 2000, Crous *et al.*, 2006). Les espèces les plus répandues à travers le monde sont : *Diplodia seriata* (téléomorphe *Botryosphaeria*

obtusa ; Shoemaker, 1964), *Diplodia mutila* (téléomorphe *Botryosphaeria mutila* ; Shoemaker, 1964), *Neofusicoccum parvum* (téléomorphe *Botryosphaeria parva* ; Pennycook and Samuels, 1985), *Neofusicoccum australe* (téléomorphe *Botryosphaeria australis* ; Slippers *et al.*, 2004), *Neofusicoccum luteum* (téléomorphe *Botryosphaeria lutea* ; Phillips *et al.*, 2002), *Fusicoccum aesculi* (téléomorphe *Botryosphaeria dothidea* ; Corda, 1829), *Lasiodiplodia theobromea* (téléomorphe *Botryosphaeria rhodina* ; Phillips, 2002). En France, les espèces les plus isolées sont *Diplodia seriata* and *Botryosphaeria dothidea* (Larignon *et al.*, 2001).

Concernant le BDA, la première espèce pathogène décrite comme étant impliquée dans le BDA dans un vignoble hongrois a été *Diplodia mutila* (téléomorphe=*Botryosphaeria stevensii*) (Lehoczky, 1974). Plusieurs autres espèces ont depuis été isolées et décrites dans d'autres vignobles européens (Larignon *et al.*, 2001; Rovesti and Montermini, 1987; Urbez-Torres *et al.*, 2006a): *B. obtusa* (anamorphe=*Diplodia seriata*), *B. parva* (anamorphe=*Neofusicoccum parvum*) et *B. dothidea* (anamorphe=*Fusicoccum aesculi*). Sur la vigne, le pouvoir pathogène de ces champignons varie selon l'espèce considérée et la souche.

Cycle biologique des *Botryosphaeriaceae*

Les différents champignons de cette famille se conservent sous forme de pycnides et/ou périthèces (lorsque la forme téléomorphe existe) localisées sur l'écorce, les plaies de taille ou les bois morts laissés au sol. Ces champignons peuvent survivre en saprophytes, ce qui leur permet d'être présents sur de nombreux substrats, source d'inoculum potentiel. Une étude, réalisée en France a montré que les conidies produites dans les pycnides sont disséminées durant la période végétative de la vigne lorsque les conditions sont humides et pluvieuses (Kuntzmann *et al.*, 2010). Elles sont observées durant la période hivernale en Californie (Urbez-Torres *et al.*, 2010). Les *Botryosphaeriaceae* pénètrent la vigne par les blessures consécutives à la taille ou le greffage et colonisent le bois. Ils y demeurent à l'état endophyte ou de pathogènes latents. Ces champignons peuvent être transmis aux plants à partir des bois issus de plants de vigne mères contaminées et pendant le processus de fabrication (Gramaje and Armengol, 2011).

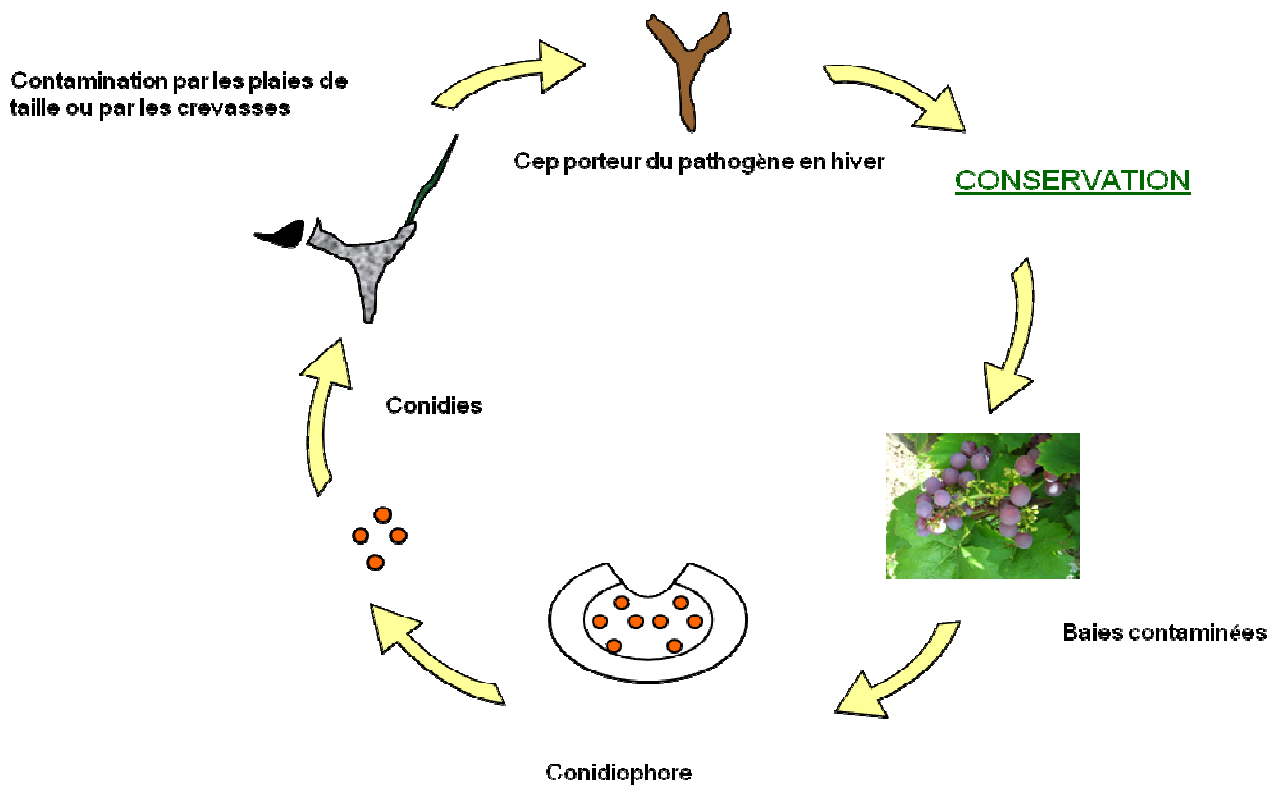


Figure 10. Cycle biologique de *Diplodia seriata*.



(Source : Jose Urbez-Torres)

Figure 11. Symptômes extérieurs causés par Botryosphaeriaceae.



(Source : Jose Urbez-Torres)

Figure 12. Nécroses dans le bois dues aux chancres à *Botryosphaeria*.

2.3) Méthode de protection vis-à-vis des maladies du bois de la vigne

La présentation de la biologie des différents agents pathogènes fongiques impliqués dans les maladies du bois met en évidence la diversité des modes d'infection, de dissémination. La variété des sources d'inoculum et le développement très lent et non visible des champignons dans le bois de vigne rendent très compliquée la mise en œuvre des méthodes de lutte. De plus, l'évolution de la réglementation des produits phytosanitaires à l'échelle européenne a conduit à l'interdiction de matière active utilisée pour la protection des plaies de taille par exemple. Les produits chimiques à base d'arsénite de sodium ont été interdits d'utilisation en 2001 en France à cause des effets cancérigènes sur l'homme et de la forte toxicité de ces produits sur l'environnement (Harmandon, 2004). De nombreuses recherches sont menées à travers le monde pour tester de nouvelles molécules utilisables en pépinière ou au vignoble. Des produits biologiques, à base de micro-organismes ou des produits naturels à base d'extraits de plantes, sont aussi à l'étude ou commercialisés. Les moyens de lutte culturaux ou prophylactiques restent les plus fréquemment les seuls moyens de lutte à disposition du viticulteur. Une synthèse des différents moyens de lutte actuels est présentée.

2.3.1) Protection chimique

La protection chimique contre les maladies du bois s'est beaucoup concentrée sur la protection des plaies de taille, voie d'entrée importante de nombreux champignons pathogènes lignocoles de la vigne. Les produits phytosanitaires contenant une ou plusieurs matières actives peuvent être appliqués soit par badigeonnage ou par pulvérisation afin de réduire les coûts. Des essais ont aussi été réalisés en injectant les produits chimiques dans le

tronc afin de garantir un effet curatif. Des préparations commerciales à base de carbendazime se révèlent efficaces pour stopper le développement des pathogènes impliqués dans l'Eutypiose et l'Esca (famille des benzimidazoles). Il en est de même pour les préparations à base de bénomyl (famille des carbamates) (Bester *et al.*, 2007; Sosnowski *et al.*, 2008; Gramaje et Armengol, 2011; Bertsch *et al.*, 2012). Ces deux substances actives sont cependant interdites en France : le bénomyl est interdit pour la composition de préparations bénéficiant d'une autorisation de mise sur le marché ; le carbendazime est interdit dans l'Union Européenne (règlement CE n°1107/2009 du 21 octobre 2009) du fait de leur dangerosité pour l'homme et l'environnement (Pitt *et al.*, 2010; Rolshausen *et al.*, 2010). D'autres matières actives montrent une efficacité *in vitro* ou au vignoble vis-à-vis des champignons ascomycètes impliqués. le tebuconazole, le prothoconazole, le fluozinam (Gramaje et Armangol, 2011). Rolshausen et al (2010) montrent l'efficacité du thiophanate méthyl vis-à-vis des champignons de la famille des Botryosphaeriaceae, de Pch et de Pal. En pépinière, les bains d'hydratation avant stockage des bois au froid ou en pré ou post greffage peuvent contenir des fongicides pour diminuer la quantité de germes infectieux et protéger les plaies du bois des infections. Les matières actives telles que le nénomyl, le carbendazim, le didecyldiméthylammonium chloride et le captan montrent des efficacités vis-à-vis de Pch et de *Phaeoacremonium* spp. (Fourie and Halleen, 2004; Fourie and Halleen, 2006; Gramaje *et al.*, 2010). Une association de deux matières actives : cyprodinil et fludioxinil ou pyradostrobine et metiram diminue l'inoculum de *Cylindrocarpon* spp., de Pch et d'espèces de Botryosphaeriaceae (Vigues *et al.*, 2010). En pépinière, les résultats sont variables et dépendent des espèces pathogènes visées (Amponsah *et al.*, 2012; Rolshausen *et al.*, 2010). De manière générale, les traitements chimiques s'avèrent très coûteux en temps et en argent. Leur efficacité est variable et elle dépendant de nombreux facteurs tels que la fréquence et les conditions d'application (les produits peuvent être lessivés par la pluie) (Calzarano *et al.*, 2004; Rolshausen and Gubler, 2005).

2.3.2) Lutte biologique

L'agent de lutte biologique *Trichoderma* spp. a fait l'objet de nombreuses études dans le cadre de la protection de la vigne vis-à-vis des maladie du bois. Ce micro-organisme ascomycète isolé du sol à des propriétés d'antagonisme et d'hyper parasitisme vis-à-vis de nombreux agents pathogènes de plante (Amreen and Kumar, 2012; Lee *et al.*, 2012; Tuao-Gava and Leal-Menezes, 2012). De plus, appliqué au sol ou sur la plante il est capable de stimuler la croissance des jeunes plants et de protéger les plantes vis-à-vis de stress abiotiques (Fourie and Halleen, 2005). Des produits commerciaux à base de *Trichoderma* sont utilisés en pépinière lors de l'élevage des plants greffés. Sur jeunes plants, Fourie *et al.* (2001) ont montré également un effet d'inhibition de *Trichoderma* sur les champignons pathogènes de la vigne : Pch, *Phaeoacremonium* spp. et *Cylindrocarpon* spp. En protection des plaies de taille, John *et al.* (2005) montrent l'efficacité de *Trichoderma* vis-à-vis d'*Eutypa lata*. Di Marco *et al.* (2004) montrent que *Trichoderma harzianum* empêche le développement de Pch. *Trichoderma atroviride* et *T. harzianum* réduisent les infections artificielles de Pch et d'espèces de Botryosphaeriaceae (Kotze *et al.*, 2011). Des plants biotisés avec *Trichoderma*

atroviride et des produits phytosanitaires ou des fertilisants enrichis avec *Trichoderma* sont aujourd'hui commercialisés. D'autres micro-organismes aux propriétés antagonistes ont également été testés : *Cladosporium herbarum*, *Fusarium lateritium*, *Aureobasidium pullulans*, *Bacillus subtilis* et *Erwinia herbicola* (Ferreira *et al.*, 1991; Schmidt *et al.*, 2001). Des molécules naturelles telles que le chitosane ou la cystéine (Nascimento *et al.*, 2007; Octave *et al.*, 2005) ont montré *in vitro* ou en serre des potentialités de protection intéressantes contre certains champignons associés aux maladies du bois. Il reste cependant à les tester en conditions réelles afin de valider leur efficacité et de pouvoir formuler des produits destinés à une mise sur le marché.

Les mesures sanitaires de prophylaxie restent largement utilisées au vignoble pour limiter le développement des maladies du bois. Le retrait des bois morts ainsi que le changement du mode de taille ont prouvé leur efficacité vis-à-vis de l'eutypiose, en regression dans de nombreuses régions viticoles (Weber *et al.*, 2007). Le rôle des sarments laissés au sol comme source d'inoculum est avancé sans qu'il y ait des preuves de leur importance. Toutefois, il est démontré que le compostage des sarments de vigne permet d'éradiquer les champignons impliqués dans les maladies du bois grâce à l'effet délétère d'une élévation de température (Lecomte *et al.*, 2006). Afin d'éviter l'arrachage des souches dépérissantes, il est possible de pratiquer le récépage ou le greffage à partir du tronc sectionné (Creaser and Wicks, 2004). Le système de taille peut favoriser ou au contraire limiter le dépérissement des souches atteintes. Le système de taille appelé Guyot-Poussart permet de conserver des trajets de sève indemne à l'opposé des plaies de taille. Il a été montré que ce système limite le développement de l'esca de la vigne (Geoffrion and Renaudin, 2002). Au vignoble, les viticulteurs réalisent de plus en plus une double taille (Deux passage successifs pour tailler la vigne). En effet, si des micro-organismes entrent par les plaies laissées par la première taille en hiver et commencent à se développer, la deuxième taille effectuée quelques mois après, avant le commencement du cycle végétatif, permet d'éliminer la partie infectée. En complément de cette méthode, des produits phytosanitaires sont appliqués sur les plaies afin de limiter l'invasion des agents pathogènes.

A la pépinière, afin de diminuer le taux d'infection des bois ou des plants de vigne, ceux-ci peuvent être traités à l'eau chaude. Ce traitement est utilisé dans certaines régions viticoles pour éliminer le phytoplasme de la flavescence dorée. Plusieurs protocoles existent. Par exemple, Les bois ou les plantes peuvent subir deux traitements à l'eau chaude (Gramaje *et al.*, 2009; Waite and Morton, 2007) : le premier d'une durée de 5 min à 54°C et le deuxième de 30 à 45 min à 50°C. Ce traitement possède cependant deux limitations : il stresse la plante pouvant mener des problèmes de reprise de la végétation pour certains plants et il ne cible pas spécifiquement les champignons pathogènes sachant que d'autres micro-organismes pour certains utiles sont également présents dans les tissus.

Toutes les méthodes présentées ont généralement une efficacité partielle parfois de courte durée alors que ces maladies se caractérisent par un développement sur une longue période qu'il est difficile de mesurer. Les coûts d'application sont en général élevés et souvent rédibitoires pour les viticulteurs surtout lorsque l'efficacité n'est pas réellement

démontrée au vignoble. Les viticulteurs sont souvent démunis devant la mortalité parfois très fréquente dans leur vigne. Même si les recherches appliquées se sont accélérées depuis plus de dix années , les résultats fiables ne sont pas à la hauteur des attentes de la profession.

Suite à l'arrêt de l'utilisation de l'arsénite, en 2001, les pouvoirs publics ont mis en place un observatoire des maladies du bois afin d'évaluer la fréquence des principales maladies du bois en France : l'esca (et BDA) et l'eutypiose à partir de 2003 dans les différents bassins de production viticole français et pour les principaux cépages. Les résultats de cette enquête menées en 2003 et 2008 fait l'objet de la partie suivante, permettant ainsi d'aborder le comportement des différents cépages dans différents contexte pédo climatiques.

Cette partie est présentée sous la forme d'une publication intitulée : « Overview of grapevine trunk diseases in France in the early 2000s ». (Acceptée)

Overview of grapevine trunk diseases in France in the early 2000s

EMILIE BRUEZ^{4,3}, PASCAL LECOMTE^{3,4}, JACQUES GROSMAN¹, BRUNO DOUBLET², CHRISTOPHE BERTSCH⁵, FLORENCE FONTAINE⁶, ADELIN UGAGLIA⁷, PIERRE-LOUIS TEISSEDRÉ⁸, Jean-Pierre DA COSTA⁹, LUCIA GUERIN-DUBRANA^{4,3} and PATRICE REY^{4,3*}

¹ DRAAF-SRAL Rhône-Alpes, 165 rue de Garibaldi BP 3202 69401 Lyon Cedex 3, France

² DRAAF-SRAL Champagne-Ardenne, 37 avenue Hoche – site les Essilards, 51686 Reims cedex 2, France.

³ INRA, ISVV, UMR1065 Santé et Agroécologie du Vignoble (SAVE), F-33140 Villenave d'Ornon, France.

⁴ Université de Bordeaux, ISVV, UMR1065 SAVE, Bordeaux Sciences Agro, F-33140 Villenave d'Ornon, France.

⁵ Université de Haute-Alsace, UFR PEPS, EA-3991 Laboratoire Vigne Biotechnologie et Environnement, 33, rue de Herrlisheim, 68008 Colmar cedex France.

⁶ Université de Reims Champagne Ardenne, UR Vignes et Vins de Champagne, EA 4707, Laboratoire Stress, Défenses et Reproduction des Plantes, 51 687 Reims France.

⁷ Université de Bordeaux, ISVV, UR GAIA, Bordeaux Sciences Agro, F-33140 Villenave d'Ornon, France.

⁸ Université de Bordeaux, ISVV, UR Oenologie, F-33140 Villenave d'Ornon, France.

⁹ IMS, Université de Bordeaux, Bordeaux sciences Agro, F-33405 Talence, France.

*E-mail : prey@bordeaux.inra.fr / patrice.rey@agro-bordeaux.fr

Summary. The National Grapevine Trunk Disease Survey was conducted in France from 2003 to 2008 to monitor the grapevine trunk diseases (GTDs), eutypa dieback and esca/black dead arm (BDA). Data collected from seven regions, 329 vineyards and 12 cultivars, were analysed. There were

great variations amongst regions in the incidence of GTDs. For esca/BDA, two groups were distinguished: vineyards in Jura and Charentes had greater incidences (93-95%) than those of Bordeaux, Alsace and Bourgogne (54-82%). Incidence increased in Charentes over the 6-year survey, with the highest values being recorded during the last 2 years. For eutypa dieback, all vineyards of Charentes were affected, with 17% to 25% of vines expressing symptoms; for the other regions, 52 to 80% of vineyards were affected, with incidences below 3%. Our study shows that certain cultivars were much more affected by esca/BDA, *e.g.* Savagnin, Trousseau, or by eutypa dieback, *e.g.* Ugni Blanc, than others. One cultivar could be significantly more attacked in one region than in another. The global health status of the vineyards was also investigated. (i) For four regions, 82% (Jura) to 87% (Alsace) of the grapevines were healthy, but this percentage decreased steadily (67%) in Charentes. (ii) Plants infected by GTDs were 32% and 18% in Jura and Charentes respectively, and only 2.9% in the Bourgogne region. (iii) The unproductive plants, *i.e.* dead, missing, replanted or restored, represented a significant part of the losses (6.6% in Charentes to 9.9% in Jura). The extension of GTDs is discussed with regard to the abiotic and biotic factors that may favour the diseases.

Keywords: esca, eutypa dieback, black dead arm, grapevine cultivar, abiotic factors, biotic factors

Introduction

The three grapevine trunk diseases (GTDs), eutypa dieback, esca decline and botryosphaeria dieback are widespread in the main vine-growing regions of the world (Armengol *et al.*, 2001; Edwards and Pascoa, 2004 ; Gimenez-Jaime *et al.*, 2006; Mugnai *et al.*, 1999; Rumbos and Rumbou, 2001; Scheck *et al.*, 1998). An increase in the incidence of GTDs over the last 10-15 years has been reported worldwide (Bertsch *et al.*, 2012; Carter 1991; Chiarappa, 2000; Graniti *et al.* 2000; Reisenzein *et al.*, 2000; Sosnowski *et al.*, 2007; Surico *et al.*, 2000; Úrbez-Torres *et al.*, 2006, 2008, 2009). In France, until the late nineties, together with eutypa dieback, the most common trunk disease was esca but, at the turn of the century, a third and new botryosphaeria disease, called black dead arm (BDA), was identified in Bordeaux (Larignon *et al.*, 2001) and in other French vine-growing areas (Panon, 2000).

Because of the threat that these diseases cause vineyards, numerous studies were carried out during the last decade to identify the causal fungi and their interactions with the vine in order to develop disease management strategies (Bertsch *et al.*, 2012; Larignon *et al.*, 2009).

As regards aetiology, the symptoms that occur in the trunk, leaves and berries have been extensively described, revealing that eutypa dieback symptoms differ markedly from those of esca and BDA, with differentiation between the latter two often proving difficult. Lecomte *et al.* (2012) reported that foliar symptoms of esca showed transitory phases that overlapped with some BDA descriptions. For esca, due to the complexity of the symptoms, changes in the definition of this disease have been reported in the literature over the last ten years (Bertsch *et al.*, 2012; Surico *et al.*, 2008). Two major simplifications in disease terminology were proposed to replace the five initial terms used for esca: wood streaking, Petri disease, young esca, esca and esca proper. The first simplification was made by Surico (2009), who suggested that the term “young esca” be replaced by “grapevine leaf stripe disease” (GLSD), so that the term “esca” only included white rot (esca) and esca proper. For the three tracheomycotic syndromes, brown wood streaking, Petri disease and GLSD, the term phaeotracheomycotic complex seemed appropriate, because it grouped the same fungi within the three symptomatically different diseases. The second simplification was made by Lecomte *et al.* (2012) when they chose not to separate esca symptoms into mild or apoplectic forms, but to use a classification based on a gradual scale of severity, starting from some leaves showing only discolorations up to complete vine wilting.

When the causal agents are considered for eutypa dieback, a well-defined fungal pathogen, *Eutypa lata*, was identified long ago (Carter, 1988) and, more recently, other species of the

diatrypaceae diatrypaceae (Trouillas and Gubler, 2010; Trouillas *et al.*, 2010). For botryosphaeria dieback, the fungi are more numerous since, according to Úrbez-Torres (2011), 21 different Botryosphaeriaceae species are associated with this disease. With esca, the situation is much more complex because it involves fungal species belonging to various species and families. Generally, esca is thought to result from the pathogenic activity of fungal species, *e.g.* *Phaeoconiella chlamydospora*, *Phaeoacremonium aleophilum*, *Fomitiporia mediterranea*; *E. lata* and *Stereum hirsutum* could also have a role in esca (Fischer, 2006; Larignon and Dubos, 1997). The involvement of other microorganisms is still a matter of speculation and recent studies indicate that diverse microflora colonise the wood of esca-diseased grapevines (Bruez *et al.*, 2011; Maher *et al.*, 2012), but their exact role remains to be determined. Finally, the origin of the various terms that define esca should be associated with the microbial species that colonize the wood of grapevines at a specific time. The activity and succession of fungal microflora that occur within the wood may lead to the complexity of the symptoms, thus generating the various terms used in the literature.

Many experiments have been carried out to reproduce the symptoms, either under controlled conditions or in the vineyard. Again, depending on the disease, various results have been obtained. For eutypa dieback, the Koch postulate is verified after *E. lata* grapevine inoculations (Moller and Kasimatis, 1978; Peros and Berger, 1994) but, for esca and botryosphaeria dieback, wood symptoms (necroses) are readily reproduced (Laveau *et al.*, 2009), although those producing the leaf symptoms are rarely obtained. This complex relationship between fungi and esca/BDA recently led Hofstetter *et al.* (2012) to state that esca is not a fungal disease, but that the presumed esca-associated fungi only decay already senescent or dead wood resulting from intensive pruning, frost or other mechanical injuries such as grafting. However, many papers have already reported that various symptoms of wood necroses, including white rot, have been obtained after inoculations of grapevines with *P. chlamydospora*, *P. aleophilum*, *F. mediterranea* or Botryosphaeriaceae in combinations or not (Rego *et al.*, 2009; Sparapano *et al.*, 2000, 2001; Surico *et al.*, 2006).

The current epidemic spread of esca dates back to the 1990s (Mugnai *et al.*, 1999), according to various European surveys for esca (Reisenzein *et al.*, 2000; Surico *et al.*, 2000) and also BDA (Larignon *et al.*, 2001; Lecomte *et al.*, 2005). Survey results provided vinegrowers and scientists with information on the evolution of these diseases. Eutypa dieback was also surveyed, for instance, in Australia (Sosnowski *et al.*, 2007). The National Grapevine Trunk Diseases Survey was established in France to monitor the evolution and determine the importance of eutypa dieback, esca and BDA during the period from 2003 to 2008. The survey was conducted in 10 French regions, on 27 varieties

of vines and for more than 600 vineyards. A preliminary result provided information on 256 individual vineyards and, at that time, the mean incidences for eutypa dieback and esca/BDA were 2.23 and 3.25%, respectively (Fussler *et al.*, 2007).

The objective of the present paper is to assess (i) the importance and the evolution of GTDs over a 6 year-period in 7 French regions. Those regions were selected because each of them has its own specific terroir features. (ii) To determine the disease incidence of specific cultivars planted in each of the regions. (iii) To compare the disease incidence of the same cultivars planted in two or three different regions. (iv) To assess the global health status of the vineyards by counting the number of healthy plants, the unproductive ones, *i.e.* dead, missing, replanted or restored grapevines, and those affected by GTDs.

Materials and methods

The survey of the French vineyards

The survey of grapevine trunk diseases was made in 329 vineyards of 7 regions of France. For each vineyard that was randomly chosen among the regions, 300 grapevines were marked and spotted. These 300 grapevines were divided in 10 locations, randomly chosen in the parcel, of 30 grapevines. During the survey, the observations were made on the same locations and on the same grapevines. For all regions, the same protocol was used. It consisted in monitoring the grapevines in each vineyard at two periods of the growing season: in June to assess symptoms of eutypa dieback and in August-September of esca/BDA. Note that as it was difficult to differentiate foliar symptoms caused by esca and BDA, the symptoms were grouped under the term esca/BDA. At the same time, dead, missing, replanted, restored and healthy (without GTD symptoms) plants were counted, they represented the unproductive grapevines. The survey was done by employees of FranceAgriMer, the French Plant Protection Agency (French Ministry of Agriculture), Institut National de la Recherche Agronomique (INRA), Institut Français de la Vigne et du Vin (IFV) and vinegrowers associations of each region.

For 5 regions: Alsace, Bordeaux, Bourgogne, Charentes and Jura, all the vineyards were monitored over the 6 years period, but some of them were not monitored one year (Alsace in 2006, Jura in 2003) or two years (Centre in 2007 and 2008). The total number of grapevine cultivars was 12 (Table 1).

For 2 other regions: Centre and Provence-Alpes Côte d'Azur (PACA), the survey was used to compare GTDs that affect the cultivar Cabernet Sauvignon planted in two regions, Bordeaux and PACA, and the cultivar Sauvignon planted in the Bordeaux, Bourgogne and Centre regions (Table 1).

For each vineyard, the information collected consisted of age, cultivar and rootstocks. Note that, for each cultivar the age value corresponds to the mean of the ages of the vineyards in 2008, in brackets are the minimum and maximum ages of the vineyards (Table 1).

Statistical analyses

The frequency of eutypa dieback and of esca/BDA corresponds to the percentage of affected

vineyards. The incidence of eutypa dieback and of esca/BDA represents the percentage of affected vines. For incidence, the confidence intervals have been calculated by using the package Hmisc of the Rsoftware (version 2.14.2) with the command “bioconf” (confidence intervals are calculated because the values are meaningless below 10%).

The plant mortality, *i.e.* the percentage of dead and missing grapevines, and the replanted and restored plants were counted too. For each result the standard deviation has been estimated.

Kruskal-Wallis test has been used to compare the data obtained with the Cabernet-Sauvignon and Sauvignon cultivars when they were planted in various regions (variances were not equal according to the Levene’s test). The pairwise Wilcoxon test has been used after the Kruskal-Wallis test to confirm the results.

The contingency table allowed us to determine if, for the same cultivar, a relation exists between the rates of esca/BDA and of eutypa dieback. The values were obtained by using Rcmdr of the R software (2.14.2). The Chi-square has been defined with the chi-square table with a liberty degree of liberty of one.

For eutypa dieback or esca/BDA symptoms, correlations between the region and the age of the grapevines and between the cultivars and the year were calculated by analysis of covariance (ANCOVA). The percentages of esca/BDA and eutypiose symptomatic expression have been transformed in logit. The *P*-values obtained by the ANCOVA shows if there is a difference or not between the variable to explain (percentage of esca/BDA and percentage of eutypiose) and the explicative variables (the age of the plant, the five regions, the year or the cultivars). ANOVA (Analysis of variance) has been used to compare the relation between the region and cultivars according to the percentage of esca and eutypa dieback attack in the vineyards. To confirm the results of the ANCOVA and the ANOVA, the LSD test has been used.

Results

Comparing frequency and incidence of grapevine trunk diseases between regions

In Charentes, frequencies of eutypa dieback were 100% in all the 27 vineyards surveyed (Fig. 1a). For the Alsace, Bordeaux and Bourgogne regions, the values were lower; they varied between 42 and 80% (Fig. 1a). From year to year, great variations were observed; for instance in Bordeaux, 80% of vineyards were affected by eutypa dieback in 2007 which reduced to 42% in 2008. In Jura, eutypa dieback was observed in 32-38% of vineyards in year 2005, 2006 and 2007, but in 2004 was reduced to only 8%.

The highest incidences of eutypa dieback in the vineyards of Charentes varied from 17.3% to 25.84% between years (Fig. 2a). From 2003 to 2008, there was a tendency for the incidence to decrease. The three highest percentages (above 21%) were recorded in 2003-2005 and the 3 lowest (below 20%) in 2006-2008. For the Alsace and Bordeaux vineyards, the incidence of eutypa dieback was always below 3.59%, the lowest value was obtained the first year of the survey (2003) and the two highest in 2007 or 2008 (Fig. 2a). In the Bourgogne vineyards, the incidence was less than 1% in 2004, 2006 and 2007; the maximum was 1.95% in 2005. For the Jura region, the incidence in the vineyards is always below 0.5%, it was of only 0.04% in 2004.

The Jura and Charentes regions had higher frequency and incidence of esca/BDA compared with that of the Alsace, Bordeaux and Bourgogne regions (Figs 1b and 2b). In the Jura vineyards, the frequency was greater than 95% and the incidence varied from 6.67% to 11.19%. In Charentes, the frequency of vineyards with esca/BDA was around 81-85% the first two years of the survey, with all vineyards affected in 2005 and in the following years frequency varied from 96% to 100%. The incidence of esca/BDA in vineyards increased each year in Charentes from 1.67% to 7.69%.

For the 3 other regions, the mean frequencies of esca/BDA were relatively similar; 66.49% in Alsace, 72.87% in Bordeaux and 67.57 in Bourgogne. For all the 5 regions, the lowest values of incidence were obtained the first year of the survey (2003) and the highest in 2007 or 2008.

Comparing incidence of grapevine trunk diseases between grapevine cultivars

In the Charentes region, the incidence of eutypa dieback in cv Ugni Blanc vines (22%) was significantly higher than the other cultivars in other regions in the survey; Cabernet Sauvignon and

Sauvignon (2.5%-3%) and all others were less than 1% (Fig. 3a).

The greatest incidence of Esca/BDA was recorded in cvs. Savagnin and Trousseau (10%) in the Jura region (Fig. 3b). Less than 2% of vines; cvs. Pinot and Chardonnay 21 in Bourgogne and in cv Merlot in Bordeaux. Four percent of Chardonnay 89 vines, planted in the department of the Yonne in Bourgogne were recorded with esca/BDA symptoms compared with 1% of cv Chardonnay 21 vines, which were planted in the department of the Côte d'Or in the same region. Except for the cultivar Sauvignon from the Bordeaux vineyards that had 7% of grapevines attacked, for the other cultivars, 3% to 6% of vines expressed esca/BDA foliar symptoms.

The incidence of esca/BDA on Cabernet-Sauvignon grapevines planted in Bordeaux (17 vineyards) and Provence-Alpes Côte d'Azur (10 vineyards) were significantly different in 2003-2006 (Kruskal-Wallis test, $P < 0.001$) but not in 2007 ($P = 0.191$) and 2008 ($P = 0.597$). The incidence for this cultivar is thus generally higher in Provence-Alpes Côte d'Azur than in Bordeaux vineyards (Fig. 4).

When the esca/BDA incidences on Sauvignon cultivar planted in the Bordeaux, Bourgogne and Centre regions are compared over the 6 years survey, no significant differences were observed (Fig. 5) according to the Kruskal-Wallis test. The pairwise Wilcoxon tests confirmed the results of Kruskal-Wallis test.

Assessment of grapevine losses from 2003 to 2008

The percentage of healthy plants in Charentes was 67.4% compared with 89% for Bourgogne, 87% in Alsace, 86% in Bordeaux and 82% in Jura. In Alsace, dead and missing vines accounted for 0.63% and 1.19% of vines, respectively. For the other regions, the incidence of dead plants was relatively similar, from 1.10% in Bourgogne to 1.67% in Charentes. The same trend was observed for the missing vines, the values varied from 2.52% in Bourgogne to 3.04% in Jura. Replanted vines were the more numerous in Jura (5.01%), Alsace (4.91%) and Bourgogne (4.60%), the lowest percentage was in Charentes with 1.21%. For restored plants, the two highest values, 1.39% and 0.91% were found in Alsace and Charentes respectively. Only 0.05% of vines were restored in Bourgogne (Table 2a).

Regarding the two GTDs surveyed, 21.2% of the vines expressed symptoms of eutypa dieback in Charentes, in the 4 other regions that value was markedly reduced (1.2% in Bourgogne, 2.1% in Bordeaux, 2.3% in Alsace), the lowest percentage was 0.3 in Jura. For esca/BDA, 8.20% of vines expressed leaf symptoms of that disease in Jura, that percentage was reduced in to half in Charentes

(4.78%) and Bordeaux (4.23%), the lowest value was obtained in Bourgogne (1.70%). In the Charentes region, there were the highest percentage of vines attacked by eutypa dieback (21.2%) and the second one for esca/BDA (4.78%). For the Jura region, esca/BDA was the higher (8.20%) but the lower for eutypa dieback (0.3%) (Table 2a).

Table 2b shows the percentages of unproductive plants, they were relatively similar for all the 5 regions since they ranged from 6.62% in Charentes to 9.92% in Jura. For vines affected by GTDs, the highest values were in Charentes (25.98%) and Jura (8.5%), note that only 2.9% of the plants are diseased in Bourgogne.

The total percentage of unproductive plants and GTDs affected grapevines across all regions was 32.6% in Charentes, compared with 11.17% in Bourgogne (Table 2b).

Link between the diseases and various parameters

For each cultivar, correlations between esca/BDA and eutypa dieback are shown on Table 3. Among the 12 cultivars studies, correlation between the two diseases was observed for the cultivars Trousseau (Jura region) and Sauvignon (Bordeaux region).

Table 4 shows that for esca/BDA and eutypa dieback expression of the symptoms, there are differences between the regions (p value <1E-10), between the years (p value <0,036) and between the cultivars (p value <2E-10),. The results have been performed by a LSD test. The age of the vineyards is not a variable associated with the percentage of symptomatic expression of eutypa dieback and esca/BDA.

The LSD test results confirmed the differences obtained by the ANCOVA and ANOVA tests.

Discussion

These results show that the GTDs, eutypa dieback and esca/BDA, are present in all five French vineyard regions. However, depending on the region, there were variations in the frequency and incidence of GTDs in the vineyards. Esca/BDA frequency was greater in Jura and Charentes, than in Bordeaux, Alsace and Bourgogne. Generally, from one year to another, the incidence did not fluctuate, except in the Charentes region where a constant increase occurred over the 6 years. For all regions, the highest frequencies were recorded during the last two years, 2007 and 2008. Does that signal the beginning of an epidemic that will develop slowly but steadily over the years? Data obtained in 2009 to 2012 from another survey performed in some French regions (Grosman and Doublet, 2012) suggest that this hypothesis is possible. However, the increase observed in the present study seems to be less than that reported by Reisenzein *et al.* (2000) in Austria. They indicated that the number of vines with external symptoms increased by an average annual rate of 2.7% in 6 years, but in certain vineyards the increase was between 2% and 20%.

Charentes was the only region with significant levels of eutypa dieback, with all its vineyards having from 17% to 25% of vine incidence symptoms for all 6 years. In the Alsace, Bordeaux and Bourgogne regions, the frequencies fluctuated over the years (from 52% to 80%) but were always lower than in Charentes. The incidences were relatively low, generally under 3% of the grapevines expressed symptoms. Jura seems relatively free of this disease since the frequency was particularly low (8% to 38%), and no more than 0.46% of grapevines expressed symptoms of eutypa dieback. So these data suggest that, unlike esca, eutypa dieback was not increasing in France. To support that observation, even in the most attacked region, Charentes, the lowest values of grapevines expressing eutypa dieback were only obtained over the last three years of the survey (2006 to 2008). However, as symptoms of eutypa dieback may fluctuate considerably, we suggest that the disease needs to be surveyed over a longer period.

To obtain an overview of the situation of GTDs in France, it should be borne in mind that, from one year to another, the same grapevines do not necessarily express foliar symptoms. So, when a period of several years is considered, the number of “foliar symptomatic vines at least once” is far more significant. Surico *et al.* (2000) indicated that, in a 6-year survey in Italy, the annual incidence of esca affected vines ranged from 11% to 19%. They found numerous different sequences of symptom expression of vines over that period; the cumulative incidence, however, was around 50%. In another Italian survey, Surico *et al.* (2006) again reported an increase in esca over time in three vineyards in Siena (50% over 11 years), Ravenna (30% over 6 years) and Florence (51% over 5). In France, Grosman and Doublet (2012) reported that in a 10-year survey in Bourgogne in a vineyard with low incidence (3-4% each year), a total of 21% of vines expressed esca/BDA at least once over that period. The expression of foliar symptoms is not neutral, as shown by Guérin-Dubrana *et al.* (2012, this

Journal issue), since the mortality due to esca is always associated with the foliar symptom expression the year before grapevine death. For eutypa dieback, symptoms are also a risk factor of death, greater or equal to that of esca. Another key question depends on the positive effect that one disease has on the other. Only two cultivars out of twelve showed a link between eutypa dieback and esca/BDA. So, with the exception of certain particular conditions, it did not seem that the two could be promoted at the same time within a vineyard.

The present study shows that the trends in symptomatic expression GTDs shown in the 6-year survey were relatively similar within each of the 5 geographic regions but that, from one region to another, frequencies and incidences differed greatly. We identified several factors that may be responsible for such differences: the cultivars, regions and year of survey. Our study clearly shows that some of the cultivars are much more attacked than others by esca/BDA, *i.e.* Savagnin, Trousseau, or eutypa dieback, *i.e.* Ugni Blanc. We observed that Cabernet Sauvignon can be significantly more attacked in a certain French region, *i.e.* Aquitaine, than in another, *i.e.* Provence-Alpes Côte d'Azur. However, for the cultivar Sauvignon, we did not observe differences concerning the grapevines planted either in the Bordeaux, Centre or the Bourgogne regions. For eutypa dieback, Sosnowski *et al.* (2007) reported that differences also occurred. They reported that foliar symptoms caused by *E. lata* on the cultivar Shiraz varied from year to year in South Australia in a 6-year survey and that, although trends were similar for vineyards within geographical regions, differences were observed between the two regions in which the experiments were conducted. All these results indicate that besides “varietal susceptibility”, other factors, such as climate and soil, are probably involved.

For other countries, White *et al.* (2011) reported that esca was found on a range of grapevine cultivars planted in South Africa, with some of them being the same as those surveyed in France, *i.e.* Chardonnay, Cabernet Sauvignon, Merlot. However, as the extent of the disease was not mentioned, it did not prove possible to compare them. A study of the susceptibility of one cultivar planted in different countries, for instance, Cabernet Sauvignon in France, Italy and the USA, Riesling in France and Austria (Reisenzein *et al.*, 2000), would certainly be useful in characterising the factors that are the more representative of each area. It would subsequently help to determine the main factors that favour GTDs.

We found that regions and the year of survey have an indirect impact on the disease. Each region has its own different climate: Alsace, for instance, has a continental climate and Bordeaux an oceanic one. Although we have not investigated that point, climate certainly has an influence on the development of GTDs. Surico *et al.* (2006) suggested that climate changes have exacerbated the esca problem. The intensity and variations of rainfalls over the years have a strong influence on the disease. Marchi *et al.* (2006) reported that, throughout a growing season, the number of grapevines that remained asymptomatic was inversely related to the rainfall in May-June or in summer. Chronic esca expression is associated with hot periods in summer following rainfalls whereas, during hot, dry summer periods, severe esca (apoplexy) was more common (Surico *et al.*, 2000).

Another important point is temperature, which may differ from one year to another. Lecomte *et al.* (2012) observed that, whatever the vineyard or year of survey in the Bordeaux region, the appearance of esca-leaf symptoms increased regularly from the beginning of June until the end of July. Afterwards, the rate of leaf symptom occurrence decreased, with certain symptoms remaining visible until September. Such an evolution of leaf symptoms could be associated with the progressive increase of mean temperatures in early summer. In this case, too, temperature may have a positive impact on fungal growth in the wood tissues.

As regards eutypa dieback, it is well known that the susceptibility of grapevine wounds to infection by *E. lata* spores is favoured by cool winter conditions (Chapuis *et al.*, 1998; Munkvold and Marois, 1995). Sosnowski *et al.* (2007) carried out a 6-year study in South Australia, to study the influence of climate on foliar symptoms of eutypa dieback on grapevines, cv Shiraz. They found that various parameters, such as winter rainfall 18 months earlier, increased temperatures in spring, and very high and very low rainfalls in October may have had an influence on the disease. These results led the authors to set up a model predicting the incidence of foliar symptoms of eutypa dieback.

One of the main results from this study is the status of vines growing in the five French regions we surveyed. It was found that 82% (Jura) to 87% (Alsace) of vines were healthy, but this percentage decreased to 67% in Charentes. These relatively low values of healthy plants should be regarded as the consequence of alterations that affected two kinds of plants. First, for plants that were affected by GTDs, there were 18% and 32% in Jura and Charentes, respectively, and only Bourgogne seems less attacked by these diseases (2.9%). Secondly, unproductive plants, *i.e.* dead, missing, replanted or restored grapevines, represented a significant part of losses in Charentes (6.6%) and Jura (9.9%). In addition to the yield losses and decrease in wine quality (Dubos and Larignon, 1987; Lorrain *et al.*, 2012) caused by GTDs, part of the unproductivity is due to these diseases, because vinegrowers

currently uproot affected plants, replanting or restoring them whenever possible. When grapevines are replanted, there is a waiting period of at least three years before the first harvest. Although the costs associated with trunk diseases are often difficult to quantify, in South Australia, eutypa dieback has been estimated to cause yield losses of at least 860 and 740 kg/ha for the Shiraz and Cabernet Sauvignon varieties (Wicks and Davies, 1999). For Shiraz alone, production losses in Australia were equivalent to 20 million Australian dollars. In California, economic losses of up to US\$260 million per annum have been attributed to the same disease (Siebert, 2001). In France, relevant assessments have not been made, but it was estimated that GTDs induced losses of around 6-7 million euros per year in the Loire-et-Cher alone. Hofstetter *et al.* (2012) estimated that the worldwide annual financial cost of the replacement of death plants due to GTDs is in excess of 1.132 billion euros. It is thus clear that these diseases are a major threat for the wine-producing regions everywhere.

To conclude, the present work indicates that GTDs are of major concern for the sustainability of certain French vineyards and vine cultivars. We have mentioned certain factors that seem to be of major importance in the development of these diseases. Further studies have to be undertaken to determine accurately their impact on the development of GTDs; presumably, other factors are involved (Lecomte *et al.*, 2011). Surico *et al.* (2004) identified the use of poor quality planting material and of fungicides as possible causes for the upsurge of esca in Tuscany, Italy. According to Peros *et al.* (2008) the use of good quality planting material and the choice of agronomic practices that favour grapevine longevity appear to be useful strategies to reduce the incidence of esca. The challenge is great because, at the same time, GTDs has created great apprehension in viticulture, as shown by recent demonstrations in France. One French deputy recently spoke of the “new phylloxera” of the 21st century in his declaration on GTDs at the National Assembly and vinegrowers called for rapid control solutions. However, because grapevine trunk diseases involve a range of biotic and abiotic factors, finding solutions would require managing most of the above-mentioned factors. A systemic approach would seemingly be needed to resolve this complex issue.

Acknowledgements

The authors acknowledge the work of field observers and farm advisers who have collected the data for the National Grapevine Wood Disease Survey. The analyses were financed by Bordeaux Sciences Agro, the Regional Council of Aquitaine, the French Ministry of Agriculture, Food-processing industry and Forest (programme CASDAR V906).

Literature cited

- Armengol J., A. Vicent, L. Torne, F. Garcia-Figueres and J. Garcia-Jimenez, 2001. Fungi associated with esca and grapevine declines in Spain: a three-year survey. *Phytopathologia Mediterranea* 40, S325–9.
- Bertsch C., M. Ramirez-Suero, M. Magnin-Robert, P. Larignon, J. Chong, E. Abou-Mansour, A. Spagnolo, C. Clément and F. Fontaine, 2012. Grapevine trunk diseases: complex and still poorly understood. *Plant Pathology* DOI: 10.1111/j.1365-3059.2012.02674.x.
- Bruez E., J. Vallance, J. Gerbore, P. Lecomte, L. Guerin-Dubrana and P. Rey, 2011. Characterization of fungal and bacterial communities that colonise the various wood tissues of healthy and esca-diseased vines. *IOBC/WPRS Working Group on “Integrated Protection and Production in Viticulture”*, Lacanau, France, 2-5 October 2011 (abstract).
- Carter M.V., 1988. *Eutypa dieback*. In: Pearson RC, Goheen AC, eds. *Compendium of Grape Diseases*. St Paul, MN, USA: APS Press, 32–4.
- Carter M.V., 1991. The status of *Eutypa lata* as a pathogen. Monograph. *Phytopathological Paper No. 32*, International Mycological Institute, Egham, UK.
- Chapuis L., L. Richard and B. Dubos, 1998. Variation in susceptibility of grapevine pruning wound to infection by *Eutypa lata* in south-western France. *Plant Pathology* 47, 463-472.
- Chiarappa L., 2000. Esca (black measles) of grapevine. An overview. *Phytopathologia Mediterranea* 39, 11-15.
- Dubos B. and P. Larignon, 1987. Esca, in: R.C. Pearson, A.C. Goheen (Eds.), *Compendium on grape diseases*, APS Publ., St Paul, Min., USA, pp. 34–35.
- Edwards J. and I.G. Pascoe, 2004. Occurrence of *Phaeoconiella chlamydospora* and *Phaeoacremonium aleophilum* associated with Petri disease and esca in Australian grapevines. *Australasian Plant Pathology* 33, 273-279.
- Fischer M., 2006. Biodiversity and geographic distribution of basidiomycetes causing esca-associated white rot in grapevine: a worldwide perspective. *Phytopathologia Mediterranea* 45, 30-42.
- Fussler L., N. Kobes, F. Bertrand, M. Mauray, J. Grosman and S. Savary, 2008. A characterization of grapevine trunk diseases in France from data generated by the National Grapevine Wood Diseases Survey. *Phytopathology* 98, 571–9.

- Gimenez-Jaime A., A. Aroca, R. Raposo, J. Garcia-Jimenez and J. Armengol, 2006. Occurrence of fungal pathogens associated with grapevine nurseries and the decline of young vines in Spain. *Journal of Phytopathology* 154, 598-602.
- Graniti A., G. Surico G. and L. Mugnai, 2000. Esca of grapevine: a disease complex or a complex of diseases? *Phytopathologia Mediterranea* 39, 16-20.
- Grosman J. and B. Doublet, 2012. Maladies du bois de la vigne. Synthèse des dispositifs d'observation au vignoble, de l'observatoire 2003–2008 au réseau d'épidémiologie-surveillance actuel. *Phytoma* 651, 31–5.
- Guérin-Dubrana L., J.C. Labrousse, S. Bastien, P. Rey and A. Gegout-Petit. 2012. Statistical analysis of grapevine mortality associated with Esca or *Eutypa* dieback foliar expression. *Phytopathologia Mediterranea* (submitted this Journal issue).
- Hofstetter V., B. Buyck, D. Croll, O. Viret, A. Couloux and K. Gindro, 2012. What if esca disease of grapevine were not a fungal disease? *Fungal Diversity* 54, 51-67.
- Larignon P. and B. Dubos, 1997. Fungi associated with esca disease in grapevine. *European Journal of Plant Pathology* 3, 147-157.
- Larignon P., R. Fulchic, L. Cere, and B. Dubos, 2001. Observation on black dead arm in French vineyards. *Phytopathologia Mediterranea* 40, S336-S342.
- Larignon P., F. Fontaine, S. Farine, C. Clement and C. Bertsch, 2009. Esca and Black Dead Arm: two major actors of grapevine trunk diseases. *Comptes Rendus de l'Académie des Sciences Biologie* 332, 765-783.
- Laveau C., A. Letouze, G. Louvet, S. Bastien and L. Guérin-Dubrana, 2009. Differential aggressiveness of fungi implicated in esca and associated diseases of grapevine in France. *Phytopathologia Mediterranea* 48, 32-46.
- Lecomte P., G. Darrietort, J.M. Liminana, G. Comont, A. Muruamendiaraz, F.J. Legorburu, E. Choueiri, F. Jreijiri, R. El Amil and M. Fermaud, 2012. New insights into esca of grapevine: the development of foliar symptoms and their association with xylem discoloration. *Plant Disease* 96, 924-934.
- Lecomte P., G. Darrietort, C. Laveau, D. Blancard, G. Louvet, J.-P. Goutouly, P. Rey and Guérin-Dubrana L., 2011. Impact of biotic and abiotic factors on the development of esca decline disease. "Integrated Protection and Production in Viticulture" *IOBC/wprs Bulletin* 67, 171-180.

- Lecomte P., M. Leyo, G. Louvet, M.F. Corio-Costet, J.-P. Gaudillere and D. Blancard, 2005. Le Black dead arm, genèse des symptômes - Observations au vignoble en Bordelais et réflexions en lien avec l'esca. *Phytoma* 587, 29-37.
- Lorrain B., I. Ky, G. Pasquier, M. Jourdes, L. Guérin-Dubrana, L. Geny, P. Rey, B. Donéche and P.L. Teissedre, 2012. Effect of Esca disease on the phenolic and sensory attributes of Cabernet Sauvignon grapes, musts and wines. *Australian Journal of Grape and Wine Research* 18, 64-72.
- Maher N., J. Piot, S. Bastien, J. Vallance, P. Rey and L. Guerin-Dubrana, 2012. Wood necrosis in Esca-affected vines: types, relationships and possible links with foliar symptom expression. *Journal International des Sciences de la Vigne et du Vin* 46, 15-27.
- Moller W.S. and A.N. Kasimatis, 1978. Dieback of grapevines caused by *Eutypa armeniacae*. *Plant Disease Reporter* 62, 254-8.
- Mugnai L., A. Graniti and G. Surico, 1999. Esca (black measles) and brown wood streaking: two old and elusive diseases of grapevines. *Plant Disease* 83, 404-17.
- Munkvold G.P. and J.J. Marois, 1995. Factors associated with variation in susceptibility of grapevine pruning wounds to infection by *Eutypa lata*. *Phytopathology* 85, 249-256.
- Panon, M.L. (2000) les maladies du bois... une bombe à retardement ? *Le Vigneron Champenois* 8, 7-9.
- Peros J.P. and G. Berger, 1994. A rapid method to assess the aggressiveness of *Eutypa lata* isolates and the susceptibility of grapevine cultivar to eutypa dieback. *Agronomie* 14, 515-23.
- Peros J.P., G. Berger and I. Jamaux-Despreaux, 2008. Symptoms, wood lesions and fungi associated with esca in organic vineyards in Languedoc-Roussillon (France). *Journal of Phytopathology* 156, 297-303.
- Rego C., A. Vaz, T. Nascimento, A. Cabral and H. Oliveira, 2009. Diseases incited by Botryosphaeriaceae fungi in Portuguese vineyards. *Phytopathologia Mediterranea* 48, 181-181.
- Reisenzein H., N. Berger and Nieder G., 2000. Esca in Austria. *Phytopathologia Mediterranea* 39, 26-34.
- Rumbos I. and A. Rumbou, 2001. Fungi associated with esca and young grapevine decline in Greece. *Phytopathologia Mediterranea* 40, S330-S335.

- Scheck H.J., S.L. Vasquez, S.J. Gubler, W.D., and Fogle, D. (1998) First report of three *Phaeoacremonium* spp. causing young grapevine decline in California. *Plant Disease* 82, 590-590.
- Siebert J. B., 2001. Eutypa: the economic toll on vineyards. *Wines Vines*, April, 50-56.
- Sosnowski M.R., Shtienberg D., Creaser ML, Wicks TJ, Lardner R, Scott ES (2007) The influence of climate on foliar symptoms of eutypa dieback in grapevines. *Phytopathology* 97: 1284-1289.
- Sparapano L., G. Bruno, C. Ciccarone and A. Graniti, 2000. Infection of grapevines by some fungi associated with esca: I. *Fomitiporia punctata* as a wood-rot inducer. *Phytopathologia Mediterranea* 39, 46-52.
- Sparapano L., G. Bruno and A. Graniti, 2001. Three-year observation of grapevines cross-inoculated with esca-associated fungi, *Phytopathologia Mediterranea* 40, 376-386.
- Surico G., G. Marchi, P. Braccini and L. Mugnai, 2000. Epidemiology of esca in some vineyards in Tuscany (Italy). *Phytopathologia Mediterranea* 39, 190-205.
- Surico G., R. Bandinelli, P. Braccini, S. Di Marco, G. Marchi, L. Mugnai and C. Parrini, 2004. On the factors that may have influenced the esca epidemic in Tuscany in the eighties. *Phytopathologia Mediterranea* 43, 136-143.
- Surico G., L. Mugnai and G. Marchi, 2006. Older and more recent observations on esca: a critical review. *Phytopathologia Mediterranea* 45, S68-S86.
- Surico G., L. Mugnai and G. Marchi, 2008. The esca disease complex. In: Ciancio A., Mukerji K.G., eds. *Integrated management of diseases caused by fungi, phytoplasma and bacteria*. Dordrecht, Netherlands: Springer Science Business Media B.V, 119-36.
- Surico G., 2009. Towards a redefinition of the diseases within the esca complex of grapevine. *Phytopathologia Mediterranea* 48, 5-10.
- Trouillas F.P. and W.D. Gubler, 2010. Pathogenicity of Diatrypaceae species in grapevines in California. *Plant Disease* 94, 867-72.
- Trouillas F., J.R. Úrbez-Torres and W.D. Gubler, 2010. Diversity of diatrypaceous fungi associated with grapevine canker diseases in California. *Mycologia* 102, 319-36.
- Úrbez-Torres J.R., G.M. Leavitt, T.M. Voegel and W.D. Gubler, 2006. Identification and distribution of *Botryosphaeria* spp. associated with grapevine cankers in California. *Plant Disease* 90, 1490-1503.

- Úrbez-Torres J.R., G.M. Leavitt, J.C. Guerrero, J. Guevara and W.D. Gubler, 2008. Identification and pathogenicity of *Lasiodiplodia theobromae* and *Diplodia seriata*, the causal agents of bot canker disease of grapevines in Mexico. *Plant Disease* 92, 519-529.
- Úrbez-Torres J.R., P. Adams, J. Kamas and W.D. Gubler, 2009. Identification, incidence, and pathogenicity of fungal species associated with grapevine dieback in Texas. *American Journal of Enology and Viticulture* 60, 497-507.
- Úrbez-Torres J.R., 2011. The status of *Botryosphaeriaceae* species infecting grapevines. *Phytopathologia Mediterranea* 50, 5–45.
- White C.H., F. Halleen and L. Mostert, 2011. Symptoms and fungi associated with esca in South Africa. *Phytopathologia Mediterranea* 50, S236–S246.
- Wicks T. and Davies K., 1999. The effect of Eutypa on grapevine yield. *Australian Grapegrower Winemaker* 426a, 15-16.

Overview of grapevine trunk diseases in France in the early 2000s

EMILIE BRUEZ^{4,3}, PASCAL LECOMTE^{3,4}, JACQUES GROSMAN¹, BRUNO DOUBLET², CHRISTOPHE BERTSCH⁵, FLORENCE FONTAINE⁶, ADELIN UGAGLIA⁷, PIERRE-LOUIS TEISSEBRE⁸, LUCIA GUERIN-DUBRANA^{4,3} and PATRICE REY^{4,3*}

¹ DRAAF-SRAL Rhône-Alpes, 165 rue de Garibaldi BP 3202 69401 Lyon Cedex 3, France

² DRAAF-SRAL Champagne-Ardenne, 37 avenue Hoche – site les Essilards, 51686 Reims cedex 2, France.

³ INRA, ISVV, UMR1065 Santé et Agroécologie du Vignoble (SAVE), F-33140 Villenave d’Ornon, France.

⁴ Université de Bordeaux, ISVV, UMR1065 SAVE, Bordeaux Sciences Agro, F-33140 Villenave d’Ornon, France.

⁵ Université de Haute-Alsace, UFR PEPS, EA-3991 Laboratoire Vigne Biotechnologie et Environnement, 33, rue de Herrlisheim, 68008 Colmar cedex France.

⁶ Université de Reims Champagne Ardenne, UR Vignes et Vins de Champagne, EA 4707, Laboratoire Stress, Défenses et Reproduction des Plantes, 51 687 Reims France.

⁷ Université de Bordeaux, ISVV, UR GAIA^{4,5} Bordeaux Sciences Agro, F-33140 Villenave d’Ornon, France.

⁸ Université de Bordeaux, ISVV, UR Oenologie, F-33140 Villenave d’Ornon, France.

Table 1. French vinegrowing regions, cultivars (age, rootstocks) and vineyards used for the survey done by the National Grapevine Trunk Disease Survey (2003-2008)

| Vine growing regions | Cultivars | Age of cultivars in 2008 | Rootstocks | Number of vineyards monitored for eutypa dieback and esca/BDA | Years | | | | | |
|----------------------|--------------------|--------------------------|--------------------------------------|---|-------|------|------|------|------|------|
| | | | | | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 |
| | Pinot Auxerrois | 27.9 (16-40) | 3309/S04/Teleki8 | 24 | X | X | X | | X | X |
| Alsace | Gewurztraminer | 25.42 (7-58) | 3309/420A/161-49/S04/34EM/Fercal/41B | 24 | X | X | X | | X | X |
| | Riesling | 27.4 (15-55) | 3309/S04/161-49 | 26 | X | X | X | | X | X |
| Bordeaux | Cabernet-Sauvignon | 24.5 (11-47) | 3309/S04/101-14/420A/Riparia | 17 | X | X | X | X | X | X |
| | Merlot | 24.7 (12-47) | 3309/S04/161-14/420A/Riparia | 17 | X | X | X | X | X | X |
| | Sauvignon | 20.1 (7-31) | 3309/S04/101-14/196-17 | 17 | X | X | X | X | X | X |
| Bourgogne | Chardonnay, 21 | 41.9 (21-78) | 3309/S04/161-14/SBB/5C | 28 | X | X | X | X | X | X |

| | | | | | | | | | | |
|-----------------------------------|------------------------|--------------|--|----|---|---|---|---|---|---|
| | Chardonnay, 89 | 25.8 (16-49) | S04/41B/Teleki | 23 | X | X | X | X | X | X |
| | Pinot noir | 42.5 (21-78) | 3309/S04/161-49/SBB | 40 | X | X | X | X | X | X |
| | Sauvignon | 26 (20-38) | 3309/S04/41B | 13 | X | X | X | X | X | X |
| Centre | Sauvignon | 23.4 (16-45) | 3309/S04/Riparia/SBB | 16 | X | X | X | X | | |
| Charentes | Ugni blanc | 26.8 (10-38) | Paulsen/Rupestris/Fercal/RSB/16 1-49/41B/R140 | 27 | X | X | X | X | X | X |
| | Poulsard | 26.7 (15-38) | 3309/S04/101-14 | 25 | | X | X | X | X | X |
| Jura | Trousseau | 22.5 (15-43) | 3309/S04/101-14 | 25 | | X | X | X | X | X |
| | Savagnin | 23.9 (14-51) | 3309/S04 | 23 | | X | X | X | X | X |
| Provence- Alpes Côte d'Azur | Cabernet- Sauvignon | 24.1 (15-29) | S04/R110/Paulsen | 10 | X | X | X | X | X | X |

Table 2a. Percentage of grapevines that were: healthy, affected by grapevine trunk diseases (eutypiose or esca/BDA), dead, missing, replanted, restored in 5 French regions (survey done from 2003 to 2008).

| | Healthy plants | Dead plants | Missing plants | Replanted | Restored plants | Eutypiose affected plants | Esca/BDA affected plants |
|-----------|----------------|-------------|----------------|-----------|-----------------|---------------------------|--------------------------|
| Alsace | 87.23 ±3 | 0.63 ±0.4 | 1.19 ±0.8 | 4.91 ±1.4 | 1.39 ±1.8 | 2.3 ±0,3 | 2.35 ±1.6 |
| Bordeaux | 85.71 ±5.6 | 1.22 ±0.9 | 3.01 ±2.4 | 2.89 ±1.4 | 0.83 ±2.1 | 2.1 ±1.2 | 4.23 ±1.7 |
| Bourgogne | 88.83 ±5 | 1.10 ±0.6 | 2.52 ±2.8 | 4.60 ±8.4 | 0.05 ±0.1 | 1.2 ±0.6 | 1.70 ±0.9 |
| Charentes | 67.40 ±9.3 | 1.67 ±0.5 | 2.83 ±0.5 | 1.21 ±0.9 | 0.91 ±1.8 | 21.2 ±5.9 | 4.78 ±1.8 |
| Jura | 82.21 ±5.5 | 1.02 ±0.9 | 3.04 ±1.1 | 5.01 ±1.8 | 0.22 ±0.4 | 0.3 ±0,2 | 8.20 ±3.0 |

Table 2b. Percentage of grapevines that were: unproductive (dead, missing, replanted, restored are together) and affected by grapevine trunk diseases (eutypiose and esca/BDA are together) in 5 French regions (survey done from 2003 to 2008).

| | Unproductive plants | GTDs affected plants | Total |
|-----------|---------------------|----------------------|-------|
| Alsace | 8.12 | 4.65 | 12.77 |
| Bordeaux | 7.95 | 6.33 | 14.28 |
| Bourgogne | 8.27 | 2.9 | 11.17 |
| Charentes | 6.62 | 25.98 | 32.6 |
| Jura | 9.92 | 8.5 | 18.42 |

Table 3. Correlation of the esca/BDA and eutypa dieback by using the contingency table chi-squared test.

| | | Data Chi-2 test | Data table | |
|-----------|--------------------|-----------------|------------|---|
| | Auxerrois | 1,79 | 3,84 | |
| Alsace | Riesling | 0,08 | 3,84 | |
| | Gewurztraminer | 0,21 | 3,84 | |
| | Sauvignon | 4,17 | 3,84 | Link between esca /BDA and eutypa dieback |
| Bordeaux | Merlot | 1,8 | 3,84 | |
| | Cabernet-Sauvignon | 0,003 | 3,84 | |
| | Chardonnay 21 | 0,7 | 3,84 | |
| Bourgogne | Chardonnay 89 | 0,002 | 3,84 | |
| | Pinot | 0,65 | 3,84 | |
| | Sauvignon | 0,04 | 3,84 | |
| Charentes | Ugni blanc | 0,001 | 3,84 | |
| | Poulsard | 3,3 | 3,84 | |
| Jura | Savagnin | 0,13 | 3,84 | |
| | Trousseau | 5,66 | 3,84 | Link between esca/BDA and eutypa dieback |

Table 4. Correlations for eutypa dieback or esca/BDA symptoms between the region and the age of the grapevines, between the cultivars and the year and between the cultivar and the region were calculated by analysis of covariance (ANCOVA). The values in red are significantly different at $\alpha= 0.05$.

P-values for the esca/BDA attack data

| | ANCOVA |
|--------|----------|
| Region | 1,63E-10 |
| Age | 0,7119 |

P-values for the eutypiose attack data

| | ANCOVA |
|--------|----------|
| Region | 4,36E-10 |
| Age | 0,9847 |

P-values for the esca/BDA attack data

| | ANCOVA |
|-----------|----------|
| Cultivars | 2,00E-16 |
| Year | 0,036 |

P-values for the eutypiose attack data

| | ANCOVA |
|-----------|----------|
| Cultivars | 2,00E-16 |
| Year | 0,0016 |

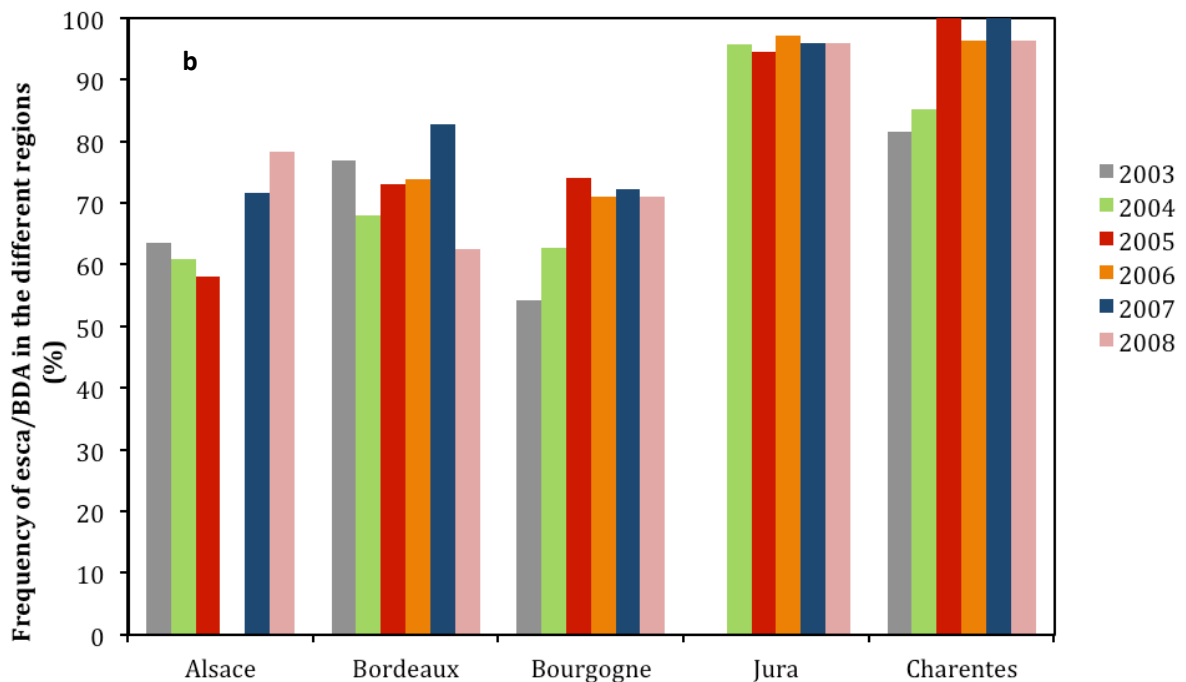
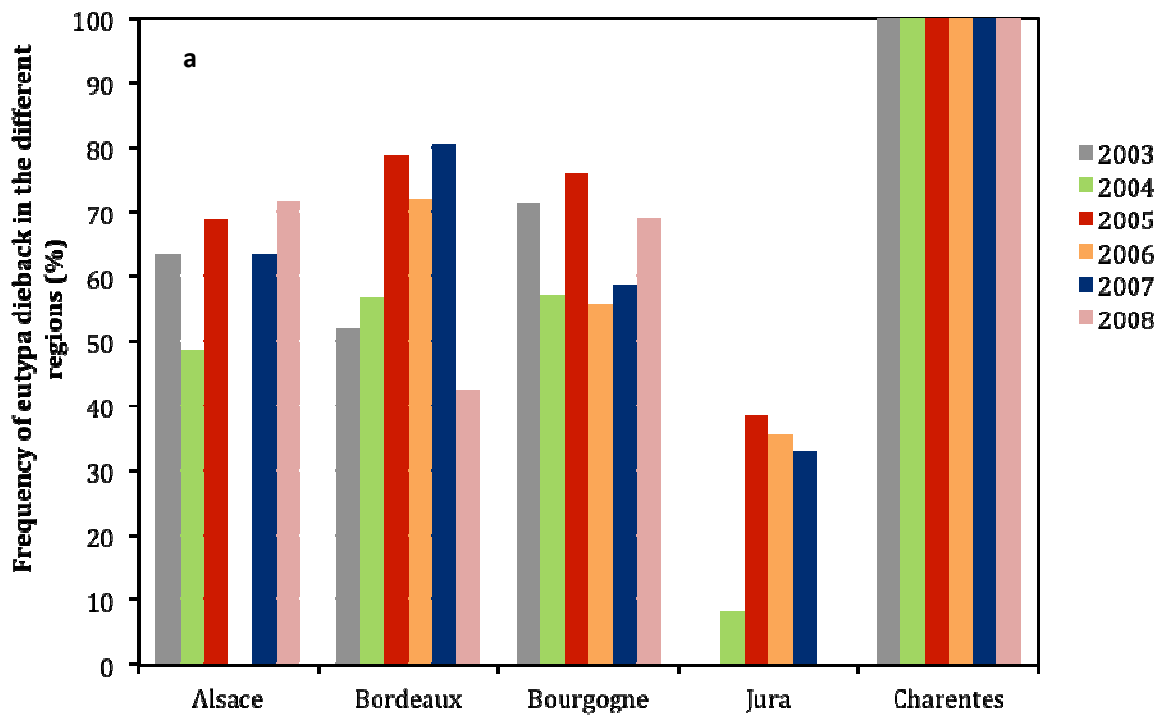


Figure 1. Frequency of eutypa dieback (a) and of esca/BDA (b) in the 5 regions, Alsace, Bordeaux, Bourgogne, Jura and Charentes surveyed over the 6 years period.

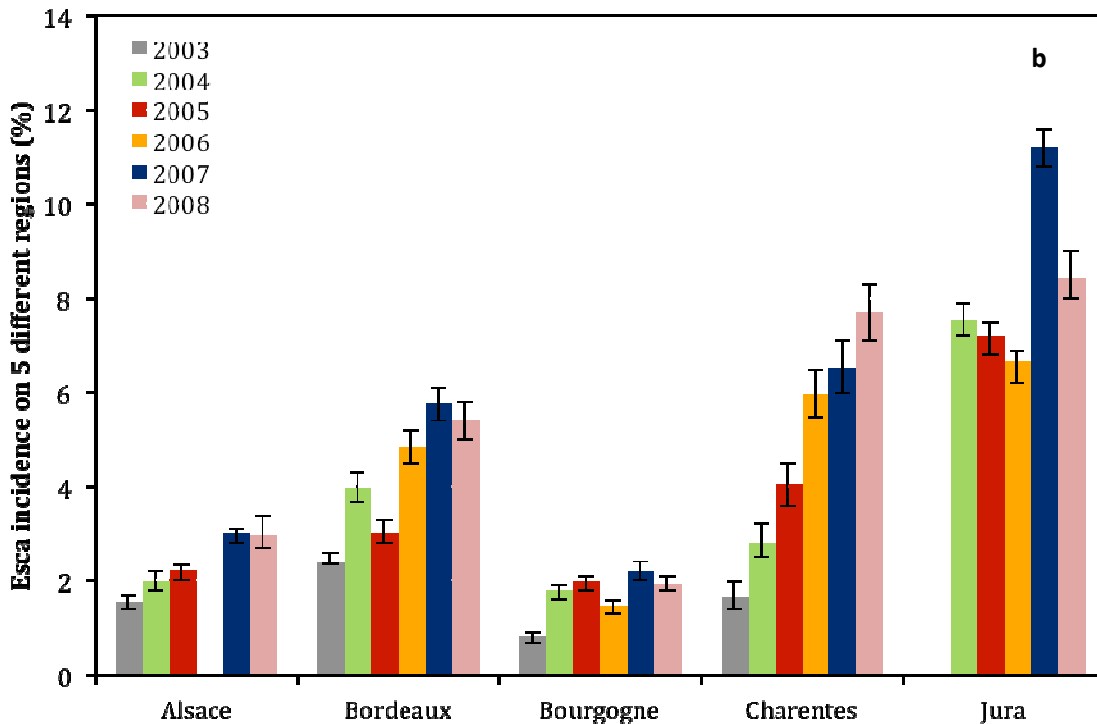
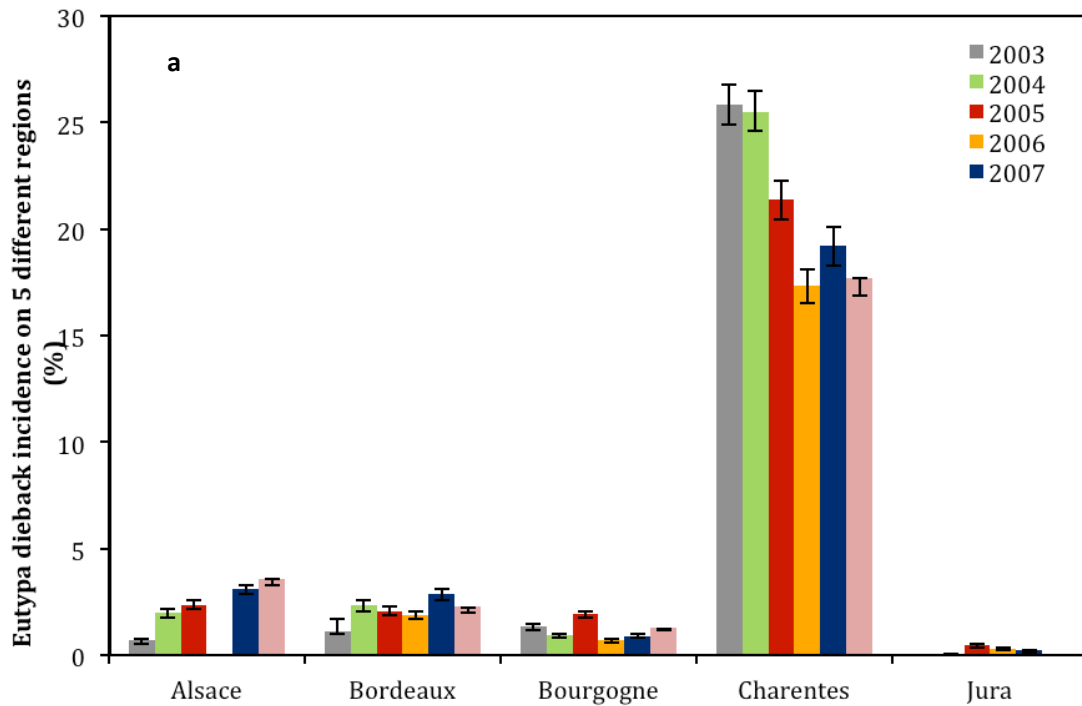


Figure. 2. Eutypa dieback (a) and Esca/BDA (b) incidences in the 5 regions : Alsace, Bordeaux, Bourgogne, Jura and Charentes surveyed over the 6 years period. The confidence intervals have been calculated. Confidence intervals are indicated on the bars.

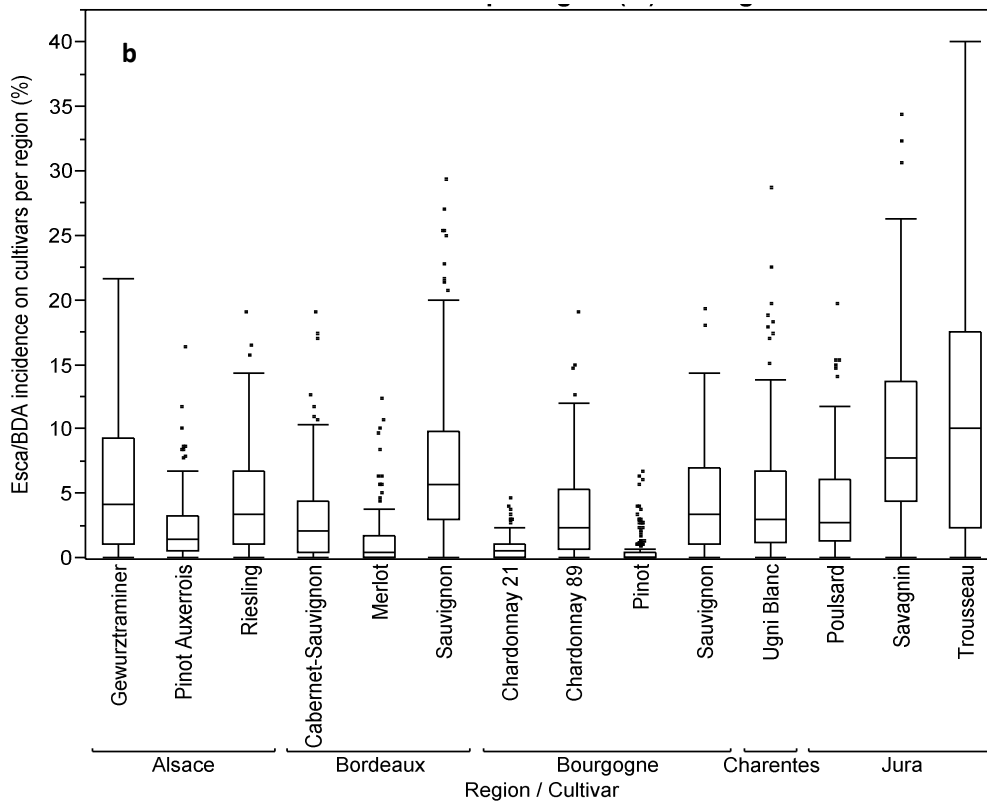
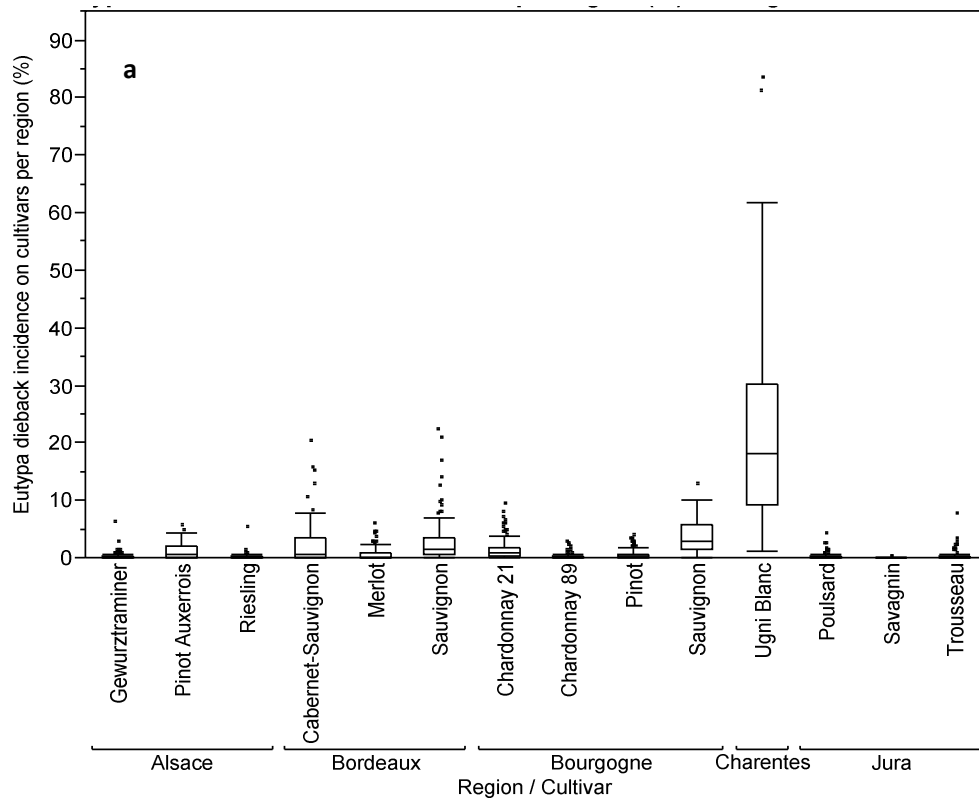


Figure 3. Eutypa dieback (a) and esca/BDA (b) incidences of different cultivars planted in the 5 regions : Alsace, Bordeaux, Bourgogne, Charentes and Jura surveyed over the 6 years period. Kruskal-Wallis test, data are expressed as a box-and-whisker plot showing median, inter-quartile range (IQR) and extreme values.

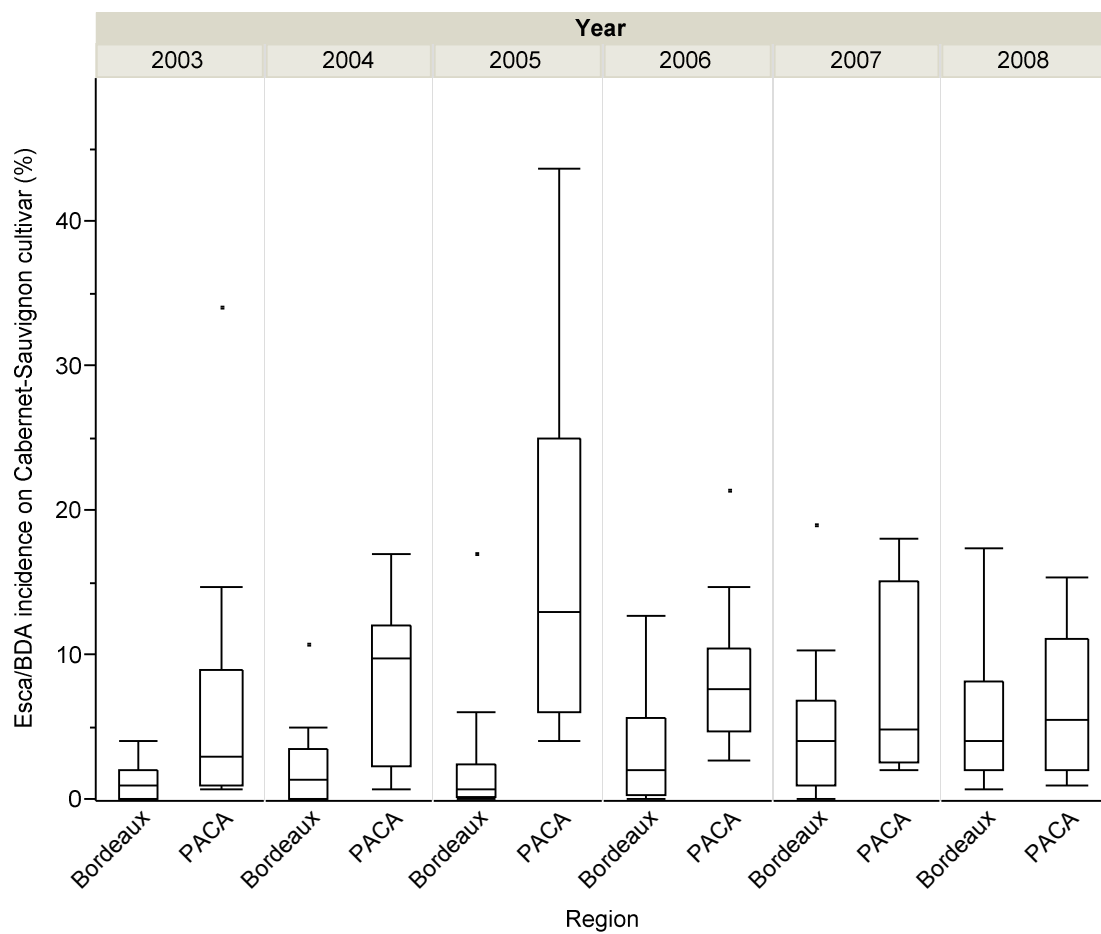


Figure 4. Esca/BDA incidence on Cabernet-Sauvignon cultivar planted in the Bordeaux and Provence-Alpes Côte d'Azur regions (PACA) regions. Kruskal-Wallis test, data are expressed as a box-and-whisker plot showing median, inter-quartile range (IQR) and extreme values.

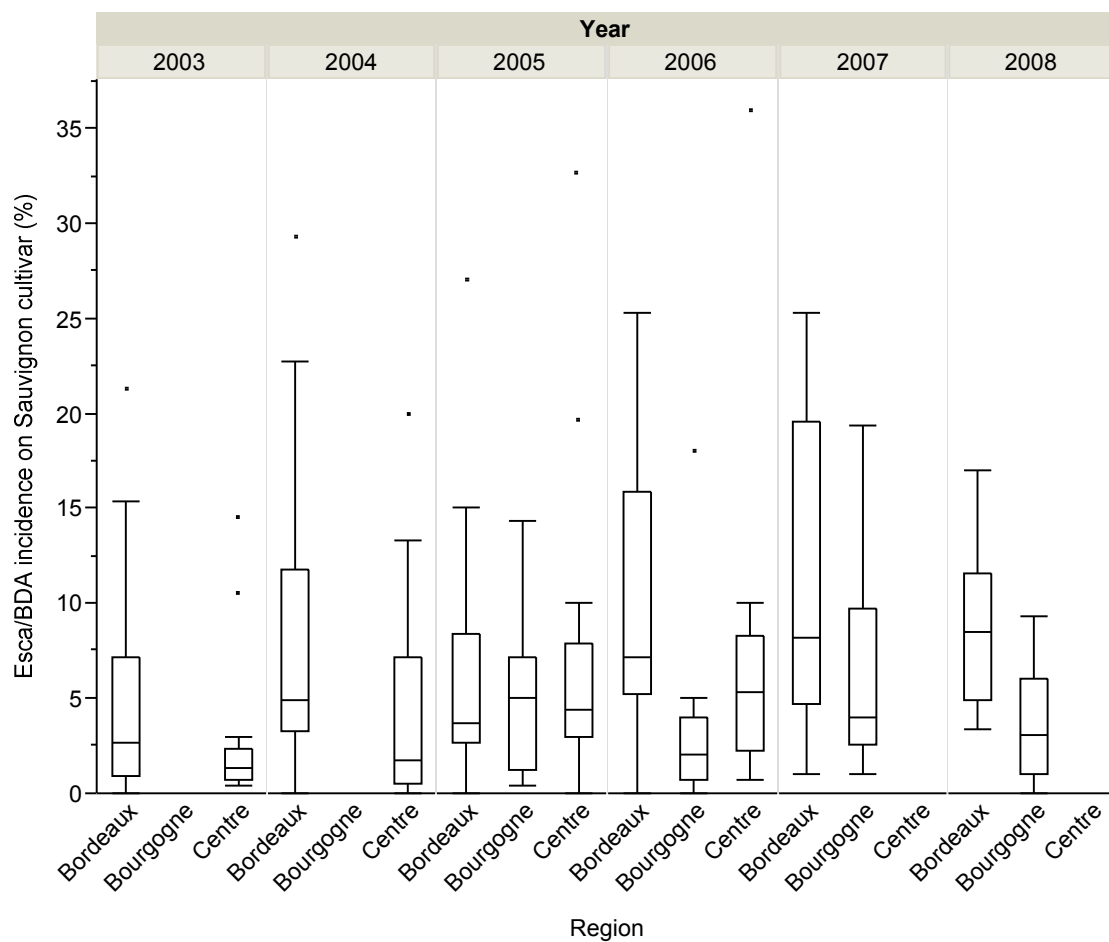


Figure 5. Esca/BDA incidence on Sauvignon cultivar planted in the Bordeaux, Bourgogne and Centre regions. Kruskal-Wallis test, data are expressed as a box-and-whisker plot showing median, inter-quartile range (IQR) and extreme values.

Présentation des objectifs de l'étude :

L'esca de la vigne, à la différence d'autres maladies cryptogamiques, présente une pathologie complexe, associée à une diversité de nécroses, de champignons pathogènes, même si trois d'entre eux sont souvent décrits comme étant les principaux dégradateurs du bois. Cette diversité a conduit à nommer plusieurs maladies selon le stade de la plante, les champignons impliqués, les symptômes observés (Surico, 2009). La difficulté de reproduire les différents symptômes, particulièrement les symptômes foliaires en conditions du vignoble ou du laboratoire induit des spéculations sur les causes réelles de cette maladie et sur l'identité des agents pathogènes. Récemment, Hofstetter *et al.* (2012) ont remis en question l'implication des champignons pathogènes décrits couramment dans la littérature dans le syndrome de l'esca. Jusqu'à présent la microflore fongique associée à l'esca a été étudiée à partir de l'isolement ou de la détection des champignons présents dans les nécroses internes associées ou non aux symptômes foliaires.

La microflore présente dans les tissus du bois non nécrosés, en relation avec les maladies du bois, n'a jamais fait, à notre connaissance, l'objet d'étude spécifique. L'analyse de la microflore par isolement microbiologique a pu parfois être réalisée en utilisant le bois non nécrosé comme « témoin ». Larignon et Dubos (1997) n'isolent aucun champignon de fragments sains alors que Mugnai *et al.* (1999) montrent que 18% de ces fragments (non colorés) sont colonisés par des champignons. Trois espèces majoritaires, bien que peu fréquentes, ont été isolées de ce type de bois : *Botryosphaeria obtusa* 6,4 %, *Phaeoconiella chlamydospora* (Pch) 3% et *Phaeoacremonium aleophilum* (Pal) 1,6 %. Aussi, Maher *et al.* (2012) isolent, de fragments de bois non colorés, le champignon Pch ainsi que des espèces de Botryosphaeriaceae et d'autres champignons plus ou moins fréquemment isolés tels que *Alternaria* spp., *Epicoccum* spp., *Fusarium* spp., *Penicillium* spp., *Trichoderma* spp. Il semble donc que les tissus apparemment sains du bois de vigne puissent être colonisés par des communautés fongiques assez complexes.

Ces informations suggèrent que l'analyse approfondie de cette microflore pourrait nous renseigner sur les premiers stades de colonisation du bois précédant la coloration et la dégradation des tissus. Plusieurs questions se posent : les différents champignons pathogènes impliqués dans ces maladies du bois, décrits dans la partie introductive, sont-ils présents dans les tissus apparemment sains. Quelle en serait la proportion comparée aux populations non pathogènes ? Cette proportion de pathogènes varie-t-elle selon la localisation des tissus ? Les tissus non colorés des bras à proximité des plaies de taille, portes d'entrées des champignons, sont-ils colonisés de la même façon que les autres tissus ? La proportion de champignons potentiellement pathogènes est-elle la même dans les différentes parties des plants (tronc ou porte-greffe) ? Les réponses à ces questions pourraient nous renseigner sur l'origine des champignons pathogènes associés à l'esca de la vigne et sur l'importance du comportement endophytique de certains champignons pathogènes comme Pch, Pal et des espèces de Botryosphaeriaceae. Enfin, l'environnement microbien influence-t-il le changement de statut d'endophyte à parasite ?

Aussi, l'objectif de ce travail de thèse a pour but de **caractériser la structure des communautés microbiennes fongiques associées aux tissus non colorés du bois**. Les tissus non colorés ne présentent pas de nécroses visibles. Cette caractérisation permettra de mieux appréhender la diversité spécifique et l'organisation fonctionnelle des communautés fongiques du bois de vigne. Au-delà de la connaissance de la fraction des champignons connus pour être pathogènes, cette étude permettra d'identifier les espèces fongiques présentes dans le bois pouvant avoir un caractère bénéfique pour la plante, soit en tant qu'antagonistes d'agents pathogènes, soit en développant des interactions mutualistes avec la plante ou en induisant des réactions de défenses chez la plante. La caractérisation de ce type de micro-organisme pourra conduire à des applications pratiques en lutte biologique par exemple.

Nous analyserons également dans ce travail de thèse, la microflore globale bactérienne, celle-ci étant peu caractérisée au niveau du tissu du bois chez la vigne et chez d'autres plantes ligneuses. Pourtant, d'après Clausen (1996), certaines bactéries présentent dans les tissus ligneux ont une capacité à dégrader le bois. Elles pourraient être les premières à intervenir dans le processus de dégradation du bois. Greaves (1971) a ainsi défini 4 types de bactéries qui vivent dans le bois : (i) celles qui affectent la perméabilité et la solidité du bois, (ii) celles qui dégradent les parois cellulaires du bois, (iii) celles qui s'allient avec des champignons pour dégrader le bois et (iv) celles qui sont saprophytes. Les genres *Bacillus* (particulièrement *B. subtilis*) et *Pseudomonas* (particulièrement *P. fluorescens*) appartiennent à la catégorie de bactéries qui altèrent le bois. D'autres espèces de *Pseudomonas* (*P. putida*) peuvent dégrader la lignine, l'un des composants principaux des parois cellulaires (Bugg *et al.*, 2011).

Dans le cadre de cette thèse, **les communautés bactériennes seront analysées globalement et comparées selon le type d'échantillon** : bois provenant de ceps ayant présentés ou non des symptômes foliaires et de différentes localisations dans le cep. **Les bactéries des différents types de tissus seront identifiées pour déterminer celles qui pourraient être impliquées dans le processus de dégradation du bois.**

Afin de répondre à nos objectifs, nous analyserons des échantillons de bois provenant de ceps de vigne d'âges différents: la première catégorie de ceps de vigne, cv. Cabernet Sauvignon, est âgé de 10 ans. Les tissus du bois de plantes qui ont exprimé des symptômes foliaires d'esca au moins 2 fois durant une période de 4 années seront analysés et comparés à ceux des plants « témoins » n'ayant jamais exprimé ces symptômes. Des ceps seront collectés chaque saison durant une année afin de prendre en compte l'effet du stade physiologique de la plante et de l'environnement sur les structures des communautés microbiennes du bois. La deuxième catégorie des ceps comprend des vignes âgées de 42 et 58 ans. Ces ceps âgés ont été étudiés afin d'évaluer les modifications des communautés fongiques en fonction de l'âge de la plante. Pour cette étude des plants non greffés d'un même cépage (le Baco blanc) provenant d'une même parcelle ont été étudiés.

Dans le but de caractériser les communautés fongiques et bactériennes et de répondre aux questions posées, plusieurs approches techniques complémentaires seront mises en œuvre et un échantillonnage adapté sera réalisé. Nous utiliserons tout d'abord une méthode dite d'empreinte moléculaire, la Single Strand Conformation Polymorphism (SSCP) visant à obtenir une « image »

globale de la diversité des communautés. Après extraction des ADNs totaux des échantillons de bois et amplification par PCR (Polymerase Chain Reaction), la technique SSCP permet d'obtenir une empreinte moléculaire des écosystèmes microbiens en séparant les molécules d'ADNr fongiques ou bactériens (selon les amorces utilisées) en fonction de leur séquence (King *et al.*, 2005). Une communauté microbienne est alors représentée sous la forme d'un profil. La complexité du profil obtenu révèle la complexité des communautés. Les profils obtenus pour chaque échantillon de tissus ligneux seront comparés entre eux. Cette analyse globale sera complétée par l'étude de la microflore cultivable par des méthodes plus classiques basées sur des isollements sur milieu de culture spécifique des champignons ou des bactéries. L'identification des taxons présents permettra d'apporter des éléments pour comprendre le fonctionnement des communautés microbiennes au sein du bois. En outre, cette méthode permettra de conserver des isolats d'intérêt pour des tests biologiques ultérieurs. Après purification des colonies, la technique de séquençage de Sanger sera appliquée afin d'identifier les taxons.

Par cette dernière technique, les micro-organismes non cultivables ne peuvent pas être identifiés. Afin de compléter la description exhaustive des communautés microbiennes, une technique de métagénomique : le pyroséquençage sera utilisée. Cette technique permet d'effectuer un séquençage rapide des ADNs présents dans l'échantillon. Elle ne nécessite pas de clonage et permet une lecture directe de la séquence obtenue après le séquençage.

Le plan de la thèse est ainsi structuré en 3 parties :

Partie 1 : Caractérisation de la microflore fongique des tissus du bois non colorés de ceps ayant ou non exprimé des symptômes foliaires d'esca

Le chapitre se compose d'une introduction méthodologique et de deux publications scientifiques qui présenteront une synthèse des travaux caractérisant la microflore fongique des tissus du bois non altéré de la vigne en utilisant les méthodes moléculaires d'empreinte moléculaire et pasteurienne.

L'objectif de cette partie est de caractériser les communautés fongiques du bois « sain » de plants asymptomatiques et symptomatiques âgés de 10 ans selon la saison : 4 périodes de prélèvements sont réalisés : hiver, printemps, été et automne. Les tissus sont prélevés dans plusieurs parties du cep : porte-greffe, tronc (extérieur et intérieur), bras. La deuxième publication rapporte les résultats d'une étude menée sur la caractérisation des microflores fongiques de ceps âgés de 42 et 58 ans d'un cépage hybride franc de pied appelé le Baco blanc. L'objectif de cette étude est de comparer les microflores des tissus des plants âgés. Ces plants ont été déracinés à partir d'une même parcelle. Le fait d'étudier un cépage hybride, permettrait aussi d'apprécier le changement des communautés de celles d'un cépage greffé, le cv. Cabernet Sauvignon.

Partie 2 : Diversité des communautés et des taxons bactériens associés au tissu ligneux de plants de vigne ayant exprimé ou non les symptômes d'esca

Le chapitre est composé d'une publication relatant les études de la microflore bactérienne des plants de vigne âgés de 10 ans utilisés dans le chapitre 1. L'objectif de ce chapitre est de

caractériser la microflore bactérienne présente dans différents tissus ligneux et de la comparer en fonction des ceps qui ont ou non exprimé les symptômes foliaires de l'esca.

L'analyse globale des communautés bactériennes par la méthode de SSCP a été réalisée sur les mêmes échantillons de bois, prélevés à différentes périodes de l'année et à différents endroits du cep. La caractérisation des différents taxons bactériens a été réalisée via la méthode pasteurienne par isolement sur milieu spécifique et par l'utilisation des plaques Biolog Ecoplate™ sur des plantes prélevés en 2012. Ces plaques permettent de déterminer la capacité de métabolisation d'un mélange de bactéries ou d'une bactérie spécifique dégradant 31 composés carbonés différents. Des informations sur la fonction de ces communautés sont ainsi obtenues.

Partie 3 : Identification des taxons fongiques et bactériens du bois de ceps de vigne par la méthode de pyroséquençage

L'objectif de cette partie est d'identifier de façon exhaustive les différents taxons bactériens et fongiques, dans les différents tissus ligneux préalablement étudiés par les autres approches. En effet, les micro-organismes isolés par la méthode pasteurienne ne sont pas représentatifs de toute la microflore présente dans l'échantillon étudié. Le pyroséquençage permet de déterminer l'ensemble des genres ou espèces de bactéries et de champignons, y compris ceux qui ne sont pas cultivables.

Ce chapitre utilise les mêmes ADNs extraits des échantillons des jeunes plants de Cabernet-Sauvignon de 10 ans qui ont été prélevés à différentes périodes. Cette méthode de métagénomique a été utilisée en complément de la méthode pasteurienne, de la méthode de Sanger et de la méthode d'empreinte moléculaire. Le pyroséquençage permet d'obtenir un grand nombre de séquences d'ADN qui peuvent être analysées afin de définir les genres (ou espèces) présents dans l'échantillon.

La discussion-conclusion permettra de synthétiser les résultats obtenus des différentes parties exposées et de mettre en avant les réponses aux questions posées : Existe-t-il des différences de microflore entre des plants symptomatiques et asymptomatiques ? Les communautés fongiques et bactériennes sont-elles spécifiques à chaque type de tissu ou à chaque partie ? Comment évoluent-elles en fonction de la physiologie de la plante ? L'ensemble des résultats sera discuté à la lumière des travaux scientifiques existant sur le sujet et des perspectives tant appliquées que fondamentales seront présentées.

Chapitre 1

Caractérisation de la microflore fongique
des tissus du bois non colorés de ceps
ayant exprimé ou non des symptômes
foliaires d'esca

Introduction

La microflore fongique globale des tissus du bois de vigne a été peu étudiée jusqu'à présent. Cependant, quelques études récentes ont porté sur la caractérisation des champignons cultivables colonisant les organes ligneux. Casieri *et al.*, (2009) ont comparé la microflore endophyte de plants greffés d'un an et ont identifié 66 taxons différents. En lien avec l'esca de la vigne, Hofstetter *et al.* (2012) ont caractérisé la microflore fongique cultivable des tissus ligneux de jeunes plants et de plantes adultes (15-30 ans) ayant exprimé ou non l'esca de la vigne. Dans cette étude, les tissus nécrosés n'ont pas été différenciés des tissus non colorés. Globalement, ces études montrent une microflore abondante et diversifiée isolée du bois de vigne, les Ascomycètes étant les taxons les plus fréquemment identifiés. Plusieurs espèces potentiellement protectrices des végétaux ou pathogènes ont été obtenues. Pour les espèces ou genre potentiellement protecteurs, les plus communs étaient *Bionectria ochroleuca* (Casieri *et al.*, 2009), *Trichoderma sp.* et *Epicoccum nigrum* (Casieri *et al.*, 2009; Hofstetter *et al.*, 2012). Dans ces mêmes études, des espèces potentiellement pathogènes ont aussi été isolées des tissus du bois de vigne, comme les *Botryosphaeria spp.* et *Aureobasidium pullulans*.

A la différence de ces études, le travail présenté dans ce chapitre vise à caractériser la microflore fongique globale des tissus non nécrosés des parties ligneuses et pérennes de plants de vigne d'âges différents. Il s'agira, chez les jeunes ceps (10 ans) ayant très peu de nécroses dans le bois, de comparer les assemblages fongiques présents chez des plants ayant ou non manifesté des symptômes d'esca. Les maladies du bois de la vigne se manifestant souvent chez des plants ayant entre 15 et 30 ans d'âge, les phénomènes de dépérissement étant plus rares chez les vignes âgées, nous avons prélevé des ceps âgés (42 et 58 ans) afin de déterminer les communautés de champignons présentes dans ces plants. Il s'agissait dans le premier cas (ceps de 10 ans d'âge) de savoir si les champignons pathogènes de l'esca étaient déjà présents dans le bois non nécrosé des ceps, pour la deuxième étude (ceps 42 et 58 ans d'âge) d'apporter des éléments permettant de comprendre pourquoi ces plants n'ont pas développé une maladie du bois de la vigne.

Afin d'étudier les microflores fongiques, deux méthodes complémentaires ont été utilisées: (i) une technique de microbiologie classique basée sur des isolements, accompagnée de la méthode de séquençage (ADN-ITS) de Sanger qui permettra d'identifier les espèces isolées. (ii) Une méthode d'empreinte moléculaire, la Single Strand Conformation Polymorphism (SSCP) servira pour analyser de manière globale les communautés microbiennes présentes dans les échantillons de bois prélevés.

Description de la technique d'empreinte moléculaire, la SSCP

La SSCP permet d'étudier de manière globale et rapide les communautés microbiennes présentes dans les échantillons de bois prélevés. Contrairement aux autres méthodes d'empreinte moléculaires, *i.e.* la T-RFLP, l'ARISA et la D/TGGE, la SSCP sépare les simples brins des

amplicons selon leur conformation tridimensionnelle différentielle. La migration électrophorétique des empreintes moléculaires est réalisée dans un capillaire présent dans le séquenceur. Lors de la réaction de PCR, une des deux amorces utilisées est marquée par un fluorochrome, ce qui aboutit au marquage d'un seul brin. Les amplicons obtenus sont dénaturés à 95°C durant 5 min. L'échantillon est ensuite mis dans de la glace afin que les brins se replient et prennent alors une forme qui est propre à chaque fragment d'ADN. La conformation est différente selon la taille de l'amplicon et la composition de l'amplicon. Les échantillons sont ensuite séparés en électrophorèse capillaire non dénaturante. Le laser détecte la fluorescence et va acquérir cette information sous la forme d'un chromatogramme.

Afin de pouvoir aligner les chromatogrammes ensemble, il faut marquer chaque échantillon à l'aide d'un étalon interne, puis utiliser le même étalon pour tous les échantillons. Un marqueur de taille appelé ROX, qui comprend un fluorochrome, est composé de 11 brins d'ADNs, est utilisé. Lors de la migration des échantillons, les ADNs présents dans le ROX vont produire des pics à intervalle régulier.

Afin d'aligner les profils, le logiciel StatFingerprints de R est utilisé ainsi que le logiciel Rcommander de R pour réaliser les ACP (Analyses en Composantes Principales). Les dossiers générés par StatFingerprints sont corrigés sous Excel.

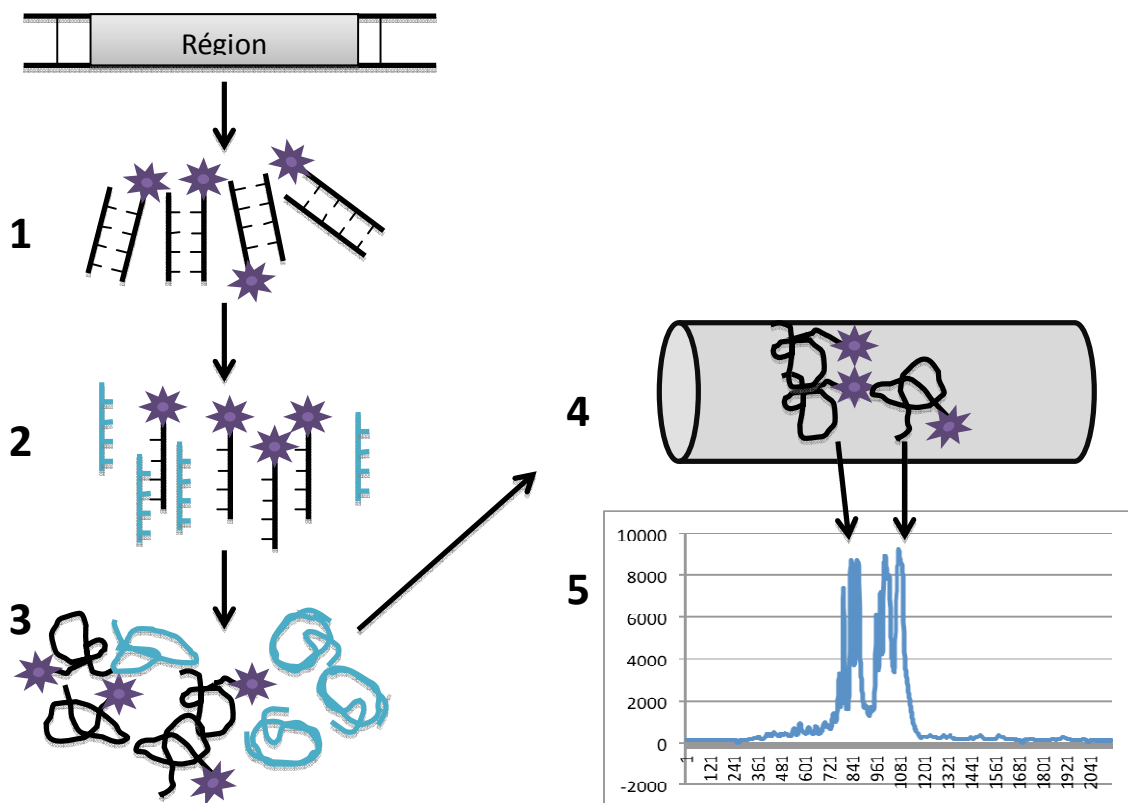


Figure 1. Principe de la SSCP

- (1) Amplicons de la PCR ayant été générés en utilisant un couple d'amorces dont une était marquée.
- (2) Dénaturation des amplicons 5 min à 95°C.
- (3) Chaque brin dénaturé est refroidi à 0°C, ce qui amène à une conformation maintenue par la présence de formamide.
- (4) Les brins passent dans le séquenceur en électrophorèse capillaire et selon leur taille et leur poids, ils seront plus ou moins vite élués.
- (5) Chromatogramme obtenu à partir des résultats donnés par le séquenceur.

Description de la technique pasteurienne d'isolement des champignons

Les approches pasteuriennes ont longtemps été les seules disponibles pour étudier les communautés microbiennes. Des échantillons environnementaux provenant de feuilles, de racines ou de bois sont désinfectés, préparés puisensemencés sur des milieux synthétiques solides sur lesquels se développent ou non les micro-organismes recherchés, *e.g.* des bactéries, des champignons ou des levures. Après incubation durant 24H, 48H, ou plus, à des températures bien définies, les micro-organismes sont isolés afin d'obtenir des cultures pures. Ils sont ensuite, lorsque c'est possible, identifiés selon des critères morphologiques. Bien que ces méthodes aient été indispensables pour l'étude et la compréhension d'organismes spécifiques, elles sont aujourd'hui difficilement applicables pour l'analyse et la caractérisation des communautés microbiennes, puisque seule une fraction de ces organismes est cultivée. En effet, il a été estimé que seul 1% des procaryotes de la plupart des environnements peuvent être cultivés (Amann *et al.*, 1995). Plusieurs explications peuvent être données : (i) les milieux synthétiques utilisés ne conviennent pas à tous les micro-organismes, (ii) les températures d'incubation ne sont pas appropriées pour la croissance des agents microbiens, (iii) une compétition peut avoir lieu entre les êtres vivants présents dans un échantillon biologique, (iv) l'état physiologique des agents microbiens, ils peuvent en effet être sous une forme conservée ou viable mais non cultivable (Garbeva *et al.*, 2004; Kirk *et al.*, 2004; Mazzola, 2004; Nocker *et al.*, 2007; Prosser, 2002). Bien que les méthodes culturales n'apportent pas toutes les informations, elles restent nécessaires et ont un intérêt indéniable. En effet, les souches identifiées et isolées peuvent être utilisées en tant que pathogène ou protecteur sur un modèle végétal bien précis. Ces méthodes sont donc complémentaires des méthodes biochimiques ou moléculaires. Elles sont aussi en développement constant comme le montre les nouvelles plaques Biolog® spécifiques pour l'analyse de micro-organismes purifiés ou d'échantillons environnementaux. Les plaques Biolog Ecoplates™ ont par exemple été développées afin de caractériser les communautés bactériennes en fonction de leur capacité à métaboliser des substrats carbonés (O'Donnell *et al.*, 2001; Ross *et al.*, 2001).

Dans le cas présent après isolement des micro-organismes, une technique de séquençage (méthode de Sanger) a été utilisée pour identifier les champignons.

Description de la méthode de séquençage (Sanger) permettant d'identifier les champignons

Le principe de la méthode de Sanger est de recopier un brin matrice par une ADN polymérase, qui est ADN dépendante. La fixation d'un oligonucléotide spécifique (amorce), complémentaire du brin matrice permet à l'ADN polymérase l'élongation d'un nouveau brin complémentaire du brin matrice dans le sens 5'-3'. L'ADN polymérase qui est un complexe enzymatique, utilise les désoxyribonucléotides libres pour recréer un nouveau brin, en formant un pont phosphodiester entre le 3'OH de la chaîne et le 5' phosphate du dNTP suivant. La réaction de Sanger repose sur l'incorporation aléatoire par cette ADN polymérase de didésoxyribonucléotides interrupteurs de chaîne (ddNTP) eux aussi présents dans le milieu réactionnel. Ces ddNTP diffèrent des dNTP par leur extrémité 3'. L'extrémité 3'OH des dNTP est remplacée par une extrémité 3'H. Cette modification empêche la formation de la liaison phosphodiester entre le ddNTP incorporé dans la chaîne et le nucléotide suivant. L'allongement de la chaîne est alors interrompu.

Dans le milieu réactionnel il y a compétition entre les dNTP et les ddNTP. Le rapport spécifique ddNTP/dNTP et l'affinité de la Taq pour chaque nucléotide sont optimisés de telle façon qu'un ddNTP soit statistiquement incorporé à toutes les positions possibles. Une migration électrophorétique du produit de cette réaction de séquence sur un gel très résolutif (polyacrylamide) va séparer tous les fragments présents en fonction de leur masse moléculaire (taille). Les plus petits fragments vont migrer plus rapidement que les grands. La grande résolution de ce gel permet de distinguer des fragments différents entre eux d'une paire de base. L'identification du ddNTP présent à l'extrémité 3' de chaque fragment déterminera la séquence nucléotidique du brin matrice initial.

De nos jours cette méthode est automatisée et ce sont des séquenceurs automatiques qui réalisent les réactions de la séquence et qui la lisent. Aussi, un traceur fluorescent est attaché à l'extrémité de l'amorce de polymérisation et permet de détecter les fragments d'ADN synthétisés. La détection des fragments ainsi synthétisés se fait en incorporant un traceur dans l'ADN synthétisé qui est attaché soit à l'oligonucléotide, soit au didésoxyribonucléotide. Une fois la réaction de séquence terminée, la taille des fragments obtenus est déterminée par une chromatographie. Le séquenceur détecte la fluorescence sortant des colonnes de chromatographie, repérant ainsi les fragments d'ADN et leur taille précise.

Une fois les séquences éditées par l'appareil, une analyse est réalisée en utilisant le logiciel Codon Code. Cette analyse via Codon Code permet d'éliminer les séquences qui n'ont pas obtenu une valeur seuil selon la qualité du chromatogramme ou la longueur de la séquence.

Une fois les champignons isolés, certains ont été identifiés par séquençage de la grande sous unité de l'ADN ribosomal, nommée ADNr 28S. D'autres zones peuvent être étudiées comme la région 5,8S ou 18S. Cette région nucléique codant pour l'ADNr 28S possède le plus grand domaine variable, elle est aussi la plus longue (3300 pb).

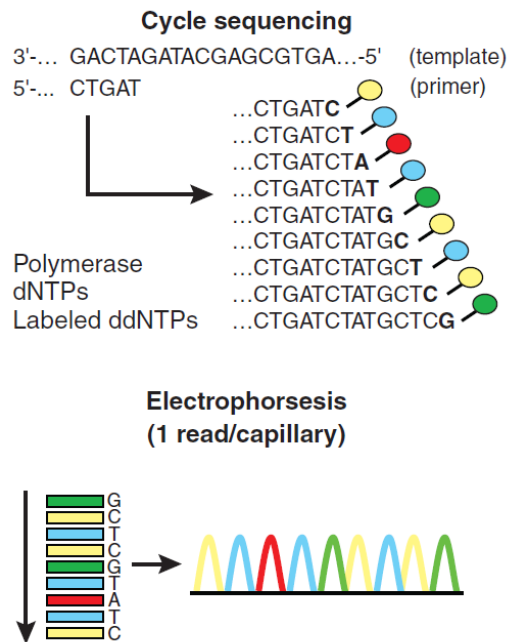


Figure 2. Principe du séquençage Sanger (Shendure and Ji, 2008)

Dans ce chapitre, la publication n°2 intitulée « **The complex relationship of fungi with esca. Analyses of the fungal communities that colonize the wood tissues of leaf-symptomatic and asymptomatic vines** » se rapporte au travail effectué sur des jeunes plants de Cabernet-Sauvignon âgés de 10 ans. Cette publication est soumise à la revue *Fungal Diversity*.

La publication n° 3 intitulée « **Old *Vitis vinifera*: when pathogenic fungal patterns of grapevine trunk diseases are not always associated with foliar disease expression or plant decline** » utilise les ceps âgés de 42 et de 58 ans. Cette publication est soumise à la revue *Phytopathology*.

Publication n°2

“The complex relationship of fungi with esca. Analyses of the fungal communities that colonize the wood tissues of leaf-symptomatic and asymptomatic vines”

Fungal Diversity (Submitted)

The complex relationship of fungi with esca. Analyses of the fungal communities that colonize the wood tissues of leaf-symptomatic and asymptomatic vines

Emilie Bruez,^{1,2} Jessica Vallance,^{1,2} Jonathan Gerbore,^{3,2,1} Pascal Lecomte,^{2,1} Lucia Guerin-Dubrana^{1,2} and Patrice Rey^{1,2*}

¹ Université de Bordeaux, ISVV, UMR1065 Santé et Agroécologie du Vignoble (SAVE), Bordeaux Sciences Agro, F-33140, Villenave d'Ornon, France

² INRA, ISVV, UMR1065 SAVE, F-33140, Villenave d'Ornon, France

³ BIOVITIS, 15400 Saint Etienne de Chomeil, France

*Correspondant author : prey@bordeaux.inra.fr

Abstract

The grapevine trunk disease, esca, is of major concern for viticulture worldwide. Our experiment aimed at comparing the fungal microflora that colonize the wood of two types of grapevines: esca-foliar symptomatic and asymptomatic plants. A fingerprint method, Single Strand Conformation Polymorphism and ITS-DNA sequencing of fungi were used. Depending on the wood samples, opposite conclusions can be drawn. In the cordons, necroses from the esca-foliar symptomatic and the asymptomatic grapevines differed, and their fungal microflora, too. However, when the fungi colonizing the trunks and rootstocks were compared, no significant differences were observed. The wood also looked healthy in the trunks and rootstocks of symptomatic and asymptomatic plants. Diverse fungal assemblages made up of potentially pathogenic and numerous plant protective/antagonistic fungi colonized these healthy wood tissues. Shifts occurred in this global fungal microflora over a year, but wood necroses did not develop during that period. The present study show that, depending on the particular parts of the plant being sampled, specific mycoflora can be associated or not with esca. So, in order to make meaningful comparisons, studying the mycoflora responsible for grapevine esca must take into account the whole pathological status of the wood of plants.

Introduction

Grapevine trunk diseases that affect vineyards are of major concern for the viticulture industry. The three main diseases, eutypa dieback, esca decline and bot cankers, cause a threat to the main vine-growing regions of the southern and northern hemispheres (Mugnai et al. 1999). An increase in the disease incidence over the last 15 years has been reported worldwide (Chiarappa 2000; Graniti et al. 2000; Grosman and Doublet 2012; Bertsch et al. 2012). For instance, the three diseases are widespread in the wine-producing regions of Italy (Romazzini et al. 2006), Spain (Gimenez-Jaime et al. 2006; Armengol et al. 2005), Greece (Rumbos and Rumbou 2001), in the USA (Scheck et al. 1998) and in Australia (Edwards and Pascoa 2004). In France, until the late nineties, besides eutypa dieback, the most common trunk disease was esca but, at the turn of the century, a third, new disease, called black dead arm (BDA), was identified in the French vine-growing areas (Larignon et al. 2001).

In the last decade, the National Grapevine Trunk Diseases Survey was established in France in order to monitor and analyze the importance and progress of the trunk diseases (period 2003 to 2008). It was reported that 50 to 83% of the plots showed symptoms of esca (Grosman and Doublet 2012). For eutypa dieback, the percentages were lower, varying from 30% to 53%. In the same plot, the number of vines showing foliar symptoms of esca was not very high, but this figure increased steadily from 1.04% in 2003 to 3.23% in 2008 (during the same period, eutypa symptoms tended to decrease). However, in order to understand the full complexity of the situation, it is essential to consider five additional points. (i) From one year to another the same vines do not necessary express foliar symptoms, so when we consider a period of several years the number of "esca-foliar symptomatic vines at least once" is far more relevant. (ii) About 10% of vines of some varieties are infected, *e.g.* Sauvignon in Aquitaine. (iii) Approximately 11% of French vineyards are unproductive, mainly because of these three diseases. (iv) Esca moderately affects the phenolic composition of grapes and decreases the sensory quality of wines, suggesting a particularly dramatic increase in the economic importance of esca if no control methods are found (Lorrain et al. 2012). (v) Regarding aetiology, the symptoms that occur in the trunk, leaves and berries have been extensively described, indicating that if eutypa dieback symptoms markedly differ from those of esca and of BDA, differentiating between these two diseases often proves rather elusive, with Lecomte et al. (2012) recently providing evidence that foliar symptoms of esca showed transitory phases which overlapped with some BDA descriptions.

Two forms of the esca disease are generally described: the chronic form, with typical foliar symptoms, *i.e.* "tiger stripes", that are expressed consecutively or not over a few years. Generally, this ends with the death of the vine (Bertsch et al. 2012). The other form, called apoplexy, is more severe, with plants dying within a few days (Larignon et al. 2009). It should be reported that, recently, Lecomte et al. (2012) chose not to separate esca symptoms into chronic or apoplectic forms, but to use a classification based on a gradual scale of severity, starting from

some leaves showing only discolorations up to complete vine wilting. It should be noted also that if “tiger stripes” constitute external foliar symptoms, internal symptoms include various necroses of wood, specifically white-rot (also called amadou), which has been associated with esca for years (Viala 1926; Graniti et al. 2000). Maher et al. (2012) recently demonstrated that chronic foliar expression of esca was associated with several internal necroses which were more extensive than those observed in asymptomatic vines. Within esca-diseased mature vines, the necroses formed a continuum within the plant, with the scion thus being like a single unit with a volume of necroses useful in determining the health status of vines.

Pathogenic fungi are assumed to be responsible for these various necroses; they grow within the wood, decay it and slowly kill the vines. Development of specific necroses, *i.e.* central necrosis, black punctate necrosis, white rot, sectorial necrosis, were associated with the multiplication of a few fungi. For instance, *Fomitiporia mediterranea* is predominant in white rot (Fischer 2002). Other fungi, *Phaeoconiella chlamydospora* and *Phaeoacremonium aleophilum* (Crous et al. 1996) are often isolated together, but *P. chlamydospora* is the most frequent in central necrosis (Mugnai et al. 1999). *Botryosphaeria* spp. is frequent in sectorial necrosis (Larignon et al. 2009; White et al. 2011). Certain reports indicated that these fungi might act simultaneously or in succession (Graniti et al. 2000). For instance, Larignon and Dubos (1997) proposed two processes of wood degradation involving a succession of fungi. The first, leading to the formation of central, light-coloured soft necrosis, included three fungi operating in sequence: *P. aleophilum* and *P. chlamydospora*, followed by *F. mediterranea*. The second leads to the development of light-coloured, soft, sectorial necrosis, primarily caused by *Eutypa lata* (also responsible for another vine wood disease, eutypa dieback), followed by *F. mediterranea*. However, the dynamics of necrotic development in woody tissues remain largely speculative. One of the most puzzling points with these fungi is that, although they can cause wood necrosis when they are inoculated on vines, the foliar symptoms are frequently lacking (Surico et al. 2006). Koch’s postulate is thus not always validated and the involvement of other microorganisms in the process of wood degradation is still the subject of speculation. Recently, the role of fungi in esca has been put into question by Hofstetter et al. (2012). Based on the comparison of the fungal microflora that colonize the wood of esca-foliar symptomatic and asymptomatic vines, they suggested that these fungi are not responsible for the disease.

In that context, the objective of our experiment was to produce complementary data that would help improve our understanding of esca development. We focused on the comparison of the fungal microflora that colonize the wood of both esca-diseased and healthy vines too. However, unlike Hofstetter et al. (2012), we took into account the pathogenic status of the wood tissues as well as the esca-foliar status of the plants. As the early development of necroses in vines is not concomitant with the development of esca-foliar symptoms, necroses can also be observed in asymptomatic vines. Consequently, apparently healthy vines, particularly mature and old vines, may have also wood necroses. So, in our sampling design, the blind method, which consists in drilling the trunks, was replaced by a vine-destructive method. This method consists in cutting the whole plant longitudinally in order to verify the status of the wood, *i.e.* necrotic or healthy. After close examination of the wood tissues, necrotic or not, the fungal microflora of

symptomatic and asymptomatic vines were compared by using a fingerprint method, *i.e.* Single Strand Conformation Polymorphism (SSCP), and DNA-ITS sequencing of the isolated fungi. The interesting point was that the plants were relatively young (10 years old) and had been surveyed for esca-foliar symptoms ever since they were planted. They had relatively fewer necroses than mature plants (15-20 years old and more), so that diverse pathogenic status samples could be compared.

Materials and methods

Plant material and sampling

Experiments were carried out on 10 years old Cabernet Sauvignon vines (*Vitis vinifera*) planted in a sandy-clay soil. The rootstock was 101 14 MG. The sampling site was located at the Luchey-Halde vineyard in Pessac Léognan (Bordeaux, France). Plants that had previously expressed or not the foliar symptoms of esca were selected and uprooted. For the foliar-symptomatic plants, we selected plants that had previously expressed esca foliar symptoms at least two times over a period of 4 years (2005 to 2009). The control plants never expressed the foliar symptoms.

In order to monitor the dynamics of the fungal microflora colonizing the wood tissues of vines, 7 asymptomatic and 7 foliar-symptomatic plants were uprooted every 12 weeks during a period of one year. Plants were sampled 4 times: in the spring (April 2010), in the summer (June 2010), in the autumn (September 2010) and in the winter (January 2011). For each vine, the various parts of the plant were cut longitudinally, *i.e.* cordons, trunks and rootstocks. Subsequently, the woods were used to isolate cultivable fungi and for extraction of the fungal DNA.

DNA extraction and fungal communities analyses

DNA extraction. For each vine collected, 10 g of non-necrotic tissues were sampled in the inner and outer parts of trunks, in the inner part of rootstocks and cordons, and from the trunk and stock barks. When white rot tissue was identified (only in esca-foliar symptomatic vines), 10 g were also collected. All these samples were ground in liquid nitrogen with a one-ball mill of Dangoumau type and kept at -80°C prior to DNA extraction.

DNA was extracted from 60-mg aliquots of woody tissues with the Indivisorb Spin Plant mini Kit (Eurobio, France) according to the manufacturer's instructions. DNA extracts were then quantified with a nanodrop (ND-1000, ThermoScientific, Labtech) and homogenized at a concentration of 10 ng. μl^{-1} .

SSCP analyses. The pair of primers recognizing the mitochondrial large subunit rDNA gene, ML1-ML2 from White et al. (1990), was used for SSCP. DNA was amplified by PCR in an Eppgradient Mastercycler (Eppendorf) in a reaction mixture (25 μl final volume) consisting of 1 μl of DNA template (10 ng. μl^{-1}), 0.2 mM of each dNTP, 1 ng. μl^{-1} of BSA (New England BioLabs), 0.2 μM of each primer, Pfu Turbo buffer 1x and 0.05 unit of Pfu Turbo DNA polymerase (Stratagene/Agilent Technologies). The cycling parameters were 95°C for 2 min followed by 30 cycles at 95°C for 1 min, 56°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. The PCR products (~250 bp) were visualized by 2% TBE agarose gel electrophoresis prior to SSCP analysis.

SSCP analyses were performed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) equipped with four 36-cm long capillaries. One microliter of a PCR product was

mixed with 18.8 μl formamide Hi-Di (Applied Biosystems) and 0.2 μl standard internal DNA molecular weight marker Genescan 400 HD ROX (Applied Biosystems). The sample mixture was denatured at 95°C for 5 min and immediately cooled on ice, and then loaded onto the instrument. The non-denaturing polymer consisted of 5.6% POP conformational analysis polymer (Applied Biosystems), 10% glycerol, EDTA buffer 10x (Applied Biosystems) and water. The migration time was set to 2000 seconds, the voltage to 15 kV and the temperature was 32°C. Samples were co-migrated with the fluorescent size standard (GeneScan-400 ROX) to allow comparison of migration profiles between samples. Patterns were aligned with StatFingerprints (version 2.0) and studied by Principal Component Analysis with R (version 2.11.1).

Fungal isolation and identification

Isolation and morphological identification of fungi. For all the esca foliar-symptomatic and asymptomatic vines collected in 2010 and 2011, plant samples consisted in 15 twigs (around 5 mm in length) of non-necrotic woody tissues from the inner and outer parts of trunks and rootstocks. Fifty six vines were sampled and 3360 pieces of wood were analyzed.

A surface sterilization method was used in order to eliminate the epiphytic fungi. The fragments were immersed in a 5% calcium hypochlorite solution during 30 seconds, washed in sterile distilled water and dried on a sterile filter paper. For each vine part, 15 sterilized twigs were placed on Malt Agar (3 pieces per plate) and incubated at 25°C in the dark. The fungal strains development was monitored over a 4 weeks period. When fungal colonies emerged from wood tissues, mycelial fragments were transferred to new Malt Agar plates. The fungi that did not grow more than 1 cm around the piece of wood over a period of one month were not isolated. Whenever possible, the taxonomic identification of the fungi was based on morphological and cultural features, and on examination of fruiting structures and conidia under the microscope. To identify the species of the most abundant genera and other isolates whose sporulation was not observed, sequencing of the internal transcribed spacer (ITS) region of the rDNA has been done. Note that the isolates were selected randomly.

Identification of fungi by sequencing the DNA ITS region. Fungal genomic DNA was isolated from fresh mycelium scraped at the surface of a Malt Agar plate with a sterile tip. Samples were freeze-dried over night (Alpha 1-4 LO plus, Bioblock Scientific) and then grinded with a little glass ball during 1 min at a frequency of 29.9 s^{-1} . Four hundred μl of CTAB (1x) were added to each sample. After an incubation at 65°C during one hour, 400 μl of chloroform-isoamyl alcohol (24:1, v/v) were added and the samples centrifuged during 30 min at 3700 rpm. The aqueous phase was transferred in a new tube and 200 μl of isopropanol were added. Samples were then kept at -20°C over night for DNA precipitation. After 20 min centrifugation at 3700 rpm, the supernatant was discarded and 500 μl of ethanol 70% were added to wash the DNA. Once the ethanol discarded, pellets were air dried and then resuspended in 50 μl of sterile distilled water.

DNA samples were sent to GATC Biotech AG (Konstanz, Germany) for sequencing of the ITS region with the primers ITS1f and ITS4 (White et al. 1990; Chanderbali et al. 2001)

Sequences were edited using CodonCode Aligner software and assigned to species based on 97% sequence similarity threshold of the ITS region. For species level identification, sequences were subjected to a bulk blastn search against the INSD (International Nucleotide Sequence Databases) as implemented in the PlutoF workbench of the UNITE database (Abarenkov et al. 2010). Four hundreds and eighty seven fungal isolates were identified by DNA sequencing of ITS region. DNA of the fungal isolates were sequenced and analyzed by PlutoF. To determine the species names, the percentage of sequence BLAST was at 99%.

Data analyses

SSCP analyses. Banding patterns were obtained from community analysis (SSCP) and all the profiles were aligned by StatFingerprints R software (The R foundation for Statistical computing, ISBN 3-900051-07-0) and they were gathered in a single numerical database before being statistically described by a global Principal Component Analysis (PCA) by R software version 2.14.2. This database consisted of 56 individuals and depending of the PCA, 108 to 208 samples. Each sampling point is calculated by analyzing 250 variables (the 250 variables represented the SSCP profile scans of the sample). PCA was performed on using the correlation coefficient of Pearson. Variables having a $\cos^2 \geq 0.5$ on one of the first third principal components (Dim1, Dim2 or Dim 3) were estimated as sufficiently well represented by the principal plane generated by this PCA.

Species diversity. For this study, we used specific diversity indexes, (i) species richness (total number of species observed), (ii) the abundance (number of isolates), (iii) Shannon's diversity index, H' (Shannon and Weaver 1963, Buckland et al. 2005), (iv) the Simpson index, D and the Evenness, J , obtained by the Shannon's equitability index and the dominance. Evenness indice was estimated to establish the closeness of equitability of species present (Gotelli and Colwell 2001). These indexes were estimated by using package Agricolaea of R software version 2.14.2.

Results

Status of the wood of esca foliar-symptomatic and asymptomatic vines

After longitudinal-sections of vines, necroses were much more important in the wood of cordons of the esca-foliar symptomatic plants than in the asymptomatic ones (Table 1). Specific tissue, the white-rot, was observed only in vines that had previously expressed esca foliar symptoms, 79% symptomatic vines had white rots in their cordons (Fig. 1A,B). Non-necrotic tissues were predominant in the trunks (Fig. 1C,D) and rootstocks (not shown) of the esca foliar-symptomatic and asymptomatic plants. Only a small dark necrosis was observed in the center of the sections, for the two types of plants (Fig. 1C,D).

Global analyses of the fungal communities

From the SSCP patterns analysis, Principal Component Analysis is obtained in order to compare the fungal communities colonizing the different types of tissues: (i) the barks of rootstocks and trunks, (ii) the non-necrotic wood tissues of rootstocks, inner trunks, outer trunks and arms, (iii) the white-rot of the vines that had expressed foliar symptoms (Fig. 2). For that global analysis, symptomatic and asymptomatic vines are pooled for each type of tissue. PCA eigenvalues indicates that the first two principal components, Dim 1 and Dim 2, explain 61.45% of the total data variance. On Dim1, the clusters representing the fungal communities of the bark samples are separated from all the other samples, respectively negative versus positive coordinates. The clusters of non necrotic tissues, *i.e.* the healthy wood of cordons, the inner trunks, the outer trunks and the rootstocks, are not separated by Dim2 but they are separated from those colonizing the barks and white rot, negative versus positive coordinates. Note that on Dim 1, the clusters of the barks are separated from the one of the white rot, negative versus positive coordinates

On Figure 3A, clusters representing the fungal communities colonizing the non-necrotic tissues (inner trunks, outer trunks, rootstocks and arms) for all the sample times (April, June, September, January) of symptomatic and asymptomatic plants are pooled. PCA eigenvalues indicates that the first two principal components, Dim 1 and Dim 2, explain 78.13% of the total data variance. The clusters of two types of plants tend to be similar because the ellipses are not clearly separated (Fig. 3A). For each sampling time, *i.e.* April, June, September and January, the clusters of symptomatic plants and asymptomatic plants are not separated and the eigenvalues indicate that for the first two principal components, Dim 1 and Dim 2, more than 70% of the data variance are explained (data not shown).

Figure 3B shows the clusters representing each of the fungal communities colonizing the wood samples taken in April, June, September and January of symptomatic and asymptomatic plants. PCA indicates that the highest two ranking components accounted for 78.13% of the variance, with the first component accounting for 68.55%. On the first axis, the cluster represents the fungal communities sampled in January is clearly separated from the communities of the

three other sampling times (April, June and September) by Dim1, positive versus negative coordinates. Ellipses obtain from the SSCP-profiles of samples from June and September display an important overlap. They are separated from the two others ellipses, *i.e.* April and December, by Dim2, positive versus negative coordinates.

Isolation and identification of fungi

In this study, we used a culture-based method to isolate the fungi associated with the non-necrotic tissues of the plants that had expressed or not esca-foliar symptoms.

From the 56 vines sampled, 3360 pieces of wood were analyzed. 574 fungal strains were isolated from esca-foliar asymptomatic plants and 612 fungal strains were isolated from esca-foliar symptomatic plants. Depending on the type of piece of wood, from none fungus to 3 fungi was isolated. The highest values of isolated fungi were in April and January, with 210 and 280 isolates, respectively. The lowest values were in June with around 115 isolates taken from the plants. When the numbers of isolates are compared between symptomatic and asymptomatic vines, no differences are observed, except in April (Fig. 4).

For each sampling time and for each type of plants, the isolates are ranked according to their division, their class, their order, their family and their genera. The Ascomycetes is the predominant division with 92.4% of the total of isolates, followed by the Zygomycetes and the Basidiomycetes which are isolated at respectively 7.2% and 0.4%. Figure 5 shows the distribution of Ascomycetes orders for each sampling period and type of plants. Thirteen orders are found and the most abundant orders are in decreased order: Hypocreales, Botryosphaeriales, Eurotiales and Pleosporales. These 4 orders are isolated at each sampling dates in the healthy wood of esca-foliar symptomatic and asymptomatic vines. Capnodiales and Heliothales were detected to a lower frequency in 8 dates of sampling. Dothideales, Pyrenulales and Sordariales are the orders detected only one time, in September for Dothideales and in January for Pyrenulales and Sordariales. The most important number of orders is obtained in September for the two types of plants and the lower in April.

Analyses of the fungi isolated from the wood of trunks and rootstocks

Distribution of the fungal genera. Figure 6 shows the distribution and frequency of isolates. Three cases are observed: fungal strains isolated more than twice (plurals), twice (doubles) or only once (singles). Globally, 35 genera were isolated from asymptomatic plants and 37 genera from esca-foliar symptomatic plants. For symptomatic plants, 19 genera were isolated several times, 6 genera twice and 10 only one time. For asymptomatic plants, 19 genera were isolated many times, 4 genera twice and 14 one time. There is no significant difference between the number of plurals, doubles and singles genera isolated from symptomatic and asymptomatic plants.

Table 2 shows the distribution of the fungi according to their class, their order, their family and their genera. A total of 48 genera are identified, the 5 most important are

Botryosphaeria, *Trichoderma*, *Penicillium*, *Bionectria* and *Fusarium*. The fungi isolated from the 48 genera represented 5 classes, 14 orders, 27 families belonging to various lineages of ascomycetes, basidiomycetes and basal fungal lineage. The majority of these isolates are Ascomycota (4 classes, 11 orders and 37 genera) followed by Basidiomycota (1 classes, 1 orders and 2 genera) and Mucoromycota (Zygomycota) (2 orders and 4 genera).

The most frequently isolated genera belong to the class of Sordariomycetes and Dothideomycetes, with a dominance of Sordariomycetes (40% of fungal isolates). Taken altogether, the two classes represented 77% of all the isolates of symptomatic and asymptomatic plants.

The genera most abundant in terms of isolates collected are: (i) *Botryosphaeria* spp. with 168 isolates for asymptomatic and 154 isolates for esca-foliar symptomatic vines (no significant difference with T-test), (ii) *Trichoderma* spp. with 132 isolates for asymptomatic and 163 for symptomatic (no significant difference with T-test), (iii) *Penicillium* spp. with 45 isolates for asymptomatic plants and 61 for symptomatic plants (no significant difference with T-test), (iv) *Bionectria* spp. with 16 isolates for asymptomatic plants and 58 for asymptomatic plants (significant difference with p-value=0.0001 with T-test) and (v) *Fusarium* with 34 isolates for asymptomatic plants and 20 for symptomatic plants (no significance differences with T-test). These 5 genera accounted for 68.8% of all the strains isolated from the wood of asymptomatic plants and 74.5% from the samples of symptomatic plants.

Figure 7 shows that more than 70% of esca-foliar symptomatic and asymptomatic plants are colonized by *Botryosphaeria* spp. and *Trichoderma* spp. For the other species, there are differences between the genera and the number of plants colonized, excepted for *Penicillium* spp., *Aspergillus* spp. and *Cladosporium* spp. where respectively 50%, 42% and 29% of the both type of plants are inhabited by these fungi. Sixty nine percent of the asymptomatic plants and 42% of symptomatic ones are colonized by *Fusarium* spp. Note that *Alternaria* spp., *Epicoccum* spp. and *Arthrinium* spp. colonize more asymptomatic plants than the symptomatics.

Fungal species isolated from the two types of plants. After random sequencing of 487 isolates (Table 4), the most frequently identified species were: *Diplodia seriata* (72 strains), *Fusarium oxysporum* (42 strains), *Bionectria ochroleuca* (33 strains) and *Neofusicoccum parvum* (32 strains). For the *Trichoderma* genus, 8 species have been identified, the two most abundant were: *Trichoderma gamsii* (31 strains) and *T. atroviride* (19 strains).

Figure 8 shows the 30 first most abundant fungal species isolated from the esca-foliar symptomatic and asymptomatic plants. *D. seriata* is the most abundant species, 58% of the strains have been isolated from the symptomatic vines and 75% from the asymptomatic ones. *F. oxysporum* (61% / 29% = asymptomatic/symptomatic), *Epicoccum nigrum* (43% / 29% = asymptomatic/symptomatic), *T. gamsii* (43% / 36% = asymptomatic/symptomatic) and *Arthrinium sacchari* (29% / 7% = asymptomatic/symptomatic) have been more isolated in asymptomatic plants than in the other type of vine. In contrast, more symptomatic plants have been colonized by *B. ochroleuca* (32% / 57% = asymptomatic/symptomatic) and *N. parvum* (29% / 46% = asymptomatic/symptomatic) than the asymptomatic plants.

Comparison of the fungal genera between esca-foliar symptomatic and asymptomatic plants

Principal Component Analysis is obtained in order to compare the fungal genera colonizing the two types of plants. PCA eigenvalues indicates that the first two principal components, Dim 1 and Dim 2, explain 44.70% of the total data variance. Except for the samples of January, the clusters of the 3 other sampling times are not separated by Dim 1 and 2 (Fig. 9A). The figure 9B shows that the clusters of the genera from esca-foliar asymptomatic and symptomatic plants tend to be separated.

Alpha diversity of the fungal genera

Eighty three species were recorded when all the observations were pooled. To calculate the biodiversity index, the number of species was adjusted with the total of samples. The fungi used for sequencing were randomly sampled, particularly for plurals taxa. Fungal biodiversity from wood of esca-foliar symptomatic and asymptomatic plants (H') were the same. Depending on sampling time, fungal biodiversity in April (H' sympto =2.05 and H' asympto=2.14) was higher than in January (H' sympto=2.87 and H' asympto=2.98). Also, dominance was not shared among a relatively large number of species because of the low value of the evenness component (J'). The values varied from 0.18 to 0.21 (Table 3).

Diversity of haplotypes

After analyses by Codon Code Software and Mega 5, 487 sequences were studied for phylogeny and statistic analyses. The 487 sequences were assembled in 43 contigs and one of them grouped the unassembled sequences. Generally each contig represented one genus or one species. One hundred and four haplotypes were defined from the 43 contigs. When the sequences of one contig were aligned, only few nucleotides changed for each species. Table 5 shows the 6 important contigs in term of sequences are *D. seriata* (72 sequences) followed by *F. oxysporum* (42 sequences), *B. ochroleuca* (33 sequences), *N. parvum* (32 sequences), *T. gamsii* (31 sequences) and *T. atroviride* (19 sequences). These 6 contigs contained 1 to 4 haplotypes. Table 5 shows that some haplotypes are detected in the two types of plants for each sampling time; for instance, for H1 and H3 of *D. seriata* and H14 of *T. gamsii*. For the other haplotypes, for which the number of sequences is also generally low, haplotypes are detected only at certain sampling time(s).

Discussion

To compare the mycoflora between esca-foliar symptomatic and asymptomatic vines, we first

verified the pathogenic status of the wood tissues to make meaningful comparisons. When the plants were longitudinally cut and opened, with the exception of a small central necrosis, the wood tissues of the trunks and the rootstocks looked similar, and were non-necrotic in all the grapevines. The only difference could be observed in the cordons: white-rot (or amadou) was extensively seen in the esca-foliar symptoms plants, for 79% of them, but not in the asymptomatic plants. This observation is in agreement with early descriptions of esca (Viala 1926; Larignon and Dubos 1997; Larignon et al. 2009), and also with Maher et al. (2012) who set up a logistic model indicating that white rot in the cordons is the best predictor for the chronic form of esca.

Study of the global fungal microflora by SSCP showed that complex microflora colonized the wood tissues of all the grapevines. However, depending on the location of the samples within the plant, marked differences could be observed or not, either in the cordons or in the trunks of asymptomatic and esca-foliar symptomatic plants.

White-rot necroses are located only in the cordons of symptomatic plants and many scientists have already reported that *F. mediterranea* is usually the predominant fungus in this zone (Fischer 2002; Berraf and Perros 2005; White et al. 2011). Sparapano et al. (2000) and Surico et al. (2006) reported that it caused healthy wood to turn into white-rot. As a specific microflora, presumably dominated by *F. mediterranea*, colonizes white-rot, that may explain why that microflora differs from those of other zones, *i.e.* barks and healthy-wood tissues, as we show by SSCP analyses.

Unlike the cordons, in the trunks and rootstocks, the woods were healthy both in esca-foliar symptomatic and asymptomatic plants, and their fungal microflora tended to be similar. This observation indicates that the method of sampling and the wood tissues used may cause the authors to differ in their conclusions about the association of fungi with esca. As we mentioned earlier, if the wood health status not verified, take samples by drilling can lead to scientists analyzing either non-necrotic samples of wood from both symptomatic and asymptomatic plants or, if the plants are older, only necrotic samples, or a combination of healthy/necrotic tissues. So it seems reasonable that, similar microflora in the esca-foliar symptomatic and asymptomatic vines have sometimes been found (Hofstetter et al. 2012). In our opinion, this key point has to be considered by scientists studying the fungal communities associated with esca.

As many papers have been devoted to the description of the mycoflora colonizing the various types of necroses (Mugnai et al. 1999; Fischer and Kassemeyer 2003; Maher et al. 2012), we have focused our attention on the fungal communities of the healthy grapevine wood tissues. The objective was to analyze these tissues before they became necrotic, usually a few years later. That would help in determining the initial factors that make a healthy wood become necrotic.

Within the trunks and rootstocks of foliar symptomatic and asymptomatic vines, the number of cultivable fungi we isolated for each sampling date varied from 115 to 280. Ascomycota fungi represented 92% of the total number of the OTUs obtained. This result is in agreement with reports that have analyzed the microflora colonizing various parts of the vines.

For instance, Casieri et al. (2009) studied the fungi colonizing the wood of disease-free vines, Mostert et al. (2000), the endophytic fungi associated with shoots and leaves, Tiedemann et al. (1988) studied those of the vascular system of rootstocks and Gonzalez and Tello (2010) those isolated from leaves, twigs and berries of *Vitis Vinifera*. With the exception of a few samples from Gonzalez and Tello (2010), all the endophytic fungi were isolated from healthy vines. Our data are also consistent with other studies dealing with the endophytic fungal communities of woody tissues of different plant hosts (Bills 1996; Stone et al. 2004; Arnold and Lutzoni 2007; Li et al. 2007; Marquez et al. 2007). Although Ascomycota species dominate, some Basidiomycota and Zygomycota were also isolated. In line with Gonzalez and Tello (2010), we did not recover any fungus from either the Hymenochaetales (*Fomitiporia* spp.) or the Stereaceae (*Stereum* spp.). The main reason is certainly because Basidiomycetes, such as *F. mediterranea* or *Stereum hirsutum*, are usually found in the necrotic tissues of grapevine plants. Stone et al. (2004) mentioned that low proportions of Basidiomycota in endophyte inventories could be due to a bias in the cultural method used to isolate the fungi. Generally, such an approach favours the occurrence of sporulating and fast-growing species rather than wood decay Basidiomycetes. In our study, as we analyzed healthy woods, the fact that we isolated numerous Ascomycota would seem reasonable.

At the fungal order level, the high proportion of Hypocreales, Botryosphaeriales, Eurotiales and Pleosporales in the trunk and rootstocks of esca-foliar symptomatic and asymptomatic grapevines suggests they are the main components of the endophytic mycota. Our results differ from those of Hofstetter et al. (2012) who isolated Pleosporales (32.5%) and Hypocreales (26.8%) from the wood of young vines (sampled from nurseries) and Pleosporales (around 27-28%) and Dothideales (around 15%) from adult plants (15-30 year-old). These differences in fungi microflora may depend on the vine part being sampled and the health status of the wood. Among the other explanations: the plant age, the soil, cultivar, climate, and other environmental factors certainly have an influence on the development of various fungal communities.

In our study, the relative abundance of each endophyte genus reflects an unequal distribution of isolate richness among genera and the low values of evenness underline that few species are abundant in the wood tissue. Unlike Shannon's index diversity (H') obtained by Gonzalez and Tello (2010), *i.e.* 3.17, our indexes were lower. But the diversity we estimated was based on the same vine variety (Cabernet-Sauvignon) and on healthy wood samples only. Therefore, in keeping with the type of samples monitored, the endophytic diversities differed too.

For the 10 most abundant genera: *Botryosphaeria*, *Trichoderma*, *Penicillium*, *Bionectria*, *Fusarium*, *Mucor*, *Rhizopus*, *Aspergillus*, *Cladosporium*, *Alternaria*, we observed no significant differences between the healthy woods of esca-foliar symptomatic and asymptomatic vines. Most of these genera were detected in the wood of at least 20% of all the plants. The most frequent genus, *Botryosphaeria*, is potentially plant pathogenic, but the majority of the other genera are non-pathogenic for plants, with some of them being potentially biocontrol agents.

As regards, *Botryosphaeria* spp., they can cause Bot canker on grapevines (Urbez-Torres et al. 2006, 2008, 2009) but also on many plants like olive trees (Chattaoui et al. 2012), peach trees (Wang et al. 2011, Beckman et al. 2011), almond trees (Inderbitzin and Michailides 2008), oak (Lynch et al. 2011). Here, *Botryosphaeria* spp. were very frequently isolated from grapevines, being in 83% and 93% of the esca-foliar asymptomatic and symptomatic plants respectively. The most frequent species was *D. seriata* (57% in symptomatic and 43% in asymptomatic plants). In the literature, contradictory results were reported: depending on the age of the grapevines and cultivar, *Botryosphaeria* species could be isolated or not. For instance, Hofstetter et al. (2012) isolated *D. seriata* from nurseries and adult vines and *N. parvum* from nursery plants. Krol (2006) did not isolate *Botryosphaeria* spp. in grapevine canes from nurseries and Gonzalez and Tello (2010) reported that *Botryosphaeria* spp. were not abundant in the vines they sampled. Again, diversity in the origin of grapevines resulted in the diversity of *Botryosphaeria* species and in the number of strains of each species.

The second, most abundant genus, *Trichoderma*, is well known for its antagonistic abilities to parasite plant pathogenic fungi and to protect many plants, including grapevine (McLean et al. 2012; Parizi et al. 2012; Perveen and Bokhari 2012; Reglinski et al. 2012). *Trichoderma* spp. strains were isolated from 75% of esca-foliar asymptomatic and 93% of symptomatic grapevines. The two most frequently isolated species were *T. gamsii* (in 42/35% of asymptomatic/symptomatic plants) and *T. atroviride* (in 25/15% of asymptomatic/symptomatic plants). The other 6 species of *Trichoderma* were isolated to a lesser extent.

In the present study, strains of two haplotypes of *D. seriata* and of one haplotype of *T. gamsii* were detected at each sampling time in both esca-foliar symptomatic and asymptomatic plants. This suggests that these two genera, in addition to being abundant, have specific haplotypes that are able to strongly colonize and persist in the wood of grapevines. Interestingly, Linaldeddu et al. (2007) reported that, when they confronted strains of *Trichoderma* and *Botryosphaeria* *in vitro*, the mycoparasite was predominant in the development of the pathogen. This finding suggests that such competition occurs in the wood of plants; however, to our knowledge no investigations have been made to determine if it shapes the two fungal communities at the vineyard.

Our results indicate that many other fungi, which colonized at least 20% of plants, have antagonistic activities. For instance, some strains of *Epicoccum nigrum*, *Alternaria alternata*, and *Fusarium oxysporum* are known as useful or promising biocontrol agents. However, it was not possible to postulate that non-pathogenic fungi are always more numerous in asymptomatic grapevines than in esca-foliar symptomatic ones. For instance, the pathogen *D. seriata* was detected in 57 % of asymptomatic and 43% of symptomatic grapevines. Furthermore, as the pathogenic status of *F. oxysporum* strains is not known, some may be plant pathogenic. So, when the cultivable mycoflora colonizing the healthy woods of the two types of plants are compared, they cannot be statistically differentiated.

Another important point is that mycoflora changed over time, with a shift in the fungal communities being observed over the year. In the rhizosphere of many plants, shifts in the genetic

structures of the fungal communities over time were also observed (Vallance et al. 2009). These shifts are frequently related to the root exudates and physiology of plants, which change over a growing season, shaping the community of microorganisms that metabolize the exudates of the plants. A relatively similar phenomenon within the trunk presumably occurs here. During the period of winter, the plant is in a latent state and there are no or only low vascular exchanges within the tissues. In spring, the plant produces the leaves and grapes, and numerous molecules are transported in the vessels. This source of food probably has an influence on the fungal communities and can, at least partly, explain the shifts observed in the microflora. Additionally, cold temperatures in winter and hot ones in summer may shape the fungal communities.

Although the mycoflora changed, significant increases over time in the pathogenic fungi or in the potentially plant protective fungi species were not observed. Whether in the trunks or in the rootstocks of grapevines, the wood necroses were very small and did not increase from one season to another. These data suggest that the global mycoflora does not have a pathogenic status, and that the equilibrium between pathogenic/antagonistic fungi seems to be in favour of the potentially plant protective fungi. One can presume that great competitions occurs between fungi and that antagonistic fungi can control, at least partly, the development of potentially pathogenic fungi over that period. However, this process does not seem sustainable because a few years later, wood necroses usually developed in the wood of mature vines (from 15 years old and more) as reported by Maher et al. (2012).

Nowadays, the scenarios that consist in describing why healthy wood of grapevines usually turns necrotic still remains to be written and the whole process demonstrated. In the present experiment, as we observed shifts over a year in the mycoflora colonizing vine wood, this suggests that, over a longer period, fungal community structures may also change. Provided that environmental factors are favourable, pathogenic fungi that have already colonized some parts of the wood of the trunks can multiply and subsequently cause necroses.

In conclusion, this study demonstrates that studying the mycoflora responsible for grapevine esca must take into account the whole pathogenic status of the plants. Otherwise, certain differences could be observed or not, depending on the wood samples used. In our opinion, the main question is not: are there differences in the microflora colonizing the wood of either apparently healthy or diseased plants? The answer is yes, because the necroses in esca-foliar symptomatic vines are more extensive and also because specific fungi colonize them, *e.g.* *F. mediterranea* in white-rot. But the real issue is how the equilibrium between the potentially plant pathogenic and potentially plant protective fungi colonizing the healthy wood breaks down over time, leading to the development of necroses. Experiments on biotic, *e.g.* bacteria, and abiotic, *e.g.* weather changes, cultural practices, factors need to be undertaken in order to understand how those factors favour the onset of the pathogenic process.

Acknowledgements

The experiment was financed by Bordeaux Sciences Agro, the Regional Council of Aquitaine, the French Ministry of Agriculture, Food-processing industry and Forest (programme CASDAR V906). We thank Pierre Darriet for the sampling of the vines at the Chateau Luchey-Halde and Sylvie Bastien for excellent technical assistance.

References

- Abarenkov K, Nilsson RH, Larsson KH, Alexander IJ, Ebenhardt U, Erland S, *et al* (2010) The UNITE database for molecular identification of fungi - recent updates and future perspectives. *New Phytol* 186: 281-285
- Armengol J, Gimenez-Jaime A, Vicent A, Garcia-Jimenez J (2005) The diseases of wood in Midi-Pyrenees Esca and BDA Etiology and importance of diseases of wood in Spain. *Progres Agri Viti* 122: 130-134
- Arnold AE Lutzoni F (2007) Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots? *Ecology* 88: 541-544
- Beckman TG, Reilly CC, Pusey PL and Hotchkiss M (2011) Progress in the Management of Peach Fungal Gummosis (*Botryosphaeria dothidea*) in the Southeastern US Peach. *Industry J Am Por Soc* 65: 192-200
- Berraf A, Peros JP (2005) Importance of *Eutypa dieback* and esca in Algeria and structure of the associated fungal community. *J Int Sci Vigne Vin* 39: 121-128
- Bertsch C, Ramirez-Suero M, Magnin-Robert M, Larignon P, Chong J, Abou-Mansour E, *et al* (2012) Grapevine trunk diseases: complex and still poorly understood. *Plant Pathol* DOI: 10.1111/j.1365-3059.2012.02674.x
- Bills GF (1996) Isolation and analysis of endophytic fungal communities from woody plants. *Am Phytopathol Soc* (APS Press)
- Buckland ST, Magurran AE, Green RE, Fewster RM (2005) Monitoring change in biodiversity through composite indices. *Philos Trans R Soc B-Biol Sci* 360: 243-254
- Casieri L, Hofstetter V, Viret O, Gindro K (2009) Fungal communities living in the wood of different cultivars of young *Vitis vinifera* plants. *Phytopathol Mediterr* 48: 73-83
- Chanderbali AS, Van der Werff H, Renner SS (2001) Phylogeny and historical biogeography of Lauraceae: Evidence from the chloroplast and nuclear genomes. *Ann Miss Bot Garden* 88: 104-134
- Chattaoui M, Rhouma A, Msallem M, Perez M, Moral J, Trapero A (2012) First Report of *Botryosphaeria obtusa* as Causal Agent of Olive Tree Branch Dieback in Tunisia. *Plant Dis* 96: 905-905
- Chiarappa L (2000) Esca (black measles) of grapevine An overview. *Phytopathol Mediterr* 39: 11-15
- Crous PW, Gams W, Wingfield MJ, VanWyk PS (1996) *Phaeoacremonium* gen nov associated with wilt and decline diseases of woody hosts and human infections. *Mycologia* 88: 786-796
- Edwards J, Pascoe IG (2004) Occurrence of *Phaeoconiella chlamydospora* and *Phaeoacremonium aleophilum* associated with Petri disease and esca in Australian grapevines. *Australas Plant Pathol* 33: 273-279
- Fischer M (2002) A new wood-decaying basidiomycete species associated with esca of grapevine: *Fomitiporia mediterranea* (Hymenochaetales). *Mycol Prog* 1: 315-324

- Fischer M, and Kassemeyer HH (2003) Fungi associated with Esca disease of grapevine in Germany. *Vitis* 42: 109-116
- Gimenez-Jaime A, Aroca A, Raposo R, Garcia-Jimenez J, Armengol J (2006) Occurrence of fungal pathogens associated with grapevine nurseries and the decline of young vines in Spain. *J Phytopathol* 154: 598-602
- Gonzalez V, Tello ML (2010) The endophytic mycota associated with *Vitis vinifera* in central Spain. *Fungal Divers* 47: 29-42
- Gotelli NJ, Colwell RK (2001) Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. *Ecol Lett* 4: 379-391
- Graniti A, Surico G, Mugnai L (2000) Esca of grapevine: a disease complex or a complex of diseases? *Phytopathol Mediterr* 39: 16-20
- Grosman J, Doublet B (2012) Maladies du bois de la vigne Synthèse des dispositifs d'observation au vignoble, de l'observatoire 2003-2008 au réseau d'épidémiosurveillance actuel. *Phytoma* 651: 31-35
- Hofstetter V, Buyck B, Croll D, Viret O, Couloux A, Gindro K (2012) What if esca disease of grapevine were not a fungal disease? *Fungal Divers* 54: 51-67
- Inderbitzin P, and Michailides TJ; (2008) High diversity of *Botryosphaeria* species from almond band and canopy cankers in California. *Phytopathology* 98: S71-S71
- Krol E (2006) Fungi inhabiting healthy grapevine canes (*Vitis* spp) in some nurseries. *Acta Agr* 59: 163-173
- Larignon P, Dubos B (1997) Fungi associated with esca disease in grapevine. *Eur J Plant Pathol* 03: 147-157
- Larignon P, Fulchic R, Cere L, Dubos B (2001) Observation on black dead arm in French vineyards. *Phytopathol Mediterr* 40: S336-S342
- Larignon P, Fontaine F, Farine S, Clement C, Bertsch C (2009) Esca and Black Dead Arm: two major actors of grapevine trunk diseases. *C R Biol* 332: 765-783
- Lecomte P, Darrieutort G, Liminana JM, Comont G, Muruamendaraz A, Legorburu FJ, *et al* (2012) New Insights into Esca of Grapevine: The Development of Foliar Symptoms and Their Association with Xylem Discoloration. *Plant Dis* 96: 924-934
- Li WC, Zhou J, Guo SY, Guo LD (2007) Endophytic fungi associated with lichens in Baihua mountain of Beijing, China. *Fungal Divers* 25: 69-80
- Linaldeddu BT, Maddau L, Franceschini A (2007) Antagonistic activity of endophytic isolates of *Trichoderma* spp, against *Botryosphaeriaceae* associated to cork oak decline. *Micologia Ital* 36: 22-29
- Lorrain B, Ky I, Pasquier G, Jourdes M, Dubrana LG, Geny L, Rey P, *et al* (2012) Effect of Esca disease on the phenolic and sensory attributes of Cabernet Sauvignon grapes, musts and wines. *Aust J Grape Wine Res* 18: 64-72
- Lynch S, Eskalen A, Zambino P, Scott T (2011) *Botryosphaeria* species complex associated with coast live oak (*Quercus agrifolia*) mortality in Southern California. *Phytopathology* 101: S111-S111

- Maher N, Piot J, Bastien S, Vallance J, Rey P, Guerin-Dubrana L (2012) Wood necrosis in Esca-affected vines: types, relationships and possible links with foliar symptom expression. *J Inter Sci Vigne Vin* 46: 15-27
- Marquez SS, Bills GF, Zabalgoceazcoa I (2007) The endophytic mycobiota of the grass *Dactylis glomerata*. *Fung Div* 27: 171-195
- McLean KL, Hunt JS, Stewart A, Wite D, Porter IJ, Villalta O (2012) Compatibility of a *Trichoderma atroviride* biocontrol agent with management practices of *Allium* crops. *Crop Protec* 33: 94-100
- Mostert L, Crous PW, Petrini O (2000) Endophytic fungi associated with shoots and leaves of *Vitis vinifera*, with specific reference to the *Phomopsis viticola* complex. *Sydowia* 52: 46-58
- Mugnai L, Graniti A, Surico G (1999) Esca (Black measles) and brown wood-streaking: Two old and elusive diseases of grapevines. *Plant Dis* 83: 404-418
- Parizi TE, Ansari M, Elaminejad T (2012) Evaluation of the potential of *Trichoderma viride* in the control of fungal pathogens of Roselle (*Hibiscus sabdariffa* L) in vitro. *Microb Pathogenesis* 52: 201-205
- Perveen K, Bokhari NA (2012) Antagonistic activity of *Trichoderma harzianum* and *Trichoderma viride* isolated from soil of date palm field against *Fusarium oxysporum*. *Afr J Microbiol Res* 6: 3348-3353
- Reglinski T, Rodenburg N, Taylor JT, Northcott GL, Chee AA, Spiers TM, *et al* (2012) *Trichoderma atroviride* promotes growth and enhances systemic resistance to *Diplodia pinea* in radiata pine (*Pinus radiata*) seedlings. *Forest Pathol* 42: 75-78
- Romanazzi G, Murolo S, Pizzichini L, Nardi S (2006) First investigations on Esca disease of grapevine in Marche region. *Universita di Bologna*
- Rumbos I, Rumbou A (2001) Fungi associated with esca and young grapevine decline in Greece. *Phytopathol Mediterr* 40: S330-S335
- Scheck HJ, Vasquez SJ, Gubler WD, Fogle D (1998) First report of three *Phaeoacremonium* spp causing young grapevine decline in California. *Plant Dis* 82: 590-590
- Shannon CE, Weaver W (1963) *The Mathematical Theory of Communication* University of Illinois Press
- Sparapano L, Bruno G, Ciccarone C, Graniti A (2000) Infection of grapevines by some fungi associated with esca I *Fomitiporia punctata* as a wood-rot inducer. *Phytopathol Mediterr* 39: 46-52
- Stone JK, Polishook JD, White JF (2004) Endophytic fungi. In: *Biodiversity of fungi* Elsevier Amsterdam: pp 241-270
- Surico G, Mugnai L, Marchi G (2006) Older and more recent observations on esca: a critical review. *Phytopathol Mediterr* 45: S68-S86
- Tiedemann S, Brendel G, Fehrmann H (1988) Investigations on endophytic fungi of grapevine with special emphasis on the vascular system of rootstocks. *J Phytopathol* 122: 147-165

- Úrbez-Torres JR, Leavitt GM, Voegel TM, Gubler WD (2006) Identification and distribution of *Botryosphaeria* spp. associated with grapevine cankers in California. *Plant Dis* 90: 1490-1503
- Úrbez-Torres JR, Leavitt GM, Guerrero JC, Guevara J, Gubler WD (2008) Identification and pathogenicity of *Lasiodiplodia theobromae* and *Diplodia seriata*, the causal agents of bot canker disease of grapevines in Mexico. *Plant Disease* 92: 519-529
- Úrbez-Torres JR, Adams P, Kamas J, Gubler WD (2009) Identification, incidence, and pathogenicity of fungal species associated with grapevine dieback in Texas. *American Journal of Enology and Viticulture* 60: 497-507
- Vallance J, Le Floch G, Deniel F, Barbier G, Levesque CA, Rey P (2009) Influence of *Pythium oligandrum* biocontrol on fungal and oomycete population dynamics in the rhizosphere. *Appl Environ Microbiol* 75: 4790-4800
- Viala P (1926) Recherches sur les maladies de la vigne Esca. *Annal Epiphyt fasc 1 et 2*:1-108
- Wang F, Zhao LN, Li GH, Huang JB, Hsiang T (2011) Identification and Characterization of *Botryosphaeria* spp Causing Gummosis of Peach Trees in Hubei Province, Central China. *Plant Dis* 95: 1378-1384
- White CL, Halleen F, Mostert L (2011) Symptoms and fungi associated with esca in South African vineyards. *Phytopathol Mediterr* 50: 236-246
- White T, Birns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Academic Press, San Diego

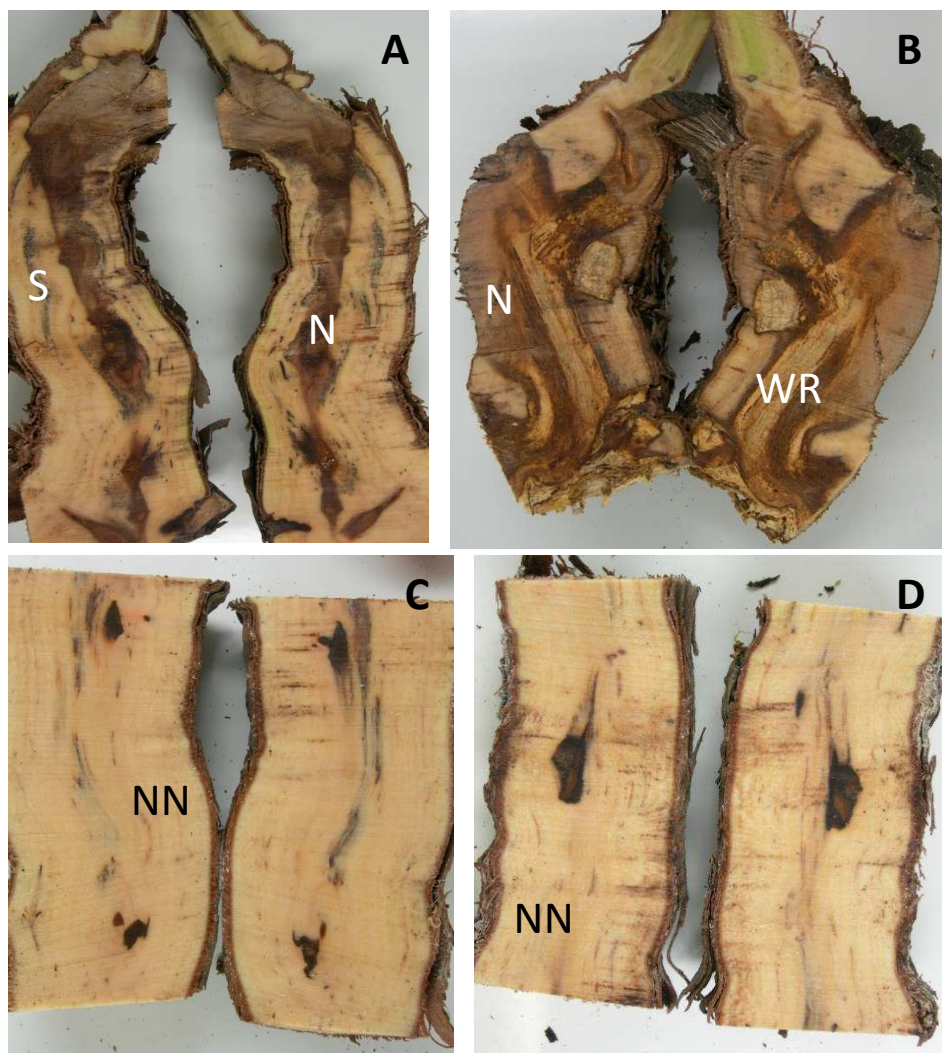


Fig. 1. Photographs of longitudinal-sections of cordons (Fig. 1A and Fig. 1B) and trunks (Fig. 1C and Fig. 1D) of plants that had expressed (Fig. 1B and Fig. 1D) or not esca-foliar symptoms (Fig. 1A and Fig. 1C). N= Necrosis tissue, NN= Non-Necrotic tissue, S= Stripes, WR=White Rot

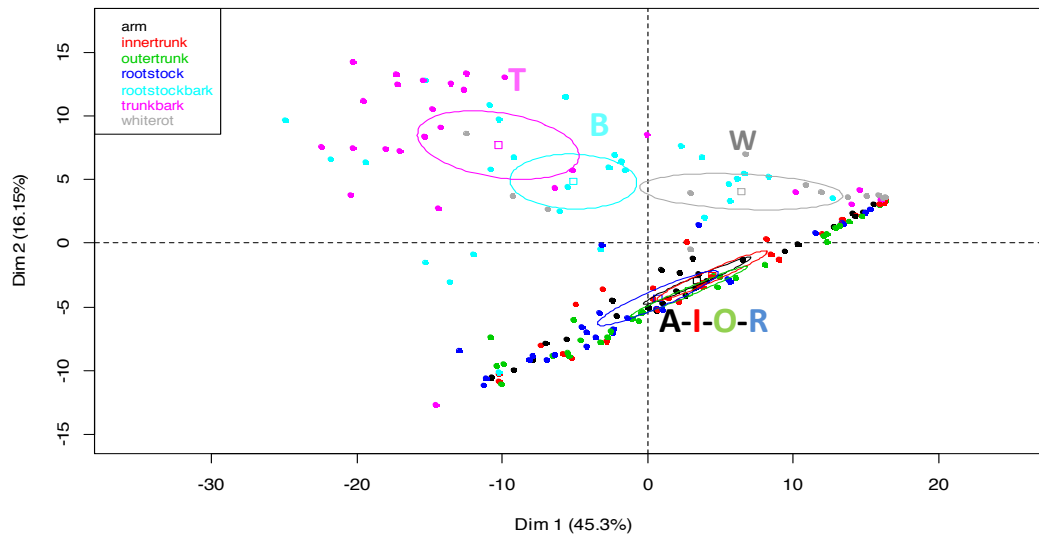


Fig. 2. Distribution of the fungal communities on the principal planes designs by the global Principal Component Analysis (PCA) obtains from numeral values of each SSCP profile. The total variation (%) explains by PCA is given in brackets.

The fungal communities of each type of wood tissues are identified by one letter: A= Arm, I= Inner trunk, O= Outer trunk, R= Rootstock, T= Trunk bark, B= Rootstock bark, W=White rot

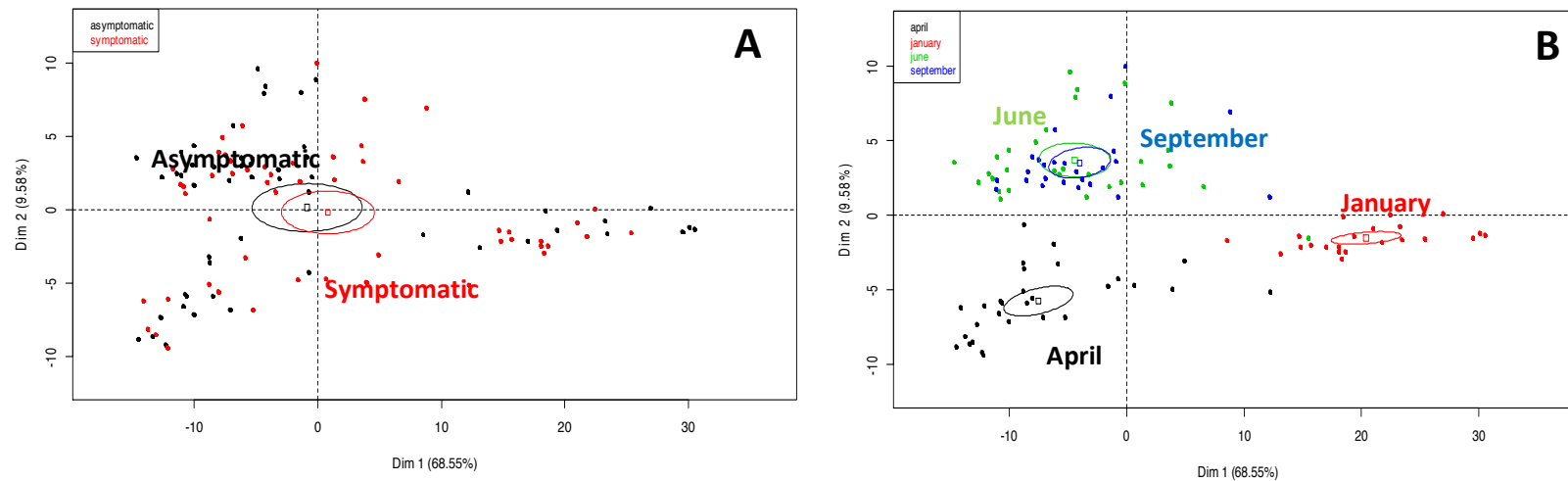


Fig. 3. Distribution of the fungal communities on the principal planes designs by the global Principal Component Analysis (PCA) obtains from numeral values of each SSCP profile. The total variation (%) explains by PCA is given in brackets.
 A. Clusters represent the fungal communities colonizing the non-necrotic tissues (inner trunks, outer trunks, rootstocks and arms) for all the sample times (April, June, September, January) of esca-foliar symptomatic and asymptomatic plants are pooled on PCA.
 B. Clusters represent each of the fungal communities colonizing the wood samples taken in April, June, September and January in the wood of the two types of vines.

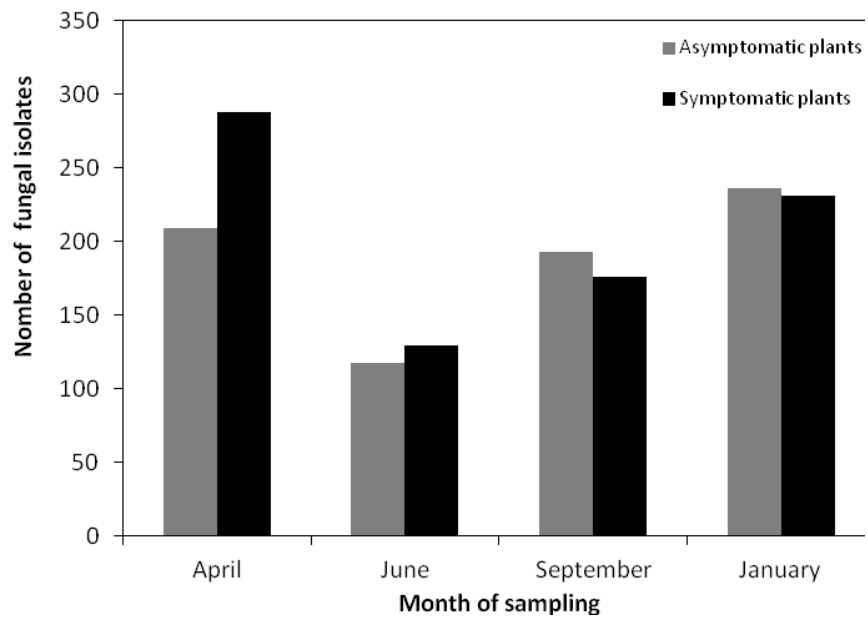


Fig. 4. Number of fungal isolates taken from the healthy wood of esca-foliar symptomatic and asymptomatic plants for the 4 sampling times.

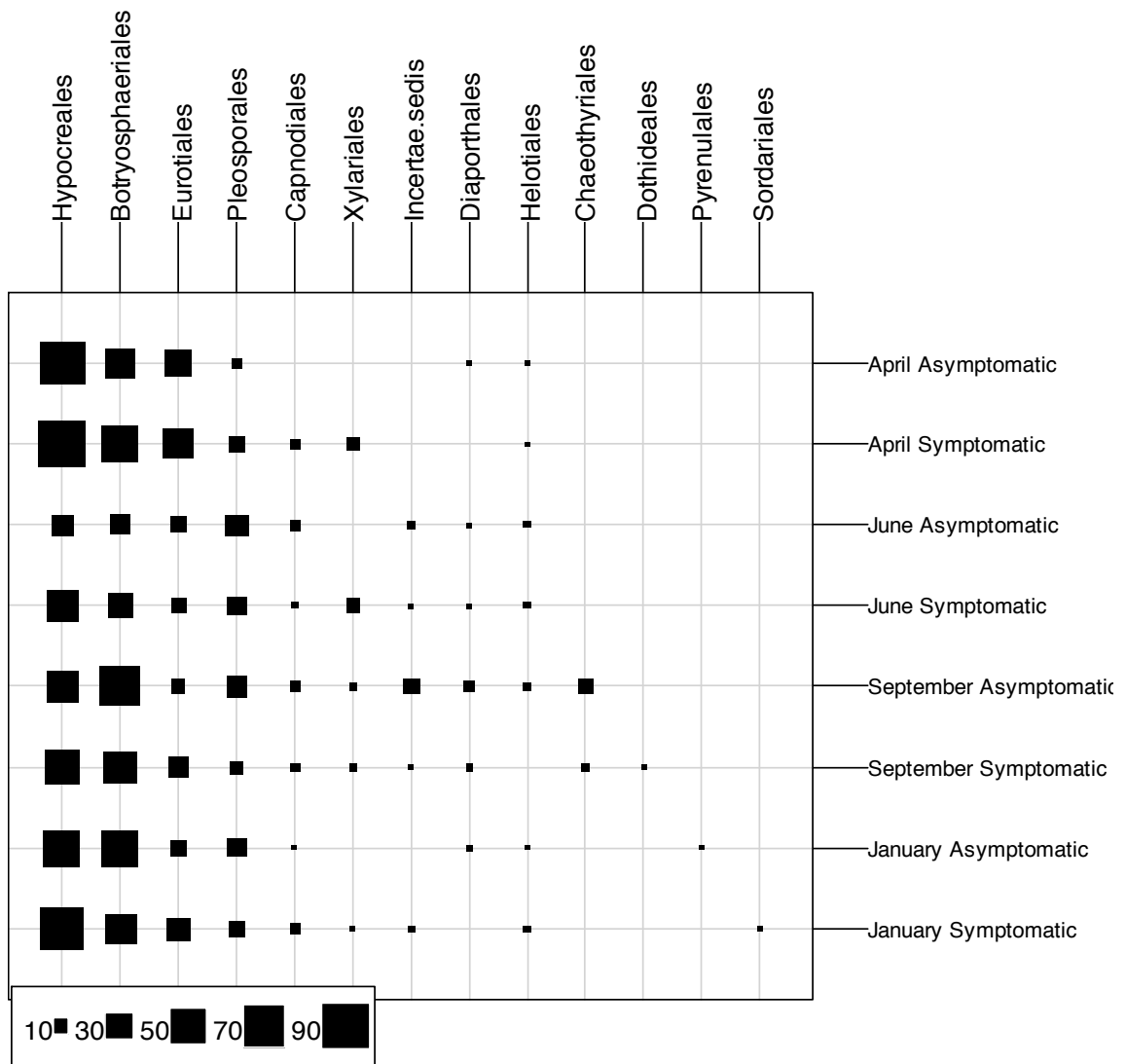


Fig. 5. Distribution of Ascomycetes orders per sampling dates and the status of the plants. A square represents the number of isolates for each sampling time.

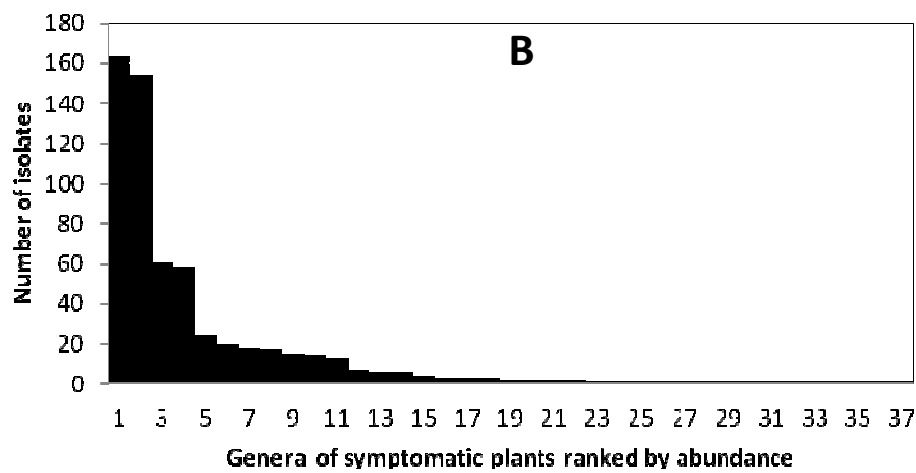
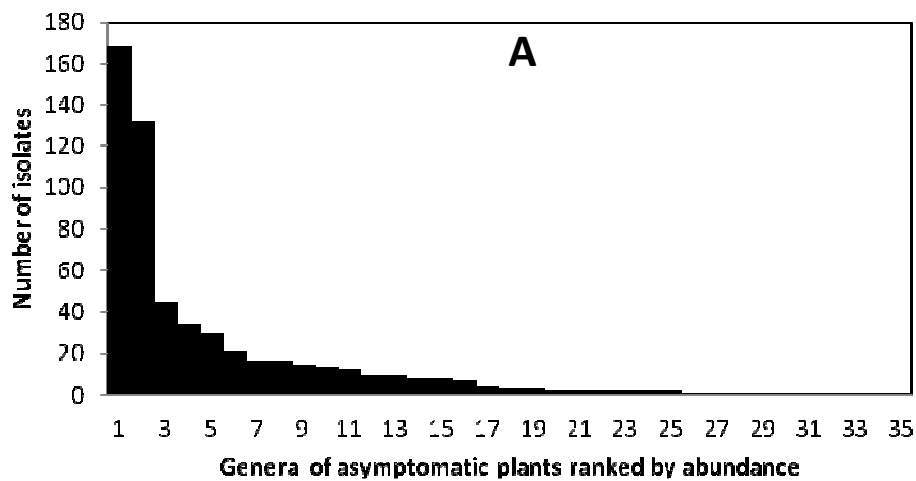


Fig. 6. Structure of fungal communities. Isolate abundances of the genera identified in the wood of esca-foliar symptomatic (Fig. 6A) and asymptomatic (Fig. 6B) plants.

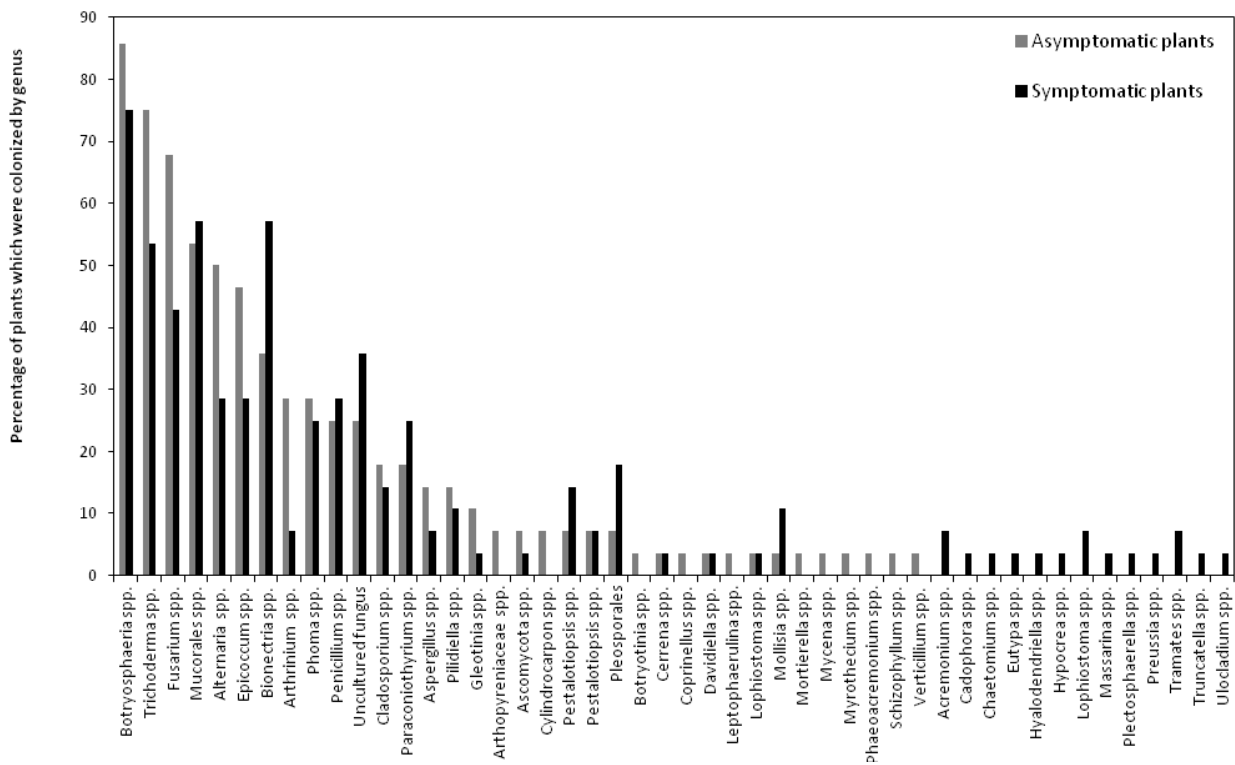


Fig. 7. Fungal genera isolated from the esca-foliar symptomatic and asymptomatic plants. The percentage represents the number of plants where the fungi have been isolated.

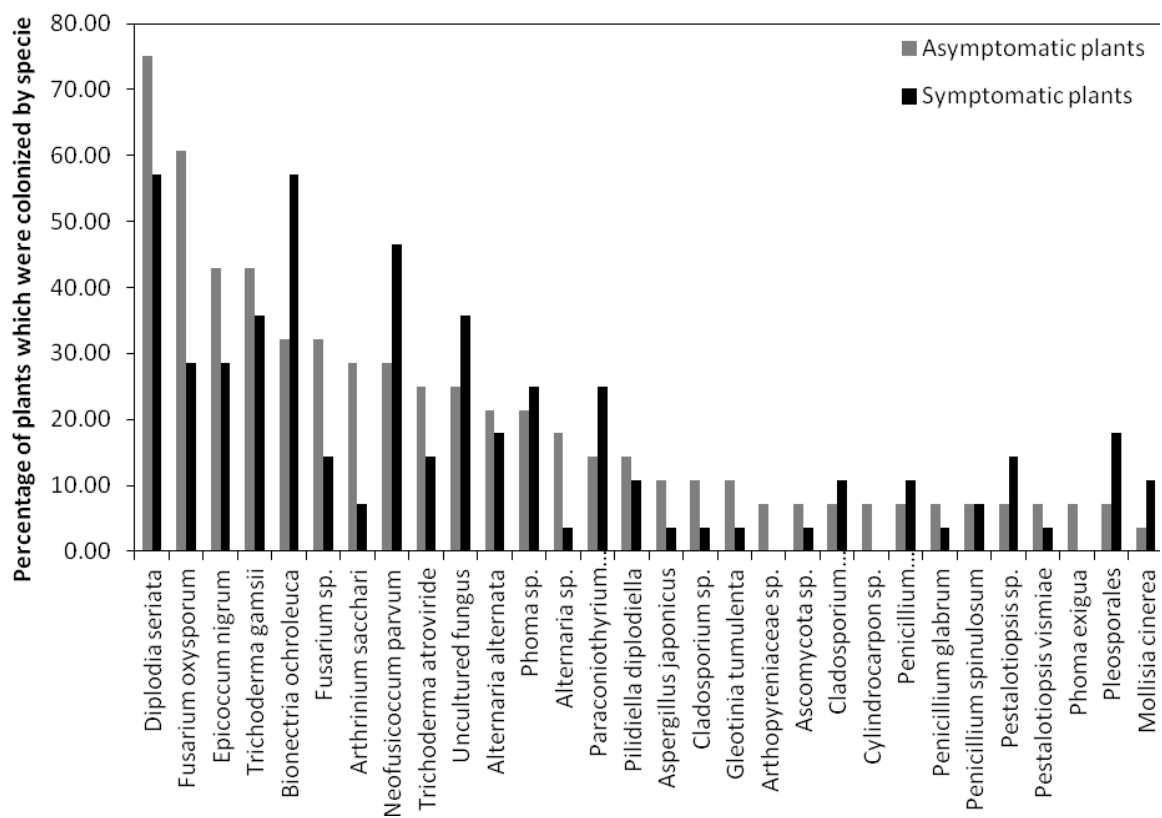


Fig. 8. The 30 first most abundant fungal species isolated from the esca-foliar symptomatic and asymptomatic plants. The percentage represents the number of plants from which the fungi have been isolated.

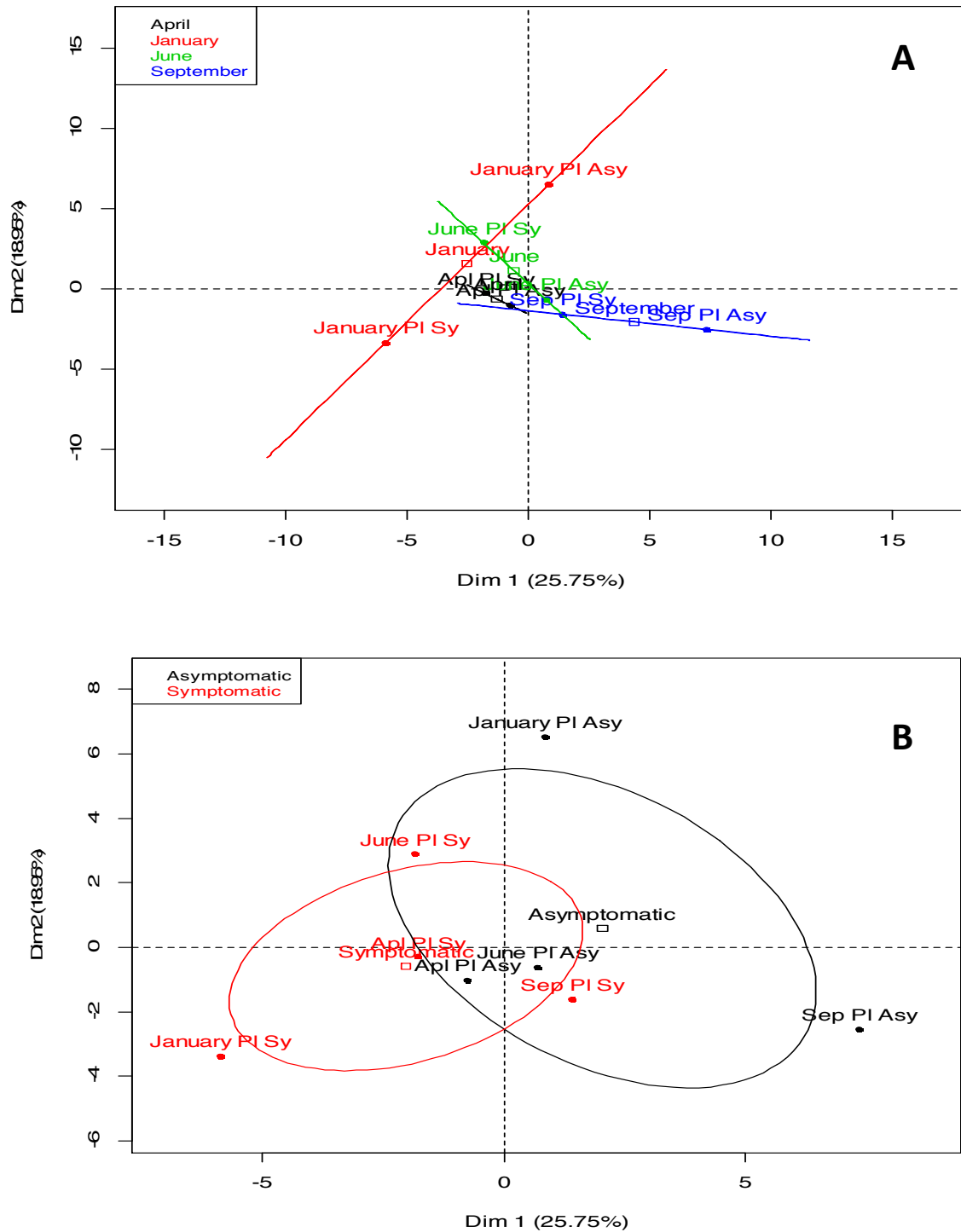


Fig. 9. Distribution of the fungal genera between symptomatic and asymptomatic plants for each sampling time. All the fungi are cultivable and have been isolated from the woods.

A. The various sampling dates are compared.

B. The pathological status of the vines is compared.

Distribution of the fungal communities is on the principal planes designs by the global Principal Component Analysis (PCA). The total variation (%) explains by PCA is given in brackets.

Table 1. Esca-foliar expression of the vines sampled. Over a 4-year period (2006 to 2009), the year of foliar symptom expression is reported for each plant (note by x). The presence of white rot in the cordons of vines is indicated by +

| Vine plant | Month of sampling | 2006 | 2007 | 2008 | 2009 | White rot in the cordons |
|------------|-------------------|------|------|------|------|--------------------------|
| R30 C72 | April | | | X | X | + |
| R36 C49 | April | | X | | X | + |
| R37 C70 | April | | X | X | X | + |
| R53 C72 | April | | X | X | X | + |
| R59 C73 | April | | X | X | X | + |
| R60 C37 | April | | X | | X | + |
| R70 C41 | April | | X | X | X | + |
| R37 C51 | June | | X | X | X | + |
| R58 C45 | June | | X | | X | |
| R60 C74 | June | | X | X | X | + |
| R67 C54 | June | | | X | X | + |
| R71 C8 | June | | | X | X | |
| R8 C8 | June | | | X | X | + |
| R85 C46 | June | | | X | X | + |
| R17 C24 | September | X | X | X | X | + |
| R34 C71 | September | | X | | X | + |
| R36 C61 | September | | X | | X | |
| R47 C82 | September | | X | X | X | + |
| R61 C36 | September | | X | X | | + |
| R66 C47 | September | | X | X | X | |
| R87 C34 | September | X | X | X | X | |
| R25 C50 | January | | X | X | | + |
| R25 C70 | January | | X | X | X | + |
| R28 C75 | January | | X | X | X | + |
| R28 C78 | January | | | X | X | + |
| R3 C52 | January | X | X | | X | + |
| R72 C11 | January | | X | X | | + |
| R8 C51 | January | X | X | | | |

Table 2. Fungal genera isolated from inner and outer rootstocks and trunks of esca-foliar symptomatic and asymptomatic vines over a cultural season.

| Class | Order | Family | Fungal genera | April 2010 | | June 2010 | | September 2010 | | January 2011 | | Total isolates |
|-----------------|------------------|---------------------|-------------------------------------|------------|-------|-----------|-------|----------------|-------|--------------|-------|----------------|
| | | | | PI Asy | PI Sy | PI Asy | PI Sy | PI Asy | PI Sy | PI Asy | PI Sy | |
| Dothideomycetes | Botryosphaerales | Botryosphaeriaceae | <i>Botryosphaeria</i> To,Ti,Ro,Ri | 36 | 50 | 16 | 24 | 62 | 42 | 54 | 38 | <u>322</u> |
| Sordariomycetes | Hypocreales | Hypocreaceae | <i>Trichoderma</i> To,Ti,Ro,Ri | 69 | 61 | 7 | 16 | 23 | 30 | 33 | 56 | <u>295</u> |
| Eurotiomycetes | Eurotiales | Trichocomaceae | <i>Penicillium</i> To,Ti,Ro,Ri | 26 | 21 | 3 | 5 | 6 | 15 | 10 | 20 | <u>106</u> |
| Sordariomycetes | Hypocreales | Bionectriaceae | <i>Bionectria</i> To,Ti,Ro,Ri | 2 | 17 | 5 | 14 | 3 | 14 | 6 | 13 | <u>74</u> |
| Sordariomycetes | Hypocreales | Nectriaceae | <i>Fusarium</i> To,Ti,Ro,Ri | 6 | 5 | 7 | 7 | 11 | 5 | 10 | 3 | <u>54</u> |
| | Mucorales | Mucoraceae | <i>Mucor</i> To,Ti,Ro,Ri | 15 | 6 | 0 | 2 | 10 | 5 | 5 | 2 | <u>45</u> |
| | Mucorales | Mucoraceae | <i>Rhizopus</i> To,Ti,Ro,Ri | 8 | 6 | 0 | 2 | 10 | 6 | 3 | 4 | <u>39</u> |
| Eurotiomycetes | Eurotiales | Trichocomaceae | <i>Aspergillus</i> To,Ti,Ro,Ri | 2 | 16 | 9 | 4 | 2 | 2 | 1 | 2 | <u>38</u> |
| Dothideomycetes | Capnodiales | Davidiellaceae | <i>Cladosporium</i> To,Ti,Ro,Ri | 0 | 5 | 5 | 2 | 3 | 2 | 1 | 5 | <u>23</u> |
| Dothideomycetes | Pleosporales | Pleosporaceae | <i>Alternaria</i> To,Ti,Ro,Ri | 1 | 2 | 3 | 1 | 8 | 2 | 4 | 1 | <u>22</u> |
| Dothideomycetes | Pleosporales | Montagnulaceae | <i>Paraconiothyrium</i> To,Ti,Ro,Ri | 0 | 2 | 5 | 7 | 0 | 0 | 4 | 4 | <u>22</u> |
| Sordariomycetes | Xylariales | Amphisphaeriaceae | <i>Pestalotiopsis</i> Ti,To,Ri | 0 | 8 | 0 | 7 | 2 | 2 | 0 | 0 | <u>19</u> |
| Dothideomycetes | Pleosporales | Didymellaceae | <i>Epicoccum</i> Ti,To,Ri | 0 | 2 | 3 | 2 | 6 | 1 | 3 | 1 | <u>18</u> |
| Sordariomycetes | Sordariales | Lasiochaeraceae | <i>Lasiosphaeria</i> To,Ti,Ro,Ri | 0 | 0 | 3 | 1 | 10 | 1 | 0 | 0 | <u>15</u> |
| Dothideomycetes | Pleosporales | Didymellaceae | <i>Phoma</i> To,Ti,Ro,Ri | 2 | 2 | 2 | 3 | 1 | 1 | 2 | 1 | <u>14</u> |
| Sordariomycetes | Diaporthales | Melanconidaceae | <i>Piliella</i> To,Ti,Ro,Ri | 1 | 0 | 1 | 1 | 4 | 2 | 2 | 0 | <u>11</u> |
| Eurotiomycetes | Chaetothyriales | Herpotrichiellaceae | <i>Phaeoconiella</i> To,Ti,Ro | 0 | 0 | 0 | 0 | 8 | 2 | 0 | 0 | <u>10</u> |
| Dothideomycetes | Pleosporales | | <i>Pleosporales</i> To,Ti,Ro,Ri | 0 | 0 | 2 | 1 | 1 | 3 | 0 | 0 | 7 |
| Dothideomycetes | Pleosporales | Arthrosporiaceae | <i>Arthrosporiaceae</i> Ti | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 2 | 4 |
| Leotiomycetes | Helotiales | ? | <i>Gloeotinia</i> To,Ro | 0 | 0 | 2 | 0 | 2 | 0 | 0 | 0 | 4 |
| Ascomycetes | Helotiales | Dermateaceae | <i>Mollisia</i> To,Ti,Ro | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 4 |
| Dothideomycetes | Botryosphaerales | ? | <i>Sphaeropsidales</i> Ti,To,Ro | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 1 | 4 |
| Ascomycetes | ? | ? | Ascomycota To,Ri | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 3 |
| Agaricomycetes | Polyporales | Polyporaceae | <i>Trametes</i> To | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 3 |
| | Hypocreales | Hypocreaceae | <i>Acremonium</i> To | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 2 |
| Leotiomycetes | Helotiales | Sclerotiniaceae | <i>Botryotinia</i> To,Ti | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 |
| Sordariomycetes | Hypocreales | Nectriaceae | <i>Cylindrocarpum</i> Ti,Ro | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 2 |
| Dothideomycetes | Capnodiales | Davidiellaceae | <i>Davidiella</i> Ro,Ri | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 2 |
| Dothideomycetes | Pleosporales | Lophiostomataceae | <i>Lophiostoma</i> spp. Ro | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 |
| Sordariomycetes | Calosphaerales | Calosphaeriaceae | <i>Phaeoacremonium</i> Ro | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 2 |
| Dothideomycetes | Pleosporales | Diothioraceae | <i>Aureobasidium</i> To | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| Leotiomycetes | Helotiales | ? | <i>Cadophora</i> Ti | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| Agaricomycetes | Polyporales | Polyporaceae | <i>Cerrena</i> Ti | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| Sordariomycetes | Sordariales | Chaetomiaceae | <i>Chaetomium</i> To | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| Sordariomycetes | Xylariales | Diatrypidae | <i>Eutypa</i> Ri | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| Leotiomycetes | Helotiales | ? | <i>Hyalodendriella</i> Ti | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| Sordariomycetes | Hypocreales | | <i>Hypocreales</i> To | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Dothideomycetes | Pleosporales | Didymellaceae | <i>Leptosphaerulina</i> Ti | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| Dothideomycetes | Pleosporales | Massariaceae | <i>Massaria</i> Ti | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| Dothideomycetes | Botryosphaerales | Botryosphaeriaceae | <i>Microdiplodia</i> Ro | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| ? | Mortierellales | Mortierellaceae | <i>Mortierella</i> Ri | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| ? | Mortierellales | Mortierellaceae | <i>Mycena</i> Ti | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| Sordariomycetes | Hypocreales | ? | <i>Myrothecium</i> Ri | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| Sordariomycetes | ? | ? | <i>Plectosphaerella</i> Ro | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| Dothideomycetes | Pleosporales | Sporormiaceae | <i>Preussia</i> Ri | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| Sordariomycetes | Xylariales | Amphisphaeriaceae | <i>Truncatella</i> Ri | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| Dothideomycetes | Pleosporales | Pleosporaceae | <i>Ulocladium</i> Ti | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Sordariomycetes | Hypocreales | ? | <i>Verticillium</i> Ro | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |

PI asy: Asymptomatic plants; PI sym: Symptomatic plants

Ti: Inner trunk; To: Outer trunk; Ri: Inner rootstock; Ro: Outer rootstock

Table 3. Alpha diversity of the fungal genera isolated from the esca-foliar symptomatic and asymptomatic vines.

| | Richness (S) | Abundance | Shannon (H) | Simpson (1-D) | Evenness (J) |
|-------------------------------|--------------|-----------|-------------|---------------|--------------|
| April symptomatic plants | 16 | 144 | 2.05 | 0.49 | 0.18 |
| April asymptomatic plants | 17 | 114 | 2.14 | 0.47 | 0.19 |
| June symptomatic plants | 24 | 104 | 2.76 | 0.36 | 0.2 |
| June asymptomatic plants | 26 | 128 | 2.51 | 0.4 | 0.2 |
| September symptomatic plants | 24 | 122 | 2.57 | 0.39 | 0.2 |
| September asymptomatic plants | 29 | 163 | 2.49 | 0.4 | 0.2 |
| January symptomatic plants | 33 | 153 | 2.87 | 0.35 | 0.21 |
| January asymptomatic plants | 33 | 141 | 2.98 | 0.34 | 0.21 |

Table 4. Fungal species ranked by sampling dates and the different part of the vines (the ITS region of these isolates were sequenced).

| Species | April symptomatic plants | | | | April asymptomatic plants | | | | June symptomatic plants | | | | June asymptomatic plants | | | | September symptomatic plants | | | | September asymptomatic plants | | | | January symptomatic plants | | | | January asymptomatic plants | | | | | | | |
|--|--------------------------|----|----|----|---------------------------|----|----|----|-------------------------|----|----|----|--------------------------|----|----|----|------------------------------|----|----|----|-------------------------------|----|----|----|----------------------------|----|----|----|-----------------------------|----|----|----|---|---|---|---|
| | Ti | To | Ri | Ro | Ti | To | Ri | Ro | Ti | To | Ri | Ro | Ti | To | Ri | Ro | Ti | To | Ri | Ro | Ti | To | Ri | Ro | Ti | To | Ri | Ro | Ti | To | Ri | Ro | | | | |
| <i>Acremonium spp.</i> (2) | | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | | 1 | | | | | | | |
| <i>Alternaria alternata</i> (13) | | 2 | | | | | 1 | | | | | | | | | | 1 | 2 | 1 | | | | | | 1 | | | | | | | | 1 | 1 | 2 | |
| <i>Alternaria brassicae</i> (2) | | | | | | | | | | | | | | | | | 1 | | | | | | | | 1 | | | | | | | | | | | |
| <i>Alternaria spp.</i> (7) | | | | | | | | | 1 | | | | | | | | 2 | | 1 | | | | | | 1 | 1 | 1 | | | | | | | | | |
| <i>Arthopyreniaceae spp.</i> (2) | | | | | | | | | | | | | 1 | | 1 | | | | | | | | | | | | | | | | | | | | | |
| <i>Arthrinium arundinis</i> (4) | | | | | | | | | | | 1 | 3 | | | | | | | | | | | | | | | | | | | | | | | | |
| <i>Arthrinium sacchari</i> (9) | | | | | | | | | | | | | | | | | | | | | | | 1 | | 2 | 3 | 3 | | | | | | | | | |
| <i>Arthrinium spp.</i> (2) | | | | | | | | | | | | | | | | | | | | | | | | | 2 | | | | | | | | | | | |
| <i>Ascomycota sp.</i> (3) | | | | | | | | | | | | | 1 | | | | | | | | 1 | | | | | | | | 1 | | | | | | | |
| <i>Aspergillus japonicus</i> (4) | | | | | | | | | | | | | | | | 1 | | | | | | | 1 | 1 | | | | | 1 | | | | | | | |
| <i>Aspergillus pseudodeflectus</i> (2) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| <i>Aspergillus ustus</i> (1) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| <i>Bionectria ochroleuca</i> (33) | 3 | 1 | 1 | | | | | | 1 | 2 | | 4 | | | | | 2 | 2 | | | 1 | 1 | | | | | | | 1 | 3 | 2 | 3 | 3 | | 1 | |
| <i>Bionectria spp.</i> (1) | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | | | | | | | | | | |
| <i>Botryosphaeria dothidea</i> (1) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 |
| <i>Botryosphaeria stevensii</i> (1) | | | | | | | | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| <i>Botryotinia fuckeliana</i> (1) | | | | | | | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| <i>Cadophora hiberna</i> (1) | | | | | | | | | | | 1 | | | | | | | | | | | | | | | | | | | | | | | | | |
| <i>Cerrena unicolor</i> (1) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 |
| <i>Chaetomium spp.</i> (1) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 |
| <i>Cladosporium cladosporioides</i> (14) | | | | | | | | | 1 | | | | 2 | | | | 3 | 1 | | | | | | | 1 | 1 | 1 | | 3 | | | | 1 | | | |
| <i>Cladosporium spp.</i> (1) | | | | | | | | | | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | |
| <i>Coprinellus spp.</i> (1) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 |
| <i>Cylindrocarpon sp.</i> (2) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 |
| <i>Davidiella tassiana</i> (2) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 |
| <i>Diplodia seriata</i> (72) | 6 | 3 | 1 | 3 | 4 | 5 | | | 3 | 4 | | | 1 | 1 | 3 | 1 | | 3 | | | 1 | 2 | 5 | 6 | 4 | 2 | 2 | 2 | 2 | 2 | 2 | 5 | 3 | | | |
| <i>Epicoccum nigrum</i> (19) | 1 | | 1 | | 1 | | 2 | | 1 | | 1 | | 3 | | | | | | | | 1 | 2 | 2 | 1 | | | | | | | 1 | 2 | | | | |
| <i>Epicoccum spp.</i> (1) | | | | | | | | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | | | |
| <i>Eutypa lata</i> (1) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 |
| <i>Fusarium equiseti</i> (1) | | | | | | | | | | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | |
| <i>Fusarium lacertarum</i> (1) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 |
| <i>Fusarium oxysporum</i> (42) | 4 | 2 | | | | | | | 1 | 1 | 4 | 1 | 1 | | 3 | | | 1 | 2 | 2 | | 2 | | | | 2 | 2 | 1 | 1 | 3 | | 5 | 3 | 1 | | |
| <i>Fusarium solani</i> (1) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 |
| <i>Fusarium sp.</i> (11) | | | | | | | | | | | | | 1 | 2 | | | | | | | 1 | | | | 1 | 2 | | | | | | | | | | 2 |
| <i>Gleotinia tumulenta</i> (4) | | | | | | | | | | | | | | | | | | | | | | | | | 2 | | | | | | | | | | | |
| <i>Hyalodendriella sp.</i> (1) | | | | | | | | | | | 1 | | | | | | | | | | | | | | | | | | | | | | | | | |
| <i>Hypocrea sp.</i> (1) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 |
| <i>Leptophaerulina chartarum</i> (1) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 |

The species in bold and underline are the most abundant species isolated and sequenced.

In brackets are the numbers of isolates sequenced per species.

Ti: Inner trunk; To: Outer trunk; Ri: Inner rootstock; Ro: Outer rootstock

Table 5. Distribution of haplotypes per fungal species for the 6 most abundant fungal species colonizing the wood of esca-foliar symptomatic and asymptomatic plants

| Fungi species | Number of sequences | Haplotypes | April symptomatic plants | April asymptomatic plants | June symptomatic plants | June asymptomatic plants | September symptomatic plants | September asymptomatic plants | January symptomatic plants | January asymptomatic plants | |
|-------------------------------|---------------------|------------|--------------------------|---------------------------|-------------------------|--------------------------|------------------------------|-------------------------------|----------------------------|-----------------------------|---|
| <i>Diplodia seriata</i> | 72 | H1 (60) | 12 | 7 | 6 | 7 | 9 | 5 | 5 | 9 | |
| | | H2 (12) | 1 | 2 | 1 | | 1 | 7 | | | |
| <i>Fusarium oxysporum</i> | 42 | H3 (40) | 5 | 3 | 6 | 6 | 4 | 5 | 3 | 8 | |
| | | H4 (1) | | | | | | 1 | | | |
| | | H5 (1) | | | | | | | | | 1 |
| | | H6 (24) | 4 | | 6 | 2 | 2 | 2 | 5 | 3 | |
| <i>Bionectria ochroleuca</i> | 33 | H7 (6) | 1 | | 1 | | | | 2 | 2 | |
| | | H8 (1) | | | | | 1 | | | | |
| | | H9 (1) | | | | | | | | | 1 |
| | | H10 (1) | | | | | 1 | | | | |
| <i>Neofusicoccum parvum</i> | 32 | H11 (23) | | 3 | 7 | 2 | 7 | | | 4 | |
| | | H12 (9) | 1 | | | | 3 | 2 | 2 | 1 | |
| <i>Trichoderma atroviride</i> | 19 | H13 (19) | 8 | 9 | | | | | 1 | 1 | |
| <i>Trichoderma gamsii</i> | 31 | H14 (28) | 2 | 1 | 2 | 6 | 5 | 4 | 4 | 4 | |
| | | H15 (3) | 1 | | | | | 2 | | | |

In brackets are the numbers of isolates sequenced per haplotype.

Publication n°3

“Old *Vitis vinifera*: when pathogenic fungal patterns of grapevine trunk diseases are not always associated with foliar disease expression or plant decline”

Phytopathology (Submitted)

Old *Vitis vinifera*: when pathogenic fungi patterns of grapevine trunk diseases are not always associated with foliar disease expression or plant decline

Emilie Bruez, Sylvie Bastien, Lucia Guerin-Dubrana, Patrice Rey

INRA, ISVV, UMR1065 Santé et Agroécologie du Vignoble (SAVE), F-33140 Villenave d'Ornon, France.

Université de Bordeaux, ISVV, UMR1065 SAVE, Bordeaux Sciences Agro, F-33140 Villenave d'Ornon, France.

Abstract. The present experiment was made with regard to the new epidemic of grapevine trunk diseases (GTDs) that is occurring worldwide. Generally old vines are free of these diseases, with only a few of them dying because of GTDs. So we focused our attention on the endophytic fungal microflora colonizing the wood functional tissues of the cultivar Baco blanc (42 and 58 year-old) in order to understand what occur in these grapevines. Grapevines were planted in the same vineyard, with the same soil, they did not express GTDs leaf symptoms the year of sampling. Molecular fingerprinting method, Single Strand Conformation Polymorphism (SSCP), shows that the global fungal microflora of 42 or 58 year-old plants tended to be different. This finding was confirmed by the cultivable strain identifications by DNA-ITS sequencing. SSCP also revealed that the fungal communities colonizing the cordon, and the various parts of the trunk, bottom, middle and top, of each plants are not clearly differentiated. Species numbers were higher in the 42 year-old plants, 41 species, than in the older plants, 30 species, but the biodiversity indexes did not allow us to differentiate the fungal endophytic of the, 42 or 58 year-old, plants. Many plant pathogens colonized the wood tissues, but in 58 year-old plants they were associated with esca, and in 42 year-old plants, with eutypa dieback. Numerous mycoparasite such as *Trichoderma* spp. were also detected in the functional wood of plants. The fact that the plants did not express the leaf symptoms of esca or eutypa dieback is discussed.

Introduction

Fungal endophytes are microorganisms inhabiting the internal tissues of plants without causing visible symptoms of disease (Hyde and Soyong, 2008; Petrini, 1991; Schulz and Boyle, 2005). They are ubiquitous, as shown by several authors, who reported that endophytes are detected in various tissues of plants, e.g. roots, woods, barks, petioles, fruits, leaves, and in almost all the plant species in which they have been sought. Fungal endophyte communities have been described, for instance, in palm trees (Fhrölich and Hyde, 1999), grapevines (Casieri *et al.*, 2009; Gonzalez and Tello, 2011; Hofstetter *et al.*, 2012; Pancher *et al.*, 2012), pine trees (Giordano *et al.*, 2009), forest trees (Arnold *et al.*, 2003; Espinosa-Garcia and Langenheim, 1990; Sun *et al.*, 2010; Suryanarayanan *et al.*, 2011). The composition of the endophyte assemblages depends on several factors: the sampling locality (Arnold *et al.*, 2001; Higgins *et al.*, 2007), the climate (Arnold and Lutzoni, 2007) and the age and pathogenic status of the plant (Arnold *et al.*, 2003). Fungal endophytes are not only associated with plants but also with lichens (Li *et al.*, 2007), mosses and fern (Petrini and Fischer, 1986), as well as sea grasses (Sanchez Marquez *et al.*, 2007; Sakayaroj *et al.*, 2010).

The ecological roles of fungal endophytes depend on the relationship they have with their hosts: they can be saprobes (Promputtha *et al.*, 2007; Tao *et al.*, 2008; Zhou and Hyde, 2001), mutualists (Carroll, 1988) or even latent pathogens (Carroll, 1988; Photita *et al.*, 2004; Sakalidis *et al.*, 2011). Thus, endophytic fungi have a pivotal role in the microbial ecosystem of the plant and in plant tolerance or resistance to biotic and abiotic stresses. They can stimulate growth of the hosts (Clay, 1986) and improve plant adaptation to water stress or resistance to pathogen damage (Arnold *et al.*, 2003; Redman *et al.*, 2002; Waller *et al.*, 2005; Saikkonen *et al.*, 2010). Many endophytes display plant protective activities, with some of them being known to be biocontrol agents. These endophytes can prevent, or at least restrict, the development of plant pathogens via direct or indirect mechanisms. (i) The direct mechanism includes the production of antibiotics that inhibit the growth of fungal phytopathogens and/or compete with them for space and nutrients. Endophyte *Trichoderma atroviride*, for instance, encodes enzyme like endochitinase, that has a high antifungal activity (Lorito and Woo, 1998a; Lorito *et al.*, 1998b). (ii) The indirect mechanism, such as the induction of plant systemic resistance, allows the plant to control pathogenic attacks (Arnold *et al.*, 2003; Gwinn and Gavin, 1992).

In plant pathology, one of the most exciting challenges is certainly to determine whether endophytic microflora change over time, and if they can impact the pathogenic status of the plant. This point could be addressed in grapevine trunk diseases (GTDs), because recent papers have demonstrated that the wood tissues of the plants are colonized by a range of endophytic fungal species, some being potentially plant protective or potentially plant pathogenic (Casieri *et al.*, 2009; Hofstetter *et al.*, 2012). Generally, this colonization is wood tissue-dependent, *i.e.* necrotic versus healthy tissues. It was reported that some fungal species are predominant within specific necroses that developed frequently within the grapevines; for instance, *Fomitiporia mediterranea* is predominant in white-rot (Fischer, 2002), *Phaeomoniella chlamydospora* is the most frequent in central necrosis (Mugnai *et al.*, 1999),

Botryosphaeria spp. are abundant in sectorial necrosis (Larignon *et al.*, 2009; White *et al.*, 2011). These fungi and necroses are associated with GTDs called esca, botryosphaeria dieback, eutypa dieback. These diseases are currently the matter of great concern for the vine growers worldwide. It was estimated that around 11% of the vineyards in France are unproductive, mainly due to trunk diseases. Recent experiment made by Bruez (2013) have shown that more than 70% of the healthy wood of relatively young (10 year-old) plants were colonized by *Botryosphaeria* spp. (potentially plant pathogenic) and *Trichoderma* spp. (potentially plant protectant).

Certain studies have determined the diversity and ecological role of endophytic fungal communities of grapevines; the 1-30 year-old plants had symptoms of various diseases, or else were asymptomatic. Halleen *et al.* (2007) isolated several hyphomycete endophytes from asymptomatic or symptomatic vascular tissues of grapevines showing Petri disease symptoms in South Africa, and tested their pathogenicity in a glasshouse and a field trial. Recently, Hofstetter *et al.* (2012) reported that fungal communities colonizing the wood of esca-foliar symptomatic and asymptomatic plants were similar. The aim of other studies was to determine which endophytic fungi colonize the wood of healthy grapevines (Casieri *et al.*, 2009), those associated with the shoots, twigs, berries and leaves (Gonzalez *et al.*, 2010; Mostert *et al.*, 2000) or with the vascular system of rootstocks (Tiedemann *et al.*, 1988). Recently, Pancher *et al.* (2012) compared the fungal endophytic communities of the lateral vine shoots of grapevines planted in vineyards, but managed using different cultural systems, *i.e.* organic or integrated pest management.

Our objective was to study the endophytic fungal microflora colonizing the wood of older grapevines. This experiment was made with regard to the new epidemic outbreak of grapevine trunk diseases that is occurring in French vineyards. It was reported that the 20-25 year-old plants were mainly attacked by GTDs (Bruez, 2013), and that older vines were generally free of these diseases, with only a few of them dying because of TGDs. Here, we focused our attention on functional wood tissues, because they are the key tissues for plant growth and development. This plant part is also important because, according to Maher *et al.* (2012) wood necroses increased in the trunks as grapevines aged. In our experiment, old plants (42 and 58 year-old) of the cultivar Baco blanc was used; that cultivar, one of the few that are not grafted in France, was planted in the same vineyard with the same type of soil. The global fungal communities of the wood of the grapevines were studied: (i) by a fingerprinting method, Single Stand Conformation Polymorphism (SSCP) and (ii) after isolation of the fungal strains, they were identified by ITS-DNA sequencing. The fungal communities of the 42 and 58 year-old plants were subsequently compared.

Materials and Methods

Plant material and sampling

Experiments were made by uprooting non-grafted Baco blanc grapevines from one vineyard from the South-West of France (Armagnac wine-producing region). The grapevines were planted in a sandy soil, called "tawny sand". Baco blanc is a hybrid of the Folle blanche (*Vitis vinifera*) and the Noah (natural hybrid of *Vitis labrusca* and *Vitis rotundifolia*). In France, the Baco blanc cultivar is the only hybrid which produce vine in France and which is the direct producers of a "controlled designation of origin" (Appellation d'Origine Contrôlée, AOC), the brandy called, Armagnac. It is planted in 2103 hectares in the South-West of France. According to the Institut Français de la Vigne et du Vin, the cultivar Baco blanc is sensitive to phylloxera, flavescente dorée, mites and downy mildew on leaves. It is tolerant to *Botrytis cinerea* and relatively tolerant to powdery mildew attacks. No data are available regarding the tolerance of the cultivar to GTDs: esca, botryosphaeria dieback and eutypa dieback.

The sampling site was located in the Capdubos vineyard in the village of Parleboscq (AOC, Armagnac), France. During the 5 past years, the harvest made by the winegrower allowed him to obtain 80-90 hl/ha. No GTDs foliar symptoms have been observed the year of the plant sampling.

In order to study the fungal microflora colonizing the wood of grapevines, 7 plants 42 year-old and 7 others 58 year-old were uprooted from the same vineyard. Then, the cordons and the trunks were cut longitudinally. The trunk of these plants was cut in three distinctive parts (bottom, middle and upper parts). The functional wood was sampled and used for the cultivable and molecular biology experiments.

DNA extraction and fungal communities analyses

DNA extraction

For each plant of grapevine collected, 10 g of necrotic tissues were sampled in the cordons, in the higher trunk, middle trunk and lower trunk. All these samples were ground in liquid nitrogen with a one-ball mill of Dangoumau type and kept at -80°C prior to DNA extraction. DNA was extracted from 60-mg aliquots of woody tissues with the Indvisorb Spin Plant mini Kit (Eurobio, France) according to the manufacturer's instructions. DNA extracts were then quantified with a nanodrop (ND-1000, Thermoscientific, Labtech) and homogenized at a concentration of 10 ng μl^{-1} .

SSCP analysis

The pair of primers recognizing the mitochondrial large subunit rDNA gene, ML1-ML2 from White and co-workers (1990), was used for SSCP. DNA was amplified by PCR in an Eppgradient Mastercycler (Eppendorf) in a reaction mixture (25 μl final volume) consisting of 1 μl of DNA template (10 ng μl^{-1}), 0.2 mM of each dNTP, 1 ng μl^{-1} of BSA (New England

BioLabs), 0.2 μ M of each primer, Pfu Turbo buffer 1x and 0.05 unit of Pfu Turbo DNA polymerase (Stratagene/Agilent Technologies). The cycling parameters were 95°C for 2 min followed by 30 cycles at 95°C for 1 min, 56°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. The PCR products (~250 bp) were visualized by 2% TBE agarose gel electrophoresis prior to SSCP analysis.

SSCP analyses were performed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) equipped with four 36-cm long capillaries. One microliter of a PCR product was mixed with 18.8 μ l formamide Hi-Di (Applied Biosystems) and 0.2 μ l standard internal DNA molecular weight marker Genescan 400 HD ROX (Applied Biosystems). The sample mixture was denatured at 95°C for 5 min and immediately cooled on ice, and then loaded onto the instrument. The non-denaturing polymer consisted of 5.6% POP Conformational Analysis Polymer (Applied Biosystems), 10% glycerol, EDTA buffer 10x (Applied Biosystems) and water. The migration time was set to 2000 seconds, the voltage to 15 kV and the temperature was 32°C.

Samples were co-migrated with the fluorescent size standard (GeneScan-400 ROX) to allow comparison of migration profiles between samples. Patterns were aligned with StatFingerprints (version 2.0) and studied by Principal Component Analysis with R (version 2.11.1).

Fungal isolation and identification

Isolation and identification on agar media

For all the grapevines, plant samples consisted of 15 portions (0,5 x 0,5 x 0,2 cm³) of non-necrotic woody tissue from the trunk (three different parts). For this work 14 vines were studied and 630 pieces of wood were analyzed.

A surface sterilization method was used in order to suppress epiphytic fungi. Thus, fragments were immersed in a 5% calcium hypochlorite solution during 30 seconds, washed in sterile distilled water and dried on a sterile filter paper. For each analyzed vine part, 15 sterilized wood pieces were placed on Malt Agar (3 pieces per plate) and incubated at 25°C in the dark. The fungal strains development was monitored during 4 weeks. When fungal colonies emerged from plant tissues into the agar, mycelial fragments were transferred to new Malt Agar plates. Whenever possible, the taxonomic identification of the endophytic fungi was based on morphological and cultural features, and on examination of fruiting structures and conidia under the microscope. To confirm the identification of the most abundant species, i.e. and define the isolates not clearly identified because sporulation failed, molecular techniques were used based on the sequencing of the internal transcribed spacer (ITS) fragment of the rDNA region. For the fungi of interest and for the most abundant fungi, some DNAs' isolates were sequenced randomly.

Identification by sequencing of the ITS region

Fungal genomic DNA was isolated from fresh mycelium scraped at the surface of a Malt Agar plate with a sterile tip. Samples were freeze-dried over night (Alpha 1-4 LO plus,

Bioblock Scientific) and then grinded with a Tissue Lyser II (Retsch, Qiagen) during 1 min at a frequency of 29.9 s^{-1} . 400 μl of CTAB (1x) were added to each sample. After an incubation at 65°C during one hour, 400 μl of chloroform-isoamyl alcohol (24:1, v/v) were added and the samples centrifuged during 30 min at 3700 rpm. The aqueous phase was transferred in a new tube and 200 μl of isopropanol were added. Samples were then kept at -20°C over night for DNA precipitation. After a centrifugation during 20 min at 3700 rpm, the supernatant was discarded and 500 μl of ethanol 70% were added to wash the DNA. Once the ethanol discarded, pellets were air dried and then resuspended in 50 μl of sterile distilled water.

DNA samples were sent to GATC Biotech AG (Konstanz, Germany) for sequencing of the ITS region with the primers ITS1f and ITS4 (White et al., 1990; Chandrabali et al., 2001). Sequences were edited using CodonCode Aligner software and assigned to species based on 97% sequence similarity threshold of the ITS region. For species level identification, sequences were subjected to a bulk blastn search against the INSD (International Nucleotide Sequence Databases) as implemented in the PlutoF workbench of the UNITE database (Abarenkov et al., 2010). 421 endophytic fungi were identified by DNA sequencing of ITS region. DNA of the sterile fungi and some DNA of interesting fungi were sequenced and analyzed by PlutoF. To determine the species names, the percentage of sequence BLAST was at 99%.

Data analysis

SSCP analysis

Banding patterns obtained from community analysis (SSCP), all profiles were aligned by StatFingerprints R software (The R foundation for Statistical computing, ISBN 3-900051-07-0) and they were gathered in a single numerical database before being statistically described by a global Principal Component Analysis (PCA) by R software version 2.14.2. This database consisted of 14 individuals but according to the PCA between 56 samples and a total of 250 variables which were the numerical values of values measured (for each sample) on the 250 SSCP profile scans. PCA was performed on using the correlation coefficient of Pearson. Variables having a $\cos^2 \geq 0.5$ on one of the first third principal components (Dim1, Dim2 or Dim 3) were estimated as sufficiently well represented by the principal plane generated by this PCA.

Species diversity

For this study, we used specific diversity indexes, (i) species richness (total number of species observed), (ii) the Abundance (number of isolates), (iii) Shannon's diversity index, H' (Shannon and Weaver 1963, Magurran, 2004), (iv) the Simpson index, D and the Evenness, J , obtained by the Shannon's equitability index and the dominance. Evenness indice was estimated to establish the closeness of equitability of species present (Gotelli and Colwell 2001). These indexes were estimated by using package *Agricolaea* of R software version 2.14.2.

Rarefaction curve, diversity estimates

Species accumulation curve was used to estimate the number of species in a given sample for non necrotic tissues of grapevines. The data of the seven plants which had the same age were pooled. The estimator of the total number of species and of the species without the singletons was calculated (Colwell, 2005).

Results

Status of the wood of the grapevines

When the plants of Baco blanc were longitudinally cut, it was observed that the trunk was empty inside, particularly the 58 year-old plants. Necrotic wood tissues were predominant, white-rot (also called amadou), a tissue specific to esca, was observed in the cordons of 4 out of 7 plants aged 58 years (not in the 42 year-old plants). The trunk non-grafted of these plants was cut in three distinctive parts (upper, middle and downy parts). The pieces of wood corresponding to the functional tissue (outer trunk wood) look healthier and non-necrotic, samples have been taken in this wood tissue.

Analyses of fungal communities

From the profiles obtained by SSCP analyses, Principal Component Analyses were obtained (i) to compare fungal communities of non-necrotic functional wood tissues of 42 and 58 year-old plants and (ii) to observe the distribution of the fungal communities in the different part of each plant.

Figure 2, PCA eigenvalues indicated that the first two principal component, Dim 1 and Dim 2, explained 62,89% of the total data variances. For each vine, samples of the arm and of the the upper-, middle- and down parts of the trunks were used. The cluster that represent the fungal microflora of 42 year-old plants is clearly separated from this of the older grapevines by Dim 1, respectively negative versus positive coordinates.

Figure 3 shows a PCA of the samples of the seven 42 year-old plants. For each plant, the SSCP profiles of the fungal microflora from the trunk (up, middle, down) and arm were compared, the first two principal components, Dim 1 and Dim 2, explained 68,38% of the data variances. Note that for each part, for instance the arm, all the samples of the 7 vines were pooled. The clusters that represent the different parts of the grapevines are not separated by Dim 1 and Dim2, respectively negative versus positive coordinates. The same result was obtained with the 58 year-old grapevines (not shown).

Colonization rate

The percentage of pieces of wood colonized by at least one fungus varied from 60 to 100% (Fig. 4.). T-test showed that there was a significantly difference between the frequencies of the plants aged 42 or 58 years (p-value= 0.0415), the highest frequencies were obtained with the younger grapevines. The highest colonization frequency, *i.e.* 100%, was found in three 42 year-old plants and in one-58 year-old. The lowest colonization, *i.e.* 60%, was obtained in one of the oldest plants.

Endophytic isolation and identification

DNA-ITS region sequencing of 422 fungal isolates has been made, 219 endophytes were obtained from the oldest plants and 203 from the younger. Among these 421 isolates, 103 were identified to the species (61 from 58 year-old and 42 from 42 year-old plants) and 318 to the genus level. Morphological and DNA-ITS region sequencing identification coincided for all the isolates.

Distribution of endophytic communities

Ascomycota was the predominant division (83%) representing 11 orders followed by the Basidiomycota (13%), 3 orders, and one order for the Zygomycota (4%). Figure 5 showed the distribution of the orders for the 14 vines. The most representative order was the Hypocreales (Ascomycota), it was identified in each plant. The other orders were in decrease the Xylariales (Ascomycota), the Eurotiales (Ascomycota) and the Diaporthales (Ascomycota). The Saccharomycetales (Ascomycota) were isolated only in the youngest plants and the Botryosphaeriales (Ascomycota) in three 58 year-old plants. The Basidiomycota were identified in the two types of plants, the highest number of isolates were in the youngest plants.

Forty genera were isolated in the 58 year-old plants and 30 in the 42 year-old plants. Isolate richness was equally distributed among endophytic species (Fig. 6). Figure 7 showed the number of isolates of the 13 most abundant species isolated from the 42 and 58 year-old plants. In the oldest plants, 4 dominant species accounted for 50% of all isolates: *Eutypa lata*, *Trichoderma sp.*, *Trichoderma harzianum*, *Penicillium sp.* For the younger plants, 5 taxa accumulated 50% of all isolates: *Phaeoacremonium aleophilum*, *Fomitiporia mediterranea*, *Penicillium sp.*, *Stereum hirsutum*, *Phaeomoniella chlamydospora* (Fig. 7). The isolate richness was a little more dispersed among the taxa. In contrast with the plurals species, there were 18 singleton isolates for the oldest plants and 8 singletons for the youngest (Fig. 6). Note that 3 species were significantly more isolated for the oldest plants: *Eutypa lata*, *Trichoderma sp.* and *Bionectria ochroleuca*. Five species colonized more specifically the youngest plants: *Phaeoacremonium aleophilum*, *Fomitiporia mediterranea*, *Stereum hirsutum*, *Coniochaete velutina* and *Scytalidium cuboideum* (Fig. 7).

Figure 8 shows the global distribution of the species of each plant. PCA eigenvalues indicates that the first two principal component, Dim 1 and Dim 2, explains 27.92% of the total data variances. The points of the plants aged 42 years are grouped conversely to those of the oldest plants (Fig. 8).

Endophytic biodiversity of the Baco's grapevines

Forty six species were identified in the grapevines of Baco, 41 species in the oldest plants and 30 species in the youngest. The species diversity of each plant were pooled and shown on the figure 9. Comparing the two types of plants, the species richness is higher for

the plants aged 58 years (12.1) than for the youngest plants (10) but there is no significant difference (Kolmogorov-Smirnov test; pvalue = 0,9379). For the two types of plants, values of Shannon-Wieder index (H') were not significantly different: 2,1 for the oldest and 1,85 for the young (KS test; pvalue= 0,2121). The index de Simpson, shows also that the index values are not significantly different (KS test; pvalue = 0,2032). The Evenness (J') shows that there is no differences between plants aged 58 years (0,49) and younger plants (0,52) (KS test; pvalue= 0,2032).

On Figure 10, species accumulation were plotted to study the relationship between the number of fungal taxa isolated and the number of plants analyzed To calculate the species accumulation curves (Sobs), the Mao-Tau estimator was used to compare the species richness with a confidence interval up to 95%. Species accumulation for the endophytic mycobiota from the Baco plants with two different ages did not approach the asymptote. But the curves representing the plurals and doubles taxa were asymptotic.

Discussion

In the present experiment, the extent to which the necroses developed in the trunk or cordons of the 42 or 58 year-old grapevines was very high. In the trunk, the wood of the inner part was totally damaged and only the outer part, corresponding to the functional tissues looked healthier. The wood samples were taken from these non-necrotic areas, either from the trunks or the cordons. In spite of the great wood degradations, the grapevines produced berries that were used to produce brandy (Armagnac) and, compared with the 5 previous years, no yield losses were noticed by the winegrower. It is also worth mentioning that, in the year of sampling, the plants did not express leaf symptoms of trunk wood diseases, *i.e.* esca, eutypa or botryosphaeria diebacks. So, these plants were appropriate for studying the fungal microflora that colonize the functional tissues of old grapevines. They were also appropriate for determining whether pathogens did or did not colonize these structures that are vital for plant growth and life.

As shown by SSCP, the global fungal microflora of 42 or 58 year-old plants tended to be different. This finding was confirmed by the cultivable strain identifications by DNA-ITS sequencing. SSCP also revealed that the fungal communities colonizing the cordon, and the various parts of the trunk, bottom, middle and top, are not clearly differentiated. This suggests that the same fungal assemblages colonize the apparently healthy wood tissues sampled. This result is in accordance with those obtained in another study by Bruez (2013), which compared the various wood tissues, necrotic or not, of younger (10 year-old) plants. The authors observed that, depending on the sampling tissues within the plant, the bark, the specific necrosis associated with esca called white-rot and the other wood tissues that looked healthy, marked differences were observed in the colonizing fungal microflora. Thus, when the results of young and old grapevines were combined, they provided evidence that fungal colonization is wood tissue dependent. As reported in the literature, other factors, such as the sampling locality (Arnold *et al.*, 2001; Higgins *et al.*, 2007) and climate (Arnold and Lutzoni, 2007) may have an influence on the endophytic fungal microflora composition. Arnold *et al.* (2003) reported that, for tropical fungal endophytes, the age and the pathogenic status of the plant may also exert an influence. In our experiment, as the grapevines were planted in the same soil in one vineyard, and were submitted to the same climate, our results suggest that, in addition to tissue-dependence, the fungal microflora colonizing the grapevine trunk is also age dependent.

From the 42 and 58 year-old grapevines, 421 fungal strains were isolated and identified by DNA-ITS-sequencing. Forty six taxa were obtained from 630 pieces of wood. Eighty three percent were Ascomycota (92.2% in 42 year-old plants; 73.6% in 58 year-old), 13% were Basidiomycota (3.2% in 42 year-old; 23.1% in 58 year-old) and 4% were Zygomycota (4.6% in 42 year-old; 3.2% in 58 year-old). This distribution pattern of Ascomycota is consistent with other studies on the endophytic communities of woody tissues or shoots and the leaves of different plant hosts (Bills, 1996; Fröhlich *et al.*, 2000; Rungjindamai *et al.*, 2008), including the grapevines (Hofstetter *et al.*, 2012; Pancher *et al.*,

2012). Previous papers have shown that the Basidiomycota are the most abundant fungi isolated when the wood decay tissue is studied (Pouska *et al.*, 2010; Schmidt *et al.*, 2011). As we have mainly sampled the most preserved wood tissues, the proportion of Ascomycota versus Basidiomycota was as expected. A similar trend has recently been found by Bruez (2013) when she studied the mycoflora of the healthy wood of young (10 year-old) grapevines.

In the Baco plants sampled, the mycoflora was composed of 46 species, with many singleton species. Species numbers were higher in the 42 year-old plants, 41 species, than in the older plants, 30 species, but the biodiversity indexes did not allow us to differentiate the fungal endophytic of the, 42 or 58 year-old, plants. However, in comparison with the number of species we obtained, those found by Casieri *et al.* (2009) and Hofstetter *et al.* (2012) were higher. Casieri *et al.* (2009) isolated endophytic fungi from healthy one year-old vines and defined 66 operational taxonomic units (OTUs). Hofstetter *et al.* (2012) isolated 158 species from 15-30 year-old grapevine wood in a single Swiss vineyard plot. These differences may be related to the necrotic process in the Baco blanc-old plants, which is certainly more advanced. As reported by Maher *et al.* (2012), the diversity of fungal communities within trunk wood necroses is low, with generally one species being predominant. Thus one may assume, that in the “apparently preserved” wood tissues of old plants the same process develops as time goes by.

In our opinion, one of the most unexpected results concerns the markedly different fungal community patterns identified in the Baco blanc-plants of different ages. Many plant pathogens colonized the wood tissues, but in 58 year-old plants they were associated with esca, and in 42 year-old plants, with eutypa dieback.

In 42 year-old plants, the pathogen *Eutypa lata* (Xylariales order) was the most prevalent fungus. *E. lata* has long been known as the pathogenic agent of grapevine eutypa dieback. Its relationship with the plants, its genetic structure (Travadon *et al.*, 2012) and its ability to produce toxins (Andolfi *et al.*, 2011) have been thoroughly investigated. *E. lata* penetrates into the plant by the wounds, moves into the xylem vessels and causes canker in the wood tissues. It is also a wood colonizer that decomposes the cellulose of the xylem (Davis *et al.*, 2003).

In 58 year-old plants, the most numerous fungi are: *Phaeoacremonium aleophilum*, then *Fomitiporia mediterranea*, *Stereum hirsutum*, *Phaeoconiella chlamydospora* and *E. lata* (also responsible of eutypa dieback). Their involvement in the development of wood necroses and, consequently in esca, have been reported by many authors (Bertsch *et al.*, 2012; Laveau *et al.*, 2009). There is general agreement, however, that esca is complex, as shown by the experiments aimed at understanding the relationship between these fungi and the plant. For instance, the use of a logical approach which consists in verifying Koch’s postulate for the fungi presumably responsible for the disease, has failed many times. This type of experiment, that is valid for many pathogens, seems to be inappropriate for esca. For all the above-listed fungi, with the exception of *E. lata*, reproducing wood symptoms (necroses) by artificial inoculations is easy, but obtaining leaf symptoms is relatively difficult (Laveau *et al.*, 2009). In fact, the disease has to be considered as a relationship between the plant and the assemblage of fungi that colonize grapevine wood at a specific time. Shifts or a succession of

species over time in these assemblages may generate the wood necrosis typical of esca. The dynamics of necrotic development in wood tissues, which have been seldom been studied, remain largely speculative (Surico *et al.*, 2000). Larignon and Dubos (1997) proposed two processes of wood degradation involving a succession of fungi. The first of these processes, leading to the formation of central, light-coloured soft necrosis, includes three fungi operating in sequence: *P. aleophilum* and *P. chlamydospora*, followed by *F. mediterranea*. The second process leads to the development of light-coloured, soft, sectorial necrosis, primarily caused by *E. lata* followed, by *F. mediterranea*. Further studies aimed at determining this complex issue still remain to be made.

Although pathogens of esca or eutypa dieback are predominant in the wood sampled, the plants did not express the leaf symptoms associated with these diseases during the year of sampling. Seven points should be considered to obtain an overview of the situation:

(i) Regarding esca, Hofstetter *et al.* (2012) recently reported that it is not caused by biotic factors but rather by abiotic factors. According to these authors, the presumed esca-associated fungi are only able to decay already senescent or dead wood resulting from abiotic stresses, intensive pruning, frost or other mechanical injuries, like grafting. This assertion disagrees with many papers that have demonstrated that necroses developed in the grapevine wood tissues inoculated by *P. aleophilum*, *P. chlamydospora*, or *F. mediterranea*, whether in combinations or not (Bertsch *et al.*, 2012). Our experiment confirms that these fungi and *E. lata* colonize alive functional wood tissues, but are they latent pathogenic endophytes or only colonizers of dead wood? Based on our results, we could also state that *E. lata* is not a pathogen, its symptoms were not observed during the year of sampling. But, as we know that this assumption is not supported by the literature (Carter, 1991), the main reason is certainly that certain conditions (see *ii*, *iii* and *iv*) are not provided for the fungus to develop and cause the disease. The same reasoning can probably be applied for esca.

(ii) From one year to another the same grapevines do not necessary express foliar symptoms of eutypa dieback or esca. So, symptomatic expression of the two diseases may have occurred in the previous years. To support this assumption, it is worth mentioning that, white-rot (also called amadou), which is the best descriptor of esca (Maher *et al.*, 2012) has only been observed in 4 out of 7 plants colonized by the “fungal communities-esca pattern”.

(iii) With esca, Maher *et al.* (2012) recently demonstrated that wood necroses formed a continuum within the plant, and that the volume of necroses is useful to determine the health status of the vines. The more the necroses developed, particularly in the functional wood tissues, the more esca developed. In the present case, this critical point is certainly not reached, and the functional wood tissues are sufficiently preserved for the plants to stay alive. Consequently, esca foliar symptoms cannot be expressed.

(iv) If pathogens colonize the apparently healthy wood tissues, potentially plant protectant fungi such as *Trichoderma* spp. do so too. In the 42 year-old plants, if the various species of the genus *Trichoderma* are counted, *Trichoderma* sp., *T. harzianum*, *T. atroviride*, their number even exceeds that of *E. lata*. *Trichoderma* spp. are known to be opportunistic and avirulent plant symbionts (Harman *et al.*, 2004) and the antagonist activity of some *Trichoderma* spp. strains against pathogenic fungi has been extensively described (Perveen and Bokhari, 2012; Schwarze *et al.*, 2012). Generally, that activity consists in mycoparasitism

and antibiosis (Brunner *et al.*, 2005; Lu *et al.*, 2004). So, in the present case, it can be assumed that *Trichoderma* spp.; similarly another mycoparasite, *Bionectria ochroleuca*, interacts in the wood with other fungi, such as esca-pathogenic fungi and *E. lata*. These interactions may create a balance in favor of the preservation of the functional wood tissues of the plant. Consequently, esca or eutypa dieback foliar symptoms cannot be expressed.

(v) Did the second process of fungal colonization hypothesized by Larignon and Dubos (1997) occur in the present study? Those authors reported that *E. lata* colonization was followed by that of *F. mediterranea*. In the 42 year-old plants we found mainly *E. lata* and a few strains of *F. mediterranea*, whereas the contrary was the case for the older grapevines. We cannot currently provide evidence of this shift, but this point is worth being mentioned.

(vi) Another fungi, *Psathyrella candolleana*, is relatively abundant in the grapevine wood (5 strains in 42 year-old plants and 10 strains in 58 year-old ones). It is known as a wood decay fungus that is able to selectively decompose carbohydrates in the wood (Fukasawa *et al.*, 2005). So, some fungal species may participate in wood degradation without displaying pathogenic behavior for the grapevine.

(vii) The cultivar used, Baco blanc, is less sensitive to some diseases, such as *B. cinerea* and, to a lesser extent to powdery mildew. Is this cultivar less sensitive to trunk wood diseases? This could be assumed to be the case, because colonization of grapevine wood by pathogens did not lead to plant decline. This point is of the utmost importance and further studies are urgently required.

The species accumulation curves observed in this survey indicated that increased sampling would have led to the discovery of a richer mycobiota. The fact that species accumulation curves plotted with data from plural species tend to have an asymptotic shape, shows that the new species which could be found by increasing sampling effort would be a unique species. Other studies reported the same kind of results (Gonzalez *et al.*, 2010; Marquez *et al.*, 2007; Marquez *et al.*, 2010; Suryanarayanan *et al.*, 2011). So, to further study the fungal assemblages, cultivable-free methods should be used, either DNA-cloning (Seena *et al.*, 2008) or pyrosequencing (Nilsson *et al.*, 2009).

To conclude, our investigation shows that the plants of different ages are colonized by fungal communities associated either with esca or eutypa-dieback diseases. However, the foliar symptoms were not observed during the year of sampling but interestingly, the harvest allowed the winegrower to make brandy. So should we consider the plant is diseased or not? Certainly, within the root tissues the microbial balance between the potentially plant protectant and plant pathogenic fungi prevents the grapevine from developing diseases. So research has to be carried out to understand how this fragile equilibrium can be used to favor plant beneficial microflora. This should open up new opportunities for allowing the plants to be protected.

References

- Abarenkov K, Nilsson RH, Larsson KH, Alexander IJ, Eberhardt U, Erland S, Hoiland K, Kjoller R, Larsson E, Pennanen T, Sen R, Taylor AFS, Tedersoo L, Ursing BM, Vralstad T, Liimatainen K, Peintner U, Koljalg U (2010) The UNITE database for molecular identification of fungi - recent updates and future perspectives. *New Phytol* **186**: 281-285
- Andolfi A., L. Mugnai, J. Luque, G. Surico, A. Cimmino and A. Evidente, 2011. Phytotoxins Produced by Fungi Associated with Grapevine Trunk Diseases. *Toxins* 3, 1569-1605.
- Arnold AE, Lutzoni F (2007) Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots? *Ecology* **88**: 541-549
- Arnold AE, Maynard Z, Gilbert GS, Coley PD, Kursar TA (2000) Are tropical fungal endophytes hyperdiverse? *Ecol Lett* **3**: 267-274
- Arnold AE, Mejia LC, Kyllö D, Rojas EI, Maynard Z, Robbins N, Herre EA (2003) Fungal endophytes limit pathogen damage in a tropical tree. *Proc Natl Acad Sci U S A* **100**: 15649-15654
- Bertsch C, Ramirez-Suerrot M, Magnin-Robert, Larignon P, Chong J, Abou-Mansour E, *et al.* (2012) Grapevine trunk diseases: complex and still poorly understood. *Plant Pathol* DOI: 10.1111/j.1365-3059.2012.02674.x.
- Bills GF (1996) *Isolation and analysis of endophytic fungal communities from woody plants*: American Phytopathological Society (APS Press).
- Bruetz E. 2013. Comparison of the fungal and bacterial communities that colonize the wood tissues of esca-foliar symptomatic or asymptomatic grapevines. PhD thesis, University of Bordeaux, France.
- Brunner K, Zeilinger S, Ciliento R, Woo SL, Lorito M, Kubicek CP, *et al.* (2005) Improvement of the fungal biocontrol agent *Trichoderma atroviride* to enhance both antagonism and induction of plant systemic disease resistance. *Appl Environ Microbiol* **71**: 3959-3965
- Carroll G (1988) Fungal endophytes in stems and leaves: from latent pathogen to mutualist symbionts. *Ecology* **69**: 2-9
- Carter M.V., 1991. The status of *Eutypa lata* as a pathogen. Monograph. Phytopathological Paper No. 32, International Mycological Institute, Egham, UK.
- Casieri L, Hofstetter V, Viret O, Gindro K (2009) Fungal communities living in the wood of different cultivars of young *Vitis vinifera* plants. *Phytopathologia Mediterranea* **48**: 73-83
- Chanderbali AS, van der Werff H, Renner SS (2001) Phylogeny and historical biogeography of Lauraceae: Evidence from the chloroplast and nuclear genomes. *Ann Mo Bot Gard* **88**: 104-134
- Clay K (1986) Induced vivipary in the sedge cyperus: virens and the transmission of the fungus *Balansia cyperi* (Clavicipitaceae). *Can J Bot-Rev Can Bot* **64**: 2984-2988
- Colwell RK. (2005) EstimateS: statistical estimation of species richness and shared species from samples, version 8. purl.oclc.org/estimates, Vol. 2005.

- Davis EC, Franklin JB, Shaw AJ, Vilgalys R (2003) Endophytic Xylaria (Xylariaceae) among liverworts and angiosperms: Phylogenetics, distribution, and symbiosis. *Am J Bot* **90**: 1661-1667
- Espinosagarcia FJ, Langenheim JH (1990) The endophytic fungal community in leaves of coastal redwood population : diversity and spatial patterns. *New Phytol* **116**: 89-97
- Fischer M (2002) A new wood-decaying basidiomycete species associated with esca of grapevine: *Fomitiporia mediterranea* (Hymenochaetales). *Mycological Progress* **1**: 315-324
- Frohlich J, Hyde KD (1999) Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic? *Biodivers Conserv* **8**: 977-1004
- Frohlich J, Hyde KD, Petrini O (2000) Endophytic fungi associated with palms. *Mycol Res* **104**: 1202-1212
- Fukasawa Y, Osono T, Takeda H (2005) Decomposition of Japanese beech wood by diverse fungi isolated from a cool temperate deciduous forest. *Mycoscience* **46**: 97-101
- Giordano L, Gonthier P, Varese GC, Miserere L, Nicolotti G (2009) Mycobiota inhabiting sapwood of healthy and declining Scots pine (*Pinus sylvestris* L.) trees in the Alps. *Fungal Divers* **38**: 69-83
- Gonzalez V, Tello ML (2010) The endophytic mycota associated with *Vitis vinifera* in central Spain. *Fungal Divers* **47**: 29-42
- Gotelli NJ, Colwell RK (2001) Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. *Ecol Lett* **4**: 379-391
- Gwinn KD, Gavin AM (1992) Relationship between endophyte infestation level of tall fescue seed lots and Rhizoctoniaeae seedling disease. *Plant Dis* **76**: 911-914
- Halleen F, Mostert L, Crous PW (2007) Pathogenicity testing of lesser-known vascular fungi of grapevines. *Austral Plant Pathol* **36**: 277-285
- Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004) Trichoderma species - Opportunistic, avirulent plant symbionts. *Nat Rev Microbiol* **2**: 43-56
- Higgins KL, Arnold AE, Miadlikowska J, Sarvate SD, Lutzoni F (2007) Phylogenetic relationships, host affinity, and geographic structure of boreal and arctic endophytes from three major plant lineages. *Mol Phylogenet Evol* **42**: 543-555
- Hofstetter V, Buyck B, Croll D, Viret O, Couloux A, Gindro K (2012) What if esca disease of grapevine were not a fungal disease? *Fungal Divers* **54**: 51-67
- Hyde KD, Soyong K (2008) The fungal endophyte dilemma. *Fungal Divers* **33**: 163-173
- Johnston PR, Johansen RB, Williams AFR, Paula Wikie J, Park D (2012) Patterns of fungal diversity in New Zealand *Nothofagus* forests. *Fungal Biol* **116**: 401-412
- Larignon P, Dubos B (1997) Fungi associated with esca disease in grapevine. *Eur J Plant Pathol* **103**: 147-157
- Larignon P, Fontaine F, Farine S, Clement C, Bertsch C (2009) Esca and Black Dead Arm: two major actors of grapevine trunk diseases. *C R Biol* **332**: 765-783
- Laveau C, Letouze A, Louvet G, Bastien S, Guerin-Dubrana L (2009) Differential aggressiveness of fungi implicated in esca and associated diseases of grapevine in France. *Phytopathologia Mediterranea* **48**: 32-46
- Li WC, Zhou J, Guo SY, Guo LD (2007) Endophytic fungi associated with lichens in Baihua mountain of Beijing, China. *Fungal Divers* **25**: 69-80

- Lorito M, Woo SL (1998) *Advances in understanding the antifungal mechanism(s) of Trichoderma and new applications for biological control*, Vol. 21.
- Lorito M, Woo SL, Fernandez IG, Colucci G, Harman GE, Pintor-Toro JA, *et al.* (1998) Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. *Proc Natl Acad Sci U S A* **95**: 7860-7865
- Lu ZX, Tombolini R, Woo S, Zeilinger S, Lorito M, Jansson JK (2004) In vivo study of Trichoderma-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. *Appl Environ Microbiol* **70**: 3073-3081
- Magurran AE (2004) *Measuring biological diversity*.: Oxford:Blackwell Science.
- Maher N, Piot J, Bastien S, Vallance J, Rey P, Guerin-Dubrana L (2012) Wood necrosis in esca-affected vines: types, relationships and possible links with foliar symptoms expression. *J Int Sci Vigne Vin* **46**: 15-27
- Marquez SS, Bills GF, Acuna LD, Zabalgoceazcoa I (2010) Endophytic mycobiota of leaves and roots of the grass *Holcus lanatus*. *Fungal Divers* **41**: 115-123
- Marquez SS, Bills GF, Zabalgoceazcoa I (2007) The endophytic mycobiota of the grass *Dactylis glomerata*. *Fungal Divers* **27**: 171-195
- Mostert L, Crous PW, Petrini O (2000) Endophytic fungi associated with shoots and leaves of *Vitis vinifera*, with specific reference to the *Phomopsis viticola* complex. *Sydowia* **52**: 46-58
- Mugnai L, Graniti A, Surico G (1999) Esca (Black Measles) and Brown Wood Streaking: Two Old and Elusive Diseases of Grapevines. *Plant Dis* **83**: 404-417
- Nilsson RH, Ryberg M, Abarenkov K, Sjökvist E, Kristiansson E (2009) The ITS region as a target for characterization of fungal communities using emerging sequencing technologies. *FEMS Microbiol Lett* **296**: 97-101
- Pancher M, Ceol M, Corneo PE, Longa CMO, Yousaf S, Pertot I, Campisano A (2012) Fungal Endophytic Communities in Grapevines (*Vitis vinifera* L.) Respond to Crop Management. *Appl Environ Microbiol* **78**: 4308-4317
- Perveen K, Bokhari NA (2012) Antagonistic activity of *Trichoderma harzianum* and *Trichoderma viride* isolated from soil of date palm field against *Fusarium oxysporum*. *Afr J Microbiol Res* **6**: 3348-3353
- Petrini O (1991) Microbial Ecology of leaves. Springer-Verlag (ed), Fungal endophytes of tree leaves, pp 179-197.
- Petrini O, Fisher PJ (1986) Fungal endophytes in *Salicornia perennis*. *Transactions of the British Mycological Society* **87**: 647-651
- Photita W, Lumyong S, Lumyong P, McKenzie EHC, Hyde KD (2004) Are some endophytes of *Musa acuminata* latent pathogens? *Fungal Divers* **16**: 131-140
- Pouska V, Svoboda M, Lepsova A (2010) The diversity of wood-decaying fungi in relation to changing site conditions in an old-growth mountain spruce forest, Central Europe. *Eur J For Res* **129**: 219-231
- Promptutha I, Lumyong S, Dhanasekaran V, McKenzie EHC, Hyde KD, Jeewon R (2007) A phylogenetic evaluation of whether endophytes become saprotrophs at host senescence. *Microb Ecol* **53**: 579-590
- Redman RS, Sheehan KB, Stout RG, Rodriguez RJ, Henson JM (2002) Thermotolerance generated by plant/fungal symbiosis. *Science* **298**: 1581-1581

- Rungjindamai N, Pinruan U, Choeyklin R, Hattori T, Jones EBG (2008) Molecular characterization of basidiomycetous endophytes isolated from leaves, rachis and petioles of the oil palm, *Elaeis guineensis*, in Thailand. *Fungal Divers* **33**: 139-161
- Saikkonen K, Saari S, Helander M (2010) Defensive mutualism between plants and endophytic fungi? *Fungal Divers* **41**: 101-113
- Sakalidis ML, Hardy GES, Burgess TI (2011) Endophytes as potential pathogens of the baobab species *Adansonia gregorii*: a focus on the Botryosphaeriaceae. *Fungal Ecol* **4**: 1-14
- Sakayaroj J, Preedanon S, Supaphon O, Jones EBG, Phongpaichit S (2010) Phylogenetic diversity of endophyte assemblages associated with the tropical seagrass *Enhalus acoroides* in Thailand. *Fungal Divers* **42**: 27-45
- Schulz B, Boyle C (2005) The endophytic continuum. *Mycol Res* **109**: 661-686
- Schmidt CS, Lorenz D, Wolf GA (2001) Biological control of the grapevine dieback fungus *Eutypa lata* I: Screening of bacterial antagonists. *J Phytopathol-Phytopathol Z* **149**: 427-435
- Schwarze F, Jauss F, Spencer C, Hallam C, Schubert M (2012) Evaluation of an antagonistic *Trichoderma* strain for reducing the rate of wood decomposition by the white rot fungus *Phellinus noxius*. *Biol Control* **61**: 160-168
- Seena S, Wynberg N, Barlocher F (2008) Fungal diversity during leaf decomposition in a stream assessed through clone libraries. *Fungal Divers* **30**: 1-14
- Shannon CE, Weaver, W. (1963) *The Mathematical Theory of Communication*: University of Illinois Press.
- Sun FF, Kuang YW, Wen DZ, Xu ZH, Li JL, Zuo WD, Hou EQ (2010) Long-term tree growth rate, water use efficiency, and tree ring nitrogen isotope composition of *Pinus massoniana* L. in response to global climate change and local nitrogen deposition in Southern China. *J Soils Sediments* **10**: 1453-1465
- Surico G, Marchi G, Braccini P, Mugnai L (2000) Epidemiology of esca in some vineyards in Tuscany (Italy). *Phytopathologia Mediterranea* **39**: 190-205
- Suryanarayanan TS, Venkatachalam A, Rajulu MBG (2011) A comparison of endophyte assemblages in transgenic and non-transgenic cotton plant tissues. *Curr Sci* **101**: 1472-1474
- Tao G, Liu ZY, Hyde KD, Liu XZ, Yu ZN (2008) Whole rDNA analysis reveals novel and endophytic fungi in *Bletilla ochracea* (Orchidaceae). *Fungal Divers* **33**: 101-122
- Tiedemann Sv, Brendel G, Fehrmann H (1988) Investigations on endophytic fungi of grapevine with special emphasis on the vascular system of rootstocks Untersuchungen uber endophytische Pilze der Rebe unter besonderer Berucksichtigung des Gefasssystems der Unterlage. *J Phytopathol* **122**: 147-165
- Travadon R, Baumgartner K, Rolshausen PE, Gubler WD, Sosnowski MR, Lecomte P, Halleen F, Peros JP (2012) Genetic structure of the fungal grapevine pathogen *Eutypa lata* from four continents. *Plant Pathol* **61**: 85-95
- Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, *et al.* (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proc Natl Acad Sci U S A* **102**: 13386-13391

- White CL, Halleen F, Mostert L (2011) Symptoms and fungi associated with esca in South African vineyards. *Phytopathologia Mediterranea* **50**: 236-246
- White T, Birns T, Lee S, and Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, Academic Press, San Diego
- Zhou DQ, Hyde KD (2001) Host-specificity, host-exclusivity, and host-recurrence in saprobic fungi. *Mycol Res* **105**: 1449-1455

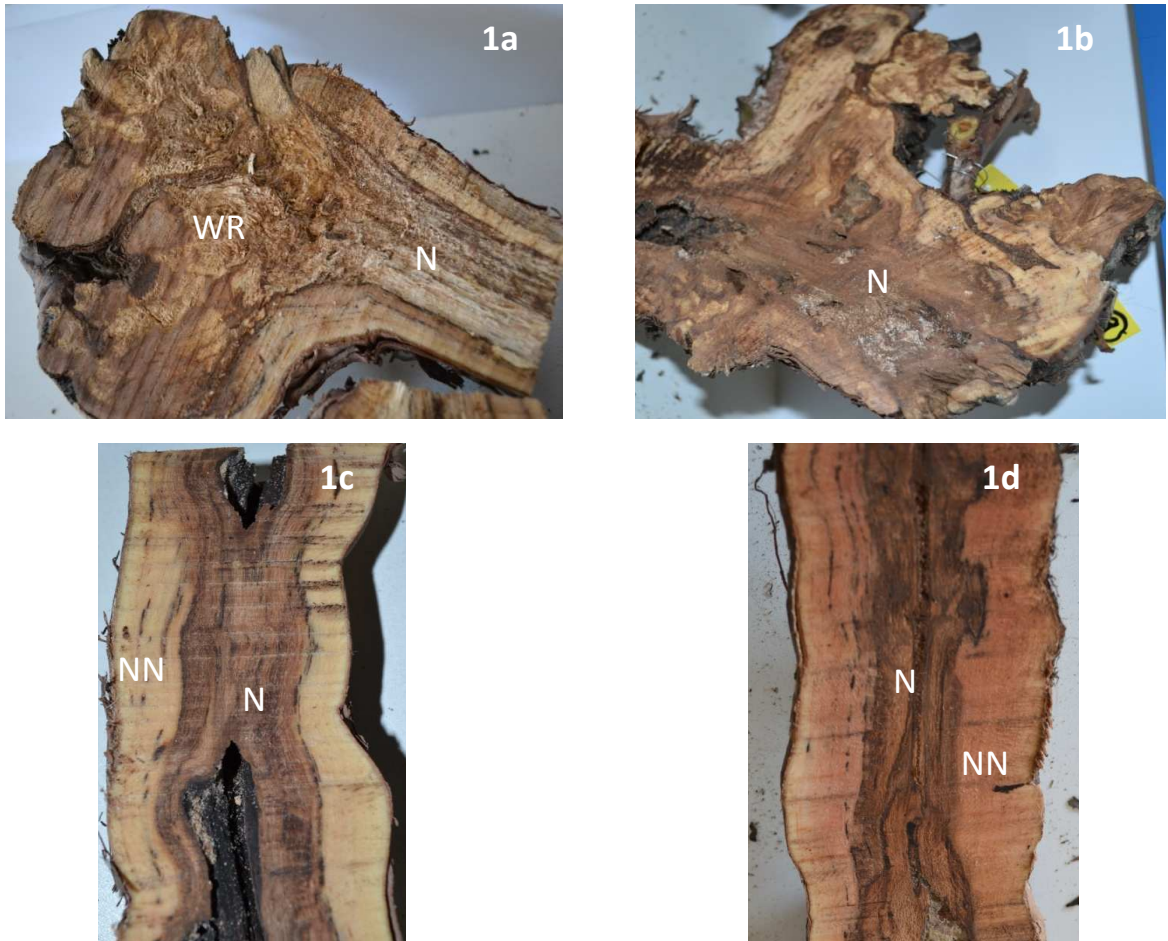


Fig. 1. Photographs of the longitudinal-sections of cordons (Fig. 1a and Fig. 1b) and trunks (Fig.1c and Fig. 1d) of grapevines aged 42 years (Fig. 1b and Fig. 1d) and 58 years (Fig. 1a and Fig. 1c). N= Necrosis tissue, NN= Non-Necrotic tissue, WR=White Rot

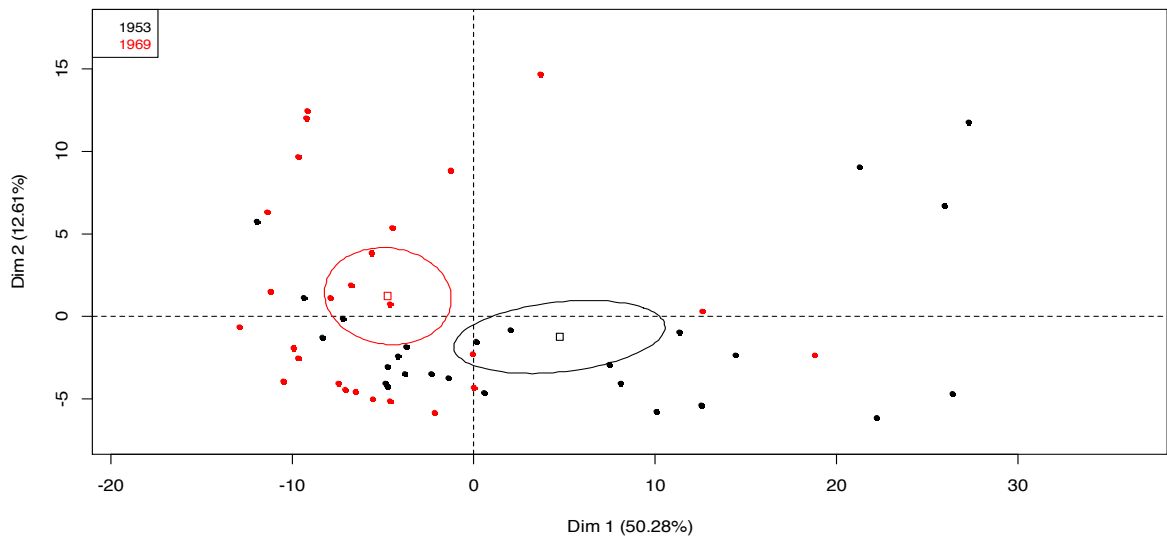


Fig. 2. Fungal microflora colonizing the arm, the upper-, middle- and downy parts of the trunks of plants aged 42 (in red) and 58 (in black) years. Distribution of the fungal communities on the principal plane designed by the global Principal Component Analysis made from numeral values of each SSCP profile. The total variation (62.89%) explained by each Principal Component is given in brackets.

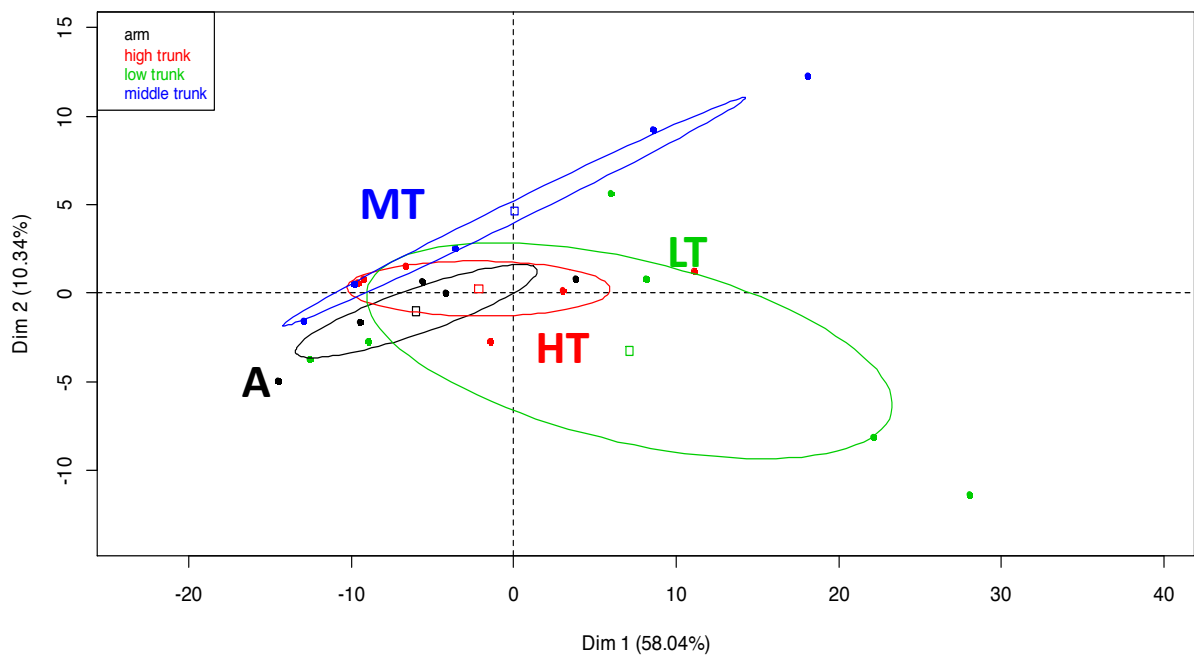


Fig. 3. Fungal microflora colonizing the trunk (upper, middle, downy parts) and the arm of the 42 year-old plants. Distribution of the fungal communities are made on the principal plane designed by the global Principal Component Analysis (obtained from numeral values of each SSCP profile). The total variation (68.38%) explained by each Principal Component is given in brackets.

The fungal communities of each type of tissue are identified by one letter: A= Arm, HT = High trunk, LT= Low trunk, MT= Middle trunk

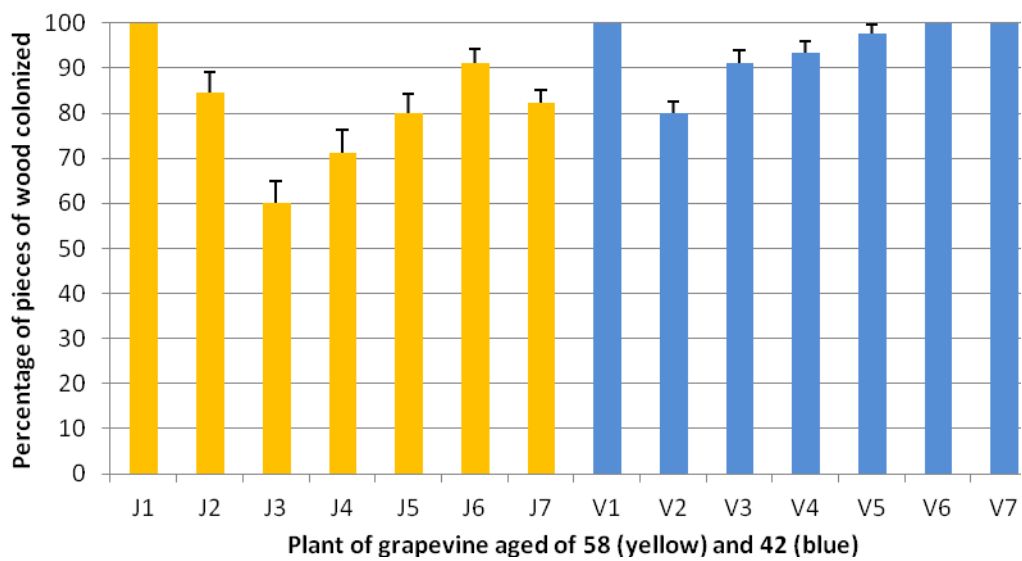


Fig. 4. Pieces of woods sampled from plants aged 42 (blue) or 58 (yellow) years and colonized by fungi.

Table 1. Endophytic fungal species isolated and sequenced from inner trunk at different part.

| Order | Family | Genus | Species | 1953 | 1969 |
|-------------------|---------------------|------------------------|----------------------|-----------|-----------|
| Xylariales | Diatrypaceae | Eutypa | lata | 34 | 12 |
| Hypocreales | Hypocreaceae | Trichoderma | sp. | 31 | 9 |
| Hypocreales | Hypocreaceae | Trichoderma | harzianum | 29 | 19 |
| Eurotiales | Trichodermaceae | Penicillium | sp. | 17 | 19 |
| Hypocreales | Bionectriaceae | Bionectria | ochroleuca | 11 | 2 |
| Mucorales | Cunninghamellaceae | Gongronella | butleri | 10 | 6 |
| Chaetothyriales | Herpotrichiellaceae | Phaeomoniella | chlamydospora | 9 | 13 |
| Helotiales | Vibrissaceae | Phialocephala | dimorphospora | 8 | 0 |
| Hymenochaetales | Hymenochaetaceae | Fomitiporia | mediterranea | 5 | 23 |
| Agaricales | Psathyrellaceae | Psytharella | candolleana | 5 | 10 |
| Helotiales | ? | Cadophora | hiberna | 5 | 3 |
| Hypocreales | Nectriaceae | Fusarium | sp. | 5 | 0 |
| Xylariales | Amphisphaeriaceae | Pestalotiopsis | disseminata | 5 | 0 |
| Calosphaeriales | Calosphaeriaceae | Phaeoacremonium | aleophilum | 4 | 31 |
| Calosphaeriales | Calosphaeriaceae | Phaeoacremonium | mortoniaceae | 3 | 3 |
| Hypocreales | Hypocreaceae | Trichoderma | hamatum | 3 | 1 |
| Botryosphaeriales | Botryosphaeriaceae | Botryosphaeria | sp. | 3 | 0 |
| Botryosphaeriales | Botryosphaeriaceae | Diplodia | seriata | 3 | 0 |
| Hypocreales | Hypocreaceae | Trichoderma | atroviride | 3 | 0 |
| Coniochaetales | Coniochaetaceae | Coniochaeta | velutina | 2 | 11 |
| Eurotiales | Trichodermaceae | Penicillium | citreonigrum | 2 | 3 |
| Xylariales | Amphisphaeriaceae | Truncatella | angustata | 2 | 1 |
| Eurotiales | Trichodermaceae | Penicillium | pancosmium | 2 | 0 |
| Russulales | Stereaceae | Stereum | hirsutum | 1 | 17 |
| Helotiales | ? | Scytalidium | cuboideum | 1 | 9 |
| Pleosporales | Montagnulaceae | Paraconiothyrium | sp. | 1 | 6 |
| Sordariales | Lasiosphaeriaceae | Arthrimum | sp. | 1 | 3 |
| Pleosporales | Sporormiaceae | Preussia | sp. | 1 | 2 |
| Pleosporales | Pleosporaceae | Pithomyces | valparadisiacus | 1 | 2 |
| Pleosporales | Didymellaceae | Epicoccum | nigrum | 1 | 1 |
| Pleosporales | Leptosphaeriaceae | Leptosphaeria | sp. | 1 | 1 |
| Eurotiales | Trichodermaceae | Penicillium | expansum | 1 | 1 |
| Pleosporales | Pleosporaceae | Alternaria | alternata | 1 | 0 |
| Pleosporales | Pleosporaceae | Alternaria | sp. | 1 | 0 |
| Sordariales | Chaetomiaceae | Chaetomium | coarctatum | 1 | 0 |
| Hypocreales | Nectriaceae | Fusarium | oxysporum | 1 | 0 |
| Pleosporales | Pleosporaceae | Lewia | infectoria | 1 | 0 |
| Pleosporales | Lophiostomataceae | Lophiostoma | corticola | 1 | 0 |
| Eurotiales | Trichodermaceae | Penicillium | commune | 1 | 0 |
| Xylariales | Amphisphaeriaceae | Pestalotiopsis | mangiferae | 1 | 0 |
| Xylariales | Amphisphaeriaceae | Seiridium | ceratosporum | 1 | 0 |
| Sordariales | Chaetomiaceae | Chaetomium | globosum | 0 | 3 |
| Helotiales | Dermateaceae | Cryptosporiopsis | sp. | 0 | 2 |

| | | | | | |
|-------------------|--------------------|---------------|----------------|---|---|
| Hypocreales | Hypocreaceae | Trichoderma | virens | 0 | 1 |
| Mucorales | Mucoraceae | Mucor | circinelloides | 0 | 1 |
| Saccharomycetales | Saccharomycetaceae | Saccharomyces | guilliermondii | 0 | 1 |

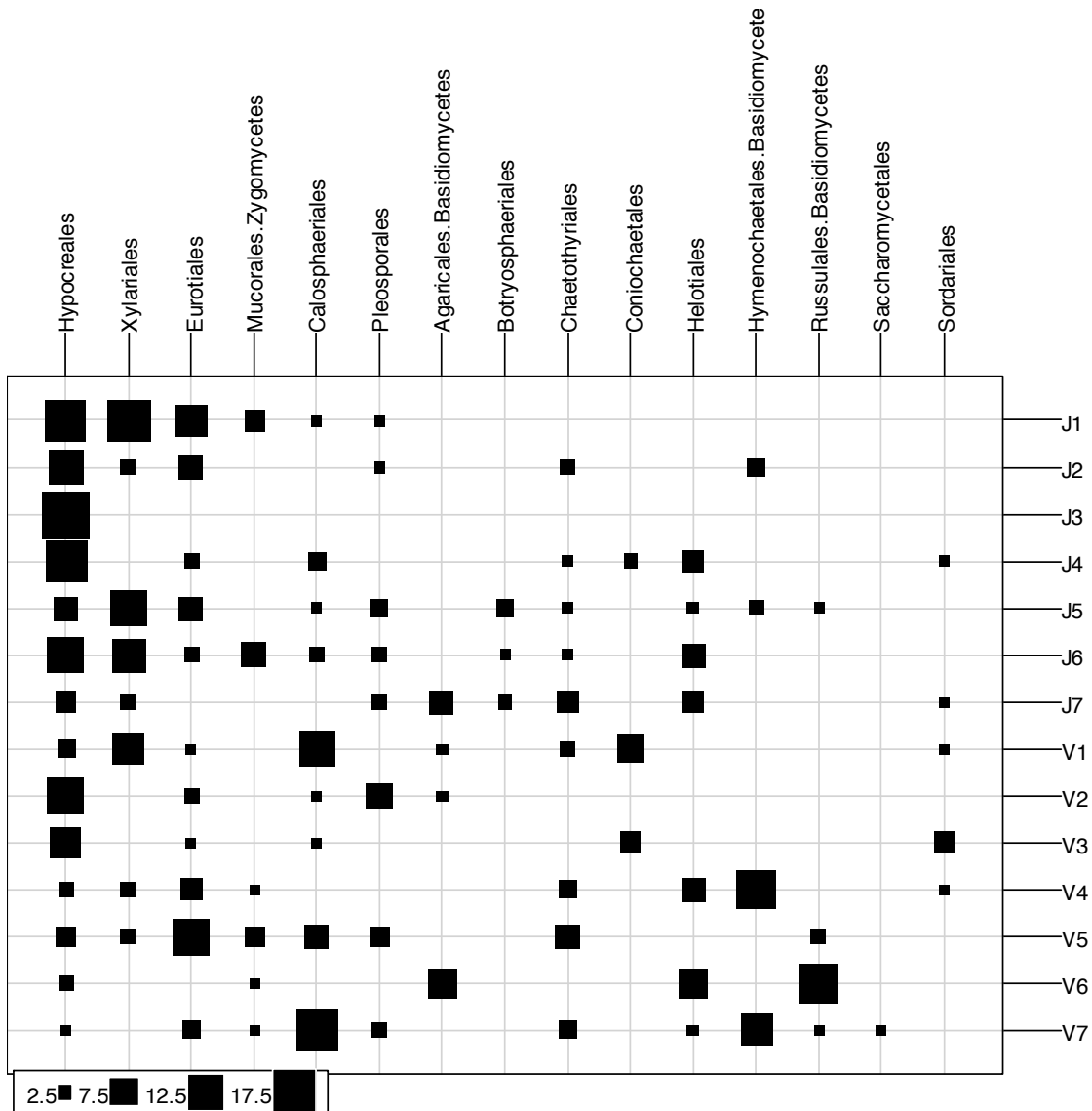


Fig. 5. Distribution of the fungi orders per plant aged 42 (7 plants coded V1 to V7) or 58 years (7 plants coded J1 to J7). A square represents the number of isolates per plant.

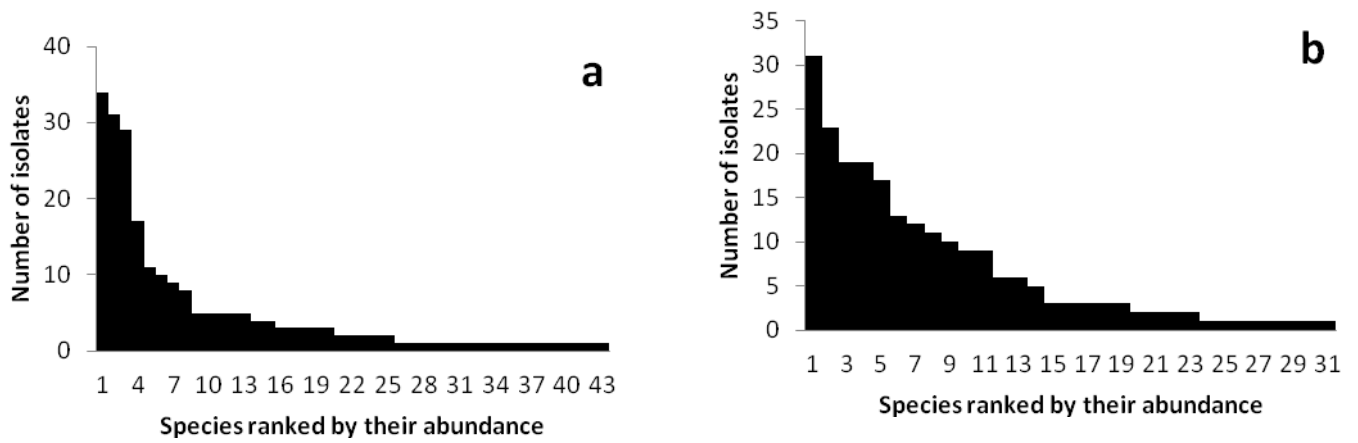


Fig. 6. Rank-order plots showing the isolate abundance of each species identified in the wood of plants aged 58 (A) and 42 years (B).

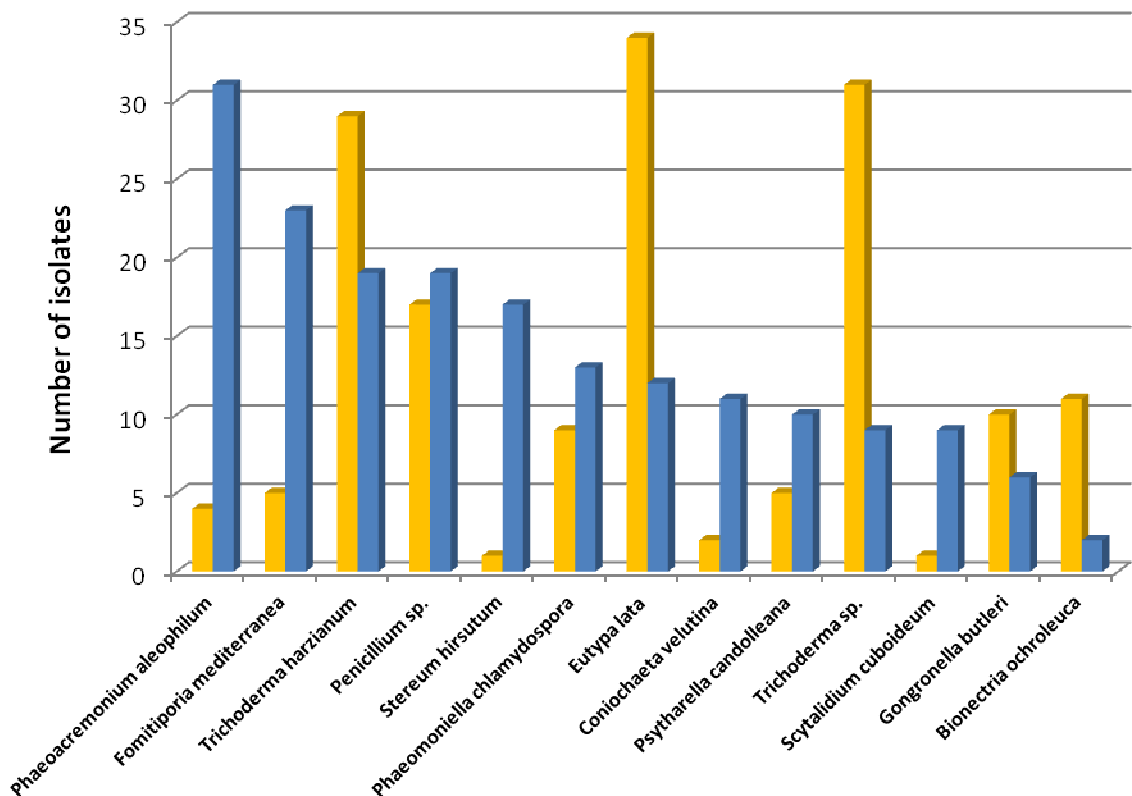


Fig. 7. Distribution of the number of isolates for the 13 principal OTUs distinguished by DNA-ITS sequencing of plants aged 42 (blue) and 58 (yellow) years

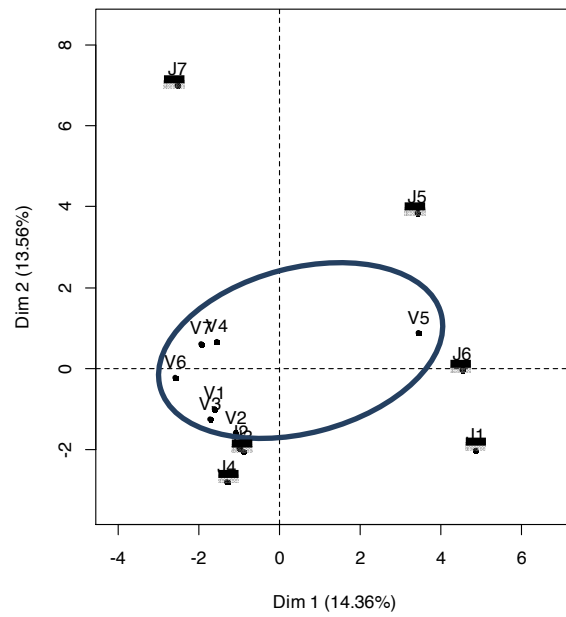


Fig. 8. Distribution of the fungal genera for the plants aged 42 and 58 year-old. The oldest pants are underlined. Distribution of the fungal communities are made on the principal plane designed by the global Principal Component Analysis (obtained from numeral values of each SSCP profile). The total variation (27.92%) explained by each Principal Component is given in brackets.

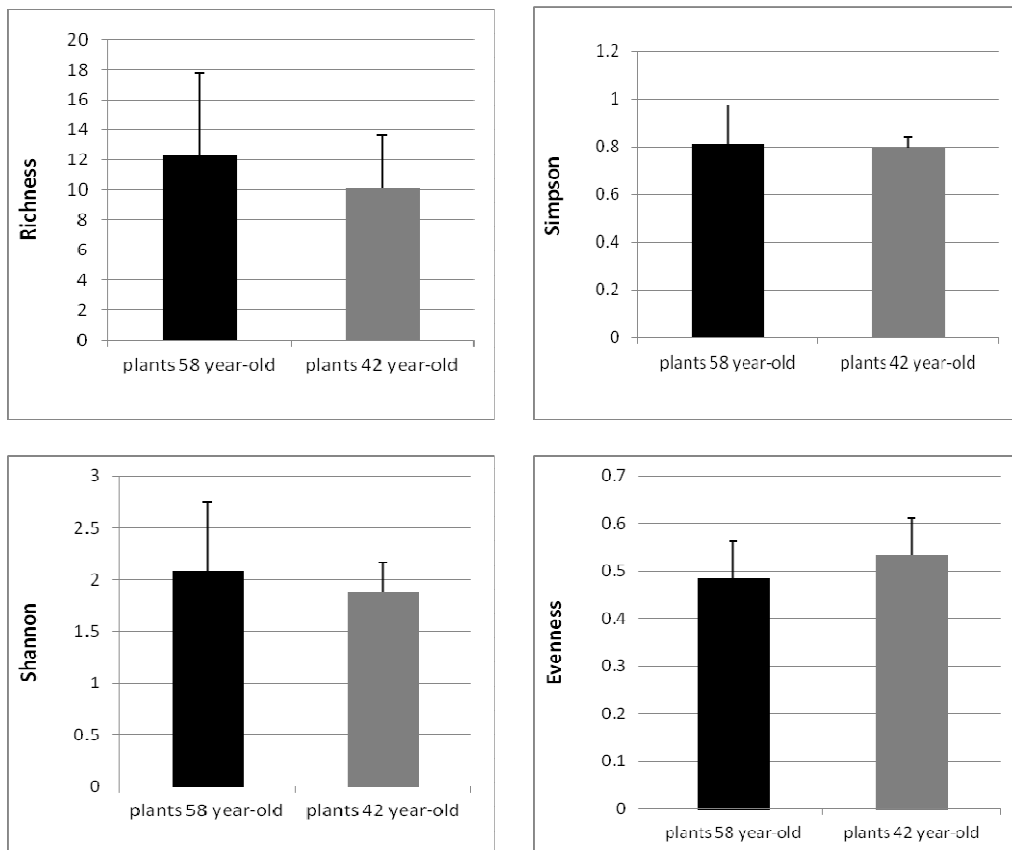


Fig. 9. Diversity indexes (Richness, Simpson, Shannon, Evenness) for the plants aged 42 and 58 years old. Data on Y axes synthesize average values of seven plants per age.

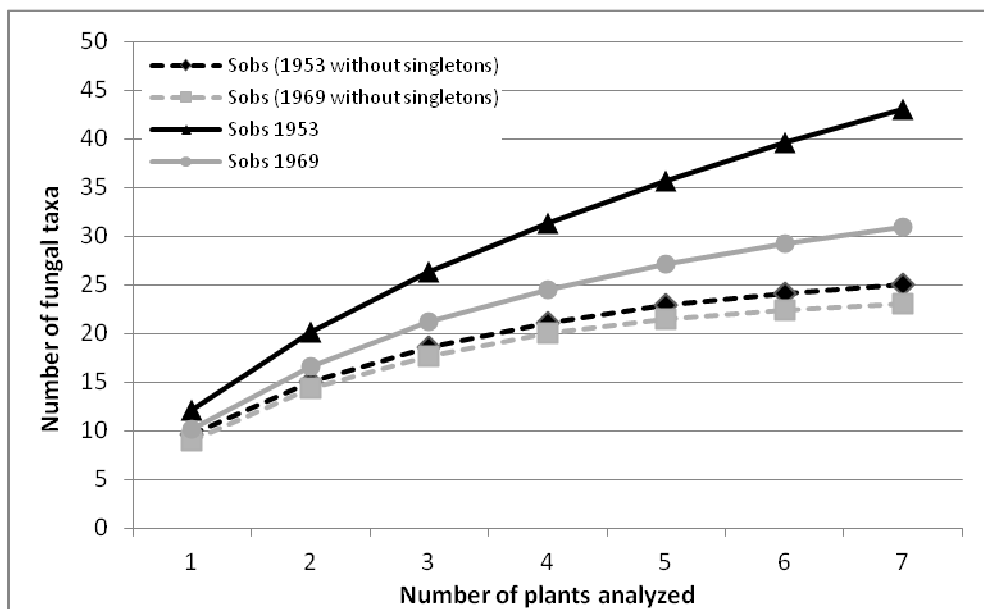


Fig. 10. Species accumulation curves showing the relationship between the number of plants analyzed and the number of fungal taxa isolated in plants aged 58 or 42 years. When all the fungi were considered (continuous lines), curves were not asymptotic but when the plurals and doubles fungal taxa were considered (broken lines), the asymptotes were obtained.

Chapitre 2

Mise en évidence de communautés bactériennes spécifiques associées aux bois de ceps de vigne ayant exprimé ou non des symptômes foliaires d'esca

Introduction

La microflore fongique colonisatrice du bois de ceps de vigne étudiée dans les publications n°2 et 3 est diversifiée et comprend de nombreuses espèces, certaines étant connues pour leur capacité à protéger les plants alors que d'autres sont associées aux maladies du bois : esca, eutypiose et chancres à *Botryosphaeria*. Toutes les parties d'une plante sont concernées: le bois du tronc, du porte-greffe et des bras. Les zones ayant développé des nécroses, par exemple l'amadou qui est une structure de bois dégradée typique de l'esca, mais aussi celles d'apparences saines sont colonisées. La diversité fongique est généralement accrue dans les zones préservées par rapport à celles nécrotiques. Une évolution de la microflore au cours du temps a aussi été observée, elle est sans doute dépendante de facteurs abiotiques (température, disponibilité en eau) ainsi que de la physiologie de la plante.

Lorsque cette microflore fongique a été étudiée (publications n°2 et 3), en particulier lorsque des isollements de champignons à partir de bûchettes de bois ont été réalisés, nous avons remarqué le développement de bactéries. Il semblait donc que le bois des plantes était colonisé par ces micro-organismes. La littérature indique cependant que les bactéries du bois de la vigne ont été très peu étudiées. Bell *et al.* (1995) ont rapporté que les vaisseaux du xylème de ceps sains étaient colonisés par de nombreuses espèces bactériennes, la plupart étant non pathogènes de *V. vinifera* alors que d'autres se sont focalisées essentiellement sur les fleurs, les baies et les racines de cette plante (Compant *et al.*, 2011). Dans tous les cas une importante diversité microbienne était signalée. L'intérêt des bactéries isolées ou non de vigne en protection des ceps a été souligné. Compant *et al.* (2008) ont indiqué que plusieurs d'entre elles ont été utilisées pour lutter contre les pathogènes foliaires de la vigne : *B. cinera*, *E. necator*, *P. viticola* ou ceux impliqués dans l'eutypiose (*E. lata*) ou l'esca (*P. aleophilum*). On peut noter que des bactéries ont montré un certain intérêt pour lutter contre les 2 principales maladies bactériennes de la vigne : *Xylella fastidiosa*, l'agent de la maladie de Pierce, qui atteint les vaisseaux du xylème et *Agrobacterium vitis*, responsable de tumeurs chez les plantes infectées. Un point à signaler est que ces pathologies sont principalement observées en Amérique du Nord, *X. fastidiosa* n'étant jusqu'à ce jour pas répandue en Europe.

Dans ce contexte et compte tenu des incertitudes sur le rôle de la microflore fongique du bois de la vigne dans le développement de l'esca. Il nous a paru judicieux d'étudier les communautés bactériennes colonisatrices du bois de cette plante. Plusieurs publications rapportent en effet, que les bactéries peuvent déstructurer le bois des arbres, ce qui facilite par la suite l'action des champignons lignicoles (Clausen, 1996; Greaves, 1971). A priori, selon Bugg *et al.* (2011) leur rôle dans la dégradation de la lignine aurait même été sous estimé. Dans la présente étude, nous avons utilisé, pour une grande partie les mêmes ceps que ceux échantillonnés lors de la publication n°2. Ils présentent en effet l'avantage d'être relativement sains au niveau du tronc, certains d'entre eux ayant exprimé des symptômes foliaires d'esca ont aussi développé de l'amadou dans les bras. La comparaison entre ceps symptomatiques foliaires esca et asymptomatiques nous permettra aussi de vérifier si certaines espèces sont associées aux plants malades ou sains. Au niveau méthodologique, notre approche a consisté à analyser la microflore bactérienne globale par SSCP, plusieurs souches ont aussi été isolées puis séquencées au niveau de l'ADNr 16S, leur fonctionnalité a été étudiée en utilisant des plaques « Biolog Ecoplates » spécifiques pour l'analyse des échantillons environnementaux. Des échantillons

de bois sains, d'autres ayant formé de l'amadou mais aussi des bandes striées nécrotiques (échantillons supplémentaires par rapport à la publication n°1) ont été utilisés.

L'ensemble de ces résultats est présenté dans la publication n°4 dont le titre est : « **Specific bacterial microflora are associated with the wood tissues of asymptomatic and foliar esca-symptomatic grapevines** ».

Publication n°4

“Specific bacterial microflora are associated with grapevine asymptomatic and esca-symptomatic wood tissues”

Applied and Environmental Microbiology (Submitted)

Specific bacterial microflora are associated with asymptomatic and esca-symptomatic wood tissues of grapevine

Emilie Bruez,^{a,b} Jessica Vallance,^{a,b} Maryam Tidjani Alou,^{a,b} Christophe Bertsch,^c Lucia Guerin-Dubrana,^{a,b} Patrice Rey^{a,b}

^a Université de Bordeaux, ISVV, UMR1065 SAVE, Bordeaux Sciences Agro, F-33140 Villenave d'Ornon, France.

^b INRA, ISVV, UMR1065 Santé et Agroécologie du Vignoble (SAVE), F-33140 Villenave d'Ornon, France.

^c Université de Haute-Alsace, UFR PEPS, EA-3991 Laboratoire Vigne Biotechnologie et Environnement, 33, rue de Herrlisheim, 68008 Colmar cedex France.

Esca is a grapevine wood disease that has long been attributed to fungi. However, although the fungal species involved in esca have been studied on many occasions, the aetiology of the disease remains poorly understood. So the involvement of other microorganisms in esca is still a matter of speculation. In the present study, we focused on bacteria because in many trees, wood-inhabiting bacteria are associated with wood decay and that they may degrade lignin. Molecular fingerprinting method, Single Strand Conformation Polymorphism (SSCP), shows that complex and specific microflora colonize the wood of both the necrotic (white-rot) and healthy wood tissues of grapevines. Note that white-rot are only in esca-foliar symptomatic plants and not in asymptomatic grapevines. Bacteria isolated from necroses and healthy wood metabolized differently the carbon substrates of the Biolog Ecoplates™, thus indicating that their trophic behaviors are different. Certain species are able or not to colonize wood necroses, for instance 70% of the total bacteria in the white-rot are *Bacillus* spp. but the order of *Enterobacteriales*, including *Pantoea agglomerans*, which is the second most isolated bacteria species, was not isolated from this necrosis. As regards the bacterial species that colonize the necrotic or the healthy wood tissues, two roles should be assigned to them: (i) a positive role, due to the biocontrol potential that many species have; a negative one, by predisposing the vine wood to fungal attacks.

INTRODUCTION

Bacteria have been described as inhabitants of the various tissues of plants (1-4). The majority of bacteria are epiphytic with many of them colonizing the rhizosphere (5), entering into the roots by passive diffusion or active selection and even proliferating within plants (6). Their mobility allows certain bacterial species to colonize the rhizosphere as well as the stems, leaves and reproductive organs (7, 8). Those species generally inhabit the plant cell cytoplasm, the intercellular spaces and the vascular system. Very frequently, bacterial populations decrease from the root parts to the berry and flower parts. Equally, many of them, having been isolated from the soil, have been also identified in various vegetative tissues (9). When they live within the plants, but without causing any visible symptoms, they are defined as endophytes (4, 10). These endophytic bacteria have been isolated from many different plants, mostly annual crops; for instance, sugar beet (11), cotton (12), rice (13, 14), soybean (15) and agronomic crops and prairie plants (16). Most of them induce beneficial effects on plants, with several reports showing that they have an agronomical interest as plant growth promoters (17) by limiting and/or preventing the attacks of plant pathogens. Backman and Sikora (18) reported that endophytes could be an emerging tool for biological control.

Even if, bacteria-colonizing plants have been the subject of an impressive number of studies, the plant studied in the present experiment, *Vitis vinifera*, has only been considered in a few publications (9, 19, 20) dedicated to the endophytic bacterial communities in grapevine. Among the tissues and vines organs those authors sampled, endophytes were in all the plant reproductive organs as well as in the other tissues such as roots, stems and leaves. For instance, Compant *et al.* (19) used cultivation methods and fluorescence *in situ* to show that endophytes colonize flowers, berries, seeds and the rhizosphere of grapevines grown under natural conditions. More endophytic bacteria were detected in the flowers and pulp of berries than in the seeds. As reported with other plants, the endophytic populations decreased from the underground parts, the roots, to the above-ground parts, the flowers.

Vineyard management is assumed to exercise a key role in the bacterial endo-colonization of the plants. Cultural practices such as pruning provide opportunities for incidental bacteria to enter accidentally the grapevine. Stomata and wounds certainly have a role too (2, 20). Some bacteria use xylem elements to spread inside plants, whereas other may have derived from some specific plant surfaces and colonized endophytically the plant reproductive organs. The potential of a plant growth-promoting rhizobacterium, *Pseudomonas* sp. (strain PsJN), to stimulate the growth and enhancement of the resistance of grapevine

transplants to gray mold caused by *Botrytis cinerea* has been investigated by (21). *B. phytofirmans* strain PsJN inoculation stimulates grapevine growth and improves its ability to withstand cold stress (22). Although many bacteria may be beneficial to the grapevine growth and health, at least two species cause severe diseases. Both of them interfere with the functioning of the xylem vessels: *Xylella fastidiosa* (Pierce's disease) infects the xylem vessels of the vine, and causes the formation of gel, preventing water from being drawn through the vine (23, 24). *Agrobacterium vitis* causes galls that hinder the function of the vascular system, thus reducing the grapevine vigor and productivity (25, 26).

In the present study, our objective was to study the bacterial microflora that colonize grapevine wood and to compare the bacterial community of asymptomatic and esca-symptomatic plants. As regards esca, this disease, along with eutypa and botryosphaeria diebacks, constitutes the grapevine trunk diseases (GTDs) that are a matter of great concern for viticulture worldwide. Although GTDs, particularly esca, have been known since antiquity, they were long considered as negligible diseases. However, during the past two decades, increased incidences of those diseases have been reported in the main vine-growing regions of the southern and northern hemispheres (27-31). According to Hofstetter *et al.* (32), the worldwide annual financial cost of the replacement of dead plants due to GTDs exceeds 1.132 billion euros. In France, it was estimated that approximately 11% of French vineyards are unproductive, mainly because of GTDs, with esca being the most frequent disease. Esca attacks the perennial organs of the plants, producing extensive wood necroses in the trunk and cordons, and typical symptoms in the leaves and berries. Ultimately, the grapevines decline and may die. Although the fungal species involved in esca have been studied on many occasions, *i.e.* *Phaeoconiella chlamydospora*, *Phaeoacremonium aleophilum*, *Fomitiporia mediterranea*, *Stereum hirsutum*, the aetiology of the disease remains poorly understood. For instance, after wood inoculations with these fungi, symptoms of necroses are usually reproduced in the wood but the leaves are frequently free of symptoms. So, the involvement of other microorganisms in esca is still a matter of speculation. In a preliminary study, Bruez *et al.* (33) indicated that diverse microflora made up of fungi and bacteria colonize the wood of both healthy and esca-diseased grapevines. Other reports indicate that in many trees, wood-inhabiting bacteria are associated with wood decay (34) and that they may degrade lignin (35). So, our aim was to provide relevant information on some questions that are still the subject of speculation: are the various parts, cordons, trunks and grapevine rootstocks colonized by bacteria? Are the typical necroses associated with esca development also

colonized by bacteria? Are there specific communities associated with esca-symptomatic plants?

To answer these questions, the present study was carried out over a 2-year period, and includes grapevines that are esca-foliar symptomatic or asymptomatic. Over this period, the structure of the microbial communities was studied by 16SrRNA-targeted polymerase chain reaction–single strand conformation polymorphism (PCR–SSCP). To fully characterize the bacterial communities inside the two kinds of grapevines, bacteria were studied by using a polyphasic approach: (1) Bacterial diversity within woods units was studied by isolating and sequencing strains from necrotic and healthy wood tissues. (2) Community-level physiological profiles (CLPP) generated with sole-carbon source-utilization tests from Biolog™ provided us with physiological data about the bacterial communities of wood. (3) The genetic structure of these microbial communities was studied by PCR–SSCP.

MATERIALS AND METHODS

Plant material and sampling design. Experiments were carried out on 10 year-old Cabernet Sauvignon vines (*Vitis vinifera*) planted in a sandy-clay soil. The rootstock was 101 14 MG. The sampling site was located at the Chateau Luchey-Halde vineyard in Méridnac (Bordeaux region, France). Plants that had previously expressed or not the foliar symptoms of esca were selected and uprooted. For the foliar-symptomatic plants, we selected plants that had previously expressed esca foliar symptoms at least two times over a period of 4 years (2005 to 2009). The control plants had never expressed the foliar symptoms during their lifetime.

In order to monitor by SSCP the dynamics of the bacterial microflora colonizing the wood tissues of vines, 4 asymptomatic and 4 esca-foliar symptomatic plants were uprooted every 12 weeks during a one-year period. Plants were sampled 5 times: in the winter (January 2010), spring (April 2010), summer (June 2010), autumn (September 2010) and again the winter of 2011 (January). For each grapevine, the cordons, the trunks and the rootstocks of the plants were cut longitudinally. Subsequently, the woods were used for extraction of the bacterial DNA.

Another sampling was made one year and a half later, in July 2012, by uprooting 4 asymptomatic and 4 esca-foliar symptomatic plants. For each plants, the cordons and the trunks of the grapevines were cut longitudinally and the woods were used to isolate cultivable bacteria and to extract the bacterial DNA.

Isolation of bacteria from the wood tissues. Samples of cordon and trunk from grapevines uprooted in July 2012 have been used. From the cordons, differences types of tissues were extracted, the white rot, the non-necrotic and the necrotic-stripe tissues. The white rot (also called amadou) is a necrosis specific of esca (36, 37), it was only present in the cordon of symptomatic plants. In the trunk, non necrotic tissues of inner trunk and outer trunk have been sampled from the two types of plant. The same wood samples were used to isolate bacteria and extract DNA. Wood samples (except the white rot) were immersed in 70% ethanol for 1 minute followed with a treatment of 2.5% calcium hypochlorite solution for 3 minutes. Because the white rot samples were immersed for 20 seconds with 70% ethanol followed by 20 seconds immersion in 2.5% calcium hypochlorite solution. Then, the samples were rinsed 3 times with sterile distilled water. To check surface sterilization, 100 μ l of the third rinsing water were plated 3 times on R2A medium. The plates were then incubated for 24 hours at 27°C and it was verified that bacterial colonies were not shown growing.

To isolate Gram+ and Gram- bacteria from the wood tissues, the R2A media has been used. Fifteen pieces of wood of each sample were placed on three plates. The plates were incubated 48 hours at 27°C. The bacterial strains isolated were transplanted until obtaining pure strains.

For the Biolog Ecoplates™ experimentation, pieces of wood of each samples were put in a liquid media, the Tryptic Soy Broth (TSB). The tubes were then incubated 48h at 27°C. After the incubation, the liquid media was distributed in the Biolog Ecoplates™ plates.

Characterization of the bacterial strains isolated from the wood samples. Two biochemical tests were used to characterize the strains isolated from the wood samples:

(i) *Gram stain*: the stains used were crystal violet (basic stain), lugol (1% iodine iodine iodide aqueous solution) and safranin. Bacteria are differentiated in two groups based on the properties of their cell walls. The gram-positive (Gram+) bacteria retain the purple stain and the gram-negative (Gram-) bacteria are decolorized and counterstained in pink. The stained bacteria observation was carried out by covering the smear with immersion oil by microscopy ($\times 1000$ lens).

(ii) *Community-level physiological profiles (CLPP)*: physiological data about the microbial population colonizing the different types of tissue were studied by Biolog Ecoplates™ (AES). These plates check simultaneously the metabolic reaction of the bacterial suspensions regarding 31 lyophilized substrates and a negative control present in triplicate on each plate. Among these 31 substrates, ten substrates contain carbon and nitrogen (including six amino-acids), two substrates contain carbon and phosphorus and nineteen contain only carbon. The inoculums were obtained by centrifuging the bacterial suspensions at 3000 rpm for thirty minutes at 15°C. The resulting centrifugates were then resuspended in 15 ml 0,85% NaCl solution. 150 μ l of the resulting bacterial suspension were inoculated in each well. The plates were then incubated at 27°C in the darkness. Optical density readings were performed at a wavelength of 590 nm after 48 hours and five days incubation (Multimo microplate reader, Synergy HT, Biotek). Each well contains besides the substrate tetrazolium salts which turn purple when they are reduced by bacterial activity. These tetrazolium salts absorbance was measured during the plate optical density reading, thus giving an estimate of the bacterial substrate degradation.

To minimize the effect of difference in bacterial mixture densities between plates, data were standardized as follows: the average well color development (AWCD) corresponding to the mean of the blanked absorbance values of the 95 wells was calculated for each plate; then, the blanked absorbance value of each well was divided by the AWCD of the corresponding

plate to get a corrected OD value (39). All corrected OD values were set to fall within 0 and 2 (boundary limits).

SSCP analysis of the bacterial communities colonizing the grapevine woods. DNA extraction from the wood samples. From the wood tissues sampled in January, April, June, September 2010 and January 2011, 10 g of non-necrotic tissues of rootstock, trunk and cordon were sampled and 10 g of white rot tissue of the cordon of symptomatic plants. For the samples of July 2012, for the esca-foliar symptomatic and asymptomatic grapevines collected, 10 g of non-necrotic tissues were sampled in the inner and outer parts of the trunks. In the cordons, 10 g of non-necrotic and necrotic stripes of wood tissues were also collected. Conversely to the grapevines uprooted in 2010 and 2011, these necrotic stripes were developed enough to be sampled (10 g of necrotic tissues). All these samples were ground in liquid nitrogen with a one-ball mill of Dangoumau type and kept at -80°C prior to DNA extraction. The DNA extraction was performed using a commercial kit Invisorb Spin Plant Mini Kit (Invitek). The extraction was done according to the manufacturer's instructions. During the sampling, wood strips were taken from the different samples and stored at -80°C . before extracting the DNA, the samples were grinded in liquid nitrogen using a beads grinder (Dangoumeau).

PCR-SSCP. DNA amplification. To study the bacterial communities, the primer couple 799f (AACMGGATTAGATACCCKG) and 1115r (AGGGTTGCGCTCGTTG) was used. The PCR amplification was performed in Mastercycler Gradient Thermocycler (Eppendorf) in a $50\ \mu\text{l}$ reaction volume containing $1\ \mu\text{l}$ of DNA ($10\ \text{ng}/\mu\text{l}$), $5\ \mu\text{l}$ of Pfu buffer 10x (Stratagene), $1\ \mu\text{l}$ of dNTP (10 mM), $1\ \mu\text{l}$ of each primer at $20\ \mu\text{M}$, $5\ \mu\text{l}$ of BSA at $10\ \mu\text{g}/\mu\text{l}$ (BioLabs), $0.5\ \mu\text{l}$ of Pfu Turbo (Stratagene) and $35.5\ \mu\text{l}$ of sterile distilled water. The following program was used: a three minutes initial denaturation at 94°C followed by 35 cycles of denaturation (45 seconds at 94°C), annealing of the primers (30 seconds at 50°C), of elongation (90 seconds at 72°C) and a final elongation at 72°C for 10 minutes.

Single Strand Conformation Polymorphism (SSCP). After being checked on agarose gel, the amplified products were prepared for the SSCP analysis: $1\ \mu\text{l}$ of PCR product was mixed with $18.8\ \mu\text{l}$ of deionized formamide (Applied Biosystem) and $0.2\ \mu\text{l}$ of internal standard (size marker GeneScan-400 ROX, Applied Biosystem). The PCR products were denatured by heating for 5 minutes at 95°C ; the samples were then cooled on ice for 10 minutes to avoid reannealing. The obtained DNA strands were separated by capillary electrophoresis and spotted using tagged primer 1115r (6-FAM-AGGGTTGCGCTCGTTG). The electrophoresis

was performed with an automatic sequencer ABI PRISM (Genetic Analyzer 3130, Applied Biosystem) with four 36 cm capillary filled with a polymer made of 5,6% POP Conformation Analysis Polymer (Applied Biosystem), 10% glycerol, EDTA buffer (10X) and water. The following migration conditions were applied: 15 kV at 30°C for 2000 seconds. The SSCP profiles obtained were then analyzed with StatFingerprints.

Identification of the bacterial strains isolated from the wood samples. *DNA extraction from the isolated bacterial strains.* Each bacterial strain was cultured in 1 ml of TSB (30 g/l) on an extraction plate and incubated 48 hours at 27°C. After the incubation period, the extraction plated is centrifuged at 3700 rpm for 30 minutes at 4°C. The supernatant is eliminated and 600 μ l of the extraction buffer (firstly heated to 65°C and 5 μ l of proteinase K/50 ml of buffer might be added if needed) was added to the centrifugate. The plate is quickly vortexed and placed in a water bath at 65°C for at least an hour. The plate was energetically shaken every ten or twenty minutes. The DNA extraction was done according to the protocol of the laboratory.

PCR. DNA amplification. The PCR amplification was performed in Mastercycler Gradient Thermocycler (Eppendorf) in 30 μ l reaction volume containing 2 μ l of DNA (10 ng/ μ l), 3 μ l of buffer (10x), 1 μ l of MgCl₂ (50 mM), 0,6 μ l of dNTP (10 mM), 0.6 μ l of the primers 799f (AACMGGATTAGATACCCCKG) and 1492r (GTTACCTTGTTACGACTT) at 10 mM, 3 μ l of BSA at 10 μ g/ μ l (BiolLabs), 0,1 μ l of Taq polymerase (SilverStar DNA polymerase, Eurogentec) and 21,1 μ l of sterile distilled water. The following program was used: a three minutes initial denaturation at 94°C followed by 30 cycles of denaturation (1 minute at 94°C), annealing of the primers (45 seconds at 50°C) of elongation (1 minute at 72°C) and a final elongation at 72°C for 8 minutes.

Sequencing and identification of bacteria. The amplicons were sequenced with the primer couple 799f (position 781 to 799 according to *Escherichia coli*'s DNAr 16S sequence)/ 1492r (position 1492 to 1510 according to *E. coli* DNAr 16S sequence) by the company GATC Biotech, Germany and specialized in sequencing. Based on the sequences obtained, identification of the bacterial strains was made by using BLAST (Basic Local Alignment Search Tool) online on the American website NCBI (National Center for Biotechnology Information). To determine the name of the species, the percentage of similarity between BLAST and our sequences was at 99%.

Data analyses. SSCP analyses. Banding patterns obtained from community analysis (SSCP), all profiles were aligned by StatFingerprints R software (The R foundation for Statistical computing, ISBN 3-900051-07-0) and they were gathered in a single numerical

database before being statistically described by a global Principal Component Analysis (PCA) by R software version 2.14.2. This database consisted of X individuals but according to the PCA X samples and a total of 250 variables which were the numerical values of values measured (for each sample) on the 250 SSCP profile scans. PCA was performed on using the correlation coefficient of Pearson. Variables having a $\cos^2 \geq 0.5$ on one of the first third principal components (Dim1, Dim2 or Dim 3) were estimated as sufficiently well represented by the principal plane generated by this PCA.

Biolog Ecoplates™ analyses. After reading the OD at 48H and 120h, data are analyzed. Each carbon substrate represents one variable, so it gives a total of 31 variables. Each sample has been three replicates. PCA was performed by using StatFingerprints R software version 2.14.2. Variables having a $\cos^2 \geq 0.5$ on one of the first third principal components (Dim1, Dim2 or Dim 3) were estimated as sufficiently well represented by the principal plane generated by this PCA.

RESULTS

Status of the wood of esca foliar-symptomatic and asymptomatic grapevines.

After longitudinal-sections of grapevines, the wood of the cordons of the esca-foliar symptomatic plants is much more necrotic than in those asymptomatic. Non-necrotic tissues were predominant in the trunks and rootstocks (not shown) of the esca foliar-symptomatic and asymptomatic plants. Necrotic stripes are observed in the cordon of two plants but a specific tissue, the white rot, is only observed in grapevines that had previously expressed esca foliar symptoms (Table 1). Seventy five percent of symptomatic vines had white rot in their cordons. Only a small dark necrosis was observed in the center of the sections, for the two types of plants (Fig. 1).

Analyses by SSCP of the bacterial communities colonizing the wood tissues of grapevines. *Global analyses of the bacterial communities over a one-year period (January 2010 to January 2011).* From the SSCP pattern analysis, PCA is obtained in order to compare the bacterial communities colonizing two types of tissues: the white rot and the non-necrotic wood tissues sampled from different parts of the esca-foliar symptomatic and asymptomatic plants (Fig.2). For that global analysis, symptomatic and asymptomatic grapevines are pooled for each types of tissue. PCA eigenvalues indicates that the first two principal component, Dim 1 and Dim 2, explain 87,27% of the total data variance. Samples of white rot are separated from the others by dim 2, respectively negative versus positive coordinates. The other samples from the rootstock, the outer and inner part of trunks and the cordons are not separated by Dim 1 or Dim 2.

Figure 3: clusters representing the bacterial communities colonizing the non-necrotic wood tissues (inner and outer trunks, rootstocks and cordons) are pooled for each sampling time (January, April, June, September 2010 and January 2011). Note that for each time, the samples of symptomatic and asymptomatic plants are pooled. PCA eigenvalues indicates that the first two components, Dim 1 and Dim 2, explain 84.28% of the total data variance. The clusters of June/January (2011) and September are clearly separated by Dim 1, respectively negative versus positive coordinates. The clusters of January (2011) and April tends to be separated by Dim 1 (negative versus positive coordinates) too. The clusters of June and September are clearly separated from the 3 others by Dim 2, respectively positive versus negative coordinates.

Figure 4 shows the clusters representing the samples (all the healthy wood tissues were pooled) from asymptomatic and esca-foliar symptomatic plants at each sampling date. PCA eigenvalues indicates that the first two components, Dim 1 and Dim 2, explain 84.09% in January (2010), 87.62%, in April, 87.36% in June, 87.62% in September and 80.65% in January (2011) of the total data variance. The clusters are not separated in January 2010, April, September and January 2011 by Dim 1 or Dim2. Only the clusters of bacterial communities colonizing esca-foliar symptomatic and asymptomatic grapevines in June are separated by Dim 1, negative versus positive coordinates.

Global analyses of the bacterial communities colonizing grapevines sampled in July 2012.

PCA of the figure 5 show the clusters representing the bacterial communities colonizing the different wood tissues of asymptomatic (Fig. 5a) and esca-foliar symptomatic (Fig 5b) plants. The PCA eigenvalues indicates that the first two components, Dim 1 and Dim 2, explain 73.76% for Fig. 5a and 85.99% for Fig. 5b of the total data variance. In the asymptomatic plants and in the cordons, the cluster of non-necrotic tissue separated from the necrotic tissue (stripes) by Dim 1, respectively negative versus positive coordinates (Fig. 5a). The clusters of the inner trunk and the outer trunk tend to be separated by Dim 1, respectively negative versus positive coordinates, too (Fig. 5a). In the esca-foliar symptomatic plants (Fig. 5b), the cluster of the white rot is separated from the 4 other types of tissue by Dim 1, negative versus positive coordinates. The 4 clusters of the inner and outer trunks, non-necrotic and necrotic (stripes) cordons, are not separated by either Dim 1 or Dim2 on Fig. 5b.

Identification of the bacteria isolated from the wood tissues. Except from one plant (Vine Symptomatic n°4), the percentage of the pieces of wood from which bacteria have been isolated was above 50%. The values varied from 40% to 85% (Fig. 6). The t-test showed that there are not significantly differences between the esca-foliar symptomatic and asymptomatic plants. (p-value =0,0913).

Figure 7 shows the distribution of the two types of bacteria Gram+ and Gram- in the plants. For 4 grapevines out of 8, Gram- are more numerous than Gram+. In one plant 50% of bacteria were Gram+ and Gram-. Note that for the asymptomatic grapevine coded Vine Asymptomatic n°4, 92% of bacteria are Gram+. When all bacteria are considered out altogether, of the 228 isolated strains, 50% are Gram- and 50% are Gram+. Forty nine percent of them are Gram+ and 51% Gram- in the asymptomatic grapevines and 51% are Gram+ and 49% Gram in the esca-foliar symptomatic plants.

Distribution of the bacteria. The 16S DNA region of 217 bacteria isolated from the pieces of wood have been sequenced and identified at the species level. 99 have been isolated from asymptomatic plants and 118 from esca-foliar symptomatic plants.

Among the most frequently isolated phylum, Firmicutes, the most numerous, bacteria are Bacillales, then Enterobacteriales and Xanthomonadales. Figure 8 shows the distribution of the orders in the 8 grapevines sampled in July 2012. Eleven orders have been defined, 7 were isolated from the asymptomatic plants and 11 orders from the esca-foliar symptomatic plants. The most representative orders are the Bacillales (60% of the total of bacteria strains) and 48% of them were isolated from the asymptomatic and 72% from esca-foliar symptomatic plants. For Enterobacteriales (26,7% of the total of bacteria strains) 55.2% of them are in the asymptomatic plants and 44.8% in the esca-foliar symptomatic plants; for the Xanthomonadales (8,8% of the total of bacteria strains) 74% of Xanthomonadales were isolated from the asymptomatic plants and 26 % from the esca-foliar symptomatic plants.

On the figure 9, the distribution of the bacteria orders isolated from the different types of wood tissues and plant parts is shown. The 3 most numerous orders: Bacillales, Enterobacteriales and Xanthomonadales are frequently detected in the grapevines sampled. Bacillales have been isolated in all the plant parts and the types of wood tissues analyzed. The same result was found with Enterobacteriales, except that they were not detected in the white rot. For the Xanthomonadales, they have not been isolated from the white rot and the non-necrotic tissue of the cordon of the esca-foliar symptomatic plants. The 8 other orders were isolated in 1 to 4 out of 9 types of wood tissues. In comparison with the 2 most numerous orders, *i.e.* Bacillales and Enterobacteriales, the values were always lower.

Twenty six genera were identified in the wood of the 8 grapevines (Fig. 10). Of these 26 genera, 15 come from the asymptomatic plants and 23 from the esca-foliar symptomatic grapevines. The nine more numerous genera have been isolated in the two types of plants. The most numerous genus (Fig. 10) is *Bacillus* (34% of the total of bacteria strains) then *Pantoea*, *Enterobacter* and *Paenibacillus*. When the distributed of the bacterial genera in the wood tissues of plants are considered it was shown that *Bacillus* is present in all the samples analysed (Fig. 11). The same result is found for *Pantoea*, except that it has not been isolated in the white rot of the cordon (Fig. 11). For *Paenibacillus* and *Brevibacillus*, they have not been detected in 2 out of 9 of the wood tissues sampled. Some of the other genera have been isolated only in a particular part or in a particular type of plant, the number of isolates are generally below 5 (Fig. 11).

Table 2 shows the distribution of the different genera/species according to the wood tissues sampled and the status of the plants. The most representative bacteria is *Bacillus* sp. (31 strains and 14% of the total of strains) with 42% in the asymptomatic plants and 58% in the symptomatic plants. Eighteen out of 20 strains isolated from the white rot belong to the *Bacillus* genera, i.e. 6 *Bacillus* sp., 5 *Bacillus ginsengihumi*, 3 *Bacillus licheniformis*, 2 *Paenibacillus* sp., 1 *Bacillus pumilus*, 1 *Brevibacillus reuszeri*. They are also isolated from the necrotic tissues and the healthy woods of plants as well.

The second most important isolated species, *Pantoea agglomerans* (26 strains, 12% of the total of strains), was isolated from the various wood tissues sampled except the white rot. But equal number of *Pantoea agglomerans* strains (13 strains) has been isolated from the two types of plants. The number of bacterial strains isolated from esca-foliar symptomatic plants was higher than in asymptomatic grapevines for *Paenibacillus* sp. (8/17 = asymptomatic/symptomatic), *Brevibacillus reuszeri* (5/12 = asymptomatic/symptomatic) and *Morganella morganii* (6/8 = asymptomatic/symptomatic).

Other genera, *Enterobacter*, *Stenotrophomonas*, *Xanthomonas*, *Erwinia*, *Agrobacterium*, are much isolated in asymptomatic plants than the esca-foliar symptomatic, but the number of strains were very low, from 8 to 2 strains. One or 2 strains of 11 genera, e.g. *Cellulomonas*, *Clostridium*, *Rhizobium*..., were only detected in esca-foliar symptomatic plants.

Note that for a group of 8 bacteria (from *Paenibacillus* sp. to *Stenotrophomonas* sp., Table 2), 19 to 11 isolated strains have been isolated from 5 to 8 of the wood tissues sampled. For the 29 other bacterial genera/species, from *Xanthomonas* sp. (6 strains) to *Xanthomonas translucens* (1 strain), strains have been isolated from the wood samples but they are detected in 1 to 4 types of wood sampled (Table 2).

Figure 12 represents the distribution of the species by their abundance. Eleven species for the two types of plants (esca-foliar symptomatic or asymptomatic grapevines) were isolated more than twice, 4 species (asymptomatic plants) and 5 species (esca-foliar symptomatic plants) were isolated twice and 14 isolates of esca-foliar symptomatic plants and 6 of asymptomatic plants are singles.

Biodiversity of the bacteria communities. The species biodiversity has been calculated and shown in the table 3. There are no significant differences between the asymptomatic and esca-foliar symptomatic plants (Wilcoxon test) for the Abundance (p-value=0,39), the Shannon index (p-value=0,48), the Simpson index (p-value=0,66) the Evenness (p-value=0,51).

Community-level physiological profiles of the bacterial communities. Figure 13 shows the pattern of the Biolog Ecoplates™ results. PCA eigenvalues indicates that the first two components, Dim 1 and Dim 2, explain 87.91% of the total data variance in Figure 13a and 87.51% of the total data variance in Figure 13b. In the asymptomatic plants, ACP on the figure 13a shows that the clusters representing the bacteria from the non-necrotic tissue of the cordon, the outer trunk and the necrotic cordon (stripes)/inner trunk tend to be separated by Dim 1, negative versus positive coordinates. In the esca-symptomatic plants, ACP on the figure 13b shows that the cluster of the white rot, the inner trunk and the necrotic (stripes) tissue of cordon are separated by Dim 1, negative versus positive coordinates. Clusters from the inner and outer trunks tend to be separated by Dim 1 too, negative versus positive coordinates. The clusters of the white rot and necrotic (stripes) tissue of cordon are separated from the non-necrotic tissue of cordon and the outer trunk by Dim 2, negative versus positive coordinates.

On Figure 14, the clusters represent the different types of wood tissue of the cordon of the asymptomatic and esca-foliar symptomatic plants. PCA eigenvalues indicates that the first two components, Dim 1 and Dim 2, explain 89.99% of the total data variance. The clusters of white rot, necrotic tissues of esca-foliar symptomatic and of asymptomatic plants are separated by Dim 1, respectively negative versus positive coordinates. The non-necrotic tissues of esca-foliar symptomatic and asymptomatic plants are not separated by Dim 1 conversely to the necrotic tissues of these plants. Cluster of non-necrotic wood tissues of symptomatic plants is separated from white rot and necrotic wood tissue of esca-foliar symptomatic plants by Dim 2, respectively positive versus negative coordinates.

DISCUSSION

Our first observation of the pathogenic status of the grapevines shows that, unlike mature (15-20 year-old) or older vines that generally display extensive wood necroses in the inner parts of the trunk (36), the wood tissues of the sampled young (10 year-old) grapevines looked relatively healthy. Both asymptomatic and foliar-esca symptomatic plants had only a small central necrosis in the trunk and rootstock. The main difference between the two types of plants depended on necroses in the arms. Necrotic stripes were observed in the two types of plants but white rot, also called amadou, was observed only in the cordons of esca-foliar symptomatic plants. This observation is in agreement with Maher *et al.* (36) and early descriptions mentioning healthy wood turning into amadou (37, 40, 41) in esca symptomatic plants. To obtain information on the structure and the physiology of the bacterial microflora colonizing the various wood tissues of esca-foliar symptomatic and asymptomatic grapevines, we studied these bacteria by SSCP, the sequencing of isolates and Biolog Ecoplates™. Our aim was also to investigate whether certain bacterial communities or species were specifically associated with the wood tissues of diseased plants.

SSCP profiles of the global bacterial microflora showed that complex microflora colonize the wood of both the necrotic and healthy wood tissues. Depending on the particular wood samples, differences could be observed between the bacterial microflora of white rot, necrotic stripes and other wood tissues that look healthy (in the trunk and in the rootstock). This observation, which is common for fungal microflora (36), has been reported here, for the first time, for the bacteria. Biolog Ecoplates™ investigations showed, too, that for the bacteria colonizing the cordons of foliar-esca symptomatic plants, bacteria metabolism is tissue-dependent. Bacteria of white-rot, necrotic stripes and healthy wood metabolized differently the carbon substrates of the Biolog Ecoplates™, thus indicating that their trophic behaviors are different.

We observed that more than 40% of the pieces of wood were colonized by at least one bacterium. Of the 217 isolated strains, 50% are Gram- and 50% are Gram+. This proportion was maintained when only bacteria from the asymptomatic and the esca-foliar symptomatic plants were counted. A different proportion was found by Bell *et al.* (20), when they studied the 84 bacterial strains they isolated from grapevines, 78% of them were Gram- and 22% Gram+. Such differences may depend on the type of tissues. In the present study, the bacteria

were isolated from various wood tissues, whereas Bell *et al.* (20) studied only the bacteria inhabiting the xylem of disease-free grapevines.

The phylum of the most representative Gram+ for the two types of plants is Firmicutes and Proteobacteria for the Gram-. Whatever the pathogenic status of the plants, the Bacillales inhabited all the wood samples, with the most abundant genus being *Bacillus*. Of the 9 most frequent species, 6 belonged to the *Bacillus* genus, *i.e.* *Bacillus sp.* (14% of the total of strains), *Paenibacillus sp.*(12%), *Brevibacillus reuszeri* (8%), *Bacillus licheniformis* (7%), *Bacillus ginsengihumi* (6%), *Bacillus pumillus* (5%). Recent publications have reported that this genus seems to be a frequent and common colonizer of the various grapevine organs. West *et al.* (9) showed that the most abundant genus in the aerial parts (mainly cane and leaf samples, a few from the wood), in the roots and on the leaves of grapevines (cultivar Chardonnay grown in an Australian vineyard, 10 year-old) is *Bacillus*. Compant *et al.*, in 2011 (19), studied the endophytic bacteria inhabiting the reproductive organs and the roots of grapevines at two different vegetation stages: at the beginning of flowering, and when the berries are ready for harvesting. The *Bacillus* genus was isolated in each sample, and *Pseudomonas* were isolated in the rhizosphere at the two vegetative states. Thus, our results are in agreement with these previous reports but we provide evidence, for the first time, that *Bacillus* is common in the wood tissues of grapevines, and that it also colonizes the wood of asymptomatic and esca-foliar symptomatic grapevine.

Our study shows that certain species are able or not to specifically colonize the white-rot. Seventy percent of the total bacteria in the white rot are *Bacillus sp.* (30%), *Bacillus gisengihumi* (25%), *Bacillus licheniformis* (15%). But the order of *Enterobacteriales*, including *Pantoea agglomerans*, which is the second most isolated bacteria species, was not isolated from amadou. *Xanthomonadales* were isolated in all the samples, excepted in the white-rot and non-necrotic tissue of the cordon of the esca-foliar symptomatic plants.

When we considered other types of wood tissues, other bacteria species and proportions were observed. The necrotic stripes of the cordons are mainly colonized by *Brevibacillus reuszeri*, which is the most abundant (15%), followed by *Bacillus sp.* (13.2%) and *Pantoea agglomerans* (11.3%) Most of the *B. reuszeri* strains were only isolated in the esca-foliar symptomatic plants. In the healthy wood tissues, *P. agglomerans* species (14% of of total bacteria strains), *Bacillus sp.* (12.5%) and *Paenibacillus sp.* (11%) are the most numerous bacteria. Considered together, these data suggest that the specific structure of the healthy or degraded wood tissues is associated with specific bacterial communities.

With the exception of specific diseases related to xylem colonization, such as *X. fastidiosa*, the study of bacteria colonizing vine wood has not been the subject of specific experiments, even if one paper did mention that bacteria colonize these tissues (31). As the comparison of bacterial communities from esca-foliar symptomatic and asymptomatic has never been done, we might wonder what the role of these bacteria is. If we refer to the species isolated from the various tissues of wood and to the specific metabolism of strains that colonize the necrotic or the healthy wood tissues, two roles should be assigned to them: (i) a positive role, due to the biocontrol potential that many species have; (ii) a negative one, by predisposing the vine wood to fungal attacks.

(i) The positive role of bacteria: *Bacillus* species and strains are the more numerous cultivable bacteria isolated from grapevine wood tissues. *Bacillus* is a well-known Gram-positive spore-forming genus that is widely distributed in the environment. *Bacillus* species have been used for decades in the biotechnology industry because they produce several molecules of interest, e.g. enzymes, antibiotics, biochemicals and various other consumer products. For some of these reasons, such as the production of several broad-spectrum antibiotics, species of *Bacillus* have great potential for biological control, and their effectiveness in controlling multiple plant diseases has been reported by many scientists (42-46). Among the most abundant *Bacillus* species we identified, *B. licheniformis* has been used to control tomato gray mold caused by *Botrytis cinerea* (47), or to reduce mango post-harvest fruit diseases (anthracnose and stem-end rot) when applied as a dip treatment in a mango pack house (48). *B. licheniformis* along with *Bacillus ginsengihumi*, *B. pulmilis*, *Bacillus* sp. are common colonizers of the rhizosphere and, recently, Trivedi *et al.* (49) isolated and characterized strains of these species, mainly associated with asymptomatic plants, on citrus roots in Florida. Certain strains of *B. licheniformis* can reduce the pathogenic effect of some fungi on maize and sunflowers (50). Several reports show that some of these *Bacillus* species are useful for controlling grapevine plant pathogens. For instance, according to Krol (51) and Lehman *et al.* (52), *B. pumilus* and *Bacillus* sp., display biocontrol activity against *B. cinerea* and *Bacillus* sp. against *Agrobacterium vitis*. However, other *Bacillus* spp., not isolated here, such as *Bacillus subtilis*, have proven their interest for controlling *E. lata* (53) or *P. chlamydospora* (54), two pathogenic fungi involved in eutypa dieback and esca, respectively.

The second most frequent bacteria species isolated in our experiment was *Pantoea agglomerans*, a Gram- bacterium, initially defined as epiphytic (55) but also able to colonize the roots (56). In our study, *P. agglomerans* was isolated from the wood necrotic stripes (not in amadou) and non-necrotic tissues of cordon and trunk as well. Although its occurrence in

the wood has not been reported before, our data suggests that *P. agglomerans* is ubiquitous in the various organs of grapevine. Compant *et al.* (19) isolated it from the endorhiza at the flowering vegetative stage of the plant, and 19% of all the bacteria collected from the xylem by Bell *et al.* (20) were *P. agglomerans* (also called *Enterobacter agglomerans*). As for the *Bacillus* species mentioned above, *P. agglomerans* displays biocontrol activity. On apple, it has been used to control post-harvest diseases caused by *Penicillium expansum*, *B. cinerea* and *Rhizopus stolonifer* (57), as well as the fire blight caused by *Erwinia amylovora* (58). As reported by Magnin-Robert *et al.* (59), Trotel-Aziz *et al.* (60-62), and Verhagen *et al.* (2011), *P. agglomerans* is also able to control *B. cinerea* infections on vine.

Due to the production of metabolites responsible for their biocontrol activities, one may assume that inside the grapevines, *Bacillus* spp. and *Pantoea agglomerans* interact with the other micro-organisms, particularly the numerous fungi species, whether plant pathogenic or not, that colonize the wood tissues and control some of them. Thus, depending on the bacterial communities and the fungi they interact with, development of esca may be delayed, prevented or not.

(ii) The negative role of bacteria: At this stage of our knowledge concerning the role of bacteria colonizing grapevine wood, the main question that remains is: are bacteria involved in the degradation of the wood and in the formation of esca typical necroses, such as amadou? In order to understand the full complexity of the situation, it is essential to consider five additional points:

1. Bacteria and degradation of wood: a recent publication suggests that a range of bacteria are able to break down lignin and that even their role in degrading the wood of trees has been underestimated (35). In our experiment, although changes in the global bacterial microflora were observed over a one-year period (from January 2010 to January 2011), they were not associated with the development of wood necroses. SSCP analyses indicated that for one sampling time (July 2011) out of four, the bacterial communities of the asymptomatic plants were different from those of the esca-foliar symptomatic ones. However, this differentiation was not observed in July 2012, although at that very time, necrotic stripes developed in the cordons. So the global bacterial communities within apparently healthy ones may be associated with esca-foliar symptomatic plants, but this phenomenon does not seem to be frequent.

2. Metabolic behavior of the wood inhabiting bacteria: SSCP profiles showed that the bacterial communities colonizing amadou were different from all the others, but those of the necrosis generally did not differ from those of the healthy tissues. Interestingly, Biolog

Ecoplates™ investigations showed that, in the cordons, the trophic behavior of the amadou-, necrotic stripes- and healthy wood-inhabiting bacteria were different. Similarly, Biolog Ecoplates™ allowed us to differentiate bacterial communities from the healthy outer and inner wood tissues of the trunks, whereas SSCP did not differentiate them. So the bacteria metabolism seems to be wood-tissue dependent, even in the apparently healthy wood tissues.

3. Are bacteria the first or second colonizers of the wood tissues? Clausen (34) reported that bacteria and actinomycetes are probably the most common wood-inhabiting microorganisms and the initial colonizers of wood of trees. However, this piece of information is lacking for the grapevine. It is only in the present paper that we have been able to show that bacteria extensively colonize the wood of 10 year-old plants. Further studies with younger plants need to be undertaken in order to verify this key point. In white-rot, we might wonder whether the bacteria participate in wood degradation or if they only benefit from wood degradation resulting from the activity of *F. mediterranea*, the fungus predominant in this necrosis. Since white-rot is reproduced after wood inoculation with this fungus (63), the second hypothesis would seem more reasonable.

4. Do bacteria prepare the wood for fungal decay? Bacteria are able to affect wood permeability, attack wood structure, or work synergistically with other bacteria and soft-rot fungi to predispose wood to fungal attack (63). Among the bacteria able to degrade lignin, we detected a certain number of them: *Sphingomonas* sp., *Erwinia* sp. and *P. polymyxa*. So this hypothesis seems worth considering, but it needs to be verified.

5. Bacteria and fungi relationships: Bacteria can control wood decay fungi but many other relationships, either positive or negative, have been reported in the literature. Kamei *et al.* (64) observed that a wood-rotting fungus, *Stereum* sp. strain TN4F, and a coexisting bacteria isolated from natural white-rot decayed wood, interacted positively. The bacterium even significantly promoted the mycelial growth of the fungi. De Boer *et al.* (65) showed, however, that wood-inhabiting bacteria numbers were drastically reduced after colonization of beech (*Fagus sylvatica*) wood blocks by the white-rot fungus *Hypholoma fasciculare*. A third, subtle scenario, was described by Valaskova *et al.* (66), in which acid-tolerant bacterial strains seemed to rely on substrates released by lignocellulolytic enzyme activities of the white-rot fungi during decay. The authors also observed no indications for antagonism (antibiosis) of the bacteria against the fungus. In grapevine wood we cannot exclude that all of these scenarios occurred but, depending on the interaction of the microbial communities, any eventual shift during the cultural season may or may not prove visible.

To conclude, our study shows that the bacteria communities extensively colonize the grapevine wood of either esca-foliar symptomatic or asymptomatic plants. Our study also provides evidence that bacteria have certainly a role in the development of esca. Depending on the relationship established between the bacterial and fungal microflora, direct or indirect roles should be considered. Determining the succession of microflora would prove helpful in understanding how esca-wood necroses develop within the grapevine.

ACKNOWLEDGMENTS

The authors acknowledge Bordeaux Sciences Agro, the Regional Council of Aquitaine, French Ministry of Agriculture, Food-processing industry and Forest (programme CASDAR V906), Fondation Poupelain for funding the research program.

REFERENCES

1. **Kobayashi DYaP, J.D.** 2000. Bacterial endophytes and their effects on plants and uses in agriculture, vol. Microbial endophytes. Marcel Dekker, New York.
2. **QuadtHallmann A, Benhamou N, Kloepper JW.** 1997. Bacterial endophytes in cotton: Mechanisms of entering the plant. *Can. J. Microbiol.* **43**:577-582.
3. **Tervet JWH, J. P.** 1948. Bacteria in the storage organs of healthy plants. *Phytopathology* **38**:960-967.
4. **Hollis JP.** 1951. Bacteria in healthy potato tissue. *Phytopathology* **41**:350-366.
5. **Compant S, Clement C, Sessitsch A.** 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology & Biochemistry* **42**:669-678.
6. **Hardoin PVO, L.S.; Van Elsas J.D.** 2008. Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol* **16**:463-471.
7. **Gray EJ, Smith DL.** 2005. Intracellular and extracellular PGPR: commonalities and distinctions in the plant-bacterium signaling processes. *Soil Biology & Biochemistry* **37**:395-412.
8. **Hallmann J.** 2001. Plant interactions with endophytic bacteria.
9. **West ER, Cother EJ, Steel CC, Ash GJ.** 2010. The characterization and diversity of bacterial endophytes of grapevine. *Can. J. Microbiol.* **56**:209-216.
10. **Hallmann J, QuadtHallmann A, Mahaffee WF, Kloepper JW.** 1997. Bacterial endophytes in agricultural crops. *Can. J. Microbiol.* **43**:895-914.
11. **Jacobs MJ, Bugbee WM, Gabrielson DA.** 1985. Enumeration, location and characterization of endophytic bacteria within sugar-beet roots. *Canadian Journal of Botany-Revue Canadienne De Botanique* **63**:1262-1265.
12. **Misaghi IJ, Donndelinger CR.** 1990. Endophytic bacteria in symptom-free cotton plants. *Phytopathology* **80**:808-811.
13. **Sun L, Qiu FB, Zhang XX, Dai X, Dong XZ, Song W.** 2008. Endophytic bacterial diversity in rice (*Oryza sativa* L.) roots estimated by 16S rDNA sequence analysis. *Microb. Ecol.* **55**:415-424.
14. **Stoltzfus JR, So R, Malarvithi PP, Ladha JK, deBruijn FJ.** 1997. Isolation of endophytic bacteria from rice and assessment of their potential for supplying rice with biologically fixed nitrogen. *Plant Soil* **194**:25-36.
15. **Kuklinsky-Sobral HL, Araujo WL, Mendes R, Pizzirani-Kleiner AA, Azevedo JL.** 2005. Isolation and characterization of endophytic bacteria from soybean (*Glycine max*) grown in soil treated with glyphosate herbicide. *Plant Soil* **273**:91-99.
16. **Zinniel DK, Lambrecht P, Harris NB, Feng Z, Kuczmarski D, Higley P, Ishimaru CA, Arunakumari A, Barletta RG, Vidaver AK.** 2002. Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Appl. Environ. Microbiol.* **68**:2198-2208.
17. **Long HH, Schmidt DD, Baldwin IT.** 2008. Native Bacterial Endophytes Promote Host Growth in a Species-Specific Manner; Phytohormone Manipulations Do Not Result in Common Growth Responses. *Plos One* **3**.
18. **Backman P, Sikora RA** 2008. Endophytes: An emerging tool for biological control. *Biological Control* **46**:1-3.
19. **Compant S, Mitter B, Colli-Mull JG, Gangl H, Sessitsch A.** 2011. Endophytes of Grapevine Flowers, Berries, and Seeds: Identification of Cultivable Bacteria, Comparison with Other Plant Parts, and Visualization of Niches of Colonization. *Microb. Ecol.* **62**:188-197.
20. **Bell CR, Dickie GA, Harvey WLG, Chan J.** 1995. Endophytic bacteria in grapevine. *Can. J. Microbiol.* **41**:46-53.
21. **Ait Barka E, Eullaffroy P, Clement C, Vernet G.** 2004. Chitosan improves development, and protects *Vitis vinifera* L. against *Botrytis cinerea*. *Plant cell reports* **22**:608-614.

22. **Ait Barka E, Nowak J, Clement C.** 2006. Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, *Burkholderia phytofirmans* strain PsJN. *Appl. Environ. Microbiol.* **72**:7246-7252.
23. **Thorne ET, Stevenson JF, Rost TL, Labavitch JM, Matthews MA.** 2006. Pierce's disease symptoms: Comparison with symptoms of water deficit and the impact of water deficits. *American Journal of Enology and Viticulture* **57**:1-11.
24. **Hopkins DL.** 1989. *Xylella fastidiosa* - xylem-limited bacterial pathogen of plants. *Annu. Rev. Phytopathol.* **27**:271-290.
25. **Vizitiu D, Dejeu L.** 2011. Crown gall (*Agrobacterium* spp.) and grapevine. *Journal of Horticulture, Forestry and Biotechnology* **15**:130-138.
26. **Burr TJ.** 2004. Grape Crown Gall Biology and Strategies for Control. *FPS Grape Program Newsletter October 2004*:16-18.
27. **Bertsch C, MR-S, M. Magnin-Robert, P. Larignon, J. Chong, E. Abou-Mansour, A. Spagnolo, C. Clément and F. Fontaine.** 2012. Grapevine trunk diseases: complex and still poorly understood. *Plant Pathology* DOI: [10.1111/j.1365-3059.2012.02674.x](https://doi.org/10.1111/j.1365-3059.2012.02674.x).
28. **Grosman J, Doublet B.** 2012. Maladies du bois de la vigne: Synthèse des dispositifs d'observation au vignoble, de l'observatoire 2003-2008 au réseau d'épidémiologie actuelle. *Phytoma* **651**:31-35.
29. **Chiarappa L.** 2000. Esca (black measles) of grapevine. An overview. *Phytopathologia Mediterranea* **39**:11-15.
30. **Graniti A, Surico G, Mugnai L.** 2000. Esca of grapevine: a disease complex or a complex of diseases? *Phytopathologia Mediterranea* **39**:16-20.
31. **Mugnai L, Graniti A, Surico G.** 1999. Esca (Black Measles) and Brown Wood Streaking: Two Old and Elusive Diseases of Grapevines. *Plant Disease* **83**:404-417.
32. **Hofstetter V, Buyck B, Croll D, Viret O, Couloux A, Gindro K.** 2012. What if esca disease of grapevine were not a fungal disease? *Fungal Divers.* **54**:51-67.
33. **Bruez E, Vallance J, Gerbore J, Lecomte P, Guerin-Dubrana L, Rey P.** 2011. Characterization of endophytic microflora colonizing wood tissues of healthy and Esca-diseased vines. *Phytopathology* **101**:S21-S21.
34. **Clausen CA.** 1996. Bacterial associations with decaying wood: A review. *International Biodeterioration & Biodegradation* **37**:101-107.
35. **Bugg TDH, Ahmad M, Hardiman EM, Rahmanpour R.** 2011. Pathways for degradation of lignin in bacteria and fungi. *Nat. Prod. Rep.* **28**:1883-1896.
36. **Maher N, Piot J, Bastien S, Vallance J, Rey P, Guerin-Dubrana L.** 2012. wood necrosis in esca-affected vines: types, relationships and possible links with foliar symptom expression. *J. Int. Sci. Vigne Vin.* **46**:15-27.
37. **Viala P.** 1926. Recherches sur les maladies de la vigne: Esca. *Annal Epiphyt fasc 1 et 2*:1-108.
38. **Janniche GS, Spliid H, Albrechtsen H-J.** 2012. Microbial Community-Level Physiological Profiles (CLPP) and herbicide mineralization potential in groundwater affected by agricultural land use. *Journal of contaminant hydrology* **140-141**:45-55.
39. **Garland JL, Mills AL.** 1991. classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Appl. Environ. Microbiol.* **57**:2351-2359.
40. **Larignon P, Fontaine F, Farine S, Clement C, Bertsch C.** 2009. Esca and Black Dead Arm: two major actors of grapevine trunk diseases. *C. R. Biol.* **332**:765-783.
41. **Larignon P, Dubos B.** 1997. Fungi associated with esca disease in grapevine. *Eur. J. Plant Pathol.* **103**:147-157.
42. **Toure Y, Ongena M, Jacques P, Guiro A, Thonart P.** 2004. Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. *J. Appl. Microbiol.* **96**:1151-1160.

43. **Collins DP, Jacobsen BJ, Maxwell B.** 2003. Spatial and temporal population dynamics of a phyllosphere colonizing *Bacillus subtilis* biological control agent of sugar beet cercospora leaf spot. *Biol. Control* **26**:224-232.
44. **Emmert EAB, Handelsman J.** 1999. Biocontrol of plant disease: a (Gram-) positive perspective. *Fems Microbiology Letters* **171**:1-9.
45. **Paul B, Chereyathmanjiyl A, Masih I, Chapuis L, Benoit A.** 1998. Biological control of *Botrytis cinerea* causing grey mould disease of grapevine and elicitation of stilbene phytoalexin (resveratrol) by a soil bacterium. *Fems Microbiology Letters* **165**:65-70.
46. **Gueldner RC, Reilly CC, Pusey PL, Costello CE, Arrendale RF, Cox RH, Himmelsbach DS, Crumley FG, Cutler HG.** 1988. Isolation and identification of iturins as antifungal peptides in biological-control of peach brown rot with *Bacillus subtilis*. *J. Agric. Food Chem.* **36**:366-370.
47. **Lee JP, Lee SW, Kim CS, Son JH, Song JH, Lee KY, Kim HJ, Jung SJ, Moon BJ.** 2006. Evaluation of formulations of *Bacillus licheniformis* for the biological control of tomato gray mold caused by *Botrytis cinerea*. *Biol. Control* **37**:329-337.
48. **Govender V, Korsten L.** 2006. Evaluation of different formulations of *Bacillus licheniformis* in mango pack house trials. *Biol. Control* **37**:237-242.
49. **Trivedi P, Spann T, Wang NA.** 2011. Isolation and Characterization of Beneficial Bacteria Associated with Citrus Roots in Florida. *Microb. Ecol.* **62**:324-336.
50. **Schmiedeknecht G, Issoufou I, Junge H, Bochow H.** 2001. Use of *Bacillus subtilis* as biocontrol agent. V. Biological control of diseases on maize and sunflowers. *Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz-Journal of Plant Diseases and Protection* **108**:500-512.
51. **Krol E.** 2006. Fungi inhabiting healthy grapevine canes (*Vitis* spp.) in some nurseries. *Acta Agrobotanica* **59**:163-173.
52. **Lehman L, McCoy RJ, Messenger BJ, Manker DC, Orjala JE, Lindhard D.** 2000. A strain of *Bacillus pumilus* for controlling plant diseases. Patent WO.
53. **Schmidt CS, Lorenz D, Wolf GA.** 2001. Biological control of the grapevine dieback fungus *Eutypa lata* I: Screening of bacterial antagonists. *J. Phytopathol.-Phytopathol. Z.* **149**:427-435.
54. **Alfonzo A, Conigliaro G, Torta L, Burruano S, Moschetti G.** 2009. Antagonism of *Bacillus subtilis* strain AG against vine wood fungal pathogens. *Phytopathol. Mediterr.* **48**:155-158.
55. **Lindow SE, Brandl MT.** 2003. Microbiology of the phyllosphere. *Appl. Environ. Microbiol.* **69**:1875-1883.
56. **Francis CA, Obratsova AY, Tebo BM.** 2000. Dissimilatory metal reduction by the facultative anaerobe *Pantoea agglomerans* SP1. *Appl. Environ. Microbiol.* **66**:543-548.
57. **Nunes C, Usall J, Teixido N, Fons E, Vinas I.** 2002. Post-harvest biological control by *Pantoea agglomerans* (CPA-2) on Golden Delicious apples. *Journal of Applied Microbiology* **92**:247-255.
58. **Kim IY, Pusey PL, Zhao YF, Korban SS, Choi H, Kim KK.** 2012. Controlled release of *Pantoea agglomerans* E325 for biocontrol of fire blight disease of apple. *Journal of Controlled Release* **161**:109-115.
59. **Magnin-Robert M, Trotel-Aziz P, Quantinet D, Biangianti S, Aziz A.** 2007. Biological control of *Botrytis cinerea* by selected grapevine-associated bacteria and stimulation of chitinase and beta-1,3 glucanase activities under field conditions. *Eur. J. Plant Pathol.* **118**:43-57.
60. **Trotel-Aziz P, Couderchet M, Biangianti S, Aziz A.** 2008. Characterization of new bacterial biocontrol agents *Acinetobacter*, *Bacillus*, *Pantoea* and *Pseudomonas* spp. mediating grapevine resistance against *Botrytis cinerea*. *Environ Exp Bot* **102**:319-336.
61. **Trotel-Aziz P, Aziz A, Magnin-Robert M, Aït Barka E, Gognies S.** 2006. Bactéries présentant une activité protectrice de la vigne contre *Botrytis cinerea*. French patent.
62. **Verhagen B, Trotel-Aziz P, Jeandet P, Baillieul F, Aziz A.** 2011. Improved Resistance Against *Botrytis cinerea* by Grapevine-Associated Bacteria that Induce a Prime Oxidative Burst and Phytoalexin Production. *Phytopathology* **101**:768-777.

63. **Sparapano L, Bruno G, Ciccarone C, Graniti A.** 2000. Infection of grapevines by some fungi associated with esca. I. *Fomitiporia punctata* as a wood-rot inducer. *Phytopathologia Mediterranea* **39**:46-52.
64. **Kamei I, Yoshida T, Enami D, Meguro S.** 2012. Coexisting *Curtobacterium* Bacterium Promotes Growth of White-Rot Fungus *Stereum* sp. *Curr. Microbiol.* **64**:173-178.
65. **De Boer W, Folman LB, Gunnewiek P, Svensson T, Bastviken D, Oberg G, del Rio JC, Boddy L.** 2010. Mechanism of antibacterial activity of the white-rot fungus *Hypholoma fasciculare* colonizing wood. *Can. J. Microbiol.* **56**:380-388.
66. **Valaskova V, Snajdr J, Gunnewiek PK, Boer Wd, Baldrian P.** 2009. Saprotrophic basidiomycete *Hypholoma fasciculare* affects fungal and bacterial community composition in colonized wood and soil. *IOBC/WPRS Bulletin* **42**:173-176.

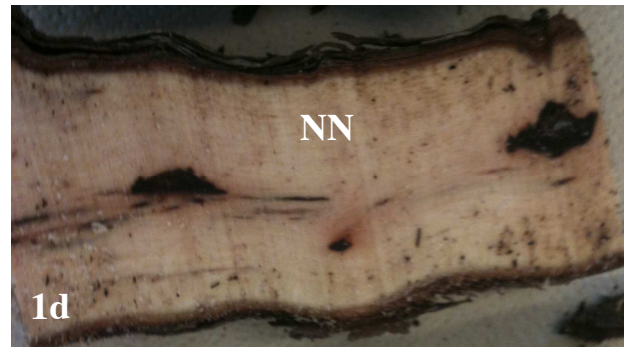
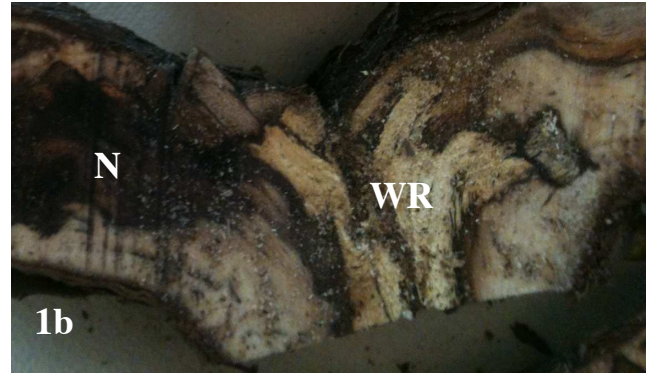


Fig. 1. Photographs of longitudinal-sections of cordons (Fig. 1A and Fig. 1B) and trunks (Fig. 1C and Fig. 1D) of plants that had expressed (Fig. 1B and Fig. 1D) or not esca-foliar symptoms (Fig. 1A and Fig. 1C). N= Necrosis tissue, NN= Non-Necrotic tissue, WR=White Rot.

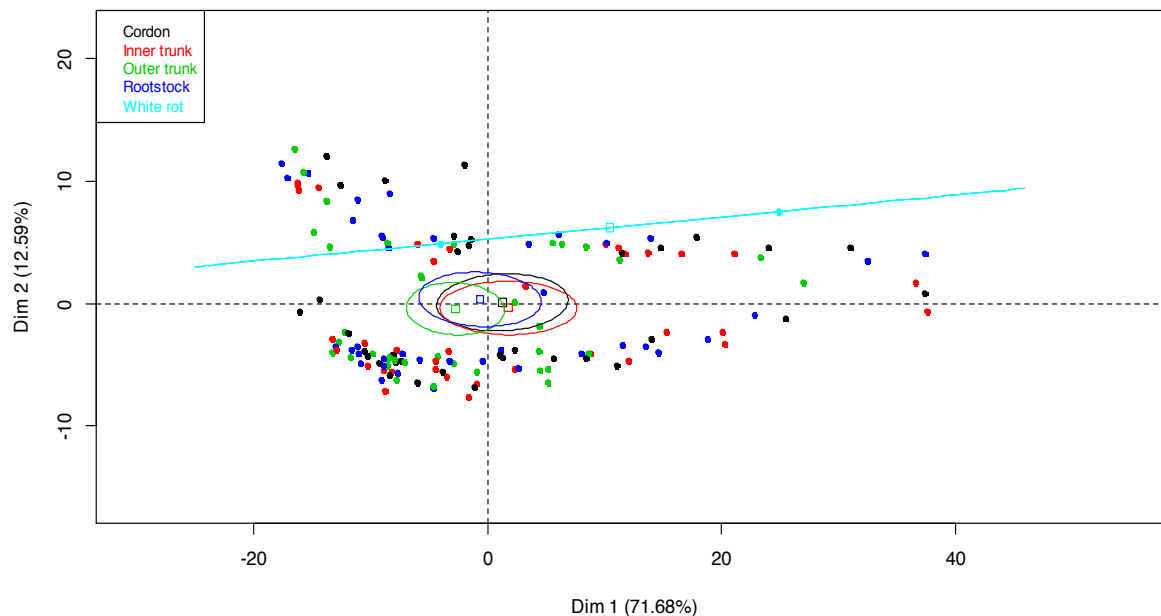


Fig. 2. Bacterial communities of the white rot and the non-necrotic tissues of the inner and outer trunks, the rootstocks and the cordons of the esca-foliar symptomatic and asymptomatic plants. All the sampling times (January 2010 to January 2011) are pooled. The distribution of the bacterial communities on the principal planes designs by the global Principal Component Analysis (PCA) are obtained from numeral values of each SSCP profile. The total variation (%) explains by PCA is given in brackets. Clusters representing the bacterial communities are identified by different colors: red= inner trunk, green= outer trunk, black= cordon, dark blue= rootstock, light blue= white rot.

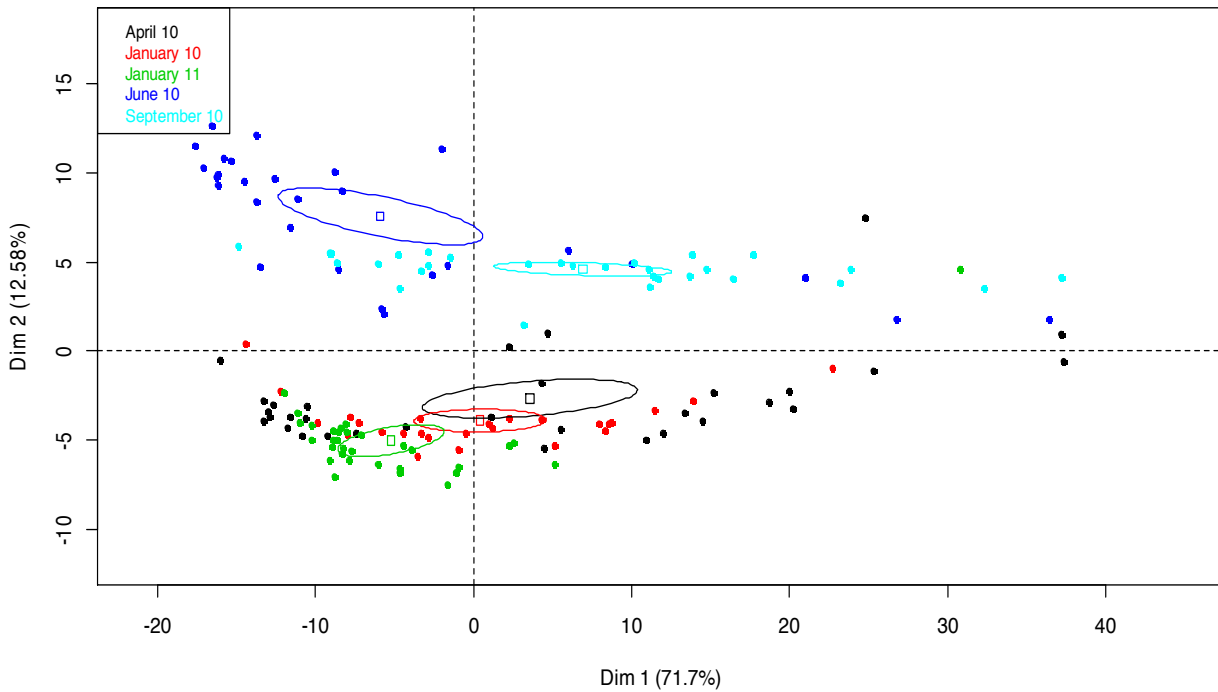


Fig. 3. Bacterial communities colonizing at each sampling time the esca-foliar symptomatic and asymptomatic plants. Note that for each sampling time, the non-necrotic wood tissues (inner and outer trunks, rootstocks and cordons) of the esca-foliar symptomatic and asymptomatic plants are pooled. The distribution of the bacterial communities on the principal planes designs by the global Principal Component Analysis (PCA) are obtained from numeral values of each SSCP profile. The total variation (%) explains by PCA is given in brackets. Clusters representing the bacterial communities are identified by different colors: red= January 2010, black= April 2010, dark blue= June 2010, light blue= September 2010, green= January 2011.

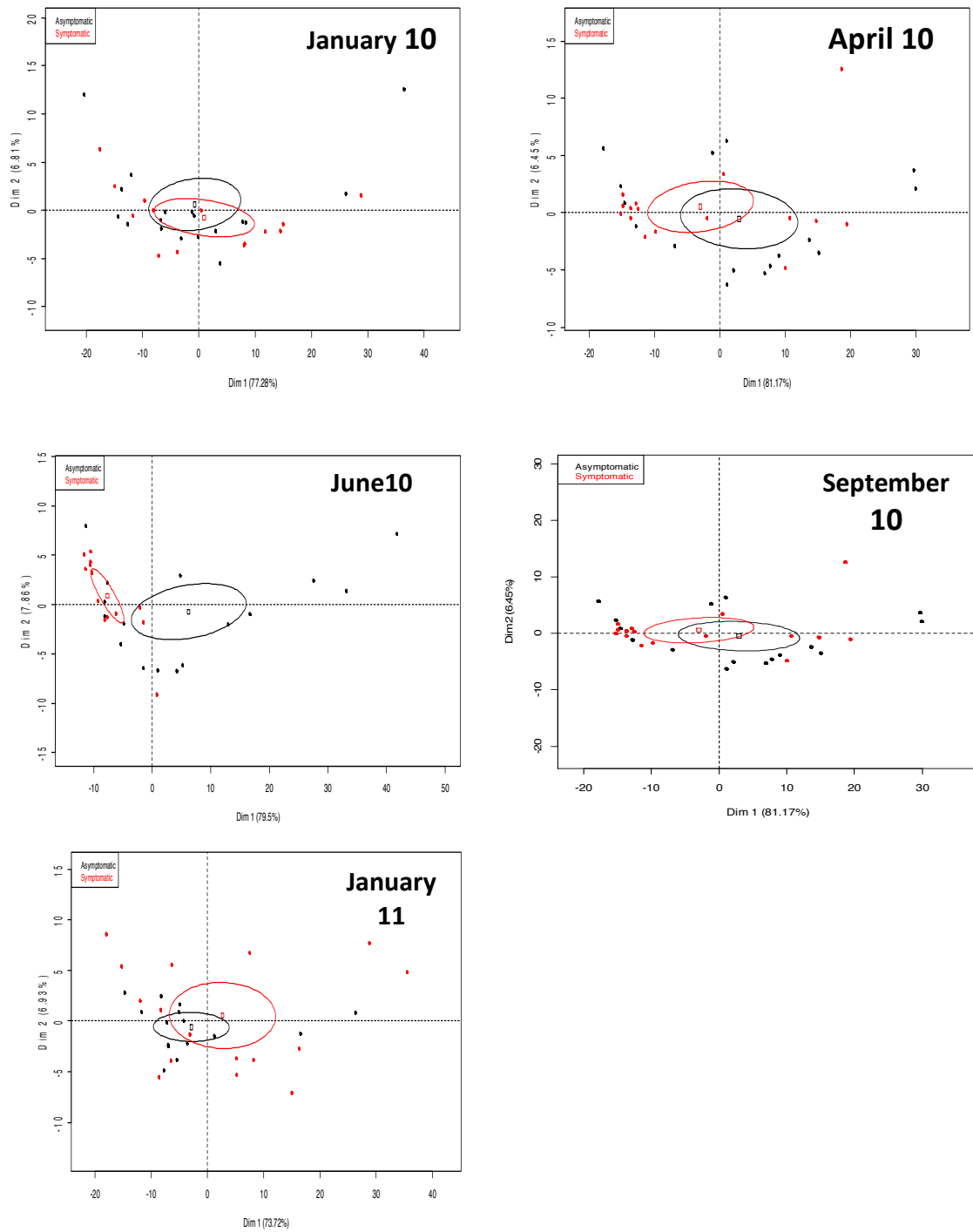


Fig. 4. Bacterial communities colonizing the non-necrotic tissues (inner trunks, outer trunks, rootstocks and cordons) for each sampling time (January, April, June and September of 2010 and January 2011) of esca-foliar symptomatic and asymptomatic plants. The distribution of the bacterial communities on the principal planes designs by the global Principal Component Analysis (PCA) is obtained from numeral values of each SSCP profile. The total variation (%) explains by PCA is given in brackets. Clusters representing the bacterial communities are identified by different colors: red=esca-foliar symptomatic plants, black= asymptomatic plants.

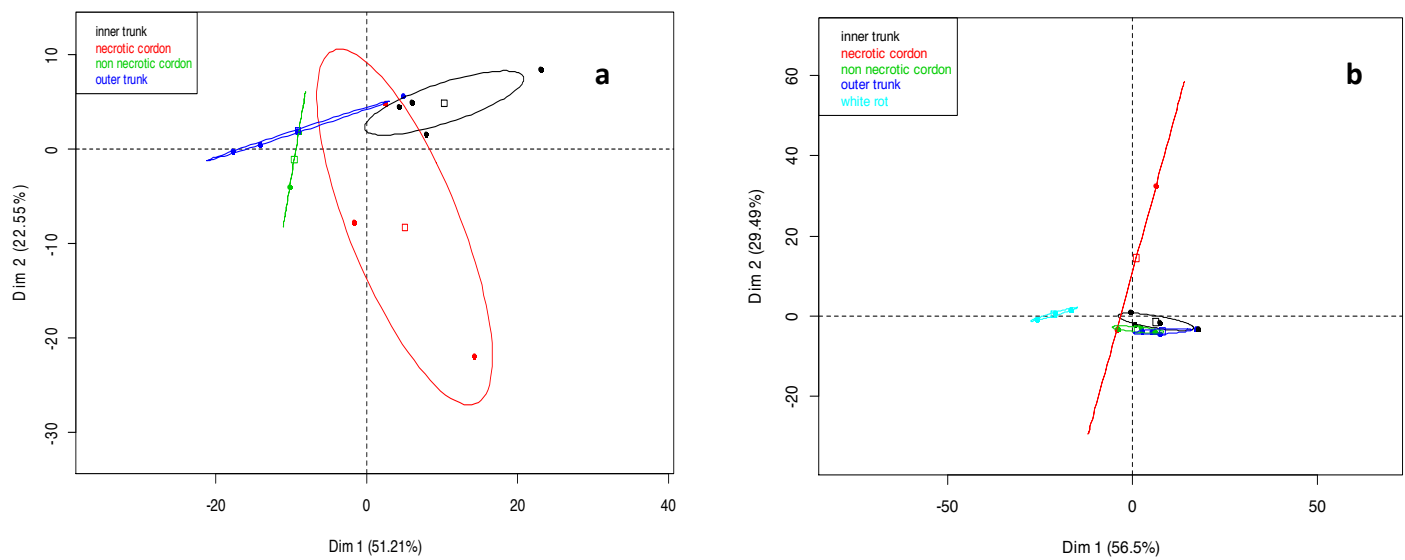


Fig. 5. Distribution of the bacterial communities on the principal planes designs by the global Principal Component Analysis (PCA) obtains from numeral values of each SSCP profiles. The total variation (%) explains by PCA is given in brackets.

- a. Clusters represent the bacterial communities colonizing the necrotic tissue (stripes) of cordon, the non-necrotic tissues of inner trunk, outer trunk and cordon of asymptomatic plants of July 2012.
- b. Clusters represent the bacterial communities colonizing the white rot of cordon, the necrotic tissue (stripes) of cordon, the non necrotic tissues of inner trunk, outer trunk and cordon of the esca-foliar symptomatic of July 2012.

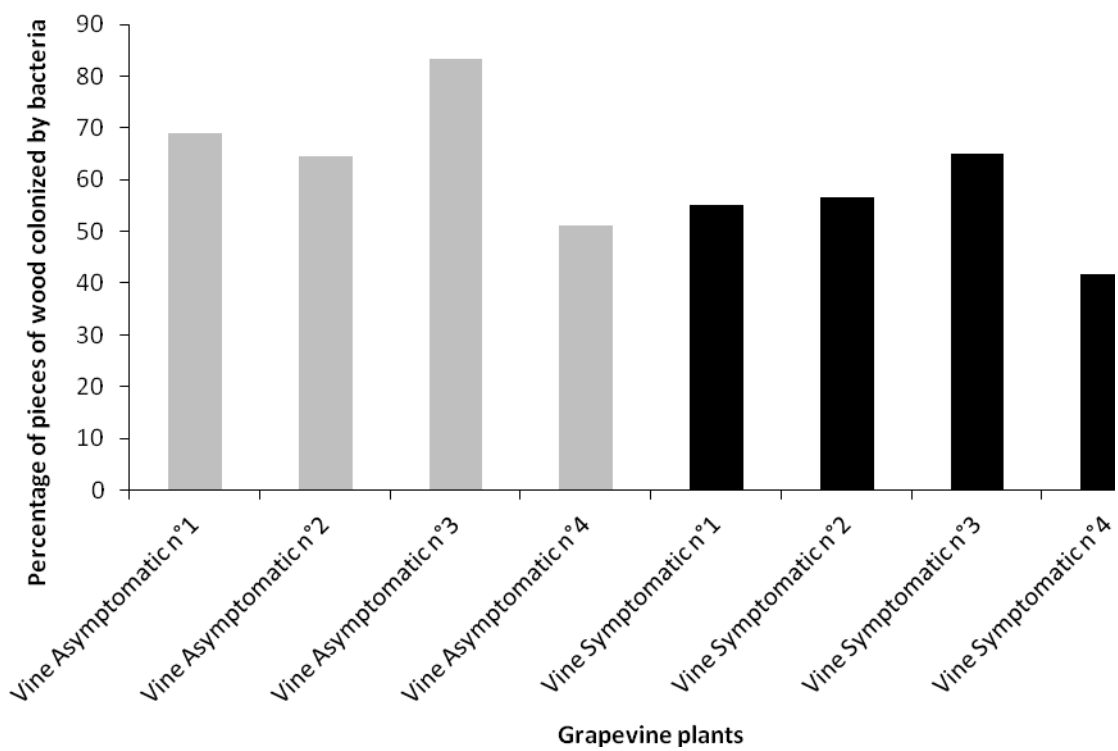


Fig. 6. Percentage of the pieces of wood colonized by bacteria. The grapevines had expressed (in black) or not (in grey) the esca-foliar symptoms. A: asymptomatic plants; S: esca-foliar symptomatic plants.

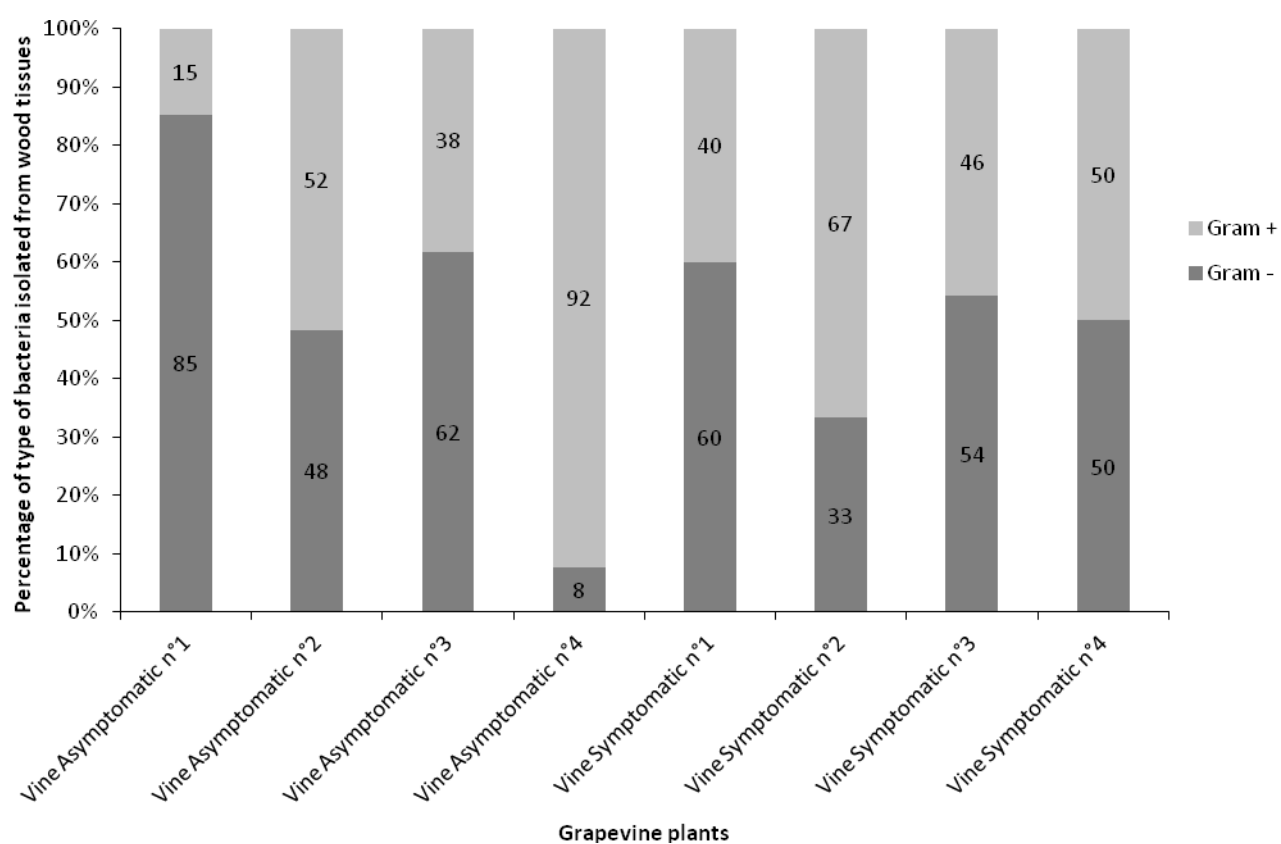


Fig. 7. Number of Gram- and Gram+ isolated from the wood tissues of grapevines. A : asymptomatic plants; S : symptomatic plants.

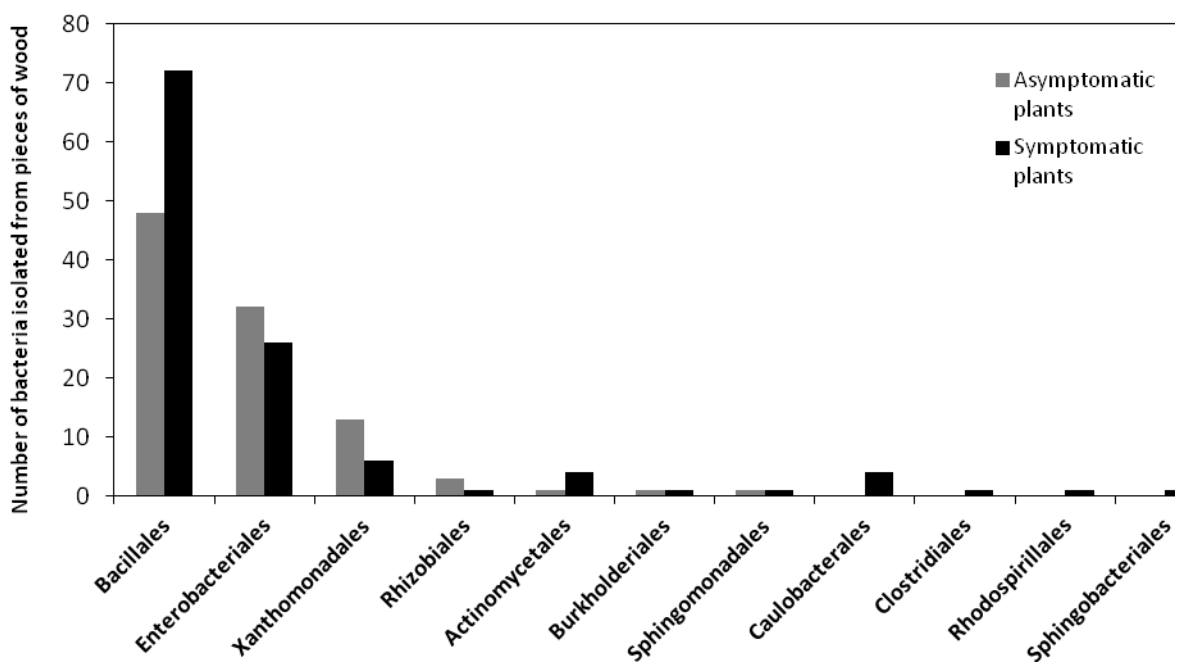


Fig. 8. Percentage of bacteria from the different identified orders. Bacteria have been isolated from the asymptomatic plants (grey) and esca-foliar asymptomatic plants (black).

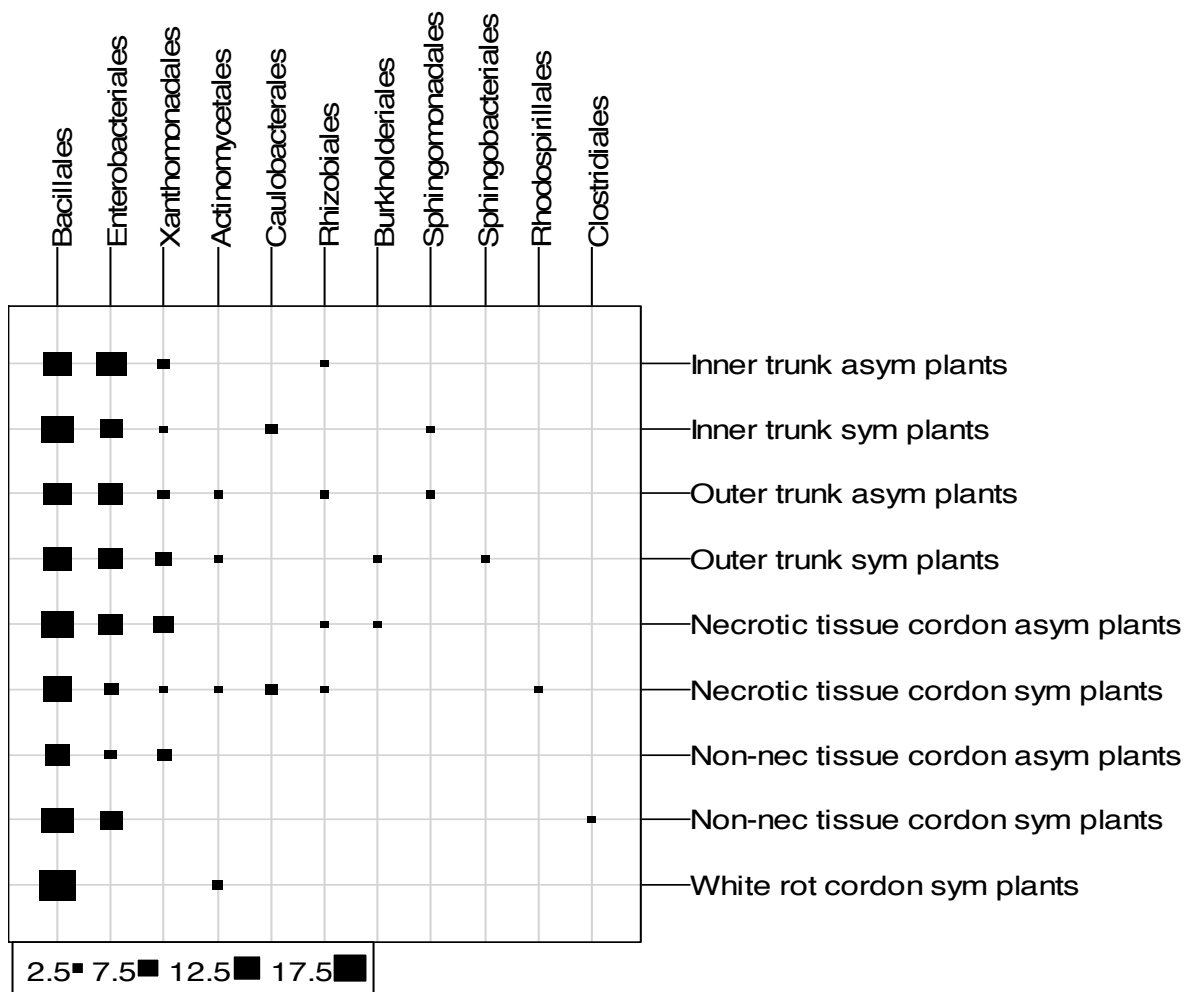


Fig. 9. Number of bacteria per order isolated from to the different wood tissues of the grapevines. Asym: asymptomatic plants; Sym: esca-foliar symptomatic plants; Non-nec: non-necrotic wood

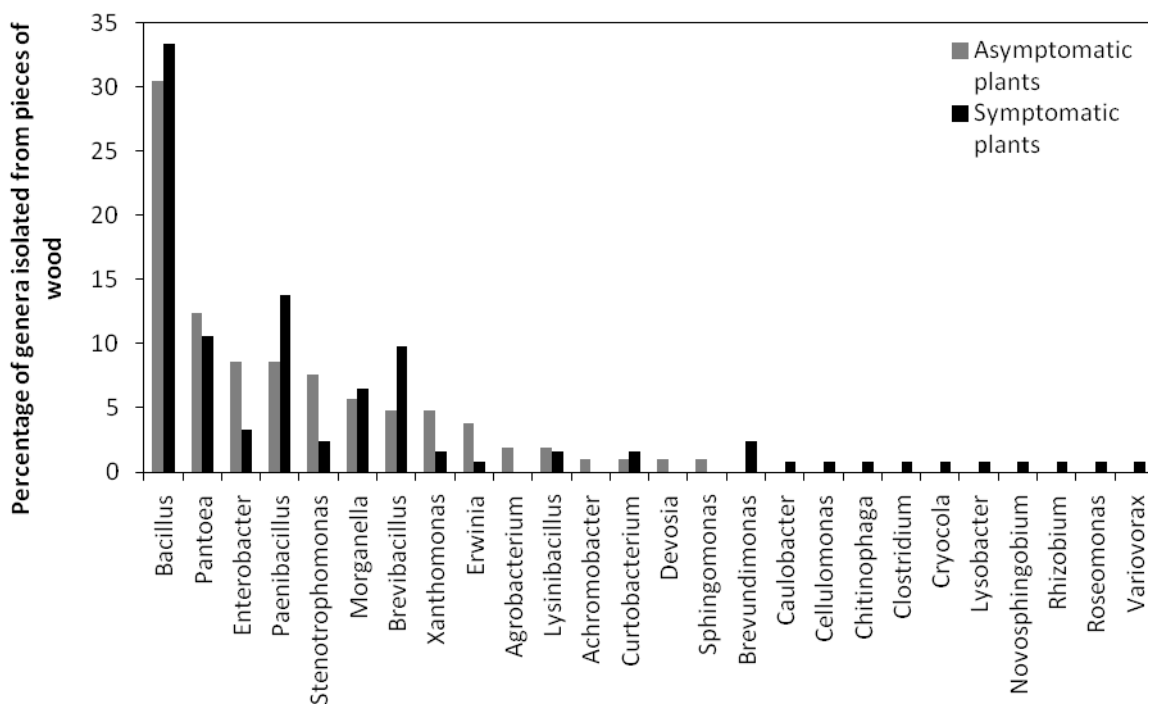


Fig. 10. Distribution of the different genera of bacteria isolated from the esca-foliar symptomatic and asymptomatic plants. Asymptomatic plants are in grey and esca-foliar asymptomatic plants in black.

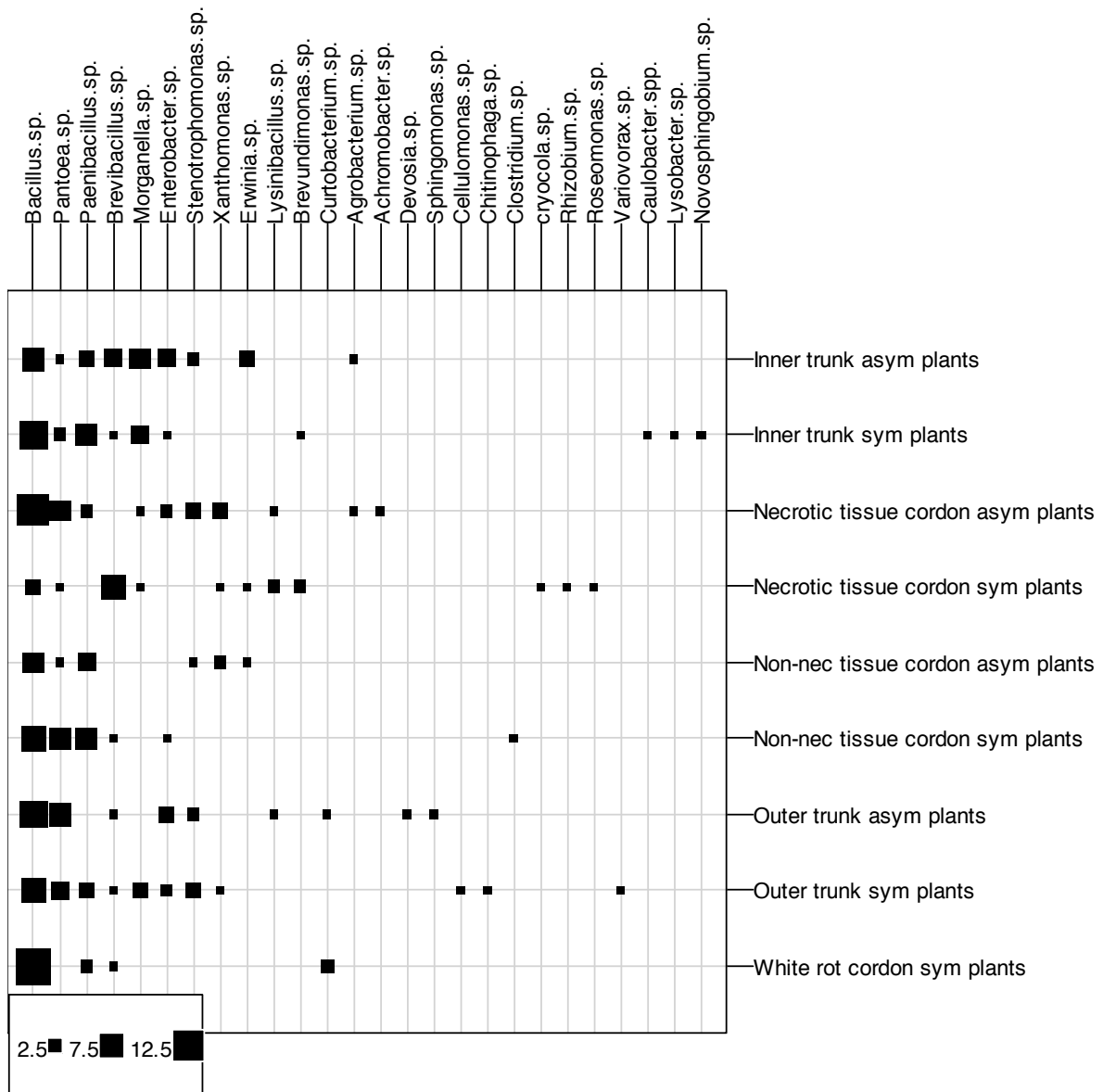
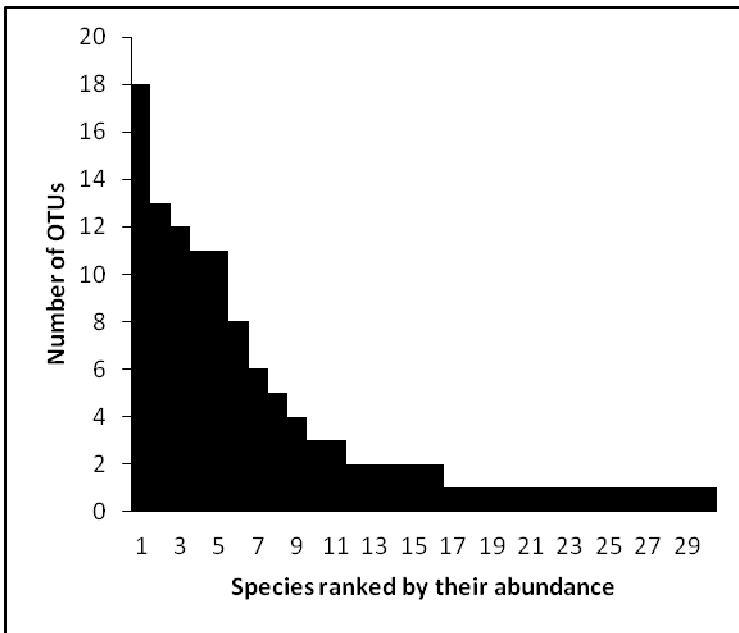
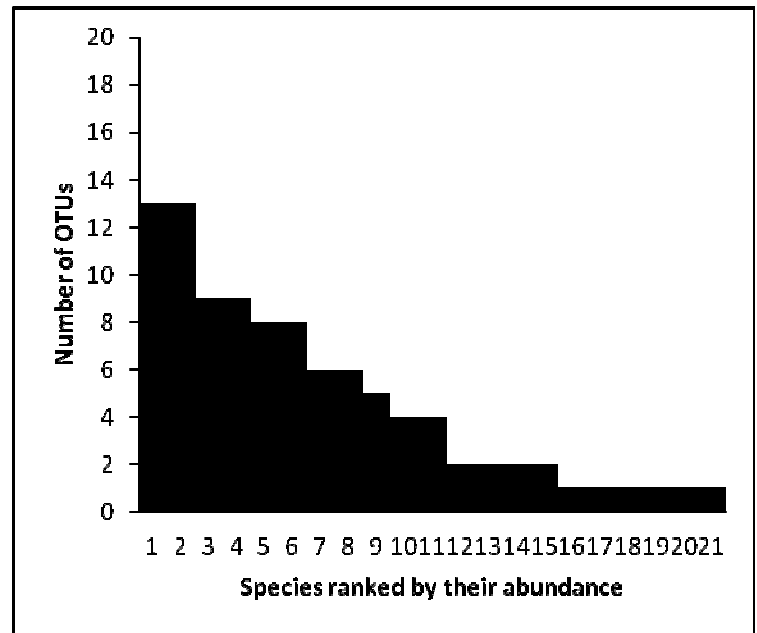


Fig. 11. Genera isolated from different part of the vines. Asym: asymptomatic plants; Sym: esca-foliar symptomatic plants; Non-nec: non-necrotic wood.



a



b

Fig. 12. Structure of bacterial communities isolated from the wood tissues. The species of bacteria are ranked by their abundance. Esca-foliar symptomatic (a) and asymptomatic (b) plants.

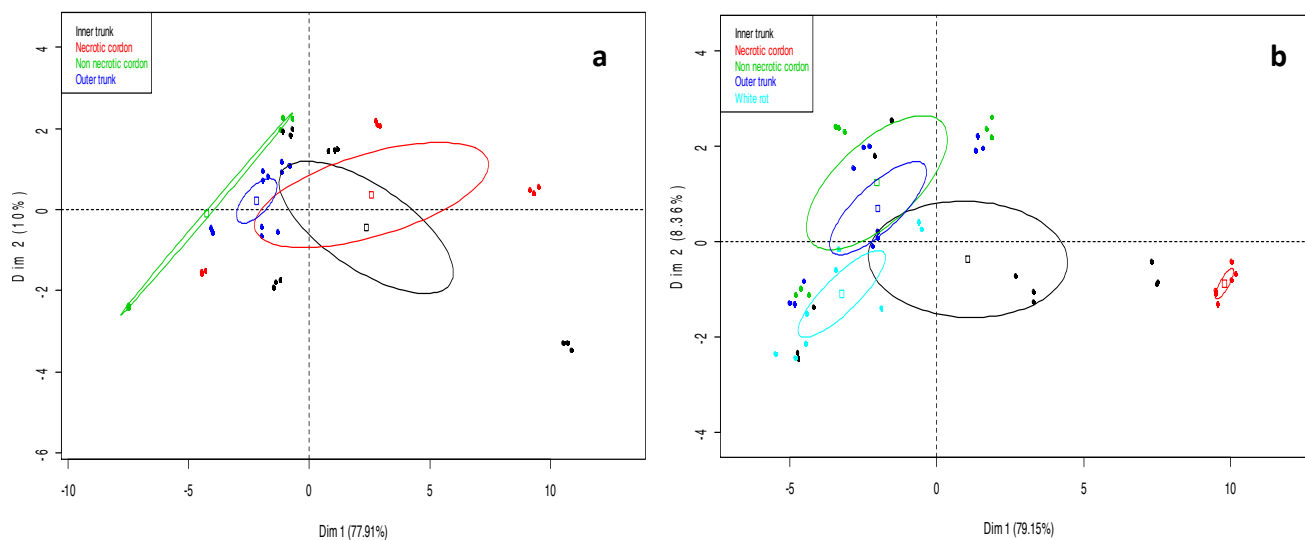


Fig. 13. Distribution of the bacterial communities on the principal planes designs by the global Principal Component Analysis (PCA) obtains from numeral values of each AWCD values from Biolog Ecoplates™. The total variation (%) explains by PCA is given in brackets. Clusters represent the bacteria taken from the plants and their ability to metabolize the substrates of the Biolog Ecoplates™. Grapevines were sampled in July 2012. Biolog Ecoplates™ were incubated 48H at 27°C in the dark.

- a. Bacteria were taken from the necrotic tissue (stripes in the cordons) and the non-necrotic wood tissues (inner trunk, outer trunk and cordon) of asymptomatic grapevines.
- b. Bacteria were taken from the white rot, the necrotic tissue (stripes in the cordons) and the non-necrotic wood tissues (inner trunk, outer trunk and cordon) of esca-foliar symptomatic grapevines.

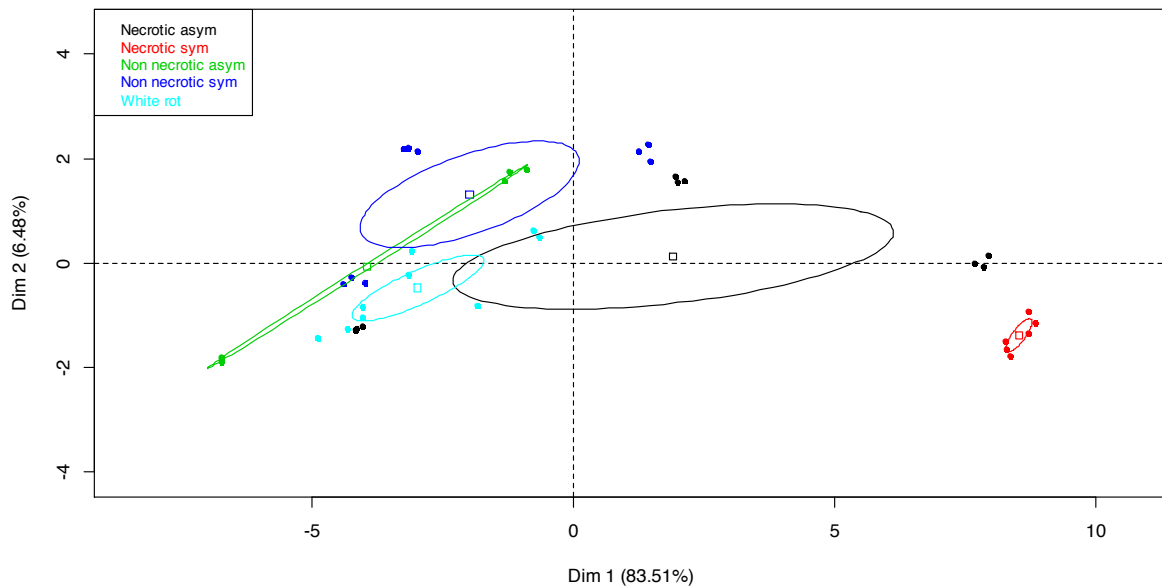


Fig. 14. Distribution of the bacterial communities on the principal planes designs by the global Principal Component Analysis (PCA) obtains from numeral values of each AWCD values from Biolog Ecoplates™. The total variation (%) explains by PCA is given in brackets. Clusters represent the bacteria colonizing the wood tissues of esca-foliar symptomatic and asymptomatic plants sampled in July 2012 and their ability to degrade the substrates of the Biolog Ecoplates™. Bacteria that colonize the various cordon-wood tissues (white rot, necrotic (stripes) and non necrotic wood tissues) are compared. Biolog Ecoplates™ were incubated 48H at 27°C in the dark. Asym= asymptomatic plants; Sym= esca-foliar symptomatic plants.

Table 1. Description of the pathological status of the grapevines wood tissues sampled from asymptomatic and esca-foliar symptomatic plants

| Vine plant | State | Necrotic tissue in cordon | | Non-necrotic tissue | | |
|------------|--------------|---------------------------|-----------------|---------------------|-------------|-------------|
| | | White rot | Necrosis tissue | Cordon | Inner trunk | Outer trunk |
| R26C33 | Asymptomatic | | X | | X | X |
| R37C18 | Asymptomatic | | X | | X | X |
| R4C8 | Asymptomatic | | X | X | X | X |
| R70C30 | Aymptomatic | | | X | X | X |
| R43C24 | Symptomatic | X | | X | X | X |
| R38C45 | Symptomatic | X | | X | X | X |
| R53C29 | Symptomatic | X | | | X | X |
| R67C24 | Symptomatic | | X | | X | X |

Table 2. Bacterial species ranked by the different part and the status of the vines (16S rDNA sequencing of the isolated bacteria).

| Bacteria species | Asymptomatic plants | | | | Symptomatic plants | | | | |
|---------------------------------------|---------------------|-------------|---------------------------|-------------------------------|--------------------|-------------|---------------------------|-------------------------------|---------------------|
| | Inner trunk | Outer trunk | Necrotic tissue of cordon | Non-necrotic tissue of cordon | Inner trunk | Outer trunk | Necrotic tissue of cordon | Non-necrotic tissue of cordon | White rot of cordon |
| <i>Bacillus sp.</i> (31) | 3 | 2 | 5 | 3 | 6 | 3 | 2 | 1 | 6 |
| <i>Pantoea agglomerans</i> (26) | 1 | 6 | 5 | 1 | 2 | 4 | 1 | 6 | |
| <i>Paenibacillus sp.</i> (19) | 3 | | 1 | 4 | 4 | 1 | | 4 | 2 |
| <i>Brevibacillus reuszeri</i> (17) | 4 | 1 | | | 1 | 1 | 8 | 1 | 1 |
| <i>Bacillus licheniformis</i> (15) | 1 | 6 | 1 | 1 | 1 | 1 | | 1 | 3 |
| <i>Morganella morganii</i> (14) | 5 | | 1 | | 4 | 3 | 1 | | |
| <i>Bacillus ginsengihumi</i> (13) | | | 1 | 1 | 2 | | | 4 | 5 |
| <i>Enterobacter sp.</i> (13) | 4 | 3 | 2 | | 1 | 2 | | 1 | |
| <i>Bacillus pumilus</i> (11) | | 1 | 5 | | | 3 | | 1 | 1 |
| <i>Stenotrophomonas sp.</i> (11) | 2 | 2 | 3 | 1 | | 3 | | | |
| <i>Xanthomonas sp.</i> (6) | | | 3 | 1 | | 1 | 1 | | |
| <i>Erwinia billingiae</i> (5) | 3 | | | 1 | | | 1 | | |
| <i>Paenibacillus lautus</i> (3) | | | | | 1 | 2 | | | |
| <i>Agrobacterium</i> (2) | 1 | | 1 | | | | | | |
| <i>Bacillus aminovorans</i> (2) | 2 | | | | | | | | |
| <i>Brevundimonas sp.</i> (2) | | | | | | | 2 | | |
| <i>Curtobacterium sp.</i> (2) | | 1 | | | | | | | 1 |
| <i>Lysinibacillus sp.</i> (2) | | 1 | 1 | | | | | | |
| <i>Lysinibacillus sphaericus</i> (2) | | | | | | | 2 | | |
| <i>Paenibacillus turicensis</i> (2) | | | | | | | | 2 | |
| <i>Achromobacter sp.</i> (1) | | | 1 | | | | | | |
| <i>Bacillus firmus</i> (1) | | | | | | | 1 | | |
| <i>Brevundimonas sp.</i> (1) | | | | | 1 | | | | |
| <i>Calaubacter sp.</i> (1) | | | | | 1 | | | | |
| <i>Cellulomonas sp.</i> (1) | | | | | | 1 | | | |
| <i>Chitinophaga sp.</i> (1) | | | | | | 1 | | | |
| <i>Clostridium sp.</i> (1) | | | | | | | | 1 | |
| <i>Cryocola sp.</i> (1) | | | | | | | 1 | | |
| <i>Curtobacterium sp.</i> (1) | | | | | | | | | 1 |
| <i>Devosia sp.</i> (1) | | 1 | | | | | | | |
| <i>Lysobacter sp.</i> (1) | | | | | 1 | | | | |
| <i>Novosphingobium sp.</i> (1) | | | | | 1 | | | | |
| <i>Paenibacillus barengoltzii</i> (1) | | | | | 1 | | | | |
| <i>Paenibacillus polymyxa</i> (1) | | | 1 | | | | | | |
| <i>Rhizobium sp.</i> (1) | | | | | | | 1 | | |
| <i>Roseomonas sp.</i> (1) | | | | | | | 1 | | |
| <i>Sphingomonas sp.</i> (1) | | 1 | | | | | | | |
| <i>Variovorax sp.</i> (1) | | | | | | 1 | | | |
| <i>Xanthomons translucens</i> (1) | | | | 1 | | | | | |
| Total of isolates | 29 | 25 | 31 | 14 | 27 | 27 | 22 | 22 | 20 |

Table 3. Alpha-diversity of the bacterial species isolated from the esca-foliar symptomatic and asymptomatic vines.

| Plant | State | Richness (S) | Abundance | Shannon (H) | Simpson (1-D) | Evenness (J) |
|--------|--------------|--------------|-----------|-------------|---------------|--------------|
| R26C33 | Asymptomatic | 12 | 25 | 2.28 | 0.87 | 0.42 |
| R37C18 | Asymptomatic | 14 | 27 | 2.53 | 0.93 | 0.47 |
| R4C8 | Asymptomatic | 14 | 34 | 2.30 | 0.87 | 0.42 |
| R70C30 | Asymptomatic | 7 | 19 | 1.71 | 0.78 | 0.32 |
| R38C45 | Symptomatic | 15 | 30 | 2.51 | 0.90 | 0.46 |
| R43C24 | Symptomatic | 12 | 33 | 2.18 | 0.86 | 0.4 |
| R53C29 | Symptomatic | 15 | 39 | 2.48 | 0.89 | 0.46 |
| R67C24 | Symptomatic | 12 | 21 | 2.24 | 0.86 | 0.41 |

Chapitre 3

Etude par pyroséquençage de la microflore fongique et bactérienne colonisant le bois de ceps de vigne

Introduction

La microflore fongique présente dans le bois de vigne a été étudiée dans les chapitres 1 et 2 en utilisant, entre autre, des approches de microbiologie classique associées au séquençage des isolats microbiens. La grande diversité des communautés fongiques et bactériennes colonisant le bois sain de la vigne a été notée, cette diversité étant nettement plus restreinte lorsqu'on considère le bois partiellement ou totalement nécrosé. La technique d'empreinte moléculaire utilisée, *i.e.* la SSCP, a montré son intérêt pour analyser rapidement un grand nombre d'échantillons. La comparaison des communautés microbiennes colonisant des tissus de bois de ceps ayant exprimé ou non des symptômes foliaires d'esca a été possible, des changements de microflore en fonction du temps ayant été mis en évidence.

La SSCP nous a également permis de sélectionner les échantillons de bois qui semblaient pertinents afin d'étudier d'une façon encore plus approfondie les communautés de micro-organismes par une technique de séquençage à haut débit. Notre choix s'est porté sur la technique de pyroséquençage 454 car elle est, depuis quelques années, largement appliquée à l'étude de l'écologie microbienne afin de caractériser les communautés fongiques et bactériennes en définissant leur richesse, diversité, composition et propriétés fonctionnelles en relation avec leurs plantes hôtes et leur environnement (Becklin *et al.*, 2012 ; Blaaid *et al.*, 2012; Cordier *et al.*, 2012). Kubartova *et al.* (2012) et Valaskova *et al.* (2009) ont notamment apporté des éléments de compréhension sur le rôle des champignons et des bactéries dans la décomposition des tissus ligneux chez différentes espèces d'arbres. D'autre part, les différents outils d'analyses utilisés consécutivement à l'obtention des résultats de pyroséquençage, *e.g.* la distance métrique Unifrac pour déterminer la beta-diversité (Lozupone and Knight, 2005), les Analyses en Coordonnées principales (ACoP), permettent de comparer de façon statistique les différentes microflore provenant d'écosystèmes et d'échantillons différents.

Description de la technique de pyroséquençage

Ronaghi *et al.* (1999) ont décrit le pyroséquençage comme une technique de séquençage, caractérisée par la révélation en temps réel de la synthèse d'un brin d'ADN. Cette technologie repose sur plusieurs avancées techniques: la PCR en émulsion, les plaques en fibre optique (PicoTiterplate, PTP), le pyroséquençage, et les technologies informatiques pour l'analyse des images (Margulies *et al.*, 2005).

Lorsque le processus de pyroséquençage est réalisé, après extraction et préparation des ADN pour le séquenceur, l'ADN génomique ou, dans notre cas, des adaptateurs sont fixés aux amplicons, par ligation, aux extrémités des fragments (Fig. 1). Des microbilles présentant à leur surface des amorces complémentaires à un des adaptateurs fixent une molécule d'ADN simple brin (ADNsb) à la fois. Les microbilles liées à l'ADNsb sont mises en émulsion en présence des réactifs de PCR (Fig. 1). Chaque gouttelette correspond à un « micropuits » qui contient une microbille liée à une molécule d'ADN. Dans chaque gouttelette est réalisée une amplification clonale du fragment lié à la bille. Après amplification et tri, les microbilles

porteurs de l'ADN amplifié sont transférées dans une plaque en fibre optique (PTP) contenant environ 1,6 millions de puits (Fig. 1). Les puits possèdent un diamètre qui assure le dépôt d'une seule microbille par puits. La PTP est ensuite placée dans une chambre conçue pour créer un flux de réactifs de séquençage au-dessus des ouvertures des puits, et pour lire la réaction de pyroséquençage (Fig.2).



Figure 1. Préparation des échantillons pour le pyroséquençage. Fragmentation de l'ADN, ajout d'adaptateurs et séparation de l'ADNdb en ADNsb ; PCR en émulsion; Transfert des microbilles dans la PTP et ajout des enzymes (immobilisées sur des microbilles) nécessaires au pyroséquençage (Source : Future virol, 2011).

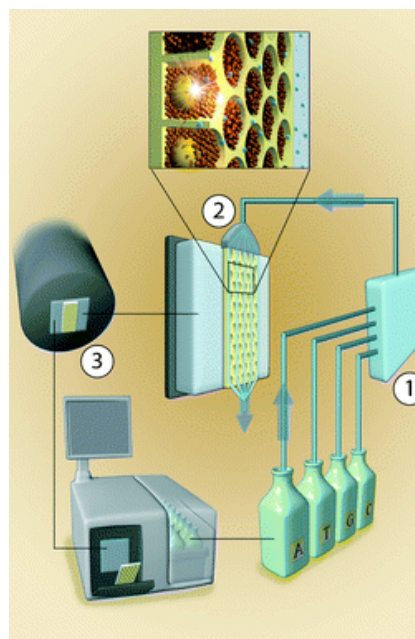


Figure 2. Appareil de pyroséquençage. (1) : assembleur de fluides; (2) : chambre de flux; (3) : caméra CCD couplée à un ordinateur (Margulies *et al.*, 2005).

La réaction de pyroséquençage se fait en suivant l'activité de l'ADN polymérase en temps réel par bioluminescence (Hyman, 1988; Nyrén, 1987; Ronaghi *et al.*, 1996; Ronaghi *et al.*, 1998). L'ADN à séquencer est hybridé avec une amorce de séquençage. Ce fragment hybridé est incubé avec un mélange d'enzymes et de réactifs immobilisés sur des microbilles (Fig. 1). Une cascade enzymatique, impliquant une ADN polymérase, une ATP sulfurylase, une luciférase et une apyrase (Fig. 3), permet de suivre la synthèse du brin d'ADN complémentaire au brin d'intérêt (Ahmadian *et al.*, 2006).

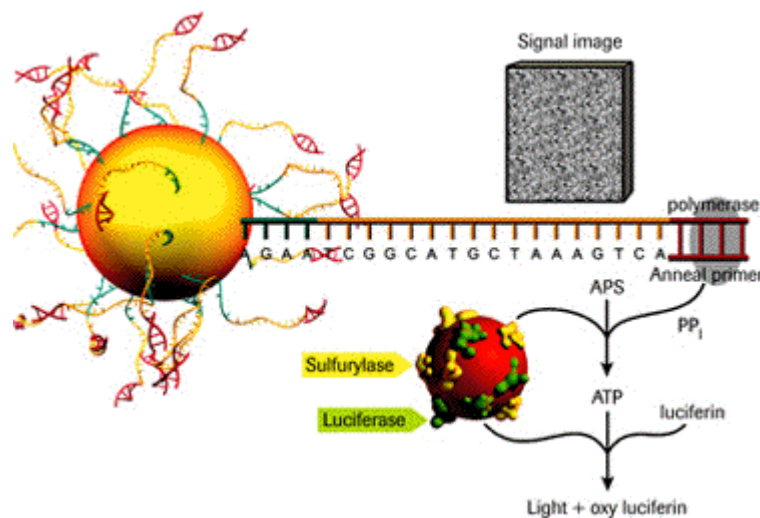


Figure 3. Représentation schématique du système enzymatique du pyroséquençage (Source : ©Roche).

Les dNTP ne sont pas ajoutés tous ensemble comme dans une réaction de séquençage Sanger, mais l'un après l'autre par flux successifs. Si le dNTP ajouté dans le milieu réactionnel correspond à celui attendu par l'ADN polymérase (complémentaire de la base du fragment d'intérêt), il est incorporé dans le brin en cours de synthèse tout en libérant un pyrophosphate inorganique (PPi) (Fig. 3). Les PPi libérés sont proportionnels en molarité au nombre de dNTP incorporés (Ronaghi *et al.*, 1996). Les PPi sont transformés en ATP par l'ATP sulfurylase (Fig. 3). En présence de luciférine, les ATP sont utilisés par la luciférase pour produire de l'oxyluciférine et émettre un signal lumineux qui peut être capté par une caméra CCD (Ronaghi *et al.*, 1998). L'intensité du signal lumineux est fonction du nombre de nucléotides incorporés sur le brin d'ADN en synthèse (Margulies *et al.*, 2005). Enfin les dNTP non incorporés ainsi que l'ATP sont dégradés par un flux contenant une apyrase. Un nouveau cycle d'addition d'un autre dNTP peut alors recommencer. La caméra CCD (Fig. 2) permet de capturer les images après addition de chaque nucléotide. Connaissant l'ordre dans lequel les 4 nucléotides sont ajoutés automatiquement, l'analyse des différentes images capturées permet de déduire la séquence des différents fragments d'ADN d'intérêt (illustré par un pyrogramme ou flowgram). Une séquence obtenue par pyroséquençage est appelée «read».

Objectif de l'étude

Dans le cas présent, en utilisant la technique de pyroséquençage 454 qui, à notre connaissance, n'a jamais servi pour étudier les communautés fongiques et bactériennes

colonisant le bois chez la vigne notre objectif est, au delà de la mise en évidence de la diversité des micro-organismes, d'apporter des informations supplémentaires permettant d'élaborer un scénario de dégradation d'un bois sain en un tissu nécrosé.

Pour notre étude, nous avons utilisé les mêmes échantillons de bois des ceps prélevés au Château Luchey-Halde (voir chapitres 1 et 2) car ils présentent l'avantage d'être peu nécrosés au niveau du tronc, d'avoir développé un tissu typique, l'amadou, qui est historiquement et scientifiquement associé à l'esca (Maher *et al.*, 2012 ; Viala, 1926). Cette diversité d'états pathologiques au niveau du bois permettra certainement de comparer les situations et d'apprécier quel est le niveau de micro-organismes potentiellement pathogènes ou non dans ces structures.

Matériels et Méthodes

1) Vignoble et échantillonnage des ceps

Cette expérimentation a été réalisée en prélevant des tissus ligneux sur des plants de *Vitis vinifera*, cépage Cabernet-Sauvignon, plantés depuis 2001 dans un sol sablo-limoneux, au Château Luchey-Halde à Pessac-Léognan. Sur cette parcelle, les symptômes foliaires typiques de l'esca sont évalués chaque année durant l'été depuis 2004. Les plants appelés « symptomatiques » sélectionnés pour la présente étude ont exprimé au moins 2 fois des symptômes foliaires d'esca entre 2006 et 2009. Les plants dits « asymptomatiques » sont des ceps témoins qui n'ont jamais exprimé ces symptômes d'esca. Quatre ceps symptomatiques et quatre plants asymptomatiques ont été prélevés lors de chaque saison : au printemps (Avril 2010), en été (Juin 2010), en automne (Septembre 2010) et en hiver (Janvier 2011) (Tableau 1). Ces ceps ont été sectionnés longitudinalement au niveau des bras, du tronc et du porte-greffe. Des échantillons de tissus non-nécrosés et d'amadou (présent uniquement dans le bras des plants symptomatiques) ont été prélevés, afin d'en extraire les ADN qui ont servi pour le pyroséquençage.

Tableau 1. Echantillonnage des plants de vigne.

Table1. Sampling of the vines.

| April 2010 | | June 2010 | | September 2010 | | January 2011 | |
|-------------|-------------|-------------|-------------|----------------|-------------|--------------|-------------|
| Asym plants | Symp plants | Asym plants | Symp plants | Asym plants | Symp plants | Asym plants | Symp plants |
| Cordon | Cordon | Cordon | Cordon | Cordon | Cordon | Cordon | Cordon |
| Trunk | Trunk | Trunk | Trunk | Trunk | Trunk | Trunk | Trunk |
| Rootstock | Rootstock | Rootstock | Rootstock | Rootstock | Rootstock | Rootstock | Rootstock |
| | White rot | | White rot | | White rot | | White rot |

Asym=Asymptomatique (Asymptomatic) ; Sym= Symptomatique (Symptomatic)

2) Extraction des ADN à partir du bois de vigne

Pour chaque échantillon, 5 à 10 g de bois ont été prélevés. Ces échantillons ont été placés dans un congélateur à -80°C dans l'attente d'être broyés à l'aide d'un Dangoumau (broyeur à bille). Une fois broyés dans l'azote liquide, les poudres d'échantillons ont été placées dans des tubes Falcon™ et conservées à -80°C. Soixante milligrammes ont été utilisés pour extraire les ADN à l'aide du kit d'extraction Indvisorb Spin Plant Mini Kit (Eurobio, France). Les ADN extraits ont été dosés à l'aide d'un NanoDrop (ND-1000, Thermoscientific, Labtech) et ils ont été homogénéisés à une concentration de 10 ng/μl.

3) Préparation des échantillons (à l'UR BIOGER de Paris Grignon)

Dans le cadre de notre étude sur les communautés bactériennes et fongiques, deux couples d'amorces ont été utilisés (Tableau 2). La PCR a été réalisée dans un volume

réactionnel de 50 μ l contenant du tampon PCR 1X, du MgCl₂ à 1.5 mM, des dNTPs à 200 μ M, du tag-primé à 0.5 μ M et de la Taq Polymérase à 2.5U. Le programme suivant a été utilisé : 5 minutes de dénaturation à 95°C suivi de 28 cycles de dénaturation (30 secondes à 95°C), d'hybridation (52 °C pour les champignons et 54°C pour les bactéries), et d'élongation (1 minute et 15 secondes pour les champignons et 45 secondes pour les bactéries) et suivi d'une élongation finale à 72°C pendant 5 minutes.

Tableau 2. Amorces sélectionnées pour le pyroséquençage.

Table 2. Primers used for the pyrosequencing.

| | Gene | Name | Other name | Sequences (5' -> 3') | References | Annealing temperature | Size of amplification |
|----------|-----------|-------|------------|------------------------|--|-----------------------|-----------------------|
| Fungi | ITS1-ITS2 | ITS1F | ITS1F | CTTGGTCATTTAGAGGAAGTAA | Gardes & Bruns, 1993; White et al., 1990 | 52°C | env. 700 pb |
| | | ITS4 | ITS4 | TCCTCCGCTTATTGATATGC | | | |
| Bacteria | ARNr 16S | 16S-F | 799f | AACMGGATTAGATACCKG | Redford et al., 2010 | 54°C | env. 350 pb |
| | | 16S-R | 1115r | AGGGTTGCGCTCGTTG | | | |

Le pyroséquençage par 454 permet de caractériser la diversité fongique et bactérienne des échantillons issus du bois de vigne. Pour cette technique, afin de réassigner quelles séquences correspondent à quel échantillon, les amorces sont marquées de façon spécifique. En effet, chaque amorce intègre une séquence barcode (MID = Multiplexes IDentifiers) de dix nucléotides en 5' qui est unique à chaque échantillon. Ce barcode permettra de trier les séquences par échantillon initial.

L'analyse d'échantillons complexes (communautés) est soumise à différents biais techniques, c'est pourquoi il a été nécessaire de réaliser des tests préalables pour tenter de les identifier. Différents nombres de cycles d'amplification ont donc été testés pour les différents marqueurs. En effet, il est préférable d'arrêter l'amplification durant la phase exponentielle (avant d'atteindre le plateau PCR) afin de ne pas modifier les proportions des différents taxa. Le nombre de cycles choisis a été 28. Un témoin artificiel composé d'un mélange d'ADN de souches pures en quantités connues, a également été amplifié avec les amorces ITS1F et ITS4 combinées à différents MID afin de déterminer si ceux-ci influaient sur le rendement PCR et sur la diversité et la proportion des taxa représentés.

Les produits de PCR ont ensuite été purifiés avec le QIAquick PCR purification kit (Qiagen) puis dosés au NanoDrop (ND-1000, Nano-Drop Technologies, Wilmington, DE) afin de vérifier la quantité et la qualité avant pyroséquençage. La taille des produits de PCR a été confirmée par électrophorèse sur gel d'agarose. Les produits de PCR purifiés ont ensuite été quantifiés plus finement en utilisant le Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Saint Aubin, France) sur un ABI Prism 7900HT (Life Technologies-Invitrogen-A-BIOSYSTEM, Villebon-sur-Yvette, France) et mélangés en quantité équimolaire pour obtenir 500 ng d'ADN total pour chaque librairie. Les amplicons regroupés en « librairies »

ont été pyroséquencés en utilisant un 454 GS FLX XL+ (454 Life Sciences – Roche Company, Branford, CT) à la plateforme GeT (Génome et Transcriptome) de Toulouse.

4) Traitement des pyroséquences et analyses

1 021 690 pyroséquences (reads) ont été obtenues pour 33 échantillons (28 échantillons naturels + 5 échantillons artificiels).

La qualité moyenne des bases séquencées est de 30.7 ± 1.6 (moyenne \pm écart-type, max = 35.0 ; min = 26.6). Les pyroséquences ont été filtrées à l'aide d'un script Python (Pyrocleaner) développé par la plateforme bioinformatique de Toulouse. Ce script permet de supprimer les séquences qui ont une longueur plus petite que la longueur moyenne moins 2 écart-types ou une longueur plus grande que la longueur moyenne plus 2 écart-types, un contenu en bases ambiguës (N) supérieur à 4% et il nettoie sur la complexité (pour une fenêtre glissante de 100 nucléotides, un pas de 5 et 40 comme limite du rapport complexité/longueur).

Toutes les étapes d'analyse des séquences fongiques et bactériennes ont été réalisées à l'aide du logiciel QIIME (Quantitative Insights Into Microbial Ecology, version 1.5.0) (Caporaso *et al.*, 2010). Les séquences ont été filtrées une deuxième fois afin d'éliminer celles dont la taille est <200 pb avec un score de qualité <25 et qui contiennent des caractères ambigus ou plus de 6 homopolymères. Une étape de « denoising » a ensuite permis de réduire le nombre d'erreurs de séquençage afin de ne pas classer certaines séquences comme des OTUs uniques additionnelles. Les séquences ont été soumises aux programmes Metaxa (Bengtsson *et al.*, 2011) et VXtractor (Hartmann *et al.*, 2010) pour les bactéries et FungalITSextractor (Nilsson *et al.*, 2010) pour les champignons afin d'extraire les régions variables V5 et V6 et les régions ITS1 et ITS2 respectivement. Une table représentative des OTUs (Unités Taxonomiques Opérationnelles) au seuil de 97% a alors été construite afin de servir pour l'assignation taxonomique et la comparaison statistique des différentes bibliothèques. Les bactéries ont été identifiées par BLAST grâce à la base de données Greengenes et les champignons grâce à la base de données UNITE.

Des courbes de raréfaction des espèces de champignons et de bactéries ont été réalisées à l'aide du logiciel EstimateS (Software win82.0) (Colwell *et al.*, 2005). Ces courbes permettent de savoir, lorsqu'elles présentent un plateau, si l'échantillonnage était suffisant pour estimer la diversité réelle.

La diversité microbienne au sein de chaque bibliothèque (alpha-diversité), exprimée par les indices de Shannon et Chao 1, a été calculée en fonction du nombre d'OTUs représentatives et du nombre de séquences qu'elles contiennent pour chaque échantillon. La structure des communautés microbiennes entre chaque bibliothèque a ensuite été comparée en générant une matrice des distances par la méthode de Bray Curtis et visualisée par Analyse en Coordonnées Principales (ACoP). Pour obtenir des ACoP, on utilise la matrice des distances ainsi que la distance des vecteurs.

Résultats

1) Distribution des séquences d'ADN obtenues

Un total de 12065 séquences d'ADNs bactériens, représentées par 593 OTUs et de 209106 séquences d'ADNs fongiques représentées par 870 OTUS a été obtenu. Le tableau 3 montre la répartition du nombre de séquences par type de cep et par temps de prélèvement. Pour les séquences bactériennes le nombre de séquences varie de 751 pour les cepts asymptomatiques d'avril à 2494 pour les cepts symptomatiques de juin. Pour le nombre de séquences fongiques, le nombre varie de 19413 à 34740 (Tableau 3).

Tableau 3. Nombre de séquences par type de plants et par temps de prélèvements.

Table 3. Number of sequences per type of plants and per sampling date.

| | | April | | June | | September | | January | |
|---------------------|---|-------------|--------------|-------------|--------------|-------------|--------------|-------------|--------------|
| Number of sequences | | Symptomatic | Asymptomatic | Symptomatic | Asymptomatic | Symptomatic | Asymptomatic | Symptomatic | Asymptomatic |
| Bacteria | Non-necrotic tissues of Cordon, Trunk and Rootstock | 1754 | 751 | 2342 | 1179 | 1565 | 803 | 1635 | 802 |
| | White rot | 245 | | 152 | | 585 | | 253 | |
| Fungi | Non-necrotic tissues of Cordon, Trunk and Rootstock | 28458 | 34740 | 26989 | 19413 | 13855 | 26679 | 29343 | 30526 |
| | White rot | 2270 | | 4289 | | 5825 | | 4191 | |

2) Analyse des communautés fongiques

2.1) Courbes de raréfactions des espèces fongiques

Au total, 674 OTUs ont été déterminées pour les champignons. Sur ces 674 OTUs, 373 ont été identifiées chez les plants symptomatiques et 400 pour les plants asymptomatiques.

Lorsque l'on observe ces courbes (Figure 1), les analyses de raréfactions montrent que le nombre d'échantillons prélevés et analysés n'est pas suffisant pour avoir une estimation de la diversité fongique réelle colonisant les différents tissus ligneux de la vigne. Le nombre d'espèces regroupées en OTUs et identifiées est donc sous-estimé.

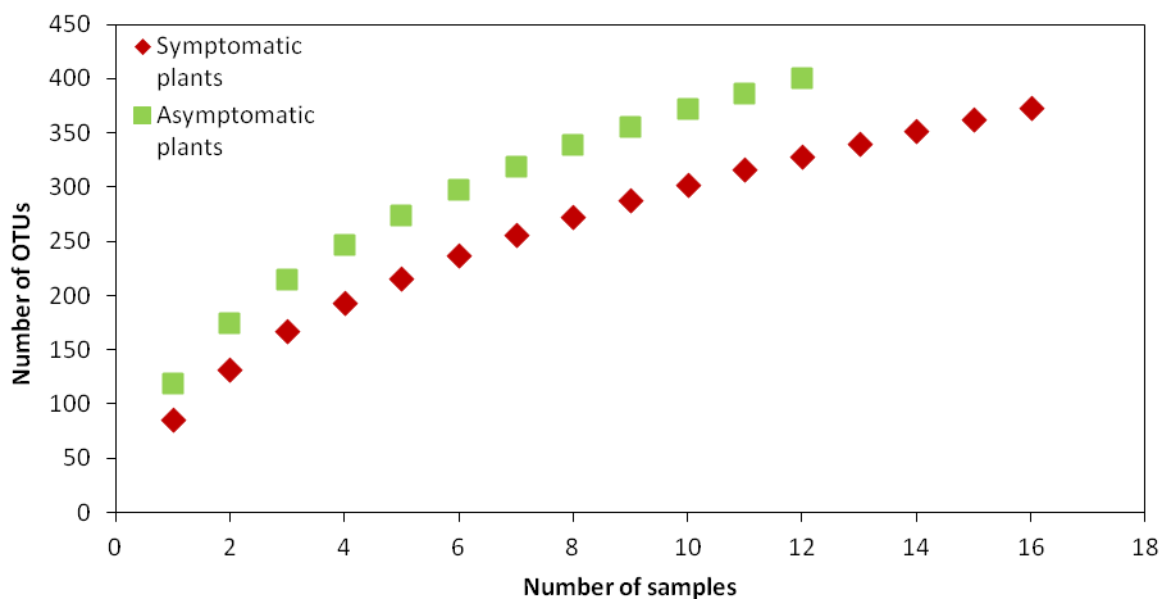


Figure 1. Courbes d'accumulation des espèces qui montre le nombre d'OTUs de champignons en ordonnées selon le nombre d'échantillons de tissus étudiés. En vert= nombre total des OTUs détectés dans les échantillons des plants sains. En rouge= nombre total des OTUs déterminés dans les échantillons des plants symptomatiques.

Figure 1. Accumulation curves of fungal species showing the number of OTUs of the different samples explained in the table 1. In green = number of OTUs of the asymptomatic plants. In red = number of the symptomatic plants.

2.2) Des phyla aux genres

Afin d'avoir une vision globale des communautés colonisant les ceps de vigne pour calculer les abondances des phyla et des genres fongiques, les ADN des différentes parties des 4 ceps asymptomatiques ou des 4 ceps symptomatiques ont été rassemblés à chaque temps de prélèvement. Comme le montre très distinctement la Figure 2, seul l'amadou est colonisé par une majorité de Basidiomycètes. Dans les autres parties, les Ascomycètes sont les plus abondants (de 68 à 87%).

La figure 3 quant à elle, montre la répartition et l'abondance des différents genres qui ont été déterminés selon les parties. Le genre *Fomitiporia* colonise essentiellement l'amadou (78%) mais est également abondant dans le bois de tronc de plants symptomatiques (16%). Le genre *Phaeoconiella* est le plus fréquent dans les bras des plants symptomatiques et asymptomatiques et dans le tronc et le porte-greffe des plants asymptomatiques. Excepté pour l'amadou, entre 6 et 15% de champignons n'ont pas pu être déterminés ne serait ce qu'au niveau du phylum, et la majorité d'entre eux sont potentiellement pathogènes comme les genres *Fomitiporia*, *Neofusicoccum*, *Phaeoconiella*, *Phaeoacremonium*, *Acremonium*, *Diplodia*, *Cryptococcus*. Seul *Bionectria*, assez abondant, est un genre identifié comme étant potentiellement protecteur.

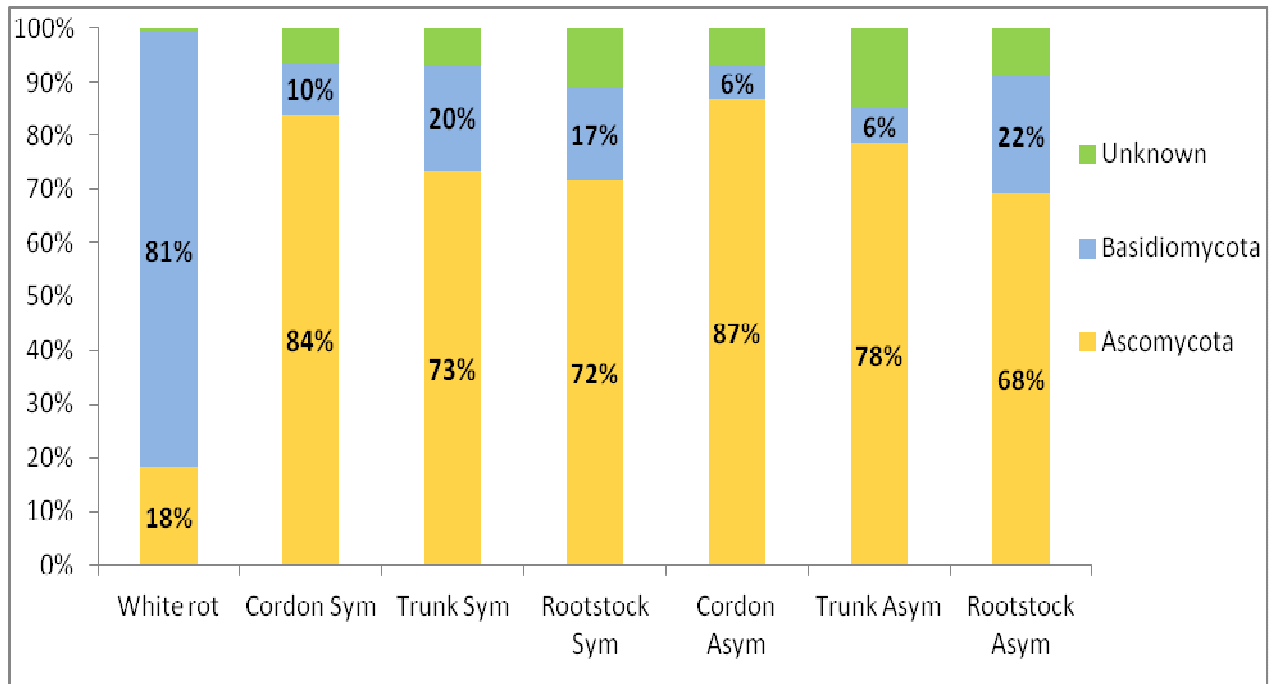


Figure 2. Abundance des différents phyla des champignons présents dans l'amadou et les tissus non nécrosés. L'abondance est représentée en pourcentage du nombre total de séquences des bactéries. Les phyla <1% n'ont pas été montrés. Sym= plants symptomatiques et Asym= plants asymptomatiques.
 Figure 2. Abundance of the different phyla of the fungi colonizing the white rot and the non necrotic tissues. Abundance is the percentage of the total number of the bacteria. Sym = Symptomatic plants and Asym = Asymptomatic plants.

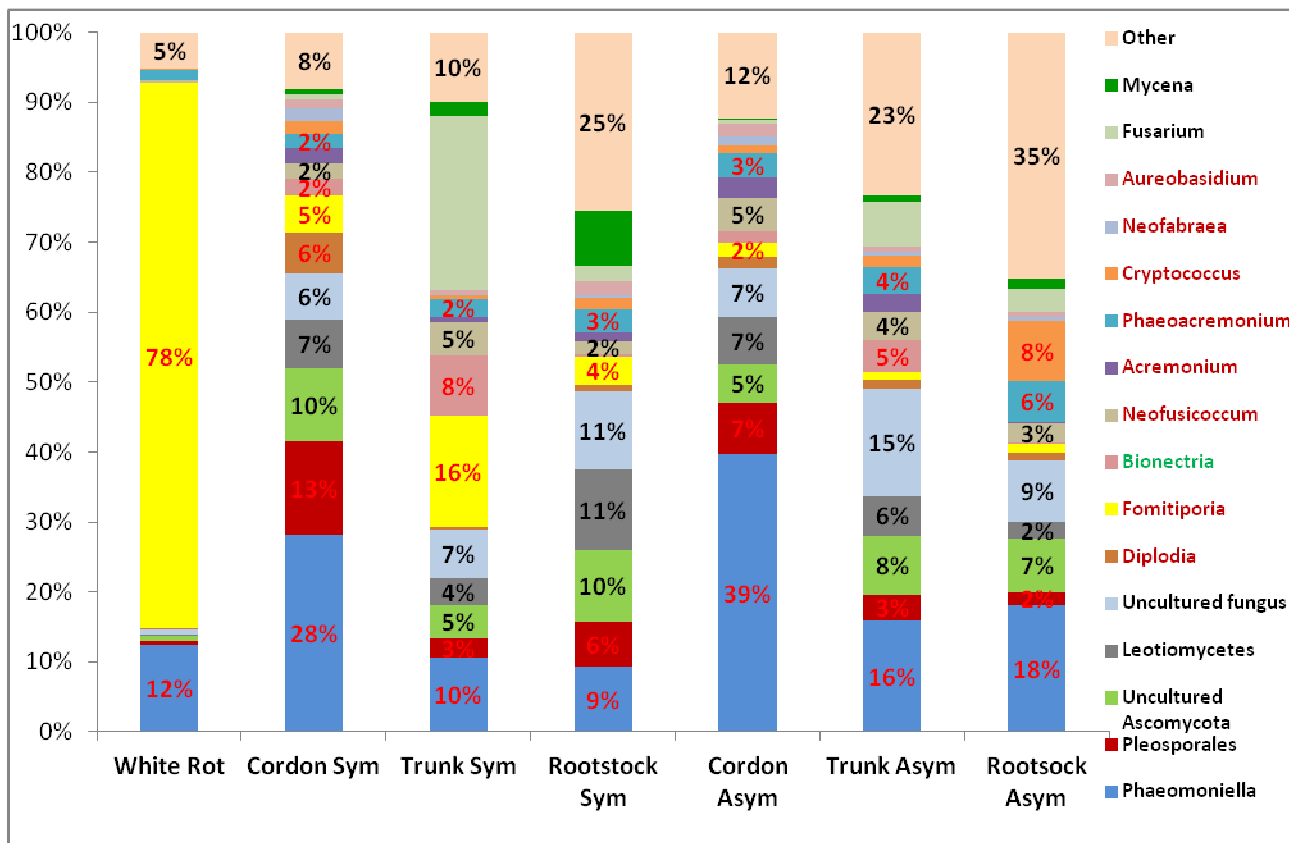


Figure 3. Abondance des différents genres de champignons présents dans l'amadou et dans les autres parties non nécrosées. Sym= plants symptomatiques et Asym = plants asymptomatiques. Les genres fongiques potentiellement pathogènes sont notés en rouge.les phyla <1% n'ont pas été montrés.

Figure 3. Abundance of the different fungal genera of the white rot and the non necrotic tissues. The genera with the percentage lower than 1% were not showed (they are in the category of "other"). Sym = Symptomatic plants and Asym = Asymptomatic plants.

2.3) Diversité des espèces

L'alpha-diversité est la diversité locale, mesurée à l'intérieur d'un système délimité. Dans notre cas, il s'agit des communautés habitant les différentes parties prélevées en avril, en juin, en septembre et en janvier.

Le tableau 4 montre la répartition des séquences selon la partie échantillonnée et le nombre total d'OTUs pour chaque échantillon. Le nombre d'OTUs est différent selon le type d'échantillon, en particulier pour lorsqu'on considère l'amadou, le nombre d'OTUs y est deux, voire trois fois plus faible que dans les parties de bois non-nécrosées. L'indice de Shannon est un indicateur de la biodiversité, c'est-à-dire qu'il indique la diversité des espèces dans un milieu donné. Les indices de Shannon sont beaucoup plus petits (de 0,72 à 2) dans l'amadou que dans les autres parties (de 2,94 à 5,27). Ces valeurs montrent donc qu'il y a moins d'espèces présentes cette nécrose typique de l'esca.

Tableau 4. Répartition du nombre de séquences et indices de diversité pour chaque échantillon.
 Table 4. Distribution of the sequences and indexes of diversity for each sample.

| Part | Reads | OTUs | Chao1 | Shannon |
|--------------------------------|-------------|-----------|-----------|-------------|
| April Sym White rot | 2270 | 62 | 87 | 2 |
| April Asym Cordon | 10944 | 130 | 143 | 3,12 |
| April Sym Cordon | 6102 | 104 | 147 | 3,77 |
| April Asym Trunk | 21684 | 185 | 212 | 4,83 |
| April Sym Trunk | 10028 | 108 | 145 | 2,94 |
| April Asym Rootstock | 2112 | 140 | 170 | 5,14 |
| April Sym Rootstock | 12328 | 187 | 206 | 4,6 |
| June Sym White rot | 4289 | 45 | 59 | 1,29 |
| June Asym Cordon | 9964 | 150 | 197 | 3,66 |
| June Sym Cordon | 3755 | 130 | 186 | 4,37 |
| June Asym Trunk | 4747 | 134 | 167 | 5,02 |
| June Sym Trunk | 11047 | 170 | 200 | 3,95 |
| June Asym Rootstock | 4702 | 158 | 195 | 4,87 |
| June Sym Rootstock | 12187 | 146 | 176 | 4,11 |
| September Sym White rot | 5825 | 47 | 54 | 1,57 |
| September Asym Cordon | 9771 | 192 | 225 | 4,8 |
| September Sym Cordon | 7161 | 183 | 208 | 4,55 |
| September Asym Trunk | 4420 | 118 | 134 | 4,43 |
| September Sym Trunk | 15302 | 190 | 230 | 5,16 |
| September Sym Rootstock | 2274 | 129 | 173 | 5,21 |
| September Asym Rootstock | 1606 | 119 | 146 | 4,42 |
| January Sym White rot | 4191 | 29 | 46 | 0,72 |
| January Asym Cordon | 9964 | 212 | 235 | 4,56 |
| January Sym Cordon | 5638 | 126 | 201 | 3,34 |
| January Asym Trunk | 1817 | 121 | 152 | 4,93 |
| January Sym Trunk | 17409 | 164 | 208 | 3,23 |
| January Asym Rootstock | 3019 | 144 | 174 | 5,27 |
| January Sym Rootstock | 6296 | 171 | 207 | 5,1 |

2.4) Comparaison des communautés fongiques en fonction des temps de prélèvement

Afin de comparer la composition fongique de chaque échantillon collecté des ceps de vigne ayant exprimé ou non des symptômes foliaires d'esca, des comparaisons phylogénétiques et phylogéniques ont été réalisées. La connaissance de la beta-diversité est nécessaire pour effectuer ces études. La notion de beta-diversité a été introduite par Whittaker (1960) comme le niveau de changements de la composition des communautés en relation avec le changement de milieu. Les communautés des échantillons interagissent entre elles contrairement à l'alpha-diversité où les communautés de chaque échantillon sont prises en compte.

Un arbre UPGMA a été réalisé à partir des données de chaque échantillon (Fig. 4). Cet arbre montre que les 4 échantillons d'amadou sont distincts des autres échantillons. Quant aux échantillons de bois non nécrosés, il ne semble pas y avoir de distinction entre les plants symptomatiques et asymptomatiques ou entre les différents temps de prélèvement. Par contre, il semblerait y avoir une catégorisation des échantillons selon le type de tissus.

Des Analyses en Coordonnées Principales ont été réalisées afin de pouvoir évaluer les similarités entre les différentes communautés fongiques. Les ACoP représentent les 28 échantillons analysés selon la méthode weighted UNIFRAC.

Les axes PC1 et PC2 représentent 56% de la variabilité totale pour les figures 5, 6 et 7. Ces figures représentent les 28 échantillons en fonction de la date de prélèvement (Fig. 5), de l'état sanitaire des ceps de vigne (Fig. 6) et des parties échantillonnées (Fig. 7). La Figure 5 ne montre pas de différence entre les dates de prélèvement selon l'axe 1 et l'axe 2 respectivement des coordonnées négatives aux coordonnées positives. La figure 6 montre que les points représentant les plants asymptomatiques seraient regroupés, indiquant que leurs diversités seraient similaires. Lorsque l'on se focalise sur les différentes parties étudiées, c'est-à-dire l'amadou, le bois non nécrosé du bras, du tronc et du porte-greffe, il y a séparation de l'amadou par rapport aux autres échantillons selon l'axe 1. Les trois parties du bois non nécrosées semblent se séparer entre le tronc et les deux autres parties selon l'axe 1 et une séparation est notable entre les échantillons du bras et du porte-greffe selon l'axe 2.

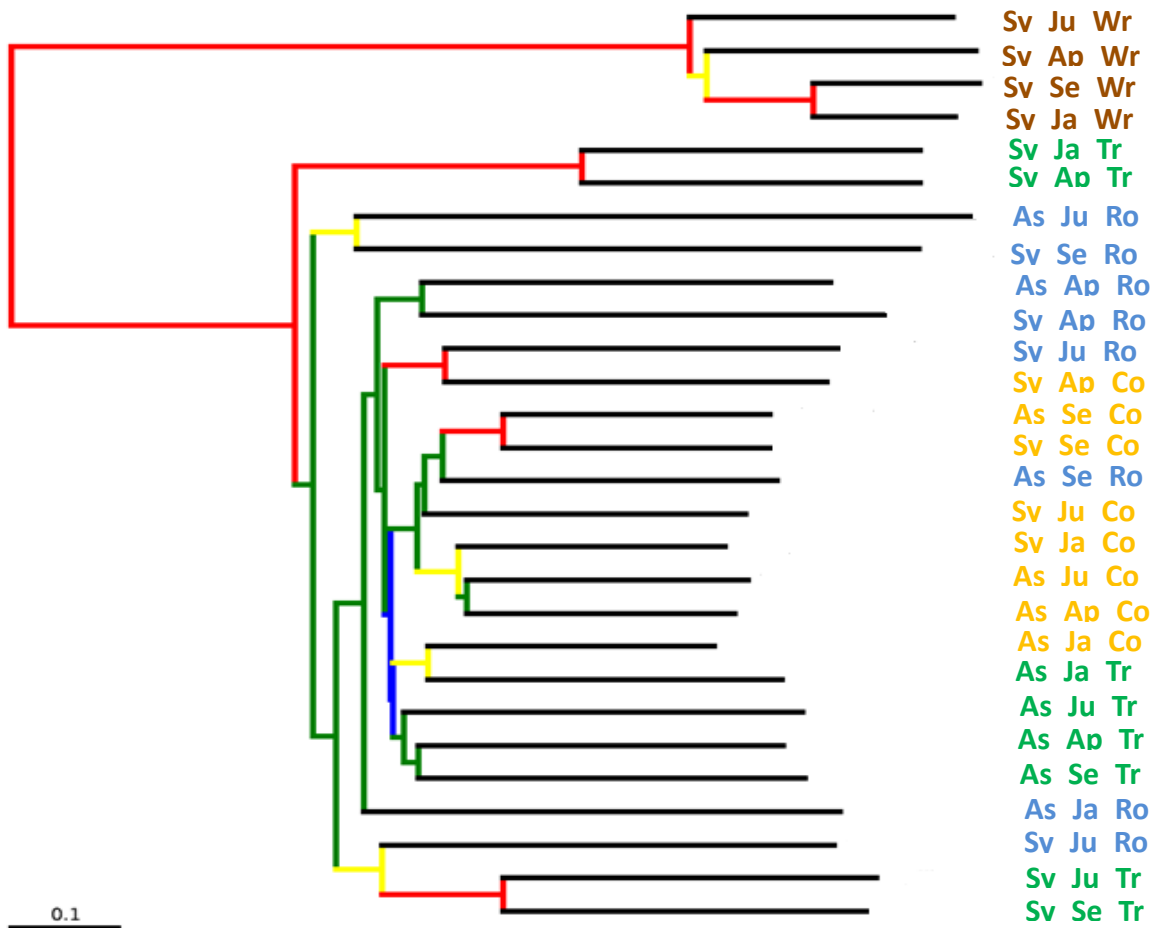


Figure 4. Arbre selon la méthode UPGMA représentant les 28 échantillons de vigne. En brun sont représentés les échantillons du bras amadou ; en orange, les échantillons du bras ; en vert, les échantillons du tronc et en bleu, les échantillons du porte-greffe.

As = plants asymptomatiques, Sy = plants symptomatiques, Ap = Avril, Ju = Juin, Se = Septembre, Ja = Janvier, Wr = Amadou, Co = Bras, Tr = Tronc, Ro = Porte-Greffe.

Figure 4. UPGMA method tree for the 28 samples. Brown = white rot samples; orange = cordon samples; green = trunk samples and blue = rootstock samples.

As= Asymptomatic plants, Sy= Symptomatic plants, Ap = April, Ju = June, Se = September, Ja = January, Wr = White rot, Co = Cordon, Tr = Trunk, Ro = Rootstock.

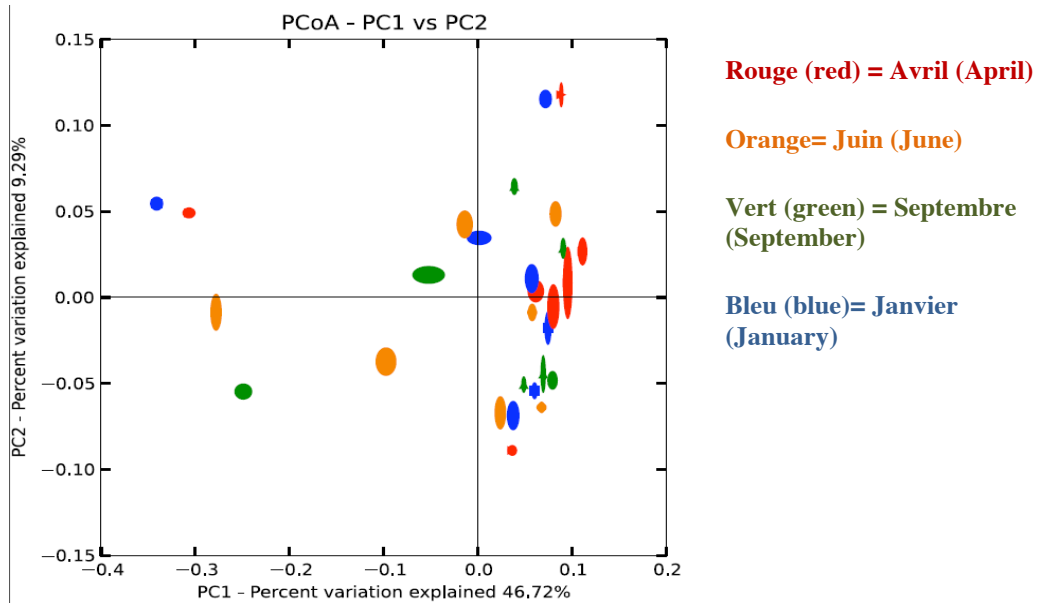


Figure 5. Analyse en Coordonnées Principales des champignons par date de prélèvement (weighted UNIFRAC). Les points en rouge représentent les échantillons d’Avril, ceux en orange, les échantillons de Juin, ceux en vert, les échantillons de Septembre et ceux en bleu sont ceux de Janvier.

Figure 5. Principal Coordinates Analysis of the fungal communities per sampling date (weighted UNIFRAC). Red = April samples; orange = June samples; green = September samples; blue = January samples.

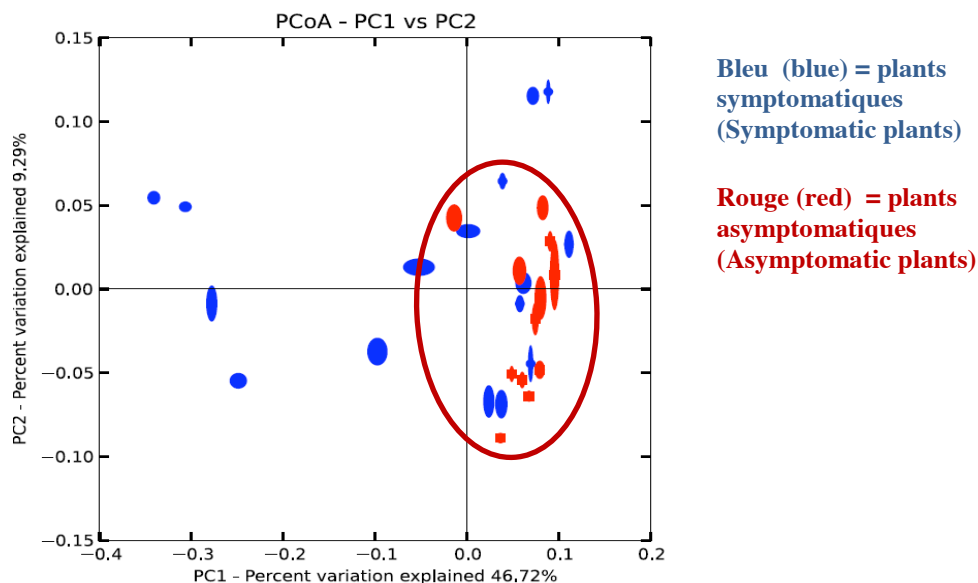


Figure 6. Analyse en Coordonnées Principales en fonction de l’état sanitaire des cepes (weighted UNIFRAC). Les points en rouge représentent les 12 échantillons qui n’ont pas exprimé les symptômes foliaires et les échantillons en bleu représentent les 16 échantillons provenant des plants symptomatiques.

Figure 6. Principal Coordinates Analysis of the fungal communities per sampling date (weighted UNIFRAC). Red= asymptomatic plants; blue= symptomatic plants.

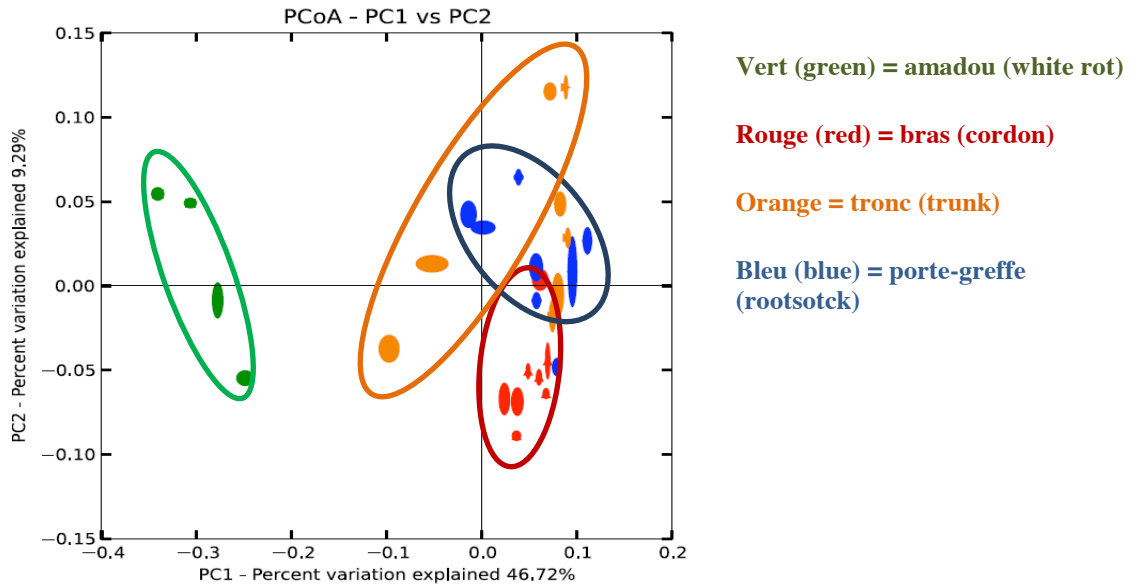


Figure 7. Analyse en Coordonnées Principales en fonction des types de prélèvements (weighted UNIFRAC). Les points en rouge représentent les échantillons des tissus non nécrosés du bras des plants symptomatiques et asymptomatiques, les points en vert représentent le bois amadou, les points orange, le tronc et en bleu le porte-greffe.

Figure 7. Principal Coordinates Analysis of the fungal communities per sampling date (weighted UNIFRAC). In green= white rot samples; in red= cordon samples; in orange= trunk samples; in blue= rootstock samples

3) Analyses des communautés bactériennes

3.1) Courbes de raréfaction des espèces bactériennes

Deux cents vingt deux OTUs représentatives des séquences des bactéries ont été déterminées. Deux cents quatre OTUs ont été identifiées chez les plants symptomatiques et 174 chez les ceps asymptomatiques. Les courbes montrent qu'un plateau est presque atteint (Fig. 8). Le nombre d'échantillons prélevés et analysés a donc permis de générer suffisamment de séquences représentatives de la diversité bactérienne au sein des différents tissus.

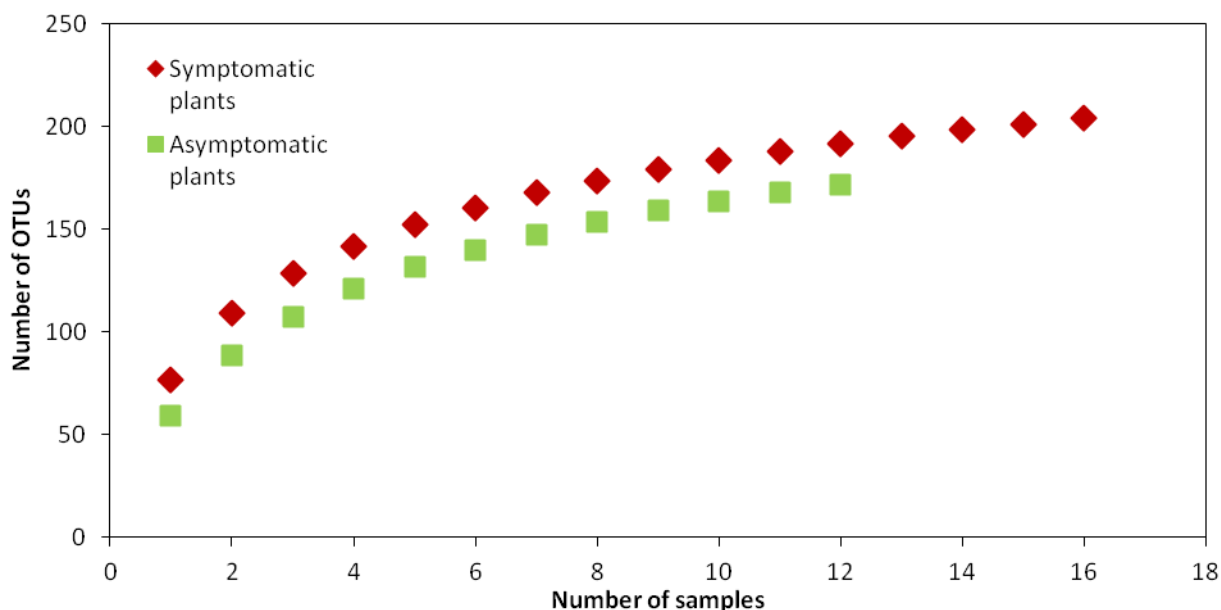


Figure 8. Courbes d'accumulation des espèces montrant le nombre d'OTUs bactériennes en ordonnées selon le nombre d'échantillons de tissus étudiés en abscisses. En vert= nombre total des OTUs détectées dans les échantillons des ceps sains. En rouge= nombre total des OTUs déterminées dans les échantillons des ceps symptomatiques.

Figure 8. Accumulation curves of bacterial species showing the number of OTUs from the different samples explained in the table 1. Green= number of OTUs of the asymptomatic plants. Red= number of the symptomatic plants.

3.2) Des phyla aux genres

Les différents échantillons analysés sont majoritairement colonisés par des bactéries appartenant à 3 phyla : des protéobactéries représentant 50 à 76% de l'effectif total, des actinobactéries et des bactéroïdètes (Fig. 9). Parmi les protéobactéries, les plus abondantes sont respectivement les alpha-, gamma- puis beta-protéobactéries. La figure 10 montre la répartition des genres parmi les échantillons d'amadou, de bois non nécrosé de bras, de troncs ou de porte-greffe provenant de ceps ayant exprimés ou non les symptômes foliaires d'esca. Le genre *Pseudomonas* est présent dans les parties du bois non nécrosé, de même que les genres *Erwinia*, *Massilia* et *Enterobacter*. Quant à l'ordre des Actinomycetales, celui-ci n'a été retrouvé que dans le bois amadou. Les *Pseudomonas* semblent être le genre majoritairement présent dans les bois non-nécrosés. En ce qui concerne le bois amadou, le genre le plus abondant est *Sphingomonas*. Globalement, les bactéries identifiées sont majoritairement des Gram -.

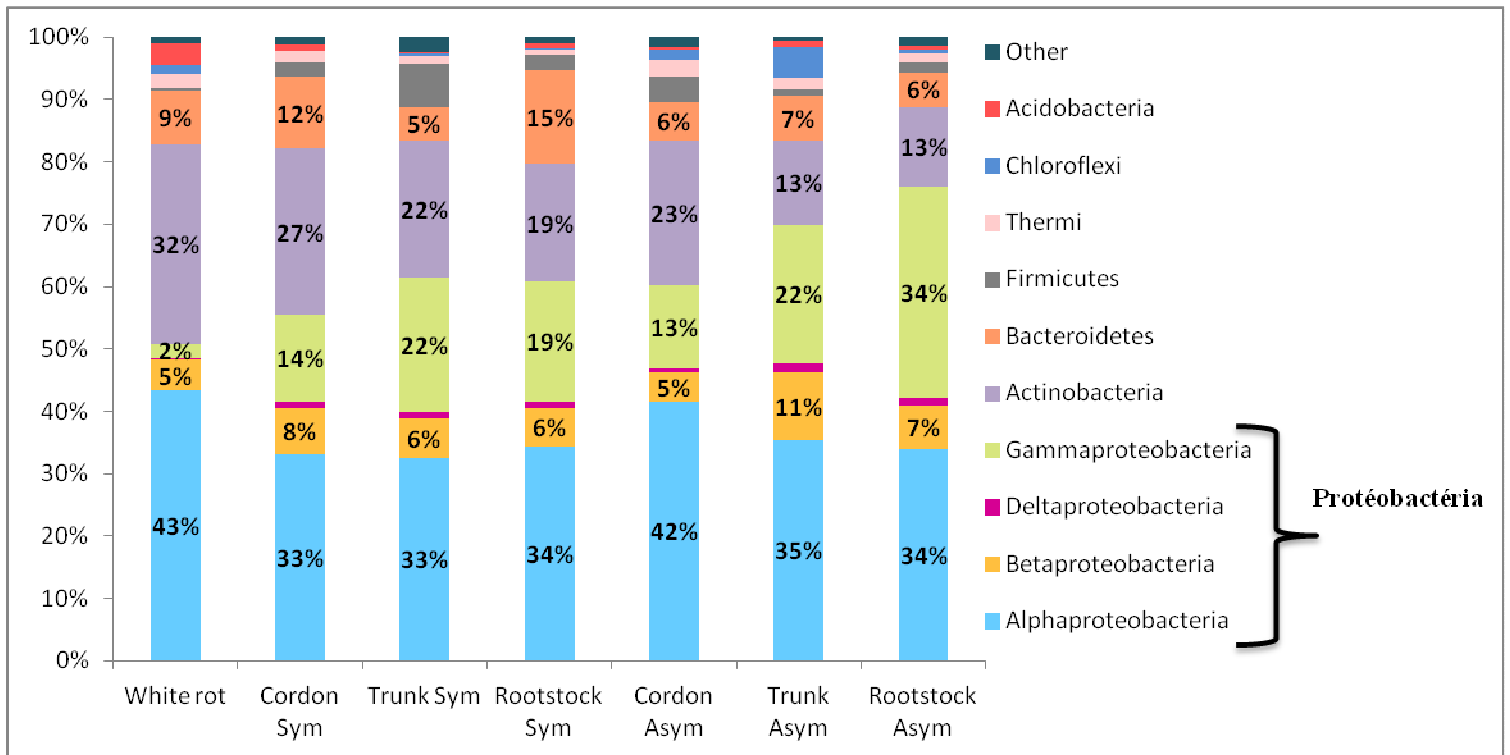


Figure 9. Abondance des différents phyla bactériens présents dans l'amadou et les tissus non nécrosés. L'abondance est représentée en pourcentage du nombre total de séquences des bactéries. Les *phyla* <1% ont été regroupés dans la catégorie « other ». Sym= plants symptomatiques et Asym= plants asymptomatiques. Sym= plants symptomatiques et Asym= plants asymptomatiques.

Figure 9. Abundance of the different phyla of the fungi determined from the white rot and the non necrotic tissues. The abundance is represented by the percentage of the total number of the bacteria. Sym = Symptomatic plants and Asym = Asymptomatic plants.

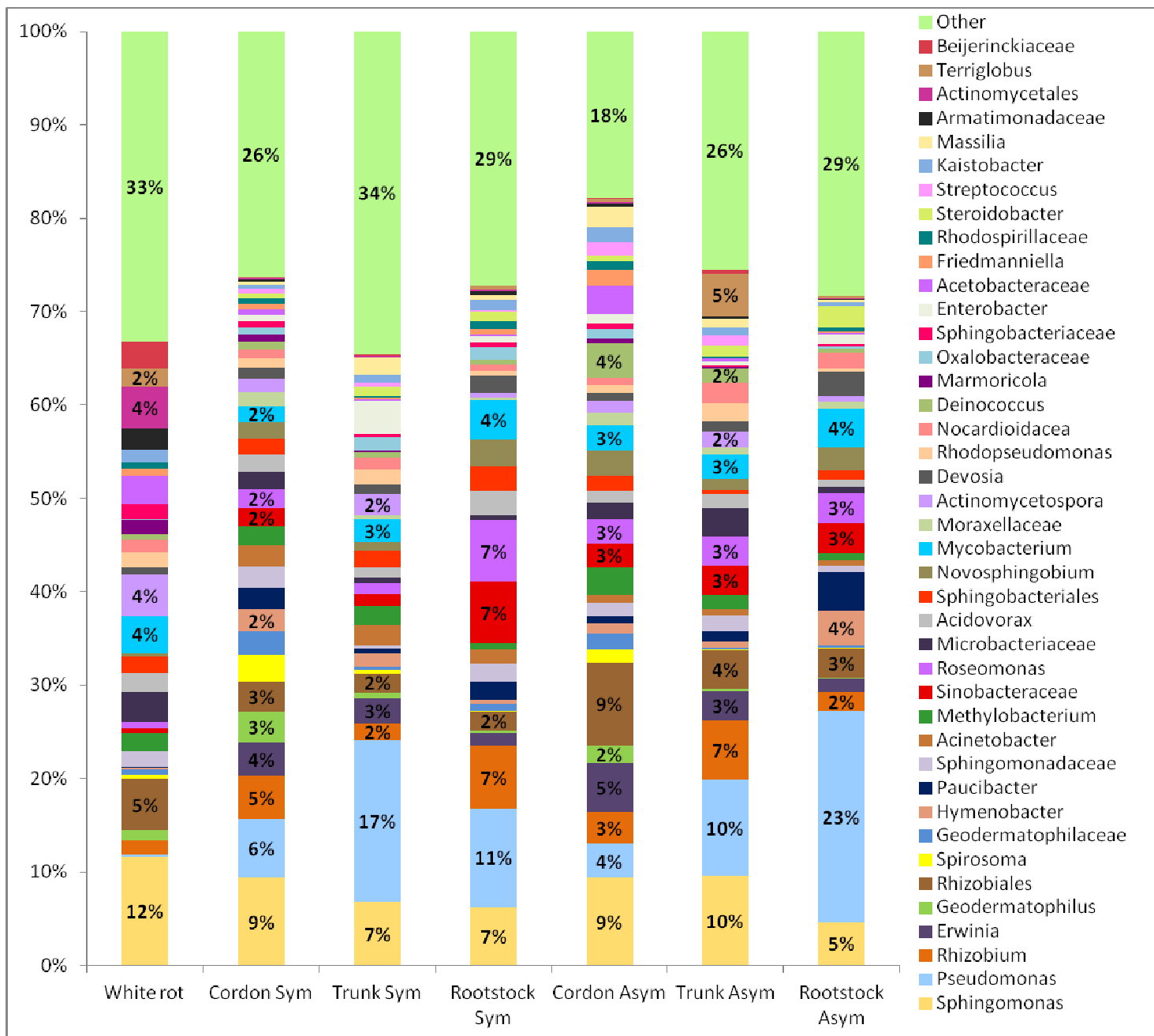


Figure 10. Abondance des différents genres bactériens présents dans l'amadou et dans les autres parties de ceps non nécrosés. Les phyla inférieurs à 1% ont été regroupés dans la catégorie « other ».

Figure 10. Abundance of the different fungal genera of the white rot and the non necrotic tissues. The genera with the percentage lower than 1% were not showed. They are in the category of "other". Sym = plants symptomatiques et Asym = plants asymptomatiques.

3.2) L'alpha diversité

La diversité des espèces pour les différents échantillons (Tableau 5) est assez élevée et ce, pour tous les types d'échantillons. En effet, les valeurs des indices de Shannon varient de 4,66 à 6,37. Seul l'échantillon du porte-greffe des plants asymptomatiques du mois de septembre a une valeur inférieure avec 2,87.

Tableau 5. Répartition du nombre de séquences et indices de diversité pour chaque échantillon.
 Table 5. Distribution of the sequences and indexes of diversity for each sample.

| Part | Reads | OTUs | Chao1 | Shannon |
|--------------------------------|------------|------------|------------|-------------|
| April Sym White rot | 204 | 70 | 120 | 5,68 |
| April Asym Cordon | 317 | 82 | 158 | 5,56 |
| April Sym Cordon | 918 | 156 | 212 | 6,2 |
| April Asym Trunk | 67 | 32 | 84 | 4,66 |
| April Sym Trunk | 205 | 64 | 210 | 5,15 |
| April Asym Rootstock | 367 | 89 | 134 | 5,45 |
| April Sym Rootstock | 671 | 114 | 185 | 5,36 |
| June Sym White rot | 152 | 63 | 127 | 5,48 |
| June Asym Cordon | 622 | 117 | 207 | 5,54 |
| June Sym Cordon | 803 | 140 | 176 | 5,95 |
| June Asym Trunk | 206 | 76 | 121 | 5,84 |
| June Sym Trunk | 1064 | 181 | 249 | 6,09 |
| June Asym Rootstock | 351 | 115 | 238 | 5,69 |
| June Sym Rootstock | 475 | 123 | 177 | 6,1 |
| September Sym White rot | 585 | 115 | 142 | 6,17 |
| September Asym Cordon | 526 | 94 | 145 | 5,8 |
| September Sym Cordon | 385 | 120 | 175 | 6,37 |
| September Asym Trunk | 119 | 28 | 60 | 4,41 |
| September Sym Trunk | 174 | 59 | 135 | 5,03 |
| September Asym Rootstock | 158 | 42 | 93 | 2,87 |
| September Sym Rootstock | 1006 | 149 | 194 | 5,6 |
| January Sym White rot | 253 | 76 | 125 | 5,35 |
| January Asym Cordon | 376 | 120 | 174 | 6,3 |
| January Sym Cordon | 622 | 135 | 186 | 6,14 |
| January Asym Trunk | 204 | 54 | 93 | 4,91 |
| January Sym Trunk | 650 | 124 | 171 | 4,84 |
| January Asym Rootstock | 222 | 70 | 169 | 5,62 |
| January Sym Rootstock | 363 | 114 | 206 | 5,57 |

3.3) La beta diversité

Les résultats de la figure 11 montrent la répartition des échantillons selon la méthode Jackknife. Il ne semble pas y avoir de regroupement des échantillons en fonction du temps de prélèvements, du type de tissus ni de l'état sanitaire des ceps. Seul un échantillon d'amadou se distingue nettement de tous autres prélèvements.

Les résultats des ACoP (Fig. 12, 13 et 14) représentent les 28 échantillons en fonction de la date de prélèvement, de l'état des ceps et des différentes parties prélevées. Les axes 1 et 2 représentent 35 % de la variabilité totale. La figure 12 montre qu'il ne semble pas y avoir de réelle différence entre les biodiversités des communautés bactériennes selon les quatre dates prélèvements. La figure 13 montre que les échantillons de bois symptomatiques se

rassemblent tandis que ceux des plants asymptotiques sont plus éparés et ce, selon les axes 1 et 2, allant des coordonnées négatives aux coordonnées positives.

La figure 14 montre qu'il y a une différence des diversités entre le bois amadou et le bois non nécrosé, selon l'axe 1, coordonnées négatives à positives. Pour les échantillons de bois non nécrosé, les biodiversités des bras semblent différer de celles du tronc et du porte-greffe selon l'axe 1, coordonnées négatives à positives.

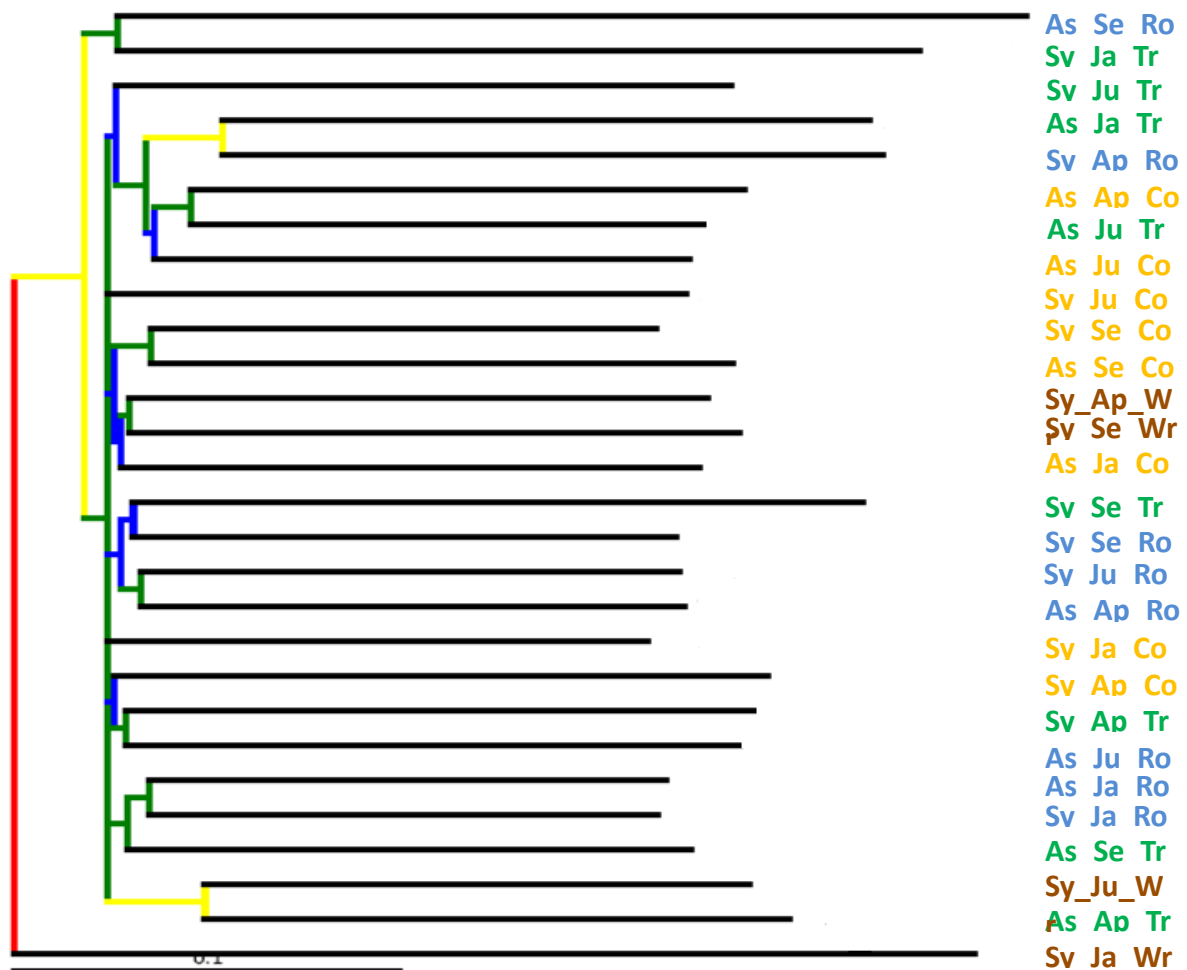


Figure 11. Arbre UPGMA selon la méthode Jackknife représentant les 28 échantillons de vigne. En brun sont représentés les échantillons du bras amadou, en orange, les échantillons du bras, en vert, les échantillons du tronc et en bleu, les échantillons du porte-greffe. As = Asymptomatique, Sy = Symptomatique, Ap = Avril, Ju = Juin, Se = Septembre, Ja = Janvier, Wr = Amadou, Co = Bras, Tr = Tronc, Ro = Porte-Greffe.

Figure 11. UPGMA method tree for the 28 samples. Brown= white rot samples; orange = cordon samples; green = trunk samples and blue = rootstock samples.

As = Asymptomatic plants, Sy = Symptomatic plants, Ap = April, Ju = June, Se = September, Ja = January, Wr = White rot, Co = Cordon, Tr = Trunk, Ro = Rootstock.

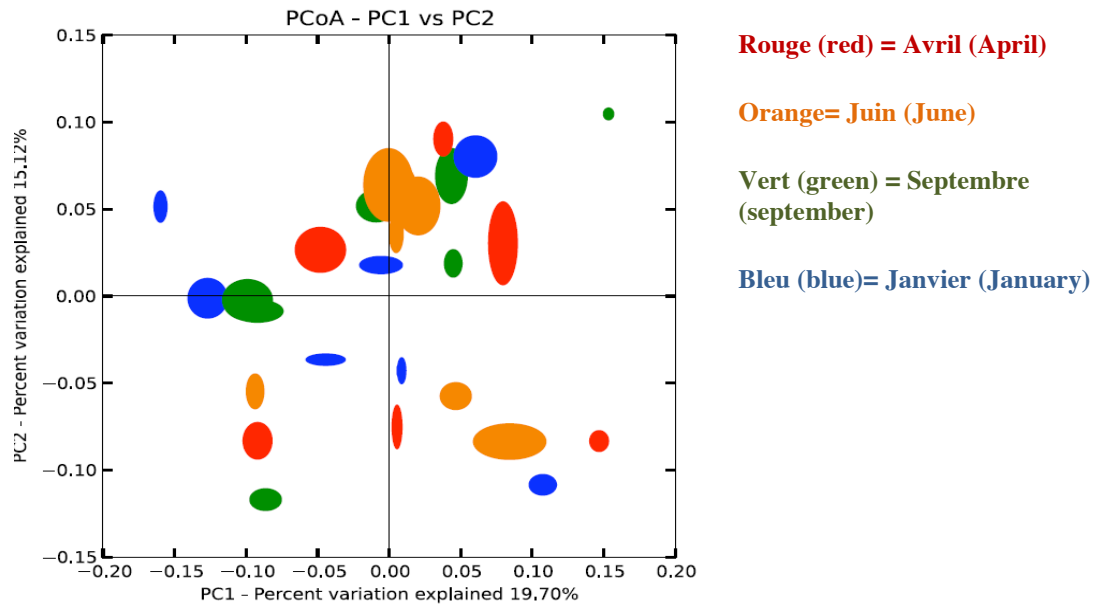


Figure 12. Analyse en Coordonnées Principales par date de prélèvement (weighted UNIFRAC). Les points en rouge représentent les échantillons d'Avril, ceux en orange, les échantillons de Juin, ceux en vert, les échantillons de Septembre et ceux en bleu sont ceux de Janvier.

Figure 12. Principal Coordinates Analysis of the fungal communities per sampling date (weighted UNIFRAC). Red = April samples; orange = June samples; green = September samples; blue = January samples.

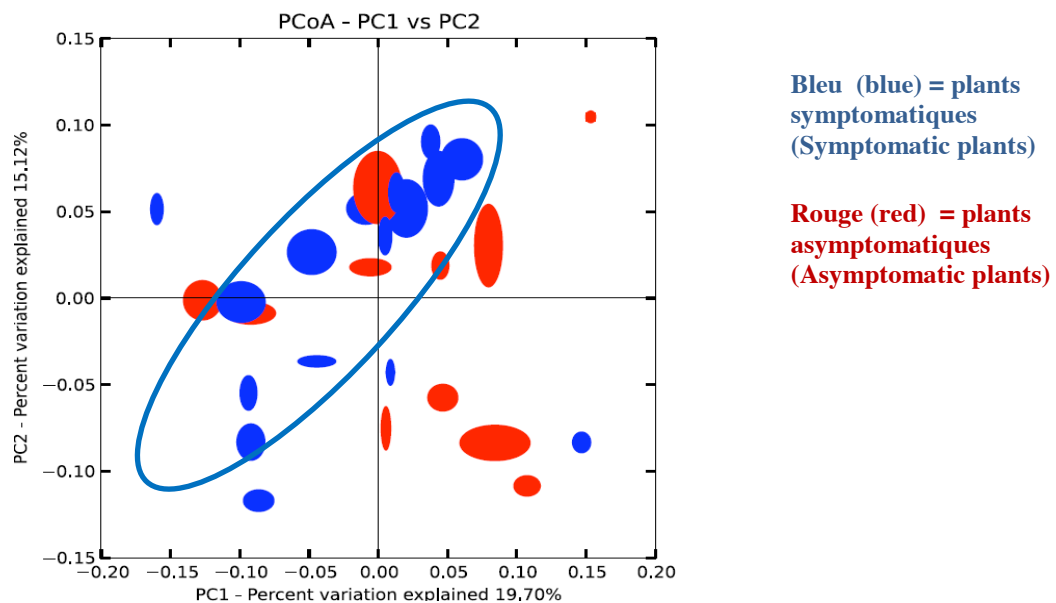


Figure 13. Analyse en Coordonnées Principales en fonction de l'état sanitaire des ceps (weighted UNIFRAC). Les points en rouge représentent les 12 échantillons qui n'ont pas exprimé les symptômes foliaires et les échantillons en bleu représentent les 16 échantillons provenant des ceps symptomatiques.

Figure 13. Principal Coordinates Analysis of the fungal communities per sampling date (weighted UNIFRAC). Red = asymptomatic plants; blue = symptomatic plants.

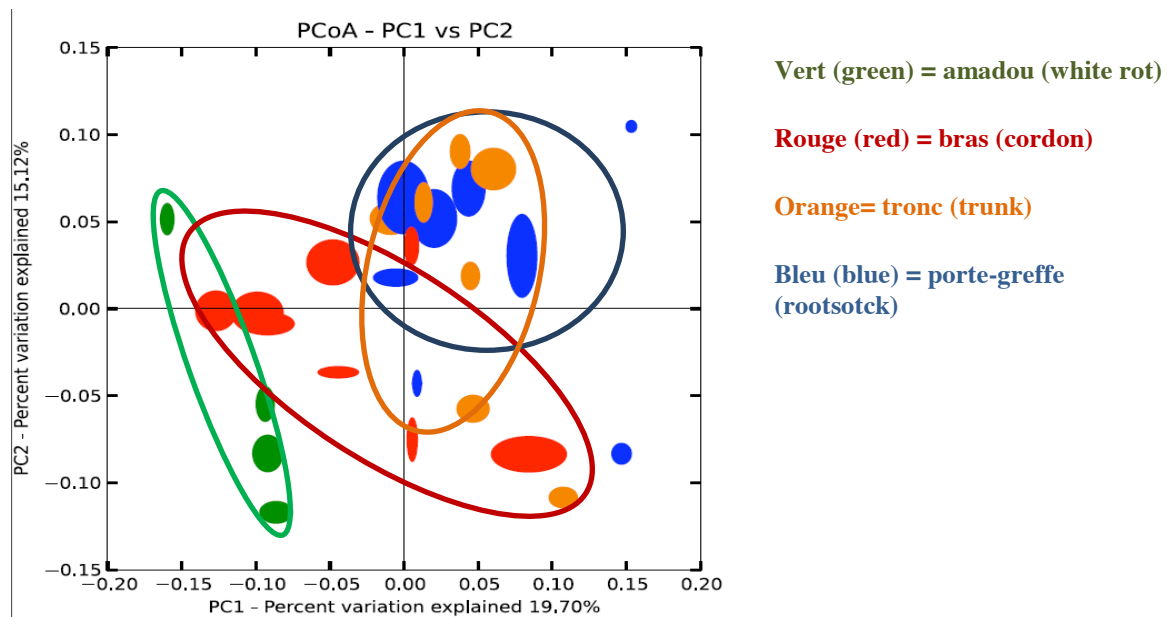


Figure 14. Analyse en Coordonnées Principales en fonction des tissus échantillonnés (weighted UNIFRAC). Les points en rouge représentent les échantillons des tissus non nécrosés du bras des ceps symptomatiques et asymptotiques, les points en vert représentent le bois amadou, les points orange, le tronc et en bleu le porte-greffe.

Figure 14. Principal Coordinates Analysis of the fungal communities per sampling date (weighted UNIFRAC). Green = white rot samples; red = cordon samples; orange = trunk samples; blue = rootstock samples.

Discussion

Il s'agit de la première étude sur la diversité bactérienne et fongique du bois de vigne qui utilise la méthode du pyroséquençage 454. Cette approche a permis, en complément des études réalisées dans les chapitres 1 et 2, de caractériser de façon plus fine la composition des assemblages de micro-organismes colonisant du bois apparemment sain ou nécrosé dans différentes parties de ceps de vigne (bras, tronc, porte-greffe) ayant exprimé ou non des symptômes foliaires d'esca et ce, en fonction des saisons.

Structure des communautés et distribution des OTUs

Les 28 échantillons de tissus de vigne ont généré un total de 12 065 séquences d'ADN bactérien, représentant 222 OTUs, et 209 106 séquences d'ADN fongique, représentant 674 OTUs. Les résultats obtenus montrent des communautés microbiennes assez diversifiées avec, de façon générale, plus d'une centaine d'OTUs dans chaque tissu analysé (Tableaux 4 et 5). De récentes études concernant le bois sain en décomposition de conifères et de palétuviers ont également mis en évidence la grande diversité des espèces fongiques et bactériennes colonisant ce type de tissus (Zhang *et al.*, 2008; Arfi *et al.*, 2012; Kubartova *et al.*, 2012). Malgré les progrès dus aux techniques de pyroséquençage qui peuvent générer plusieurs milliers de séquences, la diversité microbienne réelle est encore sous estimée. Dans la présente étude, ce phénomène est d'ailleurs plus marqué pour les champignons que pour les bactéries comme le montrent les courbes de raréfaction (Fig. 1 et 8), et ce, bien que le nombre de séquences et d'OTUs obtenues soit bien plus grand.

Les différentes analyses d'alpha et beta diversités réalisées pour comparer la structure des communautés microbiennes en fonction des saisons de prélèvement, de l'état des ceps (asymptomatiques ou symptomatiques-esca) ainsi que du type de tissus échantillonnés ont montré que c'est essentiellement l'état du bois, fortement dégradé ou non, ainsi que sa localisation dans le cep qui sélectionnent le type de micro-organismes présents.

En effet, pour les communautés fongiques, un test de Kruskal Wallis (données non montrées) réalisé sur les indices de Shannon et de Chao1 (Tableau 4) indique que la diversité des espèces est significativement moindre dans l'amadou comparé aux tissus de bois non nécrosés mais qu'elle ne varie pas en fonction des dates de prélèvement. Les Analyses en Coordonnées Principales ainsi que le dendrogramme UPGMA (Fig. 4,5,6 et 7) confirment ces résultats et montrent en plus que la répartition des espèces semble être plutôt tissus-dépendante. Les travaux réalisés par Maher *et al.* (2012) tendent aux mêmes conclusions puisqu'ils montrent que les tissus nécrosés dans le bois de vigne sont majoritairement colonisés par une espèce fongique différente selon le type de nécrose. A l'inverse Hofstetter et ses collaborateurs (2012) rapportaient que le bois de ceps ayant exprimé ou non des symptômes foliaires d'esca était colonisé par une microflore fongique identique, cette assertion semble cependant assez problématique car leur étude ne prend pas en compte l'état sanitaire du bois.

En ce qui concerne les communautés bactériennes, il n'y a pas de différences selon les dates de prélèvement (p value=0,5647). Par contre, il existe des différences marquées entre les

tissus nécrosés et ceux apparemment sains mais également entre les tissus non nécrosés des différentes parties de ceps échantillonnées, *i.e.* bras, tronc et porte-greffe. Cette répartition des espèces est également visible sur les ACoP et le dendrogramme UPGMA (Fig. 11,12,13 et 14).

Diversité taxonomique

Concernant les communautés fongiques, l'assignation taxonomique des OTUs montre que le bois non nécrosé des ceps asymptomatiques et symptomatiques (atteint par l'esca) est largement colonisé par des Ascomycètes (68 à 87% des séquences) (Fig. 2). Parmi les Ascomycètes, on note une abondance du genre *Phaeomoniella* (entre 10 à 39% des séquences), particulièrement au niveau des tissus non nécrosés des bras des ceps esca-symptomatiques et asymptomatiques (28% et 39% des séquences respectivement) (Fig. 3). Outre *Phaeomoniella*, beaucoup de genres potentiellement pathogènes de la vigne ont également été identifiés, *i.e.* *Diplodia*, *Fomitiporia*, *Neofusicoccum*, *Phaeoacremonium*, *Cryptococcus*, *Neofabraea*, *Aureobasidium*. A l'inverse, peu de genres potentiellement protecteurs des plantes ont été observés: *Acremonium* (promoteur de croissance de la plante), *Bionectria* et *Trichoderma* (champignon antagoniste, 1% à 3% des séquences obtenues). Concernant le genre *Fusarium*, son rôle est plus ambigu. Selon les espèces, voire selon les formes spéciales de *Fusarium oxysporum*, il existe des agents potentiellement pathogènes ou protecteurs de plantes. Ces informations ne sont pas vraiment en correspondance avec l'étude sur la microflore cultivable réalisée au chapitre 1, et où il semblait que plusieurs genres fongiques potentiellement non pathogènes, *e.g.* *Trichoderma*, étaient très nombreux dans le bois « apparemment » sain. Le pyroséquençage confirme la présence de ces genres mais infirme le fait qu'ils soient abondants. Au contraire, les résultats du présent chapitre indiquent que plusieurs champignons pathogènes sont déjà présents dans le bois apparemment « sain » et qu'ils représentent une part significative de la microflore fongique.

La microflore colonisant l'amadou se différencie nettement de celle des autres tissus puisque les Basidiomycètes le colonisent fortement, 81% des séquences d'ADN microbien appartenant à ce phylum. Parmi ces Basidiomycètes, 78% correspondent au genre *Fomitiporia*. La richesse des espèces colonisant ce tissu typique de l'esca est donc beaucoup moins importante que celle des tissus non nécrosés (Tableau 4). On peut également remarquer que le genre *Phaeoacremonium* est représenté dans l'amadou (1,4%). Au final, quasiment tous les champignons (91%) colonisant l'amadou sont décrits dans la littérature comme étant potentiellement pathogènes de la vigne et impliqués dans l'esca (Bertsch *et al.*, 2012). Ce résultat n'est en soi pas étonnant puisque l'espèce *F. mediterranea* a déjà été décrite comme étant majoritaire dans l'amadou par Maher *et al.* (2012) et que des inoculations avec cet agent fongique chez la vigne (Laveau *et al.*, 2009) ou le noisetier (Pilotti *et al.*, 2010) ont permis de recréer cette structure dégradée du bois.

Pour les bactéries, les résultats du pyroséquençage montrent que le phylum le plus abondant dans tous les types de bois, dégradés ou non, correspond aux Protéobactéries. Dans les tissus non nécrosés, 61% de l'ADN bactérien (tous types de tissus) appartient à ce phylum, les Alpha-protéobactéries (59%) étant le groupe le plus représenté. Dans l'amadou, les

Protéobactéries représentent 50% des séquences, le second phylum étant celui des Actinobactéries qui est présent à 32% tandis que pour les autres échantillons le pourcentage varie de 13 à 27%. Au niveau des genres bactériens, on peut remarquer une certaine différence entre ceux colonisant les tissus de bois non nécrosés et ceux nécrosés. Les genres *Pseudomonas* et *Erwinia* ne sont en effet pas identifiés dans le bois amadou, alors que la famille des Microbacteriaceae est uniquement présente dans ce tissu. Ces résultats sont cohérents avec ceux de récentes études montrant que certaines souches bactériennes (Valaskova *et al.*, 2009; Ahmad *et al.*, 2010; Bugg *et al.*, 2011) notamment isolées du sol, de tissus ligneux ou de l'intestin d'insectes ravageurs du bois avaient, in vitro, une capacité à dégrader la lignine comparable à celle de certains champignons. Ces souches appartiennent essentiellement à trois classes : Actinomycètes, Alpha- et Gamma-protéobactéries qui comprennent des genres tels que *Pseudomonas* sp., *Sphingomonas* sp. ou *Erwinia* sp. que nous avons identifié dans nos échantillons.

L'étude des microflores fongiques et bactériennes a donc mis en évidence que selon les assemblages microbiens considérés, fongiques ou bactériens, le nombre de genres principaux obtenus par rapport au nombre de séquences différait dans de fortes proportions. Chez les champignons, 13 genres (hormis les uncultured fungus, Leotiomycètes et uncultured ascomycota) représentaient au minimum 50% de toutes les séquences alors que pour les bactéries, 41 genres représentaient au minimum 66% des séquences bactériennes. Ces résultats semblent indiquer que les bactéries seraient très diversifiées dans le bois de vigne contrairement aux champignons où seuls quelques genres majoritaires seraient présents. Il a également été montré que les genres colonisant l'amadou étaient différents de ceux colonisant les tissus non nécrosés ; cette différence étant très marquée pour les champignons. Ce phénomène pourrait être expliqué par le changement de substrat résultant de l'état de dégradation du bois. En effet, pendant le processus de décomposition, la structure du bois ainsi que sa composition chimique changent avec par exemple, une diminution de la densité et du ratio C/N tandis que l'humidité et le contenu en lignine augmentent (Butler *et al.*, 2007; Laiho and Prescott, 2004; Makinen *et al.*, 2006). Ces changements résultant essentiellement des activités microbiennes influenceraient donc la composition des communautés associées en sélectionnant certaines espèces.

Références

- Ahmad M, Taylor CR, Pink D, Burton K, Eastwood D, Bending GD, Bugg TDH (2010) Development of novel assays for lignin degradation: comparative analysis of bacterial and fungal lignin degraders. *Molecular Biosystems* **6**: 815-821
- Ahmadian A, Ehn M, Hober S (2006) Pyrosequencing: History, biochemistry and future. *Clin Chim Acta* **363**: 83-94
- Arfi Y, Buee M, Marchand C, Levasseur A, Record E (2012) Multiple markers pyrosequencing reveals highly diverse and host-specific fungal communities on the mangrove trees *Avicennia marina* and *Rhizophora stylosa*. *Fems Microbiology Ecology* **79**: 433-444
- Becklin KM, Hertweck KL, Jumpponen A (2012) Host Identity Impacts Rhizosphere Fungal Communities Associated with Three Alpine Plant Species. *Microb Ecol* **63**: 682-693
- Bengtsson J, Eriksson KM, Hartmann M, Wang Z, Shenoy BD, Grelet G-A, Abarenkov K, Petri A, Rosenblad MA, Nilsson RH (2011) Metaxa: a software tool for automated detection and discrimination among ribosomal small subunit (12S/16S/18S) sequences of archaea, bacteria, eukaryotes, mitochondria, and chloroplasts in metagenomes and environmental sequencing datasets. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **100**: 471-475
- Bertsch C, Ramirez-Suero M, Magnin-Robert M, Larignon P, Chong J, Abou-Mansour E, *et al.* (2012) Grapevine trunk diseases: complex and still poorly understood. *Plant Pathol* DOI: 10.1111/j.1365-3059.2012.02674
- Blaalid R, Carlsen T, Kumar S, Halvorsen R, Ugland KI, Fontana G, *et al.* (2012) Changes in the root-associated fungal communities along a primary succession gradient analysed by 454 pyrosequencing. *Molecular Ecology* **21**: 1897-1908
- Bugg TDH, Ahmad M, Hardiman EM, Singh R (2011) The emerging role for bacteria in lignin degradation and bio-product formation. *Current Opinion in Biotechnology* **22**: 394-400
- Butler R, Patty L, Le Bayon RC, Guenat C, Schlaepfer R (2007) Log decay of *Picea abies* in the Swiss Jura Mountains of central Europe. *For Ecol Manage* **242**: 791-799
- Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**: 335-336
- Colwell RK. (2005) EstimateS: statistical estimation of species richness and shared species from samples, version 8. purl.oclc.org/estimates, Vol. 2005.
- Cordier T, Robin C, Capdevielle X, Fabreguettes O, Desprez-Loustau M-L, Vacher C (2012) The composition of phyllosphere fungal assemblages of European beech (*Fagus sylvatica*) varies significantly along an elevation gradient. *New Phytologist* **196**: 510-519

- Hartmann M, Howes CG, Abarenkov K, Mohn WW, Nilsson RH (2010) V-Xtractor: An open-source, high-throughput software tool to identify and extract hypervariable regions of small subunit (16 S/18 S) ribosomal RNA gene sequences. *Journal of Microbiological Methods* **83**: 250-253
- Hofstetter V, Buyck B, Croll D, Viret O, Couloux A, Gindro K (2012) What if esca disease of grapevine were not a fungal disease? *Fungal Diversity* **54**: 51-67
- Hyman ED (1988) A new method of sequencing DNA. *Analytical Biochemistry* **174**: 423-436
- Kubartova A, Ottosson E, Dahlberg A, Stenlid J (2012) Patterns of fungal communities among and within decaying logs, revealed by 454 sequencing. *Molecular Ecology* **21**: 4514-4532
- Laiho R, Prescott CE (2004) Decay and nutrient dynamics of coarse woody debris in northern coniferous forests: a synthesis. *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere* **34**: 763-777
- Laveau C, Letouze A, Louvet G, Bastien S, Guerin-Dubrana L (2009) Differential aggressiveness of fungi implicated in esca and associated diseases of grapevine in France. *Phytopathologia Mediterranea* **48**: 32-46
- Lozupone C and Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology* **71**: 8228-8235
- Maher, N., Piot, J., Bastien, S., Vallance, J., Rey, P., and Guerin-Dubrana, L. (2012). Wood 729 necrosis in Esca-affected vines: types, relationships and possible links with foliar 730 symptom expression. *J Inter Sci Vigne Vin* **46**: 15-27.
- Makinen H, Hynynen J, Siitonen J, Sievaneni R (2006) Predicting the decomposition of Scots pine, Norway spruce, and birch stems in Finland. *Ecological Applications* **16**: 1865-1879
- Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bembien LA, *et al.* (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**: 376-380
- Nilsson RH, Veldre V, Hartmann M, Unterseher M, Amend A, Bergsten J, *et al.* (2010) An open source software package for automated extraction of ITS1 and ITS2 from fungal ITS sequences for use in high-throughput community assays and molecular ecology. *Fungal Ecology* **3**: 284-287
- Nyren P (1987) Enzymatic method for continuous monitoring of DNA-Polymerase activity. *Analytical Biochemistry* **167**: 235-238
- Pilotti M, Tizzani L, Brunetti A, Gervasi F, Di Lernia G, Lumia V (2010) Molecular identification of *Fomitiporia mediterranea* on declining and decayed Hazelnut. *Journal of Plant Pathology* **92**: 115-129
- Ronaghi M, Karamohamed S, Pettersson B, Uhlen M, Nyren P (1996) Real-time DNA sequencing using detection of pyrophosphate release. *Analytical Biochemistry* **242**: 84-89

Ronaghi M, Uhlen M, Nyren P (1998) A sequencing method based on real-time pyrophosphate. *Science* **281**: 363-+

Ronaghi M, Nygren M, Lundeberg J, Nyren P (1999) Analyses of secondary structures in DNA by pyrosequencing. *Anal Biochem* **267**: 65-71

Valaskova V, Snajdr J, Gunnewiek PK, Boer Wd, Baldrian P (2009) Saprotrophic basidiomycete *Hypholoma fasciculare* affects fungal and bacterial community composition in colonized wood and soil. *IOBC/WPRS Bulletin* **42**: 173-176

Viala P (1926) Recherches sur els maladies de la vigne. Esca. *Annal Epiphyt fasc. 1 et 2*:1-108

Whittaker RM (1960) Vegetation of the Siskiyou Mountains, Oregon and California. *Ecological Monographs*. **30**: 279-338

Zhang HB, Yang MX, Tu R (2008) Unexpectedly high bacterial diversity in decaying wood of a conifer as revealed by a molecular method. *International Biodeterioration & Biodegradation* **62**: 471-474

Conclusions générales et Perspectives

Les maladies du bois de la vigne, *i.e.* esca, eutypiose et chancres à *Botryosphaeriae*, sont devenues en l'espace d'environ trente ans l'objet de préoccupations très importantes pour la filière viti-vinicole en France et dans le monde (Bertsch *et al.*, 2012 ; Mugnai *et al.*, 1999). Ces maladies, décrites depuis l'antiquité, principalement l'esca, étaient connues comme atteignant des ceps généralement âgés et elles n'induisaient que des dégâts jugés comme étant négligeables. La publication n° 1 intitulée « Overview of grapevine trunk diseases in France in the early 2000s » montre qu'une recrudescence et une augmentation de ces maladies est observée suivant les régions. Par exemple, le vignoble du Jura semble particulièrement atteint par l'esca et celui de Charentes par l'eutypiose. Tous les cépages sont concernés, même si des différences notables sont observées. Par exemple, le cépage Ugni Blanc est très sensible à l'eutypiose alors que le Savagnin et le Trousseau le sont par rapport à l'esca. On peut également noter que suivant les régions un même cépage peut-être atteint dans des proportions identiques par l'esca, *e.g.* le Sauvignon dans les vignobles de Bordeaux, de Bourgogne et du Centre, ou de façon différente, *e.g.* le Cabernet-Sauvignon dans le bordelais et en région Provence-Alpes Côte d'Azur.

Les facteurs responsables de ces variations ne sont à ce jour pas vraiment connus, même si des facteurs abiotiques comme la réserve utile du sol, la taille, la densité de plantation, les épisodes climatiques (alternances de périodes chaudes et pluvieuses) ... auraient un rôle. Leur hiérarchisation reste encore un vaste sujet d'étude. Il faut noter que ces pathologies induisent actuellement des pertes importantes. Il a été estimé qu'environ 6% des ceps dans le bordelais sont atteints par ces maladies, ce chiffre montant à 14% si l'on inclut les répercussions indirectes, c'est-à-dire les plants qui ont été arrachés, ceux qui sont replantés (ils ne produisent pas de baies de qualité avant 3 années), les ceps morts, ceux recépés etc. Grosman et Doublet (2012) rapportent qu'environ 11% des ceps sont actuellement improductifs en France, cette situation étant principalement imputée aux maladies du bois.

La recrudescence de l'esca en France suscite de nombreuses interrogations quant aux causes biotiques de cette maladie. Les micro-organismes, *i.e.* les communautés fongiques et bactériennes, qui sont l'objet d'étude de la présente thèse, sont cités comme étant un élément essentiel de l'esca. Les champignons colonisateurs de bois dégradés ont jusqu'à ce jour été quasi exclusivement étudiés par la communauté scientifique et de nombreuses publications ont montré qu'ils étaient responsables des différentes nécroses observées dans le bois. La plus typique, l'amadou, étant associée à l'esca et le champignon, *F. mediterranea*, étant responsable de cette dégradation (Viala 1926 ; Maher *et al.*, 2012). Une publication récente de Hosfetter *et al.* (2012) a fortement interpellé les chercheurs travaillant sur cette maladie. Leur étude montre que les pathogènes présumés de l'esca étaient répandus et détectés à la même fréquence dans les tissus de ceps ayant exprimé ou non des symptômes foliaires. Ces auteurs en ont déduit que les pathogènes fongiques étaient probablement des saprophytes dégradant du bois déjà sénescant, ou mort, résultant d'une taille intensive des ceps, du gel ou d'autres blessures mécaniques telles que le greffage. Cependant, cette affirmation est loin de faire l'unanimité car elle ne tient pas compte des nombreux travaux réalisés lors de tests de pathogénicité et qui montrent que les champignons tels que *Ph. aleophilum*, *Ph. chlamydospora* ou *F. mediterranea* sont réellement capables d'induire des nécroses dans des

tissus de bois non dégradés et vivant au moment de l'inoculation (Laveau *et al.*, 2009; Rego *et al.*, 2009; Sparapano *et al.*, 2000, 2001; Surico *et al.*, 2006). De plus Hosfetter *et al.* (2012) en perçant le bois et en ensemençant des copeaux n'avaient pas connaissance de l'état sanitaire du bois des ceps. Or Maher *et al.* (2012) ont montré que l'esca est associé à une dégradation importante du bois, ceci dans des proportions plus importante que dans les ceps asymptomatiques. Ce point est crucial car cela signifie que chez des ceps ayant le même âge, des nécroses peuvent être observées chez des plants ayant exprimés des symptômes foliaires d'esca mais aussi chez ceux asymptomatiques. La différence entre eux portant essentiellement sur le niveau de développement et l'extension des nécroses dans le bois. Pour éviter ce biais, dans la présente étude nous avons déraciné tous les ceps et analysé les tissus ligneux en tenant compte de l'état sanitaire de chacun d'eux, c'est-à-dire, nous avons vérifié si les bois étaient sains, en cours de dégradation (coloration jaunâtre) ou nécrosés.

Pour les publications « The complex relationship of fungi with esca. Analyses of the fungal communities that colonize the wood tissues of leaf-symptomatic and asymptomatic vines » et « Specific bacterial microflora are associated with grapevine asymptomatic and esca-symptomatic wood tissues » il nous a paru judicieux d'orienter l'étude vers la caractérisation d'une microflore qui n'avait pas été étudiée au préalable, celle du bois apparemment sain de ceps relativement jeunes (10 ans d'âge). Il s'agissait de déterminer quelle était les micro-organismes fongiques et bactériens colonisateurs du bois de plants où les nécroses n'étaient pas encore développées, à l'inverse de ce qui est fréquemment observé à l'intérieur des ceps plus âgés (15 ans et plus). L'intérêt aussi de ces plants de vigne est que certains avaient exprimés des symptômes foliaires d'esca, d'autres non. L'expression pathologique étant spécifiquement associée au développement d'amadou, uniquement dans les bras des ceps symptomatiques-foliaires esca. Ainsi nous avons des ceps symptomatiques et asymptomatiques ayant chacun un tronc d'apparence sain (quasiment sans nécroses) avec des « bras » ou l'amadou était présent uniquement chez ceux pour lesquels les symptômes d'esca avaient été observés.

La technique d'empreinte moléculaire, la SSCP, a d'abord était utilisée, son avantage est qu'elle permet d'obtenir une vision d'ensemble de la complexité microbienne d'un échantillon et ne nécessite pas de connaissances préalables de la composition de la microflore. Elle a été utilisée pour : (i) étudier les microflores fongiques et bactériennes colonisant le bois, (ii) comparer les échantillons selon leur provenance à l'intérieur d'un cep, *i.e.* bras, tronc, porte-greffe, (iii) suivre en fonction du temps, *i.e.* hiver (janvier), printemps (avril), été (juin) et automne (septembre), les évolutions au sein des communautés microbiennes.

Concernant le premier point (i) les profils SSCP montrent que des communautés fongiques et bactériennes très diverses colonisent et se développent dans les bois apparemment sains des ceps. (ii) Les microflores sur les écorces du tronc et du porte-greffe se différencient de celles colonisant le bois à l'intérieur des plants de vigne. Ces observations sont vérifiées pour les deux types de micro-organismes (champignons et bactéries). (iii) Les analyses réalisées avec les échantillons prélevés au cours des quatre saisons indiquent une évolution de la microflore fongique bien distincte entre janvier et avril mais moins marquée

en été (juin et septembre). Pour la microflore bactérienne, l'évolution semble relativement identique à celle observée avec les champignons. Il est important de noter que les microflores fongiques colonisant le bois sains des ceps symptomatiques ou asymptomatiques semblent présenter de nombreux recouvrements. Parfois, les microflores bactériennes se différencient entre les plants symptomatiques foliaires et ceux asymptomatiques, mais ce phénomène n'est pas fréquent (observé uniquement pour un temps de prélèvement). Ces observations concernent les microflores dans leurs globalités mais elles ne ciblent pas des espèces spécifiques qui peuvent être plus ou moins abondantes selon les mois.

Après cette observation globale de la microflore par empreinte moléculaire, les communautés microbiennes cultivables ont été étudiées en utilisant une approche combinant l'identification morphologique des isolats couplée au séquençage de la zone ADN-ITS pour les champignons ou ADNr-16S pour les bactéries.

Pour les agents fongiques, des Ascomycètes (92,4% des isolats) ont principalement été identifiés, les Zygomycètes et les Basidiomycètes représentaient uniquement 7,2% et 0,4% des isolats présents dans le bois sain des ceps. Au total 48 genres ont été isolés (35 dans les plants asymptomatiques et 37 chez ceux esca-foliaire symptomatiques). Les 5 genres fongiques les plus importants étaient: *Botryosphaeria*, *Trichoderma*, *Penicillium*, *Bionectria* et *Fusarium*. On peut noter que 70% des ceps esca-foliaire symptomatiques et des asymptomatiques étaient colonisés par des espèces de *Botryosphaeria* (potentiellement pathogènes) et de *Trichoderma* (potentiellement protectrices des végétaux). Ce résultat indique que beaucoup de champignons impliqués dans les maladies du bois, e.g. les *Botryosphaeria*, sont présents dans le bois d'apparence sain. Par contre des champignons potentiellement protecteurs du végétal sont également présents, comme les *Trichoderma* spp. Certaines souches sont connues pour leur capacité à parasiter les champignons dégradateurs du bois comme *Trametes versicolor* ou *Fomitopsis palustris* (Lee et al., 2012). Au total pour les 4 temps de prélèvements, 23% des genres isolés des bûchettes de bois de plants asymptomatiques et 27% isolés de plant symptomatiques étaient des *Trichoderma* spp. Au niveau des espèces, *Trichoderma harzianum*, *T. gamsii* et *T. atroviride* ont été isolées du bois de vigne, en quantités égales chez les plants manifestant des symptômes d'esca que chez ceux n'en manifestant pas. Le fait que ces espèces de *Trichoderma* soient naturellement présentes dans le bois sain de vigne permet de penser que de nombreuses interactions avec d'autres champignons, dont ceux potentiellement pathogènes, se passent à l'intérieur des ceps. Ceci doit certainement influencer sur les équilibres entre microflores et induire des modifications dans leurs compositions au cours du temps.

Concernant les bactéries, 50% des souches isolées étaient des Gram- et 50% des Gram+, cette proportion étant identique chez les plants symptomatiques foliaire-esca et les asymptomatiques. Le phylum le plus abondant était celui des Firmicutes (pour les Gram+) et celui des Proteobacteria (pour les Gram-). Quel que soit l'état sanitaire des plants, atteints par l'esca ou non, les Bacillales étaient isolés de tous les tissus de bois, le genre *Bacillus* était le plus abondant. Certaines espèces de bactéries étaient capables de coloniser ou non l'amadou. Ainsi 70% des souches colonisant cette nécrose typique de l'esca étaient des *Bacillus* alors que l'ordre des *Enterobacteriales*, dont *Pantoea agglomerans*, qui est l'espèce la plus

abondante parmi les bactéries, n'a jamais été détecté dans l'amadou. Il en était de même des *Xanthomonadales*. Il est important de noter que les communautés bactériennes colonisant le bois des ceps ont des aptitudes à dégrader des substrats carbonés (utilisation de plaques Biolog Ecoplates™) différentes selon les tissus où elles ont été prélevées. Par exemple, dans les bras, nous avons observé que les bactéries colonisant l'amadou, les nécroses striées et le bois apparemment sain, ont toutes des aptitudes trophiques différentes. Il en est de même des bactéries colonisant le bois non dégradé du tronc « intérieur » (au centre du cep) par rapport à celui « extérieur » (sous l'écorce). Ces résultats indiquent aussi que parfois, alors que la SSCP ne peut différencier des communautés bactériennes colonisant certains tissus, *e.g.* celles des zones du tronc appelées « extérieur » et « intérieur », les essais avec les plaques Biolog Ecoplates™ montrent que ces bactéries ont des aptitudes métaboliques très différentes.

Une question d'importance, concerne le rôle qu'exerce ces bactéries dans la préservation ou, à l'inverse, la dégradation du bois. Ce double aspect peut en effet être considéré si l'on se focalise sur les propriétés de certaines d'entre elles. *P. agglomerans*, est utilisé comme agent de biocontrôle (Nunes *et al.*, 2002 ; Kim *et al.*, 2012) pour lutter contre *B. cinerea*, *Rhizopus stolonifer* ou *Erwinia amylovora*. D'autres espèces du genre *Bacillus* comme *Bacillus sp.*, *B. licheniformis* ou *B. pumillus* ont aussi été décrites pour leur aptitude à protéger la vigne contre *B. cinerea* et *Agrobacterium vitis* (Krol, 1998) ou d'autres plantes (Lee *et al.*, 2006; Schmiedeknecht *et al.*, 2001; Trivedi *et al.*, 2011).

A l'inverse, des bactéries que nous avons isolé du bois, telle que *Sphingomonas sp.*, *Erwinia sp.* and *P. polymyxa*, peuvent dégrader la lignine. Greaves (1971) rapporte que certaines espèces affectent la perméabilité et la structure du bois, agissent en synergie avec d'autres bactéries ou champignons pour prédisposer ou préparer le bois aux attaques par des champignons dégradateurs. Chez d'autres végétaux que la vigne, des interactions positives (Kamei *et al.*, 2012), négatives (De Boer *et al.*, 2010) ou neutres ont été décrites au sein du bois. Kamei *et al.* (2012) ont montré que *Stereum sp.* souche TN4F, un agent fongique de la pourriture blanche, et une bactérie isolée de tissus dégradés de bois coexistaient et avaient une influence positive l'un sur l'autre. Selon Valaskova *et al.* (2009), certaines bactéries bénéficieraient des substrats lignino-cellulosiques libérés par l'action enzymatique d'un champignon de pourriture blanche, ce qui favoriserait leurs croissances et leurs colonisations au sein de ces tissus endommagés. Nous pouvons raisonnablement penser que ces différentes interactions et associations existent dans le bois de la vigne, elles influent sur les communautés microbiennes, façonnent leur devenir et les successions de microflore qui sont observées. Néanmoins, de futures recherches sur ce thème doivent être entreprises afin de déterminer et de comprendre les différents mécanismes mis en place.

La technique de pyroséquençage 454 utilisée (chapitre 3) a permis, en complément des études réalisées dans les chapitres 1 et 2, de caractériser de façon plus fine la composition des assemblages de micro-organismes, bactéries et champignons, colonisant le bois des jeunes ceps (10 ans d'âge). La diversité microbienne a été confirmée et précisée : 222 OTUs bactériens et 674 OTUs fongiques ont été obtenus. Les courbes de raréfaction montrent cependant qu'alors que le nombre de séquences et d'OTUs est plus grand, la diversité réelle

est sous estimée pour les champignons, ce phénomène étant nettement moins marqué pour les bactéries.

Pour ce qui est des communautés fongiques, cette technique confirme que l'amadou est un tissu colonisé par une microflore spécifique. Il est colonisé à plus de 80% par des Basidiomycetes, principalement *Fomitiporia* ce qui est en accord avec ce qui a été décrit dans la littérature (Larignon and Dubos, 1997 ; Maher *et al.*, 2012). Dans les tissus non nécrosés, ce sont essentiellement des Ascomycètes (68 à 87% des séquences) avec une prédominance du genre *Phaeomoniella* (entre 10 à 39% des séquences) au niveau des tissus non nécrosés des bras des ceps esca-symptomatiques et asymptomatiques (28% et 39% des séquences respectivement) qui a été observée. Beaucoup d'autres genres potentiellement pathogènes de la vigne ont également été identifiés, *i.e.* *Diplodia*, *Fomitiporia*, *Neofusicoccum*, *Phaeoacremonium*, *Cryptococcus*, *Neofabraea*, *Aureobasidium* et peu de genres potentiellement protecteurs des plantes, *i.e.* *Bionectria* et *Trichoderma* (champignons antagonistes, 1% à 3% des séquences obtenues). Ces résultats contrastent avec ceux obtenus dans le chapitre 1 sur la microflore fongique cultivable. Cette différence pourrait provenir de la sélection qui nous avons fait en prélevant et identifiant uniquement les champignons ayant développé un mycélium supérieur à un centimètre de diamètre en boîte de Pétri au bout d'un mois. Certains agents fongiques, ayant une croissance très rapide comme les *Trichoderma* spp., ont certainement été surestimés. De son côté, le pyroséquençage donne-t-il une quantification précise des champignons présents ? Des essais réalisés en collaboration avec l'équipe de M.H. Lebrun (UMR BIOGER, INRA) indiquent que les champignons présents dans le bois sont bien amplifiés, mais les proportions initiales des espèces amplifiées ne sont pas toujours conservées après pyroséquençage. Au final, les espèces majoritaires sont certainement bien mises en évidence, celles plus minoritaires sont représentées mais d'une façon plus aléatoire. L'ensemble donc des techniques utilisées donnent des tendances, chacune présentant des avantages et inconvénients. Au final, le résultat obtenu par pyroséquençage indique que la situation à l'intérieur des ceps est nettement plus critique que ne le laissait penser la méthode cultivable. La plupart des champignons impliqués dans l'esca sont ainsi déjà présents dans le bois non-nécrosés des ceps symptomatiques ou asymptomatiques et ceci dans des quantités assez élevées, alors que les antagonistes seraient peu nombreux.

Les résultats obtenus par pyroséquençage montrent que pour les bactéries, le phylum des Protéobactéries est le plus abondant dans tous les tissus non nécrosés (61% de l'ADN bactérien total), les Alpha-protéobactéries (59%) étant le groupe le plus représenté. Au sein de l'amadou, les Protéobactéries (50% des séquences) puis les Actinobactéries (32% des séquences) sont les phylums les plus représentés. A l'instar de ce qui a été observé avec les champignons, on peut aussi remarquer que certaines familles ou genres sont spécifiquement présents, *i.e.* les *Microbacteriaceae*, ou absent, *i.e.* *Pseudomonas* et *Erwinia*, dans l'amadou. Pour les bactéries obtenues par méthode cultivable et pyroséquençage, les résultats sont plus cohérents qu'avec les champignons, même si une parfaite correspondance n'a pas été observée. Plusieurs auteurs ont en effet rapportés que certaines souches bactériennes appartenant à trois classes : Actinomycètes, Alpha- et Gamma-protéobactéries ont une capacité certaine à dégrader la lignine (Ahmad *et al.*, 2010 ; Bugg *et al.*, 2011 ; Valaskova *et*

al., 2009). Ces souches comprennent des genres tels que *Pseudomonas*, *Sphingomonas* ou *Erwinia* que nous avons identifié dans nos échantillons par pyroséquençage et/ou méthode cultivable. La possibilité que des bactéries prédisposent ou préparent le bois aux attaques par des champignons dégradateurs semblent donc possible.

L'étude par pyroséquençage a aussi montré que les bactéries seraient très diversifiées dans le bois de vigne contrairement aux champignons. En effet, 41 genres bactériens représentaient au minimum 66% des séquences bactériennes, pour les champignons, quelques genres majoritaires étaient présents, puisque 13 genres (hormis les uncultured fungus, Leotiomycètes et uncultured ascomycota) représentaient au minimum 50% de toutes les séquences. Au final, les différentes analyses ont montré que c'est essentiellement l'état du bois, fortement dégradé ou non, ainsi que sa localisation dans le cep qui sélectionne le type de micro-organismes présents.

La dernière étude présentée dans la publication n°3 intitulée « « Old *Vitis vinifera*: when pathogenic fungi patterns of grapevine trunk diseases are not always associated with foliar disease expression or plant decline » a été réalisée afin d'étudier la microflore fongique de ceps âgés. L'expression foliaire de l'esca ou de l'eutypiose est en effet maximale chez les plants ayant entre 15 et 30 ans et semble décroître chez les ceps plus âgés, seulement quelques uns dépérissent suite à ces maladies. Dans le cas présent, le cultivar utilisé, le Baco blanc (plants de 42 et 58 ans), est un cépage hybride de pied franc qui a été conçu pour être vigoureux et produire de grandes quantités de baies. Il est aussi décrit comme étant relativement tolérant à *B. cinerea* et dans une moindre mesure à *E. necator*, sa sensibilité par rapport aux maladies du bois n'est pas vraiment connue. L'analyse du bois fonctionnel, relativement préservé, par rapport aux tissus totalement nécrosés du centre du tronc des ceps montre que la microflore est relativement peu abondante : 17 genres fongiques ont été isolés des tissus ligneux non-nécrosés de Baco blanc contre 35 genres chez les plants sains et 37 chez les jeunes plants symptomatiques esca-foliaires de Cabernet Sauvignon (publication n°2). Chez les Baco blanc, les espèces majoritaires (principalement *E. lata*) des ceps de 42 ans étaient typiques de plants atteints d'eutypiose, ceux de 58 ans étaient colonisés par des champignons impliqués dans l'esca, i.e. *Ph. aleophilum*, *F. mediterranea*, *S. hirsutum*, *Ph. chlamydospora*. Pourtant dans les 2 cas, aucun symptôme foliaire n'a été observé l'année de prélèvement des plants. Pour expliquer ce phénomène, au moins 2 raisons peuvent être avancées : (i) les tissus fonctionnels sont relativement préservés ce qui assure aux ceps un fonctionnement physiologique convenable, (ii) une microflore très abondante composée de *Trichoderma* spp. colonise aussi ce bois préservé. On peut émettre l'hypothèse qu'il se crée un équilibre entre les microflores, empêchant le développement exacerbé des champignons pathogènes et donc l'expression de l'esca ou de l'eutypiose.

En conclusion, cette étude a apporter des éléments permettant de répondre à la question suivante : les microflores sont-elles semblables chez les ceps atteints ou non par l'esca ? A notre avis, il faut considérer l'état pathologique des ceps car ce sont les nécroses

qui font la différence. Nos résultats montrent que la répartition des espèces fongiques et bactériennes est en effet tissus-dépendante. Lorsque les microflores colonisant le bois apparemment sain de plants esca-symptomatique et asymptomatique sont comparées, elles sont la plupart du temps relativement identiques ; par contre, celles colonisant les nécroses, en particulier l'amadou, se différencient de celles des tissus sains. Or, les nécroses sont plus abondantes dans les plants exprimant l'esca.

L'étude sur les plants âgés a mis aussi en évidence, l'importance de la préservation des tissus fonctionnels, et sans doute d'une microflore fongique constituée de nombreux agents mycoparasites, capables d'interagir et de détruire les champignons pathogènes eux aussi présents dans ces tissus ligneux.

Ces résultats ouvrent des perspectives de recherche sur :

- l'analyse des microflores, en particulier bactériennes, et la détermination de leur rôle dans le bois de la vigne. Les interactions entre bactéries et champignons mériteraient aussi d'être étudiées.
- Les modifications et les évolutions des communautés microbiennes au cours du temps permettraient de déterminer s'il y aura un développement ou non de nécroses dans le bois de la vigne.
- La présence d'une microflore bénéfique naturellement présente dans le bois des cepes associé à des plants ayant une tolérance à ces maladies pourrait ouvrir de nouvelles perspectives pour lutter l'esca, voire l'eutypiose, pour lesquelles aucun moyen de protection n'existe aujourd'hui.

Références bibliographiques

- Abarenkov, K, Nilsson, RH, Larsson, KH, Alexander, IJ, Ebenhardt, U, Erland, S, *et al* (2010) The UNITE database for molecular identification of fungi - recent updates and future perspectives *New Phytol* **186**: 281-285
- Abou-Mansour E, Couche E, Tabacchi R (2004) Do fungal naphthalenones have a role in the development of esca symptoms? *Phytopathol Mediterr* **43**: 75-82
- Ahmad M, Taylor CR, Pink D, Burton K, Eastwood D, Bending GD, Bugg TDH (2010) Development of novel assays for lignin degradation: comparative analysis of bacterial and fungal lignin degraders. *Molecular Biosystems* **6**: 815-821
- Ahmadian A, Ehn M, Hober S (2006) Pyrosequencing: History, biochemistry and future. *Clin Chim Acta* **363**: 83-94
- Ait Barka E, Eullaffroy P, Clement C, Vernet G (2004) Chitosan improves development, and protects *Vitis vinifera* L. against *Botrytis cinerea*. *Plant cell reports* **22**: 608-614
- Ait Barka E, Nowak J, Clement C (2006) Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, *Burkholderia phytofirmans* strain PsJN. *Appl Environ Microbiol* **72**: 7246-7252
- Alfonzo A, Conigliaro G, Torta L, Burruano S, Moschetti G (2009) Antagonism of *Bacillus subtilis* strain AG1 against vine wood fungal pathogens. *Phytopathol Med* **48**: 155-158
- Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in-situ detection of individual microbial cells without cultivation. *Microbiol Rev* **59**: 143-169
- Amponsah NT, Jones EE, Ridgway HJ, Jaspers MV (2012) Susceptibility of grapevine tissues to *Neofusicoccum luteum* conidial infection. *Plant Pathology* **61**: 719-729
- Amreen T, Kumar VBS (2012) In vitro evaluation of native *Trichoderma harzianum* against major fungal pathogen of flower and fruit crops. *Environment and Ecology* **30**: 1039-1041
- Andolfi A., L. Mugnai, J. Luque, G. Surico, A. Cimmino and A. Evidente, 2011. Phytotoxins Produced by Fungi Associated with Grapevine Trunk Diseases. *Toxins* **3**, 1569-1605
- Arfi Y, Buee M, Marchand C, Levasseur A, Record E (2012) Multiple markers pyrosequencing reveals highly diverse and host-specific fungal communities on the mangrove trees *Avicennia marina* and *Rhizophora stylosa*. *Fems Microbiology Ecology* **79**: 433-444
- Argyropoulos DS, Menachem, SB (1997) *Lignin*: Springer-Verlag.
- Armengol J, Vicent A, Torne L, Garcia-Figueres F, Garcia-Jimenez J (2001) Fungi associated with decline and infections of grapevine wood in various Spanish zones. *Plagas* **27**: 137-153

- Armengol J, Vicent A, Torne L, Garcia-Figueres F, Garcia-Jimenez J, (2001) Fungi associated with esca and grapevine declines in Spain: a three-year survey. *Phytopathologia Mediterranea* **40**: S325–329
- Armengol, J, Gimenez-Jaime A, Vicent A, and Garcia-Jimenez J (2005) The diseases of wood in Midi-Pyrenees Esca and BDA Etiology and importance of diseases of wood in Spain *Progres Agri Viti* **122**: 130-134
- Arnaud G, Arnaud M, (1931) *Traité de Pathologie Végétal. Encyclopédie Mycologique*. Lechevalier, Vol. 3 and 4
- Aroca A, Luque J, Raposo R (2008) First report of *Phaeoacremonium viticola* affecting grapevines in Spain. *Plant Pathology* **57**: 386-386
- Aroca A, Gramaje D, Armengol J, Garcia-Jimenez J, Raposo R (2010) Evaluation of the grapevine nursery propagation process as a source of *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* and occurrence of trunk disease pathogens in rootstock mother vines in Spain. *Eur J Plant Pathol* **126**: 165-174
- Arnold AE, Maynard Z, Gilbert GS, Coley PD, Kursar TA (2000) Are tropical fungal endophytes hyperdiverse? *Ecol Lett* **3**: 267-274
- Arnold AE, Mejia LC, Kyllö D, Rojas EI, Maynard Z, Robbins N, Herre EA (2003) Fungal endophytes limit pathogen damage in a tropical tree. *Proc Natl Acad Sci U S A* **100**: 15649-15654
- Arnold AE, Lutzoni F (2007) Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots? *Ecology* **88**: 541-544
- Auger J, Esterio M, Ricke G, Perez I (2004) Black dead arm and basal canker of *Vitis vinifera* cv. Red globe caused by *Botryosphaeria obtusa* in Chile. *Plant Dis* **88**: 1286-1286
- Backman PA, Sikora RA, (2008) Endophytes: An emerging tool for biological control, *Biological Control*. doi:10.1016/j.biocontrol.2008.03.009
- Becklin KM, Hertweck KL, Jumpponen A (2012) Host Identity Impacts Rhizosphere Fungal Communities Associated with Three Alpine Plant Species. *Microb Ecol* **63**: 682-693
- Beckman TG, Reilly CC, Pusey PL, Hotchkiss M (2011) Progress in the Management of Peach Fungal Gummosis (*Botryosphaeria dothidea*) in the Southeastern US Peach Industry *J Am Por Soc* **65**: 192-200
- Bell CR, Dickie GA, Harvey WLG, Chan J (1995) Endophytic bacteria in grapevine. *Canadian Journal of Microbiology* **41**: 46-53
- Bell DK, Wells HD, Markham CR (1982) In vitro antagonism of *Trichoderma* species against six fungal plant pathogens. *Phytopathology* **72**: 379-382
- Bengtsson J, Eriksson KM, Hartmann M, Wang Z, Shenoy BD, Grelet G-A, Abarenkov K, Petri A, Rosenblad MA, Nilsson RH (2011) Metaxa: a software tool for automated

- detection and discrimination among ribosomal small subunit (12S/16S/18S) sequences of archaea, bacteria, eukaryotes, mitochondria, and chloroplasts in metagenomes and environmental sequencing datasets. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **100**: 471-475
- Berraf, A, Peros, JP (2005) Importance of *Eutypa dieback* and esca in Algeria and structure of the associated fungal community *J Int Sci Vigne Vin* **39**: 121-128
- Bertelli E, Mugnai L, Surico G (1998) Presence of *Phaeoacremonium chlamydosporum* in apparently healthy rooted grapevine cuttings. *Phytopathol Mediterr* **37**: 79-82
- Bertsch, C, Ramirez-Suero, M, Magnin-Robert, M, Larignon, P, Chong, J, Abou-Mansour, E, *et al* (2012) Grapevine trunk diseases: complex and still poorly understood *Plant Pathol* DOI: 101111/j1365-3059201202674x
- Bester W, Crous PW, Fourie PH (2007) Evaluation of fungicides as potential grapevine pruning wound protectants against *Botryosphaeria* species. *Austral Plant Pathol* **36**: 73-77
- Bills, GF (1996) Isolation and analysis of endophytic fungal communities from woody plants: *Am Phytopathol Soc (APS Press)*
- Blaalid R, Carlsen T, Kumar S, Halvorsen R, Ugland KI, Fontana G, *et al.* (2012) Changes in the root-associated fungal communities along a primary succession gradient analysed by 454 pyrosequencing. *Molecular Ecology* **21**: 1897-1908
- Boubals D (1986) It is absolutely necessary to learn to double graft grapevines
Il faut absolument apprendre a surgreffer la vigne. *Progres Agricole et Viticole* **103**: 194-195
- Buckland, ST, Magurran, AE, Green, and RE, Fewster, RM (2005) Monitoring change in biodiversity through composite indices *Philos Trans R Soc B-Biol Sci* **360**: 243-254
- Bugg TDH, Ahmad M, Hardiman EM, Singh R (2011) The emerging role for bacteria in lignin degradation and bio-product formation. *Current Opinion in Biotechnology* **22**: 394-400
- Butler R, Patty L, Le Bayon RC, Guenat C, Schlaepfer R (2007) Log decay of *Picea abies* in the Swiss Jura Mountains of central Europe. *For Ecol Manage* **242**: 791-799
- Bruez E, Vallance J, Gerbore J, Lecomte P, Guerin-Dubrana L, Rey P (2011) Characterization of endophytic microflora colonizing wood tissues of healthy and Esca-diseased vines. *Phytopathology* **101**: S21-S21
- Bruez E. (2013). Comparison of the fungal and bacterial communities that colonize the wood tissues of esca-foliar symptomatic or asymptomatic grapevines. PhD thesis, University of Bordeaux, France
- Brunner K, Zeilinger S, Ciliento R, Woo SL, Lorito M, Kubicek CP, *et al.* (2005) Improvement of the fungal biocontrol agent *Trichoderma atroviride* to enhance both

- antagonism and induction of plant systemic disease resistance. *Appl Environ Microbiol* **71**: 3959-3965
- Bruno G, Sparapano L (2006) Effects of three esca-associated fungi on *Vitis vinifera* L.: III. Enzymes produced by the pathogens and their role in fungus-to-plant or in fungus-to-fungus interactions. *Physiol Mol Plant Pathol* **69**: 182-194
- Bruno G, Sparapano L (2006) Effects of three esca-associated fungi on *Vitis vinifera* L.: III. Enzymes produced by the pathogens and their role in fungus-to-plant or in fungus-to-fungus interactions. *Physiol Mol Plant Pathol* **69**: 182-194
- Bugg T, Ahmad M, Hardiman E, Singh R (2011) The emerging role for bacteria in lignin degradation and bio-product formation. *Current Opinion in Biotechnology* **22**(3): 394–400
- Burie JB, Langlais M, Calon nec A (2011) Switching from a mechanistic model to a continuous model to study at different scales the effect of vine growth on the dynamic of a powdery mildew epidemic. *Annals of Botany* **107**: 885-895
- Burr TJ (2004) Grape Crown Gall Biology and Strategies for Control. *FPS Grape Program Newsletter* **October 2004**: 16-18
- Calon nec A, Cartolaro P, Poupot C, Dubourdieu D, Darriet P (2004) Effects of *Uncinula necator* on the yield and quality of grapes (*Vitis vinifera*) and wine. *Plant Pathology* **53**: 434-445
- Calzarano F, Di Marco S, Cesari A (2004) Benefit of fungicide treatment after trunk renewal of vines with different types of esca necrosis. *Phytopathol Mediterr* **43**: 116-124
- Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**: 335-336
- Carroll G (1988) Fungal endophytes in stems and leaves: from latent pathogen to mutualist symbionts. *Ecology* **69**: 2-9
- Carter MV (1957) *Eutypa armeniaca* Hansf. & Carter, sp. nov., an airborne vascular pathogen of *Prunus armeniaca* L. in southern Australia. *Aust J Bot* **5**: 21–35
- Carter MV, Moller WJ (1974) *Eutypa* canker of apricot in Spain. *Plant Disease Reporter* **58**: 441-443
- Carter MV (1988) *Eutypa dieback*. In: Pearson RC, Goheen AC, eds. *Compendium of Grape Diseases*. St Paul, MN, USA: APS Press, 32–4
- Carter MV (1991) The status of *Eutypa lata* as a pathogen. Monograph. *Phytopathological Paper No. 32*, International Mycological Institute, Egham, UK
- Casieri L, Hofstetter V, Viret O, Gindro K (2009) Fungal communities living in the wood of different cultivars of young *Vitis vinifera* plants *Phytopathol Mediterr* **48**: 73-83

- Chadefaud M, Emberger L (1960) Les végétaux vasculaires. *Traité de Botanique* **2**: 1-1450
- Chanderbali AS, Van der Werff H, Renner SS (2001) Phylogeny and historical biogeography of Lauraceae: Evidence from the chloroplast and nuclear genomes *Ann Miss Bot Garden* **88**: 104-134
- Chapuis L, Richard L, Dubos B (1998) Variation in susceptibility of grapevine pruning wound to infection by *Eutypa lata* in south-western France. *Plant Pathology* **47**: 463-472.
- Chattaoui M, Rhouma A, Msallem M, Perez M, Moral J, Trapero A (2012) First Report of *Botryosphaeria obtusa* as Causal Agent of Olive Tree Branch Dieback in Tunisia. *Plant Dis* **96**: 905-905
- Chiarappa L (1959) Wood decay of the grapevine and its relationship with black measles disease. *Phytopathology* **49**: 510-519
- Chiarappa L (2000) Esca (black measles) of grapevine An overview *Phytopathol Mediterr* **39**: 11-15
- Choueiri E, Jrejjiri F, Chlela P, Louvet G, Lecomte P (2006) Occurrence of grapevine declines and first report of black dead arm associated with *Botryosphaeria obtusa* in Lebanon. *Plant Dis* **90**: 115-115
- Clausen CA (1996) Bacterial associations with decaying wood: A review. *Int Biodeterior Biodegrad* **37**: 101-107
- Clay K (1986) Induced vivipary in the sedge cyperus: *virens* and the transmission of the fungus. *Balansia cyperi* (Clavicipitaceae). *Can J Bot-Rev Can Bot* **64**: 2984-2988
- Cole RJ, Kirksey JW (1971) Aflatoxin G1 metabolism by *Rhizopus* species. *Journal of agricultural and food chemistry* **19**: 222-223
- Collins DP, Jacobsen BJ, Maxwell B (2003) Spatial and temporal population dynamics of a phyllosphere colonizing *Bacillus subtilis* biological control agent of sugar beet cercospora leaf spot. *Biol Control* **26**: 224-232
- Colwell RK (2005) EstimateS: statistical estimation of species richness and shared species from samples, version 8. purl.oclc.org/estimates, Vol. 2005
- Comont G, Corio-Costet M-F, Larignon P, Delmotte F (2010) AFLP markers reveal two genetic groups in the French population of the grapevine fungal pathogen *Phaeomoniella chlamydospora*. *Eur J Plant Pathol* **127**: 451-464
- Compant S, Kaplan H, Sessitsch A, Nowak J, Barka EA, Clement C (2008) Endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN: from the rhizosphere to inflorescence tissues. *FEMS Microbiol Ecol* **63**: 84-93

- Compant S, Clement C, Sessitsch A (2010) Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol Biochem* **42**: 669-678
- Compant S, Mitter B, Colli-Mull JG, Gangl H, Sessitsch A (2011) Endophytes of Grapevine Flowers, Berries, and Seeds: Identification of Cultivable Bacteria, Comparison with Other Plant Parts, and Visualization of Niches of Colonization. *Microb Ecol* **62**: 188-197
- Corde (1829) *Deutschland Flora* **2**: 111
- Cordier T, Robin C, Capdevielle X, Fabreguettes O, Desprez-Loustau M-L, Vacher C (2012) The composition of phyllosphere fungal assemblages of European beech (*Fagus sylvatica*) varies significantly along an elevation gradient. *New Phytologist* **196**: 510-519
- Cortesi P, Fischer M, Milgroom MG (2000) Identification and spread of *Fomitiporia punctata* associated with wood decay of grapevine showing symptoms of esca. *Phytopathology* **90**: 967-972
- Cortesi P, Milgroom MG (2001) Outcrossing and diversity of vegetative compatibility types in populations of *Eutypa lata* from grapevines. *Journal of Plant Pathology* **83**: 79-86
- Creaser ML, Wicks TJ (2004) Short-term effects of remedial surgery to restore productivity to *Eutypa lata* infected vines. *Phytopathol Mediterr* **43**: 105-107
- Cristinzio G (1978) Serious attacks by *Botryosphaeria obtusa* on vines in the province of Isernia. *Informatore Fitopatologico* **28**: 21-23
- Gravi attacchi di *Botryosphaeria obtusa* su vite in provincia di Isernia. *Informatore Fitopatologico* **28**: 21-23
- Crous PW, Gams W, Wingfield MJ, VanWyk PS (1996) *Phaeoacremonium* gen nov associated with wilt and decline diseases of woody hosts and human infections *Mycologia* **88**: 786-796
- Crous PW, Gams W (2000) *Phaeomoniella chlamydospora* gen. et comb. nov., a causal organism of Petri grapevine decline and esca. *Phytopathol Mediterr* **39**: 112-118
- Crous PW, Slippers B, Wingfield MJ, Rheeder J, Marasas WFO, Philips AJL, Alves A, Burgess T, Barber P, Groenewald JZ (2006) Phylogenetic lineages in the *Botryosphaeriaceae*. *Stud Mycol*: 235-253
- Damm U, Fourie P (2005) A cost-effective protocol for molecular detection of fungal pathogens in soil. *S Afr J Sci* **101**: 135-139
- Darriet P, Poupot C, Armand JM, Dubourdieu D, Clerjeau M, Glories Y, Bordeu E, Pszczolkowski P, Bugaret Y (2001) Incidence of vine sprayings with downy mildew fungicides, without parasitic fungi, on Cabernet Sauvignon grapes and wines composition. *J Int Sci Vigne Vin* **35**: 23-29

- Davis EC, Franklin JB, Shaw AJ, Vilgalys R (2003) Endophytic Xylaria (Xylariaceae) among liverworts and angiosperms: Phylogenetics, distribution, and symbiosis. *Am J Bot* **90**: 1661-1667
- De Boer W, Folman LB, Gunnewiek P, Svensson T, Bastviken D, Oberg G, *et al.* (2010) Mechanism of antibacterial activity of the white-rot fungus *Hypholoma fasciculare* colonizing wood. *Can J Microbiol* **56**: 380-388
- Del Río Conesa (2002) Study of Petri Disease in Grapevine. Proposal of a Possible Solution. *Nutri-Fitos*: 77-84
- Deliere L, Miclot AS, Sauris P, Rey P, Calonnet A (2010) Efficacy of fungicides with various modes of action in controlling the early stages of an *Erysiphe necator*-induced epidemic. *Pest Management Science* **66**: 1367-1373
- Denman S, Crous PW, Taylor JE, Kang JC, Pascoe I, Wingfield MJ (2000) An overview of the taxonomic history of Botryosphaeria, and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. *Stud Mycol*: 129-140
- Di Marco S, Calzarano F, Gams W, Cesari A (2000) A new wood decay of kiwifruit in Italy. *N Z J Crop Hortic Sci* **28**: 69-72
- Di Marco S, Calzarano F, Osti F, Mazzullo A (2004) Pathogenicity of fungi associated with a decay of kiwifruit. *Austral Plant Pathol* **33**: 337-342
- Djoukeng JD, Polli S, Larignon P, Abou-Mansour E (2009) Identification of phytotoxins from *Botryosphaeria obtusa*, a pathogen of black dead arm disease of grapevine. *Eur J Plant Pathol* **124**: 303-308
- Dubos B, Larignon, P (1987) Esca, in: R.C. Pearson, A.C. Goheen (Eds.), *Compendium on grape diseases*, APS Publ., St Paul, Min., USA, pp. 34-35
- Edwards J, Pascoe IG (2004) Occurrence of *Phaeoconiella chlamydospora* and *Phaeoacremonium aleophilum* associated with Petri disease and esca in Australian grapevines. *Australas Plant Pathol* **33**: 273-279
- Emmert EAB, Handelsman J (1999) Biocontrol of plant disease: a (Gram-) positive perspective. *FEMS Microbiol Lett* **171**: 1-9
- Eskalen A, Gubler WD (2001) Association of spores of *Phaeoconiella chlamydospora*, *Phaeoacremonium inflatipes*, and *Pm. aleophilum* with grapevine cordons in California. *Phytopathol Mediterr* **40**: S429-S432
- Espinosagarcia FJ, Langenheim JH (1990) The endophytic fungal community in leaves of coastal redwood population : diversity and spatial patterns. *New Phytol* **116**: 89-97
- Evidente A, Sparapano L, Andolfi A, Bruno G (2000) Two naphthalenone pentaketides from liquid cultures of *Phaeoacremonium aleophilum*, a fungus associated with esca of grapevine. *Phytopathol Mediterr* **39**: 162-168

- Feliciano AJ, Gubler WD (2001) Histological investigations on infection of grape roots and shoots by *Phaeoacremonium* spp. *Phytopathol Mediterr* **40**: S387-S393
- Ferreira JHS, Matthee FN, Thomas AC (1991) Biological control of *Eutypa lata* on grapevine by an antagonist strain of *Bacillus subtilis*. *Phytopathology* **81**: 283-287
- Ferreira JHS, Wyk PS, Venter E (1994) Slow dieback of grapevine: association of *Phialophora parasitica* with slow dieback of grapevines. *South African Journal of Enology and Viticulture* **15**: 9-11
- Fischer M (2002) A new wood-decaying basidiomycete species associated with esca of grapevine: *Fomitiporia mediterranea* (Hymenochaetales). *Mycol Prog* **1**: 315-324
- Fischer M, Kassemeyer HH (2003) Fungi associated with Esca disease of grapevine in Germany. *Vitis* **42**: 109-116
- Fischer M (2006) Biodiversity and geographic distribution of basidiomycetes causing esca-associated white rot in grapevine: a worldwide perspective. *Phytopathol Mediterr* **45**: S30-S42
- Fourie PH, Halleen, F (2005) Integrated strategies for proactive management of grapevine trunk diseases in nurseries. *Phytopathol Mediterr* **44**: 111
- Fourie PH, Halleen F (2002) Investigation on the occurrence of *Phaeomoniella chlamydospora* in canes of rootstock mother vines. *Austral Plant Pathol* **31**: 425-426
- Fourie PH, Halleen F (2006) Chemical and biological protection of grapevine propagation material from trunk disease pathogens. *Eur J Plant Pathol* **116**: 255-265
- Fourie PH, Halleen F, Vyver Jvd, Schreuder W (2001) Effect of *Trichoderma* treatments on the occurrence of decline pathogens in the roots and rootstocks of nursery grapevines. *Phytopathol Mediterr* **40**: S473-S478
- Fourie PR, Halleen F (2004) Proactive control of Petri disease of grapevine through treatment of propagation material. *Plant Dis* **88**: 1241-1245
- Frohlich J, Hyde KD (1999) Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic? *Biodivers Conserv* **8**: 977-1004
- Frohlich J, Hyde KD, Petrini O (2000) Endophytic fungi associated with palms. *Mycol Res* **104**: 1202-1212
- Gimenez-Jaime A, Aroca A, Raposo R, Garcia-Jimenez, J, Armengol J (2006) Occurrence of fungal pathogens associated with grapevine nurseries and the decline of young vines in Spain. *J Phytopathol* **154**: 598-602
- Francis CA, Obraztsova AY, Tebo BM (2000) Dissimilatory metal reduction by the facultative anaerobe *Pantoea agglomerans* SP1. *Appl Environ Microbiol* **66**: 543-548

- Fukasawa Y, Osono T, Takeda H (2005) Decomposition of Japanese beech wood by diverse fungi isolated from a cool temperate deciduous forest. *Mycoscience* **46**: 97-101
- Fussler L, Kobes N, Bertrand F, Mauray M, Grosman J, Savary S (2008) A characterization of grapevine trunk diseases in France from data generated by the National Grapevine Wood Diseases Survey. *Phytopathology* **98**: 571-9
- Galet P (2000) Dictionnaire Encyclopédique des Cépages. In Pratique H (ed.).
- Garbeva P, Van Veen JA, Van Elsas JD (2004) Microbial diversity in soil: Selection of microbial populations by plant and soil type and implications for disease suppressiveness. *Annual Review of Phytopathology* **42**: 243-270
- Garland JL (1996) Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. *Soil Biol Biochem* **28**:213-221
- Garland JL, Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Appl Environ Microb* **57**:2351-2359
- Geoffrion R, Renaudin I (2002) Anti-Esca pruning: a useful measure against outbreaks of this old grapevine disease. *Phytoma*: 23-27
- Gimenez-Jaime A, Aroca A, Raposo R, Garcia-Jimenez J, Armengol J (2006) Occurrence of fungal pathogens associated with grapevine nurseries and the decline of young vines in Spain. *J Phytopathol* **154**: 598-602
- Giordano L, Gonthier P, Varese GC, Miserere L, Nicolotti G (2009) Mycobiota inhabiting sapwood of healthy and declining Scots pine (*Pinus sylvestris* L.) trees in the Alps. *Fungal Divers* **38**: 69-83
- Glawe DA, Dilley MA, Moller WJ (1983) Isolation and identification of *Eutypa armeniaceae* from *Malus domestica* in Washington state. *Mycotaxon* **18**: 315-318
- Gonzalez V, Tello ML (2010) The endophytic mycota associated with *Vitis vinifera* in central Spain *Fungal Divers* **47**: 29-42
- Gotelli NJ, Colwell RK (2001) Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness *Ecol Lett* **4**: 379-391
- Govender V, Korsten L (2006) Evaluation of different formulations of *Bacillus licheniformis* in mango pack house trials. *Biol Control* **37**: 237-242
- Gramaje D, Maria Munoz R, Luisa Lerma M, Garcia-Jimenez J, Armengol J (2009) Fungal grapevine trunk pathogens associated with Syrah decline in Spain. *Phytopathol Mediterr* **48**: 396-402

- Gramaje D, Alaniz S, Abad-Campos P, Garcia-Jimenez J, Armengol J (2010) Effect of hot-water treatments in vitro on conidial germination and mycelial growth of grapevine trunk pathogens. *Annals of Applied Biology* **156**: 231-241
- Gramaje D, Armengol J (2011) Fungal Trunk Pathogens in the Grapevine Propagation Process: Potential Inoculum Sources, Detection, Identification, and Management Strategies. *Plant Dis* **95**: 1040-1055
- Graniti A (1960) Il “mal dell’esca” della vite in Puglia. *Ital Agric* **97**: 543-550
- Graniti A, Surico G, Mugnai L (2000) Esca of grapevine: a disease complex or a complex of diseases? *Phytopathol Mediterr* **39**: 16-20
- Grasso S (1969) Il “mal dell’esca” della vite (*Phellinus ignarius* Patouillard) in Sicilia. *Tecnica Agric (Catania)* **21**: 204-212
- Grasso S (1984) Infections of *Fusarium oxysporum* and *Cylindrocarpon destructans* associated with a decline of young grapevine plants in Sicily. *Informatore Fitopatologico* **34**: 59-63
- Gray EJ, Smith DL (2005) Intracellular and extracellular PGPR: commonalities and distinctions in the plant-bacterium signaling processes. *Soil Biol Biochem* **37**: 395-412
- Greaves H (1971) The bacterial factor of wood decay. *Wood Science and Technology* **5**: 6-16
- Grosman, J, and Doublet, B (2012) Maladies du bois de la vigne Synthèse des dispositifs d’observation au vignoble, de l’observatoire 2003-2008 au réseau d’épidémiosurveillance actuel *Phytoma* **651**: 31-35
- Gubler WD, Baumgartner K, Browne GT, Eskalen A, Latham SR, Petit E, Bayramian LA (2004) Root diseases of grapevines in California and their control. *Austral Plant Pathol* **33**: 157-165
- Gueldner RC, Reilly CC, Pusey PL, Costello CE, Arrendale RF, Cox RH, *et al.* (1988) Isolation and identification of iturins as antifungal peptides in biological-control of peach brown rot with *Bacillus subtilis*. *J Agric Food Chem* **36**: 366-370
- Guérin-Dubrana L, Labrousse JC, Bastien S, Rey P, Gegout-Petit A (2012) Statistical analysis of grapevine mortality associated with Esca or Eutypa dieback foliar expression. *Phytopathologia Mediterranea* (submitted this Journal issue)
- Gugino BK, Travis JW (2003) Suppression of *Cylindrocarpon destructans* utilizing compostes soil amendments. *Phytopathology* **93**: S31
- Guler-Akin MB (2005) The effects of different incubation temperatures on the acetaldehyde content and viable bacteria counts of bio-yogurt made from ewe's milk. *International Journal of Dairy Technology* **58**: 174-179
- Gwinn KD, Gavin AM (1992) Relationship between endophyte infestation level of tall fescue seed lots and Rhizoctoniaeae seedling disease. *Plant Dis* **76**: 911-914

- Halleen F, Mostert L, Crous PW (2007) Pathogenicity testing of lesser-known vascular fungi of grapevines. *Austral Plant Pathol* **36**: 277-285
- Hallmann J, QuadtHallmann A, Mahaffee WF, Kloepper JW (1997) Bacterial
- Hallmann J (2001) Plant interactions with endophytic bacteria. CABI Publishing.
- Hardoin P, van Overbeek LS, Van Elsas JD (2008) Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol* **16**: 463-471
- Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004) Trichoderma species - Opportunistic, avirulent plant symbionts. *Nat Rev Microbiol* **2**: 43-56
- Harmandon F (2004) Effets toxiques de l'arsénite de sodium sur la santé humaine et exposition des utilisateurs. *Les maladies du bois en Midi-Pyrénées*: 8-12
- Hartmann M, Howes CG, Abarenkov K, Mohn WW, Nilsson RH (2010) V-Xtractor: An open-source, high-throughput software tool to identify and extract hypervariable regions of small subunit (16 S/18 S) ribosomal RNA gene sequences. *Journal of Microbiological Methods* **83**: 250-253
- Higgins KL, Arnold AE, Miadlikowska J, Sarvate SD, Lutzoni F (2007) Phylogenetic relationships, host affinity, and geographic structure of boreal and arctic endophytes from three major plant lineages. *Mol Phylogenet Evol* **42**: 543-555
- Hofstetter V, Buyck B, Croll D, Viret O, Couloux A, Gindro K (2012) What if esca disease of grapevine were not a fungal disease? *Fungal Divers* **54**: 51-67
- Hollis JP (1951) Bacteria in healthy potato tissue. *Phytopathology* **41**: 350-366
- Hopkins DL (1989) *Xylella fastidiosa*: Xylem-limited bacterial pathogen of plants. *Annu Rev Phytopathol* **27**: 271-290
- Hua L, Li RY, Hua W, Cheng YW (2007) Discovery and identify Eutypa dieback of grapevine in china. Yangling: Northwest a & F Univ Press.
- Hyde KD, Soyong K (2008) The fungal endophyte dilemma. *Fungal Divers* **33**: 163-173
- Hyman ED (1988) A new method of sequencing DNA. *Analytical Biochemistry* **174**: 423-436
- Inderbitzin P, Michailides TJ (2008) High diversity of Botryosphaeria species from almond band and canopy cankers in California. *Phytopathology* **98**: S71-S71
- Jacobs MJ, Bugbee WM, Gabrielson DA (1985) Enumeration, location and characterization of endophytic bacteria within sugar-beet roots. *Can J Bot-Rev Can Bot* **63**: 1262-1265
- John S, Wicks TJ, Hunt JS, Lorimer MF, Oakey H, Scott ES (2005) Protection of grapevine pruning wounds from infection by *Eutypa lata* using *Trichoderma harzianum* and *Fusarium lateritium*. *Austral Plant Pathol* **34**: 569-575

- Johnston PR, Johansen RB, Williams AFR, Paula Wikie J, Park D (2012) Patterns of fungal diversity in New Zealand Nothofagus forests. *Fungal Biol* **116**: 401-412
- Kamei I, Yoshida T, Enami D, Meguro S (2012) Coexisting Curtobacterium Bacterium Promotes Growth of White-Rot Fungus Stereum sp. *Curr Microbiol* **64**: 173-178
- Keller M (2010) The Science of Grapevines: Anatomy and Physiology, Vol. 1: Academic Press
- Kim IY, Pusey PL, Zhao YF, Korban SS, Choi H, Kim KK (2012) Controlled release of *Pantoea agglomerans* E325 for biocontrol of fire blight disease of apple. *J Control Release* **161**: 109-115
- King S, McCord BR, Riefler RG (2005) Capillary electrophoresis single-strand conformation polymorphism analysis for monitoring soil bacteria. *Journal of Microbiological Methods* **60**: 83-92
- Kirk JL, Beaudette LA, Hart M, Moutoglis P, Khironomos JN, Lee H, Trevors JT (2004) Methods of studying soil microbial diversity. *Journal of Microbiological Methods* **58**: 169-188
- Kobayashi DY, Palumbo JD (2000) Bacterial endophytes and their effects on plants and uses in agriculture. Vol. Microbial endophytes, New York: Marcel Dekker :199-233
- Kotze C, Niekerk Jv, Halleen F, Mostert L, Fourie P (2011) Evaluation of biocontrol agents for grapevine pruning wound protection against trunk pathogen infection. *Phytopathol Mediterr* **50**: 247-263
- Kouyeas H (1978) Eutypa armeniacae on lemon in Greece. *Phytopathologische Zeitschrift-Journal of Phytopathology* **91**: 235-237
- Krol E (1998) Epiphytic bacteria isolated from grape leaves and its effect on *Botrytis cinerea* Pers. *Phytopathol Pol* **16**: 53-61
- Krol, E (2006) Fungi inhabiting healthy grapevine canes (*Vitis* spp) in some nurseries *Acta Agr* **59**: 163-173
- Kubartova A, Ottosson E, Dahlberg A, Stenlid J (2012) Patterns of fungal communities among and within decaying logs, revealed by 454 sequencing. *Molecular Ecology* **21**: 4514-4532
- Kuklinsky-Sobral HL, Araujo WL, Mendes R, Pizzirani-Kleiner AA, Azevedo JL (2005) Isolation and characterization of endophytic bacteria from soybean (*Glycine max*) grown in soil treated with glyphosate herbicide. *Plant Soil* **273**: 91-99
- Kuntzmann P, Villaume S, Larignon P, Bertsch C (2010) Esca, BDA and Eutypiosis: foliar symptoms, trunk lesions and fungi observed in diseased vinestocks in two vineyards in Alsace. *Vitis* **49**: 71-76

- Laiho R, Prescott CE (2004) Decay and nutrient dynamics of coarse woody debris in northern coniferous forests: a synthesis. *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere* **34**: 763-777
- Larignon P, Dubos B (1997) Fungi associated with esca disease in grapevine *Eur J Plant Pathol* **03**: 147-157
- Larignon P, Dupont J, Dubos B (2000) Esca disease. The biological background of two agents of the disease, *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora*. *Phytoma*: 30-35
- Larignon P, Fulchic R, Cere L, Dubos B (2001) Observation on black dead arm in French vineyards *Phytopathol Mediterr* **40**: S336-S342
- Larignon P, Berud F, Girardon K, Dubos B (2006) Nurseries and grapevine wood diseases. *Phytoma*: 14-17
- Larignon P, Fontaine F, Farine S, Clement C, Bertsch C (2009) Esca and Black Dead Arm: two major actors of grapevine trunk diseases *C R Biol* **332**: 765-783
- Larignon P, Coarer M, Girardon K, Berud F, Jacquet O (2009) Propagation of pioneer fungi associated with esca disease by vegetative material in French grapevine nurseries. *Phytopathol Mediterr* **48**: 177-177
- Laveau C, Letouze A, Louvet G, Bastien S, Guerin-Dubrana L (2009) Differential aggressiveness of fungi implicated in esca and associated diseases of grapevine in France. *Phytopathologia Mediterranea* **48**: 32-46
- Lecomte P, Leyo M, Louvet G, Corio-Costet MF, Gaudillere JP, Blancard D (2005) Le Black dead arm, genèse des symptômes - Observations au vignoble en Bordelais et réflexions en lien avec l'esca. *Phytoma* **587**: 29-37
- Lecomte P, Darrietort G, Defives A, Louvet G, Liminana JM, Blancard D (2006) Observations of black dead arm symptoms in Bordeaux vineyards: evolution of foliar symptoms, localization of longitudinal necroses, questions, hypotheses. *Bulletin OILB/SROP* **29**: 93-94
- Lecomte P, Darrietort G, Laveau C, Blancard D, Louvet G, Goutouly JP, Rey P, Guérin-Dubrana L (2011) Impact of biotic and abiotic factors on the development of esca decline disease. "Integrated Protection and Production in Viticulture" *IOBC/wprs Bulletin* **67**, 171-180
- Lecomte, P, Darrietort, G, Liminana, JM, Comont, G, Muruamendiaraz, A, Legorburu, FJ, *et al* (2012) New Insights into Esca of Grapevine: The Development of Foliar Symptoms and Their Association with Xylem Discoloration *Plant Dis* **96**: 924-934
- Lee JP, Lee SW, Kim CS, Son JH, Song JH, Lee KY, *et al.* (2006) Evaluation of formulations of *Bacillus licheniformis* for the biological control of tomato gray mold caused by *Botrytis cinerea*. *Biol Control* **37**: 329-337

- Lee J, Huh N, Hong JH, Kim BS, Kim GH, Kim JJ (2012) The antagonistic properties of *Trichoderma* spp. inhabiting woods for potential biological control of wood-damaging fungi. *Holzforschung* **66**: 883-887
- Lehman LJ, McCoy RJ, Messenger BJ, Manker DC, Orjala JE, Lindhard D, *et al.* (2000) A strain of *Bacillus pumilus* for controlling plant diseases. Patent WO/ 2000/058442
- Lehoczky J (1974) Black dead-arm disease of grapevine caused by *Botryosphaeria stevensii* infection. *Acta Phytopathologica Academiae Scientiarum Hungaricae* **9**: 319-327
- Levadoux L (1956) *Les populations sauvages et cultivées de Vitis vinifera L.*, Vol. 1.
- Li WC, Zhou J, Guo SY, Guo LD (2007) Endophytic fungi associated with lichens in Baihua mountain of Beijing, China. *Fungal Divers* **25**: 69-80
- Li Y, Song Y, Shi G, Wang J, Hou X (2009) Response of antioxidant activity to excess copper in two cultivars of *Brassica campestris* ssp *chinensis* Makino. *Acta Physiologiae Plantarum* **31**: 155-162
- Linaldeddu BT, Maddau L, Franceschini A (2007) Antagonistic activity of endophytic isolates of *Trichoderma* spp, against *Botryosphaeriaceae* associated to cork oak decline. *Micologia Ital* **36**: 22-29
- Lindow SE, Brandl MT (2003) Microbiology of the phyllosphere. *Appl Environ Microbiol* **69**: 1875-1883
- Long HH, Schmidt DD, Baldwin IT (2008) Native Bacterial Endophytes Promote Host Growth in a Species-Specific Manner; Phytohormone Manipulations Do Not Result in Common Growth Responses. *PLoS One* **3**
- Lorito M, Woo SL (1998) *Advances in understanding the antifungal mechanism(s) of Trichoderma and new applications for biological control*, Vol. 21
- Lorito M, Woo SL, Fernandez IG, Colucci G, Harman GE, Pintor-Toro JA, *et al.* (1998) Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. *Proc Natl Acad Sci U S A* **95**: 7860-7865
- Lorrain B, Ky I, Pasquier G, Jourdes M, Dubrana LG, Geny L, Rey P, *et al.* (2012) Effect of Esca disease on the phenolic and sensory attributes of Cabernet Sauvignon grapes, musts and wines. *Aust J Grape Wine Res* **18**: 64-72
- Lozupone C and Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology* **71**: 8228-8235
- Lu ZX, Tombolini R, Woo S, Zeilinger S, Lorito M, Jansson JK (2004) In vivo study of *Trichoderma*-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. *Appl Environ Microbiol* **70**: 3073-3081
- Luini E, Fleurat-Lessard P, Rousseau L, Roblin G, Berjeaud JM (2010) Inhibitory effects of polypeptides secreted by the grapevine pathogens *Phaeoconiella chlamydospora* and

- Phaeoacremonium aleophilum on plant cell activities. *Physiol Mol Plant Pathol* **74**: 403-411
- Luque J, Martos S, Aroca A, Raposo R, Garcia-Figueroes F (2009) Symptoms and fungi associated with declining mature grapevine plants in Northeast Spain. *Journal of Plant Pathology* **91**: 381-390
- Lynch S, Eskalen A, Zambino P, Scott T (2011) Botryosphaeria species complex associated with coast live oak (*Quercus agrifolia*) mortality in Southern California. *Phytopathology* **101**: S111-S111
- Magnin-Robert M, Trotel-Aziz P, Quantinet D, Biagianni S, Aziz A (2007) Biological control of *Botrytis cinerea* by selected grapevine-associated bacteria and stimulation of chitinase and b-1,3 glucanase activities under field conditions. *Eur J Plant Pathol* **118**:43–57
- Magurran AE (2004) *Measuring biological diversity*.: Oxford:Blackwell Science
- Maher N, Piot J, Bastien S, Vallance J, Rey P, Guerin-Dubrana L (2012) Wood necrosis in Esca-affected vines: types, relationships and possible links with foliar symptom expression. *J Inter Sci Vigne Vin* **46**: 15-27
- Makinen H, Hynynen J, Siitonen J, Sievaneni R (2006) Predicting the decomposition of Scots pine, Norway spruce, and birch stems in Finland. *Ecological Applications* **16**: 1865-1879
- Maluta DR, Larignon P (1991) Pied-noir:mieux vaut prévenir. *Viticulture* **11**: 71-72
- Marchi G, Roberti S, D'Ovidio R, Mugnai L, Surico G (2001) Pectic enzymes production by *Phaeoacremonium chlamydospora*. *Phytopathol Mediterr* **40**: S407-S416
- Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bembien LA, *et al.* (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**: 376-380
- Mazzola M (2004) Assessment and management of soil microbial community structure for disease suppression. *Annual Review of Phytopathology* **42**: 35-59
- Marquez SS, Bills GF, Zabalgoeazcoa I (2007) The endophytic mycobiota of the grass *Dactylis glomerata*. *Fung Div* **27**: 171-195
- Marquez SS, Bills GF, Acuna LD, Zabalgoeazcoa I (2010) Endophytic mycobiota of leaves and roots of the grass *Holcus lanatus*. *Fungal Divers* **41**: 115-123
- McLean KL, Hunt JS, Stewart A, Wite D, Porter IJ, Villalta O (2012) Compatibility of a *Trichoderma atroviride* biocontrol agent with management practices of *Allium* crops. *Crop Protec* **33**: 94-100
- Misaghi IJ, Donndelinger CR (1990) Endophytic bacteria in symptom-free cotton plants. *Phytopathology* **80**: 808-811
- Moller WS, Kasimatis AN (1978) Dieback of grapevines caused by *Eutypa armeniacae*. *Plant Disease Reporter* **62**: 254–8

- Moral J, Luque F, Trapero A (2008) First report of *Diplodia seriata*, the anamorph of *Botryosphaeria obtusa*, causing fruit rot of olive in Spain. *Plant Dis* **92**: 311-311
- Mostert L, Crous PW, Petrini O (2000) Endophytic fungi associated with shoots and leaves of *Vitis vinifera*, with specific reference to the *Phomopsis viticola* complex. *Sydowia* **52**: 46-58
- Mostert L, Crous PW, Groenewald JZ, Gams W, Summerbell RC (2003) *Togninia* (Calosphaeriales) is confirmed as teleomorph of *Phaeoacremonium* by means of morphology, sexual compatibility and DNA phylogeny. *Mycologia* **95**: 646-659
- Mostert L, Abeln ECA, Halleen F, Crous PW (2006) Genetic diversity among isolates of *Phaeoconiella chlamydospora* on grapevines. *Austral Plant Pathol* **35**: 453-460
- Mugnai L, Graniti A, Surico G (1999) Esca (Black measles) and brown wood-streaking: Two old and elusive diseases of grapevines. *Plant Dis* **83**: 404-418
- Munkvold GP, Marois JJ (1995) Factors associated with variation in susceptibility of grapevine pruning wounds to infection by *Eutypa lata*. *Phytopathology* **85**: 249-256
- Nascimento T, Rego C, Oliveira H (2007) Potential use of chitosan in the control of grapevine trunk diseases. *Phytopathol Mediterr* **46**: 125-125
- Nie GJ, Reading NS, Aust SD (1999) Relative stability of recombinant versus native peroxidases from *Phanerochaete chrysosporium*. *Archives of Biochemistry and Biophysics* **365**: 328-334
- Nilsson RH, Ryberg M, Abarenkov K, Sjökvist E, Kristiansson E (2009) The ITS region as a target for characterization of fungal communities using emerging sequencing technologies. *FEMS Microbiol Lett* **296**: 97-101
- Nilsson RH, Veldre V, Hartmann M, Unterseher M, Amend A, Bergsten J, *et al.* (2010) An open source software package for automated extraction of ITS1 and ITS2 from fungal ITS sequences for use in high-throughput community assays and molecular ecology. *Fungal Ecology* **3**: 284-287
- Nocker A, Burr M, Camper AK (2007) Genotypic microbial community profiling: A critical technical review. *Microbial Ecology* **54**: 276-289
- Nunes C, Usall J, Teixido N, Fons E, Vinas I (2002) Post-harvest biological control by *Pantoea agglomerans* (CPA-2) on Golden Delicious apples. *J Appl Microbiol* **92**: 247-255
- Nyren P (1987) Enzymatic method for continuous monitoring of DNA-Polymerase activity. *Analytical Biochemistry* **167**: 235-238
- O'Donnell AG, Seasman M, Macrae A, Waite I, Davies JT (2001) Plants and fertilisers as drivers of change in microbial community structure and function in soils. *Plant Soil* **232**: 135-145

- Octave S, Amborabe BE, Luini E, Ferreira T, Fleurat-Lessard P, Roblin G (2005) Antifungal effects of cysteine towards *Eutypa lata*, a pathogen of vineyards. *Plant Physiology and Biochemistry* **43**: 1006-1013
- Paillassa E, Dubos B, Larignon P, Chevrier JC (1992) Infectious inoculum of *Eutypa lata*. Its formation, development and dispersal: contributing factors. *Phytoma*: 21-23
- Pancher M, Ceol M, Corneo PE, Longa CMO, Yousaf S, Pertot I, Campisano A (2012) Fungal Endophytic Communities in Grapevines (*Vitis vinifera* L.) Respond to Crop Management. *Appl Environ Microbiol* **78**: 4308-4317
- Panon, M.L. (2000) les maladies du bois... une bombe à retardement ? *Le Vigneron Champenois* **8**: 7-9
- Parizi TE, Ansari M, Elaminejad T (2012) Evaluation of the potential of *Trichoderma viride* in the control of fungal pathogens of Roselle (*Hibiscus sabdariffa* L) in vitro. *Microb Pathogenesis* **52**: 201-205
- Pascoe I, Cottral E (2000) Developments in grapevine trunk diseases research in Australia. *Phytopathol Mediterr* **39**: 68-75
- Pascoe IG, Edwards J, Cunnington JH, Cottral EH (2004) Detection of the *Togninia* teleomorph of *Phaeoacremonium aleophilum* in Australia. *Phytopathol Mediterr* **43**: 51-58
- Paul B, Chereyathmanjiyil A, Masih I, Chapuis L, Benoit A (1998) Biological control of *Botrytis cinerea* causing grey mould disease of grapevine and elicitation of stilbene phytoalexin (resveratrol) by a soil bacterium. *FEMS Microbiol Lett* **165**: 65-70
- Pennycook SR, Samuels GJ (1985) *Botryosphaeria* and *Fusicoccum* species associated with ripe fruit rot of *Actinidia deliciosa* (Kiwifruit) in New-Zealand. *Mycotaxon* **24**: 445-458
- Peros JP, Berger G (1994) A rapid method to assess the aggressiveness of *Eutypa lata* isolates and the susceptibility of grapevine cultivar to eutypa dieback. *Agronomie* **14**: 515-23
- Peros JP, Jamaux-Despreaux I, Berger G (2000) Population genetics of fungi associated with esca disease in French vineyards. *Phytopathol Mediterr* **39**: 150-155
- Peros JP, Berger G, Jamaux-Despreaux I (2008) Symptoms, wood lesions and fungi associated with esca in organic vineyards in Languedoc-Roussillon (France). *Journal of Phytopathology* **156**: 297-303
- Perveen K, Bokhari NA (2012) Antagonistic activity of *Trichoderma harzianum* and *Trichoderma viride* isolated from soil of date palm field against *Fusarium oxysporum*. *Afr J Microbiol Res* **6**: 3348-3353
- Petrini O (1991) Microbial Ecology of leaves. Springer-Verlag (ed), Fungal endophytes of tree leaves, pp 179-197.

- Petrini O, Fisher PJ (1986) Fungal endophytes in *Salicornia perennis*. *Transactions of the British Mycological Society* **87**: 647-651
- Petzoldt CH, Sall MA, Moller WJ (1983) EUTYPA DIEBACK OF GRAPEVINES - ASCOSPORE DISPERSAL IN CALIFORNIA. *Am J Enol Vitic* **34**: 265-270
- Phillips AJL (2002) Botryosphaeria species associated with diseases of grapevines in Portugal. *Phytopathol Mediterr* **41**: 3-18
- Phillips AJL, Fonseca F, Pova V, Castilho R, Nolasco G (2002) A reassessment of the anamorphic fungus *Fusicoccum luteum* and description of its teleomorph *Botryosphaeria lutea* sp nov. *Sydowia* **54**: 59-77
- Photita W, Lumyong S, Lumyong P, McKenzie EHC, Hyde KD (2004) Are some endophytes of *Musa acuminata* latent pathogens? *Fungal Divers* **16**: 131-140
- Pilotti M, Gervasi F, Brunetti A (2005) Molecular identification of *Fomitiporia mediterranea* and *Eutypa lata*/Libertella blepharis in *Platanus x acerifolia*. *J Phytopathol* **153**: 193-202
- Pilotti M, Tizzani L, Brunetti A, Gervasi F, Di Lernia G, Lumia V (2010) Molecular identification of *Fomitiporia mediterranea* on declining and decayed Hazelnut. *Journal of Plant Pathology* **92**: 115-129
- Pitt WM, Sosnowski MR, Taylor A, Huang R, Quirk L, Hackett S, Somers A, Steel CC, Savocchia S (2010) Management of Botryosphaeria canker of grapevines. *Australian Viticulture - Practical Vineyard Management* **14**: 52-56
- Planchon JE (1887) Monographie des Ampélidées vraies. *Monographia Phanerogamerum* **5**: 305-364
- Pouska V, Svoboda M, Lepsova A (2010) The diversity of wood-decaying fungi in relation to changing site conditions in an old-growth mountain spruce forest, Central Europe. *Eur J For Res* **129**: 219-231
- Promptutha I, Lumyong S, Dhanasekaran V, McKenzie EHC, Hyde KD, Jeewon R (2007) A phylogenetic evaluation of whether endophytes become saprotrophs at host senescence. *Microb Ecol* **53**: 579-590
- Prosser JI (2002) Molecular and functional diversity in soil micro-organisms. *Plant Soil* **244**: 9-17
- QuadtHallmann A, Hallmann J, Kloepper JW (1997a) Bacterial endophytes in cotton: Location and interaction with other plant associated bacteria. *Can J Microbiol* **43**: 254-259
- QuadtHallmann A, Benhamou N, Kloepper JW (1997b) Bacterial endophytes in cotton: Mechanisms of entering the plant. *Can J Microbiol* **43**: 577-582
- Ramos DE, Moller WJ, English H (1975) Production and dispersal of ascospores of *Eutypa armeniaceae* in California. *Phytopathology* **65**: 1364-1371

- Rastogi G, Sbodio A, Tech JJ, Suslow TV, Coaker GL, Leveau JHJ (2012) Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. *Isme Journal* **6**: 1812-1822
- Redman RS, Sheehan KB, Stout RG, Rodriguez RJ, Henson JM (2002) Thermotolerance generated by plant/fungal symbiosis. *Science* **298**: 1581-1581
- Reglinski T, Rodenburg N, Taylor JT, Northcott GL, Chee AA, Spiers TM, *et al* (2012) *Trichoderma atroviride* promotes growth and enhances systemic resistance to *Diplodia pinea* in radiata pine (*Pinus radiata*) seedlings. *Forest Pathol* **42**: 75-78
- Rego C, Oliveira H, Carvalho A, Phillips A (2000) Involvement of *Phaeoacremonium* spp. and *Cylindrocarpon destructans* with grapevine decline in Portugal. *Phytopathol Mediterr* **39**: 76-79
- Rego C, Farropas L, Nascimento T, Cabral A, Oliveira H (2005) Black foot of grapevine: sensitivity of *Cylindrocarpon destructans* to fungicides. *Phytopathol Mediterr* **44**: 118-119
- Rego C, Vaz A, Nascimento T, Cabral A, Oliveira H (2009) Diseases incited by Botryosphaeriaceae fungi in Portuguese vineyards. *Phytopathologia Mediterranea* **48**: 181-181
- Reisenzein H, Berger N, Nieder G (2000) Esca in Austria. *Phytopathologia Mediterranea* **39**, 26-34
- Ren H, Lu LM, Soejima A, Luke Q, Zhang DX, Chen ZD, Wen J (2011) Phylogenetic analysis of the grape family (Vitaceae) based on the noncoding plastid trnC-petN, trnH-psbA, and trnL-F sequences. *Taxon* **60**: 629-637
- Retief E, McLeod A, Fourie PH (2006) Potential inoculum sources of *Phaeomoniella chlamydospora* in South African grapevine nurseries. *Eur J Plant Pathol* **115**: 331-339
- Reynier A (2003) *Viticulture manual*.
- Romanazzi G, Murolo S, Pizzichini L, Nardi S (2006) First investigations on Esca disease of grapevine in Marche region. Università di Bologna
- Rolshausen PE, Gubler WD (2005) Use of boron for the control of *Eutypa* dieback of grapevines. *Plant Dis* **89**: 734-738
- Rolshausen PE, Urbez-Torres JR, Rooney-Latham S, Eskalen A, Smith RJ, Gubler WD (2010) Evaluation of Pruning Wound Susceptibility and Protection Against Fungi Associated with Grapevine Trunk Diseases. *Am J Enol Vitic* **61**: 113-119
- Romanazzi G, Murolo S, Pizzichini L, Nardi S (2009) Esca in young and mature vineyards, and molecular diagnosis of the associated fungi. *Eur J Plant Pathol* **125**: 277-290
- Ronaghi M, Karamohamed S, Pettersson B, Uhlen M, Nyren P (1996) Real-time DNA sequencing using detection of pyrophosphate release. *Analytical Biochemistry* **242**: 84-89

- Ronaghi M, Uhlen M, Nyren P (1998) A sequencing method based on real-time pyrophosphate. *Science* **281**: 363-370
- Ronaghi M, Nygren M, Lundeberg J, Nyren P (1999) Analyses of secondary structures in DNA by pyrosequencing. *Anal Biochem* **267**: 65-71
- Rooney-Latham S, Eskalen A, Gubler WD (2005) Occurrence of *Togninia minima* perithecia in esca-affected vineyards in California. *Plant Dis* **89**: 867-871
- Ross N, Villemur R, Marcandella E, Deschenes L (2001) Assessment of changes in biodiversity when a community of ultramicrobacteria isolated from groundwater is stimulated to form a biofilm. *Microbial Ecology* **42**: 56-68
- Rovesti L, Montermini A (1987) A grapevine decline caused by *Sphaeropsis malorum* widespread in the province of Reggio Emilia. *Informatore Fitopatologico* **37**: 59-61
- Rumbos IC (1986) Isolation and identification of *Eutypa lata* from *Pistacia vera* in Greece. *J Phytopathol-Phytopathol Z* **116**: 352-357
- Rumbos IC (1993) Dieback symptoms on olive trees caused by the fungus *Eutypa lata*. *Bulletin OEPP* **23**: 441-445
- Rumbos I, Rumbou A (2001) Fungi associated with esca and young grapevine decline in Greece *Phytopathol Mediterr* **40**: S330-S335
- Rungjindamai N, Pinruan U, Choeyklin R, Hattori T, Jones EBG (2008) Molecular characterization of basidiomycetous endophytes isolated from leaves, rachis and petioles of the oil palm, *Elaeis guineensis*, in Thailand. *Fungal Divers* **33**: 139-161
- Saikkonen K, Saari S, Helander M (2010) Defensive mutualism between plants and endophytic fungi? *Fungal Divers* **41**: 101-113
- Sakalidis ML, Hardy GES, Burgess TI (2011) Endophytes as potential pathogens of the baobab species *Adansonia gregorii*: a focus on the Botryosphaeriaceae. *Fungal Ecol* **4**: 1-14
- Sakayaroj J, Preedanon S, Supaphon O, Jones EBG, Phongpaichit S (2010) Phylogenetic diversity of endophyte assemblages associated with the tropical seagrass *Enhalus acoroides* in Thailand. *Fungal Divers* **42**: 27-45
- Sasaki M, Kaneko Y, Oshita K, Takamatsu H, Asao Y, Yokotsuka T (1970) Studies on the compounds produced by moulds. VII. Isolation of isocoumarin compounds. *Agricultural and Biological Chemistry* **34**: 1296-1300
- Scheck H, Vasquez S, Fogle D, Gubler WD (1998) Grape growers report losses to black-foot and grapevine decline. *California Agriculture* **52**: 19-23
- Scheck HJ, Vasquez SJ, Gubler WD, Fogle D (1998) First report of three *Phaeoacremonium* spp causing young grapevine decline in California. *Plant Dis* **82**: 590-590

- Schmidt CS, Lorenz D, Wolf GA (2001) Biological control of the grapevine dieback fungus *Eutypa lata* I: Screening of bacterial antagonists. *J Phytopathol-Phytopathol Z* **149**: 427-435
- Schmidt CS, Lorenz D, Wolf GA, Jager J (2001) Biological control of the grapevine dieback fungus *Eutypa lata* II: Influence of formulation additives and transposon mutagenesis on the antagonistic activity of *Bacillus subtilis* and *Erwinia herbicola*. *J Phytopathol-Phytopathol Z* **149**: 437-445
- Schmiedeknecht G, Issoufou I, Junge H, Bochow H (2001) Use of *Bacillus subtilis* as biocontrol agent. V. Biological control of diseases on maize and sunflowers. *Z Pflanzenk Pflanzens-J Plant Dis Prot* **108**: 500-512
- Schubert M, Fink S, Schwarze FW (2008) Evaluation of *Trichoderma* spp. as a biocontrol agent against wood decay fungi in urban trees. *Biological Control* **45**: 111-123
- Schulz B, Boyle C (2005) The endophytic continuum. *Mycol Res* **109**: 661-686
- Schwarze F, Jauss F, Spencer C, Hallam C, Schubert M (2012) Evaluation of an antagonistic *Trichoderma* strain for reducing the rate of wood decomposition by the white rot fungus *Phellinus noxius*. *Biol Control* **61**: 160-168
- Serra S, Borgo M, Zanzotto A (2000) Investigation into the presence of fungi associated with esca of young vines. *Phytopathol Mediterr* **39**: 21-25
- Seena S, Wynberg N, Barlocher F (2008) Fungal diversity during leaf decomposition in a stream assessed through clone libraries. *Fungal Divers* **30**: 1-14
- Shade A, Read JS, Youngblut ND, Fierer N, Knight R, Kratz TK, Lottig NR, Roden EE, Stanley EH, Stombaugh J, Whitaker RJ, Wu CH, McMahon KD (2012) Lake microbial communities are resilient after a whole-ecosystem disturbance. *Isme Journal* **6**: 2153-2167
- Shannon, CE, and Weaver, W (1963) *The Mathematical Theory of Communication* University of Illinois Press
- Shendure J, Ji H (2008) Next-generation DNA sequencing. *Nature Biotechnology* **26**: 1135-1145
- Shoemaker RA (1964) Conidial state of some *Botryosphaeria* species on *Vitis* and *Quercus*. *Canadian Journal of Botany* **42**: 1297-1301
- Siebert JB (2001) *Eutypa*: the economic toll on vineyards. *Wines Vines*, April, 50-56
- Slippers B, Fourie G, Crous PW, Coutinho TA, Wingfield BD, Wingfield MJ (2004) Multiple gene sequences delimit *Botryosphaeria australis* sp nov from *B-lutea*. *Mycologia* **96**: 1030-1041
- Slippers B, Wingfield MJ (2007) Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. *Fungal Biology Reviews* **21**: 90-106

- Smith H, Wingfield MJ, Petrini O (1996) Botryosphaeria dothidea endophytic in Eucalyptus grandis and Eucalyptus nitens in South Africa. *Forest Ecology and Management* **89**: 189-195
- Sosnowski MR, Shtienberg D, Creaser ML, Wicks TJ, Lardner R, Scott ES (2007) The influence of climate on foliar symptoms of eutypa dieback in grapevines. *Phytopathology* **97**: 1284-1289
- Sosnowski MR, Creaser ML, Wicks TJ, Lardner R, Scott ES (2008) Protection of grapevine pruning wounds from infection by Eutypa lata. *Australian Journal of Grape and Wine Research* **14**: 134-142
- Sparapano L, Bruno G, Graniti A (2000) Effects on plants of metabolites produced in culture by Phaeoacremonium chlamydosporum, P. aleophilum and Fomitiporia punctata. *Phytopathol Mediterr* **39**: 169-177
- Sparapano L, Bruno G, Ciccarone C, Graniti A (2000) Infection of grapevines by some fungi associated with esca I Fomitiporia punctata as a wood-rot inducer. *Phytopathol Mediterr* **39**: 46-52
- Sparapano L, Bruno G, Graniti A (2001) Three-year observation of grapevines cross-inoculated with esca-associated fungi, *Phytopathologia Mediterranea* **40**: 376–386
- Stone JK, Polishook JD, White JF (2004) Endophytic fungi In: Mueller GM, Bills GF, White, JF (Eds) Biodiversity of fungi Elsevier, Amsterdam: pp 241-270
- Stoltzfus JR, So R, Malarvithi PP, Ladha JK, deBruijn FJ (1997) Isolation of endophytic bacteria from rice and assessment of their potential for supplying rice with biologically fixed nitrogen. *Plant Soil* **194**: 25-36
- Sun L, Qiu FB, Zhang XX, Dai X, Dong XZ, Song W (2008) Endophytic bacterial diversity in rice (Oryza sativa L.) roots estimated by 16S rDNA sequence analysis. *Microb Ecol* **55**: 415-424
- Sun FF, Kuang YW, Wen DZ, Xu ZH, Li JL, Zuo WD, Hou EQ (2010) Long-term tree growth rate, water use efficiency, and tree ring nitrogen isotope composition of Pinus massoniana L. in response to global climate change and local nitrogen deposition in Southern China. *J Soils Sediments* **10**: 1453-1465
- Surico G, Marchi G, Braccini P, Mugnai L (2000) Epidemiology of esca in some vineyards in Tuscany (Italy). *Phytopathologia Mediterranea* **39**: 190-205
- Surico G, Bandinelli R, Braccini P, Di Marco S, Marchi G, Mugnai L, Parrini C (2004) On the factors that may have influenced the esca epidemic in Tuscany in the eighties. *Phytopathologia Mediterranea* **43**: 136-143
- Surico, G, Mugnai, L, and Marchi, G (2006) Older and more recent observations on esca: a critical review *Phytopathol Mediterr* **45**: S68-S86

- Surico G, Mugnai L, Marchi G (2008) The esca disease complex. *In*: Ciancio A., Mukerji K.G., eds. *Integrated management of diseases caused by fungi, phytoplasma and bacteria*. Dordrecht, Netherlands: Springer Science Business Media B.V, 119–36
- Surico G (2009) Towards a redefinition of the diseases within the esca complex of grapevine. *Phytopathologia Mediterranea* **48**: 5-10
- Suryanarayanan TS, Venkatachalam A, Rajulu MBG (2011) A comparison of endophyte assemblages in transgenic and non-transgenic cotton plant tissues. *Curr Sci* **101**: 1472-1474
- Tabacchi R, Fkyerat A, Poliard C, Dubin GM (2000) Phytotoxins from fungi of esca of grapevine. *Phytopathol Mediterr* **39**: 156-161
- Tao G, Liu ZY, Hyde KD, Liu XZ, Yu ZN (2008) Whole rDNA analysis reveals novel and endophytic fungi in *Bletilla ochracea* (Orchidaceae). *Fungal Divers* **33**: 101-122
- Tegli S, Santilli E, Bertelli E, Surico G (2000) Genetic variation within *Phaeoacremonium aleophilum* and *P. chlamydosporum* in Italy. *Phytopathol Mediterr* **39**: 125-133
- Tervet JW, Hollis JP (1948) Bacteria in the storage organs of healthy plants. *Phytopathology* **38**: 960-967
- Thorne ET, Young BM, Young GM, Stevenson JF, Labavitch JM, Matthews MA, et al. (2006) The structure of xylem vessels in grapevine (vitaceae) and a possible passive mechanism for the systemic spread of bacterial disease. *Am J Bot* **93**: 497-504
- Tiedemann S, Brendel G, Fehrmann H (1988) Investigations on endophytic fungi of grapevine with special emphasis on the vascular system of rootstocks *J Phytopathol* **122**: 147-165
- Toure Y, Ongena M, Jacques P, Guiro A, Thonart P (2004) Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. *J Appl Microbiol* **96**: 1151-1160
- Travadon R, Baumgartner K, Rolshausen PE, Gubler WD, Sosnowski MR, Lecomte P, Halleen F, Peros JP (2012) Genetic structure of the fungal grapevine pathogen *Eutypa lata* from four continents. *Plant Pathol* **61**: 85-95
- Trivedi P, Spann T, Wang NA (2011) Isolation and Characterization of Beneficial Bacteria Associated with Citrus Roots in Florida. *Microb Ecol* **62**: 324-336
- Trotel-Aziz P, Aziz A, Magnin-Robert M, Aït Barka E, Gognies S (2006) Bactéries présentant une activité protectrice de la vigne contre *Botrytis cinerea*. French patent 06.06.513
- Trotel-Aziz P, Couderchet M, Biagianti S, Aziz A (2008) Characterization of new bacterial biocontrol agents *Acinetobacter*, *Bacillus*, *Pantoea* and *Pseudomonas* spp. mediating grapevine resistance against *Botrytis cinerea*. *Environ Exp Bot* **64**:21–32
- Trouillas FP, Gubler WD (2010) Pathogenicity of Diatrypaceae species in grapevines in

California. *Plant Disease* **94**: 867-72

Trouillas F, Úrbez-Torres JR, Gubler WD (2010) Diversity of diatrypaceous fungi associated with grapevine canker diseases in California. *Mycologia* **102**: 319-36

Tsujiyama S, Minami M (2005) Production of phenol-oxidizing enzymes in the interaction between white-rot fungi. *Mycoscience* **46**: 268-271

Tuao Gava CA, Leal Menezes ME (2012) Efficiency of *Trichoderma* sp isolates on the control of soil-borne pathogens in the yellow melon. *Revista Ciencia Agronomica* **43**: 633-640

Úrbez-Torres JR, Leavitt GM, Guerrero JC, Lugo JG, Gubler WD (2006) Botryosphaeria canker disease of grapevines caused by *Botryosphaeria rhodina* and *B. obtusa* in Mexico. *Phytopathology* **96**: S117-S117

Úrbez-Torres JR, Leavitt GM, Voegel TM, Gubler WD (2006) Identification and distribution of *Botryosphaeria* spp. associated with grapevine cankers in California. *Plant Dis* **90**: 1490-1503

Úrbez-Torres JR, Leavitt GM, Guerrero JC, Guevara J, Gubler WD (2008) Identification and pathogenicity of *Lasiodiplodia theobromae* and *Diplodia seriata*, the causal agents of bot canker disease of grapevines in Mexico. *Plant Disease* **92**: 519-529

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

Úrbez-Torres JR, Bruez E, Hurtado J, Gubler WD (2010) Effect of Temperature on Conidial Germination of Botryosphaeriaceae Species Infecting Grapevines. *Plant Dis* **94**: 1476-1484

Úrbez-Torres JR (2011) The status of *Botryosphaeriaceae* species infecting grapevines. *Phytopathologia Mediterranea* **5**: 5-45

Valaskova V, Snajdr J, Gunnewiek PK, Boer Wd, Baldrian P (2009) Saprotrophic basidiomycete *Hypholoma fasciculare* affects fungal and bacterial community composition in colonized wood and soil. *IOBC/WPRS Bulletin* **42**: 173-176

Vallance J, Le Floch G, Deniel F, Barbier G, Levesque CA, Rey P (2009) Influence of *Pythium oligandrum* biocontrol on fungal and oomycete population dynamics in the rhizosphere. *Appl Environ Microbiol* **75**: 4790-4800

Van Niekerk JMFPHH, F.; Crous P.W. (2006) *Botryosphaeria* spp. as grapevine trunk disease pathogens. *Phytopathol Mediterr* **45**: S43-S54

Verhagen BWM, Trotel-Aziz P, Jeandet P, Baillieul F, Aziz A (2011) Improved resistance against *Botrytis cinerea* by grapevine-associated bacteria that induce a prime oxidative burst and phytoalexin Production. *Phytopathology* **101**: 768-777

Viala P (1926) Recherches sur els maladies de la vigne Esca *Annal Epiphyt* fasc **1 et 2**:1-108

- Vignes V, Yobregat O, Barthélémy B, Dias F, Coarer M, Girardon K, *et al.* (2010) Wood decay diseases: tests of disinfection methods in French nursery. *Phytopathol Mediterr* **49**: 130-131
- Vizitiu D, Dejeu L (2011) Crown gall (*Agrobacterium* spp.) and grapevine. *Journal of Horticulture, Forestry and Biotechnology* **15**: 130-138
- Waite H, Morton L (2007) Hot water treatment, trunk diseases and other critical factors in the production of high-quality grapevine planting material. *Phytopathol Mediterr* **46**: 5-17
- Walla JA (1984) Incidence of *Phellinus punctatus* on living woody-plants in North Dakota. *Plant Dis* **68**: 252-253
- Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, *et al.* (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proc Natl Acad Sci U S A* **102**: 13386-13391
- Wang F, Zhao LN, Li GH, Huang JB, Hsiang T (2011) Identification and Characterization of *Botryosphaeria* spp Causing Gummosis of Peach Trees in Hubei Province, Central China. *Plant Dis* **95**: 1378-1384
- Weber EA, Trouillas FP, Gubler WD (2007) Double pruning of grapevines: A cultural practice to reduce infections by *Eutypa lata*. *Am J Enol Vitic* **58**: 61-66
- Wenneker M, Vink P, Van Bruggen AS (2006) *Eutypa dieback* as an important (new) disease in red currant (*Ribes rubrum*) in The Netherlands. *Communications in agricultural and applied biological sciences* **71**: 881-885
- West ER, Cothier EJ, Steel CC, Ash GJ (2010) The characterization and diversity of bacterial endophytes of grapevine. *Canadian Journal of Microbiology* **56**: 209-216
- White T, Birns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Academic Press, San Diego
- White NA, Boddy L (1992) Extracellular enzyme localization during interspecific fungal interactions. *Fems Microbiology Letters* **98**: 75-79
- White CL, Halleen F, Mostert L (2011) Symptoms and fungi associated with esca in South African vineyards *Phytopathol Mediterr* **50**: 236-246
- Whitelaw-Weckert MA, Nair NG, Lamont R, Alonso M, Priest MJ, Huang R (2007) Root infection of *Vitis vinifera* by *Cylindrocarpum liriodendri* in Australia. *Austral Plant Pathol* **36**: 403-406
- Whiteman SA, Jaspers M, Stewart A, Ridgway JJ (2004) *Phaeoemoniella chlamydospora* detection in the grapevine propagation process by species-specific PCR. *Phytopathol Mediterr* **43**: 156

- Whiteman SA, Jaspers M, Stewart A, Ridgway JJ (2005) Infested soil as a source of inoculum for *Phaeoconiella chlamydospora*, causal agent of Petri disease. *Phytopathol Mediterr* **44**: 105-106
- Whittaker RM (1960) Vegetation of the Siskiyou Mountains, Oregon and California. *Ecological Monographs*. **30**: 279-338
- Wicks T, Davies K (1999) The effect of *Eutypa* on grapevine yield. *Australian Grapegrower Winemaker* **426a**: 15-16
- Wilson D (1995) Endophyte: the evolution of a term, and clarification of its use and definition. *Oikos* **73**: 274-276
- Zhang HB, Yang MX, Tu R (2008) Unexpectedly high bacterial diversity in decaying wood of a conifer as revealed by a molecular method. *International Biodeterioration & Biodegradation* **62**: 471-474
- Zhang T, Shao M-F, Ye L (2012) 454 Pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. *Isme Journal* **6**: 1137-1147
- Zhou DQ, Hyde KD (2001) Host-specificity, host-exclusivity, and host-recurrence in saprobic fungi. *Mycol Res* **105**: 1449-145
- Zinniel DK, Lambrecht P, Harris NB, Feng Z, Kuczarski D, Higley P, *et al.* (2002) Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Appl Environ Microbiol* **68**: 2198-2208

CURRICULUM VITAE – EMILIE BRUEZ

Publications internationales à comité de lecture

Úrbez-Torres J.R., **Bruez E.**, Hurtado J., Gubler W. D. 2010. Effect of temperature on conidial germination of Botryosphaeriaceae species infecting grapevines. *Plant Disease* 94:1476-1484.

Bruez E., Lecomte P., Grosman J., Doublet B., Bertsch C., Fontaine F., Ugaglia A., Teisseidre P-L., Guerin-Dubrana L., Rey P. Overview of the grapevine trunk diseases in France in the early 2000s. *Phytopathologia mediterranea (revised version)*.

Bruez E., Vallance J., Gerbore J., Lecomte P., Guerin-Dubrana L., Rey P. The complex relationship of fungi with esca. Analyses of the fungal communities colonizing the wood tissues of leaf-symptomatic and asymptomatic vines. *Submitted to Fungal diversity*.

Bruez E., Vallance J., Tidjani Alou M., Rey P. Specific bacterial microflora are associated with grapevine asymptomatic and esca-symptomatic wood tissues. *Submitted to Applied and Environmental Microbiology*.

Bruez E., Bastien S., Guerin-Dubrana L., Rey P. Old *Vitis vinifera*: when pathogenic fungal patterns of grapevine trunk diseases are not always associated with foliar disease expression or plant decline. *Submitted to Phytopathology*.

Bruez E., Mazet F., Guerin-Dubrana L., Bertsch C., Rey P.. Pathogenic and beneficial bacteria colonizing *Vitis vinifera*. A review. *In preparation*

Actes de congrès

Bruez E., Vallance J., Gerbore J., Lecomte P., Guerin-Dubrana L., Rey P. 2011. Characterization of endophytic microflora colonizing wood tissues of healthy and Esca-diseased vines. *APS-IPPC Joint Meeting* 2011, August 6-10. Honolulu, Hawaiï, USA. *Phytopathology*, vol. 101, No 101:S21.

Gerbore J., **Bruez E.**, Vallance J., Massot M., Grizard D., Regnault-Roger C., Rey P. 2011. Characterization of biocontrol strains of *Pythium oligandrum* and control of an Esca pathogenic fungus attack. *APS-IPPC Joint Meeting* 2011, August 6-10. Honolulu, Hawaiï, USA. *Phytopathology*, vol. 101, No. 101:S21.

Gerbore J., **Bruez E.**, Vallance J., Grizard D., Regnault-Roger C. and Rey P. 2011. Protection against a vine trunk attack by *Phaeomoniella chlamydospora* is concomitant with root colonization by the oomycete, *Pythium oligandrum*. In: Working Group « Multitrophic Interactions in Soil », Landa, B. B., Navas-Cortés, J. A., Montes-Borrego, M., Steinberg C. (eds), *IOBC wprs Bulletin* vol. 71, 31-35.

Communications lors de congrès (communications orales, posters)

Gerbore J., **Bruez E.**, Vallance J., Grizard D., Regnault-Roger C. and Rey P. 2011. Protection against a vine trunk attack by *Phaeomoniella chlamydospora* is concomitant with root colonization by the oomycete, *Pythium oligandrum*. *IOBC/WPRS Working Group “Multitrophic interactions in soil”*, Cordoba, Spain, 4-7 April 2011 (Poster).

Bruez E., Vallance J., Gerbore J., Lecomte P., Guerin-Dubrana L. and Rey P. 2011. Characterization of fungal and bacterial communities that colonise the various wood tissues of healthy and esca-diseased vines. *IOBC/WPRS Working Group on “Integrated Protection and Production in Viticulture”*, Lacanau, France, 2-5 October 2011 (Communication orale).

Rey P., **Bruez E.**, Vallance J., Lecomte P., Guerin-Dubrana L., Laval V., Gautier A. and Lebrun M.H. 2011. Characterization of fungal and bacterial communities colonizing the wood tissues of healthy and Esca-diseased vines. *Workshop on wood-canker diseases*, University California Davis, Viticulture & Enology Experimental Station, 1389 Oakville Grade Road, Oakville, CA, USA, 17 – 19 october 2011 (*Communication orale*).

Brigitte L., Amselem J., Lapalu N., Gautier A., Vallance J., **Bruez E.**, Rey P., Quesneville H., Laval V. and Lebrun M.-H. 2012. Analyse bioinformatique des données de métagénomique fongique. In : *Journées Jean Chevaugéon 2012. 9^{ème} Rencontres de Phytopathologie-Mycologie de la Société Française de Phytopathologie (SFP)*, 16 au 20 janvier 2012, Aussois, France (*Communication orale*).

Bruez E., Vallance J., Gerbore J., Lecomte P., Guerin- Dubrana L. and Rey P. 2012. Endophytic microflora of wood tissues of healthy and trunk diseased- grapevines. *8th International Workshop on Grapevine Trunk Diseases*. Valencia, Spain, 18-21 June 2012. (*Communication orale*).

Gautier A., Brigitte L., Vallance J., **Bruez E.**, Lapalu N., Amselem J., Laval V., Rey P. and Lebrun M.-H. 2012. Métagénomique de la microflore du bois de la vigne. In : *Journées Jean Chevaugéon 2012. 9^{ème} Rencontres de Phytopathologie-Mycologie de la Société Française de Phytopathologie*, 16 au 20 janvier 2012, Aussois, France (*Poster*).

Gerbore J., Magnin N., **Bruez E.**, Rouxel M., Grizard D., Regnault-Roger C. and Rey P. 2012. Analysis of the plant pathogenic resistance induced in vines after the root colonization by *Pythium oligandrum*. *MACROWINE 2012 “Marcovision of viticulture wine marking and markets”*. 18-21 June, Bordeaux, France (*Poster*).

Gerbore J., Magnin N., **Bruez E.**, Vallance J., Grizard D., Regnault-Roger C. et Rey P. 2012. Utilisation de *Pythium oligandrum* pour lutter contre *Phaeomoniella chlamydospora*, un agent pathogène impliqué dans l’Esca, une maladie du bois de la vigne. *8^{ème} colloque de la Société Française de Phytopathologie*, 5 au 8 juin, Paris, France. (*Communication orale*).

Gerbore J., Magnin N., **Bruez E.**, Vallance J., Grizard D., Regnault-Roger C. and Rey P. 2012. Control of a fungus involved in grapevine trunk disease (Esca) by using the oomycete, *Pythium oligandrum*: analysis of the induced resistance. *IOBC/WPRS Working Group on “Biocontrol of plant pathogens in sustainable agriculture”*. 24-27 June, Reims, France. (*Communication orale*).

Lapalu N., Gautier A., Brigitte B., Vallance J., **Bruez E.**, Amselem J., Quesneville H., Laval V., Lebrun M.-H. and Rey P. 2013. Analysis of fungal communities associated with grapevine wood diseases, based on fungal ITS pyrosequencing. *The 27th Fungal Genetics Conference*, March 12-17, 2013, Pacific Grove, California, USA (*Poster*).