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outubro de 2019

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UMinho | 2019

Chapter 5: Concluding remarks and future perspectives

This research was partially supported by FEDER funds through COMPETE (Programa Operacional Factores de Competitividade), national funds through FCT (Fundação para a Ciência e a Tecnologia) and by Horizon 2020, the European Union's Framework Programme for Research and Innovation, within the project PRIMA/0002/2018 INTOMED - Innovative tools to combat crop pests in the Mediterranean, and PTDC/ASP-PLA/31133/2017 MicOlives - Exploiting plant induced resistance by beneficial fungi as a new sustainable approach to olive crop protection. D. Mina thanks FCT, POPH-QREN and FSE for SFRH-BD-105341/2014 grant; and also the COST Action FA1405 for two short-term scientific mission (STSM) grant.

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Tese de Doutoramento
Doutoramento em Ciências
Especialidade em Biologia

Trabalho efetuado sob a orientação da
Professora Doutora Paula Baptista
e da
Professora Doutora Teresa Lino-Neto

outubro de 2019

Acknowledgements

No decorrer desta tese de doutoramento, marcada por vários desafios (a nível pessoal e profissional) e cheia de momento de alegrias, tristezas, decisões e indecisões, foram várias as pessoas que tornaram este caminho mais fácil, sendo de extrema importância identificar os seus contributos na elaboração da mesma.

Agradeço à minha orientadora, Professora Doutora Paula Baptista, não só pela sua orientação, paciência e partilha de conhecimentos, mas também pela grande dedicação demonstrada apesar das (muitas) noites mal dormidas. Agradeço por, mesmo tendo constantemente vários assuntos em mãos, ter estado sempre presente e disponível para ajudar e tirar dúvidas à distância de uma mensagem ou chamada. Agradeço ainda o esforço dado na escrita desta tese. Sem si não teria sido possível concretizar esta tese com a qualidade que a caracteriza e a si devo o meu muito obrigado.

À minha co-orientadora, Professora Doutora Teresa Lino-Neto, pelo sorriso com que sempre me recebeu, pelos conselhos e pelo olhar crítico (e bastante construtivo) que permitiu levar a escrita da tese a bom porto. O meu muito obrigado!

Ao Professor Doutor José Alberto Pereira agradeço a oportunidade de também me ter permitido ingressar na equipa de investigação, assim como de ter posto à disposição as ferramentas necessárias à realização desta investigação. Agradeço ainda o conhecimento transmitido em campo e em laboratório que me permitiu ampliar os meus conhecimentos na área da olivicultura, e por isso o meu muito obrigado.

Aos colegas de laboratório que me acompanharam ao longo destes anos, cujo espírito de companheirismo e entreajuda tantas vezes aliviaram os "fogos" que surgiram neste percurso. Deixo assim um agradecimento especial aos elementos do laboratório das doenças da oliveira: Cristina, Cynthia, Fátima, Gisela e Teresa. Saliento ainda a importância dos "azeitólogos" (Ricardo e Nuno) e ao laboratório das pragas da oliveira (Ana, Jacinto, Maria, Rosalina), não esquecendo ainda os "estagiários" que tanto me fizeram crescer tanto a nível pessoal como profissional: Alba, Jéssica, José, Mariana, Sara, Sara, Vinícius. Muito obrigado!

Aos técnicos da Escola Superior Agrária, à D. Arminda, D. Fátima e à D. Isabel fica um obrigado por estarem sempre disponíveis para qualquer situação. Também à D. Céu, que sempre bem disposta se mostra sempre disponível para o que é preciso. Ao Sr. Manuel e ao Eng. Amílcar, pela ajuda que deram nos trabalhos de propagação da oliveira. A todos deixo um grande obrigado.

Aos meus pais por, mesmo estando longe, terem mostrado sempre aquele importante apoio e carinho, e também aos meus avós por estarem sempre disponíveis e atentos às necessidades, um muito obrigado a todos!

Por fim, não por menor importância, mas por merecer lugar de destaque, à Lara. Obrigado pelo suporte e pela força nos piores momentos. Obrigado pelo sorriso, pelo olhar e pelas oportunidades para desanuviar. Obrigado pelos conselhos, pela compreensão, ou por me chamares à razão. Sem ti este trabalho e percurso não teriam metade do valor e qualidade. Obrigado!

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

Bactérias endo- e epifticas da filosfera da oliveira com habilidades antagonistas contra a tuberculose da oliveira

A tuberculose da oliveira, causada pela bactéria *Pseudomonas savastanoi* pv. *savastanoi* (*Pss*), é uma importante doença do olival ainda sem tratamento conhecido. Esta doença afeta a parte aérea das oliveiras e caracteriza-se por um crescimento anormal dos tecidos, principalmente no tronco e ramos. Neste trabalho foi caracterizada a comunidade bacteriana que habita a filosfera da oliveira, de modo a elucidar o seu possível papel na defesa da planta contra a tuberculose da oliveira. Uma abordagem dependente de cultivo foi usada para descrever as populações da superfície (epífitos) e do interior (endófitos) de folhas, caules e nódulos de duas cultivares com diferentes suscetibilidades a esta doença. Para alguns dos isolados obtidos foi testada a sua capacidade antagonista contra *Pss* em ensaios *in vitro*, tendo os mecanismos associados a este antagonismo sido também avaliados. A eficácia do isolado mais promissor, *Bacillus amyloliquefaciens* P41, na redução do desenvolvimento da doença e na melhoria do fitness da planta foi avaliada através de ensaios *in planta*.

No geral, a comunidade bacteriana da filosfera da oliveira inclui membros pertencentes principalmente a *Proteobacteria,* em particular a *Gammaproteobacteria*. A composição bacteriana foi principalmente afetada pela cultivar do hospedeiro e em menor grau pelo órgão, que teve um maior impacto nos epífitos. Adicionalmente, cada cultivar/órgão foi aparentemente seletiva através de OTUs bacterianos específicos. A tuberculose da oliveira revelou ter também um impacto na estrutura da comunidade bacteriana, mas com diferentes efeitos, dependentes da cultivar e do habitat na planta hospedeira. Na verdade, o seu efeito foi mais notório na cultivar mais suscetível à doença e nos endófitos. Um total de 27 isolados inibiram significativamente o crescimento de *Pss*, tendo os isolados com uma maior capacidade antagonista sido isolados da cultivar suscetível. Esta capacidade antagonista deveu-se provavelmente à produção de compostos voláteis, enzimas líticas e sideróforos. *B. amyloliquefaciens* P41 reduziu a severidade da doença em até 43.7% e a população de *Pss* em até 26.8%, e simultaneamente melhorou o fitness da planta hospedeira, podendo ser possivelmente considerado um candidato promissor no controlo da tuberculose da oliveira. Estudos adicionais são necessários para identificar o papel funcional destas bactérias e dos mecanismos envolvidos na proteção da planta hospedeira contra a tuberculose da oliveira.

Palavras chave: comunidade bacteriana, interações planta-patogéne-bactéria, *Pseudomonas savastanoi pv. savastanoi*, *Olea europaea* L., susceptibilidade à doença.

Abstract

Endo- and epiphytic bacteria from olive tree phyllosphere with biocontrol abilities against olive knot

Olive knot (OK), caused by *Pseudomonas savastanoi* pv. *savastanoi* (*Pss*), is an important olive orchard disease with still no treatment known. This disease affects the aerial part of the olive trees and is characterized by overgrowth formations (knots) mainly on trunk and branches. In this work was characterized the bacterial community inhabiting the olive tree phyllosphere, in order to elucidate its possible role on plant defense against OK disease. A culture-dependent approach was used to describe the bacterial populations in (epiphytes) and on (endophytes) leaves, twigs and knots of two cultivars with different susceptibility to OK disease. Some of the isolates obtained were screened for their antagonistic effect against *Pss* in *in vitro* assays, and their mechanisms were also evaluated. The efficacy of the most promising isolate, *Bacillus amyloliquefaciens* P41, in reducing OK development and improving plant fitness was evaluated through *in planta* assays.

Overall, the bacterial community of olive tree phyllosphere comprised members belonging mainly to *Proteobacteria*, in particular *Gammaproteobacteria*. Bacterial composition was primarily impact by host cultivar, and, to a lesser extent, by plant organ which had a more control over epiphytes. In addition, each cultivar/organ apparently was selective towards specific bacterial OTUs. OK disease showed also to have an impact on the structure of bacterial communities, but with variable effects depending on the host cultivar and plant habitat. Indeed, its effect was most notorious in OK-susceptible cultivar and within endophytes. A total of 27 isolates inhibited significantly *Pss* growth, being the ones with the greatest antagonistic activity from the tissues surface of OK-susceptible cultivar. Such ability was potentially due to the production of volatile compounds, lytic enzymes and siderophores. *B. amyloliquefacients* P41 reduced OK disease's severity up to 43.7% and *Pss* population size up to 26.8% and simultaneously increased plant fitness, suggesting to be a promising candidate for controlling OK disease. More research are still required to identify the functional role of these bacteria and the mechanisms involved in conferring host plant protection to OK disease.

Keywords: disease susceptibility, microbial community, *Pseudomonas savastanoi pv. savastanoi*, *Olea europaea* L., plant-pathogen-bacteria interactions.

Table of contents

List of abbreviations

ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
BCA	Biological control agent
BLAST	Basic Local Alignment Search Tool
ºC	Celsius degrees
CoI	Co-inertia analysis
CFU	Colony forming unit
CCA	Canonical correlation analysis
DNA	Deoxyribonucleic Acid
FAO	Food and Agriculture Organization
H´	Shannon index
IAA	Indol-3-acetic acid
ITS	Internal transcribed spacer
IndVal	The indicator value
LBA	Luria Broth Agar
NCBI	National Center for Biotechnology Information
NMDS	Non-metric multidimensional analysis
OKD	Olive knot disease
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
Pss	*Pseudomona savastanoi* pv. *savastanoi*
SPSS	Statistical Package for the Social Sciences
rDNA	Ribossomal Deoxyribonucleic acid
SE	Standard error
UV	Ultraviolet light
1/D	Simpson's Reciprocal Index

CHAPTER 1.

General introduction

Part of this chapter is an adapted version of the published book chapter:

Mina D, Pereira JA, Lino-Neto T, Baptista P (2019). Exploring the phyllosphere bacterial community for improving tree crops protection. In: Kumar V, Prasad R, Kumar M, Choudhary DK (Eds.) *Microbiome in Plant Health and Disease, Challenges and Opportunities*, Springer-Verlag GmbH, pp. 35-52. doi: 10.1007/978-981-13-8495-0

1.1. Framework and objectives

Olive knot (OK) disease, caused by *Pseudomonas savastanoi* pv. *savastanoi* (*Pss*), is one of the most important diseases of olive crop (Quesada et al., 2012). This bacterial species produces tumorous galls or knots, mostly on stems and branches of olive trees, causing their death and loss of tree vigor, and thus endangering olive harvest (Quesada et al., 2012). *Pss* does not survive for long in soil, being usually found in olive tree phyllosphere as an epiphyte (Quesada et al., 2010) and/or endophyte (Marchi et al., 2009). Disease development is shown to be dependent of several factors, such as plant genotype and age, concentration of *Pss* at infection sites and their interaction with other bacterial species (Quesada et al., 2012). These interacting bacteria are usually found as epiphytic but can also occur in the knots (Marchi et al., 2006; Quesada et al., 2007; Ouzari et al., 2008). When found in olive knots together with *Pss*, some of them have been shown to either depress *Pss* growth or produce an increase in knot size (Marchi et al., 2006). Although some endophytic knot-derived bacteria seem to closely interact with *Pss*, knowledge about the diversity of endo- and epiphytic bacteria occurring in other olive tree tissues, as well as their possible antagonism against *Pss*, are still lacking. Since OK disease is difficult to control, being prevention the only reliable strategy, the use of native bacterial antagonists as biological control agents (BCA) against *Pss* to reduce OK incidence on olive crops could be of great importance. The additional advantage of exploiting beneficial traits from indigenous olive-bacteria is their natural ecological adaptation to the target niche. Moreover, under a climate change scenario, where biological control practices are predicted to be easier to deploy (Pautasso et al., 2012), there is an increasing interest to search for new BCA. Thus, the main aim of this work is to explore the bacteria community associated to the aerial olive tree tissues (*i.e.*, phyllosphere) of olive tree, in an integrative perspective, in order to designed new strategies for the control of OK disease. Specific objectives are:

1) Characterized the bacterial community inhabiting the surface (epiphytes) and interior (endophytes) of the aerial olive tree tissues (*Olea europaea* L.) of two cultivars with different susceptibilities to olive knot disease (cv. *Cobrançosa* - more tolerant; cv. *Verdeal Transmontana* - more susceptible). **Who is there?**

2) Understand the factors controlling bacterial community assemblage in olive tree phyllosphere. **Which factors contribute to their shaping?**
3) Disclose the role that bacterial community associated to the olive tree phyllosphere may have on host plant health. **What can they do?**
4) Identify and select autochthonous bacteria that could be used as BCA against OK disease. **Could they be useful?**

To achieve these objectives, this thesis is divided in five chapters:

In **chapter 1** are presented the objectives of this PhD thesis as well as several aspects related with olive knot disease, covering specifically its causal agent (taxonomy, transmission and life cycle), and their management. Emphasis is also given for the exploitation of epiphytes and endophytes inhabiting the phyllosphere of several woody tree crops in the development of new tools/approaches to manage plant diseases and improve plant health. **Chapter 2** focused on the characterization of the epiphytic and endophytic bacteria associated to olive phyllosphere and identified the possible drivers of bacterial assemblages, in particular the effect of host genotype (at cultivar level) and plant organ (objective 1 and 2). In **chapter 3** was compared the bacterial community (either epiphytic or endophytic) between healthy and *Pss*-infected olive twigs of olive tree cultivars with contrasting susceptibility to OK disease, in order to elucidate the potential role that this microbiota could have in host plant resistance/health (objective 3). In **chapter 4,** several epi- and endophytes isolated from the phyllosphere of different olive cultivars were screened in order to find suitable candidates to be exploited in the biological control of OK disease, using both *in vitro* and *in planta* assays with lignified pot-acclimated olive plants. The potential mechanisms involved in the observed antagonism were also investigated (objective 4). **Chapter 5** presented the concluding remarks of the results obtained in these studies, and future perspectives of the role of bacterial microorganisms on the protection of olive tree against OK disease.

1.2. *Pseudomonas savastanoi* pv. *savastanoi* – a concerning pathogen of olive tree

1.2.1. Pseudomonas savastanoi pv. savastanoi: taxonomy, host plant, symptoms and transmission in olive tree

Pseudomonas savastanoi pv. *savastanoi* (*Pss*) is the causal agent of olive knot (OK) disease, one of the most serious diseases affecting olive growing areas, responsible for intensive damages and leading to heavy economical losses (Young, 2004; Quesada et al., 2010). Being a gram-negative bacterium with 0.4-0.8 to 0.1-0.3 µm as size (Ramos et al. 2012), *Pss* is part of the *P. syringae* complex, which includes more than 60 pathovars and several *Pseudomonas* species as *P. amygdali, P. avellanae, P. cannabina, P. caricapapayae, P. ficuserectae, P. meliae, P. tremae* and *P. viridiflava* (Bull et al., 2010; Young, 2010, Parkinson et al., 2011). While most of the pathovars associated to this complex are generally associated to plant apoplast, causing foliar necrosis, a few number of strains shows different symptomatology by inhabiting woody plants vascular tissues (Agrios, 2005) and causing aerial tumors, namely *P. savastanoi*.

Pss has been reported as a tumor-forming microorganism in several herbaceous and woody plants belonging to different plant families as Apocynaceae (*Nerium oleander*), Myrtaceae (*Myrtus communis*) and Oleaceae family (*Jasminum officinale; Fontanesia phillyreoides*; *Olea europaea*), among others (Gardan et al., 1992; Young et al., 2004; Mirik et al., 2011).

In olive tree (*Olea europaea* L.), *Pss* typically survives as an epiphyte, forming aerial tumors, mostly on stems and branches, when specific climate conditions occur (Fig. 1.1). These knots can develop for large months, reaching more than 2.5 cm in diameter (Iacobellis, 2001) and persisting for long periods of time in the tree, being considered a chronic disease (Buonaurio et al., 2015). Although these knots are rarely present in olive fruits, tree vigor is highly reduced and compromises this culture yield by affecting olive fruits quantity and quality (Schroth et al., 1973; Young et al., 2004; Quesada et al., 2010). On wet summers, fruits were also observed to develop small brown spots with 0.5 to 2.5mm (Panagopoulos, 1993).

Figure 1.1. Olive tree branches exhibiting several branches full of tumors, symptomatic of olive knot disease (OK). Credits: Diogo Mina IPB-ESA

Although no distribution studies were performed, *Pss* has been reported all over the world on different plant hosts (Young et al., 2004; Moretti et al., 2008, CABI, 2018). The main dispersal pathway of *Pss* over long distances is strongly linked to human actions due to the transportation of infected plant material from areas where the pathogen occurs. *Pss* epiphytic lifestyle (Ercolani, 1983) associated to its ability to spread along olive xylem vessels (Marchi et al., 2009; Maldonado-González et al., 2013) eases the propagation of asymptomatic plant material (but latently infected) as an important source of *P. savastanoi* inoculum, increasing the risk of its introduction in a non-infected area (Lamichhane and Varvaro, 2013). However, over short distances, the bacterium is transmitted primarily by orchard fauna. Although no published studies confirm, birds and insects (like honeybees) are considered as agents of *Pss* transmission (Wilson, 1935; Quesada et al., 2010). Indeed, minor amounts of bacterial organisms found in the esophageal organ of olive fly *Bactrocera oleae* included *Pantoea agglomerans* (Ben-Yosef et al., 2015), a *Pss* pathogenicity associated bacteria (Buonaurio et al., 2015), suggesting the presence of *P. savastanoi* as an olive fly endosymbiont (Lloyd et al., 1986; Sood and Nath, 2002). Besides insects, rain and windblown aerosols are other probable carriers of phytopathogenic agents like *Pss*, infecting and colonizing any wound, including leaf, blossom, raceme, and peduncle scars. When more severe weather conditions occur, injuries

on bark, stems and leaves caused by frost and hail are also potential infection spots for *Pss* (Wilson, 1935). Several cultural practices like pruning and harvesting are also associated to olive knot disease short spread when instruments are not conveniently sterilized (Tous et al., 2008). The traditional "knocking down" method of olive wood harvesting is also known to cause damages in olive branches, leading to an easy way-in of *Pss* from the stick to the injury (Krueger et al., 1997). Additionally, lesions by leaf fall are also very commonly target of *Pss* infection, culminating in knots formation (Young, 2004).

1.2.2. Life cycle of Pseudomonas savastanoi pv. savastanoi in olive tree

Once the pathogen is present in the ecosystem, it can survive as an epiphytic organism for a long period, only causing disease symptoms when specific conditions occur (Fig. 1.2.). To initiate the symptomatic state of the disease, an ideal temperature of 22 to 25°C is required (Wilson, 1935; Iannotta et al., 2007). *In vitro* surviving temperature ranged from 1 to 32°C (26°C as optimal temperature), while *in vivo* it grows from 5 to 37 °C with 70 to 100% of relative humidity (Temsah et al., 2008).

Since the presence of *Pss* cells in stomata showed not promote OK disease symptoms (Surico, 1993), it is possible that the pathogen needs plant-released signals, emitted from the wounds, to initiate the infection process (Caballo-Ponce et al., 2017). Although *Pss* was observed to produce lytic enzymes (cellobiase, cellulase, peptinase and xylanase; Magie, 1963), this phenomenon was still not proved to be related with primary plant cell walls degradation. On the other hand, the secretion of IAA and cytokinin phytohormones by the pathogen on the infected area was proved to be associated to *Pss* virulence (Comai and Kosuge, 1980; Surico et al., 1985; Rodríguez-Moreno et al., 2008). These hormones stimulate a plant response, characterized by a hypertrophy and then hyperplasia of plant cells, and a following dedifferentiation of the infected tissues, producing xylem elements and periderm, which later leads to a rupture in the knot walls, exposing cavities full of *Pss* and allowing it to spread to the environment (Temsah et al., 2008; Quesada et al., 2012).

Upon entering in the host tissues, *Pss* starts to invade the intercellular spaces of cortical parenchyma. This microenvironment, which is poor in sugars and with high levels of Ca^{2+}, leads to an influx of this element to *Pss* cells, inducing the expression of pathogenic and virulent genes (Moretti et al., 2019). Once in the intercellular spaces, the pathogen is then able to

move along xylem vessels (Marchi et al., 2009; Maldonado-González et al., 2013) and produce secondary knots in other plant sites (Penyalver et al., 2006).

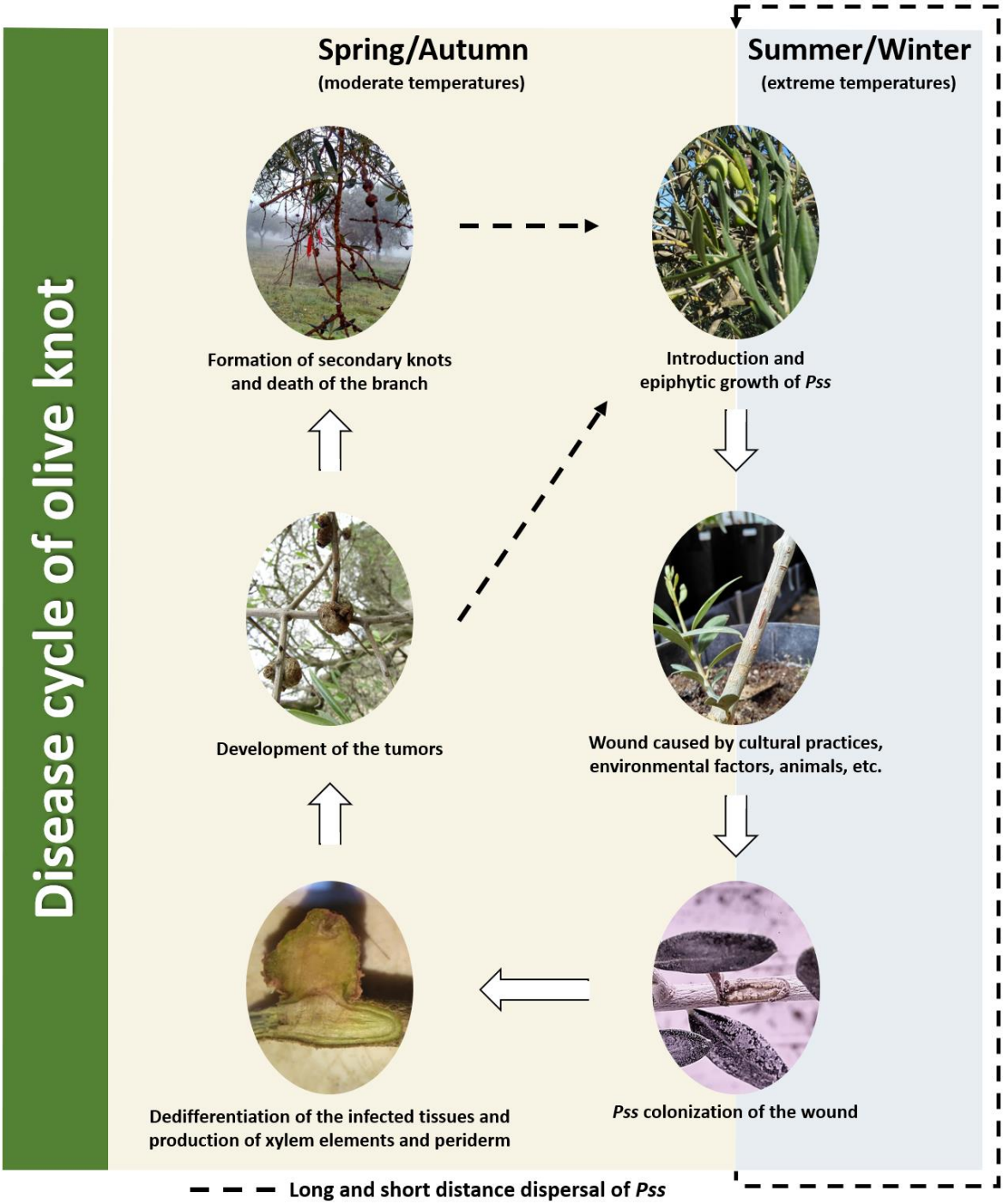

Figure 1.2. Disease cycle of olive knot caused by the bacterium *Pseudomonas savastanoi* pv. *savastanoi.*

1.2.3. Management of the pathogen in olive tree

Depending on whether the plant pathogen is already present or not in the environment, the control measures can be curative or preventive, respectively. Since it is very difficult to

eliminate a pathogen already established in an orchard, most of the available control measures are based on preventive procedures. These include, for instance, the use cultural and hygienic measures, more tolerant cultivars and chemical control (Quesada et al., 2012).

A crucial cultural control for minimizing OK disease is the use of *Pss*-free certificated plants in the establishment of new olive orchards (EPPO, 2006), in order to avoid the introduction of the pathogen in a non-infected environment. Pruning of severely affected branches could also be considered preventive maintenance for disease damage (Teviotdale and Krueger, 2004; Quesada et al., 2010), by eliminating the inoculum source. Pruning should be performed first on healthy trees to avoid contamination (Wilson, 1935) and cut branches should be burnt in the same field to avoid the dissemination of the pathogen (Trapero and Blanco, 1998). All pruning tools should also be disinfected to prevent disease transmission. These procedures should not be performed with wet weather, because the resulting wounds can serve as new infection courts. In addition, during olive harvesting, vibration methods should be preferred to the traditional branch knocking (Fig. 1.3), to avoid wounds (Krueger et al., 1999; Civantos et al., 2008).

The use of resistant cultivars could be another approach to control OK disease (Montesinos and López, 1996). However, such strategy has not been conveniently explored so far. Although several studies showed that some olive tree cultivars appear to be more tolerant (Panagopoulos, 1993; Benjama, 1994; Marcelo et al., 1999; Hassani et al., 2003; Catara et al., 2005; Penyalver et al., 2006; Nguyen et al., 2018), there are no resistant cultivars to OK disease. Representative OK-tolerant cultivars are from Spain (cvs. *Dulzal de Carmona*, *Lechín de Granada* and *Manzanilla cacereña;* Penyalver et al., 2006), Italy (cv. *Leccino;* Hassani et al., 2003) and Greece (cvs. *Kalamon* and *Megaritiki*; Panagopoulos, 1993). In Portugal, Marcelo et al. (1999) showed that, in olive plants artificially inoculated with *Pss*, cv. *Cobrançosa* was the less affected cultivar, while *Cordovil de Serpa* and *Galega Vulgar* were the most susceptible. Recently, Gomes et al. (2019) studied the fungal community associated to tolerant (cv. *Cobrançosa)* and susceptible (cvs. *Madural* and *Verdeal Transmontana*). This interaction of cultivar with *Pss* showed to shape phyllospheric fungal composition, possibly revealing how host genotype influences the role of microorganisms on *Pss* establishment and disease development.

Figure 1.3. Different methods of olive harvesting: (a) motorized branch vibrator (on the left) and traditional branch knocking with long wooden sticks (on the center); (b) mechanical vibration. Credits: Lara Pinheiro

However, all this information on cultivar OK-tolerance/susceptibility has some limitations. Indeed, those studies are not comparable among them due to different aspects that can affect the severity of olive knot disease as pathogen inoculum doses, number of inoculation sites, soil fertilization, climatic conditions, etc. (Quesada et al., 2002).

Most of the chemical treatments used to control *Pss* are copper-based compounds, described as having a toxic or bacteriostatic effect on the pathogen (Penyalver et al., 1998, Quesada et al., 2002). Although several studies propose that OK disease incidence can be managed through the reduction of the epiphytic population of *Pss* (Ercolani, 1991; Lavermicocca and Surico, 1987; Quesada et al., 2007, 2010), the effect of copper treatments on *Pss* is not completely understood.

Up to date, only a few studies focused on the control of OK disease through the use of biological control agents (BCA) (Lavermicocca et al., 2002; Rokni-Zadeh et al, 2008; Krid et al., 2010; Maldonado-González et al., 2013). Most of these studies revealed the potential of BCA for the control of *Pss* through *in vitro* conditions, but only a few tested these microorganisms through *in planta* inoculations. Maldonado-González (2013) performed inoculations in olive

plants, testing the antagonistic efficacy of root isolate *Pseudomonas fluorescens PICF7* which showed previously to control *Verticillium dahliae*, a soil-borne fungal pathogen affecting olive groves. This study concluded that *P. fluorescens PICF7* was not able to suppress OK disease but decreased *Pss* population and restrained the pathogen to inner tissues of the tumors. Nevertheless, it would be interesting to evaluate the efficacy in the field of this root endophyte, considering the biotic and abiotic conditions affecting the phyllosphere of olive tree.

1.2.4. Final conclusions

Overall, the available control measures are not enough to control *Pss* and sustainable alternatives should be further investigated. In the aboveground parts of plants, many endophytic or epiphytic bacteria are in close proximity and interact with each other. They fulfil important functions in the protection of plants from pathogenic microorganisms, improving plant health (Stone et al., 2018). Potentialities for bacteria inhabiting the aerial olive tree tissues in controlling OK disease have never been exploited, representing this strategy a practicable way to control this disease in olive groves. The few studies on the use of phyllosphere biocontrol agents on woody tree plants are revealing encouraging results (Mikiciński et al., 2016; Michavila et al., 2017) towards a future where plant diseases control could be attained without the application of chemical compounds. In addition to the use of biocontrol agents, disease suppression can be achieved by the manipulation of microbial communities through plant management practices, disclosing new sustainable ways for dealing with woody plant diseases.

1.3. Exploring the phyllosphere bacterial community for improving tree crops protection

Microbial communities on or around plants have already been described to play a pivotal role in plant growth and health (Vorholt, 2012). Such action has been mostly recognized for root-associated microorganisms (Sahu et al., 2018), while the microbial community associated to aerial parts of plants has been less studied and characterized (Carvalho and Castillo 2018). However, the aerial part of plants (phyllosphere, in *lato sensu*) has been recognized as an important habitat for microorganisms (Roat et al*.,* 2017). These microorganisms live either on the surface (usually referred as phylloplane) or inside

(endosphere) the tissues of plant organs (Carvalho and Castillo 2018). Microorganisms inhabiting the phylloplane are generally referred as epiphytes, while the ones colonizing the endosphere are referred as endophytes (Newton et al., 2010). Accordingly, the phyllosphere microbiota comprises all microorganisms living on the surface and inside of all aboveground plant tissues (Lemanceau et al., 2017a). Either in phylloplane (Meyer and Leveau 2012) or in endosphere (Ibáñez et al., 2017) of most plant species, bacteria form an important part of microbial communities, surpassing by far other microbial groups in both abundance and diversity (Lindow and Brandl 2003). The load of bacteria in leaf surface usually lies within the range of 10^6 to 10^7 cells/cm^2, up to 10^8 cells/g leaf fresh weight (Leach et al., 2017). The number of bacterial species in the phyllosphere of natural ecosystems is also enormous. Estimates of bacterial endophytes inhabiting the Brazilian Atlantic forest indicate the possible occurrence of 2 to 13 million species present in the aboveground plant parts, being almost 97% of these species undescribed (Lambais et al., 2006).

Bacteria inhabiting the phyllosphere can interact with the host plant (Kembel et al., 2012) and with other microorganisms, including both beneficial and pathogenic microbes that share the same habitat (Müller and Ruppel 2014; Leach et al., 2017). These plant–bacteria and bacteria–microbe interactions significantly influence plant performance and defense against diseases and pests (Bulgarelli et al., 2013; Rastogi et al., 2013; Ciancio et al., 2016; O'Bryan 2017). The importance of such interactions in promoting host plant defense against diseases was specially recognized in herbaceous plant species (El-Sayed et al., 2018; Rahman et al., 2018), whereas their role on woody plants protection against diseases has been less studied (Cazorla and Mercado-Blanco 2016). This would be a key knowledge for developing new strategies for agricultural tree crop protection.

In this review, the diversity and structure of bacterial communities (both endophytic and epiphytic) inhabiting the phyllosphere of economically important agricultural woody tree crops will be highlighted. Both biotic and abiotic factors that contribute to the shaping of bacterial communities will be also addressed. The potential to explore phyllosphere-associated bacteria for protecting woody crops from diseases will be discussed, either through their use as biological control agents or through their management.

1.3.1. Diversity of bacterial communities in the phyllosphere of important agricultural woody crop trees

The structure and diversity of phyllosphere bacterial communities of agricultural woody crops have been primarily studied in economic important fruit trees, such as citrus (*Citrus* sp., Araújo et al., 2002; Passera et al., 2018), apple (*Malus pumila*; Yashiro et al., 2011, 2012; He et al., 2012), banana (*Musa acuminata*/*balbisiana*; Thomas and Soly, 2009; Rossman et al., 2012), chestnut (*Castanea sativa*; Valverde et al., 2017), coffee (*Coffea arabica*/*robusta*; Vega et al., 2005), olive (*Olea europaea*; Müller et al., 2015) and stone fruits (*Prunus dulcis, P. domestica, P. salicina, P. armeniaca, P. avium, P. cerasus* and *P. persica*; Jo et al., 2014) (Table 1.1).

The phyllosphere bacterial communities associated to such crops have been analyzed by using both culture-dependent and –independent molecular approaches, such as PCR-SSCP fingerprinting, quantitative PCR, fluorescence *in situ* hybridization platforms and/or high-throughput sequencing technologies. Although a broader spectrum of bacterial colonizers can be assessed by next-generation technologies than using cultural approaches (Pham et al., 2008), PCR limitations can bias diversity studies. For example, *primers* could display different affinities to templates, inhibitory compounds could be present in different environmental samples, and plant organelle-derived RNA sequences could interfere in microbial target amplifications (Müller and Ruppel 2014).

Combined culture-dependent and -independent approaches have revealed a high degree of bacterial diversity on the phyllosphere of seven fruit tree crops, spanning a total of 104 bacterial genera, belonging to 75 families and 12 phyla (Fig. 1.4a). Globally, the bacterial communities of these fruit tree crops consisted predominantly in *Proteobacteria*, *Actinobacteria, Firmicutes* and *Bacteroidetes*, but in different proportions according to the tree species (Fig. 1.4b). For instances, the phyllosphere of *Castanea* was found to be dominated by *Actinobacteria* (Valverde et al., 2017), while *Prunus* presented up to 90% of bacteria from *Proteobacteria* (Jo et al., 2014). From the seven surveyed tree species, the phyllosphere of both *Musa* and *Citrus* showed the highest proportion of bacteria from *Firmicutes* (Araújo et al., 2002; Thomas and Soly 2009; Rossman et al., 2012; Passera et al., 2018).

Other phyla were also specific from *Citrus* spp. and *Olea* spp. phyllospheres, but at lower abundances.

Table 1.1. Woody fruit crops surveyed for phyllospheric bacterial communities. For each tree species, the bacterial community surveyed (endophytic or epiphytic), plant organ and methodological approach used are indicated.

Plant Host	Organ	Community	Method	Reference
Citrus (*Citrus* sp.)	Branches	Endophytes	Culture-dependent Culture-independent (PCR-DGGE analysis)	Araújo et al., 2002
	Leaves	Endophytes	Culture-independent (16S rRNA sequencing from ground leaf tissue)	Passera et al., 2018
Apple (*Malus pumila*)	Leaves	Epiphytes	Culture-dependent Culture-independent (DAPI and 16S rRNA gene cloning from leaves sonication extracts)	Yashiro et al., 2011
	Leaves	Epiphytes	Culture-independent (16S rRNA gene cloning from leaves sonication extracts)	Yashiro et al., 2012
	Leaves	Epiphytes Endophytes	Culture-dependent Culture-independent (macroarray hybridization)	He et al., 2012
Banana (*Musa acuminata*/ *M. balbisiana*)	Branches	Endophytes	Culture-dependent	Thomas and Soly 2009
	Fruit	Endophytes	Culture-dependent Culture-independent (see reference)	Rossman et al., 2012
Chestnut (*Castanea sativa*)	Leaves	Epiphytes	Culture-dependent	Valverde et al., 2017
Coffee (*Coffea arabica*/ *C. robusta*)	Branches Leaves Fruits	Endophytes Epiphytes	Culture-dependent	Vega et al., 2005
Olive (*Olea europaea*)	Leaves	Endophytes	Culture-independent (Illumina sequencing and qPCR)	Müller et al., 2015
Stone fruits (*Prunus dulcis*/ *P. domestica*/ *P. salicina*/ *P. armeniaca*/ *P. avium*/ *P. cerasus*/ *P. persica*)	Leaves	Epiphytes	Culture-independent (pyrosequencing)	Jo et al., 2014

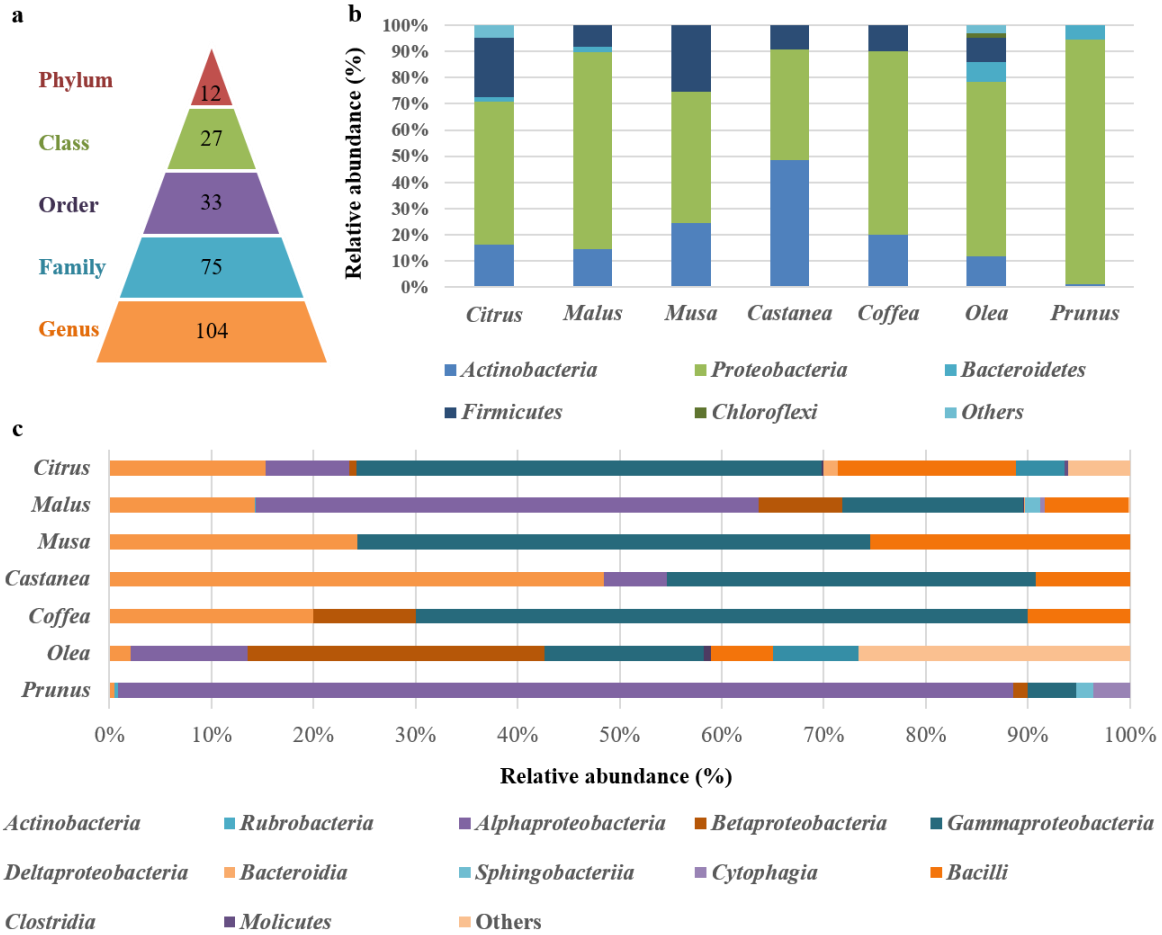

Figure 1.4. Bacterial communities associated to the phyllosphere of woody tree crop species: *Citrus* (Araújo et al., 2002; Passera et al., 2018), *Malus* (Yashiro et al., 2011, 2012; He et al., 2012), *Musa* (Thomas and Soly, 2009; Rossman et al., 2012), *Castanea* (Valverde et al., 2017), *Coffea* (Vega et al., 2005), *Olea* (Müller et al., 2015) and *Prunus* (Jo et al., 2014). (a) Number of distinct bacterial taxonomic groups detected across all tree crops; (b) Bacterial community composition, at phylum level, for each tree crop. (c) Bacterial community composition, at class level, for each tree crop.

While *Planctomycetes* was found in both cultures, a number of phyla were specifically found on *Citrus* (*Fibrobacteres, Spirochaetes* and *Tenericutes*) or *Olea (Acidobacteria, Verrucomicrobia* and *Armatimonadetes*) phyllospheres (Araújo et al., 2002; Müller et al., 2015; Passera et al., 2018).

Differences between fruit tree species become more apparent when comparing bacterial communities at class level (Fig. 1.4c). *Citrus* and *Olea* presented the highest number of classes (16), followed by *Malus* (12) and *Prunus* (6). While two bacterial classes were common among all the investigated fruit trees (*i.e.*, *Actinobacteria* and *Gammaproteobacteria*), some classes were tree species specific. Among the seven investigated tree species, the phyllosphere of *Olea* displayed the highest number of unique bacterial classes (8), followed by *Citrus* (6) and *Malus* (1). Interestingly, three bacterial classes

(*i.e*., *Cytophagia, Sphingobacteriia* and *Rubrobacteria*) were exclusively found in the phyllosphere of *Malus* and *Prunus*, suggesting that these bacteria might represent the core microbiota of *Rosaceae* family plants.

Further analysis of bacterial community composition at genus level in the phyllosphere of the studied host tree species indicates that *Bacillus, Pseudomonas, Pantoea, Micrococcus, Methylobacterium, Sphingomonas* and *Enterobacter* are highly abundant and consistently found (data not showed). Therefore, these genera are likely to represent the core bacterial community of these fruit crops.

The persistence of core members in apparently healthy trees suggests that they may be benefic to the host. Indeed, the core microbiome is considered to encompass key microbial taxa that are critical for plant health. Evolutionary processes resulted in the selection and enrichment of microbiota carrying genes with essential functions for the fitness of holobiont (*i.e*., the plant plus all associated microbiota) (Lemanceau et al., 2017a). Besides core bacterial genera, surveys on the phyllosphere of fruit tree crops also detected bacterial genera specific to a particular tree species (Rossman et al., 2012; Jo et al., 2014; Passera et al., 2018), reflecting the adaptation of bacteria to a specific environment (Lemanceau et al., 2017b).

Few studies have directly compared endophytic and epiphytic bacterial communities inhabiting the phyllosphere of woody crop trees. Despite the lack of studies comparing endo- and epiphytic bacterial communities within the same crop tree phyllosphere, Vega et al., (2005) found a higher number of bacterial species in the surface of Colombian coffee leaf than in internal leaf tissues (*i.e*. 18 *vs*. 8, respectively). The analysis of phyllospheric bacterial communities across the seven fruit tree crops (either endo- or epiphytic, or both) showed that the most abundant bacteria class in the endosphere (*i.e*., *Gammaproteobacteria*) was different from the one detected in the phylloplane (*i.e*., *Alphaproteobacteria*; Fig. 1.5a). Likewise, the bacterial community inhabiting leaves and branches, across the seven fruit tree crops, displayed a different composition (Fig. 1.5b). *Alpha*- and *Gammaproteobacteria* were dominant bacterial inhabitants of leaves, while *Actinobacteria* and *Bacilli* were common in branches. Similarly, in the phyllosphere of coffee seedlings, branches harbored greater endophytic bacterial diversity than leaves; both presenting a distinct bacterial community composition (Vega et al., 2005).

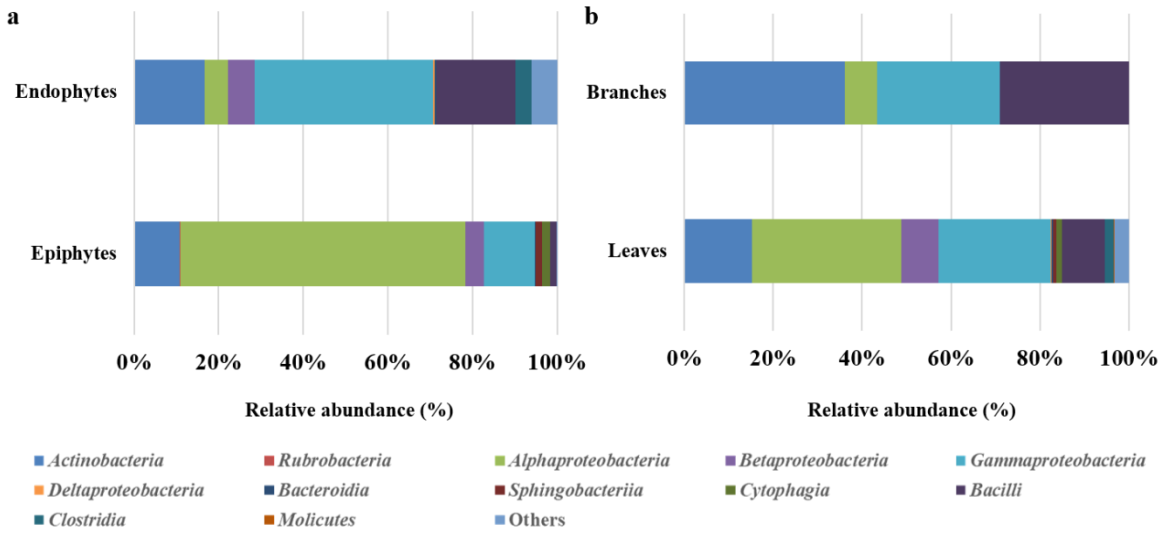

Figure 1.5. Relative abundance of surveyed bacterial classes in the phyllosphere of the seven tree crops indicated in Table 1.1. (a) Bacterial classes detected within endophytic and epiphytic communities. (b) Bacterial classes detected on leaves and branches.

Studies for disclosing the main biotic and abiotic drivers shaping bacterial communities associated to major woody crop trees phyllosphere, specifically under field conditions, are still preliminary (Laforest-Lapointe et al., 2016; Hamonts et al., 2018). Plant host species is usually one of the most important forces for the assembling of phyllospheric bacterial communities in woody tree species (Baldotto and Olivares 2008). However, the plant traits specifically involved in the selection of particular microbial epiphytic and endophytic colonizers are so far largely unknown (Kembel et al., 2014). The composition and size of phyllosphere-associated bacterial communities also depend on other biotic factors, like host age (Carper et al., 2018), development stage (Redford and Fierer 2009), host genotype (Cregger et al., 2018) and occurrence of symbiotic associations like mycorrhization (Li et al., 2018). Abiotic factors are also known to influence phyllospheric bacterial community, such as geographical location (Finkel et al., 2011; Qvit-Raz et al., 2012) and climatic factors (Carper et al., 2018). A deeper understanding of bacterial communities in the phyllosphere of woody tree crops, as well as the drivers that shape their assembling, will offer new opportunities for controlling plant diseases and improve host plant health.

1.3.2. Exploiting phyllosphere bacterial communities for woody tree crop protection

The use of bacterial isolates naturally adapted to crop species, resident microbiota and environment could provide an efficient approach to the biological control of plant diseases

under field conditions (Ozaktan et al., 2012). The search for potential bacterial biological control agents, within the same host species as the pathogen, has begun more than 40 years ago (Wrather et al., 1973; McIntyre et al., 1987). However, up to the beginning of 21st century, most of the investigation performed on woody tree crops was mainly focused on apple and pear diseases (Utkhede 1987; Janisiewicz and Roitman 1988; Vanneste 1996; Pussey 2002). Furthermore, few studies have been illustrating the biocontrol of diseases in woody tree crops by using native phyllosphere-associated bacterial members. Anyway, the antagonistic potential of phyllosphere microbiota has been explored against problematic pathogens over the last two decades, mainly through *in vitro* assays (*e.g*., Singh et al., 2004; Trivedi et al., 2010; Silva et al., 2012).

The control of woody crop diseases through the application of native phyllosphere-associated bacterial members presenting antagonistic activity, either in field or greenhouse conditions, appears to be promising (Table 1.2). The level of disease suppression achieved by application of such bacteria ranged from 27% to 86%. Most research and development efforts have been focused on isolates of *Pseudomonas* and *Bacillus* genera. *Pseudomonas* have been mostly effective for the biocontrol of bacterial diseases (*e.g*., *Erwinia* sp. and *Xanthomonas* sp.), while *Bacillus* have been mostly used for controlling fungal diseases (*e.g*., *Gnomoniopsis* sp., *Colletotrichum* sp. and *Cryphonectria* sp.). Accordingly, *Bacillus* strains are among the most exploited bacteria to be used as biocontrol agents against plant diseases (Bacon et al., 2002), in addition to their role on promoting plant growth (Pérez-Garcia et al., 2011). In recent years, there are also other bacteria that have received attention for the biocontrol of woody crop diseases, such as *Pantoea* sp. (Ozaktan et al., 2011; Gerami et al., 2013), *Serratia* sp. (Gerami et al., 2013), *Burkholderia* sp. (Silva and Costa 2014) and *Alcaligenes* sp. (Abraham et al., 2013). These genera revealed to be effective in reducing the incidence and severity of important diseases that affect several hosts, mostly pear (Gerami et al., 2013) and apple (Pusey 2002; Ozaktan et al., 2011; Mikiciński et al., 2016), but also citrus (Kupper et al., 2011; Michavila et al., 2017), banana (Silva and Costa 2014), mango (Yenjit et al., 2004), chestnut (Wilhelm et al., 1998; Pasche et al., 2016), avocado (Korsten et al., 1997), *Hevea* (Abraham et al., 2013) and pomegranate (Puneeth 2015). In particular, several *Pseudomonas* species (*i.e*., *P. graminis*, *P. agglomerans* and *P. fluorescens*) were reported to be the most promising biocontrol agents against *Erwinia amylovora* on pear (Gerami et al., 2013) and apple

(Mikiciński et al., 2016). *Bacillus subtilis* is a promising agent for controlling *Xanthomonas axonopodis* on pomegranate (Puneeth 2015).

Different mechanisms can be involved in the biological control of pathogens by these phyllospheric bacteria, although their effectiveness is still not totally understood. The antagonistic activity of *Pseudomonas* spp. towards pathogens is usually associated to the competition for nutrients (Cabrefiga et al., 2007) or to the production of secondary metabolites, such as siderophores (Duffy and Défago 1999; Sasirekha et al., 2016), antibiotics (*e.g.*, pyoluteorin and phenazines), lytic enzymes (*e.g.*, protease and celulase), hydrogen cyanide (Weller 2007; Gerami et al., 2013; Zengerer et al 2018) and antimicrobial volatile compounds (Hernández-León 2015). Also, the mechanisms used by *Bacillus* strains to control plant pathogens have been attributed to the production of antibiotics and antimicrobial compounds, such as lipopeptides and lytic enzymes (Touré et al., 2004; Huang et al., 2012; Kumar and Dubey 2012), as well as to the induction of host plant defenses (Kloepper et al., 2004). Indeed, a strong antimicrobial effect against different phytopathogenic fungi and bac teria was already reported for lipopeptides, specially from iturin A, fengycin and surfactin families (Touré et al., 2004; Ongena and Jacques 2008; Malfanova et al., 2012; Yuan et al., 2012). Sporulation of plant pathogens could be also compromised by certain *Bacillus* species, as previously reported for *B. subtilis, B. licheniformis* and *B. cereus* that were able to reduce spore germination and germ-tube elongation in *Colletotrichum gloeosporioides* (Yenjit et al., 2004). *Bacillus* can also cause morphological abnormalities in mycelium of pathogenic fungi (Chaurasia et al., 2005).

Pantoea, *Enterobacter*, *Serratia* and *Burkholderia* genera are also known to release antibiotics that are considered to be responsible for the antagonistic action against plant pathogens (Ishimaru et al., 1988; Subagio et al., 2003; Buana et al., 2014). For example, *Burkholderia* was reported to be effective in inhibiting several fungal phytopathogens of oil palm through the production of antibiotics, such as phenazine, pyrrolnitrin, pyoluteorin and 2,4-DAPG (Subagio et al., 2003; Buana et al., 2014). Expression analysis also revealed that the antagonistic effect of *Pantoea agglomerans* against fungal phypathogens is related to up- and downregulation of genes, associated to fungal defense, virulence and/or metabolic functions (Pandolfi et al., 2010). Furthermore, the suppression of phytopathogens by *Serratia* is related to a combination of mechanisms, including antibiosis (through the production of antimicrobial

compounds), parasitism (through the release of extracellular cell wall-degrading enzymes) and competition (through siderophore release) (De Vleesschauwer and Höfte 2007). The biocontrol effect of *Alcaligenes* sp. towards several phytopathogens of different herbaceous crops have been also related to the production of siderophores (Sayed and Patel 2011) and lytic enzymes, such as chitinase (Vaidya et al., 2001).

Beyond the use of biocontrol agents, disease suppression can also be achieved by the manipulation of phyllosphere microbial communities, in order to improve positive interactions with the host plant (Orozco-Mosqueda et al., 2018). In this "engineering" process, the microbial composition can be altered to maximize the benefits of the microbial social network for crop plants. To the best of our knowledge, such approach has not yet been explored in woody crop trees. Manipulation of microbiota is applied most extensively in humans for treatment of diseases (Young 2017; Larsen and Claassen 2018), and more recently in herbaceous crops for the control of root diseases (*e.g.*, Gopal et al., 2013; Martínez-Hidalgo et al., 2018). This strategy, which is largely based on the transfer of natural microbial communities (by mixing disease suppressive soils with disease conducive ones), proved to be effective in the management of several root plant diseases, including rhizoctonia root in sugar beet, potato common scab and tobacco black root rot (Gopal et al., 2013). Besides transfer of natural microbiome, soil inoculation with artificial mixture of bacterial strains with desired functions has protected *Nicotiana attenuata* from sudden-wilt disease (Santhanam et al., 2013). There are also some evidences indicating that root microbiomes can be modulated by the phytohormone salicylic acid, which role on the activation of defense responses is already well-known (Lebeis et al., 2015). Similarly, the application of bioorganic fertilizers in banana nursery pots resulted in the manipulation of the rhizospheric microbial structure and subsequently decreased the incidence of Panama disease on banana (Xue et al., 2015). Despite all these successful approaches, more research is still required to better understand the impact of synthetic/natural microbial communities, as well as different cropping practices and abiotic parameters, on the microbiome structure and how microbiome shifts are translated to plant health (Mueller and Sachs 2015). In particular, microbiome engineering for improving woody tree crop health is a largely untapped area that deserves major research efforts.

Table 1.2. Phyllospheric bacteria tested *in planta* for controlling diseases of woody tree crops. Efficiency of bacteria on preventing disease incidence (i) and severity (s) are shown.

Microorganism	Host plant	Pathogen	Assay	Efficacy (%)	Reference
Pantoea vagans	Apple	*Erwinia amylovora*	Field	54% (i)	Ozaktan et al., 2011
Pantoea agglomerans	Pear	*Erwinia amylovora*	Field	58-79% (i)	Gerami et al., 2013
Serratia sp.	Pear	*Erwinia amylovora*	Field	27-58% (i)	Gerami et al., 2013
Pseudomonas fluorescens	Apple	*Erwinia amylovora*	Field	27-36% (i)	Pussey 2002
Pseudomonas fluorescens	Pear	*Erwinia amylovora*	Field	61-75% (i)	Gerami et al., 2013
Pseudomonas protegens	Citrus	*Xanthomonas citri*	Greenhouse	78% (s)	Michavila et al., 2017
Pseudomonas graminis	Apple	*Erwinia amylovora*	Greenhouse Field	86% (s) 73% (s) /40% (i)	Mikiciński et al., 2016
Burkholderia spinosa	Banana	*Colletotrichum musae*	Field	*	Silva and Costa 2014
Alcaligenes sp.	*Hevea*	*Phytophthora meadii*	Greenhouse	34-48% (s)	Abraham et al., 2013
Bacillus licheniformis	Mango	*Colletotrichum gloeosporioides*	Greenhouse	50% (s)	Yenjit et al., 2004
Bacillus subtilis	Chestnut	*Cryphonectria parasitica*	Greenhouse	71% (s)	Wilhelm et al., 1998
Bacillus subtilis	Citrus	*Phyllosticta citricarpa*	Field	29% (s)	Kupper et al., 2011
Bacillus subtilis	Pomegranate	*Xanthomonas axonopodis*	Field	78% (s)	Puneeth 2015
Bacillus subtilis	Mango	*Colletotrichum gloeosporioides*	Greenhouse	44% (s)	Yenjit et al., 2004
Bacillus subtilis	Avocado	*Pseudocercospora purpurea*	Field	*	Korsten et al., 1997
Bacillus subtilis	Avocado	*Akaropeltopsis sp.*	Field	*	Korsten et al., 1997
Bacillus amyloliquefaciens	Chestnut	*Gnomoniopsis smithogylvyi*	Greenhouse	75% (i)	Pasche et al., 2016
Bacterium fjat	Pomegranate	*Xanthomonas axonopodis*	Field	77% (s)	Puneeth 2015

*depending on the applied treatment, different values were obtained

1.3.3. Challenges for the biocontrol of woody tree crops diseases

The efficiency of phyllospheric bacteria in controlling aerial diseases in woody tree crops, under field conditions, is often affected by several abiotic and biotic factors (Fig. 1.6).

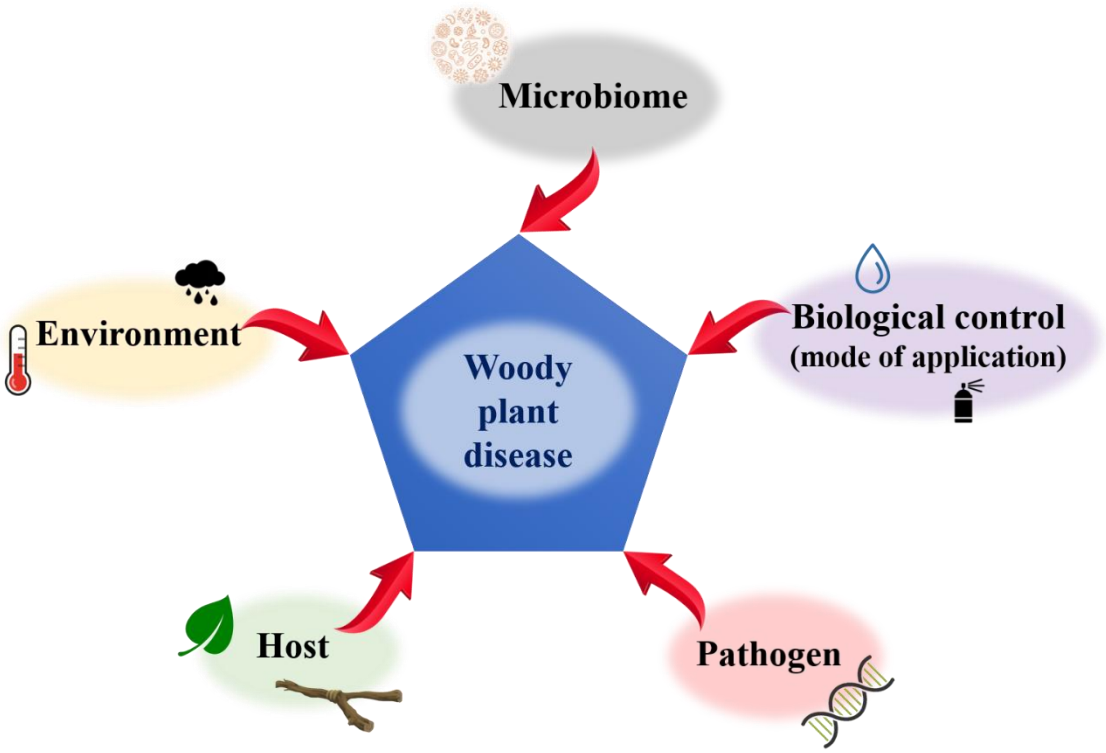

Figure 1.6. Abiotic and biotic factors with recognized influence in the efficiency of biological control against aerial diseases of woody tree crops.

Specifically, some aspects related with the biocontrol agent (*i.e*., method of their application; Silva and Costa 2004; Kupper et al 2011; Ozaktan et al., 2011), pathogen (*i.e.* strain*;* Abraham et al., 2013), host plant (*i.e.* cultivar or organ; Gerami et al., 2013; Puneeth 2015), environment (*i.e*., weather conditions; Pusey 2002) and microbiome (Xue et al., 2015), have been described to play an important role on the efficiency of biocontrol agents against diseases affecting the phyllosphere of woody crop trees.

Concerning the method of biocontrol application, Silva and Costa (2004) recognized that *Burkholderia spinosa* was more effective in suppressing the abundance of potential fungal pathogens (*Aspergillus* and *Fusarium*) on banana plants when applied as a foliar spray than as a soil drench. The application of plant regulators together with the bacterial agents was also

reported to enhance their biocontrol ability. For example, when prohexadione-calcium was applied together with *Pantoea vagans* to the phyllosphere of pear tree, a higher biocontrol of *Erwinia amylovora* was achieved (Ozaktan et al., 2011). Similarly, complex microbial inoculums can improve biocontrol activity, when compared to individual inoculums. For instance, Kupper et al., (2011) reported a synergistic effect of a complex mixture of several strains of *Bacillus subtilis* against *Guignardia citricarpa* on citrus tree.

Another limitation to effective bacterial biocontrol on woody crops is related with the specificity between the biocontrol agent and pathogenic strain. On *Hevea brasiliensis*, several strains of the same potential antagonist were reported to differently affect the same pathogenic agent (Abraham et al., 2003). In addition, depending on the host plant (*e.g.* type of cultivar) (Gerami et al., 2013) or target organ (Puneeth 2015), different effects were observed on the biocontrol of crop diseases.

The performance of a microbial control agent is widely influenced by the environment into which the antagonist is introduced. This is particularly important when biocontrol agents are applied on the above-ground parts of plants, especially for those diseases caused by air-borne microorganisms. Indeed, the phyllosphere (in particular phylloplane) is a harsh environment for microorganisms to survive. Accordingly, the destructive influence of UV light was already reported to be a limiting factor for the application of potential biocontrol bacteria (Jacobs and Sundin 2001). On the other hand, *Pseudomonas syringae pv. syringae*, a phytopathogenic and epiphytic bacteria associated to mango tree surfaces was reported to be resistant to UV radiation (Sundin et al., 1996; Cazorla et al., 2008). Environmental temperatures are also important for bacterial thriving. Pusey and Curry (2004) observed that the optimal temperature for bacterial growth on apple flowers depended on the microorganism; the temperature that allowed a population increase of the pathogen *Erwinia amylovora* was different from antagonist *Pantoea agglomerans*. Jonhson et al., (2000) obtained similar results, showing that temperatures above 12 ^{o}C lead to a successful establishment of the antagonist *P. fluorescent* A506.

A further complication for obtaining the highest biocontrol efficiency is related with the complexity of microbial communities associated with plants. Microbe-microbe interactions have a strong influence on plant-microbe interactions (Kroll et al., 2017), disguising their expected effect on plant health. Taking this into account, the understanding

of such a complex interaction (involving the host, pathogen, biocontrol agent, host microbiota and environment) would be the major key to move forward to control woody plants diseases. Novel tools and technologies are being developed to provide deeper insights into the plant microbiome, as well as into microbe-microbe and microbe-plant interactions. In a first approach, the host core microbiome (*i.e*., microbial taxa consistently present in a healthy host) of phyllospheric woody tree crop should be identified and correlated with host health. This correlation could then be ascertained by employing, for example, metatranscriptomics and metaproteomics approaches that would infer the functional properties of the host core microbial community. This knowledge could help to fully understand the impact that core microbes have on woody crop tree health, revealing also strategies for microbiome engineering.

1.3.4. Final conclusions

In the past ten years, researchers have developed a much more in depth and detailed understanding of how phyllospheric-associated bacteria can improve host plant health. However, such knowledge is so far higher for herbaceous crops than for woody crops. While more research work, both basic and applied, remains to be done, native phyllosphere-associated bacterial members are already being successfully used as biocontrol agents of some woody tree crops diseases, albeit on a small scale. Further studies are still required for enhancing the knowledge on the composition of microbial communities in the phyllosphere of woody trees, the factors shaping their assemblages, and their role/function in plant health. New approaches, as omics technologies, can provide greater advances on all these aspects. Research efforts should also be carried on for elucidating the effects of inoculation with bacterial biocontrol agents (either specific microbial strains, synthetic communities, or natural communities) on the management of woody crop diseases. Trials to identify efficient antagonists should be performed in conditions that mimics the host environment as much as possible, preferably using *in planta* assays. In this way, not only the antagonistic mechanisms that occur in natural environment would be unraveled, but the behavior of antagonistic (and pathogen) microorganisms would be evaluated (Pliego et al., 2006). Few efforts have been given on the manipulation of the microbiome to control woody crop diseases. Such approach could be further used to modulate intentionally the microbiome, recruiting disease

antagonists, a process denoted as bioengineering. This could be also an interesting option for the management of woody crop diseases, in a more sustainable way.

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CHAPTER 2.

Endophytic and epiphytic bacteria on olive tree: exploring tissue and cultivar effect

2.1. Abstract

The olive tree (*Olea europaea*) is a worldwide important culture with a great impact for the Mediterranean region. Although plant phyllosphere harbors a panoply of microorganisms known to play diverse and important roles for the plant health, the number of studies focused on the phyllospheric olive tree bacterial communities and their shaping factors are currently scarce. In the present work, surface and internal bacterial microbiota of two olive cultivars (*Cobrançosa* and *Verdeal Transmontana*) were evaluated in twigs and leaves. Although three phyla (*Proteobacteria*, *Actinobacteria* and *Firmicutes*) were consistently found in epiphytic and endophytic communities, a higher abundance of *Actinobacteria* and the presence of *Bacteroidetes* phylum was characteristic of epiphytic communities. Comparing both cultivars, cv. Verdeal Transmontana presented bacterial communities with higher richness and diversity than cv. Cobrançosa, while twigs presented higher bacterial abundance than leaves. Bacterial populations showed to be highly influenced by the cultivar, followed by the plant organ, with a number of taxa being specific to those different plant micro-environments. Altogether, the results presented here increases the knowledge on the structure of bacterial microbiome in the olive tree, which could play a significant role for olive tree be able to cope with typical Mediterranean climates (warm and dry summers and wet winters), as well as the impact that host associated variables exert on this microorganisms.

2.2. Introduction

The phyllosphere (a *sensu lato* term applied for describing the aerial parts of plants) has been recognized to be an important habitat for a myriad of microorganisms (Bringel and Couée, 2015). One of the major groups of microorganisms inhabiting this habitat, either in terms of diversity or abundance, is bacteria (Lindow and Brandl, 2003; Leach et al., 2017). They may live on the surface (generally referred as epiphytes) and/or inside (endophytes) the plant tissues (Newton et al., 2010), setting up complex microbial interactions with great impact for plant growth and productivity (Bulgarelli et al., 2013; O'Brien, 2017).

Previous studies have demonstrated that different environmental and plant-dependent factors, such as host species and plant organ, contribute to the shaping of bacterial

communities in the phyllosphere (Vorholt, 2012; Bodenhausen et al., 2013; Carper et al., 2018). Most of these studies have focused on those bacteria associated to the phyllosphere of specific host species (*e.g*., Kim et al., 2012; Lambais et al., 2014; Laforest-Lapointe et al., 2016). The variation in bacterial community composition among different genotypes from the same species has been generally overlooked. Although rare, such studies have been often limited to temperate forests (Redford et al., 2010; Laforest-Lapointe et al., 2016) or horticultural species (Hunter et al., 2010; Rastogi et al., 2012), often with contradicting results. For instances, Hunter et al. (2010) detected differences in leaf bacterial community composition among lettuce varieties, whereas Rastogi et al. (2012) did not find such differences. In addition, most of these previous studies have focused exclusively in epiphytes (Redford et al., 2010; Kim et al., 2012; Rastogi et al., 2012; Lambais et al., 2014; Laforest-Lapointe et al., 2016). Studies focusing on both epiphytic and endophytic communities are scarcer and provided limited insights into the forces shaping both bacterial communities in the phyllosphere (Hunter et al., 2010; Bodenhausen et al., 2013). The epiphytic community is faced with a poor nutrient and variable environment, characterized by the permanent changes of temperature, humidity and radiation (Bringel and Couée, 2015). The endophytic community, on the other hand, resides within a more stable environment compared to epiphytes, being the defense response of host plant the main challenge that they would probably need to face (Khare et al., 2018). There are few comparisons of epiphytic and endophytic phyllosphere bacterial communities, especially comparisons using the same plant material. Such studies performed either on *Arabidopsis thaliana* (Bodenhausen et al., 2013) or lettuce (Hunter et al., 2010) leaves indicated that bacterial epiphytes were more diverse and abundant than endophytes. If both bacterial communities inhabiting the phyllosphere are shaped by the same or different factors, or if the importance of shaping factors changes according to the plant organ remains to be elucidated.

Here, we characterize and compare the assembling of bacterial epiphytes and endophytes associated to leaves and twigs of two olive tree cultivars. Olive (*Olea europaea* L.) is a typical tree of the Mediterranean Basin (Loumou and Giourga, 2003), where around 95% of the world olive crop area is located (Barranco et al., 2008). Mediterranean-climate, characterized by severe water deficits in summer and abundant water in winter when temperatures and light are low (Belda et al., 2014), can be an extreme habitat for

phyllospheric microorganisms. The natural characteristics of these Mediterranean-climate ecosystems make them highly interesting for studying phyllosphere microbial adaptations (Lopez-Llorca and Macia-Vicente, 2009). Climate change scenarios foresee temperatures increases in many Mediterranean regions (Gualdi et al., 2013), revealing the importance of such studies. Most research on olive tree phyllosphere microbiota have mainly focused on fungal communities, either exclusively on endophytic (Fisher et al., 1992; Sia et al., 2013; Torres et al. 2013; Martins et al., 2016) or both endophytic and epiphytic populations (Gomes et al., 2018). As far as we know, there are only one study focusing on archaeal and bacterial diversity in olive tree phyllosphere (Muller et al., 2015). Using olive tree growing in Mediterranean-climate ecosystem, the present work seeks to answer the following questions: (i) How do bacterial communities differ in diversity and composition between two host genotypes (at cultivar level) and two plant organs (leaves and twigs)? (ii) Does host genotype (at cultivar level) and plant organ affect the assembling of endophytic and epiphytic bacterial communities in a similar way? (iii) Can we determine indicator communities associated with cultivar and plant organ? The bacterial communities structure in olive tree phyllosphere was determined using a culture-dependent approach (followed by the identification of rRNA 16S barcodes) foreseeing a possible application of those microbiota on future interaction studies.

2.3. Material and Methods

2.3.1. Study site and sample collection

Sampling was performed from September to October 2015, in three olive orchards located in Mirandela, Portugal, at coordinates N 41º 32.593'; W 07º 07.445' (orchard 1), N 41º 32.756'; W 07º 07.590' (orchard 2) and N 41° 29.454'; W 07° 30.398' (orchard 3). In the selected orchards, trees were planted with 7 x 7 m spacing and were managed following integrated production guidelines (Malavolta and Perdikis, 2012). In each orchard, 7 olive trees of cv. *Cobrançosa* and 7 olive trees of cv. *Verdeal Transmontana* were randomly selected, resulting in the evaluation of 21 olive trees from each cultivar. Apparently healthy branches of each tree were randomly collected with sterilized shears and gloves, placed into sterile roll bags and brought to the lab on ice. Plant material was stored at 4 °C up to processing that occurred within the next 24h (for epiphytes) or 72h (for endophytes).

2.3.2. Bacterial isolation

From two different branches of each tree, around 1 g of leaves and twigs were detached and used to isolate epiphytes and endophytes. Leaves and twigs were separately immersed in 10 mL of peptone water (10 g/L peptone, 5 g/L sodium chloride) and shaken gently on a rotary shaker (100 rpm), for one hour, at room temperature. Aliquots of 100 µL of the microbial suspension were plated in triplicate onto Luria Bertani (LB) agar medium (10 g/L peptone, 5 g/L yeast extract, 5 g/L sodium chloride, 10 g/L agar) and incubated at 25^{o}C in the dark until bacterial growth. Daily observations were performed, in order to isolate and count bacterial colonies. The number of colonies (CFU; Colony Forming Units) present on 1 cm^2 surface area of leaves/twigs was transformed to log CFU per cm^2. To estimate leaf and twig surface areas, the ellipse ($A = \pi abx2$) and cylinder ($A = 2\pi rh + 2\pi r^2$) equations were respectively used, where A is the area, a and b are the corresponding longitudinal and transverse axes of the leaf, and r and h are the radius and height of the twig segments. The obtained average area for leaves was 39.5 ± 11.4 cm^2 for cv. *Cobrançosa* and 37.7 ± 13.0 for cv. *Verdeal Transmontana*, and for twigs was 11.0 ± 3.6 cm^2 for cv. *Cobrançosa* and 11.0 ± 2.3 for cv. *Verdeal Transmontana*.

From the same plant material used to isolate epiphytes, five segments of twigs and five leaves from each tree were randomly selected and used to isolate endophytic bacteria. For this, leaves and twigs were first surface disinfected through sequential immersion in 70% (v/v) ethanol for 1 min, 3% (v/v) sodium hypochlorite for 1 min, and then rinsed three times (1 min each) with sterile distilled water. To ensure the efficiency of the sterilization protocol, the surface of each leaf and twig were imprinted onto LB agar medium. Each fragment was cut into five pieces (*ca*. 5 x 5 mm), which were then transferred to LB agar medium for allowing endophytes growth. Plates were incubated at 25 ^{o}C in the dark. Daily observations were performed in order to count and isolate the bacterial colonies growing out from the plant tissues segments.

Altogether, in this work a total of 4.200 plant segments were used for evaluating endophytic communities, resulting from replicates in the following experimental design: 3 orchards x 2 olive tree cultivars x 7 trees x 2 plant organs x 5 plant segments.

2.3.3. Bacterial identification

Bacterial isolates were first grouped by colony morphology (color, form, elevation and edges). Two representatives of each morphotype were selected for molecular identification using V1-V4 regions from 16S rRNA. Genomic DNA was extracted and purified using *REDExtract-N-Amp™ Plant PCR* kit (Sigma, Poole, UK) following manufacturer instructions. The extracted genomic DNA was used as template for V1–V4 region amplification, using the forward V1F (5'- AGAGTTTGATCCTGGCTCAG-3') and reverse V4R (5'- TACNVGGGTATCTAATCC-3') primers for 16S amplicon region (Cai et al., 2013). Amplifications occurred in a MyCycler™ Thermocycler (Bio-Rad) thermocycler, using 50 µL PCR reactions, which contained 7 µL of 10x buffer, 2.5 µL of 25 mM $MgCl_2$, 1 µL dNTPs of 10 mM, 1 µL of each primer (10 µM), 3 µL of DNA extract and 0.25 µL of DFS-*Taq* DNA Polymerase (5 U/µL) (BIORON GmbH). Cycling conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 50 sec, 45°C for 30 sec, 72°C for 90 sec, with a final extension of 72°C for 5 min. The amplified products were purified and sequenced at *Macrogen Inc.* (Seoul, South Korea). Taxonomic identification was performed by using the NCBI database (http://www.ncbi.nlm.nih.gov) and BLAST analysis sorted by higher identity score and lowest E-value. For sequence identities >98%, the genus and species were accepted; for sequence identities between 95% and 97%, only the genus was accepted; and for sequence identities <95%, isolates were labelled as 'unknown' bacteria (Yarza et al., 2014). The obtained sequences are available at GenBank. Pure cultures of each identified isolate were deposited and are preserved in the culture collection of the Mountain Research Centre (CIMO), Instituto Politécnico de Bragança (Portugal).

2.3.4. Diversity and community analysis

Epiphytic and endophytic bacterial diversity in each olive tree phyllosphere was assessed by evaluating the abundance (*i.e.*, relative number of isolates), richness (*i.e.*, number of operational taxonomic units - OTUs), and diversity by computing Simpson's Reciprocal Index (1/D) in *Species Diversity and Richness* v. 4.0 (Seaby and Henderson, 2006). Diversity values of the whole, epiphytic, and endophytic bacterial communities associated to cvs. *Cobrançosa* and *Verdeal Transmontana* are presented as the mean of replicates (*i.e.*, tree), displaying respective SE values. Means were compared by using an analysis of variance (ANOVA) with

SPSS v. 22 (SPSS, 2013), and the significant differences among means were determined by Tukey's test (p<0.05).

Non-metric multidimensional scaling (NMDS) was carried out to determine the similarity in bacterial community composition among host cultivars (*i.e*., *Cobrançosa* and *Verdeal Transmontana*) and plant organ (*i.e*., leaves and twigs). This analysis was performed for the whole, epiphytic and endophytic bacterial communities, by using two similarity indexes. Jaccard's similarity index compares samples based on presence/absence differences (Magurran, 2013), while Bray-Curtis coefficient takes into account not only the presence/absence of bacterial species but also their abundance (Clarke, 1993). NMDS calculates a stress value (Kruskal's stress), which assesses how well the derived ordination fits the given dissimilarities. According to Clarke (1993), Kruskal's stress values less than 0.2 represent plots with good ordination. Analysis of similarity (ANOSIM) was used to determine if differences in bacterial composition among samples are statistically significant. This analysis was performed from Bray-Curtis distance matrices (obtained from raw abundance data) with 999 permutations. ANOSIM generates an *R*-value ranging from 0 (completely similar) to 1 (completely dissimilar) and a *p*-value (significant level below 0.05) (Clarke and Gorley, 2015). Both NMDS and ANOSIM analyses were performed by using the *Community Analysis Package* v. 4.0 (Henderson and Seaby, 2007). The relative abundance of bacterial families that exhibited a significant (p<0.05) differential abundance across host cultivar and/or plant organ were represented in a heatmap using the *heatmap.2* function in the *gplots* package of *R* software (R Core Team, 2018).

Indicator Value (IndVal) analysis (Dufrêne and Legendre, 1997) was used to identify bacterial OTUs that are characteristic (habitat specialists) of each host cultivar and plant organ. This method identifies indicator species based on their specificity (*i.e*., uniqueness) to a particular habitat (A) and their frequency in that habitat (B). The *IndVal* values were computed by *R* software, using the function *multipatt* from *indicspecies* package (Cáceres, 2013). Only bacterial genera with significant (p<0.05) *IndVal* values > 0.3 were considered, as this latter value can be regarded as a good threshold for habitat specialization (Dufrêne and Legendre, 1997).

2.3.5. Factors driving bacterial communities in olive tree phyllosphere

A co-inertia analysis (CIA) coupled with Monte Carlo permutation tests was used to determine whether epiphytic and endophytic bacterial communities were similarly affected by host cultivar and plant organ. This analysis establishes a co-structure between sets of variables (host cultivar and plant organ) that are linked by the same bacterial genera (Dolédec, 1994). For performing this analysis, the bacterial abundance (at genus level) was used for the *dudi.pca* and *coinertia* functions in the *ade4* package (Dray and Dufour, 2007) of *R* software (R Core Team, 2018). Using the same package, the *table.value* function was used to visualize the results in a factorial map. To assess the significance of CIA results, Monte Carlo permutation tests were used for obtaining a *RV*-coefficient. This coefficient, which varies between 0 and 1, gives an indication of the correlation between two data tables: the closer the coefficient to 1, the stronger the correlation between tables (Josse et al., 2008). To estimate the proportion of bacterial community variation explained by host cultivar and plant organ, variation partitioning analysis was performed with *vegan* package using *varpart* function, in *R* software. The significance of each fraction was tested using the *anova* function, applied on the object resulting from a previous canonical correspondence analysis (CCA) using the *cca* function. These analyses were performed for the whole, epiphytic and endophytic bacterial communities.

2.4. Results

2.4.1. Composition and diversity of epiphytic and endophytic bacterial communities

A total of 421 bacterial isolates belonging to 89 bacterial operational taxonomic units (OTUs) were recovered from both leaves and twigs of olive trees from cvs. *Cobrançosa* and *Verdeal Transmontana* (Fig. S2.1). A larger consortium of epiphytic bacteria (65 OTUs, 30 genera, 17 families, 10 orders, 7 classes, and 4 phyla) was found when compared to endophytic bacteria (45 OTUs, 16 genera, 12 families, 9 orders, 5 classes, and 3 phyla) (Fig. S2.2). On average, the number of epiphytic OTUs per tree was 1.3-fold significantly higher (p<0.001) than the number of endophytes (Table S2.1). Only 21 OTUs were shared by both bacterial communities, representing 24.1% of the total number of identified OTUs (Fig. S2.2).

Across all samples, four distinct prokaryotic phyla were detected (*Proteobacteria, Actinobacteria, Firmicutes* and *Bacteroidetes*), although more than 83% of total OTUs belonged to *Proteobacteria* and *Actinobacteria* phyla (Fig. S2.1). Epiphytic bacterial community was mostly composed by members belonging to *Proteobacteria* phylum (60.8% of the total epiphytic bacteria strains), mainly from *Gammaproteobacteria* class (55.7%), in which the most abundant order was *Pseudomonadales* (38.2%) (Fig. S2.2a). The second most abundant phylum was *Actinobacteria* (22.7%) and only included *Actinomycetales* members, which was then followed by *Firmicutes* (14.3%). The most abundant endophytic bacteria also belonged to the *Proteobacteria* phylum (71.6% of the total identified endophytes), 74.5% of which were from *Gammaproteobacteria* class, mostly including members of *Pseudomonadales* and *Enterobacteriales* orders (64.6 and 34.7% of the corresponding class) (Fig. S2.2b). Other taxa were represented by less than 26%.

2.4.2. Bacterial diversity differs among host cultivars and plant organs

The bacterial abundance (relative number of isolates), richness (number OTUs/tree) and alpha diversity (Simpson's index) differed significantly among olive tree cultivars, but these differences were greater for endophytes when compared to epiphytes (Fig. 2.1; Table S2.1). For epiphytic community, the identified bacterial abundance and alpha diversity on cv. *Verdeal Transmontana* were 1.5-fold and 1.2-fold higher (p<0.01), respectively, when compared to cv. *Cobrançosa*. These differences were higher for endophytes, which also presented in cv. *Verdeal Transmontana* a higher abundance (up to 2.2-fold, p<0.01) and alpha diversity (up to 2.4-fold, p<0.001), also exhibiting a significant higher richness (up to 2.8-fold, p<0.001) when compared to cv. *Cobrançosa*.

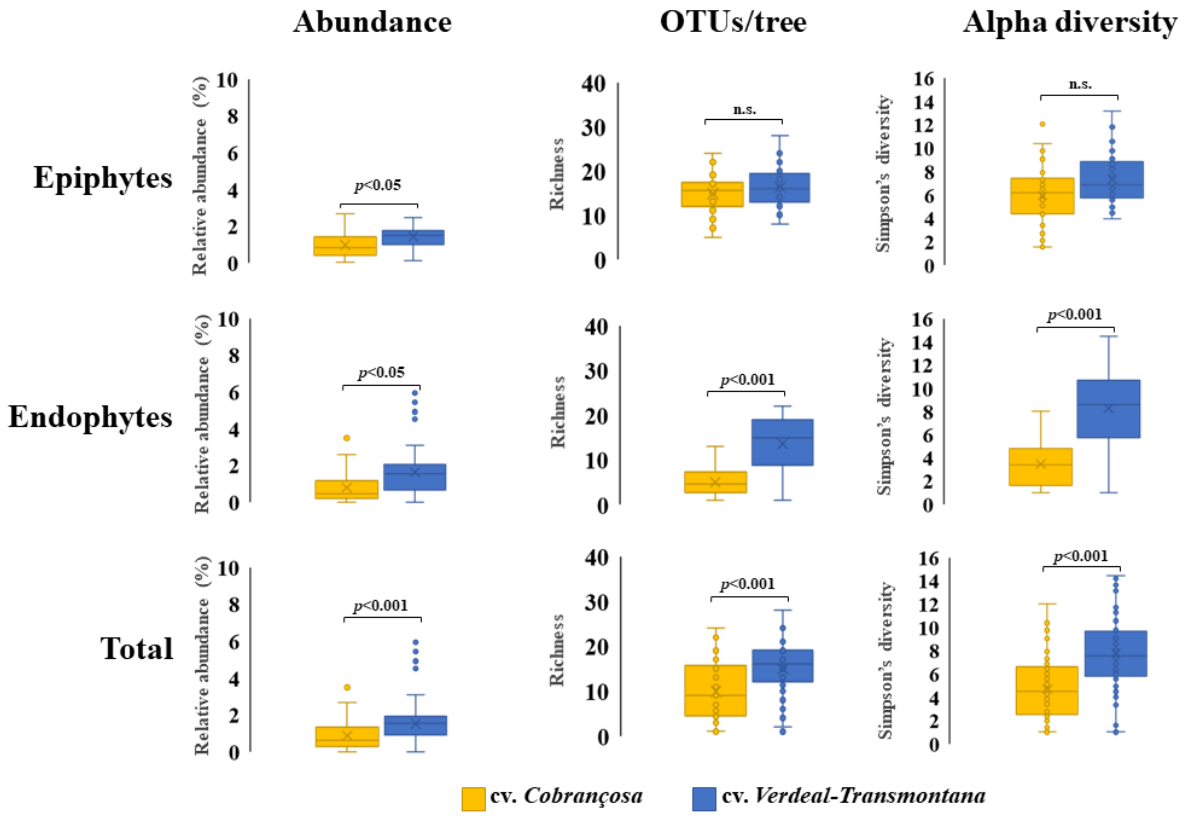

Figure 2.1. Comparison of epiphytic, endophytic and whole bacterial communities between *Cobrançosa* and *Verdeal Transmontana* cultivars, regarding their abundance (relative abundance *per* tree), richness (number of OTUs/tree) and alpha diversity (Simpson's index). Box plots depict medians (central horizontal lines), the inter-quartile ranges (boxes), 95% confidence intervals (whiskers), and outliers (dots). Significant differences between pairs of values are showed over horizontal lines. (n.s., not significant).

Leaves and twigs exhibited different bacterial abundances, displaying twigs a higher abundance (up to 1.7-fold, $p<0.001$) when compared to leaves (Fig. 2.2; Table S2.1). This increase was mostly due to an increase on the abundance of epiphytic community (up to 1.8-fold, $p<0.001$), since the endophytic bacterial community almost remained unchanged in both organs. In contrast, a significant reduction (1.2-fold, $p<0.001$) on the number of isolated epiphytic OTUs/tree was detected on twigs compared to leaves, revealing a higher representation of each OTU in twigs. In any case, no significant differences were detected for alpha diversity in both organs. The endophytic bacterial abundance, richness and diversity were not significant between leaves and twigs.

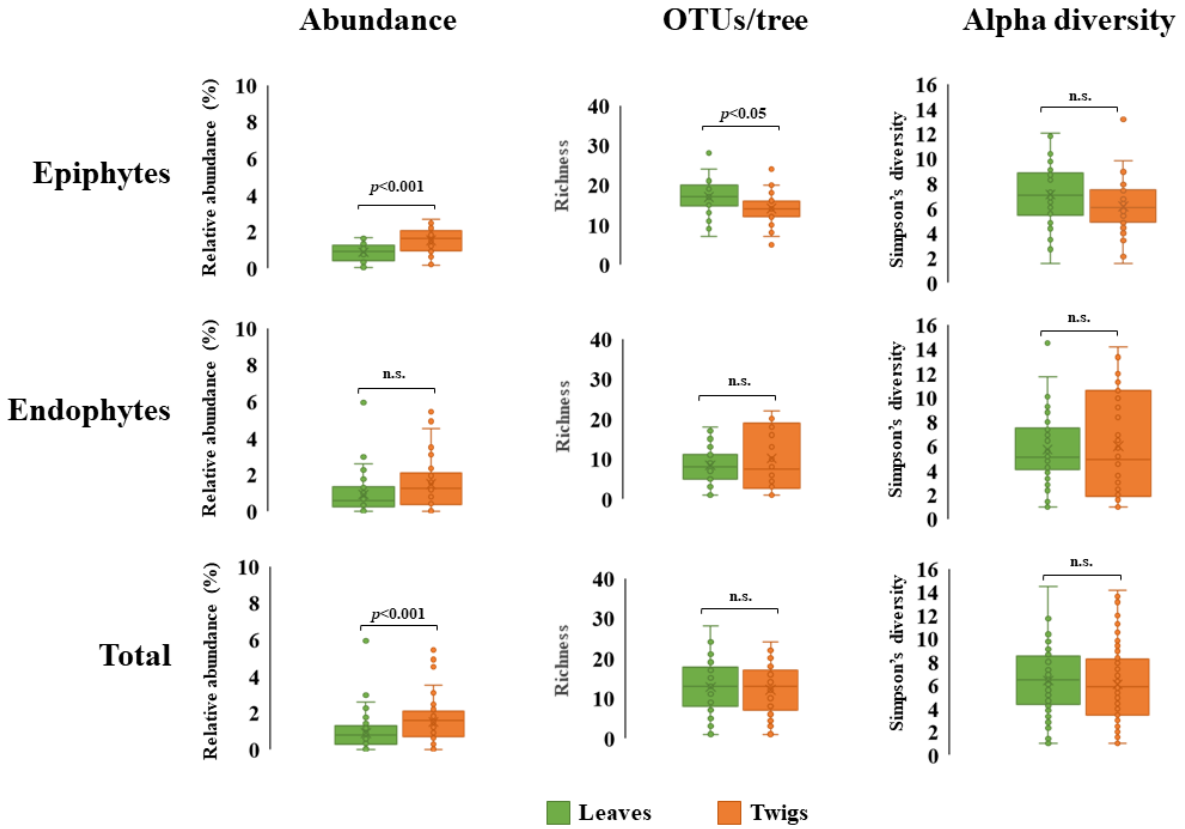

Figure 2.2. Comparison of epiphytic, endophytic and whole bacterial communities in leaves and twigs, regarding their abundance (relative abundance *per* tree), richness (number of OTUs/tree) and alpha diversity (Simpson's index). Box plots depict medians (central horizontal lines), the inter-quartile ranges (boxes), 95% confidence intervals (whiskers), and outliers (dots). Significant differences between pairs of values are showed over horizontal lines. (n.s., not significant).

The whole bacterial communities present on leaves and twigs of cvs. *Cobrançosa* and *Verdeal Transmontana* were significantly distinct, as indicated by non-metric multidimensional scaling (NMDS) plots (Fig. 2.3), taking into account different similarity measures of bacterial communities (Bray-Curtis coefficient and Jaccard's similarity indexes). A clearer separation of bacterial communities was noticeable when the ordination was based on the Jaccard's similarity index (Kruskal stress = 0.14), which only considers the presence/absence of bacterial OTUs disregarding their abundance (Magurran, 2013). This was also the case of epiphytic communities (Kruskal stress = 0.13), but not with the endophytic community that was better discriminated when using the Bray-Curtis coefficient (Kruskal stress = 0.14) that also considers the abundance of each bacterial OTU. This reveals that the abundance of endophytes is an important factor to take into consideration for endophytic

communities. Moreover, while bacterial epiphytes were clearly separated considering olive cultivar and organ, this separation was not so well observed on bacterial endophytes.

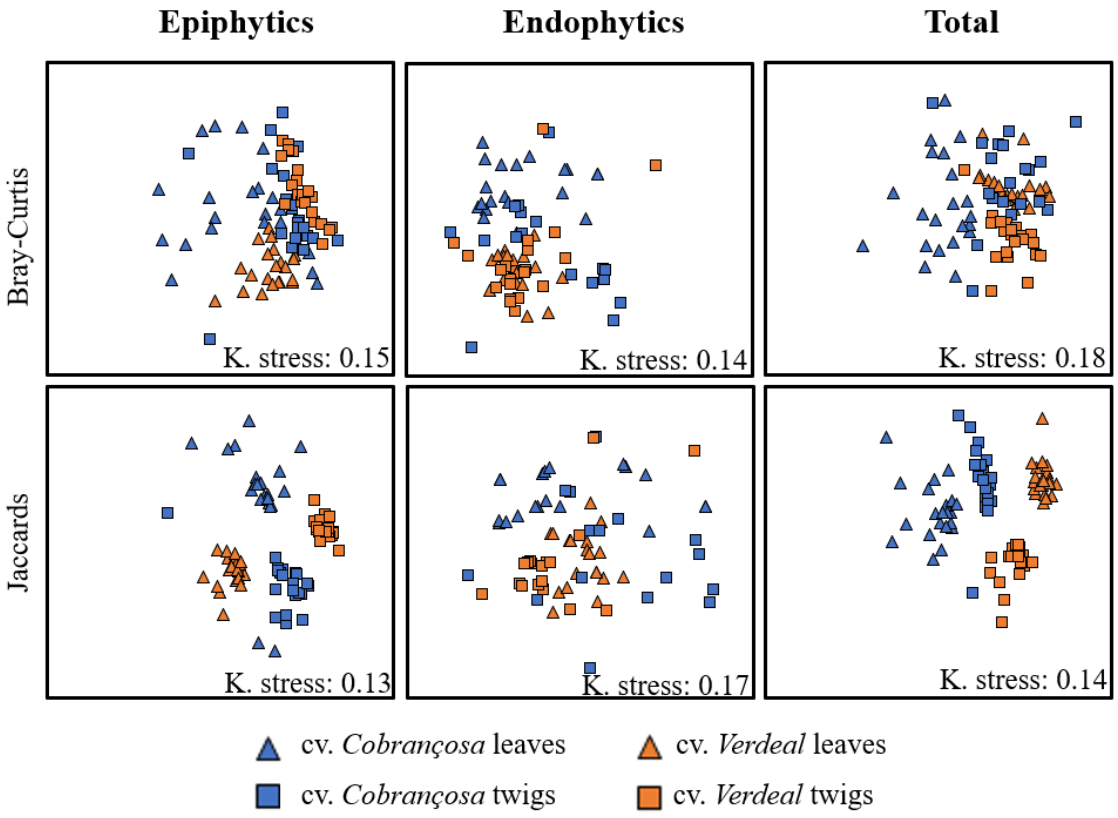

Figure 2.3. Non-metric multidimensional scale (NMDS) plots corresponding to the clustering of epiphytic, endophytic and whole bacterial communities. Cluster analysis was performed with two different community similarity measures, namely, Bray–Curtis coefficient (raw abundance data) and Jaccard's index (binary data). Bacterial communities from different olive tree cultivar (*Cobrançosa* or *Verdeal-Transmontana*) and plant organ (leaves or twigs) are represented by different colors/shapes.

The analysis of similarities (ANOSIM) using Bray-Curtis coefficients also revealed distinct bacterial communities from cv. *Cobrançosa* and cv. *Verdeal Transmontana* (R = 0.312, p = 0.001; Table S2.2). However, differences between cultivars were greater within endophytes (R = 0.390, p = 0.001) than within epiphytes (R = 0.207, p = 0.001), and greater in leaves (R = 0.591, p = 0.001) than in twigs (R = 0.469, p = 0.001). The endophytic community colonizing leaves displayed the greatest differentiation among both cultivars (R = 0.624, p = 0.001). These differences could be due to the enrichment of cv. *Cobrançosa* on bacteria belonging to *Caulobacteriaceae* and *Xanthomonadaceae* families, while cv. *Verdeal*

Transmontana was mostly inhabited by bacteria from *Staphylococcaceae, Alcaligenaceae* and *Paenibacillaceae* families (Fig. 2.4a).

The composition of bacterial communities on leaves was distinct from those on twigs (R = 0.252, $p = 0.001$; Table S2.2), but was more dissimilar for cv. *Verdeal Transmontana* ($R = 0.708$, $p = 0.001$) than for cv. *Cobrançosa* ($R = 0.357$, $p = 0.001$). While cv. *Verdeal Transmontana* leaves/twigs dissimilarities were greatest within epiphytes ($R = 0.787$, $p = 0.001$), in cv. *Cobrançosa* the dissimilarities were greatest within endophytes ($R = 0.386$, $p = 0.001$). Such differences could mainly be due to the enrichment of twigs on bacteria belonging to *Paenibacillaceae* and depletion on *Alcaligenaceae, Corynebacterineae* and *Staphylococcaceae*, when compared to leaves. Depending on its epiphytic or endophytic plant habitat, *Microbacteriaceae* and *Caulobacteriaceae* bacterial abundance also contributed to leaves/twigs dissimilarities (Fig. 2.4b).

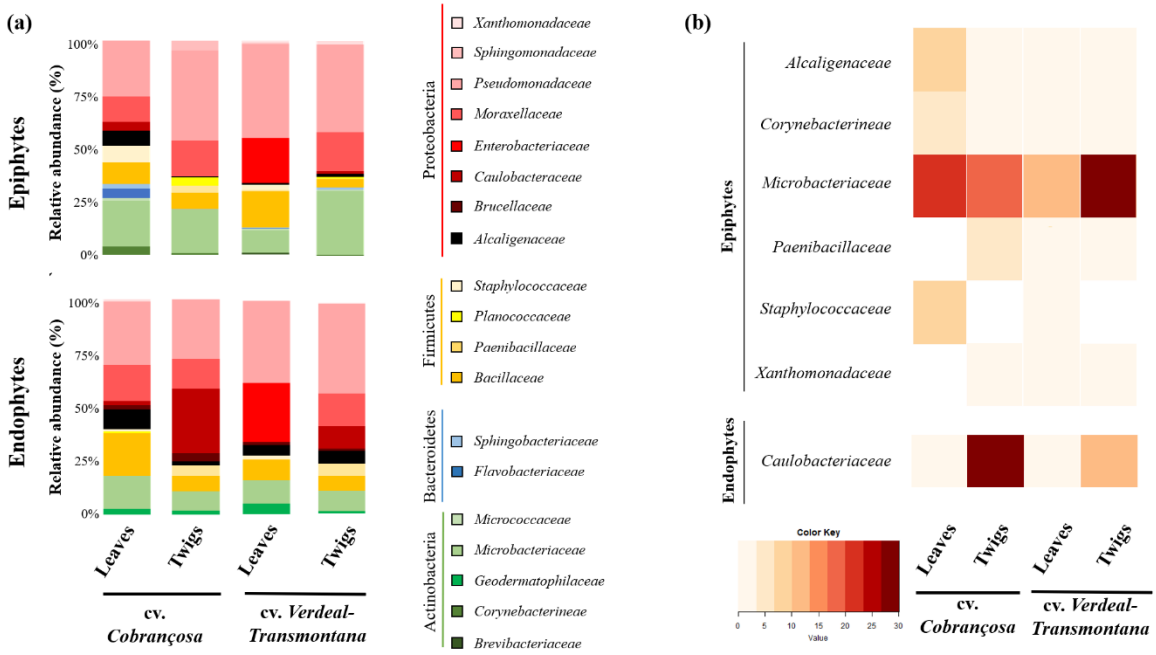

Figure 2.4. Relative abundance of bacterial families (and respective phyla) of epiphytes and endophytes present in leaves and twigs of olive tree cv. *Cobrançosa* and cv. *Verdeal-Transmontana*. **(a)** Relative abundance of bacterial families; **(b)** Relative abundance of bacterial families that exhibited significant ($p<0.05$) differential abundance across host cultivar and plant organ. In b, displayed differences were only detected on epiphytic or on endophytic environment, not on both.

2.4.3. Bacterial composition is primarily shaped by host cultivar and then by plant organ

For testing the relationships between bacterial communities and host cultivars or plant organs, in order to assess whether epiphytic and endophytic bacterial communities were similarly influenced by both variables, a co-inertia analysis was performed. The plant habitat (*i.e*., internal and external plant tissues) revealed to influence the structure of the whole bacterial community (RV=0.901; $p = 0.002$), explaining 5.0% of the variation in their composition (Table S2.3). The results also showed that epiphytic and endophytic bacterial communities were similarly affected by host cultivar ($RV = 0.847$, $p = 0.002$ and $RV = 0.966$, $p = 0.003$, respectively), but differently influenced by plant organ. Indeed, higher significant co-inertia coefficients were found for epiphytic ($RV = 0.931$, $p = 0.003$) when compared to endophytic ($RV = 0.739$, p = 0.003) bacterial communities regarding plant organs. The proportion of variation in bacterial communities that could be explained by host cultivar or plant organ factors, as evaluated by a variation partitioning analysis, corroborated these results. While host cultivar accounted for an almost similar variation on epiphytic (7.7%, $p = 0.005$) and endophytic (8.0%, $p = 0.005$) bacterial communities, the plant organ only explained 2.2% ($p = 0.005$) of the endophytic bacterial composition variation in contrast with 6.3% ($p = 0.005$) of epiphytic variation (Table S2.3). Co-inertia analysis also revealed that the bacterial genera that contributed most to bacterial communities distinction in different plant organs were *Staphylococcus* (within epiphytes) and *Ochrobactrum* (within endophytes), which were linked with leaves (Fig. 2.5). *Curtobacterium* (within epiphytes) and *Brevundimonas* (within endophytes) were positively correlated with twigs. Host plant cultivars were mostly differentiated by epiphytes belonging to *Frondihabitans* and *Xanthomonas* genera, which were related with cv. *Cobrançosa* and cv. *Verdeal Transmontana*, respectively.

2.4.4. Habitat specialists are present in phyllosphere-associated bacterial communities

An indicator species analysis was carried out in order to identify the characteristic bacterial OTUs from a specific habitat type (*i.e*., host cultivar and plant organ). In total, 42 bacterial OTUs (out of 89 OTUs, 47.2%) displayed significant ($IndVal > 0.3$, $p < 0.05$) habitat preference, being 23 epiphytes and 19 endophytes (Table S2.4). Most of these indicator species were present in leaves [cv. *Cobrançosa* (12) and cv. *Verdeal Transmontana* leaves (17)], contrasting with those present in twigs [cv. *Cobrançosa* (4) and cv. *Verdeal*

Transmontana leaves (9)]. The best indicator bacterial OTUs of cv. *Cobrançosa* (*IndVal*>0.7) were the epiphytes *Bacillus megaterium*, *Bacillus subtilis*, *Curtobacterium oceanosedimentum*, *Pantoea vagans*, and the endophytes *Pseudomonas aeruginosa*, *Pseudomonas graminis* and *Brevundimonas* sp..

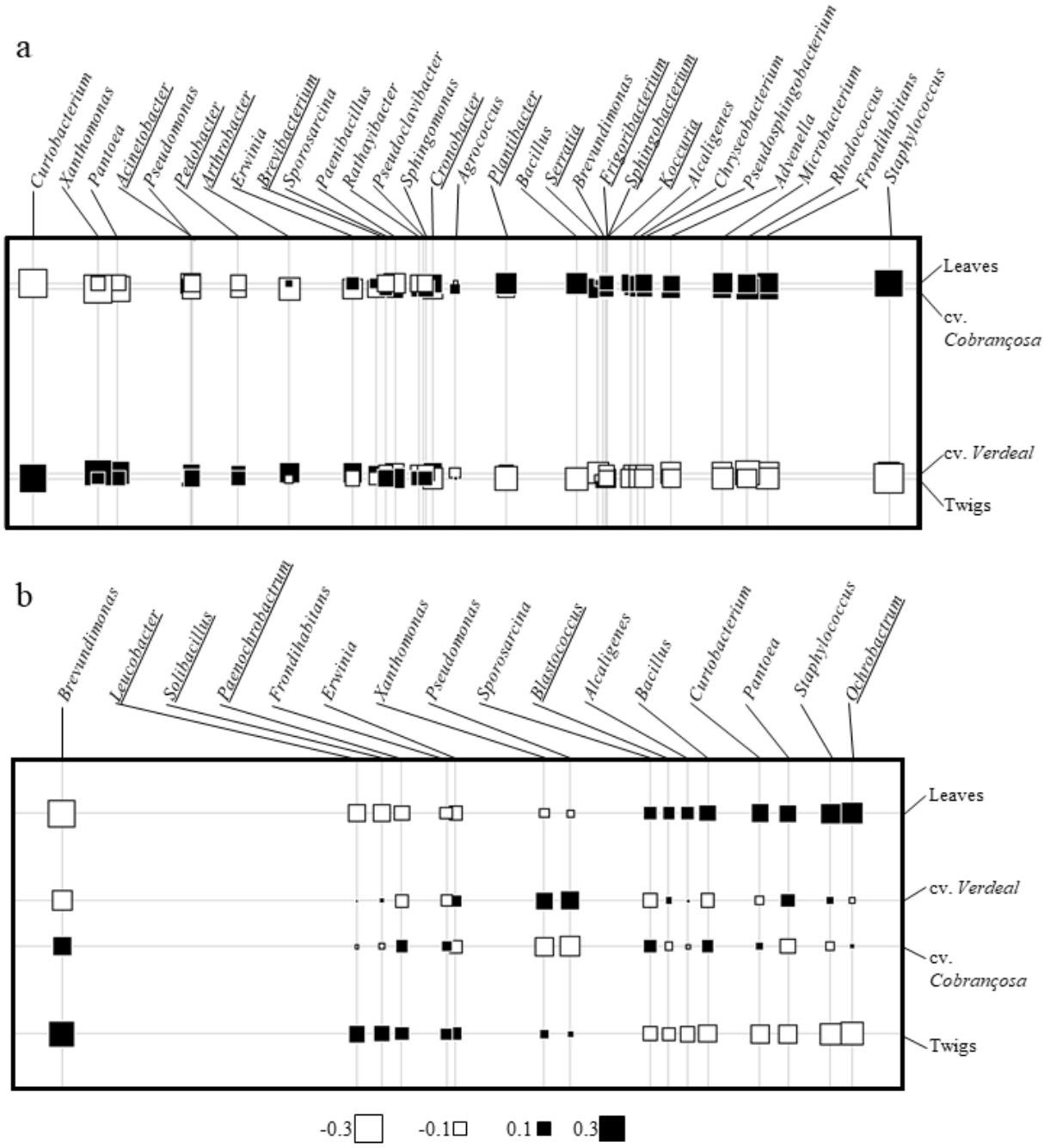

Figure 2.5. Co-inertia factorial map of (a) epiphytic and (b) endophytic olive tree bacterial communities, presenting positive (■) and negative (□) relationships with cultivars (Cobrançosa vs. Verdeal-Transmontana) and plant organs (leaves vs. twigs). The square size indicates the degree of relatedness between variables (host cultivar or plant organ) and bacterial community. Underlined genera are exclusive from each community.

Concerning the cv. *Verdeal Transmontana*, the best indicator species were the epiphytes *Pseudomonas poae*, *Bacillus cereus*, *Erwinia olea*, *Erwinia aphidicola*, *Curtobacterium herbarum*, *Pseudomonas lutea*, *Pseudomonas septica*, and the endophytes *Pantoea vagans*, *Pantoea brenneri* and eight *Pseudomonas* OTUs.

2.5. Discussion

Olive trees are highly adapted to low water availability and increased temperature conditions (Connor, 2005). Their survival ability could be partially related with a significant reservoir of beneficial microorganisms on their phyllosphere. Our results revealed that olive trees growing in the Mediterranean region, where drought conditions are usual and are even becoming more prevalent, are colonized on the phyllosphere by bacterial members belonging to four phyla. *Proteobacteria* (in particular *Gammaproteobacteria* class), followed by *Actinobacteria* and *Firmicutes*, were the most diverse and abundant phyla, while the presence of bacteria belonging to *Bacteriodetes* phylum was scarce. Müller et al. (2015) similarly found a high abundance of members belonging to *Proteobacteria, Actinobacteria* and *Firmicutes* phyla in the endosphere of olive leaves collected from different Mediterranean locations. When analyzing the epiphytic leaf community of chestnuts (Valverde et al., 2017) and other perennial species (Vokou et al., 2012) of the Mediterranean region, a predominance of *Proteobacteria*, *Actinobacteria* and *Firmicutes* was similarly observed. All these studies have focused on endophytes, ignoring the epiphytes, or vice-versa.

Members of detected phyla, in particular of *Actinobacteria* and *Firmicutes*, often prevail in arid environments (*e.g.*, Makhalanyane et al., 2015; Coleman-Derr et al., 2016; Sun et al., 2018) due to their ability to resist to UV-radiation and desiccation (Vikram et al., 2016). Their resistance has been mostly attributed to their ability to produce photoprotective pigments (Stankovic et al., 2012) and to repair UV-damages through multiple mechanisms (Makarova et al., 2001). In addition, their ability to produce spores allows their survival in harsh environmental conditions (Swick et al., 2013). Hence, these features displayed by bacteria inhabiting the olive tree phyllosphere are likely to increase their resilience and to help the host plant to cope abiotic stresses associated to Mediterranean climate. Indeed, the microorganisms are thought to have an influential role in governing key bioprocesses under extreme conditions (Makhalanyane et al., 2015). For example, the phyllospheric bacteria have

already been reported to protect the host plant from drought and high temperature (reviewed in Hussain et al., 2018), which are considered serious abiotic stresses of crop plants in the Mediterranean region.

In this work, the olive plant habitat (internal *vs*. external plant tissues) revealed to be determinant for the bacterial community structure, as described previously for other plant species, such as *Quercus ilex* (Peñuelas et al., 2012), and others non-perennial or Mediterranean species (Hunter et al., 2010; Bodenhausen et al., 2013). Differences on nutrients and/or environmental conditions between internal and external olive tree tissues could have influenced the selection of specific bacterial OTUs, giving rise to different bacterial communities within epiphytes and endophytes. In particular, a greater abundance for *Actinobacteria* and the exclusive presence of *Bacteroidetes* were observed within epiphytes as compared to endophytes colonizing the olive tree phyllosphere. This effect has been previously observed in the phyllosphere of several plant species (Agler et al., 2016; Wagner et al., 2016). The greater exposition to environmental conditions on the surface of olive leaves/twigs, as compared to internal plant tissues, could explain the dominance of bacterial members belonging to resistant phyla to desiccation and radiation within the epiphytic communities. As the Mediterranean regions are expected to be heavily impacted by climate change (Vessela et al., 2017), the elucidation of bacterial taxa function in internal and external tissues of olive tree phyllosphere would be important to delineate future lines of action.

The diversity and composition of the whole bacterial community inhabiting the olive tree phyllosphere was significantly different between host genotypes (at cultivar level), suggesting a degree of host control over bacterial communities. Since the surveyed olive cultivars are growing close to one another and with the same management practices, the differences found on bacterial diversity and composition among cultivars are most probably related to differences on chemical/physical properties of both surveyed cultivars. Indeed, leaves of cvs. *Cobrançosa* and *Verdeal Transmontana* have already revealed differences on several physical and chemical parameters (Meirinhos et al., 2005; Malheiro et al., 2015; 2016), and such features have long been considered to influence phyllospheric bacterial colonization (Lindow and Brandl, 2003; Yadav et al., 2005; Ruppel et al., 2008; Lemanceau et al., 2017). Thus, each olive tree cultivar apparently displays specific traits that govern phyllosphere-associated microbial assembly, as verified on olive fungal community by Gomes et al. (2019).

This is consistent with other studies performed on bacterial communities associated to the phyllosphere of coffee (Vega et al., 2005) and cotton (Adams and Kloepper, 2002). Additionally, our results suggest that host plant probably has more control over colonization of internal than of external tissues. Specific plant genotype traits, such as defence compounds production, have already been showed to act as habitat filters by influencing the establishment of microbial species within plant tissue (reviewed by Saunders et al., 2010). The slightly low influence of host cultivar on epiphytic community composition may be related to the higher susceptibility of epiphytes to environmental factors when compared to endophytes, as previously observed for fungal community inhabiting the olive tree phyllosphere (Gomes et al., 2018).

The plant organ (leaves *vs.* twigs) was found to significantly affect the composition of bacterial communities in the olive tree phyllosphere, as reported in previous studies for other plant species, like *Vitis vinifera* (Company et al., 2011), *Coffea arabica* (Vega et al., 2005), *Pinus flexilis* (Carper et al., 2018) or *Populus* (Cregger et al., 2018). This effect was greater within the epiphytic than within the endophytic bacterial communities, which could be related with the greater differences between leaves and twigs on their surfaces, when compared to the internal plant tissues. Indeed, the surface of both olive organs differ greatly on morphological traits and microenvironmental conditions (Férnandez, 2014), which were already known to influence the bacterial colonization of phyllosphere (Lemanceau et al., 2016). In comparison with twigs, olive leaves are exposed to more radiation and subjected to more desiccation, which are detrimental factors for bacterial colonization of leaf surfaces (Vorholt, 2012). The higher abundance of bacterial epiphytes observed in twigs than in leaves support our hypothesis. In contrast, the reduced effect of plant organ on endophytic assemblage is probably due to the similarities of endospheric environment among leaves and twigs.

The bacterial specificity in each olive tree organ was found to be dependent on host cultivar. In cv. *Verdeal Transmontana*, bacterial epiphytes exhibited a higher degree of organ specificity than endophytes, while in cv. *Cobrançosa* the opposite was observed. This differential colonization patterns may be related to the variations on the physical (biometric measurements, Malheiro et al., 2015) and chemical (flavonoid compounds, fatty acid profiles and plant volatiles; Meirinhos et al., 2005; Malheiro et al., 2015; 2016) features detected in both cultivars. Therefore, each host cultivar seems to have its own foliar/twig features, which

would select for specific epiphytic/endophytic bacterial communities. This hypothesis is corroborated by the high number of bacterial genera that were found to be positively associated with a specific cultivar/plant organ. Similarly, a high number of bacterial OTUs (at species level) that could be considered as specialists of one specific cultivar/plant organ (*i.e*., that prefer one specific host cultivar) was found. Regarding the host cultivar, bacterial OTUs characteristic of cv. *Verdeal Transmontana* was higher (26) than that of cv. *Cobrançosa* (16), suggesting a stronger effect of the former cultivar in selecting specific bacteria. The bacterial recruitment by plant has been mostly described for the rhizosphere (Patel et al., 2015; Schulz-Bohm et al., 2017; Tena, 2018), while studies reporting phyllosphere selection are still lacking. From our findings, the bacterial recruitment occurring in the phyllosphere seems to be mainly affected by the host genotype, both for epiphytes or endophytes selection.

The phyllosphere of both cultivars seem to recruit a greater number of beneficial rather than pathogenic bacterial OTUs. This finding is in accordance with previous studies that showed a higher recruitment of beneficial microbes by the plant to obtain the maximum mutualistic benefits, not only under standard but also under stressful conditions (Vorholt, 2012; Ortega et al., 2016). Indeed, most of the indicator bacteria of cv. *Verdeal Transmontana* comprise common plant beneficial members reported to have potential *i*) to increase host resistance to climatic stresses (*P. frederiksbergensis*; Subramanian et al., 2016), *ii*) to improve plant growth (*Curtobacterium herbarum*, Díez-Méndez et al., 2017; *P. lutea*, Peix et al., 2004), and *iii*) to control a broad range of plant pathogens [*Pantoea vagans* (Pusey, 2002, Walterson et al., 2015); *Bacillus cereus (Shafi et al*., 2016); *Pseudomonas orientalis* (Zengerer et al., 2018)]. Associated to this cultivar, several members of the fluorescent *Pseudomonas* genus (*P. poae*, *P. baetica*, *P. congelans*, *P. fluorescens*, *P. mandelii*) were also found, which have been reported to control several plant pathogens (Müller et al., 2016). *Cobrançosa* cultivar had also several associated isolates described as potential antagonists of phytopathogens, such as *Bacillus megaterium* (Mohamed et al., 2017), *Bacillus subtilis* (Torres et al., 2016), *Pantoea vagans* (Sharifazizi et al., 2017) and *Pseudomonas graminis* (Mikiciński et al., 2016). Other isolates associated to *Cobrançosa* cultivar have been described to protect plants on stressful environments (*Curtobacterium oceanosedimentum*, Khan et al., 2019). In contrast, microorganisms described as plant pathogens were detected on cv. *Cobrançosa*

(*Pseudomonas aeruginosa*, Bose et al., 2016). Future investigations should be conducted targeting on the ecological roles of these bacterial specialists in Mediterranean ecosystems.

In conclusion, in this work the bacterial communities of olive tree phyllosphere revealed to be primarily impacted by host cultivar and, to a lesser extent, by plant organ. However, while host cultivar affects in a similar way the composition of endophytic and epiphytic bacterial community, the plant organ has greater influence on epiphytic than on endophytic bacterial community structure. Each olive cultivar/plant organ apparently was selective towards specific bacterial OTUs. The ecological roles of these bacterial needs to be studied in the future, because they might be important in supporting olive tree survival in Mediterranean regions.

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2.7. Supporting Information

The following Supporting Information is available for this chapter:

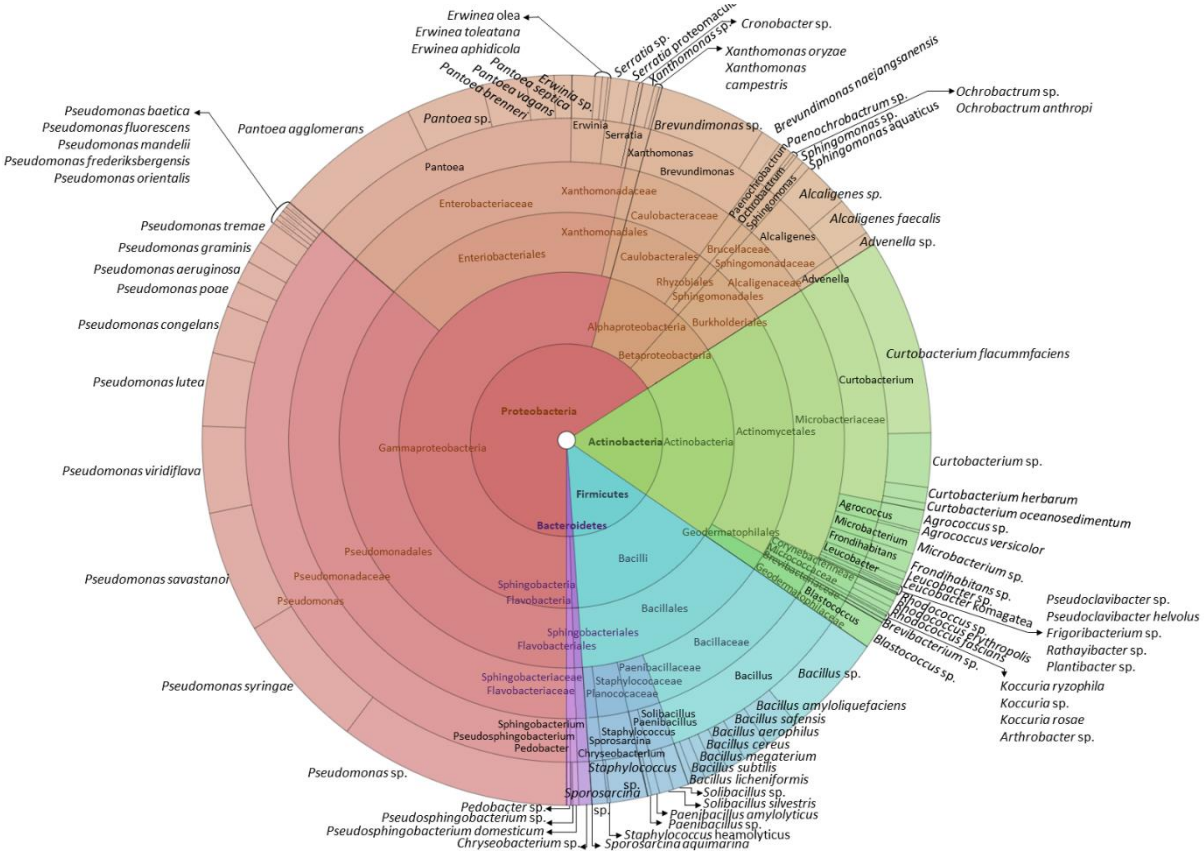


Figure S2.1. Krona chart showing the relative abundance of operational taxonomic units (OTUs) detected on olive tree phyllosphere.

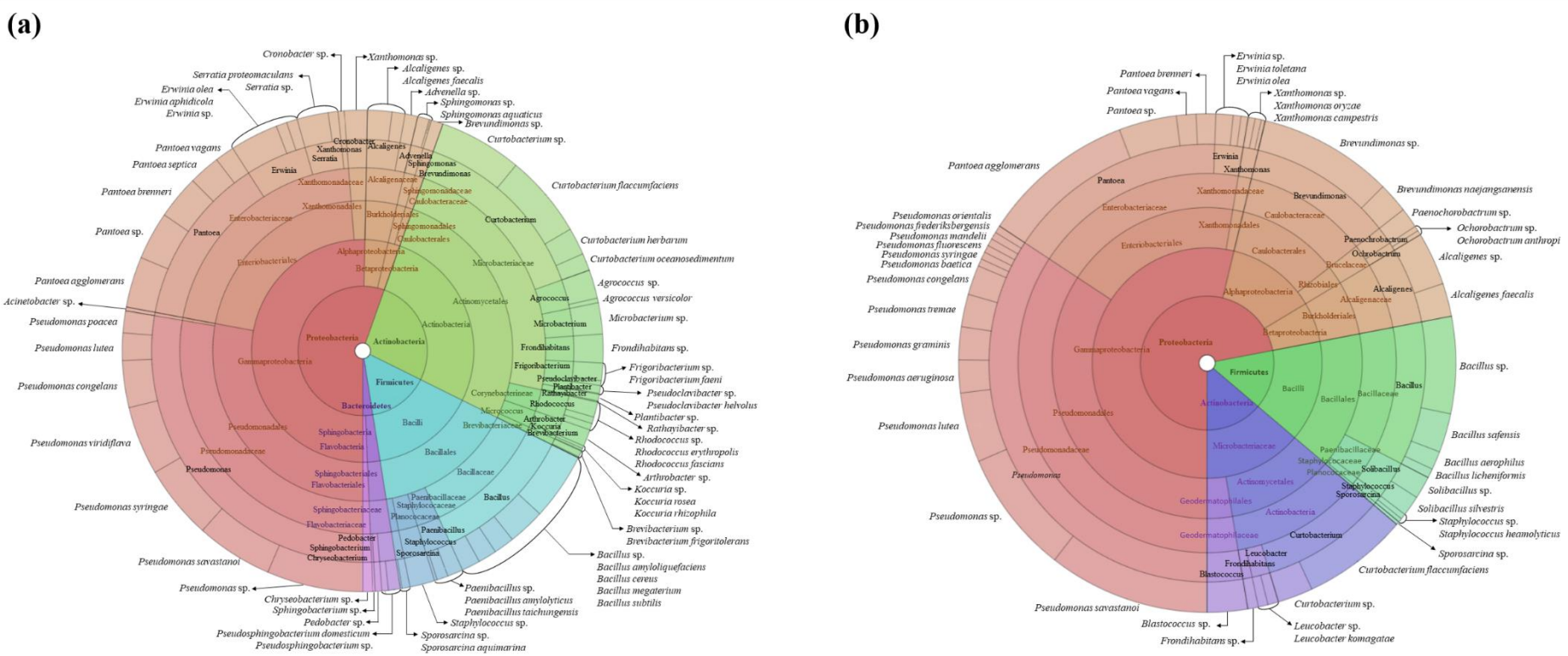


Figure S2.2. Krona chart representing the relative abundance of operational taxonomic units (OTUs) detected in the epiphytic (a) and endophytic (b) communities from olive tree phyllosphere.

Table S2.1 - Abundance and diversity of bacterial epiphytes and endophytes detected on each olive tree organ from both cultivars (*Cobrançosa* and *Verdeal Transmontana*). Values are presented as means ± SE (n=21 for each cultivar; n=42 for total). Simpson's indexes (1/D) of each bacterial community are presented in brackets. Different superscript letters denote statistically significant differences (p<0.05) among olive plant organs/cultivars.

		cv. *Cobrançosa*			cv. *Verdeal Transmontana*			Total		TOTAL
		Leaves	Twigs	Total	Leaves	Twigs	Total	Leaves	Twigs	
Epiphytes	Isolates/tree (log CFU/cm^2)	6.3±0.9^{a}	12.0±1.6^{b,c,d}	9.1±1.0^{a,b}	10.1±0.9^{a,b,c}	16.8±1.0^{d}	13.5±0.9^{c,d}	8.2±0.7^{a,b}	14.4±1.0^{c,d}	11.3±0.7
	OTUs/tree	16.2±0.9^{a,b}	13.5±0.8^{a}	14.8±0.6^{a,b}	17.9±0.8^{a,b}	14.7±0.8^{a,b}	16.3±0.6^{a,b}	17.0±0.6^{b}	14.1±0.6^{a}	15.6±0.4
	Total number of OTUs	44	42	58	46	35	53	62	49	65
	Simpson's index (1/D)	6.2±0.6^{a,b} (20.8)	5.7±0.5^{a} (13.4)	5.9±0.4^{a} (21.3)	8.0±0.4^{b} (20.6)	6.6±0.5^{a,b} (14.2)	7.3±0.3^{a,b} (20.3)	7.1±0.4^{a,b} (20.9)	6.2±0.3^{a} (13.4)	6.6±0.3 (21.3)
Endophytes	Isolates/tree (log CFU/cm^2)	8.9±2.0^{a}	15.0±3.4^{a,b,c}	12.0±2.0^{a}	19.4±4.6^{a,b,c}	30.5±5.0^{c}	25.0±3.4^{b,c}	14.2±2.6^{a,b,c}	22.8±3.2^{a,b,c}	18.5±2.1
	OTUs/tree	5.6±0.5^{a}	4.4±0.8^{a}	4.9±0.5^{a}	11.3±1.0^{b,c,d}	16.0±1.5^{d}	13.6±1.0^{c,d}	8.4±0.8^{a,b}	10.2±1.2^{b,c}	9.3±0.7
	Total number of OTUs	18	17	27	31	25	38	37	28	45
	Simpson's index (1/D)	4.1±0.4^{a,b} (11.7)	2.8±0.4^{a} (6.7)	3.5±0.3^{a} (10.3)	7.3±0.6^{c,d} (13.1)	9.2±0.9^{d} (17.8)	8.3±0.5^{d} (18.6)	5.7±0.4^{b,c} (15.0)	6.0±0.7^{b,c} (14.7)	5.8±0.4 (15.1)
Whole community	Total number of OTUs	50	48	68	63	48	74	80	64	89
	Simpson's index (1/D)	5.0±0.4 (21.9)	4.3±0.4 (12.8)	4.7±0.3 (19.8)	8.6±0.5 (16.7)	7.0±0.4 (16.8)	7.8±0.3 (21.4)	6.4±0.3 (18.9)	6.1±0.3 (14.3)	6.2±0.2 (22.5)

Table S2.2. Analysis of similarity (ANOSIM), based on Bray-Curtis distance, between bacterial communities (total, epiphytic and endophytic) inhabiting leaves and twigs of cvs. *Cobrançosa* and *Verdeal Transmontana*. *R*-statistics values are displayed (all are significant at $p<0.001$).

	cv. *Cobrançosa* vs. cv. *Verdeal*			**Leaves vs. twigs**		
	Leaves	Twigs	Total	cv. *Cobrançosa*	cv. *Verdeal*	Total
Epiphytic community	0.556	0.304	0.207	0.249	0.787	0.279
Endophytic community	0.624	0.447	0.390	0.386	0.545	0.264
Whole community	0.591	0.469	0.312	0.357	0.708	0.252

Table S2.3. Variation partitioning of host cultivar (cv. *Cobrançosa vs*. cv. *Verdeal Transmontana*) and plant organ (leaves vs. twigs) to achieve bacterial composition variance. ANOVA analyses to test significant differences (*p*-value) were obtained by Canonical Correlation Analysis (CCA). All *Varpart* values are significant at $p<0.01$.

Target	**Factor**	***Varpart* (%)**
Whole community	Plant habitat (internal/external plant tissues)	5.0
	Cultivar	4.9
	Plant organ	1.7
Epiphytic community	Cultivar	7.7
	Plant organ	6.3
Endophytic community	Cultivar	8.0
	Plant organ	2.2

Table S2.4. List of epiphytic and endophytic bacterial indicator genera in leaves and twigs of cvs. *Cobrançosa* and *Verdeal Transmontana*. The *IndVal* values were calculated according to A (a measure of specificity) and B (a measure of fidelity) factors. *IndVal* values displayed in bold and underlined are significant at $p<0.001$, displayed in bold are significant at $p<0.01$ and displayed in italics are significant at $p<0.05$.

Community	Olive cultivar	Plant compartment	Indicator species	A	B	*IndVal*
Epiphytes	*Cobrançosa*	Leaves	*Bacillus megaterium*	1.000	0.810	**0.900**
			Bacillus subtilis	1.000	0.810	**0.900**
			Curtobacterium oceanosedimentum	1.000	0.762	**0.873**
			Serratia proteamaculans	1.000	0.429	**0.655**
			Frondihabitans sp.	0.781	0.524	**0.640**
			Rhodococcus erythropolis	1.000	0.286	**0.535**
			Rhodococcus fascians	1.000	0.286	**0.535**
			Advenella sp.	0.807	0.286	*0.480*
			Chryseobacterium sp.	0.945	0.238	*0.474*
			Frigoribacterium faeni	1.000	0.191	**0.436**
		Twigs	*Pantoea vagans*	1.000	0.762	**0.873**
			Paenibacillus amylolyticus	0.900	0.333	**0.548**
	Verdeal Transmontana	Leaves	*Pseudomonas poae*	1.000	1.000	**1.000**
			Bacillus cereus	1.000	0.952	**0.976**
			Erwinia olea	1.000	0.524	**0.724**
			Erwinia aphidicola	1.000	0.524	**0.724**
			Cronobacter sp.	0.975	0.238	**0.482**
			Plantibacter sp.	0.703	0.238	*0.409*
			Paenibacillus taichungensis	1.000	0.143	*0.378*
		Twigs	*Curtobacterium herbarum*	1.000	1.000	**1.000**
			Pseudomonas lutea	1.000	1.000	**1.000**
			Pseudomonas septica	0.724	0.904	**0.809**
			Agrococcus versicolor	1.000	0.238	**0.488**
Endophytes	*Cobrançosa*	Leaves	*Pseudomonas aeruginosa*	1.000	0.619	**0.787**
			Pseudomonas graminis	1.000	0.619	**0.787**
		Twigs	*Brevundimonas sp.*	0.873	0.571	**0.706**
			Erwinia toletana	1.000	0.238	**0.488**
	Verdeal Transmontana	Leaves	*Pantoea vagans*	1.000	0.762	**0.873**
			Pantoea brenneri	1.000	0.762	**0.873**
			Pseudomonas baetica	1.000	0.762	**0.873**
			Pseudomonas congelans	1.000	0.762	**0.873**
			Pseudomonas syringae	1.000	0.762	**0.873**
			Pseudomonas fluorescens	1.000	0.762	**0.873**
			Pseudomonas mandelii	1.000	0.762	**0.873**
			Pseudomonas frederiksbergensis	1.000	0.762	**0.873**
			Ochrobactrum anthropi	1.000	0.191	**0.436**
		Twigs	*Pseudomonas tremae*	1.000	0.905	**0.951**
			Bacillus licheniformis	1.000	0.476	**0.690**
			Brevundimonas naejangsanensis	1.000	0.476	**0.690**
			Xanthomonas campestris	1.000	0.238	**0.488**
			Leucobacter komagatae	1.000	0.191	**0.436**

CHAPTER 3.

Impact of plant genotype and plant habitat in shaping bacterial pathobiome: a comparative study in olive tree

3.1. Abstract

Plant-inhabiting microorganisms interact directly with each other which effect is being recognized to influence the disease process. However, the role of the host plant and plant habitat in shaping pathobiome composition and their implications for host susceptibility/resistance to a particular disease, are currently unknown. For the elucidation of these questions was chose as a model system the olive knot (OK) disease, which is caused by *Pseudomonas savastanoi* pv. *savastanoi* (*Pss*). Thus, both epiphytic and endophytic bacterial community of asymptomatic and OK-symptomatic twigs of olive cultivars of varying susceptibilities to OK disease, were investigated by molecular identification of cultivable isolates. Our results indicate that OK disease is the main driver of the bacterial community causing changes on their diversity, abundance and composition. The microbiota most perturbed was found in the OK-susceptible cultivar and in the endophytic communities. Plant habitat (epiphytes *vs*. endophytes) also showed an important role in shaping microbial community assemblage, in particular in symptomatic twigs of the OK-susceptible cultivar. Host cultivar had little effect on the bacterial microbial community composition, being the bacterial community assemblage of OK-symptomatic twigs more affected. Overall, the pathobiome seems to result from an intricate interaction between *Pss*, the resident bacteria, and the host. Specific bacterial genera were associated to the presence and absence of OK disease in each cultivar, and their ability to trigger disease should be studied in the future.

3.2. Introduction

It is now well established that plants harbor a complex microbial community (microbiota) that provides numerous health benefits (Compant et al., 2019). From the various mechanisms employed by microbes to improve host plant health, microbe-microbe interactions seem to play fundamental roles (Kim et al., 2011). Indeed, there are some studies indicating that within plant microbiota, the pathogens established multiple interactions, either positive or negative, with other microorganisms that may influence or drive the disease process (*e.g.*, Vayssier-Taussat et al., 2014; Busby et al., 2016; Jakuschkin et al., 2016). Such consortium of microbes, which play a direct role in the causation of disease, has been recently termed as pathobiome (Vayssier-Taussat et al., 2014). Although in this concept the pathogenic agent is view integrated within its biotic environment (Vayssier-Taussat et al., 2014), the role

of the host plant in shaping pathobiome and their implications for host susceptibility/resistance to a particular disease, have not yet been studied. The structure of plant-associated microbiota is plant genotype dependent (Whipps et al., 2008 Bodenhausen et al., 2014; Wagner et al., 2016). We hypothesized that these distinct microbial compositions among plant genotypes and hence microbial interactions may lead to different pathobiomes. Another critical question is if pathobiome composition depends on plant habitat. Plant-associate microorganisms have the ability to colonize whether the surface (epiphytic) or the internal (endophytic) plant tissues (Turner et al., 2013). However, if the microbial interactions in the surface of plants tissues may lead a different pathobiome from the ones in the interior of the same plant tissues, is largely unknown. Microbiota comparisons (either epiphytic or endophytic) between healthy and disease plant tissues of cultivars with contrasting susceptibility to diseases, could be helpful to elucidate these questions (Wille et al., 2018). Such approach, besides by providing insight on the potential role of microbiota in plant resistance, could additionally contribute for the identification of microbial strains that can be used as "probiotic" to drive the plant microbiota to a pathogen-resistant composition. In the Humans for instance, faecal microbial transplantation to treat diseases has been recognized as a promising therapy (Weingarden et al., 2015).

Pseudomonas savastanoi pv. *savastanoi* (*Pss*) is the causal agent of the olive knot (OK) disease, which is one of the major threats to olive tree (*Olea europaea* L.) production in most olive growing regions of the world, in particular Mediterranean region (Quesada et al., 2010, 2012). *Pss* lives epiphytically on the surface of olive organs (Quesada et al., 2010), and under favorable weather conditions, *Pss* population increase and penetrate olive tissues, leading to the formation of tumorous overgrowths (Quesada et al., 2012). These knots, deeply colonized by *Pss* microcolonies, are the main symptoms of the disease, which appear mostly on olive tree twigs, branches and trunks (Ramos et al., 2012). Several non-pathogenic bacterial species from these knots have been reported to cooperate with the *Pss* for increasing disease severity (Hosni et al., 2011; Passos da Silva et al., 2014; Buonaurio et al., 2015). These knots, then, can provide a great model system to study the impact of plant host and plant habitat on the pathobiome structure. So far, no resistant olive tree genotype has been found, but, is common that olives of different cultivars exhibit different degree of susceptibility to OK disease (Iannotta et al., 2005; Penyalver et al., 2006; Godena et al., 2012). For instances, among the

most important Portuguese commercial olive cultivars, *Cobrançosa* is less susceptible to OK than the cv. *Verdeal Transmontana* (Gomes et al., 2019). These two cultivars present simultaneously asymptomatic twigs and knots in the same olive tree, also making this system suitable for studying the impact of host genotype on the "health microbiota" versus "disease microbiota" (*i.e*., pathobiome) structure.

Here, we investigated the epiphytic and endophytic bacterial community of asymptomatic twigs and knots of olive cultivars of varying susceptibilities to OK (cvs. *Cobrançosa* and *Verdeal Transmontana*), by PCR identification of cultivable isolates, to answer the following questions: (i) May host cultivar shape the associated pathobiome community? (ii) Is pathobiome composition variable according to the plant habitat (epiphyte *vs*. endophyte)? (iii) Is there any bacterial consortium associated to asymptomatic ("healthy-promoting microbiota") or to knots ("disease-promoting microbiota") and are these linked to cultivars susceptibility to OK disease? Such approach allows us to capture complex pathogen-microbe-plant interactions to predict links between microbial community and disease/healthy states, and to elucidate the possible contribution of such links for the different susceptibilities of cultivars to OK disease. The isolation of cultivable bacteria will further allow to study the contribution of phyllospheric bacteria to the development or inhibition of olive knot disease through bioassay experiments.

3.3. Material and Methods

3.3.1. Asymptomatic and diseased twigs sampling

Sample collection was performed during spring 2015 in two olive orchards located in Mirandela, northeast of Portugal, at coordinates N 41º 32.593'; W 07º 07.445' (orchard 1) and N 41º 32.756'; W 07º 07.590' (orchard 2). These orchards contain two olive cultivars of varying susceptibilities to OK disease (*i.e*., cv. *Verdeal Transmontana* is more susceptible than cv. *Cobrançosa*; Gomes et al., 2019), growing together, within 7 m of each other, under identical environmental conditions and management practices (integrated production guidelines; Malavolta and Perdikis, 2018). In each orchard, seven olive trees of each cultivar were randomly selected and both asymptomatic and OK-symptomatic twigs were collected, from the same branch, at mid-canopy height with sterilized shears and gloves. The collected

samples were placed individually into sterile roll bags, brought to the lab on ice, and then stored at 4°C until bacterial isolation, which was performed within one week.

3.3.2. Epiphytic and endophytic bacterial isolation

After removing the leaves from twigs, the epiphytic bacteria were isolate from pieces of five twigs and knots (each one with *ca*. 1-gram weight) cut from asymptomatic and symptomatic twigs, respectively. These plant tissues segments were individually immersed in 9 mL peptone water (10g/L peptone, 5g/L sodium chloride) and shaken for one hour at 100 rpm at room temperature. Aliquots of 1 ml of bacterial suspension was then incorporated in triplicate in 10 mL of Luria Bertani (LB) agar medium (10g/L peptone, 5g/L yeast extract, 5g/L sodium chloride, 10g/L agar) and incubated at 25 ^{o}C in the dark until bacterial growth. Daily observations were performed in order to isolate and count the bacterial colonies (CFU, Colony Forming Units). For isolation, single colonies were picked up, cultured in sterile LB plates and stored at 4 ^{o}C when full growth was observed. The abundance of epiphytes was expressed as log CFU/cm^2 representing the number of colonies *per* cm^2 of twig/knot surface. Surface of healthy twigs and knots were measured based on cylinder ($A=2\pi rh+2\pi r^2$) and sphere ($A=4\pi r^2$) area equations respectively, where *A* is the area, *r* is the radius and *h* is the height of tissues segments. The average twig and knot segments area were 11.0±3.6 and 2.9±1.3 cm^2, respectively, for cv. *Cobrançosa*, and 11.0±2.3 and 2.9±1.2 cm^2, respectively, for cv. *Verdeal Transmontana*.

Endophytes were isolated from the same twig/knot segments used to isolated epiphytes. For this, tissues segments were surface sterilized by immersion in 70% (v/v) ethanol for 1 min, followed by 3% (v/v) sodium hypochlorite for 1 min and then rinsed three times in sterile distillated water (1 min each). After drying, each twig/knot was cut in segments (ca. 4-5mm). Five sterilized segments per twig/knot were aseptically transferred to Petri dishes containing LB medium, in quintuple, and incubated at 25 ^{o}C in the dark until bacterial growth. A total of 7000 plant tissue segments were used to isolated bacterial endophytes. As for the epiphytes, dishes were daily monitored and single colonies emerging from tissues segments were counted and subculture to LB medium in order to obtain pure cultures.

3.3.3. DNA isolation and 16S rDNA sequencing

Taxonomic identification of the bacterial was performed by using both morphological and molecular approach. Groups of bacterial strains were firstly formed based on morphological similarity (such as color, size, shape, opacity, elevation, margin surface) and cultural features of their colonies. Then, from each group, two morphotypes were selected for molecular identification, raising a total of 294 bacterial isolates. Bacterial DNA was extracted using REDExtract-N-Amp™ Plant PCR kit (Sigma, Poole, UK) following manufacturer instruction and used for PCR amplification of V1 - V4 regions from 16S rRNA. For PCR reaction, 3µL of extracted DNA was used in a 50µL mixture containing 0.25µL of each dNTPs at 10mM, 7µL of 10x buffer, 2.5µL of 25mM $MgCl_2$, 0.25µL of DFS-Taq DNA Polymerase (5 units/ µL) and 1µl of each primer at 10 µM (V1F: 5'- AGAGTTTGATCCTGGCTCAG-3'; V4R: 5'- TACNVGGGTATCTAATCC-3') (Cai et al., 2013). Amplifications occurred in a MyCycler™ Thermocycler (Bio-Rad) using the following PCR program: 94°C for 5 min, followed by 35 cycles of 94°C for 50 sec, 45°C for 30 sec, 72°C for 90 sec, with a final extension of 72°C for 5 min. PCR product was sequenced by Macrogen Inc. (Seoul, South Korea) and taxonomic identification was performed by using the NCBI database (http://www.ncbi.nlm.nih.gov) and BLAST analysis sorted by higher identity score and lowest E-value to species (when identity presented a value >98%) or genus (for 95% to 97% identity) level. For sequence identities <95%, isolates were labelled as 'unknown' bacterial. Bacterial isolates identified were preserved in the culture collection of the Mountain Research Centre (CIMO), Instituto Politécnico de Bragança.

3.3.4. Data analysis

Asymptomatic (*i.e*., twig) and symptomatic (*i.e*., knots) twigs of each cv. *Cobrançosa* and *Verdeal Transmontana* were compared in terms of their epiphytic or endophytic bacterial community in order to assess if their variation is affected by plant host and plant habitat. In all the analysis performed, was excluded *Pss* in order to reflect the true bacterial community changes accurately (*i.e*., that is not due to an overabundance of the pathogen *Pss* in the symptomatic twigs).

3.3.4.1. Bacterial diversity

Diversity of bacterial epiphytes and endophytes in asymptomatic and OK-symptomatic twigs was assessed by evaluating the abundance (relative number of isolates *per* tree), richness (number of operational taxonomic units - OTUs *per* tree) and Shannon-Wiener Index (H), by using OTUs abundance matrix in R software (R Core Team, 2018). The percentage changes on these diversity parameters occurring on OK-symptomatic twigs in relation to asymptomatic twigs was calculated by using the formula: changes (%) = ((symptomatic twigs - asymptomatic twigs)/symptomatic twigs) x 100. These changes are presented as the mean of replicates (*i.e*., tree = 14, for each cultivar), displaying respective SE values, for the epiphytic and endophytic bacterial communities. Differences among means were determined by an analysis of variance (ANOVA) with R software, and the averages were compared using Tukey's test ($p<0.05$).

3.3.4.2. Bacterial community composition

Non-metric multidimensional scaling (NMDS) was performed using Bray-Curtis index with normalized OTU matrix, in order to calculate the average dissimilarity in the composition of bacterial community associated to each cultivar due to different twig status (asymptomatic *vs*. OK-symptomatic) and plant habitat (epiphytic *vs*. endophytic). Kruskal's stress was used to estimate model's goodness of fit, with a commonly acceptable value when lower than 0.2 (McCune and Grace, 2002). A one-way analysis of similarity (ANOSIM) was also performed, using Bray–Curtis distance matrices, to find significant differences between the bacterial community groups observed in NMDS ordination. This analysis generates a *p*-value (significant level below 0.05) associated to an *R*-value, which range from 0 (completely similar) to 1 (completely different) (Clarke and Gorley, 2015). Both NMDS and ANOSIM analysis were performed using *vegan* (Oksanen et al., 2018) package (*metaMDS* and *anosim* functions, respectively) in R software. The average percentage of bacterial abundance at different taxonomic levels (phylum, family and genus) was calculated across asymptomatic and OK-symptomatic twigs, in order to reveal which taxonomic group change more among the samples. Differences among means were determined by an analysis of variance (ANOVA) with R software, and the averages were compared using Tukey's test ($p<0.05$).

3.3.4.3. Factors driving bacterial communities

In order to assess the contribution of host cultivar (cv. *Cobrançosa vs*. *Verdeal Transmontana*), twig status (asymptomatic *vs*. OK-symptomatic) and plant habitat (epiphytic vs. endophytic) to the bacterial community variation, was performed a variation partitioning analysis using *varpart* function included in the *vegan* package, in R software, with normalized OTU matrix. The significance of each fraction was tested using the *anova.cca* function.

3.3.4.4. Identification of bacterial consortium associated to each host cultivar and twig status

A Multiple Factor Analysis (MFA) was used to simultaneously identify bacterial genera associated to a specific host cultivar and twig status. For this analysis, only the epiphytic and endophytic bacterial genera with the greatest power to separate asymptomatic from OK-symptomatic and cv. *Cobrançosa* from cv. *Verdeal Transmontana* twigs were used. These bacterial genera were identified by using a Random Forest analysis, which was computed with the R *RandomForest* package (Cutler et al., 2007). The importance of bacterial genera to distinguishing bacterial populations was measure by considering the decrease in mean Gini. A higher mean decrease in Gini will imply a higher importance (Breiman, 2001). MFA was computed with the R software, by using the *FactoMineR* (Le et al., 2008) package and performed for epiphytic and endophytic communities. Bacterial genera and variables were graphically represented on the two first dimensions. Then, Spearman correlations were performed through R *corrplot* (Wei and Simko, 2017) package to check the correlation of pre-selected epiphytic and endophytic bacterial genera with the relative abundance of *Pss.*

3.4. Results

3.4.1. Bacterial community associated to olive twigs

The isolation of bacterial from asymptomatic and OK-symptomatic twigs of 28 olive trees of two cultivars (*Cobrançosa* and *Verdeal Transmontana*) yielded a total of 312 isolates, belonging to 66 bacterial operational taxonomic units (OTUs), 31 genera and 17 families, mostly from the *Proteobacteria* and *Actinobacteria* phyla (76.3% and 18.2% of the total bacteria isolates, respectively) (Fig. S3.1.). Considering all the bacterial OTUs obtained from the olive tree, 68.2% and 56.1% were found on the surface and interior of twig tissues,

respectively. The epiphytic bacterial communities were predominantly dominated by members belonging to genera *Pseudomonas* and *Curtobacterium* accounting together 83.2% of total abundance of epiphytes, whereas *Pseudomonas* and *Pantoea* were dominant in the endophytic community (accounting together 71.3% of the total endophytic isolates) (Fig. S3.1.).

3.4.2. Comparison of bacterial community between asymptomatic and OK-symptomatic twigs

The abundance and diversity of bacterial varied between asymptomatic and OK-symptomatic twigs, but differences depend on the host cultivar and plant habitat (Fig. 3.1.; Fig. S3.2.). Indeed, epiphytic bacterial abundance reduced significantly ($p<0.001$) from asymptomatic to OK-symptomatic twigs in a similar amount in both cultivars (up to 85.6%), whereas within endophytic community was observed the opposite.

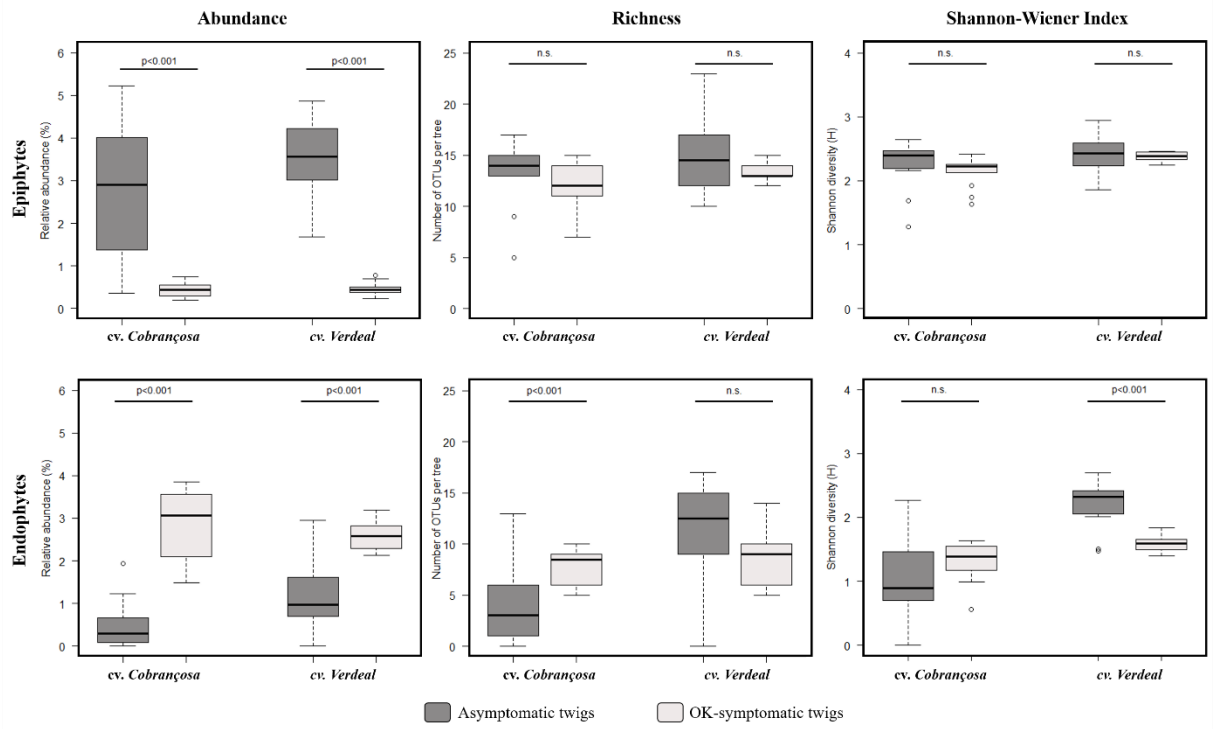
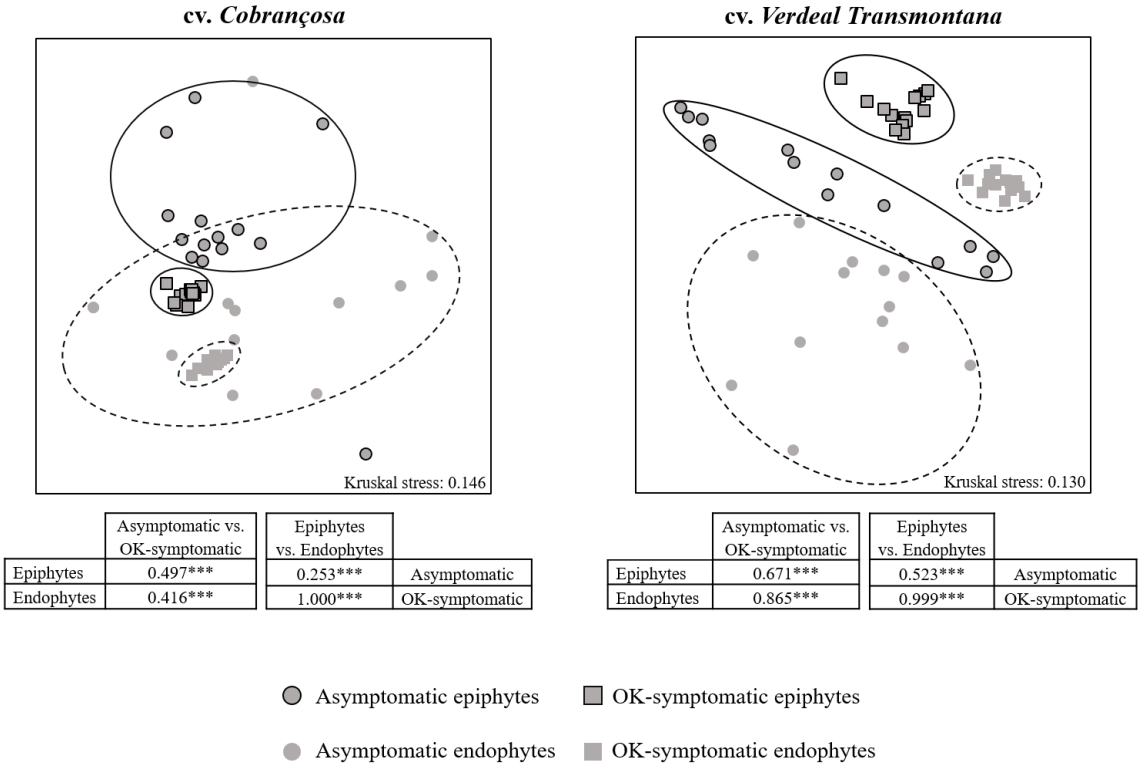


Figure 3.1. Comparison of bacterial diversity between asymptomatic and OK-symptomatic twigs, either within endophytic or epiphytic communities of each olive tree cultivar (*Cobrançosa* and *Verdeal Transmontana*). Diversity at community level was evaluated by determining abundance, richness and by using Shannon–Wiener index. Box plots depict medians (central horizontal lines), the inter-quartile ranges (boxes), 95% confidence intervals (whiskers), and outliers (black dots). Significant differences between pairs of values are represented over horizontal lines.

This increase on endophytic abundance was significantly (p<0.05) greater on cv. *Cobrançosa* (83.3%) compared to cv. *Verdeal Transmontana* (52.9%). The bacterial diversity determined by the species richness and the Shannon diversity index was significantly different between asymptomatic and OK-symptomatic twigs only within the endophytic community, but with different trends depending on cultivar. In the cv. *Cobrançosa*, the richness of endophytes increased significantly (p<0.001) from asymptomatic to OK-symptomatic twigs, whereas in the cv. *Verdeal Transmontana* was observed an opposite result for Shannon diversity.

The NMDS plots and ANOSIM analysis (Fig. 3.2.), based on Bray-Curtis index, showed that the whole bacterial community composition differs significantly (ANOSIM R=0.255, p<0.001) between asymptomatic and OK-symptomatic twigs. These differences were higher in the OK-susceptible cv. *Verdeal Transmontana*, either within epiphytic (R=0.671, p<0.001) or endophytic (R=0.865, p<0.001) communities, than in the OK-tolerant cv. *Cobrançosa* (R=0.497 and R= 0.416 with p<0.001, respectively).



	Asymptomatic vs. OK-symptomatic	Epiphytes vs. Endophytes	
Epiphytes	0.497***	0.253***	Asymptomatic
Endophytes	0.416***	1.000***	OK-symptomatic

	Asymptomatic vs. OK-symptomatic	Epiphytes vs. Endophytes	
Epiphytes	0.671***	0.523***	Asymptomatic
Endophytes	0.865***	0.999***	OK-symptomatic

Figure 3.2. Nonmetric multidimensional scaling (NMDS) plots and ANOSIM test for the bacterial assemblages in twigs of olive tree cvs. *Cobrançosa* and *Verdeal Transmontana* due to different twig status (asymptomatic vs. OK-symptomatic) and plant habitat (epiphytic vs. endophytic). Bray-Curtis coefficient was used as a measure of similarity between populations and Kruskal's stress values are presented (values less than 0.2 represent good ordination plots). ANOSIM test showed the R-statistics (R) and the statistical significance, which is denoted by asterisks (* p<0.05; ** p<0.01; *** p<0.001).

For both olive tree cultivars, the dissimilarity found on bacterial composition between epiphytic and endophytic communities was greater in OK-symptomatic twigs (*R*=1.000 and *R*=0.999 with *p*<0.001, for cv. *Cobrançosa* and *Verdeal Transmontana*, respectively) than in asymptomatic twigs (*R*=0.253 and *R*=0.523 with *p*<0.001, for cv. *Cobrançosa* and *Verdeal Transmontana*, respectively).

Taxonomic differences of epiphytic and endophytic bacterial between asymptomatic and OK-symptomatic twigs were identified by comparing their relative abundance at the phylum, family and genus level (Fig. 3.3.). Overall, both asymptomatic and OK-symptomatic twigs of the two olive tree cultivars were dominated by bacterial isolates belonging to *Pseudomonadaceae* family (*Proteobacteria* phylum), accounting 26.8% and 51.7%, respectively of the total isolates obtained in each sample type.

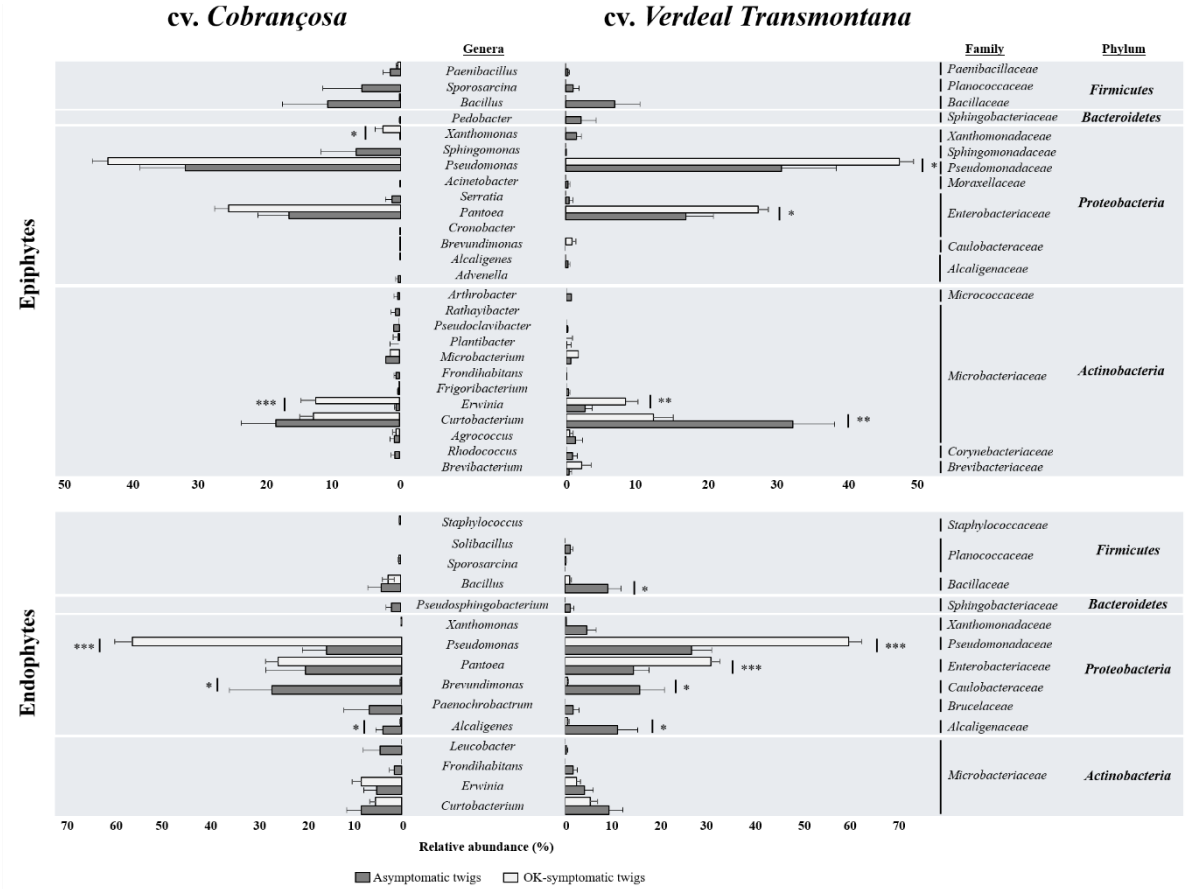


Figure 3.3. Comparison of the relative abundance of bacterial community at phylum, family and genus level between asymptomatic and OK-symptomatic twigs of olive tree cvs. *Cobrançosa* and *Verdeal Transmontana.* Each value is expressed as mean ± standard error (n = 14). Statistically differences between pairs of values are showed by asterisks (* *p*<0.05; ** *p*<0.01; *** *p* <0.001).

Microbacteriaceae and *Enterobacteriaceae* were the second most representative families of asymptomatic and OK-symptomatic twigs, representing together 24.4% and 27.3% of the total isolates in each sample type, respectively. Bacterial communities of each cultivar were differently affected by OK disease. In cv. *Cobrançosa*, a significant increase in *Xanthomonas* (up to 106.4-fold), *Erwinia* (25.5-fold) and *Pseudomonas* (3.6-fold), and a significant decrease on *Brevundimonas* (140.0-fold) and *Alcaligenes* (16.4-fold) were observed in OK-symptomatic twigs in relation to asymptomatic twigs.

In cv. *Verdeal Transmontana* was observed a significant increase in the abundance of *Erwinia* (up to 3.2-fold), *Pseudomonas* (up to 1.5-fold) and *Pantoea* (up to 1.6-fold), as well as a significant decrease on *Curtobacterium* (up to 2.6-fold). In this cultivar, *Bacillus* and *Alcaligenes* genera were only present on asymptomatic twigs, while *Brevundimonas* genus was only isolated from OK-symptomatic tissues. Moreover, the number of bacterial genera that disappeared with OK disease was greater in cv. *Verdeal Transmontana* (in total 19) when compared to cv. *Cobrançosa* (in total 15).

3.4.3. Variance partitioning of community composition variations

The relative importance of host cultivar, twig status and plant habitat on variations in bacterial community composition was estimated by variation partitioning analysis (Table S3.1.). Results showed that bacterial composition in twigs was mainly explained by twig status and plant habitat, being responsible for 7.3% and 7.1% of the total variation, respectively. The amount of variance explained by twig status was greater in cv. *Verdeal Transmontana* (20.5%) and in endophytic (11.7%) communities, while plant habitat affects mainly the bacterial composition in cv. *Verdeal Transmontana* and in symptomatic twigs, explaining 13.8% and 26.8% of species composition variance, respectively. Host cultivar, which explained 3.6% of the total community variation, had a higher influence on OK-symptomatic (24.4%) twigs and epiphytic (14.7%) communities.

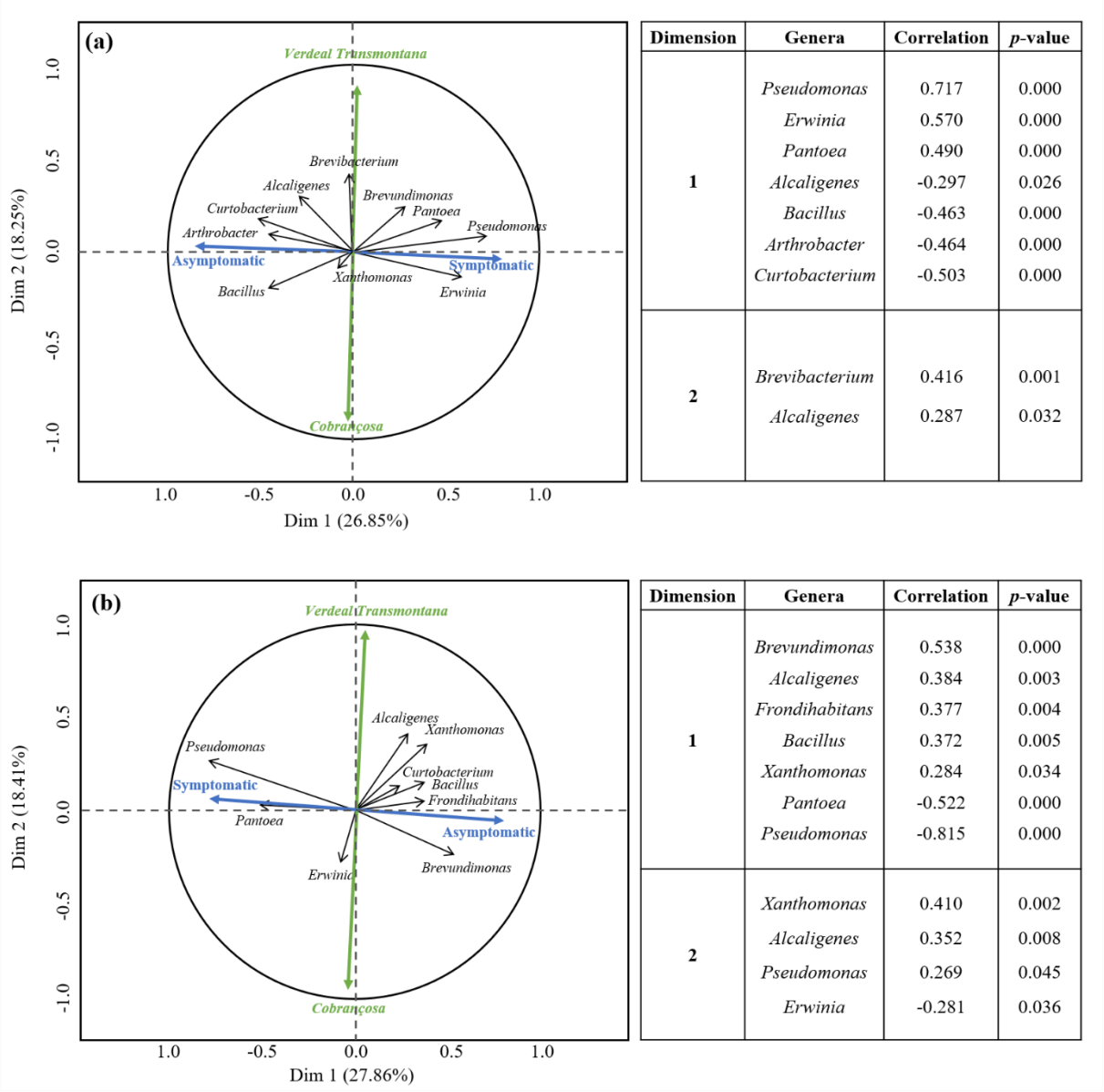
3.4.4. Association between bacterial communities, olive cultivar and twig status

One goal of this study was the identification of a set of bacterial genera associated to asymptomatic or to OK-symptomatic twigs and elucidate if these consortia could explain

differences in susceptibility of different olive tree cultivars to OK disease. To more accurately predict such relationships a random forest analysis was employed to rank bacterial genera importance to distinguish either asymptomatic from OK-symptomatic twigs (Fig. S3.3.) or cv. *Cobrançosa* from cv. *Verdeal Transmontana* (Fig. S3.4.). The most important bacterial genera were selected according to their Gini coefficient value, *i.e*. the higher its value the greater is its importance (Breiman, 2001; Cutler et al., 2007). Overall, ten and nine different bacterial genera were identified to be the most important variables for distinguishing asymptomatic from OK-symptomatic twigs (Fig. S3.3.) and cv. *Cobrançosa* from cv. *Verdeal Transmontana* (Fig. S3.4.), respectively. These bacterial genera were selected and used to perform a Multiple Factor Analysis (MFA), in order to find relationships between bacterial genera and presence/absence of OK symptoms and/or susceptibility/tolerance of cultivar to OK disease (Fig. 3.4.). In this analysis, the first dimension showed a clear dichotomy between asymptomatic and OK-symptomatic twigs either within epiphytic (Fig. 3.4a) or endophytic (Fig. 3.4b) bacterial communities. Positive values on the first dimension are linked to OK-symptomatic twigs in the case of epiphytic bacterial communities, while for endophytic communities the positive values are associated to asymptomatic twigs. From the bacterial genera characteristic of the first dimension, *Pseudomonas, Erwinia* and *Pantoea* in the epiphytic community, as well as *Pantoea* and *Pseudomonas* in the endophytic community, were positively correlated with the presence of OK disease.

This result is corroborated by the significantly positive correlation of these genera with *Pss* abundance (Table S3.2.). On the other hand, *Alcaligenes, Bacillus, Arthrobacter* and *Curtobacterium* in the epiphytic community, as well as *Brevundimonas*, *Alcaligenes*, *Frondihabitans*, *Bacillus* and *Xanthomonas* in the endophytic community, were positively correlated with asymptomatic twigs. Some of these bacterial genera were also found to be negatively correlated with *Pss* abundance (Table S3.2.).

Likewise, the second dimension of the MFA ordination of both epiphytic and endophytic bacterial communities clearly separated the two olive cultivars (Fig. 3.4.). From the bacterial genera characteristic of the second dimension, *Brevibacterium* and *Alcaligenes* in epiphytic community, as well as *Xanthomonas, Alcaligenes* and *Pseudomonas* in endophytic community, were positively correlated with cv. *Verdeal Transmontana*. *Erwinia* was the only one found to be specifically associated to cv. *Cobrançosa*.



Dimension	Genera	Correlation	*p*-value
1	*Pseudomonas*	0.717	0.000
	Erwinia	0.570	0.000
	Pantoea	0.490	0.000
	Alcaligenes	-0.297	0.026
	Bacillus	-0.463	0.000
	Arthrobacter	-0.464	0.000
	Curtobacterium	-0.503	0.000
2	*Brevibacterium*	0.416	0.001
	Alcaligenes	0.287	0.032

Dimension	Genera	Correlation	*p*-value
1	*Brevundimonas*	0.538	0.000
	Alcaligenes	0.384	0.003
	Frondihabitans	0.377	0.004
	Bacillus	0.372	0.005
	Xanthomonas	0.284	0.034
	Pantoea	-0.522	0.000
	Pseudomonas	-0.815	0.000
2	*Xanthomonas*	0.410	0.002
	Alcaligenes	0.352	0.008
	Pseudomonas	0.269	0.045
	Erwinia	-0.281	0.036

Figure 3.4. Circle plot of Multiple Factor Analysis (MFA) correlations of bacterial abundance among cultivars (*Cobrançosa* and *Verdeal Transmontana*) and twig status (asymptomatic and OK-symptomatic) applied to epiphytic (a) and endophytic (b) bacterial communities of olive tree. On the right part of the figure is showed the correlation table between each dimension and bacteria genera.

3.5. Discussion

In this work, both the epiphytic and endophytic bacterial community was compared between healthy and *Pss*-infected olive twigs of olive tree cultivars with contrasting susceptibility to OK disease. Overall, we intend to disclose the role that host plant-microbe-pathogen interaction may have in the development of OK disease and the underlying pathobiome.

3.5.1. May host cultivar shape the associated pathobiome community?

Changes on bacterial abundance, diversity and composition, in particular of endophytes, between asymptomatic and OK-symptomatic twigs, showed to differ according to olive tree cultivar. A similar result was obtained by Gomes et al. (2019) when analysing the effect of host cultivars on the fungal communities of asymptomatic and OK-symptomatic twigs of cvs. *Cobrançosa* and *Verdeal Transmontana*. The consistent results observed in these two studies greatly strengthen the evidence that host genotype (at cultivar level) could drive changes in their microbial community when the plant became infected by the pathogen *Pss*. This hypothesis is reinforced by the observed greater contribution of host cultivar in bacterial assemblage in OK-symptomatic twigs (24.4%) when compared to asymptomatic twigs (2.5%), as similarly observed for the fungal community in the same olive tree cultivars (Gomes et al., 2019). The role of host plant in structuring both rhizosphere and root endosphere bacterial communities in response to pathogen attack was already reported (Berendsen et al., 2012; Zamioudis and Pieterse, 2012), and we hypothesized that the same could occur in olive tree phyllosphere upon *Pss* infection. In these studies, plants subject to pathogen attack have been proposed to recruit protective bacteria to suppress pathogens in the rhizosphere (Berendsen et al., 2012; Sébastien et al., 2015). Such effect in our pathosystem should be further studied.

Differences on bacterial composition between asymptomatic and OK-symptomatic twigs were greater in the OK-susceptible cv. *Verdeal Transmontana* compared to OK-tolerant cultivar. Thus, the interaction of cultivar and *Pss* seems to influence the establishment of pathobiome communities in olive knots. Similarly, previous studies have suggested that differences on microbial abundance and diversity between asymptomatic and symptomatic tissues, were possibly related to the susceptibility of the plant host to a certain disease (Lacava et al., 2004; Suhaimi et al., 2017; Zhao et al., 2017; Hamonts et al., 2018). Here, the composition of the pathobiome resulted possibly from changes in plant metabolism induced by the *Pss*, which were probably greater in cv. *Verdeal Transmontana* when compared to cv. *Cobrançosa*. Indeed, plants can defend themselves against pathogens by a variety of mechanisms that enable the detection of pathogen invasion and the activation of a defense response (Andersen et al., 2018). This defensive response is highly complex. Overall, it involves cellular reprogramming characterized by altered plant metabolism that may result in the biosynthesis of compounds with defensive function, including counterattacking the pathogen

invasion (reviewed by Tugizimana et al., 2018). There are still gaps in understanding the dynamism and complexity of such metabolic alterations (Tugizimana et al., 2018), but recent studies indicate that this response is cultivar dependent (Tugizimana et al., 2019). Thus, we hypothesized that the greater differences on bacterial composition between asymptomatic and OK-symptomatic twigs in cv. *Verdeal Transmontana* when compared to cv. *Cobrançosa* may be due to differential metabolite changes among the two cultivars triggered upon *Pss* infection. However, further studies should be conducted to confirm this hypothesis.

The different result observed among cultivars may also be due to *Pss* itself. In mammalian host, bacterial pathogens have been showed the capacity to alter their environment/habitat to their favor, either producing a unique niche or creating a barrier to competing microbes (Wilson et al., 2019). In the rhizosphere, bacteria have similarly showed to alter the soil environment in an extent to favor certain microbial species over others (Scharf et al., 2016). In our study, the different changes observed among cultivars upon *Pss* infection, may also reflect changes made by the pathogen *Pss* to the microhabitat, probably as a result of their interaction with the resident bacteria (pathogen-bacteria interaction), which is different between cultivars. Indeed, both cultivars had a different initial bacterial community and the interaction that they establish with *Pss* may probably lead to higher community fluctuation in cv. *Verdeal Transmontana* than in cv. *Cobrançosa*. Although the results presented here are in accordance with the accepted idea that host microbiome is a key for plant capability to overcome a pathogen attack (Rodriguez et al., 2019; Vannier et al., 2019), this assumption still needs to be confirmed with further work.

3.5.2. Is pathobiome composition variable according to the plant habitat (epiphyte vs. endophyte)?

Changes in bacterial diversity and composition between asymptomatic and OK-symptomatic twigs were greater for endophytes than for epiphytes, thus suggesting a greater sensitivity of endophytes to *Pss*. While no comparative studies are available considering the effect of a plant disease in host bacterial epiphytic and endophytic community composition, Gomes et al. (2019) obtained an opposite result, with epiphytic fungal communities being highly affected than endophytic communities by bacterial disease. In the cv. *Cobrançosa*, *Pss* seems to increase the diversity of endophytic bacterial while in cv. *Verdeal Transmontana* was observed the opposite. Altogether, the results suggested that these changes of bacterial

diversity in the presence of *Pss* could result from the production of specific compounds by the interaction established between pathogen-host plant or pathogen-native bacterial community that could benefit or inhibit specific bacterial endophytes. In fact, the capacity of *Pseudomonas* to affect the growth and density of other bacterial has been shown in different organisms, including plants (Tashiro et al., 2013; Pandey et al., 2014; Zohara et al., 2016). For example, in a recent study on kiwifruit vines was observed that *Pseudomonas syringae* pv. *actinidae* infection affected the epiphytic bacterial community structure and diversity (Purahong et al., 2018). These changes on bacterial community composition have been reported to be a consequence of the cooperation and competition behaviors of *Pseudomonas* species with other microorganisms (Dandekar et al., 2013; Tashiro et al., 2013). Based on our results, such pathogen effect seems to have greater impact in endophytic bacterial community when compared to epiphytic community. Not only diversity, but also the abundance of bacterial endophytes was increased in the presence of *Pss*. Endophytically, *Pss* cells are organized in clusters together with biofilm layers (Temsah et al., 2008). The formation of this biofilm provides several beneficial properties to bacterial cells inhabiting it, such as social cooperation, resource capture and protection from antimicrobials (Flemming et al., 2016), which may explain the increase of bacterial abundance in *Pss*-infected twigs.

3.5.3. Is there any bacterial consortium associated to asymptomatic ("healthy-promoting microbiota") or to knots ("disease-promoting microbiota") and are these linked to cultivars susceptibility to OK disease?

In this study, a number of bacterial genera were found to be associated with asymptomatic or symptomatic twigs of each cultivar. Among the genera most associated to OK-symptomatic twigs, both *Pantoea* and *Erwinia*, were already been reported to occur in olive knots and suggested to be crucial for the development of OK disease (Passos da Silva et al., 2014). For example, both *Pantoea agglomerans* and *Erwinia toletana,* which have been found very often to be associated with the olive knot (Fernandes and Marcelo, 2002; Marchi et al., 2006; Quesada et al., 2007; Ouzari et al., 2008; Hosni et al., 2011; Passos da Silva et al., 2014; Buonaurio et al., 2015), when inoculated together with *Pss* revealed to increase the tumors size in olive trees (Marchi et al., 2006; Hosni, 2010). Strains of *P. agglomerans* were also described as potentials tumor inducers in other plant species, such as gypsophila

(Cooksey, 1986), beet (Burr et al., 1991), Douglas fir (DeYoung et al., 1998), wisteria (Opgenorth et al., 1994), and cranberry (Vasanthakumar and McManus, 2004). It is still unclear how exactly *P. agglomerans* and *E. toletana* modulates the OK-disease severity. A number of studies provided evidence for an N-acyl homoserine lactones-mediated cross-talk between *Pss*, *P. agglomerans* and *E. toletana* through quorum sensing system, which resulted in changes of *Pss* virulence (Hosni et al., 2011; Caballo-Ponce et al., 2018) and definitely playing an important role on the development of OK disease. Interestingly, apart from *Pantoea* and *Erwinia*, one other bacterial genus (*i.e*. *Pseudomonas*) was found to be associated with OK-symptomatic twigs, considering both epiphytic and endophytic bacterial communities. Complex of *Pseudomonas* sp. were previously reported to be associated to plant diseases in different crops, such as *Solanum lycopersicum* (Kůdela et al., 2010), *Prunus* (Ruinelli et al., 2019), *Citrus* (Beiki et al., 2016) and *Mango* (Gutiérrez-Barranquero et al., 2019). In our study, the frequent presence of *Pseudomonas* sp. with *Pss* in olive knots, suggests that this consortium is very stable and that both organisms probably benefits from the presence of each other. Further work needs to be done to establish whether this *Pseudomonas* microflora contributes to the OK disease caused by *Pss*.

The number of bacteria genera associated with asymptomatic twigs was higher when compared to OK-symptomatic twigs. Among the genera associated to asymptomatic twigs, *Bacillus* have been identified as the most promising in improving plant growth and controlling plant diseases (Radhakrishnan et al., 2017). In fact, there are many studies indicating the ability of *Bacillus* spp. to inhibit microbial pathogen growth either in soil or in plant tissues (Caulier et al., 2018; Radhakrishnan et al., 2017; Shafi et al., 2016). In olive, a few numbers of species belonging to this genus were demonstrated to have a high antagonistic potential not only against *Verticillium dahliae* (Muller et al., 2015; Cabanás et al., 2018) but also against *Pss* (Krid et al., 2010, 2012). In these two studies, *Bacillus* spp. isolated from olive leaves were tested against the pathogen with promising results in both *in vitro* and *in planta* assays. A strong association was also detected between *Alcaligenes*, *Brevundimonas, Curtobacterium* and *Arthrobacter* with asymptomatic tissues. Although *Alcaligenes* include clinically relevant strains (Sonnenberg et al., 2012; Tena et al., 2015), some studies have been reporting the bacteriostatic and fungistatic activity (biocontrol activity) of some members of this genus against an array of plant pathogens (Sayyed et al., 2009; Kavroulakis et al. 2010; Yokoyama et

al., 2013). Species belonging to *Brevundimonas* were previously observed to confer fitness benefits to host plants, being indicated as a potential soil bioremediator (Singh et al., 2016) and plant growth promotor (Kumar and Gera, 2014). However, members of this genus are frequently known as causing severe infections in humans (Cao et al., 2015; Swain and Rout, 2017; Ryana and Pembroke, 2018), compromising its use in various beneficial applications, including in the control of plant diseases. Members of *Curtobacterium* have been mainly described as plant pathogen (Junior et al., 2012) and have been found as an endophyte on some woody plant species, such as coffee (Vega et al., 2005), orange and tangerine (Araujo et al., 2002). However, there are also reports showing the biocontrol potential of some members of this genus. For example, in citrus plants, *C. flaccumfaciens* revealed to inhibit *Xyllela fastidiosa*, a xylem-inhabiting pathogen responsible for problematic disease symptoms in several woody hosts species (Carlucci et al., 2013, Amanifar et al., 2014; Bucci et al., 2018; Saponari et al., 2019). *Verticillium dahliae,* another pathogen affecting the roots of olive trees, was also inhibited by *C. flaccumfaciens* in *in vitro* assays (Berg et al., 2005). *Arthrobacter* includes a large number of species that are widespread in nature, in particular in soil, and with great importance in environmental and industrial applications (Zhang et al., 2015; He et al., 2017; Prum et al., 2018). Apart from nitrogen fixation (Fernández-González et al., 2017), members of the genus *Arthrobacter* were also showed antagonism towards several plant pathogens and the capacity to inhibit plant diseases (Velázquez-Becerra et al., 2013; Zhang et al, 2018). The role of these bacterial genera associated to asymptomatic twigs, on olive tree's defense against OK disease remains a topic for further study.

In summary, we showed that olive bacterial community was changed by OK disease. This effect was most notorious within endophytes than within epiphytes and was dependent on the host cultivar. Indeed, we observed a greater effect of the OK disease on bacterial community assemblage associated with cv. *Verdeal Transmontana* than with cv. *Cobrançosa*. Overall, the composition of bacterial community in olive knots seems to result from complex interactions between host plant-*Pss*-native bacteria. Our work also identified key bacterial genera that could play an important role in the susceptibility/tolerance of cultivars to OK disease. Understanding the mechanisms of interaction (cooperation vs. competition) and communication of these bacteria with *Pss* will shed light on the role of these bacteria on the process of OK disease development.

3.6. References

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3.7. Supporting Information

The following Supporting Information is available for this chapter:

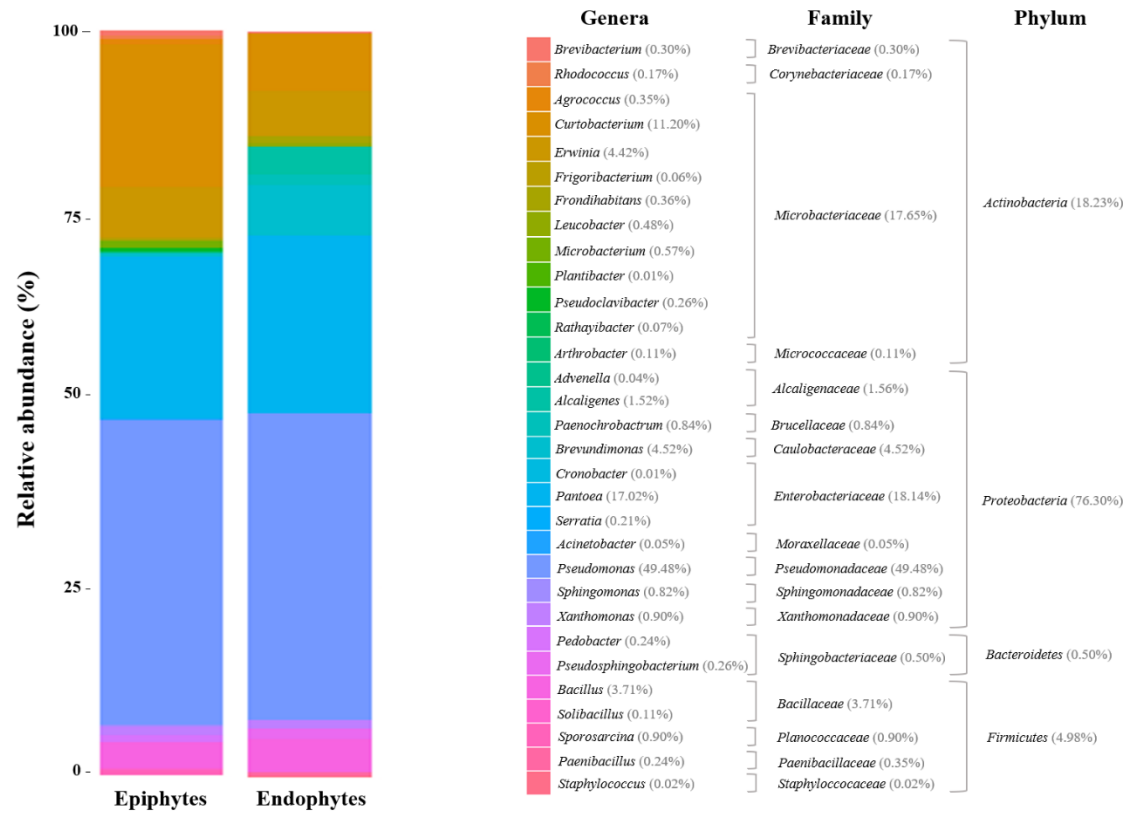

Figure S3.1. Relative abundance (%) of the global epiphytic and endophytic bacteria community isolated from twigs of olive tree, at phylum, family and genus levels.

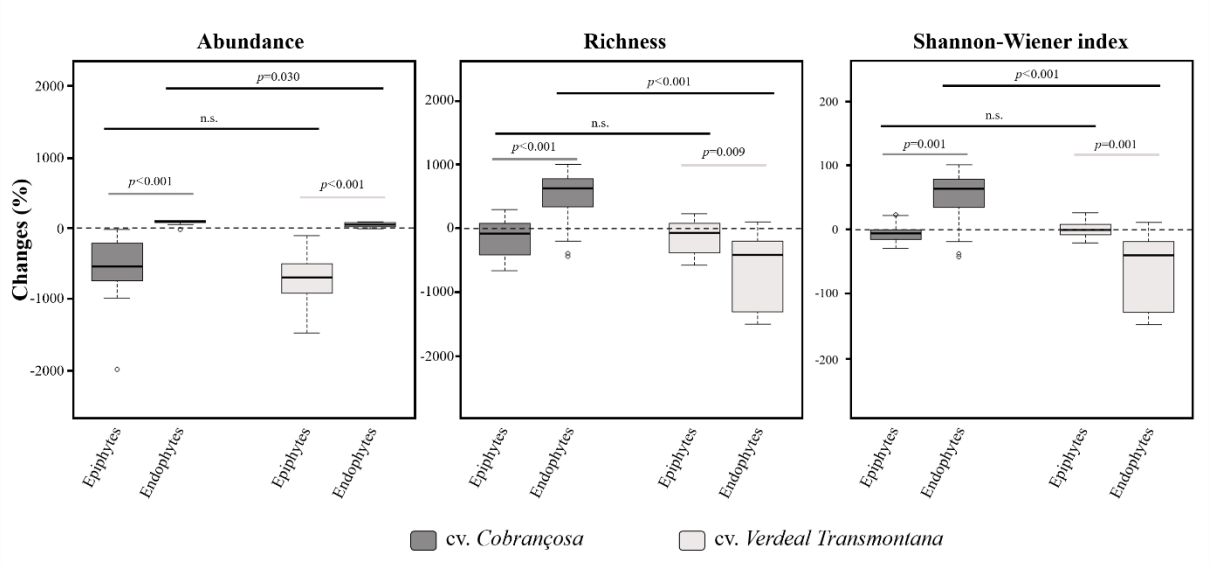

Figure S3.2. Changes (%) on epiphytic and endophytic bacterial abundance, richness and diversity, occurring on OK-symptomatic twigs in relation to asymptomatic twigs, of each olive tree cultivar (*Cobrançosa* and *Verdeal Transmontana*). Boxplots depict medians (central horizontal lines), the inter-quartile ranges (boxes), 95% confidence intervals (whiskers), and outliers (dots). Statistically differences between pairs of values are showed over horizontal lines. Abbreviation: n.s., not significant.

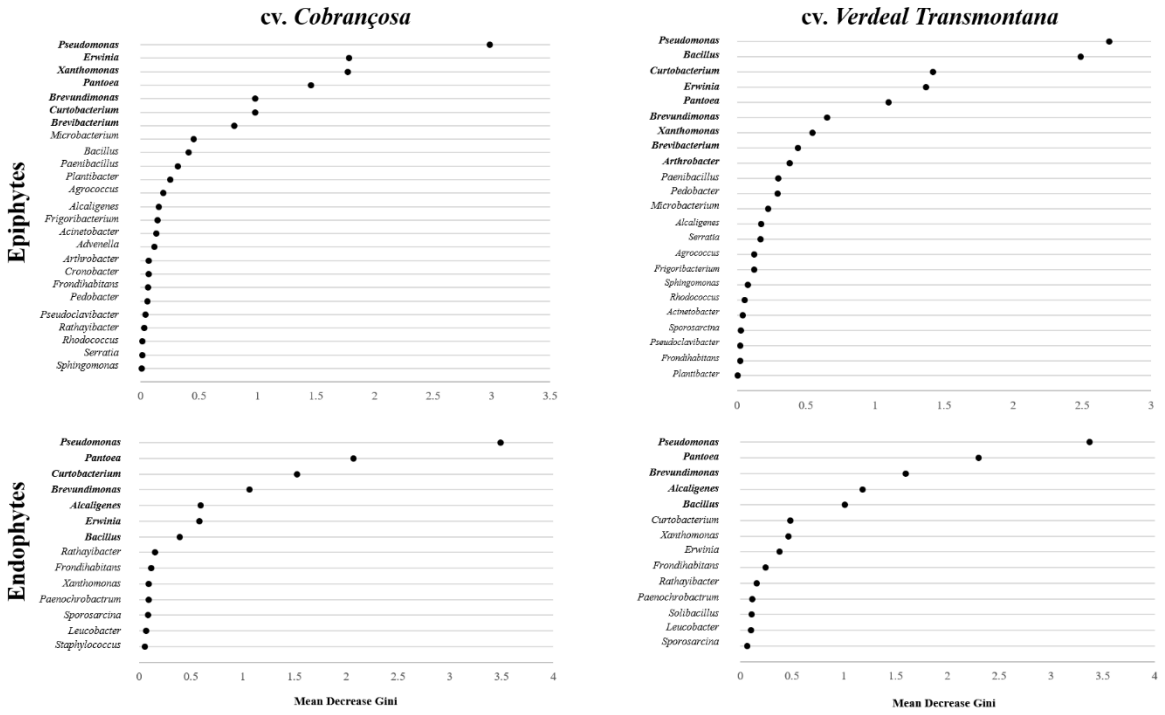

Figure S3.3. Ranking of relative importance of each bacterial genera to distinguish among asymptomatic and OK-symptomatic twigs of both cvs. *Cobrançosa* and *Verdeal Transmontana*, within epiphytic and endophytic bacterial communities. Mean Decrease Gini value measure the importance of bacterial genera, with highest values representing the best predictors. Genera in bold were considered as the main relevant to distinguish twig status.

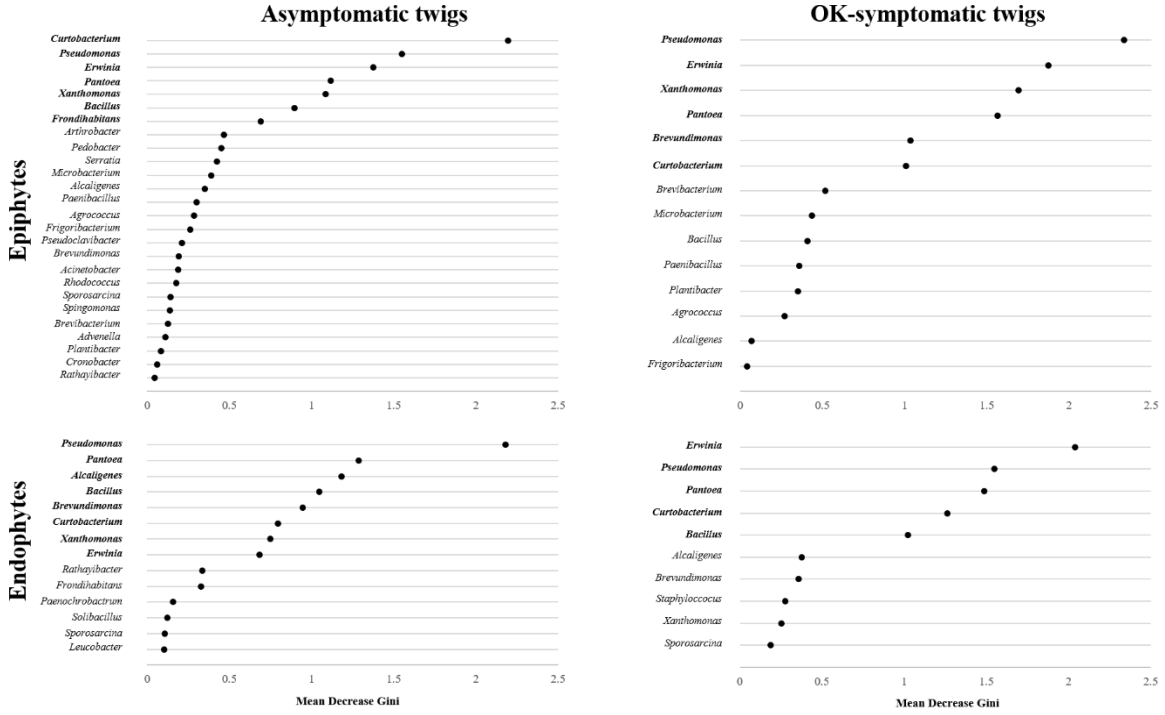

Figure S3.4. Ranking of relative importance of each bacterial genera to distinguish among cvs. *Cobrançosa* and *Verdeal Transmontana* on asymptomatic and OK-symptomatic twigs, within epiphytic and endophytic bacterial communities. Mean Decrease Gini value measure the importance of bacterial genera, with highest values representing the best predictors. Genera in bold were considered as the main relevant to distinguish host cultivars.

Table S3.1. Total variance (v*arpart*) explained by host cultivar (*Cobrançosa vs. Verdeal Transmontana*), twig status (asymptomatic *vs*. Olive knot-symptomatic) and plant habitat (epiphytic *vs*. endophytic) on the bacterial community composition. ANOVA analysis was performed to test the significant differences (*p*-value).

Effect	Target	Varpart (%)	*p*-value
Host cultivar (*Cobrançosa* vs. *Verdeal Transmontana*)	Total community	3.6%	0.005
	Symptomatic community	24.4%	0.005
	Asymptomatic community	2.5%	0.005
	Epiphytic community	14.7%	0.005
	Endophytic community	10.6%	0.005
Twig status (Asymptomatic *vs*. Olive knot symptomatic)	Total community	7.3%	0.005
	cv. *Cobrançosa* community	8.4%	0.005
	cv. *Verdeal* community	20.5%	0.005
	Epiphytic community	4.7%	0.005
	Endophytic community	11.7%	0.005
Plant habitat (Epiphytic *vs*. Endophytic)	Total community	7.1%	0.005
	cv. *Cobrançosa* community	11.4%	0.005
	cv. *Verdeal* community	13.8%	0.005
	Symptomatic community	26.8%	0.005
	Asymptomatic community	7.5%	0.005

Table S3.2. Spearman correlation analysis between bacterial genera abundances and *Pseudomonas savastanoi pv. savastanoi* abundance within epiphytic and endophytic communities of each olive tree cultivar (cv. *Cobrançosa* and cv. *Verdeal Transmontana*). Only significant correlation values are presented.

Plant habitat	Plant cultivar	Genera	Correlation coefficient	*p*-value
Epiphytic communities	cv. *Cobrançosa*	*Pseudomonas*	0.629	0.016
		Erwinia	0.708	0.005
	cv. *Verdeal Transmontana*	*Pantoea*	0.622	0.018
		Pseudomonas	0.570	0.033
Endophytic communities	cv. *Cobrançosa*	*Bacillus*	-0.663	0.009
		Curtobacterium	-0.529	0.049
	cv. *Verdeal Transmontana*	*Pseudomonas*	0.674	0.008
		Brevundimonas	-0.566	0.035

CHAPTER 4.

Screening the olive tree phyllosphere: seek and find for a potential antagonist against *Pseudomonas savastanoi* pv. *savastanoi*

4.1. Abstract

Olive knot (OK) is a widespread bacterial disease, caused by *Pseudomonas savastanoi* pv. *savastanoi* (*Pss*), which currently has not effective control methods. The use of naturally occurring microbial antagonists, such as bacteria, as biocontrol agents could be a strategy to manage this disease. The objective of this work was to identify those bacteria from olive tree phyllosphere able to antagonize *Pss* by *in vitro* and *in vivo* experiments. Elucidation of their modes of action and of potential relationship between antagonism and bacteria origin were investigated as well. To this end, sixty bacteria strains isolated from the surface and inner tissues of leaves, twigs and knots of two olive cultivars of varying susceptibilities to OK were screened for their antagonistic effect *in vitro* against *Pss.* A total of 27 bacterial strains were able to significantly inhibit *Pss* growth, being this effect linked to bacteria origin. Strains from OK-susceptible cultivar and colonizing the surface of plant tissues showed the strongest antagonistic potential. The antagonistic activity was potentially due to the production of volatile compounds, lytic enzymes and siderophores. *Bacillus amyloliquefaciens* P41 was the most effective antagonistic strain and their capacity to control OK disease was subsequently assayed in *in vivo* experiments. This strain showed to significantly reduce OK disease's severity (43.7%), knots weight (55.4%) and population size of *Pss* (26.8%), and simultaneously to increase shoot dry weight (55.0%) and root water content (39.6%) of *Pss*-infected olive plantlets. Bacterial isolates characterized in this study, in particular *B. amyloliquefaciens* P41, may be considered promising biocontrol candidates for controlling OK disease.

4.2. Introduction

Olive knot (OK) disease, caused by the bacterium *Pseudomonas savastanoi* pv. *savastanoi* (*Pss*), is a serious threat to olive production worldwide, especially in Mediterranean countries (Quesada et al., 2007; Moretti et al., 2008). In this disease occurs the formation of overgrowths (tumorous galls or knots) mainly on the branches and twigs of olive trees (Rodríguez-Moreno et al., 2008; Quesada et al., 2010; Ramos et al., 2012). These galls cause the decline and death of branches, leading to serious losses in terms of yield and olive oil quality (Tjamos et al., 1993; Quesada et al., 2012). Control

of olive knot disease is difficult, being mainly based on the removal of infected branches by pruning and foliar sprays with copper-based compounds (Quesada et al., 2012). With limited available options for OK disease control, the use of biocontrol agents, such as bacteria, represents a promising environmentally-friendly strategy for the management of the disease. Indeed, several bacterial, including *Pseudomonas* (Zadeh et al., 2008; Krid et al., 2010), *Bacillus* (Krid et al., 2010, 2012) and *Rhizobium* (Kacem et al., 2009), have already showed antagonistic activity against *Pss* under *in vitro* conditions. This antimicrobial activity was attributed to the production of bacteriocins by *Rhizobium* (Kacem et al., 2009) and *Pseudomonas* (Lavermicocca et al., 2002). However, other compounds produced by these three bacterial genera might also be involved in the inhibition of *Pss* as previously reported to occur for other phytopathogens (*e.g*., Kumar et al., 2012, Sasirekha et al., 2016; Zengerer et al., 2018). These include, for instances, siderophores (Sasirekha et al., 2016), lytic enzymes, antibiotics, hydrogen cyanide (Weller, 2007; Gerami et al., 2013; Zengerer et al., 2018), lipopeptides (Touré et al. 2004) and antimicrobial volatile compounds (Hernández-León, 2015). Nevertheless, in *in planta* assays, *Pseudomonas* did not suppress OK disease development (Maldonado-González et al., 2013) and *Bacillus* showed variable efficacy in reducing the weight of knots according to the strain (Krid et al., 2012; Ghanney et al., 2016). Thus, for a most successful identification of biocontrol agents is recommended to perform both *in vitro* and *in vivo* experiments (De Silva et al., 2019). Ideally, this screening process should include native microorganisms, which are already adapted to the crop, the resident microbiota and the environment in which it is to be used (Ozaktan et al. 2012). This is of particular importance for olive tree phyllosphere-associated bacterial community, as most of their members living either in the surface (as epiphytes) or in the interior of plant tissues (as endophytes) are unique to their host genotype and/or plant organ (unpublished results). Indeed, we have previously demonstrated that the phyllosphere of two olive genotypes with different degrees of susceptibility to OK disease (*i.e., cv*. *Cobrançosa* and cv. *Verdeal Transmontana*, being the former less susceptible to OK) have its own bacterial community (unpublished results). Bacterial community composition of olive tree leaves has also shown to differ from the ones inhabiting the twigs, of the same olive tree. As far as we known, no studies have examined if the

antagonistic effects of a specific bacterial strain against to pathogen are linked to their origin in terms of host susceptibility and/or plant organ.

In the present study, was evaluated the antagonistic activity of epiphytic and endophytic bacterial isolated from leaves, twigs and knots of two olive cultivars of varying susceptibilities to OK (*cvs*. *Cobrançosa* and *Verdeal Transmontana*), against *Pss* through *in vitro* assays. Their mode of action, by the production of lytic enzymes, siderophores and antibacterial volatile compounds, was investigated as well. *In planta* assays (olive pot experiments) were further performed to evaluate the ability of the most antagonistic isolate to control OK disease. This study aims to answer the following questions: i) Is antagonistic effect of the bacteria against *Pss* linked to their origin in terms of host (*i.e*, genotype susceptibility to OK), plant organ (*i.e*., leaf, twig, knot) and/or plant habitat (epiphyte *vs*. endophyte)? ii) Which mechanisms are involved in the antagonistic effect displayed by native bacterial against *Pss*? iii) What is the potential of native bacterial in controlling OK disease development and in reducing *Pss* population on olive phyllosphere? By combining physiological aspects of antagonistic bacterial agents with host plant characteristics (susceptibility, type of tissue, habitat) we expected to increase the success and the regularity of the effectiveness of biological control conferred by these bacterial strains against olive knot disease.

4.3. Material and methods

4.3.1. Bacterial isolates and inocula production

The epiphytic and endophytic bacterial isolates tested for their antagonistic effect against *Pss* were obtained from the microbial collection of the Mountain Research Centre (CIMO), Instituto Politécnico de Bragança (Portugal). These isolates were originally isolated between 2016 and 2017 from symptomless leaves and twigs, and knots of olive tree cvs. *Cobrançosa* and *Verdeal Transmontana*, collected in Mirandela (Northeast of Portugal), as stated in Mina et al. (submitted, 2019). From this collection, stored in 30% (v/v) glycerol at −80 °C, was selected for this study a total of 60 bacterial isolates, 5 of each population (*i.e*., plant cultivar, plant organ, and plant habitat) (Table S4.1.). *Pseudomonas savastanoi* pv. *savastanoi* strain EnVN39 was obtained from the

same bacterial collection. It was isolated from the inner tissues of active knots of naturally infected olive trees cv. *Verdeal Transmontana* (Mirandela, Portugal) and previously molecularly identified by sequencing a portion of the *ptz* gene by using primers *Pss1* (5'-TGGGTTGCTACTTGTACCGGA-3') and *Pss2* (5'-CCGTGTACTACGTTCAGCGAG-3') (Basim and Ersoy, 2001). The bacterial inoculum used in the assays was prepared from these frozen stocks by transferring bacterial cells onto Luria Bertani agar (LBA) medium (10g/L peptone, 5g/L yeast extract, 5g/L sodium chloride, 10g/L agar). The bacterial was grown at room temperature for two days, and bacterial cells produced were used to prepare inoculum for the subsequent studies. For this, bacterial cells were scraped from the agar plates with a sterile rod, suspended on 5 ml liquid LB medium and shaken on a rotary shaker (100 rpm) for 24 hours at room temperature. Bacterial cell densities were adjusted spectrophotometrically (optical density at 600 nm, OD_{600}=0.5) to a concentration of 10^8 CFU/ml using LB liquid media, before used as an inoculum in *in vitro* and *in planta* assays.

4.3.2. *Antagonistic activity in vitro*

Assessment of the antagonistic activity of the 60 bacterial isolates against *Pss* was performed through the establishment of dual cultures. Two sterile filter paper discs (5 mm diameter) were placed separated 3 cm apart on the surface of Petri dishes (9 cm diameter) containing 10 ml of LBA medium. Subsequently, each disc was impregnated with 5µl of *Pss* or the antagonist and left to dry in laminar flow cabinet. Control plates were performed with single inoculated disc (for each antagonist and *Pss*). Plates were incubated at 25 ± 2 °C in the dark, and for each bacterial isolate-*Pss* combination, five replicates were done and the whole experiment was repeated twice. Daily measurements of the internal radius (*i.e.*, the radial growth towards the interacting bacterial colony) were performed (in millimeters) with a transparent ruler for each testing bacteria. These measurements were performed until no growth was observed for at least of one of the interacting species. The obtained data was used to calculate the percentage of growth rate reduction for both pathogen and antagonist, comparatively to the control plates, by using the following equation: $[(G_C-G_{DC})/G_C] *100$,

where G_C is the growth rate of the *Pss*/antagonist colony in control plates and G_{DC} is the growth rate of the *Pss*/antagonist colony in the dual-culture assay.

4.3.3. Mechanisms associated with the antagonistic activity

Bacterial strains having antagonistic activities (*i.e*., that inhibited more than 50% of *Pss* growth and were not significantly inhibited by *Pss*; Table S4.2.) were further studied for the production of different compounds related to phytopathogen biocontrol, including antibacterial volatile compounds, lytic enzymes (lipase and protease) and siderophores.

4.3.3.1. Volatile assay

The volatile assay was designed and performed in order to evaluate the potential effect of the volatiles produce by the antagonistic on *Pss* growth. This assay was performed in Petri dishes (9 cm diameter) containing 10 ml of LBA medium, and in which 1 cm wide strip of agar was removed from the mid portion of media. In one side of this plate was placed in the center one sterile filter paper disc (5 mm diameter) impregnated with 5µl of *Pss*, and in the opposite side was spread over the agar 5µl of the antagonist using sterile cotton swab. Addition of the sterile liquid LB medium instead of antagonistic isolate was used as control. The plates were sealed with parafilm and incubated at 25 ± 2 °C in the dark. After an incubation period (determined in dual-culture assay), at 25 ± 2ºC in the dark, *Pss* colony area was measured and compared with the control plates by using the formula presented in the above section.

4.3.3.2. Lytic enzymes

Assessment of protease and lipase production was performed through dual-culture technique, in LBA medium containing the respective enzyme substrate, according to Maria et al. (2010). Briefly, for protease activity, dual cultures were established on medium amended with 0.4% (w/v) gelatin (Prolabo) at pH 6.0. After an incubation period (determined in dual-culture assay), at 25 ± 2ºC in the dark, the plates

were flooded with saturated aqueous ammonium sulphate (Prolabo), and the undigested gelatin precipitated with ammonium sulphate. The appearance of a clear area around the colony is indicative of protease activity. For lipase activity, dual cultures were established on medium supplemented with 1% (v/v) Tween 20 (Aldrich). A clear zone around the colony indicates lipase-positive antagonist. Control plates were performed as described previously in the dual culture assay. The level of enzyme activity was evaluated by using the formula: Enzyme activity = D-d, where D is the area of colony plus clearing zone, and d is the area of colony, in mm^2.

4.3.3.3. Siderophore

Siderophore production was evaluated according to Perez-Miranda et al. (2007), with slight differences. For this, Chrome azurol S (CAS) reagent was first prepared by mixing 60.5 mg CAS, 72.9 mg hexadecyltrimetyl ammonium bromide (HDTMA), 30.24 g Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) and 1 mM $FeCl_3.6H_2O$ in 10 mM HCl 10 mL. CAS reagent was amended with agarose (0.9%, w/v) and after sterilization 10 mL of this solution was plated in 90 mm Petri dishes, followed by an application of a 10 mL LBA medium overlay. Dual cultures were then established in these prepared plates. Control plates were performed as described previously in the dual culture assay. After an incubation period (determined in dual-culture assay), at 25 ± 2ºC in the dark, was measure the orange zone formed around the bacterial colonies, which is indicative of siderophore production. The level of siderophore production was evaluated by using the same formula previously described for enzyme activity.

In all the described assays, there were five replicated plates for each antagonistic-*Pss* combination and the whole experiment was repeated twice. For each antagonist it was calculated the percentage of increase on the production of lytic enzymes and siderophore in the presence of the pathogen by using the equation $[(A_{DC}-A_C)/A_C]*100$, where A_C is the area of the halo in control plates and A_{DC} is the area of the halo in the dual-culture assay.

4.3.4. In planta assay

The bacterial strain which exhibited the greatest inhibitory effect on *Pss* growth in the dual-culture assay (isolate P41 - *Bacillus amyloliquefaciens*) was selected for *in planta* assay (pot experiments). This study aims to assess the biocontrol ability of this isolate *in planta* against OK disease development, without compromising plant growth. Indeed, direct activation of plant defense is commonly associated with reduced growth (Stenberg et al. 2017), and therefore the impact of isolate P41 on plant growth should be studied as well.

4.3.4.1. Production of olive plantlets

Pot experiments were conducted with 2-year-old olive plantlets cv. *Cobrançosa* obtained from propagation of semi-woody cuttings. To improve rooting, the base of cuttings was treated with 3000 ppm indole-3-butyric acid (IBA), and further placed into a greenhouse on basal heated benches filled with sand and perlite mixture (1:1). Cuttings were automatically sprayed for 10 seconds, every 40 minutes; and were kept under greenhouse conditions (day/night thermal regime of 23º/18º ± 2ºC, 10 h light/14 h dark photoperiod and 70 ± 10% relative humidity) for three months. Rooted cuttings were then selected and transplanted to plastic pots of two liters filled with the same growth mixture as before and were further maintained for 2 years under the same greenhouse conditions as for rooting. During this period, plants were irrigated every 2 days.

4.3.4.2. Plant inoculation

Both *Pss* and antagonistic bacterial inoculum were prepared as described above in LB media, but containing 1% (w/v) of agar. Inoculation procedure was adapted from Penyalver et al. (2006) with minor changes. A V-shaped wound of 1 cm long (with about 2 mm deep by 5 mm wide) was made on the middle part of the main stem with a sterile scalpel and inoculated with 10µl of the selected antagonist (P41), or *Pss*, or the combination of both antagonist+*Pss*. Controls were inoculated with 10µl sterile LB culture medium containing 1% (w/v) of agar. Each wound was wrapped with Parafilm,

which was removed one week later. For each treatment and control, a total of 30 olive plantlets were inoculated, in a total of 120 plants. All the plants were maintained at 26 ^{o}C, 8 h light/16 h dark photoperiod and 70% of relative humidity, and were watered when needed.

4.3.4.3. Parameters evaluated

Disease rating was visually recorded after 14, 28, 42, 56 and 70 days *post*-inoculation (DPI) by using the 1-6 severity scale of Matas et al. (2012), where 1 = no knots; 2 = mild thickening of the wound; 3 = small knot at the base of the wound; 4 = small knots at both the base and the top of the wound; 5 = knot covering the wound completely; 6 = knot larger than the wound (Fig. S4.1). OK disease development was monitored in 10 arbitrarily selected trees of each treatment, which were kept marked throughout the assay. The data obtained were used to calculate the area under the disease progress curve (AUDPC) for each treatment using the formula (Madden et al., 2007):

$$\mathrm{AUDPC} = \sum_{\mathrm{i}=1}^{\mathrm{n}-1} [(Y_i + Y_{i+1})/2](\mathrm{t_{i+1}} - \mathrm{t_i})$$

where Y_i represents disease severity (1 to 6 scale) on the i^{th} date, t_i is the time in days at the i^{th} observation, and n is the total number of observations which OK disease development was recorded. From the remaining 20 plants *per* treatment, five plants were randomly selected in each time point mentioned above and brought to the laboratory, for estimation of knot weight *per* plant, enumeration of *Pss* population sizes, and evaluation of plant growth. The collected plants were firstly separated into leaves, stem and roots. The obtained stems were then used to estimate the average knot fresh weight *per* plant, by weighting stem fragments with 1 cm long sampled in the inoculation site. The obtained fragments were further used to estimate *Pss* population densities. For this, stem cut in small pieces, were immersed in 5 mL of peptone water (10g/L peptone, 5g/L sodium chloride) and shaken for 10 minutes at 100 rpm at room temperature. Aliquots of 1 mL of the suspension were plated in triplicate in 10 mL of PVF-1 medium (10 ml/L glycerol, 30g /L sucrose, 2.5 g/L Difco casamino acids, 1.96 g/L $K_2HPO_4.3H_2O$,

0.4g/L $MgSO_4.7H_2O$, 0.4 g/L SDS, 16 g/L agar, pH adjusted to 7.1 with HCl) (Surico et al., 1989) and incubated at 25 ± 2 ^oC, in the dark, until bacterial growth. Daily observations were performed in order to count the fluorescent bacteria *Pss* colonies. Results are presented as CFU *per* mL. For each plant were also measured total shoot height and root length. Shoot height was registered on the beginning of the assay and its increment was evaluated by a new height measurement on each harvesting period. Stems and roots from the previous plants were separately used to determine fresh weight (fw), oven-dried at 60ºC for three days, and then weighed again to determine dry weight (dw). Water content was expressed as percentage and determined by dry weight/fresh weigh ratio. Leaves neighboring the inoculation site were used for measuring the photosynthetic pigment contents. Chlorophyll a (chl a), chlorophyll b (chl b) and carotenoids (car) contents were determined spectrophotometrically after methanolic extraction of fresh leaves, according to Ozerol and Titus (1965). Total chlorophyll was calculated by the sum of both chlorophyll a and b content. Results are presented as mg of pigment *per* g of leaf.

4.3.5. Statistical analysis

Statistical analyses were carried out using R software (R Core Team, 2018). To evaluate how plant habitat, plant organ and plant cultivar are related with antagonistic potential, a Multiple Factor Analysis (MFA) was performed by using the *FactoMineR* (Le et al., 2008) R package. Data from *in vitro* and *in planta* assays were analyzed by multifactorial analysis of variance (ANOVA) and means were compared using Tukey *post-hoc* test at *p*-value <0.05 by using *agricolae* package (functions *aov* and *TukeyHSD,* respectively).

4.4. Results

Phyllosphere bacteria inhabiting the surface and the inner part of olive tissues and isolated from two olive cultivars with different susceptibility to OK disease (cv. *Cobrançosa* – more tolerant, and cv. *Verdeal Transmontana* – more susceptible) and plant compartments (leaves, twigs and knots) were tested *in vitro* against the pathogen

Pss (Table S4.1.). The 60 strains tested belonged to 40 different bacterial species, and to 22 genera.

4.4.1. Bacterial colonizing different cultivars and plant habitat displayed different inhibitory effects against Pss

From a total of 60 isolates, almost 50% (27 isolates from which 15 were epiphytes and 12 were endophytes) were able to significantly inhibited *Pss* growth, with percentage inhibitions ranging from 15.8 to 85.2%. (Fig. 4.1.; Table S4.2.). Most of the isolates that inhibited significantly *Pss* growth belonged to *Pseudomonas* and *Bacillus* genera, representing together 33.3% of the antagonists. The number of bacterial strains with capacity to inhibited significantly *Pss* growth were very similar irrespective of plant organ from which they were isolated (9 isolates/organ). By contrast, differences were found among cultivars, being cv. *Verdeal Transmontana* showed a higher number of antagonistic bacteria than cv. *Cobrançosa* (15 *vs*. 12 isolates, respectively).

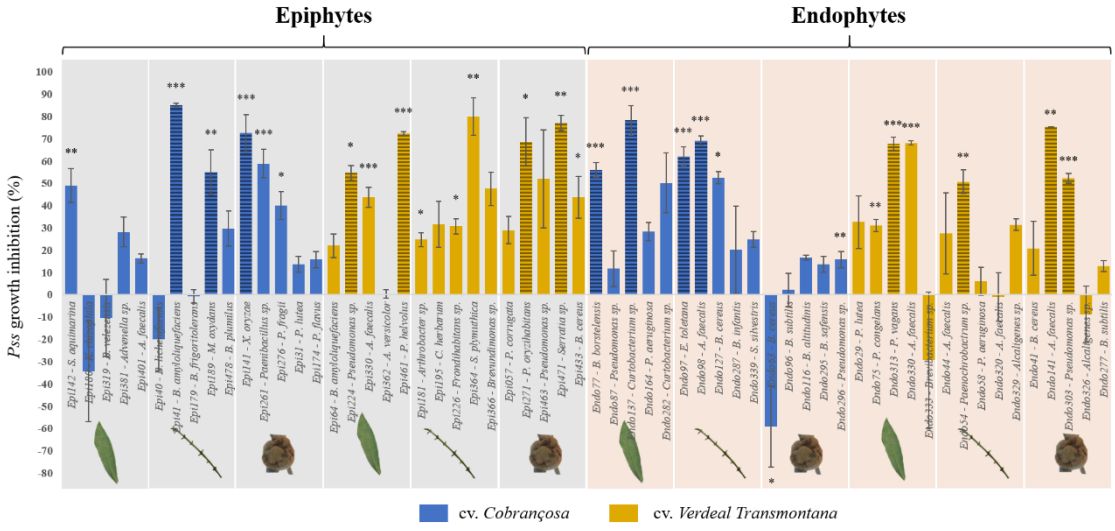

Figure 4.1. Inhibition of *Pseudomonas savastanoi* pv. *savastanoi* (*Pss*) growth in relation to control (%) after challenging by different epiphytic and endophytic bacterial isolated from leaves, twigs and knots of olive tree cvs. *Cobrançosa* and *Verdeal Transmontana* in the dual-culture assays. Horizontal lines indicate the bacterial species used on the evaluation of inhibition mechanisms. Statistically significant differences comparatively to control plate (*Pss* single culture) are indicated by an asterisk (*p<0.05; **p<0.01; ***p<0.001).

According to the MFA analysis, the inhibition of *Pss* growth by the antagonists were highly significantly correlated with Dimension 1 (R^2=0.77, p<0.001) (Fig. 4.2.). The qualitative variables (cultivar, habitat and organ) were associated to Dimension 2. The potential to inhibit *Pss* growth was more associated to plant cultivar (R^2=0.59, p<0.001) and habitat (R^2=0.42, p<0.001), than plant organ (R^2=0.26, p=0.007) from which the antagonistic strain was isolated. Indeed, the bacterial isolates from cv. *Verdeal Transmontana* showed more potential to inhibited *Pss* growth (estimated coefficient=0.64, p<0.001) than the ones isolated from cv. *Cobrançosa*. Epiphytes presented higher antagonistic potential (estimated coefficient=0.46, p<0.001) than endophytes. Considering plant organs, twigs showed to be related to isolates with higher antagonistic ability (estimated coefficient=0.55, p=0.002).

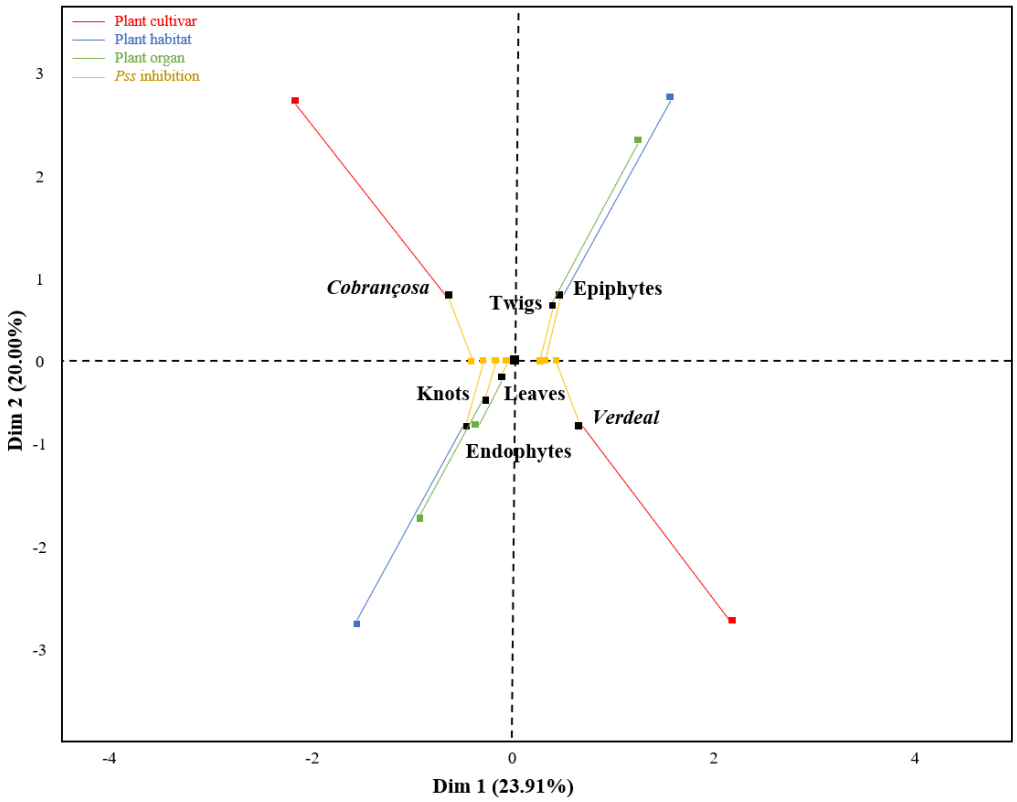

Figure 4.2. Individual factor map obtained with the Multiple Factor Analysis (MFA) showing the association of *Pss* growth inhibition ability of different bacterial isolates among plant cultivars (*Cobrançosa* and *Verdeal Transmontana*), organs (leaves, twigs and knots) and habitats (epiphytes and endophytes). The first two dimensions represent 43.91% of the total variance. Dimension 1 is associated to the inhibition ability of the antagonists (contribution of 77.3%, p<0.001), while Dimension 2 is associated to habitat (contribution of 42.7%, p<0.001), cultivar (41.4%, p<0.001) and organ (15.9%, p<0.001).

4.4.2. Production of volatiles, lytic enzymes and siderophores by antagonists inhibited Pss growth

The production of antibacterial volatile compounds, lytic enzymes (lipase and protease) and siderophores by bacterial isolates when in co-culture with *Pss* were studied, as an attempt to clarify the potential mechanisms behind such activity. This study was performed only for the bacterial strains that inhibited more than 50% of *Pss* growth and were not significantly inhibited by *Pss* (Table S4.2.). In total, 15 antagonistic isolates (7 epiphytes and 8 endophytes), belonging to 12 genera (*Pseudomonas, Pseudoclavibacter, Serratia, Bacillus, Microbacterium, Xanthomonas, Pantoea, Paenochrobactrum, Alcaligenes, Brevibacillus, Curtobacterium* and *Erwinia*), accomplish these criteria. From the 15 antagonistic isolates, eight were able to significantly affect the growth of *Pss* through volatile emission, being P41, P461 and D144 the isolates that showed the highest inhibition rates (36.5±5.8%, 34.2±7.0% and 32.3±10.41%, respectively, when compared to control) (Fig. 4.3).

When compared to control, only three epiphytic isolates increased significantly the production of lipase when challenged by *Pss* pathogen: P271 (47.5±2.1%), P141 (47.1±6.1%) and P41 (26.6±4.8%). Protease production was increased significantly only by the two endophytic isolates D54 (87.9±27.2%) and D144 (33.3±4.0%) when in co-culture with *Pss*, when compared to control. The production of siderophores by bacterial antagonists was variable according to the co-culture, being in some cases increased [D144 (54.4±8.0%), D97 (35.1±8.3%) and P41 (26.4±5.4%)] or decreased [P461 (-55.8±0.81%)] significantly as compared to control (single inoculated disc).

4.4.3. P41 significantly affected the development of OK disease and increase the growth of disease plants

During the initial screening, *Bacillus amyloliquefaciens* (P41 bacterial isolate), exhibited the greatest inhibitory effect against *Pss* and showed to be the greatest producer of compounds related to phytopathogen biocontrol. This feature makes this strain an ideal candidate to be explored as biological control agent against *Pss*.

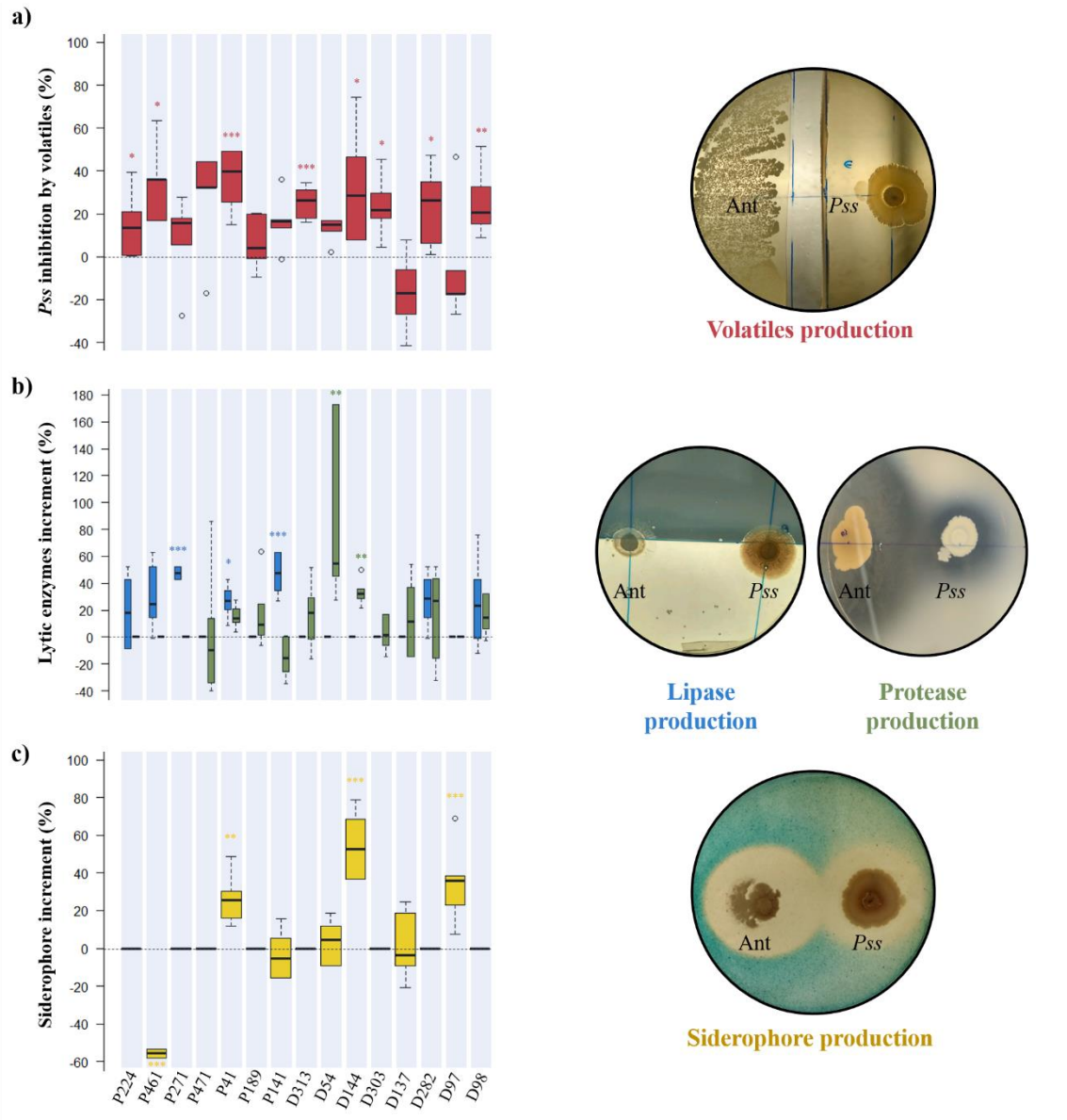

Figure 4.3. Compounds related to phytopathogen biocontrol produced by antagonistic bacterial in the dual-culture assays. **(a)** The production of antibacterial volatile compounds by antagonists was assess by estimating their inhibitory effect on *Pseudomonas savastanoi* pv. *savastanoi* (*Pss*) growth (%). The **(b)** lytic enzymes (lipase-blue, and protease-green) and **(c)** siderophores produced by antagonists were expressed as the percentage of their increase in relation to the control plates. Statistically significant differences from control plates are indicated by an asterisk (*p<0.05; **p<0.01; ***p<0.001). Representative plates with both antagonist (Ant) and the pathogen (*Pss*) used to detect the different compounds related to phytopathogen biocontrol are presented, in the right.

Therefore, we have selected this isolate to determine its capacity to suppress OK disease development in *in planta* assays. This effect was evaluated through determination of AUDPC for disease severity, knots weight and pathogen abundance on the inoculated area of olive plants treated with *Pss* or P41+*Pss* (Fig. 4.4).

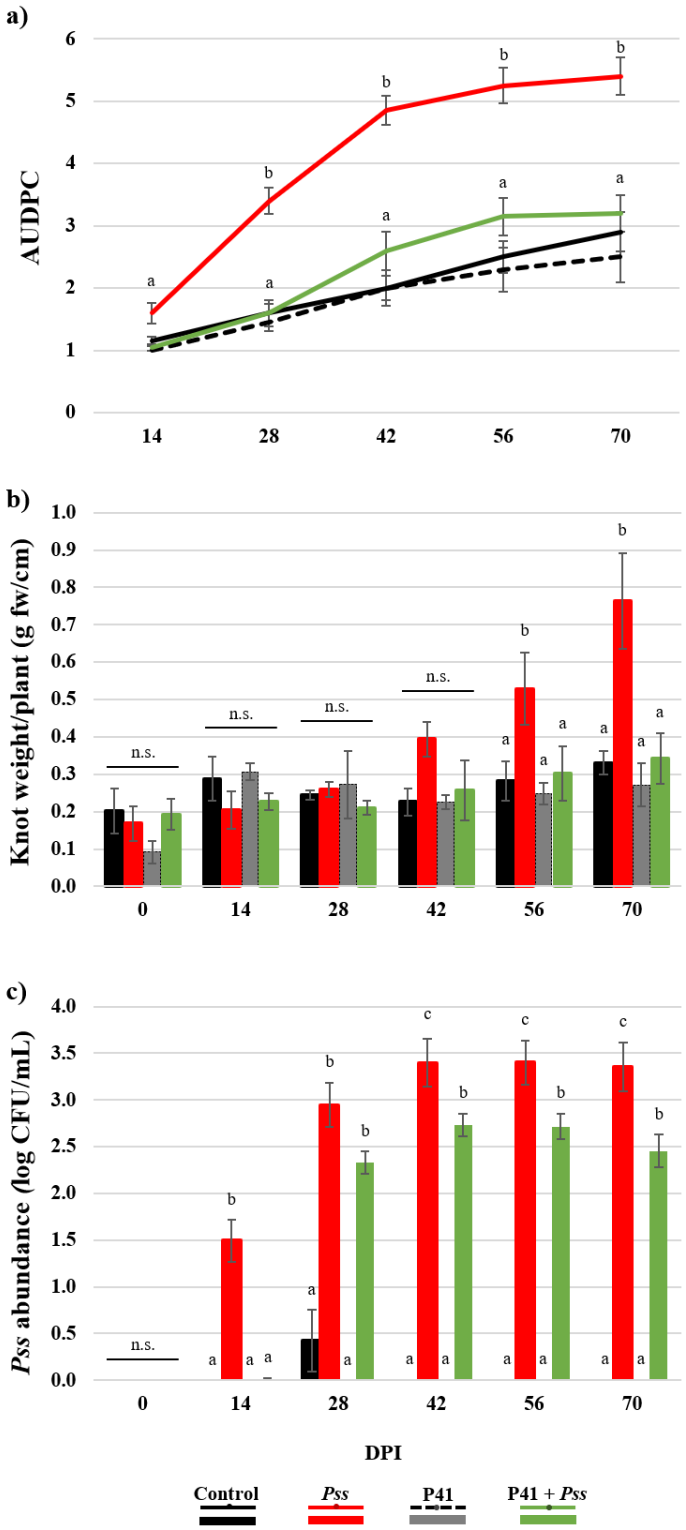

Figure 4.4. Effect of *Bacillus amyloliquefaciens* (strain P41) on olive knot disease development in *in planta* assays after 14, 28, 41, 56 and 70 days post-inoculation (DPI): **(a)** Area under disease progress curve – AUDPC**, (b)** knot fresh weight *per* plant (mg fw/cm) and **(c)** *Pseudomonas savastanoi* pv. *savastanoi* (*Pss*) populations density (log CFU/mL) in olive plantlets inoculated with LB medium (control), the pathogen (*Pss*), the antagonist (P41) and both antagonist and pathogen (P41+*Pss)*. Data is presented as means ± SE (n=10 for severity; n=5 for the knots weight and *Pss* density). Statistically significant (p<0.05) differences between the four treatments, in each day, are indicated by different letters (n.s.-non-significant).

Non-inoculated plants and inoculated with P41 were used as controls. The AUDPC, which estimates the amount of disease severity along the study, was significantly ($p<0.01$) higher in olive plantlets inoculated with *Pss* when compared to plantlets inoculated with P41+*Pss* and controls (Fig. 4.4a). This result was observed after 14 days post-inoculation and was maintained until the end of the assay. Overall, the AUDPC in P41+*Pss* plantlets was significantly lower up to 1.8-fold when compared to plantlets inoculated solely with *Pss*. Plantlets treated exclusively with *Pss* also showed significantly ($p<0.05$) higher knot weight (up to 2.2-fold) and *Pss* abundance (up to 1.3-fold) when compared to plants inoculated with P41+*Pss*, after 56 and 14 days post-inoculation, respectively (Fig. 4.4b and c). These results were observed until the end of the assay. Although the capacity of P41 in reducing OK disease development, it was observed that some of the plants exclusively treated with the antagonist P41 developed OK symptoms as well (Fig. 4.4a). However, the amount of disease developed in this treatment did not significantly differ from plantlets non inoculated (negative control). The biocontrol effect of microbial agents against phytopathogens has been suggested to potentially compromise plant growth (Huot et al., 2014). Therefore, several plant growth parameters, including plant growth, shoot and root height, dry weight, water content as well as leaves pigments content were evaluated on the same treatments after 14, 28, 42, 56 and 70 days pot-inoculation (Fig. 4.5.; Table S4.3.).

In general, it could be seen that the growth of plantlets exclusively treated with P41 or P41+*Pss* was not significantly reduced when compared to control (non-inoculated) when considering all the growth parameters evaluated, with exception of the shoot height. After 70 DPI, non-inoculated plants (control) showed significantly ($p<0.001$) higher shoot height compared to other treatments. There was no significant difference on the growth parameters evaluated between plantlets inoculated with P41 and with P41+*Pss*. By the end of the assay, the inoculation of plants with P41 alone and with P41+*Pss*, showed significantly ($p<0.01$) higher shoot dry weight (up to 2.8-fold and 2.2-fold, respectively) and root water content (up to 1.3-fold and 1.7-fold, respectively), when compared to plants inoculated solely with *Pss*. No significant differences were observed on chlorophyll a, b, total and carotenoids contents between treatments (Table S4.3.).

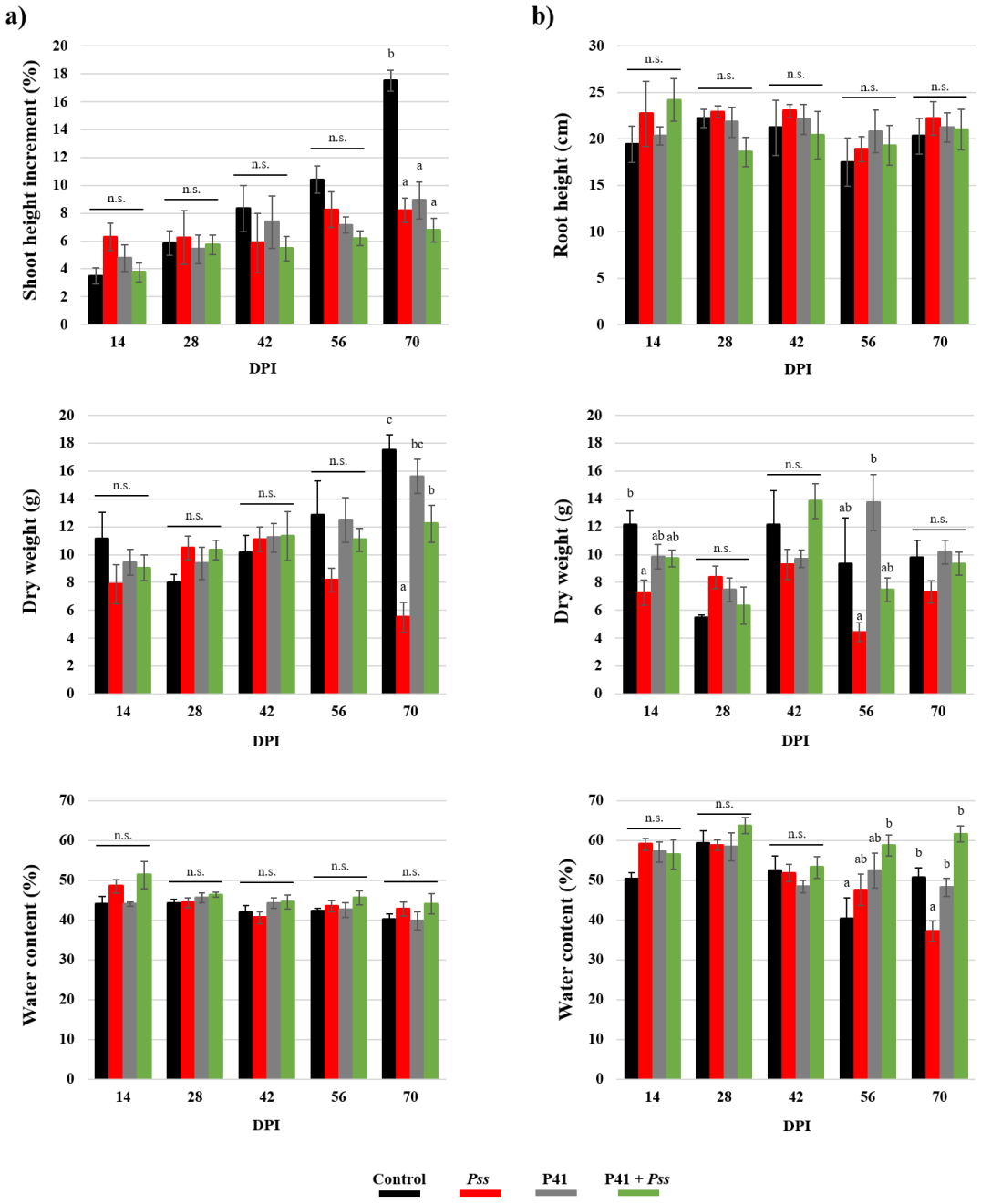

Figure 4.5. Effect of *Bacillus amyloliquefaciens* (strain P41) on olive plant growth in *in planta* assays after 14, 28, 41, 56 and 70 days post-inoculation (DPI). Plant growth parameters were evaluated at the level of the **(a)** shoots and **(b)** roots of olive plantlets inoculated with LBA medium (control), *Pseudomonas savastanoi* pv. *savastanoi* (*Pss*), antagonist (P41) or both antagonist and pathogen (P41+*Pss*). Values, in each day, are expressed as mean ± SE (n=5). Statistically significant ($p<0.05$) differences between the four treatments, in each day, are indicated by different letters (n.s.-non-significant).

4.5. Discussion

In this study, we tested the biocontrol potential of several bacteria isolated from the olive tree phyllosphere against *Pss*, by combining *in vitro* and *in planta* assays. These isolates are good candidates for biocontrol of OK disease because of their adaptation to the host and the environment (Mercado-Blanco and Bakker, 2007).

In this study was showed for the first time that bacterial strains have differences on the effectiveness in inhibiting *in vitro* growth of *Pss*, depending on their origin. Bacterial strains isolated from OK-susceptible cultivar cv. *Verdeal Transmontana* displayed higher antagonistic effect against *Pss* than the ones isolated from OK-tolerant cv. *Cobrançosa*. Therefore, we hypothesized that isolates from these two cultivars may have different modes of action to protect host plant from *Pss* infection. Microbial antagonists may use a variety of mechanisms against phytopathogens broadly classified into direct or indirect, depending on the requirement of interspecies physical contact or not, respectively (Hajek and Eilenberg, 2018). Probably, isolates from cv. *Cobranços*a stimulate/prime the immunity of host plant to combat *Pss* invasions, while isolates from cv. *Verdeal Transmontana* seems to act directly against the pathogen. However, this assumption still needs to be confirmed with further work. We also found that epiphytes have a higher inhibition potential against *Pss* than endophytes, which makes epiphytes as the most promising for the biocontrol of OK disease. This aspect is of particular importance for this pathosystem since the infection of olive tree is believed to be cause by the epiphytic *Pss* (Quesada et al., 2010). From the 27 isolates which significantly inhibited *Pss*, one third belonged to *Bacillus* and *Pseudomonas*. These two genera have been already reported to be the most promising biocontrol agents of several plant diseases (Shafi et al., 2017), in particular the ones affecting the roots (Li et al., 2013; Chowdhury et al., 2015; Fan et al., 2018; De Vrieze et al., 2018). The use of these two genera as biocontrol agents in the phyllosphere has been less studied than in the rhizosphere. A number of studies have reported the effectiveness of *Bacillus* and *Pseudomonas* strains isolated from the phyllosphere of different woody crops, namely lemon (Michavila et al., 2017) and apple (Mikiciński et al. 2016) cultures, to control bacterial pathogens that infects aboveground organs of these two crops. Interestingly, isolates belonging to *Pseudomonas* (Zadeh et al., 2008; Krid et al., 2010) and *Bacillus*

(Krid et al., 2010, 2012) were previously showed to be effective in antagonizing *Pss* in *in vitro* assays, as observed in our work.

The *in vitro* inhibition of *Pss* by most of the antagonistic bacterial tested was probably caused by the induction of volatile organic compounds (VOCs). Indeed, eight bacterial strains out of the 15 strains that antagonize *Pss*, increased the production of VOCs. The antibacterial activity of the VOCs produced by microbial antagonists was previously reported and proposed as an effective biological control strategy against several phytopathogens (Ossowicki et al., 2017; Bui et al., 2019). These compounds may act as promoter of plant defence responses (Erb, 2018) and as inhibitor of phytopathogen growth (Enespa and Chandra, 2017). The microbial VOCs, due to their highly diffusible capacity, are considered ideal for the biocontrol because they do not require the contact between the antagonist and the pathogen to perform their activity (Contarino et al., 2019). Besides VOCs, both lytic enzymes and siderophores could also be potential involved in the inhibition of *Pss* growth. The lytic enzymes lipase or protease were induced in five bacterial antagonists in the presence of *Pss*. Previous studies have similarly reported strong enhancement of lipase and protease (Trivedi et al., 2008; Amaresan et al., 2012; Geetha et al., 2014) in bacterial antagonistic during *in vitro* interaction with several plant pathogens. Bacterial cell membranes are primarily composed of lipids and proteins (Barák and Muchová, 2013). Thus, we hypothesized that the bacterial antagonistic tested may inhibited *Pss* growth by excreting lipase and protease enzymes that degraded cellular components of the pathogen. Three of the antagonists tested were also increased the production of iron binding ligands, called siderophores, in the presence of *Pss*. These siderophores may sequester iron from the culture medium, making probably iron unavailable to the *Pss* and therefore restricting its growth. Iron is essential for growth and pathogenesis of almost all species of phytopathogenic bacteria (Shanmugaiah et al., 2015; Pi and Helmann, 2018. Suppression of phytopathogens by bacterial antagonists through siderophore-mediated competition for iron have been already reported either in *in vitro* (Akter et al., 2016) or in field (Gull et al., 2012; Sasirekha and Srividya, 2016) conditions.

Among the different bacterial strains tested, P41 strain, identified as *Bacillus amyloliquefaciens*, showed the most promising antagonistic traits against *Pss* under *in*

vitro conditions. This strain, isolated from twigs of cv. *Cobrançosa*, displayed the highest antimicrobial activity against *Pss*. Previous studies have similarly identified this species as one of the most efficient in inhibiting either bacterial (Abdallah et al., 2018) or fungal (Freitas et al., 2019) plant pathogens in *in vitro* conditions. In the present study, the mechanisms involved in *Pss* inhibition by *B. amyloliquefaciens* P41 probably include secretion of inhibitory compounds (VOCs and lipase) and competition for nutrients (through the production of siderophores). These mechanisms were previously observed by other authors for this species (Kejela et al., 2016; Verma et al., 2016; Jamali et al., 2018). Genome analysis of *B. amyloliquefaciens* revealed that this species is capable to produce other secondary metabolites aimed to suppress plant pathogens or to enhance/mediate defense response of host plant against plant pathogens (Chowdhury et al., 2015). In agreement with *in vitro* assays, the inoculation of olive plantlets with *B. amyloliquefaciens* P41 showed to reduce significantly OK disease's severity, knots weight and population size of *Pss* as compared to pathogen inoculated and un-inoculated control. Likewise, other studies have demonstrated the beneficial effects of *B. amyloliquefaciens* on disease suppression on other crops, such as tomato (Gautan et al., 2019), apple (Zhang et al., 2015), pistachio (Siahmoshteh et al., 2017) and lettuce (Chowdhury et al., 2013). As far as we known, this study illustrates for the first time the potential role of *B. amyloliquefaciens* as biological agent for controlling OK disease. This is of great significance because some strains of *B. amyloliquefaciens* are already commercially available (e.g. Serenade, Bayer Crop Science; RhizoVital®42, Abitep GmbH) for use as biocontrol agents against plant pathogens (Chowdhury et al., 2015). In olive culture, biological control of *Pseudomonas savastanoi* pv. *savastanoi* have been underexploited and only a few studies demonstrated some efficiency of an olive phyllospheric bacteria against this pathogen in *in planta* conditions. Krid et al. (2012) and Ghanney et al. (2016) were able to reduce the *Pss* population and the size of knots by inoculating with *Bacillus subtilis* F_1 (isolated from olive leaves) and *Bacillus mojavensis* A-BC-7 (isolated from the olive phylloplane), respectively, but only six olive trees were tested. Maldonado-González et al., 2013 examined the interaction between endophytic antagonist *Pseudomonas fluorescens* PICF7 (previously isolated from olive roots and efficiently used as an antagonist of olive verticillosis, Mercado-Blanco et al., 2004)

against *Pss* observing a reduction of *Pss* at early times after co-inoculation, revealing important findings concerning the way pathogen interacts and moves within host tissues.

Besides its biocontrol traits, several strains of *B. amyloliquefaciens* have been previously described as plant growth promoters (Shao et al., 2014; Asari et al., 2016; Abdallah et al., 2018). Contrasting with these reports, in the present study, was observed that *B. amyloliquefaciens* P41 did not promote plant growth when compared to un-inoculated treatment. Indeed, they did not differ significantly for most of the plant growth parameters evaluated. Only treatments involving the inoculation of microorganisms (either pathogen or P41, or both) showed a significant decrease of shoot height increment when compared to un-inoculated treatment. Plants are known to hold a limited reservoir of resources that, in the presence of biotic or abiotic stresses, activate their immune response locally or systemically, affecting the normal plant growth and development, in a phenomenon known as growth–defense tradeoff (Walling, 2009; Huot et al., 2014). Thus, microbial inoculation of olive plantlets may probably induce this growth–defense tradeoff phenomenon, and therefore reducing shoot height. However, when *B. amyloliquefaciens* P41 was inoculated onto olive plantlets, either alone or in combination with *Pss*, was observed to enhance significantly both shoot dry weight and root water content when compared to plants inoculated solely with *Pss*.

Although several studies reported the ability of *B. amyloliquefaciens* to improve plant development (Shao et al., 2014; Ben Abdallah et al., 2018) or protect it against plant pathogens (Zhang et al., 2015; Siahmoshteh et al., 2017), only a few studies revealed both skills on the same strain for a specific culture (Qiao et al., 2014; Asari et al., 2016). The results presented here suggests that *B. amyloliquefaciens* P41 is able to promote plant growth and reduce the detrimental effects of various stresses caused by *Pss* infection.

In conclusion, we have showed that olive phyllosphere harbors a number of bacterial strains with great potential to be used as BCA against *Pss*. The strongest antagonistic potential was ascribed to bacteria inhabiting the twigs surface of cv. *Verdeal Transmontana*. In particular, *Bacillus amyloliquefaciens* P41, showed great

potential in the management of OK disease, by simultaneously promoting plant growth and reducing disease´s severity of *Pss*-infected olive plantlets. Although promising results were obtained from using this strain, further experiments are needed to determine its effectiveness under field conditions, and with different cultivars. The biocontrol mechanisms displayed by this strain also need to be deeply investigated.

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4.7. Supporting Information

Figure S4.1. Development stages of olive knot disease on olive tree plants artificially inoculated with *Pss.* Scale: 1 = no knots; 2 = mild thickening of the wound; 3 = small knot at the base of the wound; 4 = small knots at both the base and the top of the wound; 5 = knot covering the wound completely; 6 = knot larger than the wound.

Table S4.1. Origin of the bacterial strains assayed in this work. Epiphytic and endophytic bacteria were isolated from different *Olea europaea* organs (leaves -L, healthy twigs -T and knot -K) and cultivars (*Cobrançosa* -C and *Verdeal Transmontana* -V).

Cultivar	Organ	Epiphytes		Endophytes	
		Isolate	Species	Isolate	Species
C	L	P142	*Sporosarcina aquimarina*	D77	*Brevibacillus borstelensis*
C	L	P180	*Kocuria rhizophila*	D87	*Pseudomonas* sp.
C	L	P319	*Bacillus velezensis*	D137	*Curtobacterium* sp.
C	L	P381	*Advenella* sp.	D164	*Pseudomonas aeruginosa*
C	L	P401	*Alcaligenes faecalis*	D282	*Curtobacterium* sp.
C	T	P40	*Bacillus licheniformis*	D97	*Erwinia toletana*
C	T	P41	*Bacillus amyloliquefaciens*	D98	*Alcaligenes faecalis*
C	T	P179	*Brevibacterium frigoritolerans*	D127	*Bacillus cereus*
C	T	P189	*Microbacterium oxydans*	D287	*Bacillus infantis*
C	T	P478	*Bacillus plumilus*	D339	*Solibacillus silvestris*
C	K	P141	*Xanthomonas oryzae*	D63	*Bacillus cereus*
C	K	P261	*Paenibacillus* sp.	D96	*Bacillus subtilis*
C	K	P276	*Pseudomonas fragii*	D116	*Bacillus altitudinis*
C	K	P31	*Pseudomonas lutea*	D295	*Bacillus safensis*
C	K	P174	*Plantibacter flavus*	D296	*Pseudomonas* sp.
V	L	P64	*Bacillus amyloliquefaciens*	D29	*Pseudomonas lutea*
V	L	P224	*Pseudomonas* sp.	D75	*Pseudomonas congelans*
V	L	P330	*Alcaligenes faecalis*	D313	*Pantoea vagans*
V	L	P362	*Agrococcus versicolor*	D330	*Alcaligenes faecalis*
V	L	P461	*Pseudoclavibacter helvolus*	D333	*Brevibacterium* sp.
V	T	P181	*Arthrobacter* sp.	D44	*Alcaligenes faecalis*
V	T	P195	*Curtobacterium herbarum*	D54	*Paenochrobactrum* sp.
V	T	P226	*Frondihabitans* sp.	D58	*Pseudomonas aeruginosa*
V	T	P364	*Serratia plymuthica*	D320	*Alcaligenes faecalis*
V	T	P366	*Brevundimonas* sp.	D329	*Alcaligenes* sp.
V	K	P57	*Pseudomonas corrugata*	D41	*Bacillus cereus*
V	K	P271	*Pseudomonas oryzihabitans*	D144	*Alcaligenes faecalis*
V	K	P463	*Pseudomonas* sp.	D303	*Pseudomonas* sp.
V	K	P471	*Serratia* sp.	D326	*Alcaligenes* sp.
V	K	P433	*Bacillus cereus*	D277	*Bacillus subtilis*

Table S4.2. Growth inhibition of *Pss* and bacterial isolates in dual culture, relatively to the controls (single-cultures of *Pss* and bacterial isolates). Epiphytic and endophytic bacteria were isolated from different *Olea europaea* organs (leaves -L, healthy twigs - T and knot -K) and cultivars (*Cobrançosa* -C and *Verdeal Transmontana* - V). Datasets in bold represent the most promising isolates, selected for the evaluation of the antagonism associated mechanisms. Statistical significance: *p <0.05; **p <0.01; ***p <0.001.

Epiphytes			**Endophytes**		
Isolate	*Pss*	Bacterial isolate	Isolate	*Pss*	Bacterial isolate
P142	49.00±7.54**	31.75±1.61	D77	56.06±3.30***	48.68±4.11
P180	-34.43±22.54	29.89±28.46	D87	11.74±8.02	57.66±3.93***
P319	-10.36±17.37	-21.28±13.29	**D137**	**78.22±6.54*** **	**-108.11±17.53****
P381	28.19±6.61	69.46±1.57**	D164	28.34±3.97	-7.67±3.35
P401	16.38±2.04	48.24±7.90*	**D282**	**50.18±13.42**	**14.36±2.50**
P40	-20.10±14.07	55.14±21.97	**D97**	**62.12±4.22*** **	**-6.20±3.32**
P41	**85.21±0.81*** **	**-150.63±24.15****	**D98**	**69.21±2.09*** **	**-25.37±10.23**
P179	-0.63±2.97	49.53±3.82**	D127	52.49±2.76*	71.27±6.92**
P189	**54.96±10.10****	**2.69±11.37**	D287	20.19±19.52	65.50±3.49**
P478	29.78±7.87	42.86±6.25	D339	24.81±3.44	3.54±4.83
P141	**72.52±8.26*** **	**19.68±4.62**	D63	-59.06±18.16*	71.14±11.96**
P261	58.82±6.36***	61.74±19.07*	D96	2.21±7.47	-4.64±14.52
P276	40.02±6.22*	66.32±10.02*	D116	16.57±1.10	30.09±2.37
P31	12.14±4.73	24.36±4.92	D295	13.74±3.61	12.62±8.9
P174	14.55±4.21	19.93±6.98	D296	15.75±3.55**	-10.88±4.42
P64	22.04±5.32	23.14±6.13	D29	32.58±11.76	23.02±5.04
P224	**54.64±3.32***	**16.75±7.91**	D75	31.06±2.73**	11.29±15.63
P330	43.72±4.53***	4.41±14.46**	**D313**	**67.63±2.99*** **	**-1.72±9.96**
P362	0.32±1.96	43.20±3.59	D330	68.16±1.05***	45.60±1.06**
P461	**72.38±0.92*** **	**-33.52±34.55**	D333	-29.28±30.58	70.45±4.32
P181	24.71±3.11*	44.20±3.85	D44	27.52±18.12	8.81±1.04**
P195	31.68±10.28	20.15±3.11	**D54**	**50.76±5.3****	**19.25±2.24**
P226	30.73±3.38*	47.68±9.76	D58	6.10±6.21	67.85±0.29*
P364	79.97±8.45**	85.61±2.42*	D320	-1.10±10.27	11.80±7.16
P366	47.54±7.57	86.78±8.14**	D329	31.46±2.48	-12.92±1.69
P57	29.01±6.06	35.36±2.86	D41	20.85±12.01	35.84±2.86
P271	**68.70±10.77***	**16.95±9.64**	**D144**	**75.37±0.31****	**25.26±2.79**
P463	51.91±22.01	10.57±36.69	**D303**	**52.37±2.15*** **	**-14.62±10.26**
P471	**77.10±3.5****	**38.83±13.45**	D326	-8.84±12.63	54.14±3.68***
P433	43.72±9.3*	62.33±1.18***	D277	12.91±2.44	34.19±9.06

Table S4.3. Contents of chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*), total chlorophyll and carotenoids (car) on leaves of olive plantlets after 14, 28, 41, 56 and 70 days post-inoculation (DPI) LBA medium (control), *Pseudomonas savastanoi* pv. *savastanoi* (*Pss*), antagonist (P41) or both antagonist and pathogens (*Pss* + P41). Values, in each day, are expressed as mean ± SE (n=5). Different letters indicate statistically significant differences between treatments, in each day.

Treatments	DPI				
	14	28	42	56	70
Chlorophyll a (mg/g)					
Control	1.09 ± 0.18^{a}	1.32 ± 0.12^{b}	1.40 ±0.07^{b}	1.20 ± 0.17^{a}	1.58 ± 0.20^{a}
Pss	1.25 ±0.15^{a}	1.16 ±0.18^{b}	1.11 ±0.15^{b}	1.69 ±0.16^{a}	1.67 ±0.12^{a}
P41	0.90 ± 0.12^{a}	1.06 ± 0.08^{a}	0.92 ± 0.06^{a}	1.27 ± 0.10^{a}	1.21 ± 0.10^{a}
Pss + P41	1.29 ± 0.12^{a}	1.32 ± 0.04^{b}	1.54 ± 0.27^{b}	1.68 ± 0.07^{a}	1.63 ± 0.13^{a}
Chlorophyll b (mg/g)					
Control	0.48 ± 0.06^{a}	0.50 ± 0.05^{a}	0.55 ± 0.02^{a}	0.50 ± 0.06^{a}	0.67 ± 0.09^{a}
Pss	0.52 ± 0.09^{a}	0.46 ± 0.07^{a}	0.45 ± 0.05^{a}	0.70 ± 0.07^{a}	0.74 ± 0.07^{a}
P41	0.35 ± 0.04^{a}	0.44 ± 0.03^{a}	0.39 ± 0.03^{a}	0.55 ± 0.04^{a}	0.53 ± 0.05^{a}
Pss + P41	0.51 ± 0.05^{a}	0.54 ± 0.02^{a}	0.62 ± 0.11^{a}	0.71 ± 0.04^{a}	0.69 ± 0.06^{a}
Total chlorophyll (mg/g)					
Control	1.57 ± 0.24^{a}	1.82 ± 0.17^{a}	1.94 ± 0.09^{a}	1.70 ± 0.23^{a}	2.26 ± 0.29^{a}
Pss	1.78 ± 0.23^{a}	1.62 ± 0.25^{a}	1.56 ± 0.21^{a}	2.39 ± 0.23^{a}	2.41 ± 0.19^{a}
P41	1.26 ± 0.16^{a}	1.50 ± 0.11^{a}	1.31 ± 0.09^{a}	1.82 ± 0.14^{a}	1.74 ± 0.15^{a}
Pss + P41	1.80 ± 0.16^{a}	1.86 ± 0.06^{a}	2.16 ± 0.38^{a}	2.39 ± 0.11^{a}	2.32 ± 0.19^{a}
Carotenoid (mg/g)					
Control	0.20 ± 0.04^{a}	0.27 ± 0.02^{a}	0.27 ± 0.01^{ab}	0.23 ± 0.03^{a}	0.31 ± 0.04^{a}
Pss	0.26 ± 0.03^{a}	0.24 ± 0.03^{a}	0.22 ± 0.03^{ab}	0.33 ± 0.03^{a}	0.32 ± 0.02^{a}
P41	0.21 ± 0.03^{a}	0.23 ± 0.02^{a}	0.19 ± 0.02^{a}	0.25 ± 0.02^{a}	0.24 ± 0.02^{a}
Pss + P41	0.27 ± 0.02^{a}	0.26 ± 0.01^{a}	0.31 ± 0.04^{b}	0.32 ± 0.01^{a}	0.30 ± 0.02^{a}

CHAPTER 5.

Concluding remarks and future perspectives

5.1. Concluding remarks and future perspectives

One of the main diseases affecting olive orchards all over the world is olive knot (OK) disease, caused by *Pseudomonas savastanoi* pv. *savastanoi* (*Pss*) (Quesada et al., 2012). This disease is characterized by the development of tumors on the above-ground tissues of the plant, mainly trunk and twigs. Although disease symptoms in fruits are rare, OK disease is responsible for a decrease in tree vigor and a reduction on number, size and quality of olive fruits, compromising the yield of this crop (Young, 2004; Quesada et al., 2010). Olive knot cannot be eradicated once it is established in an orchard, and therefore its control is based on preventive measures (Teviotdale and Krueger, 2004; Quesada et al., 2010, 2012). Previous works have revealed the role of plant-resident microorganisms on the defense of the plant against several diseases on both herbaceous and woody plant species (Cazorla and Mercado-Blanco 2016; Rahman et al., 2018). The main aim of this PhD thesis was to evaluate the bacterial community associated to the olive tree phyllosphere, both epiphytic and endophytic communities, and elucidate its possible role on plant defense against OK disease. Therefore, at first, studies were conducted to characterize the bacterial communities inhabiting the surface and the interior of leaves and twigs tissues of two olive cultivars ("Who it there?") and evaluate which factors shape these microbial assemblages ("Which factors contribute to their shaping?"). In subsequent studies, bacterial communities were related with plant susceptibility to OK disease ("What can they do?"), and their potential role in the control of OK disease was evaluated in *in vitro* and *in plant* assays ("Could they be useful?").

5.2. Who is there?

The epiphytic and endophytic bacterial community associated to the phyllosphere of asymptomatic olive tree revealed to be rich and abundant in both organs (leaves and twigs) and cultivars (cv. *Cobrançosa* and *Verdeal Transmontana*). Overall, the bacterial community comprised four phyla (*Proteobacteria, Actinobacteria, Firmicutes* and *Bacteroidetes*) and 19 families, in a total of 89 OTUs. *Pseudomonadales* and *Actinomycetales* were the most abundant families inhabiting the surface of plant tissues,

while *Pseudomonadales* and *Enterobacteriales* were the most abundant families colonizing the interior of plant tissues.

5.3. Which factors contribute to their shaping?

In the present work were tested the influence of different cultivars (cv. *Cobrançosa* vs. *Verdeal Transmontana*), plant organs (leaves vs. twigs), symptomatology (asymptomatic vs. OK-symptomatic twigs) and plant habitat (epiphytic vs. endophytic) on bacterial assemblage in olive tree phyllosphere. Results showed that host cultivar was a key factor structuring the whole bacterial community of olive tree phyllosphere, suggesting an important role of plant-derived metabolites in the bacterial community composition of phyllosphere (chapter 2). The contribution of host cultivar in bacterial assemblage was most notorious in OK-symptomatic twigs (chapter 3). Thus, apart plant-derived metabolites, the plant immune system as well, could probably affect the composition of bacterial communities associated with the olive knots. Plant organ was most important in shaping epiphytes than endophytes, being leaves and twigs displayed distinctive epiphytic bacterial assemblages (chapter 2). These differences are probably related to the variable physic-chemical properties displayed between leaves and twigs. Results from chapter 3, also showed that the presence of OK disease and plant habitat contribute to the shaping of bacterial communities in olive tree phyllosphere. These results suggest that the presence of the pathogen and the physic-chemical properties of the surface and/or inner plant tissues may exert some influence on bacterial community composition of olive twigs.

5.4. What can they do?

In this study, the comparison of bacterial community (either epiphytic or endophytic) between asymptomatic and disease olive tree tissues of cultivars with contrasting susceptibility to OK disease, was performed in order to better understand the impact of the interaction between host plant-pathogen-resident bacteria on OK disease development (chapter 3). Results indicate that OK disease disturb the resident bacterial communities of twigs, by changing significantly their composition, diversity and

abundance. However, this effect was variable depending on the host cultivar and plant habitat, being most notorious on the OK-susceptible olive tree cultivar and within endophytes. In particular, a reduction on epiphytic bacterial abundance was observed in symptomatic twigs, while within endophytes was observed the opposite. The presence of OK disease also showed to increase the diversity of endophytes in OK-tolerant cultivar, whereas an opposite result was observed in OK-susceptible cultivar. Thus, the bacterial community in olive knots has probably resulted from complex interactions between *Pss*, host plant and its indigenous bacteria. During this interaction, probably is produced an array of metabolites that could drive the bacterial community in olive knots. Changes on these metabolites are likely to facilitate the invasion of bacterial species not typically resident in olive twigs, with greater effect in OK-susceptible cultivar than in OK-tolerant cultivar. A number of bacterial genera were highly associated to the presence or absence of OK disease in each cultivar. While *Pantoea*, *Erwinia*, *Pseudomonas* were positively correlated with the presence of OK disease, the genera *Alcaligenes, Arthrobacter*, *Bacillus, Brevundimonas*, *Curtobacterium*, *Frondihabitans* and *Xanthomonas* were positively correlated with asymptomatic twigs. From these, *Erwinia* was the only one found to be specifically associated to OK-tolerant cultivar, being most of the remaining genera associated to OK-susceptible cultivar. Thus, it is likely that these bacterial genera might be critical for the establishment of the pathogen in olive twigs and could also play an important role in the susceptibility/tolerance of cultivars to OK disease.

5.5. Could they be useful?

To answer this question, the antagonist effect of 60 bacterial strains isolated from the surface and inner tissues of leaves, twigs and knots of two olive cultivars of varying susceptibilities to OK was evaluated *in vitro* assays against *Pss* (**chapter 4**). Almost half of the tested bacteria (45%) showed to significantly inhibit the growth of *Pss* (up to 85.2% in relation to control), being this effect linked to the bacteria origin. In fact, the bacteria inhabiting the surface of plant tissues and associated to OK-susceptible cultivar, displayed the highest inhibition activity. This inhibitory effect was potentially due to the production of volatile compounds, lytic enzymes and siderophores. Among

the antagonistic isolates, *Bacillus amyloliquefaciens* P41 showed the highest inhibitory effect, and thus, its ability on the control of OK disease was tested in *in planta* assays. Compared to non-inoculated control, P41 was able to reduce OK disease's severity (up to 43.7%) and population size of *Pss* (up to 26.8%), and simultaneously to increased plant fitness, suggesting as a promising biocontrol candidate for controlling OK disease.

Overall, the community of microorganisms inhabiting the olive phyllosphere was shown to result from a complex interaction established between plant-Pss-resident bacteria. This study also brings into focus the importance of these interactions to olive tree health and, in particular, to the development of OK disease. However, there is still a requirement for research to unravel the intricacies of communication between all members of this multipartite interaction, that lead to assembly the pathobiome in olive twigs. In particular, the exact function of the bacteria positively associated to asymptomatic or OK-symptomatic twigs, in olive tree health should be studied and carefully examined by using metatranscriptome sequencing, metaproteome and metabolome analysis. The exact mechanism by which P41 interfered with *Pss* should also be further investigated.

5.6. References

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