UNIVERSITÉ
DE REIMS
CHAMPAGNE-ARDENNE

Universidade de Aveiro Departamento de Biologia
Ano 2017

Cátia Carvalho Pinto

VineMicrobiome: uma análise aprofundada do microbioma natural da vinha (Vitis vinifera L.)

VineMicrobiome: a deep analysis of the natural microbial community of Vitis vinifera L.

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada em regime de cotutela com a Universidade de Reims Champagne- Ardenne sob a orientação científica da Doutora Gabriela Moura, Professora Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro, da Doutora Ana Catarina Gomes do Instituto de Investigação Interdisciplinar e do Centro de Neurociências e Biologia Celular da Universidade de Coimbra e da Professora Florence Fontaine do Laboratório de Stress, Defesa e Reprodução de Plantas da Universidade de Reims Champagne-Ardenne.

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To my family

o júri

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palavras-chave Microbioma da vinha, doenças do lenho (GTDs), microrganismos benéficos, Fito_S127B, Fito_F278, colonização da vinha, mecanismos de defesa da planta, proteção da vinha.

resumo

A vinha, *Vitis vinifera* L., abriga naturalmente um ecossistema microbiano complexo ou microbioma, tais como microrganismos neutros, fitopatogenos ou benéficos. Entre os fitopatogenos, aqueles implicados nas doenças do lenho da videira (GTDs) são responsáveis pelas doenças mais destrutivas, para o qual não existem tratamentos altamente eficazes. Por outro lado, os microrganismos benéficos (BCAs) podem desempenhar papéis específicos na proteção das plantas contra estes agentes. Neste sentido, o atual desafio consiste em compreender como estes BCAs interagem com a planta e qual o seu potencial biotecnológico para o desenvolvimento de novas estratégias de proteção da planta. Neste contexto, o objetivo deste estudo visou primeiramente analisar profundamente as comunidades microbianas associadas com a videira ao longo do seu ciclo de crescimento até à fermentação do vinho e, em seguida, compreender as interações entre vinha-BCAs-GTDs. Para isso, dois potenciais BCAs isolados da videira foram testados contra diferentes espécies de Botryosphaeriaceae e, em seguida, caracterizados relativamente ao seu potencial de colonização, de indução dos mecanismos de defesa da planta, na presença ou não do fitopatogeno (*D. seriata* F98.1), e análise do respetivo genoma.

Os resultados demonstraram que o microbioma da videira é altamente dinâmico ao longo do ciclo de crescimento da planta. Como esperado, a biodiversidade microbiana é maior nos solos, e estas comunidades diferem significativamente daquelas presentes nas folhas e mostos vínicos. Contudo, alguns destes microrganismos são partilhados, o que sugere a existência de um microbioma comum. Diferentes isolados foram obtidos, pertencendo na sua maioria ao género *Bacillus, Streptomyces* e *Aureobasidium.* A videira é naturalmente colonizada por microrganismos com potencial antagonista de várias espécies de Botryosphaeriaceae. Entre estes, destacam-se os isolados *Streptomyces* sp. Fito_S127B e *A. pullulans* Fito_F278, que foram selecionados como potenciais BCAs. Estes microrganismos produzem diferentes enzimas extracelulares importantes para as atividades de controlo biológico e são capazes de colonizar com sucesso a videira: Fito_S127B coloniza a rizosfera, enquanto que Fito_F278 coloniza desde as raízes até às folhas. A inoculação artificial da videira com *D. seriata* F98.1 mostrou que o comprimento das lesões necróticas causadas pelo fitopatogeno são significativamente reduzidas na presença de Fito_S127B. Em contrapartida, a espécie Fito_F278 foi menos eficaz. Estes BCAs ativaram algumas respostas de defesa específicas da videira, o que permitiu uma resposta mais rápida e sólida da planta contra o agente fitopatogénico. A análise do genoma destes microrganismos permitiu averiguar diferentes genes que codificam compostos bioativos altamente importantes para o controlo biológico.

De uma forma geral, este estudo abrange novos conhecimentos relativos à estrutura das comunidades microbianas associadas à videira e às suas interações. Para além disso, destaca que a videira ostenta naturalmente microrganismos com um controlo biológico promissor e que estes podem promover respostas de defesa importantes na planta. Neste sentido, estes resultados permitem não só uma melhor compreensão das interações da videira-BCAs-GTDs, mas também representam um forte contributo e avanço para o desenvolvimento de novas estratégias da gestão da vinha, tais como as doenças do lenho.

keywords

Grapevine microbiome, GTDs, beneficial microorganisms, Fito_S127B, Fito_F278, grapevine colonisation, plant defence mechanisms, grapevine protection.

abstract

Vitis vinifera L. is a widely cultivated fruit crop, that naturally harbours a complex microbial ecosystem or plant microbiome, such as neutral, phytopathogenic or beneficial microorganisms. Among phytopathogens, those implied in Grapevine Trunk Diseases (GTDs) are responsible for the most destructive diseases worldwide, and currently no highly effective treatments are available. Beneficial microorganisms (BCAs) may play specific roles on plant protection against phytopathogens though, the present challenge is to understand how such BCAs interact with plant and their biotechnological potential for development of innovation strategies. In this context, the aim of this study was firstly to unveil the microbial communities associated with grapevine along its growth cycle until wine fermentation and, secondly, to better understand the grapevine – BCAs – GTDs interactions. Two potential BCAs isolated from grapevine were tested against Botryosphaeriaceae species and then deep characterized, namely for their colonisation potential, induction of defence mechanisms in grapevine, in the presence or not of *D. seriata* F98.1, and their genome analysis.

Results showed that grapevine microbiome was very dynamic along the growth cycle. As expected, the microbial biodiversity was higher in soils, and these microbial communities differed significantly from those of leaves and wine musts. A proportion of microbial communities was shared within these structures, suggesting the existence of a core microbiome. Several isolates were then obtained from grapevine which mostly belonged to *Bacillus*, *Streptomyces* and *Aureobasidium* genera. Some of them significantly decreased in vitro the mycelium growth of several Botryosphaeriaceae species, such as *Streptomyces* sp. Fito_S127B and *A. pullulans* Fito_F278 which were highly effective and thus selected as potential BCAs. These strains showed to produce a high range of extracellular enzymes with biocontrol value, and were able to successfully colonize grapevine: Fito_S127B was an epiphyte from rhizosphere, while Fito_F278 colonised grapevine from roots to leaves. The artificial inoculation of green stems with *D. seriata* F98.1 on cutting plants showed that the necrotic lesions length caused by the pathogen was significantly reduced by Fito_S127B, in contrast to Fito_F278, which was less effective. Furthermore, these BCAs activated some specific defence responses of grapevine, allowing a more rapid and solid response of plant against the pathogen. The genome analysis also showed that these BCAs strains are an important source of bioactive compounds of biocontrol value.

Overall, this study brought new insights on the structure of microbial communities of grapevine and their interactions. Moreover, highlighted that grapevine is a natural source of microorganisms with a promising biocontrol against GTDs, and that they can promote plant defence responses. Thus, these findings provide not only a better understand of the grapevine- BCAs- GTDs interactions but also a strong contribution to future GTDs management strategy**.**

mots-clés

Microbiome de la vigne, MDB, microorganismes bénéfiques, Fito_S127B, Fito_F278, colonisation de la vigne, mécanismes de défense, protection

résumé

La vigne est une culture fruitière largement cultivée, qui abrite naturellement un microbiome complexe, i.e. colonisée par des microorganismes neutres, phytopathogènes ou bénéfiques. Parmi les phytopathogènes, ceux associés aux maladies du bois (MDB) induisent des maladies très destructrices, et les traitements disponibles pour les contrôler ont actuellement une efficacité partielle. Les microorganismes bénéfiques (BCAs) peuvent jouer un rôle spécifique dans la protection des plantes contre les phytopathogènes et le défi actuel est de comprendre comment ces microorganismes interagissent avec les plantes et leur potentiel biotechnologique pour le développement de stratégies innovantes. Dans ce contexte, l'objectif de cette étude était d'abord de caractériser les communautés microbiennes associées à la vigne tout au long de son cycle végétatif jusqu'à la fermentation du vin et, d'autre part, de mieux comprendre les interactions entre la vigne- BCA – MDB. Pour cela, deux potentiels BCAs isolés de la vigne ont été testés contre des espèces de Botryosphaeriaceae et leur potentiel de colonisation, d'induction de mécanismes de défense dans la vigne, en présence ou non de *D. seriata* F98.1, ont été caractérisés ainsi que l'analyse de leur génome.
Les résultats ont montré que le microbiome de la vigne était très dynamique au cours de son cycle végétatif. Comme prévu, la biodiversité microbienne était plus élevée dans les sols, et les communautés variaient entre le sol, les feuilles et les moûts de vin. Une proportion de communautés microbiennes était similaire dans ces structures, ce qui suggère l'existence d'un microbiome commun. Plusieurs isolats ont été sélectionnés à partir de vignes et appartenaient principalement aux genres *Bacillus*, *Streptomyces* et *Aureobasidium*. Certains d'entre eux ont considérablement diminué la croissance du mycélium de plusieurs espèces de Botryosphaeriaceae, telles que *Streptomyces* sp. Fito_S127B et *A. pullulans* Fito_F278 qui ont été sélectionnés comme BCAs. Ces souches ont montré qu'elles produisaient une gamme élevée d'enzymes extracellulaires intéressantes pour le biocontrôle et ont pu coloniser avec succès la vigne : Fito_S127B était une épiphyte du système racinaire de la vigne, tandis que Fito_F278 pouvait coloniser l'ensemble de la plante, des racines aux feuilles. L'inoculation artificielle des tiges avec *D. seriata* F98.1 a montré que la longueur des nécroses causées par l'agent pathogène a été significativement réduite par Fito_S127B, contrairement à Fito_F278 qui était moins efficace. De plus, ces BCAs sont capables d'activer certaines réponses de défense de la vigne, permettant une réponse plus rapide et plus forte de la plante contre le pathogène. L'analyse du génome a également montré que ces souches sont une source des composés bioactifs, importants pour le biocontrôle.
Dans l'ensemble, cette étude a apporté de nouvelles connaissances sur la structure des communautés microbiennes de la vigne et leurs interactions. De plus, elle a confirmé que la vigne est une source naturelle de microorganismes prometteurs pour une gestion biologique des MDB et qu'ils peuvent promouvoir les réponses de défense des plantes. Ainsi, ces résultats fournissent non seulement une meilleure compréhension des interactions entre la vigne et les BCAs-MDB, mais aussi une forte contribution à la future stratégie de gestion durable des MDB.

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ABBREVIATIONS

Φ PSII	Optimal quantum yield of PSII
ABA	Abscisic acid
Avr	Avirulence gene
B.C.	Before Christ
BABA	β-Aminobutyric acid
BCA	Biological Control Agent
CWDE	Cell wall degrading enzymes
DELFs	Defensin-like proteins
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
EC	European Community
EF1	Elongation factor 1
ET	Ethylene
EU	European Union
FD	Flavescence Dorée
GST	Glutathione S-transferase
GTD	Grapevine Trunk Diseases
ha	Hectare
HCN	Hydrogen cyanide
hpi	Hours post inoculation
HR	Hypersensitive response
HSP	Heat shock protein
IPM	Integrated Pest Management
IAA	Indole-3-acetic acid
ISR	Induced Systemic Resistance
JA	Jasmonic acid
LPs	Lipopolysaccharides
kg	Kilogram
kha	Thousand of hectares
MAMP	Microbial associated molecular patterns
MAP	Mitogen-activated protein
MDB	Maladie du Bois
mha	Million of hectares
mhl	Million of hectoliters
MTI	MAMP-triggered immunity
PAL	Phenylalanine-ammonium liase
PAMP	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Pierce's disease
PGP	Plant growth promoting
PGPR	Plant growth promoting rhizobacteria
PRs	Pathogenesis-related proteins
PRRs	Pattern recognition receptors
PS II	Photosystem II
PVPP	Poly(vinylpolypyrrolidone)
R&D	Research and Development
RLs	Rhamnolipids
RNA	Ribosomal ribonucleic acid
ROS	Reactive Oxygen Species
SA	Salicylic acid
SAR	Systemic Acquired Resistance
STS	Stilbene synthase
TCA	Trichloroacetic acid
Tub	Tubulin
Trp	Tryptophan synthase
US	United States

OPENING-REMARKS

Vitis vinifera L. is one of the oldest cultivation crop, with nutritional and health benefits and, by far with a great economic impact worldwide. Considering the area under vines, Spain has the biggest vineyard surface worlwide, followed by China, France, Italy or Turkey (OIV, 2016).

As other plants, grapevine is susceptible to diverse phytopathogen attacks which compromise grapevine productivity and the longevity of vines, affecting the rooting capacity and vitality of plants and, ultimately, leading to plant mortality (Ferreira *et al.,* 2004). Nowadays, the most threatening phytopathogens in vineyards are those implied on Grapevine Trunk Diseases (GTDs), namely Esca (*Phaeomoniella chlamydospora* and several *Phaoacremonium* species), Botryosphaeria dieback (*Botryosphaeria dothidea*, *Diplodia seriata*, *Diplodia mutila*, *Neofusicoccum parvum, Ladiodiplodia theobromae*) and Eutypa dieback (*Eutypa lata*). Indeed, GTDs are the most devastating diseases of grapevine worldwide for the past three decades and, since the ban of sodium arsenite in 2001, no highly effective means of control are available (Larignon *et al.,* 2009; Bertsch *et al.,* 2012; Fontaine *et al.,* 2016b). In addition to GTDs, other well widespread fungal pathogens in vineyards comprise those responsible for the powdery (*Erysiphe necator*) and downy mildews (*Plasmopara viticola*), and grey mould (*Botrytis cinerea*) (Armijo *et al.,* 2016).

It is known that grapevine hosts a wide range of microorganisms, neutral, beneficial or pathogenic, collectively referred as the grapevine microbiome or plant's second genome. Though this microbial complexity is not yet fully discovered and it is of great interest to unveil this microbial community in order to understand the dynamic and interactions of these populations on grapevine. Contrary to pathogenic microorganisms, the neutral or beneficial microorganisms could have a positive effect on plants, without causing disease symptoms (Lodewyckx *et al*., 2002; Pinto and Gomes, 2016). These are potential microorganisms since they can contribute to the plant's growth, can have antagonistic activity against several phytopathogens and can reinforce the natural plant defences (Berg, 2009; Compant *et al.,* 2010). Thus, such microorganisms can potentially be used as biological control agents (BCAs) to control phytopathogens, reducing significantly the application of chemical fungicides in vineyards, and thus preserving the microbial biodiversity of grapevine (Pinto *et al.,* 2014; Pinto and Gomes, 2016). The present challenge is to understand how such microorganisms interact with plant and their biotechnological potential for the development of innovation strategies.

In this context, this thesis aims to understand the grapevine-microbiome interactions, and to explore the biotechnological potential of beneficial microorganisms, with the ultimate goal to contribute to a more efficient and more sustainable viticulture. Thus, this study combines combines multidisciplinary approaches, such as metagenomic, molecular biology or microbiology, to deeply

characterize the natural microbiome of grapevine, to identify and characterize potential BCAs from grapevine and, also to study the interactions between grapevine – BCAs - phytopathogens, namely those responsible for GTDs such as *Botryosphaeriaceae* species. For this, two-pillar strategies were adopted during this thesis project, which were carried out in two host laboratories, namely Genomics Unit from Biocant - Portugal, for the grapevine-associated microbiome characterization, and the Research Unity of Vines and Wines of Champagne (URVVC) from the University of Reims Champagne Ardenne - France, for the analysis of the biotechnological potential of beneficial microorganisms and, thus, the plant-BCAs- GTDs interactions.

In general, this thesis is presented over four chapters. Thus, Chapter 1 describes the state-of-the-art on the current knowledge about the importance of the grapevine-microbial interactions and their impact on both grapevine performance, production and protection. This chapter include two review publications, namely one on the microbiome associated with grapevine (Publication 1) and the other on the effects of grapevine trunk diseases on vine physiology (Publication 2). Thereafter, results and discussion are presented in two-pillar strategies, and in the form of chapters:

Pillar A: Deep characterization of the natural grapevine-associated microbiome

This pillar includes the Chapter II - Characterization of the grapevine microbiome, and the Chapter III – Selection of potential BCAs. Results from Chapter II are included in three publications that describes not only the relationship between grape cultivars and microbiome structure but also the temporal evolution of these microbial communities from vines to wine (Publication 3, 4 and 5); Chapter III also includes three publications that focus on the analysis of the draft genomes of potential BCAs (Publication 6, 7 and 8).

Pillar B: The biotechnological potential of beneficial microorganisms

This pillar includes the Chapter 4 – Phytoprotector potential of two selected BCAs against GTDs agents. Results from this Chapter are included in one publication that focuses particularly on the plant-microbial interactions within the *A. pullulans* Fito_F278 strain (Publication 9).

AVANT-PROPOS

Vitis vinifera L. est l'une des plus anciennes cultures, avec des bénéfices nutritionnels et pour la santé, et avec un important impact économique dans le monde. Concernant la surface viticole, l'Espagne possède la plus grande surface de vignoble dans le monde, suivie de la Chine, de la France, de l'Italie ou de la Turquie (OIV, 2016).

Comme beaucoup de plantes, la vigne est sensible aux attaques de diverses phytopathogènes qui ont un impact négatif sur la productivité et la longévité de la plante, affectant sa capacité d'enracinement, sa vitalité, et au final, conduisant à sa mortalité (Ferreira *et al.*, 2004). Actuellement, les phytopathogènes les plus menaçants dans le vignoble sont ceux associés aux maladies du bois (MDB) à savoir, Esca (*Phaeomoniella chlamydospora* et plusieurs espèces de *Phaoacremonium*), Botryosphaeria dieback (*Botryosphaeria dothidea*, *Diplodia seriata*, *Diplodia mutila*, *Neofusicoccum parvum*) et Eutypa dieback (*Eutypa Lata*). En effet, au cours des trois dernières décennies, les MDB sont devenues les maladies les plus dévastatrices de la vigne dans le monde entier et, depuis l'interdiction de l'arsénite de sodium en 2001 en France, aucun moyen de contrôle aussi efficace n'est disponible (Larignon *et al*., 2009 ; Bertsch *et al*., 2012 ; Fontaine *et al*., 2016b). En plus des MDB, d'autres agents pathogènes sont aussi problématiques dans le vignoble comme celui associé à l'oïdium (*Erysiphe necator*), au mildiou (*Plasmopara viticola*) et à la pourriture grise (*Botrytis cinerea*) (Armijo *et al*., 2016).

La vigne est colonisée par de nombreux microorganismes, neutres, bénéfiques et pathogènes, désignés collectivement comme le microbiome de la vigne ou le deuxième génome de la plante. Cette complexité microbienne n'est pas encore pleinement découverte. Par conséquent, il est d'un grand intérêt de mieux connaître cette communauté microbienne dans le but de comprendre la dynamique et les interactions de ces populations chez la vigne. Contrairement aux microorganismes pathogènes, les microorganismes neutres ou bénéfiques pourraient avoir un effet positif sur les plantes, sans causer de symptômes en lien avec une maladie (Lodewyckx *et al*., 2002 ; Pinto et Gomes, 2016). Ce sont des microorganismes bénéfiques potentiels, car ils peuvent contribuer à la croissance de la plante, avoir une activité antagoniste contre les phytopathogènes et peuvent renforcer les défenses naturelles des plantes (Berg, 2009 ; Compant *et al.*, 2010). Ainsi, ces microorganismes peuvent potentiellement être utilisés comme agents de biocontrôle (BCA) pour maîtriser les phypathogènes, qui permettront de réduire de manière significative l'application de fongicides dans les vignobles et, aussi préserver la biodiversité microbienne de la vigne (Pinto *et al*., 2014 ; Pinto and Gomes, 2016). Le défi actuel est de comprendre comment ces microorganismes interagissent avec les plantes et leur potentiel biotechnologique pour le développement de stratégies innovantes.

Dans ce contexte, cette étude vise à comprendre les interactions entre la vigne et les microorganismes et à explorer le potentiel biotechnologique des microorganismes bénéfiques, dans le but ultime de contribuer à une viticulture plus efficace et plus durable. Ainsi, cette étude combine des approches multidisciplinaires, telles que la metagénomique, la biologie moléculaire ou la microbiologie, pour caractériser précisement le microbiome naturel de la vigne, pour identifier et caractériser des BCAs isolés de la vigne et aussi pour étudier les interactions entre la vigne- BCAs – phytopathogènes, à savoir les responsables des MDB tels que le Botryosphaeria dieback. Pour cela, deux stratégies de travail à deux axes ont été adoptées lors de ce projet de thèse, qui ont été réalisées dans deux laboratoires, à savoir l'Unité de Génomique du Biocant-Portugal, pour la caractérisation du microbiome de la vigne, et l’Unité de Recherche Vigne et Vins de Champagne (URVVC) France, pour l'analyse du potentiel biotechnologique des microorganismes et, par conséquent, l'interaction de la vigne-BCAs-MDB.

En général, cette thèse est présentée sur quatre chapitres. Ainsi, le Chapitre 1 décrit l'état de l'art sur les connaissances actuelles de l'importance des interactions entre les vignes et les microorganismes et leur impact sur la performance, la production et la protection de la vigne. Ce chapitre comprend deux publications, à savoir une sur le microbiome associé à la vigne (Publication 1) et l'autre sur les effets des maladies du bois sur la physiologie de la vigne (Publication 2). Par la suite, les résultats et la discussion sont présentés selon deux axes et sous forme de chapitres :

Axe A : Caractérisation profonde du microbiome naturel associé à la vigne

Cet axe comprend le Chapitre II - Caractérisation du microbiome de la vigne et le Chapitre III - Sélection des BCA potentiels. Les résultats du Chapitre II sont inclus dans trois publications qui décrivent non seulement la relation entre les cépages et la structure des microbiomes, mais également l'évolution temporelle de ces communautés microbiennes des vignes au vin (Publications 3, 4 et 5) ; Le Chapitre III comprend également trois publications liées à l’étude du génome des BCAs potentiels (Publications 6, 7 et 8).

Axe B : Le potentiel biotechnologique des microorganismes bénéfiques

Cet axe comprend le Chapitre 4 - Potentiel de phytoprotection de deux BCA sélectionnés contre les MDBs. Les résultats de ce chapitre sont inclus dans une publication qui se concentre particulièrement sur les interactions entre la vigne et la souche *A. pullulans* Fito_F278 (Publication 9).

Chapter I

INTRODUCTION

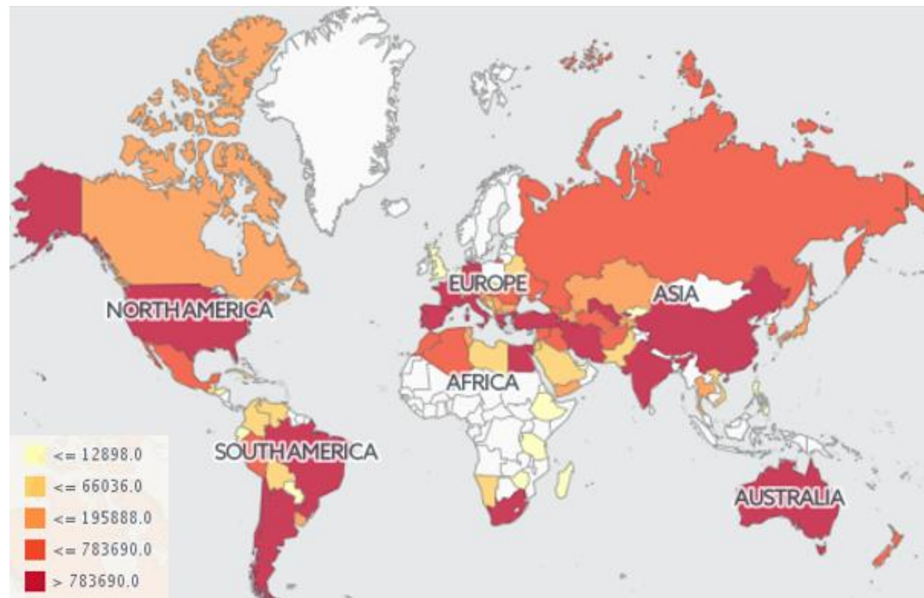

Figure 1: Worldwide production of grapes in 2014 **(FAOSTAT, 2014).** The production quantities of grapes by country are showed in tons. In 2014, a total of 74.5 million of tons of grapes were produced worldwide.

1.1. *Vitis vinifera:* a worldwide economically important crop

Grapevine (*Vitis vinifera* L.), a woody perennial plant, belongs to the genus *Vitis* that comprises around 60 *Vitis* species. Among this genus, *Vitis vinifera* acquired a significant economic impact although other species, such as *V. rupestris, V. riparia* and *V. berlandieri* are used as rootstocks due to their resistance against important pathogens such as *Phylloxera,* downy and powdery mildews (Terral *et al*., 2010). *Vitis vinifera* comprises two subspecies namely, *V. vinifera* ssp *vinifera* (or *sativa*), a domesticated form, and its wild relative *V. vinifera* ssp *sylvestris* (This *et al.*, 2006; Garcia and Revilla, 2013). This classification is based on morphological differences such as leaves, flowers, fruit clusters, berry shape or seeds, habitat or mating system. In particular, while the wild grapevine is dioecious with anemophilous pollination and has small leaves with variable forms, small and thin fruits, the domesticated form is self-pollinating or hermaphrodite and produces abundant bunches with bigger berries (Levadoux, 1956; Bouby and Marinval, 2001; This *et al.,* 2006; Zohary *et al.,* 2012).

Grapevine, together with olive, date fruit and fig were the earliest cultivated and domesticated fruit crops (Zohary and Spiegel-Roy, 1975; Zohary *et al.,* 2012). The grapevine domestication seems linked to wine discovery, though the origin and historical biogeography of grapevine domestication still unclear. Archaeological data suggest a primary domestication in the Neolithic period, probably in the fourth millennia B.C., and in the geographical area of Near East or the Transcaucasian region (Zohary and Spiegel-Roy, 1975; This *et al.,* 2006; Terral *et al*., 2010; Myles *et al.,* 2011). Over time, grapevine was spread by human's civilizations to the South-eastern Mediterranean regions, Palestine, Southern Lebanon and Jordan (Zohary and Spiegel-Roy, 1975). Then, in the third millennium B.C., domesticated grapevines appeared in the Near East, Southern Greece, Cyprus and Egypt, in the beggining of the second millennium B.C. in the Southern Balkans and, in the half of the second millennium B.C., grapevines were found in Southern Italy (Bouby and Marinval, 2001; McGovern and Robert, 2003). In the second part of the first millennium B.C. grapevine appeared in Northern Italy, Southern France, Spain and Portugal (Levadoux, 1956; Bouby and Marinval, 2001). Uncertainly remains the hypothesis of secondary domestication events along the Mediterranean area that may have happened, i.e., areas where wild grapevine was domesticated initially (Grassi *et al.,* 2003; This *et al.,* 2006; Myles *et al.,* 2011).

Vitis vinifera L. is nowadays present in all continents except in Antarctica. In particular, such geographic distribution occurs in the central and southern regions of Europe, in the western regions and middle east of Asia, China, in Mediterranean coast of Africa, South Africa, North America (such as California, British Columbia, Ontario, Québec), South America (Chile, Argentina, Uruguay, Peru and Brazil), Australia and New Zealand (Figure 1). Currently, the wild form of grapevine is rare and can be

Table 1: Worldwide area under vines in 2015 (OIV, 2016)

kha	**2011**	**2012**	**2013**	**2014**	**2015**
Spain	1 032	1 017	1 021	1 022	1 021
China	633	709	760	799	830
France	796	792	793	791	786
Italy	720	713	705	690	682
Turkey	508	497	504	502	497
USA	413	412	422	419	419
Argentina	219	222	224	226	225
Iran	239	240	227	221	223
Portugal	206	206	208	211	217
Chile	191	192	192	192	211
Romania	170	162	157	154	192
Australia	133	135	133	132	149
Moldavia	110	110	110	110	140
South Africa	102	102	102	102	130
India	119	120	119	120	120
Brazil	90	91	90	89	85
New Zealand	37	38	38	38	39
Total	**7 489**	**7 494**	**7 536**	**7 539**	**7 511**

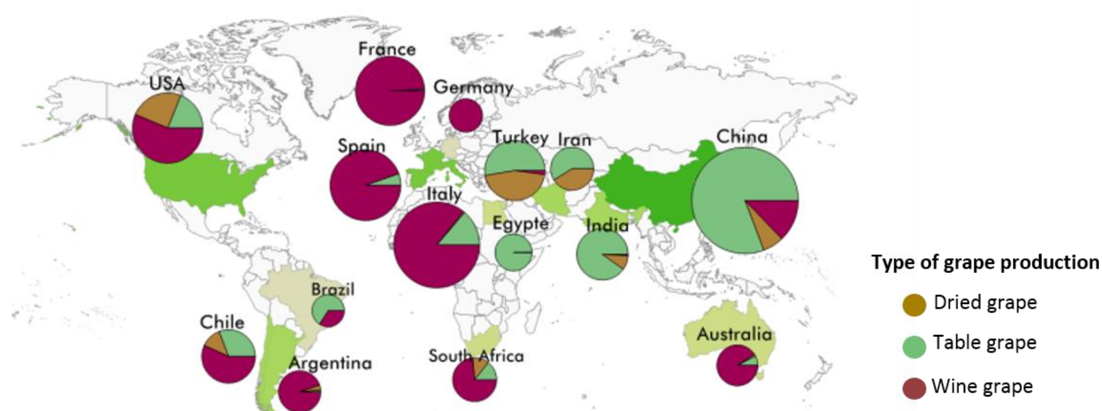

***Figure 2: Major grape producers worldwide by type of grape production* (OIV, 2016).** The non-alcoholic grape juice and spirituous beverages are not represented.

found along the temperate Mediterranean basin and from Portugal to Turkemenistan, Uzbekistan, Tajikistan, along the Danube and Rhine rivers or northern forests of Tunisia (Arnold *et al.*, 1998; McGovern and Robert, 2003; This *et al.,* 2006). *V. vinifera* contains more than 6 000 grape varieties worldwide and many different clonal varieties though only few are of commercial importance (Bouby and Marinval, 2001).

From the economic perspective, *Vitis vinifera* L. is one of the most important crops worldwide and is estimated to be implemented in a total of 7.5 mha in 2015, with the production of 75.7 million of tonnes of grapes (Figure 1) and 259 mhl of wine (OIV, 2016). Europe (35.8%) and Asia (35.4%) are the largest producers of grapes followed by America continent (19.9%), Africa (6.2%) and Oceania (2.7%) (FAOSTAT, 2014).

Considering the area under vines, 5 countries represents 50% of the worldwide vineyard namely, Spain (14%) which has the biggest vineyard surface area, followed by China (11%), France (10%), Italy (9%) and Turkey (7%) (OIV, 2016) (Table 1). Portugal has the 9th world largest surface area under vines, despite being the 11th producer, which unveils a production problem. Since 2000, European vines surface area reduced considerable and China, a new world wine country, has lately demonstrated an upward trend. This decline may be explained in part by the European farm policies and by the increase of vineyard surface areas in the rest of the world (Bordiga, 2016).

The great majority of total world grape production is destined for wine production (48%) but also for table grapes (36%), dried grape (8%), non-alcoholic grape juice and spirituous beverage (8%) (OIV, 2016). In Europe, grape production is almost exclusively for wine production while other countries as China, Turkey, India or Iran produce mostly table grapes (Figure 2).

Since ancient times, wine production and consumption have been related to social and cultural aspects and, nowadays, is within the frame of a diet and lifestyle. Moreover, in many countries, such as in Portugal and France, the viticulture is an important cultural heritage and a cultural identity.

1.2. The importance of the grapevine microbiome and the plant-microbial interactions on the plant development and wine production

The consagration and success to produce quality wines relies upon a complex and incessant interaction of several factors in vineyard and during the wine fermentation process. In vineyards, the grapevine is conditioned by biotic and abiotic factors and by viticulture management practices. The biotic factors include the microbial communities such as plant pathogens (bacteria, fungi, phytoplasma or virus) and pests (insects as grape phylloxera, several beetle’s species or grape berry moth) while the

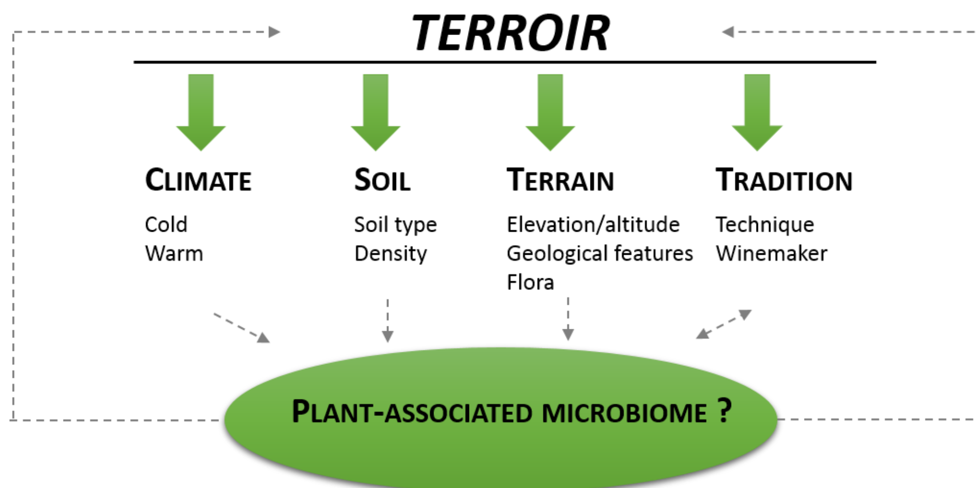

Figure 3: Overview of the terroir definition **(Adapted from Resolution OIV/VITI 333/2010; Gilbert *et al.*, 2014).**

abiotic factors comprise the climatic (temperature, wind, rainfall, sunlight) and edaphic conditions (soil, nutrients, pH, salinity) or even the phytotoxicity of pesticides and atmospheric pollution (Amaro, 2003). Given the wine fermentation, the associated microorganisms within grapes and wine musts, together with the oenological practices and cellar technology are remarkable for the wine process. Altogether, the association of region's climate, specific soil type, landscape characteristics, oenological techniques and biodiversity features clearly define the distinctive characteristics of such wine region and thus, the *terroir* (Resolution OIV/VITI 333/2010) (Figure 3). Notoriously, the microbial communities associated with grapevine may also have a direct or indirect role in the organoleptic properties of wine and, within this general context, the microbial consortium could integrate the *terroir* definition (Figure 3). Although this is still discussible as remains the questions of how significant are soil microbiome or region-specific microorganisms in defining *terroir* (Barata *et al.,* 2012; Gilbert *et al.,* 2014).

Considering the biotic factors, the grapevine, as other plants, is naturally colonised by a myriad of microorganisms, which together form its microbiome, also referred as the plant's second genome (Berendsen *et al.,* 2012; Turner *et al.,* 2013; Berg *et al.,* 2014). The grapevine-associated microorganisms are in close interaction with the plant and both are inseparable entities since they entwined in their ecology and evolution (Vandenkoornhuyse *et al.,* 2015). In this regard, both the plant and its associated microbiota can be considered as meta-organism or holobionts (Berg *et al.,* 2014; Vandenkoornhuyse *et al.,* 2015). The biogeochemical processes, soil fertility, plant health, productivity, plant growth and resistance strongly depend on the equilibrium of these microbial communities however, this equilibrium could be compromised and rely on the host genotype, plant diversity, soil type, climate or agriculture practices (Philippot *et al.,* 2013; Hartmann *et al.,* 2015; Van Der Heijden and Hartmann, 2016). These drivers can shape the microbial structure and encourage a better adaptation of certain microorganisms in detriment of others. Thus, the plant microbiome, the plant-microbial interactions and the microbial dynamics from the vineyards until the wine making process are critical and will consequently influence the quality and organoleptic properties of wines (Turner *et al*., 2013; Berg *et al*., 2014; Pinto *et al*., 2014).

Given the overwhelming range of plant-associated microorganisms such as bacteria, yeasts, filamentous fungi, archaea or protists, some of them have the potential to promote beneficial interactions with the plant by promoting its growth and development, enable the availability of limiting nutrients (such as solubilisation of phosphate, production of siderophores, fixation of nitrogen), tolerance to abiotic stress, reinforce the natural plant immune response, or even to promote the plant protection against pathogens (Van der Heijden *et al.,* 2008; Mendes *et al.,* 2013). Conversely, microorganisms can also have negative effects on plant growth and productivity by competing together for nutrients, transform nutrients into inaccessible forms to plants or by acting as pathogens (Van der Heijden *et al.,* 2008). In turn, grapevine ensures a protected environment and plant nutrients.

The microbial communities can colonize either inside (endophytes) and the surface (epiphytes) of plant tissues at both rhizosphere (belowground) and phyllosphere (aboveground) (Lindow and Brandl, 2003; Whipps *et al.,* 2008; Philippot *et al.,* 2013; Turner *et al*., 2013). The phyllosphere represents the largest microbial habitat in plants and is of a great importance from the environmental and agronomical point of view as these microorganisms are involved in the cycling of elements, remediation of residual pesticides, can act as phytostimulators or plant protectors (Whipps *et al.,* 2008; Pinto *et al.,* 2016). Herein, and particularly in the leaves, bacteria are the most abundant microorganisms (Lindow and Brandl, 2003; Müller and Ruppel, 2013) though the ubiquitous black yeast-like fungus *Aureobasidium pullulans* also has a great population size (Pinto *et al.,* 2014) and has been notorious for its antagonist potential and biocontrol of post-harvest diseases of fruits (Castoria *et al.,* 2001). In opposition to the belowground parts, such as soil and roots, the phyllosphere is a hostile environment characterized by nutrient and water limitations, high range of temperatures, UV exposure and presence of reactive oxygen species (ROS) (Lindow and Brandl, 2003; Müller and Ruppel, 2013). Thus, the aboveground microorganisms are suitable to explore the microbial ecology (Lindow and Brandl, 2003) and as they are well adapted and more resistant to the biotic and abiotic stresses, presenting an important biotechnological potential (Müller and Ruppel, 2013).

Recent studies, from our laboratory and others, have clearely demonstrated that *i)* grapevine soils until wine have similar microbial signatures (Zarraonaindia *et al.,* 2015) and *ii)* wine-growing regions have specific microbial communities (Bokulich *et al.,* 2014; Pinto *et al.,* 2015). Altogether, these studies are helpful to demystify the origin of the plant associated microorganisms and to better understand the role of this native microbiota in the organoleptic qualities of wine and the uniqueness of regional wines. Thus, the holistic approach of the microbial consortium and function is of utmost importance. The deep knowledge and understanding of the identity, ecology and the role of the microbiome on ecosystem functioning and plant productivity, encompasses a biotechnological potential to implement a sustainable management strategy for vineyards (Pinto *et al.,* 2016), emphasizing the *terroir* (Figure 3) and promising the quality and identity of wines. Exploring the grapevine microbiome is, thus a long way to answer to such questions of how to use these microbial communities to predict plant diseases and how these communities can produce valuable wine styles.

Publication 1- *Vitis vinifera* microbiome: from basic research to technological development

Within the context of the *Vitis vinifera*-associated microbiome research, a review was published in the Biocontrol journal (Pinto and Gomes, 2016). Overall, the state-of-the-art of the grapevine microbiome research worldwide is presented and an overview of the plant colonisation, the impact of the viticulture practices on the microbial communities and the potential role of these microbial structures for vineyards management are deeply discussed.

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Vitis vinifera microbiome: from basic research to technological development

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Abstract Plants naturally harbours a complex microbial ecosystem or plant microbiome, as neutral, beneficial or pathogens microorganisms, that are in a close interaction with the plant. The balance of these interactions is a key element for plant health, plant growth and productivity although several factors as ecological and environmental factors represents important drivers of the microorganism's community. Herein, a review on plant microbiome is presented, and the case study of *Vitis vinifera* (grapevine) is presented as an example of the application of the study of a woody plant microbiome. Overall, new ecologically and sustainable strategies for agriculture are needed. The exploitation of the natural microbiome associated with plants and the identification of novel potential strains with plant benefits and biocontrol potential represent a challenge and a technological development for crops protection.

Keywords Biological control agents · Plant microbiome · Grapevine microbiome

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Introduction

The plant microbiome interacts with the plant and plays important roles in plant growth, its health status and, consequently, guarantees the balance of the plant's ecosystem. Further, these plant-associated microorganisms influence its nutrition or resistance to abiotic and biotic stresses (Vandenkoornhuyse et al. 2015). The microbiome dynamics and distribution is conditioned by plant's genotype, species, age and health status, as well as by environmental factors including farming management practices and crop protection (Lindow and Brandl 2003; Whipps et al. 2008; Berendsen et al. 2012). The plant-associated microorganisms colonize both plant surfaces and inner tissues and, due to the direct and important impact on plants, these microorganisms are also referred as the plant second genome (Berendsen et al. 2012; Turner et al. 2013; Berg et al. 2014).

The complexity of the plant microbiome is not yet fully understood and a gap of the knowledge concerning the microorganisms that colonize plants, their plant-microbial interactions and microbiome shaping is a challenge for scientific community, where questions about the ecology, functions and significance of these microorganisms arise. In fact, an increasing interest has emerged to unravel the microbial communities associated with plants, as the exploitation of this natural microbiome is expected to contribute to a better understanding of the plant-microbial interactions, the processes evolved on plant adaption and the

development of new ecologically and sustainable strategies for agriculture (Fig. 1). Indeed, some microbial inoculants are already commercially available as a consequence of the increasing interest to innovate in agriculture practices (Gardener and Fravel 2002). These microbial inoculants included bacteria belonging to the genera *Bacillus, Pseudomonas* or *Streptomyces* and of fungi *Candida, Thrichoderma* or *Gliocladium* (Gardener and Fravel 2002).

For long, the study of the plants' microbiome has been dependent on cultivation-dependent approaches, which have a limited range of analysis, as it does not allow for identification of all microorganisms present in such sample. The recent emergence of cultivation-independent approaches, such as those based on next-generation sequencing, provided significant advances for exploring the plant microbiome and its microbial interactions, at their natural environment (Müller and Ruppel 2014; Pinto et al. 2014). The majority of these culture-independent studies are based on the 'omics' approaches such as metagenomic, metatrancriptomic or metaproteomic (Turner et al. 2013). These methodologies allowed for the deep knowledge of the total microbial biodiversity and revealed a higher community complexity than previously reported with cultivation-dependent approaches (Müller and Ruppel 2014). Further, they allowed to unveil an unrecognized biodiversity that was not previously described in the DNA databases (Turner et al. 2013; Pinto et al. 2014). Generally, the study of prokaryotes is based on the analysis of the 16S ribosomal RNA and the eukaryotes on the 18S or the hypervariable internally transcribed spacer region (ITS). The majority of the published studies focus on the analysis of the microbial communities associated with rhizosphere and on the endophytic bacteria, across different plant species.

In this review, we will focus both on the plant microbiome and on the state-of-the-art of grapevine microbiome, a woody perennial plant, presented here as a case-study. For this, the impact of different viticulture practices on the natural microbial ecosystem and the current perspectives to develop innovative sustainable approaches for grapevine management, based on the microbial biodiversity management, will be also discussed.

General overview of plant microbiome

The plant-microbiome and its importance

There are around 300,000 plant species worldwide, all colonized by a myriad of microorganisms, embedded in a complex micro-ecosystem. Several factors affect the plant-microbial associations as plant species, age, physiology, environment, soil type, crop management or presence of plant pathogens. The plant microbiome is composed of different microorganisms that could be neutral, beneficial or pathogenic to the plant, and which colonize different plant organs and are in permanent interaction (Turner et al. 2013). Amongst them, bacteria and fungi are the mostly abundant and

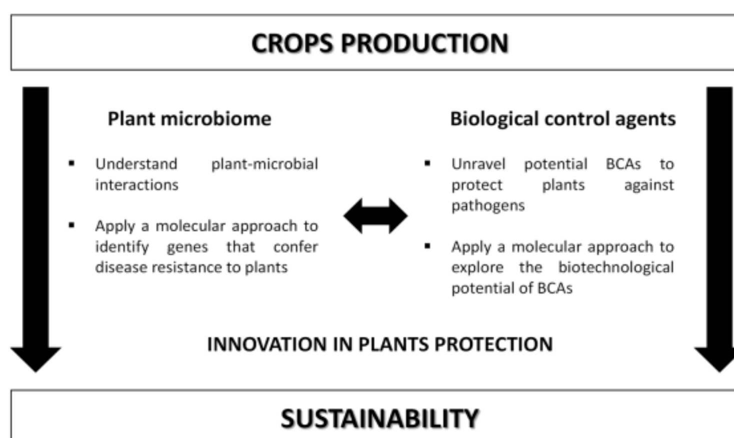

Fig. 1 Exploited plant microbiome for crops protection. General vision to develop new sustainable strategies for crop protection through the deep characterization of the plant microbiome and identification of potential biological control agents (BCAs), naturally associated with plants

can colonize both the phyllosphere, the external surface and interior of the plant (Whipps et al. 2008; Newton et al. 2010), and the rhizosphere (Philippot et al. 2013; Turner et al. 2013). Depending on their localization, these can be classified as epiphytes, if present on the plant's surface, or as endophytes, if present in the internal plant tissues (Hallmann et al. 1997; Porras-Alfaro and Bayman 2011). The endophytes are ubiquitous and can be latent or active colonizers (Hallmann et al. 1997), though it is not yet well understood how these populations impact on the plant health and function (Porras-Alfaro and Bayman 2011).

Indeed, endophytes have been thought to be plant pathogens, but it is now known that they could also be beneficial or neutral microorganisms (Hallmann et al. 1997). However, and under specific conditions, they can have a negative impact on the plant (Porras-Alfaro and Bayman 2011). Interestingly, most of endophytes derived from rhizosphere and from the soil, although they can also derived from phyllosphere or even from cultural practices of plant propagation and pruning (Hallmann et al. 1997; Compant et al. 2010; West et al. 2010). To penetrate the plant tissues, these microorganisms make use of their hydrolytic enzymes, such as cellulases and pectinases (Hallmann et al. 1997). The first report related to the existence of endophytes microorganisms on plants and their soil origin hypothesis, was referred by M.L.V. Galippe in 1887 (Galippe 1887a, b; Compant et al. 2012). Interestingly, West et al. (2010), who analysed both endophytic and epiphytic bacteria from different parts of grapevine, showed that many of the epiphytes isolated were from the same genera or species as endophytic bacteria. According to their results, they hypothesised that epiphytes become endophytes, and that their entry occurs randomly across the plant (West et al. 2010). Further, these microorganisms could remain only on a specific plant tissue or move across the plant, through the xylem vessels or by colonizing intercellular spaces (Lamb et al. 1996; Compant et al. 2005; Turner et al. 2013).

Interestingly, studies focusing on the rhizosphere microbiome, from a wide range of plant species, have unveiled a similar distribution of microorganisms at the phylum level, where generally the Proteobacteria phylum was the most abundant, with bacteria from Pseudomonadaceae or Burkholderiaceae families (DeAngelis et al. 2009; Philippot et al. 2013; Berg et al. 2014). Also, other groups from Actinobacteria or Firmicutes phyla were abundant. Indeed, the rhizosphere—the soil adjacent to the plant roots—is an intriguing and dynamic environment: for one hand the root exudates modulate the associated microbial populations, and on the other hand the present microbial communities also modulates the plant's response. Also, the soiĺs physicochemical properties affect the plant's physiology and root exudates, and its associated microbiome (Philippot et al. 2013). Indeed, the microorganisms present in the soil may express a set of enzymes and/or metabolites that will solubilize nutrients, providing them to the plant, and, thus, stimulating its growth and health status (Bloemberg and Lugtenberg 2001; Bakker et al. 2013; Philippot et al. 2013).

At the plant phyllosphere, most of the published studies are focused on the microbial biodiversity of leaves, the largest microbial habitat. In contrast to the rhizosphere, phyllosphere is poorer in nutrients and faces several external stress factors that have an impact on the exposed areas of these structures (Newton et al. 2010; Turner et al. 2013). The plant-associated microorganisms from phyllosphere are distinct from those of rhizosphere as a consequence of the physicochemical composition and surrounding environment, which strongly modulates the structure of the microbial ecology and its dynamics (Lindow and Brandl 2003). Bacteria are the prevailing microorganisms followed by fungi (Lindow and Brandl 2003; Whipps et al. 2008; Newton et al. 2010; Müller and Ruppel 2014). Most of the leaves' colonizing microorganisms are commensals (Müller and Ruppel 2014), who are actively involved in the cycling of elements, in remediation of residual pesticides or pollutants, and consequentially they impact on the plant development. Additionally, these microorganisms may act as phytostimulators or even as phytoprotectors against plant pathogens (Müller and Ruppel 2014). Regarding their taxonomy, microorganisms from the Proteobacteria phylum have been described to be dominant, though also Firmicutes or Actinobacteria have been reported (Turner et al. 2013; Pinto et al. 2014).

Some of the colonizing microorganisms can provide benefits to the plant, as they may contribute to its growth, or may reduce its biotic or abiotic stress, by displaying an antagonistic activity against plant pathogens or even reinforcing the natural plant defences. These microorganisms have the potential

to be used in agriculture as control plant pathogens, thus reducing the application of chemical compounds, or even as biofertilisers or biostimulants for agriculture (Berg 2009; Compant et al. 2010). Some bacteria as *Azospirillum*, *Burkholderia, Rhizobium* were described as plant growth promoters (Compant et al. 2005; Berg 2009), while other genera as *Bacillus, Pseudomonas* or *Streptomyces* were reported to influence plant health (Berg 2009). Further, some endophytes were considered as promising biocontrol agents (Hallmann et al. 1997). Altogether, the plant microbiome encompasses a biotechnological potential, which can play a role on the implementation of sustainable crop management strategies (Berg et al. 2014; Pinto et al. 2014).

From plant microbiome to the plant-microbial interactions

The plant-microbial interactions are far from being completely understood, thus the deep understanding of the structure of microbial communities, their function and their interactions with the plant are a present challenge. Further, unveiling these can be of great impact for plant protection (Whipps 2001).

Recent studies on plant-associated microorganisms have demonstrated that these were more diverse than previously thought, and that plants interacted with both beneficial, neutral and pathogenic microorganisms (Müller and Ruppel 2014). Further, such interactions were shown to be very important as they influenced the plant health status, which could result either on a disease scenario, with negative repercussions to plant productivity and reproduction, or on a plant growth promotion and protection (Fig. 2). Also, these interactions impacted on the soil quality. Altogether, it is now accepted that the vitality of plants is clearly dependent on a balanced microbial ecosystem (Pinto et al. 2014).

The plant-microbial interactions can be beneficial, neutral or harmful and three general interactions are described (Mercado-Blanco and Bakker 2007; Turner et al. 2013). The first type of direct interaction is between plants and pathogenic microorganisms (Fig. 2). This leads to a disease scenario, and the normal physiology and vitality of the plant is compromised. The second type of interaction occurs between the plant and neutral or beneficial microorganisms (Fig. 2), where the former may stimulate the plant growth by increasing the nutrient availability for plants through the synthesis of siderophores for iron sequestration, phosphorous (P) solubilisation, and fixation of the atmospheric nitrogen (N) to plants or hormonal stimulation. Also, these microorganisms can improve the soil structure and quality, and induce the plant defence mechanisms through a systemic acquired resistance (SAR) or induced systemic resistance (ISR) (Bloemberg and Lugtenberg 2001; Lodewyckx et al. 2002; Compant et al. 2005; Adesemoye et al. 2009; Berg 2009). Interestingly, these microorganism might protect the plant from abiotic stresses such as salinity, drought, increase of CO_2 levels or temperature (Compant et al. 2005; Berg 2009; Compant et al. 2010). Overall, this interaction is based on a mutualist symbiosis (Mercado-Blanco and Bakker 2007). The third type include the interaction between pathogens and beneficial microorganisms (Fig. 2). The beneficial microorganisms may have an antagonistic effect against the pathogen through different mechanisms as direct competition for space and nutrients, degradation of the virulence factors of pathogen that will inhibit the growth and development of pathogens, or even production of active metabolites, namely antibiotics, antimicrobial compounds, lytic enzymes or siderophores. In this context, some of these microorganisms can be regarded as potential biocontrol agents.

Overall, the interaction between microorganisms involves different modes of action as antibiosis, competition, parasitism, as production of cell wall degrading enzymes, and induced resistance (Whipps 2001). Obviously, and independently from the nature of each interaction, the consequences are important for the plant phytosanitary status.

When things go wrong: plant diseases

As mentioned above, some microorganisms may be deleterious to the plants. In the last years, several reports have focused on the emerging infectious diseases (EIDs) of crops and forest trees, as they can be unexpected, can have devastating impacts on plant cultures, and even can get epidemic (Vurro et al. 2010).

The development of an infectious episode normally involves a susceptible host-plant, a virulent pathogen and a favourable environment, which, all together, compose the disease triangle (Francl 2001; Fletcher et al. 2010). Regarding plant diseases, the most

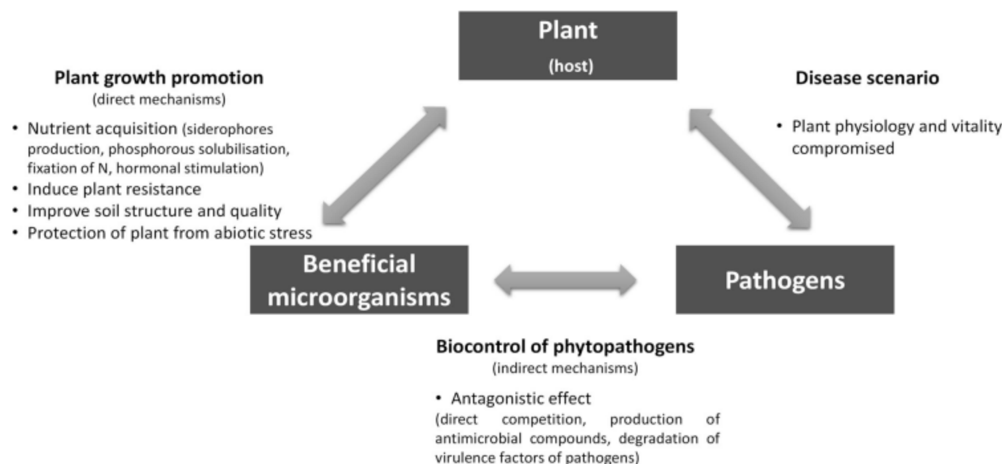

Fig. 2 The plant-microbial interactions could be beneficial, neutral or harmful to the plant and can evolve (i) a disease scenario (plant—pathogen), which affects the normal physiology and vitality of the plant, (ii) a plant growth promotion (plant-beneficial microorganisms) through different mechanisms or pathways as siderophores production, phosphorous (P) solubilisation, nitrogen fixation or hormonal stimulation and (iii) biocontrol of the pathogens (beneficial microorganisms—pathogens) through an antagonistic effect

susceptible plant groups are cereals (wheat, rice or maize), tubers (potato, cassaca, yam or taro) and vegetable crops (dry beans, peas, lentils, cabbage or brassicas) (Fletcher et al. 2010). An important example of the impact of pathogens is the case of *Phytophthora infestans*, the responsible agent of potato late blight that significantly affected the Ireland productions during the nineteenth century, and caused a severe famine (Fletcher et al. 2010; Vurro et al. 2010). Another, the fungus *Cochliobolus miyabeanus*, was responsible for a destructive disease of rice crop, and caused the famine in East Bengal in the 1940s (Strange 2003). Regarding wheat, the third most-produced cereal, it is significantly affected by the pathogen *Puccinia graminis* sp. *tritici,* which cause the stem rust, and by *Fusarium graminearum,* a *Fusarium* head blight responsible. Across woody plants, the plant canker disease caused by the bacteria *Pseudomonas syringae* is one of the most serious diseases that affects a high range of plants worldwide as kiwifruit, nectarine, tomato, apricot, hazelnut, coffee, apple, olive or forest trees (Lamichhane et al. 2014; Bartoli et al. 2014). Also, the bacterium *Xanthomonas campestris* pv. *musacearum* is responsible for the diseases in banana fruits across Africa countries (Vurro et al. 2010).

The worldwide spread of plant diseases is dependent on different mechanisms or pathways, such as efficient vectors for disease dissemination, and is conditioned by climatic and environmental factors. For instance, the case of the Citrus Tristeza Virus (CTV) was firstly reported in the South America and then it was disseminated for other world regions by the aphid vector *Toxoptera citricidus*; or the Cassava Mosaic Virus that infects cassava plants in Africa, which is spread by the vector *Bemisia tabaci* (Vurro et al. 2010). Generally, and like the animal and human emerging infectious diseases, these diseases can be defined as new infections or infections that have existed and, due to several factors, rapidly increased their incidence (Morse 1995).

Guaranteeing the plant health status is a challenge, as often there are no available treatments to control some of these diseases, which negatively affect agricultural production and, consequently, cause significant losses in industry and a serious impact on both social and global economy. It is important to emphasize that ecological and environmental factors, as well as the intensive and changing agriculture practices and the introduction of new planting material, are important drivers of the stability of the plant's microbiome (Morse 1995; Morens et al. 2004; Vurro et al. 2010;

Pautasso et al. 2012). These clearly impact the natural microbial biodiversity (Pinto et al. 2014) and create an opportunity to intensify the appearance of novel pathogens or even of novel resistant pathogens (Morse 1995).

The adaption of pathogens to new habitats may enable the appearance of disease in a new host (Morse 1995). Likewise, the genomic evolution of microorganisms, through the DNA modifications, such as mutations, deletions, duplications and translocations may result in a change on their phenotype and thus contributing to increase their virulence, or even leading to the emergence of a novel pathogenic phenotype (Arber 2008). An important example is the bacterium *Xylella fastidiosa*, which is one of the most important emerging pathogen worldwide, with an enormous host range (over 300 plant species), and which causes devastating diseases in a wide range of crops, including olive trees, citrus, peach, grapevine, coffee, almonds or plum fruits. This gram-negative bacteria is xylem-limited and it is disseminated by spittlebug, cicadas or leafhopper vectors (Purcell 1996, Hopkins 2005; EFSA 2015). The plant symptoms vary according to the host species or climatic conditions and, generally, consist of plant drying and the wilting of the leaves and fruits, which ultimately leads to the plant's death as a consequence of the obstruction of the xylem vessels (Hopkins 2005). For long, *Xylella fastidiosa* was confined and well distributed across the American continent including Canada, USA, Mexico, Brazil, Paraguay or Argentina. Recently, it was reported in Taiwan, Iran and at Europe (EFSA 2015). At the European Union (EU), *X. fastidiosa* is considered a quarantine organism and the first outbreak was reported in 2013 in the Puglia region (Italy) affecting olive trees (*Olea europea*). The spittlebug *Philaenus spumarius*, abundant on olive trees, was identified as the vector responsible for the pathogen dissemination (EFSA 2015). Further, a different subspecies of *X. fastidiosa* was reported in 2015 in the south of France, affecting ornamentals plants.

Worldwide preventive measures have been set up to control the epidemic spread of pathogenic microorganisms, which undergo from early diagnosis and pathogen identification, to monitor the diseases epidemiology and to prevent the pathogen's spreading. In the case of the *Xylella fastidiosa*, where there is no currently treatment or control available, emergency measures have been taken to avoid and eradicate the disease's dissemination. These protective measures include a rigorous control of the imported plants from non-EU countries, prophylactic measures at pruning, fertilisation or irrigation steps, removal of infected plants and control of the insect vector through phytosanitary treatments (EFSA 2015).

Interestingly, the case for *Xylella fastidiosa* is a good example where knowledge is urgently need to act against and thus to halt its spread and to preserve the affected crops. Indeed, the knowledge of the natural microbiome associated with plants and the understanding of the plant-microbial interactions might provide significant advances to understand the pathogens. Further, this knowledge might support the development of diagnostic tests and the development of focused and effective plant treatments based on a sustainable approaches.

Case study: the grapevine microbiome

The grapevine and its microbiome

Grapevine (*Vitis vinifera* L.), a woody perennial plant, is a globally economic and social important crop. It is estimated that the total area of vineyards is of 7.16 million hectares worldwide, with a production of 27 million of tonnes of wine (FAOSTAT 2013). In Europe, Spain has the highest area of vineyards, followed by France, Italy and Portugal (EUROSTAT 2014). The regional characteristics, climate, soil, plant genotype and cultivars and even the microbial richness constitutes a network responsible for the production of unique wines.

As a plant, grapevine hosts several microorganisms and the plant-microbial interactions may influence the plant health status, plant growth and, ultimately, may affect the wine production and quality (Pinto et al. 2014; Zarraonaindia and Gilbert 2014). Regarding the microbial communities associated with grapevine, special attention has been focused on the endophytes, namely on the profile of the bacterial diversity (Supplementary Table 1) by applying both cultivation dependent and independent approaches (Bulgari et al. 2009; West et al. 2010; Compant et al. 2011; Marasco et al. 2013; Baldan et al. 2014; Bulgari et al. 2014; Yousaf et al. 2014). Further, the endophytic fungal communities (Supplementary Table 2) have been

analysed (Casieri et al. 2009; Martini et al. 2009; González and Tello 2011; Cosoveanu et al. 2014; Pinto et al. 2014), as well as the arbuscular mycorrhizal fungal (AMF) communities (Schreiner and Mihara 2009; Lumini et al. 2010; Magurno et al. 2010; Holland et al. 2013), as they contribute for grapevine performance through the nutrients uptake, root and crop growth, drought tolerance or even plant protection (Baumgartner 2006). Also, attention has been paid on the microbial community on its woody tissues, as a consequence of the increasing incidence of grapevine wood diseases, whose etymology is still poorly understood (Larignon and Dubos 1997; Casieri et al. 2009; Bertsch et al. 2012; Bruez et al. 2014; Fontaine et al. 2015). In fact, these grapevine trunk diseases (GTD) are the most destructive diseases of grapevines and the *Botryosphaeria* dieback, Eutypa dieback and Esca are the three major prevalent diseases. The current diagnostic is difficult and sometimes inconclusive due to the complexity of symptoms. Furthermore, there are no treatments available to control these diseases (Bertsch et al. 2012; Fontaine et al. 2015).

In general, bacteria from Proteobacteria phylum are the most abundant across different structures from grapevine plants. Regarding leaves, Bulgari et al. (2009) analysed the endophytic bacteria of *Vitis vinifera* cv. Barbera (an Italian variety) using a cultivation-independent approach. This study has unveiled a dominance of the γ-Proteobacteria phylum, namely of the Enterobacteriaceae family with the *Pantoea agglomerans* as major species. Interestingly, this microorganism was previously isolated from grapevine xylem and was considered as a biocontrol agent. In fact, a commercially product is available based on the *P. agglomerans* strain E325, isolated from 'Gala' apple blossoms, which has been successfully applied for control of fire blight from apple and pear caused by *Erwinia amylovora* (Pusey et al. 2008). When using cultivation-dependent methods, the most abundant microorganisms were *Curtobacterium* (Actinobacteria phylum), *Bacillus* and *Enterococcus* (Firmicutes phylum) (Bulgari et al. 2009). Comparatively, Bulgari et al. (2014), analysed the effect of the phytoplasmas on the endophytic bacterial from grapevine leaves and showed that phytoplasmas could alter the microbial biodiversity. West et al. (2010) analysed endophytic bacteria of a *V. vinifera* cv. Chardonnay, from a ten years old vineyard located in New South Wales, and the most abundant microorganisms were *Bacillus* spp., *Pseudomonas* spp. and *Curtobacterium* spp.. They also reported for the first time *Streptomyces* spp. as an endophyte of grapevine. In their work, they have isolated more bacteria from roots and base of the vines when compared with the aerial parts of the plant, which has also been observed in more recent studies (Zarraonaindia et al. 2015).

Recently, the complete analysis of the grapevine microbiome, both bacterial and fungal biodiversity, naturally associated with grapevine leaves of *V. vinifera* cv. Tempranillo were characterized through a cultivation-independent approach based on the 454 sequencing (Pinto et al. 2014). This study has unveiled the dynamics of microbial biodiversity across the grapevine vegetative cycle and the abundance of the prokaryotic Proteobacteria phylum and the eukaryotic Ascomycota phylum. Amongst bacteria, the Enterobacteriaceae, Streptococcaceae or Pseudomonadaceae families were the most abundant, whereas the *Aureobasidium*, *Sporormiella*, *Alternaria* and *Guignardia* genera were the most abundant among the eukaryotic population. Interestingly, entomopathogens, such as *Zoophthora radicans* and *Pandora neoaphidis*, were also uncovered (Pinto et al. 2014). Considering its temporal variation, the eukaryotic biodiversity decreased along the vegetative cycle and, importantly, the application of chemical treatments on vineyard strongly modulated the fungal dynamics (Pinto et al. 2014). Results also indicated that the bacterial biodiversity is more stable during the plant growth stage (Pinto et al. 2014). In another study (Zarraonaindia et al. 2015), samples of *V. vinifera* cv. Merlot shown that the microbiome of leaves, grapes and flowers was dominated by the Proteobacteria phylum, and that *Pseudomonas, Sphingomonas* and *Methylobacterium* genera were the most abundant on leaves and grapes, whilst flowers were dominantly colonized by *Pseudomonas* and *Erwinia* spp. (Zarraonaindia et al. 2015).

Regarding the plant's roots, it is widely accepted that they influence the microbial community associated with the rhizosphere, the transition zone between roots and soil, in particularly that their exudates and morphology shape their microbiome, thus acting as a niche, under unique selective environment (Berg and Smalla 2009). However, and contrarily to other crops, there is a lack of information on grapevine-root associated microorganisms (Steenwerth et al. 2008;

Marasco et al. 2013). For the understanding of the plant-root associated microorganisms, it is important to take into account both rhizosphere and soil. Indeed, the soil is considered as an important reservoir of microbial biodiversity, which plays a key role on soil richness and constitutes an important factor for the plant́s growth and development. The microbial biodiversity of soil is influenced by several factors, including its chemical and physical composition, its depth, the plant roots and the root-derived carbon, and even the soil's management. A recent study using samples collected from *V. vinifera* cv. Merlot reported that the bacterial microorganisms associated with soil and roots were significantly influenced by the soil's pH and C:N ratio (Zarraonaindia et al. 2015). Overall, microorganisms from the Proteobacteria phylum were the most abundant and the genera *Planctomycetes* spp. and *Actinobacteria* spp. had the greater abundances on soil and roots, respectively. Further, the authors found that rare microorganisms present in soil and roots, as *Pasteurellales, Staphylococcus, Gluconobacter* or *Streptococcus,* were also present in grape and leaf samples at higher abundances (Zarraonaindia et al. 2015). Similarly, the effect of soil morphology and root depth and structure on microbial communities in a Pinot Noir vineyard from California was assessed (Steenwerth et al. 2008). While fungal communities increased with depth in soils containing roots, the abundance of Gram-negative bacteria decreased, which was probably due to the lower pH of soils. In contrast, no changes were obtained for Gram-positive bacteria and actinomycetes. Regarding the AMF, Holland et al. (2013) showed that AMF associated with Merlot and Shiraz varieties is dependent on grape variety and inter-row vegetation. Also, Schreiner and Mihara (2009), evinced that the age of the vineyard affected the phylotypes associated with Pinot Noir variety and those decreased with vineyard age. Further, soil characteristics and land-use are drivers of the AMF structure (Schreiner and Mihara 2009; Lumini et al. 2010).

Concerning the biogeographic characterization of the microbial community structure associated with grapevine, studies have mainly been focused on grape samples. In a recent work, Bokulich et al. (2014) demonstrated that both Chardonnay and Cabernet Sauvignon varieties, collected across the four major wine regions in California, USA displayed a specific associated microbiome, suggesting a regional pattern. Also, the grape cultivar and year of production influenced this microbial structure. Conversely, other studies were inconclusive for the regional impact on plant microbiome. Moreover, the biogeographical microbial patterns associated with bulk soil (Zarraonaindia et al. 2015) or roots were also recently identified (Marasco et al. 2013).

Colonization of microorganisms across grapevine plants

The ability of microorganisms, both pathogenic and beneficial, to move within the vine tissues has been studied on the last years by different groups (Tarbah and Goodman 1987; Compant et al. 2005, 2008; Thorne et al. 2006; Chatelet et al. 2006; Rolli et al. 2015). Concerning the plant pathogens, some move through the plant for systematic infection and different studies were carried out to understand their movement and the disease spreading. Researchers reported the movement of *Agrobacterium tumefaciens* biovar 3, the crown gall agent, through the xylem vessels of two grape cultivars, namely Chancellor and Catawba, after inoculation of the shoots (Tarbah and Goodman 1987). Interestingly, the development of tumours at 24 cm from the inoculation point, suggested the vascular movement of the bacteria. To better understand the microorganism's movement across xylem, Thorne et al. (2006) studied the movement of *Yersinia enterocolitica*, strain GY5232 with a *lux* operon, under transpiring conditions, by monitoring it using X-ray films in *V. vinifera* cv. Chardonnay and *Muscadinia rotundifolia* cv. Cowart. The results reported similar distribution of bacteria across grape varieties where the presence of the bacteria was uniformly distributed in the first veins and randomly distributed across the second and tertiary veins. Further, dark spots along veins were observed, which would indicate the presence of bacteria aggregates. Also Chatelet et al. (2006) observed the spread of *Xylella fastidiosa,* responsible for Pierce's disease on grapevine, tagged with *gfp* across grapevine leaves of *V. vinifera* cv. Chardonnay. Interestingly, the needle-inoculation of leaves unveiled the presence of bacteria in the leaf lamina but not in the leaf margins, where the first symptoms of disease appear. Further, after the inoculation at the petiole level, a small amount of bacteria was detected at the leaf lamina, but none was detected when inoculation took place near to the leaf

margin. Thus, this study clearly demonstrated that the rapid colonization of bacteria was due to the primary xylem.

Regarding the beneficial microorganisms, Compant et al. (2005, 2008) followed the colonization of *V. vinifera* L. cv. Chardonnay plantlets containing five developed leaves by both wild-type and mutant *Burkholderia* sp. strain PsJN, a plant growth-promoting bacterium, tagged with green fluorescent protein (*gpf*) or *gus*-tagged. During its colonization, the bacteria was firstly found on root surfaces and then migrated to the internal tissues from roots and xylem vessels until reached the fifth leaf after 72 h post-inoculation, thus suggesting a spread through the transpiration pathway. In the study of Rolli et al. (2015), the *gfp*-labelled *Acinetobacter* and *Pseudomonas* strains were able to colonize both Arabidopsis and grapevine rhizoplane after 1 h of exposure to the bacteria and were even detected after seven and 21 days post-inoculation.

Impact of the viticulture practices on the microbial dynamics

Viticulture has been the agro-sector with the most intensive use of pesticides and with a major diffusion of fungicides. Concerning the harmful effects of pesticides to humans, animals, environment and microbial ecosystems, a legislative framework to reduce the use and impact of pesticides has been applied within the European Union. Different viticulture practices are nowadays applied, namely conventional, integrated pest management (IPM) and organic practices that may impact crop quality and health. In conventional practices, the chemical or synthetic pesticides and inorganic fertilizers are routinely applied. Consequently, the microbial biodiversity, both pathogens and beneficial microorganisms, are affected and an imbalance on the microbial ecosystem is observed. Generally, a greater impact occurs on the fungal communities (Newton et al. 2010; Pinto et al. 2014) and the development of pesticides resistance can often occur (Newton et al. 2010). The IPM management practices allow for the sustainable application of pesticides. Regarding the organic practices, these are highly appreciated but a paradox is installed as copper-based products are allowed for plant protection. This leads a high soil contamination, which in turn affects the soil microbial communities, namely those that are sensitive to copper, and so can cause damage to plants and induce phytotoxicity (Berg et al. 2005; Esparza 2006; Schmid et al. 2011). Indeed, copper is an essential micronutrient, but when present at higher concentrations is toxic for organisms and aquatic environments (Pietrzak and McPhail 2004; Berg et al. 2005). Since copper residues levels are persistent and accumulates on vineyards soils, due to the long-term application (Pietrzak and McPhail 2004), preventive measures should be applied.

The effect of different viticulture managing practices on bacterial (Supplementary Table 3) and fungal (Supplementary Table 4) biodiversity structure has been deeply studied by several authors (Dell'Amico et al. 2008; Lejon et al. 2008; Grube et al. 2011; Schmid et al. 2011; Pancher et al. 2012; Setati et al. 2012; Corneo et al. 2013; Campisano et al. 2014; Martins et al. 2014; Pinto et al. 2014; Fernández et al. 2015; Setati et al. 2015; Vega-Avila et al. 2015). Schmid et al. (2011) compared both endophytes and epiphytes present in different structures of the grapevine phyllosphere of *V. vinifera* cv. Sauvignon Blanc from a vineyard in Austria, and observed that the black fungi *Aureobasidium pullulans,* an ubiquitous plant colonizer, was the prevailing microorganism under organic practices. These results are in accordance with Grube et al. (2011) that identified both *A. pullulans* and *Epicoccum nigrum,* suggesting that these microorganisms are copper-tolerant. Conversely, the basidiomycetous yeast *Sporidiobolus pararoseus* has been associated with the conventional system. Also, the authors observed that both *Cladosporium* sp. and *Alternaria tenuissima* were found on both systems (Schmid et al. 2011). Regarding the bacterial community, in other recent studies the genera *Bacillus* sp. and *Pseudomonas* sp. were detected at both organic and conventional systems, whereas microorganisms from *Staphylococcus* sp. and *Frigoribacterium* sp. genera were exclusively isolated from organic and conventional practices, respectively (Grube et al. 2011). Moreover, it was demonstrated that the epiphytic yeast from grape berries were significantly higher on organical than on conventional practices, and that the viticulture practices impacted the microbial community with special focus for the copper-based products (Martins et al. 2014). In fact, this study elucidated a negative correlation between the copper levels and the microbial communities.

Recently, both bacterial (Campisano et al. 2014) and fungal (Pancher et al. 2012) endophytes associated with *V. vinifera* cv. Merlot and Chardonnay, from organic and IPM practices in Italy, were analysed and differences across the viticulture practices were found, though no significant differences were obtained between grapevine cultivars. Concerning bacterial microorganisms, a dominance of *Mesorhizobium, Caulobacter* and *Staphylococcus* genera was detected in the organic practices, whereas the *Ralstonia, Burkholderia* and *Stenotrophomonas* genera were the most abundant on IPM vineyards (Campisano et al. 2014). Interestingly, the fungal endophytes showed that isolates of *Alternaria* sp, *Epicoccum nigrum* and *Aureobasidium pullulans* were observed on both viticulture practices, while the majority of the species as *Botryosphaeria obtusa*, *Botryosphaeria dothidea, Truncatella angustata, Neofusicoccum parvum* or *Phoma herbarum* were isolated from IPM vineyards. Possible factors explaining such microbial distribution are the application of fungicides on the IPM practices, which shaped the structure of these microbial communities (Pancher et al. 2012). Also, the bacterial communities of the grapevine rhizosphere from Argentinean cultivars, from both organic and conventional systems, were analysed (Vega-Avila et al. 2015) and the organic vineyards showed a major abundance of *Firmicutes, Acidobacteria, Verrucomicrobia* and *Planctomycetes,* while the conventional vineyards were characterized by the presence of *Proteobacteria* and *Bacteroidetes*.

The effect of soil organic status on bacterial and fungal populations in a French vineyards has also been investigated (Lejon et al. 2008), and a differential response of these microbial communities to copper suggested a differential toxicity. Likewise, the bacterial communities in a former vineyard soil, from Italy, was assessed in order to verify the influence of long-term copper contamination on these populations (Dell'Amico et al. 2008). To achieve this, two sites of the vineyard contaminated with different concentrations of copper both higher than the EU recommended limits, were sampled and analysed by DGGE. Globally, this study has showed Firmicutes as the dominant phylum in both types of samples. However, the less copper-polluted sample was dominated by Gram-positive bacteria as *Bacillus megaterium* or *Paenibacillus,* whereas the samples from soils with higher copper levels were dominated by Gram-negative bacteria, namely *Alcaligenes, Sphungobacterium, Brevundimonas* and *Stenotrophomonas* (Dell'Amico et al. 2008).

Exploiting grapevine microbiome for vineyards management

Looking for phytoprotectors at the vineyard for grapevine protection constitutes a step forward for grapevine management, fostering their sustainability. The exploitation of the grapevine microbiome would allow not only to understand the balance between microorganisms and elucidate their positive interactions, but also to identify potential strains with plant benefits that could be applied as biocontrol agents, plant growth promoters or bio-fertilizers. The genome sequencing of these microorganisms will allow for the accession of their biotechnology potential, through the identification of potential genes and, consequently, it will allow for the understanding on the pathways evolved on genomic adaptation, pathogenicity, stress-tolerance and metabolic function, as antimicrobial compounds and enzymes productions (Bloemberg and Lugtenberg 2001; Berg et al. 2013). Further, these phytoprotectors could synthetize bioactive secondary metabolites with particular interest for novel drug discovery, or even to be applied as elicitors for plant defence induction (Compant et al. 2013; Delaunois et al. 2014). Among these metabolites, alkaloids, flavonoids, terpenoids, steroids or even the antibiotics are the most produced drugs for general industry (Guo et al. 2008).

Contrary to pesticides, the application of phytoprotectors microorganisms as biological control agents (BCAs), or their bioactive compounds, encompass several advantages as these microbial inoculants are environmentally safe, show a smaller risk for human and animal health and even for environment, and allow for a reduction of agrochemical inputs. Also, and importantly, BCAs are effective for a targeted activity for plant diseases control, thus allowing a reduced development of pathogen resistance and the balance between microbial ecosystems is preserved (Bloemberg and Lugtenberg 2001; Berg 2009; Adesemoye et al. 2009). The application of BCAs is permitted in both conventional and integrated pest management practices (Berg 2009). However, there are some significant barriers to the entrance of BCAs into the market, such as the registration of a biocontrol agent,

due to the risk assessments, and its long and costly development process. Thus, the application of NGS techniques will be useful to better describe the pathogenicity risks and the regulatory pathways of these potential biocontrol agents (Mercado-Blanco and Bakker 2007; Berg et al. 2013).

Regarding the phytoprotectors of *Vitis vinifera*, they are naturally present at vineyard, where they are well adapted to the stressful conditions of the vineyard's environment, namely high temperature range, UV radiation, intense sunlight, nutrient conditions or even vineyard management. Thus, these microorganisms clearly represent an advantage to use them as phytoprotectors or biological control agents for crop protection against different grapevine pathogens. Replacing pesticides by these alternatives will conserve the land for grape production and guaranty a balanced microbial ecosystem.

Conclusion

The massive exploitation of the natural resources by agriculture and forestry industries has led to an imbalance of the biodiversity, including of the naturally existing microbial communities. To preserve this microbial biodiversity is urgent and imperative as some of these microorganisms are beneficial to plants, and are responsible for several pathways as the nitrogen fixation, solubilisation of phosphate, production of siderophores, or even plant protection against pathogens or plant growth. Thus, unravelling the plant microbiome and the plant-microbial interactions is a very actual challenge which might pave the way to the development of new technological approaches for the improvement of agriculture productivity and sustainability. Altogether, and taking advantage of the NGS approaches, advances in plant microbiome expertise will allow to identify and exploit microorganisms with promising characteristics to plant and to identify new genes and enzymes capable to be use as new strategies for crop protection.

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Compliance with ethical standards

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Cátia Pinto is PhD student working on the natural microbial communities associated with grapevine and on biocontrol of grapevine diseases (with a special focus on grapevine trunk diseases – GTDs) by using beneficial microorganisms.

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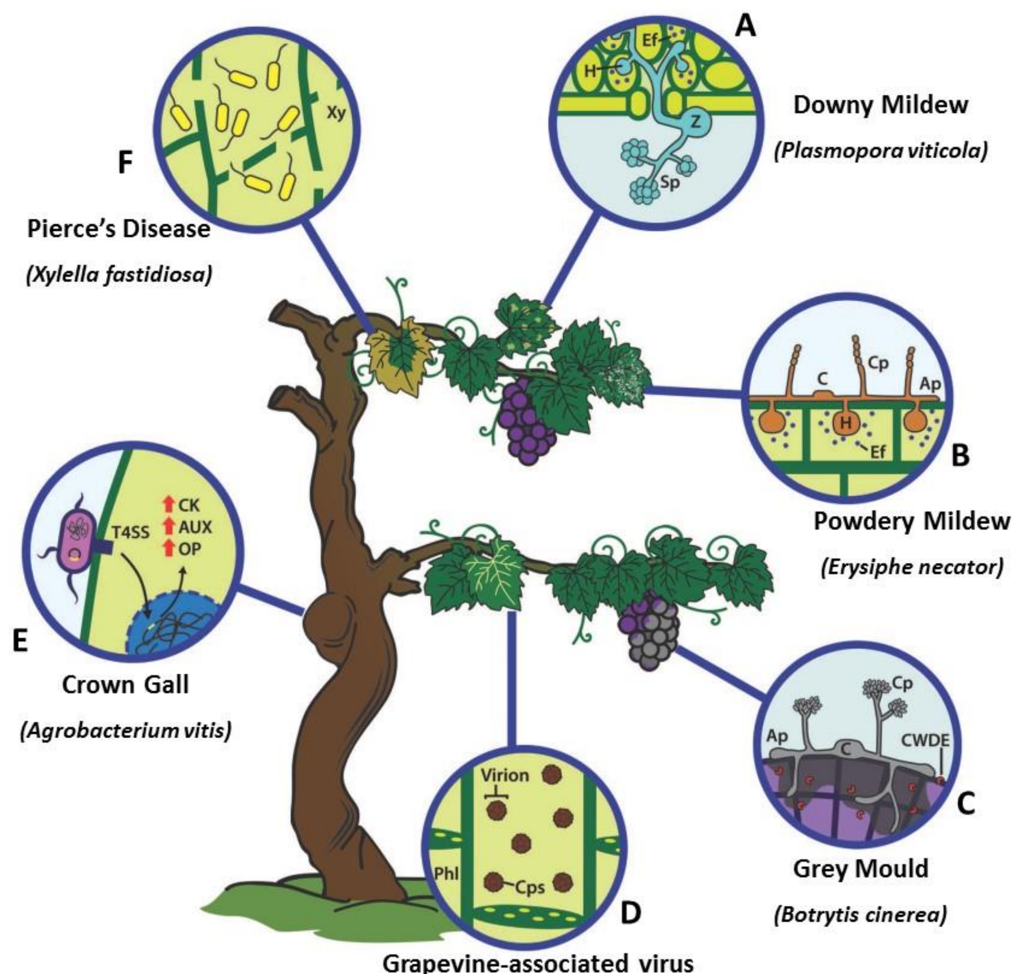

Figure 4: General overview of some important grapevine diseases, infection strategy by pathogens and plant symptoms **(Adapted from Armijo *et al*., 2016).** Grapevine is susceptible to different pathogens attack such as fungi (A, B, C), bacteria (E, F), virus (D) or phytoplasma. Herein are presented the infection strategy and plant symptoms of some of the major grapevine diseases worldwide. The downy mildew (A) disease is caused by the infection of the *Plasmopora viticola* zoospores (Z) through the stoma, followed by the sporangium (Sp) production; the powdery mildew (B) is caused by the *E. necator* when their conidia infect the epidermal layers. Both downy and powdery mildews develop haustoria (H) and secret virulence factors (Ef) into to the plant, manipulating the plant defence response and are biotrophic pathogens; The grey mould (C) infection occurs when the *B. cinerea* conidia (C) germinate and penetrate the plant tissue, followed by a secretion of cell wall degrading enzymes (CWDE) causing a tissue necrosis. Thus, *B. cinerea* is a necrotrophic pathogen; The grapevine-associated virus (D) are microorganisms that live in the phloem (Phl), whose infection can cause different symptoms in grapevine; The crown gall (E) is caused by the bacteria *A. vitis* through the injection of tDNA by the type-IV secretion system (T4SS) that is integrated in the grapevine genome. This infection induces the synthesis of cytokinins (CK), auxins (AUX) and opines (OP); The Pierce's disease (F) is caused by *X. fastidiosa* that grows and accumulates across the xylem vessels (Xy) and is transmitted by insect vectors. Ap – appresorium; Cp – conidiophore; Cps – capside. (Armijo *et al.,* 2016).

1.3. Major grapevine diseases and their impact on plant development and wine production

Grapevine is vulnerable to several pathogens infection and, depending on weather conditions, temperature, growing season, plant genotype and susceptibility of vines or the pathogen location in the plant, grapevine could face different severities of diseases. Indeed, and according to the temperature, a range between 20°C and 28°C is an optimal temperature for pathogens growth and, consequently to disease development.

The disease outbreaks in grapevine can be caused by bacteria, fungi, phytoplasmas or virus and the rapid and accurate identification of these pathogens is important to prevent diseases and their further dissemination. Globally, downy mildew, powdery mildew and grey mould, caused by *Plasmopora viticola, Erysiphe necator* and *Botrytis cinerea*, respectively (Figure 4) are some of the most important worldwide grapevine diseases (Armijo *et al.,* 2016). Grapevine Trunk Diseases (GTDs), Flavescence Dorée (FD) and Pierce's disease (PD) are three most known diseases that have become a major concern and a challenge for wine industry, and for which there are no effective treatments available to control their spread.

The causal agent of downy mildew (*Plasmopara viticola*) is an obligate parasite that cause serious damages on grape clusters of all grape varieties (Figure 4). *P. viticola* can survive in the dead leaves during the winter as oospores or sexual spores until spring where the increasing of temperature (above 10°C), rainfall and humidity will allow the development of the fungi (Carisse *et al.,* 2006). The first symptoms of disease are observed on leaves from 5 to 7 days after plant infection and include yellow circular spots, also called as oil spots. Then, and under favourable conditions, a white downy fungal growth (the sporulation of the pathogen) is observed on the lower leaf surfaces (Carisse *et al.,* 2006). Inflorescences and berries may also be symptomatic and harbour the pathogen. Thus, the higly affected influorescences can become yellow, brown or dry completely and the infected berries will dry and die. Likewise, *Erysiphe necator*, the causal agent of powdery mildew, is an obligate parasite that can survive overwinters as cleistothecia, a structure that contains ascopores or sexual spores (Carisse *et al.,* 2006). Powdery mildew can develop even in the absence of free-water and under warm weather, and the progression of symptoms can be observed on leaves at both upper and undersides, through grey-white mildew that will become dark gray spots overtime (Carisse *et al.,* 2006; Magarey, 2010), and inflorescences and berries could become infected by showing a grey to whitish powder (Carisse *et al.,* 2006). Importantly, those berries infected in later periods are more susceptible for further *Botrytis* infection (Carisse *et al.,* 2006). Regarding grey mould, *B. cinerea* can infect over 200 plant hosts worldwide (Williamson *et al.,* 2007). In grapevine, *B. cinerea* remains overwinter as sclerotia or mycelium in the plant debris until the spring time, where spores can then infect grape tissues (Carisse *et al.,* 2006). The grey mould disease occurs frequently on ripe berries

close to harvest period and rainfalls and high humidity are essential for fungus infection (Viret *et al.,* 2004). The symptoms occur mainly on berries, though it can also occur on leaves, causing necrotic brown spots, and on inflorescences, causing drought or even latent infections that will be visible at the veraison (Carisse *et al.,* 2006). In berries, the spores cover the berries's surface and progressively occupies the entire cluster. Indeed, as in all the other susceptible cultures, *B. cinerea* causes important damage and economical losses in viticulture, by promoting their rot, and leading to an undesirable quality of grapes and wines.

Presently, GTDs are considered the most destructive diseases of grapevine worldwide and their expansion is increasing in all wine-producing countries. The three main GTDs are Esca disease, Botryosphaeria dieback and Eutypa dieback, and all mainly attack the perennial organs of grapevine (Bertsch *et al.,* 2012; Fontaine *et al.,* 2016a). Esca is a complex disease and a major problem in Europe, that result from the activity of different fungi namely, *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum* and *Fomitiporia mediterranea*. Though, *Eutypa lata* and *Stereum hirsutum* may also be involved (Larignon *et al.,* 2009; White *et al.,* 2011). The Botryosphaeria dieback is caused by botryosphaeriaceae species such as *Botryosphaeria dothidea*, *Diplodia seriata*, *Diplodia mutila*, *Neofusicoccum parvum* or *Lasiodiplodia theobromae* (Úrbez-Torres, 2011) and the Eutypa dieback or eutypiosis is mainly caused by the *Eutypa lata* though *Eutypa leptoplaca*, *Cryptovalsa ampelina*, *Diatrypella* sp. or *Eutypella* spp. may be envolved. In the young grapevine decline, Petri disease and Black-foot disease are the most impacting diseases (Gramaje and Armengol, 2011). The Petri disease is caused by *Phaeomoniella chlamydospora* and other species of the genus *Phaeoacremonium,* while the Black-foot disease is caused by *Cylindrocarpon* spp. and *Campylocarpon* spp. (Gramaje and Armengol, 2011). The general symptoms of GTDs in the wood include sectorial or central necrosis, with brown strips or cankers, and the leaves, may present a discoloration or drying (Larignon *et al.,* 2009). In turn, the symtpoms associated with young grapevine decline include an atrophied growth of the plant, reduced vigor, retarded or absent sprouting, shortened internodes, sparse and chlorotic foliage with necrotic margins and dieback, or even sunken necrotic root lesions (Gramaje and Armengol, 2011). The early identification of these diseases is difficult since lesions are inside of the plant and the visible symptoms usually take several years to develop (Fontaine *et al.,* 2016a). Regarding the vine and disease management, the sodium arsenite, used in the effective control of Esca, was banned in 2003 in all winemaking countries due to its environmental and human toxicity (Spinosi *et al.,* 2009). Consequently, nowadays no effective treatments are available and, as result, the OIV established in 2006 a resolution with preventive measures to contain the proliferation of these wood diseases (resolution OIV-VITI 2/2006). Presently, the main challenges to control these diseases focus on the deep study of factors that explain the grape cultivars resistance, to exploit the potential of microorganisms for biological control and to develop other protection products based on natural

compounds (Fontaine *et al.,* 2016b). Considering that pruning wounds are a principle point of entry of pathogens in plant, the development of treatments that focus on this area associated with cultural and sanitation methods are important strategies to control the GTDs spread. Indeed, alternative and innovative solutions are urgently required, since GTDs cause the death of vines on a shorter or long-term and are responsible for high economic costs associated with the replacement of dead grapevines (Fontaine *et al.,* 2016b). In fact, it is estimated that such replacement represents a cost of more than 1.5 billion dollars per year (Hofstetter *et al.,* 2012).

Flavescence Dorée (FD), caused by a phytoplasma and transmitted by the leafhopper *Scaphoideus titanus* Ball*,* a severe grapevine yellow disease and a threat to European vineyards, where the damages result in vines lost (Belli *et al.,* 2010). This is in fact a quarantine disease, firstly reported on Europe in the south-west of France in 1950 and then spreaded to other viticultural regions in France, northern Italy (1964) and neighbouring European countries (Caudwell, 1990; Belli *et al.,* 2010). The symptoms of FD are the same of grapevine yellows diseases, which difficult its differentiation. Overall, symptoms include leaf yellowing or reddening during the summer, desiccation of both inflorescence and bunches, premature leaf fall or presence of black spots in the new canes (Belli *et al.,* 2010). Although infected plants usually die, it has been reported that some may recover, though remaining less productive.

Pierce's disease is a plant quarantine disease caused by the bacteria *Xylella fastidiosa,* which is spread through the grapevine xylem by insect vectors such as sharpshooters (*Homalodisca coagulate*), leafhoppers (*Cicadellidae* family) and spittlebugs (superfamily *Cercopoidea*) that feed on the plant (Figure 4) (Armijo *et al.,* 2016). This bacterium was firstly reported in California in 1892 by Newton Pierce and since then caused serious damages in Calofornia's vineyards, in both cultivated and wild plants (Hopkins, 2005; EFSA, 2015). The symptoms include leaves chlorosis and scorching, wilting and dring of fruits, uneven maturation of canes and delay in bud development and the bacteria multiplication may result in the grapevine death within 1-2 years after the initial infection (Hopkins, 2005; EPPO, 2016). In EU, the *X. fastidiosa* was firstly detected in 2013 on olive trees in the Puglia region of Italy, and then in 2015 was discovered in ornamental plants in France namely, in Corsica island and in the mainland (Alpes-Maritimes) (EFSA, 2015; EPPO, 2016). These focuses are under official control through restricted EU emergency measures (EFSA, 2015). Until now, no records of PD in vineyards were found in EU.

Overall, grapevine diseases cause important damages to both leaves and grapes and are associated with a significant reduction of the plant yield and grape's quality that causes important economic losses in the wine sector. The early diagnostic of symptoms and identification of pathogens and insect vectors are important steps to prevent their dissemination and, thus, the grapevine diseases. However, their precoce detection is sometimes difficult given that some diseases express similar symptoms in plant, and that different diseases can be present at the same time, or even that weather conditions,

nutritional deficiencies or pesticides can produce symptoms similar to the diseases (Carisse *et al.,* 2006). With regard to the pathogens, the better knowledge of the epidemiological development of the pathogens agents and the plant-pathogen interactions are some of the key factors to improve their control.

Publication 2- The effects of grapevine trunk diseases (GTDs) on vine physiology

As referred above, GTDs are the most destructive grapevine diseases worldwide and no currently control is available. Due to the importance of this subject for the wine sector, a review was published in the European Journal of Plant Pathology (Fontaine *et al.,* 2016a) and in which the author of this PhD thesis (Cátia Pinto) was actively involved and wrote the introduction and the impacts of GTDs on berry maturation sections of the publication. Overall, the state-of-the-art of the GTDs and the effects of GTDs on vine physiology (such as trunk, stem, leaves and grapes) are here discussed.

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The effects of grapevine trunk diseases (GTDs) on vine physiology

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Abstract Esca disease as well as Botryosphaeria and Eutypa dieback cause considerable economic problems for vineyards worldwide, and currently, no efficient treatment is available to control these diseases. For these three grapevine trunk diseases (GTDs), the main physiological effects reported concern carbohydrate metabolism and defence responses in the different organs of vine. In the trunk, a depletion of starch reserves in woody tissues is associated with fungal colonization; in the leaves, where pathogens are not present, the carbohydrate metabolism is also affected as revealed by a decline of the photosynthetic rate. A consequence of these disturbances is a lower pool of carbon reserves that might contribute to a decrease of plant development and vigour during the subsequent year. Other metabolic activities such as lipid and amino acid metabolism are down regulated. The perturbation of these primary metabolisms is often associated with the induction of defence responses. The development of biochemical barriers resulting from the accumulation of both tyloses and gummosis is observed during the infection of the wood causing blockage of the xylem vessels and thus limiting the fungal invasion. Their progression in the wood is also inhibited by the formation of polyphenol-rich reaction zones and by the accumulation of pathogenesis-related proteins, and the oxidative burst and the production of reactive oxygen species. Additionally, detoxification processes of the vine are involved; this reaction could be linked to the production of extracellular compounds by GTD agents some of which are phytotoxic. As a consequence, the sensory quality of berries and probably the wine made from these berries decrease. This review presents an overview of the physiological modifications described in vines affected by GTDs.

Keywords Berries · Botryosphaeria dieback · Esca disease · Eutypa dieback · Green stem · Leaf · Trunk

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Introduction

The grapevine (*Vitis vinifera*) is a very important worldwide crop of great economic significance although it is highly subjected to different pathogen attacks that can impact negatively on the plant health status and, consequently, reduce the yield and quality of grapes. Presently grapevine trunk diseases (GTDs) are the most destructive diseases worldwide as there is currently no control. These diseases negatively affect the vineyard heritage and cause serious economic losses in the wine industry. The three main GTDs are Esca disease, Botryosphaeria dieback and Eutypa dieback, which normally attack the perennial organs, at all the stages of grapevine growth.

Infection can be as propagation material or pruning wounds, cause symptoms in woody tissues, on leaves and berries and in a number of cases leading to the vine death (Larignon et al. 2009; Bruez et al. 2014). Generally, symptoms normally appear on at least 7 years old grapevine although symptoms in younger vines are also possible as occurs in the young Esca (Petri disease) or young vine decline (Bruez et al. 2014). Considering a replacement of 1 % of the plants per year, it is estimated that the replacement of the dead plants due to the GTDs is above 1.5 billion dollars (Hofstetter et al. 2012). The impact of GTDs is proportional to the vineyard age and the most severe symptoms appear on the older vineyards (Gubler et al. 2005). The severity and impact of GTDs differ across cultivars, although there are no resistant cultivars to these diseases (Gubler et al. 2005; Surico et al. 2006). GTDs are characterized by the presence of different species of a main pathogen or a complex of causal pathogens that grow within the wood, causing wood discoloration and decay (Bertsch et al. 2013; Bruez et al. 2014; Luque et al. 2014).

The Esca disease is described as a disease complex comprising several syndromes (Surico et al. 2008; Surico 2009; Bertsch et al. 2013) among which 'grapevine leaf stripe disease' (GLSD) and Esca are the most widespread in vineyard. GLSD is a tracheomycotic syndrome mainly caused by *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingfield and L. Mugnai) P.W. Crous and W. Gams and several *Phaeoacremonium* species. Symptoms of GLSD occur both in the woody tissues of trunk and branches, as well as in leaves and berries. The most common wood symptoms (observable in mother vine stocks, rooted cuttings or the trunk and branches of standing vines) comprise several forms of discoloration, among which black wood streaking involving single or several xylem vessels and areas with darkened or brown necrosis circumscribing the pith are most commonly observed (Fig. 1) or longitudinal xylem brown stripes under the bark (Larignon and Dubos 2001; Lecomte et al. 2012). External symptoms of GLSD are characterized by spots that appear between the veins or along the edges of the leaves, and these expand and become confluent to finally result in chlorotic orangy or red and necrotic strips, with only a narrow green stripe along the midrib. The term 'Esca' commonly refers to the white rot occurring in the trunk and branches of mature standing vines and that is caused by *Fomitiporia mediterranea* and/or other basidiomycetes. 'Esca proper', usually encountered in mature vineyards, indicates the co-occurrence of GLSD and Esca on the same vine (Surico 2009; Bertsch et al. 2013). 'Apoplexy', consisting of the partial or complete sudden wilting of the crown, mainly occurs on GLSD and/or Esca proper-affected plants (Surico et al. 2008; Letousey et al. 2010). The disease spread occurs by dispersion of spores by wind and rain. Varieties vary in susceptibility; Tempranillo, also known as Tinta Roriz or Aragonez, is very susceptible to both types of Esca disease (Almeida 2007). Other grape cultivars such as Cabernet Sauvignon, Thompson Seedless, Sauvignon blanc, Mourvèdre, Ugni blanc, Cinsault or Trousseau are more susceptible to Esca proper when compared with Merlot, Pinot Noir, Carignan or Roussane (Edwards et al. 2001; Feliciano et al. 2004; Larignon et al. 2009; Lorrain et al. 2012).

Botryosphaeria dieback is caused by several *Botryosphaeriaceae* species, the most commonly isolated being *Botryosphaeria dothidea*, *Diplodia seriata, Diplodia mutila*, *Neofusicoccum parvum* or *Lasiodiplodia theobromae* (Úrbez-Torres 2011). Botryosphaeria dieback is widely distributed and was described in Australia, Brazil, Chile, Egypt, France, India, Italy, Lebanon, Mexico, Portugal, South Africa, Spain and United States of America (Gubler et al. 2005; Úrbez-Torres 2011). The Botryosphaeria dieback agents are considered as the causal agent of trunk cankers and decline of grapevine. Wood symptoms of the disease consist of brown wood streaking, internal necrotic lesions of the wood, wood discoloration ("brown stripe") in the outer xylem, and perennial cankers (Fig. 2). External symptoms consist in bud necrosis or their mortality, leaf spots, dead arms, shoot dieback and bunch rot, (Larignon 2001; Niekerk et al. 2004; Úrbez-Torres 2011; Spagnolo et al. 2014b). The disease spread occurs with the release of spores in the growing season, which are disseminated by rain and wind. The grape varieties such as Cabernet Sauvignon, Cabernet Franc or Sauvignon Blanc are described to be susceptible to Botryosphaeria dieback, whereas Merlot is less susceptible (Larignon et al. 2009).

Eutypa dieback or eutypiosis is mainly caused by the species *Eutypa lata* that infects grapevine pruning wounds although other microorganisms as *Eutypa leptoplaca, Cryptovalsa ampelina, Diatrypella* sp. or *Eutypella* spp. were also observed on Eutypa dieback-affected plants (Gubler et al. 2005). The infection spread occurs with the release of ascospores by episodes of rain and wind, at springtime (Leavitt 1991). The symptoms

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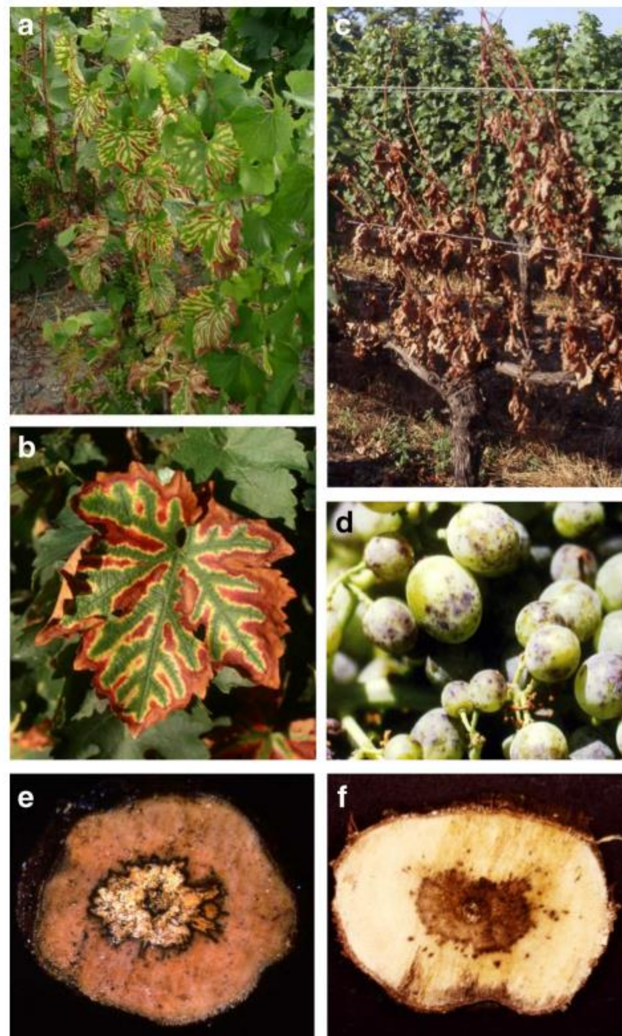

Fig. 1 Disease symptoms of Esca disease in grapevine. (**a**,**b**) typical tiger-like necrosis and chlorosis on leaves. (**c**) apoplexy, characterized by dieback of one or more shoots. (**d**) spotting on grappe berries. (**e**,**f**) cross section of trunk showing white rot (**e**) and black wood streaking. (**f**). Authors of pictures (**a**) Fontaine Florence, and other, Philippe Larignon, IFV

of Eutypa dieback on wounds include a necrotic canker on the wood that starts at the wound and moves across the branch (Gubler et al. 2005; Almeida 2007) (Fig. 3). Stunted and weak growth of shoots is also observed and the leaves are small, chlorotic and with marginal necrosis (Leavitt 1991). The evolution of the disease can lead to the plant death in 3 to 5 years after the visualization of the first symptoms (Almeida 2007). There are differences in varietal susceptibility, the varieties as Cabernet Sauvignon, Chasselas, Chenin, Cinsaur, Mauzac, Muscadelle, Négrette, Sauvignon or Ugni blanc are highly sensitive and Alicante Bouschet, Chardonnay, Gewürztraminer and Jurançon are susceptible. Others such as Cabernet franc, Carignane, Colombard, Duras, Gamay, Malbec, Mourvèdre, Pinot Meunier and Portugais bleu are moderately susceptible and Aligoté,

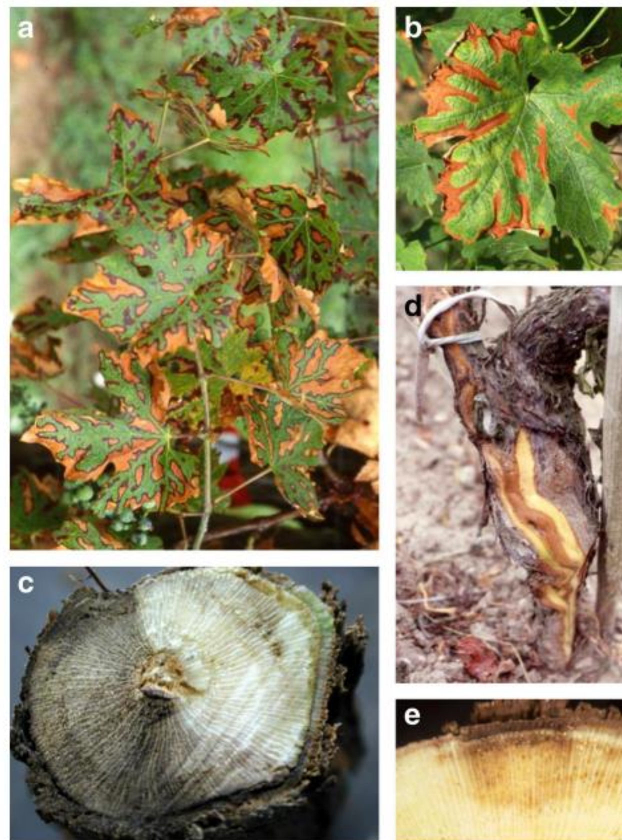

Fig. 2 Disease symptoms of Botryosphaeria dieback in grapevine. (**a**,**b**) typical orange spots on the margins of the leaf. (**c**) cross section of trunk showing a grey wedge-shape canker. (**d**) brown stripe under the bark, which may also be observed in Esca disease. (**e**) cross section of trunk showing brown streaking. Author of pictures is Philippe Larignon, IFV

Merlot, Sémillon, Sylvaner, Grolleau or Petit Verdot are tolerant to disease (Bertsch et al. 2013).

GTDs are widely distibuted over the world. Before 2001, when the application of sodium arsenite was

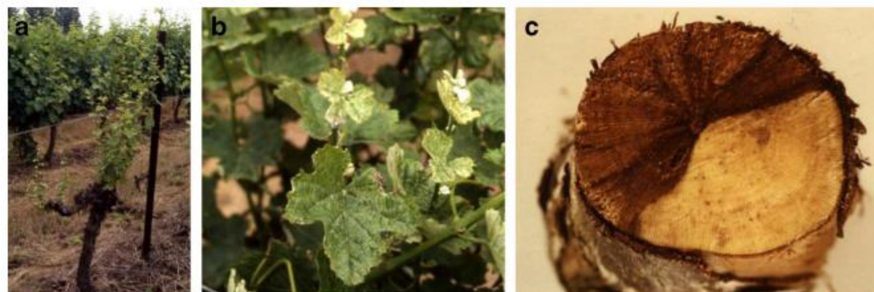

Fig. 3 Disease symptoms of Eutypa dieback in grapevine. (**a**,**b**) stunted shoots and leaves characteristic of Eutypa dieback foliar symptoms. (**c**) cross section of wood showing a wedge of discoloured tissue. Author of pictures is Philippe Larignon, IFV

allowed, the incidence of GTDs, namely Esca disease in Portugal, Hungary, France and Germany was at 20, 11, 15 and 19 %, respectively (Mugnai et al. 1999). Currently, it is estimated that 13 % of the vineyards are unproductive in France (Bruez et al. 2014). In Spain, 7 % of plants infection with GTDs at the Castilla and Léon region is reported in 2006 (Martin and Cobos 2007). In Portugal, the GTDs were reported in the early 1990s and the incidence of GTDs is most important in Vinho Verde, Dão and Alentejo appellations. In Italy, Esca disease impacts different wine regions such as Tuscany, Sicily and Apulia. In general, the average of annual increase of GTDs incidence in Italy is estimated to be 4–5 % (Mugnai et al. 1999). Countries outside Europe, i.e. California, USA, it is estimated that 64 and 55 % of Sauvignon Blanc and Chardonnay are infected with Botryosphaeria dieback, respectively (Gubler et al. 2005). In British Columbia, Canada, a low incidence of Esca (0.2 %) and young vine decline (8 %) was observed (Úrbez-Torres et al. 2014). In Chile, it was observed that amongst 694 wood samples with GTD symptoms, collected from 67 vineyard regions, *P. chlamydospora* (85 %) and *Botryosphaeriaceae* species (56 %), namely *D. seriata*, were the most frequently isolated microorganisms (Diaz et al. 2013). Also, table grapes *Botryosphaeriaceae* species were the most observed and the disease incidence varied between 22 and 69 % according to the vine age (11 to 20 years old, respectively) (Morales et al. 2012). In New Zealand, it was estimated that for 43 vineyards distributed over six wine regions showed 88 % of *Botryosphaeriaceae* species infection and *N. luteum* and *N. parvum* were the most prevalent microorganisms (Baskarathevan et al. 2012). Finally, for China, an emerging country of wine production, vineyards are highly affected specially by Botryosphaeria dieback (Yan et al. 2013).

The earlier diagnostics of GTDs is rather difficult due to the lack of external plant symptoms, as on leaves or berries, or otherwise because of the complexity of symptoms; in some cases it is possible to observe symptoms of more than one of the GTDs in the same plant or to confound symptoms with different GTDs. Furthermore, the symptoms and virulence of some diseases, such as Botryosphaeria dieback, could differ across countries or even in different regions from the same country (Niekerk et al. 2004; Úrbez-Torres 2011). The disease diagnostic is also difficult because a period of latency before the appearance of symptoms after the pathogen attack (Larignon et al. 2009). A recent work of Bruez et al. (2014) analyzing the microbial community present on wood tissues of Cabernet Sauvignon with 10-year-old vines, showed no significant differences between asymptomatic and symptomatic plants foliar Esca disease symptoms.

It has been suggested that the recent and substantial dissemination of GTDs around vineyards is due to changes in the cultural practices namely, the reduction of the sanitary care of vine propagation material and poor protection of pruning wounds (Graniti et al. 2000; Almeida 2007). Others factors such as climate, temperature and periods of rain (humidity), and vine age are known to increase the vine susceptibility to GTD infection. Also, fertile soils, intense fertilization, vigorous rootstock, grapevine cultivar and plant physiology conditions influence the dissemination of GTDs (Graniti et al. 2000; Surico et al. 2004; Surico et al. 2006). Furthermore, the only treatment available to control GTDs previously was based on the application of sodium arsenite. But due to its considerable toxicity and also because it is considered as a carcinogen, this compound was banned in 2001 (Almeida 2007; Larignon et al. 2009). An increase in foliar symptoms was noticed in plants treated with bioactivators or biostimulants, and was probably due to an increase in plant physiological process, as a result of which fungal toxins might be translocated more readily to the leaf (Calzarano et al. 2007; Di Marco and Osti 2009). Inversely, fosetyl Al treatments caused a decrease of foliar symptoms expression correlated with a decrease of photosynthesis and leaf transpiration (Di Marco et al. 2011). Nevertheless, there are presently no available treatments for GTDs control and the current prevention is based on the application of prophylactic measures including trunk renewal and rejuvenative surgery. The developing of new sustainable strategies for vineyard management is urgently required.

In this review, we will focus on the impact of GTDs on vine physiology. Physiological disturbances were firstly described by cytological analysis before studies of transcriptomic, proteomic and metabolomic. In fact, the “omics” approaches lead to a greater understanding by target specific analyses such as the study of the plant gene expression. This review presents the current knowledge reported on the impact of the 3 main GTDs on the berries, leaves, the green stem and the trunk.

Impacts of GTDs on berry maturation

The great majority of studies on berry maturation are related with the Esca disease. It is well suggested that the berry and leaf symptoms due to the Esca infection might be caused by phytotoxic metabolites or other compounds produced by the causal agents that are transported inside the plant to the aerial parts (Mugnai et al. 1999). The studies focus on the impact of Esca disease on berry maturation at harvest period ; and, we could note that at present, few information are available on the early stages of the reproductive development, what areas well as on the effects on the inflorescence development and on the flowering rate.

Esca disease impacts on the normal maturation of berries and also flavonoid metabolism, which is responsible for the production of anthocyanins and tannins (Lorrain et al. 2012). As a consequence, both sugar and phenolic contents and thus the sensory quality of berries and wines decrease (Mugnai et al. 1999; Calzarano et al. 2001, 2004, Calzarano et al. 2008; Lorrain et al. 2012; Bruez et al. 2014). According to Lorrain et al. (2012), who analysed bunches of Cabernet Sauvignon from the Bordeaux region in 2009 and 2010, the presence of Esca disease decreased significantly the skin concentration of catechin, epicatechin and anthocyanins when compared with healthy berries. However, no differences were observed for the total phenol and tannin contents. Moreover, when grapes showed a proportion of Esca >25 %, a decrease of the sugar content of musts and an increase of the titratable acidity, nitrogen assimilation, increase of the mineral levels, resveratrol, tartaric acid and malic acid were observed (Calzarano et al. 2008; Lorrain et al. 2012). The increase of nitrogen is suggested by the increase of amino-acids such as proline, as a response to the plant stress caused by Esca disease infection (Calzarano et al. 2008). Furthermore, the Esca disease-affected grapes reported an activation of the defence genes such as the pathogenesis-related proteins (PR-P) PR-5, PR-10 and the polyphenol oxidase (PPO), when compared with the control (Pasquier et al. 2013). Esca disease-affected grapes are subject to an oxidative stress that impacts proteins as aldehyde dehydrogenase 2 (ALDH2) and cysteine synthase which in turn influence the synthesis of compounds with oenological interest (Pasquier et al. 2013). Considering the severe form of GTDs Esca, the impact on the epidermis of the berries is characterized by dark and purple spots until maturity and even can be transformed some cases turning into necrosis (Larignon and Dubos 1997; Almeida 2007). Different coloured spots may occur on berries and the incidence of symptoms could differ across grape clusters and affect only some berries or different cluster of berries. The spots on berries could be explained by the diffusion of different enzymes or toxins (White 2010; Andolfi et al. 2011; Abou-Mansour et al. 2015) through the vascular system (Mugnai et al. 1999). Moreover, the grape symptoms could appear without any symptoms expression on leaves (Leavitt 1991; Mugnai et al. 1999).

Botryosphaeria dieback can lead to fruit rot and drop (Úrbez-Torres 2011). According to the symptomatology of disease, either the mild or the severe form; with the mild form it wilting of the inflorescence and grape clusters has been observed, whereas the severe form is characterized by the drying of inflorescences and grape clusters or a complete death of the shoots (Larignon et al. 2001). For Eutypa dieback, berries are a smaller size and they ripen unevenly (Bertsch et al. 2013).

Physiological modifications on leaves

On leaves, the studies to date focus mainly on both Esca and Eutypa dieback. For Esca disease, leaf symptoms can occur in mild or severe forms (Chiarappa 1959; Larignon and Dubos 1997; Mugnai et al. 1999; Bertsch et al. 2013). In the mild form, the leaves at the beginning of summer, at pea size stage, present chlorotic interveinal areas that later become necrotic. The severe form corresponds to a sudden leaf wilting within a few days. In the case of the mild form, Esca greatly affects grapevine physiology already in symptomatic leaves (Felgueiras et al. 2007; Petit et al. 2006). Foliar symptoms are associated with both stomatal closure and alteration of the photosynthetic apparatus as revealed by: (i) a decrease in CO_2 assimilation, a decrease of the transpiration, and a significant increase in intercellular CO_2 concentration; (ii) a strong drop in both the maximum fluorescence yield and the effective Photosystem II quantum yield; and (iii) a reduction of total chlorophyll, but a stable carotenoid content (Petit et al. 2006; Magnin-Robert et al. 2011). A similar decrease of chlorophyll content and fluorescence was described in symptomatic leaves of *in vitro* plants infected with Pch or *Phaeoacremonium angustius* (Santos et al. 2005). Moreover, a gradual decline of net photosynthesis (P_n) was observed in the asymptomatic leaves of

symptomatic Esca canes (Petit et al. 2006; Magnin-Robert et al. 2011). Indeed, the intensity of gas exchange alteration is correlated to foliar symptoms development. No alteration of photosynthetic parameters was detected in asymptomatic canes. Conversely, within symptomatic ones, a slight decline of photosynthesis and stomatal conductance is observed in leaves without symptoms, whereas leaves with chlorosis symptoms are more strongly affected. Alteration of the photosynthetic apparatus could be detected two months before the appearance of foliar symptoms as observed in Cabernet sauvignon (Christen et al. 2006). The reduced photosynthesis could be due to lower activity of the photosynthetic enzymes such as carbonic that anhydrase catalyses the conversion of CO_2 to HCO_3, or enzymes used in the breakdown of the photoassimilates (Baker et al. 1997). In accordance with a decline in P_n, anatomical studies highlighted damage of the organelles and a decrease in starch grains in asymptomatic leaves of symptomatic canes. In the green parts of symptomatic leaves, strands of less dense cytoplasm separated the large translucent areas of the cells. Plastids contained small starch grains and underdeveloped grana, and thylakoids were elongated. Consequently, these damages were accompanied by a decrease in carbohydrates (Lima et al. 2010). Additionally, the damaged intracellular structures were more extensive in the chlorotic parts of the symptomatic leaves, as the tonoplasts were disrupted (Valtaud et al. 2009a). In addition to the photosynthetic apparatus alteration, defence mechanisms were also affected especially the glutathione pool, defence proteins, such as PR-proteins, and phenolic compounds (Valtaud et al. 2009a). An accumulation of resveratrol and other phenolic compounds was observed in asymptomatic leaves, which then increased during symptom emergence (Lima et al. 2010; Valtaud et al. 2011; Magnin-Robert et al. 2011; Lambert et al. 2013; Calzarano et al. 2014). Altogether, these observations indicate (i) alterations of the leaf cells occur before the development of visible symptoms and (ii) a rerouting of carbon and energy from primary to secondary metabolism for symptomatic leaves.

Water transport could be also hampered in Esca-diseased plants through xylem dysfunction. Apoplexy expression is often correlated to an excess of water in the soil combined with hot weather, leading to a dramatic imbalance between transpiration (stomatal opening) and absorption (Surico et al. 2006). The involvement of water stress has been investigated in several studies (Marchi et al. 2006; Edwards et al. 2007a,b). Several tools could be used to estimate drought influence, such as chlorophyll fluorescence measurement (Christen et al. 2006), thermal imaging (Grant et al. 2007; Möller et al. 2007), or stomatal conductance measurement (Edwards et al. 2007a,b). In vineyards, a considerable decline of both gas exchanges and water use efficiency was observed in visually healthy leaves of GLSD-affected grapevines 7 days before an apoplectic event. Additional analysis indicated that photosynthesis disturbance was mainly due to non-stomatal factors because the stomata closure decreased as internal leaf CO_2 concentrations increased (Letousey et al. 2010). In contrast, Edwards et al. (2007b,c) observed an increase of the leaf stomatal conductance, which led directly to a water deficit (estimated by lower water potentials) in response to *P. chlamydospora* infections in 3-year-old potted grapevines that were maintained in greenhouse conditions. A comparison of transient fluorescence in Esca-affected and drought-stressed plants revealed two different functional behaviour patterns of the photosystem II, suggesting that GLSD infection cannot simply be interpreted as a water deficit (Christen et al. 2007; Letousey et al. 2010). In the meantime, significant declines in chlorophyll fluorescence and photosynthesis-related gene expression in leaves were also observed 7 days before the apoplectic event (Letousey et al. 2010).

Leaves of Eutypa lata-infected vines are chlorotic, cupped and tattered with marginal necrosis and dead interveinal tissue (Möller et al. 1974). Anatomical studies show an alteration of the ultrastructure of the tissue, implying chloroplast degradation, cytoplasm lysis, and endomembrane breakdown for the severely affected leaves (Philippe et al. 1992; Valtaud et al. 2009b). Moreover, Camps et al. (2010) reported that the most abundant genes that were regulated during the asymptomatic phase were associated with energy metabolism, especially with the light phase of photosynthesis. The structural and molecular decline of the photosynthetic system can be responsible, at least partly, for the death of the plant. In a complementary way, Eutypa dieback leads to a lowering of the water content and to an accumulation of abscisic acid in the leaves (Koussa et al. 2002); and a decline of specific free polyamines (Rifai et al. 2005). These changes may lower the permeability of the membranes and, as a consequence, modify exchanges with the environment, which in turn possibly intensifies the dehydration of the infected

leaves during their development (Koussa et al. 2002). The limitation of exchanges results in a stomatal closure, in a higher concentration of abscisic acid in the guard cells and in an alteration of vascular tissues of the infected plants.

Perturbations on green stems

Whether green shoots of a diseased plants exhibit symptoms or not, carbohydrate reserves in these organs are reduced during the winter rest as a consequence of the impairment of total leaf photosynthesis rate during the previous vegetative season. The following year, the lower pool of reserves might contribute to a significant decrease in plant growth, as well as an overall loss in plant vigor (defined as number of buds, number of inflorescences, fruit set, and grape quality), (Petit et al. 2006). Structural modifications in green stems of plants affected by GLSD or Eutypa dieback have also been documented (Fleurat-Lessard et al. 2013). These authors reported the occurrence of three out of four walls of the scheme of COmpartmentalization of Decay in Trees (CODIT, Shigo 1982). They also observed cambium damage and modification of vascular member differentiation as well as the presence of highly damaged suberized sheets during the vegetative season. It was hypothesized that suberin deposition, which normally is elicited by a fungal attack, could be directly or indirectly modified by fungal metabolites in the stems of grapevine affected by GLSD or Eutypa dieback (Fleurat-Lessard et al. 2013).

The most accredited hypothesis to explain the appearance of GTD foliar symptoms considers the translocation of fungal toxic metabolites from woody tissues to the foliage via the green stem (Andolfi et al. 2011; Bertsch et al. 2013; Abou-Mansour et al. 2015). With the attempt to retrieve further information about physiological changes occurring in grapevines affected by trunk diseases, Spagnolo et al. (2012) performed a two dimensional electrophoresis (2-DE)-based proteomic study on green stems of apoplectic and Esca proper-affected plants. Important proteome variations specifically related to apoplexy or GLSD symptoms were observed for proteins involved in primary metabolism and energy, stress response and defence response in symptomatic and asymptomatic stems of diseased plants. The catabolic ATP synthesis seemed to be especially increased in stems of diseased plants as proteins involved in cellular respiration like succinate dehydrogenase, pyruvate decarboxylase and cytosolic malate dehydrogenase were found to be over accumulated. This is consistent with the fact that carbohydrate metabolism plays an important role in the activation of plant defence response (Rojas et al. 2014; Bolton 2009), which is a cost-intensive mechanism (Berger et al. 2007). Proteins of the antioxidant system were also differentially expressed. Among them, there was a gluthatione S-transferase (GST phi class), which was over accumulated in stems from diseased plants. Up-regulation of the gene encoding for GST tau (*GST1*) was reported by Letousey et al. (2010) and Magnin-Robert et al. (2011) in leaves of apoplectic and GLSD-affected plants, respectively. Valtaud et al. (2009) emphasised the importance of leaf glutathione metabolism on GLSD-affected plants and observed the over accumulation of mRNAs encoding GSTU1 and GSTF2, as well as of the correspondent proteins before the appearance of GLSD symptoms. Considering the importance of glutathione in plant stress responses and its protective role to oxidative bursts (Kmives et al. 1998), a role of the GST system in the direct detoxification of fungal metabolites and/or in the cellular detoxification after the oxidative burst related to it, was proposed (Frova 2003; Valtaud et al. 2009a; Spagnolo et al. 2012). On the other hand, the down accumulation of two other enzymes of the antioxidant system in symptomatic stems of Esca proper affected plants, namely superoxide dismutase and ascorbate peroxidase (Spagnolo et al. 2012), could indicate that the grapevine antioxidant system, as well as the accumulation of other stress-related proteins, is insufficient or not completely efficient in counteracting the oxidative stress linked to the appearance of GLSD symptoms. In the same study, Spagnolo and co-workers also found two pathogen related (PR)-5 family proteins, namely thaumatin- and osmotin-like (van Loon et al. 2006) to be abundantly expressed in diseased plants. Up-regulation of genes encoding for thaumatin- and osmotin-like proteins were reported in leaves of rooted cuttings artificially infected with the main Eutypa dieback agent *Eutypa lata* (Camps et al. 2010). Members of the PR-5 family are known to have antifungal (Monteiro et al. 2003) and anti-oomycete (Colditz et al. 2007) properties, and may also have function in plant resistance (van Loon et al. 2006; Kortekamp 2006). Relative to their antifungal activity, the increased synthesis of thaumatin- and osmotin-like in stems of apoplectic and Esca proper-

affected plants could either be the result of the translocation of toxic fungal metabolites from woody tissues to the foliage via the transpiration stream (Andolfi et al. 2011; Bertsch et al. 2013; Abou-Mansour et al. 2015), or mean that fungal propagules are effectively present within symptomatic stems (Spagnolo et al. 2012) although no isolation of GTD agents from green stems or leaves of naturally infected field-grown grapevines has been reported so far. Finally, two small heat shock proteins were down-accumulated in symptomatic stems of Esca proper-affected plants. Among heat shock proteins (HSPs), small HSPs (smHSPs) are regarded as molecular chaperones accumulating in response to stresses, especially heat (Waters et al. 1996; Yang et al. 2011), by binding partially denatured proteins for preventing irreversible protein inactivation and aggregation (Waters et al. 1996). Small HSPs may act as molecular chaperones, resulting in the maintenance of cellular conditions suitable for inducible plant defence responses (Maimbo et al. 2007) and there are reports indicating their involvement in response to various abiotic or biotic stresses (Al-Whaibi 2011). HSPs function may extend beyond their chaperone activity, limiting the damage that results from ROS accumulation (Gurley 2000). Therefore, the decreased accumulation observed by Spagnolo et al. (2012) could depend from the same cause provoking the impairment of the antioxidant system. In another two dimensional electrophoresis (2-DE)-based proteomic study, Spagnolo et al. (2014a) looked at identifying the most highly sensitive period of grapevine to the Botryosphaeria dieback agents *N. parvum* and *D. seriata* after artificial infection on green stems of field-grown vines. Among the three phenological stages considered, namely G stage (separated clusters), flowering and veraison, the flowering was the period of highest sensitivity possibly as consequence of the high metabolic activity oriented towards developing inflorescences (Lebon et al. 2008; Petit et al. 2009). Indeed, a general trend of down accumulation of stress- and defence-related proteins was observed in stems inoculated with *N. parvum* or *D. seriata*, while strongest responses to the infection were activated during the G stage through the over accumulation of primary metabolism proteins, stress- and defence-related proteins. Results of pathogenicity tests performed in the same study corroborated those of the proteomic analysis, as lowest and highest mean lesion lengths were recorded for the G stage and the flowering, respectively.

Differential changes in several metabolisms according to the discolouration in trunk

The first extensive study relying on physiological variations occurring in the trunk wood of grapevines affected by trunk diseases was recently performed by Magnin-Robert et al. (2014). Using a (2-DE)-based proteomic approach, these authors described changes occurring at proteome level in the black streaked or asymptomatic trunk wood of apoplectic or Esca proper-affected standing vines. Results of that study highlighted that proteome changes were more related to the wood symptom than to external symptoms (GLSD or apoplexy). This observation was enabled by the fact that plants without foliar symptoms (control plants) showed the same trunk wood discolorations observed in symptomatic plants. However, proteome variations linked to the presence of foliar symptoms were also noted. In the meantime, results from fungal isolation indicated that inoculum of GLSD agents in symptomatic wood of diseased plants was likely greater than in the symptomatic wood of control plants. This gave confirmation of the relationship between wood discolorations and the expression of foliar symptoms, which in turn represents an important association between fungal pathogens isolated from woody tissues and foliar symptom development. As for green stems (Spagnolo et al. 2012), the differentially expressed proteins documented by Magnin-Robert et al. (2014) belonged to primary metabolism, stress tolerance and defence response. Proteins of the phenylpropanoid pathway (chalcone flavone isomerase, isoflavone reductase, leucoanthocyanidin dioxygenase), antioxidant system (peroxidase, superoxide dismutase, glutathione S-transferase) and several PR-proteins were among those differentially accumulated. Phenylpropanoid compounds have a role in plant defence and their functions range from preformed or inducible physical and chemical barriers against infection to signal molecules involved in local and systemic signalling for defence gene induction (Dixon et al. 2002). The activation of the antioxidant system serves to protect plant cells against ROS which are generated during the HR of plant defence (Tuzun and Somanchi 2006). Other proteins found to be differentially expressed in the work of Magnin-Robert et al. (2014) were PR-proteins such as thaumatin-like protein, endochitinase, β-1,3-endoglucanase, PR-10 and PR-17 (van Loon et al. 2006). Setting aside thaumatin-like protein, whose

functions are described in the previous paragraph, endochitinase and β-1,3-endoglucanase represent the most important PR families induced in response to interactions with fungi (Colditz et al. 2007). These PR proteins exhibit inhibitory effects on the growth of fungi while depolymerising polysaccharides of mycelial walls (Colditz et al. 2007). Over expression of all the above proteins was especially observed in the black streaked trunk wood of diseased plants, clearly indicating that a defence response, strictly related to the presence of fungal pathogens, is activated in woody tissues and this is more intense in plants showing foliar symptoms. On the other hand, similar to what was observed in green stems (Spagnolo et al. 2012), smHSPs were found to be over accumulated in the asymptomatic wood and down accumulated especially in the black streaked wood of Esca proper-affected plants. This result is another indication about the possible role of smHSPs in plant resistance (Yang et al. 2011). The related hypothesis raised by Magnin-Robert et al. (2014) was that the accumulation of these proteins is impaired by an excessive oxidative stress condition associated to the disease (see previous paragraph). All together, these results led the authors to conclude that quantitative and/or qualitative proteome alterations in the black streaked wood of plants expressing foliar symptoms are not enough for avoiding their appearance. On the other hand, those proteins over regulated in asymptomatic wood could be regarded as the limiting factor in symptomatic wood for avoiding foliar symptom development.

Although no grapevine variety is known at present to be resistant to GTDs, a disease susceptibility ranking based on the percentage of foliar symptom expression was suggested for some cultivars (Bruez et al. 2013; Murolo and Romanazzi 2014). With the attempt to observe the physiological changes eventually related to the different susceptibility, Spagnolo et al. (2014b) performed another (2-DE)-based proteomic study aimed at exploring the variations occurring in the trunk wood of three grapevine cultivars: 'Chardonnay' (less susceptible), 'Mourvèdre' and 'Gewurztraminer' (more susceptible) (Grosman and Doublet 2012). Notably, the attention was drawn to the wood discoloration considered as being strictly related to Botryosphaeria dieback, namely the brown stripe (Larignon et al. 2001; Bertsch et al. 2013; Spagnolo et al. 2014b). Contrary to the black wood streaking (Magnin-Robert et al. 2014), brown stripe is solely found in plants showing foliar symptoms, thus samples from control plants consisted of only asymptomatic wood whereas samples from diseased plants consisted of both brown stripe and asymptomatic wood. Several PR proteins (PR2, PR5, PR17) and other defence-related proteins like polyphenol oxidase (PPO) were found to be more abundant in the brown striped wood of the three cultivars. PPO is wound-inducible and is involved in plant resistance (Thipyapong et al. 2007) through the production of phytoalexins, phenols, and lignins (Constabel et al. 1995; Thipyapong et al. 1995). However, other defence- and stress-related proteins (s-adenosylmethionine synthetase SAMS), major latex protein (MLP), PR10, smHSPs and one member of the antioxidant system (SOD) were down accumulated in the brown striped wood. SAMS, which was also found to be down accumulated in symptomatic green stems as well as in the black streaked trunk wood of apoplectic and Esca proper-affected vines (Spagnolo et al. 2012; Magnin-Robert et al. 2014), produces S-adenosylmethionine (SAM). SAM is the major methyl group donor in plants (Deytieux et al. 2007) and lead to the biosynthesis of polyamines (Roje et al. 2006) and ethylene (Tsunezuka et al. 2005). Polyamines participate in responses to biotic and abiotic stresses (Hussain et al. 2011). Ethylene, a stress hormone, plays a role in plant disease resistance pathway (Tsunezuka et al. 2005; Arimura et al. 2002). It is therefore reasonable to hypothesize that an ethylene- and/or polyamine-mediated response was limited in these cases by the decreased expression of SAMS. In the same study, Spagnolo et al. (2014b) quantified total phenolic and stilbene compounds in asymptomatic and brown striped trunk wood. It has been reported that levels of phenolic compounds increase in the discoloured wood of Esca disease-affected grapevines (Agrelli et al. 2009; Amalfitano et al. 2000, 2011) and a number of studies suggest that phenolic compounds, and especially stilbenes, could play a role in limiting the development of fungi in grapevine wood (Lambert et al. 2012 and references therein, Lima et al. 2012). In the study of Spagnolo et al. (2014b), levels of total phenolics were found to be the highest in the brown striped wood of the three cultivars while some difference was noted when considering the specific stilbenes. When looking at the different proteome changes in the brown striped wood depending of the cultivar (Spagnolo et al. 2014b), the primary metabolism seemed to be particularly impaired in Chardonnay. In 'Gewurztraminer', the glycolysis and citrate cycle pathways seemed to be over regulated while a deficiency of the antioxidant system and an over regulation of some amino acid metabolism appeared to occur in 'Mourvèdre'.

The authors concluded that: i) the low abundance of some defence- and stress-related proteins in the brown stripe probably contributes to make the global response insufficient to avoid the brown stripe as well as foliar symptoms of Botryosphaeria dieback; ii) the different susceptibility of the three cultivars could be explained, at least in part, by the diverse expression of various proteins involved in the primary metabolism, defence or stress response, as well as the accumulation of specific stilbenes.

Different responses associated to the susceptibility of grapevine cultivars to GTD were also reported by Lambert et al. (2013). These authors observed an earlier and stronger activation of defence response, particularly with regard to induction of the phytoalexin pathway genes *PAL* and *STS*, and a higher accumulation of stilbene compounds and some PR proteins for two cultivars less susceptible to Esca disease ('Merlot and 'Carignan') as compared to a susceptible one ('Cabernet Sauvignon'). Still, after a detailed comparative morphological and anatomical study on lignified stems from three grapevine cultivars Pouzoulet et al. (2014) advanced the hypothesis that susceptibility to Esca disease could depend from vessel dimension. Merlot, the most tolerant cultivar, showed the lowest mean vessel diameter, whereas 'Thompson Seedless', the most susceptible, showed the greatest mean vessel diameter. 'Cabernet Sauvignon', the intermediate cultivar in terms of susceptibility, showed an intermediate vessel diameter value. The authors proposed that plants carrying vessels of small diameter like Merlot, might be able to restrict the spread of toxins and bud cells in a quicker and more efficient manner than plants carrying wider vessels like 'Cabernet Sauvignon' and 'Thompson Seedless' through a more rapid and efficient plugging of infected vessels by tyloses and gels.

Conclusion

Characterizing the impact of GTDs on grapevine physiology is a key step for understanding the mechanisms that lead to disease development and the appearance of symptoms, and thus the development of effective strategies to control GTDs. GTDs affect the main organs of vines including trunk, green stems, leaves and berries (Fig. 4); no information on their effect in the inflorescence development and the root system is available. In

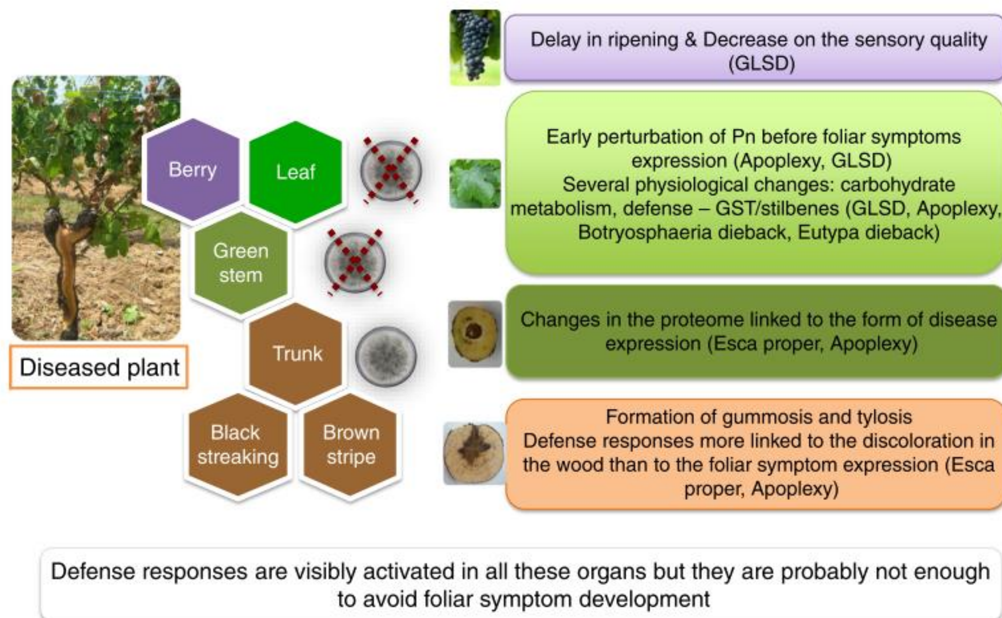

Fig. 4 Summary of the main physiological modifications in organs of grapevines affected by Esca disease, Botryosphaeria dieback or Eutypa dieback

the trunk inhabited by the pathogens, carbon metabolism seems to be strongly affected and is associated with an induction of stress/defence responses. These latter tend to strengthen the plant cell wall, maintain the osmotic and redox balance, destroy the fungal cell walls and resist pathogen infection. In green stems and leaves, several modifications of both primary and secondary metabolisms were also reported. As a consequence of all these perturbations, both sugar and phenolic content and thus the sensory quality of wine decrease. We could underline that the responses of vine differ according to the different foliar symptoms, as well as to the cultivars. Over the last 10 years, a huge information was obtained on the impact of GTDs on vine physiology, but the relationship between the xylem-inhabiting fungi, their production of extracellular compounds and the foliar symptoms emergence still remain unclear and need to be further investigated.

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1.4. Plant infection strategies and mechanisms of grapevine defense

In phytopathology, the development of a plant disease is conditioned not only by the presence of a pathogen, but also by a susceptible host and a favourable environment (Figure 5). Likewise, the time element is absolutely required for a plant disease scenario and for this reason, this factor should also be considered. This triangular relationship is referred as the disease triangle (Stevens, 1960; Francl, 2001) and the control and mitigation of one of these factors results in the effective prevention of the diseases (Francl, 2001). Even though the environment-host-pathogen interactions are complex, this conceptual model can be used to predict plant diseases outcomes.

Changes of environmental conditions, such as climate or precipitation, impact the physiology of the host and provide conditions for a greater or lower development of pathogens (Grulke, 2011). In turn, host and pathogen interact between them (Figure 5) and both are somewhat flexible to these environmental conditions (Grulke, 2011).

Plant pathogens have specific infection strategies and lifecycles, and can be classified such as necrotrophics, biotrophics and hemibiotrophics. The necrotrophic pathogens obtain nutrients from necrotic or death plant tissues, promoted by the secretion of lytic enzymes and phytotoxins, while the biotrophic microorganisms obtain nutrients from living plant tissues. The hemibiotrophic microorganisms can act as biotrophic pathogens in early stages of infection which then evolve to necrotrophic (Glazebrook, 2005; Armijo *et al.,* 2016).

Among grapevine pathogens, *B. cinerea*, responsible for the grey mould, is an example of a necrotrophic microorganism (Williamson *et al.,* 2007), whereas the powdery mildew and downy mildew, caused by *E. necator* and *P. viticola,* respectively, are obligate biotrophic microorganisms. Considering the necrotrophic *B. cinerea,* this is a variable and ubiquous fungus, who can live as a parasite in green tissues or as a saprophyte in dead tissues (Armijo *et al.,* 2016), and can infect grapevine by a direct penetration of the mycelium through skin pores or grape damages, or by an invasion through the flowers receptacle in which the pathogen is in a latent state until the berry ripening (Viret *et al.,* 2004). Overall, the conidial germination of *B. cinerea* requires the contact with a solid surface for nutrient acquisition followed by the development of the appressorium structure that secrets lytic enzymes, such as cutinases and lipases, to allow the penetration of *B. cinerea* (Figure 4). Then, inside of the plant tissues occurs an oxidative burst and secretion of cell wall degrading enzymes (CWDE) including pectinases such as exo – and endo-polygalacturonases, pectin methylesterases, pectate lyases, cellulases and hemicellulases, that together with the production of oxalic acid that causes the decomposition and necroses of tissues (Kars *et al.,* 2005; Williamson *et al.,* 2007; Armijo *et al.,* 2016). The biotrophic powdery mildew, *E. necator*, is an obligate fungus, who depends on the photosynthetic tissues of grapevine (Qiu *et al.,* 2015). Thus, a conidiospore germinates on the

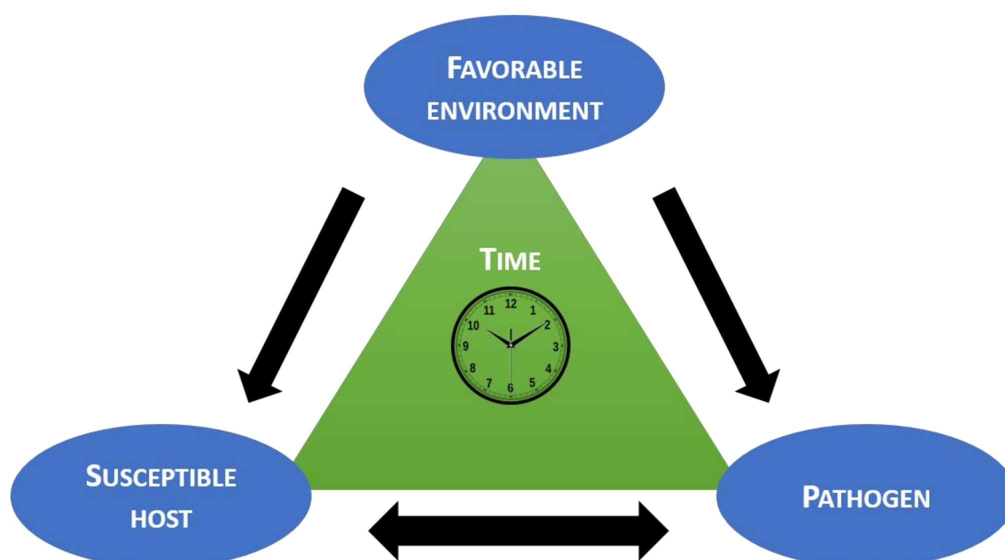

Figure 5: The plant disease triangle **(Adapted from Francl 2001 and Grulke 2011).** Inter-relationship of the three causal factors of diseases: favourable environment, susceptible host and pathogen. Though, as time is absolutely required for a plant disease scenario this factor is also considered by plant pathologists. Indeed, environmental conditions impact the physiology of the plant host (by changing the magnitude of plant growth and fitness including the quantity and quality of plant tissues and plant responses to pathogens) and provide optimal or slighter conditions for pathogen's development. In turn, pathogens are dependent on the nutritional conditions of the susceptible host and on auspicious conditions for the pathogens attack; and the performance of the plant host can improve the plant response against pathogens through the plant resistance, tolerance and defence response (Grulke, 2011).

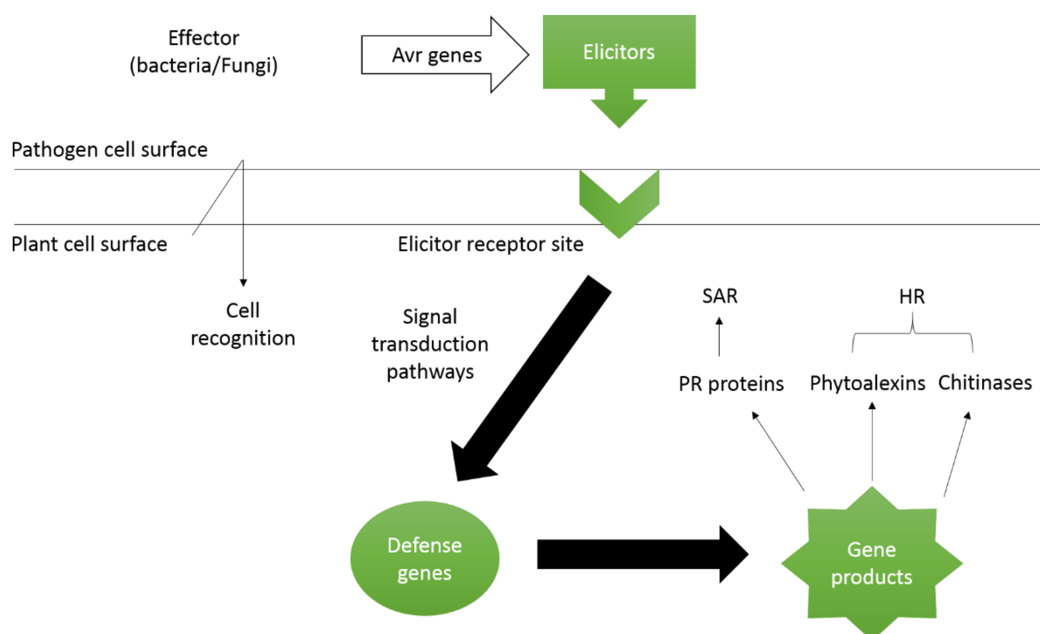

Figure 6: General overview of the primary immune response of plant under a pathogen attack **(Thakur and Sohal, 2013).** Generally, the chemical defense in plants is activated by elicitors that leads to the gene production such as PR-proteins that are responsible for the systemic acquired resistance (SAR) or phytoalexins and chitinases that activate the hypersensitive response (HR).

epidermis of such tissues to form the appressorium, which then penetrates the cell wall and the epidermal cell of the plant to form the haustorium (Figure 4). This structure promotes the molecules exchange between the pathogen and the host cells, where the pathogen both acquires nutrients (such as hexoses, amino acids, vitamins) and secrets proteins to suppress the plant defense response (Qiu *et al.,* 2015). The success of this infection leads to the spread of the pathogen via hyphae across the surface, with production of more appressoria and haustoria structures.

The first barrier to pathogen attacks is the grapevine cell wall. This structure is very heterogenous and composed by interconnected structures of polysaccharides (cellulose, hemicellulose and pectin), proteins and polyphenols. In response to the pathogens attack, plant promotes a down-regulation of photosynthesis and a down- or up- regulation of primary metabolism, essential for the plant growth and development (Rojas *et al.,* 2014), and activates defense mechanisms, through an innate immune system of each cell, under a systemic signal (Jones and Dangl, 2006). Generally, after the pathogen recognition via elicitor molecules (Figure 6), the defense response includes the production of antimicrobial metabolites or proteins (such as phytoalexins), the synthesis of defense enzymes, the accumulation of pathogenesis-related (PR) proteins, the production of ROS, and the production of callose and lignin to reinforce the plant cell wall structure (Glazebrook, 2005). However, the defense responses must be regulated in an appropriated way as its activation requires an abundant supply of energy, causing deleterious effects on the plant growth (Glazebrook, 2005; Rojas *et al.,* 2014).

Overall, the plant immune system (Figure 6) can rely on the use of transmembrane pattern recognition receptors (PRRs) that detect directly the microbial – or pathogen- associated molecular patterns (MAMPS or PAMPs), and on a gene-for-gene recognition using polymorphic nucleotide binding – leucine rich repeat domains (NB-LRR) products, which are encoded by resistance (R) genes (Jones and Dangl, 2005; Zipfel and Felix 2005). The former strategy acts within the cell and is only effective against the biotrophic and hemibiotrophic pathogens (Glazebrook, 2005), as the plant defence response results in the cell death.

The MAMP-triggered immunity (MTI) defence responses occur through signalling actions that include ion fluxes, mitogen-activated protein (MAP) kinase cascade activation and ROS production (Farace *et al.,* 2015). MAMPs from bacteria include flagellin, EF-Tu, peptidoglycans, lipopolysaccharides (LPSs) and rhamnolipids (RLs), whereas from fungi include chitin and elicitins (Farace *et al.,* 2015; Fesel and Zuccaro, 2016). Thus, the stimulation and activation of plant primary immune response occur from the action of elicitors (Figure 6) which are equivalent to PAMPs (Zipfel and Felix, 2005) and include compounds such as proteins, glycoproteins, glycans, lipids and synthetic molecules (Garcia-Brugger *et al.,* 2006; Thakur and Sohal, 2013). According to their origin and molecular structure, the elicitors are classified as physical or chemical, biotic or abiotic, complex or defined (Thakur and Sohal, 2013) and are either constituents of the pathogen or released from the

plant or pathogen cell walls by hydrolytic enzymes (Garcia-Brugger *et al.,* 2006). Among them, the most studied elicitors are the oligogalacturonides, chitosan, β-heptaglucosan, lipopolysaccharides, elicitins (namely cryptogein), Avr genes (Avr2, Avr4, Avr5, Avr9), Pep-13, Flg22, xylanase, BcPG1, AvrPto (Garcia-Brugger *et al.,* 2006). In the gene-for-gene recognition, R gene-mediated resistance recognizes the pathogen-derived signals encoded by avirulence genes (Avr), and defense response can lead to an oxidative burst through the rapid production of ROS or result in a hypersensitive response (HR) through the cell death. Such response limits the pathogen growth by restricting its access to nutrients, followed by an activation of the salicylic acid (SA)-dependent signalling, leading to the expression of pathogenesis-related (PR) proteins (Glazebrook, 2005; Qiu *et al.,* 2015). Other responses may involve either ethylene (ET) or jasmonate (JA) signalling pathways (Glazebrook, 2005). Indeed, both SA and JA may inhibit the expression of some genes, while the induction of others may require ET and JA. In grapevine, the mechanism of defense against necrotroph microorganisms is generally mediated by JA and ET pathways (Glazebrook, 2005; Garcia-Brugger *et al.,* 2006) and the induction of the genes related with phytoalexin biosynthesis (phenylalanine ammonia lyase – PAL) and stilbene synthase also occurs.

Grapevine can synthetize defense proteins such as PR proteins, defensin-like proteins (DELFs), and proteins involved in the detoxification of ROS. This type of defense response would occur during the powdery mildew infection, though is not exclusive to this pathogen (Armijo *et al.,* 2016). Grapevine also contains cell wall-associated proteins such as polygalacturonase-inhibiting proteins (PGIPs), to reduce the pectin degradation caused by pathogens. However, in some cases, such as in the *B. cinerea* infections, despite the induction of PGIPs expression, the infection process may be too fast for allowing the accumulation of sufficient amounts of PGIPs to protect the plant (Kars *et al.,* 2005). Furthermore, an accumulation of phytoalexins in grapes, such as α-viniferin and trans-resveratol and oxidative burst would occur (Aziz *et al.,* 2003). Considering GTDs, the affected grapevines display several defence mechanisms to inhibit the diseases progression, which includes an accumulation of PR-proteins, formation of polyphenol-rich reaction zones, oxidative burst, production of ROS, induction of phytoalexin pathway (such as *PAL* and *STS* genes) or accumulation of resveratrol in leaves, amongst others (Fontaine *et al.,* 2016a). However, and despite such myriad of different responses, the defense against Botryosphaeria dieback agents, namely *N. parvum* and *D. seriata*, are weaker during the flowering phase of the growth cycle, which may be possible related with the high metabolic activity of the plant, as a consequence of the inflorescences development (Spagnolo *et al.,* 2014; Spagnolo *et al.,* 2017). Indeed, the plant growth cycle influences carbohydrate storages in the plant and, consequently, may influence the pathogen infection.

Other effective resistance responses of grapevine include the SA, JA and systemic acquired resistance (SAR) (Figure 6). The SAR is acquired when an avirulent microorganism colonizes the plant or when the plant resists to an earlier infection caused by a pathogen, thus developing resistance

through the SA-regulated defense responses (Glazebrook, 2005; Thakur and Sohal, 2013). Moreover, some microorganisms, as *B. subtillis,* can produce different cyclic lipopeptides (LPs) that are involved in induce systemic resistance (ISR) activation.

Overall, the grapevine susceptibility to the pathogens infection depends on grapevine genotype and their resistance, severity of disease, type of pathogens, degree of pathogen colonization, plant microbiome and climate conditions.

1.5. Management of grapevine diseases and the importance to develop new solutions for protection

Viticulture is one of the agricultural sectors that uses the greatest amount of chemical pesticides. Indeed, an average of 21 kg of pesticides per hectare (ha) are used in EU vineyards. These products are mostly applied by spraying the plant canopy, with a great dispersion into the environment, thus acting as an important source of contamination (Endure, 2010). Among the chemical control, fungicides are the most applied products (19.5 kg/ha) followed by herbicides (1.28 kg/ha) and insecticides (0.30 kg/ha). Overall, it is estimated that 38% of the total volume of pesticides are applied in vineyards and around 76% of them are used to control powdery mildew diseases. In EU, France is the most prolific user of pesticides by applying 20% of the total pesticides and 30% of fungicides in viticulture, in a country where vineyards accounted only with 3% of the agriculture area (Aubertot *et al.,* 2005).

Pesticides are the most common products to protect grapevine from devastating diseases. Indeed, these products allow for the protection and intensive production of crops and because of their relatively low cost and easy application they are the most applied products on crops protection (Aubertot *et al.,* 2005). Importantly, the constant dependence of these synthetic compounds causes negative effects to humans, animals and several environmental implications such as accumulation of pesticides in the natural resources, water pollution, increase of greenhouse emissions or soil erosion with loss of fertility, loss of drainage and crop support and decrease of biodiversity (Tilman *et al*., 2002; Sabatier *et al.,* 2014; Barzman *et al.,* 2015). Furthermore, this leads to sources of pesticide-resistant pathogen strains (Amaro, 2003), which are of major concern.

With the constant increase of world population, the continued exploitation of environmental resources and non-renewable natural resources for food production is unavoidable (Godfray *et al.,* 2010), thus imprinting a general environmental stress of high risk at all levels. Indeed, an increasing concern to reduce chemical compounds in agriculture led to an entire reform of the legislative framework of pesticides within EU, with an important focus on the placing of plant protection products

on the market (Regulation (EC) No 1107/2009 that replaced the Directive 91/414/EEC), the need of a sustainable use of pesticides (Directive 2009/128/EC) and the control of the maximum residue levels of pesticides (regulation (EC) No 396/2005) (ECPA, 2013). As a consequence of these regulations, some products like copper salt were restricted in their use by the European Community (EC) (Regulation (EC) No 473/2002), to avoid their accumulation in soils and sediments, while others, as sodium arsenite used in the GTDs control, were even banned due to their high ecotoxicological risks (Spinosi *et al.,* 2009). Furthermore, it was demonstrated that the long-term use of the herbicide glyphosate had effects on soil erosion and was associated with the remobilisation of a banned pesticide dichlorodiphenyltrichloro-ethane (DDT) stored in vineyard soils (Sabatier *et al.,* 2014).

Importantly, different viticulture practices may be used, including the conventional, the integrated pest management (IPM) and the organic viticulture. In the conventional management, both pesticides and inorganic fertilizers are used, whereas in the IPM the plant protection is based on a sustainable application of control products to minimize the effects on agro-ecosystems and encouraging natural pest control mechanisms (Barzman *et al.,* 2015; Pinto and Gomes, 2016). In the last years, the organic viticulture has gained importance as it represents an alternative to conventional viticulture. Thus, the organic viticulture aims at protecting the natural balance of the vineyards and their surroundings by using limited amounts, or even zero pesticides, by substituting synthetic fertilisers by organic manure, or by using crop rotation (Hole *et al.*, 2005). Herein, the impacts on flora, soil fertility and plant- and soil- associated microorganisms are lower when compared with the conventional systems (Hole *et al.,* 2005; Schmid *et al.,* 2011). Nevertheless, in the organic vineyards the copper and sulphur treatments are still allowed for plant protection and are often used for the control of downy mildew. Copper is an essential micronutrient, but at high levels can cause damage on vine and contaminate the surrounding soils, thus monitoring its levels in grapes are of utmost importance.

The development of low-input agricultural systems, with reduced usage of synthetic compounds, and the introduction of new ecological alternatives is, at this moment, the biggest challenge for a modern and more sustainable wine and grape industry, with increased and improved crop yield without compromising the environment and ecosystem health. It was demonstrated that the low use of chemical pesticides rarely decreased the productivity of arable farms in France, which proves that a better management of these products can be achieved (Lechenet *et al.,* 2017). Furthermore, alternatives to pesticides do exist, but these are not always obvious and some need to be accepted by markets. Among them, the cultural methods (such as crop rotation), the use of genetic resistant/ tolerant grapevine varieties, biotechnical methods (pheromone mating disruption or biogenic elicitors), or biological methods (biological control agents -BCAs) are promising alternatives to chemical pesticides and harmless methods for plant diseases control (Aubertot *et al.,* 2005). Considering the crop rotation, this is not a solution for vineyards management and is generally applied

in arable crops or vegetables. Furthermore, there are few resistant grapevine cultivars to major pathogens and new insights in the genome of resistant cultivars are still needed for the better understanding of their potential in further breeding programs. Thus, and regarding the biological methods, the biological control is an environmental-friendly strategy that consists in the use of living microorganisms as BCAs, such as bacteria, fungi or virus, to suppress the activities and the development of general or specific plant pathogens (Pal and Gardener, 2006). These potential microorganisms are defined as microbial biopesticides and their application is regulated by the Regulation (EC) No 1107/2009 (Glare *et al.,* 2012; Villaverde *et al.,* 2014). Indeed, BCAs have their origin in nature, which constitutes a lower risk to environment, humans and animals when compared to chemical pesticides (Villaverde *et al.,* 2014). Furthermore, the natural substances produced by BCAs (secondary metabolites, enzymes, phytotoxins or elicitors) can also be applied for biological control (Bailey and Falk, 2011). However, and despite the multiple advantages of this biological strategy, the application of BCAs still involves high costs, requires technical skills and *in vivo* results are not always consistent.

Overall, to ensure a better link between higher quality and more durable viticulture practices, it is important to put together not only the low-input of pesticides or environmental-friendly strategies in viticulture management, but also the application of good cultural practices such as pruning and training vines. The correct management of these conditions will reduce the disease incidence in grapevine and will contribute to a more sustainable management of vineyards and to their safeguard. Nevertheless, to develop new and successful crop protection products is important to firstly identify targets and sources of such products, as well as the delivery systems to be adopted (Figure 7).

1.5.1. From grapevine microbiome to grapevine protection: exploit microorganisms with BCAs potential

It has already been demonstrated that the conventional management of the vineyards has significant impact on the grapevine's associated microorganisms (Pinto *et al*., 2014), both beneficial and pathogens, and is an important shaper of such microbial communities. Indeed, at the system level, both the soil and the plant microbiome play important roles in soil processes, where the abundance and the equilibrium of the microbial population will determine the plant's health status and, consequently, its productivity, yield and grape quality. Importantly, the soils of vineyards are the basis for *terroir* and are strictly connected with wine quality and wine identity, therefore preserving both their fertility and their quality is central to the identity of wines. Thus, the biological control is

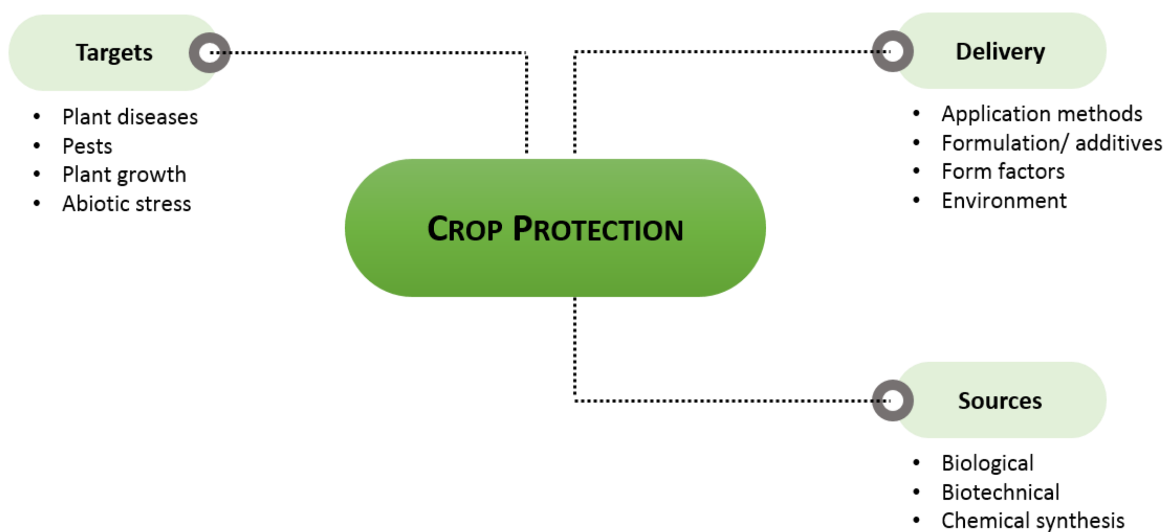

Figure 7: Important points to consider in crop protection **(Olson, 2015).** The development of a crop production product requires a prior analysis and identification of the target, type of product to develop (sources) and the delivery system to be adopted.

considered an ecological alternative to chemical pesticides application, which enhances the preservation of the natural microbial resources associated with plant and is, undoubtedly a new sustainable strategy for vineyards management.

With the ongoing development of plant microbiome sequencing, the characterization and the identification of natural and beneficial microorganisms with biocontrol potential from grapevine is a present challenge for viticulture management (Sébastien *et al.,* 2015; Pinto and Gomes, 2016). As previously described, beneficial microorganisms are natural colonisers of the plant and due to their antagonistic activities can be applied as BCAs against several grapevine pathogens. Furthermore, these microorganisms can contribute to plant's growth and can reinforce the natural plant defences. Indeed, these microorganisms can synthetize a myriad of antimicrobial metabolites or can be applied as elicitors to activate the plant defense responses. Among them, the elicitors as salicylic acid, methyl salicylate, benzothiadiazole, benzoic acid or chitosan are related with the activation of several defense-related enzymes in plants (Thakur and Sohal, 2013). Thus, the deep knowledge and the complete genome sequencing of these BCAs represents a powerful strategy to access to their biotechnological potential by identifying key genes and important pathways involved on their antagonistic activities or on plant growth promotion. In this light, the grapevine microbiome is a source of new BCAs (Sébastien *et al.,* 2015) and the knowledge of them are of utmost importance for the development of new environmental friendly and ecologically management solutions.

1.5.2. The mode of action of BCAs

The first application of BCAs occured in 1835 through the aplication of fungal spores of *Beauveria bassiana* to control insect pathogens (Olson, 2015). Since then, BCAs have aroused great interest as an alternative to conventional pesticides. The principles of BCAs' application to plant diseases control are based on a balanced management of pathogens, through a direct or indirect mechanisms, and to ensure the equilibrium of the natural microbial population. Contrarly to chemical pesticides, the application of BCAs has important advantages as these microorganisms are environmental-friendly, are natural microorganisms, can promote plant growth benefits, have low risk to develop pathogen-resistant strains and the impact on non-target microorganisms is reduced (Villaverde *et al.,* 2014). Furthermore, some BCAs have a broad spectrum activity against different pathogens which enhances its potencial in diseases control.

Each potential BCA develops diverse interactions with host and pathogens. Indeed, beneficial microorganisms can develop positive interactions with plant and promote their growth through nutrient acquisition (by phosphate solubilisation or nitrogen fixation), induce plant-resistance,

improve soil structure and quality, fix nitrogen or protect plant from abiotic stress. These BCAs are denominated as plant growth promoters (PGP) and those bacteria inhabitants of rhizosphere are denominated as plant growth promoting rhizobacteria (PGPR) (Lugtenberg and Kamilova, 2009; Beneduzi *et al.,* 2012). The direct plant growth includes biofertilization, stimulation of root growth, rhizoremediation and plant stress control (Lugtenberg and Kamilova, 2009). On the other hand, the interactions between BCAs and pathogens result in a biocontrol activity that may involve different modes of action such as antibiosis, competition, parasitism, cell wall degrading enzymes or plant-inducing resistance (Lo, 1998; Pal and Gardener, 2006; Jamalizadeh *et al.,* 2011; Pinto and Gomes, 2016). Often, more than on mode of action may be implicated in biocontrol. The deep knowledge of the strategies and mode of action applied by BCAs within pathogens and plants may provide useful information to select specific microorganisms and to improved their efficacy in biocontrol activities. The mode of action involved in biocontrol will be described separetly in the following paragraphs.

Antibiosis

The antibiosis involves the production of antibiotics (volatile or non-volatile compounds), lytic compounds, enzymes or bacteriocins by biocontrol microorganisms that are effective to suppress one or more plant pathogens (Riley and Wertz, 2002; Pal and Gardener, 2006; Jamalizadeh *et al.,* 2011).

Some antibiotics produced by BCAs are utmost importance such as agrocin 84 produced by *Agrobacterium radiobacter*, bacillomycin D, fengicin, iturin, mycosubtilin or Zwittermicin A produced by different *Bacillus* species, 2,4-diacetylphloroglucinol (Phl), phenazines, pyoluteorin, pyrrolnitrin or oomycin A produced by *Pseudomonas fluorescens* species, herbicolin produced by *Pantoea agglomerans*, xanthobaccin A produced by *Lysobacter* sp., pseudane produced by *Burkholderia cepacia,* streptomycin or kanamycin produced by *Streptomyces* genus and gliotoxin or gliovirin produced by *Trichoderma virens* (Lo, 1998; Pal and Gardener, 2006). In general, the antibiotics are low-molecular weight compounds that may be effective at low concentrations. Indeed, a set of *Bacillus, Streptomyces* and *Trichoderma* species are promisors BCAs due to their ability to produce bioactive secondary metabolites with antibacterial and antifungal properties. The antibiotics produced by BCAs target cellular functions of the pathogen by impacting on their DNA replication, RNA synthesis, cell wall synthesis and protein synthesis (Procópio *et al.,* 2012). Beyond antibiotics, BCAs can also produce hydrolytic componds (chitinases, proteases, glucanases, cellulases or hemicellulases) that hydrolyze the pathogens' fungal cells and other secondary metabolites such as hydrogen cyanide (HCN), produced by many *Pseudomonas* species, that are highly toxic for the aerobic microorganisms, causing the block of the cytochrome oxidase pathway (Pal and Gardener, 2006; Beneduzi *et al.,* 2012). Likewise, the lipopeptide biosurfactants produced by *Pseudomonas* and *Bacillus* species are encompassed in biocontrol (Beneduzi *et al.,* 2012).

For instance, bacteriocins are toxins produced by bacteria and with a killing spectrum against closely related bacteria (Riley and Wertz, 2002). Among gram-negative bacteria, the colicins (produced by *Escherichia coli* strains), pyocins (*Pseudomonas aeruginosa*) or marcescins (*Serratia marcescens*) are the most representative (Riley and Wertz, 2002). The bacteriocins from gram-positive bacteria are more diverse than those found in gram-negative strains and those from *Bacillus* spp. have broader spectra of inhibition (Beneduzi *et al.,* 2012).

In the light of the antibiosis, the genome analysis of potential BCAs is a step up to exploit new genes coding antibiotics and secondary metabolites with relevance for pathogens suppression.

Competition

A successful colonization of plant niches by microorganisms include an effective competition of the space and nutrients (carbohydrates, nitrogen, oxygen) available. The competition is the most common mechanism within biocontrol activity. Overall, nutrients are frequently limited on soils or plant surfaces (Pal and Gardener, 2006) and BCAs have an efficient uptake of essential nutrients such as siderophores production. Indeed, the competition for iron demonstrates the importance of siderophores production in biological control.

The siderophores (iron carriers) are low molecular weight iron chelators secreted by bacteria, fungi or even by monocotyledonous plants that have a very high affinity for ferric ionic from soils or surrounding environments, allowing its solubilisation (Alexander and Zuberer, 1991; Andrews *et al.,* 2003; Pal and Gardener, 2006). Iron is the 4th most important element, essential for many biological processes such as photosynthesis, respiration, oxygen transport, N_2 fixation, methanogenesis, H_2 production and consumption, trichloroacetic acid (TCA) cycle, DNA biosynthesis and gene regulation and thus, imperative for plant growth and development (Andrews *et al.,* 2003). Though iron has a limited bioavailability in nature since it is often present as an insoluble form. Indeed, under aerobic conditions at a neutral or alkaline pH, Fe is present as a reduced and unstable form (Fe^{2+}) which is readily oxidized to the oxidized ferric form (Fe^{3+}), that is insoluble for plants and microorganisms and can be potentially toxic (Alexander and Zuberer, 1991; Andrews *et al.,* 2003; Beneduzi *et al.,* 2012). Overall, the siderophores production allows the formation of the complex ferric-siderophore that is then binded to specific membrane receptors, taken up by the cell, where the iron is reduced (Fe^{3+} to Fe^{2+}), and returned to the cell surfaces (Alexander and Zuberer, 1991; Andrews *et al.,* 2003). Each microorganism has specific siderophore-mediated iron uptake strategies (Figure 8) (Andrews *et al.,* 2003).

Several siderophores were identified and they are classified according to their functional groups, structural features and type of ligands across four classes namely, carboxylate, hydroxamates, phenol catecholates and pyoverdines (Beneduzi *et al.,* 2012). Some well known siderophores are enterobactin

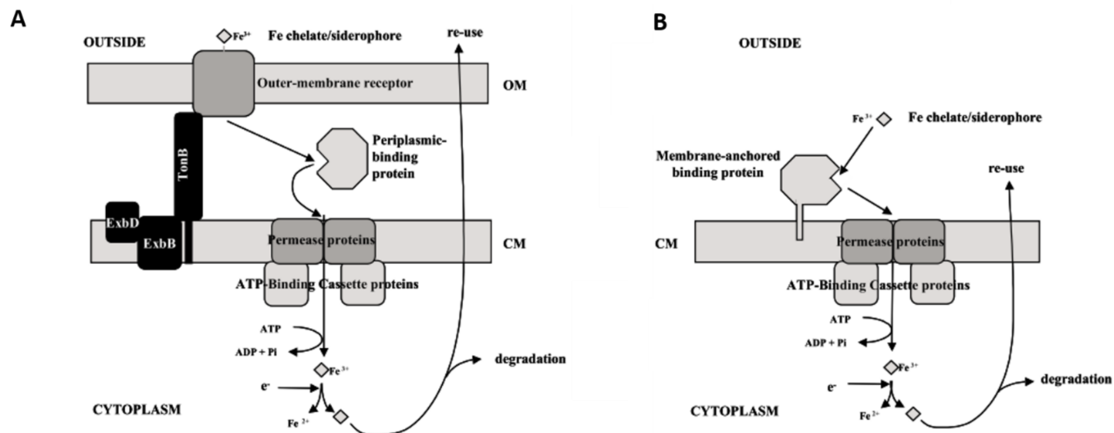

Figure 8: General overview of the siderophore-based iron acquisition in Gram-negative (A) and Gram-positive (B) bacteria **(Andrews *et al.,* 2003).** In Gram-negative bacteria (A) the complex ferric-siderophore is bindind to specific outer membrane (OM) receptors that is driven by cystosolic membrane (CM) and mediated by the energy-transducting TonB-ExbB-ExbD system. Then, the periplasmic binding proteins move the ferric-siderophores to the CM ATP-binding cassette (ABC) transporters and deliver to cytoplasm, where the complex will be reduced. In turn, Gram-positive bacteria (B) lack the OM and do not require the TonB-ExbB-ExbD system. Thus, the ferric-siderophores penetrate directly the CM, through the binding-protein-dependent ABC permeases, to cytoplasm where the complex will be then reduced (Andrews *et al.,* 2003).

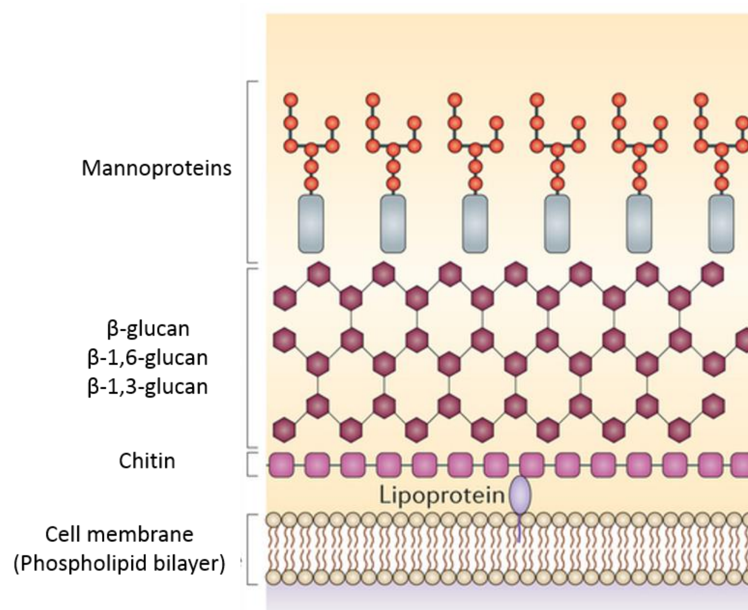

Figure 9: Schematic overview of the fungal cell wall composition **(Brown *et al.,* 2015).** The fungal cell wall mainly consists of polysaccharides such as mannoproteins, β-glucan namely, β-1,3 and β-1,6-glucan adjacent to chitin and the chitin, that is located close to the cell membrane. The most abundant β-glucan in the fungal cell wall is the β-1,3-glucan (Fesel and Zuccaro, 2016).

(found in *Escherichia coli* or *Salmonella typhimurium*), ferrichrome (*Aspergillus, Ustilago, Penicillium*), pseudobactin (*Pseudomonas* sp.), ferribactin (*Pseudomonas* fluorescens), cepabactin (*Pseudomonas cepacian*), pyoverdine (*Pseudomonas aeruginosa*) or schizokein (*Bacillus subtilis*). In the light of biocontrol, the siderophores production by BCAs confers a clear competition for the carbon sources available, allowing their colonisation across plant, in detriment of other microorganisms, and by improving the plant development.

Parasitism

The parasitism consists when BCAs feed on or within pathogen, resulting on its destruction or lysis structure (Lo, 1998; Jamalizadeh *et al.,* 2011). This direct parasitism is particular important in soil-borne diseases and at a lesser extent in foliar diseases (Jamalizadeh *et al.,* 2011). Going forward, some biocontrol fungi can develop a parasitism with other pathogenic fungi by using cell-wall-degrading enzymes such as chitinases, glucanases and β-1,3-glucanases as a strategy to disrupt the host cell walls (Lo, 1998; Jamalizadeh *et al.,* 2011). These microorganisms are referred as mycoparasites and can be effective to the plant diseases control. Indeed, BCAs such as *Aureobasidium pullulans* can produce extracellular exochitinase and β-1,3-glucanases in the presence of pathogens, suggesting that these enzymes have an important role in the biological control activities (Handelsman and Stabb, 1996; Castoria *et al.,* 2001; Vero *et al.,* 2009). Other well known mycoparasites include the BCAs *Pythium oligandrum* or *Trichoderma* species (Handelsman and Stabb, 1996). Overall, the strategies involved during the mycoparasitism include four steps namely, chemotropic growth, recognition, attachment and cell wall degradation and penetration (Lo, 1998). Thus, the chemotropic growth consists on the growth of the mycoparasite across the target pathogen, that produces chemical compounds which are chemoattractant for the mycoparasite. Then, the recognition step involves the interaction of both surface receptors of pathogens (lectins) and mycoparasites (carbohydrate) and the attachment and cell wall degradation is characterized by the production of enzymes such as chitinases, proteases and β-1,3-glucanases by the mycoparasite against pathogens. Finally, the penetration occurs when the mycoparasite produces appresoria-like structures to penetrate the host pathogen (Handelsman and Stabb, 1996; Lo, 1998).

Cell wall degrading enzymes

The cell wall degrading enzymes produced by BCAs are involved in the decline of fungal pathogens, and those such as chitinases and glucanases have been gained significance in agriculture or environmental management (Manjula and Podile, 2005). The cell wall of fungal pathogens contains various layers and the inner skeletal layer is constituted by β-1,6- glucan, β-1,3- glucan and chitin (Figure 9). Thus, hydrolytic enzymes such as lipase, chitinase β-1,4-N-acetyl-glucosaminidase

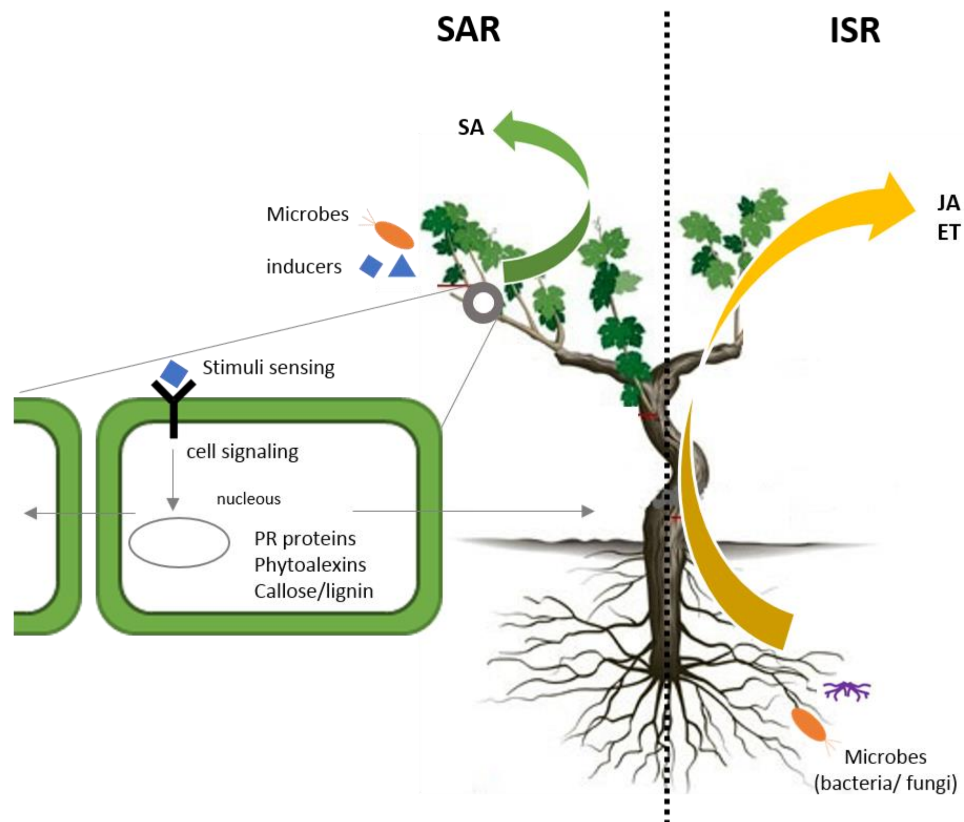

Figure 10: Representation of the systemic resistance induced in plants - SAR and ISR **(Adapted from Burketová *et al.,* 2015).** The systemic acquired resistance (SAR) pathway is induced by necrotizing pathogens attacks, non-pathogens microorganisms (BCAs), other natural or synthetic compounds (inducers) or even foliar treatments of plants with microbe-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs). After the recognition of these compounds, the plant cell triggers a signalling and defence responses that includes the production of pathogenesis-related proteins (PR), phytoalexins or other antimicrobial compounds and reinforcement of the plant cell walls with lignin and callose. The SAR pathway is mediated by salicylic acid (SA). Herein, this local response can also promote the production of mobile signals that will be transported *via* xylem to promote other defence responses in distal parts of the plant. In turn, the induced systemic resistance (ISR) can be induced by plant-associated microorganisms such as those colonizers of the root systems. The ISR pathway is mediated by jasmonic acid (JA) and ethylene (ET). Both SAR and ISR pathways can act additively to enhance a better defence level of the plant when compared with their protection alone (Lo, 1998; Beneduzi *et al.*, 2012 Burketová *et al*., 2015).

(NAGase), β-1,3-glucanase, β-glucosidase or proteases produced by BCAs are relevant as they can hydrolyse the host cell walls, causing the pathogen decline. Studies have demonstrated the importance of these enzymes on biological control (Lo, 1998; Manjula and Podile, 2005; Geraldine *et al.,* 2013). In this light, the efficiency by *Trichoderma* spp. in controlling the white mold caused by *Sclerotinia sclerotiorum* were mostly due to the cell wall degrading enzymes production namely, NAGase and β-1,3-glucanase and parasitism (Geraldine *et al.,* 2013).

<u>Plant-inducing resistance</u>

Plants can express different responses according to the chemical stimuli of BCAs or pathogen microorganisms. These stimuli may induce or condition the plant host defenses by increasing their tolerance and resistance against a pathogen and subsequent infection episodes (Pal and Gardener, 2006). In agreement to the chemical stimuli, the induction of plant defenses can be local or systemic.

BCAs can induce a hypersensitive response in plant through a mechanism denominated as induced systemic resistance (ISR) (Figure 10). This response is mediated by JA and/or ethylene pathways. Several rhizobacteria strains such as PGPR have been identified as elicitors of the plant host defenses and are associated with a ISR (Jamalizadeh *et al.,* 2011). Another induced response consists on a systemic acquired resistance (SAR) (Figure 10) that is mediated by SA, and that leads to the expression of PR proteins and other defense responses that include the synthesis of lytic enzymes (chitinases, glucanases, peroxidases), phytoalexins or other antimicrobial compounds, reinforcement of the plant cell walls through lignification and deposition of callose (Lo, 1998; Jamalizadeh *et al.,* 2011). The SAR pathway can be induced by necrotroph pathogens, BCAs and other natural or synthetic compounds and contrary to the ISR, SAR is characterized by a local response (Lo, 1998). Though, this local response can be transferred to distal parts of the plant through mobile signals, promoting a systemic plant defense (Conrath,2011; Burketová *et al.,* 2015).

BCAs can produce siderophores, lipopolysaccharides, salicylic acid or other volatile substances that acts as a stimulus promoting either a SAR or ISR response in plant and, consequently, providing its protection against a large spectrum of pathogens (Pal and Gardener, 2006). The protection mediated by ISR is less than those obtained through SAR pathways and is partly dependent on the plant genotype (Bloemberg and Lugtenberg, 2001; Beneduzi *et al.,* 2012). Both SAR and ISR can thus act additively promoting a better protection level of the plant when compared with their protection alone (Beneduzi *et al.,* 2012).

The activation of both SAR and ISR pathways after an external stimulus such as those from BCAs or pathogens can lead to a priming process (Conrath, 2011). After a stimulus, the plant is in alert and activates its defense mechanisms, becoming primed plants. Priming represents a plant immunological memory that will enable plant to respond more rapidly and efficiently to subsequent low levels of

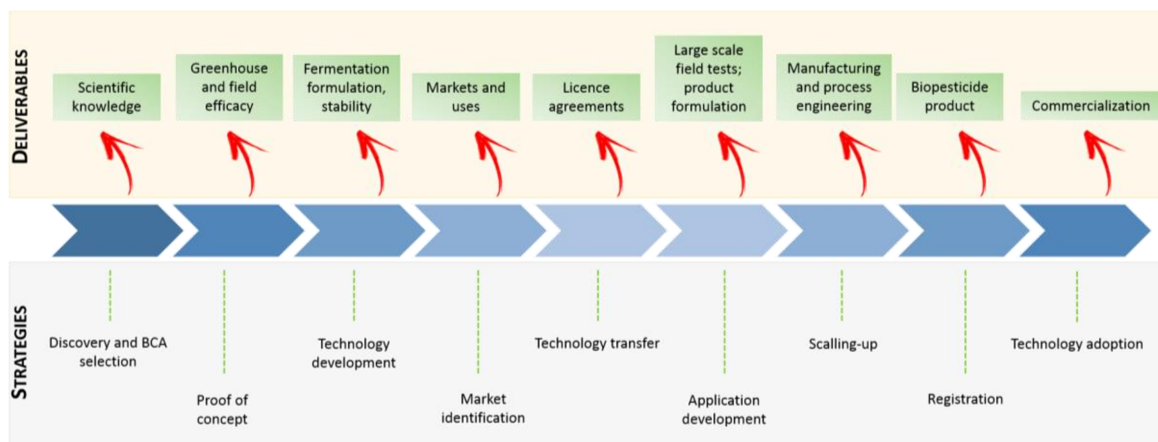

Figure 11: Overview of the biopesticides scientific innovation chain **(Bailey *et al.*, 2009).**

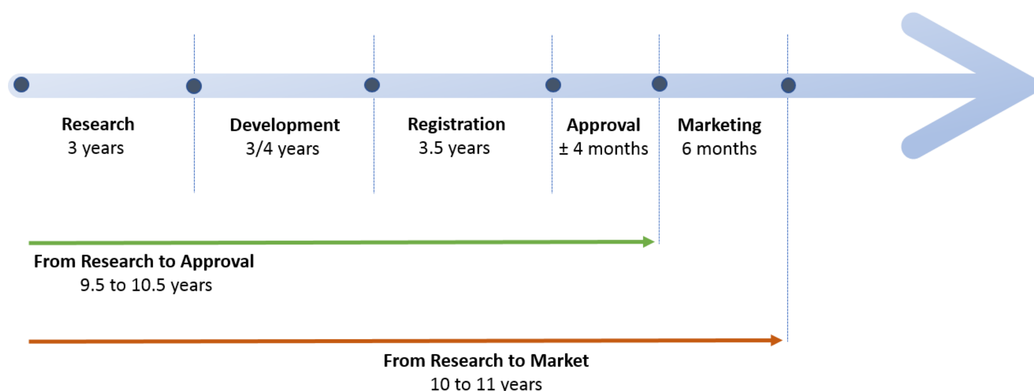

Figure 12: Chronological and logical steps associated with the development and registration of a biopesticide product **(ECPA, 2013).**

pathogens or abiotic stress by activating its defense response and which results in an increasing of resistance and stress tolerance (Conrath, 2011; Mauch-Mani *et al.,* 2017). Defense priming presents less energy costs to the plant and is a promising method for biocontrol (Conrath, 2011).

1.5.3. Challenges in the commercialization of BCAs

The high investment need in the research and development (R&D) of a new biological control product represents the first great challenge to develop a biopesticide (Figures 11 and 12). Furthermore, the registration process of these products in the EU is a long and expensive procedure which may constitute an important delay for their commercialization (Figure 11).

As previously referred, the BCAs application is an advantageous strategy for a sustainable viticulture management as they are naturally present in nature, are adapted to stress conditions such as UV and drought, have limit or null toxicity and the environmental impact is minimized (Koul, 2011). Furthermore, and contrarily to chemical pesticides, BCAs can be applied in different crop management strategies and can even be applied in alternation with other control products. However, it is important to be aware that BCAs must be cultivable microorganisms so that they can be used in future crops management (Müller and Ruppel, 2004). It is estimated that less than 1% of potential BCAs results in successful products (Glare *et al.,* 2012). Considering that these are living organisms, the efficacy of these biological products may not be constant and sometimes occurred at rather limited levels. Consequently, the formulation and storage conditions must be carefully selected in order to guarantee their biological activity as well as their shelf-life and stability. The formulation of a biopesticide should be designed considering the characteristics of the microorganism, the delivery application of the product and ensures its stability. Thus, formulations can be performed as powder, granules or aqueous suspensions (Bailey and Falk, 2011). Despite of their mode of action, some BCAs have a very specific activity against plant pathogens, representing a disadvantage of these products and limiting their widespread (Koul, 2011; Glare *et al.,* 2012). Indeed, a deep knowledge of the activity spectra of these microorganisms, their mode of action, manufacturing methods (such as fermentation or synthesis), shelf life and stability, and delivery options are some important strategies to consider for improving their application and competition in the biopesticides market (Bailey and Falk, 2011).

The registration process of a future biological control product may agree with a set of data requirements and only microorganisms or its metabolites that pose low or zero risks of pathogenicity or toxicity to environment and to non-target microorganisms could be a subject of authorization (Chandler *et al.,* 2011). In opposite to the US legislation, a registration of a biopesticide in Europe follows the same regulatory framework as those for chemical products namely, according to the

Regulation (EC) No 1107/2009 (ECPA, 2013; Huber, 2016). Depending on their active substance, a biopesticide product can be of a microorganism (BCAs), biochemical (secondary metabolites of plants or microorganisms; plant extracts; yeast fermentation products) or semiochemical (pheromone or other chemical signal produced by an organism) origin (Chandler *et al.,* 2011; Koul, 2011; Olson, 2015; Huber, 2016). Information such as mode of action or broad spectrum of the biopesticide candidate must be provided during registration. Furthermore, more than 100 specific tests are also performed to evaluate their physical and chemical properties, analytical methods, toxicity and metabolism, environmental and eco-toxicological tests, evaluation of residues in food and their efficacy (ECPA, 2013). The approval process of biopesticide product includes the approval of the active substance at EU and the formulated product registering in each Member State (ECPA, 2013). In Europe, the registration of a plant protection product has an average delay of 3.5 years and the approval of a low risk biopesticide product can take up to 120 days (ECPA, 2013; Huber, 2016) (Figure 12).

The time-long and tightening regulatory restrictions applied in EU reflects the number of biopesticides actually available in the market. Whilst US have more than 430 registered biopesticides, the EU only have almost 100 registered products that includes 43 microorganisms (Appendix 1: Table S1), 30 pheromones and semiochemicals and 25 plant extracts and other alternatives (Weidenauer, 2015 – personal communication). Among the EU registered BCAs, 33% are bacteria, 42% fungus, 7% yeasts and 19% virus (Appendix 1: Table S1). Currently, it is estimated that biopesticides represent only about 5% of the total pesticides volume market though this industry is growing and with an estimated compound annual growth rate of 8.64% (Olson, 2015; Timmusk *et al.,* 2017). Worldwide, the biopesticides are dominated by microbial biopesticides namely, bacterium-based products and fungi products (Glare *et al.,* 2012), accounting about 90% of total biopesticides market (Koul, 2011) while biofertilizers are dominated by nitrogen-fixing organisms such as *Rhizobium* spp., *Actinorhizobium* spp., *Azotobacter* spp. and *Azospirillum* spp. (Timmusk *et al.,* 2017). North America is one of the largest appliers of biocontrol products followed by Europe and Asia (Koul, 2011; Olson, 2015; Timmusk *et al.,* 2017). Among Europe, Spain, Italy and France are the countries with major application of these products.

In the last years, important agrochemical companies such as Bayer Crop Sciences have been moving to the commercialization of biopesticides products through investments in new R&D programs and innovation deals with other companies/ start-ups. This evidence a new trend of crops management and the response of these companies to a sustainable demand by producers. Actually, different biopesticides are available in the market and the application of these products is segmented into different types and across crops.

Among microbial biopesticides products, the most applied are the bioinsecticides namely, those based on the bacterium *Bacillus thuringiensis* (Bt). This BCA produces a toxin (Bt toxin) that disrupts

the insect gut and can also produce antibiotics such as Zwittermycin A that potentializes its insecticidal activity (Chandler *et al.,* 2011; Olson, 2015). Due to the success of this biopesticide, about 75% of biopesticide market consist on Bt-based products (Olson, 2015). Other efficacious biofungicides are based on *Bacillus subtilis* species, that present a broad spectrum against multiple fungal pathogens, *Bacillus pumilus* to control downy and powdery mildews, *Streptomyces* sp. to control a broad range of plant diseases, or even the *Trichoderma* spp., which is applied in different soil-borne diseases or to target the grey mould of grapes caused by *Botrytis cinerea* (Marrone, 2002; Glare *et al.,* 2012). Some of successful biopesticides are also based on microbial bioactive compounds such as the Serenade® that is based on a *B. subtilis* QST-713 strain and on lipopetide compounds such as iturins, agrastatins and surfactins with antimicrobial properties that are produced by the microorganism during fermentation process, under controlled conditions (Marrone, 2002).

Indeed, new alternative of crop protection methods such as microbial biopesticdes represents a bright business opportunity. Though, the high costs associated with R&D and the need of a repetitive number of treatments in crops, for an optimal efficacy of the product, are two constraints that influence the choice of these products on vineyard's management. Improvements in the formulation of BCA products, technological progress to reduce manufacturing costs and a faster legislation process may encourage the biopesticides R&D and their further widespread use of these products over different management programs by increasing their popularity and competition with conventional pesticides.

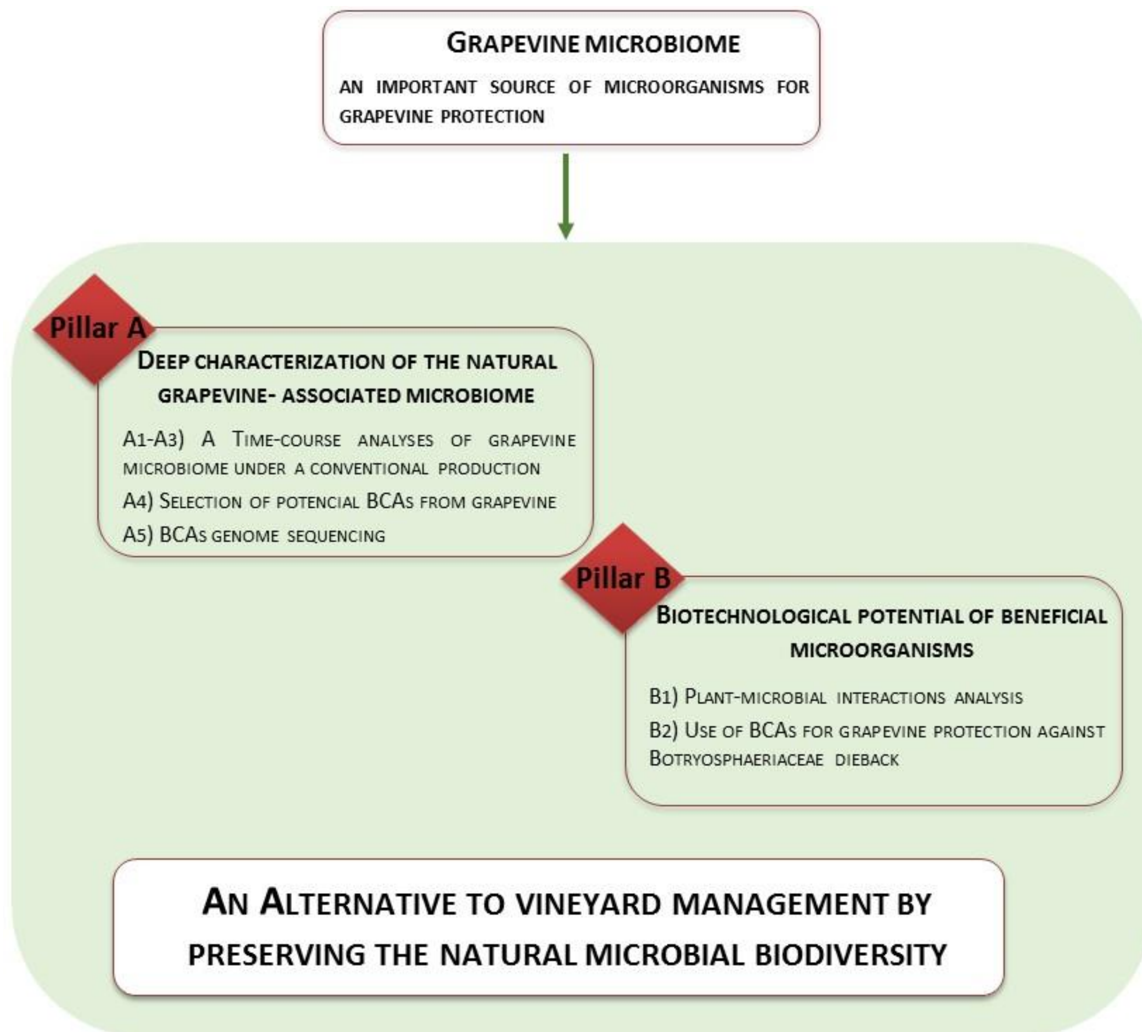

***Figure 13: Pert diagram of the current PhD project*.** General overview of the PhD project: main goals and corresponded axis.

1.6. Objectives

The natural microbiome associated with grapevine, also referred as the plant's second genome, is determinant for plant health, productivity and, consequently, will influence the quality of the final product (Turner *et al.,* 2013; Pinto *et al.,* 2016). Currently, the interactions between plant-microorganisms are far from being completely understood. Indeed, grapevine is naturally colonised by a myriad of microorganisms, both beneficial, neutral and pathogens, and preserving their equilibrium is of utmost importance. Nevertheless, this microbial equilibrium is affected by several external factors, of which the most disrupter is the constant application of chemical pesticides in vineyards (Pinto *et al.,* 2014), and for this reason the development of sustainable control methods is an urgent need. Thus, the deep understanding of these microbial resources, their function and their interactions with the plant constitutes an important step to explore the evolution of these communities across grapevine growth cycle, to discover BCAs with biotechnological potential to develop new sustainable solutions for vineyard protection, and by enhancing a decrease of the conventional pesticides. Under this framework, the general objective of this thesis is to fully understand the grapevine-microbiome interactions, and to explore the biotechnological potential of beneficial microorganisms, with the ultimate goal to contribute to a more efficient and more sustainable viticulture (Figure 13).

To achieve this overarching objective, we have defined a two-pillar strategy, each of which with clear specific objectives (Figure 13):

<u>Pillar A: Deep characterization of the natural grapevine-associated microbiome</u>

This pillar is focused on the analysis of the natural grapevine microbiome associated with different grape varieties, and on the understanding of the forces that shape plant-microbial interactions. The specific objectives for this pillar are:

A1) to deep characterize the structure and dynamics of the microbial communities, both eukaryotic and prokaryotic, associated from the vineyard to the wine (soils, leaves and wine musts samples);
A2) to understand the relationship between grape cultivars and microbiome structure;
A3) to understand the temporal evolution of these microbial communities (over the grapevine vegetative cycle, fermentation evolution and grapevine seasons);
A4) to isolate, identify and characterize potential BCAs from grapevine;
A5) to explore the biotechnological potential of BCAs through their genome analysis.

Pillar B: The biotechnological potential of beneficial microorganisms

This pillar is focused on the analysis of the plant-microbial interactions and on the protection potential of grapevine BCAs against GTDs. The specific objectives for this pillar are:

B1) to understand the colonisation capacity of BCAs over grapevine plantlets;

B2) and to explore the impact and the potential use of BCAs for grapevine protection against Botryosphaeriaceae dieback, namely *Diplodia seriata* F98.1, under greenhouse conditions.

Altogether, this work aims at contributing to a more efficient and more sustainable management of grapevine, where the application of BCAs is proposed for the preservation of the natural microbial biodiversity associated with grapevine.

1.6.1. Thesis outline

Overall, the work herein carried out, as well as its results and their discussion are presented across three chapters:

Chapter II: Characterization of the grapevine microbiome

This chapter explores the pillar A (Figure 13), namely the main goals from A1 to A3. The natural microbial communities associated from the vineyard to the wine are unveiled and deeply characterized through a metagenomic approach. For the grapevine microbiome analysis, a vineyard from Bairrada Appellation (Portugal) of 10 ha with different grape varieties, of which the most significant are Tinta Roriz (TR), Touriga Nacional and Baga, was selected and both soils and leaves were collected for two consecutive growing seasons. Samples were collected before and after the phytosanitary treatments and across the grapevine vegetative cycle. Given the wine fermentation microbiome analysis, six Portuguese wine appellations, namely Minho, Douro, Dão, Bairrada, Estremadura and Alentejo were selected and, for each appellation, the three most representative grape varieties were considered for sampling. The wine microbiome was fully characterized as regards the analysis of three stages of fermentation, namely Initial musts (IM), and Start and End of alcoholic fermentations (SF and EF, respectively). Results from this chapter are included in three publications that describes not only the relationship between grape cultivars and microbiome structure but also the temporal evolution of these microbial communities:

- **Publication 3:** Cátia Pinto, Diogo Pinho, Susana Sousa, Miguel Pinheiro, Conceição Egas, Ana C. Gomes. Unravelling the diversity of grapevine microbiome. *PloS One*, 2014, 9: e85622. doi:10.1371/journal.pone.0085622. This paper is among the top 10% most cited *Plos One* articles and currently have a total of 62 citations.

- **Publication 4:** Cátia Pinto, Valéria Custódio, Miguel Pinheiro, Conceição Egas, Ana C. Gomes., Vine Microbiome: the microbial diversity associated with diferente Portuguese grape varieties. Manuscript for submission to the American Society for Microbiology Journal.

- **Publication 5:** Cátia Pinto, Diogo Pinho, Remy Cardoso, Valéria Custódio, Joana Fernandes, Susana Sousa, Miguel Pinheiro, Conceição Egas and Ana C. Gomes. Wine fermentation microbiome: a landscape from different Portuguese wine appellations. *Frontiers in Microbiology*, 2015, 6: 905. doi: 10.3389/fmicb.2015.00905. This paper currently has a total of 30 citations.

Chapter III: Selection of potential BCAs

The present chapter is included in the pillar A (Figure 13) and explore the mail goals A4 and A5. Herein, several isolates from grapevine are tested for their biocontrol potential towards important grapevine pathogens such as *B. cinerea* and *Botryosphaeriaceae* dieback agents (*Diplodia seriata* and *Neofusicoccum parvum*), under *in vitro* conditions. Three potential BCAs, namely *Streptomyces* sp. Fito_S127B strain, *Aureobasidium pullulans* Fito_F278 and *Bacillus amyloliquefaciens* Fito_F321 are then characterized for their mode of action during biocontrol activities. Furthermore, their capacity to produce extracellular enzymes, to solubilise phosphate, to produce siderophores, and their physiological traits and effect on non-target microorganisms are also explored. In addiction, the draft genome of these BCAs is presented, which provides insights of their biotechnological potential and mechanisms involved in biocontrol. This chapter includes three publications related to the draft genome of these promising BCAs:

- **Publication 6:** Cátia Pinto, Susana Sousa, Hugo Froufe, Conceição Egas, Christophe Clément, Florence Fontaine, Ana C. Gomes, Draft genome sequence of *Streptomyces* sp. Fito_S127B strain, a soil microorganism from *Vitis vinifera* microbiome with a promising biotechnological importance. Manuscript in preparation.

- **Publication 7:** Cátia Pinto, Susana Sousa, Hugo Froufe, Conceição Egas, Christophe Clément, Florence Fontaine, Ana C. Gomes, Draft genome sequence of *Aureobasidium pullulans* strain

Fito_F278, a resident microbiota of grapevine with biocontrol potential against GTDs. Manuscript for submission to the Genome Announcements Journal

- **Publication 8:** Cátia Pinto, Susana Sousa, Hugo Froufe, Conceição Egas, Christophe Clément, Florence Fontaine, Ana C. Gomes, Draft genome sequencing of *Bacillus amyloliquefaciens* strain Fito_F321, an endophyte microorganism from *Vitis vinifera* with biocontrol potential. Manuscript for submission to the Standard in Genomic Sciences Journal.

Chapter IV: Phytoprotector potential of two selected BCAs against GTDs agents

This chapter explores the pillar B (Figure 13). Herein, results from the plant-microbial interactions are presented namely, the colonisation capacity of two selected BCAs (*Streptomyces* sp. Fito_S127B and *A. pullulans* Fito_F278) across plantlets cv. Chardonnay, and their potential use for grapevine protection against *Botryosphaeriaceae* species, in particular *D. seriata* F98.1. Thus, for the follow-up of BCAs colonisation, a molecular assessment is presented by using strain-specific primers, which were designed through their whole genome analysis. Given the grapevine protection, results from a 4-month greenhouse assay performed in cutting plants of *V. vinifera* cv. Chardonnay are explored and several parameters analysed namely, photosystem II, necrotic lesions length of green stems caused by the pathogen, follow-up of BCAs and pathogen colonisation over time and analysis of the plant expression genes involved in different signalling pathways (PR proteins, phenylpropanoid metabolism, detoxication and stress tolerance, cell wall compounds, water stress). This chapter includes a fully description of the plant- BCAs- *D.* seriata interaction, and one publication that focuses particularly on the plant-microbial interactions within the *A. pullulans* Fito_F278 strain:

- **Publication 9:** Cátia Pinto, Valéria Custódio, Mariana Nunes, Aurélie Songy, Fanja Rabenoelina, Barbara Courteaux, Christophe Clément, Ana C. Gomes, Florence Fontaine., Biocontrol potential and grapevine colonisation by natural microbial resources of grapevine: a case study of *Aureobasidium pullulans* strain Fito_F278. Manuscript for submission to the Applied and Environmental Microbiology journal.

Chapter I (French version)

INTRODUCTION

Introduction generale

Vitis vinifera : une culture économique mondiale importante

La vigne (*Vitis vinifera* L.), une plante ligneuse, appartient au genre *Vitis* qui comprend environ 60 espèces de *Vitis*. Parmi ce genre, *Vitis vinifera* a acquis un impact économique important, bien que d'autres espèces telles que *V. rupestris*, *V. riparia* et *V. berlandieri* soient utilisées comme porte-greffes en raison de leur résistance à d'importants agents pathogènes tels que le Phylloxera, oïdium et mildiou (Terral *et al*., 2010). *V. vinifera* comprend deux sous-espèces, soit *V. vinifera* ssp *vinifera* (ou *sativa*), une forme domestiquée, et son sauvage *V. vinifera* ssp *sylvestris* (This *et al*., 2006 ; Garcia and Revilla, 2013).

La vigne, avec l'olive, la datte et la figue, étaient les premières cultures fruitières cultivées et domestiquées (Zohary et Spiegel-Roy, 1975 ; Zohary *et al.*, 2012). La domestication de la vigne semble liée à la découverte du vin, bien que l'origine et la biogéographie historique de sa domestication ne soient toujours pas claires. Aujourd'hui la vigne est présente sur tous les continents sauf en Antarctique. En particulier, elle est cultivée dans les régions centrales et méridionales de l'Europe, dans les régions occidentales et au Moyen-Orient de l'Asie, de la Chine, de la côte méditerranéenne d'Afrique, d'Afrique du Sud, d'Amérique du Nord (comme la Californie, la Colombie-Britannique, l'Ontario, le Québec), Amérique du Sud (Chili, Argentine, Uruguay, Pérou et Brésil), Australie et Nouvelle-Zélande (Figure 1). Actuellement, sa forme sauvage est rare et peut être trouvée sur le long du bassin méditerranéen tempéré du Portugal au Turkménistan, en Ouzbékistan, au Tadjikistan, le long du Danube et du Rhin ou des forêts du nord de la Tunisie (Arnold *et al.,* 1998 ; McGovern, 2004 *et al.*, 2006). *V. vinifera* contient plus de 6 000 variétés de raisins dans le monde entier et de nombreuses variétés clonales différentes, mais seulement quelques-unes sont d'importance commerciale (Bouby et Marinval, 2001). En fait, la vigne est une des cultures les plus importantes dans le monde et, de loin, la plus important sur le plan économique. Une superficie totale de vignes de 7.5 mha en 2015 a été estimée, soit une production de 75.7 millions de tonnes de raisins (Figure 1) et 259 mhl de vin (OIV, 2016). L'Europe (35,8%) et l'Asie (35,4%) sont les plus grands producteurs de raisins suivis par le continent Américain (19,9%), l'Afrique (6,2%) et l'Océanie (2,7%) (FAOSTAT, 2014). Compte tenu de la superficie des vignes, 5 pays représentent 50% du vignoble mondial, à savoir l'Espagne (14%) qui possède la plus grande superficie viticole, suivie de la Chine (11%), de la France (10%), de l'Italie (9%) et de la Turquie (7%) (OIV, 2016) ; le Portugal a la neuvième (Tableau 1). La grande majorité de la production mondiale totale de raisins est destinée à la production viticole (48%) mais aussi aux raisins

de table (36%), aux raisins secs (8%), aux jus de raisin non alcoolisés et aux boissons spiritueuses (8%) (OIV, 2016). En Europe, la production de raisin est presque exclusivement destinée à la production du vin tandis que d'autres pays, comme la Chine, la Turquie, l'Inde ou l'Iran produisent principalement des raisins de table (Figure 2).

Depuis l'antiquité, la production et la consommation du vin ont été liées aux aspects sociaux et culturels. De plus, dans de nombreux pays, comme le Portugal et la France, la viticulture est un patrimoine culturel important et a une identité culturelle.

L'importance du microbiome de la vigne et les interactions plantes-microorganismes sur le développement de la plante et la production du vin

La consécration et le succès de la production de vins de qualité reposent sur une interaction complexe de plusieurs facteurs au vignoble et pendant le processus de fermentation du vin. Dans le vignoble, la vigne est conditionnée par des facteurs biotiques et abiotiques et par des pratiques culturables. Les facteurs biotiques comprennent les communautés microbiennes telles que les agents pathogènes des plantes (bactéries, champignons, phytoplasmes ou virus) et les parasites (insectes comme le phylloxère du raisin, plusieurs espèces de coléoptères ou mollusques) alors que les facteurs abiotiques comprennent le climat (température, vent, pluviométrie, lumière) et les conditions édaphiques (sol, nutriments, pH, salinité) ou même la phytotoxicité des pesticides et la pollution atmosphérique (Amaro, 2003). Compte tenu de la fermentation du vin, les microorganismes associés aux raisins et aux moûts de vin, ainsi que les pratiques œnologiques et la technologie de la cave, sont remarquables pour le processus du vin. Dans l'ensemble, l'association du climat de la région, du type de sol spécifique, des caractéristiques du paysage, des techniques œnologiques et des caractéristiques de la biodiversité définissent clairement les caractéristiques spécifiques d'une région viticole et donc le *terroir* (Résolution OIV/VITI 333/2010) (Figure 3). A noter que, les communautés microbiennes associées à la vigne peuvent également avoir un rôle direct ou indirect dans les propriétés organoleptiques du vin et, dans ce contexte général, le consortium microbien pourrait intégrer aussi la définition de *terroir* (Figure 3). Cependant, ce sujet est encore discutable, en raison des questions concernant la pertinence du microbiome du sol ou de microorganismes spécifiques à la région dans la définition de *terroir* (Barata *et al.*, 2012 ; Gilbert *et al.*, 2014).

Compte tenu des facteurs biotiques, la vigne, comme d'autres plantes, est naturellement colonisée par une myriade de microorganismes nommée par microbiome ou deuxième génome de la plante (Berendsen *et al.*, 2012 ; Turner *et al.,* 2013 ; Berg *et al.*, 2014). Ces microorganismes sont en

interaction avec la plante et les deux sont des entités inséparables puisqu'elles se retrouvent dans leur écologie et leur évolution (Vandenkoornhuyse *et al*., 2015). À cet égard, la plante et son microbiome associé peuvent être considérés comme un méta-organisme ou des holobionts (Berg *et al.*, 2014, Vandenkoornhuyse *et al.*, 2015). Cependant, les processus biogéochimiques, la fertilité des sols, la santé des plantes, la productivité, la croissance et la résistance de la plante dépendent fortement de l'équilibre de ces communautés microbiennes. En effet, cet ’équilibre est dépendent du génotype de la plante et sa diversité, du type de sol, du climat ou des pratiques viticoles (Philippot *et al*., 2013, Hartmann *et al*., 2015, Van Der Heijden et Hartmann, 2016). Ces facteurs peuvent façonner la structure microbienne et encourager une meilleure adaptation de certains microorganismes au détriment des autres. Ainsi, le microbiome de la plante, les interactions plantes-microorganismes et la dynamique microbienne présent depuis le vignoble jusqu'à la fabrication du vin sont importants et influenceront par conséquent la qualité et les propriétés organoleptiques des vins (Turner *et al*., 2013 ; Berg *et al*., 2014 Pinto *et al*., 2014).

Compte tenu de la diversité des microorganismes associés aux plantes comme les bactéries, les levures, les champignons filamenteux, les archéa ou les protistes, certains d'entre eux ont le potentiel de promouvoir des interactions bénéfiques avec la plante en favorisant sa croissance et son développement, permettant la disponibilité de nutriments limitants (tels que la solubilisation du phosphate, la production de siderophores, la fixation de l'azote), la tolérance au stress abiotique, le renforcement de la réponse immunitaire des plantes naturelles, voire la promotion de la protection des plantes contre les agents pathogènes (Van der Heijden *et al*., 2008, Mendes *et al*., 2013). À l'inverse, les microorganismes peuvent également avoir des effets négatifs sur la croissance et la productivité des plantes en étant en compétition pour les nutriments, transformant les nutriments en formes inaccessibles vers les plantes ou en agissant comme agents pathogènes (Van der Heijden *et al*., 2008). À son tour, la vigne assure un environnement protégé et mes à disposition des nutriments.

Des études récentes ont exploré le microbiome associé aux sols de la vigne jusqu'au vin et ont identifié des microorganismes communs (Zarraonaindia *et al*., 2015). Des régions viticoles distinctes ont également montré des communautés microbiennes spécifiques (Bokulich *et al*., 2014, Pinto *et al*., 2015). Au final, ces études sont utiles pour démystifier l'origine des microorganismes associés à la plante et pour mieux comprendre leur rôle dans les qualités organoleptiques du vin et l'unicité des vins régionaux. Ainsi, l'approche holistique du consortium microbien et de sa fonction est de la plus haute importance. La connaissance et la compréhension approfondie de l'identité, de l'écologie et du rôle du microbiome sur le fonctionnement de l'écosystème et la productivité végétale englobent un potentiel biotechnologique pour mettre en œuvre une stratégie de gestion durable des vignobles (Pinto et Gomes, 2016), mettant l'accent sur le *terroir* (Figure 3) et promettant la qualité et l'identité des vins. L'exploration du microbiome de la vigne est donc un moyen de répondre à de telles questions

sur la façon d'utiliser ces communautés microbiennes pour prédire les maladies des plantes et comment ces communautés peuvent produire des vins uniques.

Dans le cadre de la recherche du microbiome associée à la vigne, une synthèse bibliographique a été publiée dans le journal Biocontrol (Pinto et Gomes, 2016) - Publication 1. Dans l'ensemble, l'état de l'art de la recherche sur les microbiomes de la vigne à l'échelle mondiale est présenté et un aperçu de la colonisation des plantes, de l'impact des pratiques viticoles sur les communautés microbiennes et du rôle potentiel de ces structures microbiennes pour la gestion des vignobles sont discutés.

Les principales maladies de la vigne et leur impact sur le développement des plantes et la production viticole

La vigne est vulnérable à plusieurs infections pathogènes. En fonction des conditions météorologiques, de la température, de la saison, du génotype et de la sensibilité des vignes ou de l'emplacement des agents pathogènes dans la plante, la vigne pourrait être confrontée à différentes maladies. En effet, et selon la température par exemple, une variation entre 20°C et 28°C est une température optimale pour la croissance des agents pathogènes et, par conséquent, le développement de la maladie.

Les maladies dans la vigne peuvent être causées par des bactéries, des champignons, des phytoplasmes ou des virus et l'identification rapide et précise de ces agents pathogènes est importante pour prévenir leur propagation. Globalement, le mildiou, l'oïdium et la pourriture grise, provoquées par *Plasmopora viticola*, *Erysiphe necator* et *Botrytis cinerea*, respectivement (Figure 4) sont quelques-unes des maladies les plus importantes de la vigne (Armijo *et al*., 2016). Pourtant, les maladies du bois (MDB), la Flavescence Dorée (FD) et la maladie de Pierce (PD) soint trois maladies bien connues et sont devenues une préoccupation majeure et un défi pour l'industrie viticole car il n'existe aucun traitement efficace pour contrôler leur propagation.

En ce qui concerne les MDB, actuellement ces maladies sont considérées comme les plus destructrices de la vigne dans le monde entier et leur expression augmente dans tous les pays viticoles. Les trois MDB principales sont l'Esca, Botryosphaeria dieback et Eutypa dieback, qui attaquent principalement les organes pérennes de la vigne (Bertsch *et al*., 2012 ; Fontaine *et al*., 2016a). D'autres MDB, comme la maladie de Petri et la maladie du pied noir, affectent les jeunes vignobles contribuant ainsi à leur déclin (Gramaje et Armengol, 2011). L'Esca est une maladie complexe et un problème majeur en Europe, qui résulte de l'activité de différents champignons, nommément *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum* et *Fomitiporia mediterranea*. *Eutypa lata* et *Stereum*

hirsutum peuvent également être impliqués (Larignon *et al.*, 2009 ; White *et al.*, 2011). Le Botryosphaeria dieback ou dépérissement est causé par des espèces de botryosphaeriaceae telles que *Botryosphaeria dothidea*, *Diplodia seriata*, *Diplodia mutila*, *Neofusicoccum parvum* ou *Lasiodiplodia theobromae* (Úrbez-Torres, 2011) et Eutypa dieback ou eutypiosis est principalement causée par *Eutypa lata*. Cependant, les espèces comme *Eutypa leptoplaca*, *Cryptovalsa ampelina*, *Diatrypella* sp. ou *Eutypella* spp. peuvent-être aussi impliquées. Dans le déclin des jeunes vignes, la maladie de Petri est causée par *Phaeomoniella chlamydospora* et d'autres espèces du genre *Phaeoacremonium,* alors que la maladie du pied noir est causée par *Cylindrocarpon* spp. et *Campylocarpon* spp. (Gramaje et Armengol, 2011). Les symptômes généraux des MDB dans le bois comprennent la nécrose sectorielle ou centrale avec des bandes marronnes ou des chancres et, dans les feuilles, y compris une décoloration et un déssèchement (Larignon *et al*., 2009). À leur tour, les symptômes associées au déclin de la jeune vigne incluent une croissance atrophiée de la plante, une vigueur réduite, un débourrement retardé ou absente, des entre-noueds raccourcis, un feuillage chlorosée et épais avec des marges nécrotiques et un déssèchement (Gramaje et Armengol, 2011). De plus, les vignes affectées ont des racines nécrosée (Gramaje et Armengol, 2011). L'identification précoce de ces maladies est difficile car les lésions sont à l'intérieur de la plante, dans le bois, et les symptômes visibles prennent généralement plusieurs années pour s'exprimir (Fontaine *et al*., 2016a).

En ce qui concerne les moyens de control, l'arsénite de sodium utilisé pour contrôler l'Esca, était un moyen efficace mais a été interdit en 2003 dans tous les pays viticoles d'Europe en raison de sa toxicité (Spinosi *et al*., 2009). Par conséquent, aucun traitement aussi efficace n'est disponible et, en conséquence, l'OIV a établi en 2006 une résolution avec des mesures préventives pour limiter la prolifération de ces maladies (résolution OIV-VITI 2/2006). À l'heure actuelle, les principaux défis liés à leur contrôle se concentrent sur l'étude approfondie des facteurs expliquant la résistance des cultivars, d'exploiter le potentiel des microorganismes pour le biocontrôle et pour développer d'autres produits de protection à base de composés naturels (Fontaine *et al*., 2016b). Étant donné que les plaies de taille sont un moyen d'entrer des agents pathogènes dans la vigne, le développement de traitements axés sur leur protection est l'une des stratégies importantes pour maîtriser la propagation des MDB. En effet, de nouvelles solutions de contrôle sont rapidement nécessaires puisque les MDB causent la mort de vignes à court ou à long terme et sont responsables des coûts économiques élevés associés au remplacement de vignes mortes (Fontaine *et al*., 2016b). En fait, il est estimé que ce remplacement de vignes représente un coût de plus de 1.5 milliard de dollars par an (Hofstetter *et al*., 2012).

Dans l'ensemble, les maladies de la vigne causent des dommages importants aux feuilles et aux raisins et sont associées à une réduction significative du rendement des plantes et de la qualité du raisin qui provoque des pertes économiques importantes dans le secteur du vin. Le diagnostic précoce

des symptômes et l'identification précoce des agents pathogènes et des insectes vecteurs sont des étapes importantes pour prévenir leur dissémination et, par conséquent, prévenir les maladies de la vigne. Cependant, leur diagnostic précoce est parfois difficile car certaines maladies présentent des symptômes similaires dans les plantes, différentes maladies peuvent être présentes en même temps ou des facteurs tels que les conditions météorologiques, les déficiences nutritionnelles ou les pesticides peuvent produire des symptômes similaires aux maladies (Carisse *et al.,* 2006). En ce qui concerne les agents pathogènes, la meilleure connaissance du développement épidémiologique de ces microorganismes et des interactions plantes-pathogènes sont quelques-uns des points clés pour améliorer leur contrôle.

Comme mentionné ci-dessus, les MDB sont les maladies les plus destructrices de la vigne dans le monde et actuellement aucun contrôle efficace n'est disponible. En raison de l'importance de ce sujet pour le secteur vitivinicole, une synthèse bibliographique a été publiée dans l'*European Journal of Plant Pathology* (Fontaine *et al*., 2016a) et dans laquelle l'auteur de cette thèse (Cátia Pinto) a été activement impliqué et a écrit l'introduction et les impacts des MDB sur la maturation des baies. Dans l'ensemble, l'état de l'art des MDB et les effets des MDB sur la physiologie de la vigne (tels que le tronc, la tige, les feuilles et les raisins) sont discutés dans la publication 2.

Les stratégies d'infection des plantes et les mécanismes de défense de la vigne

En phytopathologie, le développement d'une maladie végétale est conditionné non seulement par la présence d'un agent pathogène, mais aussi par un hôte sensible et un environnement favorable (Figure 5). De même, le facteur temps est absolument nécessaire pour un scénario de maladie et, pour cette raison, ce facteur est pris em compte. La relation triangulaire est appelée de triangle de la maladie (Stevens, 1960 ; Francl, 2001) et l'élimination d'un de ces facteurs entraîne la prévention des maladies des plantes (Francl, 2001). Même si les interactions entre l'environnement-hôte-pathogène sont complexes, ce modèle conceptuel peut être utilisé pour prédire les résultats des maladies des plantes.

Les changements des conditions environnementales telles que le climat ou les précipitations, affectent la physiologie de la plante et fournissent des conditions pour un développement plus ou moins important des agents pathogènes (Grulke, 2011). À leur tour, la plante et l'agent pathogène interagissent entre eux (Figure 5) et les deux sont quelque peu flexibles à ces conditions environnementales (Grulke, 2011).

Les pathogènes ont des stratégies d'infection spécifiques et des cycles de vie, et peuvent être classés comme nécrotrophes, biotrophes et hémi-biotrophes. Les agents pathogènes nécrotrophiques

obtiennent des nutriments provenant des tissus de cellules nécrotiques ou mortes, favorisés par la sécrétion d'enzymes lytiques et de phytotoxines, tandis que les microorganismes biotrophes obtiennent des nutriments provenant des tissus végétaux vivants. Les microorganismes hémi-biotrophes peuvent agir comme agents pathogènes biotrophes aux premiers stades de l'infection puis évoluer ensuite vers le nécrotrophique (Glazebrook, 2005 ; Armijo *et al*., 2016). La première barrière d'attaque des agents pathogènes est la paroi cellulaire de la vigne. Cette structure est très hétérogène et composée de structures interconnectées de polysaccharides (cellulose, hémicellulose et pectine), de protéines et de polyphénols. En réponse à l'attaque des agents pathogènes, la plante favorise une régulation négative de la photosynthèse et une régulation positive ou négatif du métabolisme primaire, indispensable à la croissance et au développement de la plante (Rojas *et al.*, 2014). De plus, la plante active les mécanismes de défense par un système immunitaire de chaque cellule et sur un signal systémique (Jones et Dangl, 2006). En général, après la reconnaissance des agents pathogènes à travers désignées éliciteurs (Figure 6), la réponse de défense comprend la production de métabolites ou de protéines antimicrobiennes (telles que les phytoaléxines), la synthèse des enzymes de défense, l'accumulation de protéines liées à la pathogenèse (PR), la production d'espèces réactives de l'oxygène (ROS) et production de callose et de lignine pour renforcer la structure de la paroi cellulaire de la plante (Glazebrook, 2005). Cependant, les réponses de défense doivent être réglementées de manière appropriée, car son activation nécessite beaucoup d'énergie, ce qui peut provoquer des effets néfastes sur la croissance de la plante (Glazebrook, 2005 ; Rojas *et al*., 2014).

Dans l'ensemble, le système immunitaire de la plante (Figure 6) peut s'appuyer sur l'utilisation de récepteurs de reconnaissance transmembranaires (PRRs) qui détectent directement les modèles moléculaires associés aux microbes ou pathogènes (MAMPS ou PAMP), et à une reconnaissance de gènes en utilisant des molécules polymorphes de nucléotides, comme des domaines à répétition riche en leucine (NB-LRR), qui sont codés par des gènes de résistance (R) (Jones et Dangl, 2005 ; Zipfel et Felix 2005). Cette stratégie agit dans la cellule et n'est efficace qu'avec les agents pathogènes biotrophes et hémi-biotrophes (Glazebrook, 2005), car la réponse à la défense de la plante entraîne la mort cellulaire.

Les réponses de défense de l'immunité déclenchée par le MAMP (MTI) se produisent par des actions de signalisation comprenant des flux ioniques, une activation en cascade de protéines activées par mitogène (MAP) et une production de ROS (Farace *et al*., 2015). Les MAMP des bactéries comprennent flagellin, EF-Tu, peptidoglycans, lipopolysaccharides (LPS) et rhamnolipides (RL) et les champignons comprennent la chitine et les élicitines (Farace *et al*., 2015, Fesel et Zuccaro, 2016). Ainsi, la stimulation et l'activation de la réponse immunitaire primaire des plantes proviennent de l'action des éliciteurs (Figure 6) qui sont équivalents aux PAMP (Zipfel et Felix, 2005) et qui comprennent des composés tels que des protéines, des glycoprotéines, des glycanes, des lipides et des molécules

synthétiques (Garcia-Brugger *et al.*, 2006, Thakur et Sohal, 2013). Selon leur origine et leur structure moléculaire, les éliciteurs sont classés comme physiques ou chimiques, biotiques ou abiotiques, complexes ou définis (Thakur et Sohal, 2013), et sont des constituants de l'agent pathogène ou libérés par la paroi cellulaire de la plante ou des pathogènes par des enzymes hydrolytiques (Garcia-Brugger *et al.*, 2006). Parmi eux, les éliciteurs les plus étudiés sont les oligogalacturonides, le chitosan, le β-heptaglucosan, les lipopolysaccharides, les élicitines (à savoir la cryptogéine), les gènes Avr (Avr2, Avr4, Avr5, Avr9), Pep-13, Flg22, xylanase, BcPG1, AvrPto (Garcia -Brugger *et al.*, 2006). Dans la reconnaissance du gène-pour-gène, la résistance médiée par le gène R reconnaît les signaux dérivés des pathogènes codés par les gènes d'avirulence (Avr) et la réponse de la défense peut conduire à un éclatement oxydatif grâce à la production rapide de ROS ou à une réponse hypersensible (HR) à travers de la mort cellulaire. Une telle réponse limite la croissance des agents pathogènes en diminuant son accès aux nutriments, suivie d'une activation de la signalisation dépendante de l'acide salicylique (SA), ce qui conduit à l'expression de protéines liées à la pathogenèse (PR) (Glazebrook, 2005 ; Qiu *et al.*, 2015). D'autres réponses peuvent concerner des voies de signalisation d'éthylène (ET) ou de acid jasmonate (JA) (Glazebrook, 2005). En effet, SA et JA peuvent inhiber l'expression de certains gènes tandis que l'induction d'autres peut nécessiter ET et JA. Chez la vigne, le mécanisme de défense contre les microorganismes nécrotrophiques implique généralement les voies JA et ET (Glazebrook, 2005 ; Garcia-Brugger *et al.*, 2006) et l'induction des gènes liés à la biosynthèse de phytoalexine (phénylalanine ammoniaque lyase - PAL) et stilbène synthase se produit également.

La vigne peut synthétiser des protéines de défense telles que les protéines liées à la pathogenèse (PR), les protéines de type défensif (DELF) et les protéines impliquées dans la détoxication des ROS. Ce type de réponse de défense se produirait pendant l'infection de l'oïdium mais n'est pas exclusif de ce pathogène (Armijo *et al.*, 2016). La vigne contient également des protéines associées à la paroi cellulaire telles que des protéines inhibitrices de la polygalacturonase (PGIP), afin de réduire la dégradation de la pectine causée par les agents pathogènes. Bien que l'infection d'agents pathogènes induise une expression de PGIP, cette infiltration, telle que *B. cinerea*, serait si rapide qu'elle ne permet pas l'accumulation de quantités suffisantes de PGIP pour protéger la plante (Kars *et al.*, 2005). En outre, une accumulation de phytoalexines dans les raisins, l'α-viniferine et le trans-resveratol dans les feuilles et un éclatement oxydatif se produiraient (Aziz *et al.*, 2003). En ce qui concerne les MDBs, les vignobles affectés présentent plusieurs mécanismes de défense pour inhiber la progression de ces maladies dont une accumulation de protéines PR, la formation de zones de réaction riches en polyphénols, le burst oxydatif, la production de ROS, l'induction de la voie des phytoalexines (comme les gènes PAL et STS) ou l'accumulation de resvératrol dans les feuilles (Fontaine *et al.*, 2016a). Les réponses de défense de la vigne aux agents de dépérissement, à savoir, *N. parvum* et *D. seriata*, sont faibles lors de la phase de floraison du cycle végétative de la plante. Cela peut être en lien avec l'activité métabolique élevée

de la plante, en conséquence du développement des inflorescences (Spagnolo *et al*., 2014, Spagnolo *et al.*, 2017). En effet, le cycle végétatif de la plante influence les réserves de glucides et, par conséquent, peut influencer l'infection par les agents pathogènes. D'autres réponses de résistance efficaces de la vigne comprennent le SA, le JA et la résistance acquise systémique (SAR) (Figure 6). La SAR est acquise lorsqu'un microorganisme non-virulent colonise la plante ou lorsque la plante résiste à une infection antérieure causée par un agent pathogène ; à son tour la plante développe une résistance à travers les réponses de défense régulées par le SA (Glazebrook, 2005 ; Thakur et Sohal, 2013). D'autres microorganismes comme *B. subtillis* peuvent produire différents lipopeptides cycliques (LP) impliqués dans l'activation de la résistance systémique (ISR).

Dans l'ensemble, la sensibilité de la vigne à l'infection par des agents pathogènes dépend de son génotype et de leur résistance, de la sévérité et du type de pathogène et de leur degré de colonisation mais aussi des conditions climatiques.

La gestion des maladies de la vigne et l'importance de développer de nouvelles solutions de protection

Parmi les autres cultures, la viticulture est l'agro-secteur qui applique la plus grande quantité de pesticides chimiques. En effet, une moyenne de 21 kg de pesticides par hectare (ha) est utilisée dans l'UE. Ces produits sont principalement appliqués par méthodes de pulvérisation, et une grande dispersion de ces produits dans l'environnement entraîne des risques importants de contamination (Endure, 2010). Parmi le contrôle chimique, les fongicides sont les produits les plus appliqués (19.5 kg/ha) suivis d'herbicides (1.28 kg/ha) et d'insecticides (0.30 kg/ha). Dans l'ensemble, il est estimé que 38% du volume total de pesticides sont appliqués dans le vignoble et environ 76% d'entre eux sont utilisés pour lutter contre l'oïdium. Dans l'UE, la France, pays où les vignobles ne représentient que 3% de la superficie agricole, est l'utilisateur le plus prolifique de pesticides en appliquant 20% du total des pesticides et 30% des fongicides dans la viticulture (Aubertot *et al*., 2005).

Avec l'augmentation constante de la population mondiale, l'exploitation continue des ressources environnementales pour la production alimentaire est encore inévitable (Godfray *et al.*, 2010). Cependant, les ressources naturelles telles que le sol ou l'eau, sont des ressources non renouvelables. Un souci croissant de réduire les composés chimiques dans l'agriculture a conduit à un cadre législatif des pesticides au sein de l'UE. Ainsi, ces préoccupations soulignent la mise sur le marché des produits phytosanitaires (règlement (CE) n°1107/2009 qui a remplacé la directive 91/414/CEE), la nécessité d'une utilisation durable des pesticides (directive 2009/12/CE) et le contrôle des niveaux maximaux de résidus de pesticides (règlement (CE) n°396/2005) (ECPA, 2013). En conséquence de ces règlements,

certains produits comme le cuivre ont été limités dans leur utilisation par la communauté européenne (CE) (règlement (CE) n°473/2002) pour éviter leur accumulation dans les sols et les sédiments. D'autres, comme l'arsénite de sodium utilisé dans le contrôle des MDB, ont même été interdits en raison de leur risque éco toxicologique (Spinosi *et al*., 2009). En outre, il a été démontré que l'utilisation à long terme de l'herbicide glyphosate a des effets sur l'érosion des sols et ce produit est associé à la remobilisation d'un pesticide interdit le dichlorodiphényltrichloroéthane (DDT), que reste stocké dans des sols viticoles (Sabatier *et al*., 2014).

La réduction des composés synthétiques et l'introduiction de nouvelles alternatives écologiques constituent un nouveau défi pour une industrie viticole moderne et durable, afin d'augmenter et d'améliorer le rendement des cultures sans compromettre l'environnement et la santé des écosystèmes. Il a été démontré que la faible utilisation des pesticides chimiques diminue rarement la productivité des fermes arables en France, ce qui prouve qu'une meilleure gestion de ces produits peut être obtenue (Lechenet *et al*., 2017). En outre, des alternatives aux pesticides existent également, mais elles ne sont pas toujours évidentes. Parmi eux, les méthodes culturales (telles que la rotation des cultures), les vignes résistantes, les méthodes biotechnologiques (phéromones ou éliciteurs biogéniques) ou les méthodes biologiques (agents de lutte biologique - BCAs) sont des alternatives prometteuses aux pesticides chimiques et sont des méthodes inoffensives pour le contrôle des maladies des plantes (Aubertot *et al*., 2005). Compte tenu de la rotation des cultures, cette solution n'est pas une solution pour la gestion des vignobles et est généralement appliquée dans les cultures arables ou les légumes. En outre, il existe peu de cépages résistants aux agents pathogènes et de nouvelles recherches dans le génome des cultivars résistants doivent encore être menées pour mieux comprendre leur potentiel dans des programmes de production. Ainsi, et en ce qui concerne les méthodes biologiques, le biocontrôle est une stratégie respectueuse du l'environnement et qui consiste à utiliser des microorganismes vivants tels que des bactéries, des champignons ou des virus pour supprimer les activités et le développement des agents pathogènes généraux ou spécifiques (Pal et Gardener, 2006). Ces microorganismes potentiels sont définis comme des biopesticides microbiens et leur application est réglementée par le règlement (CE) n° 1107/2009 (Glare *et al*., 2012 ; Villaverde *et al*., 2014). En effet, les BCA sont originaires de la nature et leur utilisation constitue un moindre risque pour l'environnement, les humains et les animaux par rapport aux pesticides chimiques (Villaverde *et al*., 2014). En outre, les substances naturelles produites par les BCAs (métabolites secondaires, enzymes, phytotoxines ou éliciteurs) peuvent également être appliquées pour le biocontrôle (Bailey et Falk, 2011). Cependant, et malgré les multiples avantages de cette stratégie, l'application de BCAs implique toujours des coûts élevés, nécessite des compétences techniques et les résultats *in vivo* ne sont pas toujours cohérents.

Dans l'ensemble, et afin d'assurer un meilleur lien entre la qualité et les pratiques durables en viticulture, il est important de mettre en place non seulement un faible apport de pesticides ou des stratégies respectueuses de l'environnement dans la gestion de la vigne, mais aussi mettre en place l'application de bonnes pratiques culturales telles que l'élagage et la production des vignes. La bonne combinaison de ces conditions réduira l'incidence de la maladie dans la vigne et contribuera à une gestion durable des vignobles et à leur sauvegarde. Ainsi, pour le développement d'un nouveau produit de protection végétale il est important d'identifier d'abord les cibles et les sources du produit et le système d'application à adopter (Figure 7).

Du microbiome à la protection de la vigne : exploiter les microorganismes avec un potentiel de biocontrôle

Il a déjà été démontré que la gestion conventionnelle de la vigne affecte tous les microorganismes associés à la plante (Pinto *et al*., 2014). En effet, le microbiome associé aux sols et aux plantes joue un rôle important dans les processus du sol, et l'abondance et l'équilibre de la population microbienne détermineront l'état de santé de la plante et, par conséquent, la productivité, le rendement et la qualité des produits finaux. Les sols des vignobles sont la base du *terroir* et sont strictement liés à la qualité et à l'identité du vin. La préservation de la fertilité et de la qualité des sols est une clé importante pour conserver l'identité des vins régionaux. Ainsi, le biocontrôle est considéré comme une alternative écologique à l'application de pesticides, qui améliore la préservation des ressources microbiennes naturelles associées aux plantes et constitue, sans aucun doute, une nouvelle stratégie durable pour la gestion des vignobles.

Avec le développement du séquençage des microbiomes, la caractérisation et l'identification de microorganismes naturels et bénéfiques avec potentiel de biocontrôle constituent un défi pour la gestion de la viticulture (Sébastien *et al*., 2015, Pinto et Gomes, 2016). Comme décrit précédemment, les microorganismes bénéfiques sont des colonisateurs naturels de la plante et, en raison de leurs activités antagonistes, peuvent être appliqués sous la forme de BCA contre plusieurs agents pathogènes de la vigne. En outre, ces microorganismes peuvent contribuer à la croissance de la plante et peuvent renforcer leurs défenses naturelles. En effet, ces microorganismes peuvent synthétiser une myriade de métabolites antimicrobiens ou peuvent être appliqués en tant qu'éliciteurs pour activer les réponses de la défense des plantes. Parmi ceux-ci, les éliciteurs comme l'acide salicylique, le salicylate de méthyle, le benzothiadiazole, l'acide benzoïque ou le chitosan sont liés à l'activation de plusieurs enzymes liées aux défenses des plantes (Thakur et Sohal, 2013). Ainsi, la connaissance

approfondie et le séquençage complet du génome de ces BCA représentent une stratégie puissante pour accéder à leur potentiel biotechnologique, en identifiant des gènes clés et les voies importantes impliquées dans leurs activités antagonistes ou sur la promotion de la croissance des plantes. De ce fait, le microbiome de la vigne est une source potentielle de nouveaux BCAs (Sébastien *et al*., 2015) et leur connaissance est d'une importance capitale pour le développement de nouvelles solutions de gestion écologique.

Le mode d'action des BCAs

La première application des BCA s'est produite en 1835 par l'application de spores fongiques de *Beauveria bassiana* pour contrôler des insectes pathogènes (Olson, 2015). Depuis, les BCA ont suscité un grand intérêt en tant que solution de rechange aux pesticides conventionnels. Les principes de l'application des BCA au contrôle des maladies des plantes reposent sur une gestion équilibrée des agents pathogènes, par des mécanismes directs ou indirects, pour assurer l'équilibre de la population microbienne naturelle. Contrairement aux pesticides, l'application des BCAs présente des avantages importants, car ces microorganismes sont respectueux de l'environnement, sont des microorganismes naturels, peuvent favoriser les bénéfices de croissance des plantes, présentent un faible risque de développer des souches résistantes aux agents pathogènes et l'impact sur les microorganismes non ciblés est réduit (Villaverde *et al.*, 2014). En outre, certains BCA ont une large activité de spectres contre différents agents pathogènes qui améliore son potentiel dans le contrôle des maladies.

Chaque potentiel BCA développe diverses interactions avec la plante et les agents pathogènes. En effet, les microorganismes bénéfiques peuvent développer des interactions positives avec les plantes et favoriser leur croissance grâce à l'acquisition de nutriments (par la solubilisation du phosphate ou la fixation de l'azote), induire leur résistance, améliorer la structure et la qualité du sol, fixer l'azote ou protéger les plantes du stress abiotique. Ces bactéries sont dénommées promoteurs de la croissance des plantes (PGP) et ces microorganismes de la rhizosphère sont appelés de rhizo bactéries que favorisent la croissance des plantes (PGPR) (Lugtenberg et Kamilova, 2009 ; Beneduzi *et al.*, 2012). La croissance directe des plantes comprend la biofertilisation, la stimulation de la croissance des racines, la rhizo remédiation et le contrôle du stress végétal (Lugtenberg et Kamilova, 2009). D'autre part, les interactions entre les BCA et les agents pathogènes entraînent une activité de biocontrôle qui peut impliquer différents modes d'action tels que l'antibiose, la compétition (Figure 8), le parasitisme, les enzymes dégradant de la paroi cellulaire (Figure 9) ou la résistance induisant les plantes (Figure 10) (Lo, 1998 ; Pal et Gardener, 2006 Jamalizadeh *et al*., 2011 ; Pinto et Gomes, 2016). Souvent, plus qu'un mode d'action peut être impliqué dans le biocontrôle. La connaissance approfondie de ces stratégies

et du mode d'action utilisé par les BCAs vis-à-vis des agents pathogènes et des plantes peuvent fournir des informations utiles pour sélectionner des microorganismes spécifiques et pour améliorer leur efficacité dans les activités de biocontrôle.

Les défis dans la commercialisation des BCAs

Le besoin d'un investissement dans la recherche et le développement (R&D) d'un nouveau produit de biocontrôle représente le premier grand défi pour développer un biopesticide (Figures 11 et 12). En outre, le processus d'inscription de ces produits dans l'UE est une procédure longue et coûteuse qui peut constituer un retard important pour leur commercialisation (Figure 11).

Comme indiqué précédemment, l'application des BCAs est une stratégie avantageuse pour une gestion durable de la viticulture, car elles sont naturellement présentes dans la nature, adaptées aux conditions de stress telles que les UV et la sécheresse, ont une toxicité limite ou nulle et l'impact environnemental est minimisé (Koul, 2011). En outre, et contrairement aux pesticides, les BCAs peuvent être appliqués dans différentes stratégies de gestion des cultures et peuvent même être appliqués en alternance avec d'autres produits de contrôle. Cependant, il est important de savoir que les BCAs doivent être des microorganismes cultivables afin qu'ils puissent être utilisés dans la gestion des cultures futures (Müller et Ruppel, 2004). Il est estimé que moins de 1% des produits de biocontrôle sont réussis (Glare *et al*., 2012). Étant donné que ce sont des organismes vivants, l'efficacité de ces produits biologiques peut ne pas être constante et se traduit parfois à des niveaux d'efficacité plutôt limités. Par conséquent, les conditions de formulation et de stockage doivent être soigneusement sélectionnées afin de garantir leur activité biologique ainsi que leur durée de conservation et leur stabilité. La formulation d'un biopesticide devrait être conçue compte tenu des caractéristiques du microorganisme, de l'application de livraison du produit et de sa stabilité. Ainsi, les formulations peuvent être réalisées sous forme de poudre, de granulés ou de suspensions aqueuses (Bailey et Falk, 2011). Malgré leur mode d'action, certains BCA ont une activité très spécifique contre les agents pathogènes des plantes, ce qui représente un inconvénient de ces produits et leur limitation de leur diffusion (Koul, 2011 ; Glare *et al*., 2012). En effet, une connaissance approfondie des spectres d'activité de ces microorganismes, leur mode d'action, les méthodes de fabrication (telles que la fermentation ou la synthèse), la durée de vie et la stabilité sont des stratégies importantes à prendre en compte pour améliorer leur application et leur concurrence (Bailey et Falk, 2011).

Le processus d'enregistrement d'un futur produit de contrôle biologique peut convenir d'un ensemble d'exigences en matière de données. Seulement les microorganismes ou ses métabolites qui présentent des risques faibles ou nulles de pathogénicité ou de toxicité pour l'environnement et les

microorganismes non ciblés peuvent être soumis à l'autorisation (Chandler *et al*., 2011). Contrairement à la législation américaine, l'enregistrement d'un biopesticide en Europe suit le même cadre réglementaire que celui des produits chimiques, conformément au règlement (CE) n°1107/2009 (ECPA, 2013 ; Huber, 2016). Selon leur substance active, un biopesticide peut être un microorganisme (BCA), un produit biochimique (métabolites secondaires de plantes ou de microorganismes, extraits de plantes, produits de fermentation de levure) ou semi-chimique (phéromone ou autre produit chimique produit par un organisme) (Chandler *et al*., 2011 ; Koul, 2011 ; Olson, 2015 ; Huber, 2016).

En effet, une nouvelle alternative aux méthodes de protection des cultures telles que les biopesticides microbiennes représente une brillante opportunité de réussite. Cependant, les coûts élevés associés à la R&D et la nécessité d'un nombre répétitif de traitements dans les cultures, pour une efficacité optimale du produit, sont deux contraintes qui influencent le choix de ces produits sur la gestion du vignoble. Les améliorations apportées à la formulation des produits BCA, les progrès technologiques pour réduire les coûts de fabrication et un processus de législation plus rapide peuvent encourager la R&D des biopesticides et leur utilisation généralisée par différents programmes de gestion.

Objectifs de la thèse

Le microbiome naturel associé à la vigne, également appelé comme le deuxième génome de la plante, est lié à la santé végétale, à la productivité et, par conséquent, influencera la qualité du produit final (Turner *et al*., 2013 ; Pinto *et al*., 2016). Actuellement, les interactions entre les microorganismes et la plante sont loin d'être complètement comprises. En effet, la vigne est naturellement colonisée par une myriade de microorganismes, à la fois bénéfiques, neutres et pathogènes, et la préservation de leur équilibre est de la plus haute importance. Cependant, cet équilibre microbien est affecté par l'application constante de pesticides dans les vignobles (Pinto *et al*., 2014), ainsi de nouvelles méthodes de contrôle durable sont nécessaires. La compréhension approfondie de ces ressources microbiennes, de leurs fonctions et de leurs interactions avec la plante constitue une étape importante pour explorer l'évolution de ces communautés à travers le cycle végétatif de la vigne et découvrir des BCAs avec un potentiel biotechnologique afin de développer de nouvelles solutions durables pour la protection du vignoble et ainsi diminuer l'utilisation de pesticide. Dans ce contexte, l'objectif général de cette thèse est de comprendre pleinement les interactions entre la vigne et le microbiome et d'explorer le potentiel biotechnologique de microorganismes bénéfiques, dans le but de contribuer à une viticulture plus efficace et plus durable (Figure 13).

Pour atteindre ces objectifs, nous avons défini une stratégie en deux axes, chacun avec des objectifs précis et clairs (Figure 13) :

<u>Axe A : Caractérisation profonde du microbiome naturel associé à la vigne</u>

Cet axe consisté en l'analyse du microbiome naturel de la vigne associée à différents cépages et sur la compréhension des interactions plantes-microorganismes. Les objectifs spécifiques de cet axe sont les suivants :

A1) caractérisation de la structure et de la dynamique des communautés microbiennes, eucaryotes et procaryotes, associées de la vigne au vin (sur des échantillons de sols, des feuilles et moûts de vin);
A2) comprendre la relation entre les cépages et la structure des microbiomes ;
A3) comprendre l'évolution temporelle de ces communautés microbiennes (en lien avec le cycle végétatif de la vigne, évolution de la fermentation et pendant des années successives) ;
A4) isoler, identifier et caractériser les BCA potentiels de la vigne ;
A5) explorer le potentiel biotechnologique des BCA sélectionnés grâce à l'analyse de leur génome.

Axe B : Le potentiel biotechnologique des microorganismes bénéfiques

Cet axe s'est concentré sur l'analyse des interactions plantes-microorganismes et sur le potentiel de protection des BCAs de la vigne contre les MDBs. Les objectifs spécifiques de cet axe sont les suivants :

B1) comprendre la capacité de colonisation des BCAs à l'aide du modèle *vitro*-plant de la vigne ;
B2) explorer l'impact et l'utilisation potentielle des BCAs pour la protection de la vigne contre le dépérissement lié à *Diplodia seriata* F98.1 ; expérimentation réalisée en serre.

Ce travail vise à contribuer à une gestion plus efficace et plus durable de la vigne, où l'application des BCAs est proposée pour la préservation de la biodiversité microbienne naturelle associée à la vigne.

Résumé de la thèse

Dans l'ensemble, les travaux présentés ici, ainsi que ses résultats et leur discussion sont présentés dans trois chapitres :

Chapitre II: Caractérisation du microbiome de la vigne

Ce chapitre explore l'axe A (Figure 13), à savoir les principaux objectifs de A1 à A3. Les communautés microbiennes naturelles associées de la vigne au vin sont idéntifiées et caractérisées par une approche métagénomique. Pour l'analyse des microorganismes associées à la vigne, un vignoble de l'appellation de Bairrada (Portugal) de 10 ha avec différents cépages, dont les plus significatifs sont Tinta Roriz (TR), Touriga Nacional (TN) et Baga, a été sélectionné et les sols et les feuilles ont été collectés pendant deux années consécutives. Les échantillons ont été prélevés avant et après les traitements phytosanitaires et au cours du cycle végétatif de la vigne. Compte tenu de l'analyse des microorganismes associés à la fermentation du vin, six appellations de vin en Portugal ont été sélectionnés, à savoir le Minho, le Douro, le Dão, la Bairrada, l'Estrémadure et l'Alentejo. Par chaque appellation, les trois cépages les plus représentatifs ont été considérés pour l'échantillonnage. Ensuite, le microbiome du vin a été entièrement caractérisé à travers l'analyse de trois étapes de la fermentation, à savoir les moûts initiaux (IM), et le début et la fin des fermentations alcooliques (SF et EF, respectivement).

Les résultats de ce chapitre ont fait l'objet de trois publications qui décrivent la relation entre les cépages et la structure des microbiomes, mais également l'évolution temporelle de ces communautés microbiennes :

- **Publication 3 :** Cátia Pinto, Diogo Pinho, Susana Sousa, Miguel Pinheiro, Conceição Egas, Ana C. Gomes. Unravelling the diversity of grapevine microbiome. *PloS One*, 2014, 9 : e85622. doi:10.1371/journal.pone.0085622. Cette publication fait parties des 10% des articles les plus cités de *Plos One* et compte actuellement avec 62 citations.

- **Publication 4 :** Cátia Pinto, Valéria Custódio, Miguel Pinheiro, Conceição Egas, Ana C. Gomes., Vine Microbiome: the microbial diversity associated with different Portuguese grape varieties. Manuscrit for submission to the American Society for Microbiology journal.

- **Publication 5 :** Cátia Pinto, Diogo Pinho, Remy Cardoso, Valéria Custódio, Joana Fernandes, Susana Sousa, Miguel Pinheiro, Conceição Egas, Ana C. Gomes. Wine fermentation microbiome: a landscape from different Portuguese wine appellations. *Frontiers in Microbiology*, 2015, 6 : 905. Doi : 10.3389/fmicb.2015.00905. Cette publication compte actuellement avec 30 citations.

Chapitre III : Sélection des BCA potentiels

Le présent chapitre est inclu dans l'axe A (Figure 13) et explore les objectifs A4 et A5. Ici, plusieurs isolats obtenus de la vigne sont testés pour leur potentiel de biocontrôle vers des agents pathogènes importants de la vigne tels que *B. cinerea* et Botryosphaeriaceae (*Diplodia seriata* et *Neofusicoccum parvum*), dans des conditions *in vitro*. Trois BCAs potentiels, à savoir *Streptomyces* sp. Fito_S127B, *Aureobasidium pullulans* Fito_F278 et *Bacillus amyloliquefaciens* Fito_F321 sont ensuite caractérisées pour leur mode d'action lors des activités de biocontrôle. En outre, leur capacité à produire des enzymes extracellulaires, à solubiliser le phosphate, à produire des sidérophores, leurs caractéristiques physiologiques et leurs effets sur les microorganismes non visés sont également explorés. Enfin, le génome de ces BCAs est présenté ce qui fournit des informations sur leur potentiel biotechnologique et leurs mécanismes impliqués dans le contrôle biologique. Ce chapitre comprend trois publications liées à l'étude du génome de ces potentiels BCAs :

- **Publication 6 :** Cátia Pinto, Susana Sousa, Hugo Froufe, Conceição Egas, Christophe Clément, Florence Fontaine, Ana C. Gomes, Draft genome sequence of *Streptomyces* sp. Fito_S127B strain, a soil

microorganism from *Vitis vinifera* microbiome with a promising biotechnological importance. Manuscrit en préparation.

- **Publication 7 :** Cátia Pinto, Susana Sousa, Hugo Froufe, Conceição Egas, Christophe Clément, Florence Fontaine, Ana C. Gomes, Draft genome sequence of *Aureobasidium pullulans* strain Fito_F278, a resident microbiota of grapevine with biocontrol potential against GTDs. Manuscrit à soumettre au Genome Announcements Journal

- **Publication 8 :** Cátia Pinto, Susana Sousa, Hugo Froufe, Conceição Egas, Christophe Clément, Florence Fontaine, Ana C. Gomes, Draft genome sequencing of *Bacillus amyloliquefaciens* strain Fito_F321, an endophyte microorganism from *Vitis vinifera* with biocontrol potential. Manuscrit à soumettre au Standard in Genomic Sciences Journal.

Chapitre IV : Potentiel de phytoprotection de deux BCAs sélectionnés contre les MDBs

Ce chapitre explore l'axe B (Figure 13). Les résultats de l'interaction plante-microorganismes sont présentés, à savoir, la capacité de colonisation des deux BCAs sélectionnés (*Streptomyces* sp. Fito_S127B et *A. pullulans* Fito_F278) à l'aide de plants *in vitro* cv. Chardonnay, et leur utilisation potentielle pour la protection de la vigne contre des espèces de Botryosphaeriaceae, en particulier *D. seriata* F98.1. Ainsi, pour le suivi de la colonisation des BCAs, une identification moléculaire est présentée en utilisant des amorces spécifiques de la souche, qui ont été conçues grâce à leur analyse complète du génome. Ensuite, une expérimentation en serre de 4 mois a été effectuée avec des boutures cv. Chardonnay, et plusieurs paramètres ont été analysés, à savoir le photosystème II, les lésions nécrotiques de la tige causées par le pathogène, la colonisation de la plante par les BCAs et les agents pathogènes, ainsi que l'analyse de l'expression de gènes de la vigne impliqués dans différentes voies de signalisation (protéines PR, métabolisme des phénylpropanoïdes, désintoxication et tolérance au stress, composés de la paroi cellulaire, stress hydrique). Ce chapitre comprend une description complète de l'interaction plante-BCAs-*D. seriata* et dont une publication qui se concentre particulièrement sur les interactions entre la vigne et la souche *A. pullulans* Fito_F278 :

- **Publication 9 :** Cátia Pinto, Valéria Custódio, Mariana Nunes, Aurélie Songy, Fanja Rabenoelina, Barbara Courteaux, Christophe Clément, Ana C. Gomes, Florence Fontaine., Biocontrol potential and grapevine colonisation by natural microbial resources of grapevine : a case study of *Aureobasidium pullulans* strain Fito_F278. Manuscrit à soumettre à Applied and Environmental Microbiology Journal.

RESULTS AND DISCUSSION

Chapter II

CHARACTERIZATION OF THE GRAPEVINE MICROBIOME

Chapter outline

Grapevine, as other plants, is an organism that harbours a myriad of microbial resources that are in a constant interaction with the plant. These associated microorganisms can be beneficial, neutral or pathogens, and are collectively referred as the plant microbiome (Beneduzi *et al.,* 2012; Turner *et al.,* 2013; Pinto and Gomes, 2016). The microbial communities are of utmost importance as they can form beneficial or harmful relationships with grapevine, which may have a direct or indirect effect on plant health and productivity (Berg, 2009; Pinto *et al.,* 2016). As a consequence, both wine production and wine quality will be affected. Beneficial microorganisms can promote the plant growth and stress tolerance, improve plant nutrition, reduce the plant pathogens and induce plant resistance, while phytopathogens are responsible for several plant diseases (Berg, 2009; Berg *et al.,* 2016). Studies have shown the importance of the root microbiome in plant protection, in particular against soil-borne pathogens (Weller *et al.,* 2002; Berg *et al.,* 2016). Herein, the main involved mechanisms include either a direct interaction with phytopathogens or an indirect interaction *via* the plant through a stimulation of its immune system (Lugtenberg and Kamilova, 2009). Thus, the role of the beneficial plant-associated microorganisms is of utmost interest for crops management as either biofertilizers or BCAs (Compant *et al.,* 2010). Indeed, under the challenge of sustainable agriculture practices, this is an important aspect. In addition to the functional context of these microorganisms, recent studies also highlighted the importance of the autochthonous grapevine-associated microorganisms in the winemaking process (Knight *et al.,* 2015; Pinto *et al.,* 2015; Bokulich *et al.,* 2016; Belda *et al.,* 2017). In this context, the grapevine microbiome should be recognized not only as a natural reservoir of potential BCAs for protection, but also as a source of microorganisms with oenological value for the valorisation of regional wines. Thus, the grapevine microbiome must be considered towards a holistic view.

In this chapter, the microbiome associated from the vineyard to wine was explored through a metagenomic approach. Given the grapevine microbiome, a temporal and spatial analysis of the microbial communities associated with soils and leaves from different grape cultivars namely, Tinta Roriz (TR), Touriga Nacional (TN) and Baga, was achieved. Overall, samples were collected over two consecutive growing seasons (2010 and 2011), before and after the phytosanitary treatments, and across the vegetative growth of grapevine. Given the wine fermentation microbiome, six Portuguese wine appellations, namely Minho, Douro, Dão, Bairrada, Estremadura and Alentejo were selected and, for each appellation, the three most representative grape varieties were considered for sampling. The wine microbiome was fully characterized as regards the analysis of three stages of fermentation, namely Initial musts (IM), and Start and End of alcoholic fermentations (SF and EF, respectively).

Results from this chapter are included in three publications, namely publications 3, 4 and 5. Overall, results showed that grapevine microbiome was very dynamic along the growth cycle of the plant – where the eukaryotic biodiversity decreased and the bacterial increased. Though, such differences were more pronounced at leaves than in soils. Effectively, this suggested that the abiotic factors shaped these microbial communities, namely the application of phytosanitary products. Comparactively, a decrease of the microbial biodiversity occurred within the fermentation process as a result of the selective environment created over the spontaneous wine fermentation. Overall, the eukaryotic population from soils, leaves and wine musts was characterized by the Ascomycota and Basidiomycota phylum, while the bacterial population from soils was dominated by the Proteobacteria, Actinobacteria and Acidobacteria phylum, leaves by Firmicutes, Proteobacteria and Actinobacteria and wine musts by Proteobacteria, Actinobacteria and Firmicutes. As expected, a higher microbial biodiversity in soils than in leaves and wine musts was found and, specific microbial communities were identified over these structures. Although, a proportion of microorganisms were shared between them, suggesting the existence of a core microbiome. Interestingly, wine-associated microorganisms were identified in both soils and leaves. Despite being at very low levels (<1%), the results suggested that these microorganisms are natural colonizers of the vine, even before the appearance of berries. Given the wine musts, namely the IM, a biogeographical correlation for the microbial communities was identified between wine appellations suggesting that each wine region contains specific microbial communities.

Overall, these findings added further evidences about the complete microbiome landscape of vineyard and wine fermentations. Furthermore, highlighted not only the analysis of the plant-microbial interactions and its importance for the equilibrium of the grapevine microecosystem, but also the potential role of endogenous microorganisms on the uniqueness of regional wines.

Contexte

La vigne, tout comme d'autres plantes, est un organisme qui abrite une myriade de ressources microbiennes qui sont constamment en interaction avec la plante. Ces microorganismes peuvent être bénéfiques, neutres ou pathogènes, et sont collectivement désignés sous le terme microbiome (Beneduzi *et al*., 2012 ; Turner *et al*., 2013 ; Pinto et Gomes, 2016). Les communautés microbiennes sont d'une importance capitale car elles peuvent constituer des relations bénéfiques ou nuisibles pour la vigne, ce qui peut avoir un effet direct ou indirect sur la santé et la productivité végétale (Berg, 2009 ; Pinto *et al.*, 2016). En conséquence, la production et la qualité du vin va alors dépendre du comportement de ces communautés microbiennes. D'une part, les microorganismes bénéfiques contribuent largement à la croissance de la plante, favorisent sa tolérance au stress, améliorent également sa nutrition, favorisent une réduction des agents pathogènes et permettent à la plante d'être plus résistante. D'autre part, les agents pathogènes sont responsables de plusieurs maladies (Berg, 2009 ; Berg *et al*., 2016). Des études ont montré l'importance du microbiome racinaire dans la protection des plantes, en particulier contre les agents pathogènes du sol (Weller *et al*., 2002, Berg *et al*., 2016). Dans ce cas, les principaux mécanismes impliqués comprennent une interaction directe avec les agents pathogènes ou une interaction indirecte *via* la plante grâce à une stimulation de son système immunitaire (Lugtenberg et Kamilova, 2009). Ainsi, le rôle des microorganismes bénéfiques associés aux plantes est d'un grand intérêt pour la gestion des cultures, afin qu'ils agissent en tant que biofertilisants, ou comme agents de lutte biologique (BCA) (Compant *et al*., 2010). En effet, dans une logique visant à mettre en avant des productions agricoles durables, c'est un aspect qui est important à souligner. En plus du contexte fonctionnel de ces microorganismes, des études récentes ont également mis en évidence l'importance des microorganismes d'origine autochtone associés à la vigne dans le processus de vinification (Knight *et al*., 2015 ; Pinto *et al*., 2015 ; Bokulich *et al*., 2016 ; Belda *et al*., 2017). Dans ce contexte, le microbiome de la vigne devrait être reconnu non seulement comme un réservoir naturel de BCAs potentiels pour la protection de la vigne, mais aussi comme source de microorganismes de valeur œnologique pour la valorisation des vins régionaux. Ainsi, le microbiome de la vigne doit être orienté vers une approache holistique.

Dans ce chapitre, le microbiome associé à la vigne et au vin a été exploré par une approche métagénomique. Compte tenu du microbiome de la vigne, une analyse temporaire et spatiale des communautés microbiennes associées aux sols et aux feuilles de différents cépages, à savoir Tinta Roriz (TR), Touriga Nacional (TN) et Baga, a été réalisée. Dans l'ensemble, les échantillons ont été recueillis pendant deux années de croissance consécutives (2010 et 2011), avant et après les traitements phytosanitaires de la vigne, et au cours du cycle végétatif de la plante. En ce qui concerne le microbiome associé à fermentation du vin, six appellations de vin en Portugal ont été sélectionnés, à

savoir le Minho, le Douro, le Dão, la Bairrada, l'Estrémadure et l'Alentejo et, pour chaque appellation, les trois cépages les plus représentatifs ont été considérés pour l'échantillonnage. Ensuite, le microbiome du vin a été entièrement caractérisé à travers l'analyse de trois étapes de la fermentation, à savoir les moûts initiaux (IM), et le début et la fin des fermentations alcooliques (SF et EF, respectivement).

Les résultats de ce chapitre sont inclus dans trois publications, à savoir les publications 3, 4 et 5. Dans l'ensemble, les résultats ont montré que le microbiome de la vigne était très dynamique au cours du cycle végétatif de la plante - où la biodiversité eucaryote a diminué et celle des bactéries a augmenté. Cependant, ces différences étaient plus prononcées dans les feuilles que dans les sols. Effectivement, cela a suggéré que les facteurs abiotiques ont façonné ces communautés microbiennes, à savoir notemment l'application de produits phytosanitaires. Par comparaison, une diminution de la biodiversité microbienne s'est produite dans le processus de fermentation en conséquence du l'environnement sélectif créé lors de la fermentation spontanée du vin. Dans l'ensemble, la population eucaryote des sols, des feuilles et des moûts du vin a été caractérisée par les phylum Ascomycota et Basidiomycota, alors que la population bactérienne des sols était dominée par les Proteobacteria, Actinobacteria et Acidobacteria, les feuilles par les Firmicutes, Proteobacteria et Actinobacteria et les moûts du vin par les Proteobacteria, Actinobacteria et Firmicutes. Comme prévu, une plus grande biodiversité microbienne a été observée dans les sols que dans les feuilles et les moûts du vin et, des communautés microbiennes spécifiques ont été identifiées sur ces structures. Bien qu'une proportion de ces communautés ait été partagée entre eux, ce qui suggère l'existence d'un microbiome commun. De plus, des microorganismes liés au vin ont été identifiés dans les sols et les feuilles. En dépit d'être à des niveaux très bas (<1%), ces résultats ont suggéré que ces microorganismes sont des colonisateurs naturels de la vigne, même avant l'apparition des baies. Compte tenu des moûts du vin, à savir IM, une corrélation biogéographique des communautés microbiennes a été identifiée entre les différent appellations de vin suggérant que chaque région viticole contient des communautés microbiennes spécifiques.

Dans l'ensemble, ces résultats ont apporté des notions precises sur le microbiome de la vigne et du vin. En outre, ont mis en évidence non seulement l'analyse des interactions plantes-microorganismes et leur importance pour l'équilibre du micro-écosystème de la vigne, mais aussi le rôle potentiel des microorganismes endogènes sur l'unicité des vins régionaux.

Publication 3- Unravelling the diversity of grapevine microbiome

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Unravelling the Diversity of Grapevine Microbiome

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Abstract

Vitis vinifera is one of the most widely cultivated fruit crops with a great economic impact on the global industry. As a plant, it is naturally colonised by a wide variety of both prokaryotic and eukaryotic microorganisms that interact with grapevine, having either beneficial or phytopathogenic effects, who play a major role in fruit yield, grape quality and, ultimately, in the evolution of grape fermentation and wine production. Therefore, the objective of this study was to extensively characterize the natural microbiome of grapevine. Considering that the majority of microorganisms are uncultivable, we have deeply studied the microflora of grapevine leaves using massive parallel rDNA sequencing, along its vegetative cycle. Among eukaryotic population the most abundant microorganisms belonged to the early diverging fungi lineages and Ascomycota phylum, whereas the Basidiomycota were the least abundant. Regarding prokaryotes, a high diversity of Proteobacteria, Firmicutes and Actinobacteria was unveiled. Indeed, the microbial communities present in the vineyard during its vegetative cycle were shown to be highly structured and dynamic. In all cases, the major abundant microorganisms were the yeast-like fungus *Aureobasidium* and the prokaryotic Enterobacteriaceae. Herein, we report the first complete microbiome landscape of the vineyard, through a metagenomic approach, and highlight the analysis of the microbial interactions within the vineyard and its importance for the equilibrium of the microecosystem of grapevines.

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Introduction

Vitis vinifera naturally hosts a reservoir of microorganisms that interact with it and can be transferred to grapes and then into the winery where, ultimately, it may affect the wine production and compromise its quality. Those microorganisms can be beneficial, neutral or pathogenic to the plant [1,2].

The grapevine is also colonized by other ubiquitous microorganisms known as epiphytes and endophytes, who could have beneficial or neutral effects on plants, without causing disease symptoms [3]. Some of these microorganisms are even considered as natural biocontrol agents due to their ability to protect the plant against phytopathogens and reinforce the natural plant defences [4].

Indeed, grapevine is susceptible to several phytopathogens attacks with negative impact on vine longevity, affecting the plant vitality [5]. Altogether, they compromise the normal physiological properties of the grapevine and its vigour, resulting in a decrease of fruit yield and quality, and thus undermining the expected economic return of the vineyard. The most critical phytopathogens agents are fungi, viruses and phytoplasmas [6,7].

The balance of the grapevine microecosystem is influenced by biotic and abiotic factors and also by spatial and temporal fluctuations. In addition, the chemical treatments also affect the grapevine microbiome and are responsible for the appearance of pesticide-resistant pathogen strains [8]. Therefore, a complete survey of the grapevine's microbial ecology, under natural conditions, is of outmost importance because the grape production and quality can be affected by the vineyard's active microbial community [9].

Indeed, phytopathogens have a direct negative impact on grapevine and cause blighting, shrivelling, vine decay and tissue damage [10]. Moreover, the microbial secondary metabolites as mycotoxins, produced by some moulds, are toxic metabolites that may later contaminate the wines. An example of a relevant mycotoxin present in wines, with highest impact in red wines, is the ochratoxin_A (OTA) that is produced by *Aspergillus* spp. and *Penicillium* spp. [11,12]. On the other hand, the microbial community can activate the plant defence pathways, inducing the accumulation of pathogenesis-related (PR) proteins of grapevine as a protection against fungal pathogen attacks or other biological stresses [5]. In fact, it is well known that the accumulation of such PR proteins as chitinases and taumatin-like proteins will later affect the wine clarity and stability [5,13].

Altogether, microorganisms are important for the equilibrium of ecosystems, although little is known about the magnitude and variability of those populations under natural conditions [14]. Indeed, the majority of studies characterizing the microbial diversity rely on classical microbiological approaches. However, cultivation-independent molecular techniques are now starting to be widened, and metagenomics, the study of all indigenous biota

from samples, represents a powerful tool for assessing the microbial communities [15–17].

In our study we have used 454 Next-Generation Sequencing (NGS) to sequence the rDNAs of all microorganisms present in the vine's samples. With these data we will be able to identify both abundant and rare microorganisms present on the vineyard and therefore unravel the dynamics of microbial population during the grapevine vegetative cycle.

Materials and methods

Sampling procedures and DNA extraction

A vineyard with 10 ha located in Bairrada appellation (Cantanhede, Portugal) was chosen for this study (Figure S1a). The sampling was authorized by the private owner, who is fully acknowledged in this paper, and no specific permissions were required for this activity. Also, the field study did not involve endangered or protected species. In order to obtain the deepest insight on the microbial biodiversity, we have collected both healthy (asymptomatic) and diseased leaves from *V. vinifera* cv Tempranillo (also known as Aragonez and Tinta Roriz). For this study, we have decided to sample leaves as they are the biomarkers for the phytosanitary status of plant, are the most abundant, and are the organ of highest surface of the plant. Comparatively to other structures as fruits or flowers, which are not always present, the leaves are the most permanent structures, thus allowing the study along the vegetative cycle of the plant. Furthermore, leaves are more convenient to sample than the wood, which would require cuttings and thus would jeopardize the vitality of the vine. The leaf samples were repeatedly collected during the vegetative cycle from May to July in a total of 10 samplings, from T1 to T10. Sampling was done in 5 different vines distributed in the vineyard, before and after chemical treatments (Figure S1b). The sampling was carried out always from the same vines all over the experiment, in order to minimize sources of variability within this study. A total of 50 leaves were collected and stored at −80°C for subsequent DNA extraction. The DNA, from individual grapevine leaf samples, was extracted using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Amplification of prokaryotic and eukaryotic population and pyrosequencing

A PCR amplicon library was built with the extracted DNA. The PCR primers were designed to target the V6 region of the 16S rDNA for prokaryotic population analysis and the ITS2 and D2 rDNA sequences for eukaryotic population study. A preliminary analysis of our results demonstrated that species obtained with D2 and ITS2 region sequencing are different and that the common taxonomic organisms are low (Figure S2). Therefore, here in we have sequenced and analysed both regions to have the most complete landscape of the vineyards microbiome.

Distinct PCR reactions were performed for V6, D2 and ITS2 region. The amplification of the PCR products was carried out in a 30 μL reaction mix containing 1× reaction buffer (USB, Affymetrix), 0.2 mM of $MgCl_2$ (USB, Affymetrix), 0.2 mM dNTPs (Bioron), 2 μL of DNA, 1 unit of FideliTaq DNA Polymerase (USB, Affymetrix) and 0.4 μM of the eukaryotic forward and reverse specific primer or 0.8 μM of prokaryotic primers. Both eukaryotic and prokaryotic regions were amplified with primers containing the 454 Life Science's sequence adaptors (5′-CGTATCGCCTCCCTCGCGCCATCAG-3′), a barcode with 8 nucleotides which allowed the pooling of multiple samples for pyrosequencing, and the universal primers. The ITS2 region was amplified with the primers ITS2_F 5′-GCATCGATGAAGAACGC-3′ and ITS2_R 5-'CCTCC GCTTATTGATATGC-3', the D2 region was amplified with D2_F 5′AAGMACTTTGRAAAGAGAG-3′ and D2_R 5′-GGTCCGTGTTTCAAGACG-3′ and the V6 region with the primers V6_F 5′-ATGCAACGCGAAGAACCT-3′ and V6_R 5′-TA GCGATTCCG ACTTCA-3′. Cycling conditions consisted of an initial denaturation step at 94°C for 5 min; followed by 25 cycles at 94°C 35 s, 50°C 35 s and 68°C for 40 s; and a final extension for 5 min at 68°C. The PCR amplicons were analysed with the HT DNA 5000 SE30 Chip for the LabChip 90 (Caliper LifeSciences, USA). The PCR products were purified with the High Pure 96 UF Cleanup Plates (Roche) and then their quality and quantity were assessed by fluorimetry, using the PicoGreen® dsDNA quantitation kit (Invitrogen, USA). Afterwards, samples were pooled together in equimolar amounts, and the fragments in the amplicon library were bound to beads under conditions that favour one fragment per bead. The fragments in the amplicon library were subject to an emulsion PCR and the resulting DNA library beads were deposited into the PicoTiterPlate (PTP) for high-throughput pyrosequencing using the Genome Sequencer FLX System Instrument (454 Life Sciences, Roche) at Biocant, Portugal. All sequences obtained from this work are publically available in NCBI platform with the accession number SRP029989.

Data analysis

The raw data was analysed by an automatic annotation pipeline implemented at the Bioinformatics Unit of Biocant. The sequence reads obtained were sorted by identification TAGs and quality filters were applied in order to remove low-quality reads. We have eliminated (*i*) sequences with less than 120 pb, (*ii*) sequences that contained unresolved nucleotides (>2), (*iii*) masked sequences with more than 50% of low complexity areas [18], (*iv*) chimera sequences detected using UChime [19]. Sequences were then grouped according to their phylogenetic distance of 3% [14] and grouped in Operational Taxonomic Units (OTU) through USearch [20]. The consensus sequences were automatically obtained by this software. These pairwise distances served as input to Mothur package [21] for the generation of rarefaction curves (richness of population analysis) and the calculation of the population diversity analysis estimator Chao1 (α diversity). Consensus sequences for each OTU were blasted against curate databases which allowed for taxonomic annotation. Prokaryotic microorganisms were searched on Ribosomal Database Project II (RDP) database [22], whereas eukaryotic microorganisms were identified on the nt@ncbi/SILVA database. After BLAST, the best hits were selected and subjected to another quality control: only the sequences with an alignment greater than 60% and an e-value lower than $1e^{-5}$ were selected and applied for a bootstrap test with 100 replicates, which were obtained by seqBoot from Phylip package [23]. Only those sequences with an identity greater than 70% were accepted, while all the others were considered new sequences.

Eukaryotic and prokaryotic data were analyzed to determine the minimum significant difference ($\rho<0.05$) between biodiversity (Chao1) and one-way analysis of variance (ANOVA) was performed by employing SPSS 20.0 (IBM, US). Normality tests (Shapiro-Wilk) were carried out for each month of collections (May, June, July) and for interval betweenchemical treatments. As all groups followed the normal distribution, a T-test was used.

The microbial communities present from T1 to T10 were compared at family level for prokaryotic microorganisms and at genus level for eukaryotic population through the sequence reads analysis. Thus, microbial population comparisons were carried out

using these taxa. Nevertheless, in some cases it was possible to achieve a sound identification of the species sequence (table S1), mostly for the eukaryotic population, which are also herein discussed.

To analyze the community composition, a log-transformed (log $_{10}$(x+1)) of microbial community was performed. Then, to compare the microbial community structure across the different times of collection (T1–T10), a Principal Component Analysis (PCA) was performed with Bionumerics 6.5 (Applied Maths NV, Belgium). The scores and loadings values were exported and both plots were designed in Excel 2010 (Microsoft, USA). Metastats [24] was used to detect differentially abundant taxa in two microbial populations (microbial population before and after chemical control and during vegetative cycle – May, June, July) and to assess the significance of the observed differences in microbial community. The heat maps were done using Bionumerics 6.5 (Applied Maths NV, Belgium).

Results

Microbial population diversity and richness of grapevines

The objective of this work was to assess the microbial community from grapevine leaves, during the vineyard's vegetative cycle, using a culture independent approach. To achieve this we have undergone a DNA massive parallel sequencing of 16S rRNA gene and D2 and ITS2. Throughout the vegetative cycle of the grapevine, a total of 50 leaf samples were collected from V. vinifera cv Tempranillo and samples were collected before and after the application of chemical treatment according to the calendar of Figure S1b. The deep sequencing of microbial communities originated a total of 142 096 sequences, of which 139 034 sequences passed the Quality Control filters, which represented 97.9% of the obtained sequences (Table 1). For eukaryotic microorganisms we have obtained 79 398 sequences (38 187 identified with D2 region and 41 211 with ITS2) and for prokaryotic we have obtained 59 636 sequences (Table 1). The number of reads per sample ranged from 2070 to 9462 sequences. All the high-quality sequence reads were grouped at a genetic distance of 3% and generated a total of 1 043 OTUs for ITS2, 895 for D2 and 1 242 for V6. On average, we have obtained 97±11 and 124±7 OTUs for eukaryotic and prokaryotic microorganisms, respectively.

The diversity of eukaryotic and prokaryotic populations was compared between samples by rarefaction curves analysis (Figure 1). This allowed us to measure the deepness of our experiments and to characterize the microbial community [25]. Rarefaction curves showed that a good coverage of the entire community was achieved. Therefore, we are aware that despite unveiling a complex and rich microbial structure, there still exists a hidden biodiversity within the vineyard, which we were not able to expose (Table 1).

For each sample we have determined its expected richness (Chao1 index). In our analysis, we have predicted a total richness ranging from 179±17 (eukaryotic microorganisms) to 203±15 (prokaryotic population). By comparing the obtained number of OTUs with its predicted Chao1, we were able to determine the coverage of our experiments. The richness estimators indicated that 54.4±2.2 % and 62.7±2.7 % of the eukaryotic and prokaryotic diversity was uncovered, respectively (Table 1).

In order to assess to the microbial biodiversity during the plant's vegetative cycle, the Chao1 was determined (Figure 2). Interestingly, the Chao1 varied during the vegetative cycle of grapevine and the sequencing of ITS2 regions exposed a higher biodiversity at May and a lower biodiversity at July when compared with D2 and V6 regions.

Microbial community composition

The eukaryotic microbiome of the grapevine leaves was mainly characterized by a high amount of microorganisms from Early diverging fungal lineages (27.9%), Ascomycota phylum (26.3%) and Basidiomycota (16.9%), and at much lower abundances we have also identified microorganisms from Chytridiomycota, Blastocladiomycota and Rozella phyla, which all summed up represent 4.3% of the microbial population. The unknown eukaryotic sequences corresponded to 24.7%, meaning that these sequences were not assigned to any microorganism during the BLAST process (Figure S3a).

Again, our results also reinforce the need for simultaneously sequencing of both eukaryotic regions which was crucial because their discriminating power is rather different, where some organisms are only identified by one of these regions (Figure S4).

Regarding the microbial community, the grapevine showed a dominance of microorganisms that belonged to early diverging fungal lineages namely, *Rhizopus*, *Mucor* and the entomopathogens *Zoophthora* and *Pandora*. Among *Rhizopus* and *Pandora*, these microorganisms were very dynamic along the vegetative cycle and showed to have a higher dominance on July. *Rhizopus* is responsible for the sour rot of grapes and for post-harvest diseases in close association with others as *Penicillium*, *Alternaria* or *Diplodia* [26,27]. The *Mucor* population decreased along the vegetative cycle and, as *Rhizopus*, is an important genera associated with post-harvest diseases of table grapes [28]. Finally, the entomopathogens *Zoophthora radicans* and *Pandora neoaphidis* are insect-pathogenic fungi that infect and kill a variety of insects, including pests [29].

Indeed, some of these early diverging fungi lineages are known to affect the functional insect biodiversity, rather than to impact directly on the physiology of grapevine. For this reason, from now on we will focus on microorganisms belonging to the Ascomycota and Basidiomycota phyla.

Of these, the most dominant genera were *Aureobasidium*, *Sporormiella* and *Alternaria* from Ascomycota phylum (Figure 3a) and the phytopathogen *Guignardia*, which had higher abundances at T1, T2, T3, T5 and T6. At lower abundance, we have identified other genera as *Kurtzmanomyces*, *Colacogloea*, *Lewia*, *Ustilago*, *Puccinia* and *Cronartium*. The eukaryotic community of T1 was the most complex and biodiverse of all samples and, interestingly, such biodiversity consistently decreased during the vegetative cycle. As mentioned above, *Aureobasidium* was dominant, which is in agreement with previously published studies that reported these species as the most abundant in similar eukaryotic communities [30,31].

The dominant phylum among prokaryotic community was Proteobacteria with 31.2% and the Firmicutes with 29.4%. The least abundant phylum was Actinobacteria with 19.4% (Figure S3b). At the class level, the microbial communities were mostly characterized by Gammaproteobacteria (18.8%), Bacilli (18.1%), Betaproteobacteria (12.6%), Actinobacteria (12.1%), Alphaproteobacteria (11.2%), Negativicutes (9.9%), unknown microorganisms (8.7%) and a minor abundance of other class bacterial which all summed up represents 8.6% (Figure S3c).

Bacterial community (Figure 3b) was mostly dominated by Streptococcaceae, Enterobacteriaceae, Pseudomonadaceae and Moraxellaceae families followed by Leuconostocaceae, Comamonadaceae, Veillonellaceae, Xanthomonadaceae, Sphingomonadaceae and Neisseriaceae

Although the microbial community seemed to be similar from T1 to T10, the relative abundances varied during the vegetative

Table 1. Total sequences obtained for eukaryotic (ITS2 and D2) and prokaryotic (V6) microbial community for all samples (T1–T10).

					0.03 distance			
Time Points	Target region	Total reads	High quality	OTU obtained	OTU that passed the blast	CHAO 1	ACE	Coverage (%)
T1	D2	3310	3284	118	111	173	171.37	64.2%
	ITS2	6958	6858	187	176	244	233.70	72.1%
	V6	9528	9462	91	88	127	125.49	69.4%
T2	D2	3371	3342	175	170	272	373.87	62.4%
	ITS2	5630	5454	244	228	392	518.71	58.1%
	V6	8258	8197	130	127	219	327.75	58.1%
T3	D2	3544	3511	109	99	212	208.34	46.6%
	ITS2	3176	3034	148	138	254	353.58	54.4%
	V6	6254	6127	134	126	233	355.03	54.1%
T4	D2	6758	6680	91	85	250	379.68	33.9%
	ITS2	4117	3828	91	84	186	280.17	45.2%
	V6	5534	5464	111	107	152	183.03	70.3%
T5	D2	4094	4071	72	69	116	116.77	59.5%
	ITS2	2765	2648	48	46	106	369.32	43.4%
	V6	6728	6627	169	163	242	254.01	67.2%
T6	D2	3902	3872	109	106	199	340.45	53.3%
	ITS2	3354	3197	121	115	245	358.78	46.9%
	V6	5763	5723	132	128	225	297.95	56.9%
T7	D2	2096	2070	48	47	87	123.31	54.3%
	ITS2	3712	3485	61	58	106	213.35	54.7%
	V6	3923	3872	104	103	176	206.29	58.6%
T8	D2	3325	3304	83	78	165	207.70	47.3%
	ITS2	3751	3576	74	73	110	109.81	66.5%
	V6	4383	4274	149	142	268	386.75	52.9%
T9	D2	4543	4487	72	68	146	192.89	46.5%
	ITS2	4518	4292	65	65	106	138.57	61.6%
	V6	5615	5553	157	146	250	304.77	58.3%
T10	D2	3612	3566	67	62	89	100.74	69.5%
	ITS2	5197	4839	61	60	127	186.99	47.2%
	V6	4377	4337	116	112	138	144.16	81.4%
Total	**D2**	38555	38187	944	895	1710	2215.11	52.3%
	ITS2	43178	41211	1100	1043	1875	2762.97	55.6%
	V6	60363	59636	1293	1242	2030	2585.22	61.2%
	Eukaryotic	81733	79398	2044	1938	3585	4978	54.4±2.2%
	Prokaryotic	60363	59636	1293	1242	2030	2585	62.7±2.7%
	TOTAL	**142096**	**139034**	**3337**	**3180**	**5616**	**7563**	

OTUs and estimated species (Chao1) were determined at a genetic distance of 3% using Mothur. The coverage obtained was also determined as being the ratio between the observed OTUs and the estimated Chao1 (OTUs/Chao1).
doi:10.1371/journal.pone.0085622.t001

cycle. For example while T1 was characterized by the major abundance of Pseudomonadaceae and Sphingomonadaceae, T10 was characterized by the dominance of Streptococcaceae and Enterobacteriaceae.

To analyze the dynamics and relationships among the entire microbial communities (eukaryotic and prokaryotic) present from T1 to T10, a Principal Component Analysis (PCA) was carried out. Figure 4a shows that this separated samples into twogroups, in terms of similarity degree. The first cluster grouped the sampling times T4, T6 and T8, which corresponded to those samples collected after chemical treatment, whereas the second cluster groups T2, T3, T5, T7 and T10, which were collected both before (T3,T5, T7) and after chemical treatments (T2, T10), and have no correlation with chemical treatment or collection time. Indeed, the separation into these 2 clusters is mainly explained by the presence of Enterobacteriaceae, Pseudomonadaceae, Streptococcaceae, *Alternaria* and *Sporormiella*, in the first cluster, and the presence of *Aureobasidium* in the second cluster (Figure 4b). Furthermore T1, which does not belong to any of the clusters formed by the hierarchical clustering based on a Pearson correlation matrixes, is

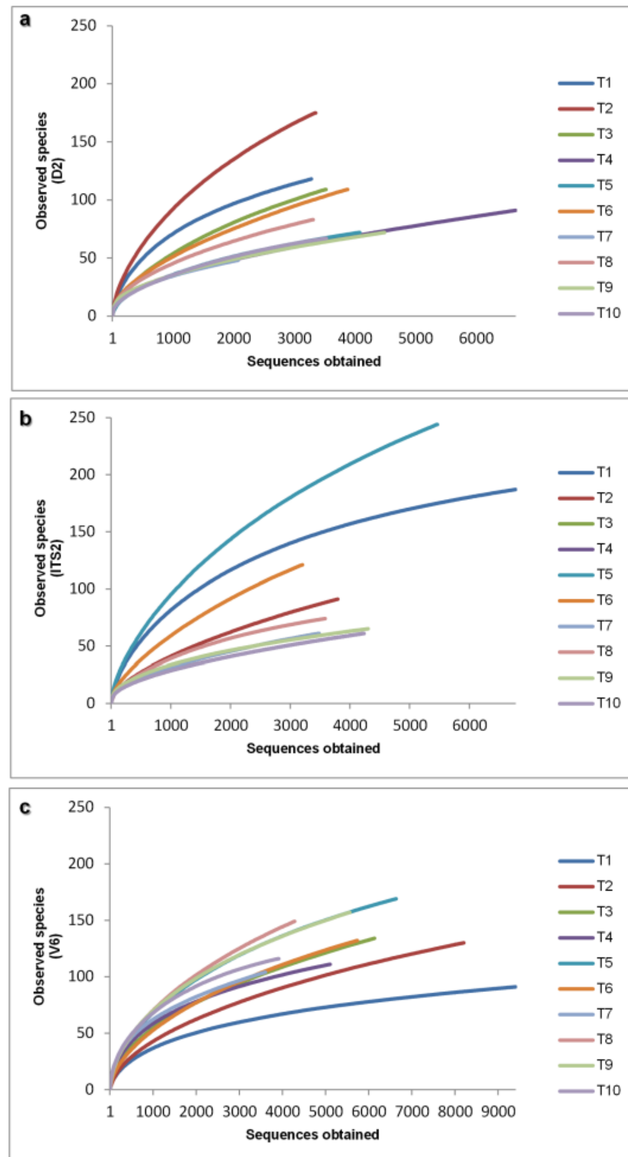

Figure 1. Rarefaction curves at a genetic distance of 3% for each sample (T1–T10). D2 (a) and ITS2 (b) sequences both from the analysis of 26S rRNA and ITS regions of eukaryotic population present in the sample and V6 sequences (c) from the analysis of 16S rRNA of prokaryotic diversity.
doi:10.1371/journal.pone.0085622.g001

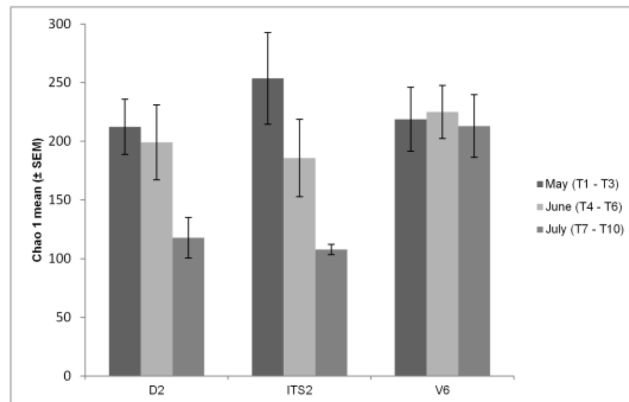

Figure 2. Biodiversity dynamics associated with D2, ITS2 and V6 during the vegetative cycle of grapevine. The means of Chao1 index ± SEM are represented in the graph. Significance was assessed with one-way analysis of variance (ANOVA) and $p<0.05$ was set as statistic significant level. No significant differences were obtained for D2, ITS2 and V6 regions among May, June and July.
doi:10.1371/journal.pone.0085622.g002

mainly characterized by the major abundances of Guignardia, a phytopathogens, and Pseudomonadaceae.

Impact of the chemical treatments on microbial community

The chemical treatments affected the vineyard's microbial population and the comparison among microbial community using Metastats [24] revealed differences between communities ($\rho<0.05$) (Figure 5a). In general, chemical treatments had a negative impact on the balance between phytopathogens and phytoprotectors in the *V. vinifera* microbiome (Figure S5), and a significant decrease on population was observed after the first treatment on May (Figure 5b), when there was the highest microbial biodiversity in the vineyard. Considering the eukaryotic community ($\rho<0.05$) we found significant differences in the populations of *Alternaria, Bulleromyces, Claviceps, Cryptovalsa, Diaporthe, Guignardia, Lewia, Pleurophoma, Puccinia, Sporormiella, Stemphylium, Sydowia* and *Ustilago* (Figure 5a; Table S2).

Aureobasidium, the most abundant eukaryotic genus, showed a relative abundance of 7.1% and 4.1% before and after chemical treatments, respectively. *Sporormiella* (6.1 and 5.1%) and the phytopathogens *Alternaria* (3.9 and 4.2%) and *Guignardia* (3.3 and 3.0%) were also abundant (Figure S6a). Interestingly, we have identified a negative correlation between *Aureobasidium* and *Alternaria*: when *Aureobasidium* is present, *Alternaria* is reminiscent and vice-versa. *Alternaria* is mainly present after the chemical treatment when *Aureobasidium* is less abundant, which suggest that *Aureobasidium* have a protector effect on plant and its abundance on microbial community is clearly affected by chemical control. Among the rare eukaryotic genera, we found *Filobasidiella, Diaporthe* (the teleomorph of *Phomopsis viticola*), *Cryptovalsa, Stemphylium, Candida, Phomopsis, Botryotinia, Dothichiza, Bulleromyces* and *Dioszegia*. Interestingly, among this low abundance microorganisms, we have observed *Botryosphaeria dothidea* (0.23%), which is a phytopathogen associated with grapevine trunk disease that causes the decline of grapevine, limiting vineyard longevity and productivity [32].

In our analysis, *Saccharomyces, Hanseniaspora* and *Metschnikowia* were also identified in leaves at T3, T6 and T8, though at low levels ($<$ 1%). Our data demonstrated that despite being at low levels, these microorganisms are natural colonizers of the vine, even before the appearance of grape fruits and after chemical treatments.

When chemical control with the active element sulfur is applied (Table S3), a drastic impact on the yeast flora is observed [33], and this explains the observed decrease of Aureobasidium, Rhodotorula or Candida by the end of May. In general, the whole yeast community was affected by these chemical treatments (Figure 5b). Furthermore, the application of the chemical treatments supplemented with folpet (applications between T3–T4, T5–T6 and T7–T8) showed an impact mostly in Aureobasidium during May and June which is in accordance with the study of Cabras et al. [34]. Folpet is generally used to control downy mildew, powdery mildew and grey mold infections which are the most devastating grapevine diseases [35]. In our study we did not find the most damaging phytopathogens of grapevine namely, downy mildew (*Plasmopara viticola*) and powdery mildew (*Uncinula necator*) (syn. *Erysiphe necator*) [36,37] which might be explained by the constant chemical control of these phytopathogens (Table S3).

Our experimental vineyard was also subjected to the treatments against black rot disease (*Guignardia bidwelli*) and phomopsis cane and leaf spot (*Phomopsis viticola*). As expected, after the chemical control the abundance of these phytopathogens decreased dramatically (Figure 5; table S1). Interestingly, only a chemical treatment was applied against *G. bidwelli* and we have observed that during the vegetative cycle new infections have emerged on the vineyard (Figure S5b).

Concerning the prokaryotic population, we found significant differences across Bacillaceae, Corynebacteriaceae, Enterobacteriaceae, Halomonadaceae, Leuconostocaceae, Microbacteriaceae, Moraxellaceae, Propionibacteriacea, Pseudomonadaceae, Sphingomonadaceae, Streptococcaceae and Xanthomonadaceae families ($\rho<0.05$) (Figure 5a; Table S2).

The most abundant family was Enterobacteriaceae, with a relative abundance of 7.1% and 7.0% before and after chemical treatments, respectively (Figure S6b). Also, Streptococcaceae (7.0% and 7.3%), Pseudomonadaceae (6.5% and 6.1%), Moraxellaceae (5.8% and 6.2%) and Comamonadaceae (5.0% and 4.7%), were

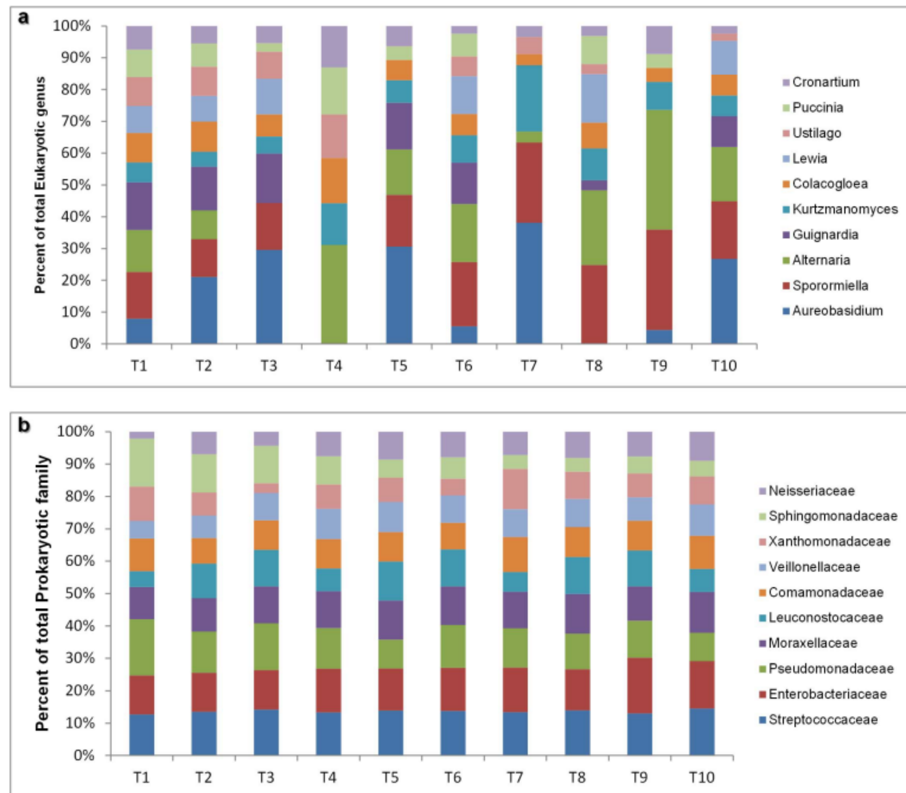

Figure 3. Eukaryotic (a) and prokaryotic (b) microbial community distribution from T1 to T10. Relative abundance of the 10 most abundant eukaryotic and prokaryotic microorganisms through the genus and family analysis, respectively.
doi:10.1371/journal.pone.0085622.g003

among the most abundant before and after chemical treatments of the vineyards, respectively. Other families, as Methylobacteriaceae, Oxalobacteraceae, Nocardioidaceae, Rhodobacteraceae or Bacillaceae were also detected, though with relative abundances below 2%, and were considered as rare microorganisms. In general, the applied chemicals affected the bacterial community and a decreased on the relative abundance was observed after chemical treatments for Enterobacteriaceae, Pseudomonadaceae, Comamonadaceae or Xanthomonadaceae families.

In the grapevine bacterial population, both lactic acid bacteria (LAB) and acetic acid bacteria (AAB) were identified among other bacterial groups. In wine production LAB, especially *Oenococcus oeni*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* are of outmost importance because of the malolactic fermentation. In our samples, the identified LAB belonged to Carnobacteriaceae, Enterococcaceae, Leuconostocaceae and Streptococcaceae families. From the Carnobacteriaceae it was observed *Trichoccus* and *Atopostipes* genus and from the Enterococcaceae family *Enterococcus* (mainly *E. italicus*) was also observed. From the Leuconostocaceae we observed *Leuconostoc* (*L. citreum* and *L. fallax* species) and *Weisella* (*W. confuse*) and, finally, from the Streptococcaceae family, *Streptococcus* and *Lactococcus* were identified. However, most of these bacteria are not associated with the winemaking process. We have also detected microorganisms from the Lactobacillaceae family mainly, *Lactobacillus salivarius*, though this population was rare (table S1). Like previous reports on biodiversity of grapes, we did not identify *O. oeni* in grape leaves [38,39]. Overall, our data show an increase of LAB from May to July (Figure 5b).

Regarding the AAB, we have detected in our samples microorganisms from the Acetobacteraceae family, mainly *Acidisoma*, *Gluconacetobacter* and *Roseomonas* genus. Remarkably, the AAB was predominantly present on May and June in a positive correlation with the presence of *Botryotinia*. This finding reinforces the positive correlation between AAB and *Botrytis* infection in the vineyards [40], [41].

Distribution and interactions of microbial community across vegetative cycle

The eukaryotic microbial community was very dynamic during the vegetative cycle (Figure 5b) and presented significant

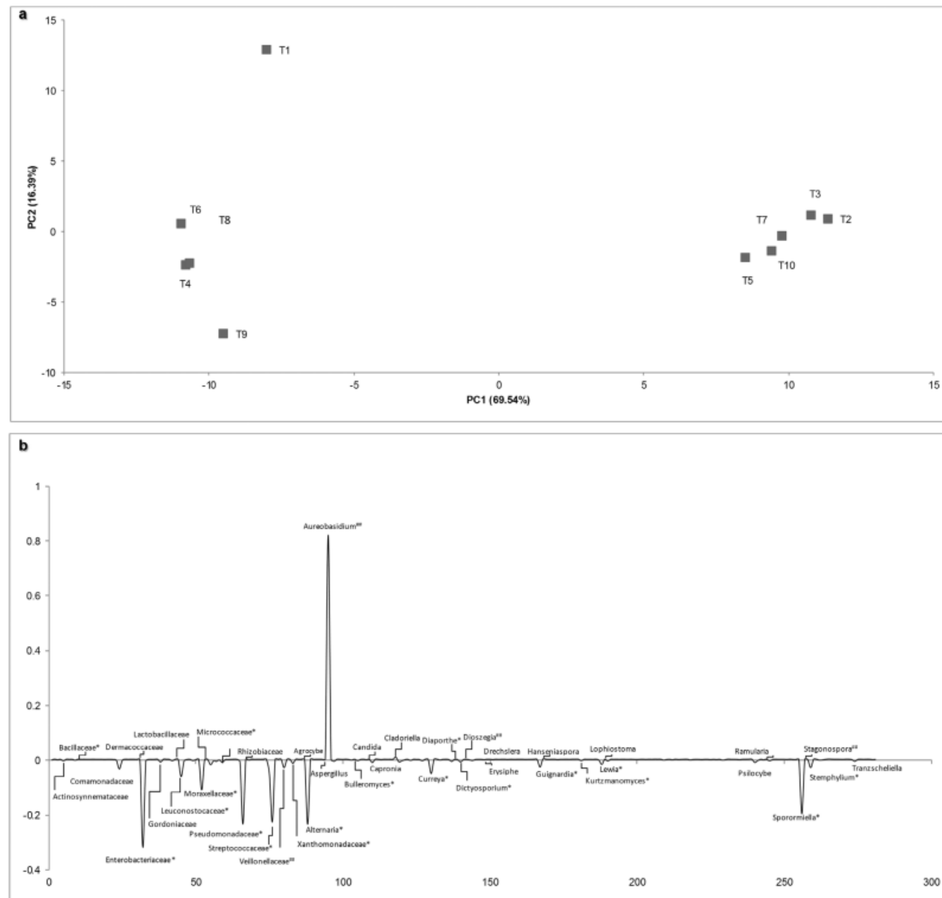

Figure 4. PCA biplot diagram and loading plot of microbial community across sampling time (T1–T10), based on sequence abundance of eukaryotic genus and bacterial family. (a) The PCA diagram is shown and the percentages of data variability explanation are shown in the upper of X and Y axis and more than 85% of the variability in the data is accounted. (b) Loading plot of principal component 1 showing variables that explain variability across eukaryotic genus and prokaryotic family during the vegetative cycle of grapevine (T1–T10). The significant differences were observed in Metastats for eukaryotic genus or prokaryotic families and are represented with asterisk (*) and microbial community that are identified with ## are considered false discovery rate.
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alterations in its structure ($\rho<0.05$). Among phytopathogens, differences were found in *Guignardia*, *Diaporthe* or *Phomopsis* and between phytoprotectors differences were found in *Aureobasidium* and *Rhodotorula* (table S4).

Concerning the 10 most abundant eukaryotic communities on May, June and July (Figure S7a, 7b and 7c), we observed that on these months the most abundant microorganisms were *Aureobasidium* and *Alternaria*. Furthermore, in May *Guignardia* was the most abundant phytopathogen though *Bensingtonia*, *Claviceps*, *Ustilago*, *Alternaria or Curreya* were also present. On June, *Sporormiella* and *Alternaria* showed an increase and a decrease of *Guignardia* from May to July was detected. Then, on July *Alternaria*, *Aureobasidium* and *Sporormiella* were the most abundant microorganisms and an increase of *Alternaria*, *Aureobasidium* and *Sporormiella* was observed.

Prokaryotic population was also very dynamic and significant differences ($\rho<0.05$) across the microorganisms as Enterobacteriaceae, Pseudomonadaceae, Streptococcaceae, Sphingomonadaceae, Moraxellaceae, Leuconostocaceae were observed (Figure 5b and table S4).

Of the 10 most abundant prokaryotic communities (Figure S7d, 7e and 7f) we observed that on May Pseudomonadaceae, Streptococcaceae, Sphingomonadaceae and Enterobacteriaceae dominated the microbial consortia. On June, Streptococcaceae was the most abundant family followed by microorganisms from

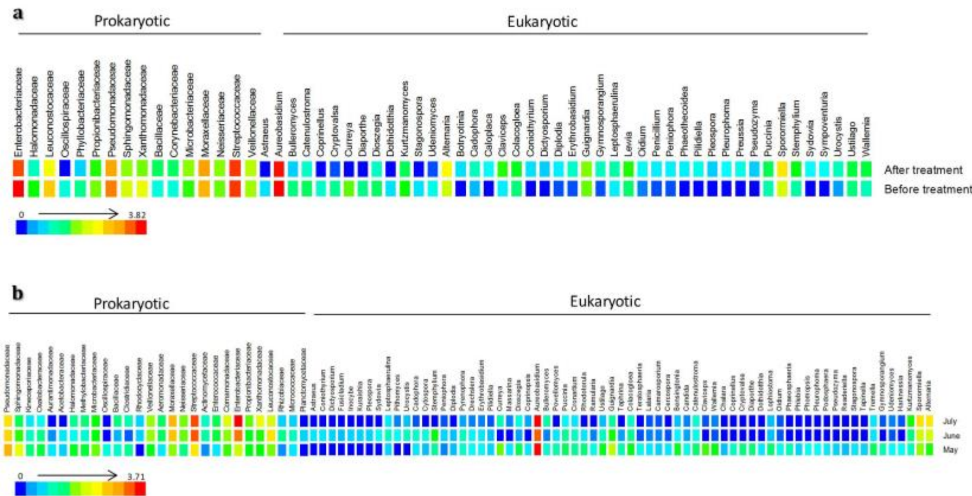

Figure 5. Variation on the abundance of the significant eukaryotic genus and prokaryotic family (p<0.05) as affected by chemical treatment (a) and grapevine vegetative cycle (b). The variation on population during the grapevine vegetative cycle could be interpreted through the variation of the square's color where the red square indicates a higher number of reads and a blue square denotes a reduce number of reads of a specific microorganism. Only the significant population is shown (p<0.05).

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the Enterobacteriaceae, Moraxellaceae and Pseudomonadaceae families. Finally, on July, the most abundant families were Enterobacteriaceae and Streptococcaceae.

Discussion

In this work we have uncovered the microbial biodiversity of grapevine leaves through metagenomic approaches and the interpretation of microbial communities showed to be essential to understand the balance of phytopathogens and beneficial microorganisms. Such understanding could represent a crucial step for the development of environmental friendly strategies for plant protection and grape production.

Grapevine phytoprotectors vs. phytopathogens

The abundant eukaryotes and prokaryotes identified belonged to the *Aureobasidium* genus and Enterobacteriaceae family, respectively, which is in agreement with previous published studies [39]. These microorganisms play an important role in the microbial consortium of vineyards and grapes and are thought to be beneficial. They have specific modes of action as induction of host resistance and production of glucanases, chitinases and proteases, which makes them excellent antagonists and also beneficial microorganisms [42].

According to our data, the prevalence of *Aureobasidium* genus is due to the presence of *A. pullulans*. Previous data related that *A. pullulans*, *Epicoccum nigrum*, *Rhodotorula* and *Candida* dominate the consortia of grapes and together are the most abundant anti-phytopathogen microorganisms [31,43–45]. Further, published data also refers that *A. pullulans* has antagonistic activity against moulds, namely *Botrytis* and certain bacterial as *Bacillus* [31], which may explain the lower prevalence of *Bacillus* genus in our results.

In our samples we have also detected *Bulleromyces*, namely *B. albus* and *Dioszegia* spp. The former is referred as a beneficial microorganism with antagonistic activities and with the capacity to produce extracellular polysaccharides [46] and the latter was described to be associated with arbuscular mycorrhizal fungi, revealing a beneficial action [47,48]. Beyond these, also the yeasts *Sporobolomyces* and *Candida*, have been reported to have antifungal effects [49]. Those yeasts do not have any enological interest and are described as natural inhabitants of the vineyard [9].

Interestingly, a wide diversity of eukaryotic phytopathogens was present in the analysed leaf samples amongst which *Rhizopus*, *Lewia*, *Alternaria*, *Diaporthe*, *Phomopsis*, *Cryptovalsa*, *Stemphylium*, *Ustilago* and *Botryotinia*. Some of these phytopathogens as *Phomopsis* (*Phomopsis* type 2), *Cryptovalsa* (*C.ampelina*) and *Botryotinia* are commonly associated with diseases in the vineyard [50,51]. Other microorganisms as *Lewia*, *Alternaria*, namely, *A. solani* and *Stemphylium*, (*S. solani*) have been reported as phytopathogens of different crop cultures as wheat, sorghum, pistachio, potatoes and tomatoes [52,53].

It is worthwhile to notice the emergence of *Guignardia* (*G. bidwelli*) on grapevines, which was one of the most abundant phytopathogens in our samples, has been recently detected in some Portuguese vineyards and it was noticed for the first time on Bairrada appellation during the 2006 vine campaign [54]. *G. bidwelli* causes the black rot and, according to the severity of the disease, the qualitative and quantitative performance of the vineyard could be drastically affected. To date, *G. bidwelli* is restricted to some viticulture regions and in Portugal their occurrence is higher at Bairrada and Alentejo appellations [55].

Among prokaryotic consortia, we have also identified potential antagonistic microorganisms. According to previous studies, the most well-known and reported bacterial antagonists are species of *Pseudomonas* (*Pseudomonadaceae* family), *Burkholderia* (*Burkholderiaceae*),

Bacillus (*Bacillaceae*), *Serratia* and *Pantoea* (*Enterobacteriaceae*) and *Actinomycetes* (*Actinomycetaceae*) [56,57]. It is well documented that bacterial strains belonging to *Streptococcus* (*Streptococcaceae* family) also display antagonistic activity against fungal pathogens [56]. In our samples, all these bacterial were detected although *Burkholderiaceae* where not abundant.

Additionally, the LAB found in our study are referred to be widespread in fermentable materials and, because of their potential of acidification, they prevent the growth of phytopathogenic microorganisms and have inhibitory effects on yeasts [38,58].

Furthermore, a wide diversity of bacterial microorganisms cohabit with grapevine but do not cause adverse effects, with exception of *Pseudomonas syringae* and *Xanthomonas campestris* pv. *viticola* that are described as phytopathogens but were not detected on our analysis. However, and with exception of some bacteria that have impact in wine production, most of the bacterial population that we found on grapevine are not integrated in the wine microbial consortium and do not influence wine quality [59].

Microorganism's modulation in the vineyards

The vineyard's microbial population showed to be very dynamic across the vegetative cycle and a high biodiversity was unveiled. Nevertheless, a sharp decline in eukaryotic biodiversity was observed during the grapevine ripening which can be explained by spatial and temporal fluctuations, biotic and abiotic factors or other conditional factors as chemical treatments [59]. Beyond these, viticulture practices, grapevine varieties, age of vines, grapevine ripening and vectors are all known to influence the microbial ecosystem, microbial dispersal and even microbial performance [45,60]. Nevertheless, microbial community is ubiquitous and some of them are responsible to maintain the ecosystem function.

Despite the unveiled high biodiversity, the observed balance between the different microorganisms and the dynamics across grapevine ripening, there is still a large gap in the knowledge of the functional diversity and significance of microbial community-plants interaction on grapevine. In fact, the co-existence of different microbial population generates competition for the nutrients, different interactions are established and enzymatic compounds are produced. Indeed, the latter could have a toxic effect on other species, and thus have antifungal properties [43,58,61].

The deep analysis of the microbial consortia revealed statistically significant differences in eukaryotic and prokaryotic diversity within chemical treatments and during the vegetative cycle, which will have a direct and indirect effect on grapevine community composition. Indeed, among eukaryotic population the early diverging fungal lineages and Ascomycota phyla and the prokaryotic Proteobacteria showed higher abundances in vineyards.

Altogether, this work reinforced the importance of studying the natural biodiversity of grapevine and highlighted the need of a more detailed study of the microbial interactions on plant. Furthermore, the grapevine microbial consortia showed to contain both beneficial and phytopathogenic microorganisms which will have a significant influence on the vine performance and also on the wine quality. Our data contribute to the characterization of the biodiversity of grapevines and to the analysis of biomarkers with the potential to unveil the plant health status.

Supporting Information

Figure S1 Vineyard chosen for study and chemical treatments calendar. (a) Sample collection was done in the 5 vines, throughout the 10 time points (T1 to T10). To ensure reliable results, all samples were collected from the same vines. (b)The time intervals of samples collection was defined according to the chemical treatments calendar, over the 3 months of trial. The leaves were collected before and closed to the chemical treatment application (green plot) and after the chemical treatment (red plot).
(PNG)

Figure S2 Venn diagram showing the observed species for ITS2 and D2 region and common species. The number of reads and the OTUs obtained are showed for both regions. Taxonomic classification was defined by 97% of sequence similarity. To determine which region of the 26S rDNA would be most suited for the metagenomic analysis of eukaryotic microorganisms, a preliminary test was carried out where within the same sample we have targeted both ITS2 and D2 regions. After this analysis, we have obtained 123 observed microorganisms for ITS2 and 121 for D2 region although, just 41 microorganisms were common to both regions.
(TIF)

Figure S3 Microbial community distribution over the vegetative cycle of grapevine. Relative abundance of the eukaryotic microorganisms (a) that were mostly characterized by Early diverging fungi and Ascomycota phyla. The prokaryotic community (b) was characterized by Proteobacteria and Firmicutes and at the class level (c) by Gammaproteobacteria and Bacilli.
(TIF)

Figure S4 Relative abundance of the number of reads during the grapevine vegetative cycle of the eukaryotic population. Detailed description of the relative abundance of D2 (a) and ITS2 sequences (b) during sampling collection. A deep analysis of D2 region showed that early diverging fungal lineages were only identified by D2 sequencing and also this region identified predominantly microorganisms designated as others. According to ITS2, the major relative abundance was of Ascomycota and unknown microorganisms.
(TIF)

Figure S5 Effect of chemical treatment application on specific microorganisms. The balance of microbial community is affected by chemical treatments and a decrease of both phytopathogens (a,b) and phytoprotectors (c,d) is observed. The chemical control was applied between the intervals T1 and T2, T3 and T4, T5 and T6, T7 and T8, T9 and T10. The arrows indicate the application of chemical treatments with known direct effect on presented microorganisms.
(TIF)

Figure S6 Relative abundance (%) of eukaryotic genus and prokaryotic family before and after the chemical treatment. The most abundant phylogenetic groups (>2%) of eukaryotic genus (a) and prokaryotic family (b).
(TIF)

Figure S7 Microbial Composition of May (a and d), June (b and e) and July (c and f). Relative abundance of the 10 most abundant eukaryotic (a, b and c) and prokaryotic (d, e and f) microorganisms for each month, through the genus and family analysis, respectively.
(TIF)

Table S1 Species observed on eukaryotic and prokaryotic community. Distribution of the microbial microorganisms identified both eukaryotic and prokaryotic, during the vegetative cycle of grapevine. For each microorganism, a consensus ID, number of reads, alignment lenght, alignment start, score and the respective e-value for the blast are shown.
(XLSX)

Table S2 Impact of chemical treatments application on eukaryotic and prokaryotic community. Application of Metastat to compare and to detect differences between microbial communities (both eukaryotic and prokaryotic) with chemical treatment application.
(XLSX)

Table S3 Active elements of chemical treatments. Chemical treatments calendar with their respective active elements. The interval of application of such chemical treatment is also shown.
(XLSX)

Table S4 Distribution and interactions of microbial community across vegetative cycle. Application of Metastat to compare and to detect differences between microbial communities (both eukaryotic and prokaryotic) during the vegetative cycle of grapevine.
(XLSX)

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Author Contributions

Conceived and designed the experiments: CP ACG. Performed the experiments: CP SS. Analyzed the data: CP DP MP. Contributed reagents/materials/analysis tools: SS MP CE ACG. Wrote the paper: CP ACG.

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Publication 4- Vine Microbiome: the microbial diversity associated with different Portuguese grape varieties

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The supporting information of this publication is available in the Appendix 2 section.

Abstract

Plants naturally harbor a complex microbial community that constitutes the plant microbiome, and which are in a constant interaction with plant. Indeed, these microorganisms are important for plant performance, plant health or plant stress tolerance and may have a direct or indirect effect on plant vitality and productivity. The aim of this study was to deep explore the microbial communities associated with soils and leaves of Baga, Tempranillo (TR) and Touriga Nacional (TN) grape varieties over the grapevine growth cycle and growing seasons, by using a metagenomic approach.

Results showed that grapevine-associated microorganisms are very dynamic and highly complex. Overall, soils and leaves were characterized by specific microbial signatures, in which the major explanatory variables were *Aureobasidium, Fusarium, Alternaria, Lophiostoma, Diplodia* or *Cadophora* in soils, and *Candida*, *Alternaria, Lewia*, *Debaryomyces*, *Saccharomyces* and *Aureobasidium* in leaves. Given the bacterial communities, Sphingomonadaceae and Nocardioidaceae families were the major explanatory variables in soils, and Streptococcaceae and Enterobacteriaceae in leaves. Furthermore, each grapevine structure shared a microbial profile, which suggests that grapevine is a reservoir of a core microbiome. Interestingly, most of these common microorganisms belonged to low-abundance population. As expected, soils were characterized by the higher and more stable microbial biodiversity, suggesting their importance as a major microbial reservoir of grapevine. In contrast, a particular microbial distribution was found in leaves, namely a decrease of the eukaryotic biodiversity and an increase of the bacterial communities during the grapevine ripening. Indeed, these communities varied across grapevine growth cycle and were affected by the phytochemicals application, though these were not the only explained variables for this variation. In fact, grape varieties were also responsible for this microbial shaping. This was particularly observed in soils, were each cultivar assembled a specific eukaryotic microbial cluster.

Overall, results highlighted that the grapevine-associated microorganisms significantly differed over the grapevine structures (soils and leaves), and those microbial biodiversity decreased as distance from soils increased. Furthermore, an important microbial profile was preserved over the grapevine structures and growing seasons, suggesting the existence of a core microbiome in which may have an important functional ecology that promoted a stable and consistent plant-association.

Introduction

Plants naturally encompass several microbial communities that constitutes the plant microbiome. These microorganisms are important for plant performance, plant health or plant stress tolerance and may have a direct or indirect effect on plant vitality and productivity (Zilber-Rosenberb and Rosenberg, 2008; Berg, 2009; Berendsen *et al*., 2012; Turner *et al.,* 2013).

The microbial communities are of utmost importance as they can form beneficial or harmful relationships with plants and thus promote or induce the plant growth, plant defense mechanisms and stress resistance, or be associated with plant diseases (Berg, 2009; Pinto *et al*., 2016). Depending on the plant organs or structures, these microorganisms may be involved in different processes such as carbon cycle, nitrogen-fixation, nutrient uptake or antibiotic and active compounds production. Further, the microbial richness and its dynamics will be conditioned by biotic, abiotic factors and host. Regarding the microorganisms from phyllosphere (above-ground), these are highly dynamic as a consequence of changes in temperature, UV exposure, low nutrient content, crops management practices or even by temporal and spatial fluctuations (Lindow and Brandl, 2003; Whipps *et al.,* 2008; Turner *et al*., 2013). On other hand, microorganisms from rhizosphere (below-ground) are highly modulated by root exudates and by the physicochemical properties of the soil (Philippot *et al.*, 2013; Heijden and Hartmann *et al.,* 2016; Lareen *et al.,* 2016). Related to this, the role of the microbial biodiversity on the ecosystem functioning has been study and different hypothesis presented (Naeem *et al.,* 2002; Hooper *et al.,* 2005; Heijden and Hartmann *et al.,* 2016). Hence the plant and their associated microbiome are intrinsically connected and cannot be dissociated, altogether form the holobiome. For genomic evolution, the holibiome is considered as the unit of selection and as an adaptation process (Zilber-Rosenberg and Rosenberg, 2008; Guerrero *et al.,* 2013, Vandenkoornhuyse *et al.,* 2015).

Grapevine (*Vitis vinifera* L.) is one of the most cultivable fruit crop in the world with a significant impact on economy and society. For some countries, viticulture encompasses a cultural identity, heritage and tradition. Guaranty the grapevine protection against microbial pathogens is crucial to keep vines healthy and productive and consequently, to avoid economical losses. Actually, the majority of grapevine management practices mostly depends in the application of pesticides and, and among crops, grapevine has the major incidence of these chemicals (Pinto *et al.,* 2016). Previously studies highlighted that these compounds cause a disturbance on microbial biodiversity (Newton *et* al., 2010; Pancher *et al.,* 2012; Pinto *et al*., 2014). Thus, understanding the natural microbial communities associated with grapevine may create an opportunity to discover potential beneficial microorganisms proficient to promote beneficial plant-microbe interactions and even to protect the plant through pathogen inhibition or suppression. Further, as this resident microbiota presents low risks on the

environment and is well adapted to the vineyards conditions, this may provide an advantage and a promotion of a sustainable viticulture, thus reducing the input of agrochemicals.

Herein, this study addresses a deep characterization of the microbial communities associated with soils and leaves of Baga, Tempranillo (TR), also known as Aragonez or Tinta Roriz and Touriga Nacional (TN) grape varieties, using a metagenomic approach. With this study, we aimed to determine (i) the dynamics of both eukaryotic and bacterial communities along the grapevine growth cycle, (ii) to correlate the microbial structure among different grapevine niches and (iii) whether grape varieties harbor a specific microbial profile. Overall, this study deep characterizes the grapevine-associated microorganisms and represents an opportunity to better understand and to identify potential microorganisms and microbial correlations that could be applied for further sustainable viticulture management practices, in a short or long-term.

Material and methods

Sample collection

Superficial soils and grapevine leaves were collected from a vineyard in Bairrada Appellation (Cantanhede, Portugal) across three grape varieties namely, Baga, Tempranillo (TR; also known as Aragonez or Tinta Roriz) and Touriga Nacional (TN). For the superficial soils, 8 plants (2 plants of each grape variety) were selected and samples were collected only at 2011, in a total of 7 samplings. Regarding the leaves, these were collected over two consecutive growing seasons – 2010 and 2011. For this, 18 plants (8 plants cv Baga; 5 plants cv TR and 5 plants cv TN) were selected and samples collected from May to July at 2010 and from April to September 2011, in a total of 10 (from T1 to T10) and 15 samplings (from T1 to T15), respectively. Samples were collected before and after the application of chemical treatments in vineyard, and were performed always in the same vines, to minimize the sources of variability (Appendix 2: Figure S1; Table S1). Superficial soils samples (n=56) and grapevine leaves (n=450) were then stored at -80ºC for subsequent DNA extraction.

DNA extraction and pyrosequencing

For this study, no sterilization procedure of leaves samples was performed prior to the DNA extraction, and both endophytes and epiphytes microorganisms were analysed. The genomic DNA from soils and leaves was extracted using the QIAamp® DNA Stool Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and as previously described by Pinto *et al*., 2014. The amplicon generation of both prokaryotic (bacterial V6 region of 16S rDNA) and eukaryotic (fungal ITS2 and D2) microorganisms was performed according to the methodology of Pinto *et al*., 2014. Superficial

soils were only analysed for V6 and ITS2 region, while leaves were analysed for both V6, ITS2 and D2 regions. The V6 region was amplified with the primers V6_F (5'- AT GCA ACG CGA AGA ACC T-3') and V6_R (5'-TA GCG ATT CCG ACT TCA-3'), the ITS2 region with the ITS2_F (5'- GC ATC GAT GAA GAA CGC-3') and ITS2_R (5'-CC TCC GCT TAT TGA TAT GC-3'), and D2 region with the D2_F (5'-AA GMA CTT TGR AAA GAG AG-3') and D2_R (5'-GG TCC GTG TTT CAA GAC G-3'). All primers contained a 454 Life Science's sequence adaptors (5'-CGT ATC GCC TCC CTC GCG CCA TCA G-3'), a barcode with 8 nucleotides and the universal primer. For the sequencing, the PCR products were pooled together in equimolar amounts, and the fragments in the amplicon library were bound to beads under conditions that favour one fragment per bead. The fragments in the amplicon library were subject to an emulsion PCR and the resulting DNA library beads were deposited into the PicoTiterPlate (PTP) for high-throughput pyrosequencing using the GS-FLX System Instrument (454 Life Sciences, Roche) at Biocant, Portugal.

Data analysis

The sequences reads obtained were analysed with MetaBiodiverse, an in-house automatic annotation pipeline (Vaz-Moreira *et al*., 2011; Egas *et al*., 2012; Pinto *et al*., 2014). The raw data was split by identification of barcode sequences and quality filtered through the elimination of (i) short sequences (<120 pb), (ii) sequences containing more than two ambiguous bases (N), (iii) masked sequences with more than 50% of low complexity areas (Sogin *et al*., 2006) and (iv) chimera sequences, detected with UChime (Edgar *et al*., 2011). Sequences were then grouped into Operational Taxonomic Units (OTU) through USearch version 6.0.307 (Edgar, 2010), at a phylogenetic distance of 0.03 (Sharpton *et al*., 2011). The Mothur package (Schloss *et al*., 2009) was applied to generate the rarefaction curves (richness of population analysis) and to calculate the population diversity analysis estimator Chao1 (α diversity). The taxonomical annotation of each OTU was queried against the Ribosomal Database Project II (RDP; Cole *et al.,* 2009) and nt@ncbi/SILVA database for prokaryotic and eukaryotic assignment, respectively. The best hits were then selected and subjected to another quality control where only the sequences with an alignment greater than 60% and an e-value less than $1e^{-50}$ were selected and further applied for a bootstrap test with 100 replicates, using the seqBoot from Phylip package (Felsenstein, 1989). Only those sequences with an identity greater than 70% were reported, while all the others went up the taxonomy levels until reached 70%.

The microbial population comparison was carried out through the analysis of the sequence reads at the family and genus level for prokaryotic and eukaryotic population, respectively. The population abundances were log-transformed ($\log_{10}(x+1)$) and used to calculate a Bray-Curtis resemblance matrix. The data was then explored through a principal coordinate analysis (PCO), analysed by PERMANOVA

for significant differences and by SIMPER to identify the taxa responsible for similarities and dissimilarities between samples/groups, by using the Primer E software version 6 (Clarke and Gorley, 2006). The microbial communities' sizes were separated by grape varieties and plant structure through a Venn diagram by using the Venny 2.1 software (Oliveros, 2007).

Data availability

To date, the project has not yet been submitted, and therefore does not have an accession number.

Results

Herein, the microbiome associated with soil and grapevine leaves of different grape varieties namely, Baga, Tempranillo (TR) and Touriga Nacional (TN) was assessed by a DNA massive parallel sequencing of both ITS2 and D2 regions, for eukaryotic microorganism's identification, and 16S rRNA gene, for bacterial analysis. Samples were collected along the grapevine growth cycle; soils were collected only during 2011 growing season, while leaves were collected before and after the application of chemical treatments in the vineyard, over two growing seasons - 2010 and 2011 (Appendix 2: Table S1).

Considering the soils, after filtering the low-quality reads, a total of 254,949 sequence reads were obtained (123,176 eukaryotic and 131,773 prokaryotic) from a total of 56 superficial soils samples (Table 1). An average of 287±16 OTUs and 636±34 OTUs were generated for eukaryotic and prokaryotic, respectively. Considering the expected richness, it was predicted a total of 384±21 and 1107±61 for the eukaryotic and prokaryotic community, respectively. A good coverage of the population was achieved, namely 71.20% for the eukaryotic population and 57.82% for bacteria (Table 1).

Regarding the leaves, a total of 541,457 sequence reads (320,848 eukaryotic and 220,609 prokaryotic) were obtained from 180 samples collected during the 2010 season and a total of 781,527 sequence reads (491,642 eukaryotic and 289,885 prokaryotic) generated from 270 samples collected at 2011 (Table 1). These sequences were grouped at a genetic distance of 3% and, in average generated 108±5 OTUs for eukaryotic and 118±5 OTUs for prokaryotic at 2010. At 2011 growing season, an average of 60±10 OTUs and 66±7 were obtained for both eukaryotic and prokaryotic, respectively. The expected richness (Chao 1 index) was determined for each sample and, in general was predicted a total richness ranging from 180±8 (eukaryotic population) to 191±10 (prokaryotic population) at 2010, and

from 77±13 (eukaryotic) to 85±9 (prokaryotic) at 2011 (Table 1). Consequently, the coverage of this study was determined by comparing the obtained OTU with its predicted Chao 1 index. Thus, the richness indicators showed that 57.09±1.10% and 61.98±1.70% of the eukaryotic and prokaryotic diversity, respectively, was uncovered at 2010, while at 2011, 78.93±2.8% and 78.36±2.78% was uncovered for eukaryotic and prokaryotic, respectively. Altogether, these results showed a good coverage of the entire microbial community's present at leaves although, there still exists a hidden biodiversity within the vineyard which were not able to unveil (Table 1).

Overall eukaryotic and bacterial communities

Soil samples were characterized by a higher microbial biodiversity when compared with leaves (Figure 1; Table 1). Furthermore, this biodiversity was more stable in soils, where no significant fluctuations of both eukaryotic and bacterial OTUs were found across different sampling time-points (Figure 1A). In addition, no strong correlation was found between the eukaryotic (R^2= 0.15) or bacterial (R^2= 0.28) observed OTUs and grapevine growth cycle. Contrary to soils, leaves where characterized by a more dynamic microbial biodiversity across the plant cycle, with a significant decrease of the eukaryotic biodiversity and an increase of the prokaryotic communities over time (Figure 1B and 1C). These fluctuations were observed for both grapevine growing seasons (Figure 1B and 1C). Thus, the observed microbial biodiversity in leaves showed a strong correlation with the grapevine growth cycle: while the eukaryotic OTUs were negatively associated with grapevine growth cycle (R^2= 0.81 and R^2= 0.71 in 2010 and 2011, respectively), the bacterial OTUs were positively associated (R^2= 0.62 and R^2= 0.80 in 2010 and 2011, respectively).

The analysis of the eukaryotic community showed two dominant phyla across soil and leaves samples (Figure 2A). Overall, the Ascomycota phylum was the major abundant at both soils (46%) and leaves (35%), followed by the Basidiomycota phylum that accounted a similar relative abundance across both samples (Figure 2A). Curiously, a high amount of Early diverging fungal lineages was uncovered at leaves (21%) and many of sequences were not assigned to any microorganism during the Blast process. Those where designed as "unknown" and, as expected, these were most abundant in soils (46%) than in leaves (39%). At class level, soils were clearly dominated by Dothideomycetes (18%) and Sordariomycetes (13%), while at leaves the Dothideomycetes (29%) where the most abundant. Both soils and leaves contained mostly *Aureobasidium,* which accounted a relative average of 8% and 44%, respectively.

Table 1: Total sequences obtained for fungal (D2 and ITS2) and bacterial (V6) communities for soils and grapevine leaves samples. The OTUs and the estimated species (Chao 1) were determined at a genetic distance of 3% and, the coverage determined as being the ratio between the observed OTUs and the estimated Chao 1.

		2010 (Leaves)					2011 (leaves)					2011 (Soil)				
Grape variety	**Target Region**	**Total reads**	**High quality**	**OTU obtained (mean ± SEM)**	**Estimated species (mean ± SEM)**	**Coverage % (mean ± SEM)**	**Total reads**	**High quality**	**OTU obtained (mean ± SEM)**	**Estimated species (mean ± SEM)**	**Coverage % (mean ± SEM)**	**Total reads**	**High quality**	**OTU obtained (mean ± SEM)**	**Estimated species (mean ± SEM)**	**Coverage % (mean ± SEM)**
TR	D2	38555	38187	94.40 ± 11.32	171.01 ± 20.15	53.75 ± 3.34	56692	55054	57.93 ± 12.58	77.73 ± 16.13	74.00 ± 3.27	--	--	--	--	--
	ITS2	43178	41211	110.00 ± 20.49	187.52 ± 30.27	55.02 ± 3.05	41594	38657	49.33 ± 14.25	59.34 ± 16.50	82.19 ± 2.60	21078	19643	259.57 ± 30.19	259.57 ± 30.19	72.61 ± 1.15
	V6	60363	59636	129.30 ± 7.73	203.03 ± 16.03	62.72 ± 2.86	69522	67164	74.40 ± 9.15	98.29 ± 12.37	77.27 ± 2.48	23265	22540	545.00 ± 66.68	984.43 ± 125.79	55.84 ± 1.65
TN	D2	37159	36763	73.90 ± 9.59	128.26 ± 20.82	58.29 ± 4.37	85598	84401	63.00 ± 8.20	87.90 ± 11.30	72.80 ± 3.50	--	--	--	--	--
	ITS2	46693	44207	99.30 ± 14.39	153.02 ± 15.91	60.85 ± 3.47	54746	49965	48.30 ± 8.40	62.50 ± 12.00	81.10 ± 3.10	38452	36033	285.00 ± 43.46	397.06 ± 58.59	71.19 ± 1.56
	V6	44197	43616	109.00 ± 12.52	180.73 ± 25.88	61.94 ± 3.09	76105	75307	66.10 ± 6.40	85.10 ± 8.20	78.90 ± 3.70	34879	33715	722.57 ± 81.64	1269.46 ± 141.91	57.17 ± 1.75
Baga I	D2	37087	36814	101.70 ± 5.83	176.39 ± 13.15	54.90 ± 2.31	86855	85098	75.73 ± 10.78	98.66 ± 14.09	77.33 ± 2.57	--	--	--	--	--
	ITS2	43153	41460	125.40 ± 14.38	206.20 ± 23.93	57.78 ± 3.20	81564	70316	54.00 ± 7.77	66.85 ± 9.15	79.32 ± 2.30	35107	32992	330.57 ± 22.49	330.57 ± 22.49	74.84 ± 2.26
	V6	52184	51724	109.10 ± 8.50	201.44 ± 22.31	55.17 ± 4.07	69062	67334	63.87 ± 6.91	82.76 ± 8.20	76.98 ± 3.03	38320	37230	712.14 ± 60.42	1242.03 ± 95.98	57.14 ± 1.14
Baga II	D2	37906	37469	117.20 ± 13.55	196.97 ± 18.03	54.01 ± 2.24	71242	69555	75.13 ± 9.81	91.72 ± 11.68	81.62 ± 2.31	--	--	--	--	--
	ITS2	45405	44737	142.40 ± 11.87	216.70 ± 18.63	61.92 ± 2.31	44660	38596	53.40 ± 9.07	68.84 ± 13.81	83.12 ± 2.73	36572	34508	261.57 ± 29.78	261.57 ± 29.78	77.50 ± 2.66
	V6	66175	65633	122.60 ± 10.77	178.11 ± 17.40	68.08 ± 2.59	80774	80080	60.40 ± 6.67	75.14 ± 8.08	80.29 ± 1.89	39547	38288	550.43 ± 34.06	913.19 ± 70.58	60.84 ± 1.54
Total	D2	150707	149233	96.80 ± 5.61	168.16 ± 9.65	55.24 ± 1.56	300387	294108	67.95 ± 10.33	88.99 ± 13.30	76.44 ± 2.92	--	--	--	--	--
	ITS2	178429	171615	119.28 ± 7.94	190.86 ± 11.64	58.89 ± 1.52	222564	197534	51.25 ± 9.89	64.40 ± 12.86	81.43 ± 2.67	131209	123176	284.18 ± 16.22	383.76 ± 21.28	71.20 ± 0.00
	V6	222919	220609	117.50 ± 5.03	190.82 ± 10.15	61.98 ± 1.70	295463	289885	66.20 ± 7.27	85.33 ± 9.20	78.36 ± 2.78	136011	131773	635.78 ± 33.96	1106.64 ± 60.65	57.82 ± 0.81
	Eukaryotic	329136	320848	108.04 ± 4.99	179.51 ± 7.62	57.06 ± 1.10	522951	491642	59.60 ± 10.11	76.70 ± 13.08	78.93 ± 2.80	131209	123176	284.18 ± 16.22	383.76 ± 21.28	71.20 ± 0.00
	Prokaryotic	222919	220609	117.50 ± 5.03	190.82 ± 10.15	61.98 ± 1.70	295463	289885	66.20 ± 7.27	85.33 ± 9.20	78.36 ± 2.78	136011	131773	635.78 ± 33.96	1106.64 ± 60.65	57.82 ± 0.81

Bacterial populations associated with soils (Figure 2B) were mostly characterized by Proteobacteria (50%) phylum, followed by Actinobacteria (21%), Acidobacteria (15%), Verrucomicrobia (3%), Planctomycetes (2%) and Firmicutes (1%). In lower abundances, phyla Gemmatimonadetes (0.7%), Nitrospirae (0.5%), Cyanobaceteria (0.4%), Deinococcus-Thermus (0.1%), Chloroflexi (0.08%), Bacteroidetes (0.04%), Chlamydiae (0.01%), Armatimonadetes (0.01%) and Ignavibacteria (0.01%) were observed and grouped in the “Others” group. Contrarily, leaves were characterized by Firmicutes (46%) and Proteobacteria (44%) phylum, an in lower extent by Actinobacteria (4%). Al class level, soils had a greater relative abundance of Betaproteobacteria (22%) and Alphaproteobacteria (16%) from Proteobacteria phylum, while leaves were mostly characterized by Bacilli (43%) and Gammaproteobacteria (35%) from Firmicutes and Proteobacteria phylum, respectively. Moreover, the Oxalobacteraceae (9%) was the dominant family in soils, while leaves contained mostly Streptococcaceae (32%) and Enterobacteriaceae (22%) families.

Grapevine was a reservoir of a core microbiome

By comparing the eukaryotic communities from both soils and leaves of 2011, 27.6% of microorganisms were identified as common elements and included *Aureobasidium, Alternaria, Botryosphaeria, Cryptovalsa, Filobasidiella, Fusarium* or, surprisingly, the wine-yeasts *Hanseniaspora, Metschnikowia* and *Saccharomyces*, which were present in lower abundances (Table S2). Unique microbial signatures were identified in each structure (soil=176, leaves =131) and, in general these where present in low relative abundances. Furthermore, and among soils, only 18.8% of microorganisms were common elements across Baga, TR and TN varieties. Among them, Baga and TN were those that shared more taxa. Among leaves, and considering both growing seasons (2010 and 2011), 32.1% of microbiota were common across cultivars.

Contrarily to the eukaryotic microorganisms, the bacterial populations were more conserved across plant structures and grape varieties. Thus, bacterial communities showed that 52.1% were common microorganisms among soils and leaves, and included the Acetobacteraceae, Actinomycetaceae, Bacillaceae, Comamonadaceae, Enterobacteriaceae or Pseudomonadaceae families (Table S2). Unique microorganisms were also identified on soils (52) and leaves (15), though they showed a relative low abundance. Furthermore, 57.6% and 51.2% of microorganisms from soils and leaves, respectively were common signatures across the grape varieties in analysis.

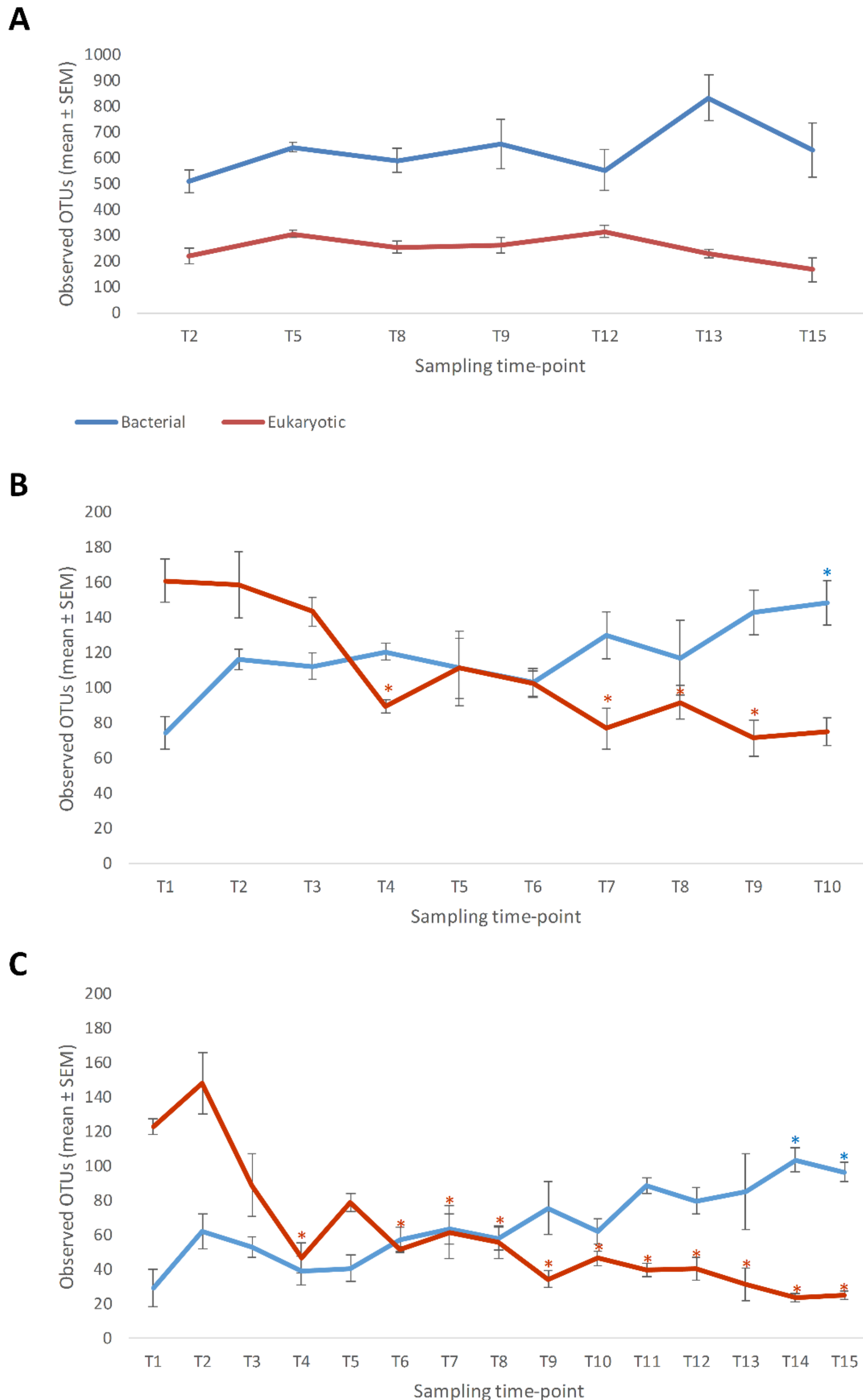


Figure 1: Fluctuations on microbial biodiversity were observed across grapevine growth cycle. Shown are the average of observed OTUs (mean ± SEM) of (A) soil, (B) leaves at 2010 season and (C) leaves at 2011 season for both bacterial (blue) and eukaryotic (red) communities. *indicates significantly different means (p<0.05).

Soil and leaves were characterized by specific microbial signatures

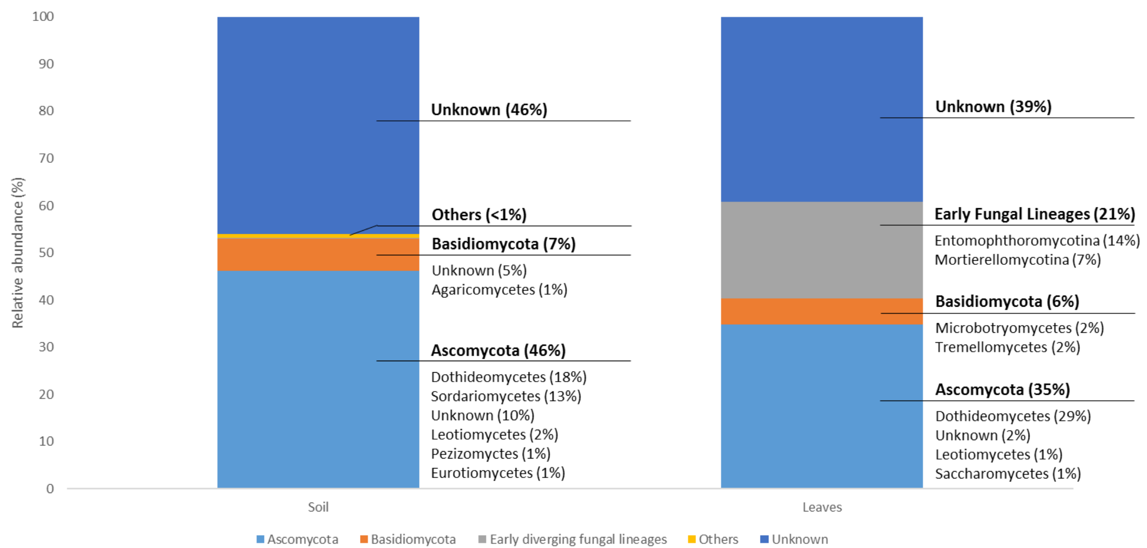
The grapevine structure clearly influenced the associated microbial communities (Figure 3). Indeed, sample type (soils and leaves) was the major explanatory variable of microbial communities and this was higher in bacterial communities (74.8%) (Figure 3B), when compared with eukaryotic population (39.9%) (Figure 3A). The perMANOVA tests showed significant differences (p<0.05) across sample type for both microbial populations in analysis (Table 2).

Considering the average of similarity of groups, soils (eukaryotic: 41.74; bacterial: 76.66) had higher values comparing to leaves (eukaryotic: 37.46; bacterial: 58.85), and the average similarity was superior in bacterial communities (Table S3).

Regarding the eukaryotic microbial signatures, soils were mainly characterized by unknown microorganisms, *Aureobasidium, Fusarium, Alternaria, Lophiostoma, Diplodia* or *Cadophora* that, altogether contributed with 52% for the group similarity, while leaves were mainly characterized by unknown microorganisms, *Candida, Alternaria, Lewia, Debaryomyces, Saccharomyces* and *Aureobasidium* which contributed with 90% for the group similarity (Table S3). An average of dissimilarity of 79.87 was found between soil and leaves, and microorganisms that contributed for this separation were *Aureobasidium*, *Fusarium, Lophiostoma* or *Diplodia* that have a higher abundance in soils, *Alternaria* with a similar average of abundance in both groups, and *Candia, Lewia* or *Debaromyces* in leaves.

Among the bacterial communities of soil, the similarity of the group was due to the Sphingomonadaceae, Nocardioidaceae, Comamonadaceae, Oxalobacteraceae, Micrococcaceae or Intrasporangiaceae families, which accounted with 30% for the group similarity (Table S3). Among leaves, Streptococcaceae, Enterobacteriaceae, Moraxellaceae, Leuconostocaceae or Comamonadaceae families contributed with approximately 59% for group similarity. The dissimilarity between groups (71.68) were due to Nocardioidaceae, Oxalobacteraceae, Intrasporangiaceae, Micrococcaceae, Pseudonocardiaceae or Sphingomonadaceae families that have higher average of abundance in soils and Streptococcaceae, Moraxellaceae, Enterobacteriaceae or Leuconostocaceae families in leaves.

A



B

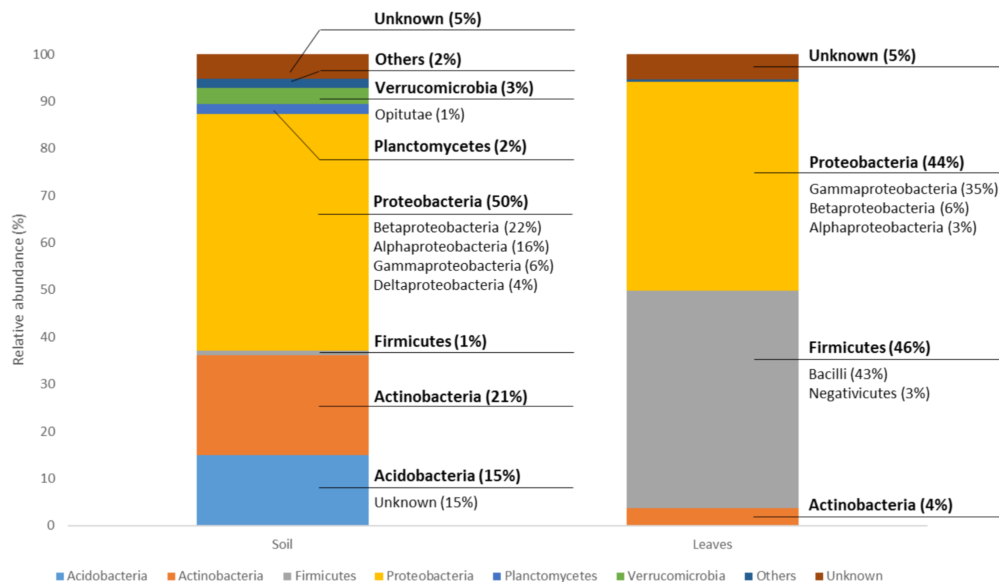


***Figure 2: Relative abundance (%) of the eukaryotic (A) and bacterial (B) communities at the phylum and class level associated with soils and leaves across different grape varieties*.** Only groups with an average abundance of >1% are shown, with exception with “Others” in soil samples from Figure 2A and 2B. In Figure 2A, Others group (<1%) corresponded to Chytridiomycota (0.5%), Early diverging fungal lineages (0.2%) and Glomeromycota (0.2%) phyla and in Figure 2B included the phyla Gemmatimonadetes (0.7%), Nitrospirae (0.5%), Cyanobaceteria (0.4%), Deinococcus-Thermus (0.1%), Chloroflexi (0.08%), Bacteroidetes (0.04%), Chlamydiae (0.01%), Armatimonadetes (0.01%) and Ignavibacteria (0.01%).

At soil level, each grape variety assembled specific fungal communities

Overall, soils were characterized by *Aureobasidium* (8.1%), *Fusarium* (6.9%), *Alternaria* (2.0%) and *Boeremia* (1.4%) which presented the highest relative abundances. At species level, these included *Aureobasidium* sp., *Fusarium nelsonii, Fusarium oxysporum, Fusarium solani, Alternaria* sp. and *Boeremia exigua*. Although, a large number of unknown microorganisms dominated the eukaryotic microbial consortium (71.7%) of soils. Among bacteria, Oxalobacteraceae (9.1%), Sphingomonadaceae (7.6%), Nocardioidaceae (6.4%), Comamonadaceae (5.6%), Micrococcaceae (2.7%), Intrasporangiaceae (1.9%), Microbacteriaceae (1.8%), Rhodospirillaceae (1.7%) and Pseudonocardiaceae (1.7%) families, together with unknown microorganisms (43.9%), accounted to the 82% of total of the bacterial communities. Thus, and considering the genus level, *Massilia, Sphingomonas, Nocardioides, Marmoricola, Arthrobacter, Terrabacter, Tetrasphaera, Agromyces, Microbacterium, Skermanella* or *Actinomycetospora* were the microorganisms with higher relative abundances.

The microbial communities varied across grapevine growth cycle, although the sampling time-points and treatments intervals applied in vineyard did not totally explained the observed variation in both eukaryotic and bacterial communities (Table 2). Interestingly, grape varieties were the major explanatory variable of eukaryotic (30.8% explained) (Figure 4A) and bacterial communities (35.7% explained) (Figure 4B) of soils and, the perMANOVA tests showed significant differences ($P<0.05$) across them (Table 2). Though, no significant differences were found across Baga variety (I and II).

As observed for the eukaryotic population (Figure 4A), each grape variety is defined by a specific cluster. Thus, the eukaryotic and bacterial communities responsible for similarities within each grape variety and dissimilarities between them, were analyzed by using SIMPER analysis (Table S4). The average of similarity was similar across grape varieties though was higher in bacterial (TR: 79.68%; TN: 80.32%; Baga I: 79.80%; Baga II: 74.92%) than in eukaryotic population (TR: 49.36%; TN:48.03%; Baga I: 50.42%; Baga II: 47.90%). Consequently, the average of dissimilarity was higher in the formers. Thus, and regarding the eukaryotic communities, TR cultivar was mainly characterized not only by *Aureobasidium* and *Fusarium,* which contributed with 16.63% for the group similarity, but also by *Entrophospora, Hypocrea, Penicillum, Bionectria, Sebacina* and *Cryptovalsa*. TN was also characterized by *Fusarium* and *Aureobasidium,* but also by *Cytospora, Alternaria, Chalara, Stachybotrys, Pilidium* and *Coniella,* while Baga was characterized by *Alternaria*, *Diplodia*, *Lophiostoma, Trametes* and *Clathrosphaerina* (Table S4). The analysis of dissimilarity of both eukaryotic and bacterial communities revealed that several microorganisms contributed to the dissimilarity of grape varieties, which is explained by the microbial niches that are preferentially associated for each grape variety.

Table 2: Results of perMANOVA analysis based on the Bray-Curtis dissimilarities for eukaryotic and bacterial communities from soil and leaves in relation to grape varieties, sampling time-point and treatments applied in vineyard.

			Eukaryotic communities					Bacterial communities				
Structure	**Season**	**Test**	**df**	**SS**	**MS**	**Pseudo-F**	***p***	**df**	**SS**	**MS**	**Pseudo-F**	***p***
Soil	2011	Grape variety	3	14459	4819.7	3.74	0.001	3	1875.7	625.25	2.708	0.001
		Sampling time-point	6	9132.8	1522.1	1.1811	0.073	6	1639.1	273.19	1.215	0.093
		Treatment application	2	3682.6	1841.3	1.4755	0.047	2	515.68	257.84	1.1056	0.332
Leaves	2010	Grape variety	3	8739.1	2913	1.6727	0.002	3	2587.7	862.56	1.7182	0.003
		Sampling time-point (month)	2	10521	5260.6	3.0207	0.001	2	2660.6	1330.3	2.65	0.002
		Treatment application	1	3045.9	3045.9	1.7069	0.024	1	577.54	577.54	1.126	0.294
	2011	Grape variety	3	9465.1	3155	1.6102	0.003	3	1830.1	610.05	0.55761	0.901
		Sampling time-point (month)	5	31769	6353.8	3.2427	0.001	5	13732	2746.4	2.5103	0.008
		Treatment application	1	2517.6	2517.6	1.3109	0.142	1	346.25	346.25	0.34815	0.952
	2010 vs 2011	Season (year)	1	65726	65726	34.764	0.001	1	5936.6	5936.6	7.5887	0.001
Soil vs Leaves	2011	Grape variety	3	13499	4499.8	2.4701	0.001	3	2379.7	793.22	1.0009	0.434
		Plant structure	1	52167	52167	28.637	0.001	1	73046	73046	92.175	0.001

Df= degrees of freedom; SS= sum of squares; MS= mean of sum squares; Pseudo-F = F value by permutation and *P* indicates statistical significance ($p < 0.05$). The *p*-values are based on 999 permutations.

Within leaves, bacterial communities were more similar over time than eukaryotic communities

The overall microbial biodiversity at leaves level was higher in 2010 (Figure 1B) when compared to 2011 growing season (Figure 1C; Table 1), which suggest that environmental conditions impacted on the microbial communities. Furthermore, the eukaryotic community of T1 was the most biodiverse and such microbial biodiversity consistently decreased across the grapevine growth cycle, while bacterial population increased (Figure 1B and 1C).

Curiously, leaves collected at 2010 showed a considerable dominance of microorganisms belonging to early diverging fungal lineages, such as *Mortierella, Mucor, Rhizopus,* and the entomopathogens *Pandora* and *Zoophthora.* Altogether, these microorganisms accounted with a relative abundance of 41% of the total population and were identified through the amplification of the D2 region. Indeed, some of the early diverging fungi lineages, such as *Pandora* and *Zoophthora*, are known to affect the functional insect biodiversity, rather than to impact directly on the physiology of grapevine (Pinto *et al.,* 2014). Considering that these microorganisms were not detected during the 2011 growing season, from now on we will focus only on microorganisms from Ascomycota and Basiodiomycota phylum.

The 10 most abundant microorganisms of leaves accounted approximately to 95 and 98% of the eukaryotic population on 2010 and 2011. In 2010, leaves were mainly characterized by *Aureobasidium* (29.3%), *Alternaria* (3.70%), *Sporormiella* (2.67%), *Coleosporium* (1.29%), *Guignardia* (1.07%), *Penicillium* (0.79%), *Ustilago* (0.74%), *Kurtzmanomyces* (0.37%) or *Lewia* (0.36%), while at 2011, leaves contained *Rhodotorula* (3.54%), *Aureobasidium* (3.12%), *Cochliobolus* (1.86%), *Saccharomyces* (0.95%), *Alternaria* (0.95%), *Candida* (0.27%), *Filobasidiella* (0.21%), *Metschnikowia* (0.18%) or *Tremella* (0.15%). At species level, these included *Aureobasidium* sp., *Alternaria sp., A. triticina, A. brassicicola, A. solani, Sporormiella* sp., *C. phellodendri*, *C. senecionis*, *G. bidwellii, P. canescens, P. radicum, Ustilago* sp., *U. cynodonti, K. nectairei* and *Lewia* sp. during the 2010, while 2011 included *Rhodotorula* sp., *R. fujisanensi, R. nothofagi, R. laryngis, Aureobasidium* sp., *A. pullulans, Cochliobolus* sp., *S. cerevisiae, Alternaria* sp., *A. triticina, A. brassicicola, Candida* sp., *C. tropicalis, Filobasidiella* sp., *Metschnikowia* sp., *M. pulcherrima* and *Tremella* sp.. However, many unknown microorganisms dominated the eukaryotic microbial consortium of leaves at 2010 (54.66%) and 2011 (86.99%), and these were mainly obtained through the ITS2 region amplification.

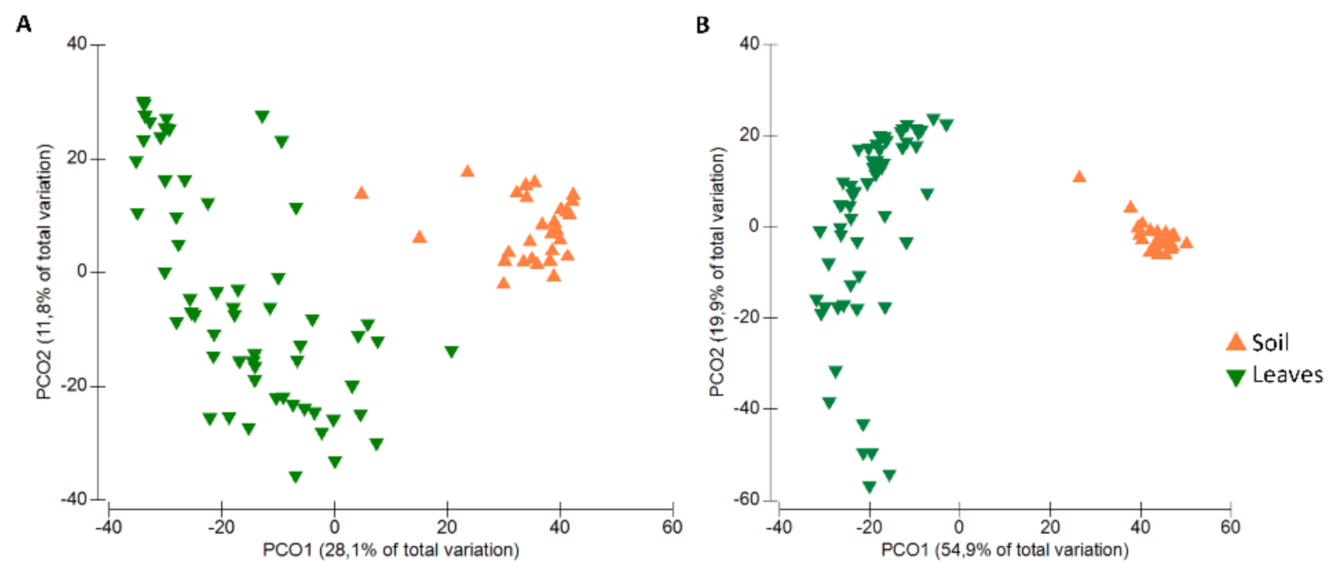


Figure 3: Microbial communities were significantly different among soil and leaves samples. Shown are Principal coordinates analysis (PCOA) of (A) eukaryotic and (B) bacterial communities from soil and leaves, based on Bray-Curtis similarity.

Considering the most abundant bacterial microorganisms, this community's structure was similar across growing seasons, though contained variations on their abundance. Thus, Enterobacteriaceae (relative abundance of 20.87% and 16.04% at 2010 and 2011, respectively) and Streptococcaceae (20.22%; 34.53%) families were the most abundant, followed by Moraxellaceae (8.86%; 9.37%), Pseudomonadaceae (4.57%; 2.67%), Comamonadaceae (4.05%; 2.60%), Leuconostocaceae (3.80%; 2.47%), Xanthomonadaceae (3.44%; 2.00%), Veillonellaceae (2.65%; 2.27%) and Sphingomonadaceae families (1.67%; 1.82%). At genus level those mostly included, *Pantoea*, *Serratia*, *Morganella*, *Erwinia, Citrobacter* and *Raoultella* from Enterobacteriaceae family, *Lactococcus* and *Streptococcus* (Streptococcaceae), *Acinetobacter* and *Enhydrobacter* (Moraxellaceae), *Pseudomonas* (Pseudomonadaceae), *Variovorax, Acidovorax, Pelomonas, Comamonas* and *Delftia* (Comamonadaceae), *Weissella* and *Leuconostoc* (Leuconostocaceae), Stenotrophomonas and *Rudaea* (Xanthomonadaceae)*, Veillonella* (Veillonellaceae) and Sphingomonas (Sphingomonadaceae).

Comparing the microbial communities of both seasons, 53% and 64% of the eukaryotic and bacterial microorganisms respectively, where common to both growing seasons. This demonstrates and reinforces an existing core microbiome in the plant which is preserved across time. Moreover, the microorganisms included exclusively to each season were microorganisms with lower abundances (rare microbial population) within the microbial consortium.

Sampling-time and grapevine management were drivers of the microbial communities of leaves

The overall distribution and dynamics of the entire microbial communities was carried out by a Principal Coordinate Analysis (PCO). Analysis supported an evolution of both eukaryotic (Figure 5A and 5B) and bacterial (Figure 6A and 6B) population according to the sampling-time/month. Also, microbial communities were clustered according to the growing season (Figure 5C and 6C). Indeed, and in contrast to the microbial population of soils, sampling time modulated significantly ($p<0.05$) the eukaryotic and bacterial biodiversity of leaves and, together with growing seasons (2010 and 2011) those were important drivers of microbial communities (Figure 5 and 6; Table 2). Differences ($p<0.05$) between the eukaryotic and bacterial communities associated to each grape variety were also found. Furthermore, the application of chemical treatments affected the vineyard's microbial population, especially the eukaryotic community (Table 2).

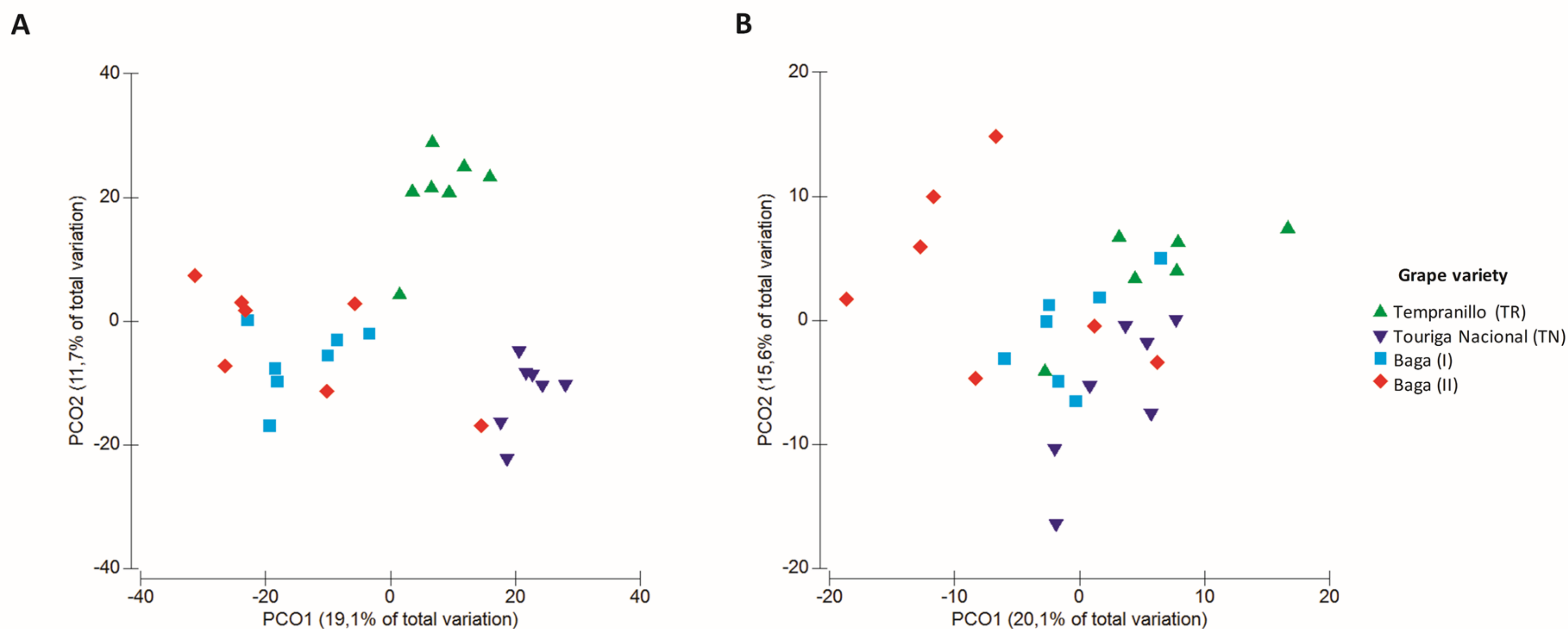


Figure 4: Microbial communities showed differences across grape varieties from soil samples. Shown are Principal coordinates analysis (PCoA) of (A) eukaryotic and (B) bacterial communities based on Bray-Curtis similarity.

Important winemaker microorganisms were detected in soils and leaves

Our data demonstrated that microorganisms involved during the wine making process, such as the *Saccharomyces, Hanseniaspora* and *Metschnikowia,* were retrieved in soils and leaves, though at low relative abundance (<0.6%). Indeed, an interestingly gradient of these microorganisms was observed: the relative abundance increases from soil to leaves. Given the bacterial communities, both lactic acid (LAB) and acetic acid (AAB) bacteria were also identified at soils and leaves. The identified LAB belonged to the Carnobacteriaceae, Enterococcaceae, Leuconostocaceae or Streptococcaceae families, while AAB belonged to Acetobacteraceae family. Contrary to the identified yeasts, these bacterial microorganisms are not involved with the winemaking process.

Discussion

Herein is presented a deep analysis of both eukaryotic and prokaryotic communities associated with different grape varieties, such as Baga, Tempranillo (TR) and Touriga Nacional (TN), over the grapevine growing cycle and two growing seasons.

Overall, grapevine showed specific microbial signatures associated with soils and leaves, which is in line with previous observations (Ottesen *et al.,* 2013; Zarraonaindia *et al.,* 2015). Among them, the major explanatory variables were *Aureobasidium, Fusarium, Alternaria, Lophiostoma, Diplodia* or *Cadophora* in soils, and *Candida*, *Alternaria, Lewia*, *Debaryomyces*, *Saccharomyces* and *Aureobasidium* in leaves; and given the bacterial communities, these differences were explained by Sphingomonadaceae and Nocardioidaceae families in soils, and Streptococcaceae and Enterobacteriaceae in leaves. Indeed, this may suggest the major adaptability and/or preference of those microorganisms for such specific plant niches (Martins *et al.,* 2013). Furthermore, soils-associated microorganisms were more stable than those from leaves. In fact, grapevine canopy faces different environmental conditions such as extreme temperatures, rainfall or UV light, which together with the low water availability and nutrients this may constitute a limiting factor for the microbial development (Lindow and Brandl, 2003; Whipps *et al.,* 2008). Our results also showed a decrease of the eukaryotic biodiversity associated with grapevine leaves during the grapevine ripening, and an increase of the bacterial biodiversity. This observed distribution agrees with previous results (Pinto *et al.,* 2014), and shows that the application of phytosanitary treatments posed a selective pressure on the microbial ecosystem of the grapevine, altering it, and thus contributing to its variation, especially on eukaryotic communities. In fact, phyllosphere is the principal target of agricultural management

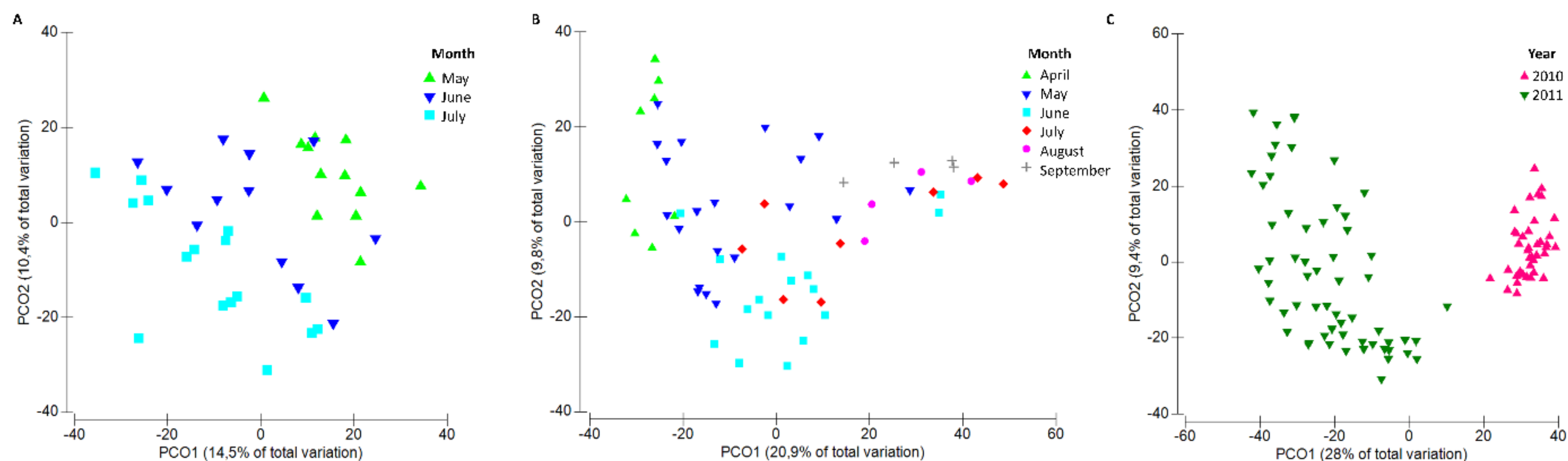


Figure 5: Principal coordinates analysis (PCoA) of the eukaryotic microbial communities based on the genus level analysis. PCoA of the microbial communities across (A) 2010 season (B) 2011 and (C) plot of the fungal community structure and distribution during both grapevine campaigns (2010 and 2011).

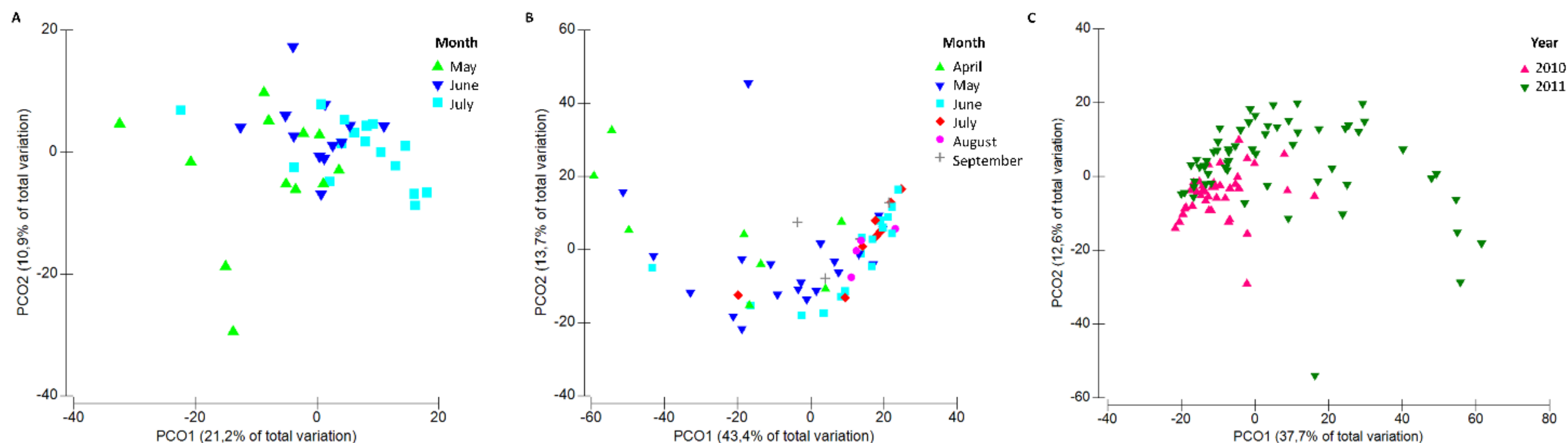


Figure 6: Principal coordinates analysis (PCoA) of the bacterial communities based on the family level analysis. PCoA of the microbial communities across (A) 2010 season (B) 2011 and (C) bacterial communities' distribution during both grapevine campaigns (2010 and 2011).

regimes (Comitini and Ciani, 2008), constituting a detrimental effect on the plant microbial ecosystem (Pinto *et al.,* 2014; Pinto and Gomes, 2016; Porter and Setati, 2016). Considering the importance of this subject, a great deal of interest on the analysis of the impact of farming practices on soil and plant-associated microbial communities has been investigated across different crops (Cordero-Bueso *et al.,* 2011; Hartmann *et al.,* 2015; Abdelfattah *et al.,* 2016; Porter and Setati, 2016; Morrison-Whittle *et al.,* 2017).

In this study, soils showed a significantly higher microbial biodiversity then those of leaves. Although, both comprise an important shared microbial profile, suggesting the existence of a core microbiome. Indeed, recent studies described soils as a primary reservoir of plant-associated microorganisms (Zarraonaindia *et al.,* 2015), and suggests that plant then select specific microorganisms to prosper to other plant structures/ niches, such as roots or leaves (Berendsen *et al.,* 2012). However, and despite these evidences, the origin of the epiphytes microorganisms over the plant remain poorly understood and is a highly complex subject. Interestingly, unique microorganisms were identified in both soils and leaves, though this population was present in low relative abundances. A possible explanation is based on the microbial competition. However, and considering that a large proportion of OTUs corresponds to a rare microbial population this may emphasizes that these communities can fulfil important ecosystem functions, such as nitrogen – fixation, or other important metabolic processes (Dawson *et al.,* 217).

Overall, soils were characterized by the eukaryotic *Aureobasidium, Fusarium*, *Alternaria* and *Boeremia*, while leaves were composed mainly by *Aureobasidium, Alternaria, Rhodotorula* or *Sporormiella*. These results reinforce that *Aureobasidium* is a resident microbiota of grapevine, which is in line with other studies (Sabate *et al*., 2002; Grube *et al*., 2011; Barata *et al*., 2012; Pinto *et al*., 2014). Considering that *Aureobasidium* is an important biocontrol agent of several post-harvest diseases caused mostly by *Botrytis cinerea* (Ippolito *et al.,* 2000; Castoria *et al.,* 2001), this present study highlighted that grapevine is naturally colonized by microorganisms with biocontrol capacity and who may be considered as the first protective barrier of plants against the phytopathogens or other abiotic stresses. Given the bacterial communities, the Oxalobacteraceae, Sphingomonadaceae and Nocardioidaceae were the most abundant families in soils, while Enterobacteriaceae and Streptococcaceae were the most abundant in leaves.

In the current study, differences in the microbial communities from different grape varieties was showed, suggesting a potential microbial profile of each cultivar. From the ecological and biotechnological point of view, these differences may or not have an impact on the plant, namely by influencing either the resistance or the susceptibility of cultivars to diseases. Indeed, previous studies have already demonstrated that grape varieties influence the grape must- associated microbiota, both fungal and bacterial communities (Bokulich *et al.,* 2014). Interestingly, it has also been demonstrated

that other different plant species growing on the same soils harbor different rhizosphere microbial communities (Berg *et al.,* 2006; Berendsen *et al.,* 2012), which reinforce the role of plants and even plant species on shaping their associated microbiome. Beyond these, the age of plants and their growing cycle are known to influence the microbial ecosystem, microbial dispersal and microbial performance (Lindow and Brandl, 2003; Garijo *et al.,* 2011; Pinto *et al.,* 2014). Moreover, the growing seasons are also important drivers of the microbial communities (Bokulich *et al.,* 2014).

Interestingly, in both soils and leaves were observed microorganism involved in the winemaking process, notably the *Saccharomyces* and non-*Saccharomyces* such as *Hanseniaspora* and *Metschnikowia*. Despite these microorganisms were present at a low-abundance, these are natural colonizers of the grapevine even before the appearance of berries (Pinto *et al.,* 2014). Thus, this may suggest that soils may be a primary source of winemaker microorganisms (Zarraonaindia *et al.,* 2015; Belda *et al.,* 2017).

In conclusion, the microbial consortia associated with grapevine significantly differed between soils and leaves, and such microbial biodiversity decreased as distance from soils increases. Furthermore, an important microbial profile was preserved over the grapevine structures and growing seasons, suggesting the existence of a core microbiome in which may have an important functional ecology that promoted a stable and consistent plant-association. Moreover, results also highlighted that grapevine is a potential source of endogenous beneficial microorganisms with BCAs profile or even of oenological interest.

Competing interests: The authors declare that they have no competing commercial interest in relation to the submitted work.

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Authors’ contributions: Conceived and designed the experiments: CP and ACG. Performed the experiments: CP, VC. Analysed the data: CP, MP. Contributed with reagents, materials, analysis tools: MP, CE, ACG. Wrote the paper: CP. Review the paper: ACG.

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Publication 5- Wine fermentation microbiome: a landscape from different Portuguese wine appellations

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Wine fermentation microbiome: a landscape from different Portuguese wine appellations

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Grapes and wine musts harbor a complex microbiome, which plays a crucial role in wine fermentation as it impacts on wine flavour and, consequently, on its final quality and value. Unveiling the microbiome and its dynamics, and understanding the ecological factors that explain such biodiversity, has been a challenge to oenology. In this work, we tackle this using a metagenomics approach to describe the natural microbial communities, both fungal and bacterial microorganisms, associated with spontaneous wine fermentations. For this, the wine microbiome, from six Portuguese wine appellations, was fully characterized as regards to three stages of fermentation – Initial Musts (IM), and Start and End of alcoholic fermentations (SF and EF, respectively). The wine fermentation process revealed a higher impact on fungal populations when compared with bacterial communities, and the fermentation evolution clearly caused a loss of the environmental microorganisms. Furthermore, significant differences ($p < 0.05$) were found in the fungal populations between IM, SF, and EF, and in the bacterial population between IM and SF. Fungal communities were characterized by either the presence of environmental microorganisms and phytopathogens in the IM, or yeasts associated with alcoholic fermentations in wine must samples as *Saccharomyces* and non-*Saccharomyces* yeasts (as *Lachancea, Metschnikowia, Hanseniaspora, Hyphopichia, Sporothrix, Candida,* and *Schizosaccharomyces*). Among bacterial communities, the most abundant family was Enterobacteriaceae; though families of species associated with the production of lactic acid (Lactobacillaceae, Leuconostocaceae) and acetic acid (Acetobacteriaceae) were also detected. Interestingly, a biogeographical correlation for both fungal and bacterial communities was identified between wine appellations at IM suggesting that each wine region contains specific and embedded microbial communities which may contribute to the uniqueness of regional wines.

Keywords: grape microbiology, wine spontaneous fermentation microbiome, industrial metagenomics

Introduction

The knowledge and the understanding of the microbial *terroir* – how the microbiome contributes to the natural environment of grapes and to the identity of wine, is a process that starts at the vineyards, at the harvest of grapes, and then evolves along the different stages of fermentation (Van Leeuwen and Seguin, 2006; Bokulich et al., 2013). Indeed, it is known that grapes harbor

a complex microbiome, including a high range of filamentous fungi, yeasts and bacteria with different physiological and metabolic characteristics (Pretorius, 2000; Fleet, 2003; Barata et al., 2012). The microflora of the grapes is highly variable, mostly due to the influence of external factors as environmental parameters, geographical location, grape cultivars and application of phytochemicals on the vineyards (Pretorius, 2000; Cadez et al., 2010; Pinto et al., 2014). These microbial communities play an important role during the winemaking process, as they metabolize the sugars from the grapes and produce a whole set of secondary metabolites that influence the wine aromatic quality (Fleet, 2003). In fact, the natural diversity of those metabolic pathways, and the contribution of the different microorganisms involved on the fermentation process, is well documented (Setati et al., 2012). Therefore, unveiling the microbial biodiversity of grapes and during their fermentation will expand our understanding on fermentation dynamics, on its control (Bisson, 1999; Bisson and Butzke, 2000) and may also contribute to the identification of novel starter cultures (Fleet, 2008; Ciani et al., 2010).

The spontaneous wine fermentation is carried out by indigenous microbiota (Heard, 1999; Pretorius, 2000; Ciani et al., 2006; Renouf et al., 2007). Species of *Metschnikowia, Candida, Hanseniaspora*, *Pichia, Lachancea* (*Kluyveromyces*), and *Saccharomyces* are often present at the initial stages of wine fermentations and form the dominant consortium (Cocolin et al., 2000; Mills et al., 2002; Fleet, 2008). However, during the wine fermentation, the ethanol content increases and *Saccharomyces cerevisiae* strains dominate the alcoholic fermentation (AF; Fleet, 2008). Additionally, a deacidification may occur, by conversion of malic acid into lactic acid. This process is known as malolactic fermentation (MLF) and is due to the activity of lactic acid bacteria (LAB; Lonvaud-Funel, 1999; Lerm et al., 2011). The LAB species associated with MLF generally belong to the *Oenococcus, Pediococcus, Lactobacillus,* and *Leuconostoc* genera (Lonvaud-Funel, 1999). Indeed, MLF mainly influences the organoleptic characteristics and the aging of wines (Lonvaud-Funel, 1999). On the other hand, acetic acid bacteria (AAB) may cause a negative impact on the winemaking process, due to the production of undesirable metabolites, as acetic acid, thus affect negatively the quality of wine and so are considered spoilage microorganisms (Zoecklein et al., 2000).

The majority of the wine microbiology studies focus on the characterization of *S. cerevisiae* strains (Pretorius, 2000; Fleet, 2008; Nisiotou et al., 2011). Nevertheless, recent studies based on culture-independent methods, started to explore the microbial communities associated with wine grapes (Bokulich et al., 2013; Taylor et al., 2014). It is widely accepted that unveiling the indigenous microbial community associated with particular grape varieties, from specific locations, could represent an important source of distinctive metabolites and introduce an authenticity *terroir* to the region (Heard, 1999; Jolly et al., 2006; Fleet, 2008). The biogeographical distribution of the wine associated microorganisms has been recently investigated in vineyards from different regions of California (Bokulich et al., 2013), New Zealand (Taylor et al., 2014), and in conventional, biodynamic, and integrated vineyards of South Africa (Setati et al., 2012). These studies allowed for a better spatial and temporal characterization of the wine grapes microbiome and brought new insights of its dynamics and biodiversity. Also, other biogeography wine studies have been previously published focusing on *S. cerevisiae* (Schuller et al., 2012). Nevertheless, there is still a lack of knowledge on the diversity and the dynamics of microbial communities as a whole– from the wine grapes until the wine fermentation, which can now be obtained using high-throughput sequencing technologies and metagenomics approaches that allow for the identification of both non-cultivable microorganisms, and of less represented species.

In this work, a total of six different Portuguese wine appellations were considered to analysis and high-throughput sequencing was used to unveil the wine microbiome present at initial musts (IM), and start and end of alcoholic fermentations (SF and EF, respectively). This work aims to understand the dynamics of microbial communities across spontaneous wine fermentations and also to reveal the biogeographic distribution of grape and wine microbiomes of Portuguese wine appellations.

Materials and Methods

Grape Sampling, Laboratory-Scale Fermentation, and DNA Extraction

The grape samples were collected during the 2010 vintage, from six different Portuguese appellations, namely, Minho (Mi), Douro (Dr), Dão (D), Bairrada (B), Estremadura (E), and Alentejo (Al). For each appellation, the three most representative grape varieties were considered for sampling, with exception of Minho where only two grape varieties were considered (**Supplementary Figure S1**). For all regions, the sampling was carried out 1 day prior the harvest. The sampling was authorized by private wine producers, who are fully acknowledged in this paper, and no specific permissions were required for this activity. Also, the field study did not involve any endangered or protected species.

For each appellation, one vineyard (farm) with different grape varieties was selected, and for each grape variety, 2 kg of healthy and undamaged grapes were collected. Grapes were collected from multiple bunches of different grapevines, randomly distributed across the vineyard in order to assure the representativeness of the sampling. These samples were collected into sterile plastic bags and transported to the laboratory chilled on ice. In total, 17 grape samples were collected, crushed and allowed for laboratory-scale fermentation (spontaneous AF) under aseptic conditions and acclimatised at 21°C, at the Genomics Unit from Biocant. For each sample, the microbial diversity was analyzed at three stages: IM, corresponding to the juice of crushed grapes; start of alcoholic fermentation (SF) and end of alcoholic fermentation (EF), which corresponded to the weight loss of 5 and 70 g/L of sugar, respectively. The SF and EF where daily monitored through weighting. At each stage, 50 mL of wine must were collected and centrifuged at 4000 rpm for 10 min. The respective microbial pellets were collected, washed twice with 0.9% NaCl and re-suspended with glycerol. A total

of 51 samples ($n = 17 \times 3$ fermentation stages) were stored at −80°C for DNA extraction. The DNA from each individual sample was extracted using the DNeasy Plant mini kit (QIAGEN, USA), according to the manufacturer's instructions, with a prior cell rupture using glass beads in Tissue Lyser (Qiagen, USA), to assure full disruption of microbial cells.

rDNA Library Construction and Pyrosequencing

A PCR amplicon library was built for each individual sample. For a better discrimination of the entire microbial community present during the fermentation process, rDNA sequences from both prokaryotic and eukaryotic microorganisms were amplified, using PCR primers that were designed to target three distinct regions. The V6 hypervariable region of the 16S rRNA was used for the identification of prokaryotic microorganisms (Sogin et al., 2006) and the D2, from the 26S rRNA, and ITS2 regions (White et al., 1990) for eukaryotic identification. The sequence-specific portions of the used primers were: V6_F 5′-ATGCAACGCGAAGAACCT-3′ and V6_R 5′-TAGCGATTCCGACTTCA-3′ of V6 region; D2_F 5′-AAGMACTTTGRAAAGAGAG-3′ and D2_R 5′-GGTCCGTGT TTCAAGACG-3′ of D2 region; and ITS2_F 5′-GCATCGATG AAGAACGC-3′ and ITS2_R 5′-CCTCCGCTTATTGATAT GC-3′ of ITS2 region. Additionally, the fusion primers also contained a specific Roche 454 adaptor sequence and a multiplex identifier sequence with eight nucleotides, which allows the pooling of amplicons.

All PCR reactions were carried out in 30 µL reaction mix containing 2 µL of DNA template, 1.5 units of FastStart High Fidelity Taq DNA polymerase (Roche, USA), 1x reaction buffer with $MgCl_2$ (1.8 mM) incorporate (Roche, USA), 0.2 mM dNTPs (Bioron, Germany) and 0.8 µM of the forward and reverse primers for V6 region or 0.4 µM of forward and reverse primers for D2 and ITS2 regions. For prokaryotes amplification, cycling conditions consisted in a first denaturation step at 94°C for 5 min followed by 20 cycles with a denaturation step at 94°C for 35 s, annealing at 50°C for 35 s and an extension at 72°C for 40 s. A final extension cycle at 72°C for 5 min was applied. The cycling conditions applied for eukaryotic microorganisms were the same, but the PCR consisted in 25 cycles. The amplification success was assessed by electrophoresis using the HT DNA 5K/RNA LabChip for the LabChip 90 (Caliper Life Sciences, USA). The PCR reaction products were then purified with the High Pure 96 UF Cleanup Plates (Roche, USA) and quantified using the PicoGreen® dsDNA quantitation kit (Invitrogen, USA). Samples were pooled together according to the number of DNA molecules, in equimolar concentrations and submitted for pyrosequencing using the GS FLX Titanium platform (454 Life Sciences, Roche) at Biocant, Portugal. The raw data obtained was deposited in NCBI platform with the accession number SRA097159.

Bioinformatic Data Analysis

Raw sequence reads were processed with MetaBiodiverse, an automatic annotation pipeline fully implemented at Genoinseq of Biocant (Vaz-Moreira et al., 2011; Egas et al., 2012; Pinto et al., 2014). Briefly, the raw data obtained was split through the identification of barcode sequences and quality filters were applied to remove low quality reads. Thus, (*i*) short sequences (<120 bp), (*ii*) sequences containing more than two undetermined nucleotides (N), (*iii*) masked sequences with more 50% of low complexity areas (Sogin et al., 2006) and (*iv*) chimera sequences, detected using UChime were removed (Edgar et al., 2011). All sequences with a distance value below 0.03, which corresponds to the species-level threshold (Sharpton et al., 2011), were grouped in operational taxonomic units (OTUs) through USearch, version 6.0.307 (Edgar, 2010). The Mothur package (Schloss et al., 2009) was used to generate rarefaction curves (richness of population analysis) and to calculate the population diversity analysis estimator Chao1 (α diversity). For the taxonomic annotation, each generated consensus sequences were queried by BLAST on curated databases. The Ribosomal Database Project II (RDP; Cole et al., 2009) was used for prokaryotic microorganisms assignment and the nt@ncbi/SILVA database for eukaryotic classification. After BLAST, the best hits were selected and subjected to another quality control. All sequences with an alignment of less than 40% or with an *E*-value greater than $1e^{-50}$ were rejected. Sequences that passed the quality check were subjected to a bootstrap test with 100 replicates, using the seqBoot application from the Phylip package (Felsenstein, 1989). The OTU identification process implemented provided a high level of confidence in taxon assignment of each sequence. The process assessed the correct *E*-values scores, went through the taxonomy path and identified the lowest common taxonomy level in the bootstrap process. Only those sequences with an identity greater than 70% were reported, while all the others went up the taxonomy levels until reached 70%.

Statistical Analyses

To determine the minimum significant difference ($p < 0.05$) in the biodiversity (Chao1) of IM, SF and EF samples, one-way analysis of variance (ANOVA) was performed using SPSS 20.0 (IBM, US). Shapiro-Wilk normality tests were carried out for each eukaryotic and prokaryotic phylogenetic group. As most groups did not follow the normal distribution, Friedman and Sign tests (pairwise comparisons) were used. The microbial communities were compared at family level for prokaryotic population and at genus level for eukaryotic population through the sequence reads analysis. Thus, microbial population comparisons were carried out using these taxa.

Sequence reads data matrixes of the 97% similarity grouped bacterial and fungal OTUs, produced by Metabiodiverse, were normalized by the total reads obtained for each analyzed sample, and then log(X+1) transformed and used to calculate a Bray–Curtis resemblance matrixes. The data obtained for the three fermentation stages were (i) explored by principal coordinate analysis (PCO), (ii) tested by Analysis of Similarities (ANOSIM) for significant differences and (iii) analyzed by SIMPER to identify the taxa responsible for similarity between samples within each group and dissimilarities between groups, using Primer E software version 6 (Clarke and Gorley, 2006). The same analyses were performed to explore and test the influence of wine appellations on microbiome although, for each fermentation

stage, individual matrixes were created in order to remove the "fermentation stage" variable.

Results

Diversity and Richness of Microbial Communities

In this study, we assessed and compared the microbial community of IM, and the Start and End of wine alcoholic fermentations (SF and EF, respectively), from six Portuguese appellations by DNA massive parallel sequencing of 16S rDNA for bacteria, and both, ITS2 and D2 for fungal analysis. Two target regions were used for the fungal population identification as previous experiments demonstrated that these combination would allow for the highest coverage of eukaryotic organisms (Pinto et al., 2014).

The deep sequencing of microbial communities generated a total of 1,180,106 sequences of ITS2, D2, and V6 regions from IM, SF, and EF (**Table 1** and **Supplementary Table S1**). A total of 1,160,482 sequences passed the quality control parameters, representing an acceptance of 98.3% of high quality sequences (723,474 eukaryotic sequences: 313,919 reads for ITS2 region and 409,555 for D2 region; and 437,008 prokaryotic sequences). The clustering of the sequences at a phylogenetic distance of 3% generated a total of 1,034 OTUs for ITS2, 1,099 for D2, and 1,461 for V6. The number of OTUs from both eukaryotic and prokaryotic communities decreased along the fermentation.

The diversity of microbial community was compared by rarefaction curve analysis (**Supplementary Figure S2**) and the ratio between the number of the obtained and the expected OTUs (predicted by Chao1) was used to determine the coverage for the microbial communities: it was of 73.7 ± 2.0% for ITS2 region, 71.7 ± 1.9% for D2 region and 65.1 ± 1.9% for V6 region (**Supplementary Table S1**).

In order to assess the variations of microbial biodiversity, the Chao1 richness estimator was used to compare the three fermentation stages at both domain and phylum levels. In general, and as expected, a decrease of richness was observed over the spontaneous wine fermentation for both fungi and bacteria, at the analyzed taxonomical levels (domain and phylum; **Figure 1**). Considering the domain (**Figure 1A**), no significant differences were found for the three rDNA regions. At the phylum level, significant differences ($p < 0.05$) in the Basidiomycota between all stages of fermentation were observed (both for ITS2 and D2 regions), and in the Ascomycota population differences were between SF and EF, but not between IM and SF (**Figure 1B**). For the bacterial population, a decrease in biodiversity was observed but no significant differences were detected (V6 rDNA region). A clear relationship was observed between the microbial community biodiversity and the stage of fermentation. Interestingly, the variations of biodiversity, which were observed along the fermentation stages, revealed a higher impact on the structure of the eukaryotic population, when compared with the prokaryotic communities. Moreover, regarding the microbial biodiversity, the prokaryotic population was richer than the eukaryotic population.

General Characterization of Microbial Communities

The dominant phylum across the entire eukaryotic population was Ascomycota (42.4%), though it also contained Basidiomycota (17.7%), and other fungi, as Chytridiomycota phylum (0.2%) and *basal fungal lineages* (5.6%). Also, a considerable number of unidentified microorganisms (34.1%) were mostly present at IM (**Figure 2A**).

TABLE 1 | Total sequences obtained for eukaryotic (ITS2 and D2) and prokaryotic (V6) microbial community for IM, SF, and EF samples.

		No. Reads		0.03 distance		
Sampling point	**Target region**	**Total**	**High quality**	**OTU obtained (mean ± SEM)**	**Estimated species (mean ± SEM)**	**Coverage (mean ± SEM)**
IM	ITS2	119876	116064	68 ± 6	100 ± 9	68.83 ± 2.26%
	D2	131837	129652	71 ± 6	110 ± 10	66.54 ± 2.52%
	V6	145796	145051	78 ± 12	134 ± 21	60.30 ± 3.19%
SF	ITS2	114993	111075	33 ± 3	47 ± 5	74.44 ± 3.62%
	D2	145559	143100	36 ± 3	56 ± 7	68.63 ± 3.29%
	V6	159940	159054	56 ± 9	83 ± 13	66.92 ± 3.28%
EF	ITS2	90207	86780	20 ± 1	29 ± 4	77.74 ± 4.10%
	D2	138156	136803	19 ± 2	25 ± 2	79.82 ± 3.23%
	V6	133742	132903	54 ± 9	81 ± 12	68.15 ± 3.48%
	Eukaryotic	740628	723474			
	Prokaryotic	439478	437008			
	Total	**1180106**	**1160482**			

Operational taxonomic units (OTUs) and estimated species (chao1) were determined at a genetic distance of 3% using Mothur. The coverage obtained was also determined as being the ratio between the observed OTUs and estimated Chao1 (OTUs/Chao1). A detailed table with indication of the samples origin is provided as ***Supplementary Table S1****.*

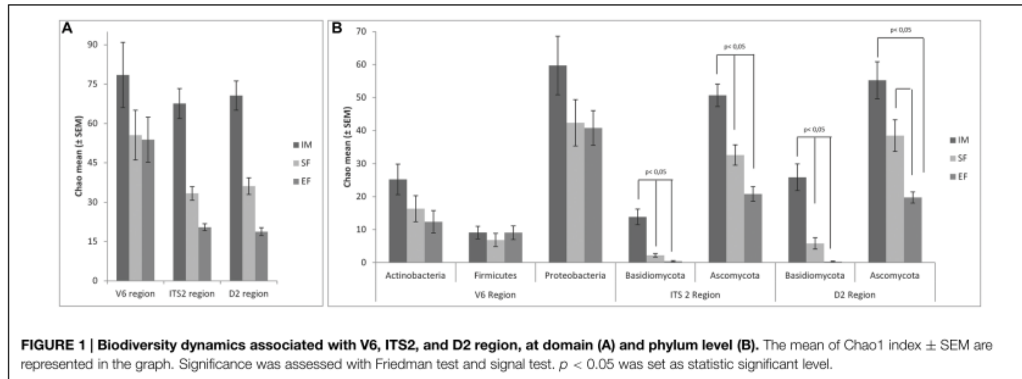

FIGURE 1 | Biodiversity dynamics associated with V6, ITS2, and D2 region, at domain (A) and phylum level (B). The mean of Chao1 index ± SEM are represented in the graph. Significance was assessed with Friedman test and signal test. $p < 0.05$ was set as statistic significant level.

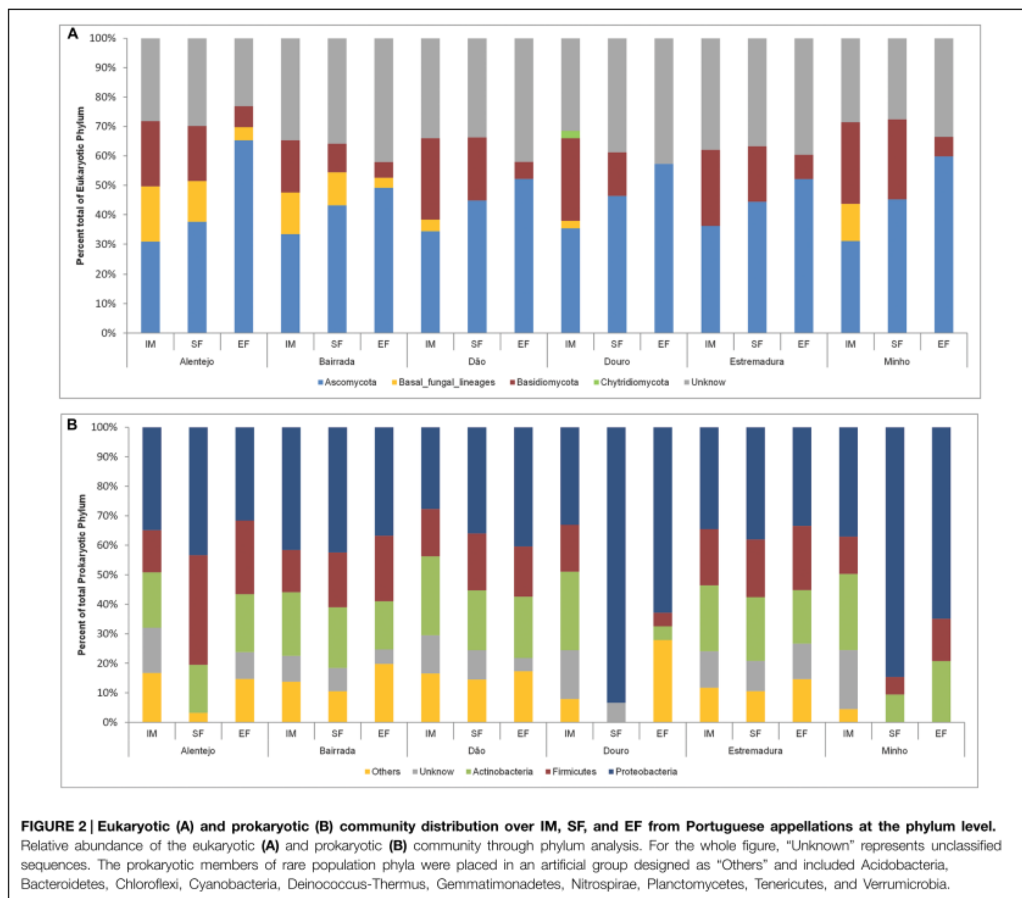

FIGURE 2 | Eukaryotic (A) and prokaryotic (B) community distribution over IM, SF, and EF from Portuguese appellations at the phylum level. Relative abundance of the eukaryotic **(A)** and prokaryotic **(B)** community through phylum analysis. For the whole figure, "Unknown" represents unclassified sequences. The prokaryotic members of rare population phyla were placed in an artificial group designed as "Others" and included Acidobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Gemmatimonadetes, Nitrospirae, Planctomycetes, Tenericutes, and Verrumicrobia.

In all samples, the dynamics of microbial populations at phylum level were very similar. Nevertheless, the relative abundances varied along the fermentation and across Portuguese appellations (**Figure 2A**). Microorganisms belonging to Basidiomycota phylum decreased during the fermentation process. To better understand such population dynamics, the relative abundance at class level was analyzed. The entire microbial community was mostly characterized by Saccharomycetes (22.9%), Dothideomycetes (16.2%), Leotiomycetes (12.9%), Microbotryomycetes (9.6%), and Schizosaccharomycetes (7.7%; **Figure 3A**).

Concerning the prokaryotic communities, the dominant phyla were Proteobacteria (41.6%), Actinobacteria (19.2%), and Firmicutes (17.9%; **Figure 2B**). The members of under-represented phyla were grouped together in the artificial group "Other" (12.4%) and included Acidobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Gemmatimonadetes, Nitrospirae, Planctomycetes, Tenericutes, and Verrumicrobia. As a reflection of the microbial community dynamics, and as seen in eukaryotic microorganisms, the relative abundances of all prokaryotic communities varied in both time and space. Along the spontaneous wine fermentations, it was possible to observe an increase of microorganisms belonging to the Proteobacteria phylum (**Figure 2B**), thus indicating that samples are losing their environmental characteristics. Regarding the prokaryotic classes, microorganisms from Gammaproteobacteria (27.9%), Betaproteobacteria (15.9%), Alphaproteobacteria (14.8%), Actinobacteria (13.2%), and Bacilli (11.5%) were identified (**Figure 3B**).

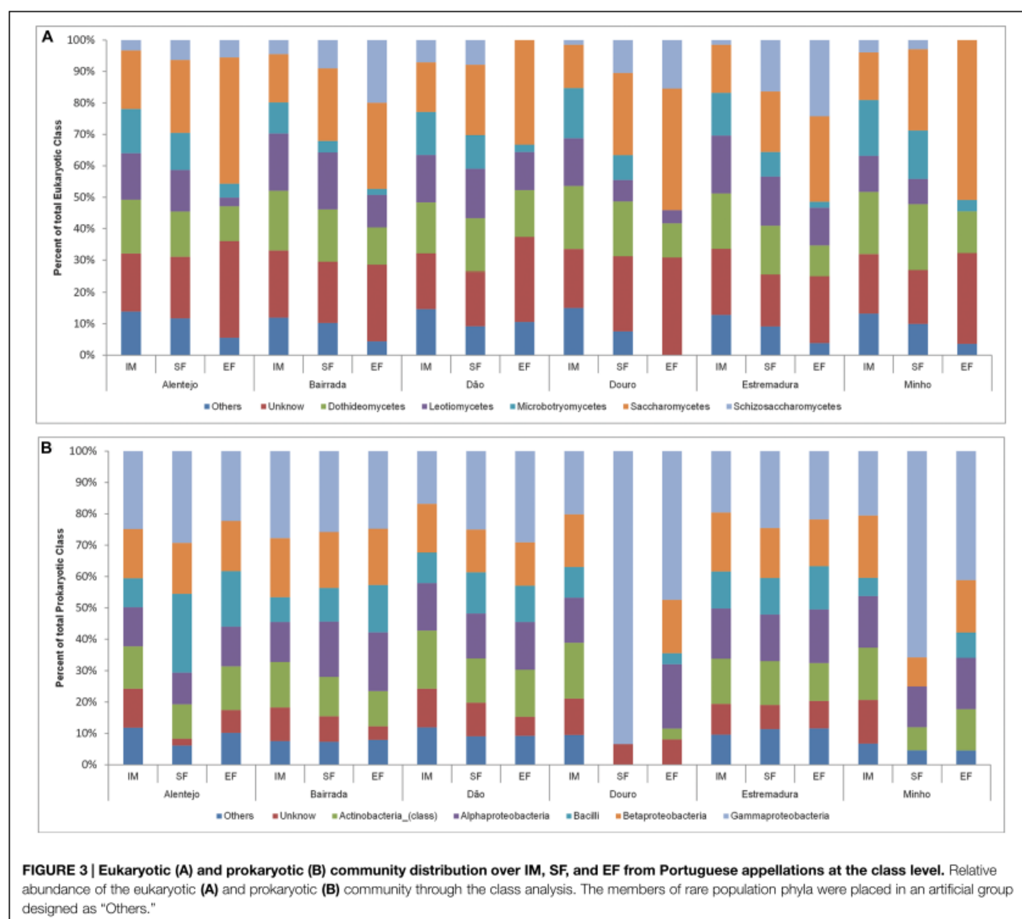

FIGURE 3 | Eukaryotic (A) and prokaryotic (B) community distribution over IM, SF, and EF from Portuguese appellations at the class level. Relative abundance of the eukaryotic **(A)** and prokaryotic **(B)** community through the class analysis. The members of rare population phyla were placed in an artificial group designed as "Others."

The Landscape of Microbial Communities Throughout Wine Fermentation

The dynamics of microbial communities present at IM, SF, and EF of samples from different Portuguese wine appellations were explored by principal coordinates analysis (PCO; **Figure 4**). For both fungal (**Figure 4A**) and bacterial communities (**Figure 4B**), samples were grouped according to their fermentative stage, where the first axis explains 48 and 52.3% of the total variation, respectively. Interestingly, SF samples were mixed with both IM and EF and, indeed this stage is a transition between IM and EF. As expected, the distribution of the microbial community composition is affected by fermentation. Significant differences (Fungi: $R_{ANOSIM} = 0.512$, $p = 0.001$; Bacteria: $R_{ANOSIM} = 0.170$, $p = 0.002$) between IM, SF, and EF samples were observed for a global test. Conversely, no significant differences were observed between SF and EF samples of the bacterial communities ($R_{ANOSIM} = 0.155$, $p = 0.954$) when analyzed by pairwise tests.

The fungal and bacterial microorganisms responsible for the similarities within each group, and the dissimilarity between the different stages of fermentation, were analyzed using SIMPER analysis (**Supplementary Table S2**). The average of similarity within each group increased over the fermentation process for both fungal (IM: 39.84%; SF: 42.27%; EF: 64.19%) and bacterial community (IM: 42.64%; SF: 48.36%; EF: 46.96%). Further, the fungal communities of IM samples were mainly characterized by the environmental yeasts *Aureobasidium* and *Rhodotorula*, which contributed with 64.55% for the group similarity. Other microorganisms, such as *Hanseniaspora*,

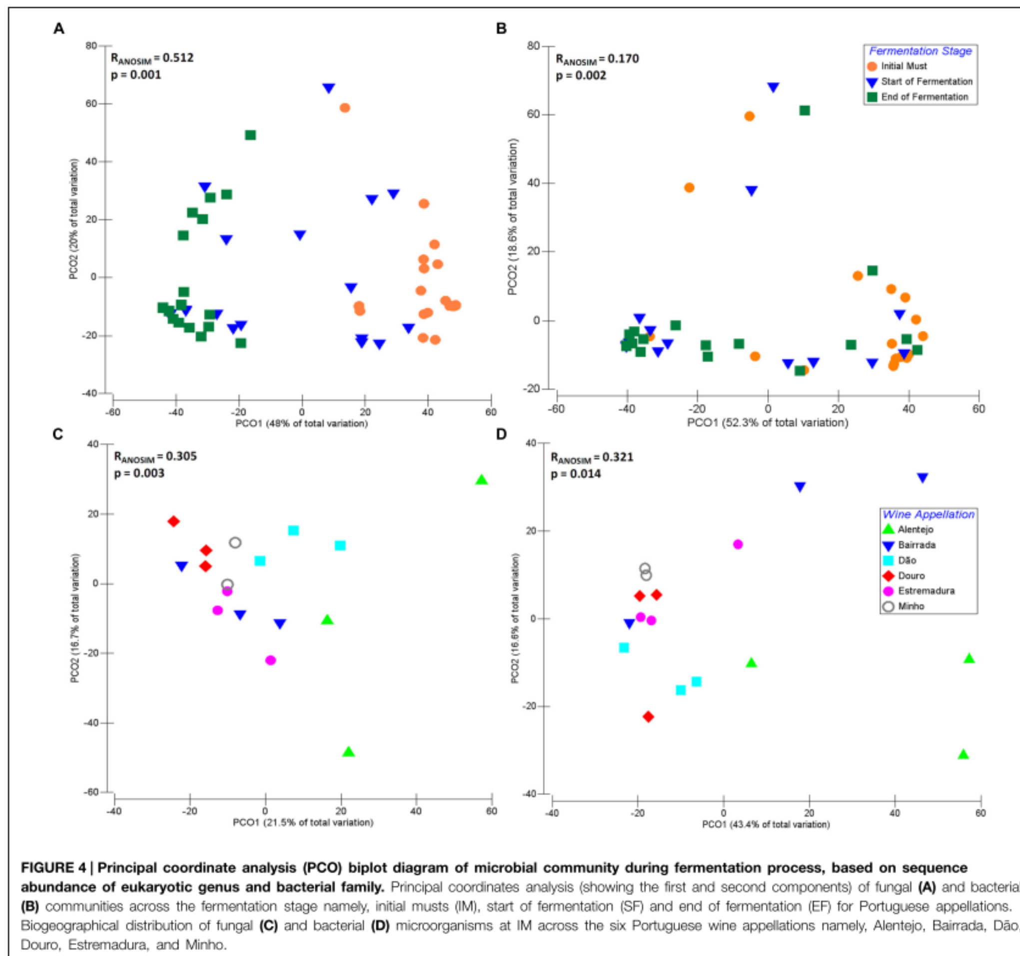

FIGURE 4 | Principal coordinate analysis (PCO) biplot diagram of microbial community during fermentation process, based on sequence abundance of eukaryotic genus and bacterial family. Principal coordinates analysis (showing the first and second components) of fungal **(A)** and bacterial **(B)** communities across the fermentation stage namely, initial musts (IM), start of fermentation (SF) and end of fermentation (EF) for Portuguese appellations. Biogeographical distribution of fungal **(C)** and bacterial **(D)** microorganisms at IM across the six Portuguese wine appellations namely, Alentejo, Bairrada, Dão, Douro, Estremadura, and Minho.

Saccharomyces, *Lachancea*, *Botryotinia*, *Alternaria*, *Aspergillus*, *Metschnikowia*, *Filobasidiella,* and *Candida* contributed with 25.80% for the group similarity. Regarding the bacterial community at IM, Enterobacteriaceae, Pseudomonadaceae, Microbacteriaceae, Comamonadaceae families contribuited with 52.68% for group similarity, followed by Oxalobacteraceae, Sphingomonadaceae, Xanthomonadaceae, Nocardioidaceae, Methylobacteriaceae, Halomonadaceae, Propionibacteriaceae, Rhodobacteraceae, Micrococcaceae, Acetobacteraceae, which all together contributed with 38.25%.

The analysis of similarity of the fungal community at SF and EF revealed that fewer microorganisms contributed to the similarity of groups when compared with IM, which is explained by the evolution of the fermentative process. In fact, the microbial community tended to be more similar and less diverse at EF. At SF, the microorganisms *Saccharomyces*, *Hanseniaspora*, *Aureobasidium,* and *Lachancea* contibuted with 91.91% for group similarity, and at EF the *Saccharomyces* and *Hanseniaspora* microorganisms contributed with 91.19%. The same behavior was observed for bacterial communities where Enterobacteriaceae, Halomonadaceae, Comamonadaceae, Pseudomonadaceae, and Xanthomonadaceae families contributed with 91.44% of similarity for SF group, whereas Enterobacteriaceae, Comamonadaceae, Acetobacteraceae, Xanthomonadaceae, Pseudomonadaceae, and Oxalobacteraceae families contributed with 91.44% for EF group similarity.

Regarding the comparison between IM, SF, and EF groups of fungal communities, a higher dissimilarity value was obtained for IM vs. EF (86.53%) followed by IM vs. SF (73.84%) and SF vs. EF (53.44%), where microorganisms belonging to the *Lachancea, Saccharomyces, Hanseniaspora, Aureobasidium, Schizosaccharomyces, Candida, Metschnikowia, Torulaspora, Rhodotorula,* and *Alternaria* genera contributed for the dissimilarity of the groups. Furthermore, the diferences of the dissimilary were less pronounced for the bacterial community when compared with fungal population: IM vs EF (66.09%), IM vs SF (66.05%), and SF vs EF (50.51%). Micoorganisms belonging to the Halomonadaceae, Enterobacteriaceae, Pseudomonadaceae, Comamonadaceae, Oxalobacteraceae, Microbacteriaceae, Sphingomonadaceae, Acetobacteraceae, and Xanthomonadaceae familes were those that mostly contributed for the dissimilarity of groups (**Supplementary Table S2**).

Microbiome of Wine Appellations

In order to understand the biogeographical distribution of microbial populations, the microbiome associated with the six Portuguese appellations was individually compared for IM, SF, and EF, for both bacterial and fungal communities (**Figures 4C,D**). Significant differences were observed across wine appellations for IM samples (Fungi: $R_{\mathrm{ANOSIM}} = 0.305$, $p = 0.003$; Bacteria: $R_{\mathrm{ANOSIM}} = 0.321$, $p = 0.014$). For both fungal (**Figure 4C**) and bacterial communities (**Figure 4D**), samples were grouped according to their similarity, where the first axis explain 21.5 and 43.4% of the total variation, respectively. The SIMPER analysis (**Supplementary Table S2**) revealed that the average of similarity within each wine appellation was higher at Minho for both bacterial (76.20%) and fungal (63.21%) communities, followed by Estremadura (50.49 and 51.99% for bacterial and fungal populations, respectively), Bairrada (40.81 and 51.77%), Douro (49.68 and 50.68%), Dão (59.74 and 45.29%), and Alentejo (51.54 and 23.98%). The SF samples (fungi: $R_{\mathrm{ANOSIM}} = 0.060$, $p = 0.320$; bacteria: $R_{\mathrm{ANOSIM}} = 0.073$, $p = 0.271$) and EF samples (fungi: $R_{\mathrm{ANOSIM}} = -0.039$, $p = 0.596$; bacteria: $R_{\mathrm{ANOSIM}} = 0.093$, $p = 0.199$) did not show any significant differences.

Regarding the fungal microorganisms that contributed for each wine appellation, the genus *Aureobasidium* dominated and contributed for an average of 44.39% appellations similarity (**Supplementary Table S2**). Interestingly, it was observed a regional effect on the contribution of other microorganisms: at Alentejo appellation *Lachancea* prevailed, contributing for 21.44% of region's similarity; in the Estremadura appellation *Rhodotorula* and *Botryotinia* contributed for 37.96% of the similarity; the Bairrada appellation was characterized by the presence of *Hanseniaspora* and *Ramularia,* who contributed for 18.86% of the regional similarity; the Dão appellation was characterized by the presence of microorganisms from the *Lachancea* and *Rhodotorula* genera (29.07% of similarity); within Douro appellation, *Rhodotorula* and *Erysiphe* contributed with 21.29% for the similarity; and finally, the Minho appellation was characterized by *Rhodotorula* and *Alternaria* (40% of similarity; **Supplementary Table S2**). In general, the fungal populations of IM were characterized by ubiquitous genera as *Aureobasidium, Rhodotorula, Hanseniaspora, Alternaria, Metschnikowia, Saccharomyces, Candida, Ramularia, Penicillium, Lewia, Filobasidiella, Leptosphaerulina,* and *Schizosaccharomyces*, forming the principal structure of the microbial populations (**Figure 5A**).

In SF samples, an increase of *Saccharomyces* population was observed in all regions. Nevertheless, Alentejo had the highest abundance of *Lachancea* and Minho was characterized by having the richest biodiversity, which included *Hanseniaspora, Lachancea, Metschnikowia,* and *Aureobasidium.* Expectedly at EF the dominant genus was *Saccharomyces*, but still some regional differences were observed: samples from Alentejo, Douro, and Minho presented a similar composition (*Saccharomyces* and *Lachancea*), while Bairrada and Dão were mostly composed by *Saccharomyces*. Samples from Estremadura region contained high amounts of both *Saccharomyces* and *Schizosaccharomyces.*

Regarding the bacterial community, the families of Halomonadaceae and Enterobacteriaceae contributed with 91.93% for the Alentejo appellation similarity whereas at Bairrada region, Enterobacteriaceae and Pseudomonadaceae contributed with 75.78%. At Dão appellation, Microbacteriaceae, Oxalobacteraceae, and Enterobacteriaceae contributed with 36.83% and Comamonadaceae, Enterobacteriaceae, Oxalobacteraceae, and Microbacteriaceae families with 52.35% for Douro region similarity. Finally, at Estremadura, Enterobacteriaceae, contributed with 22.47% and at Minho appellation, Oxalobacteraceae, Pseudomonadaceae, and or Enterobacteriaceae with 45.39% for the similarity. It is interesting to notice that the bacterial families responsible for the regional similarities were mostly environmental, and are not related with the oenological process.

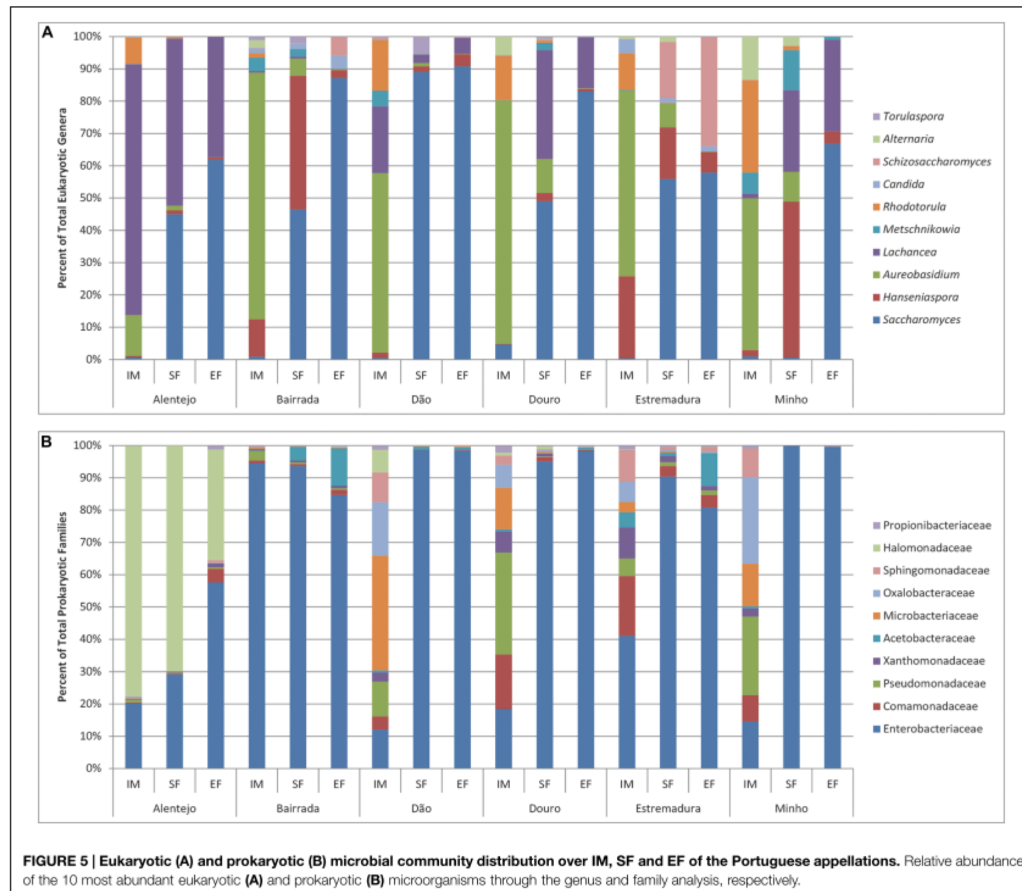

FIGURE 5 | Eukaryotic (A) and prokaryotic (B) microbial community distribution over IM, SF and EF of the Portuguese appellations. Relative abundance of the 10 most abundant eukaryotic **(A)** and prokaryotic **(B)** microorganisms through the genus and family analysis, respectively.

In general, the bacterial community was observed to differ across the appellations at IM samples. Additionally, grapes from Alentejo and Bairrada appellations presented the most distinct bacterial profiles (**Figure 5B**). Regarding SF and EF samples, Enterobacteriaceae was ubiquitous to all appellations. Bairrada and Estremadura were also characterized by high amounts of Acetobacteriaceae, while samples from Alentejo presented a unique microbiome characterized by the Halomonadaceae family (**Figure 5B**).

Regarding the most abundant bacterial family, Enterobacteriaceae, microorganisms from the genus *Pantoea* were found in all samples, whereas *Klebsiella* was only detected at IM and SF, and *Tatumella* was only identified at SF and EF samples. Also, bacteria belonging to the Microbacteriaceae family as *Curtobacterium* and *Frigobacterium* were detected in all samples and *Leifsonia* only at IM samples. Concerning all samples, the bacterial genera *Gluconobacter* (Acetobacteraceae) and *Leuconostoc* (Leuconostocaceae) were also abundant, which was expected as they have been long related with wine fermentations. *Variovorax* (Comamonadaceae); *Carnimonas*, *Halotalea*, and *Zymobacter* (Halomonadaceae); *Massilia* (Oxalobacteraceae); *Pseudomonas* (Pseudomonadaceae); and *Sphingomonas* (Sphingomonadaceae) were also extensively detected in all samples.

Discussion

The aims of this work were to characterize and to compare the diversity of the microbial communities during spontaneous wine fermentations and across different wine Portuguese appellations. To achieve this, high-throughput sequencing was used to fully characterize both eukaryotic and prokaryotic communities from samples collected from six Portuguese wine regions.

Wine fermentations are known to harbor a heterogeneous population of microorganisms. In this work, a diverse set of microbial communities was identified, where the most abundant phyla were Proteobacteria and Ascomycota from prokaryotic and eukaryotic populations, respectively. As expected, a clear relationship was observed between the microbial community and fermentation stage. The biodiversity across the fermentation process decreased for both prokaryotic and eukaryotic communities as a result of the selective environment created over the spontaneous wine fermentation. Interestingly, the variations of biodiversity along this process revealed a higher impact on the fungal community structure, when compared with the bacterial populations. Furthermore, the prokaryotic populations were more diverse than the eukaryotic populations.

In this study, the most abundant eukaryotic microorganisms at IMs were *Aureobasidium* (*A. pullulans*), *Rhodothorula* (*R. nothofagi*), *Hanseniaspora* (*H.uvarum*), and *Lachancea* (*L. thermotolerans*). A diverse set of bacterial population was also uncovered, where Enterobacteriaceae (namely, *Pantoea,* and *Klebsiella*) and Pseudomonadaceae (namely, *Cellvibrio,* and *Pseudomonas*) were the most abundant families. This is in line with the previous reported by Bokulich et al. (2013), where microorganisms as *Cladosporium* spp., *A. pullulans, H. uvarum* were detected as the major eukaryotic population in the IMs, and as regards to prokaryotic population, Lactobacillales, Pseudomonadales, or Enterobacteriales were also identified.

The high microbial biodiversity within IM samples was mostly due to environmental microorganisms derived from vineyard. Indeed, several detected microorganisms, namely, *Botryotinia, Phomopsis, Aspergillus, Penicillium, Aureobasidium, Rhodotorula,* Enterobacteriaceae, or *Sphingomonas,* were previously described on grapevine leafs and grape surfaces and some of them are even refereed as inhabitant of grapes (Mills et al., 2008; Martins, 2012; Bokulich et al., 2013; Pinto et al., 2014). Also, *Saccharomyces* was detected at IMs, which suggests that this community comes from grapes, reinforcing findings from Bokulich et al. (2013), Pinto et al. (2014), and Taylor et al., 2014.

Regarding the origin of spoilage microorganisms, there has been a vivid discussion on whether or not these are present at the vineyards, where grapes are the principal source for wine contamination and deterioration (Renouf et al., 2005), or otherwise, winemaking equipment is the source of spoilage microorganisms (Couto et al., 2005). For instance, it is considered that *Dekkera*/*Brettanomyces*, the lactic and AAB are the most important wine spoilage microorganisms (Bartowsky et al., 2003; Beneduce et al., 2004; Cocolin et al., 2004). In this study, *Dekkera*/*Brettanomyces bruxellensis* was not detected, which is in line with the study of Suárez et al. (2007), who reported that this spoilage yeast is mainly present in winemaking equipment with deficient cleaning; and is opposed to the findings reported by Renouf and Lonvaud-Funel (2007). Still, these results *per se* do not yet allow for a clear conclusion on their origin. In the other hand, LAB and AAB were detected at low abundances, but *Oenococcus oeni*, a LAB extensively used to carry out the MLF, was not detected. Additionally, filamentous fungi (molds) were identified on IMs: *Alternaria, Aspergillus, Botrytis, Cladosporium, Penicillium,* or *Rhizopus*, which are undesirable for wine quality (Toit and Pretorius, 2000). *Aspergillus* (*A. niger*) and *Penicillium* (*P. glabrum* and *P. brevicompactum*) were found in all the appellations considered in this work. However, and along fermentations, these molds disappeared, which supports the observations that they are sensitive to the wine fermentation conditions (Blesa et al., 2006).

From the IM to the wine, sequential stages of microbial development were observed, as result of fermentation activities (Fleet et al., 1984; Jolly et al., 2003). An initial growth of non-*Saccharomyces,* such as *Hanseniaspora, Torulaspora, Metschnikowia,* and *Pichia* at SF was followed by a decrease or even a disappearance of these yeasts at the EF and, conversely, the increase of *S. cerevisiae* was evidenced. A similar kinetic pattern was also observed on prokaryotic community, where in transition from IM to SF, Enterobacteriaceae family increased, and then decreased from SF to EF, specifically in Bairrada, Dão, and Estremadura appellations.

In spontaneous wine fermentations, *S. cerevisiae* was dominant despite the high abundance of *Hanseniaspora* and *Lachancea*. Yeasts associated with wine fermentation such as *Metschnikowia* (*M. pulcherrima and M. viticola*), *Torulaspora* (*T. delbrueckii*), *Schizosaccharomyces* (*S. japonicus*), *Candida* (*C. zemplinina*), *Issatchenkia* (*I. terricola*), and, less frequently, *Pichia* (*P. kluyveri* and *P. kudriavzevii*) were also detected. However, their relative abundances varied according to their appellation of origin. Indeed, each appellation presented characteristic microbial communities, with different abundances of non-*Saccharomyces* and specific patterns of microbial communities. Interestingly, *Schizosaccharomyces* (*S. japonicus*) was also detected, even at later stages, and was present at higher abundances in the Estremadura region. This yeast is characterized by having a high fermentative capacity at high temperatures (optimal growth around 30°C), and by being resistant to SO_2 and to the stringent conditions of fermentation (Torija et al., 2001). Regarding *Torulaspora delbrueckii,* it was found until EF, and it has been previously reported to survive until later stages of fermentation and to produce lower levels of acetic acid (Ciani et al., 2006). Interestingly, samples which presented higher abundance of this microorganism also generally had higher abundance of AAB namely, *Gluconobacter* (*G. oxydans*).

Among bacterial communities, during the fermentation, Enterobacteriaceae was the most abundant family (namely, *Tatumella* sp.). Nisiotou et al. (2011) also showed that Enterobacteriaceae persists in fermentation, and Ruiz et al. (2010) also confirmed its prevalence at beginning, mid and final stages of MLFs in different Spanish wineries. This raises the question if these bacteria interact with fermenting yeasts and, if so, in what degree can this microbial population influence (negatively or positively) the organoleptic proprieties of wine. The bacterial populations were found to be less dynamic than the eukaryotic populations in the later stages of fermentation process, and their geographic profiles were more similar: it was observed a clear dominance of Enterobactereaceae family at all appellations but Alentejo, where microorganisms from Halomonadaceae family were also presented with high abundance. The Bairrada and Estremadura appellations were also characterized by

the presence of microorganisms from the Acetobacteraceae family. Among the LAB, high amounts of *Lactobacillus* (Lactobacillaceae), *Leuconostoc* (Leuconostocaceae), *Lactococcus,* and *Streptococcus* (Streptococcaceae) were detected. Additionally, *Facklamia* (Aerococcaceae), *Carnobacterium, Dolosigranulum, Granulicatella,* and *Trichococcus* from Carnobacteriaceae family, *Enterococcus* (Enterococcaceae) and *Weisella* as *W. cibaria* (Leuconostocaceae) were also detected, but at lower abundances. Interestingly, and with exception of *Weisella*, those specific microorganisms had not been previously isolated from musts and wines (König and Fröhlich, 2009).

To investigate whether or not there is a geographic imprint on the wine fermentation microbiome, a PCO was performed for each fermentation stage in order to evaluate differences according to wine appellation. Interestingly, significant differences ($p < 0.05$) were observed for both fungal and bacterial microbial communities at IM between wine appellations. These results are consistent with those reported by Bokulich et al. (2013), who observed differences in the microbial community structure across wine appellations from California. Over the fermentation process, the initial microbiome associated with each wine appellation disappears and, as a consequence, the biogeographic profile was lost (no significant differences were observed for SF and EF). As observed, this microbiome is characterized by the presence of environmental microorganisms, which constituted a signature of each Portuguese wine regions. Moreover, these results also suggested that the initial microbial community could strongly contribute to the uniqueness of the wines derived from each specific wine appellation. Furthermore, each wine appellation presented its own pattern of biodiversity that varied in terms of the microbial abundance. This finding is of special interest when considering the non-saccharomyces population at the SF, whom have been acknowledged for their metabolic contribution to the final wine sensorial properties (Romano et al., 2003; Jolly et al., 2014), which reinforces their role on the regional attributes of wines. These findings open new horizons to dissect how microbiomes affect wine properties and support the need to unveil the endogenous microflora of such regions and explore its natural microbial populations in order to produce valuable wines styles.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00905

Figure S1 | Portugal map with the appellations and grape varieties chosen for study. Sample collection was done in 6 appellations – Minho (M), Douro (Dr), Dão (D), Bairrada (B), Estremadura (E), and Alentejo (Al). The grave varieties collected were Al, Alvarinho; Ax, Alfrocheiro; B, Baga; J, Jean; L, Loureiro; P, Piriquita; T, Trincadeira; TF, Touriga Franca; TN, Touriga Nacional; and TR, Tinta Roriz (also known Aragonez).

Figure S2 | Rarefaction curves at a genetic distance of 3% for each sample (IM, SF, and EF). D2 **(A)** and ITS2 **(B)** sequences both from the analysis of 26S rRNA and ITS regions of eukaryotic population present in the sample and V6 sequences **(C)** from the analysis of 16S rRNA of prokaryotic diversity. The IM, start fermentation (SF) and end of fermentation (EF) are represented by the blue, yellow and green color, respectively.

Table S1 | Total sequences obtained for eukaryotic (ITS2 and D2) and prokaryotic (V6) microbial communities for IM, SF, and EF from different wine appellations. Operational taxonomic units (OTUs) and estimated species (Chao1) were determined at a genetic distance of 3% using Mothur. The coverage obtained was also determined as being the ratio between the observed OTUs and the estimated Chao1 (OTUs/Chao1).

Table S2 | Analysis of the similarity and dissimilarity across wine fermentation stages and wine appellations. The similarity and dissimilarity across wine fermentation stages namely, initial musts (IM), start of fermentation (SF), and end of fermentation (EF) and wine appellations were calculated through the SIMPER analysis.

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Chapter III

SELECTION OF POTENTIAL BCAS

Chapter outline

Grapevine is naturally colonised by a myriad of microbial communities. Among them, the beneficial grapevine- associated microorganisms play a significant role in supporting the vine health and growth, through direct or indirect mechanisms. These microorganisms can positively interact with the plant by stimulating the plant growth, by reducing the pathogen infection or by reducing the abiotic plant stress, without causing disease symptoms (Lodewyckx *et al*., 2002; Lugtenberg and Kamilova, 2009; Compant *et al.,* 2010, Pinto and Gomes, 2016). Thus, beneficial microorganisms are of utmost interest for agriculture management either as promotors of the plant growth (PGP) and/or biofertilizers or as promotors of plant protection by acting as BCAs (Bloemberg and Lugtenberg, 2001; Lugtenberg and Kamilova, 2009; Armijo *et al.,* 2016). Contrarily to the application of pesticides, the application of beneficial microorganisms in agricultural systems ensures an equilibrium of the natural microbial population of the plant. In this context, the application of potential BCAs against important grapevine diseases, such as grey mould (*Botrytis cinerea*) or GTDs, may be considered. Indeed, these are devastating diseases, responsible for great losses within productivity and yield (Amijo *et al.,* 2016; Fontaine *et al.,* 2016b). Given the GTDs, actually there are no highly effective treatments to control them, which reinforces the need to develop urgently new control strategies (Fontaine *et al.,* 2016b).

In this chapter, the biocontrol potential of natural grapevine isolates was addressed towards important grapevine pathogens, in attempt to select potential BCAs against GTDs, under *in vitro* conditions. Selected BCAs were then characterized for their mechanisms involved in biocontrol, namely capacity to produce volatiles compounds and hydrolytic enzymes (amylases, cellulases, lipases, pectinases, proteases and ureases), phosphate solubilisation and siderophores production. Also, their tolerance to pH and salinity conditions, their effects on non-target microorganisms, their growth curves and a molecular identification were achieved. Later on, and in order to deeply address the biotechnological potential, their genome was sequenced and analysed.

Results demonstrated that of the 202 isolates, 15% and 19% reduced significantly ($p<0.05$) the mycelium growth of *B. cinerea* and *D. seriata,* respectively*.* On its hand, three isolates, namely *Streptomyces* sp. Fito_S127B strain, *Aureobasidium pullulans* Fito_F278 and *Bacillus amyloliquefaciens* Fito_F321 were highly effective against several GTDs agents. The success of these biocontrol activities occurred by a direct confrontation between the pathogen and the BCAs, suggesting that antibiosis and competition for nutrients and space were the mechanisms used by these BCAs. Meanwhile, our strains may produce volatiles which were able to inhibit the pathogen growth or retarding its sporulation (as observed with Fito_F278). Among hydrolytic enzymes, cellulases showed a higher enzymatic index among strains although they were also able to produce amylases, lipases, pectinases and proteases.

Overall, isolates were tolerant to pH conditions though only Fito_F278 and Fito_F321 were more tolerant to salinity levels. Going forward, Fito_F278 strain showed an unexpected effect against non-target microorganisms by means of inhibiting their growth. This suggests a clear competition of Fito_F278 towards other strains, when present in higher abundance. The genome sequencing of these BCAs allowed to predict important protein-coding genes involved in biocontrol activities such as angolamycin or siderophore in Fito_S127B, alcohol dehydrogenases, tannases, cellulases, ureases, trehalose-phosphatase, glucanases or chitinases in Fito_F278 and bacillaene, difficidin, macrolactin, surfactin, fengycin and siderophore in Fito_F321 strain.

Overall, results highlighted that grapevine is naturally colonised by beneficial microorganisms which can act as a first barrier against the phytopathogens attack. Furthermore, the most effective biocontrol strains, such as Fito_S127B, Fito_F278 and Fito_F321, can produced a set of enzymes or other compounds with interest not only for biocontrol activities but also for PGP. Thus, this study reinforces the potential use of these strains against GTDs and offers an alternative to the application of pesticides in grapevine management. Furthermore, highlighted the importance of an accurate molecular identification of BCAs strains for future regulatory authorization. The genome sequencing of each BCA suggested that these strains represent undoubtedly a source of novel bioactive compounds of biocontrol value, which must be further explored.

Contexte

La vigne est naturellement colonisée par de nombreuses communautés microbiennes. Parmi eux, les microorganismes bénéfiques jouent un rôle important dans la santé et la croissance de la plante, grâce à des mécanismes directs et/ou indirects. Ces microorganismes peuvent interagir positivement avec la plante en stimulant sa croissance, en réduisant l'infection par les agents pathogènes ou en réduisant les stress abiotiques, sans provoquer de symptômes pathologiques (Lodewyckx *et al*., 2002; Lugtenberg et Kamilova, 2009; Compant *et al*., 2010 ; Pinto et Gomes, 2016). Ainsi, les microorganismes bénéfiques sont d'un grand intérêt pour la gestion de l'agriculture soit en tant que promoteurs de la croissance végétale et/ou biofertilisants, soit en tant que promoteurs de la protection des plantes en agissant comme agents de biocontrôle (BCA) (Bloemberg et Lugtenberg, 2001 ; Lugtenberg et Kamilova, 2009 ; Armijo *et al*., 2016). Contrairement à l'application de pesticides, l'application de microorganismes bénéfiques dans les systèmes agricoles assure un équilibre de la population microbienne naturelle de la plante. Dans ce contexte, nous pouvons envisager l'application de BCAs contre d'importantes maladies de la vigne, à savoir la pourriture grise (*Botrytis cinerea*) ou les MDB. En effet, ce sont des maladies dévastatrices, responsables de pertes importantes en productivité et rendement (Amijo *et al*., 2016 ; Fontaine *et al*., 2016b). Concernant les MDB, aucun traitement hautement efficace pour les contrôler n'est actuellement disponible, ce qui renforce la nécessité de développer de nouvelles stratégies de contrôle (Fontaine *et al*., 2016b).

Dans ce chapitre, le potentiel de biocontrôle d'isolats naturels de la vigne a été testé *in vitro* vis-à-vis d'agents pathogènes de la vigne, dans le but de sélectionner des BCAs potentiels contre les MDB. Les BCA sélectionnés ont ensuite été caractérisés pour leurs mécanismes impliqués dans le biocontrôle, à savoir la capacité à produire des composés volatils et des enzymes hydrolytiques (amylases, cellulases, lipases, pectinases, proteases et ureases), la solubilisation du phosphate et la production de siderophores. En outre, leur tolérance aux conditions de pH et de salinité, leurs effets sur des microorganismes non ciblés, leurs courbes de croissance et leur identification moléculaire ont été étudiés. Afin d'approfondir leur potentiel biotechnologique, leur génome a été séquencé et analysé.

Les résultats ont démontré que sur les 202 isolats, 15% et 19% ont réduit de manière significative (p <0,05) la croissance du mycélium de *B. cinerea* et *Diplodia seriata*, respectivement. Trois isolats, à savoir *Streptomyces* sp. Fito_S127B, *Aureobasidium pullulans* Fito_F278 et *Bacillus amyloliquefaciens* Fito_F321 ont été très efficaces contre plusieurs agents des MDB. Le succès de ces activités de biocontrôle à été révélé par une confrontation directe entre le pathogène et les BCAs, ce qui suggère que l'antibiose et la concurrence pour les nutriments et l'espace étaient les mécanismes utilisés par ces BCAs. Ces souches peuvent aussi produire des substances volatiles capables d'inhiber la croissance

des agents pathogènes ou de ralentir leur sporulation (comme observé pour Fito_F278). Parmi les enzymes hydrolytiques, les cellulases ont montré un indice enzymatique plus élevé parmi les souches, bien qu'elles soient également capables de produire des amylases, des lipases, des pectinases et des proteases. Dans l'ensemble, les isolats étaient tolérants aux conditions de pH, mais seuls Fito_F278 et Fito_F321 étaient les plus tolérants aux niveaux de salinité. La souche Fito_F278 a montré un effet inattendu contre les microorganismes non ciblés en inhibant leur croissance. Cela suggère une concurrence claire de la souche Fito_F278 vers d'autres souches, lorsqu'elle est présente dans une plus grande proportion. Le séquençage du génome de ces BCAs a permis d'indentifier des gènes codant pour des protéines impliquées dans des activités de lutte biologique, telles que l'angolamycine ou le sidérophore pour Fito_S127B, les alcools déshydrogénases, les tannases, les cellulases, les uréases, la tréhalose-phosphatase, les glucanases ou les chitinases pour Fito_F278 et le bacillaène, macrolactine, surfactine, fengycine et siderophore pour Fito_F321.

Dans l'ensemble, les résultats ont mis en évidence que la vigne est naturellement colonisée par des microorganismes bénéfiques qui peuvent constituer une première barrière contre l'attaque des pathogènes. De plus, les souches les plus efficaces, telles que Fito_S127B, Fito_F278 et Fito_F321, peuvent produire un ensemble d'enzymes ou d'autres composés intéressant non seulement pour les activités de biocontrôle, mais aussi pour la promotion de la croissance des plantes. Ainsi, cette étude renforce l'utilisation potentielle de ces souches contre les MDB et offre une alternative à l'application de pesticides dans la gestion de la vigne. En outre, nous pouvons souligner l'importance d'une identification moléculaire précise des BCAs pour les futures autorisations réglementaires. Le séquençage du génome de chaque BCA a suggéré que ces souches représentent sans aucun doute une source de nouveaux composés bioactifs de valeur de biocontrôle, qui doivent être plus explorés.

Screening of potential BCAs from grapevine microbiome to control grapevine diseases: a special focus on *Botryosphaeria* dieback

Plants, including grapevine, are naturally colonised by a myriad of microorganisms which could be neutral, beneficial or pathogenic. Furthermore, those plant-microbial interactions may have a direct or indirect effect on plant growth and plant health status (Pinto and Gomes, 2016).

Given the grapevine diseases, both grey mould and grapevine trunk diseases (GTDs) are devastating diseases of grapevine, responsible for great losses within productivity and yield. Briefly, grey mould is caused by the necrotrophic *Botrytis cinerea* Pers. Fr. which causes important damages on berry clusters (Amijo *et al.,* 2016). Indeed, *B. cinerea* integrates the top 10 of plant pathogens (Dean *et al.,* 2012), which is not surprising as this pathogen can infect more than 200 host plants such as fruit crops, vegetables and ornamental plants, causing severe damages at both pre- and post-harvest (Williamson *et al.,* 2007; Dean *et al.,* 2012). In vineyards, this pathogen is particular worrisome during flowering to bunch period or from *veraison* to harvest. Climatic conditions such as warm wet weather and rainfalls promotes favourably the development of the disease (Viret *et al.,* 2004). Regarding the GTDs, the three main diseases are Esca disease, Botryosphaeria dieback and Eutypa dieback, which mainly attack the perennial organs of grapevine (Bertsch *et al.,* 2012; Fontaine *et al.,* 2016a). Several pathogenic fungi are associated with these diseases. Thus, esca disease is caused by *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum* and *Fomitiporia mediterranea* or even *Eutypa lata* and *Stereum hirsutum* (Larignon *et al.,* 2009; White *et al.,* 2011); Botryosphaeria dieback by botryosphaeriaceae species such as *Botryosphaeria dothidea*, *Diplodia seriata*, *Diplodia mutila*, *Neofusicoccum parvum* or *Lasiodiplodia theobromae* (Úrbez-Torres, 2011); and the Eutypa dieback or eutypiosis is mainly caused by the *Eutypa lata* though *Eutypa leptoplaca*, *Cryptovalsa ampelina*, *Diatrypella* sp. or *Eutypella* spp. may be involved. Overall, *N. parvum* and *P. chlamydospora* have been shown to be the most virulent pathogens within GTDs (Laveau *et al.,* 2009). Actually, no effective control measures are available to control GTDs. The current methods applied are not curative but preventive, and include some nursery measures before planting, to maintain good hygiene and wound protection (Gramaje and Armengol, 2011; Gramaje and Di Marco, 2015), hot water treatment, preventive culture measures in vineyards (removing burning brunches, dead vines, or pruning residues) or develop training systems and trunk renewal practices (Fontaine *et al.,* 2016b). Thus, alternatives are urgently needed to effectively control these diseases.

With regards to beneficial microorganisms these are of great deal of interest as they fulfil a significant role in plants accessibility to nutrients, improving plant nutrition, and can carry out a pathogen defence through an antagonistic activity, or by stimulating the plant defence responses through hormone-mediated signalling pathways as jasmonic acid (JA), ethylene (Et) or salicylic acid

(SA). Thus, these microorganisms can act as promotors of the plant growth (PGP) or being involved in plant protection by acting as biocontrol agents (BCAs) (Bloemberg and Lugtenberg, 2001; Lugtenberg and Kamilova, 2009; Armijo *et al.,* 2016). Several mechanisms are involved in the suppression of pathogens and which includes antibiosis, competition, parasitism, cell-wall-degrading enzymes or plant-inducing resistance (Pal and Gardener, 2006; Jamalizadeh *et al.,* 2011). Briefly, the antibiotics production (such as fengicin, iturin, streptomycin or kanamycin), bacteriocins, siderophores production, volatile organic compounds (VOCs) or other enzymes involved in the cell-wall- degrading such as chitinases, glucanases, lipases or proteases can be efficiently produced by beneficial microorganisms and determine the success of their biocontrol activities. Contrarily to the application of pesticides in agricultural crops, the application of these beneficial microorganisms ensures an equilibrium of the natural microbial population of the plant. Furthermore, the risk to develop pathogen-resistant strains and the impact on non-target microorganisms is reduced or even null (Villaverde *et al.,* 2014). Indeed, pesticides protect plants from pathogen attacks, improve crop production and avoid great yield losses in worldwide crops (Tanaka and Ōmura, 1993). Though, the routinely application of these compounds causes seriously harmful effects on environment, health problems on humans or animals and even causes an imbalance of the natural microbial communities of grapevine (Berg, 2009; Pinto *et al.,* 2014). Additionally, pesticide residues are persistent in nature and may be detectable in wines. In the last years, search for ecologically management strategies for agriculture, in alternative to chemical control, are growing (Berg, 2009). Thus, the plant-associated microorganisms, namely beneficial communities, represents a potential biocontrol strategy. In fact, some BCAs are already commercially available. However, new attempts are needed to improve their efficacy after field release, notably the microbial formulations. Indeed, this must be carefully designed to improve the BCAs' shelf-life and to avoid their cell death after field delivery, as a consequence of the sharp change of the environmental conditions.

Herein, in this study, we demonstrated the natural antagonistic capacity of isolates from grapevine against important diseases such as *B. cinerea* and *Botryosphaeriaceae* dieback agents, associated with GTDs. A set of promisor's biocontrol microorganisms namely, *Streptomyces* sp. (Fito_S127B), *Aureobasidium pullulans* (Fito_F278) and *Bacillus amyloliquefaciens* (Fito_F321) were selected and deep analysed for their mechanisms involved in biocontrol, namely: (1) capacity to produce volatile compounds; (2) their biocontrol potential after cell deactivation; (3) production of hydrolytic enzymes, siderophores production, solubilisation of phosphate and tolerance to pH and salinity conditions; (4) their physical interaction within non-target microorganisms; (5) characterization of their growth curves for a small-scale production of strains to test their efficiency under controlled conditions, and (6) their molecular identification.

Material and methods

Sampling

Samples were collected from four vineyards located in Bairrada Apellation at Cantanhede, Portugal over two vine seasons namely, from April to September 2011 and September 2012. Sampling was randomly performed across vineyards and from different grape cultivars and included soil, roots, leaves, stems and berries. The biological material was then placed in sterile Falcons or sterile bags, transported to the laboratory, stored in the refrigerator at 4°C until use and processed up to 24 hours after collection.

Isolation of microorganisms

Samples of roots, leaves, stems and berries were (a) homogenised and crushed in a sterile saline solution (0.85%) with a sterile pestle or (b) previously surface sterilized with 70% ethanol for 5 min, followed by 1% sodium hypochlorite and washed three times in sterile MiliQ water. Except for roots, each treated sample was spread (0.1mL) over the surface of PDA (Merck) or YPD (Yeast Extract-Peptone-Dextrose: Yeast extract 10 $g.L^{-1}$; Glucose 20 $g.L^{-1}$; Peptona 20 $g.L^{-1}$; Agar 20 $g.L^{-1}$) and incubated at 28°C for 48h or 72h. Root samples were serially diluted before spread in culture media. Soil samples were mixed, suspended in sterile MiliQ water, homogenized by vortexing and serially diluted up to 10^{-6}, spread (0.1mL) over the surface of PDA and incubated at 28°C for 48h or 72h. Then different colonies were selected and pricked in new culture plates and incubated at 28°C for 48h. The isolation process was repeated until to obtain pure cultures. Each isolate selected was further assigned to a specific isolation code. For long-term preservation, each isolate was stored in Cryovials containing PDB (Formedium) or YPD broth with 80% glycerol for bacterial or yeast isolates at -80°C.

Antifungal assays (preliminary screening)

A preliminary screening of the antagonistic activity of the 202 obtained isolates against *Botrytis cinerea* strain 630 and *Diplodia seriata* strain Fito_F14, was performed to select potential biocontrol microorganisms. The *B. cinerea* strain 630 is a strain collection of the University of Reims Champagne-Ardenne (France) and *D. seriata* strain Fito_F14 was isolated at 2011 from grapevine leaves in the Bairrada appellation, Portugal. The PDA plates were inoculated centrally with a 3-mm diameter of mycelium agar disk of fungal pathogen with 7 days old and then four different isolates with 48/72h old were deposited at 2 cm away from the border of the Petri dish. Cultures were incubated in triplicate at 28°C and followed for 7 days. Herein the mycelium inhibition was not calculated.

The potential antagonist microorganisms were then selected for dual tests. For this, a 3-mm diameter plug of pathogen was transferred to 2.5 cm from the border of the plate and each isolate

inoculated at 180° from the pathogen and at the same distance from the border. Plates inoculated only with pathogen served as control. The assay was performed in triplicate and plates were incubated at 28°C and followed for 7 days. The antifungal activity was calculated based on the percent relative of the pathogen' mycelium inhibition through the formula (MI%): MI% = 100* (Mfg-Mga)/Mfg, where Mfg corresponds to the pathogen' mycelium free growth and Mga to the mycelium growth of pathogen in the presence of the antagonistic microorganism. Isolates showing the highest antagonistic activity in the dual tests have been selected to further study their biocontrol effect against Botryosphaeriaceae species.

Validation of biocontrol isolates against Botryosphaeria dieback

The biocontrol potential of the previously selected isolates was then determined by dual tests against Botryosphaeria dieback agents namely, *D. seriata* (strains F98.1 (Robert-Siegwald *et al.,* 2017) and Ds99.7) and *N. parvum* (strains Np Bt-67, Np Bourgogne and Np SV) (strains collection of the University of Reims Champagne Ardenne). The strain F98.1 was isolated from Syrah cultivar (Pyrénées-Orientales, France), Ds99.7 from Clairette (Rhône-Alpes, France), Np Bt-67 from Fernão Pires (Estremadura, Portugal), Np Bourgogne from Chardonnay (Bourgogne, France) and Np SV isolated from Syrah (Bouches-du-Rhône, France). The antagonistic tests were performed according to the methodology described above and followed until 15 days. A diary registration of the mycelium growth was measured and the area (cm^2) of pathogen mycelium inhibition determined by using the Image J software. The percent relative of the pathogen' mycelium inhibition was calculated through the formula: MI% = 100* (Mfg-Mga)/Mfg, where Mfg corresponds to the pathogen' mycelium free growth (control) and Mga to the mycelium growth of pathogen in the presence of the antagonistic microorganism. From the results obtained, only three isolates were selected for further analysis, namely Fito_S127B, Fito_F278 and Fito_F321.

Effect of strains volatiles on fungal biocontrol

The effect of volatile compounds emitted by Fito_S127B, Fito_F278 and Fito_F321 on the mycelial inhibition growth of *D. seriata* Fito_F14 strain was measured. For this, Petri dishes were inoculated both at top and bottom parts. At the top of the Petri dish, each strain was inoculated individually on a line across the entire diameter of the Petri dish and in specific culture media, namely Fito_S127B on MyM (Maltose-Yeast Extract-Malt Extract: maltose 4 $g.L^{-1}$, yeast extract 4 $g.L^{-1}$, malt extract 1 $g.L^{-1}$, agar 18 $g.L^{-1}$), Fito_F278 on YPD (Yeast Extract-Peptone-Dextrose: Yeast extract 10 $g.L^{-1}$; Glucose 20 $g.L^{-1}$; Peptona 20 $g.L^{-1}$; Agar 20 $g.L^{-1}$) and Fito_F321 on LB agar (Duchefa Biochemie). In turn, at the bottom of the Petri dish, a 3-mm diameter of mycelium agar disk containing the fungal pathogen (*D. seriata* Fito_F14 strain) with 7 days old was inoculated centrally in PDA medium. Plates were sealed with

parafilm and incubated at 28°C for 15 days. Herein, the fungal pathogen was exposed, without a direct physical contact, to the volatile compounds emitted by each strain in the top of Petri dish. A diary registration of the mycelium growth was measured and the area (cm^2) of pathogen mycelium inhibition determined by using the Image J software. The percent relative of the pathogen' mycelium inhibition was calculated according to the formula described above.

Characterization of the biocontrol potential of strains after cellular deactivation

Different methodologies were tested for deactivation of the selected isolates (Fito_S127B, Fito_F278 and Fito_F321). Among them, the deactivation protocol for Fito_S127B consisted on applying 1 mL at 10^6 CFU/mL of fresh colonies at 80°C during 5 min followed by 1 min on ice and the strain Fito_F278 was deactivated after cell disruption (1.5 mL at 10^6 CFU/mL) through the bead beating method (3 cycles of 1 min each, interspersed with 1 min on ice). The Fito_F321 was tested for these both methodologies and also for an incubation in high steamy heat for 10, 20, 30, 40 or 60 min. After deactivation, 0.5 mL at 10^6 CFU/mL of each strain was individually homogenised on 15 mL of PDA at ± 50°C. After solidification, the pathogen *D. seriata* Fito_F14 was incubated centrally in the plates with a 3-mm diameter of mycelium agar disk of fungal pathogen with 7 days old. Plates were incubated at 28°C and followed for 15 days. Negative control consisted on the free growth of the pathogen. A diary registration of the mycelium growth was measured and the area (cm^2) of pathogen mycelium inhibition determined by using the Image J software and according to the methodology referred above.

Physiological and biochemical characterization of the selected isolates

The selected microorganisms (Fito_S127B; Fito_F278 and Fito_F321) were evaluated for their ability to produce siderophores, solubilize phosphates and to produce hydrolytic enzymes (amylases, cellulases, lipases, pectinases, proteases and ureases). The siderophore production was determined by applying the method of Chrome Azurol S agar assay test (CAS) according to Alexander and Zuberer (1991) and positive results were observed through the yellow halo formation around colonies. For both tests, plates were incubated until a period of 10 days at 28°C and in triplicate. The phosphate solubilisation was evaluated in Pikovskaya culture medium (glucose 10 g.L^{-1}; NaCl 0.2 g.L^{-1}; $(NH_4)_2(SO_4)$ 0.5 g.L^{-1}; yeast extract 0.5 g.L^{-1}; $MnSO_4$ 0.1g.L^{-1}; $MgSO_4$ 0.1 g.L^{-1}; agar 20 g.L^{-1} and $Ca_3(PO_4)$ 5 g.L^{-1} that was sterilized separately) and the degradation halo (clear zone) around colony corresponded to a positive activity.

Given the hydrolytic enzymes, amylases were assessed by spot inoculation of each selected strain in PDA at 28°C for 48h which was then flooded with 5 mL iodine solution for 2 min. Cellulases production was assessed according to Kasana *et al*. (2008). For this, strains were spot inoculated in CMC agar ($NaNO_3$ 2 g.L^{-1}; K_2HPO_4 1 g.L^{-1}; $MgSO_4$ 0.5 g.L^{-1}; KCl 0.5 g.L^{-1}; carboxymethylcellulose (CMC)

sodium salt 2 g.L^{-1}; peptone 0.2 g.L^{-1}; agar 17 g.L^{-1}) at 28°C for 48h and then flooded with 5 mL of iodine solution for 2 min. The lipase production was confirmed through the spot inoculation of strains in PDA supplemented with 1% Tween-20 (Hasan et al., 2013), a lipid substrate, and incubated at 28°C for 48h. The capacity to hydrolyse pectin was assessed by spot inoculation of Fito_F278 in nutrient agar (NA) (peptone 5 g.L^{-1}; beef extract 3 g.L^{-1}; NaCl 5 g.L^{-1}; Agar 15 g.L^{-1}; pH 6.8) supplemented with 0.2% of pectin, incubation at 28°C for 48h and then flooded with 5 mL of iodine solution for 2 min. Briefly, the proteolytic activity was confirmed according to Hasan et al. (2013). Thus, strains were spot inoculated in Petri dishes with NA supplemented with 1% of gelatin, a protein source, and incubated at 28°C for 48h. The urease screening was detected according to Seeliger (1956), with some modifications. The Christensen's culture media (peptone 1 g.L^{-1}; glucose 1 g.L^{-1}; NaCl g.L^{-1}; KH_2PO_4 2 g.L^{-1}; phenol red 0.012 per 1L; agar 20 g.L^{-1}; pH 6.8) was distributed in 1.5mL microtubes and a drop of 20% urea solution, sterilised by filtration, was added. Strains were inoculated and incubated at 28°C until a period of 5 days. The urea hydrolysis causes a colour change of the media from orange-yellow to pinkish red (Seeliger 1956). Overall, each enzymatic activity was determined in specific culture media and results were expressed by positive activity, when a clear zone around strain colony was observed, or negative activity. Then, the enzymatic index (EI) was calculated by the relationship between the average diameter of the degradation halo (clear zone) and the average diameter of the colony growth. All enzymatic activity tests were performed in triplicate and in each experiment, each strain was inoculated twice. The negative control consisted of a Petri dish containing the specific culture media without strain inoculation.

Furthermore, the strains tolerance to growth under salinity and different pH conditions was also evaluated. For this, each strain was streaked on their specific culture medium adjusted with different salt concentrations (0%, 2%, 4%, 6%, 8%, 10%, 12% and 14% of NaCl) and incubated for 72h at 28°C. For pH analysis, each culture medium was adjusted with 5, 6, 7, 9 and 11 and strains were then incubated for 48h at 28ºC. The specific culture media of each isolate were as follows: Fito_S127B were streaked on MyM (Maltose-Yeast Extract-Malt Extract: maltose 4 g.L^{-1}, yeast extract 4 g.L^{-1}, malt extract 1 g.L^{-1}, agar 18 g.L^{-1}), Fito_F278 on YPD (Yeast Extract-Peptone-Dextrose: Yeast extract 10 g.L^{-1}; Glucose 20 g.L^{-1}; Peptona 20 g.L^{-1}; Agar 20 g.L^{-1}) and Fito_F321 on LB agar (Duchefa Biochemie). The observations of strains to salinity and pH tolerance were recorded as positive (+, growth) or negative (-, no growth) and expressed as CFU/mL after counting cells method. Experiments were performed in triplicate.

Effects of the selected isolates on non-target microorganisms

Each selected isolate (Fito_S127B, Fito_F278 and Fito_F321) was firstly growth in both solid and liquid specific medium. Growth conditions on liquid medium were performed according to the

methodology presented in the next step. After that, each isolate was adjusted at a concentration of 1 x10^6 CFU/mL and 500 µL were then homogenised individually with 16 mL of PDA at ± 50°C in new Petri dishes. After medium solidification, each strain was spot inoculated (with a 3-mm diameter of agar containing the strain) at a 2.5 cm from the border of the plate, incubated at 28°C and followed for 5 days. All tests were performed in triplicate and in each experiment, each strain was inoculated in four spots. Control conditions consisted on the normal growth of each strain in PDA medium. The non-target effects on microorganisms were observed through halo formation around colonies or inhibition of microorganism's growth.

Determination of the growth curves of the selected strains

For the analysis of the growth curve of Fito_S127B, five culture media were initially tested, namely Tryptic Soy Broth (TSB, Sigma), LB (Duchefa Biochemie), MyM broth, 2xYT broth (bacto trytone 16 g.L^{-1}; bacto yeast extract 10 g.L^{-1}; NaCl 5 g.L^{-1}, at pH7) (Sohoni *et al.,* 2012) and glycerol yeast extract (glycerol 4mL.L^{-1}; yeast extract 6 g.L^{-1}; peptone 1 g.L^{-1}) (Khopade *et al.,* 2012). The strain was previously inoculated for 7 days at 28°C in MyM agar medium to allow its sporulation. At the end of this period, the inoculated plates were dipped with a 20% sterilized glycerol solution to allow the releasing of the spores from the cell culture (Sohoni *et al.,* 2012). Subsequently, a pre-culture was carried out in a 500 mL erlenmeyer by adding 2mL of the spore suspension to 40 mL of each culture medium under test. These pre-cultures were incubated at 28°C and 150 rpm and the optical density (OD) analysis at 450 and 600 nm was recorded during the first 24h of incubation. Overall, the medium 2xYT broth at pH7 was the one selected to carried out the growth curve analysis of Fito_S127B strain. For this, after dipped the fresh colonies of Fito_S127B with a 20% sterilized glycerol solution, a first pre-culture was carried out in a 500 mL Erlenmeyer flask containing sterilized glass beads (180 µm, Sigma) by adding 2mL of the spore suspension to 40 mL of 2xYT (pH7). After 36h of incubation at 28°C and 150 rpm, 1 mL of this pre-culture 1 was transferred to another 100 mL Erlenmeyer flask containing 50 mL of 2xYT medium (pH7) and glass beads (pre-culture 2). After 30h of incubation at 28°C and 150 rpm, a pre-culture 3 was established under the same conditions as those established in pre-culture 2. After 18h of incubation, the cell culture itself was established. Thus, 1 mL of pre-culture 3 was transferred to a new Erlenmeyer containing 50 mL of 2xYT medium (pH7) and glass beads and incubated at 28°C and 150 rpm. The growth kinetics were monitored by reading the OD at 450 and 600 nm at 0, 3 ,6 ,9 ,12, 24 ,27 ,30 ,33 and 48 hours' post incubation (hpi) and by plating the cell suspension in MYM medium to estimate the cell concentration (CFU/mL) through plate counting method.

Regarding the Fito_F278 strain, this was initially inoculated at 28°C for 48h in YPD medium. Subsequently, a colony was selected and incubated in approximately 20 mL of YPD medium for 6h at 28°C and at 150 rpm, to check the growth of the yeast. At this time, a pre-culture was prepared through

1 mL of the above prepared yeast suspension in 10 mL of liquid YPD medium and incubated overnight at 28 °C and 150 rpm. After incubation (±15h), the OD was measured and adjusted to a final concentration of 0.1. Then, 5 mL of the above pre-culture was added to 50 mL of liquid YPD medium and the growth kinetics were monitored by reading OD at 450 and 600 nm at 0, 1, 2, 4, 8, 12, 24, 28 and 32 hpi, and by plating the cell suspension in YPD medium to estimate the cell concentration (CFU/mL) through plate counting method.

Given the Fito_F321 strain, the strain was initially growth on LB agar medium for 48h at 28°C. At this time, a pre-culture was established by inoculating a colony into 20 mL of liquid LB medium. After an incubation, overnight at 28°C and 150 rpm, the culture itself was established. Thus, 5 mL of the pre-culture was added to 45 mL of liquid LB medium and the growth kinetics was monitored by reading the OD at 450 and 600 nm at 0, 1, 2, 4, 8, 12, 24, 28 and 32 hpi and by plating the cell suspension in LB agar medium to estimate the cell concentration (CFU/mL) through plate counting method.

For all strains, each growth curve was repeated twice. Furthermore, for the plate counting method, two serial dilutions and two plates per dilution were carried out. The estimated strain growth kinetics was determined based on the correlation between the average of counting cells (CFU/mL) and the average of OD (650 nm).

Molecular identification of microbial isolates

For both bacterial and yeasts isolates, genomic DNA was extracted from fresh cultures grown on PDA medium. Extractions were performed using the Wizard Genomic DNA Purification kit (Promega, Madison, USA), following the standard protocol for bacteria or yeasts. The DNA integrity and quality was checked by 0.8% agarose gel electrophoresis and by using NanoDrop spectrophotometer (Thermo Scientific, USA), respectively.

For bacterial isolates, the 16S rDNA was amplified by using 27F (5'-AGAGTTTGATCACTGGCTCAG-3') and 1492R (5'-TACGGCTTACCTTGTTACGACTT-3') primers, and for yeasts isolates the ITS region was amplified by using the ITS1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') primers (White *et al.,* 1990). Distinct PCR reactions were carried out for each region. Thus, reactions for 16S rDNA identification were carried out in 25 µL reaction mixture containing 1x reaction buffer (USB, Affymetrix), 1.7 mM of $MgCl_2$ (USB, Affymetrix), 0.2mM dNTPs (Bioron), 1U of FideliTaq DNA Polymerase (USB, Affymetrix), 0.2 µM of forward and reverse primers and 2 µL of genomic DNA. The ITS reactions were carried out in 25 µL reaction mixture containing 1x reaction buffer (Biocant own buffer), 2 mM $MgCl_2$, 0.2 mM dNTPs (Bioron), 1U of Taq DNA Polymerase (Biocant own taq DNA polymerase), 0.4 µM of forward and reverse primers and 2 µL of genomic DNA. The reactions conditions for 16S rDNA were: 94°C for 4 min, (94°C for 30s, 50°C for 30s and 72°C for 45s) x 25 cycles followed by a final extension at 72°C for 5 min. For ITS region, cycling conditions consisted: 95°C for

6 min, (94°C for 40s, 53°C for 40s and 72°C for1 min) x 35 cycles followed by an incubation at 72°C for 5 min.

The amplified PCR products were visualized on a 0.8% (w/v) agarose gel stained with ethidium bromide and the nucleic acid concentration and quality was achieved by using the NanoDrop spectrophotometer (Thermo Scientific, USA). PCR products were then purified with Illustra Exostar kit (GE Healthcare Life Sciences) and sequenced using the 3500 Genetic Analyser (Applied Biosystems) at Biocant, Portugal. The obtained sequences were identified using the BLAST search against NCBI and Ribosomal Database Project (RDP).

Statistical analysis

The Principal Component Analysis (PCA) for microbial population distribution analysis was performed by using Primer E software version 6 (Clarke and Gorley, 2006) and XLSTAT software's. The significance of differences between samples in the antagonistic activity, enzymatic activity and physiological traits was analysed by using the SPSS software version 20.0 (SPSS, Inc., Armonk, NY) through one-way analysis of variance (ANOVA), followed by Bonferroni *post hoc* analysis (a powerful analysis when testing small numbers of comparisons), and a confidence limit of 95% was applied. The assumptions of ANOVA were determined through Shapiro-Wilk test ($p>0.05$), for normality test, and Levene's test ($p>0.05$) for homogeneity of variances in the residuals. In cases where the assumptions for a parametric ANOVA were rejected, the non-parametric Kruskal-Wallis test was applied. Standard error of the mean (SEM) were calculated for all mean values. In the growth curves of isolates, the standard error was determined.

Table 1: General distribution of microbial isolates across grapevine samples.

	Soil	Root	Leaf	Berry	Must	Total
Aureobasidium pullulans	0	0	5	2	0	**7**
Bacillus amyloliquefaciens	2	2	8	0	0	**12**
Bacillus cereus	1	0	0	0	1	**2**
Bacillus methylotrophicus	0	0	1	0	0	**1**
Bacillus sp.	3	1	6	1	1	**12**
Bacillus subtilis	3	1	0	0	1	**5**
Burkholderia sp.	1	0	0	0	0	**1**
Cryptococcus magnus	0	0	1	0	0	**1**
Hanseniaspora uvarum	0	0	0	0	1	**1**
Metschnikowia pulcherrima	0	0	0	1	2	**3**
Paenibacillus sp.	1	0	0	0	0	**1**
Pantoea sp.	0	0	0	1	0	**1**
Pseudomonas sp.	1	0	0	0	0	**1**
Saccharomyces cerevisiae	0	0	0	0	1	**1**
Streptomyces chartreusis	0	0	1	0	0	**1**
Streptomyces coelicolor	2	0	0	0	0	**2**
Streptomyces sp.	5	0	0	0	0	**5**
Ustilago cynodontis	0	0	1	0	0	**1**
Total	**19**	**4**	**23**	**5**	**7**	**58**

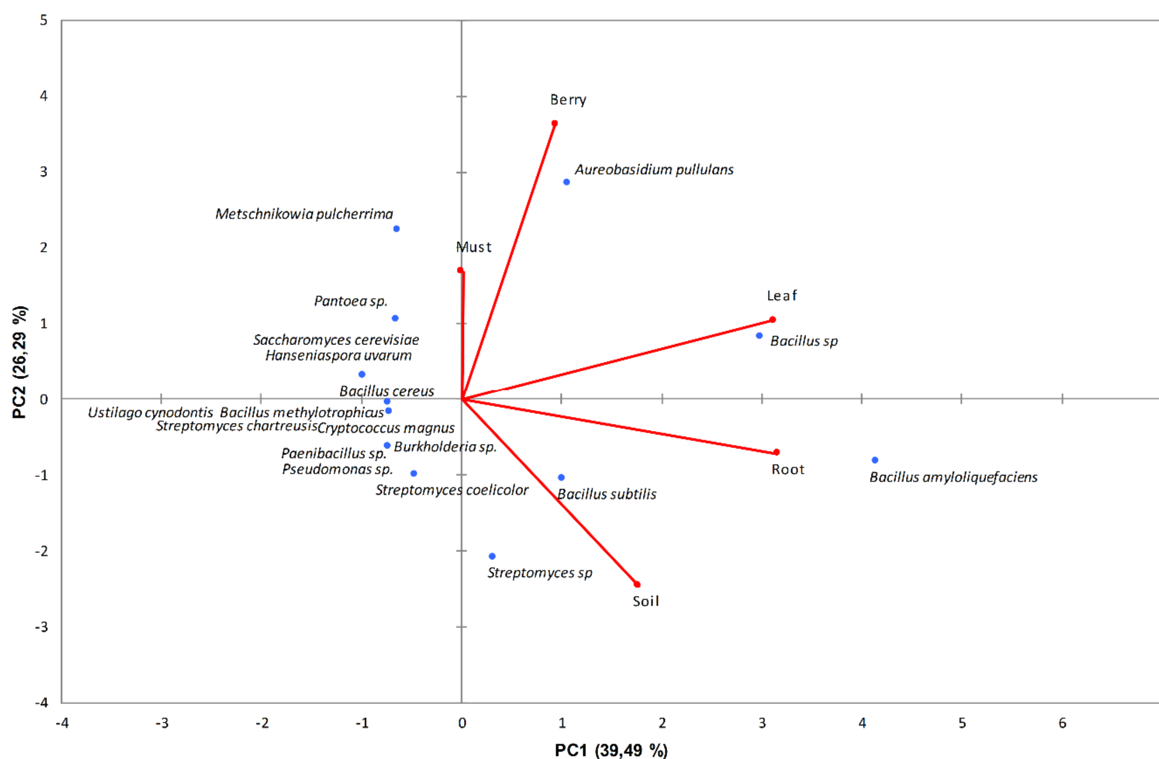

Figure 1: PCA biplot of microbial isolates diversity and their distribution across grapevine structure. In the PCA biplot, based on a Pearson correlation coefficient, the variance explained by each PCA axis is given in the parentheses.

Results

Distribution of isolates

A total of 202 isolates were obtained from different grapevine samples in analysis, namely soil, root, leaf, berry and must. Among them, mostly of microorganisms were isolated from soil (n=82) and leaves (n=62) as a consequence of the initial number of samples collected. At a lesser extent, isolates were obtained from berries (n=26), musts (n=21) and roots (n=10) samples. Therefore, only microorganisms (n=58) that showed a potential antagonistic activity against grapevine pathogens were further identified by molecular means and are presented from now on. Overall, the major abundant isolates were *Bacillus* (n=32), *Streptomyces* (n=8) and *Aureobasidium* (n=7), accounting with 55.2%, 13.8% and 12.1%, respectively. Though others such as *Metschnikowia* (n=3) and *Burkholderia, Cryptococcus, Hanseniaspora, Paenibacillus, Pantoea, Pseudomonas, Saccharomyces* and *Ustilago* (n=1) were also isolated (Table 1).

The distribution of these microorganisms was confined to specific grapevine location showing its adaptability to specific plant niches (Figure 1). Thus, soils were mostly characterized by *Streptomyces* microorganisms, namely *Streptomyces* sp. and *S. coelicolor* and followed by *Bacillus* such as *Bacillus* sp., *B. subtilis* and *B. amyloliquefaciens*. Root isolates were characterized by *B. amyloliquefaciens* though *Bacillus* sp. and *B. subtilis* were also isolated while leaves were characterized mainly by *B. amyloliquefaciens, Bacillus* sp. and *A. pullulans.* Other isolates such as *B. methylotrophicus, Cryptococcus magnus, S. chartreusis* and *Ustilago cynodontis* were also obtained. Berries and must samples were characterized by yeasts such as *A. pullulans* (berries) and *Metschnikowia pulcherrima* (berries and musts). Berries were also characterized by the presence of *Bacillus* sp., *M. pulcherrima* and *Pantoea* sp. while musts samples contained *Hanseniaspora uvarum, Saccharomyces cerevisiae* and the bacteria *B. cereus, B. subtilis* and *Bacillus* sp. (Figure 1).

General antifungal activity

All the 202 isolates were tested for their inhibitory activity against *B. cinerea* strain 630 and *D. seriata* strain Fito_F14, under *in vitro* conditions. After the pre-screening tests, positive isolates were selected and tested against the same fungal pathogens but under dual culture tests. Results showed that 15% (n=31) and 19% (n=39) of isolates inhibited ($p<0.05$) the mycelium growth of *B. cinerea* and *D. seriata,* respectively (Table 2). Overall, mostly of isolates showed a mycelium inhibition value up to 60% and the great majority of the antagonistic microorganisms belonged to the genus *Bacillus.* Indeed,

Table 2: In vitro antagonistic activity of different grapevine isolates against the pathogens Botrytis cinerea strain 630 and Diplodia seriata strain Fito_F14. Results of the inhibition of the pathogen's mycelial growth (%) after 7 days of inoculation are presented by Mean ± SEM values, and isolates with significant antagonistic activity (p<0.05) are presented. [#]corresponds to isolates which antagonistic activity do not differ significantly.

Isolates	Species	Isolation source	Inhibition of the pathogen's mycelial growth (%)	
			***Botrytis cinerea* (strain 630)**	***Diplodia seriata* (Fito_F14)**
Fito_F7	*Bacillus* sp.	Leaf	0	69.01 ± 0.48
Fito_S36	*Streptomyces coelicolor*	Soil	71.65 ± 0.00	22.22 ± 10.27#
Fito_S39	-	Soil	41.73 ± 0.64	0
Fito_S48	*Streptomyces coelicolor*	Soil	16.01 ± 13.07#	65.50 ± 4.16
Fito_S49	*Streptomyces* sp.	Soil	0	68.42 ± 1.65
Fito_M82A	*Bacillus subtilis*	Must	69.29 ± 1.11	76.02 ± 2.08
Fito_U88	*Pantoea* sp.	Berry	42.52 ± 0.64	0
Fito_S93	*Bacillus* sp.	Soil	0	59.06 ± 1.72
Fito_S122	*Bacillus subtilis*	Soil	67.72 ± 1.29	77.19 ± 1.43
Fito_S124	*Bacillus toyonensis*	Soil	0	59.65 ± 1.65
Fito_S126	*Streptomyces* sp.	Soil	0	66.08 ± 0.48
Fito_S127B	*Streptomyces* sp.	Soil	40.16 ± 1.70#	86.55 ± 1.72
Fito_M139	*Bacillus cereus*	Must	0	63.74 ± 0.48
Fito_M141	*Saccharomyces cerevisiae*	Must	40.94 ± 0.00	0
Fito_S151	-	Soil	47.24 ± 2.80	0
Fito_C	*Bacillus cereus*	Laboratory	0	70.76 ± 0.48
Fito_L159	*Bacillus methylotrophicus*	Laboratory	64.57 ± 2.23	0
Fito_L160	*Bacillus methylotrophicus*	Laboratory	68.50 ± 0.64	0
Fito_R203	*Bacillus* sp.	Root	0	76.03 ± 2.90
Fito_F224	*Bacillus methylotrophicus*	Leaf	68.50 ± 1.29	0
Fito_S227	Pseudomonas sp.	Soil	0	69.59 ± 2.90
Fito_S230	*Bacillus amyloliquefaciens*	Soil	66.93 ± 1.93	76.02 ± 0.95
Fito_S234	*Bacillus amyloliquefaciens*	Soil	68.50 ± 0.64	80.70 ± 0.83
Fito_S247	*Bacillus subtilis*	Soil	67.72 ± 0.64	78.36 ± 1.26
Fito_F251	*Bacillus amyloliquefaciens*	Leaf	68.5 ± 0.64	80.70 ± 1.43
Fito_F252	*Bacillus amyloliquefaciens*	Leaf	64.57 ± 2.23	81.87 ± 0.48
Fito_F264	*Bacillus amyloliquefaciens*	Leaf	0	77.78 ± 1.72
Fito_R270	*Bacillus subtilis*	Root	58.27 ± 1.29	66.67 ± 1.43
Fito_R271	*Bacillus amyloliquefaciens*	Root	61.42 ± 0.64	77.19 ± 2.48
Fito_F278	*Aureobasidium pullulans*	Leaf	41.73 ± 0.64	29.82 ± 4.14
Fito_L282	*Pseudomonas chlororaphis*	Laboratory	74.80 ± 0.64	74.26 ± 1.26
Fito_F289	*Bacillus amyloliquefaciens*	Leaf	47.24 ± 2.57	83.63 ± 0.48
Fito_F290	*Bacillus* sp	Leaf	66.14 ± 0.64	81.87 ± 0.95
Fito_F293	*Bacillus* sp.	Leaf	80.31 ± 2.32	78.95 ± 2.19
Fito_R304	*Bacillus amyloliquefaciens*	Root	59.84 ± 0.00	83.62 ± 1.26
Fito_F305	*Streptomyces chartreusis*	Leaf	0	71.93 ± 0.83
Fito_F310	-	Leaf	0	26.90 ± 3.13
Fito_F315	*Bacillus amyloliquefaciens*	Leaf	65.35 ± 0.64	80.12 ± 1.26
Fito_F317	*Bacillus amyloliquefaciens*	Leaf	66.93 ± 1.11	78.95 ± 0.83
Fito_F319	*Bacillus* sp.	Leaf	77.95 ± 4.50	76.61 ± 0.95
Fito_F321	*Bacillus amyloliquefaciens*	Leaf	62.20 ± 3.34	87.72 ± 4.30
Fito_S331	-	Soil	59.84 ± 1.11	0
Fito_S332	*Streptomyces* sp.	Soil	19.42 ± 7.93#	87.72 ± 0.83
Fito_S333	-	Soil	0	29.24 ± 18.14
Fito_S341	*Bacillus subtilis*	Soil	66.14 ± 0.64	81.87 ± 0.48
Fito_F350	*Bacillus* sp.	Leaf	70.08 ± 0.64	76.02 ± 0.48
Fito_Fr356	-	Berry	52.76 ± 1.13	66.08 ± 1.90
Fito_Fr359	*Bacillus* sp.	Berry	0	45.61 ± 0.83

the isolates Fito_F293 and Fito_F321, both from *Bacillus* genera, were the strains with a major inhibitory activity against *B. cinerea* and *D. seriata* with an inhibition of the pathogen's mycelial growth of 80.31 ± 2.32% and 87.72 ± 4.30%, respectively. Although, other microorganisms such as *Streptomyces, Pseudomonas, Aureobasidium* and *Pantoea* also showed an antagonistic potential against these pathogens. The yeast *Saccharomyces cerevisiae* strain Fito_M141, isolated from must samples, showed an antagonistic activity against *B. cinerea,* accounting with a reduction of 40.94% of the pathogen's mycelial growth. Therefore, only representative isolates of each genus and with the highest inhibition against *D. seriata* strain Fito_F14 were selected for further antagonistic tests against *Botryosphaeriaceae* species, and included Fito_F321 (*B. amyloliquefaciens*), Fito_S127B (*Streptomyces* sp.), Fito_S234 (*B. amyloliquefaciens*), Fito_L282 (*P. chlororaphis*) and Fito_F278 (*A. pullulans*) strains.

The biocontrol activity against Botryosphaeria species

The antagonistic capabilities of Fito_S127B (*Streptomyces* sp.), Fito_S234 (*B. amyloliquefaciens*), Fito_F278 (*A. pullulans*), Fito_L282 (*P. chlororaphis*) and Fito_F321 (*B. amyloliquefaciens*) strains were assessed by mycelium inhibition growth of five fungal pathogens responsible for the Botryosphaeria dieback, namely *D. seriata* strains F98.1 (Robert-Siegwald *et al.,* 2017) and Ds99.7 and *N. parvum* strains Np Bt-67, Np Bourgogne and Np SV. Antagonistic tests were carried out through dual culture assays. In general, strains reduced significantly ($\rho<0.05$) the mycelium growth of all pathogenic fungi (Table 3; Figure 2) and distinct inhibition zones were developed (Appendix 3: Figure S1).

Overall, Fito_S127B strain showed the highest means of antagonistic activity against Ds99.7 (27.60 ± 0.85%), Np Bt67 (34.80 ± 3.07%), Np Bourg. (35.99 ± 0.94%) and Np SV (30.06 ± 1.61%). Contrarily, Fito_L282 strain showed lower means of the mycelium inhibition growth against F98.1 (20.75 ± 1.05%), Np Bt67 (9.16 ± 1.10%) and Np Bourg. (20.48 ± 3.91%) and Fito_F278 strain showed a minimum inhibition against Ds99.7 (7.97 ± 0.78%) and Np SV (11.50 ± 3.42%). Therefore, Fito_F278 showed the highest inhibition values against F98.1 strain. Both Fito_S234 and Fito_F321 strains, *B. amyloliquefaciens* species, showed similar means of all pathogen's inhibition. Regarding the pathogen Ds99.7, the antagonistic means obtained were smaller when compared with the other pathogens as this was a more aggressiveness strain.

Considering the biocontrol activity, only the strains Fito_S127B, Fito_F278 and Fito_F321 were selected for a deep characterization.

Table 3: Antagonistic activity of the isolates Fito_S127B, Fito_S234, Fito_F278, Fito_L282 and Fito_F321 against different pathogens responsible for the Botryosphaeria dieback. Results of the inhibition of the pathogen's mycelial growth (%) after 15 days of inoculation are presented by Mean ± SEM values, and isolates with significant antagonistic activity (*p*<0.05) compared with control (pathogen' mycelium free growth) are presented. #corresponds to isolates which antagonistic activity do not differ significantly from control.

Isolate	Specie	Inhibition of pathogen's mycelium growth (%)				
		Diplodia seriata F98.1 strain	*Diplodia seriata* Ds99.7 strain	*Neofusicoccum parvum* Np Bt67 strain	*Neofusicoccum parvum* Np Bourg strain	*Neofusicoccum parvum* Np SV strain
Fito_S127B	*Streptomyces* sp.	29.47 ± 5.98	27.60 ± 0.85	34.80 ± 3.07	35.99 ± 0.94	30.06 ± 1.61
Fito_S234	*Bacillus amyloliquefaciens*	28.48 ± 2.34	17.35 ± 2.13	23.05 ± 0.99	24.64 ± 2.72	23.41 ± 1.66
Fito_F321		26.83 ± 0.40	15.03 ± 1.22	23.47 ± 0.30	26.91 ± 1.66	27.65 ± 1.15
Fito_F278	*Aureobasidium pullulans*	33.50 ± 0.62	7.97 ± 0.78	17.69 ± 2.75	26.53 ± 4.09	11.50 ± 3.42#
Fito_L282	*Pseudomonas chlororaphis*	20.75 ± 1.05#	10.76 ± 1.43	9.16 ± 1.10#	20.48 ± 3.91	16.98 ± 1.13

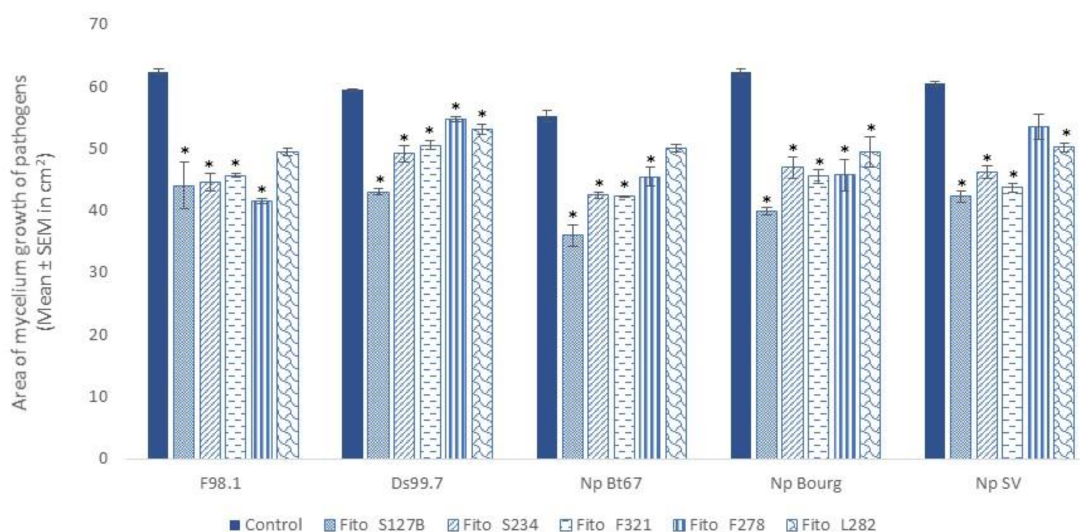

Figure 2: The antagonistic potential of grapevine isolates expressed by the area of the mycelium growth of pathogens (cm^2). The antagonistic potential of Fito_S127B (*Streptomyces* sp.), Fito_S234 (*B. amyloliquefaciens*), Fito_F278 (*A. pullulans*), Fito_L282 (*P. chlororaphis*) and Fito_F321 (*B. amyloliquefaciens*) strains were assessed by mycelium inhibition growth of five fungal pathogens responsible for the Botryosphaeria dieback, namely *Diplodia seriata* strains F98.1 (Robert-Siegwald *et al.,* 2017) and Ds99.7 and *Neofusicoccum parvum* strains Np Bt-67, Np Bourgogne and Np SV. Control corresponded to the free growth of each pathogen. Results corresponded to the area of the mycelium growth of pathogens after 15 days of inoculation and are expressed as Mean ± SEM. *Correspond to strains that reduced significantly the area of the fungal growth (*p*<0.05).

Effect of strains volatiles on the biocontrol of *D. seriata*

Volatile compounds produced by Fito_S127B, Fito_F278 and Fito_F321 strain had a retarding effect on the mycelial growth of *D. seriata* Fito_F14 strain though this was observed only until the third day after inoculation (dpi) (Figure 3). Besides this mycelial growth inhibition of pathogen, after the sixth dpi the pathogen eventually grew, occupying the entire plate.

Overall, the volatiles emitted by Fito_F278, *A. pullulans*, were the ones that significantly inhibited the growth of the pathogen, when compared with the antagonistic activity of the other strains in analysis. Thus, Fito_F278 showed an antagonistic activity against Fito_F14 of 32.30 ± 2.57% at 1 dpi, 34.97 ± 4.61% at 2 dpi and 21.28 ± 5.89% at 3dpi (Appendix 3: Table S1). Curiously, when the pathogen is exposed to the volatiles emitted by Fito_F278, changes are observed in the sporulation of the pathogen, namely a retarding effect (Appendix 3: Figure S2).

Effect of cell deactivation on biocontrol activities

The cell deactivation of Fito_S127B and Fito_F278 strains was carried out to measure the biocontrol capacity of killed strains against *D. seriata* Fito_F14 strain. Both Fito_S127B and Fito_F278 lose their antagonistic activity after cell deactivation, and no significant differences were found on the inhibition of the mycelium growth of pathogen (Figure 4). For the Fito_F321 strain, different protocols have been tested for cell deactivation, namely high temperature, cell disruption by using the bead beating method and incubation in high steamy heat, though none of them proved to be effective and spores from Fito_F321 were still viable (similar to the control condition).

Evaluation of isolates for their physiological and biochemical traits

With regards to siderophores and phosphate solubilisation, only *A. pullulans* Fito_F278 strain and *B. amyloliquefaciens* Fito_F321 strain were able to produce, under *in vitro* conditions (Table 4). According to the enzymatic activity, all strains produced amylase, cellulase, lipase, pectinase and protease though the urease activity was not detected (Table 4; Figure 5). Overall, Fito_S127B showed a major enzymatic index of amylase and lipase while Fito_F278 showed a major enzymatic index of pectinases and proteases and a slightly smaller activity for amylase. Fito_F321 showed a smaller enzymatic index of lipase, pectinase and protease. Given the cellulase, this was the enzyme with a higher enzymatic index for all strains studied.

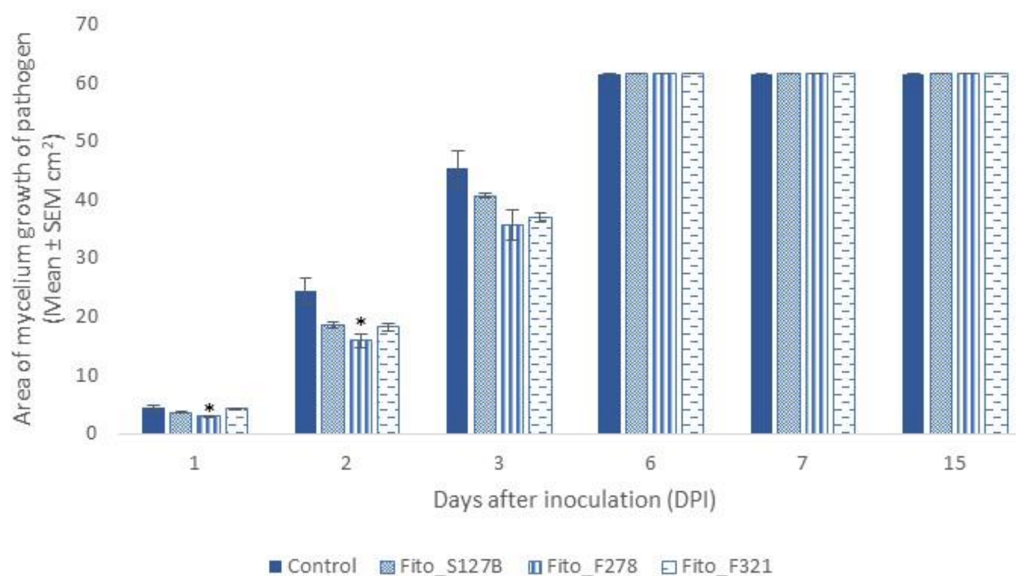

Figure 3: Effect of the strains volatile compounds against D. seriata Fito_F14 strain. The antagonistic potential of the volatile compounds emitted by Fito_S127B (*Streptomyces* sp.), Fito_F278 (*A. pullulans*) and Fito_F321 (*B. amyloliquefaciens*) strains on the mycelium inhibition growth of the pathogen *D. seriata* Fito_F14 strain. Results corresponded to the area of the mycelium growth of pathogen after 15 days of inoculation and are expressed as Mean ± SEM. *Correspond to strains that reduced significantly the area of the fungal growth (p<0.05).

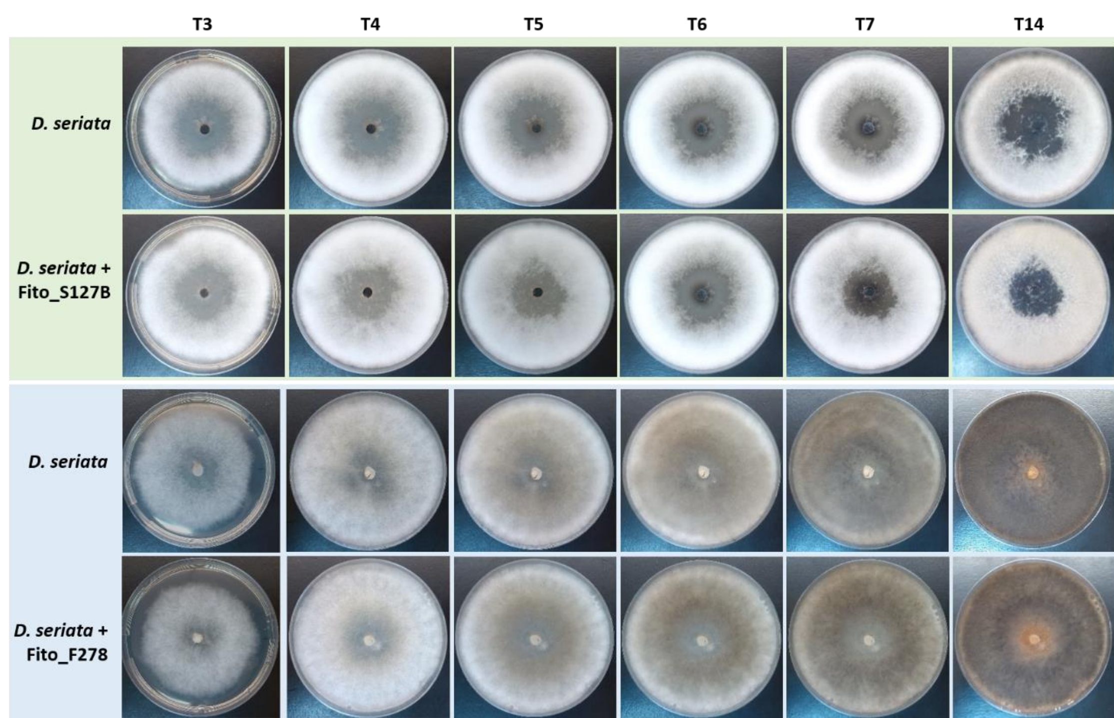

Figure 4: The antagonistic potential of Fito_S127B and Fito_F278 strains after cell deactivation. Analysis of the effect of cell deactivation of Fito_S127B and Fito_F278 strains on the mycelium inhibition growth of *Diplodia seriata* Fito_F14 strain. Herein, each deactivated strain was homogenised with PDA at ± 50°C and after solidification, the pathogen *D. seriata* Fito_F14 was incubated centrally in the Petri plates and the antagonistic potential followed for 15 days.

Six different pH (range from 5 to 11) and eight salinity levels (0 to 14%) were investigated for their influence on strains tolerance. Results indicated that Fito_S127B and Fito_F278 were able to grow from pH 5 to 11 though Fito_F321 was only able to grow at pH 6 to 9 (Table 4). The morphology of Fito_F278 and Fito_F321 colonies were slightly altered with the increasing of pH, which became smaller. Further, no significant differences were found on Fito_F278 and Fito_F321 strains abundance (CFU/mL) under the different pH analysed, contrary to Fito_S127B where significant differences ($p<0.05$) were founded for pH 9 and 11 (Appendix 3: Table S2). Given the salinity levels, Fito_S127B was able to grow only up to 2%, indicating that this strain had the lowest salinity tolerance. On the other hand, Fito_F278 and Fito_F321 were able to grow up to 8% and 6%, respectively (Table 4). Interestingly, the morphology of colonies from all strains was altered with NaCl, becoming smaller by increasing the NaCl concentration in the culture medium. Overall, statistical analysis showed that salinity levels impact significantly ($p<0.05$) the strains abundance (CFU/mL) (Appendix 3: Table S2).

Non-target effects of the potential BCAs

The non-target effects of the potential selected BCAs (Fito_S127B, Fito_F278 and Fito_F321) were tested against the same microorganisms to evaluate their interaction and their possible competition for the same niche. Overall, when Fito_S127B is homogenised with PDA (higher microbial abundance), does not have an impact on the growth of the non-target microorganisms, namely Fito_F278 and Fito_F321 (Figure 6). The same behaviour is observed when Fito_F321 is homogenised with PDA. Though, the colonies' size of the non-target microorganisms, both Fito_S127B and Fito_F278, are smaller when compared with the control (Figure 6). Furthermore, the co-inoculation of Fito_F321 with Fito_S127B results in the appearance of clear zones in which the development of Fito_F321 does not occur (antagonistic effect). Conversely, when Fito_F278 is homogenised in PDA (higher microbial abundance), there is no development of both Fito_S127B and Fito_F321 (antagonistic effect) (Figure 6). Clear zones are also observed resulting from the co-inoculation of Fito_F278 and Fito_F321.

Optimization process of the growth curve of the Fito_S127B strain

To address the growth curve of Fito_S127B strain, five culture media were initially tested, namely TSB, LB, MyM broth, 2xYT broth (pH7) and glycerol yeast extract. After a first assay containing 2mL of a spore suspension in each culture medium, results showed that those culture media without any sterilise glass beads, namely TSB and LB, the strain formed pellets that prevent it from measuring the OD (Figure 7). On the other hand, culture media containing glass beads resulted in smaller pellets of Fito_S127B (Figure 7), and both 2xYT and glycerol yeast extract were the most effective culture media.

Table 4: Production of siderophores, phosphate solubilisation, extracellular enzymes (amylase, cellulase, lipase, pectinase, protease and urease) and effect of pH and salinity on the growth of Fito_S127B, Fito_F278 and Fito_F321 strains. The "+" indicates activity (halo observation for siderophores and phosphate solubilisation or growth under pH and salinity conditions) and "-"indicates no activity; The enzymatic activity is expressed according to the degradation halo formation size: (+) halo ≤0.4 cm; (++) halo 1.0 cm; (+++) halo 1.0-2.0 cm and (++++) halo > 2.0 cm.

Conditions	**Strains**		
	Fito_S127B	Fito_F278	Fito_F321
Biochemical characterization			
Siderophore	-	+	+
Phosphate solubilisation	-	+	+
Enzymatic activity			
Amylase	++	++++	+++
Cellulase	++++	++++	++++
Lipase	++	++	+++
Pectinase	+++	++++	++++
Protease	++	+	+++
Urease	-	-	-
pH			
Standard (6.5)	+	+	+
5	+	+	-
6	+	+	+
7	+	+	+
9	+	+	+
11	+	+	-
Salinity levels (% NaCl)			
Standard (0%)	+	+	+
2%	+	+	+
4%	-	+	+
6%	-	+	+
8%	-	+	-
10%	-	-	-
12%	-	-	-
14%	-	-	-

In order to minimize these pellets, an optimization of Fito_S127B growth kinetics was carried out by applying different pre-cultures in 2xYT and glycerol yeast extract culture media. Results demonstrated that, the incorporation of glass beads in culture media together with three pre-cultures before the culture itself was relevant to decrease considerably the pellets of the strain (Figure 8). Furthermore, the 2xYT medium showed more effective results (Figure 8) and was therefore selected to determine the growth curve of Fito_S127B.

The growth curve determination of the selected strains

In figure 9 are presented the growth curves of Fito_S127B, Fito_F278 and Fito_F321. The growth curves of each strain were carried out on specific culture media and determined together by reading the optical density (OD) and the use of plate count method. With these both methods were possible to predict the average of cell density of each strain, according to the OD. Thus, for Fito_S127B it was estimated that one OD corresponds to an average of cell density of 2.37×10^6 CFU/mL, for Fito_F278 corresponds to 4.77×10^5 CFU/mL and for Fito_F321 corresponds to 1.02×10^7 CFU/mL. Furthermore, with the growth curve of each strain was also possible to determine a formula to estimate at a given OD the number of hours' post-inoculation (hpi) required to obtain a desirable strain concentration.

Overall, each strain showed a specific growth curve. Notably, the exponential phase of growth was more rapidly attained by Fito_F321 (after 4 hpi), followed by Fito_S127B (6 hpi) and Fito_F278 (12 hpi) (Figure 9). In general, the exponential phase ranged between 8h (Fito_F321) to 17h (Fito_S127B) and thereafter strains entered a stationary phase followed by a death phase.

Molecular identification of grapevine isolates

The sequencing of 16S rRNA gene and ITS region allowed to identify the grapevine isolates that showed antagonistic activities against grapevine pathogens including *D. seriata* Fito_F14 and *B. cinerea* strain 630 (Table 1; Table 2). Given the selected isolates, Fito_S127B identified as *Streptomyces* sp., exhibited 99% of sequence identity with *Streptomyces* sp. R97-2 strain (KC329482.1); Fito_F278, an *Aureobasidium pullulans*, exhibited 98% identity with *A. pullulans* Y11 strain (KC897669.1) and Fito_F321, a *Bacillus amyloliquefaciens*, showed 100% sequence identity with *Bacillus amyloliquefaciens* B15 (CP014783.1) strain and *Bacillus velezensis* GH1-13 strain (CP019040.1).

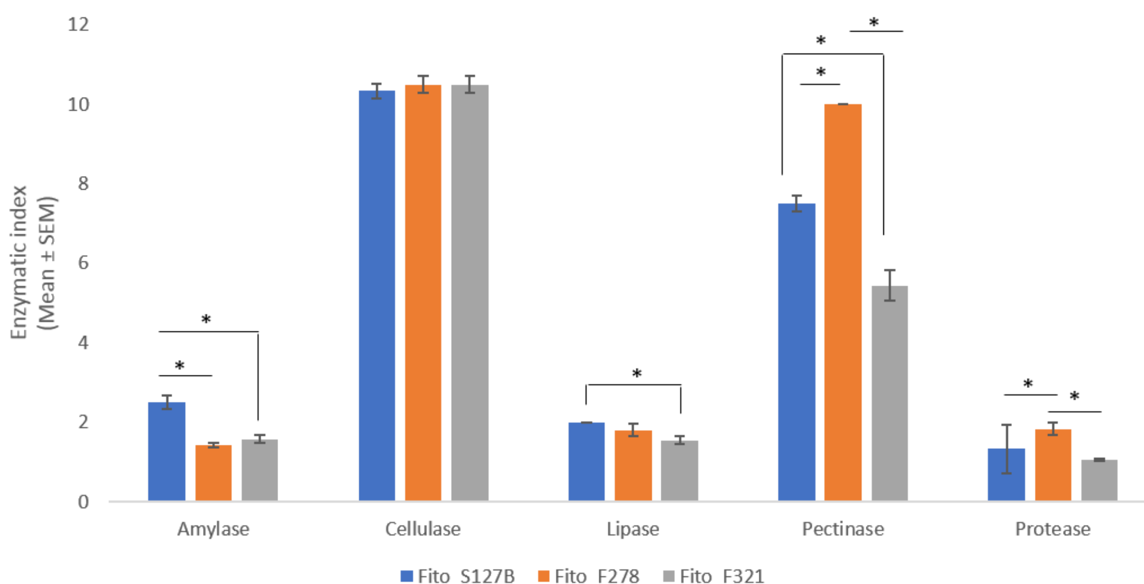

Figure 5: Comparative analysis of extracellular enzyme activity (amylase, cellulase, lipase, pectinase and protease) in Fito_S127B, Fito_F278 and Fito_F321 strains. The production of extracellular enzymes was observed by a clearing zone developed around the colony. The enzymatic index was calculated through the ratio between the diameter of the clarification zone and the diameter of colony (Dh/Dc) (Garcia *et al.,* 2007). Results are expressed as Mean ± SEM. *Correspond to significant differences among the activity of different strains p<0.05.

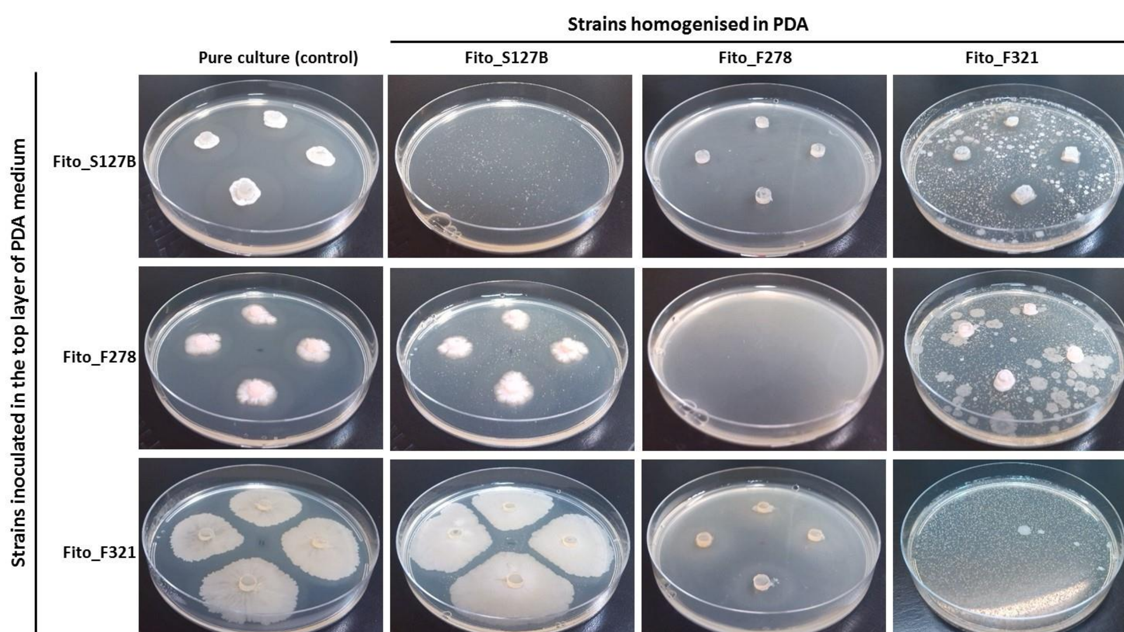

Figure 6: Screening of the non-target effects between the Fito_S127B, Fito_F278 and Fito_F321 strains. Herein, each strain was individually homogenised with PDA medium (500 µL at 10^6 CFU/mL) and then, after solidification of the medium, each strain was inoculated at the top layer of PDA. The non-target effects were observed after five days of inoculation through the growth or inhibition growth of the inoculated strains.

Discussion

Grapevine is naturally colonised by microorganisms with a biocontrol potential

In this study, a set of microbial isolates obtained from a vineyard located in Cantanhede, Portugal were investigated for their antagonistic activities against important grapevine diseases such as those responsible for the grey mould and Botryosphaeriaceae dieback.

The microbial isolates were obtained from different structures of grapevine such as soil, root, stem, leaves, berries and musts and their distribution supported that both below- and aboveground parts of grapevine where dominated by specific microorganisms (Table 1; Figure 1). The observed microbial distribution confirmed the major adaptability and preference of these microorganisms for such specific plant niches (Martins *et al.,* 2013; Zarraonaindia *et al.,* 2015). Indeed, the microbial composition and abundance are generally shaped by the physico-chemical conditions, by the plant itself and by agricultural practices (Marschner *et al.,* 2004; Philippot *et al.,* 2013). Thus, some factors such as pH, plant fertilisation, soil type, nutrient conditions, plant age and genotype are important drivers of these microbial communities (Heijden *et al.,* 2008).

Of the isolated microorganisms, samples were dominated by *Bacillus* (55.2%), *Streptomyces* (13.8%) and *Aureobasidium* (12.1%). Overall, belowground samples, namely soil and roots, were dominated by *Streptomyces* sp. and *Bacillus* sp., while the aboveground samples were dominated by *Bacillus* sp. and *Aureobasidium pullulans*. Given the *Bacillus,* this genus showed to be well distributed across all grapevine samples which is in line with previous studies (West *et al.,* 2010; Compant *et al.,* 2011; Martins *et al.,* 2013). Indeed, the distribution of this genus is not restricted to soil or plant roots and can be found all over the plant (Boriss, 2011). Furthermore, as they have the capacity to produce biofilms this is a competitive strategy that allow their efficient plant colonization (Boriss, 2011). The *Streptomyces* sp. is a soil bacterium, belonging to the class actinobacteria, which have important roles on nutrient recycling, such as carbon and nitrogen sources, and the ability to produce bioactive secondary metabolites as antibiotics or extracellular enzymes (Doumbou *et al.,* 2001; Inbar *et al.,* 2005; Sousa *et al.,* 2008; Procópio *et al.,* 2012; Seipke *et al.,* 2012). Regarding *Aureobasidium,* this is an ubiquitous black yeast that colonises different plants (Deshpande *et al.,* 1992). In vineyards, *Aureobasidium* is part of the dominant microbial consortium which is being typically associated with the plant phyllosphere, grapes and musts though could also be present in soils, wood or pruning wounds (Sabate *et al.,* 2002; Grube *et al.,* 2011; Barata *et al.,* 2012; Setati *et al.,* 2012; Pinto *et al.,* 2014; Fischer *et al.,* 2016).

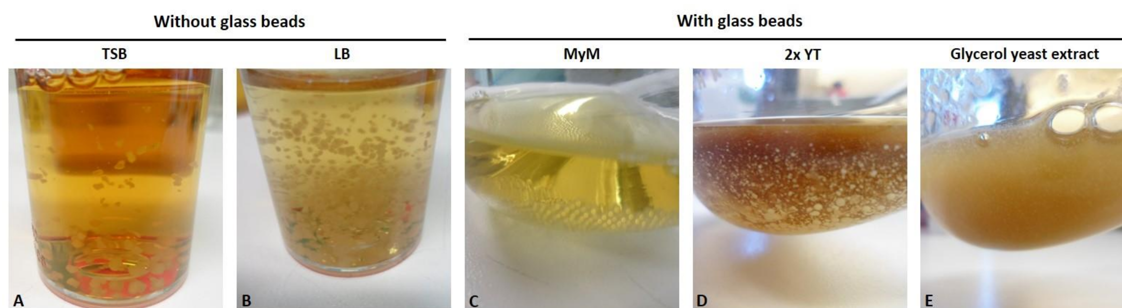

Figure 7: Growth of Fito_S127B strain under (A) TSB, (B) LB, (C) MyM, (D) 2xYT and (E) glycerol yeast extract culture media. Herein, the growth of Fito_S127B strain was tested and compared under culture media that did not contained sterile glass beads, such as TSB and LB, and which contained glass beads, namely MyM, 2xYT (pH 7) and glycerol yeast extract.

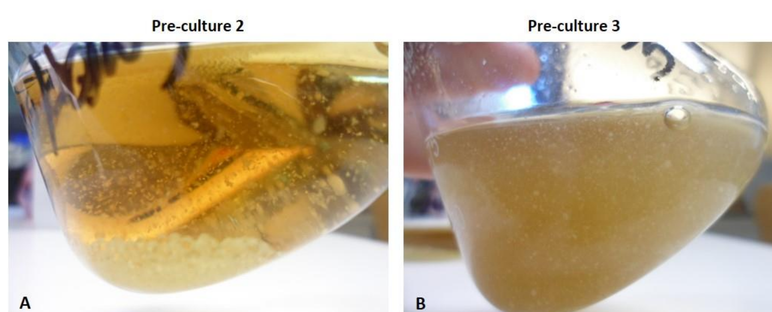

Figure 8: Comparison of Fito_S127B strain growth over different pre-cultures. The growth of Fito_S127B strain was tested within different pre-cultures overtime and results showed that pellets are reduced from pre-culture 2 (A) to pre-culture 3 (B). Herein, the microbial growth of Fito_S127B occurred in the 2xYT (pH7) medium.

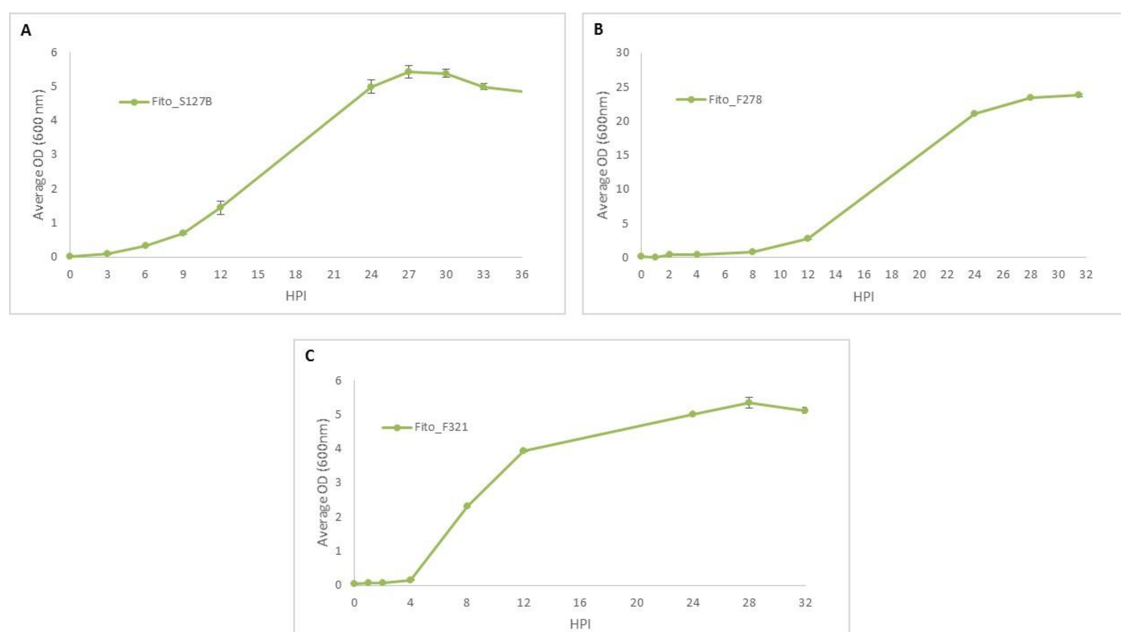

Figure 9: Growth curve obtained for the (A) Fito_S127B, (B) Fito_F278 and (C) Fito_F321 strains. The growth curves of each strain were carried out in specific culture medium namely, Fito_S127B was growth on MyM, Fito_F278 on YPD and Fito_F321 on LB. The optical density (OD) was measured at a wave length of 600 nm across different hours' post-inoculation (hpi). The error bars represent the standard error.

Herein, of the 202 isolates, 31 and 39 showed a significant inhibition ($p<0.05$) on the growth of the pathogens *B. cinerea* strain 630 and *D. seriata* Fito_F14 strain*,* respectively (Table 2). Among them, the great majority of antagonistic microorganisms belonged to the genus *Bacillus* though others such as *Streptomyces, Pseudomonas, Pantoea* or the yeasts *Aureobasidium* and *Saccharomyces* were recorded. In literature, the biocontrol potential of microorganisms from these genera has been documented. Different biopesticides are available for commercialization and included some microorganisms such as *B. amyloliquefaciens, B. firmus, B. pumilus, B. subtilis, B. thuringiensis, Pseudomonas, Streptomyces, Gliocladium, Phytium, Trichoderma* sp., *A. pullulans* or *S. cerevisiae* (EU pesticides database, 2017). Indeed, several *Bacillus* species are recognized for their broad biocontrol range against several plant pathogens (EPA, 2006; Boriss, 2011) such as *Pseudocercospora musae*, responsible for the banana leaf spot, *Colletotrichum musae,* a post-harvest anthracnose agent (Fu *et al.,* 2010), *Eutypa lata,* the causal agent of dieback in grapevines (Ferreira *et al.,* 1991), *B. cinerea,* a grey mould disease of grapevine (Paul *et al.,* 1998), *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, important bacterial pathogens of rice (Wu *et al.,* 2015), among others. The success of this genus seems to be associated with its genetic and metabolic diversity, with the production of antimicrobial compounds and enzymes, and the capacity to form resistant endospores which allow them to colonise different environments and being a versatile microorganism within their mode of action (Baruzzi *et al.,* 2011; Wu *et al.,* 2015). Regarding *Streptomyces,* a microorganism from the soil ecosystem and well adapted to this highly competitive environment, is considered a plant-growth promoter and with the ability to suppress soil-borne pathogens (Seipke *et al.,* 2012). As *Bacillus* species, *Streptomyces* may produce chitinases to degrade chitin, a structural component of fungi, for energy source and as a biocontrol mechanism (Hoster *et al.,* 2005). Promising *Streptomyces* microorganisms have been reported as potential biocontrol agents (Doumbou *et al.,* 2001; Loqman *et al.,* 2009; Couillerot *et al.,* 2014). Among grapevine, the potential antagonist of *Streptomyces* has been studied in the control of *B. cinerea* (Ilic *et al.,* 2007; Williamson *et al.,* 2007; Loqman *et al.,* 2009). Interestingly, in our study a negative correlation of the antagonistic effect of different *Streptomyces* isolates were found: when the inhibition of *B. cinerea* growth occurs, the inhibition of *D. seriata* is reminiscent or even null, and vice-versa. In 1993, it was estimated that approximately 60% of bioactives metabolites of *Streptomyces* origin were applied in agriculture, in the form of insecticides and herbicides (Tanaka and Ōmura, 1993). Going forward, in this study, the *Pseudomonas chlororaphis* Fito_L278 strain showed to be highly effective against both *B. cinerea* and *D. seriata*. *Pseudomonas* is recognized as a root colonizer, a plant growth promoter microorganism and with a biocontrol activity. An example of an efficient biocontrol agent is the *Pseudomonas* sp. DSMZ 13134 strain, a biopesticide commercially available which is applied for potatoes, vegetables, grass and lupin (Buddrus-Schiemann *et al.,* 2010). Given the *Pantoea* sp. Fito_U88 strain, isolated from berries, only showed a certain

potential antagonist against *B. cinerea.* Although this strain did not show any antagonism against *D. seriata,* a study of Haidar and collaborators (2016) showed that strains of *Pantoea agglomerans,* isolated from grape berries, were able to reduce the necrotic lesions of the wood caused by *N. parvum*. In addition to isolates of *P. agglomerans* obtained from grape berries, this species was also isolated from tissue of trunk and cordon of both Esca symptomatic and non-symptomatic grapevine plants (Bruez *et al.,* 2015). Regarding the *Aureobasidium pullulans* Fito_F278 strain, in our study this isolate showed a higher antagonistic effect against *B. cinerea* (41.73 ± 0.64%) than to *D. seriata* (29.82 ± 4.14%). In general, the biocontrol potential of *A. pullulans* is associated with post-harvest diseases of apple fruits, strawberries, kiwifruit or table grapes, caused mostly by *B. cinerea* (Ippolito *et al.,* 2000; Castoria *et al.,* 2001; Schena *et al*., 2002). To best of our knowledge, there is only one study available that applied *A. pullulans* to control GTDs, namely to the infection of grapevine wounds, caused by *Eutypa lata* (Munkvold and Marois, 1993). From the oenology point of view, this is an irrelevant yeast though its biocontrol properties, biotechnological potential, combined with its ubiquitous presence in nature may represent a strategy to the control of grapevine diseases, namely GTDs. Regarding *S. cerevisiae*, this strain only showed a biocontrol effect towards *B. cinerea,* which is in line with previously studies (Raspor *et al.,* 2010). Indeed, this is of utmost interest, as *S. cerevisiae,* while a microflora associated with berries, may represent a natural barrier on grapes against grey mould infection, through a competition for space and nutrients. Moreover, the production of cell wall-degrading enzymes, production of antifungal compounds such as killer toxins, induction of host resistance and mycoparasitism may be the mechanisms normally associated with its biocontrol properties (reviewed in El-Tarabily and Sivasithamparam, 2006).

Overall, it is important to keep in mind that the antagonist response under *in vitro* conditions may vary depending on the culture medium used and the growth conditions applied.

Grapevine is an important source of microorganisms with control potential of GTDs

According to the antagonistic results obtained, five isolates (*Streptomyces* sp. Fito_S127B strain, *B. amyloliquefaciens* Fito_S234 and Fito_F321 strains, *A. pullulans* Fito_F278 strain and *P. chlororaphis* Fito_L282 strain), were then selected to check their biocontrol potential against Botryosphaeria dieback agents, namely *D. seriata* (F98.1 and Ds99.7 strains) and *N. parvum* (Np BT-67, Np Bourgogne and Np SV strains). *Botryosphaeriaceae* dieback is an important GTD, causing trunk cankers and decline of grapevine (Fontaine *et al.,* 2016a). Previously studies showed that *D. seriata* was consistently and highly isolated in plants with both typical esca symptoms and decline symptoms, at Spanish vineyards (Armengol *et al.,* 2001). These observations were similar in France and in Italy (Larignon and Dubos, 1997; Mugnai *et al.*, 1999).

In our study, all the selected strains reduced effectively the mycelium growth of pathogens (Table 3; Figure 2). Among them, results allowed us to detect *Streptomyces* sp. Fito_S127B strain as the most efficient isolate, which significantly (p<0.05) reduced the mycelial growth of all pathogens. Overall, *Streptomyces* have been implicated in the biocontrol of several phytopathogens (Seipke *et al.,* 2012; Inbar *et al.,* 2005; Loqman *et al.,* 2009; Evangelista-Martínez, 2014) though, and to the best of our knowledge, this is the first study that showed the antagonistic effect of *Streptomyces* isolates against Botryosphaeria dieback agents. A similar study also reported the effective biocontrol effect of *Streptomyces* sp. strains towards *Eutypa lata,* on both agar media and grape wood (Schmidt *et al.,* 2001). The antagonistic results suggested that the mechanisms involved in the suppression of the pathogens growth may be antibiosis, which is in line with other studies (Loqman *et al.,* 2009). It is referred that some mechanisms of *Streptomyces* involved in biocontrol are associated with the secondary metabolites production and competition with pathogens for nutrients and space (Inbar *et al.,* 2005; Tarkka *et al.,* 2008). To address the importance of bioactive compounds produced by *Streptomyces* on biocontrol activities, a study of Couillerot and collaborators (2014) showed that the main bioactive metabolites produced by *S. anulatus* S37, namely streptochlorin, nigericin and piericidin were able to reduce the impact growth of *B. cinerea* in dual confrontation tests and on *V. vinifera* L. plantlets. As referred above, *Streptomyces* sp. are soil microorganisms, that play an important role on nutrients recycling and are regulators of plant productivity and plant growth (Doumbou *et al.,* 2001; Inbar *et al.,* 2005; Tarkka *et al.,* 2008; Sousa *et al.,* 2008; Procópio *et al.,* 2012; Seipke *et al.,* 2012). Considering the antagonistic potential of Fito_S127B against some GTDagents, the confirmation of the protective effect on plant through soil inoculations must be achieved to better understand how this microorganism interacts with grapevine and how it can develop a remote defence mechanism against these pathogens. Given the *B. amyloliquefaciens* Fito_S234 and Fito_F321 strains also decreased significantly the growth of pathogens (p<0.05). As previously referred, *Bacillus* species have been already described as biocontrol agents of GTDs, namely against *Eutypa lata* (Ferreira *et al.,* 1991; Schmidt *et al.,* 2001) and have been used in pruning wounds protection against *N. austral, N. parvum, D. seriata, L. theobromae, E. lata, P. chlamydospora* or *P. viticola* (Kotze *et al.,* 2011). A survey in 146 European nurseries showed that *Bacillus* sp. was applied in three nurseries during the grapevine propagation processes (Gramaje and Di Marco, 2015). The *A. pullulans* Fito_F278 strain significantly reduced the mycelium growth of *Botryosphaeria* dieback agents and the highest levels of antagonistic activity were observed against *D. seriata* strain F98.1 (33.51±0.62%) and *N. parvum* strain Np Bourgogne (26.53±4.09%). Contrarily, *D. seriata* strain Ds99.7, which is the high aggressiveness strain, was the less susceptible to the mycelium inhibition (7.80±0.78%). The antagonistic effect of *A. pullulans* was already reported (Bertsch *et al.,* 2012) though, and to best of our knowledge, there is only one study available that applied *A. pullulans* to control GTD namely, the infection of grapevine wounds

against *Eutypa lata* (Munkvold and Marois, 1993). In this study, two field experiments were performed in California region: the first in 1990 at Thompson Seedless vineyard and the second in 1991 in a cv. Chenin Blanc vineyard. A set of natural occurring microorganisms was applied to test their efficacy as biocontrol agents and, *A. pullulans* significantly reduces infection, with a reduction superior to 50% compared to control treatment, only in the first field. Lastly, *P. chlororaphis* Fito_L282 strain, together with Fito_F278 strain, showed the lowest values of antagonism against all pathogens which ranged from 9.16 ± 1.10% (Np Bt67) to 20.75 ± 1.05% (F98.1). Previously biocontrol effect of *Pseudomonas* strains against *E. lata* were effective reported on agar media and ineffective on wood*,* under *in vitro* conditions (Schmidt *et al.,* 2001).

So far, microorganisms such as *Bacillus subtilis*, *Fusarium lateritium, Erwinia herbicola, Cladosporium herbarum, Trichoderma atroviride* (Esquive®, a product commercially available in France), *Pythium oligandrum, A. pullulans* and *Rhodotorula rubra* or natural molecules (chitosan, cysteine) are some of the products that have been tested against GTDs, alone or in combination with fungicides (Bertsch *et al.,* 2012). Though some were tested only either *in vitro* or in nurseries (Bertsch *et al.,*2012). Thus, a great deal of interest emerges in those five tested isolates from this study to further develop new strategies to effective control the Botryosphaeria dieback. Based on our results, only the strains *Streptomyces* sp. Fito_S127B, *A. pullulans* Fito_F278 and *B. amyloliquefaciens* Fito_F321 were further characterized.

Isolates showed an efficient antagonistic effect only in a direct confrontation

In the present study, Fito_S127B, Fito_F278 and Fito_F321 showed an effective reduction of the mycelial growth of *D. seriata* Fito_F14 pathogen under dual culture tests (direct confrontation). Though, this protective effect was only observed when the biocontrol strains were alive (Table 2). Indeed, and after a cell deactivation, these microorganisms completely lose their biocontrol potential (Figure 4), and the pathogen normally grew. Given the Fito_F321, different protocols were tested for cell deactivation (such as high temperature, cell disruption by using the bead beating method or incubation on high steamy heat), though the strain showed to be highly resistant to all of them. Overall, this is utmost interest as allow us to analyse the resistance of our strains to further extreme conditions (such as temperature) and to address possible formulations that will improve their shelf-life.

Furthermore, our strains were able to produce volatile compounds (not identified in this study) with an antagonistic effect towards the pathogen growth. Though this antagonistic activity was observed until the third dpi (Figure 3) and, after then, these volatiles were not efficient to limit the growth of pathogens. A study of Tyc and collaborators (2015) suggested that the loss of production of volatile compounds during a microbial interaction, is influenced by the interspecific interactions between microorganisms. Overall, the volatiles emitted by Fito_F278 were those that more

significantly inhibited ($p<0.05$) the pathogen and showed to have a retarding effect on its sporulation. Given the study of Francesco *et al.* (2015), the volatiles compounds produced by *A. pullulans* L1 and L8 strains were effective against five pathogens namely, *B. cinerea, Colletotrichum acutatum, P. expansum, P. digitatum* and *P. italicum*. The volatile compounds identified were 2-phenyl, 1-butanol-3-methyl, 1-butanol-2-methyl and 1-propanol-2-methyl and were mainly produced in the first 96h of growth. Among them, the 1-propanol-2-methyl was the volatile least active while 2-phenyl was the most active (Francesco *et al.,* 2015). Another study identified a total of 45 volatile compounds produced by an *A. pullulans* strain isolated from the grape-associated microorganisms. These compounds were detected after 48 hpi and were mostly alcohols and aldehydes, normally associated with the aroma profile of red wines (Verginer *et al.,* 2010). Given the *Bacillus* and *Streptomyces* species, they are well known to produce volatile compounds against phytopathogens (Yuan *et al.,* 2012; Mallaiah and Muthamilan, 2015; Wang *et al.,* 2013; Cordovez *et al.,* 2015). Across GTDs, the inhibitory effect against *P. chlamydospora, P. aleophilum, E. lata, P. viticola, L. theobromae, D. seriata, N. australis* and *N. parvum,* was compared by means of volatile and non-volatile compounds produced by *Trichoderma atroviride.* Results showed that the volatiles had a higher inhibition on pathogens (inhibition ranging from 23.6% for *L. theobromae* to 72.4% for *P. viticola*) when compared with non-volatile compounds (inhibition ranging from 7.5% for *N. parvum* to 20.6% for *L. theobromae*) (Kotze, 2008). Other study also showed the capacity of *Trichoderma* strains to produce volatile and non-volatile compounds against *E. lata* (John *et al.,* 2004).

Indeed, the volatile compounds are gaseous secondary metabolites, which allow the microbial communication and antagonistic interactions (Tyc *et al.,* 2015). Although, the volatiles emitted by microorganisms strongly depends on the culture media and growth conditions (Schulz *et al.,* 2004; Verginer *et al.,* 2010). Soil bacteria are recognized for their volatiles compounds, which can also stimulate plant growth (such as 2,3-butanediol or acetoin), elicit induced systemic resistance (ISR) or induced systemic tolerance (IST) in plants (Ryu *et al.,* 2003; Ryu *et al.,* 2004) or being involved in biofilm formation, drug resistance or virulence (indole and derivatives such as quinolones and (S)-3-hydroxytridecan-4-one) (Lee and Lee, 2010). Though, the production of these compounds can be influenced by interactions with other microorganisms (Tyc *et al.,* 2014). Despite these compounds, several studies focus on the non-volatiles compounds (Foster and Bell, 2012).

Overall, the volatile compounds emitted by our strains could play an important role in the antagonistic activity against GTDs. However, it will be firstly necessary to identify these volatiles and to test different growth substrates to improve the efficacy of these compounds on biocontrol activities.

The biochemical and physiological traits of strains are intrinsically associated with their biocontrol activities

To address the biochemical and physiological traits of Fito_S127B, Fito_F278 and Fito_F321, different *in vitro* analysis was performed in specific medium to determine the capacity of strains to solubilise phosphate, to produce siderophores and extracellular enzymes (such as amylase, cellulase, lipase, pectinase, protease and urease) and their tolerance to pH conditions and salinity levels. With exception of Fito_S127B, the other strains were able to solubilise phosphate and to produce siderophores. Although Fito_S127B did not produce siderophores under *in vitro* conditions, the genome analysis (presented in the next section of Chapter 3) allowed to predict 4 siderophores gene clusters with an 83% similarity with desferrioxamine B gene cluster. Indeed, a study of Gopalakrishnan *et al.* (2014) also confirmed the siderophores production by several actinomycetes strains, under *in vitro* conditions.

Furthermore, our results showed that all strains produced a high range of important extracellular enzymes, which are important to hydrolyse the pathogen cell wall. Though, the urease was not detected. The enzymatic activity is important for the biocontrol potential of these microorganisms. Indeed, it is referred that mechanism such as phosphate solubilisation, nitrogen fixation, production of degrading enzymes (such as amylases, proteases or hemicellulases), phytohormones (auxin, cytokinin, gibberellins and polyamines) and volatile compounds may act as growth stimulants (ethylene and 2,3- butanediol) and are normally associated with PGP microorganisms (Boriss *et al.,* 2011). The siderophores production is also an indirect mechanism implicated on the plant growth promotion (Mendes *et al.,* 2013). This is of utmost interest and additional assays regarding the phytohormones analysis and identification of volatiles of our strains will be further necessary to confirm or not their PGP potential. Overall, *Streptomyces* as PGP was already being reported across different crops such as tomato (El-Tarabily, 2008) or wheat (Sadeghi *et al.,* 2012). It is well recognized the biochemical importance of *Streptomyces* and their thermophilic activity (Kim *et al.,* 1998). Furthermore, both *Streptomyces* and *Bacillus* strains were observed in grapevine roots and were associated as PGP (Karagöz *et al.,*2012; Marasco *et al.,* 2013). Given the *A. pullulans,* its PGP traits, namely through the ability to produce IAA, and its antifungal activity on dark chestnut soil were also achieved (Ignatova *et al.,* 2015).

Regarding the pH, all strains were able to growth at different pH, except for Fito_F321 that did not grow at pH 5 and pH 11. Both Fito_F278 and Fito_F321 showed to be salt tolerant though Fito_S127B was more sensible. Contrarily to Fito_S127B, the high salinity tolerance of *Streptomyces* strains was demonstrated in previous studies (Sadeghi *et al.,* 2012; Gopalakrishnan *et al.,* 2014). Thus, a study of Sadeghi *et al.* (2012) demonstrated that the growth rate of *Streptomyces* strains only decreased significantly after an exposure up to 300 mM NaCl. Overall, the morphological forms of

Fito_S127B colonies were affected by means of salinity conditions which agrees with other morphological studies (reviewed in Desphande et al., 1992; Gaur et al., 2010).

The identification of both biochemical and physiological traits allowed to accomplish that these three strains have a broad portfolio of enzymatic activity and have the capacity to develop and survive under harsh environmental conditions. Thus, and together with their biocontrol potential, this is a great deal of interest to integrate these strains in a further disease management program.

***A. pullulans* Fito_F278 strain showed an unexpected effect against non-target microorganisms**

So far, many studies analyse the biocontrol effect of strains against phytopathogens, though little is known about the effect of these potential biocontrol agents on other biocontrol strains (Winding *et al.,* 2004). In this study, the growth of *Streptomyces* sp. Fito_S127B and *B. amyloliquefaciens* Fito_F321 strains was inhibited after a co-inoculation with *A. pullulans* Fito_F278. Results suggested a clear competition of Fito_F278 towards other strains, when present in higher abundance. It is recognized that microorganisms can secrete several enzymes, carried out a communication via quorum sensing or competition that can promote or inhibit the growth of the surrounding microorganisms (Elias and Bahin, 2012; Foster and Bell, 2012). Thus, these interactions could be mutually positive or synergistic, such as those observed between Fito_S127B and Fito_F321, or antagonistic (negative), as the interaction of Fito_F278 with Fito_S127B and Fito_F321. Furthermore, microorganisms that have the capacity to form biofilms may critically have an impact on the development and shape of the microbial communities (Elias and Bahin, 2012). Indeed, in our study, was observed that both Fito_F278 and Fito_F321 can develop biofilms, under specific growth conditions (data not shown), reflecting its prone to competition. These observations may be to some extent justify why these microorganisms constitute the most dominant microbial communities associated with grapevine and their well distribution across both below- and above-ground parts of the plants. It is suggested that the co-habitation between microorganisms may occur through different spatial organizations, namely (a) separate microcolonies, (b) co-aggregation or (c) layering (Elias and Bahin, 2012).

The pairwise interactions analysed in this study were effective to demonstrate the potential of cooperation between different biocontrol strains and the possibility to use them as co-inoculations in further grapevine management treatments.

The characterization of growth curves is essential for a small-scale production of strains

To carry out an analysis of the growth kinetics of a microorganism it is essential to firstly select the appropriate culture medium and optimize the growth conditions (such as pH, temperature or shaking), as these factors may have a significant effect in the improving of strain production as well as on the productivity of its antibiotics or other secondary metabolites (Khopade *et al.,* 2012; Sohoni *et al.,*

2012). In our study, the growth curves of each strain where characterized and a small-scale optimization in low volumes of microbial cultivation was implemented. Given Fito_S127B, the most effective strategy consisted on directly inoculate spores in the production medium (2xYT medium, pH7), containing glass beads, followed by several cultures. This procedure allowed to decrease the pellets formed by the strain and to improve the growth cycle, by reducing the lag phase period. The bacteria Fito_F321 and the yeast Fito_F278 showed a great growth performance on LB and YPD medium, respectively. Although the biomass produced by each strain was not quantified, the growth curves indicated that each strain as a specific kinetic profile. Thus, Fito_S127B and Fito_F278 strains showed a logistic profile, while Fito_F321 showed a fast-acceleration/ slow-deceleration profile (Mitchell *et al.,* 2004).

These first insights on the empirical microbial growth curves were essential to characterize the kinetics of each strain and represents a step forward to further obtain microbial biomass, by applying a fermentation process. Indeed, this is a crucial step for future applications of these microorganisms as BCAs, under greenhouse or field conditions.

The molecular identification of grapevine isolates allowed an accurate identification

Overall, sequencing of both 16S rRNA gene and ITS region allowed the identification of grapevine isolates. These regions have been intensively used in the identification of prokaryotic and eukaryotic microorganisms as they are evolutionary conserved regions across microorganisms. The molecular methods, together with the biochemical and physiological analysis of these potential biocontrol microorganisms allowed their accurate identification and characterization. In fact, and considering that these are wild-type strains, the unambiguous identification of these potential BCAs, before and after their further introduction into the vineyards, is a prerequisite for future regulatory authorization (Hintz *et al.,* 2001; Felici *et al.,* 2008).

Conclusion

Overall, our results highlight that natural isolates from grapevine have a promisor biocontrol activity which may constitute a first physical barrier in grapevine, preventing plant from the phytopathogen attacks. Thus, the management of these specific microbial communities could be potentially applied in further sustainable strategies for grapevine, contributing for a decrease or even replace the chemical pesticides to environmental- friendly products. Furthermore, grapevine is a source of microorganisms that can provide an efficient biocontrol effect against GTDs. The mode of action of these microorganisms includes antibiosis and a competition for nutrients and space. Though their highly enzymatic activity, associated with siderophore production, phosphate solubilisation and tolerance to pH and salinity conditions make them not only good biocontrol candidates but also adapted microorganisms to harsh environmental conditions. Thus, results indicated that *Streptomyces* sp. Fito_S127B, *Bacillus amyloliquefaciens* Fito_F321, and to a lesser exent *Aureobasidium pullulans* Fito_F278, are good candidates to develop a biocontrol product or a combination of BCAs against *Botryosphaeriaceae* dieback. Although, *in vivo* studies, such as with *in vitro* plants of grapevine and greenhouse assays, will be crucial to better understand the plant- beneficial microbial interactions and the suitability of these microorganisms in biocontrol applications. Based on this, the Chapter 4 presents a more detailed characterization of these microorganisms under *in vivo* conditions.

Genome sequencing of potential BCAs

In the last years, an increasing trend of BCAs' genome research was observed. Briefly, the genome sequencing of a BCA is a crucial step to better understand their mechanisms applied during biocontrol activities and to decipher their biotechnological value. Thus, the genome sequencing is a valuable shortcut to find and analyse genes of interest in a simple and quickly way and, to understand out these entire genomes work. However, it is important to note that genes and genomes represent distinct levels of genetic organization: where genes codes proteins and RNA, and genomes codes the structure of genetic works (Heng *et al.,* 2011).

From the plant protection point of view, the BCAs' genome provides an efficient and powerful tool to investigate the molecular mechanisms involved in the interaction between BCAs and plant, and the mechanisms involved in biocontrol, namely potential virulence genes or prediction of antibiotics or other secondary metabolites with biotechnological potential and industrial interest. Furthermore, genomes allow a phylogenetic analysis of these strains with other closed related microorganisms. Going forward, and considering the increased interest by the application of BCAs in agriculture applications, the unambiguous identification of these potential microorganisms, before and after their introduction into the environment, is a prerequisite for their further regulatory authorization (Hintz *et al.,* 2001; Felici *et al.,* 2008). Thus, the strain-specific identification is a valuable mean to monitor their presence and persistence on plants, to follow-up their colonisation across plants and to understand their epidemiological development and its interactions with the host and environment (Larena and Melgarejo, 2009). In this context, the genome sequencing of these BCAs represents an important tool to discover specific markers/ genes that allow an effective identification of these microorganisms from intra- and inter-species.

As previously referred, the draft genome sequencing of three selected BCAs isolated from grapevine, namely *Streptomyces* sp. Fito_S127B, *A. pullulans* Fito_F278 and *B. amyloliquefaciens* Fito_F321 is here described.

Publication 6 - Draft genome sequence of *Streptomyces* sp. Fito_S127B strain, a soil microorganism from *Vitis vinifera* microbiome with a promising biotechnological importance

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The supporting information of this publication is available in the Appendix 4 section.

Abstract

Here, the draft genome sequence of *Streptomyces* sp. Fito_S127B strain was reported, an isolate from the *Vitis vinifera* soil. This strain has the capacity to colonize grapevine roots at epiphytic level, to improve the grapevine fitness and to have a biocontrol potential against important grapevine diseases such as trunk diseases (GTDs). The total draft genome size was 8.37 Mb, with a high G+C content (72.9%) and with 7,207 protein-coding genes identified, 3 CRISP regions, 67 tRNAs and 4 rRNA genes. The secondary metabolites predicted included a total of 61 gene clusters. The most abundant were T1PKS (16), NRPS (13), siderophores (4) and terpenes (4) gene clusters. Among them, 2 genes shared 100% similarity with ectoine and angolamycin gene (T1PKS) which are notorious for conferring bacterial resistance against osmotic stresses and biocontrol activity, respectively. Other important putative genes involved in biocontrol and plant growth promotion were identified. Overall, the genome analysis of Fito_S127B strain highlights the biotechnological potential of this strain and its promising applicability on future agricultural management programs.

Key-words
Genome sequencing, *Streptomyces* sp., Fito_S127B strain, Grapevine-associated microorganism, soil isolate; biocontrol

Abreviations: GTD, Grapevine Trunk Disease; PDA, Potato Dextrose Agar; MYM, Maltose-Yeast Extract-Malt Extract

Introduction

The *Streptomyces* are the largest genus among prokaryotes microorganisms. This is a complex group of actinomycetes, Gram-positive bacteria, mycelium-forming and with a high GC – rich content. *Streptomyces* are soil bacteria with a prominent ecological role on the mineralization process in nature and, thus in the recycling of carbon and nitrogen sources. Furthermore, they produce a wide range of bioactive metabolites such as antifungals, antivirals, antitumoral or mainly antibiotics, which are of commercial interest to medicine and agricultural industry (Manteca and Sanchez, 2009; Procópio *et al.,* 2012; Seipke *et al.,* 2012). Actually, 80% of the antibiotics are from *Streptomyces* origin and it is predicted that only 10% of the bioactive compounds of *Streptomyces* have been discovered (Guo *et al.,* 2008; Procópio *et al.,* 2012). Due to the significance of this genus, an intensive isolation and characterization has been achieved (Guo *et al.,* 2008). Curiously, *Streptomyces* has a characteristic smell of soil that is due to the secondary metabolite geosmin, a well conserved trait among this genus (Gust *et al.,* 2003; Seipke *et al.,* 2012).

The beneficial traits of *Streptomyces* are well known. Indeed, the production of bioactive compounds is essential for their biocontrol activities against phytopathogens and for the plant growth promotion (PGP), through the auxin production and by increasing the nutrient assimilation for plant host by means of siderophores, phosphate solubilisation or nitrogen fixation (Seipke *et al.,* 2012; Gopalakrishnan *et al.,* 2013). For this reason, *Streptomyces* are important biocontrol agents (BCAs) against several plant diseases. Endophytic *Streptomyces* were also isolated and their strategy consists on a first colonization of plant roots followed by a plant host invasion (Taechowisan *et al.,* 2003). So far, only some rare *Streptomyces* species are plant pathogens (Seipke *et al.,* 2012).

In the present study, we have obtained the draft genome sequence of *Streptomyces* sp. Fito_S127B strain, a soil isolate of grapevine (*Vitis vinifera*) with a promising biocontrol potential against GTDs, the most widely dangerous grapevine diseases with no currently efficient control strategies (Fontaine *et al.,* 2016).

Organism information

Classification and features

In the course of the characterization of the grapevine microbiome to identify new isolates with biocontrol potential, *Streptomyces* sp. Fito_S127B strain was isolated from a soil sample of *Vitis*

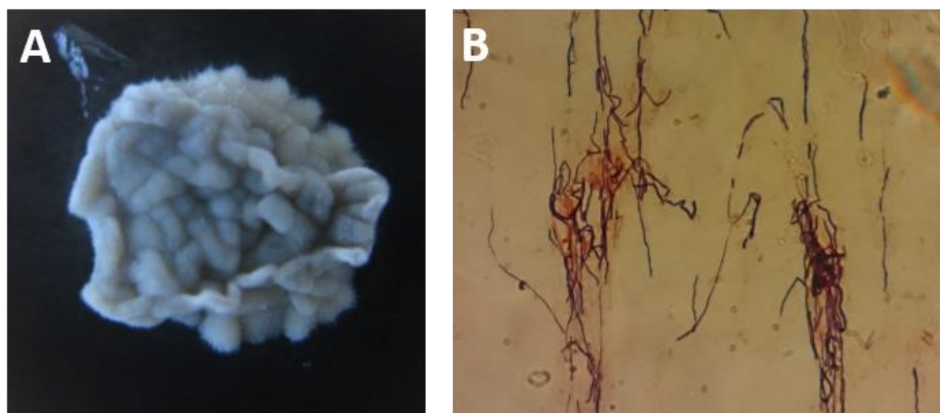

Figure 1: Analysis of the Streptomyces sp. Fito_S127B strain. Fito_S127B strain analysis on A) PDA growth and on B) an optical microscopy, under a 1000x amplification after Gram staining.

Table 1. Classification and general features of Streptomyces sp. Fito_S127B strain, according to the MIGS recommendations (Field *et al*., 2008).

MIGS ID	Property	Term	Evidence code[a]
	Classification	Domain *Bacteria*	TAS [Woese *et al.* 1990]
		Phylum *Actinobacteria*	TAS [Garrity and Holt, 2001]
		Class Actinobacteria	TAS [Stackebrandt *et al.*, 1997]
		Order *Actinomycetales*	TAS [Buchanan, 1917; Skerman *et al.*, 1980; Stackebrandt *et al.*, 1997; Zhi *et al.*, 2009]
		Family *Strepromycetaceae*	TAS [Waksman and Henrici, 1943; Skerman *et al.*, 1980; Stackebrandt *et al.*, 1997; Kim *et al.*, 2003; Zhi *et al.*, 2009]
		Genus *Streptomyces*	TAS [Waksman and Henrici, 1943; Skerman *et al.*, 1980; Witt and Stackebrandt, 1990; Wellington *et al.*, 1992]
		Species *Streptomyces* sp.	NAS
		Strain: Fito_S127B	
	Gram stain	Gram-positive	IDA
	Cell shape	Branched mycelia	IDA
	Motility	Not reported	IDA
	Sporulation	Sporulating	IDA
	Temperature range	unreported	
	Optimum temperature	28°C	IDA
	pH range; Optimum	5-11, 6.5	IDA
	Carbon source	Not reported	IDA
MIGS-6	Habitat	Soil, grapevine	IDA
MIGS-6.3	Salinity	0-2% (w/v) NaCl	IDA
MIGS-22	Oxygen requirement	Aerobic	NAS
MIGS-15	Biotic relationship	Free-living	IDA
MIGS-14	Pathogenicity	Non-pathogen	IDA
MIGS-4	Geographic location	Cantanhede, Portugal	IDA
MIGS-5	Sample collection	2011	IDA
MIGS-4.1	Latitude	unreported	
MIGS-4.2	Longitude	unreported	
MIGS-4.4	Altitude	unreported	

[a] Evidence codes – IDA: Inferred from Direct Assay; TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project (Ashburner *et al*., 2000).

vinifera, during the 2011 vine campaign at Bairrada appellation – Cantanhede, Portugal. Briefly, the soil was mixed, suspended in sterile MiliQ water, homogenized by vortexing and samples were then serially diluted up to 10^{-6}, spread (0.1mL) over the surface of PDA and incubated at 28°C for 48h or 72h. Sub-cultures were then performed until obtaining pure colonies that were further assigned to a specific isolation code. Overall, *Streptomyces* sp. Fito_S127B strain is a Gram-positive bacterium with aerial mycelium and which may produce spores at maturity (Figure 1). This strain can growth at pH range between 5-11 (with an optimal growth at pH 6.5) and under salinity conditions up to 2% (w/v) NaCl (optimum 0% NaCl). The general features of this strain are presented in Table 1.

The sequencing of the 16S rRNA region followed by a Blast search on RDP (Ribosomal Database Project) and NCBI databases allowed to identify and confirm this strain as *Streptomyces* sp.. A phylogenetic tree (Figure 2) was reconstructed on the basis of the 16S rRNA gene with other taxonomy close *Streptomyces* species obtained after BLAST by using GGDC web server (Meier-Kolthoff *et al.,* 2013) through the DSMZ phylogenomics pipeline, adapted to single genes (Meier-Kolthoff *et al*., 2014). Briefly, after a multiple sequence alignment created with MUSCLE (Edgar, 2004), a maximum likelihood (ML) and a maximum parsimony (MP) trees were inferred with RAxML (Stamatakis, 2014) and TNT (Goloboff et al., 2008), respectively. Regarding ML, a fast bootstrapping in conjunction with the autoMRE bootstopping criterion (Pattengale et al., 2010) and subsequent search for the best tree was used. MP criterion were conducted with PAUP* (Swofford, 2002) using 1000 bootstrapping replicates. The phylogenomic trees were then edited on the graphical viewer FigTree version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Genome sequencing information

Genome project history

An ongoing project with this isolate showed that Fito_S127B strain has the capacity to colonize grapevine roots at epiphytic level under *in vitro* and greenhouse conditions and to improve the performance of plantlets, including the plant growth. Furthermore, antagonistic tests with this strain showed a promising biocontrol potential against GTDs namely, Botryosphaeriaceae species by reducing the mycelial growth of pathogens. This prompted us to perform the genome sequencing of Fito_S127B strain to elucidate the potential genes involved in biocontrol activities. The draft genome sequence of Fito_S127B strain was performed at Biocant (Portugal), using the GS FLX+ system (Roche, 454 Life Sciences), though to date this draft genome sequence has not yet been deposited at NCBI Genbank. A summary of the project is summarized in Table 2.

Figure 2: Phylogenetic tree highlighting the position of Streptomyces sp. Fito_S127B strain relative to phylogenetic close strains. The phylogenetic tree was inferred from the 16S rRNA gene matrix under maximum likelihood (ML) and the GTR + GAMMA model. The *Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3 strain (CP003838) was used as an outgroup. The branches are scaled in terms of the expected number of substitutions per site. Numbers at the branches are bootstrapping (1,000 times) support values (when larger than 60%) from ML (left) and MP (right). The GenBank accession numbers are shown in parentheses.

Growth conditions and genomic DNA preparation

Streptomyces sp. Fito_S127B strain was grown on Maltose-Yeast Extract-Malt Extract (MYM) medium (0.4% maltose, 0.4% yeast extract, 1% malt extract, 1.8% agar, pH 7) at 28°C. Then the genomic DNA was extracted by using the Wizard Genomic DNA purification kit (Promega, Madison, USA), following the standard protocol for Gram-positive bacteria. The size and DNA integrity was checked by 0.8% agarose gel electrophoresis, the concentration determined by using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) and quality assessed with NanoDrop spectrophotometer (Thermo Scientific, USA).

Genome sequencing and assembly

A DNA library was constructed through 1mg of high-quality genomic DNA. For this, the genomic DNA was fragmented by nebulization and the sequencing adaptors ligated to create double stranded DNA libraries. After quality assessment by using High sensitivity DNA Analysis Kit (Agilent Technologies) and library titration with KAPA Library Quantification Kit (Kapa Biosystems), the final genome fragments were pyrosequenced in the GS FLX+ system (Roche, 454 Life Sciences), using GS FLX Titanium Sequencing Kit XL+ at Biocant (Cantanhede, Portugal). The sequencing reads were assembled with the GS Assembler, version 2.9 (Roche, 454 Life Sciences) using the default parameters. The *de novo* read assembly yielded 519 contigs, with an average size of 17,237 bp and a N50 of 30,979 bp and generated a genome of 8.37 Mb (8,367,629bp) with an average G+C content of 72.9%.

Genome annotation

The structural and functional annotation was performed using the Prokaryotic Genome Prediction (PGP) pipeline (Egas *et al.,* 2014). Thus, this pipeline predicted the non-coding RNA genes and miscellaneous features by using tRNAscan-SE 2.0 (Lowe and Eddy, 1997; Schattner *et al.,* 2005), RNAmmer (Lagesen *et al.,* 2007) and PILERCR (Edgar, 2007). Then the coding sequences (CDS) were predicted with Prodigal (Hyatt *et al.,* 2010) and corrected by the PGP pipeline based on the GenePRIMP algorithm (Pati *et al.,* 2010). The functional annotation of protein coding genes was carried out through InterProScan (Zdobnov and Apweiler, 2001) against Pfam database (Finn et al., 2008), TIGRFAM (Haft et al., 2003), Hamap (Pedruzzi et al., 2013), PIRSF (Wu et al., 2004), PRINTS (Attwood et al., 2012), SMART (Letunic et al., 2012), SUPERFAMILY (Wilson et al., 2009), ProSite (Sigrist et al., 2010) databases and RPS-BLAST against Clusters of Orthologous Groups (COG) database (Tatusov et al., 1997). The product name of the identified coding sequences (CDSs) was assigned by using Pfam database, TIGRFAM and COG annotation (Mavromatis *et al.,* 2009). The CDSs that were not assigned to a specific product with these databases were named as hypothetical proteins.

Table 2. Project information.

MIGS ID	Property	Term
MIGS 31	Finishing quality	Draft-genome
MIGS-28	Libraries used	KAPA Library Quantification Kit
MIGS 29	Sequencing platforms	GS FLX Titanium Sequencing Kit XL+
MIGS 31.2	Fold coverage	-
MIGS 30	Assemblers	GS Assembler, version 2.9
MIGS 32	Gene calling method	Prodigal, GenePRIMP
	Locus Tag	Not realeased
	Genbank ID	Not realeased
	Genbank Date of Release	Not realeased
	GOLD ID	-
	BIOPROJECT	Not realeased
MIGS 13	Source Material Identifier	Fito_S127B
	Project relevance	PGP, Biocontrol, Grapevine, GTD

Table 3. Genome statistics.

Attribute	Value	% of Total[a]
Genome size (bp)	8,367,629	100
DNA coding (bp)	7,085,087	84.67
DNA G+C (bp)	6,100,002	72.90
DNA scaffolds	-	-
Total genes	7296	100
Protein coding genes	7225	99.03
rRNA	4	0.05
tRNA	67	0.92
Genes assigned to COGs	3782	51.84
Genes with Pfam domains	2277	31.21
Genes with transmembrane helices	0	0.00
CRISPR repeats	3	0.04

[a]The total is based on either the size of genome in base pairs or the total number of genes in the predicted genome

Genome properties

The total genome size of the *Streptomyces* sp. Fito_S127B strain is 8,367,629bp and the GC content is 72.9% (Table 3; Figure 3), which is similar to other sequenced genomes from this genus (Appendix 4: Tale S1) with comparable isolation sources. Interestingly, this is a genus with a high GC content and with a genome size that may vary according to the *habitat* that each microorganism occupies. Thus, and according to this data, *Streptomyces* sp. isolated from compost samples seems to have a smaller genome size (5 Mb) when compared with those *Streptomyces* sp. isolated from soils, plants or water sources (8 Mb) (Appendix 4: Tale S1). Though, to achieve more exact conclusions is necessary a more exhaustive analysis among the 439 *Streptomyces* sp. available genomes in the NCBI platform, of which 19 are assigned as complete genomes (https://www.ncbi.nlm.nih.gov/genome/genomes/13511). Going forward, a total of 7,225 coding genes were predicted in the Fito_S127B strain genome, 3 CRISP regions, 67 tRNA genes and 4 rRNA genes. The predicted protein encoding genes showed a total length of 7,085,087 bp which represents 84.67% of the total genome size. The classification of genes into COGs functional categories is shown in Table 4.

Insights from the genome sequence

A total of 153 metabolic pathways were identified using the KEGG annotation and the majority of proteins-coding genes were involved in the metabolism namely, carbohydrate, amino acid, energy, nucleotide or metabolism of cofactors and vitamins, genetic information procession and environmental information processing. As expected, the analysis also revealed protein-coding genes involved in the biosynthesis of secondary metabolites. Overall, the metabolic pathways identified are included within several metabolism pathways (such as alanine, aspartate and glutamate, glycine, serine and glutamate, cysteine and methionine, pyruvate, fructose, mannose, galactose, ascorbate and aldarate, starch and sucrose, propanoate, butanoate, methane, nitrogen, sulphur or biotin), glycolysis, tricarboxylic acid cycle (TCA cycle) - also known as Krebs cycle, fatty acid biosynthesis, glucosinolate, antibiotic biosynthesis (streptomycin, acarbose and validamycin, ansamycins, vancomycin, monobactam, carbapenem, phenazine or glucosinolate) and even degradation pathways (atrazine, benzoate, aminobenzoate, chloroalkane, caprolactam, lysine, limonene, pinene or geraniol). Interestingly, and regarding the biosynthesis of secondary metabolites, phenazine is of significant interest due to its impacts on bacterial interactions and biotechnological processes namely, its contribution to the biofilm formation and survival of bacteria or influence in plant growth and elicitation of an induced systemic resistance (Pierson III and Pierson, 2010).

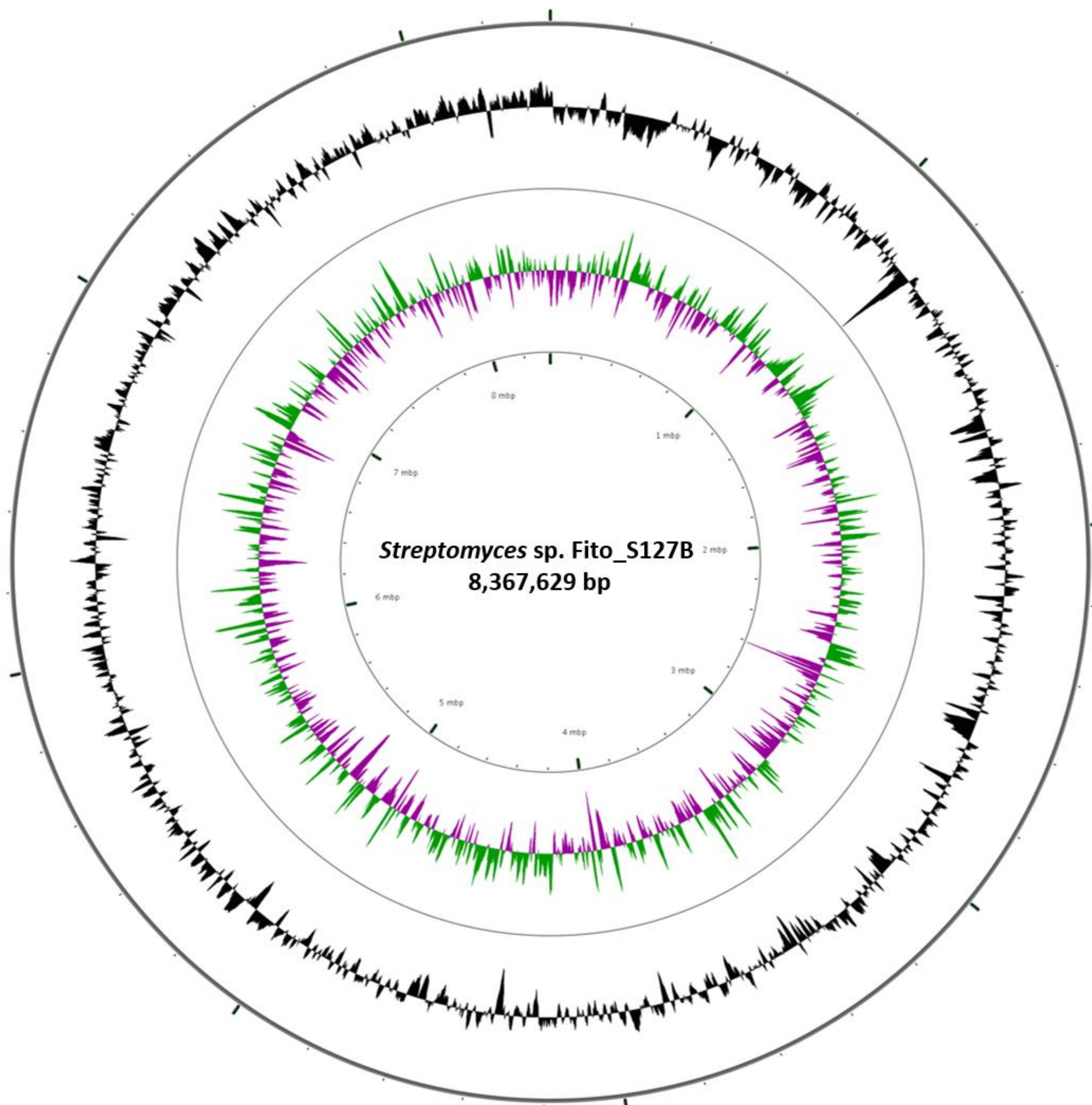

Figure 3: Circular map of the Streptomyces sp. Fito_S127B strain genome. Circle display (from the outside to center): circle 1 shows the G+C % content (black) and circle 2 shows the GC skew (green represents above average and violet below average). The map was generated with CGView (Stothard and Wishart, 2005).

The secondary metabolite genes clusters were predicted using antiSMASH 4.0 (Weber *et al*., 2015), and included a total of 61 gene clusters (Appendix 4: Table S2) namely, 16 T1PKS, 13 NRPS, 4 siderophore, 4 terpenes, 3 butyrolactone, 2 amglyccycl, 2 melanin, 2 T1PKS-lantipeptide-NRPS, 1 bacteriocin, 1 bacteriocin-NRPS, 1 ectoine, 1 lantipeptide, 1NRPS-T1PKS, 1 T2PKS, 1 T3PKS, 1 Terpene-Lassopeptide-NRPS, 1 Transatpks, 3 others and 3 other KS. Of these clusters, 2 genes shared 100% similarity with ectoine and angolamycin gene (T1PKS) (Appendix 4: Table S3). Ectoine is a natural compound that confers resistance and adaptation to extreme osmotic stress environments (salt or temperature) and is encoded by the cluster genes ectA, ectB and ectC (Bursy *et al.,* 2008). In turn, the angolamycin, firstly isolated from *Streptomyces eurythermus* in soil samples from Angola, Congo and Switzerland*,* is an important antibiotic that inhibit the microorganism's growth such as gram-positive and protozoa (Korzybski *et al.,* 1967; Vasquez, 1967). Among siderophore, we founded an 83% similarity with desferrioxamine B gene cluster and among terpenes and Terpene-Lassopeptide-Nrps, a 92% similarity with hopene and 85% similarity with isorenieratene gene clusters were reported, respectively. The hopene have stabilizing functions in bacterial membranes, protecting against water loss, and isorenieratene is an aromatic carotene with antioxidative properties and normally present in green photosynthetic bacteria and few actinomycetes (Krügel *et al.,* 1999). By far, through the ResFinder- 2.1 (Zankari *et al.,* 2012) it was possible to predict that Fito_S127B has one antimicrobial resistance gene to oleandomycin conferred by oleC gene (94.38% of identity).

Interestingly, a protein involved in the biosynthesis of mitomycin was identified. This is recognized for its antibacterial (against gram-positive or gram-negative) and antitumor activity.

A comparison of the *Streptomyces* genomes with RAST server (Aziz *et al.,* 2008) revealed that *Streptomyces avermitilis* MA-4680 is the closest neighbour of Fito_S127B strain followed by *Streptomyces coelicolor* A3(2) (Figure S1). Though, the pairwise genome comparisons performed by using the JSpecies WS web server (Richter and Roselló-Móra, 2009) to estimate the average of nucleotide identity (ANI) between genomes using the MUMmer software (ANIm) showed that a major ANIm was obtained with *Streptomyces sp.* e14 (87.24%) with a genome alignment of 37.68% (Appendix 4: Table S4). The *Streptomyces avermitilis* MA-4680 and *Streptomyces coelicolor* A3(2) had an ANIm of 85.97% and 86.35%, respectively. Furthermore, a deep comparison of the metabolic or function parts of *Streptomyces* sp. Fito_S127B with *Streptomyces avermitilis* MA-4680 by using the RAST database (Aziz *et al.,* 2008), indicated that 263 genes were unique to *Streptomyces* sp. Fito_S127B. Thus, and taking as example the virulence, diseases and defense category, genes involved in copper resistance protein D, vancomycin B-type resistance protein or cobalt-zinc-cadmiun resistance protein CzcD were identified. Others from the nitrogen metabolism were also found and included the nitrilase or ammonia assimilation, which are recognized for their important roles within plant-microbial interactions such as defence, detoxification or plant growth.

Table 4. Number of genes associated with general COG functional categories.

Code	Value	%age[a]	Description
J	164	2.27	Translation, ribosomal structure and biogenesis
A	1	0.01	RNA processing and modification
K	445	6.16	Transcription
L	107	1.48	Replication, recombination and repair
B	1	0.01	Chromatin structure and dynamics
D	22	0.30	Cell cycle control, Cell division, chromosome partitioning
V	73	1.01	Defense mechanisms
T	167	2.31	Signal transduction mechanisms
M	128	1.77	Cell wall/membrane biogenesis
N	0	0.00	Cell motility
U	28	0.39	Intracellular trafficking and secretion
O	84	1.16	Posttranslational modification, protein turnover, chaperones
C	265	3.67	Energy production and conversion
G	367	5.08	Carbohydrate transport and metabolism
E	393	5.44	Amino acid transport and metabolism
F	87	1.20	Nucleotide transport and metabolism
H	163	2.26	Coenzyme transport and metabolism
I	215	2.98	Lipid transport and metabolism
P	170	2.35	Inorganic ion transport and metabolism
Q	196	2.71	Secondary metabolites biosynthesis, transport and catabolism
R	475	6.57	General function prediction only
S	231	3.20	Function unknown
-	3443	47.65	Not in COGs

[a]The total is based on the total number of protein coding genes in the genome.

Conclusion

In this study, the genome of *Streptomyces* sp. strain Fito_S127B, a natural microbial resource of the soil microbiome associated with grapevine, was characterized. This 8Mb genome size showed a high number of coding sequences (7,207) which encoded genes with a significant biotechnological importance. Among them, some secondary metabolites are highlighted such as ectoine (T1PKS), angolamycin or siderophore (desferrioxamine B gene cluster) for their role in bacterial resistance against osmotic stress and biocontrol activity. Furthermore, the potential of this strain on plant growth promotion was also addressed. Overall, and from a biotechnological point of view, the genome information disclosed in this study will be further used to deep investigate new genes that target new antibiotics and other bioactive compounds with notorious biocontrol potential to be future applied on grapevine diseases management namely, against GTDs.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

CP, FF and ACG designed research. CP performed the experiments and SS was associated with DNA isolation and purification. CP and HF were evolved on the Bioinformatic analysis. Contributed reagents/materials/analysis tools: SS, CE, CC, FF and ACG. Wrote the paper: CP, FF and ACG. All authors read and approved the final manuscript.

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Publication 7- Draft genome sequence of *Aureobasidium pullulans* strain Fito_F278, a resident microbiota of grapevine with biocontrol potential against GTDs

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Running title: Draft genome sequence of *Aureobasidium pullulans* Fito_F278

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Abstract

Aureobasidium pullulans is an ubiquitous black-yeast with a biotechnological importance and used in the biocontrol of post-harvest diseases of fruits. Here, we present the draft genome sequence of *A. pullulans* strain Fito_F278, a resident microorganism from grapevine and a promisor biocontrol agent against trunk diseases (GTDs).

Aureobasidium pullulans (de Bary) G. Arnaud is an ubiquitous black-yeast widely distributed across different plants and environments and a well-adapted microorganism (Chi *et al.,* 2009; Martini *et al.,* 2009; Grube *et al.,* 2011; Pinto *et al.,* 2014). This is one of the most abundant microorganisms of grapevine and is considered as a resident microbiota (Grube *et al.,* 2011; Pinto *et al.,* 2014). *A. pullulans* is a polymorphic microorganism that according to environmental conditions, can grow as budding yeast or as mycelia (Gaur *et al.,* 2010). Furthermore, *A. pullulans* is of a biotechnological significance with a high production of hydrolytic enzymes including amylases, chitinases, β-1,3-glucanases, xylases, proteases, cellulases, lipases and mannases, extracellular polysaccharides (EPS), especially pullulan, siderophores and single cell proteins (SCP) (Deshpande *et al.,* 1992; Ippolito *et al.,* 2000; Chi *et al.,* 2009; Gaur *et al.,* 2010). In this light, the high enzymatic versatility together with its resistant mechanisms and competition for nutrients and space, constitute the mode of action of this microorganism, contributing to its successful in plant colonisation and biocontrol activities (Ippolito *et al.,* 2000; Schmid *et al.,* 2011). *A. pullulans* is, thus an effective microorganism against different post-harvest diseases of fruits (Ippolito *et al.,* 2000; Castoria *et al.,* 2001; Schena *et al.,* 2002; Mounir *et al.,* 2007; Schmid *et al.,* 2011), wheat diseases caused by *Fusarium culmorum* (Wachowska and Glowacka, 2014) or even against various GTDs agents (Munkvold and Marois, 1993; Pinto *et al.,* in press). Due to its biocontrol success, biocontrol products are already available on the market (EU pesticides database, 2017).

The strain Fito_F278 was isolated during the 2012 vine campaign at Bairrada appellation – Cantanhede, Portugal from leaves of *Vitis vinifera,* infected with downy mildew. After microbial isolation, the ITS region was amplified and sequenced to confirm species identity and the BLASTn search identified the isolate as *Aureobasidium pullulans,* that showed a 98% identity with *A. pullulans* strain Y11 (GenBank: KC897669.1). Then, the draft genome sequence of Fito_F278 strain was performed at Biocant (Portugal), using the GS FLX Titanium Sequencing Kit XL+. The sequencing reads were assembled with the GS Assembler, version 2.9 (Roche, 454 Life Sciences) using the default parameters, resulting in a genome assembly comprising 821 contigs with an average contig length of 37,376 bp (longest contig with 741,690 bp and the smallest with 100 bp). The draft genome sequence

of Fito_F278 strain contains 30,686,389 bp, covering a total 30.68Mb, with an average G+C content of 50.35%. A total of 322 tRNAs, using the tRNAscan-SE 2.0 (Lowe and Eddy, 1997), and a predicted number of 14,438 coding-sequences (CDSs) were identified. Other genome sequences of *A. pullulans* strains are available. Overall, the genome size of Fito_F278 strain is similar in size to *A. pullulans* var. *pullulans* isolated from hypersaline waters of Sečovlje solar saltern (Slovenia) (29.62 Mb; G+C: 50.02%) though the genome is four times less fragmented (209 contigs) and it carries 11,844 CDSs. Curiously, the genome size of the IMV 00882 strain, isolated from Kirovograd region soil (Chernobyl) is completely distant in size from these ones (40.99 Mb; G+V: 51%; contigs: 879).

The genome analysis of Fito_F278 strain using antiSMASH 4.0 (Weber et al., 2015) identified 21 metabolites gene clusters. Among them, Fito_F278 encoded 2 NRPS clusters (nonribosomal peptides), 5 PKS clusters (polyketide synthases), 1 hybrid PKS-NRPS cluster, 4 terpene and other secondary metabolites (in a total of 9). Overall, the *A. pullulans* Fito_F278 strain reveals genes that coding enzymes such as amylase, alcohol dehydrogenases, tannases, cellulases, ureases, trehalose-phosphatase, glucanases and chitinases, which some of them are commercially important or involved in biocontrol activities. Other genes implicated in virulence factors such as phospholipases or beta-lactamases were also predicted as well as a number of cutinases. At the biotechnological point of view, cutinases are enzymes that hydrolyse the plant cuticle, and were firstly discovered in fungal pathogens (Nyyssölä, 2015). Genes coding for catechol dioxygenase were also identified and are of utmost importance for the biodegradation of aromatic molecules in the environment such as pollutants (Justice, 2004; Chan *et al.,* 2012). Curiously, a gene coding for the luciferase-like monooxygenase was even identified.

Overall, the draft genome sequence of *A. pullulans* Fito_F278 is a step forward to find new genes encoding important bioproducts with biotechnological potential for biocontrol or other industrial activities.

Accession number(s). To date, the project has not yet been submitted, and therefore does not have an accession number.

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Publication 8- Draft genome sequence of *Bacillus amyloliquefaciens* strain Fito_F321, an endophyte microorganism from *Vitis vinifera* with biocontrol potential

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The supporting information of this publication is available in the Appendix 5 section.

Abstract

Bacillus amyloliquefaciens strain Fito_F321 is a naturally occurring strain in vineyard, with the ability to colonise grapevine, which unveils a naturally biocontrol potential against phytopathogens of grapevine, including those responsible for the Botryosphaeria dieback, a GTD disease. The genome size of *B. amyloliquefaciens* strain Fito_F321 is 3,856,229 bp, with a GC content of 46,54% and that contains 3,697 protein-coding genes, 86 tRNA coding genes and 5 rRNA genes. The draft genome of Fito_F321 strain allowed to predict a set of bioactive compounds as bacillaene, difficidin, macrolactin, surfactin and fengycin that due to their antimicrobial activity are of utmost importance for biocontrol of grapevine diseases.

Key-words

Genome sequencing, *Bacillus amyloliquefaciens*, Fito_F321 strain, Grapevine-associated microorganism, Biocontrol, Endophytic microorganism

Abreviations: GH, glycoside hydrolase; GTD, Grapevine Trunk Disease; LPs, lipopeptides

Introduction

Bacillus amyloliquefaciens is a species from the genus *Bacillus*, and closely related to *Bacillus subtilis* [1]. Together with other different *Bacillus* species, such as *B. subtilis*, *B. pumilus* or *B. thuringiensis*, *B. amyloliquefaciens* has been reported to develop beneficial relationships with plants by promoting the plant growth, improving resistance to environmental stress or having important biological activities for plant diseases control [2,3,4]. This specie produces a variety of antimicrobial compounds, as bacteriocins, antifungal compounds as lipopeptides (LPs), namely iturins and fengycins, and siderophores [5,6]. Given its biocontrol potential, aligned with its physiological characteristics, namely resistant spores to both UV light and heat, long shelf life and their advantageous characteristics for formulation, this microorganism is an environmental-friendly alternative to agrochemicals. Indeed, some of *B. amyloliquefaciens* strains are thus commercially available as biocontrol agents.

Altogether these characteristics prompted us to explore the *Bacillus amyloliquefaciens* strain Fito_F321, which was isolated from grapevine leaves by our laboratory in the Bairrada appellation - Portugal, and that was a naturally occurring strain in vineyard. In this study, we have obtained the draft genome sequencing of *Bacillus amyloliquefaciens* strain Fito_F321, analysed it and compared with known genome sequences, in order to improve the knowledge of genes evolved in the interaction with grapevine and with antimicrobial activity interest for further viticulture and agronomic applications.

Organism information

Classification and features

B. amyloliquefaciens strain Fito_F321 was isolated from *Vitis vinifera* cv. Merlot at Bairrada appellation – Cantanhede, Portugal during the 2012 vine campaign. The samples collection was authorized by the private owner, who is fully acknowledged in this paper, and no specific permissions were required for this activity. Briefly, leaf tissues were homogenised in a sterile saline solution (0.85% NaCl) with a sterile pestle. The bacteria isolate was then obtained after plating the homogenised leaves on PDA medium and incubated for 24h at 28°C. Sub-cultures were then carried out on the same culture medium until obtaining pure colonies that were further assigned to an isolation code. Microscopy analysis showed that *B. amyloliquefaciens* strain Fito_F321 is a Gram-positive, rod shape and aerobic microorganism (Figure 1). The classification and general features of *B. amyloliquefaciens* strain Fito_F321 are presented in Table 1.

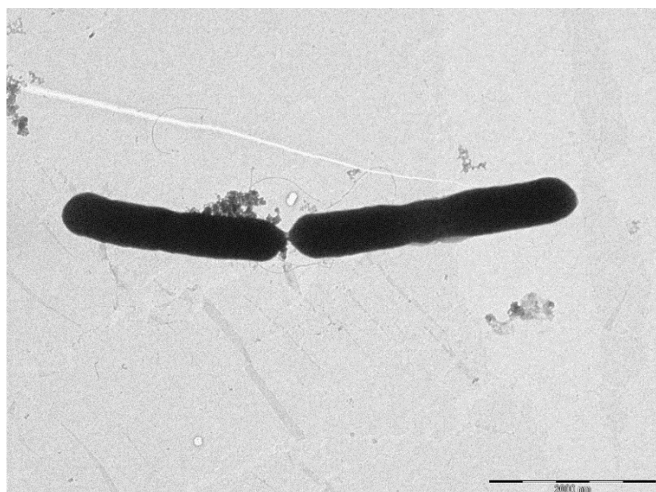

Figure 1: Transmission electron micrograph of Bacillus amyloliquefaciens strain Fito_F321. Bar: 2µm.

Table 1. Classification and general features of Bacillus amyloliquefaciens strain Fito_F321, according to the MIGS recommendations [7].

MIGS ID	Property	Term	Evidence code[a]
	Classification	Domain *Bacteria*	TAS [9]
		Phylum *Firmicutes*	TAS [10,11,12]
		Class Bacilli	TAS [13, 14]
		Order *Bacillales*	TAS [11]
		Family *Bacillaceae*	TAS [11, 15]
		Genus *Bacillus*	TAS [11, 16]
		Species *Bacillus amyloliquefaciens*	TAS [1, 17]
		Strain: Fito_F321	
	Gram stain	Gram-positive	IDA
	Cell shape	Rod-shaped	IDA
	Motility	Motile	NAS
	Sporulation	Spore-forming	NAS
	Temperature range	unreported	
	Optimum temperature	28°C	IDA
	pH range; Optimum	6-9, 6.5	IDA
	Carbon source	Organic carbon source	NAS
MIGS-6	Habitat	Leaf, grapevine	IDA
MIGS-6.3	Salinity	0-6% (w/v); salt tolerant	IDA
MIGS-22	Oxygen requirement	Aerobic	NAS
MIGS-15	Biotic relationship	free-living	IDA
MIGS-14	Pathogenicity	Non-pathogen	NAS
MIGS-4	Geographic location	Cantanhede, Portugal	IDA
MIGS-5	Sample collection	2012	IDA
MIGS-4.1	Latitude	unreported	
MIGS-4.2	Longitude	unreported	
MIGS-4.4	Altitude	unreported	

[a] Evidence codes – IDA: Inferred from Direct Assay; TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project [8].

The 16S rRNA sequence of both *B. amyloliquefaciens* strain Fito_F321 and other closely related species available on NCBI database were then selected for phylogenetic analysis. The phylogenetic tree and similarities of the 16S rRNA were inferred by using the GGDC web server using the DSMZ phylogenomic pipeline [18], adapted to single genes. In brief, a multiple sequence alignment was created with MUSCLE [19] and the maximum likelihood (ML) and maximum parsimony (MP) trees were inferred from the alignment with RAxML [20] and TNT [21], respectively. Regarding ML, a fast bootstrapping in conjunction with the autoMRE bootstopping criterion [22] and subsequent search for the best tree was used. MP criterion were conducted with PAUP* [23] using 1000 bootstrapping replicates. The phylogenomic trees were then edited on the graphical viewer FigTree version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) and is shown on Figure 2. The phylogeny analysis revealed that 21 type strains were divided in different groups and the *B. amyloliquefaciens* strain Fito_F321 clustered in the same group with *B. amyloliquefaciens* UMAF6614 and *B. amyloliquefaciens* subsp. *plantarum* NAU-B3, close to *B. amyloliquefaciens* SQR9, *B. subtilis* ATCC 19217 and *B. amyloliquefaciens* subsp. *plantarum* TrigoCor1448. Furthermore, *B. amyloliquefaciens* strain Fito_F321 was clearly distinct from *B. amyloliquefaciens* LL3 and *B. amyloliquefaciens* DSM7. Herein *B. subtilis* ATCC 19217 clustered with *B. amyloliquefaciens* strains but not in the same clade of *B. subtilis* strains.

Genome sequencing information

Genome project history

B. amyloliquefaciens strain Fito_F321 was selected for sequencing as a part of an ongoing project that focus on the deep characterization of the grapevine-associated microorganisms and their natural biocontrol potential. Thus, its specific biocontrol activity against important grapevine pathogens as grey mould or grapevine trunk diseases (GTDs) and its physic and biochemical characteristics such as capacity to growth on different pH and salinity conditions, production of siderophores, phosphate solubilisation and high enzymatic activity, were the drivers for its sequencing.

Sequencing of *B. amyloliquefaciens* strain Fito_F321 genome was performed at Biocant, Portugal and the draft genome sequencing project has been deposited at the NCBI platform under the accession number PRJNA360208 and Biosample ID SAMN06205151. A summary of the project is shown in Table 2.

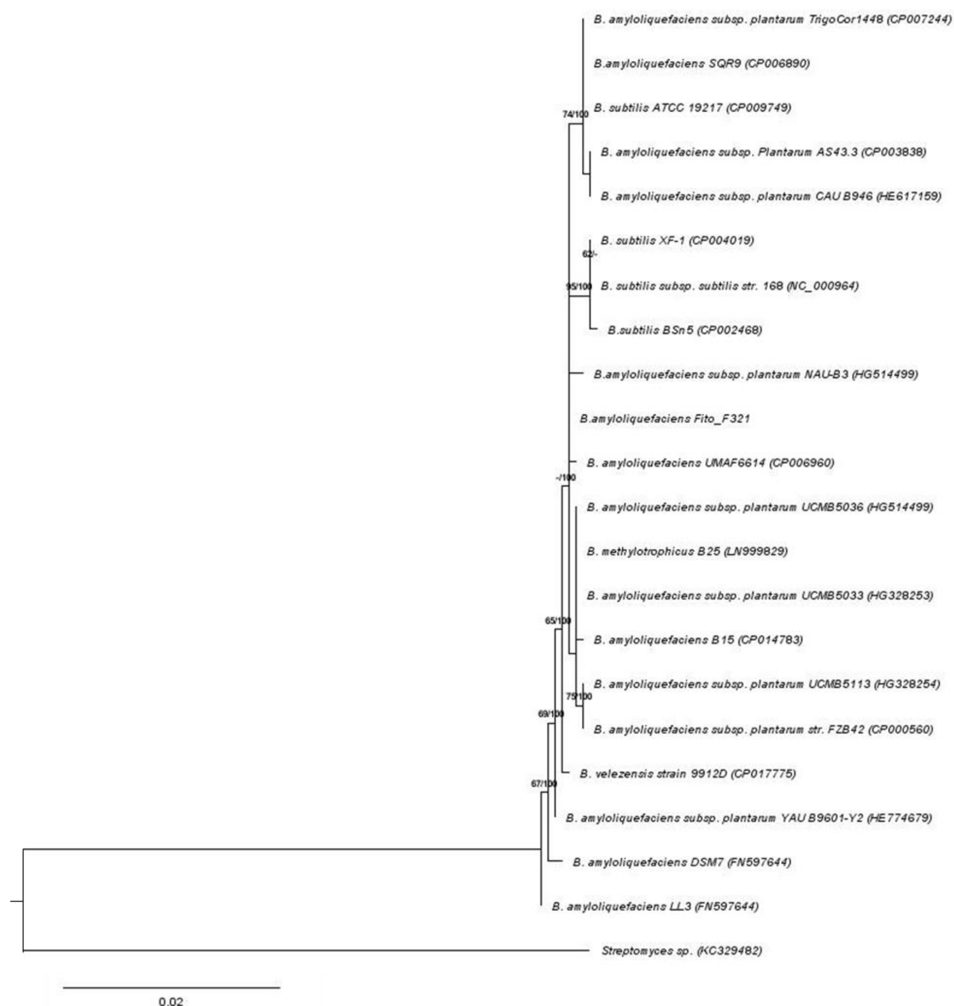

Figure 2: Phylogenetic tree inferred from the 16S rRNA gene matrix under maximum likelihood (ML), under the GTR + GAMMA model. Rooting was done with *Streptomyces* sp. (KC329482). The branches are scaled in terms of the expected number of substitutions per site. Numbers at the branches are bootstrapping support values (when larger than 60%) from ML (left) and MP (right). The GenBank accession numbers are shown in parentheses.

Table 2. Project information.

MIGS ID	Property	Term
MIGS 31	Finishing quality	Draft-genome
MIGS-28	Libraries used	KAPA Library Quantification Kit
MIGS 29	Sequencing platforms	GS FLX Titanium Sequencing Kit XL+
MIGS 31.2	Fold coverage	41x
MIGS 30	Assemblers	GS Assembler, version 2.9
MIGS 32	Gene calling method	Prodigal, GenePRIMP
	Locus Tag	BVY13
	Genbank ID	PRJNA360208
	Genbank Date of Release	02/05/2017
	GOLD ID	-
	BIOPROJECT	SAMN06205151
MIGS 13	Source Material Identifier	Fito_F321
	Project relevance	Biocontrol, Grapevine, GTD

Growth conditions and genomic DNA preparation

B. amyloliquefaciens strain Fito_F321 was grown in Luria-Agar medium at 28°C for 24h. The genomic DNA was extracted by using the Wizard Genomic DNA Purification kit (Promega, Madison, USA), following the standard protocol for Gram- positive bacteria. The DNA integrity was checked by 0.8% agarose gel electrophoresis, the concentration was determined by using Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) and quality assessed with NanoDrop spectrophotometer (Thermo Scientific, USA).

Genome sequencing and assembly

A DNA library was built through 1mg of high-quality genomic DNA. Briefly, genomic DNA was fragmented by nebulization and the sequencing adaptors ligated to create double stranded DNA libraries. After quality assessment by using high sensitivity DNA analysis kit (Agilent Technologies) and library titration with KAPA library quantification kit (Kapa Biosystems), the final genome fragments were pyrosequenced in the GS FLX+ system (Roche, 454 Life Sciences), using GS FLX Titanium Sequencing Kit XL+ at Biocant (Cantanhede, Portugal). The sequencing reads were assembled with the GS Assembler, version 2.9 (Roche, 454 Life Sciences) using the default parameters. The sequencing produced 285,879 reads with an average length of 580 bases. The final assembly yielded 59 contigs, a genome coverage of 41% and generated a genome of 3.86 Mb.

Genome annotation

The structural and functional annotations were performed using the PGP pipeline (Prokaryotic Genome Prediction) [24]. Prediction of non-coding RNA genes and miscellaneous features were performed with the PGP pipeline by using tRNAscan-SE [25], RNAMMer [26] and PILERCR [27]. The coding sequences (CDS) were predicted with Prodigal [28] and automatically corrected by PGP pipeline based on the GenePRIMP algorithm [29]. Functional annotation of protein coding genes was carried out under PGP pipeline in InterProScan [30] against Pfam database [31], TIGRFAM [32], Hamap [33], PIRSF [34], PRINTS [35], SMART [36], SUPERFAMILY [37], ProSite [38] databases and RPS-BLAST against Clusters of Orthologous Groups (COG) database [39]. The product name of the identified coding sequences (CDSs) was assigned by using Pfam database, TIGRFAM and COG annotation [40]. The CDSs that were not assigned to a specific product with these databases were named as hypothetical proteins.

Table 3. Genome statistics.

Attribute	Value	% of Total[a]
Genome size (bp)	3,856,229	100
DNA coding (bp)	3,424,790	88.81
DNA G+C (bp)	1,794,204	46.53
DNA scaffolds	54	-
Total genes	3846	100
Protein coding genes	3657	98.09
RNA genes	95	2.47
Pseudo genes	94	2.44
Genes in internal clusters	NA	-
Genes with function prediction	2790	72.54
Genes assigned to COGs	2697	70.12
Genes with Pfam domains	3241	84.27
Genes with signal peptides	248	6.45
Genes with transmembrane helices	2500	65.00
CRISPR repeats	0	0.00

[a]The total is based on either the size of genome in base pairs or the total number of genes in the predicted genome

Table 4. Number of genes associated with general COG functional categories.

Code	Value	%age[a]	Description
J	158	4.32	Translation, ribosomal structure and biogenesis
A	0	0.00	RNA processing and modification
K	227	6.21	Transcription
L	97	2.65	Replication, recombination and repair
B	1	0.03	Chromatin structure and dynamics
D	34	0.93	Cell cycle control, Cell division, chromosome partitioning
V	43	1.18	Defense mechanisms
T	105	2.87	Signal transduction mechanisms
M	136	3.72	Cell wall/membrane biogenesis
N	41	1.12	Cell motility
U	40	1.09	Intracellular trafficking and secretion
O	78	2.13	Posttranslational modification, protein turnover, chaperones
C	156	4.27	Energy production and conversion
G	191	5.22	Carbohydrate transport and metabolism
E	269	7.36	Amino acid transport and metabolism
F	78	2.13	Nucleotide transport and metabolism
H	122	3.34	Coenzyme transport and metabolism
I	117	3.20	Lipid transport and metabolism
P	149	4.07	Inorganic ion transport and metabolism
Q	85	2.32	Secondary metabolites biosynthesis, transport and catabolism
R	306	8.37	General function prediction only
S	264	7.22	Function unknown
-	960	26.25	Not in COGs

[a]The total is based on the total number of protein coding genes in the genome.

Genome properties

The genome statistics are provided in Table 3 and Figure 3, and genome visualisation was performed on Artemis version 16.0.0 [42]. The draft genome sequencing of *B. amyloliquefaciens* strain Fito_F321 was distributed across 59 contigs with an estimated genome size of 3,856,229 bp and an average of GC content of 46.53%. The genome analysis showed that Fito_F321 strain' genome contained 3,657 protein coding genes predicted, 86 tRNA, 5 rRNA and without any CRISP elements. The predicted protein encoding genes showed a total length of 3,424,790 bp which represents 88.81% of the total genome size. Of these, 2,697 proteins were assigned to a COG functional category across 20 categories (Table 4). The majority of protein-coding genes were assigned as function unknown (264 proteins) and general function prediction only (306 proteins), which all together represents 15.59% of the protein encoding genes (Table 4). The proteins not assigned in COGs (960 proteins) represent 26.25% and the amino acid transport (269 proteins), transcription (227 proteins) and carbohydrate transport and metabolism (191 proteins) were the followed categories with 7.36%, 6.21% and 5.22%, respectively. Interestingly, the defense mechanisms included 43 protein-coding genes, which represents about 1% of the annotated genome, and included β-lactamase (class C), multi-drug efflux pumps as ATP-binding cassette (ABC) transport and the multidrug and toxic compound extrusion (matE), antimicrobial peptides (AMPs) and lanthionine synthetase component C-like protein (LANCL).

Insights from the genome sequence

A total of 111 metabolic pathways were identified using the KEGG annotation and included, several metabolism pathways (as alanine, aspartate and glutamate, fructose, mannose, galactose, glutathione, methane, nitrogen, pyruvate, sulphur, tryptophan or starch and sucrose), glycolysis, TCA cycle, fatty acid biosynthesis, glucosinolate biosynthesis, antibiotic biosynthesis (neomycin, kanamycin, gentamicin, puromycin, streptomycin or tetracycline) or degradation pathways of noxious compounds (atrazine, benzoate, bisphenol, dioxin, ethylbenzene, limonene, pinene, naphthalene, polycyclic aromatic hydrocarbon or toluene). In general, the metabolic pathways identified showed that the majority of protein-coding genes are involved in the metabolism, that includes amino acid metabolism, biosynthesis or other secondary metabolites, carbohydrate metabolism, energy metabolism, glycan biosynthesis and metabolism, lipid metabolism, metabolism of cofactors and vitamins, metabolism of other amino acids, metabolism of terpenoids and polyketides, nucleotide metabolism or xenobiotics biodegradation and metabolism, and genetic information procession (Figure 4).

B. amyloliquefaciens Fito_F321
3,856,229 bp

Figure 3: Circular map of the B. amyloliquefaciens strain Fito_F321 genome. Circle display (from the outside to center): circle 1 shows the G+C % content (black) and circle 2 shows the GC skew (green represents above average and violet below average). The map was generated with CGView [41].

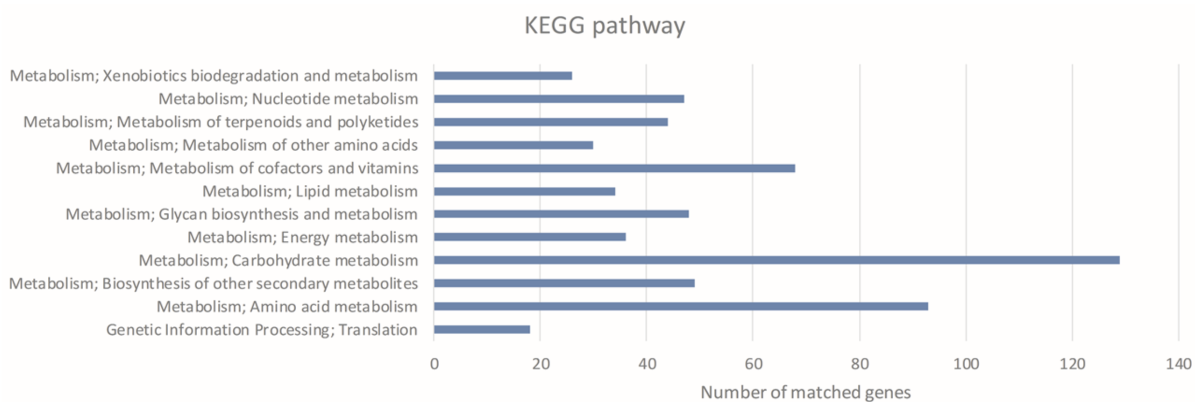

Figure 4: KEGG annotation of protein-coding genes.

Among the identified genes, some of them are involved with plant growth promotion, namely the gene nitrogen fixation protein NifU and others involved on the iron complex transport system were detected. Another feature of *B. amyloliquefaciens* strain Fito_F321 is the gene S-ribosylhomocysteinase (LuxS), indicating that this strain produces autoinducer 2 (AI-2), an extracellular molecule with function of quorum sensing. Regarding antimicrobial resistance, the genome of Fito_F321 encodes multiple drug resistance transporters as EmrB, bcr_cfla and a putative tetB protein, a tetracycline resistance. The polysaccharide intercellular adhesin (PIA) synthetic pathway was also identified and may play a critical role on the biofilm formation by this strain [43]. Also, different glycoside hydrolase (GH) families were found and included Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (GH1), 6-phospho-beta-glucosidase/alpha-galactosidase (GH4), cellulase (GH5), GH11, GH16 and Beta-xylosidase (GH43). These enzymes hydrolyse the glycosidic bonds of glycosides, glycans and glycoconjugates and they have an important function in the catabolism of carbohydrate metabolism contributing to the generation of carbon sources [44]. In the meantime, genes encoding virulence factors as hemolysins and related proteins containing CBS domains, or even pectate and pectin lyases were detected. These are lytic enzymes that disrupt the pectic compounds present in the structure of the plant cell wall, and depending of the interaction between plant-microorganism, they may have a positive interaction as they could act as elicitors of the plant response.

According to the genome analysis using antiSMASH 3.0 [45], 14 secondary metabolites gene clusters were identified. Among them, *B. amyloliquefaciens* strain Fito_F321 encoded 4 PKS clusters (polyketide synthases), 2 NRPS clusters (nonribosomal peptides) and 2 hybrid PKS-NRPS clusters. Thus, 3 types of antibacterial polyene PKs are produced and comprised bacillaene, difficidin and macrolactin, 2 types of lipopeptides (LPs) as fengycin and surfactin, antifungal active compounds, and the siderophore or bacillibactin. In addition, the remaining 6 clusters were predicted to produce secondary metabolites including terpene, ladderane, lantipeptide or microcin (Appendix 5: Table S1).

To further characterize the extent of which *B. amyloliquefaciens* strain Fito_F321 differentiate from other strains, a genome comparison of *B. amyloliquefaciens* strain Fito_F321 with other related species of *Bacillus* spp. was carried out by using both GGDC 2.1 web server [46] available at http://ggdc.dsmz.de/, using the DSMZ phylogenomics pipeline [18], to estimate the DNA-DNA hybridization (DDH), and the JSpecies WS web server [47] to estimate the Average Nucleotide Identity (ANI) through pairwise comparisons of genomes. The estimate DDH was calculated with the formula two at the GGDC website, which is the recommended for draft genomes and the ANI values were calculated using the MUMmer software (ANIm) as described by Richter and Roselló-Móra (2009) [47]. This analysis allowed to calculate the intergenomic distances between genomes and the probability of belonging to the same species. For this, 22 strains with sequenced genome were download from the

NCBI database. This general comparison is shown in Appendix 5: Table S2 and the intergenomic distances, through the DDH estimate and ANI in Appendix 5: Table S3. The results showed that *B. amyloliquefaciens* strain Fito_F321 had a lower distance with *B. amyloliquefaciens* SQR9 with a DDH estimate of 90.60% and a probability that corresponds to the same species of 96.01%. These results were also supported by the ANI analysis where both strains reached a similarity of 98.91%, with 95.86% of the aligned genome. Despite this, the probability for being the same subspecies is lower than 79%, meaning that these strains do not belong to the same subspecies. The same comparative results were obtained for all the remaining strains in analysis. *B. subtilis* ATCC 19217 is the second strain with a lower distance to *B. amyloliquefaciens* strain Fito_F321, followed by *B. amyloliquefaciens* FZB42 with a similarity of 98.85 and 98.40%, respectively (Appendix 5: Figure S1). Furthermore, *B. amyloliquefaciens* DSM7, *B. amyloliquefaciens* LL3, *B. amyloliquefaciens* TA208 and the expected *B. subtilis* subsp. *subtilis* 168, *B. subtilis* XF-1, *B. subtilis* BSn5 and *B. pumilus* SAFR-032 showed the lower DDH estimate and, thus, the bigger intergenomic distances with *B. amyloliquefaciens* strain Fito_F321.

Conclusions

In this study, we have characterized the genome of *B. amyloliquefaciens* strain Fito_F321, a natural grapevine-associated microorganism, which was isolated from grapevine leaves. Given its genomic and physiological characteristics, this microorganism may provide an interesting model to study the plant-microbial interactions and their role in grapevine protection. This draft genome is slightly smaller (3.86 Mb) when compared to others of the same species although, and together with *B. amyloliquefaciens* subsp. *plantarum* UCMB5036 and *B. amyloliquefaciens* FZB42 they share not only a similar genome size but also approximately the same number of protein-coding sequences. However, the similarities among genomes showed that *B. amyloliquefaciens* strain Fito_F321 is highly similar with *B. amyloliquefaciens* SQR9, an isolate from the cucumber rhizosphere, with a DDH value of 90.60% and a ANIm value of 98.91%, while the genome similarity with *B. amyloliquefaciens* FZB42 and *B. amyloliquefaciens* subsp. *plantarum* UCMB5036 have a DDH value of 85.90% and 84.90%, respectively.

The predicted gene compounds of *B. amyloliquefaciens* strain Fito_F321 as bacillaene, difficidin, macrolactin, surfactin, fengycin and siderophore, together with other protein-coding genes herein presented, are of utmost importance for its biocontrol activity and could explain its positive plant-microbial interactions, as well as its role on the natural protection of vineyard. Thus, these gene clusters suggest that the Fito_F321 strain can produce bioactive compounds of biocontrol value, which

represents a source of novel bioactive compounds and that may be essential for the grapevine protection in pursue a more sustainable viticulture.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CP, FF and ACG designed research. CP performed the experiments and SS did DNA isolation and purification. CP and HF were evolved on the Bioinformatic analysis. Contributed reagents/materials/analysis tools: SS, CE, CC, FF and ACG. Wrote the paper: CP, FF and ACG. All authors read and approved the final manuscript.

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Chapter IV

PHYTOPROTECTOR POTENTIAL OF TWO SELECTED BCAS AGAINST GTDS AGENTS

Chapter outline

GTDs seriously affect the viticulture worldwide, causing great economic impacts. Furthermore, since sodium arsenite was banned no long-term or effective alternatives are available to control GTDs, which reinforces the need to develop new strategies. In this context, BCAs emerge as a friendly and sustainable management strategy. As previously elucidated, the biocontrol potential of BCAs against phytopathogens relies on several mechanisms such as antibiosis, competition for space or nutrients, parasitism or by producing cell wall degrading enzymes (Pal and Gardener, 2006; Jamalizadeh *et al*., 2011). Furthermore, BCAs can also act indirectly by promoting a plant-inducing resistance. This is an early activation of defensive responses of plants, called as priming effect, that allow a plant protection against further biotic and abiotic stresses (Conrath *et al.,* 2001; Conrath, 2011). Moreover, some of these beneficial microorganisms can also stimulate the plant growth. For this reason, these microorganisms are of great interest for application in agriculture as biofertilizers or as BCAs (Compant *et al.,* 2010). Though, and comparatively to chemicals, the application of BCAs is sometimes considered less effective. Thus, one of the most important prerequisites of a successful BCAs relies on its competent colonisation of plants (Maurer *et al.,* 2013). Indeed, the understanding of the BCAs' colonization processes is important not only to predict their interactions with plant host but also to verify their capacity to establish themselves in the plant environment after field applications (Compant *et al.,* 2010).

In the previous chapter, a set of promising BCAs against *Botryosphaeriaceae* species were identified. Among them, three isolates were selected and deeply characterized, namely *Streptomyces* sp. Fito_S127B, *Aureobasidium pullulans* Fito_F278 and *Bacillus amyloliquefaciens* Fito_F321. These are wild-type strains all isolated from grapevine, namely Fito_S127B from soil and Fito_F278 and Fito_F321 from leaves. Despite their promising biocontrol under *in vitro* conditions, their capacity to colonize and to protect grapevine was not elucidated. Thus, in this chapter the ability of Fito_S127B and/or Fito_F278 to colonise plantlets of cv. Chardonnay was firstly assessed. Then their biological control against *Diplodia seriata,* a Botryosphaeria dieback agent, was analysed in cutting plants of *Vitis vinifera* cv. Chardonnay. Over three growing seasons, a 4-month greenhouse assay was performed and, parameters such as the necrotic lesions length, analysis of PSII and expression of genes involved in different signalling pathways (PR proteins, phenylpropanoid metabolism, detoxication and stress tolerance, cell wall compounds, water stress), to determine the plant responses under the interaction between plant – *D. seriata* – Fito_S127B and/or Fito_F278, were investigated. Furthermore, a molecular assessment by using strain-specific primers, designed through the genome analysis of each BCA, was carried out to follow-up the BCAs colonisation across plants.

Results showed that Fito_S127B successfully colonised superficial roots and showed a plant growth promoting (PGP) potential, and Fito_F278 was able to colonize from the roots to the leaves, at both epiphytic and endophytic level. The follow-up of these BCAs colonisation by using strain-specific primers was a simple and rapid strategy to identify them after the plant inoculation Thus, primers targeting the helix-turn-helix protein domain (*HxlR* gene) were selected to monitor Fito_S127B and glutathione-S-transferase (*GST* gene) for Fito_F278. The specificity tests with non-target strains showed 100% and 79% of specificity of *HxlR* for Fito_S127B and GST for Fito_F278, respectively. Given the greenhouse trials, results showed that the necrotic lesions length caused by the phytopathogen was significantly reduced by Fito_S127B (29.44%), in contrast to Fito_F278 which was less effective. Gene expression analysis showed that both BCAs activated some specific defence responses of grapevine, and highlighted that these strains may have promoted a priming effect. Additionally, the gene expression of plants inoculated with *D. seriata* was higher induced when plants were pre-treated with Fito_S127B. The follow-up of strains colonisation after their soil release showed that their survival was limited and not always systematic. Fito_S127B was recovered mainly from soils and up to two months of its inoculation (T3+ 4 weeks), while Fito_F278 was recovered from soils up to one month (T3+1 week) and after then from roots (T3+ 4 weeks). In fact, these results are different with those from grapevine plantlets where a successful colonization of both strains was achieved.

Overall, results suggested that *Streptomyces* sp. Fito_S127B was a promising BCA of GTDs, namely *Botryosphaeriaceae* agents. This strain induced effectively some defence mechanisms of grapevine, allowing a more rapid and solid response of plant against *D. seriata*. Furthermore, Fito_S127B was also a competent colonizer of grapevine rhizosphere. The follow-up of BCAs colonisation through a molecular assessment, by using strain-specific primers, showed to be an important step for an unambiguous and easy identification of these strains. This approach allowed a rapid monitorization of BCAs after their release under greenhouse conditions, and can be successfully apply in other future field applications.

Contexte

Les MDB affectent gravement la viticulture dans le monde entier, causant d'importants impacts économiques. Après l'interdiction de l'arsénite de sodium, aucune alternative à long terme ou aussi efficace n'est disponible pour contrôler les MDB, ce qui renforce la nécessité de développer des nouvelles stratégies. Dans ce contexte, les BCAs apparaissent comme une stratégie écologique et durable à utiliser dans les vignobles. Comme précédemment énoncé, le potentiel de biocontrôle des BCAs contre les agents pathogènes consiste en plusieurs mécanismes tels que l'antibiose, la concurrence pour l'espace ou les nutriments, le parasitisme ou la production d'enzymes dégradant la paroi cellulaire (Pal et Gardener, 2006 ; Jamalizadeh *et al*., 2011). En outre, les BCAs peuvent également agir indirectement en favorisant une résistance induite des plantes. Il s'agit d'une activation précoce des réponses défensives des plantes, appelées potentialisation ou priming, qui permet une protection de la plante contre les stresses biotiques et abiotiques (Conrath *et al*., 2001 ; Conrath, 2011). Certains de ces microorganismes bénéfiques peuvent également stimuler la croissance des plantes. Pour cette raison, ces microorganismes sont d'un grand intérêt pour l'application dans l'agriculture en tant que biofertilisants ou comme BCAs (Compant *et al*., 2010). Cependant, et comparativement aux pesticides, l'application des BCAs est parfois considérée comme peu efficace. Ainsi, une des conditions des plus importantes pour l'efficacité des BCAs dépende notemment de sa capacité à coloniser les plantes (Maurer *et al.,* 2013). En effet, la compréhension des processus de colonisation des BCAs est très importante non seulement pour prédire leurs interactions avec la plante, mais aussi pour vérifier leur capacité à s'établir dans l'environnement de la plante après des applications sur le terrain (Compant *et al.,* 2010).

Dans le chapitre précédent, un ensemble de BCAs prometteurs contre des espèces de *Botryosphaeriaceae* a été identifié. Parmi eux, trois isolats ont été choisis et caractérisés en détail, à savoir *Streptomyces* sp. Fito_S127B, *Aureobasidium pullulans* Fito_F278 et *Bacillus amyloliquefaciens* Fito_F321. Ce sont des souches de type sauvage toutes isolées de la vigne, à savoir Fito_S127B dans le sol et Fito_F278 et Fito_F321 au niveau des feuilles. Malgré leur potentiel promotteur en tant que biocontrôle lors de tests *in vitro*, leur capacité à coloniser et à protéger la vigne n'a pas été élucidée. Ainsi, dans ce chapitre, la capacité de la souche Fito_S127B et / ou Fito_F278 à coloniser des plantules de cv. *Chardonnay* a été évaluée. Ensuite, leur potentiel en tant que BCA contre *Diplodia seriata*, un agent responsable pour le dépérissement de la vigne, a été analysé à l'aide de boutures de *Vitis vinifera* cv. *Chardonnay*. Au cours de trois saisons de végétation, un essai en serre de 4 mois a été réalisé et différents paramètres ont été étudiés tels que la longueur des lésions nécrotiques, l'analyse de l'activité du PSII et l'expression des gènes impliqués dans différentes voies de signalisation (protéines PR, métabolisme des phénylpropanoïdes, détoxication et la tolérance au stress, les composés de la

paroi cellulaire, le stress hydrique), a fin de déterminer les réponses des plantes dans l'interaction vigne - *D. seriata* - Fito_S127B et / ou Fito_F278. En outre, une analyse moléculaire à l'aide des amorces spécifiques de chaque souche, conçues à partir de l'analyse du génome de chaque BCA, a été effectuée pour suivre la colonisation de ces microorganismes dans la plante.

Les résultats ont montré que Fito_S127B a colonisé avec succès les racines superficielles et peut stimuler la croissance des plantes (PGP), alors que Fito_F278 a pu coloniser la plante des racines vers les feuilles, au niveau épiphyte et endophytique. Le suivi de cette colonisation a été réalisée à l'aide d'amorces spécifiques, une stratégie simple et rapide pour identifier ces microorganismes après leur inoculation dans les plantes. Ainsi, les amorces ciblant le domaine de protéine hélice-tour-hélice (gène HxIR) ont été sélectionnées pour identifier la souche Fito_S127B et le glutathion-S-transférase (gène GST) pour Fito_F278. Les tests de spécificité avec des souches non visées ont montré une spécificité de 100% et 79% de HxIR pour Fito_S127B et GST pour Fito_F278, respectivement. Les résultats des essais en serre ont montré que la longueur des lésions nécrotiques causée par l'agent pathogène a été considérablement réduite par Fito_S127B (29.44%), contrairement à Fito_F278 qui était moins efficace. L'analyse de l'expression des gènes a montré que ces BCAs ont activé quelques réactions de défense spécifiques de la plante ; nous pouvouns souligner que ces souches ont favorisé un effet de potentialisation. En outre, l'expression génétique des plantes inoculées avec *D. seriata* était plus élevée lorsque les plantes étaient prétraitées avec Fito_S127B. Le suivi de la colonisation des souches après leur inoculation au niveau du sol, a montré que leur survie était limitée dans le temps et pas toujours systématique : Fito_S127B a été rétrouvé principalement dans le sol et jusqu'à deux mois après son inoculation (T3 + 4 semaines), tandis que Fito_F278 a été détecté dans le sol jusqu'à un mois (T3 + 1 semaine) et au niveau des racines (T3 + 4 semaines). Ces résultats sont différents de ceux obtenus lors des expérimentations *in vitro* plants.

Dans l'ensemble, les résultats suggèrent que *Streptomyces* sp. Fito_S127B est un agent prometteur de biocontrôle des MDB, à savoir les agents associés au *Botryosphaeria* dieback. Cette souche induit efficacement certains mécanismes de défense de la vigne, permettant une réponse plus rapide et plus solide de la plante contre *D. seriata*. En outre, Fito_S127B est également un colonisateur compétent de la rhizosphère de la plante. Le suivi de la colonisation des BCAs grâce à l'elaboration d'amorces spécifiques de chaque souche, a été une étape importante pour une identification sans ambiguïté et facile de ces souches. Effectivement, cette approche a permis une surveillance rapide des BCAs après leur inoculation au niveau des plantes dans des conditions de serre ; elle pourra également être utilisée pour des applications futurs sur le terrain.

Impact of potential selected two BCAs in grapevine protection against GTDs

GTDs are the most destructive diseases, affecting vineyards worldwide. These trunk pathogens can remain in plants for several years before symptoms appear in foliage level, which difficult its early identification (Di Marco *et al.,* 2000). Several alternatives have been proposed to control these diseases after the banning of sodium arsenite, and include proactive measures such as pre-treatment of propagation material with benomyl, hot water or *Trichoderma* spp. (Fourie and Halleen, 2004; Martin *et al.,* 2009; Gramaje and Di Marco, 2015; Halleen and Fourie, 2016), vine cuttings or pruning wound protection with *Trichoderma* spp., (Di Marco *et al.,* 2004), fungicides as fosetyl Al, (Di Marco *et al.,* 2000) or chitosan (Nascimento *et al.,* 2007). Though no long-term or effective alternatives are already available, which reinforces the need to develop new strategies such as BCAs.

The infection and development of GTDs in grapevine, like others phytopathogens, interfere with the primary metabolism of the plant, namely carbon metabolism which is involved in grapevine yield and vigour (Petit *et al.,* 2006; Petit *et al.,* 2009). Perturbations on this metabolism may cause modifications on the photosynthetic activity and chlorophyll fluorescence of the plant which, in turn, may disturb the carbon balance, affecting the reserves on the storage organs and the carbon nutrition of the plant (Petit *et al.,* 2006). In addition to phytopathogens, also drought (Flexas *et al.,* 2002) or even phytotoxicity of agrochemicals (Petit *et al.,* 2009) can interfere with photosynthesis rate. Indeed, in response to these biotic and environmental stresses, plants can develop different physical and chemical defence strategies. Among them, a decrease of the photosynthesis rate occurs as a strategy to overcome these stresses (Chapin *et al.,* 2003). Thus, this decrease is caused by the stomatal closure due to the water stress induced by the phytopathogen and to a disturbance in the metabolic pathway of the photosynthesis such as Rubisco or carbonic anhydrase, which catalyse the conversion of CO_2 into HCO_3 (Nogués *et al.,* 2002; Petit *et al.,* 2006). Furthermore, a stimulation of plant defence responses may also occur. Thus, a response to a phytopathogen attack, may result in an upregulation of defence genes, namely pathogenesis-related (PR) proteins such as β-1,3-glucanases and quitinases, a greater activity of phenylalanine-ammonium liase (*PAL*), polyphenol oxidase (*PPO*) or guaiacol peroxidase (*GPX*) and a repression of photosynthetic genes such as ribulose-1,5-biphosphate carboxylase small subunit (*RbcS*) (Garcia *et al.,* 2003; Berger *et al.,* 2004; Petit *et al.,* 2009). Another defence mechanisms consist in the production of reactive oxygen species (ROS) to destroy the pathogen, through the activation of the detoxication mechanisms such as glutathione metabolism (Valtaud *et al.,* 2009), rapid and localized cell death (hypersensitive response, HR) and formation of chemical barriers by accumulation of phytoalexins (stilbene such as resveratrol and ε- viniferin) or other antimicrobials secondary metabolites (tannins or flavonoids) (Jeandet *et al.,* 2002; Martin *et al.,* 2009; Magnin-Robert *et al.,* 2011). Given the *PAL* gene it is suggested that is essential in regulating

salicylic acid (SA) biosynthesis and, consequently triggering a systemic acquired resistance (SAR) (Mauch-Mani and Slusarenko, 1996). Moreover, *PAL* is a precursor of stilbene synthase (*STS*) (Jeandet *et al.,* 2002).

It has been suggested that BCAs may be involved in the activation of defensive responses of the plant, protecting them against future phytopathogens attack. This induced resistance is called as priming effect (Conrath *et al.,* 2011; Conrath, 2011). Indeed, plant growth promoter rhizobacteria (PGPR) can induce a systemic resistance (ISR) in plants (Choudhary and Johri, 2009), or the recognition of an avirulent microorganism can elicit an oxidative burst leading to an induction of defence genes and a HR, that will then develop a SAR in plant (Alvarez *et al.,* 1998). Since grapevine is an important host of beneficial microorganisms, exploring this population is a step forward to identify potential BCAs from grapevine for grapevine protection.

Considering the increased interest in the development and application of new BCAs, the unambiguous identification of these potential microorganisms, before and after their introduction into the environment, is a prerequisite for their further regulatory authorization (Hintz *et al.,* 2001; Felici *et al.,* 2008). Thus, the strain-specific identification is a valuable mean to monitor their presence and persistence on plants, to follow-up their colonisation across plants and to understand their epidemiological development and its interactions with the host and environment (Larena and Melgarejo, 2009). Overall, strains identification was initially carried out by applying conventional methods such as classic microbiology techniques, using general or selective agar medium and subsequent characterization of their morphological traits (Larena and Melgarejo, 2009). However, these methods are limited, do not allow the identification of closely related strains and are time-consuming and laborious. Molecular assessment such as DNA fingerprinting (Random amplified polymorphism DNA - RAPD) or enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody were also applied for strains identification or phylogenetic classification though, these methods are likewise time-consuming and require considerable expertise (Felici *et al.,* 2008; Fujimoto *et al.,* 2010; Endo *et al.,* 2012). Nowadays, and to overcome all these limitations, PCR-based methods for strains-specific identification have been developed. Indeed, the molecular methods are the more efficient, sensitive and rapid tools for strains identification and do not require the target microorganisms to be cultured for detection (Knight, 2000; Larena and Melgarejo, 2009). Among them, the 16S rRNA and ITS regions are conserved regions among species and, thus, the mostly convenient target regions for bacterial and fungal/ yeasts identification, respectively (White *et al.,* 1990). However, they do not allow to differ closely related strains due to the high sequence homology in the variable regions (Sattler *et al.,* 2014). Thus, strain-specific identification using strain-specific primers is a possible alternative to these regions and a powerful strategy to identify and even quantify specific strains from different biological materials (Fujimoto *et al.,* 2010).

This study attempted firstly to understand the biocontrol potential of *Streptomyces* sp. Fito_S127B and *Aureobasidium pullulans* Fito_F278 against *Diplodia seriata,* a Botryosphaeria dieback agent, under greenhouse conditions. To achieve this, the colonisation capacity of these potential BCAs, the necrotic lesions length, the analysis of PSII and the expression of genes involved in different signalling pathways (PR proteins, phenylpropanoid metabolism, detoxication and stress tolerance, cell wall compounds, water stress), to determine plant responses to the interaction between plant – *D. seriata* – Fito_S127B and/or Fito_F278, were investigated. Secondly, this study aimed to develop a quick and reliable PCR-based method for detection and follow-up of the BCAs colonisation across grapevine plants, after their *in vivo* inoculation. For this, strain-specific primers were developed on the basis of a comparison of genes retrieved from their whole-genome sequence (WGS) with other closely related strains.

Material and methods

Colonization bioassay

Determination of BCAs growth and inoculum preparation

Given the Fito_S127B strain, fresh colonies were growth in MYM medium (Maltose-Yeast Extract-Malt Extract: maltose 4 $g.L^{-1}$, yeast extract 4 $g.L^{-1}$, malt extract 1 $g.L^{-1}$, agar 18 $g.L^{-1}$) and then dipped with a 20% sterilized glycerol solution. A first pre-culture was carried out in a 500mL Erlenmeyer flask containing sterilized glass beads (180 µm, Sigma) by adding 2mL of the spore suspension to 40mL of 2xYT (bacto trytone 16 $g.L^{-1}$; bacto yeast extract 10 $g.L^{-1}$; NaCl 5 $g.L^{-1}$, at pH7) (Sohoni *et al.,* 2012). After 36h of incubation at 28°C and 150 rpm, 1mL of this pre-culture 1 was transferred to another 100mL Erlenmeyer flask containing 50mL of 2xYT medium (pH7) and glass beads (pre-culture 2). After 30 h of incubation, a pre-culture 3 was established under the same conditions as those established in pre-culture 2. Then, after 18h of incubation, the cell culture itself was established. Thus, 1mL of pre-culture 3 was transferred to a new Erlenmeyer containing 50mL of 2xYT medium (pH7) and glass beads and incubated at 28°C and 150 rpm. The cell concentration (CFU/mL) was then estimated by reading the optical density (OD) at 600 nm.

Regarding the Fito_F278 strain, this was initially inoculated at 28°C for 48h in YPD (Yeast Extract-Peptone-Dextrose: yeast extract 10 $g.L^{-1}$; glucose 20 $g.L^{-1}$; peptona 20 $g.L^{-1}$; agar 20 $g.L^{-1}$) medium. Subsequently, a colony was selected and incubated in approximately 20mL of YPD medium for 6 h at 28°C and at 150 rpm, to check the growth of the yeast. At this time, a pre-culture was prepared through 1mL of the above prepared yeast suspension in 10mL of liquid YPD medium and incubated overnight

at 28°C and 150 rpm. After incubation (±15h), the OD was measured and adjusted to a final concentration of 0.1. Then, 5mL of the above pre-culture was added to 50mL of liquid YPD medium. The cell concentration (CFU/mL) was then estimated by reading the OD at 600 nm.

To prepare the final cell suspension, each strain was firstly collected by centrifugation (4,500 rpm for 10 min at 4°C) and washed twice with phosphate-buffered saline solution (PBS: NaCl 8 $g.L^{-1}$; KCl 0.2 $g.L^{-1}$; Na_2HPO_4 1.44 $g.L^{-1}$; KH_2PO_4 0.24 $g.L^{-1}$; pH7.5). The concentration of the strain inoculum was then adjusted to approximately $3x10^8$ CFU/mL with PBS at pH7.5 for Fito_S127B and $1x10^6$ CFU/mL of Fito_F278 strain. The strain measures were based on the OD at 600 nm and concentrations calculated as previously described in Chapter 3.

Plant material, growth conditions and inoculation

A bioassay was performed to verify the ability of 2 selected BCAs, namely *Streptomyces* sp. Fito_S127B and *Aureobasidium pullulans* Fito_F278, to colonize grapevine plantlets. *Vitis vinifera* L. cv Chardonnay (clone 7535) used in this bioassay were micropropagated by nodal explants in culture tubes with 25 mm diameter, containing 15mL of Martin Medium (Martin *et al.,* 1987). Plants were grown in a growth chamber under white fluorescent light (200 $µmol.m^{-2}.s^{-1}$), 16h photoperiod and at a temperature constant of 26°C (Compant *et al.,* 2005).

Plantlets with five-week-old were then selected and for each experiment, three conditions were performed, namely (a) control, (b) plants inoculated with Fito_S127B and (c) plants inoculated with Fito_F278. Plant inoculation was carried out by dipping the roots during 10s in a 5mL of strain suspension in PBS at pH7.5 or only PBS at pH7.5 (control). Plants were then carefully transferred to Magenta Box containing 100 mL of semi-solid Martin Medium (Martin *et al.,* 1987) and incubated in the growth chamber as described above. Each Magenta Box contained 2 plants. Overall, each condition contained n = 15 uniform plants and the experiment was repeated three times.

Analysis of grapevine colonization by the selected strains

The effect of strains on the plant health status and their capacity to colonize grapevine was compared with control plants at 4, 7 and 14 days' post inoculation (dpi). For each sampling time, 5 plants of each condition were selected and removed from the growth medium. Samples were then pooled together and 2 biological replicates were performed and analysed by (a) classic microbiology, (b) molecular techniques and (c) microscopy analysis on a three-dimensional (3D) microscope VHX-2000 (Z100x100). For each replicate, root and leaves fresh weights were determined to further estimate the effect of colonization (CFU/g of fresh weight).

Given the (a) classic microbiology analysis, both epiphytic and endophytic colonization were analysed by plate counting method. For this, roots and leaves of each condition were sampled, pooled

as described above, weighted and gently rinsed in sterile distilled water. For the epiphytic colonization, samples were ground with a sterile pestle containing 1mL of PBS, then transferred to an Eppendorf microcentrifuge tubes and vortexed at room temperature (RT). After that, 10-fold serially dilutions were carried out for roots and 100µL was cultured on MyM medium for Fito_S127B analysis, YPD for Fito_F278 or Luria-Agar (LB) for control treatment. Leaves samples were directly cultured on each respective culture medium. For the endophytic colonization analysis, both roots and leaves were surface sterilized with 70% ethanol for 1 min, followed by 0.6% sodium hypochlorite for 3 min and washed four times in sterile distilled water. Samples were then ground in 1mL of PBS pH7.5 and macerated with a sterile pestle. After a vortex and a 10-fold serially dilution, samples were cultured on the respective culture medium as described above. Leaves samples were directly cultured on each respective culture medium. To ensure the efficacy of the sterilization step, 100µL of the last wash solution of each condition was cultured on the respective medium. For both analyses, colonies were counted after 48 to 72h of incubation at 28°C. Data related to the microbial densities by using CFU were transformed to logarithmic values before analysis.

Going forward, for the (b) molecular analysis, roots and leaves from each condition and sampling time were collected. Roots were gently rinsed in sterile distilled water, dried and then stored at -80°C until processed. The methodology applied for molecular analysis is fully described posteriorly in the molecular validation of the BCAs strains colonization in the molecular validation subsection from material and methods.

To address the (c) microscopy analysis, both fresh roots and leaves surfaces were observed on a three-dimensional (3D) microscope VHX-2000 (Z100x100) and photographed with an automatic photographic system 3D VHX-H3M. To avoid traces of strains suspensions in roots and leaves, samples were gently rinsed in sterile distilled water before observations.

<u>Greenhouse bioassay</u>

General overview of the greenhouse assay

To address the biocontrol potential of *Streptomyces* sp. Fito_S127B and *A. pullulans* Fito_F278 against *D. seriata* F98.1 (Robert- Siegwald *et al.,* 2017), a Botryosphaeria dieback agent, a greenhouse assay was carried out across three different vegetative seasons, namely from 2014 to 2016. Experiments consisted on an artificial inoculation of the plant's green stems with a plug containing the pathogen' mycelium or a PDA plug (control treatments). *D. seriata* F98.1 was isolated from symptomatic vines of Syrah variety in the Pyrénées Orientales, France. A total of 6 experimental conditions were tested, namely: (a) control plants (without pathogen), (b) plants inoculated with

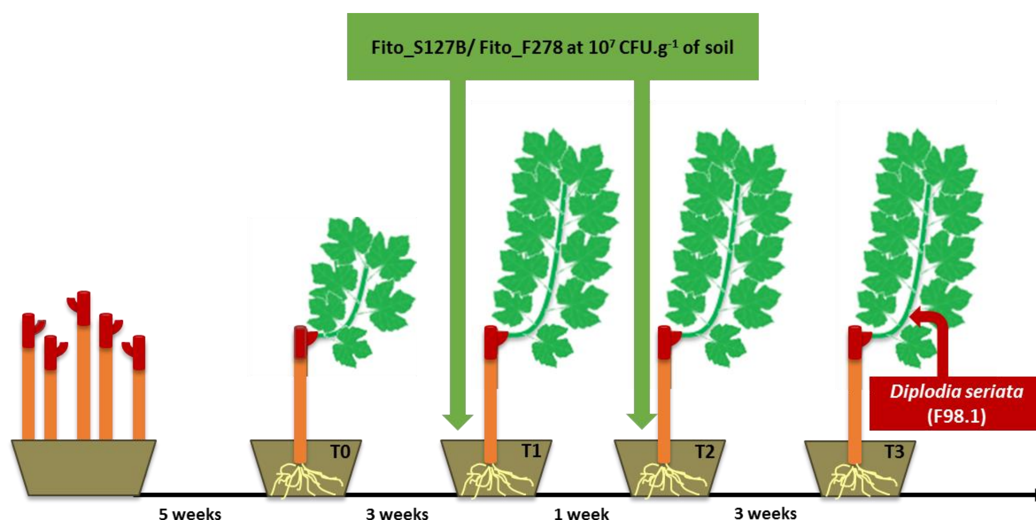


Figure 1: General overview of the cutting model (Spagnolo et al., 2017) applied in the greenhouse bioassays. Tests were carried out with *V. vinifera* cv. Chardonnay in order to address the interaction and biocontrol potential of *Streptomyces* sp. Fito_S127B and *A. pullulans* Fito_F278 against *D. seriata* F98.1, a Botryosphaeria dieback agent. Cuttings were planted in commercial soil. After 5 weeks (T0), cuttings were individually transferred for new pots containing 250g of commercial soil. Strains inoculations were performed at T1 and T2 through a soil inoculation at an approximately concentration of 1x10^{7} CFU.g^{-1} of soil. After three weeks (T3), the pathogen was artificially inoculated at green stems, namely in the third internode.

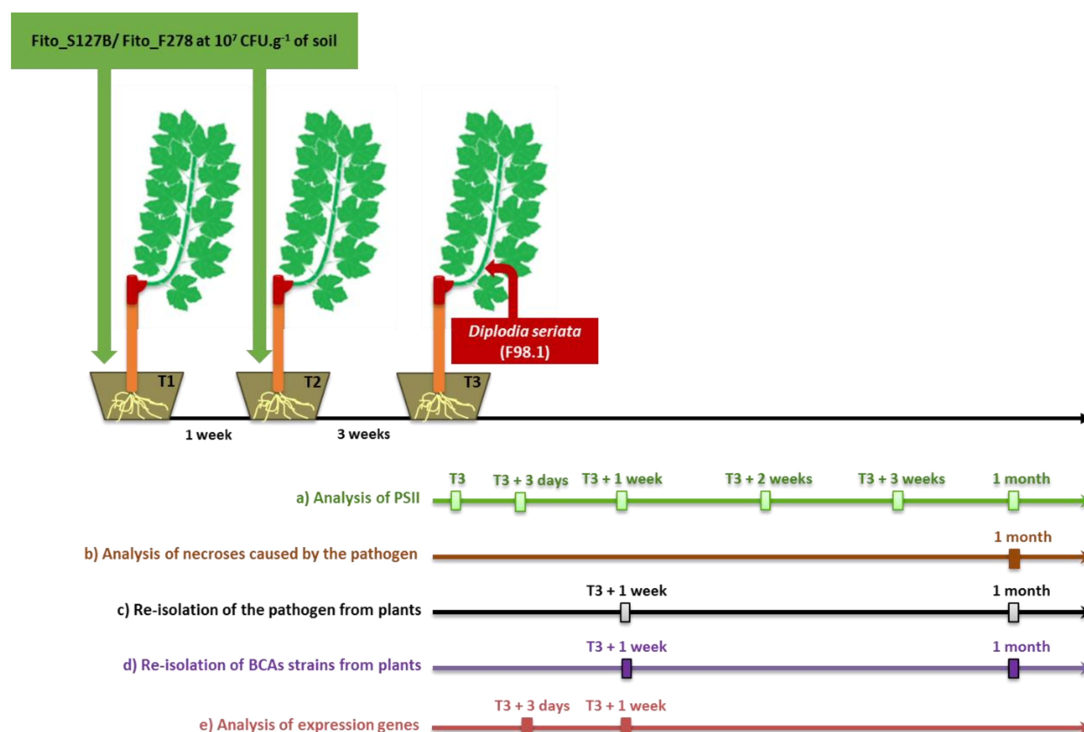


Figure 2: General overview of the parameters analysed after artificially plants inoculation and their respective time schedule. A total of five parameters were analysed after the artificial inoculation of the plants with pathogen. The analysis was as follows: analysis of PSII; b) analysis of the necrotic lesions length of green stems caused by the pathogen; c) re-isolation of the pathogen from green stems; d) re-isolation of BCAs from cutting plants; and e) analysis of the gene expression of eight selected genes. For each parameter, the time schedule is indicated in the figure.

Fito_S127B, (c) Fito_F278, (d) *D. seriata* F98.1, (e) *D. seriata* F98.1 + Fito_S127B and (f) *D. seriata* F98.1 + Fito_F278. During the 2016 growing season, two extra conditions were added, namely (g) plants co-inoculated with both Fito_S127B + Fito_F278 and (h) plants inoculated with *D. seriata* F98.1 + Fito_S127B + Fito_F278. Herein, the Fito_S127B was inoculated at the soil level, while Fito_F278 inoculated at leaves. This was a preliminary assay that aimed to understand the effect of combining different BCAs on grapevine protection and to compare results with individually inoculations.

Overall, a total of ten biological replicates was carried out for each condition, except for 2016 growing season where twelves replicates were applied.

Determination of the BCAs growth and inoculum preparation

The BCAs growth and inoculum preparation was carried out as previously described in the determination of strains growth and inoculum preparation at the colonization bioassay methodology. The strain concentration was adjusted to approximately $1x10^7$ $CFU.g^{-1}$ of soil, for both strains. Measures were based on the OD at 600 nm and cell concentrations calculated as previously described in Chapter 3.

Plant material, growth conditions and inoculation

Vine branches were firstly collected from *V. vinifera* cv. Chardonnay plants aged of 31 years old at the Moët & Chandon vineyard located at Epernay, France. Then, branches were sectioned into cuttings comprising 3 buds and disinfected in a 0.05% cryptonol bath for 4h at 28°C. After that, a growth hormone, namely 0.0035% 2.5-dichlorobenzoic acid, was applied to the upper bud to protect cuttings from drying and to keep their activity. Cuttings were stored at 4°C and protected from the light until their use. Cuttings were then emerged in a 0.05% cryptonol bath to allow their rehydration and disinfection. After proceeding with an approximately 5 mm section at the lower end of cuttings these were emerged into a 1-butyric acid solution (AIB) at 1 $g.L^{-1}$ for 30s to promote the root formation. Cuttings were planted in commercial soil (Figure 1) and placed in a greenhouse chamber. The greenhouse conditions were as follows: temperatures of 24°C during the day and 18°C during the night and a relative humidity of 60%. The general methodology of vines inoculation is presented on Figure 1. Thus, after five weeks (T0), cuttings were individually transferred for new pots containing 250g of commercial soil. Then, after three weeks, strains were firstly individually inoculated (T1) at the soil level by applying 30mL of strain solution at a concentration of $1x10^7$ $CFU.g^{-1}$ of soil. A second strain inoculation (T2) was repeated after one week and under the same conditions. Given the two extra tested conditions in 2016, 30mL of Fito_S127B ($1x10^7$ $CFU.g^{-1}$ of soil) was inoculated at soil, while 5mL of Fito_F278 ($1x10^6$ CFU/mL) was inoculated at leaves by applying a spray method. To prevent the rapid drying of Fito_F278 after its foliar inoculation, all co-inoculated plants were placed individually

in sterile protective plastic bags, which were then removed after 15 days from the first plant inoculation.

Going forward, three weeks after the second BCA inoculation, a plug containing the pathogen or a PDA plug (control treatments) was individually inoculated in green stems (T3), according to the cutting model described by Spagnolo *et al.,* (2017). For this, each cutting stem was firstly surface-sterilized with a cotton soaked with 70% alcohol before infection. Then, stems were longitudinally wounded at the third internode and a 5-mm plug containing the pathogen mycelium, with 5 day-old, was here inoculated. Control plants (without pathogen inoculation) were inoculated with a 5-mm PDA plug.

Treated stems were covered with a humid Parafilm® to ensure an effective infection which was then removed after 15 dpi.

Characterization of the interaction between grapevine-BCAs-phytopathogen

After the artificial inoculation of the grapevines with pathogen, a total of five different parameters were analysed (Figure 2), namely: a) analysis of PSII; b) analysis of the necrotic lesions length of green stems; c) re-isolation of the pathogen from green stems; d) re-isolation of BCAs from cutting plants; and e) analysis of the gene expression of 8 selected genes. The methodology applied for each analysis is shown below.

a) Analysis of photosystem II (PSII)

Herein, measurements of the chlorophyll *a* fluorescence levels, namely the activity of photosystem II (PSII), a sensitive and reliable marker of the plant's early responses to stresses (Chapin III *et al.,* 1993; Letousey *et al.,* 2010), was determined by using the pulse amplitude modulated (PAM) fluorometers. The optimal quantum yield of PSII electron transport (ϕPSII) was automatically calculated by the formula ($[F_m - F_0]/F_m$), where F_0 is the minimal fluorescence and F_m the maximal fluorescence (Genty *et al.,* 1989). This indicated the amount of light absorbed by the chlorophyll associated with the PSII (Petit *et al.,* 2006). Thus, a decline of ϕPSII may be associated with a down-regulation of the electron transport (Nogués *et al.,* 2002; Petit *et al.,* 2006). Measures were performed in all cutting plants at the first (L1) and forth leaf (L4) above the point of inoculation (PI) with the pathogen or PDA plug (control), at T3, T3+3 days, T3+1 week, T3+2 weeks, T3+3 weeks and T3+4 weeks.

b) Analysis of the necrotic lesions length of green stems

The observations of wood lesions were carried out after 1 month (T3+4 weeks) of the pathogen inoculation. For this, the area of wounds at the PI was calculated by multiplying the length and width of necrosis. Measures were performed for all plants and results were expressed by mean ± standard error of the mean (SEM).

c) Re-isolation of the pathogen from green stems

Green stems artificially inoculated with the pathogen were used for the re-isolation tests at T3+1 week and T3+4 weeks, as described by Larignon and Dubos (1997). For this, for each timepoint four plants of each condition (both inoculated or not with pathogen) were analysed. Plant woods were firstly passed into the flame, for a surface sterilisation, and then cut longitudinally. Six necrotic pieces of wood per plant were plated into malt extract agar (MEA, 20 $g.L^{-1}$) supplemented with 0.015% of sulfate streptomycin and incubated at 24°C for a minimal of 7 days. At T3+1 week, only the PI was analysed while at T3+ 4 weeks, isolations were carried out from the PI, 1cm above the PI (PI + 1 cm) and 1cm below the PI (PI – 1cm). Fungal isolates were then analysed based on their morphology and their identity were confirmed by molecular techniques, namely through sequencing of ITS region.

d) Re-isolation of BCAs from cutting plants

To address if the BCAs were still present and alive in grapevines after one (T3+1 week) and two months (T3+4 weeks) of their inoculation in plants, different grapevine samples, such as soil, roots and leaves, were simultaneously analysed by classic microbiology methods and by molecular analysis, through DNA extraction and amplification with BCAs-specific primers. The rhizosphere was analysed only by classic microbiology methods.

Given the classic microbiology techniques, and considering soil samples, 0.1g of soil was weighted and 0.9mL of sterile distilled water added and gently vortexed. Then 10-fold serially dilutions were performed and 100µL were cultured on the respective culture medium namely, LB for control and *D. seriata* F98.1 conditions, MyM for Fito_S127B and *D. seriata* F98.1 + Fito_S127B conditions and YPD for Fito_F278 and *D. seriata* F98.1 + Fito_F278 conditions. Rhizosphere was analysed by emerging 0.1g of roots in 0.9mL of sterile distilled water and gently vortexed. After 10-fold serially dilutions, 100µL were cultured on the respective culture medium as described above. Regarding roots and leaves, these samples were surface sterilised to analyse the endophytic colonisation. This methodology was carried out only with the classic microbiology methods. The sterilization process was as follows: 70% ethanol for 1 min, followed by 0.6% sodium hypochlorite for 3 min and washed four times in sterile distilled water. Samples were then ground in 1mL of PBS pH7.5 and macerated with a sterile pestle. A 10-fold serially dilution was performed for roots while leaves were plated directly in the respective culture media. To ensure the efficacy of the sterilization step, 100µL of the last wash solution of each treatment was cultured on the respective culture medium. Isolates were counted after 48 to 72h of incubation at 28°C. Overall, three plants were analysed for each condition and timepoint and two replicates were carried out for each sample or dilution. The obtained isolates were then analysed by their morphology and their identity confirmed by molecular techniques, namely sequencing of 16S rDNA or ITS region or amplification with strain-specific primers.

Table 1: Primers of genes analysed by real-time reverse – transcription polymerase chain reaction (Spagnolo *et al*., 2014; Spagnolo *et al*., 2017).

Matrix	Function	Gene	Primer Sequences	Genbank or TC TIGR* Accession number
Green stem	Housekeeping genes	*ADH2* (Alcohol dehydrogenase 2)	5'- GACCATGTTCTTCCTGTATTCAC-3' 5'- GTAGCACCAAGACCTGTAGAG-3'	XM_002281263.1
		60SRP (60S Ribosomal Protein L18)	5'- ATCTACCTCAAGCTCCTAGTC -3' 5'- CAATCTTGTCCTCCTTTCCT -3'	XM_002270599
	Phenylpropanoid metabolism	*STS* (Stilbene sythase)	5'- AGGAAGCAGCATTGAAGGCTC -3' 5'- TGCACCAGGCATTTCTACACC -3'	FJ851185
	Detoxification and stress tolerance	*Hahl* (Haloacid dehalogenase hydrolase)	5'- CCCTCAGGATAGCCAACATCA -3' 5'- AGGTGCCAACCAGAACTGTGT -3'	XM_002270599.1
		HSP (alpha crystalline heat shock protein)	5'- TCGGTGGAGGATGACTTGCT -3' 5'- CGTGTGCTGTACGAGCTGAAG -3'	XM_002272382
	Defense proteins	*Gluc* (β-1,3 glucanase)	5'- TCAATGGCTGCAATGGTGC -3' 5'- CGGTCGATGTTGCGAGATTTA -3'	DQ267748
	Aquaporin	*PIP2.2* (aquaporin plasma membrane intrinsic protein 2-2)	5'- GGTTCAGTCTCCATTGCACATG -3' 5'- TTGGCAGCACAGCAGATGTAT -3'	XM_002271336
	Cell wall compounds	*fascAGP* (fasciclin-like arabinogalactan protein)	5'- CGAAACCCCAAAGCCTAAGAA -3' 5'- GAAAACACAAAGGGGTTGCA -3'	XM_002280793.2
Leaves	Housekeeping genes	*EF1* (EF1-α elongation factor)	5'- GAACTGGGTGCTTGATAGGC -3' 5'- AACCAAAATATCCGGAGTAAAAGA -3'	GU585871
		39SRP (39S Ribosomal protein L41-A)	5' GACTGACTTCAAGCTTAAACC -3' 5' GATATAACAGGGAATACAGCAC-3'	XM_002285709.1
	Detoxification and stress tolerance	*Hahl* (Haloacid dehalogenase hydrolase)	5'- CCCTCAGGATAGCCAACATCA -3' 5'- AGGTGCCAACCAGAACTGTGT -3'	XM_002270599.1
		GST5 (Glutathione S-transferase 5)	5'- GCAGAAGCTGCCAGTGAAATT -3' 5'- GGCAAGCCATGAAAGTGACA -3'	XM_002277883
	Defense proteins	*Gluc* (β-1,3 glucanase)	5'- TCAATGGCTGCAATGGTGC -3' 5'- CGGTCGATGTTGCGAGATTTA -3'	DQ267748
		PR6 (Serine-protease inhibitor 6)	5'- AGGGAACAATCGTTACCCAAG -3' 5'- CCGATGGTAGGGACACTGAT -3'	AY156047

*Available at http://www.jcvi.org/cms/research/projects/tdb/overview/

Regarding the molecular analysis, soils, roots and leaves from each condition were collected. For the molecular analysis, both epiphytic and endophytic population was analysed. Roots were gently rinsed in sterile distilled water, dried and then all samples were stored at -80°C until processed. The methodology applied for molecular analysis is fully described posteriorly in the molecular validation of the BCAs strains colonization of cuttings plants subsection from material and methods. Overall, three plants were analysed for each condition and timepoint. Then, each sample type was pooled together before DNA extraction.

e) RNA extraction, Real-Time RT-PCR and gene expression profile

The analysis of gene expression was carried out for green stems and leaves samples. Given the leaf samples, the first (L1) and the fourth leaf (L4) above the PI were collected from each treatment at T3+3 days and T3+1 week. Leaves were collected separately, immediately frozen in the field with liquid nitrogen and stored at -80°C until use. Green stems were collected upward the PI at T3+1 week and stored as previously refereed. A total of four plants were analysed per condition and timepoint. Before RNA extraction, samples from each condition were polled together and grounded to a fine powder in liquid nitrogen. Both L1 and L4 were grounded separately.

Total RNA extraction was carried out from 50mg of powdered leaves or 2 x 50mg of powdered green stems by using the Plant RNA Purification Reagent (Invitrogen, France). The RNA pellet was re-suspended in 15µL (leaves) or 2 x 8µL (green stems) of RNase free water and then treated with RD1 DNase enzyme (Promega Corp., Madison). The RNA integrity was checked by 0.8% agarose gel electrophoresis and the quantity determined by measuring the absorbance at 260 nm (Biowave DNA Spectrophotometer, Biochrom WPA). The RNA concentration was then adjusted to 100 ng/µL.

The reverse transcription was carried out on 150ng of total RNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific, Inc.) and the reaction was carried out under the conditions: 30 min at 42°C follow with 2 min at 95°C. After that, a PCR reaction for the amplification of the actine from vine was carried out to check the success of the reverse transcription. The reaction was performed as follows: 94°C for 3 min, (94°C for 30s, 60°C for 30s and 72°C for 30s) x 30 cycles followed by a final extension at 72°C for 10 min. The DNA amplification and integrity was checked by 1% agarose gel electrophoresis.

The Real-time PCR was then carried out by using the ABsolute Blue qPCR SYBR Green ROX mix (Thermo Fisher Scientific Inc.), according to the manufacturer's protocol, and in the CFX96 thermocycler system (Bio-Rad, Hercules, CA, USA). All reactions were carried out in duplicate in 96-well plates (15 µL per well) containing 7.5µL of 1x SYBR Green I mix (including Taq polymerase, dNTPs and SYBR Green dye), 1.4µL of forward and reverse primers (at 3µM) (Table 1), 1.1µL of MiliQ water and 5µL of a 1:10 dilution of reverse transcript RNA. Negative controls consisted of Mili-Q water.

Conditions consisted of the following thermal profile: denaturation at 95°C for 15s and amplification with 95°C for 10s and 60°C for 45s for 40 cycles. The melting curves were performed from 65-95°C at 0.5° $C.s^{-1}$, and allowed to address the specificity of each amplification. Results were analysed with CFX Manager Software version 3.0 (Biorad Laboratories). The relative gene expression was determined with the formula fold induction $2^{-\Delta\Delta Ct}$, where ΔΔCt = (Ct GI [unknown sample]- Ct GI [reference sample]) – (Ct reference genes [unknown sample]- Ct reference genes [reference sample]). The GI is the gene of interest. Results were normalized with reference genes, namely *60SRP* and *ADH2* for stems and *EF1a* and *60SRP* for leaves, and expressed in relation to the control sample where have a 1x expression of the interest gene. Overall, results were expressed as mean ± standard deviation (SD) of three independent experiments, realized in duplicates. The relative expression of the genes is considered up- or down-regulated when changes in their expression were >2-fold or <0.5-fold, respectively. The target genes used for analysis are listed in Table 1.

Molecular validation

Molecular identification of fungal isolates

The genomic DNA was extracted by applying the CTAB method. Briefly, fungal isolates were firstly growth in PDA plates at 25°C until 7 days. Then, cell walls of fungal mycelia were broken down by using glass beads (180µm, Sigma) in an Eppendorf tube. The CTAB 2x extraction buffer was added, samples were centrifuged at 5,000 x g for 10 min at 4°C, and the supernatant collected into a new tube. This step was repeated and then samples were incubated at 65°C for 1h in a bath with shaking. After centrifugation at 13,000 rpm for 5 min at room temperature (RT), the supernatant was collected into a new tube, chloroform was added and tubes were homogenized by inversion and centrifuged under the same conditions as referred above. The DNA was precipitated with cold isopropanol (-20°C), carefully homogenized, and stored at -20°C at least 2h or overnight. After centrifugation at 13,000 rpm for 30 min at 4°C, DNA pellet was washed with cold 70% ethanol and centrifuged at 7,000 rpm for 5 min at 4°C. DNA pellet was then allowed to dry and DNA was dissolved in 35µL of sterile Mili-Q water. The DNA quantity was measured through the absorbance at 260 nm (Biowave DNA Spectrophotometer, Biochrom WPA).

The ITS region was amplified by using the ITS4 (5'- TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG) primers (White *et al.,* 1990). PCR reaction was carried out in 25µL reaction mixture containing 1x Dream Taq buffer with $MgCl_2$ (Thermo Fisher Scientific), 0.2mM dNTPs mix (Thermo Fisher Scientific), 0.5mM $MgCl_2$, 1.25U of Dream Taq DNA Polymerase (Thermo Fisher Scientific), 0.2µM of forward and reverse primers and 2µL of genomic DNA. The reactions conditions

were: 94°C for 3 min, (94°C for 30s, 50°C for 30s and 72°C for 30s) x 30 cycles followed by a final extension at 72°C for 7 min. The amplified PCR products were visualized on a 0.8% (w/v) agarose gel stained with ethidium bromide and the nucleic acid concentration and quality was achieved by using the NanoDrop spectrophotometer (Thermo Scientific, USA). PCR products were then purified with Illustra Exostar kit (GE Healthcare Life Sciences) and sequenced using the 3500 Genetic Analyser (Applied Biosystems) at Biocant, Portugal. The obtained sequences were identified using the BLAST search against NCBI database.

Molecular identification of bacterial and yeasts isolates obtained from cutting plants

The genomic DNA of bacterial and yeasts isolates were extracted by using the Wizard Genomic DNA Purification kit (Promega, Madison, USA), following the standard protocol for bacteria or yeasts, respectively. The DNA quantity was measured through the absorbance at 260 nm (Biowave DNA Spectrophotometer, Biochrom WPA).

A first PCR amplification was carried out to amplify a barcoding region, namely 16S rDNA and ITS region for bacterial and yeasts isolates, respectively. Thus, the 16S rDNA was amplified by using the 27F (5'-AGAGTTTGATCACTGGCTCAG-3') and 1492R (5'-TACGGCTTACCTTGTTACGACTT-3') primers, and the ITS region was amplified by using the ITS4 (5'- TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG) primers (White *et al.,* 1990). Distinct PCR reactions were carried out for each region. Thus, PCR reactions were carried out in 25µL reaction mix containing 1x Dream Taq buffer with $MgCl_2$ (Thermo Fisher Scientific), 0.2mM dNTPs mix (Thermo Fisher Scientific), 0.2µM of forward and reverse primers, 1.25U of Dream Taq DNA Polymerase (Thermo Fisher Scientific) and 2µL of genomic DNA. PCR reactions for detection of yeasts isolates also contained 0.5mM $MgCl_2$. Cycling conditions consisted in a first denaturation step at 94°C for 4 min or 94°C for 3 min for 16S rDNA and ITS region, respectively which were then followed by 30 cycles with a denaturation step at 94°C for 30s, annealing at 50°C for 30s and an extension at 72°C for 30s. A final extension cycle at 72°C for 5 min or 72°C for 7 min was applied for 16S rDNA and ITS region, respectively. The amplified PCR products were visualized on a 0.8% (w/v) agarose gel and isolates with interest were sequenced for their molecular identification. The sequencing conditions were the same as previously described on the molecular identification of fungal isolates subsection.

Then, a second PCR amplification was carried out for each isolate by using specific primers of Fito_S127B and Fito_F278 strain. This approach allowed us to quickly identify whether these bacterial and yeasts isolates would be the BCAs strains previously inoculated in the cutting plants. PCR reactions are fully described in the subsection molecular approach to follow-up the BCAs colonization across grapevine plantlets.

Table 2: Strain-specific primers.

Strain	Target gene	Primer code	Primer sequences	Amplification size (bp)
Fito_S127B (*Streptomyces* sp.)	*trpB* (tryptophan synthase beta chain)	trpB_FS127B	5' - GTACCGCCTCGTCGTACAG -3'	480
		trpB_RS127B	5'- GAACCTGGATTTCGCGGGG- 3'	
	hpB (helix-turn-helix protein domain)	hpb_FS127B	5'- CTGCGAATTCGGTGAGGGC- 3'	250
		hpb_RS127B	5'- GGGTGAACTTTCCGTACGC- 3'	
	HxIR (helix-turn-helix protein domain)	HxIR_F127B	5'- CGTGCTTGGCGAGGATGC- 3'	200
		HxIR_RS127B	5'- TGGCCGCCACGAAGGATC- 3'	
Fito_F278 (*A. pullulans*)	*trp α* (tryptophan synthase subunit alpha)	trp_F(F278)	5'- CTATCGCCCTGAAGAACG- 3'	400
		trp_R2(F278)	5'- GATCTGGGAGTGCAGTGT- 3'	
	GST (glutathione S-transferase)	GST_F(F278)	5'- GCTGACCGCAATTCGCATAC- 3'	800
		GST_EF1R(F278)	5'- GTTGCTCATGAAGGTGAGGG- 3'	
	EF1 (elongation factor 1)	EF1TU_F(F278)	5'- CTCTACAGGAAGCCGCCG- 3'	500
		EF1TU_R(F278)	5'- CAAGGGCAGTTGGAGCTG- 3'	
	Tub (tubulin)	Tub_F(F278)	5'- GTTCCGGCTTGGGCTCATAC- 3'	300
		Tub_R2(F278)	5'- GCGGACATGACTGTGCTGAC- 3'	
Fito_F321 (*B. amyloliquefaciens*)	*trpB* (tryptophan synthase beta chain)	trpB_F321	5'- CAAAAGTGGTGCCAGTCTTA- 3'	200
		trpB_R321	5'- CGGCTTTGCGGAGCTGTTC- 3'	

Table 3: Strain-specific primers used in the nested-PCR amplifications.

Strain	Target gene	Primer code	Primer sequences	Amplification size (bp)
Fito_S127B (*Streptomyces* sp.)	*HxIR* (helix-turn-helix protein domain)	HxIR_F1 ext.	5'- GACGTGCTCCACGACCATC- 3'	350
		HxIR_RS127B	5'- TGGCCGCCACGAAGGATC- 3'	
		HxIR_FS127B	5'- CGTGCTTGGCGAGGATGC- 3'	150
		HxIR_R1 int.	5'- CTGGTCGGCGAGAAGTAC- 3'	
Fito_F278 (*A. pullulans*)	*GST* (glutathione S-transferase)	GST_F(F278)	5'- GCTGACCGCAATTCGCATAC- 3'	800
		GST_EF1R(F278)	5'- GTTGCTCATGAAGGTGAGGG- 3'	
		GST_F2(F278)	5'- CTGTCGGTGCCCTTGAGGA- 3'	500
		GST_EF1R1(F278)	5'- CGTCGTTGTACTTGTAGTCC- 3'	

Design of strain-specific primers for BCAs identification based on whole genome data

The whole genome sequencing (WGS) of BCAs previously presented in Chapter 3 allowed not only to carry out a genome analysis of each potential selected BCAs but also to design strain-specific primers for both inter- and intra- species analysis. For this, after exploring the genome of each strain on Artemis Version 16.0 software, a group of genes were retrieved, subjected to a BLASTn search on the NCBI platform and sequences were extracted from the corresponding WGS for a more detailed analysis. Thus, the selected nucleotide sequences for Fito_S127B were *hpb* and *HxlR* (helix-turn-helix protein domain) and *trpB* (tryptophan synthase beta chain); and for Fito_F278 were *EF1* (elongation factor 1), *GST* (Glutathione S-transferase C), *trpα* (tryptophan synthase subunit alpha) and *Tub* (tubulin). Although in this study the *in vivo* results for Fito_F321 strain were not presented, the nucleotide sequence in analysis was *trpB* (tryptophan synthase beta chain). The retrieved single gene sequences were imported to BioEdit version 7.2.5 (Hall, 1999) for sequence alignment in order to search discriminative nucleotides. Strain-specific primers were designed and their properties were analysed with Oligo Analyzer version 1.5 (Gene Link). The target genes are presented in Table 2.

After a PCR optimization, the sensitivity and specificity of each primer set was determined. The sensitivity analysis was tested using a set of target strains for detection, while PCR specificity was achieved by examining the potential of these primers to detected and distinguish *Streptomyces* sp. Fito_S127B and *A. pullulans* Fito_F278 against 6 and 14 non-target strains, respectively. The best strain-specific primers were then selected for each strain.

Molecular approach to follow-up the BCAs colonization across grapevine plantlets

The genomic DNA of roots and leaves were extracted by using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and final DNA was eluted in 50µL of Buffer AE. The molecular identification of Fito_S127B strain was carried out through the amplification of *HxlR* gene by using the HxlR_FS127B and HxlR_RS127B primers (Table 2), and Fito_F278 identified through the amplification of the *GST* gene with GST_F(F278) and GST_EF1R(F278) primers (Table 2). PCR reactions were carried out in 25µL reaction mix containing 1x Dream Taq buffer with $MgCl_2$ (2 mm) (Thermo Scientific, US), 0.2mM dNTPs (Thermo Scientific, US), 0.2 µM of each primer, 1.25 U of Dream Taq DNA polymerase (Thermo Scientific, US) and 2 µL of genomic DNA. PCR reactions for detection of Fito_S127B also contained 10% DMSO. Cycling conditions consisted in a first denaturation step at 94°C for 4 min followed by 30 cycles with a denaturation step at 94°C for 30s, annealing at 58°C (Fito_F278 strain) or 60°C (Fito_S127B) for 30s and an extension at 72°C for 45s. A final extension cycle at 72°C for 5 min was applied. The amplified PCR products were visualized on a 0.8% (w/v) agarose gel stained with ethidium bromide.

Molecular approach to follow-up the BCAs colonization across cutting plants

The genomic DNA of soils, roots and leaves from cutting plants were extracted by using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The final DNA of leaves was eluted in 50µL of Buffer AE, while DNA from soil and roots was eluted in 100µL. The molecular confirmation of BCAs strains among cutting plants was carried out by a nested-PCR reaction, using a set of strain-specific primers (Table 3). Both PCR reactions were carried out in a 25 µL reaction mix. Before the PCR amplifications, the genomic DNA from soil samples was diluted (1:10 or 1:100) and treated with 10% PVPP.

The first PCR reaction contained 1x Dream Taq buffer with $MgCl_2$ (2 mm) (Thermo Scientific, US), 0.2mM dNTPs (Thermo Scientific, US), 0.2µM of each primer (Fito_S127B strain: HxIR_F1 ext. and HxIR_RS127B; Fito_F278: GST_F(F278) and GST_EF1R(F278)), 1.25 U of Dream Taq DNA polymerase (Thermo Scientific, US) and 0.5µL of genomic DNA. PCR reactions for detection of Fito_S127B strain also contained 10% DMSO and reactions for Fito_F278 detection contained 0.5mM $MgCl_2$. Cycling conditions consisted in a first denaturation step at 94°C for 4 min followed by 20 cycles with a denaturation step at 94°C for 30s, annealing at 58°C (Fito_F278 strain) or 60°C (Fito_S127B) for 30s and an extension at 72°C for 45s. A final extension cycle at 72°C for 5 min was applied. The amplified PCR products were visualized on a 0.8% (w/v) agarose gel stained with ethidium bromide, to verify the amplification of the positive control (C+: containing gDNA from pure BCAs colonies) and the non-contamination of the negative control (C-: containing sterile water instead of gDNA samples). Then, 0.5µL of the PCR product from the first PCR reaction was used directly as template for the second PCR reaction. PCR amplification mix was the same as described above, with exception of the set of primers used (Fito_S127B strain: HxIR_FS127B and HxIR_R1 int.; Fito_F278: GST_F2(F278) and GST_EF1R1(F278)). The target amplification was initiated at 94°C for 4 min followed by 30 cycles with a denaturation step at 94°C for 30s, annealing at 57°C (Fito_F278 strain) or 58°C (Fito_S127B) for 30s and an extension at 72°C for 45s. A final extension cycle at 72°C for 5 min was applied. Amplified PCR products were then analysed on a 0.8% (w/v) agarose gel stained with ethidium bromide.

Statistical analysis

To determine whether necrotic lesions length and PSII of cutting plants inoculated with *D. seriata*, *D. seriata* + Fito_S127B and/or Fito_ F278 and Fito_S127B and/or Fito_F278 were significantly different from control plants, a one-way analysis of variance (ANOVA), followed by Bonferroni *post hoc* analysis were carried out by using the GraphPad Prism version 5.01 software. In cases of the null hypothesis was rejected, the non-parametric test Kruskal-Wallis followed by the Dunn's multiple comparison test was carried out. Differences at $p<0.05$ were considered significant. For a better analysis of PSII, the outliers were removed and results were presented as means ± SD for the three growing seasons

studied (2014, 2015, 2016). Necrotic lesions length results were expressed as mean ± SEM for each growing season.

Data related to the microbial densities by using CFU were transformed to logarithmic values before statistical analysis. The methodology applied was the same as previously described for necrotic lesions length and PSII analysis.

Results

Colonization bioassay

Colonization of *in vitro* plants of grapevine by BCAs strains

To address the plant-microbial interactions and plant colonisation by the selected BCAs, namely *Streptomyces* sp. Fito_S127B and *A. pullulans* Fito_F278, roots of plantlets cv Chardonnay were dipped in a strain solution ($3x10^8$ CFU/mL and $1x10^6$ CFU/mL for Fito_S127B and Fito_F278, respectively) and then allowed to grow under *in vitro* conditions, in a photoperiod chamber. Assays showed that Fito_S127B was able to colonize superficial roots (Figure 3A) and was not detected at leaves. Though, the CFU count at root surfaces decreased considerably (approximately 8,500-fold) when compared with the initially strain solution inoculated. Overall, this colonisation remained stable over time with an average of 10^4 CFU/mL or 4.48 ± 0.14 log CFU/g FW. Meanwhile, it was observed that in certain circumstances, Fito_S127B was detected in the internal tissues of roots. These observations occurred at 7 dpi (10^2 CFU/mL) and 14 dpi (10^1 CFU/mL) (data not shown), though this colonisation was not systematic. A further deep microscopy analysis of roots (Figure 5) showed rupture of root tissues which may explain this colonisation. Fito_F278 (Figure 3B) was detected at root surfaces (10^7 CFU/mL), internal root tissues (10^4 CFU/mL) and leaf surfaces (10^4 CFU/mL) at 4dpi. The CFU count at root surfaces increased 74-fold (10^7 CFU/mL), when compared to the initially strain inoculation. At 7 dpi, the CFU count was similar for both internal root tissues and leaf surfaces, while an increase of 13-fold was detected at root surfaces. At 14 dpi, was observed a general decrease of the microbial strain density over all tissues in analysis. Fito_F278 was also able to colonise the internal tissues of leaves, even if in very low quantities (data not shown), though this colonization was not systematic.

In addition, results showed that these BCAs impacted on the plantlet performance and health status (Figure 4). Thus, plantlets inoculated with Fito_S127B showed a similar or even a better growth performance when compared with the non-inoculated plantlets (control). Indeed, the microscopy analysis showed that the development of plantlets roots inoculated with this strain was higher when compared with control, and with a higher number of secondary roots (Figure 5). Furthermore, rupture

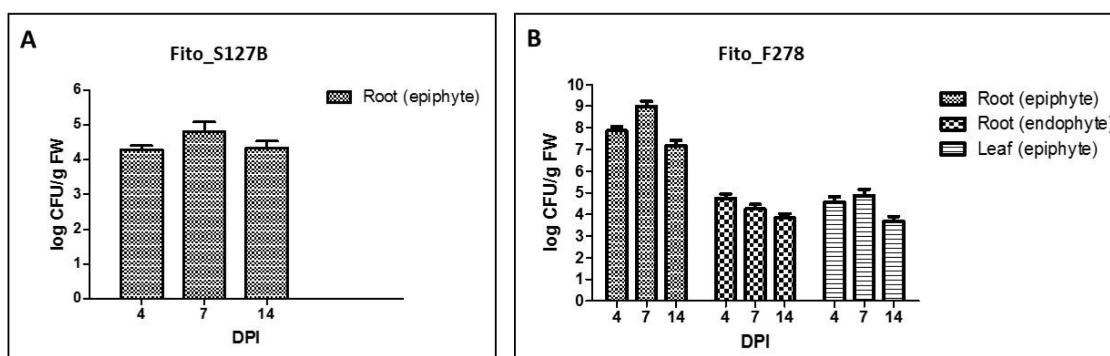


Figure 3: Analysis of (A) Fito_S127B and (B) Fito_F278 colonisation of *in vitro* plants of *Vitis vinifera* cv. Chardonnay at 4, 7 and 14 days' post-inoculation (dpi). The CFU count was log-transformed and values are means ± SEM. FW, fresh weight. No significant differences on CFU count were obtained at 4,7 and 14 dpi for each plant tissue in analysis.

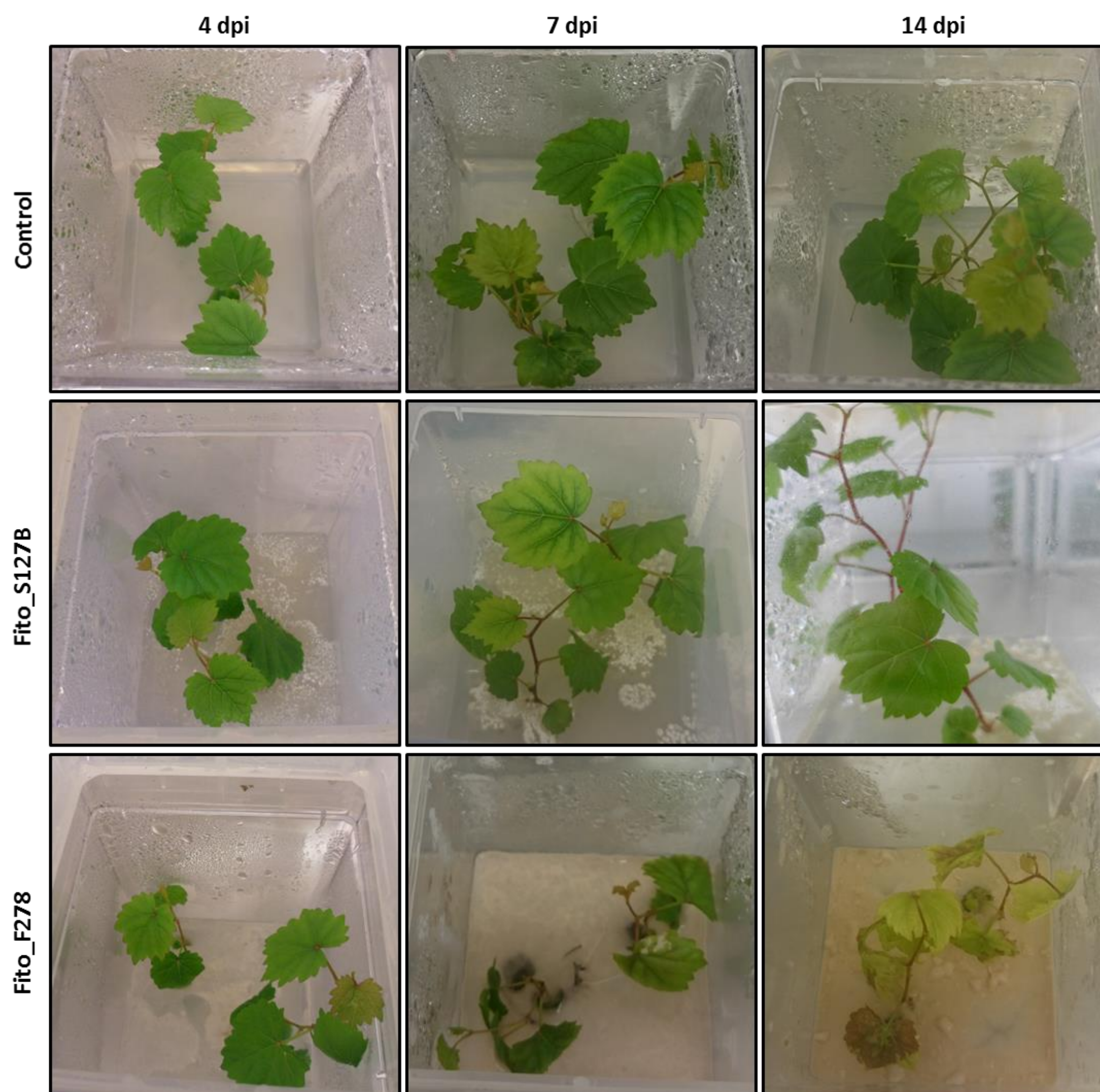


Figure 4: I*n vitro* plants of *V. vinifera* cv. Chardonnay inoculated or not with BCAs. Control plants (without inoculation) and plants inoculated with Fito_S127B and Fito_F278 strains were followed after 4, 7 and 14 days' post-inoculation (dpi).

of root tissues was observed at 7dpi, which may have constituted an entry channel of this strain to the internal root tissues. The morphology of leaves from these plants was similar to those of control plants (Figure 5).

Contrarily, plants inoculated with Fito_F278 showed a negative impact on plant growth and development (Figure 4). This was clearly observed from 7dpi. Overall, symptoms included fragility and stagnation of the plant growth (Figure 4), a strain biofilm at the plant roots level (Figure 4 and 5A) and a discoloration and spot necrosis on leaves (Figure 5B). Then, the plant health status worsened until 14 dpi. Herein, plantlets were clearly fragile (Figure 4), with short and dark roots and with a general discoloration and spots on leaves (Figure 4 and 5). The nutrients competition between plantlets and strain, together with the high inoculum concentration of Fito_F278, appeared to have a harmful effect on plantlets.

The molecular analysis confirmed the classic microbiology results

To validate the grapevine colonisation by Fito_S127B and Fito_F278 through a molecular assessment, a PCR amplification was performed for roots (Figure 6A and 7A) and leaves (Figure 6B and 7B) at 4, 7 and 14 dpi. Given the Fito_S127B, PCR results (Figure 6) are in line with the re-isolation data (Figure 3A), confirming the presence of this strain only at roots. Herein, plants inoculated with Fito_S127B were subjected to an amplification with Fito_F278-specific primers to confirm the specificity of these primers and to discard the non-cross-contamination of plantlets.

Regarding plants inoculated with Fito_F278, PCR results (Figure 7) are in concordance with the re-isolation data (Figure 3B), except for 4 dpi where Fito_F278 was not detected in leaves by molecular means. As previously achieved, plants inoculated with Fito_F278 strain were also subjected to an amplification with Fito_S127B-specific primers to confirm their specificity and to discard the non-cross-contamination of plantlets (data not shown).

<u>**Greenhouse bioassay: a three years' survey**</u>

The BCAs inoculation did not affect the grapevine photosynthesis

The overall analysis of the PSII over the three growing seasons (2014, 2015 and 2016) showed that the plant inoculation with *D. seriata* (Ds), BCAs and both *D. seriata* + BCAs did not have a significant effect on photosynthesis, when compared with non-inoculated plants (control condition) (Figure 8). Though, at T3+3 days a significant perturbation of photosynthesis was observed for Fito_S127B, Ds, Ds + Fito_S127B and Ds + Fito_F278 conditions, through an increase of the PSII activity.

Figure 5: 3D-microscopic observations of *in vitro* plants cv. Chardonnay inoculated or not with BCAs. Control plants (without inoculation) and plants inoculated with Fito_S127B and Fito_F278 strains were followed after 4, 7 and 14 days' post-inoculation (dpi) for both (A) roots and (B) leaves. The arrow indicates a rupture on the root tissue inoculated with Fito_S127B. Bars = 100 µm.

Similar results were observed on the preliminary assays using a co-inoculation of both BCAs, namely inoculation of Fito_S127B at roots and Fito_F278 at leaves (data not shown). Herein, differences ($p<0.05$) of PSII activity were found for Fito_S127B + Fito_F278 condition at T3+3 days and for Ds + Fito_S127B + Fito_F278 at T3 and T3+3 days.

Fito_S127B reduced the necrotic lesion lengths on green stems caused by *D. seriata*

In the biocontrol assays, green stems (cv. Chardonnay) artificially inoculated with *D. seriata* F98.1 showed typical lesions caused by the pathogen, at the inoculation zone. These results contrasted with plants inoculated with a sterile PDA plug, which only presented a wound due to the intentional lesion caused in green stems. Overall, and as expected, these plants (control, Fito_S127B and Fito_F278 conditions) showed a lower lesions surface length when compared with cuttings containing the pathogen (Figure 9).

Considering only green stems artificially inoculated with *D. seriata*, it was observed that plants inoculated with Fito_S127B showed a lower necrotic lesions length over all growing seasons (Figure 9). Indeed, the mean of lesions length of Ds condition was 0.32 ± 0.02 cm^2 (2014), 0.22 ± 0.02 cm^2 (2015) and 0.36 ± 0.02 cm^2 (2016), while plants inoculated with Fito_S127B showed a mean of lesions length of 0.13 ± 0.02 cm^2 (2014), 0.18 ± 0.02 cm^2 (2015) and 0.32 ± 0.02 cm^2 (2016). Thus, Fito_S127B significantly ($p<0.05$) reduced the necrotic lesion in 2014 by 58.93%. In 2015 and 2016, a decrease was also observed and accounted with a 18.32% and 11.50% of reduction, respectively. Although this was not statistically significant. Overall, and considering the average of the three growing seasons under analysis, Fito_S127B showed a significantly reduction of 29.44% of the necrotic lesion lengths caused by Ds F98.1, suggesting that this strain efficiently protected cuttings against *D. seriata*. Conversely, plants inoculated with Fito_F278 did not show any influence in reducing the necrotic lesions length caused by the pathogen (Figure 9). In any case, and considering the average of the three growing seasons, the mean of lesions length in plants inoculated with Fito_F278 were slightly higher (0.32 ± 0.03 cm^2) when compared with those plants only inoculated with pathogen (0.31 ± 0.02 cm^2).

Given the preliminary results using a co-inoculation of both BCAs, it was observed a significantly ($p<0.05$) reduction of 35.86% of the necrotic lesion lengths, when compared with plants only inoculated with pathogen (data not shown). Indeed, while cuttings co-inoculated with both BCAs showed a mean lesions length of 0.23 ± 0.01 cm^2, plants inoculated with Ds showed a mean lesions length of 0.36 ± 0.02 cm^2.

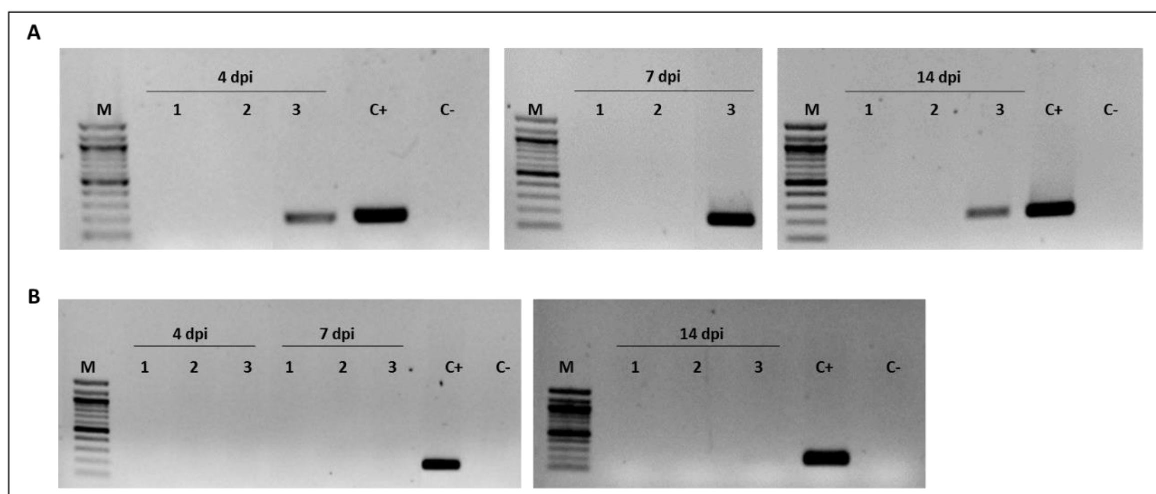


Figure 6: Follow-up of the Fito_S127B colonisation at roots (A) and leaves (B) at 4, 7 and 14 dpi through a PCR amplification with strain-specific primers. PCR amplification of *HxlR* (helix-turn-helix protein domain) gene, showing a 200pb amplicon, of both non-inoculated (control) and inoculated plants at roots (A) and leaves (B). M: 100 bp DNA ladder (NEB, UK); Lane 1: control plants (non-inoculated plants); Lane 2: plants inoculated with Fito_F278 strain; Lane 3: plants inoculated with Fito_S127B; C+: positive control (gDNA of pure Fito_S127B); C-: negative control (sterile water instead of gDNA).

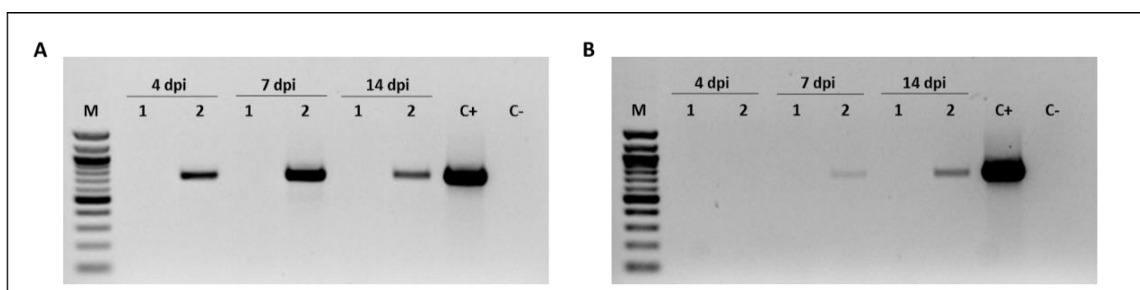


Figure 7: Follow-up of the Fito_F278 colonisation at roots (A) and leaves (B) at 4, 7 and 14 dpi through a PCR amplification with strain-specific primers. PCR amplification of *GST* (Glutathione S-transferase C) gene, showing a 750pb amplicon, of both non-inoculated (control) and inoculated plants at roots (A) and leaves (B). M: 100 bp DNA ladder (NEB, UK); Lane 1: control plants (non-inoculated plants); Lane 2: plants inoculated with Fito_F278; C+: positive control (gDNA of pure Fito_F278); C-: negative control (sterile water instead of gDNA).

D. seriata was recovered from cutting plants after one month of its artificial inoculation

Results showed that *D. seriata* was recovered from green stems at the PI. This re-isolation was higher at T3+4 weeks than at T3+1 week (Table 4), which may be justified by the time required for the development of the pathogen inside of green stems. Though, the re-isolation rates differed across growing seasons. Given the T3+4 weeks, *D. seriata* was not re-isolated from Ds+ Fito_S127B and Ds + Fito_F278 conditions only at 2014. Regarding the preliminary results using a co-inoculation of both BCAs, the re-isolation rate of *D. seriata* was of 25% at T3+1 week and 75% at T3+4 weeks. Regarding the low recovering rate of the pathogen, especially at Ds condition, this did not mean that the pathogen was not present in green stems. Indeed, observations of the tissues necrosis may confirm that they were caused by the presence of the pathogen.

As expected, *D. seriata* was not recovered from control conditions (control, Fito_S127B and Fito_F278 conditions), though other fungal microorganisms were isolated from the PI. The majority of such isolates were identified as *Acremonium* sp. though, others as *Aspergillus cristatus* (control condition), *Cladosporium tenuissimum* (Fito_S127B condition) and *Phialemonium inflatum* (Fito_F278 condition) were identified.

BCAs were recovered from cutting plants after one and two months of their soil inoculation

One of the challenges of this study was to achieve if the inoculated BCAs were able to survive in cutting plants one (T3+1 week) and two months (T3+4 weeks) after soil inoculation. For this, different grapevine samples such as soil, roots and leaves were collected and analysed by both classic microbiology techniques and direct molecular analysis, through DNA extraction and amplification with strain-specific primers. The endophytic population of roots and leaves samples, as well as from rhizosphere, was analysed only by classic microbiology techniques. Herein, results from rhizosphere are not presented as the obtained isolates were not identified as Fito_S127B or Fito_F278.

Results showed that BCAs strains were present in soils and roots (Table 5). Though, their distribution was not systematic over conditions and growing seasons. Given plants inoculated with Fito_S127B (Table 5), this strain was identified in soils at T3+1 week in Fito_S127B (2015 and 2016) and Ds + Fito_S127B (2016) condition, and at T3+4 weeks in Fito_S127B (2014, 2015 and 2016) and Ds + Fito_S127B (2015). This strain was also detected at roots in Fito_S127B condition, at both timepoints over 2015. Overall, the detection of Fito_S127B in soil confirmed not only its survival capacity up to two months after its inoculation but also its preference for this highly competitive environment in terms of microbial biodiversity. In contrast, Fito_F278 (Table 5) was not detected in 2014 however, it was identified in soil samples at T3+1 week in Fito_F278 (2015) and Ds + Fito_F278 (2015 and 2016) conditions. Furthermore, this strain was also detected at roots at T3+1 week in Fito_F278 (2015) and

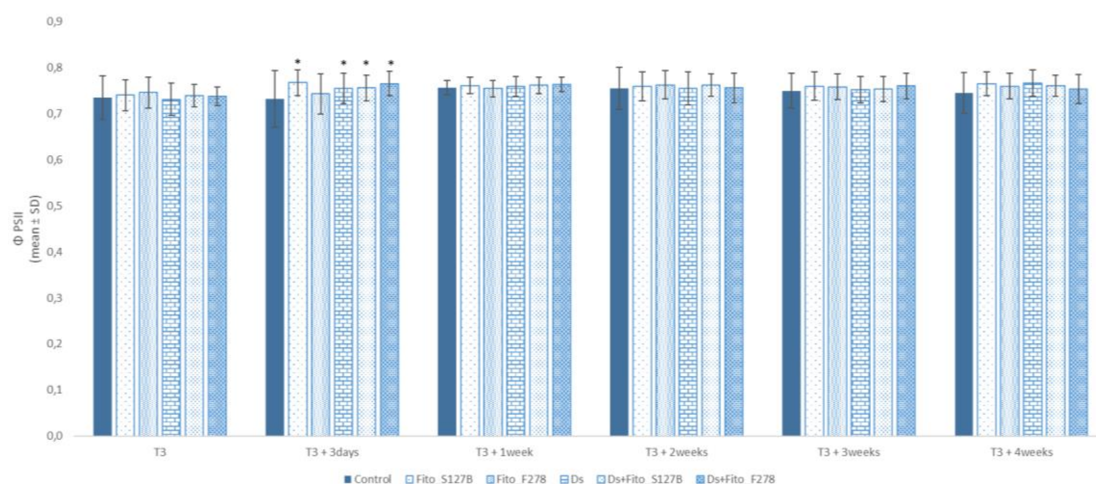


Figure 8: Analysis of the PSII for each condition overtime. Ds corresponds to the pathogen *D. seriata* F98.1. Significant differences (p<0.05) were determined by a one- way analysis of variance (ANOVA) followed by a Bonferroni *post hoc* test. In cases where the null hypothesis was rejected, the non-parametric test Kruskal-Wallis followed by the Dunn's multiple comparison test was carried out. *Significant differences when compared with control condition. Results are means ± SD of three growing seasons studied (2014, 2015, 2016).

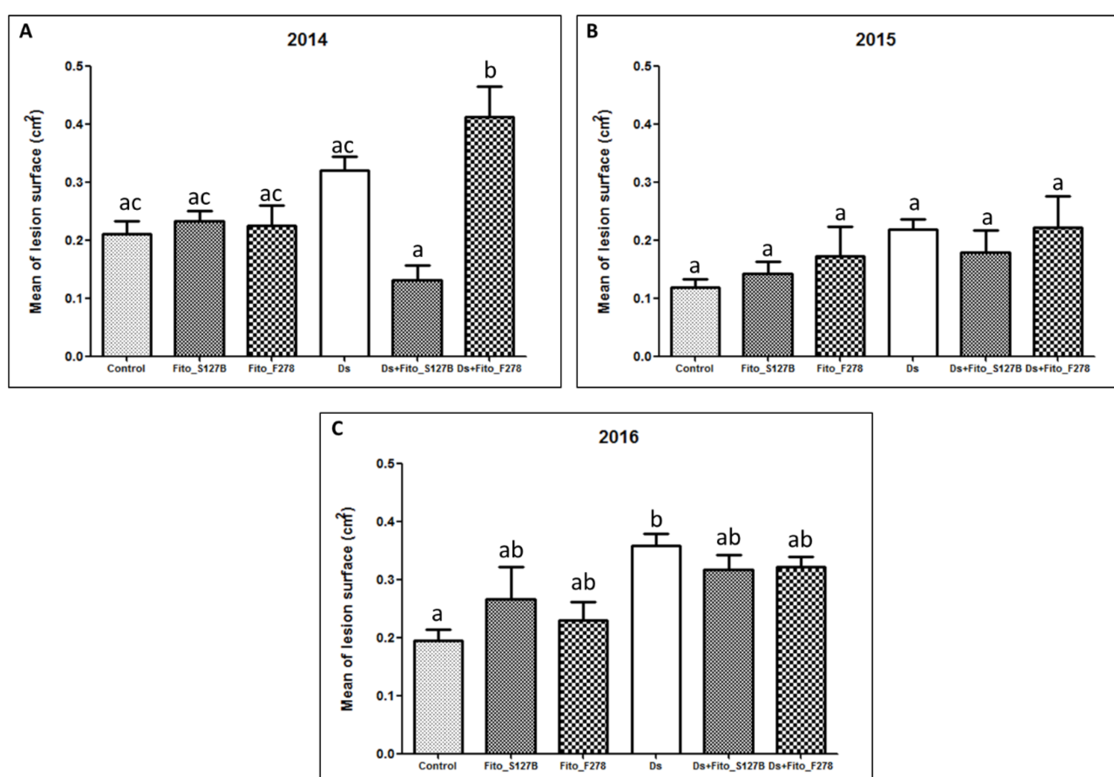


Figure 9: Analysis of the necrotic lesion surfaces of green stem cuttings (cv. Chardonnay) after artificial inoculation. Results of the three growing seasons are here presented, namely at 2014 (A); 2015 (B); and 2016 (C). Results are means ± SD of the necrotic lesion surfaces (cm^2) of green stem cuttings measured one month (T3+4 weeks) after their artificial inoculation with sterile PDA plug (control; Fito_S127B and Fito_F278 conditions) or with *D. seriata* F98.1 (Ds; Ds+Fito_S127B and Ds+Fito_F278 conditions). In the graphics, Ds corresponds to *D. seriata* F98.1. The significant differences (p<0.05) were determined by a one- way analysis of variance (ANOVA) followed by a Bonferroni *post hoc* test. In cases where the null hypothesis was rejected, the non-parametric test Kruskal-Wallis followed by the Dunn's multiple comparison test was carried out. The same letter above columns is not significantly different according to p<0.05.

Ds+ Fito_F278 (2016) conditions and detected in both conditions at T3+4 weeks of 2016. Results from 2016 growing season demonstrated that this strain was isolated inside of roots, confirming its endophytic potential. Overall, results suggested that Fito_F278 was able to survive in soils up to one month after its inoculation, and then roots seems to have constituted a favourable environment to its survival for longer periods.

None of the strains were detected at leaves by both classic microbiology and molecular analysis. However, Fito_F278 was detected in 2014 at green stems at T3+1 week (data not shown). This may confirm the endophytic potential of this strain, even if it was not a systematic process. Regarding the preliminary results with both BCAs inoculation, Fito_F127B was isolated from soil samples and Fito_F278 was detected by molecular analysis in leaves for both conditions (Fito_S127B + Fito_F278 and Ds+ Fito_S127B + Fito_F278) and timepoints (T3+1 week and T3+4 weeks). At roots, strains were only detected by molecular analysis: Fito_S127B was identified only at T3+1 week in the Ds+ Fito_S127B + Fito_F278 condition and Fito_F278 strain in Fito_S127B + Fito_F278 and Ds+ Fito_S127B + Fito_F278 conditions, at T3+1 week and T3+4 weeks, respectively.

General population density recovered from the re-isolation steps

During the re-isolation steps, the population density obtained was estimated for 2015 and 2016 growing seasons, through the CFU count. As previously exposed, the microbial isolates from roots and leaves samples were related to the endophytic microorganisms. As expected, soils, rhizosphere and roots samples showed a higher population density when compared with leaves. Overall, and considering the average of both growing seasons, the population density of soils showed an average of 10^8 CFU/g of soil, rhizosphere had 10^7 CFU/g of roots and the endophytic population of roots and leaves showed an average of 10^6 and 10 CFU/g of tissue, respectively. Indeed, these results allowed to have an idea about the population density across cuttings ecosystem, though did not allow an assessment of the general biodiversity. Thus, to investigate the effect of BCAs inoculation on microbial biodiversity associated with soil or root samples, a more in-depth study will be necessary, such as polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) or a metagenomics approach.

Table 4: Re-isolation rate (%) of the D. seriata F98.1 from green stems cuttings (cv. Chardonnay) at T3+1 week and T3+4 weeks after artificial inoculation.

	T3 + 1 week			T3 + 4 weeks		
	2014	**2015**	**2016**	**2014**	**2015**	**2016**
Ds	33%	25%	0	67%	0	75%
Ds + Fito_S127B	0	0	0	0	25%	75%
Ds + Fito_F278	0	0	25%	0	50%	100%

The re-isolation of the pathogen was carried out from the point of inoculation (PI) at T3+1 week and at the PI, PI+1cm and PI- 1cm at T3+4 weeks. In 2014, isolates were carried out from 3 plants/ replicates while at 2015 and 2016, isolates were carried out from 4 plants. As expected, the pathogen was not recovered from control conditions, namely control, Fito_S127B and Fito_F278 conditions.

Table 5: Re-isolation rate (%) of the Fito_S127B and Fito_F278 strains from soil, roots and leaves samples at T3+1 week and T3+4 weeks.

		Soil		Root		Leaf	
Growing season	**Condition**	**T3+1week**	**T3+4weeks**	**T3+1week**	**T3+4weeks**	**T3+1week**	**T3+4weeks**
2014	Fito_S127B	-	+	-	-	-	-
	Ds+Fito_S127B	-	-	-	-	-	-
2015	Fito_S127B	+	+	+*	+*	-*	-*
	Ds+Fito_S127B	+	+#	-*	-*	-*	-*
2016	Fito_S127B	+#	+#	-	-	-	-
	Ds+Fito_S127B	-	-	-	-	-	-
2014	Fito_F278	-	-	-	-	-	-
	Ds+Fito_F278	-	-	-	-	-	-
2015	Fito_F278	+*	-*	+*	-*	-*	-*
	Ds+Fito_F278	+*	-*	-*	-*	-*	-*
2016	Fito_F278	-	-	-	+	-	-
	Ds+Fito_F278	+#	-	+#	+#	-	-

The re-isolation of the Fito_S127B and Fito_F278 were carried out from soil, roots and leaves samples from all conditions at T3+1 week and T3+4 weeks, over 2014, 2015 and 2016. Herein are presented the results obtained by both classic microbiology techniques and a direct molecular analysis of grapevine samples with strain-specific primers. For classic microbiology techniques, isolations were carried out from three individual plants, while for the molecular analysis three grapevine samples of each timepoint were pooled for DNA extraction. Results obtained from classic microbiology techniques for roots and leaves, are related to the endophytic population while results from molecular analysis contains both epiphytic and endophytic microorganisms. All the obtained isolates were identified by Sanger sequencing and/or confirmed with strain-specific primers amplification.
It is important to note that in 2014, the direct molecular analysis was performed only for soil samples. Results from rhizosphere are not presented as the obtained isolates were not identified as Fito_S127B or Fito_F278 strains.
*only analysed by a direct molecular analysis
#only positive for classic microbiology techniques

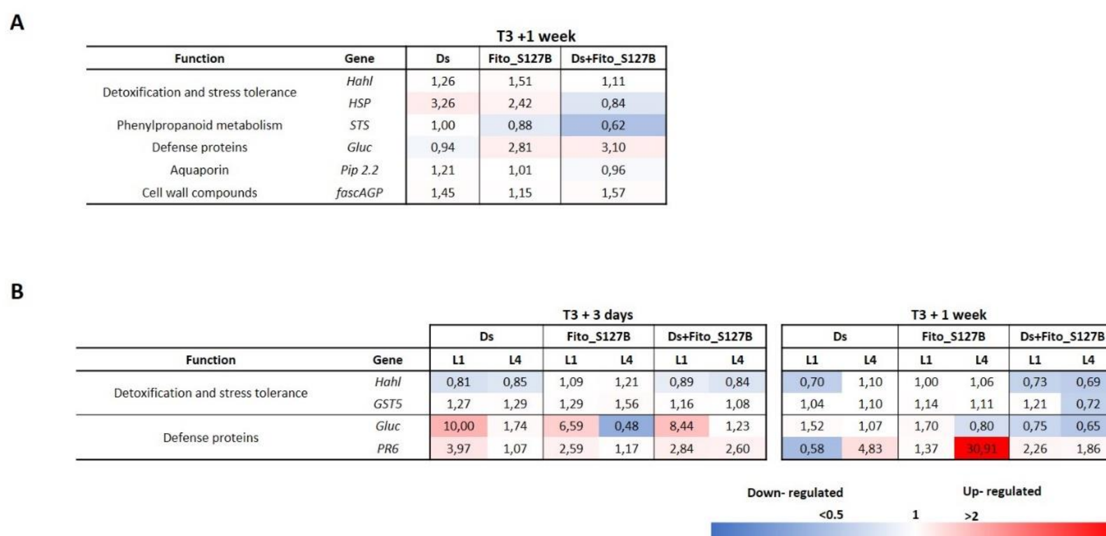
Assessment of plant defence responses at stem and leaves by qRT-PCR

The expression analysis of a set of 8 genes (Table 1) was compared at stem and leaves for all conditions conducted in this study. For that, both the effect of *D. seriata* infection and Fito_FS127B and/or Fito_F278 on grapevine responses were assessed at stem (T3+1 week) and leaves (T3+3 days and T3+1 week) by qRT-PCR, over three growing seasons. The target genes in analysis included genes encoding the phenylpropanoid metabolism (*STS*), proteins involved in the detoxication process and stress tolerance (*Hahl, HSP, GST5*), defence proteins (*Gluc, PR6*), cell wall compounds (*fascAGP*) and water stress (*PIP 2.2*).

Comparing plants inoculated with Fito_S127B, results showed that at the stem level (Figure 10A), genes such as *Hahl, STS, PIP 2.2* and *fascAGP* presented similar levels of relative expression among all conditions. Though, an upregulation of *Gluc* was observed on all conditions containing Fito_S127B. Furthermore, the relative expression levels of *HSP* in grapevine were upregulated in plants inoculated with *D. seriata* and Fito_S127B. Regarding the leaves (Figure 10B), the *Hahl* and *GST5* genes presented similar levels of relative expression among all conditions and timepoints and an upregulation of defence genes (*Gluc* and *PR6)* was observed only at T3+3 days and for leaves closest to the artificial inoculation point of pathogen/ PDA plug. At T3+1 week, relative expression reached similar values across conditions, with exception of the *PR6* gene that still induced. Interestingly, this upregulation was observed on the top leaves (L4) for Ds and Fito_S127B condition. Overall, these results suggested that Fito_S127B strain could induce resistance against *D. seriata* by an upregulation of the defence pathways (*Gluc* and *PR6)*.

Going forward, results from plants inoculated with Fito_F278 showed that at green stems (Figure 11A), genes such as *Hahl, STS, Gluc, PIP 2.2* and *fascAGP* presented similar levels of relative expression among all conditions. With exception of the *Gluc* gene, these results were similar to those obtained with plants inoculated with Fito_S127B. An upregulation of the *HSP* gene was observed for Ds and Ds+Fito_F278 conditions. At leaves (Figure 11B), the expression levels of *Hahl* and *GST5* genes were similar across all conditions and timepoints, which are in accordance with results obtained in grapevines inoculated with Fito_S127B. Grapevine also showed an upregulation of defence genes (*Gluc* and *PR6*). Thus, and regarding *Gluc,* an upregulation was observed only at T3+3 days for leaves closest to the artificial inoculation point of pathogen/ PDA plug. Although, plants inoculated with both Ds + Fito_F278 still had their defence mechanisms activated at T3+1 week. Contrary, the expression levels of *PR6* gene were upregulated across time.

The preliminary results obtained with both BCAs inoculation in cutting plants, showed that the relative expression of *Hahl, PIP 2.2* and *fascAGP* genes at green stems presented similar levels of relative expression among conditions (data not shown). Furthermore, an upregulation of *Gluc* was



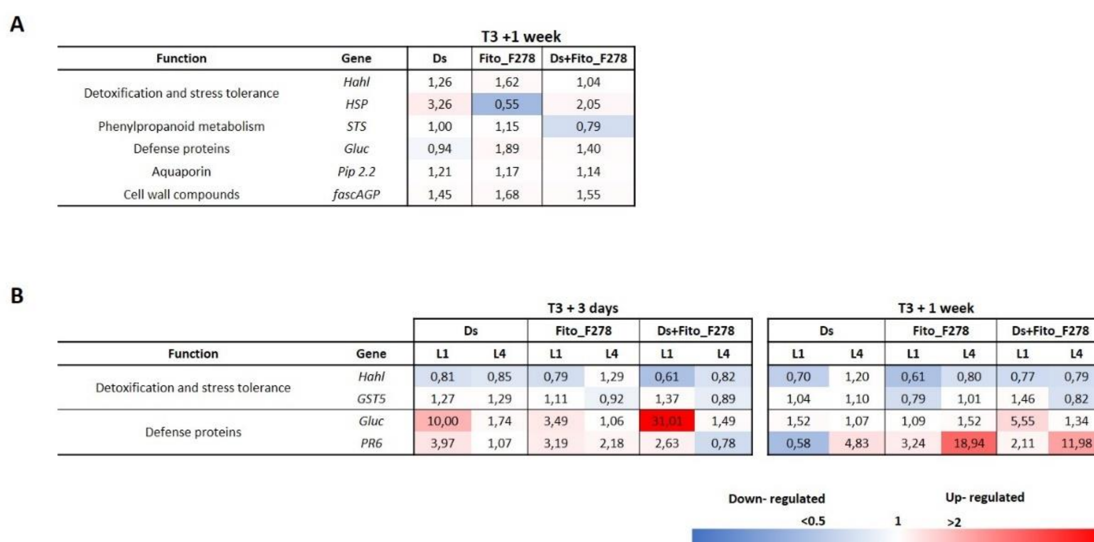
A

Function	Gene	T3 +1 week Ds	Fito_S127B	Ds+Fito_S127B
Detoxification and stress tolerance	*Hahl*	1,26	1,51	1,11
	HSP	3,26	2,42	0,84
Phenylpropanoid metabolism	*STS*	1,00	0,88	0,62
Defense proteins	*Gluc*	0,94	2,81	3,10
Aquaporin	*Pip 2.2*	1,21	1,01	0,96
Cell wall compounds	*fascAGP*	1,45	1,15	1,57

B

		T3 + 3 days						T3 + 1 week					
		Ds		Fito_S127B		Ds+Fito_S127B		Ds		Fito_S127B		Ds+Fito_S127B	
Function	Gene	L1	L4	L1	L4	L1	L4	L1	L4	L1	L4	L1	L4
Detoxification and stress tolerance	*Hahl*	0,81	0,85	1,09	1,21	0,89	0,84	0,70	1,10	1,00	1,06	0,73	0,69
	GST5	1,27	1,29	1,29	1,56	1,16	1,08	1,04	1,10	1,14	1,11	1,21	0,72
Defense proteins	*Gluc*	10,00	1,74	6,59	0,48	8,44	1,23	1,52	1,07	1,70	0,80	0,75	0,65
	PR6	3,97	1,07	2,59	1,17	2,84	2,60	0,58	4,83	1,37	30,91	2,26	1,86

Figure 10: Gene expression levels in green stems (A) and leaves (B) from plants inoculated or not with Fito_S127B and *D. seriata* F98.1. Transcript accumulation of *Hahl, HSP, STS, Gluc, PIP 2.2* and *fascAGP* genes in stems (A) and *Hahl, GST5, Gluc* and *PR6* genes in leaves (B) was determined by qRT-PCR at T3+3 days (only for leaves) and T3+1 week after artificially inoculation of pathogen at green stems. Herein, Ds corresponds to *D. seriata* F98.1. Results represent the relative expression levels ($\Delta\Delta C_t$) of reported conditions in relation to the control (plants not inoculated; data not shown). Values shown are means of three independent repetitions (each repetition with two technical replicates). The expression of a given gene was considered up- or down-regulated when the value of relative expression was >2-fold or <0.5-fold compared to the control, respectively.



A

Function	Gene	T3 +1 week Ds	Fito_F278	Ds+Fito_F278
Detoxification and stress tolerance	*Hahl*	1,26	1,62	1,04
	HSP	3,26	0,55	2,05
Phenylpropanoid metabolism	*STS*	1,00	1,15	0,79
Defense proteins	*Gluc*	0,94	1,89	1,40
Aquaporin	*Pip 2.2*	1,21	1,17	1,14
Cell wall compounds	*fascAGP*	1,45	1,68	1,55

B

		T3 + 3 days						T3 + 1 week					
		Ds		Fito_F278		Ds+Fito_F278		Ds		Fito_F278		Ds+Fito_F278	
Function	Gene	L1	L4	L1	L4	L1	L4	L1	L4	L1	L4	L1	L4
Detoxification and stress tolerance	*Hahl*	0,81	0,85	0,79	1,29	0,61	0,82	0,70	1,20	0,61	0,80	0,77	0,79
	GST5	1,27	1,29	1,11	0,92	1,37	0,89	1,04	1,10	0,79	1,01	1,46	0,82
Defense proteins	*Gluc*	10,00	1,74	3,49	1,06	31,01	1,49	1,52	1,07	1,09	1,52	5,55	1,34
	PR6	3,97	1,07	3,19	2,18	2,63	0,78	0,58	4,83	3,24	18,94	2,11	11,98

Figure 11: Gene expression levels in green stems (A) and leaves (B) from plants inoculated or not with Fito_F278 and *D. seriata* F98.1. Transcript accumulation of *Hahl, HSP, STS, Gluc, PIP 2.2* and *fascAGP* genes in stems (A) and *Hahl, GST5, Gluc* and *PR6* genes in leaves (B) was determined by qRT-PCR at T3+3 days (only for leaves) and T3+1 week after artificially inoculation of pathogen at green stems. Herein, Ds corresponds to *D. seriata* F98.1. Results represent the relative expression levels ($\Delta\Delta C_t$) of reported conditions in relation to the control (plants not inoculated; data not shown). Values shown are means of three independent repetitions (each repetition with two technical replicates). The expression of a given gene was considered up- or down-regulated when the value of relative expression was >2-fold or <0.5-fold compared to the control, respectively.

observed on Fito_S127B + Fito_F278 condition. The analysis of the *HSP* gene did not lead to any conclusion. At the leaves, *Hahl* and *GST5* were similar across all conditions and timepoints and an upregulation of the defence genes *Gluc* and *PR6* were reached and maintained over the timepoints. These results seem to be in agreement with those previously presented however, it is important to note that these are preliminary results and would be necessary to repeat the assay for a more robust analysis. Although not mentioned, the relative expression levels of *HSP* and *Lac17* were also determined in leaves. Contrary to results obtained for *HSP* gene at green stems, the amplification melt curves of leaves samples showed dimers. For *Lac*17 gene, involved in the secondary metabolites, many of the samples did not amplified. For this reason, these genes were not considered in analysis.

Development of strain-specific primers for identification of Fito_S127B and Fito_F278 strains

The Fito_S127B-specific primer targeting the tryptophan synthase beta chain (*trpB)* and helix-turn-helix protein domain, namely *hpb* and *HxIR* genes produced an approximately 480, 250 and 200bp amplicon, respectively (Table 2 and 6). Results obtained by using primers that amplified the *trpB* and *HxIR* genes showed a 100% of specificity with Fito_S127B and any non-target isolates (n = 6) were amplified. In contrast, primers for *hpb* gene could amplify non-target microorganisms (Table 6). Given the obtained results, the strain-specific primers targeting the *HxIR* gene were then selected for the detection and identification of the Fito_S127B in grapevine assays. The best PCR efficiency consisted in using 10% DMSO in the PCR reaction and applying an annealing temperature of 60°C, as these primers are highly rich in GC content (66.7%).

Given the Fito_F278, the tested set of primers targeting the glutathione S-transferase (*GST*), elongation factor 1 (*EF1*), tryptophan synthase alpha chain (*trpα*) and tubulin (*Tub*) genes produced an approximately, 800, 500, 400 and 300bp amplicon, respectively (Table 2 and 6). The specificity analysis showed that primers for *EF1*, *trpα* and *Tub* genes did not give accurate results as they amplified non-target microorganisms, including other *A. pullulans* strains (Table 6). Primers amplifying the *GST* gene showed a specificity of 79%, as other *A. pullulans* strains were amplified. Though, these strains may be probably closely related or even the same strain as Fito_F278. Once the best results were obtained with primers for *GST* gene amplification, these were then selected as strain-specific primers for Fito_F278 analysis. Notwithstanding, strain-specific primers were likewise developed for *B. amyloliquefaciens* Fito_F321 despite this strain was not used on bioassays. Primers targeting the tryptophan synthase beta chain (*trpB)* resulted in a 200bp amplicon (Table 2 and 6). Additionally, results showed a 78% of specificity of strain-specific primers as non-target microorganisms were amplified (n=5 from a total of 23 isolates). As previously referred, these amplified strains may be probably very close or even the same strain as Fito_F321.

Table 6: Analysis of the sensitivity and specificity of the strain-specific primers using non-target microorganisms isolated from vineyards

Strain	Target gene	Primer code	**PCR conditions**		***In vitro* analysis of the sensitivity and specificity of strain-specific primers**			
			Amplification size (bp)	**Annealing temperature (°C)**	**No.of target strains detected***	**Sensitivity (%)****	**No of non-target strains detected**	**Specificity (%)*****
Fito_S127B (*Streptomyces* sp.)	*trpB* (tryptophan synthase beta chain)	trpB_FS127B trpB_RS127B	480	60°C	2 (n=2)	100	0 (n= 6)	100
	hpB (helix-turn-helix protein domain)	hpb_FS127B hpb_RS127B	250	60°C	3 (n= 3)	100	1 (n= 4)	75
	HxIR (helix-turn-helix protein domain)	HxIR_F127B HxIR_RS127B	200	60°C	2 (n= 2)	100	0 (n= 6)	100
Fito_F278 (*A. pullulans*)	*trp α* (tryptophan synthase subunit alpha)	trp_F(F278) trp_R2(F278)	400	51°C	1 (n= 1)	100	6 (n= 14)	57
	GST (glutathione S-transferase)	GST_F(F278) GST_EF1R(F278)	800	58°C	1 (n= 1)	100	3 (n= 14)	79
	EF1 (elongation factor 1)	EF1TU_F(F278) EF1TU_R(F278)	500	59°C	1 (n= 1)	100	7 (n= 14)	50
	Tub (tubulin)	Tub_F(F278) Tub_R2(F278)	300	60°C	1 (n= 1)	100	5 (n= 14)	64
Fito_F321 (*B. amyloliquefaciens*)	*trpB* (tryptophan synthase beta chain)	trpB_F321 trpB_R321	200	56°C	3 (n= 3)	100	5 (n= 23)	78

*The target strains correspond to pure cultures or a mix of samples containing the target strains, namely Fito_S127B, Fito_F278 and Fito_F321.

**The sensitivity of the strain-specific primers was calculated through the formula: sensitivity (%)= (ts/Tts)*100, where ts is the number of target strains detected and Tts is the total number of target strains tested.

*** The specificity of the strain-specific primers was calculated through the formula: specificity (%) = (nts/Tnts)*100, where nts is the number of non-target strains undetected and the Tnts is the total number of non-target strains tested.

Discussion

***Streptomyces* sp. Fito_S127B is a coloniser of the below-ground parts of grapevine and with a PGP potential**

Before analysing the biocontrol potential of a microorganism, it is important to understand it capacity to colonize plants and to what extent its interactions with plant are positive, in particular to the plant growth promotion and plant health status. For this, an *in vitro* bioassay with plantlets of cv. Chardonnay inoculated at the roots with BCAs strains, namely *Streptomyces* sp. Fito_S127B and *A. pullulans* Fito_F278, was carried out.

Fito_S127B was successfully re-isolated only from superficial grapevine roots, elucidating its preference to colonise the below-ground parts of plant. Though the microbial strain density founded at roots (average of 10^4 CFU/mL) was considerably lower than the initially plants inoculation (10^8 CFU/mL). These results may explain that Fito_S127B was able to colonize root surfaces but its proliferation occurred at a slower rate. Furthermore, in some cases, it was observed small rupture of root tissues which constituted an entrance for this strain, allowing its penetration and colonisation inside of root tissues. Although, it is important to note that these observations were not systematic and may therefore be influenced (or not) by its proliferation rate. Indeed, S*treptomyces* spp. are predominantly filamentous and ubiquitous soil bacteria though can colonize nearby roots and even to penetrate plant cells (Seipke *et al.,* 2012). Endophytic *Streptomyces* microorganisms were already being described (Coombs and Franco, 2003; Franco *et al.,* 2007; Golinska *et al.,* 2015; Franco *et al.,* 2016), nevertheless the abundance and diversity of these endophytic colonisers may be depended on plant species, soils types or environmental conditions, including the microbial load and biodiversity (Govindasamy *et al.,* 2014). These endophytic microorganisms are associated as biological control agents (Misk and Franco, 2011; El-Tarabily *et al.,* 2009) and plant growth promoters (Hasegawa *et al.,* 2006; El-Tarabily, 2008; El-Tarabily *et al.,* 2009). Given grapevine, a study of Loqman *et al.,* (2009) showed that *Streptomyces* strains were able to establish sufficient endophytic populations in grapevine plantlets and West *et al.,* (2010) isolated and identified endophytic *Streptomyces* sp. residing within grapevine tissues, namely at roots and canes.

Our study also demonstrated that plants inoculated with Fito_S127B showed a considerable development of roots, namely secondary roots, when compared with non-inoculated plants (control). Indeed, several *Streptomyces* sp. microorganisms are plant beneficial and effective growth promoter microorganisms, via several mechanisms such as nutrient uptake, plant growth

hormones production or other bioactive compounds (Seipke *et al.,* 2012; Golinska *et al.,* 2015). The increase of root and shoot dry weight of plants such as soybean, alfalfa, cucumber or tomato, by some actinomycetes strains, was already been reported (Xiao *et al.,* 2002; El-Tarabily, 2008; El-Tarabily *et al.,* 2009). *Streptomyces* sp. are also effective against plant pathogens and even inducers of plant defence responses (Hasegawa *et al.,* 2006; Franco *et al.,* 2007; Conn *et al.,* 2008). Furthermore, these microorganisms have important roles in recycling of organic matter and are important producers of several bioactive compounds such as antibiotics, with commercial interest to medicine and agricultural industry (Procópio *et al.,* 2012; Seipke *et al.,* 2012).

***A. pullulans* Fito_F278 is an endophyte microorganism but its colonisation leads to an unexpected effect in grapevine plantlets**

Given the *A. pullulans* Fito_F278, results suggested that this strain could colonize the host upon inoculation, from the roots to the leaves and both at the epiphytic and endophytic level. This is in agreement with previous studies that reported *A. pullulans* as a widespread epiphyte and an endophyte of different plants, including grapevine (Pugh and Buckley, 1971; Martini *et al*., 2009). This microbial proliferation occurred up to 7 dpi, and after this period the microbial strain density decreased, as a consequence of the bioavailability of nutrients. Fito_F278 was also able to colonise the internal tissues of leaves, thought this colonisation was not systematic (data not shown). Considering that this is a potential BCA, the ability to colonize grapevine tissues in a successful way is essential to prevent plant diseases and to control the pathogen spread (Chow *et al.,* 2017). Furthermore, its rapid colonisation and proliferation capacity may be related not only with its adaptability to colonise different environments but also as a competing strategy for space and nutrients against the surrounding microbial communities, in particular against phytopathogens.

Going forward, in our study any positive influence of Fito_F278 on plantlets was found in terms of plant growth and development, namely after 7 dpi. Indeed, microorganisms can impact positively or negatively the plant growth and health. However, results from this study were not expected as *A. pullulans* is a naturally an abundant resident microbiota of grapevine (Martini *et al.,* 2009; Pinto *et al.,* 2014; Pinto *et al.,* 2015) and an important biological control agent. Furthermore, it is recognized that endophytes microorganisms have a symbiotic association with the host plants, not causing any apparent symptoms of disease, and may even provide benefits to plant such as nutrient acquisition or by producing a variety of bioactive compounds or enzymes, important for plant growth and development (direct mechanisms) and protection against phytopathogens (indirect mechanisms) (Wilson, 1995; Dutta *et al.,* 2014; Nair and Padmavathy, 2014; Santoyo *et al.,* 2016).

Thus, a possible justification for the obtained results could be (i) the high initial CFU plant inoculation (10^6 CFU/mL) performed and (ii) the high content of carbon source present in the culture medium (sucrose content at 3% (w/v)). Altogether, these may lead to the rapid colonisation and proliferation of Fito_F278, followed by the production of a biofilm layer and probably of extracellular compounds. Consequently, this competition for nutrients limited the nutrients available for plants and favoured the magnitude of plant symptoms.

The BCAs inoculation and the development of *D. seriata* did not affect the grapevine photosynthesis

The photosynthesis was not affected in leaves from plants inoculated with BCAs, *D. seriata* or BCAs + *D. seriata*, during the period under analysis. Though, is important to note that plants from this study were young plants, analysed up to a maximum period of one month (T3+4 weeks) after the artificial inoculation of the phytopathogen at green stems. Furthermore, no visual symptoms of disease caused by the phytopathogen were observed on leaves. Meanwhile, other previous studies on grapevine have reported that photosynthesis was drastically affected in leaves with esca symptoms (Petit *et al.,* 2006), in pre-apoplectic vines plants (Letousey *et al.,* 2010; Magnin-Robert *et al.,* 2011), in water stressed plants infected with Botryosphaeriaceae spp. (Niekerk *et al.,* 2011) or even in leaves infected with downy and powdery mildew (Moriondo *et al.,* 2005). The alterations in photosynthesis were coupled with a decline of the photosynthetic rate, namely PSII activity, decrease of gas exchange, changes in chlorophyll fluorescence and repression of photosynthesis-related genes (Letousey *et al.,* 2010; Magnin-Robert *et al.,* 2011). Thus, perturbations on the carbon metabolism, especially on photosynthesis, occurred as a consequence of the reorientation of the carbohydrates by the pathogens and the development of chlorotic and necrotic areas in leaves after the pathogens infection (Letousey *et al.,* 2010). In this sense, the decrease of photosynthesis, namely PSII is directly proportional to the disease progression (Nogués *et al.,* 2002). Modifications on photosynthesis may be induced not only by phytopathogens but also by drought (Flexas *et al.,* 2002) or chemicals (Petit *et al.,* 2009).

Streptomyces* sp. Fito_S127B had a significant inhibitory effect on *D. seriata

Previous results presented in Chapter 3, showed the biocontrol potential of *Streptomyces* sp. Fito_S127B and *A. pullulans* Fito_F278 against important grapevine diseases, such as *B. cinerea* and several Botryosphaeriaceae species, under *in vitro* antagonistic tests. Herein, the biocontrol potential of both strains was assessed under *in vivo* conditions through the artificially inoculation

of grapevine cutting stems (cv. Chardonnay) with the phytopathogen *D. seriata* F98.1. Fito_S127B did not totally prevent the pathogen growth on cutting plants, though an important reduction of the necrotic lesions length of plants was observed. Overall, and in average, this strain showed a significantly reduction (p<0.05) of 29.44% of the necrotic lesions length caused by *D. seriata*, suggesting that this strain efficiently protected cuttings against the pathogen. This is of utmost interest, considering that this is a soil bacterium that colonizes the plant roots and which can trigger a biocontrol activity at a distant point. Interestingly, and considering the antagonistic results from Chapter 3, Fito_S127B showed to be only effective in controlling the pathogen growth under a direct confrontation. Thus, in this present study, this effective protection response seems to be partly associated with its interactions with the plant, namely by promoting a signalling defence pathway in plant, as discussed below. Going forward, and considering that Fito_S127B is a soil microorganism with a biocontrol potential against different phytopathogens, this may also represent an opportunity and protective measure for vineyard soils, in case of these soils constitute a main source of inoculum for grapevine infections (Travadon *et al.,* 2015; Flontaine *et al*., 2016b).

Regarding Fito_F278, and in contrast to the antagonistic results, plants inoculated with this strain did not show a reduction of the necrotic lesions caused by the phytopathogen. Conversely, Fito_F278 even tended to increase these necrotic lesions length. A similar result was also observed by Haidar *et al.,* 2016 in grapevine cuttings inoculated with *B. pumilus* (S35) or *Xanthomonas* sp. (S45), together with the pathogen *N. parvum*. Despite Fito_F278 seems to have failed the plant protection against *D. seriata*, an up-regulation of plant defence pathways (discussed below) was observed. Furthermore, and as discussed on Chapter 3, this strain may also produce volatile compounds with biocontrol interest. Preliminary results using a co-inoculation with both BCAs, namely Fito_S127B at soil and Fito_F278 at leaves, showed a significantly reduction (p<0.05) of the plant necrotic lesions length caused by *D. seriata* of 35.86% (data not shown). Thus, the soil inoculation strategy adopted in this cutting model for the biocontrol study of Fito_F278 did not maximized its biocontrol potential.

As referred in the previous chapter, and to best of our knowledge, only one study applied *A. pullulans* to control GTD namely, the infection of grapevine wounds against *Eutypa lata* (Munkvold and Marois, 1993). Herein, two field experiments were performed in California region, namely the first in 1990 at Thompson Seedless vineyard and the second in 1991 in a cv. Chenin Blanc vineyard. A set of natural occurring microorganisms was applied through a wound inoculation, to test their efficacy as biocontrol agents. Among them, *A. pullulans* significantly reduced infection, with a reduction superior to 50% compared to control treatment though, this reduction was only observed

in the first field. So far, and to date, we have demonstrated for the first time the effectiveness of *Streptomyces* sp. and *A. pullulans* as BCAs against Botryosphaeriaceae dieback agents, namely *D. seriata*, in grapevine cuttings under greenhouse conditions. Recently, other different study was carried out to control Botryosphaeriaceae species, namely *N. parvum* (Haidar *et al.,* 2016). Herein, a total of 46 bacterial strains were applied to control *N. parvum*, using grapevine cuttings and under greenhouse conditions. Results showed that *Pantoea agglomerans* and *Enterobacter* sp. reduced the necrosis length caused by the phytopathogen, in which *P. agglomerans* reduced the necrotic lesions by 43.5%. Altogether, these both studies allow not only a model to study the grapevine-BCAs-pathogen interactions but also constitute a great advance and new perspectives for the control of Botryosphaeriaceae infection by applying BCAs in grapevine cuttings, under greenhouse conditions.

BCAs were recovered from cutting plants up to one and two months after their inoculation, though their survival was not systematic

Both Fito_S127B and Fito_F278 were able to effectively colonize grapevine plantlets. When these strains were inoculated in soils of cutting plants they were recovered up to one and two months after their inoculation at soils and roots. Though, their survival over conditions and growing seasons was limited and not always systematic. Even so, and in general, Fito_S127B was recovered mainly from soils and up to two months (T3+4 weeks) of its inoculation, while Fito_F278 was recovered from soils up to one month (T3+1 week) and then from roots (T3+4 weeks). These results suggested that each strain presented a preference for a certain habitat or even a survival strategy. Thus, Fito_S127B was undoubtedly a soil colonizer, while Fito_F278 seemed to prefer roots for colonization after longer periods, due to the root exudates. Overall, these results underlined that the successful application of BCAs may be limited, even if applied with relatively controlled conditions, as a consequence of the external conditions that they are exposed and in which directly or indirectly impact their physiological adaptation, proliferation and survival rate (van Elsas *et al.,* 1998). Furthermore, the microbial communities present in soils, notably the commercial soils used in this study, may exercise some competition over the inoculated BCAs, resulting in undesirable effects.

Interestingly, in the preliminary assay performed with both BCAs, where all the co-inoculated plants were placed individually in sterile protective plastic bags during the inoculations period and for 15 days, strains were detected in their inoculation focus, namely at soil (Fito_S127B) and leaves (Fito_F278) over all timepoints in analysis. This may suggest that the protective plastic bag confined

a humid and conducive environment, allowing the strains colonization and proliferation across plants. In fact, both biotic and abiotic factors may have an important role on the initial settlement rate of the introduced strains and, thus, influence their colonization and proliferation effectiveness. Furthermore, it was already demonstrated that increasing the number of BCAs released in the field did not always improve or increase the pathogens control (Crowder, 2007). Thus, understanding the environmental and host factors are crucial to maximize the BCAs colonization and, consequently improve the disease control efficacy.

The grapevine inoculation with BCAs suggested an activation of defence pathways

To further access the effect of *D. seriata* infection and Fito_S127B and/or Fito_F278 colonisation in young cutting plants and the biocontrol potential of these BCAs, the gene expression analysis of eight genes were compared at stem and leaves level. The target genes included genes encoding the phenylpropanoid metabolism (*STS*), proteins involved in the detoxication process and stress tolerance (*Hahl, HSP, GST5*), defence proteins (*Gluc, PR6*), cell wall compounds (*fascAGP*) and water stress (*PIP 2.2*).

The artificial inoculation of cutting plants with *D. seriata* induced the upregulation of genes concerned the detoxication and stress tolerance (*HSP*) at stem and defence genes (*Gluc, PR6*) at leaves. Interestingly, similar results were obtained in plants inoculated with Fito_S127B, though also had an induction of *Gluc* at stem. Then, for *D. seriata* + Fito_S127B condition, only genes concerning the defence proteins (*Gluc, PR6*) were induced in both stem and leaves. Plants inoculated with Fito_F278 only had an induction of PR genes (*Gluc, PR6*) at leaves, while *D. seriata* + Fito_F278 had a similar expression to the plants inoculated individually with the pathogen. Curiously, for plants inoculated individually, and with exception of Fito_F278, both PR genes were induced at T3+3 days for the leaf closer to the artificial inoculation point (L1) and then, at T3+1 week, only *PR6* was induced at more distant leaves (L4), suggesting a signalling mobilization across the plant. Conversely, when plant was inoculated with *D. seriata* + Fito_S127B condition, leaded to an upregulation of the *PR6* gene across both leaves at T3+3 days and then this induction was concentrated only at L1, suggesting a defence mechanism closer to the artificial inoculation point. Plants inoculated with Fito_F278, showed an induction of *PR6* on both leaves (L1 and L4) and for both timepoints, acting as a signalling molecule activating defence responses across grapevine cells. Indeed, a similar behaviour was reported to plants inoculated with *D. seriata* + Fito_F278 at T3+1 week.

Beyond the above-mentioned genes, the expression of genes involved in the detoxification processes (*Hahl, GST5*), water stress (*PIP 2.2*) and cell wall compounds (*fascAGP*) was not affected for all conditions.

The expression of some of these genes was already been reported (Reis *et al.,* 2016; Spagnolo *et al.,* 2017). Considering the genes analysed at stem level (*Hahl, HSP, STS, Gluc, Pip 2.2* and *fascAGP*), our results are in line with a previous study performed on asymptomatic (AP) cv. Tempranillo plants artificially inoculated with *D. seriata* F98.1 (Reis *et al.,* 2016). Though, when the same results are compared with symptomatic plants (SP), the gene expression values are different. Indeed, in our study, no foliar symptoms caused by the *D. seriata* infection were observed.

Going forward, and looking in particular to the phenylpropanoid metabolism, the stilbene synthase gene (*STS*) was not induced by *D. seriata* or BCAs at the stem level. A similar result was obtained by Reis *et al.,* (2016) on plants inoculated with *D. seriata* F98.1. Conversely, a study of Spagnolo *et al.,* (2017) reported an induction of *STS* gene in *D. seriata* and *N. parvum* artificially infected stems. Also, Liswidowati *et al.,* (1991) showed an induction of *STS* genes in grapevine cell suspensions elicited with *B. cinerea*, while Douillet-Breuil *et al.* (1999) observed a resveratrol accumulation on grapevine leaves treated with UV-C irradiation. Indeed, the induction of *STS* gene in plants often occurs in response to biotic or abiotic stresses (Jeandet *et al.,* 2002). The phenylpropanoids have defensive functions in plant through preformed or inducible physical and chemical barriers against infection to signal molecules involved in local and systemic signalling for defence gene induction (Dixon *et al.,* 2002*a*). Among phenolic compounds, stilbenes are involved in plant defence mechanisms against wood diseases by establishing a chemical barrier to limit the pathogen growth. However, the antimicrobial activity of these compounds depends on the phytopathogen (Lambert *et al.,* 2012). Progresses on gene transfer in plants of genes involved in plants' defence mechanisms against phytopathogens, such as *STS* genes have been reported (Hain and Grimmig, 2000; Jeandet *et al.,* 2002).

Given genes involved in the detoxification and stress tolerance, no modifications of *Hahl* and *GST5* expression were detected under the tested conditions. A similar trend was reported for AP stem and SP leaves of plants inoculated with *D. seriata* (Reis *et al.,* 2016) and on stems artificially inoculated with *N. parvum,* at different phenological stages (Spagnolo *et al.,* 2017). In contrast, the *GST1* expression was induced in pre-symptomatic leaves of esca-affected vines (Letousey *et al.,* 2010; Magnin-Robert *et al.,* 2011) and the expression of other enzymes in the phi and tau *GST* classes were induced in leaves before the appearance of esca visible symptoms, and then decreased once the disease became established (Valtaud *et al.,* 2009). The induction of this gene may suggest

an oxidative stress caused by GTDs and, the expression of *GSTs* suggests that this gene can be used as early marker of esca infection in grapevines. Another study reported that the induction of *GST* gene was higher in plants inoculated with both *P. chlamydospora* + *P. oligandrum,* a potential BCAs of esca disease, than those plants infected only with the pathogen (Yacoub *et al.,* 2016). In this case, these results proposed that the BCAs promote the priming, allowing a more intensive response of the plant against the pathogen infection (Yacoub *et al.,* 2016). The gluthatione S-transferase (*GST*) enzyme detoxify potential endogenous toxic metabolites produced during the oxidative stress, such as lipid peroxides (Letousey *et al.,* 2010). Indeed, this enzyme has a special attention regarding herbicide detoxification in plants, as they are crucial to remove toxins from the cytoplasm of plant leaves (Dixon *et al.,* 2002b; Valtaud *et al.,* 2009). The GSTs can be dived into different classes and, among them, tau and phi are the most numerous and inducible following an exposure of plants to stresses (Dixon *et al.,* 2002b). Thus, the tau GSTs may detoxify toxins by tau-transferases, while phi GSTs may protect plant against oxidation via the peroxidase and transferase activity (Valtaud *et al.,* 2009). In fact, glutathione is important for plant stress responses, such as an oxidative burst, and a decrease of glutathione in early plant stress stages could have detrimental effects on further appropriate stress responses (Valtaud *et al.,* 2009). Similar to previous studies, an upregulation of *HSP* gene was detected in green stems of plants inoculated with *D. seriata* (Reis *et al.,* 2016) and grapevine affected by esca proper and apoplexy (Spagnolo *et al.,* 2012). Conversely, no modifications of *HSP* expression was recorded in *N. parvum* artificially-inoculated stems at different phenological stages of grapevine (Spagnolo *et al.,* 2017). Curiously, also the Fito_S127B strain induced the expression of *HSP* in green stems though, no modifications were observed in plants inoculated with *D. seriata* + Fito_S127B, suggesting that this strain helped on grapevine responses against phytopathogen infection. The expression of heat shock protein (*HSP*) is correlated with response to stress, especially heat (Water *et al.,* 1996). Several HSPs have molecular chaperone function that bind partially folded or denatured proteins to prevent irreversible protein aggregation and inactivation (Water *et al.,* 1996).

Regarding the defence proteins (*Gluc, PR6*), an upregulation of both PR genes was observed in leaves in response to *D. seriata* inoculation. These results are in accordance with previous studies showing an induction of PR genes, such as *PR6*, *PR10*, Gluc or quitinases, on leaves, green stems and wood of plants affected by GTDs species (Valtaud *et al.,* 2009; Letousey *et al.,* 2010; Magnin-Robert *et al.,* 2011; Spagnolo *et al.,* 2012; Reis *et al.,* 2016; Yacoub *et al.,* 2016; Spagnolo *et al.,* 2017). Furthermore, the PR genes were induced in plants inoculated with Fito_S127B and/or Fito_F278 and phytopathogen + BCAs, reinforcing that these BCAs may stimulate the plant defence

responses. Altogether, these results indicated that grapevine may perceive signals as a result of the phytopathogen infection, and react to them by triggering defence pathways. These PR-proteins could be translocated across plants through phloem, allowing a better efficacy of plant responses (Bortolotti *et al.,* 2005). Furthermore, β-1,3- glucanases and quitinases are known to inhibit the mycelium growth of a wide range of fungal pathogens, through the degradation of their cell wall. Thus, the upregulation of *Gluc* on green stems from plants inoculated with *D. seriata* + Fito_S127B may be implicated with the decrease of the lesions length caused by the phytopathogen.

The expression of both aquaporin (*PIP 2.2*) and cell wall compounds (*fascAGP*) were not affected on green stems of cuttings inoculated with *D. seriata* or Fito_S127B and/or Fito_F278. These results are in accordance with a previous study on green stems of AP and SP plants inoculated with *D. seriata* strains and *N. parvum* (Reis *et al.,* 2016) and in pre-apoplectic grapevine leaves (Letousey *et al.,* 2010). Meanwhile, a repression of *PIP* 2.2 gene on both AP and SP leaves and *fasc*AGP on green stems was recorded on plants inoculated with *N. parvum* (Reis *et al.,* 2016), and a repression of *PIP* 2.2 was also observed in drying leaves after the appearance of esca symptoms (Letousey *et al.,* 2010). These results suggested that GTDs infection may perceive a water stress signal, especially on the latest steps of the disease (Letousey *et al.,* 2010). In any case, the expression of *PIP* 2.2 appeared to be affected at leaves and may be related with the photosynthesis disruption, as a consequence of GTD infection. *PIP,* a plasma membrane intrinsic protein, is an aquaporin for transcellular water transport across the plasma membrane and which have a crucial role on plant water relations, namely in water balance and water use efficiency (Tyerman *et al.,* 2002). Furthermore, these aquaporins are associated with plant tolerance to biotic or abiotic stresses (drought, salinity). The fasc*AGP* genes are thought to accumulate in response to elicitor molecules released by the phytopathogen and, thus, play a role in plant defence (Reis *et al.,* 2016).

Overall, our results suggested that both Fito_S127B and Fito_F278, activated the defence pathways of grapevine. Furthermore, Fito_S127B induced the *Gluc* expression in green stems and Fito_F278 over accumulated *PR6* in plant, which leaded to assume that these strains could promote a physiological condition in plant, namely the priming, and, thus, allowing grapevine to mobilize intensive defence reactions against phytopathogen infections. However, defence responses in plants inoculated with both *D. seriata* + Fito_S127B strain were generally stronger in stems if compared to the corresponding plants inoculated with phytopathogen + Fito_F278. These differences, together with the gene expression results at leaves, could have determined the lower lesions lengths recorded in *D. seriata* + Fito_S127B condition. In this sense, these results emphasize the promising biocontrol potential of Fito_S127B.

Strain-specific primers allowed a rapid and reliable identification of the BCAs colonization across grapevine plants

The reliable strain-specific identification is an important step to track the BCAs' efficacy to survive under *in vivo* assays and to follow-up their colonization capacity across plants, after their *in vivo* inoculation. The monitoring methods of a strain can rely into microscopy – based, cultivation-base, immunology and DNA-based techniques (van Elsas *et al.,* 1998). In the present study, the cultivation-based and DNA-based assessments were combined for the same analysis. Thus, in the cultivation-based assessment the tracking of both Fito_S127B and Fito_F278 were estimated by plating in semi-selective growth medium followed by a CFU count, which allowed the detection of viable and culturable microbial cells. However, and regarding the cutting plants, both target and non-target microorganisms were obtained, the distinction of strains was sometimes difficult and the microbial density naturally present in the biological material may have limited or even inhibited the growth of inoculated BCAs, because of their rapid development. Moreover, this was a time-consuming methodology (Felici *et al.,* 2008). To overcome this non-specific analysis, DNA-based methods were applied as these methods allow a more reliable and rapid strain identification. Although, and despite the advantages of this assessment, there is no discrimination between viable, dead cells or other cell-free DNA present in the biological material and, consequently, the microbial density may be overestimated (Felici *et al.,* 2008).

Going forward, strain-specific primers were developed for *Streptomyces* sp. Fito_S127B strain, *A. pullulans* Fito_F278 and even for *B. amyloliquefaciens* Fito_F321 to discriminate them from other microorganisms and to monitor their plant colonisation across grapevine plantlets and cutting plants. For this, several DNA sequences retrieved from their genome, and coding for different genes, were identified, aligned and compared across other closely related genomes available in public databases, to find unique nucleotides or DNA sequences that differentiate the target strains from all others. Given the Fito_S127B, three strain-specific primers targeting the tryptophan synthase beta chain (*trpβ*) and helix-turn-helix protein domain (*hpb and HxlR*) were developed. Among them, specificity tests with non-targeting strains showed that two set of primers were 100% Fito_S127B specific, namely those targeting the *trpβ* and *HxlR* gene. In order to minimize the number of PCR reactions only primers targeting the *HxlR* gene were selected to monitor the Fito_S127B. The helix-turn-helix protein domain is the most widely distributed family of DNA-binding proteins. The *HxlR* gene, located in this domain and upstream of the *hxlAB* operon, encodes two enzymes in the ribulose monophosphate pathway responsible for the detoxification of formaldehyde. The formaldehyde, which is toxic to all microorganisms, results from the

degradation of the organic compounds containing methyl or methoxy groups, such as lignin and pectin (Yurimoto *et al.,* 2005; Hingston *et al.,* 2015). Thus, the expression of *HxlR* gene, which is induced by the presence of formaldehyde, will increase the survival of this strain under these stresses. Regarding the Fito_F278, four sets of primers targeting the glutathione S-transferase (*GST*), elongation factor 1 (*EF1*), tryptophan synthase alpha chain (*trpα*) and tubulin (*Tub*) gene were developed. Despite the high sensitivity of these primers, the specificity tests demonstrated that they did not amplified other fungal, bacterial or plant DNA though they could amplify some non-target strains, notably some *A. pullulans*. However, these strains may probably be closely related or even the same strain as Fito_F278. Thus, and once the best results were obtained with primers amplifying *GST*, these were selected as strain-specific primers of Fito_F278 strain. *GST* is mainly involved in the detoxification process and tolerance of microorganisms to oxidative stress (Sheehan et al., 2001; McGoldrick et al., 2005). At last but not the least, a strain-specific primer targeting the tryptophan synthase beta chain (*trpβ*) was developed for *B. amyloliquefaciens* Fito_F321. A primers specificity of 78% was obtained due to the amplification of other *Bacillus* strains. Though, and as previously referred, these amplified non-target strains may probably be the same as Fito_F321 strain. The *trpβ* gene was already used as a housekeeping gene in phylogenetic studies of *Bacillus* species (Liu *et al.,* 2013). The synthesis of L-tryptophan is important for the survival and replication of most bacteria (Merino *et al.,* 2008). T*rpβ* catalyses the last step of the tryptophan biosynthesis from indole and serine. Interestingly, the biosynthesis of the auxin indole-3-acetic acid (IAA), which promotes the plant growth, is dependent on the presence of tryptophan (Idris *et al.,* 2007). Thus, the expression of genes involved in the biosynthesis of tryptophan and tryptophan- dependent synthesis of IAA may influence the IAA available and, consequently, the plant- growth promotion.

Contrary to PCR results obtained for the Fito_S127B and Fito_F278 detection within grapevine plantlets, in cutting plants non-specific amplification bands (data not shown) were obtained. To overcome this, a nested-PCR method was then carried out and new internal primers for each gene were designed. This strategy proved to be fundamental and more sensitive to identify these BCAs in plants. In a general way, with the molecular assessment, we demonstrated that Fito_S127B was able to successfully colonize roots from grapevine plantlets, while Fito_F278 colonized both roots and leaves. In cutting plants, the Fito_S127B was identified on soils and even on roots after two months of their plant inoculation (T3+4 weeks). Although, this colonization was not observed for all tested conditions and growing seasons. The Fito_F278 was detected in soils up to one month after its inoculation (T3+1 week) and then in roots, which seems to have constituted a favourable environment to its survival for longer periods. As previously referred, this colonization was not

observed for all tested conditions and growing seasons. Despite not previously mentioned, an attempt was made to identify both epiphytic and endophytic colonisation of BCAs across grapevine plantlets by using the strain-specific primers. However, PCR results were not reliable due to the poor DNA quality obtained and the PCR-inhibitory substances interfered with amplification.

The development of strain-specific primers and genomic markers were already being reported to detect BCAs. Thus, a study of Felici *et al.* (2008) used RAPDs to generate sequence-characterized amplified region (SCAR) markers as molecular probes to monitor the population of *B. subtilis* 101 strain in the rhizosphere of tomato. Other studies also detected bacteria such as *Azospirillum brasilense* FP2 strain (Stets *et al.,* 2015), fungi, namely *Epicoccum nigrum* 282 strain (Larena and Melgarejo, 2009), the protective *Fusarium oxysporum* Fo47 strain (Edel-Hermann *et al.,* 2011), *Trichoderma atroviride* 11 strain (Hermosa *et al.,* 2001) and yeasts such as *A. pullulans* L47 strain (Schena *et al.,* 2002). Additionally, some of these studies identified and quantified the specific strain densities by applying a real-time PCR (Larena and Melgarejo, 2009; Edel-Hermann *et al.,* 2011; Stets *et al.,* 2015).

Conclusion

So far, and to date, we have demonstrated for the first time the biocontrol potential of *Streptomyces* sp. Fito_S127B and *A. pullulans* Fito_F278 against Botryosphaeriaceae dieback agents, in grapevine cuttings under greenhouse conditions. In conclusion, *Streptomyces* sp. Fito_S127B strain, a soil isolate, successfully colonised grapevine roots and was able to protect young vines against *D. seriata*. This is a great deal of interest as this strain may be a promising biocontrol agent against GTDs. On the other hand, *Aureobasidium pullulans* Fito_F278, a resident microorganism from grapevine microbiome with endophytic potential, was the less effective under greenhouse conditions. In fact, the efficacy loss of BCAs in the field has been reported. Thus, keep the reproducibility of *in vitro* results from the laboratory to the field, together with improvements on microbial formulations to maximize the microbial performance and shelf-life, is a present challenge to guarantee the successful efficacy of BCAs.

Findings also showed that Fito_S127B and Fito_F278 activated specific defence responses of grapevine, which may have promoted a physiological condition in plant called priming. However, this must be further investigated to better understand the signalling pathway applied, namely if was a SAR through priming for salicylic acid (SA)-dependent defence, ISR through priming for jasmonic-acid (JA) and ethylene (ET) -dependent defence or by BABA-IR through ABA – and

phosphoinositide (PI)-dependent signalling. Moreover, the lower lesions lengths caused by the phytopathogen in plants inoculated with *D. seriata* + Fito_S127B, emphasizes not only the biocontrol potential of Fito_S127B but also its role in the activation of plant defence mechanisms, helping the plant to respond more rapidly to fungal development.

Concerning the strain-specific identification, the PCR assay developed in this study by using strain-specific primers was an efficient, simple, rapid, and reliable tool for screening and identification of both Fito_S127B and Fito_F278 strains, under plant colonization processes. Furthermore, we showed that combining both cultivation-based methods and conventional PCR was a useful strategy not only to easily detect and identify these strains but also to quantify the viable BCAs cells in grapevine. These results provide a basis for a future development of an effective real-time PCR method to identify and to monitor quantitively the Fito_S127B and Fito_F278 strains following its release in field trials.

Publication 9- Biocontrol potential and grapevine colonisation by the natural microbial resources of grapevine: a case study of *Aureobasidium pullulans* strain Fito_F278

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The supporting information of this publication is available in the Appendix 6 section.

Abstract

Grapevine is associated with natural microbial resources, whose interactions may have direct or indirect effects on plant growth and phytosanitary status. Some microorganisms have a natural ability to improve plant nutrition, tolerance to abiotic stresses or even to suppress grapevine pathogens. Indeed, grapevine is strongly threatened by several diseases outbreaks such as trunk diseases (GTDs), the most devastating diseases worldwide. Considering that plants are a potential source of biocontrol microorganisms, the exploitation of these communities is of utmost interest for further viticulture management strategies.

This study aimed to evaluate the biocontrol potential of natural microbial isolates from below- and above-ground parts of grapevine against *Botrytis cinerea* and Botryosphaeriaceae species. Their biocontrol potential was achieved by measuring the inhibition of pathogen growth (antibiosis tests) followed by the evaluation of hydrolytic enzymes (amylase, cellulase, lipase, pectinase, protease and urease), siderophores production, phosphate solubilisation, physiological traits (NaCl, pH) and patterns of grapevine colonisation.

Results showed that each plant ecosystem was dominated by specific microorganisms, confirming their adaptability to these niches. Overall, the major isolates were *Bacillus* (55.2%), *Streptomyces* (13.8%) and *Aureobasidium* (12.1%) and of the 202 obtained isolates, 15% and 19% were effective against *B. cinerea* and *D. seriata,* respectively ($p<0.05$). The most efficient biocontrol microorganism belonged to the *Bacillus* genera though *Streptomyces, Pseudomonas,* and *Aureobasidium* also suppressed the pathogen growth. Considering that *A. pullulans* is an abundant microorganism of grapevine, the Fito_F278 strain, which belonged to this species, was then selected. This strain significantly reduced ($p<0.05$) the growth of several Botryosphaeriaceae species and was able to produce siderophores, hydrolytic enzymes and to solubilize phosphate. The pectinolytic and cellulolytic activities showed the higher enzymatic index. Furthermore, Fito_F278 strain was able to grow at pH values between 5 and 11, in NaCl concentrations of up to 8% and to colonise grapevine at both endophyte and epiphyte level.

Grapevine is a natural source of biocontrol microorganisms and *A. pullulans* strain Fito_F278 is one of the resident microbiota. The biotechnological potential associated with this strain may determine the success of its biocontrol against a wide range of plant pathogens especially Botryosphaeriaceae.

Key words: Grapevine microbiome; trunk diseases; biological control; *Aureobasidium pullulans*; plant colonisation

Introduction

Grapevine, as all other plants, represents a natural reservoir of microbial resources, embedded in a complex micro-ecosystem, which the permanent interaction within plant may have a direct or indirect impact on plant growth and on sanitary quality (Pinto and Gomes, 2016). Consequently, these microbial communities, which may be pathogenic, neutral or beneficial, may influence the quality of bunches and the sensorial and organoleptic properties of wine (Fleet, 2003; Compant *et al.,* 2013; Pinto *et al.,* 2015). The relationship between plant and microorganisms constitutes a mutual interaction, as in turn plant ensures a protected environment and plant nutrients, that allow the development of these microbial communities (Baldan *et al.,* 2015).

Grapevine is largely attacked by different pathogens. Powdery mildew, downy mildew and grey mould, caused by *Erysiphe necator, Plasmopara viticola* and *Botrytis cinerea*, respectively, are important fungal pathogens (Armijo *et al.,* 2016). Although, the grapevine trunk diseases (GTDs), such as Esca, Botryosphaeriae dieback and Eutypiosis, are of utmost concern to wine industry since GTDs are the most destructive grapevine diseases worldwide (Mugnai *et al.,* 1999; Larignon *et al.,* 2009; Bertsch *et al.,* 2012; Fontaine *et al.,* 2016a). These fungal pathogens, infect the perennial organs and grow within the woody tissues, causing internal wood necrosis designated as black streaked, central or sectorial necrosis and white-rot, a typical woody necrosis associated with Esca disease (Larignon and Dubos, 1997; Bruez *et al.,* 2016). The external symptoms on leaves and berries may be a consequence of extracellular compounds that are transported by the transpiration stream (Mugnai *et al.,* 1999; Guérin-Dubrana et al. 2013). Among GTDs, the most frequent is Esca, a disease complex, as different fungal pathogens are associated and including *Phaeomoniella chlamydospora, Phaeocremonium minumum* and *Fomitiporia mediterranea*. Moreover, *Eutypa lata* and *Sterum hirsutum* may be also involved (Mugnai *et al.,* 1999; Bertsch *et al.,* 2012). Botryosphaeria dieback is caused by *Botryosphaeriaceae* species as *Botryosphaeria dothidea, Diplodia seriata, Diplodia mutila* or *Neofusicoccum parvum* and are the causal agents of trunk cankers and decline of grapevine (Larignon *et al.,* 2009; Úrbez-Torres, 2011; Fontaine *et al.,* 2016a). Eutypiosis or Eutypa dieback is caused by *Eutypa lata* although *Eutypa leptoplaca, Cryptovalsa ampelina, Diatrypella* spp. or *Eutypella* spp. may also be involved (Gubler *et al.,* 2005; Fontaine *et al.,* 2016a).

As symptoms and disease's diagnostic is rather difficult and complex, because one or several pathogenic fungi could be involved in the same plant, grapevines can become unproductive and

the vineyard replacement is imperative. According to the severity of disease appearance, plant decline and death may occur in a few years after the pathogen(s) attack or even in a few days, such as apoplexy, a severe form of GTDs (Larignon and Dubos, 1997; Guérin-Dubrana *et al.,* 2013). So far, there are no efficient treatments available to limit GTD emergence which constitutes a global threat to wine heritage and with negative repercussions at social and economic level (Bertsch *et al.,* 2012; Fontaine *et al.,* 2016a; Fontaine *et al.,* 2016b). Thus, one of the alternatives to prevent GTDs is to use beneficial microorganisms with biocontrol potential.

Indeed, beneficial microorganisms play a significant role in plants accessibility to nutrients, improving plant nutrition, in pathogen defence through an antagonistic activity, or by stimulating the plant defence responses through hormone-mediated signalling pathways as jasmonic acid (JA), ethylene (Et) or salicylic acid (SA). In general, and depending on their mode of action, these microorganisms are referred as biofertilizers or phytostimulators, when promote the plant growth, and as biocontrol when associated with plant protection (Berg, 2009; Bloemberg and Lugtenberg, 2001; Baldan *et al.,* 2015; Armijo *et al.,* 2016). The biofertilization is related to nitrogen fixation and some of the most efficient microorganisms belong to the genera *Allorhizobium*, *Azorhizobium, Bradyrhizobium, Burkholderia, Rhizobium* or *Mesorhizobium* but others such as *Acetobacter* and *Azetobacter* are also known as nitrogen-fixing microorganisms (Bloemberg and Lugtenberg, 2001). Phytostimulation consists in enzymes secretion such as auxins like the indole-3-acetic acid (IAA), cytokinins, gibberellins or ethylene, that promote the plant growth (Bloemberg and Lugtenberg, 2001). Among microorganisms, the *Azospirillum* spp. genus is a well characterized phytostimulator. In the biocontrol activity, antagonistic microorganisms control the fungal pathogens or bacteria growth, by several mechanisms as a direct antibiosis through production of antimicrobial compounds and volatile organic compounds (VOCs), by degradation of pathogen's virulence factors, competition for space and nutrients, competition for minerals as iron through siderophores production, or by inducing plant resistance (Bloemberg and Lugtenberg, 2001; Whipps 2001; Compant *et al.,* 2005; Berg, 2009). Indeed, several microorganisms such as *Bacillus* spp*., Pseudomonas* spp*., Streptomyces* spp. or the fungal genera such as *Ampelomyces* and *Trichoderma* are well known antagonists and some of them are commercially available (Bloemberg and Lugtenberg, 2001; Berg, 2009). The application of these biocontrol agents consists in a sustainable management that will reduce the use of chemical fertilizers and pesticides which ones disturb clearly the natural microbial population including beneficial communities (Pinto *et al.,* 2014).

In the last years, the characterization of the grapevine microbiome has been object of study. In general, and among bacteria, the Proteobacteria and Firmicutes phylum are the most abundant

across grapevine and include mainly the *Pseudomonas, Pantoea* or *Bacillus* genera whereas, among fungal communities, *Aureobasidium pullulans, Rhodotorula* or *Alternaria* are some of the most abundant eukaryotic population (reviewed in Pinto and Gomes, 2016). The better knowledge of these natural microbial resources and their interactions with grapevine will allow the identification and characterization of beneficial microorganisms with biocontrol potential from and for grapevine protection and, thus, promote advances in its management.

In the present study, a deep analysis of the microbial resources associated with grapevine was performed. For this, the population structure of grapevine and their biological control potential against fungal pathogens as *Botrytis cinerea* and *Botryosphaeria* species were firstly explored. Then an extended analysis of *Aureobasidium pullulans* strain Fito_F278, one of the most abundant microorganism from grapevine, was achieved in order (i) to better understand its natural abundance on plant, (ii) to evaluate its efficacy to protect the plant against grapevine pathogens namely, those responsible for Botryosphaeria dieback, (iii) to characterize its biochemical potential and (iii) to determine the epiphytic and endophytic patterns of grapevine colonisation.

Material and methods

Isolation, identification and characterization of potential antagonistic microorganisms

Sampling site and microbial isolation

Samples were collected across four vineyards located in the Bairrada Appellation at Cantanhede – Portugal from April to September in 2011 and September in 2012. The sampling was randomly assigned across vineyards and samples such as soil, roots, leaves, stems and berries were collected from different grapevine varieties. Briefly, the plant tissues were (a) homogenised in a sterile saline solution (0.85%) with a sterile pestle or (b) previously surface sterilized with 70% ethanol for 5 min, followed by 1% sodium hypochlorite and washed three times in sterile MiliQ water. Microorganisms were then isolated on PDA (Merck) or YPD (Yeast Extract-Peptone-Dextrose: Yeast extract 10 g.L^{-1}; Glucose 20 g.L^{-1}; Peptona 20 g.L^{-1}; Agar 20 g.L^{-1}) and incubated at 28°C for 48h. Then different colonies were selected and pricked in new culture plates and incubated at 28°C for 48h. The isolation process was repeated until obtain pure cultures. For long-term preservation, each isolate was stored in Cryovials containing PDB (Formedium) or YPD broth with 80% glycerol for bacterial or yeasts isolates and 20% glycerol for fungi at -80°C.

***In vitro* assessment of antifungal capacity**

A preliminary screening of the antagonistic activity of a total of 202 obtained isolates against *Botrytis cinerea* strain 630, from the University of Reims Champagne-Ardenne (France), and *Diplodia seriata* strain Fito_F14, isolated at 2011 from grapevine in the Bairrada appellation, was performed in order to pre-select potential isolates with antagonistic activity. For this, a 3-mm diameter of mycelium agar disk of fungal pathogen with 7 days old was placed at the center of a new PDA plate and four different isolates with 48/72h old deposited at 2 cm away from the border of the Petri dish. Cultures were incubated in triplicate at 28°C and followed for 7 days. Herein the mycelium inhibition was not calculated.

After a pre-screening test, the potential antagonist microorganisms were selected and a co-culture test performed. For this, a 3-mm diameter plug of pathogen was transferred to 2.5 cm from the border of the plate and each isolate inoculated at 180° from the pathogen and at the same distance from the border. Plates inoculated only with the pathogen served the control. The assay was performed in triplicate and plates were incubated at 28°C and followed for 7 days. The inhibitory effect of each isolated strain against the pathogen was calculated based on the percent relative of mycelium inhibition through the formula (MI%): MI% = 100* (Mfg-Mga)/Mfg, where Mfg corresponds to the mycelium free growth and Mga to the mycelium growth in the presence of the antagonistic microorganism.

Further, the antagonistic capacity of *Aureobasidium pullulans* strain Fito_F278 against Botryosphaeria dieback namely, *Diplodia seriata* (strains F98.1 (Robert-Siegwald *et al.,* 2017) and Ds99.7) and *Neofusicoccum parvum* (strains Np Bt-67, Np Bourgogne and Np SV) was tested according to the methodology described above. The inhibitory effect of Fito_F278 against fungal pathogens was calculated through the area of pathogen mycelium growth over time by using the Image J 1.50b software (National Institutes of Health, USA).

Biochemical and physiological characterization of *Aureobasidium pullulans* strain Fito_F278

The selected microorganism was tested for its capacity to produce siderophores and to solubilise phosphate under *in vitro* conditions. Siderophore production was determined by applying the method of Chrome Azurol S agar assay test (CAS) according to Alexander and Zuberer (1991). The siderophore production was observed through the yellow halo formation around colonies.

The phosphate solubilisation was analysed with Pikovskaya culture medium (Glucose 10g/L; NaCl 0.2g/L; $(NH_4)_2(SO_4)$ 0.5g/L; Yeast extract 0.5g/L; $MnSO_4$ 0.1g/L; $MgSO_4$ 0.1g/L; Agar 20g/L and $Ca_3(PO_4)$ 5g/L that was sterilized separately) and the degradation halo (clear zone) around colony

corresponded to a positive activity. For both tests, plates were incubated until a period of 10 days at 28°C and in triplicate.

The enzymatic activity was analysed for amylase, cellulase, lipase, pectinase, protease and urease production. Each enzymatic activity was determined in specific culture media and results were expressed by positive activity, when a clear zone around strain colony was observed, or negative activity. Then, the enzymatic index (EI) was calculated by the relationship between the average diameter of the degradation halo (clear zone) and the average diameter of the colony growth. All enzymatic activity tests were performed in triplicate and in each experiment, Fito_F278 was inoculated twice. The negative control consisted of a Petri dish containing the specific culture media without strain inoculation.

The amylolytic capability of Fito_F278 was assessed by spot inoculation of the strain in PDA at 28°C for 48h which was then flooded with 5 mL iodine solution for 2 min. Cellulases production was assessed according to Kasana *et al*. (2008). For this, Fito_F278 was spot inoculated in CMC agar ($NaNO_3$ 2 g.L^{-1}; K_2HPO_4 1 g.L^{-1}; $MgSO_4$ 0.5 g.L^{-1}; KCl 0.5 g.L^{-1}; carboxymethylcellulose (CMC) sodium salt 2 g.L^{-1}; peptone 0.2 g.L^{-1}; agar 17 g.L^{-1}) at 28°C for 48h and then flooded with 5 mL of iodine solution for 2 minutes. The lipase production was confirmed through the spot inoculation of the strain in PDA supplemented with 1% Tween-20 (Hasan *et al.,* 2013), a lipid substrate, and incubated at 28°C for 48h. The capacity to hydrolyse pectin was assessed by spot inoculation of Fito_F278 in nutrient agar (NA) (peptone 5 g.L^{-1}; beef extract 3 g.L^{-1}; NaCl 5 g.L^{-1}; Agar 15 g.L^{-1}; pH 6.8) supplemented with 0.2% of pectin, incubation at 28°C for 48h and then flooded with 5 mL of iodine solution for 2 minutes. Briefly, the proteolytic activity was confirmed according to Hasan *et al*. (2013). Fito_F278 was spot inoculated in Petri dishes with NA supplemented with 1% of gelatin, a protein source, and incubated at 28°C for 48h. The urease screening was detected according to Seeliger (1956), with some modifications. The Christensen's culture media (peptone 1 g.L^{-1}; glucose 1 g.L^{-1}; NaCl g.L^{-1}; KH_2PO_4 2 g.L^{-1}; phenol red 0.012 per 1L; agar 20 g.L^{-1}; pH 6.8) was distributed in 1.5mL microtubes and a drop of 20% urea solution, sterilised by filtration, was added. The strain was then inoculated and incubated at 28°C until a period of 5 days. The urea hydrolysis causes a colour change of the media from orange-yellow to pinkish red (Seeliger, 1956).

Furthermore, strain was also characterized for different physiological traits namely, its capacity to growth on different pH and salinity conditions. For pH analysis, the strain was streaked in YPD medium and adjusted with pH 5, 6, 7, 9 and 11 and incubated for 48h at 28°C. For the salinity effect, the strain was streaked again in YPD medium and adjusted with different salt concentrations (0%,

2%, 4%, 6%, 8%, 10%, 12% and 14% of NaCl), and incubated for 72h at 28°C. Experiments were performed in triplicate.

Molecular identification of isolates with antagonistic potential

The genomic DNA of isolates were extracted by using the Wizard Genomic DNA Purification kit (Promega, Madison, USA), following the standard protocol for bacteria or yeasts. The DNA integrity and quality was checked by 0.8% agarose gel electrophoresis and by using NanoDrop spectrophotometer (Thermo Scientific, USA), respectively.

The molecular identification of each isolate was performed after sequencing of the 16S rDNA for bacteria identification, by using forward (5'-AGAGTTTGATCACTGGCTCAG-3') and reverse (TACGGCTTACCTTGTTACGACTT) primers, and the ITS region for yeasts identification, with ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') primers (White *et al.,* 1990). PCR Reactions were carried out in 25 µL reaction mix and distinct PCR reactions were performed for 16S rDNA and ITS analysis. Thus, reactions for 16S rDNA identification contained 1x reaction buffer (USB, Affymetrix), 1.7 mM of $MgCl_2$ (USB, Affymetrix), 0.2mM dNTPs (Bioron), 1U of FideliTaq DNA Polymerase (USB, Affymetrix), 0.2µM of forward and reverse primers and 2 µL of genomic DNA. The ITS reactions containing 1x reaction buffer (Biocant own buffer), 2mM $MgCl_2$, 0.2mM dNTPs (Bioron), 1U of Taq DNA Polymerase (Biocant own taq DNA polymerase), 0.4µM of forward and reverse primers and 2µL of genomic DNA. The 16S rDNA cycling conditions were a first step at 94°C for 4 min followed by 25 cycles with a denaturation step at 94°C for 30s, annealing at 50°C for 30s and extension at 72°C for 45s, and a final extension cycle at 72°C for 5 min. For ITS region, cycling conditions consisted in a first denaturation step at 95°C for 6 min followed by 35 cycles of a 94°C for 40s, 53°C for 40s and 72°C for 1 min, and a final extension cycle at 72°C for 5 min. PCR products were purified with Illustra Exostar kit (GE Healthcare Life Sciences) and sequenced using the 3500 Genetic Analyser (Applied Biosystems) at Biocant, Portugal.

<u>Colonization bioassay</u>

Plant material and growth conditions

A bioassay was performed to verify the ability of *Aureobasidium pullulans* strain Fito_F278 to colonize *in vitro* plants of grapevine. Plantlets of *Vitis vinifera* L. cv Chardonnay clone 7535 used in this bioassay were propagated by nodal explants in culture tubes with 25 mm diameter, containing

15mL of Martin Medium (Martin *et al.,* 1987). Plants were grown in a growth chamber under white fluorescent light (200µmol.m^{-2}.s^{-1}), 16h photoperiod and at a temperature constant of 26°C (Compant *et al.,* 2005).

Inoculation of *in vitro* plantlets of grapevine

Plantlets with five-week-old were then selected and for each experiment, two conditions were performed, namely (a) control and (b) plants inoculated with Fito_F278 strain. Each condition contained *n*=15 uniform plants and the experiment was repeated three times.

Plant inoculation was performed by dipping the plant roots during 10s in a 5mL of strain suspension in PBS at pH 7.5 (1 x 10^6 CFU/mL of Fito_F278) or PBS at pH 7.5 (control treatment). Plants were then carefully transferred to Magenta Box containing 100mL of semi-solid Martin Medium (Martin *et al.,* 1987) and incubated in the growth chamber as described above. Each Magenta Box contained 2 plants.

Grapevine colonization analysis

The effect of strain on the plant health status and its colonization capacity was compared with control plants at 4, 7 and 14 days' post root inoculation (dpi). For each sampling time, 5 plants of each condition were selected, pooled together and 2 biological replicates were performed and analysed by classic microbiology and molecular techniques. For each replicate, root and leaves fresh weights were determined. Therefore, fresh plant root and leaves from control and inoculated plants were also collected for three-dimensional (3D) microscopy analysis (VHX-2000 (Z100x100)).

For classic microbiology analysis, the epiphytic and endophytic colonization of plants were analysed by plate counting. For this, roots and leaves of each condition were sampled, weighted and rinsed in sterile distilled water. The epiphytic colonization was analysed after performed 10-fold serially dilutions and by plating 100µL on YPD for Fito_F278 or Luria-Agar (LA) for control. For the endophytic colonization analysis, both roots and leaves were surface sterilized with 70% ethanol for 1 min, followed by 0.6% commercial bleach for 3 min and washed four times in distilled water. Samples were then ground in 1mL of PBS pH 7.5 and macerated. After a vortex and a 10-fold serially dilution, samples were cultured on the respective culture medium as described above. To ensure the efficacy of the sterilization step, 100µL of the last wash solution of each condition was cultured on the respective culture medium. For both analysis, colonies were counted after 48 to 72h of incubation at 28°C.

For molecular analysis, roots and leaves from each condition and sampling time were stored at -80°C until processed.

Molecular validation of the strains colonization

The genomic DNA of roots and leaves were extracted by using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

To confirm the strain colonization of plants, strain-specific primers pairs were designed for Fito_F278 strain. For this, after the whole genome sequencing (WGS), strain-specific primers were designed through an *in-silico* genome analysis by BLASTn search of discriminative nucleotide sequences. Of them, the gene encoding the Glutathione S-transferase (GST) was the best strain-specific sequences to identify Fito_F278 strain. The sequence-specific primers were: GST_F 5'-GCTGACCGCAATTCGCATAC-3' and GST_EF1R 5'-GTTGCTCATGAAGGTGAGGG-3'. PCR reactions were carried out in 25 µL reaction mix containing 2 µL of genomic DNA, 1.25 U of Dream Taq DNA polymerase (Thermo Scientific, US), 1x Dream Taq buffer with $MgCl_2$ (2 mm), 0.2mM dNTPs (Thermo Scientific, US) and 0.2 µM of each primer. Cycling conditions consisted in a first denaturation step at 94°C for 4 min followed by 30 cycles with a denaturation step at 94°C for 30s, annealing at 58°C for 30s and an extension at 72°C for 45s. A final extension cycle at 72°C for 5 min was applied.

Statistical analysis

The Principal Component Analysis (PCA) for microbial population distribution analysis was performed by using Primer E software version 6 (Clarke and Gorley, 2006) and XLSTAT software's. The significance of difference between samples in the antagonistic activity, physiological traits and grapevine colonisation was analysed using the SPSS software version 20.0 (SPSS, Inc., Armonk, NY) and XLSTAT software by one-way analysis of variance (ANOVA), followed by Tukey and Bonferroni *post hoc* analysis, and a confidence limit of 95% was applied. The assumptions of ANOVA were determined through Shapiro-Wilk test (p>0.05), for normality test, and Levene's test (p>0.05) for homogeneity of variances in the residuals. In cases where the assumptions for a parametric ANOVA were rejected, the non-parametric Kruskal-Wallis test was applied. Standard error of the mean (SEM) were calculated for all mean values. For the plant colonisation analysis, the colonies of each sample were transformed to the logarithmic scale.

Results

Microbial diversity

Over the 2011 and 2012 *Vitis vinifera* growth cycle, a total of 202 isolates were obtained from soil, root, stem, leaves, berries and musts samples. In this study, most of microorganisms were isolated from the soil and leaves, as a consequence of the initial number of samples collected. Only isolates (*n*=58) that showed an antagonistic potential against different grapevine pathogens (data not showed) were further identified by molecular techniques. Among them, results showed that each plant sample where dominated by specific microorganisms which confirmed their adaptability to specific plant location (Figure 1).

The most commonly isolated genera were *Bacillus* (n=32), *Streptomyces* (n=8) and *Aureobasidium* (n=7), accounting for 55.2%, 13.8% and 12.1%, respectively. The soil isolates were mostly *Streptomyces* microorganisms, identified as *Streptomyces* sp. and *S. coelicolor,* followed by *Bacillus* sp. and *B. subtilis*. Others as *B. amyloliquefaciens, B. cereus*, *Burkholderia* sp., *Paenibacillus* sp. or *Pseudomonas* sp. were also isolated. The root isolates were identified as *Bacillus amyloliquefaciens, Bacillus* sp. and *B. subtilis* and at leaves, isolates were mostly *B. amyloliquefaciens, Bacillus* sp. and *Aureobasidium pullulans.* Though, others were identified and included *B. methylotrophicus, Cryptococcus magnus*, *Streptomyces chartreusis* and *Ustilago cynodontis.* The isolates obtained from berries and musts where particularly yeasts namely, *Aureobasidium pullulans* (berries) and *Metschnikowia pulcherrima* (berries and musts). Others as *Hanseniaspora uvarum*, *Saccharomyces cerevisiae* and the bacteria *Bacillus* sp., *B. subtilis* and *B. cereus,* were also isolated in musts and both *Pantoea* sp. and *Bacillus* sp. in berries (Figure 1).

Evaluation of the antifungal activity

The antagonistic capacity of grapevine isolates was tested against *Botrytis cinerea* (strain 630) and *Diplodia seriata* (Fito_F14) (Figure 2). Approximately, 15% (n=31) and 19% (n=39) of isolates, respectively, inhibited the mycelium growth of pathogens ($p<0.05$) through the co-culture assay (Appendix 6: Figure S1; Table S1). Among them, isolates Fito_F293 and Fito_F321, both from *Bacillus* genera, were the strains with a major inhibitory activity against *B. cinerea* and *D. seriata,* respectively. Although, others from *Streptomyces, Pseudomonas* and *Aureobasidium* genus also inhibited the pathogens growth. The yeast *Saccharomyces cerevisiae* showed an antagonistic activity especially against *B. cinerea.*

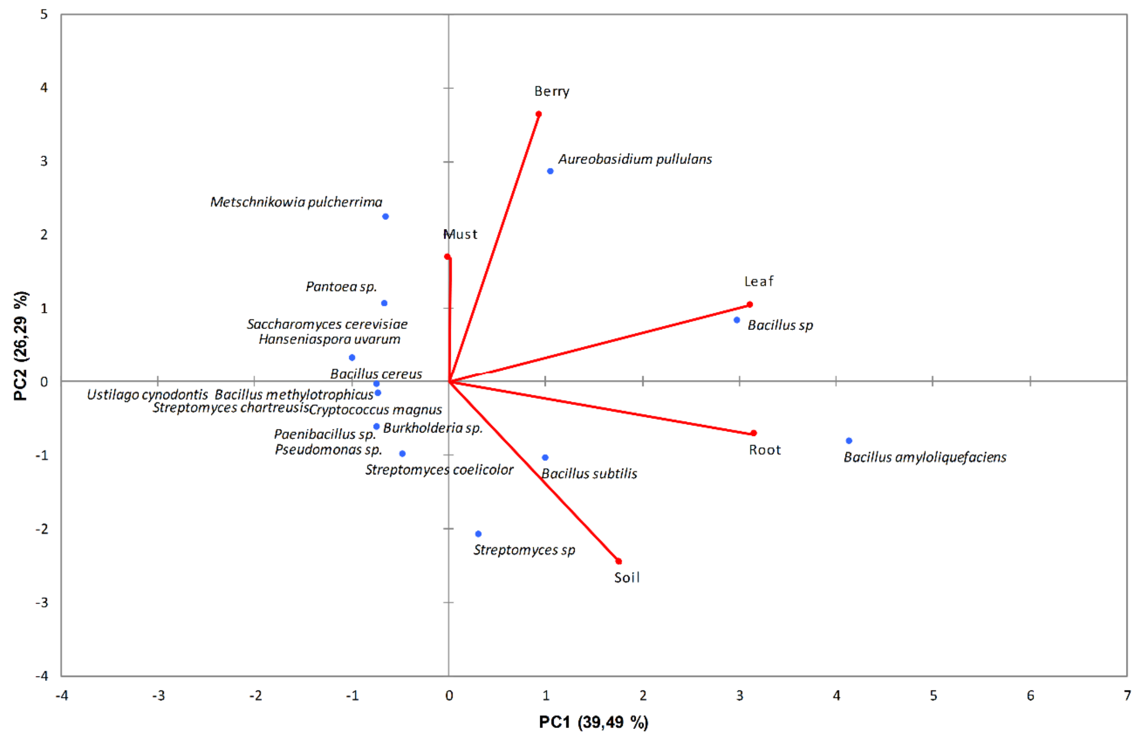

***Figure 1: PCA biplot of microbial isolates diversity and their distribution across grapevine structure*.** In the PCA biplot, based on a Pearson correlation coefficient, the variance explained by each PCA axis is given in the parentheses.

Among all, the isolate Fito_F278, an *Aureobasidium pullulans* strain, was chosen for further studies considering its natural high abundance in grapevine microbiome (Pinto *et al.,* 2014), abroad antagonistic activity over a high range of phytopathogens (Ippolito *et al.,* 2000; Castoria *et al.,* 2001) and the benefit, to better understand its interaction with grapevine.

The interaction and antagonistic activity of *A. pullulans* strain Fito_F278 was then assayed against different strains responsible of Botryosphaeria dieback, a GTD agent, namely *D. seriata* (strains F98.1 (Robert-Siegwald *et al.,* 2017) and Ds99.7) and *Neofusicoccum parvum* (strains Np Bt-67, Np Bourgogne and Np SV) through a co-culture test. Details of the pathogens origin and antagonistic activity observed against all pathogens tested are shown in Table 1 and Figure 2. The strain Fito_F278 reduced significantly the mycelium growth of all pathogenic fungi (p<0.05). This inhibition is early observed, 2 or 3 days after inoculation, and is clearly notorious after the 4-day post inoculation (Appendix 6: Figure S2). The strains more susceptible to mycelium inhibition were *D. seriata* strain F98.1 (33.51 ± 0.62%) and *N*. *parvum* strain Np Bourgogne (26.53 ± 4.09%). Contrarily, *D. seriata* strain Ds99.7 (7.80 ± 0.78%) was the less susceptible to inhibition by Fito_F278.

Evaluation of *A. pullulans* (Fito_F278) for enzymatic production and physiological traits

A. pullulans strain Fito_F278 was able to produce siderophores and to solubilize the phosphate under *in vitro* conditions (Table 2). Fito_F278 presented enzymatic activity that ranged between the pectinolytic activity (10.00 ± 0.00), cellulolytic (10.50 ± 0.20), proteolytic (1.83 ± 0.15), lipolytic (1.81 ± 0.15) and amylolytic (1.42 ± 0.05). Although, the urease activity was not detected (Table 2). Further, Fito_F278 grown under a gradient of pH ranging from 5 to 11 (Table 3) and no significant differences were found on the strain abundance (CFU/mL) under the different pH in analysis. However, the morphology of colonies was slightly altered, becoming smaller at pH 9. Under salinity conditions, Fito_F278 was able to grow up to 8% NaCl (Table 3). Significant differences (p<0.05) of strain abundance (CFU/mL) were found between standard conditions (0% NaCL) and 4%, 6% and 8% NaCl, respectively. In the meantime, the morphology of colonies was altered with NaCl, becoming smaller by increasing the NaCl concentration in the culture medium.

Colonization of *in vitro* plants of grapevine by *A. pullulans* (Fito_F278)

To address the plant colonisation capacity of Fito_F278, roots of plantlets cv Chardonnay were dipped in a Fito_F278 strain solution at 10^6 CFU/mL and then allowed to grow under *in vitro* conditions, in a photoperiod chamber. At 4 days' post root inoculation, the strain CFU count at root

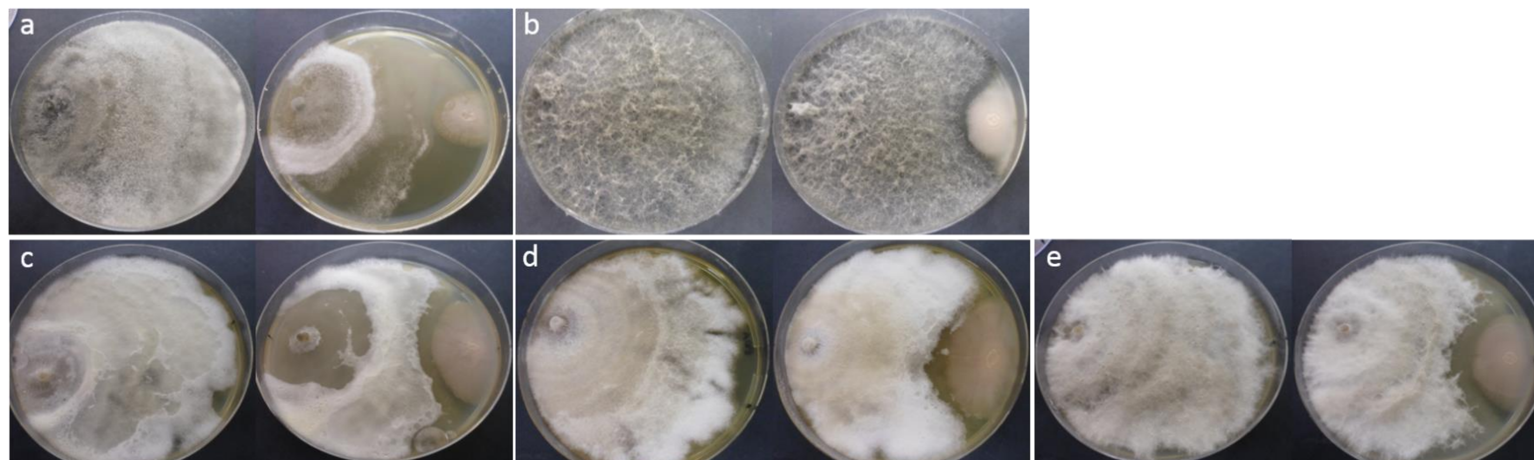

Figure 2: In vitro antagonistic activity of Aureobasidium pullulans strain Fito_F278, a natural occurring strain from Vitis vinifera, against fungal pathogens responsible for the Botryosphaeria dieback. Antagonistic activity against (A) *Diplodia seriata* strain F98.1, (B) *Diplodia seriata* strain Ds99.7, (C) *Neofusicoccum parvum* strain Np Bt-67, (D) *Neofusicoccum parvum* strain Np Bourgogne and (E) *Neofusicoccum parvum* strain Np SV.

Table 1: Evaluation of the antagonistic activity of Aureobasidium pullulans strain Fito_F278 against fungal pathogens responsible for the Botryosphaeria dieback. Results of the area of pathogen's growth (cm^2) and the inhibition of the pathogen's mycelial growth (%) after 14 days of inoculation are presented by Mean ± SEM values. *Fito_F278 reduced significantly the area of the fungal growth (p<0.05).

Fungal pathogen				Area of pathogen growth (cm^2)		
	Strain	**Isolation source**	**Country**	**Free pathogen growth (control)**	**Pathogen growth with Fito_F278 inoculation**	**Inhibition of pathogen's mycelium growth (%)**
Diplodia seriata	F98.1	Syrah	Pyrénées-Orientales, France	62.42 ± 0.46	41.51 ± 0.39*	33.51 ± 0.62
	Ds 99.7	Clairette	Rhône-Alpes, France	59.47 ± 0.20	54.73 ± 0.46*	7.80 ± 0.78
Neofusicoccum parvum	Np Bt-67	Fernão Pires	Estremadura, Portugal	55.22 ± 0.99	45.45 ± 1.52*	17.69 ± 2.75
	Np Bourgogne	Chardonnay	Bourgogne, France	62.27 ± 0.59	45.75 ± 2.54*	26.53 ± 4.09
	Np SV	Syrah	Bouches-du-Rhône, France	60.43 ± 0.44	53.48 ± 2.07*	11.50 ± 3.42

surfaces increased 74-fold (7.40x10^7 CFU/mL), when compared to the initially strain solution inoculated (Figure 3). Fito_F278 strain was detected at the internal root tissues (5.52x10^4 CFU/mL) and at leaf surfaces (3.64x10^4 CFU/mL). At 7 dpi, the CFU count was similar for both internal root tissues and leaf surface, while 13-fold higher levels were detected at root surface. At 14 dpi, a decrease on root and leaf colonisation, for both external and internal tissues was observed. Curiously, Fito_F278 was also able to colonise the internal tissues of leaves even if in very low quantities (data not shown) and this colonization was not systematic. Despite the increase and the decrease of CFU count, no statistical differences were found over time for each plant tissue in analysis.

Regarding the plant-microbial interaction effect on the *in vitro* plants health status, the plant inoculation with Fito_F278 strain had repercussive effects on the plant growth and development compared to control plants (Figure 4). Symptoms such as stagnation of the plant growth (Figure 4), a strain biofilm at the plant roots level (Figure 5A) and discoloration and spot necrosis on the leaves (Figure 5B), appeared from 7 dpi. These symptoms evolved, and at 14 dpi the plantlet was clearly fragile, with short and dark roots and with several spots on the leaves (Figures 4 and 5). The nutrients competition between plantlets and strain, together with the high inoculum concentration of Fito_F278, appears to have a hurtful effect on the plants.

Design of strain-specific primer for *A. pullulans* strain Fito_F278

The sequencing of the Fito_F278 genome strain allowed to select gene sequences for intra and inter-species discrimination and, thus to develop a strain-specific primer. The glutathione S-transferase (GST) gene, corresponding to a class of enzymes which employ glutathione (GSH) in several reactions and have an antioxidant function (Sheehan *et al.,* 2001; McGoldrick *et al.,* 2005), was chosen for Fito_F278 identification. After blasted the gene sequence against different strains, the strain-specific primer was designed, the PCR conditions optimized and the sensitivity and specificity determined. The PCR amplification of Fito_F278 originated an expected band with 750 bp and the PCR analysis with DNA samples from other different strains did not give rise to any DNA amplification (data not shown), which confirmed the specificity of these primers.

In order to validate the grapevine colonisation by Fito_F278, a PCR amplification was performed for roots (Figure 6A) and leaves (Figure 6B) at 4, 7 and 14 dpi (Figure 6). The PCR results agree with the re-isolation data (Figure 3) except at 4 dpi where Fito_F278 was not detected in leaves.

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Table 2: Production of siderophores, phosphate solubilisation and extracellular enzymes (amylase, cellulase, lipase, pectinase, protease and urease) by Aureobasidium pullulans strain Fito_F278.

	Reaction result	**Enzymatic index**
Siderophore	+	-
Phosphate solubilisation	+	-
Amylase	++++	1.42 ± 0.05
Cellulase	++++	10.50 ± 0.20
Lipase	++	1.81 ± 0.15
Pectinase	++++	10.00 ± 0.00
Protease	+	1.83 ± 0.15
Urease	-	-

The "+" indicates activity (halo observation) and "-" indicates no activity; The enzymatic activity is expressed according to the degradation halo formation size: (+) halo ≤0.4 cm; (++) halo 1.0 cm; (+++) halo 1.0-2.0 cm and (++++) halo > 2.0 cm. The enzymatic index (EI) was calculated by the relationship between the average diameter of the degradation halo and the average diameter of the colony growth. Results of EI are presented by Mean ± SEM values.

Table 3: Evaluation of the physiological traits (pH and salinity) on the growth of Aureobasidium pullulans strain Fito_F278.

	Condition	**General result**	**log (CFU/mL)**
pH levels	5.0	+	5.55 ± 0,07
	6.0	+	5.71 ±0.08
	6.5 (standard)	+	5.68 ± 0.06
	7.0	+	5.65 ± 0.05
	9.0	+	5.49 ± 0.06
	11.0	+	5.44 ± 0.04
Salinity levels (% NaCl on culture medium)	0% (standard)	+	6.79 ± 0.02
	2%	+	6.64 ± 0.03
	4%	+	6.53 ± 0.07*
	6%	+	6.51 ± 0.03*
	8%	+	6.43 ± 0.02*
	10%	-	-
	12%	-	-
	14%	-	-

The "+" indicates the growth and "-" indicates without growth. The strain growth was log transformed and results are presented by Mean ± SEM values. *Significant differences ($p<0.05$) of strain abundance (CFU/mL) when compared with standard conditions (0% NaCl).

Discussion

Herein an analysis of the cultivable microbial resources of grapevine and their potential to naturally protect grapevine against important diseases were investigated. Further, our focus was on the deep characterization of *Aureobasidium pullulans* strain Fito_F278 to understand its potential as biocontrol agent against GTDs, its relevance in grapevine colonisation and to develop specific-strain primers to monitor its plant colonisation.

Population structure of grapevine reveals a natural biocontrol potential

Overall, a set of isolates from different structures of grapevine such as soil, root, stem, leaves, berries or musts where obtained and identified. Although a small percentage of microbial microorganisms were isolated, which is not fully representative from grapevine microbiome, these isolates agreed with those previously reported (Barata *et al.,* 2012; Pinto *et al.,* 2014). Both below- and above-ground samples where dominated by specific microorganisms which confirm their major adaptability and preference for certain niches across grapevine (Martins *et al.,* 2013; Zarraonaindia *et al.,* 2015). Such microbial distribution is influenced by several factors such as physico-chemical conditions (pH, plant fertilisation, soil type), nutritional characteristics of below- and above-ground parts of the plant or plant age. Thus, both soil and plant produce a selection pressure on their associated microbial communities (Marschner *et al.,* 2004). This is in agreement with a study of Marschner and collaborators (2004) in which the experimental data suggested the strong effect of soil type on the rhizosphere microorganisms from barley and cucumber.

In general, our samples were dominated by *Bacillus* (55.2%), *Streptomyces* (13.8%) and *Aureobasidium* (12.1%). Among them, *Bacillus* sp. was consistently present in all ecosystems, which is in agreement with previous reports (West *et al.,* 2010; Compant *et al.,* 2011; Martins *et al.,* 2013). Indeed, species from this genus are of biotechnological interest since they can act as BCAs or even stimulate the plant growth and health, through several antibiotics or enzymes that they can actively produce (Baruzzi *et al.,* 2011). Furthermore, they can synthetize resistant endospores, allowing its successful colonisation (Baruzzi *et al.,* 2011). Overall, belowground samples (soils and roots) were dominated by *Bacillus* sp. and *Streptomyces* sp., while the aboveground samples were dominated by the yeast-like fungus *A. pullulans*. In fact, *Streptomyces* sp. is ubiquitous in soils and have an important role in mineralization processes (Manteca and Sanchez, 2009; Seipke *et al.,* 2012). In vineyards, *A. pullulans* is considered as a resident microbiota that can be associated with both below- and aboveground parts of the plant (Sabate *et al.,* 2002; Grube *et al.,* 2011; Barata *et al.,* 2012; Pinto *et al.,* 2014) though, in this study no isolates were obtained from soils or root samples.

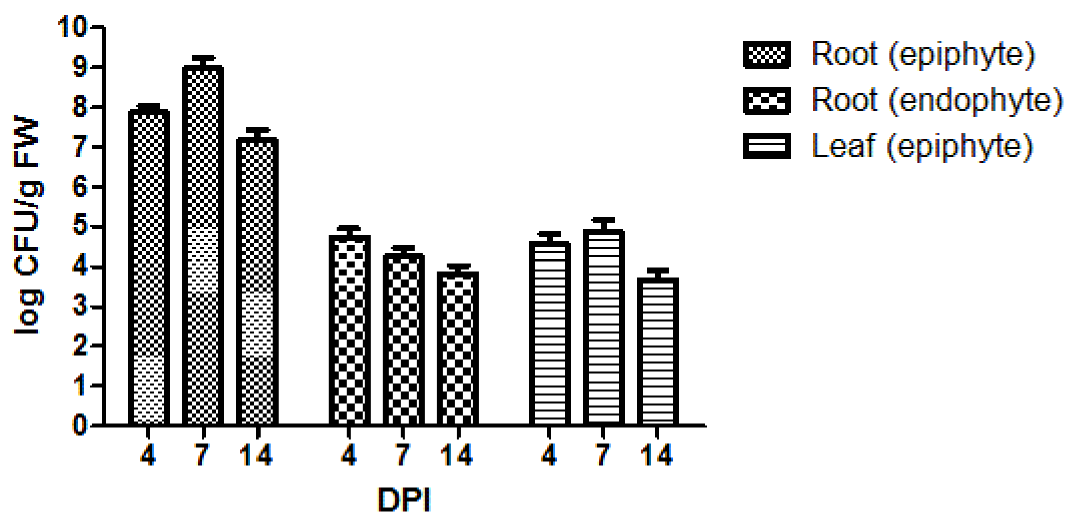

Figure 3: Analysis of Aureobasidium pullulans Fito_F278 colonisation of in vitro plants of Vitis vinifera cv. Chardonnay at 4, 7 and 14 days post-inoculation (dpi). The CFU count was log-transformed and values are means ± SEM. FW, fresh weight. No significant differences on CFU count were obtained at 4,7 and 14 dpi for each plant tissue.

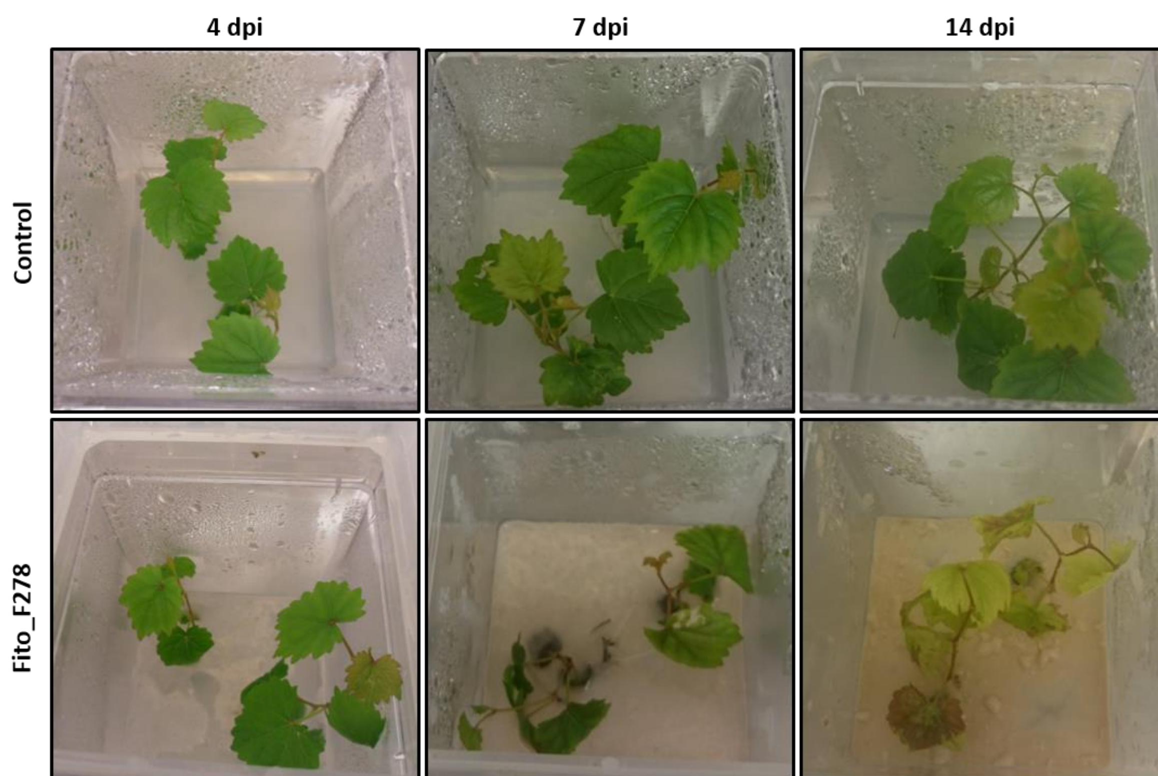

Figure 4: In vitro plants of Vitis vinifera cv. Chardonnay. Control plants (without inoculation) and plants inoculated with Fito_F278 strain where followed after 4, 7 and 14 days post-inoculation (dpi).

In fact, it is important to notice that this study is not representative of the entire microbial community and the dominance of certain microorganisms across samples may had influenced the obtained isolates. Going forward, and as expected, oenologically-important microorganisms such as *Saccharomyces cerevisiae* and the non-*Saccharomyces* yeasts *Metschnikowia pulcherrima* and *Hanseniaspora uvarum,* all involved in the vinification process of wine, were isolated from berries and musts (Sabate *et al.,* 2002; Setati *et al.,* 2012; Barata *et al.,* 2012). Musts samples also showed environmental bacteria such as *Bacillus* that derived from vineyard (Pinto *et al.,* 2015).

To decipher the biocontrol potential of the naturally occurring microbial resources of grapevine, antagonistic tests were performed and highlighted their high biocontrol potential against *B. cinerea* and Botryosphaeriaceae, by reducing the pathogen's mycelium growth. This reinforces that grapevine is a natural source of biocontrol agents and that these populations constitute a primary physic barrier against phytopathogens which can further enhance the plant immune system during a phytopathogen attack. Among them, isolates from *Bacillus* genera were the most highly effective for reducing such phytopathogens. Indeed, *Bacillus* species are recognised for their biocontrol activity against different plant diseases and several studies underline its importance (Fu *et al.,* 2010; Borriss, 2011; Ji *et al.,* 2013; Qiao *et al.,* 2014; Aziz *et al.,* 2015). Though, also *Streptomyces* sp. showed a promising biocontrol capacity against *D. seriata,* namely Fito_F14 stain, an important GTD agent. Like *Bacillus* spp., *Streptomyces* sp. are plant beneficial and growth promoter microorganisms (Seipke *et al.,* 2012). Furthermore, they have the ability to produce a high range of antibiotics and secondary metabolites which are therefore important not only for agriculture (such as biocontrol or biofertilizer) but also for human medicine or food production (Seipke *et al.,* 2012). Concerning yeasts, both *A. pullulans* and *S. cerevisiae* showed a biocontrol potential. Contrary to *S. cerevisiae*, *A. pullulans* is an irrelevant yeast in the vinification process but has a high biotechnological potential and can suppress plant pathogens. The biological control activity of *S. cerevisiae* has been already reported against *Fusarium oxysporum*, a pathogen of sugar beet plants (Shalaby and El-Nady, 2008) or against *Coletotrichum acutatum*, the causal agent of postbloom fruit drop, a devastating disease of citrus (Lopes *et al.,* 2015).

***Aureobasidium pullulans* strain Fito_F278 is an antagonist of GTDs agents**

As previously reported, *Aureobasidium pullulans* dominated the microbial consortia of grapevine and is recognized by its high range distribution over plant, which could be found in pruning wounds, wood, leaves, grapes and musts (Munkvold and Marois, 1993; Martini *et al.,* 2009; Pinto *et al.,* 2014; Pinto *et al.,* 2015; Fisher *et al.,* 2016). Indeed, this black-yeast, is an

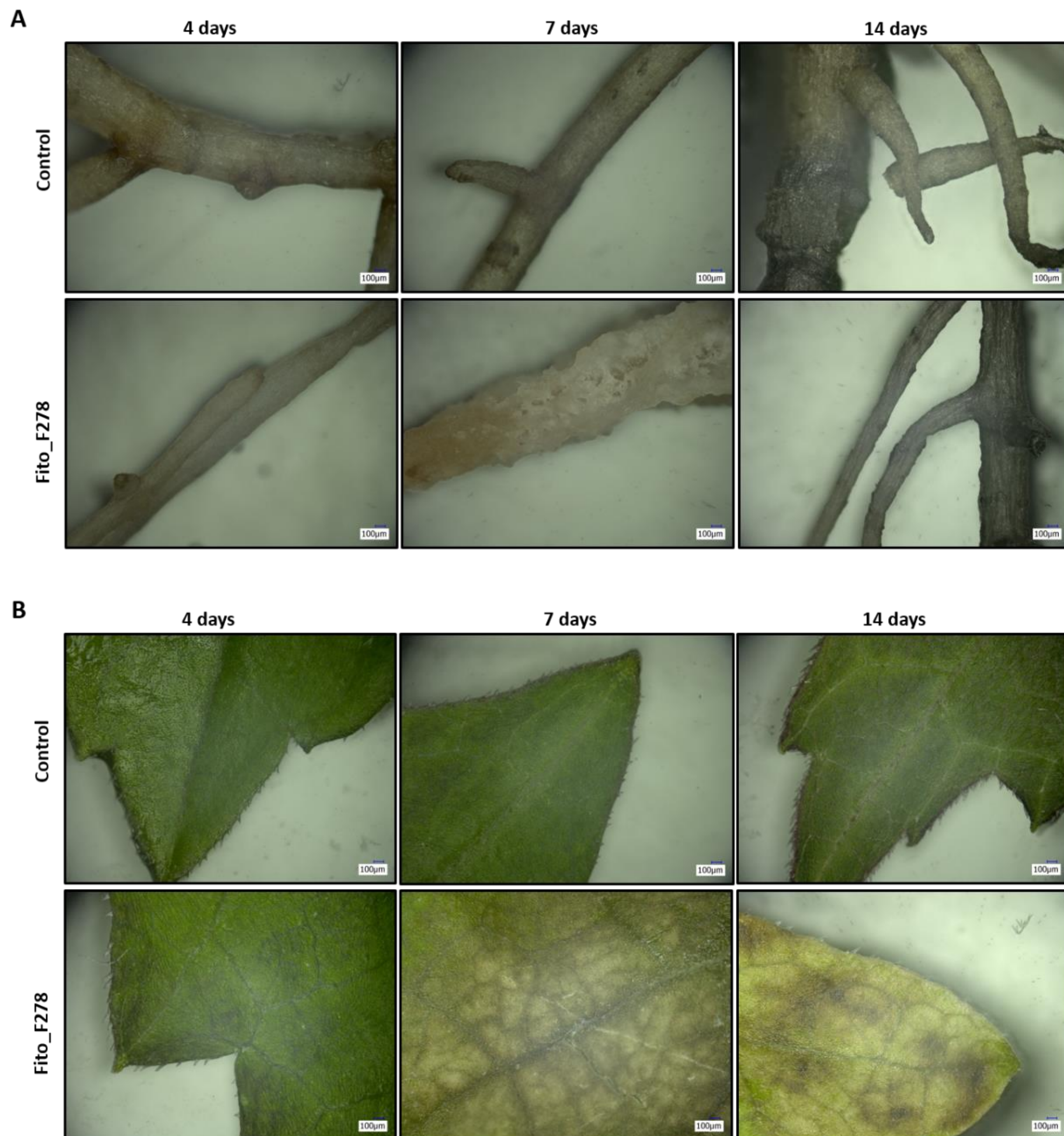

Figure 5: 3D-microscopic analysis of control and in vitro plants cv. Chardonnay inoculated with Fito_F278 at 4, 7 and 14 days post-inoculation. Microscopy analysis of (A) roots and (B) leaves. Bars = 100 µm.

ubiquitous and a natural occurring microbial resource of *Vitis vinifera* (Sabate *et al.,* 2002; Martini *et al.,* 2009; Grube *et al.,* 2011; Barata *et al.,* 2012; Pinto *et al.,* 2014) and other plants as apple, cucumber, cabbage and also found on cereal grains, food products or water (Desphande *et al.,* 1992; Vero *et al.,* 2009).

In our study, *A. pullulans* strain Fito_F278 significantly reduced the mycelium growth of Botryosphaeria dieback agents, when compared to the control, and under *in vitro* conditions. The highest levels of antagonistic activity were observed for *Diplodia seriata* strain F98.1 and *Neofusicoccum parvum* strain Np Bourgogne with a mycelium inhibition of 33.51±0.62% and 26.53±4.09%, respectively. Contrarily, *D. seriata* strain Ds99.7, which is the high aggressiveness strain, was the less susceptible to the mycelium inhibition (7.80±0.78%). The mode of action visually used by *A. pullulans* strain Fito_F278 for the antagonistic response supports a clear evidence in competition of space and nutrients against pathogens by means of a huge growth of colonies. To date, there is only one study available that applied *A. pullulans* to control GTD namely, the infection of grapevine wounds against *Eutypa lata*, a GTD agent (Munkvold and Marois, 1993). In this study, two field experiments were performed in California region: the first in 1990 at Thompson Seedless vineyard and the second in 1991 in a cv. Chenin Blanc vineyard. A set of natural occurring microorganisms was applied to test their efficacy as biocontrol agents and, among them, *A. pullulans* significantly reduced the infection, with a reduction superior to 50% compared to control treatment, only in the first field.

In general, strains of *A. pullulans* are mainly reported as important biocontrol agents of post-harvest diseases of apple fruit (*B. cinerea* and *Penicillium expansum*), cherry tomato, kiwifruit (*B. cinerea*), sweet cherry (*B. cinerea* and *Monilia laxa*), strawberries or table grape (*B. cinerea, P. expansum, Rhizopus stolonifera* and *Aspergillus niger*) (Ippolito *et al.,* 2000; Castoria *et al.,* 2001; Schena *et al.,* 2002; Bencheqroun *et al.,* 2007; Vero *et al.,* 2009). Other studies revealed that *A. pullulans* reduced the *Fusarium* head blight (FHB), a devastating disease of common wheat caused mainly by *Fusarium culmorum,* with a decrease of disease severity of 21.67% (Wachowska and Glowacka, 2014). In this regard, and due to its high versatility in the control of several plant pathogens, *A. pullulans* is of utmost interest and can be potentially applied in the vineyard for the simultaneous control of a broad spectrum of phytopathogenic agents.

Furthermore, our results showed that *A. pullulans* strain Fito_F278 produced a high range of important extracellular enzymes such as amylase, cellulase, lipase, pectinase and proteinase, under *in vitro* conditions which can hydrolyse the pathogen cell wall. In addition, Fito_F278 produced siderophores, solubilised the phosphate, grew at different pHs and was resistant to high salinity

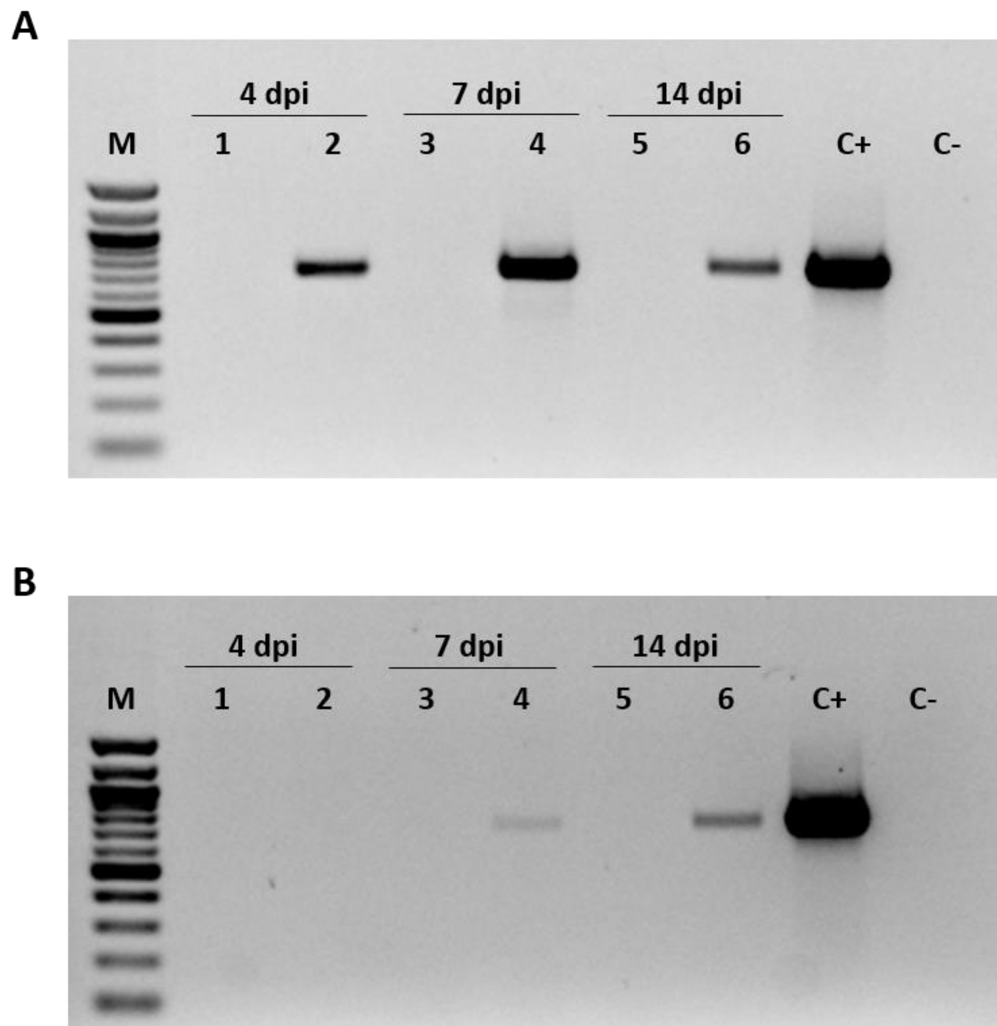

Figure 6: Detection of the Fito_F278 strain colonisation at the root (A) and leaves (B) at 4, 7 and 14 dpi through a PCR amplification. PCR amplification of Glutathione S-transferase (GST) gene, showing ~750pb amplicon, of both non-inoculated (control) and inoculated plants at root (A) and leaves (B). M: 100 bp DNA ladder (NEB, UK); Control plants (non-inolcuated plants): lanes 1, 3 and 5; Plants inoculated with Fito_F278 strain: lanes 2, 4 6. C+: positive control (gDNA of pure Fito_F278 strain); C-: negative control (sterile water instead of DNA).

conditions. The morphological forms of *A. pullulans* colonies were affected by means of pH and salinity conditions which agrees with other morphological studies (reviewed in Desphande *et al.,* 1992; Gaur *et al.,* 2010). Regarding the pH, temperature and nutrient sources (carbon or nitrogen), studies referred that these have an important role on different products biosynthesis such as the exopolysaccharide pullulan or glucan, by means of stimulation or suppression (Gaur *et al.,* 2010; Singh *et al.,* 2012). Altogether, this physico-biochemical profile may be related with its activity as a biocontrol agent, its tolerance and thus, a well-adapted microorganism. The ability of *A. pullulans* to produce such enzymes and to be tolerant to salt concentrations and pH has already been reported (Buzzini and Martini, 2002; Zalar *et al.,* 2008). The recognition of the antimicrobial metabolites of *A. pullulans* associated with high enzymatic activity, the production of exopolysaccharides including the biodegradable extracellular polysaccharide (EPS) pullulan, used in food production, and its resistance to a high range of temperatures, defined this microorganism as a biotechnological potential and an important biocontrol candidate (Desphande *et al.,* 1992; Singh *et al.,* 2008; Vero *et al.,* 2009; Gaur *et al.,* 2010; Gostincar *et al.,* 2014). Furthermore, *A. pullulans* is a copper and suffer tolerant microorganism (Grube *et al.,* 2011; Schmid *et al.,* 2011; Pinto *et al.,* 2014), which ensures its prevalence in the conventional viticulture.

The rapid colonisation of *A. pullulans* leads to an unexpected effect in grapevine plantlets

Results showed that Fito_F278 strain colonized plantlets of grapevine from the roots to the leaves and this colonisation was both epiphyte and endophyte; this has been confirmed by strain-specific primers. This is in agreement with previous studies that reported *A. pullulans* as a widespread epiphyte and an endophyte of different plants, including grapevine (Martini *et al.,* 2009). However, we have observed that *A. pullulans* can colonise the internal tissues of leaves (data not shown), although very few CFU were isolated and the colonization was not systematic.

Amazingly, in our study any positive influence of the *A. pullulans* strain Fito_F278 on the plantlets was found in terms of plant growth and development after 7 dpi. A high initial CFU plant inoculation (10^6 CFU/mL) was performed and this could be related with the magnitude of plant symptoms. Although, and considering that the *in vitro* culture media is strongly rich in carbon source (sucrose content at 3% (w/v)), this may indicate that a competition for nutrients appears to be an important mechanism used by *A. pullulans.* A rapid colonisation, the production of both biofilm layer and extracellular compounds may be promoted, thus limiting the nutrients available for plants and favouring the appearance of plant symptoms. A study of Singh and collaborators (2012) showed that an optimal concentration of sucrose at 3% (w/v) in a fermentation batch and

at 42°C resulted in a higher production of exopolysaccharide namely, pullulan when compared with other carbon sources as fructose, glucose, lactose or xylose. The capacity of microorganisms to produce biofilms is associated with a direct promotion of plant growth (Timmusk *et al.,* 2017). Though, for *A. pullulans* may be related to the colonization capacity of several niches, guaranteeing its adaptability and survival. In this regard, this rapid colonisation and proliferation may be related to the effective biocontrol through competition.

Monitoring the *A. pullulans* plant colonisation by using strain-specific primers

After plant inoculation, *A. pullulans* was monitored to evaluate its colonisation across grapevine tissues and to understand if external constraints impact or not the colonisation. For this, strain-specific primers for detection of *A. pullulans* strain Fito_F278 were developed. Although ITS region is the most commonly used for species identification (White *et al.,* 1990), this is a conserved region among species and does not confine an intra-species distinction. Thus, the glutathione S-transferase (GST) gene, mainly involved in the detoxification process and tolerance to oxidative stress (Sheehan *et al.,* 2001; McGoldrick *et al.,* 2005), was here used since it allowed an intra-species discrimination among *A. pullulans* strains. The specificity tests (data not shown) showed that the selected primers did not amplified other fungal, bacterial or plant DNA and only *A. pullulans* was amplified.

Specific primers for *A. pullulans* targeting the ITS2 region were also developed by Martini *et al*. (2009) to detect endophytic colonization of these microorganisms on grapevine leaves and shoots. However, and as the ITS2 region is conserved across *A. pullulans* strains, these primers only allowed an inter-species identification (Martini et al, 2009). Schena *et al*. (2002) analysed also the genetic variability of different *A. pullulans* strains by RAPD and synthetized a sequence-characterized amplified region (SCAR) primers. Other household genes are described in literature for phylogenetic analysis of *A. pullulans* and encode proteins such as actin (ACT), β-tubulin (BTUB), translation elongation factor 1α (EF1α), calmodulin (CAL), elongase (ELO), NAD-dependent glycerol-phosphate dehydrogenase (GPD) or RNA polymerase 2 largest subunit (RPB2) (Zalar *et al.,* 2008; Gostincar *et al.,* 2014).

Indeed, a molecular analysis together with viable cell count allowed a fine and accurate analysis of our strain colonisation. Therefore, molecular detection is more sensitive and faster than CFU method and, thus, a more advantageous method. Furthermore, and considering that *A. pullulans* is an ubiquitous microorganism and highly numerous, these strain-specific primers will be clearly

useful for further intra and inter-species distinction and, thus, a rapid identification of our strain on future greenhouse or field experiments.

To conclude, grapevine is colonised by a myriad of microorganisms with a natural biocontrol potential against *B. cinerea* and Botryosphaeria species. This suggests that plants have a natural barrier against fungal pathogens attack. Furthermore, understanding the principles within microbe-microbe and plant-microbial interactions will provide new insights to generate a set of potential biocontrol communities in grapevine ecosystem for viticulture management. The effectiveness of the biological control of *A. pullulans* against different Botryosphaeria dieback agents is thus a step forward on preventing GTDs diseases and a certain novelty measure since, and to the best of our knowledge, there is no application of *A. pullulans* to control these diseases. However, further trials in grapevine plants are need using *A. pullulans* alone or in combination with other biocontrol agents.

Author Contribution

CP conducted the design of experiment, experimental work and writing of the manuscript. VC was associated with the isolation of grapevine isolates and performed their identification and characterization. MN was associated with the physico-chemical characterization of Fito_F278 strain. FR helped for molecular analysis and validation. BC prepared the *in vitro* plants of grapevine for this study. CC reviewed the manuscript and contributed with consumables. ACG and FF, supervised, coordinate the experiments, contributed with consumables, write and critically revised the manuscript.

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CONCLUDING REMARKS AND PERSPECTIVES

The study of the natural microbiome associated with plants, often referred as the plant's second genome (Turner *et al.,* 2013), is of utmost importance as these communities are in a close interaction with plant. In this context, the overall findings of this thesis documented the importance to exploit the grapevine-associated microorganisms as they may hold important leads for the grapevine protection against phytopathogens, such as those implied in GTDs, allowing the development of sustainable management strategies.

Firstly, this work added further evidences about the complete grapevine microbiome, from vineyard landscapes until wine fermentation, and allowed for the better understanding of the plant-microbial interactions (Chapter 2). The used holistic approach to uncover the microbial diversity from the vine to wine has unveiled highly complex and dynamic microbial communities, where either each plant structure (soils and leaves) or each fermentation process harboured unique and distinctive microbial communities, though they all embedded an essential core microbiome. Differences in the microbial communities from different grape varieties and wine appellations have been showed, suggesting a potential microbial profile of each cultivar and a biogeographical correlation. From the ecological and biotechnological point of view, differences between grape varieties may or not have an impact on the plant, namely by influencing either the resistance or the susceptibility of cultivars to diseases; however, from the oenological perspective, these differences are of particular importance as they may influence the wine characteristics and/or uniqueness of regional wines. In relation to the microbial biodiversity, soils were more biodiverse than leaves or wine musts, suggesting their importance as a major microbial reservoir of grapevine. Indeed, this study provided evidences that the application of phytosanitary treatments poses a selective pressure on the microbial ecosystem of the grapevine, altering it, and thus contributing to its variation, especially on eukaryotic communities, where a decrease of biodiversity is clearly notorious during the grapevine ripening. Therefore, it was demonstrated that the equilibrium of the grapevine microecosystems are clearly compromised by the usage of phytochemicals and, consequently, plants may even become more susceptible to further phytopathogen attacks or other threats. In this context, both plant health and productivity may be affected. Moreover, results on grapevine microbiome also reinforced that both microbial communities and plant are inseparable entities, on a symbiotic relation, since one influences the other and vice-versa.

Finally, the deep characterization of the microbial communities associated with grapevines offered not only a better understanding of the plant-microbial interactions, but also allowed for the identification of potential BCAs, that could be further used as biocontrol and/ or elicitor of the natural defences of plant (Chapters 3 and 4). Indeed, our results suggested that grapevine is a natural source of BCAs, who may be considered as the first protective barrier of plants against external threats, notably the phytopathogens. Three grapevine isolates, namely *Streptomyces* sp. Fito_S127B, *Aureobasidium pullulans* Fito_F278 and *Bacillus amyloliquefaciens* Fito_F321, were here identified as antagonistic microorganisms against important grapevine diseases, in particular against *Botryosphaeriaceae* species, GTD agents, and thus are herein regarded as potential BCAs. The *in vitro* characterization of these strains confirmed their direct antimicrobial action against phytopathogens, as well as their ability to produce a set of enzymes (cellulases, amylases, lipases, pectinases and proteases) or other compounds of high biotechnological potential not only for biocontrol activities but also for PGP. This was further confirmed by genome sequencing of the selected microorganisms, which has unveiled genes coding putative bioactive compounds of biocontrol value. Importantly, these wild-strains successfully colonized grapevine, which is of utmost interest since one of the prerequisites of a successful BCA relies on its competence for the colonisation of plants. Our results thus reported that Fito_S127B, a soil isolate, was a colonizer of grapevine rhizosphere, while Fito_F278, a leaf isolate, colonised grapevine from roots to leaves, at both epiphytic and endophytic level. In addition, the follow-up of these BCAs colonisation through a molecular assessment, by using strain-specific primers, showed to be an important step for an unambiguous and easy monitorisation and/or identification of these strains, after their release under greenhouse conditions. From the point of view of grapevine protection, Fito_S127B strain was undoubtedly the most effective against *Botryosphaeriaceae* agents, both under *in vitro* and *in vivo* conditions and, thus, can be regarded as high potential BCA. In fact, Fito_S127B showed to be able not only to reduce the plant necrosis caused by the artificial inoculation of the pathogen (*D. seriata*), but also to effectively induce some defence mechanisms of grapevine, allowing a more rapid and solid response of plant against *D. seriata*. Furthermore, Fito_S127B can survive in the plant rhizosphere up to two months after its soil inoculation, which ensures a continuous interaction with the plant.

Overall, the outcomes of this thesis provide new knowledge on the grapevine microbiome and represent an important break-through on the understanding of the plant-BCAs-GTDs interactions. Altogether, these results set the grounds of valorisation of the vineyard's microorganisms as BCAs for future viticulture management, by ensuring an equilibrium of the natural microbial population

of the plant. Therefore, there are still unanswered questions that must be clarified. In this context, the results described in this thesis open new avenues for a future R&D, as follows:

1) <u>Grapevine microbiome</u>:

Herein the associated microbiome with vines and wines from conventional vineyards system was fully described, along the grapevine growing cycle and growing seasons. However, it will be interesting to perform a further characterization of the grapevine microbiome associated with organic vineyards, in order to have a holistic farm approach. Altogether, this will allow to compare and to understand the input of different agricultural systems on the natural microbial communities, and to exploit other potential beneficial microorganisms with a BCA potential. Indeed, the impact of the conventional farming systems in the environment and microbial biodiversity was reported (Hole *et al.,* 2005; Schmid *et al.,* 2011; Pinto *et al.,* 2014; Pinto and Gomes, 2016). Here, an imbalance of the microbial ecosystem and a loss of the microbial biodiversity occur as a consequence of the constant application of phytosanitary treatments (Pinto *et al.,* 2014). In contrast, in organic farming systems, where most of the synthetic chemicals are prohibited and the input of pesticides is highly reduced, the microbial ecosystem is preserved. Moreover, it is suggested that this farming system may benefit biodiversity (Hole *et al.,* 2005). In fact, it was reported that the relative abundances of some microorganisms such as *Aureobasidium pullulans* were higher in organic vinyeards than in conventional systems (Schmid *et al.,* 2011), and the management practices may have been a contributing factor in determine the associated microbial communities (Abdelfattah *et al.,* 2016). For this reason, the organic farming systems are an important source of potential BCAs. Considering that in the last decades the proportion of organically produced vineyards increased (Willer, 2008; Eurostat, 2015), the in-depth study of the microbial communities associated with these vineyards can be of a such interest to exploit new beneficial microorganisms with biocontrol potential and, thus, to develop new environmental friendly strategies for grapevine protection. Indeed, this offers a competitive advantage as these microorganisms are well adapted to these vineyards, to the surrounding environment and to the highly microbial competition.

2) <u>Grapevine-BCAs-pathogens interactions</u>:

The research on the interaction between beneficial isolates from grapevine and *Botryosphaeriaceae* pathogens allowed to identify promising antagonistic strains, such as *Streptomyces* sp. Fito_S127B, *A. pullulans* Fito_F278 and *B. amyloliquefaciens* Fito_F321, and to

conclude about their biotechnological potential. Although, further evidences on their modes of action against phytopathogens shall be elucidated and deep characterized. Antibiosis seemed to be the principal mechanism used by these strains when confronted with pathogens by a dual culture, which emphasises the need to identify the secondary metabolites and/or antimicrobial compounds involved. In fact, these natural substances produced by BCAs may be of relevance for phytopathogens suppression and are of a great deal of interest for further biological control strategies (Bailey and Falk, 2011). Another important issue is to address the potential use of these beneficial microorganisms on the detoxification of phytotoxins produced by *Botryosphaeriaceae* agents. Indeed, these phytopathogens produce a high-molecular weight hydrophilic compounds with toxic properties to plants (Andolfi *et al.,* 2011; Abou-Mansour *et al.,* 2015), such as *D. seriata* F99.2 that produces three melleins, namely (3R, 4R)-4-hydroxymellein, (3R)-7-hydroxymellein and (3R,4R)-4,7,-dihydroxymellein (Andolfi *et al.,* 2011). Previous studies have already documented the potential use of the beneficial bacterium *Bacillus subtilis* PTA-271 (Trotel-Aziz *et al.,* 2008) to degrade two major phytotoxin compounds, terremutin and mellein, produced by Botryosphaeriaceae (Trotel-Aziz *et al.,* 2017). Other BCAs and their detoxification enzymes were also able to degrade deoxynivalenol (DON), a commonly detected *Fusarium* mycotoxin with toxic effects on animals and humans, and with a risk to the food chain (Tian *et al.,* 2016a; Tian *et al.,* 2016b). Strains such as *B. licheniformis* and *B. subtilis* were able to degrade DON under anaerobic conditions (Cheng *et al.,* 2010), different *Trichoderma* strains inhibited the mycotoxin production (Tian *et al.,* 2016b) and *Bacillus* sp. showed to produce the deepoxidase, an important detoxification enzyme (Li *et al.,* 2011). Considering that the draft genome sequencing of the potential BCAs identified in this study is already available, this will allow an *in-silico* prediction of the detoxification genes of these microorganisms.

Moreover, and given the interaction between grapevine-BCAs, the direct mechanisms involved shall also be considered. Results showed that Fito_S127B strain can act both as a BCA or plant defence inducer, though the plant pathways induced by this strain were not fully addressed. In this context, the analysis of the gene expression in grapevine of the JA, ET and ABA genes, in response to Fito_S127B inoculation, shall be addressed. These genes are important regulators in plant defence- signalling pathway and are involved in the induction of plant ISR and, thus, a priming effect (Loon, 2007; Conrath, 2011). Furthermore, results with plantlets inoculated with Fito_S127B also showed that this strain tends to improve the plant’s performance and even their growth. Thus, the analysis of the ACC deaminase, IAA production or other phytohormones, shall be carried out as

these are intimately involved in the plant growth promotion (Loon, 2007; Hardoim *et al.,* 2008; Souza *et al.,* 2015; Santoyo *et al.,* 2016).

Altogether, the exploitation of both direct and indirect mechanisms applied by these beneficial microorganisms in their interactions with grapevine and phytopathogens, will set a better understanding of their potential on protecting grapevine from biotic and abiotic stresses (Souza *et al.,* 2015).

3) Grapevine protection:

Collectively, our results supported the potential use of a set of promising BCAs for grapevine protection, and clearly highlighted the potential of the *Streptomyces sp.* Fito_S127B. However, a large -scale of efforts is still needed until this/ these BCA(s) could be integrated under the vineyard management. Indeed, this work allowed to conclude about the capacity of these BCAs to colonize grapevine, to protect grapevine against *D. seriata,* and other *Botryosphaeriaceae* species*,* under greenhouse conditions, and to induce the plant defence responses, after a soil inoculation. Although, future studies on the optimizing the use of these BCAs to improve their biocontrol against *Botryosphaeriaceae* species are of a great deal of interest as, different types of BCAs' application may yield different plant protection responses. Thus, and to address this, we shall (1) to cover new strategies of BCAs' application in the field; (2) to assess whether combined use of BCAs as well as with natural molecules and even biofungicides lead to a higher biocontrol efficacy; (3) to understand the potential of these BCAs as a preventive or curative agents of grapevine diseases; and (4) to conduct field studies. Thus, and given the (1) strategies of BCAs' application, methods such as spraying the grapevine canopy with BCAs formulations must be considered since these are the most applied and preferential methods in vineyards. Meanwhile, results from this study evaluated the grapevine protection by applying individual BCAs inoculation. Although results were promising, some degree of inconsistency in biocontrol was observed between growing seasons, as a consequence of some variable conditions. Indeed, it was already reported that in comparison with pesticides, some biological products may have a lower efficacy and sometimes their activity is variable due to the ecological parameters (Fjelsted and Ehlers, 2011; Xu *et al.,* 2011). To overcame this, future studies by (2) using combined BCAs strains and/or natural molecules, and biofungicides shall be considered as an attempt to exploit potential synergistic effects among them and, thus, to improve the biocontrol efficacy of grapevine diseases, namely GTDs (Xu *et al.,* 2011). Previous studies have already showed good results in combined treatments against GTDs, applied at various time-points during the propagation process of grapevine in nurseries (Halleen and Fourie, 2016).

Among them, results using an integrated treatment of benomyl, hot water treatment, didecyldimethylammonium chloride (Sporekill, ICA International Chemicals Pty. Ltd) and strains of *Trichoderma harzianum* (Trichoflow-T^{TM}, Agrimm Technologies Ltd.) showed to be highly effective and, thus, recommended for use in nurseries (Halleen and Fourie, 2016). Furthermore, the dual application of both *Burkholderia phytofirmans* strain PsJN::*gfp*2x with the systemic profungice genpliconil "SM 26", reduced the lesions sizes caused by the artificial infections of *Neofusicoccum parvum* in cuttings cv Chardonnay and Sauvignon (Spagnolo *et al.,* 2016). Other studies also reported that the combined application of BCAs with fungicides, such as *P. fluorescens* Pf2 and acibenzolar-S-methyl (ASM), or endophytic BCAs, such as *Bacillus* sp. and *Serratia marcescens,* were more effectively to suppress the bacterial wilt in tomato (Abo-Elyousr *et al.,* 2012; Barretti *et al.,* 2012; Yuliar *et al.,* 2015). Another important aspect comprises (3) to understand if this/ these BCA(s) strain(s) can be applied as a preventive or curative agents of grapevine diseases, namely GTDs. In this context, proactive measures shall be performed in nurseries, by means of a prior inoculation of the planting material with these promising BCAs, and/or in the field, through pruning wounds treatments. Indeed, proactive management of GTDs infection in grapevine nurseries was already been reported and most of them apply *Trichoderma* sp. formulations, or also *Bacillus* sp. and *Glomus intraradices* (Fourie, 2004; Gramaje and Di Marco, 2015; Halleen and Fourie, 2016), alone or in combination with other compounds. Furthermore, (4) field studies shall be conducted to evaluate the efficacy of these BCAs strains under uncontrolled conditions, as well as their survival and shelf-life.

4) <u>BCA registration</u>:

Several lines of evidence suggested that among the studied BCAs, the Fito_S127B is a very promising strain not only as a BCA but also as a plant defence inducer and/or priming agent. In addition, its capacity to produce several bioactive compounds of biocontrol and biotechnological value are clearly remarkable. Thus, and by answering to the previously questions, one of the next challenges will be to carry out a register of the Fito_S127B strain, namely a patent. This is especially important as, and to the better of our knowledge, this is the first report that evidence the effectiveness of a *Streptomyces* sp. microorganism against Botryosphaeriaceae dieback agents, under greenhouse conditions.

CONCLUSIONS GENERALES ET PERSPECTIVES

Le microbiome naturel associé aux plantes constitue son deuxième génome (Turner *et al*., 2013) et il est d'une importance capitale car ces communautés sont en interaction étroite avec les plantes. Dans ce contexte, les principaux résultats de cette thèse ont révélé l'importance d'exploiter les microorganismes associés à la vigne, car ce sont des pistes prometteuses pour la protection de la plante contre les pathogènes, telles que ceux impliqués dans les MDB, permettant d'élaborer des stratégies de gestion durable.

Nos travaux ont apporté de nouvelles informations sur le microbiome du vignoble jusqu'à la fermentation du vin et ont permis une meilleure compréhension des interactions entre la vigne – microorganismes (Chapitre 2). En considérant une vision globale des microorganismes associés à la vigne et au vin, nos résultats ont montré que ces communautés microbiennes étaient très complexes et dynamiques au cours du cycle de croissance de la plante. Chaque composé (sols et feuilles) et processus de fermentation abritaient des communautés microbiennes distinctes, et certains microorganismes clés y étaient présents. Cependant, et malgré cette spécificité, un microbiome de base a été identifié. Les résultats ont montré des différences dans les communautés microbiennes de différents cépages et d'appellations de vin, ce qui suggère un profil microbien potentiel de chaque cépage et une corrélation biogéographique. Du point de vue écologique et biotechnologique, les différences entre les cépages peuvent ou non influencer la résistance ou la sensibilité des cultivars aux maladies ; du point de vue œnologique, ces différences ont une importance particulière puisqu'elles peuvent influencer les caractéristiques du vin et/ou l'unicité des vins régionaux. Compte tenu de la biodiversité microbienne, les sols étaient plus riches en biodiversité que les feuilles ou les moûts de vin, ce qui suggère leur importance en tant que réservoir microbien majeur de la vigne. Effectivement, cette étude a montré que l'application de traitements phytosanitaires a pu constituer un changement dynamique sur l'écosystème microbien naturel, ce qui contribue à la variation du microbiome. Ceci a notamment concerné les communautés eucaryotes, où une diminution de cette biodiversité est clairement visible lors de la maturation de du raisin. Ainsi, l'équilibre du micro-écosystème de la vigne a été clairement compromis, et la plante peut être plus sensible à d'autres attaques de pathogène ou d'autres menaces. Dans ce contexte, la santé des plantes et la productivité peuvent être affectées. En outre, les résultats sur le microbiome de la vigne ont également renforcé l'idée que les communautés microbiennes et les plantes sont des entités inséparables, puisque l'une influence l'autre et vice-versa.

Enfin, la caractérisation profonde des communautés microbiennes associées à la vigne a permis non seulement une meilleure compréhension des interactions plantes-microorganismes, mais aussi permis d'identifier des BCAs prometteurs, qui peuvent être davantage utilisés comme agents de biocontrôle et/ou stimulation des défenses naturelles de la plante (Chapitres 3 et 4). En effet, les résultats ont suggéré que la vigne est une source naturelle de BCAs, et ces microorganismes peuvent représenter la première barrière protectrice des plantes contre les stress biotiques, notamment les champignons. Trois isolats prometteurs de la vigne, à savoir *Streptomyces* sp. Fito_S127B, *Aureobasidium pullulans* Fito_F278 et *Bacillus amyloliquefaciens* Fito_F321, ont été identifiés comme des microorganismes antagonistes contre d'importantes maladies de la vigne, en particulier contre les espèces de Botryosphaeriaceae, agents responsables de MDB. La caractérisation *in vitro* de ces souches a confirmé leur effet antimicrobien direct contre les agents pathogènes et leur potentiel à produire un ensemble d'enzymes (cellulases, amylases, lipases, pectinases et proteases) ou d'autres composés intéressants non seulement pour les activités de biocontrôle mais aussi pour un effect PGP. Néanmoins, leur séquençage du génome a également suggéré un potentiel biotechnologique et révélé d'autres nouveaux composés bioactifs de valeur importante pour le biocontrôle. Ces souches sauvages ont colonisé avec succès la vigne, ce qui est d'un grand intérêt car l'un des préalables d'un BCA peut reposer sur sa capacité à coloniser des plantes. Les résultats ont donc montré que Fito_S127B, un isolât du sol, était un colonisateur compétent de la rhizosphère de la vigne, tandis que Fito_F278, un isolât de la feuille, a colonisé la vigne des racines aux feuilles, à la fois épiphytique et endophytique. En outre, le suivi de leur colonisation par une évaluation moléculaire, en utilisant des amorces spécifiques de chaque souche, a montré une étape importante pour une surveillance et une identification sans ambiguïté de ces souches après leur inoculation dans les plantes, en serre. Du point de vue de la protection de la vigne, la souche Fito_S127B était la plus efficace contre les agents de Botryosphaeriaceae, en conditions *in vitro* et *in vivo* ; par conséquent, cette souche est un BCA très prometteur. En effect, Fito_S127B s'est révélé capable non seulement de réduire la taille des nécroses des plantes causée par l'inoculation artificielle du pathogène (*D. seriata*), mais aussi d'induire efficacement certains mécanismes de défense de la vigne, permettant une réponse plus rapide et plus forte de la plante contre *D. seriata*. De plus, Fito_S127B peut survivre dans la rhizosphère de la plante jusqu'à deux mois après son inoculation au niveau du sol, ce qui assure une interaction continue avec la plante.

Dans l'ensemble, les résultats de cette thèse ont apporté des nouvelles connaissances sur le microbiome de la vigne et représentent une étape importante dans la compréhension des interactions entre plantes-BCA-MDB. Néanmoins, une valorisation des microorganismes du

vignoble, comme les BCAs, dans la gestion future du vignoble en assurant un équilibre de la population microbienne naturelle de la plante est à définir. Ainsi, il reste encore des questions sans réponse qui doivent être clarifiées. Dans ce contexte, les résultats décrits dans cette thèse ouvrent des nouvelles opportunités pour une future recherche et développement, et concernant :

1) Microbiome de la vigne :

Dans cette thèse, le microbiome associé au vignoble et au vin d'un système conventionnel de production a été décrit en détail, au cours du cycle de croissance de la vigne et des années. Cependant, il sera intéressant d'effectuer une autre caractérisation du microbiome de la vigne associée aux vignobles de production organique, afin d'avoir une approche holistique sur les types de production. Cela permettra de comparer et de comprendre l'apport de différents systèmes agricoles sur les communautés microbiennes naturelles et d'exploiter d'autres microorganismes potentiels avec un potentiel BCA. En effet, l'impact des systèmes agricoles conventionnels dans l'environnement et la biodiversité microbienne ont été signalés (Hole *et al.,* 2005 ; Schmid *et al*., 2011 ; Pinto *et al*., 2014 ; Pinto et Gomes, 2016). Ici, un déséquilibre du micro-écosystème et une perte de la biodiversité microbienne se produisent probablement en lien avec l'application constante de traitements phytosanitaires (Pinto *et al.,* 2014). En revanche, dans les systèmes d'agriculture organique, où la plupart des produits chimiques sont interdits et l'apport de pesticides est fortement réduit, le micro-écosystème est préservé. Il est ainsi suggéré que ce système agricole puisse favoriser la biodiversité (Hole *et al*., 2005). En fait, il a été rapporté que les abondances relatives de certains microorganismes tels que *Aureobasidium pullulans* étaient plus élevées dans les vignes organiques que dans les systèmes classiques (Schmid *et al.,* 2011), et que les pratiques de gestion ont peut-être contribué à déterminer le microbiome associé (Abdelfattah *et al*., 2016). Pour cette raison, les systèmes d'agriculture organique sont une source importante de BCAs. Étant donné que, au cours des dernières décennies, la proportion des vignobles organiques a augmenté (Willer, 2008 ; Eurostat, 2015), l'étude approfondie des communautés microbiennes associées à ces vignobles peut avoir un tel intérêt pour exploiter de nouveaux microorganismes bénéfiques avec un potentiel de biocontrôle et, donc développer des nouvelles stratégies durables pour la protection de la vigne. Cela offre donc un avantage concurrentiel car ces microorganismes sont bien adaptés à ces vignobles, à l'environnement et à la compétition entre les microorganismes.

2) <u>Interactions plante-BCAs-pathogènes :</u>

L'étude sur l'interaction entre les souches bénéfiques de la vigne et les pathogènes associés au Botryosphaeriaceae a permis d'identifier des souches antagonistes prometteuses, telles que *Streptomyces* sp. Fito_S127B, *A. pullulans* Fito_F278 et *B. amyloliquefaciens* Fito_F321, et de conclure sur leur potentiel biotechnologique. Toutefois, des notions supplémentaires sur leurs modes d'action contre les agents pathogènes doivent être élucidées et mieux caractérisées. L'effet antibiose semblait être le principal mécanisme utilisé par ces souches lorsqu'elles sont confrontées à des agents pathogènes lors d'une confrontation directe *in vitro*, ce qui met l'accent sur la nécessité d'identifier les métabolites secondaires et/ou les composés antimicrobiens impliqués. Ces produits naturels produits par les BCAs peuvent être pertinents pour la suppression des agents pathogènes et sont très intéressants pour d'autres stratégies de lutte biologique (Bailey et Falk, 2011). Un autre problème important est d'analyser l'utilisation potentielle de ces microorganismes bénéfiques sur la désintoxication des phytotoxines produites par les agents de Botryosphaeriaceae. En effet, ces agents pathogènes produisent des composés hydrophiles de haut poids moléculaire ayant des propriétés toxiques pour les plantes (Andolfi *et al*., 2011 ; Abou-Mansour *et al*., 2015), tels que *D. seriata* F99.2 qui produit trois melléines, à savoir (3R, 4R) -4-hydroxyméline, (3R) -7-hydroxyméline et (3R, 4R) -4,7, -dihydroxyméline (Andolfi *et al*., 2011). Des études précédentes ont déjà montré l'utilisation potentielle de la bactérie *Bacillus subtilis* PTA-271 (Trotel-Aziz *et al*., 2008) pour dégrader deux composés majeurs de phytotoxine, à savoir la terrémutine et la melléine, produits par des Botryosphaeriaceae (Trotel -Aziz *et al.,* 2017). D'autres BCAs et leurs enzymes de détoxication ont également permis de dégrader le désoxynivalénol (DON), une mycotoxine de *Fusarium* généralement détectée et qui a des effets toxiques sur les animaux et les humains et, donc avec un risque pour la chaîne alimentaire (Tian *et al*., 2016a ; Tian *et al*., 2016b). Des souches telles que *B. licheniformis* et *B. subtilis* ont pu dégrader le DON dans des conditions anaérobies (Cheng *et al*., 2010), différentes souches de *Trichoderma* ont inhibé la production de mycotoxines (Tian *et al*., 2016b) et *Bacillus* sp. produit la deepoxydase, une importante enzyme de désintoxication (Li *et al*., 2011). Étant donné que le génome des trois BCAs potentiels identifiés dans cette thèse est déjà disponible, cela permettra une prédiction *in silico* des gènes de désintoxication de ces microorganismes.

En outre, et compte tenu de l'interaction entre les plantes-BCAs, les mécanismes directs impliqués doivent également être pris en considération. Les résultats ont montré que la souche Fito_S127B peut agir à la fois comme un BCA ou comme un stimulateur de défense des plantes, bien que les voies végétales induites par cette souche n'aient pas été entièrement abordées. Dans

ce contexte, l'analyse de l'expression des gènes de la plante, à savoir ceux associés aux voies du JA, ET et ABA, en réponse à l'inoculation Fito_S127B, doit être abordée. Ces gènes sont des régulateurs importants dans la voie de la signalisation des défenses des plantes et sont impliqués dans l'induction de l'ISR de la plante et, par conséquent, un effet de potentialisation (Loon, 2007 ; Conrath, 2011). Nos résultats avec des *vitro*-plants inoculées avec Fito_S127B ont également montré que cette souche tend à améliorer la performance de la plante et même sa croissance. Ainsi, les analyses de l'ACC déaminase, de la production d'IAA ou d'autres phytohormones doivent être menées car elles sont intimement impliquées dans la promotion de la croissance des plantes (Loon, 2007 ; Hardoim *et al.*, 2008 ; Souza *et al.*, 2015 ; Santoyo *et al.*, 2016).

Dans l'ensemble, l'étude des mécanismes directs et indirects appliqués par ces microorganismes bénéfiques pendant leurs interactions avec la plante et les agents pathogènes permettra de mieux comprendre leur potentiel de protection de la vigne contre les stresses biotiques et abiotiques (Souza *et al.*, 2015).

3) Protection de la vigne :

Collectivement, les résultats ont soutenu l'utilisation potentielle d'un ensemble de BCAs prometteurs pour la protection de la vigne et ont clairement mis en évidence le potentiel de la souche *Streptomyces* sp. Fito_S127B. Cependant, des efforts sont encore nécessaires jusqu'à l'intégration de ces BCA(s) dans la gestion durable du vignoble. En effet, ce travail a permis de conclure sur la capacité de ces BCAs à coloniser la vigne, à protéger la vigne contre *D. seriata* en serre, et d'autres espèces de Botryosphaeriaceae, et à induire des réponses de la défense des plantes, après une inoculation au niveau du sol. Des études futures sur l'optimisation de l'utilisation de ces BCAs pour améliorer leur biocontrôle contre les espèces de Botryosphaeriaceae présentent un intérêt considérable, car différents types d'applications de BCAs peuvent donner lieu à différentes réponses de protection des plantes. Ainsi, et pour y remédier, nous devrons (1) analyser des nouvelles stratégies d'application des BCAs sur le terrain ; (2) évaluer si l'utilisation combinée de BCAs ainsi que des molécules naturelles et même de fongicides entraîne une efficacité de biocontrôle plus élevée ; (3) comprendre le potentiel de ces BCAs en tant qu'agents préventifs ou curatifs des maladies de la vigne ; et (4) mener des études sur le terrain. Ainsi, et compte tenu des stratégies (1) de l'application des BCAs, des méthodes telles que la pulvérisation du feuillage avec des formulations de BCAs doivent être considérées car ce sont les méthodes les plus appliquées et préférées dans les vignobles. De plus, les résultats de cette étude ont évalué la protection de la vigne en appliquant l'inoculation individuelle des BCAs. Bien que les résultats soient prometteurs,

des incohérences dans l'utilisation du biocontrôle ont été observés entre les années de croissance de la plante, en raison de certaines conditions variables. En effet, il a déjà été rapporté que, par rapport aux pesticides, certains produits biologiques peuvent avoir une efficacité inférieure et parfois leur activité est variable en raison des paramètres écologiques (Fjelsted et Ehlers, 2011 ; Xu *et al*., 2011). Pour remédier à cela, des études futures par (2) l'utilisation de souches et/ou de molécules naturelles combinées et les fongicides doivent être considérées comme une tentative d'exploiter les effets synergiques potentiels entre eux et, par conséquent, d'améliorer l'efficacité de biocontrôle des maladies de la vigne, à savoir les MDB (Xu *et al*., 2011). Des études précédentes ont déjà montré de bons résultats dans les traitements combinés contre les MDBs appliqués à différents moments du processus de propagation de la vigne, dans les pépinières (Halleen et Fourie, 2016). Parmi eux, les traitements combinant du benomyl, un traitement à l'eau chaude, du chlorure de didécyldiméthylammonium (Sporekill, ICA International Chemicals Pty. Ltd) et des souches de *Trichoderma harzianum* (Trichoflow-TTM, Agrimm Technologies Ltd.) se sont révélés très efficaces et, par conséquent, recommandés dans les pépinières (Halleen et Fourie, 2016). Par ailleurs, l'application combinée de la souche de *Burkholderia phytofirmans* PsJN :: gfp2x avec le profungicide fenpiconil «SM 26», a réduit la taille des lésions causées par les infections artificielles de *N. parvum* dans les boutures cv Chardonnay et Sauvignon (Spagnolo *et al.,* 2016). D'autres études ont également montré que l'application combinée de BCAs avec des fongicides, tels que *P. fluorescens* Pf2 et acibenzolar-S-methyl (ASM), ou BCAs endophytiques, tels que *Bacillus* sp. et *Serratia marcescens*, ont efficacement supprimé le fléau bactérien chez la tomate (Abo-Elyousr *et al*., 2012; Barretti *et al*., 2012 ; Yuliar *et al.,* 2015). Un autre aspect important comprend (3) si cette ou ces souches BCA (s) peuvent être appliquées comme des agents préventifs ou curatifs de maladies de la vigne, à savoir les MDB. Dans ce contexte, des mesures préventives doivent être effectuées dans les pépinières, au moyen d'une inoculation préalable du matériel de plantation avec ces BCAs et/ou sur le terrain, grâce à des traitements des plaies. En effet, la gestion proactive de l'infection par les MDBs dans les pépinières de vigne a déjà été signalée et la plupart d'entre elles appliquent des formulations à la base de *Trichoderma* sp., ou encore *Bacillus* sp. et *Glomus intraradices* (Fourie, 2004 ; Gramaje et Di Marco, 2015 ; Halleen et Fourie, 2016), seuls ou en combinaison avec d'autres composés. Enfin, (4) des études sur le terrain doivent être menées pour évaluer l'efficacité de ces souches BCAs sur des conditions incontrôlées, ainsi que leur survie et leur durée de conservation dans l'environnement.

4) Homologation de BCAs :

Plusieurs résultats ont suggéré que parmi les BCAs étudiés, Fito_S127B est une souche très prometteuse non seulement comme agent de biocontrôle, mais aussi comme inducteur des défenses de la plant et/ou agent de potentialisation. Sa capacité à produire plusieurs composés bioactifs, importants pour le biocontrôle et biotechnologie, est clairement remarquable. Ainsi, et pour répondre aux questions citées précédemment, l'un des défis suivants consistera à effectuer un enregistrement de la souche Fito_S127B, à savoir un brevet. Ceci est particulièrement important car, cette thèse est la première étude en mettre en évidence l'efficacité de biocontrôle des microorganismes de l'espèce *Streptomyces* sp. contre des agents du dépérissement de la vigne, en serre.

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Appendix

Appendix 1: List of the EU approved and pending microbial biopesticides

Table S1: List of the EU approved and pending microbial biopesticides (European Union pesticides database, 2017).

	Microorganism	Category	Date of approval	Expiration of approval
A) Approved microorganisms				
Bacteria	*Bacillus amyloliquefaciens* MBI 600	FU	16/09/2016	16/09/2026
	Bacillus amyloliquefaciens subsp. *plantarum* D747	FU	01/04/2015	31/03/2025
	Bacillus firmus I-1582	NE	01/10/2013	30/09/2023
	Bacillus pumilus QST 2808	FU	01/09/2014	31/08/2024
	Bacillus subtilis strain QST 713	BA, FU	01/02/2007	30/04/2018
	Bacillus thuringiensis subsp. *aizawai* strains ABTS-1857 and GC-91	IN	01/05/2009	30/04/2019
	Bacillus thuringiensis subsp. *israeliensis* (serotype H-14) strain AM65-52	IN	01/05/2009	30/04/2019
	Bacillus thuringiensis subsp. *kurstaki* strains ABTS 351, PB 54, SA 11, SA12 and EG 2348	IN	01/05/2009	30/04/2019
	Bacillus thuringiensis subsp. *tenebrionis* strain NB 176 (TM 14 1)	IN	01/05/2009	30/04/2019
	Beauveria bassiana strains ATCC 74040 and GHA	IN	01/05/2009	30/04/2019
	Pseudomonas chlororaphis strain MA342	FU	01/10/2004	30/04/2018
	Pseudomonas sp. strain DSMZ 13134	FU	01/02/2014	31/01/2024
	Streptomyces K61 (formerly *S. griseoviridis*)	FU	01/05/2009	30/04/2019
	Streptomyces lydicus WYEC 108	FU, BA	01/01/2015	31/12/2024
Fungus	*Ampelomyces quisqualis* strain AQ10	FU	01/04/2005	31/07/2017
	Coniothyrium minitans strain CON/M/91-08 (DSM 9660)	FU	01/01/2004	31/10/2017
	Gliocladium catenulatum strain J1446	FU	01/04/2005	31/07/2017
	Lecanicillium muscarium (formerly *Verticillium lecanii*) strain Ve6	IN	01/05/2009	30/04/2019

(continued…)	**Microorganism**	**Category**	**Date of approval**	**Expiration of approval**
	Metarhizium anisopliae var. *anisopliae* strain BIPESCO 5/F52	IN	01/05/2009	30/04/2019
	Paecilomyces fumosoroseus strain Fe9901	IN	01/10/2013	30/09/2023
	Paecilomyces lilacinus strain 251	NE	01/08/2008	31/07/2018
	Phlebiopsis gigantea (several strains)	FU	01/05/2009	30/04/2019
	Pythium oligandrum M1	FU	01/05/2009	30/04/2019
	Trichoderma asperellum (formerly *T. harzianum*) strains ICC012, T25 and TV1	FU	01/05/2009	30/04/2019
	Trichoderma asperellum (strain T34)	FU	01/06/2013	31/05/2023
	Trichoderma atroviride (formerly T. *harzianum*) strains IMI 206040 and T11	FU	01/05/2009	30/04/2019
	Trichoderma atroviride strain I-1237	FU	01/06/2013	31/05/2023
	Trichoderma atroviride strain SC1	FU	06/07/2016	06/07/2031
	Trichoderma gamsii (formerly *T. viride*) strain ICC080	FU	01/05/2009	30/04/2019
	Trichoderma harzianum strains T-22 and ITEM 908	FU	01/05/2009	30/04/2019
	Trichoderma polysporum strain IMI 206039	FU	01/05/2009	30/04/2019
	Verticillium albo-atrum (formerly *Verticillium dahliae*) strain WCS850	FU	01/05/2009	30/04/2019
Yeast	*Aureobasidium pullulans* (strains DSM 14940 and DSM 14941)	FU, BA	01/02/2014	31/01/2024
	Candida oleophila strain O	FU	01/10/2013	30/09/2023
	Saccharomyces cerevisiae strain LAS02	FU	06/07/2016	06/07/2031
Virus	Cydia pomonella Granulovirus (CpGV)	IN	01/05/2009	30/04/2019
	Helicoverpa armigera nucleopolyhedrovirus (HearNPV)	IN	01/06/2013	31/05/2023
	Mild Pepino Mosaic Virus isolate VC 1	EL	29/03/2017	29/03/2032
	Mild Pepino Mosaic Virus isolate VX 1	EL	29/03/2017	29/03/2032
	Pepino mosaic virus strain CH2 isolate 1906	EL, VI	07/08/2015	07/08/2030
	Spodoptera exigua nuclear polyhedrosis virus	IN	01/12/2007	30/11/2017

(continued…)	Microorganism	Category	Date of approval	Expiration of approval
	Spodoptera littoralis nucleopolyhedrovirus	IN	01/06/2013	31/05/2023
	Zucchini Yellow Mosaik Virus, weak strain	EL	01/06/2013	31/05/2023
B) Pending microorganisms				
Bacteria	*Bacillus amyloliquefaciens* AH2	FU		
	Bacillus amyloliquefaciens strain FZB24	FU		
	Bacillus subtilis IAB/BS03	FU		
	Chromobacterium subtsugae PRAA4-1T	IN		
	Pasteuria nishizawae Pn1	NE		
Fungus	*Beauveria bassiana* IMI389521	IN		
	Beauveria bassiana PPRI 5339	IN		
	Beauveria bassiana strain 147	IN		
	Beauveria bassiana strain NPP111B005	IN		
	Fusarium sp. L13	FU		
	Purpureocilium lilacinum PL 11	NE		

*Categories: BA -Bactericide; EL – Elicitor; FU – Fungicide; IN – Insecticide; NE – Nematicide

Appendix 2: Supporting information of the Publication 4 – Vine Microbiome: the microbial diversity associated with different Portuguese grape varieties

Figure S1: Vineyard used in this study and the respective grape varieties in analysis. Soils (**X**), sampling was done in 2 vines randomly selected for each grape variety and across 7 time points (T2, T5, T8, T9, T12, T13 and T15) during 2011 season. In the case of the leaves (●), the sample collection was done in 5 vines randomly selected for each grape variety and across 10 (T1 to T10) and 15 (T1-T15) time points during 2010 and 2011, respectively. For both vine campaigns, leaves were collected before and after chemical treatments application. To ensure reliable results, all samples were collected from the same vines.

Table S1: Calendar of sampling and respective time-points for both 2010 and 2011 growing seasons. Soil samples were collected only at 2011 and in a total of 7 samplings (T2, T5, T8, T9, T12, T13 and T15). Grapevine leaves were collected over two consecutive years, in a total of 10 (T1 to T10) and 15 sampling (T1 to T15) in 2010 and 2011, respectively. Leaves were collected before (BT) and after (AT) the application of chemical treatments.

Time-point	2010		2011	
T1	BT	May	BT	April
T2	AT	May	BT	April
T3	BT	May	AT	May
T4	AT	June	BT	May
T5	BT	June	AT	May
T6	AT	June	BT	May
T7	BT	July	AT	May
T8	AT	July	BT	June
T9	BT	July	AT	June
T10	AT	July	BT	June
T11	--	--	AT	June
T12	--	--	BT	July
T13	--	--	AT	July
T14	--	--	Extra sampling	August
T15	--	--	Extra sampling	September

Appendix 3: Supporting information of the Chapter 3 – Screening of potential BCAs from grapevine microbiome to control grapevine diseases: a special focus on *Botryosphaeriaceae* dieback

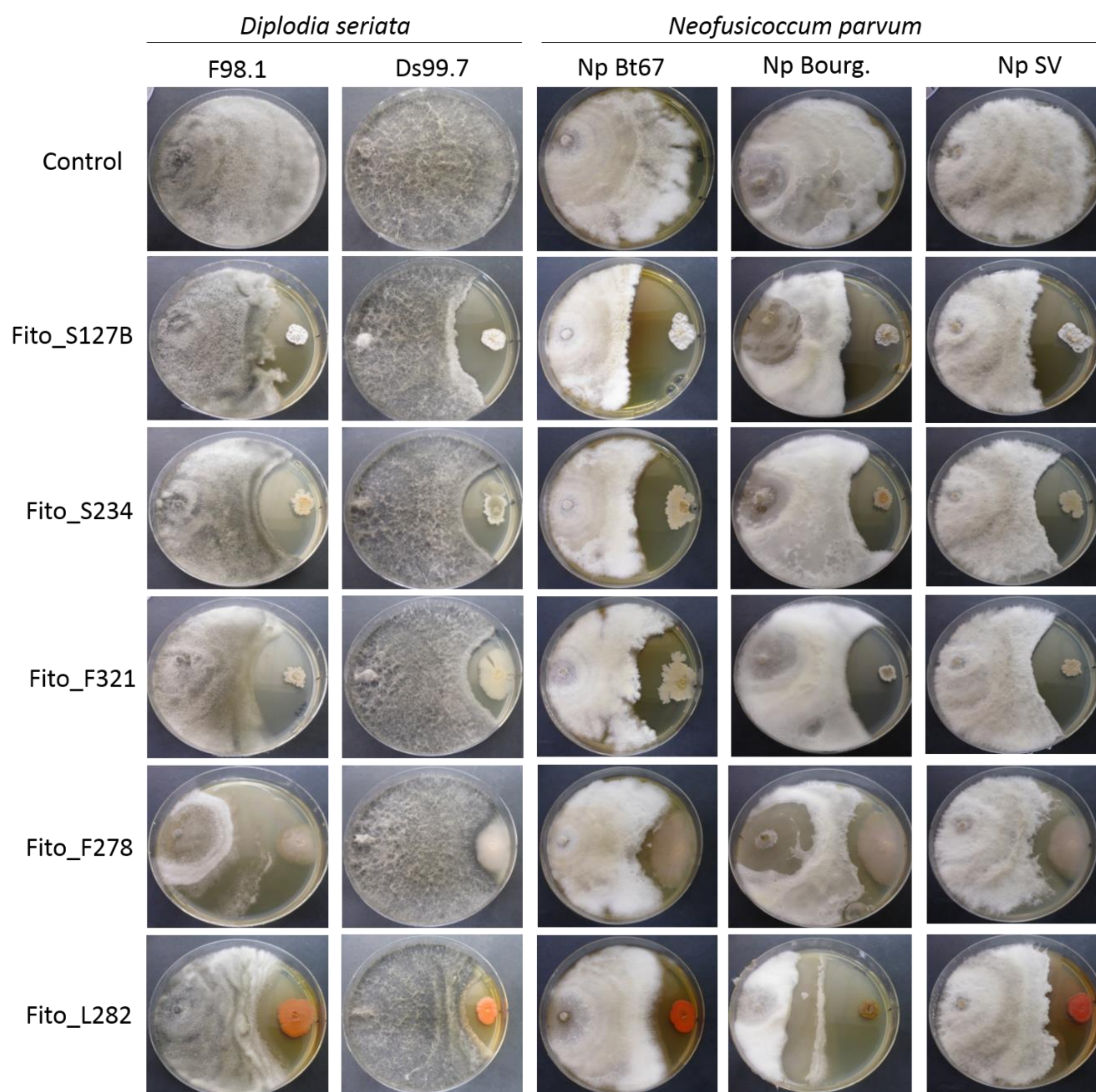

Figure S1: Inhibition of Botryosphaeriaceae dieback agents by grapevine isolates. Inhibition of *Diplodia seriata* strains F98.1 (Robert-Siegwald *et al.,* 2017) and Ds99.7 and *Neofusicoccum parvum* strains Np Bt-67, Np Bourgogne and Np SV by Fito_S127B (*Streptomyces* sp.), Fito_S234 (*B. amyloliquefaciens*), Fito_F278 (*A. pullulans*), Fito_L282 (*P. chlororaphis*) and Fito_F321 (*B. amyloliquefaciens*) strains, 15 days after incubation at 28°C.

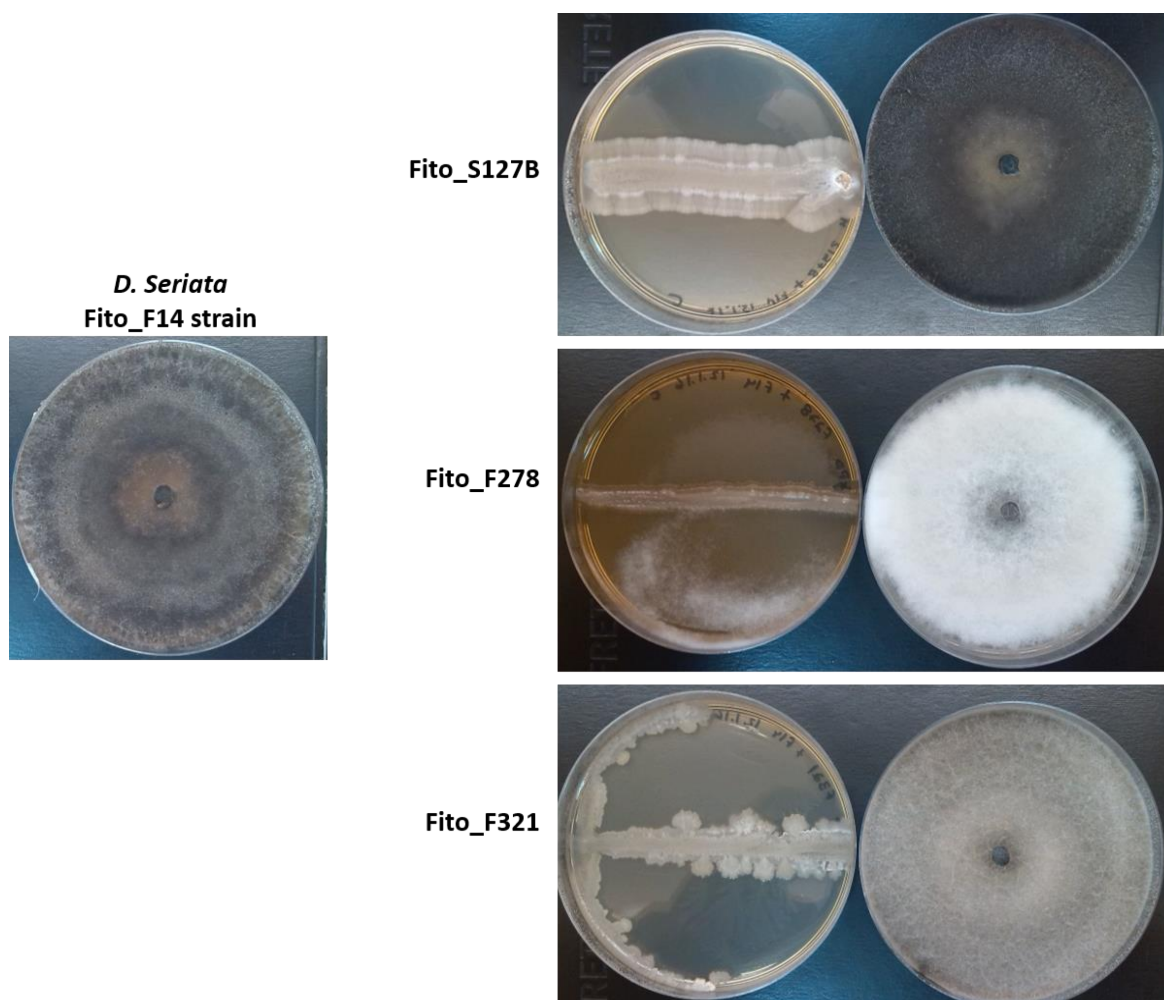

Figure S2: Effect of the strains volatile compounds against D. seriata Fito_F14 strain after 15 days of inoculation. The antagonistic potential of the volatile compounds emitted by Fito_S127B (*Streptomyces* sp.), Fito_F278 (*A. pullulans*) and Fito_F321 (*B. amyloliquefaciens*) strains on the mycelium inhibition growth of the pathogen *D. seriata* Fito_F14 strain. The plate designated as *D. seriata* Fito_F14 strain is related to the control plate (free growth of the pathogen).

Table S1: Effect of the strains volatile compounds on the mycelial inhibition growth of the pathogen D. seriata Fito_F14 strains. Results of the inhibition of the pathogen's mycelial growth (%) are presented by Mean ± SEM values, and isolates with significant antagonistic activity (*p*<0.05) compared with control (pathogen' mycelium free growth) are presented with an *. The – indicates no inhibition of the pathogen mycelial growth.

		Inhibition of pathogen's mycelium growth (%)					
Isolate	**Specie**	**1 DPI**	**2 DPI**	**3 DPI**	**6 DPI**	**7 DPI**	**15 DPI**
Fito_S127B	*Streptomyces* sp.	18.84 ± 2.97*	23.80 ± 1.88*	10.25 ± 0.71	-	-	-
Fito_F278	*Aureobasidium pullulans*	32.3 ± 2.57*	34.97 ± 4.61*	21.28 ± 5.89*	-	-	-
Fito_F321	*Bacillus amyloliquefaciens*	3.69 ± 2.12	25.55 ± 2.27*	18.44 ± 1.93	-	-	-

Table S2: Evaluation of the physiological traits (pH and salinity) on the growth of Fito_S127B, Fito_F278 and Fito_F321 strains. The strain growth was log transformed and results are presented by Mean ± SEM values. *Significant differences (p<0.05) of strain abundance (CFU/mL) were then compared with standard conditions (0% NaCl).

log (CFU/mL)	**Strains**		
	Fito_S127B	Fito_F278	Fito_F321
pH			
Standard (6.5)	4.15 ± 0.00	5.68 ± 0.06	8.50 ± 0.14
5	4.16 ± 0.01	5.55 ± 0.07	0.00 ± 0.00
6	4.17 ± 0.00	5.71 ± 0.08	8.35 ± 0.24
7	4.15 ± 0.00	5.65 ± 0.05	8.40 ± 0.12
9	3.84 ± 0.01*	5.49 ± 0.06	7.83 ± 0.01
11	3.74 ± 0.02*	5.44 ± 0.04	0.00 ± 0.00
Salinity levels (% NaCl)			
Standard (0%)	6.86 ± 0.02	6.79 ± 0.02	7.90 ± 0.05
2%	5.97 ± 0.05*	6.64 ± 0.03	7.62 ± 0.02*
4%	0.00 ± 0.00	6.53 ± 0.07*	7.35 ± 0.01*
6%	0.00 ± 0.00	6.51 ± 0.03*	6.83 ± 0.04*
8%	0.00 ± 0.00	6.43 ± 0.02*	0.00 ± 0.00
10%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
14%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Appendix 4: Supporting information of the Publication 6 – Draft genome sequence of *Streptomyces* sp. Fito_S127B strain, a soil microorganism from *Vitis vinifera* microbiome with a promising biotechnological importance

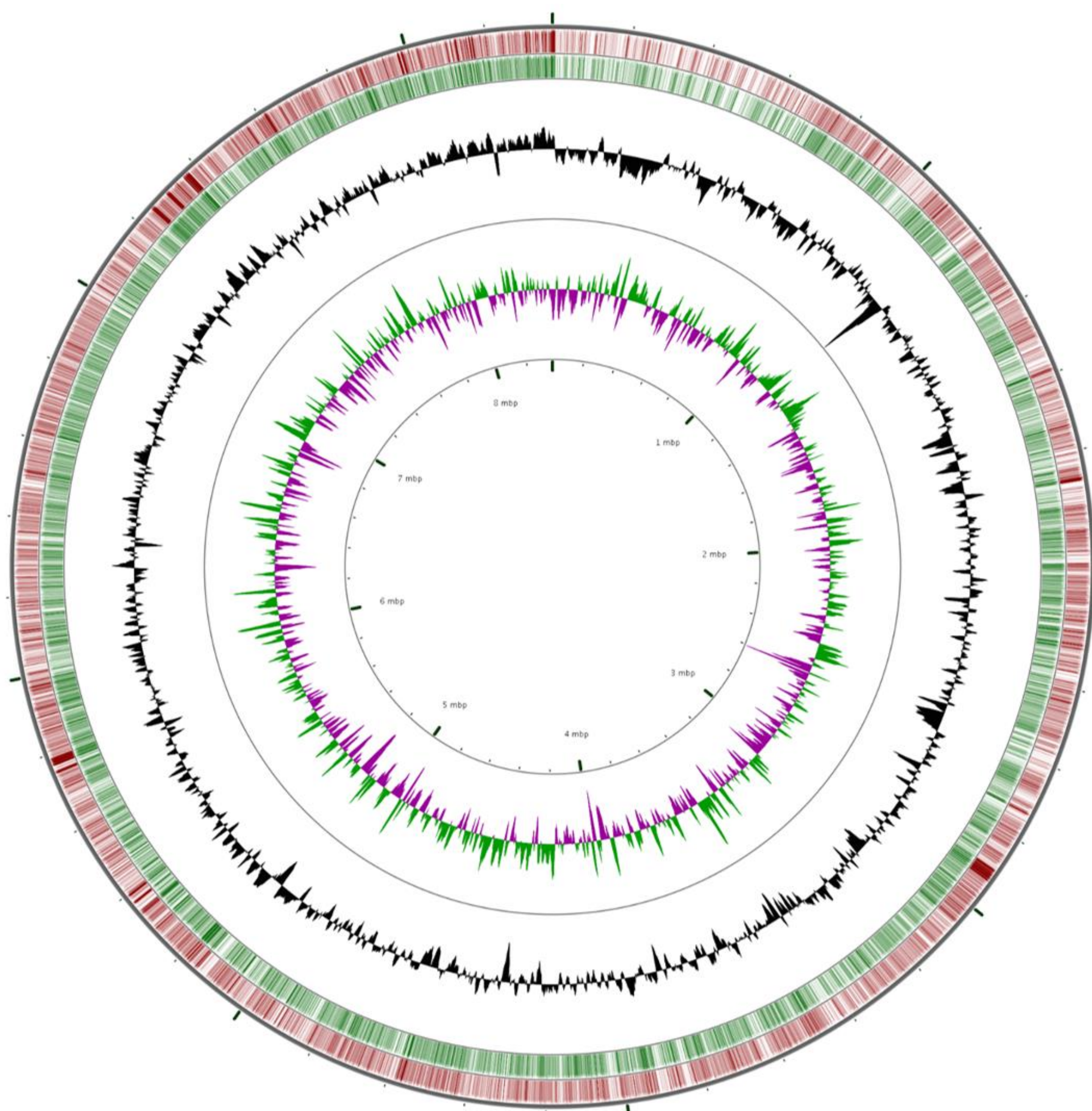

Figure S1: Circular map representing the Blastn comparison of the Streptomyces sp. Fito_S127B strain genome with S. avermitilis MA-4680 and S. coelicolor A3(2), the two closest neighbour genomes identified by RAST database. Circles display (from the outside to center): circle 1 shows *S. avermitilis* MA-4680 (red colour); circle 2 shows *S. coelicolor* A3(2) (green colour); circle 3 shows the G+C % content (black), and circle 5 shows the GC skew (green represents above average and violet below average). The map was generated with CGView (Stothard and Wishart, 2005).

Table S1: General comparison of the genome statistics of other sequenced genomes from Streptomyces genus. The detailed information was obtained from the Genome Assembly and Annotation Report available on the NCBI platform.

Strain	Isolation source	Country	GB acession number	Genome size (Mb)	G+C content (%)	Protein-coding sequences	tRNA coding genes	rRNA
Streptomyces sp. Fitp_S127B	Soil (*Vitis vinifera*)	Portugal	--	8,37	72,90	7,207	67	4
Streptomyces sp. Mg1	Soil	Alaska (USA)	CP011664.1	8,72	72,17	7,393	71	21
Streptomyces sp. SPMA113	Soil	Thailand	BDFA00000000.1	11,60	71,10	8,852	64	5
Streptomyces sp. 769	Soil	China	CP003987.1	10,34	71,62	8,423	67	21
Streptomyces sp. CdTB01	Soil	China	CP013743.1	10,19	71,55	8,812	70	18
Streptomyces sp. 4F	Soil	China	CP013142.1	8,05	72,3	6,813	68	18
Streptomyces sp. JHA26	Soil	Japan	BDJC00000000.1	7,34	72,50	6,748	68	-
Streptomyces sp. SirexAA-E	Soil	-	CP002993.1	7,41	71,7	6,333	64	19
Streptomyces avermitilis MA-4680	Soil	-	BA000030.4	9,03	70,72	7,582	68	6
Streptomyces coelicolor A3	Soil	-	AL645882.2	8,67	72,12	7,825	63	6
Streptomyces specialis GW41-1564	Soil	-	FAXE01000000	5,87	72,20	5,550	53	5
Streptomyces sp. TP-A0874	Compost	Japan	BBZK00000000.1	5,10	71,40	4,180	61	3
Streptomyces sp. F-3	Compost	China	BDDR01000001.1	5,31	71,90	6,041	69	3
Streptomyces sp. EN16			MJAF00000000.1	8,58	71,50	7,302	74	11
Streptomyces sp. EN27	Endophytes wheat plants	Australia	MJAG00000000.1	7,56	71,60	6,350	14	66
Streptomyces sp. EN23			MJAI00000000.1	7,44	71,60	6,304	65	15
Streptomyces sp. TOR3209	Tomato rhizosphere	-	AGNH00000000.1	8,07	72,59	7,564	73	4
Streptomyces sp. TP-A0867	Leaf (*Allium tuberosum*)	Japan	BBON00000000	9,90	71,80	8,385	68	
Streptomyces sp. CFMR 7	Rubber tree	Malaysia	CP011522.1	8,31	72,07	6,829	65	18
Streptomyces sp. PAMC26508	Lichen Cladonia borealis	Antarctic	CP003990.1	7,53	71,1	6,380	68	18
Streptomyces sp. TP-A0598	Seawater	Japan	BBNO00000000	8,32	71,00	7,240	75	
Streptomyces sp. MWW064	Marine sediment	Thailand	BBUY00000000	7,90	70,80	7,135	71	
Streptomyces sp. CNQ-509	Marine sediment	USA	CP011492.1	8,04	73,1	6,559	57	15
Streptomyces sp. PTY087I2	Tunicate	Panama	LZRD00000000.1	8,17	71,50	6,782	66	18
Streptomyces sp. AW19M42	Internal organs of sea squirt	Norway	CBRG000000000.1	8,01	70,60	7,727	62	8

Table S2: *Secondary metabolite gene clusters identified.*

Secondary metabolite	Gene clusters
Amglyccycl	2
Bacteriocin	1
Bacteriocin-Nrps	1
Butyrolactone	3
Butyrolactone-Otherks	1
Ectoine	1
Lantipeptide	1
Lantipeptide-T1pks-Nrps	2
Melanin	2
Nrps	13
Other	3
Otherks	1
Siderophore	4
T1pks	16
T1pks-Nrps	1
T1pks-Otherks	1
T2pks	1
T3pks	1
Terpene	4
Terpene-Lassopeptide-Nrps	1
Transatpks	1
Total	**61**

Table S3: Secondary metabolite gene clusters identified and the respective most similar known clusters.

Contig	Secondary metabolite	From	To	Most similar known cluster	MIBiG BGC-ID
4	Amglyccycl	29050	50276	Validamycin biosynthetic gene cluster (14% of genes show similarity)	BGC0000722_c1
4	Amglyccycl	93197	114441	-	-
376	Bacteriocin	1	3668	-	-
64	Bacteriocin-Nrps	1	32936	Informatipeptin biosynthetic gene cluster (42% of genes show similarity)	BGC0000518_c1
63	Butyrolactone	20975	31778	-	-
95	Butyrolactone	402	11361	Rabelomycin biosynthetic gene cluster (8% of genes show similarity)	BGC0000262_c1
313	Butyrolactone	1	5943	Griseoviridin / viridogrisein biosynthetic gene cluster (5% of genes show similarity)	BGC0000459_c1
11	Butyrolactone-Otherks	1	34358	SF2575 biosynthetic gene cluster (6% of genes show similarity)	BGC0000269_c1
80	Ectoine	14040	24444	Ectoine biosynthetic gene cluster (100% of genes show similarity)	BGC0000853_c1
412	Lantipeptide	1	2204	-	-
114	Lantipeptide-T1pks-Nrps	1	22701	Splenocin biosynthetic gene cluster (25% of genes show similarity)	BGC0001216_c1
122	Lantipeptide-T1pks-Nrps	1	21128	Landepoxcin biosynthetic gene cluster (11% of genes show similarity)	BGC0001202_c1
77	Melanin	18723	29262	Istamycin biosynthetic gene cluster (4% of genes show similarity)	BGC0000700_c1
202	Melanin	1644	12024	Melanin biosynthetic gene cluster (42% of genes show similarity)	BGC0000908_c1
5	Nrps	1	30026	WS9326 biosynthetic gene cluster (7% of genes show similarity)	BGC0001297_c1
7	Nrps	53089	96358	Meilingmycin biosynthetic gene cluster (6% of genes show similarity)	BGC0000093_c1
22	Nrps	12690	59681	Mannopeptimycin biosynthetic gene cluster (22% of genes show similarity)	BGC0000388_c1
26	Nrps	1	44816	Arginomycin biosynthetic gene cluster (10% of genes show similarity)	BGC0000883_c1
27	Nrps	1	32755	-	-
71	Nrps	1174	31160	-	-
76	Nrps	953	30086	Griseoviridin / viridogrisein biosynthetic gene cluster (5% of genes show similarity)	BGC0000459_c1
109	Nrps	1	23867	Skyllamycin biosynthetic gene cluster (4% of genes show similarity)	BGC0000429_c1
116	Nrps	1	22263	Phosphinothricin biosynthetic gene cluster (6% of genes show similarity)	BGC0000406_c1
187	Nrps	1	14565	Eponemycin biosynthetic gene cluster (21% of genes show similarity)	BGC0000345_c1
188	Nrps	1	14560	Capreomycin biosynthetic gene cluster (6% of genes show similarity)	BGC0000316_c1
260	Nrps	1	9284	-	-
317	Nrps	1	5793	Azinomycin B biosynthetic gene cluster (8% of genes show similarity)	BGC0000960_c1
1	Other	95205	138735	Oxazolomycin biosynthetic gene cluster (9% of genes show similarity)	BGC0001106_c1
12	Other	9846	53871	Laspartomycin biosynthetic gene cluster (4% of genes show similarity)	BGC0000379_c1
133	Other	1	19803	Echosides biosynthetic gene cluster (11% of genes show similarity)	BGC0000340_c1
298	Otherks	1	6876	-	-
5	Siderophore	85984	99403	-	-
13	Siderophore	1	9194	-	-
38	Siderophore	29297	42482	-	-
75	Siderophore	10880	22649	Desferrioxamine B biosynthetic gene cluster (83% of genes show similarity)	BGC0000940_c1
1	T1pks	1	26614	Apoptolidin biosynthetic gene cluster (30% of genes show similarity)	BGC0000021_c1
19	T1pks	1	29554	Concanamycin A biosynthetic gene cluster (21% of genes show similarity)	BGC0000040_c1
117	T1pks	1	22237	-	-
118	T1pks	1	22230	-	-
126	T1pks	1	20719	Isorenieratene biosynthetic gene cluster (18% of genes show similarity)	BGC0001227_c1
136	T1pks	1	19582	-	-
137	T1pks	1	19393	Chlorothricin biosynthetic gene cluster (13% of genes show similarity)	BGC0000036_c1
238	T1pks	1	10572	Monensin biosynthetic gene cluster (20% of genes show similarity)	BGC0000100_c1
243	T1pks	1	9992	Erythromycin biosynthetic gene cluster (9% of genes show similarity)	BGC0000054_c1
249	T1pks	1	9688	Tetrocarcin A biosynthetic gene cluster (11% of genes show similarity)	BGC0000162_c1
250	T1pks	1	9665	Incednine biosynthetic gene cluster (6% of genes show similarity)	BGC0000078_c1
277	T1pks	1	7932	-	-
334	T1pks	1	5047	Angolamycin biosynthetic gene cluster (100% of genes show similarity)	BGC0000018_c1
336	T1pks	1	5003	-	-
366	T1pks	1	4054	-	-
375	T1pks	1	3719	Nigericin biosynthetic gene cluster (50% of genes show similarity)	BGC0000114_c1
15	T1pks-Nrps	1	63329	Azinomycin B biosynthetic gene cluster (44% of genes show similarity)	BGC0000960_c1
37	T1pks-Otherks	1	46199	Sanglifehrin A biosynthetic gene cluster (6% of genes show similarity)	BGC0001042_c1
167	T2pks	1	15673	Spore pigment biosynthetic gene cluster (75% of genes show similarity)	BGC0000271_c1
192	T3pks	1	14394	Flaviolin biosynthetic gene cluster (75% of genes show similarity)	BGC0000902_c1
20	Terpene	7170	29209	Kanamycin biosynthetic gene cluster (19% of genes show similarity)	BGC0000705_c1
89	Terpene	1	12434	Herboxidiene biosynthetic gene cluster (3% of genes show similarity)	BGC0001065_c1
90	Terpene	3982	26489	Hopene biosynthetic gene cluster (92% of genes show similarity)	BGC0000663_c1
120	Terpene	4987	21808	Marinacarboline biosynthetic gene cluster (23% of genes show similarity)	BGC0001137_c1
3	Terpene-Lassopeptide-Nrps	1	110851	Isorenieratene biosynthetic gene cluster (85% of genes show similarity)	BGC0000664_c1
246	Transatpks	1	9924	Tetronasin biosynthetic gene cluster (9% of genes show similarity)	BGC0000163_c1

Table S4: Pairwise genome comparisons between Streptomyces sp. Fito_S127B strain with other genomes from Streptomyces sp. genus. The average of nucleotide identity (ANI) between genomes were calculated using MUMmer software (ANIm) by using the JSpecies WS web server (Richter and Roselló-Móra, 2009).

			ANIm		
Strain	**GB acession number**	**Isolation source**	**ANIm (%)**	**Aligned (%)**	**Aligned (bp)**
Streptomyces sp. e14	ACUR00000000.1	-	87.24	37.68	3152837
Streptomyces ghanaensis ATCC 14672	ABYA00000000.1	Soil	86.76	36.55	3058604
Streptomyces griseoflavus Tu4000	ACFA00000000.1	-	86.58	35.58	2976959
Streptomyces sp. CdTB01	CP013743.1	Soil	86.57	42.43	3550573
Streptomyces sp. TOR3209	AGNH00000000.1	Tomato rhizosphere	86.56	37.79	3162524
Streptomyces viridochromogenes DSM 40736	ACEZ00000000.1	-	86.48	37.52	3139246
Streptomyces sp. 4F	CP013142.1	Soil	86.37	35.58	2977530
Streptomyces lividans TK24	CP009124.1	-	86.36	37.97	3177178
Streptomyces coelicolor A3(2)	AL645882.2	Soil	86.35	38.18	3194726
Streptomyces scabiei 87.22	FN554889.1	-	86.02	31.36	2623696
Streptomyces avermitilis MA-4680	BA000030.4	Soil	85.97	35.11	2938062
Streptomyces sp. 142MFCol3.1	AUKV00000000.1	-	85.92	34.53	2889085
Streptomyces clavuligerus ATCC 27064	ADGD00000000.1	-	85.43	13.50*	1129471
Streptomyces sp. 769	CP003987.1	Soil	85.28	19.06*	1595049
Streptomyces sp. C	ACEW00000000.1	-	85.27	19.45*	1627293
Streptomyces sp. Mg1	CP011664.1	Soil	85.15	20.19	1689703
Streptomyces sp. CFMR 7	CP011522.1	Rubber tree	85.15	21.48	1797213
Streptomyces sp. SirexAA-E	CP002993.1	Soil	85.01	20.88	1747162
Streptomyces sp. PAMC26508	CP003990.1	Lichen Cladonia borealis	84.88	19.58*	1638216
Streptomyces sp. AW19M42	CBRG000000000.1	Internal organs of sea squirt	84.86	20.68	1730725
Streptomyces specialis GW41-1564	FAXE01000000	Soil	84.41	10.68*	893906
Streptomyces sp. CNQ-509	CP011492.1	Marine sediment	84.35	13.93*	1165501

*Suspicious alignment.

Appendix 5: Supporting information of the Publication 8 – Draft genome sequence of *Bacillus amyloliquefaciens* strain Fito_F321, an endophyte microorganism from *Vitis vinifera* with biocontrol potential

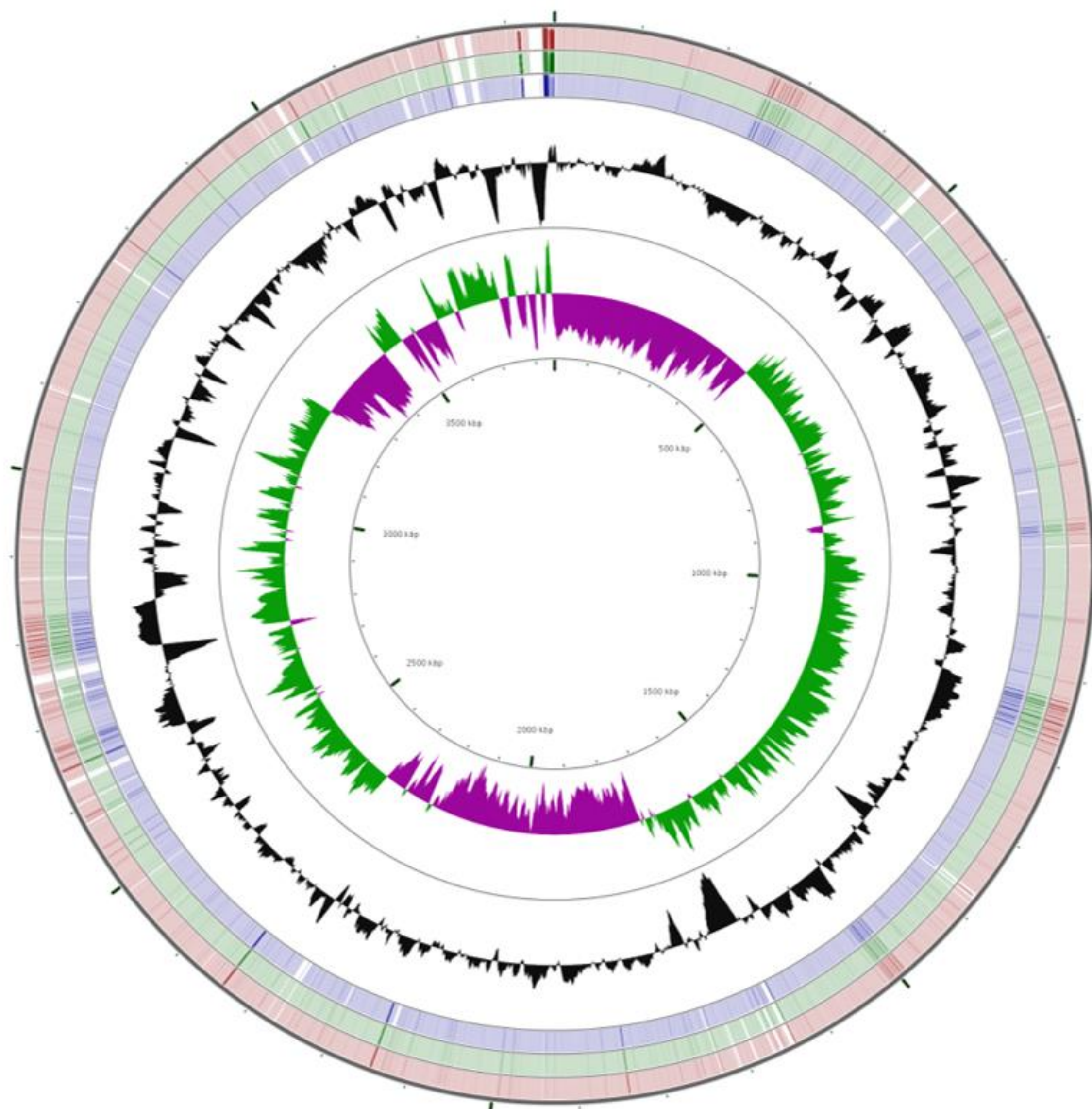

Figure S1: Circular map representing the Blastn comparison of the B. amyloliquefaciens strain Fito_F321 genome with B. amyloliquefaciens SQR9, B. subtilis ATCC 19217 and B. amyloliquefaciens FZB42, the three genomes with a higher DDH estimate. Circles display (from the outside to center): circle 1 shows *B. amyloliquefaciens* SQR9 (red colour); circle 2 shows *B. subtilis* ATCC 19217 (green colour); circle 3 shows *B. amyloliquefaciens* FZB42 (blue colour); circle 4 shows the G+C % content (black), and circle 5 shows the GC skew (green represents above average and violet below average).

Table S1: Secondary metabolite gene clusters identified.

Secondary metabolite	Gene clusters
Bacteriocin-Nrps	1
Ladderane	1
Lantipeptide	1
Microcin	1
Nrps	1
Nrps-Transatpks	1
Nrps-Transatpks-Bacteriocin	1
Other	1
Otherks	1
T3pks	1
Terpene	2
Transatpks	2

Table S2: Comparative analysis of the genome features of B. amyloliquefaciens strain Fito_F321 with others Bacillus spp.

Strain	GB acession number	Genome size (bp)	G+C content (%)	Protein-coding sequences	Percent of coding region (%)	tRNA coding genes	rRNA
B. amyloliquefaciens strain Fito_F321	PRJNA360208	3,856,229	46,54%	3,697	89%	86	5
B. amyloliquefaciens SQR9	CP006890	4,117,023	46,10%	4,078	89%	72	7
B. subtilis strain ATCC 19217	CP009749	3,959,897	46,45%	3723	90%	72	7
B. amyloliquefaciens FZB42	CP000560	3,918,589	46,40%	3,693	88%	89	10
B. methylotrophicus UCMB5113	HG328254	3,889,530	46,71%	3656	90%	89	10
B. amyloliquefaciens subsp. plantarum AS43.3	CP003838	3,961,368	46,60%	3,861	89%	89	10
B. methylotrophicus UCMB5033	HG328253	4,071,167	46,19%	3912	90%	86	10
B. amyloliquefaciens subsp. plantarum YAU B9601-Y2	HE774679	4,242,774	45,85%	3,989	87%	91	10
B. velezensis TrigoCor1448	CP007244	3,957,904	46,53%	3,755	89%	77	8
B. amyloliquefaciens UMAF6614	CP006960	4,005,145	46,49%	3,807	90%	82	9
B. amyloliquefaciens subsp. plantarum NAU-B3	HG514499	4,196,170	45,99%	4,077	90%	92	10
B. amyloliquefaciens subsp. plantarum UCMB5036	HF563562	3,910,324	46,60%	3,660	90%	89	10
B. amyloliquefaciens B15	CP014783	4,006,754	46,47%	3,836	90%	89	10
B. velezensis 9912D	CP017775	4,206,167	NA	NA	NA	NA	NA
B. amyloliquefaciens subsp. plantarum CAU B946	HE617159	4,019,861	46,51%	3,823	89%	95	10
B. methylotrophicus B25	LN999829	3,862,757	46,69%	3,674	90%	68	7
B. amyloliquefaciens DSM 7	FN597644	3,980,199	46,10%	3,921	87%	94	10
B. amyloliquefaciens LL3	CP002634	4,001,985	45,70%	4,228	88%	72	8
B. amyloliquefaciens TA208	CP002627	3,937,511	45,83%	4,089	88%	70	6
B. subtilis subsp. subtilis 168	NC_000964	4,214,630	43,50%	4,106	87%	86	10
B. subtilis XF-1	CP004019	4,061,186	43,80%	3,853	84%	77	9
B. subtilis BSn5	CP002468	4,093,599	43,85%	4,145	89%	83	11
B. pumilus SAFR-032	CP000813	3,704,465	41,30%	3679	89%	69	7

*NA means that the information is not already available.

Table S3: Comparative analysis of the genome distances between B. amyloliquefaciens strain Fito_F321 with other Bacillus spp., through the DDH method and ANI.

		DDH method					**ANIm**		
Strain	**GB acession number**	**Distance**	**DDH estimate**	**Probability that DDH > 70% (same species)**	**Probability that DDH > 79% (same subspecies)**	**Difference in % G+C**	**ANIm (%)**	**Aligned (%)**	**Aligned (bp)**
B . amyloliquefaciens SQR9	CP006890	0.0114	90.6%	96.01%	66.28%	0.44	98.91%	95.86%	3.696.425
B. subtilis ATCC 19217	CP009749	0.012	90.1%	95.83%	65.39%	0.09	98.85%	95.73%	3.691.546
B. amyloliquefaciens FZB42	CP000560	0.0167	85.9%	94.14%	58.4%	0.06	98.4%	95.22%	3.671.944
B. methylotrophicus UCMB5113	HG328254	0.0169	85.7%	94.06%	58.08%	0.17	98.38%	95.39%	3.678.467
B. amyloliquefaciens subsp. plantarum AS43.3	CP003838	0.0172	85.4%	93.9%	57.5%	0.05	98.37%	96.4%	3.717.580
B. methylotrophicus UCMB5033	HG328253	0.0172	85.4%	93.93%	57.63%	0.35	98.37%	96.6%	3.725.087
B. amyloliquefaciens subsp. plantarum YAU B9601-Y2	HE774679	0.0172	85.4%	93.92%	57.6%	0.68	98.36%	95.83%	3.695.453
B. velezensis TrigoCor1448	CP007244	0.0172	85.3%	93.9%	57.51%	0,00	98.36%	95.09%	3.666.890
B. amyloliquefaciens UMAF6614	CP006960	0.0174	85.2%	93.84%	57.3%	0.05	98.35%	95.78%	3.693.329
B. amyloliquefaciens subsp. plantarum NAU-B3	HG514499	0.0175	85.1%	93.8%	57.16%	0.55	98.35%	95.93%	3.699.366
B. amyloliquefaciens subsp. plantarum UCMB5036	HF563562	0.0177	84.9%	93.69%	56.79%	0.06	98.26%	96.17%	3.708.705
B. amyloliquefaciens B15	CP014783	0.018	84.7%	93.56%	56.34%	0.07	98.28%	96.51%	3.721.654
B. velezensis 9912D	CP017775	0.0204	82.5%	92.33%	52.43%	0.51	98.08%	93.71%	3.613.601
B. amyloliquefaciens subsp. plantarum CAU B946	HE617159	0.0233	80,00%	90.61%	47.82%	0.03	97.73%	96.1%	3.705.801
B. methylotrophicus B25	LN999829	0.0234	80,00%	90.57%	47.74%	0.17	97.75%	96.01%	3.702.384
B. amyloliquefaciens DSM 7	FN597644	0.0604	55.3%	35.9%	7.84%	0.45	94.15%	88.6%	3.416.734
B. amyloliquefaciens LL3	CP002634	0.0613	54.8%	34.25%	7.42%	0.83	94.08%	88.75%	3.422.356
B. amyloliquefaciens TA208	CP002627	0.0614	54.8%	34.17%	7.4%	0.71	94.08%	88.93%	3.429.250
B. subtilis subsp. subtilis 168	NC_000964	0.2147	20.5%	0%	0%	3.02	84.14%	25.67%	990.037
B. subtilis XF-1	CP004019	0.215	20.4%	0%	0%	2.68	84.02%	25.88%	997.82
B. subtilis BSn5	CP002468	0.2157	20.4%	0%	0%	2.69	84.1%	25.63%	988.23
B. pumilus SAFR-032	CP000813	0.2436	18,00%	0%	0%	5.25	85.02%	3.09%	119.224

Appendix 6: Supporting information of the Publication 9- Biocontrol potential and grapevine colonisation by the natural microbial resources of grapevine: a case study of *Aureobasidium pullulans* strain Fito_F278

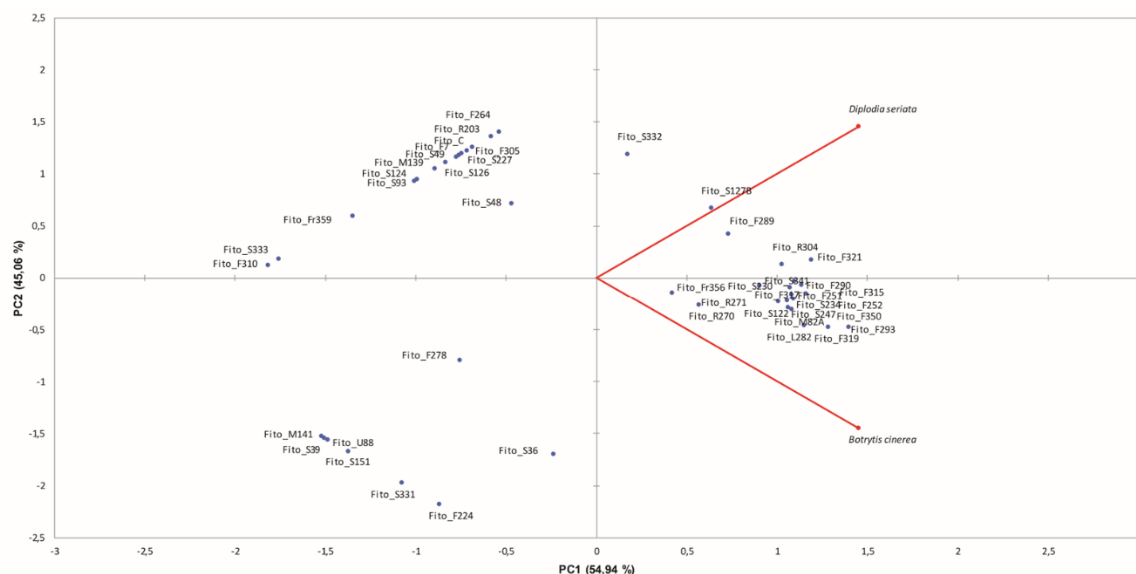

Figure S1: Principal Component Analysis (PCA) biplot of the co-culture assay showing the antagonistic effect of the grapevine isolates (p<0.05) against Botrytis cinerea strain 630 and Diplodia seriata (Fito_F14) pathogens. In the PCA biplot, based on a Pearson correlation coefficient, the variance explained by each PCA axis is given in the parentheses. Only isolates with a significant difference of antagonism are accounted (p<0.05).

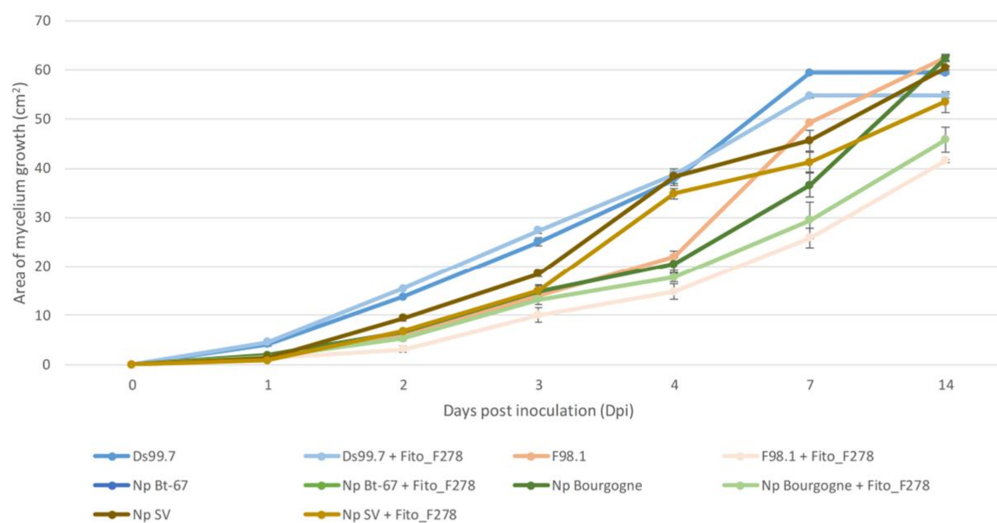

Figure S2: Kinetics of the area of the mycelium growth (cm^2) of free pathogen growth (control) and pathogen growth when inoculated with Fito_F278 strain. Results of the area of pathogen's growth (cm^2) from 0 to 14 days after inoculation are presented by Mean ± SEM values. The fungal pathogens in analysis are *Diplodia seriata* strains F98.1 and Ds99.7 and *Neofusicoccum parvum* strains Np Bt-67, Np Bourgogne and Np SV.

Table S1: In vitro antagonistic activity of different grapevine isolates against the pathogens Botrytis cinerea strain 630 and Diplodia seriata (Fito_F14). Results of the inhibition of the pathogen's mycelial growth (%) after 7 days of inoculation are presented by Mean ± SEM values, and isolates with significant antagonistic activity (p<0.05) are presented. #corresponds to isolates which antagonistic activity do not differ significantly.

	Inhibition of the pathogen's mycelial growth (%)	
Isolates	***Botrytis cinerea* (strain 630)**	***Diplodia seriata* (Fito_F14)**
Fito_F7	0	69.01 ± 0.48
Fito_S36	71.65 ± 0.00	22.22 ± 10.27#
Fito_S39	41.73 ± 0.64	0
Fito_S48	16.01 ± 13.07#	65.50 ± 4.16
Fito_S49	0	68.42 ± 1.65
Fito_M82A	69.29 ± 1.11	76.02 ± 2.08
Fito_U88	42.52 ± 0.64	0
Fito_S93	0	59.06 ± 1.72
Fito_S122	67.72 ± 1.29	77.19 ± 1.43
Fito_S124	0	59.65 ± 1.65
Fito_S126	0	66.08 ± 0.48
Fito_S127B	40.16 ± 1.70#	86.55 ± 1.72
Fito_M139	0	63.74 ± 0.48
Fito_M141	40.94 ± 0.00	0
Fito_S151	47.24 ± 2.80	0
Fito_C	0	70.76 ± 0.48
Fito_L159	64.57 ± 2.23	0
Fito_L160	68.50 ± 0.64	0
Fito_R203	0	76.03 ± 2.90
Fito_F224	68.50 ± 1.29	0
Fito_S227	0	69.59 ± 2.90
Fito_S230	66.93 ± 1.93	76.02 ± 0.95
Fito_S234	68.50 ± 0.64	80.70 ± 0.83
Fito_S247	67.72 ± 0.64	78.36 ± 1.26
Fito_F251	68.5 ± 0.64	80.70 ± 1.43
Fito_F252	64.57 ± 2.23	81.87 ± 0.48
Fito_F264	0	77.78 ± 1.72
Fito_R270	58.27 ± 1.29	66.67 ± 1.43
Fito_R271	61.42 ± 0.64	77.19 ± 2.48
Fito_F278	41.73 ± 0.64	29.82 ± 4.14
Fito_L282	74.80 ± 0.64	74.26 ± 1.26
Fito_F289	47.24 ± 2.57	83.63 ± 0.48
Fito_F290	66.14 ± 0.64	81.87 ± 0.95
Fito_F293	80.31 ± 2.32	78.95 ± 2.19
Fito_R304	59.84 ± 0.00	83.62 ± 1.26
Fito_F305	0	71.93 ± 0.83
Fito_F310	0	26.90 ± 3.13
Fito_F315	65.35 ± 0.64	80.12 ± 1.26
Fito_F317	66.93 ± 1.11	78.95 ± 0.83
Fito_F319	77.95 ± 4.50	76.61 ± 0.95
Fito_F321	62.20 ± 3.34	87.72 ± 4.30
Fito_S331	59.84 ± 1.11	0
Fito_S332	19.42 ± 7.93#	87.72 ± 0.83
Fito_S333	0	29.24 ± 18.14
Fito_S341	66.14 ± 0.64	81.87 ± 0.48
Fito_F350	70.08 ± 0.64	76.02 ± 0.48
Fito_Fr356	52.76 ± 1.13	66.08 ± 1.90
Fito_Fr359	0	45.61 ± 0.83