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Exploitation of endophytes of centenarian olive trees in the management of olive knot disease

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Supervised by

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Table of Content

Abstract

Endophytes are microorganisms that spend most of their life cycle within plant tissues and they are recognized to play important roles in plant protection against abiotic and biotic stresses. Some recent studies have been showed that ancient plants are inhabited by a greater number of rare endophytes and may represent a rich repository of potential biocontrol agents. Thus, in this work the effect of the application of a consortium of bacterial endophytes, retrieved from centennial olive trees, on the development of olive knot (OK) disease, was evaluated. The mechanisms responsible for the control of OK by this consortium of microorganisms was also evaluated by studying the changes in the indigenous stem endophytic bacterial community, through 16S rRNA gene amplicon-based metagenomic analysis. Accordingly, endophytes were extracted from stems of centennial olive trees using a Histodenz gradient, and used to inoculated one-year-old olive plantlets. One week later, these plants were inoculated with the causal agent of OK, the bacterium *Pseudomonas savastanoi* pv. *savastanoi* (*Pss)*. Results showed that the inoculation with bacterial endophytes reduced significantly both the incidence (up to 1.6-fold) and severity (up to 2.1-fold) of OK, when compared to plants inoculated exclusively with the pathogen *Pss*. This effect is likely due to changes caused by bacterial endophytes on stem associated-bacteria composition, being noticed a decrease on *Pseudomonas*, Methylobacterium and *Mycoplasma* and an increase on Micrococcaceae, Rikenellaceae and *Corynebacterium*, when compared to plants inoculated uniquely by *Pss*. Likewise, the inoculation of plants uniquely with *Pss* affected greatly the bacterial community assembly of the stem and potentially beneficial bacteria. The bacterial inoculum was dominated by the symbiotic genus *Mycoplasma*, being the first time reported on olive tree. Overall, the results suggest that centenary olive trees may represent an important source of endophytes with valuable biocontrol proprieties. The inoculation of olive plants with endophytes from ancient trees could be an innovative strategy to manage olive knot disease. Despite these promising results, further studies are needed to better understand their mechanisms of action.

Keywords: *Pseudomonas savastanoi* pv. *savastanoi*, bacterial community, biological control, incidence, severity.

Resumo

Os endófitos são microrganismos que colonizam os tecidos internos das plantas a maior parte do seu ciclo de vida, e são conhecidos por conferirem proteção às plantas contra stresses bióticos e abióticos. Recentemente, alguns estudos mostraram que plantas centenárias possuem um maior número de endófitos raros e, por isso, podem constituir um repositório de potenciais agentes de biocontrolo. Assim, neste trabalho foi avaliado o efeito da aplicação de um consórcio de endófitos bacterianos, isolado de oliveiras centenárias, no desenvolvimento da tuberculose da oliveira (OK). Os mecanismos subjacentes ao controlo desta doença por parte do consórcio bacteriano foram igualmente estudados, pela avaliação de alterações na comunidade bacteriana indigena dos caules através de análise metagenómica (gene 16S rRNA). Assim, os endófitos foram extraídos de caules de oliveiras centenárias usando Histodenz e usados para inocular plântulas de oliveira com um ano de idade. Uma semana depois, estas plantas foram inoculadas com o agente causal da OK, a bactéria *Pseudomonas savastanoi* pv. *savastanoi* (*Pss*). Os resultados mostraram que a inoculação com endófitos bacterianos reduziu significativamente tanto a incidência (até 1,6 vezes) quanto a severidade (até 2,1 vezes) de OK, quando comparada com plantas inoculadas exclusivamente com o patogénico *Pss.* Este efeito deveu-se, provavelmente, a alterações na composição da população nativa dos caules causada pelos endófitos bacterianos, tendo-se observado um decréscimo em *Pseudomonas*, Methylobacterium e *Mycoplasma* e um aumento em Micrococcaceae, Rikenellaceae e *Corynebacterium*, quando comparado com plantas inoculadas exclusivamente com *Pss*. Da mesma forma, a inoculação de plantas exclusivamente com *Pss* afetou significativamente a estrutura da comunidade bacteriana nativa do caule, incluindo bactérias potencialmente benéficas. O inóculo bacteriano mostrou apresentar grande abundãncia do género simbiótico *Mycoplasma*, tendo sido reportado pela primeira vez em oliveira. No geral, os resultados sugerem que os endófitos das oliveiras centenárias podem representar uma importante fonte de agentes de biocontrolo. A inoculação de plantas de oliveira com endófitos de árvores antigas pode ser uma estratégia inovadora para controlar a doença da tuberculose da oliveira. Apesar destes resultados promissores, são necessários mais estudos para perceber melhor o mecansimo de ação destes endófitos.

Palavras-chave: *Pseudomonas savastanoi* pv. *savastanoi*, comunidade bacteriana, controlo biológico, incidência, severidade

Index of figures

List of Abreviations

- **ANOSIM:** Analysis of similarities
- **ANOVA:** Analysis of variance
- **AUDPC:** Area under the disease progress curve
- **DPI:** Days post inoculation
- **NMDS:** Non-Metric Multidimensional Scaling
- **OK:** Olive knot.
- **OTU:** Operational taxonomic unit
- ***Pss*****:** *Pseudomonas savastanoi* pv. *savastanoi*

1 -

Framework and objectives

1. Framework and objectives

Olive knot (OK), caused by *Pseudomonas savastanoi* pv. *savastanoi* (*Pss*), is one of the most important diseases of olive crop worldwide (Quesada et al., 2010). This disease produces tumorous galls or knots, mostly on stems and branches of olive trees, causing their death and loss of tree vigor (Penyalver et al., 2006). Like many other bacterial diseases, the control of olive knot is very difficult. This is mainly due to the difficulty in the eradication of the pathogen once established in an orchard (Quesada et al., 2012). Thus, most of the available control measures against olive knot are based on preventive procedures. These are very diverse and include, for instances, cultural practices that reduced the levels of pathogen inoculum or the rate of disease spread, and chemical sprays, essentially carried out with copper compounds, with low efficacy (Quesada et al., 2012).

Microbial communities inhabiting the internal plant tissues (referred as endophytes) have already been described to play a pivotal role in the protection of plants from pathogenic microorganisms, improving plant health (Datlof et al., 2017). Such action has been mostly recognized for herbaceous plant species (Ab Rahman et al., 2018), whereas their role on woody plants protection against diseases has been less studied (Cazorla and Mercado-Blanco, 2016). This beneficial effect provided by endophytes to host plants open the possibility on their application for the management of diseases. Most of the research carried out in this regard have been tested the protective effect granted by endophytes to plants by mean of single-species inoculants (reviewed by Dutta et al., 2014). However, it is expected that the use of a combination of endophytes will improve the efficacy of the biocontrol activity of endophytes. Indeed, such strain combination could not only bring functional complementary as well as increase their survival to harsh conditions (De Vrieze et al., 2018).

The main aim of this work is to evaluate the efficacy of the application of a consortium of endophytic microorganisms on the control of olive knot disease. The endophytes tested were retrieved from one centennial olive tree with tolerance to OK. There are no resistant olive cultivars to OK disease. However, several studies showed that some olive tree cultivars appear to be more tolerant than others (e.g. Boussadia,et al., 2008). Similarly, in an olive orchard located in Mirandela (Northeast of Portugal) was identified the presence of a centennial olive tree with a higher tolerance to OK than nearby trees from the same olive grove (Rodrigues, 2018). Thus, this olive tree could be a good source of endophytic multi-species inoculant to be used in a biocontrol strategy against the OK disease. Moreover, this approach is highly favorable because these endophytes are already present in the hosts environment, and thus well adapted to environmental stresses common in their micro-habitat (Rabiey et al., 2019). Using this strain consortium might therefore lead to enhance the efficacy of the biocontrol activity of endophytes.

To reach this goal, the following specific objectives were set:

1) Evaluate the effect of the inoculation of olive plantlets with an endophytic inoculum retrieved from one centennial olive tree with tolerance to OK, on the control of olive knot disease.
2) Evaluate the impact of the inoculation on the indigenous stem endophytic bacterial community composition, with an attempt to elucidate the mechanism of biocontrol by means of microbial interaction.

2 - Introduction

2. Introduction

2.1. Olive tree: geographical distribution, production and constrains

The cultivated olive tree (*Olea europaea* L. subsp. *europaea* var. *europaea*) is one of the most important crop species in the Mediterranean region (Fig. 1) (Besnard, Cornille and Terral, 2018). The olive fruit is consumed as food either as table olives, or used for oil production, a product widely appreciated in the world, especially for its health benefits (Conte et al., 2020). Although it is recognized that the cultivation of olive trees is quite ancient, the exact domestication history of the olive tree is still unknown (Besnard, Cornille and Terral, 2018a). It has been thought that cultivated varieties were derived from the wild olive tree, so-called oleaster (*O. europaea* var. *sylvestris*) (Gros-Balthazard et al., 2019). This species is believed to originate from Asia Minor, which then spread to Greece, being endemic to the Mediterranean region (Aranda et al.,2011). Nowadays, olive crop is spread over Mediterranean-type climate regions (Besnard, Cornille and Terral, 2018b). In the last decade, olive oil and table olive production have increased worldwide because of the development of modern orchards, intensification of the traditional ones and expansion into new crop producing areas (Férnandez-Escobar, 2013). In Portugal, olive-derived products are a major contributor for the national economy. With an olive production of 876 215 tons in 2017, Portugal ranks 9th in the world (Fig. 1) and 4th in the EU, just after Spain, Italy and Greece (FAOSTAT, 2020). The largest part of the Portuguese olive plantation area is in the hinterland, mainly in the Alentejo and Trás-os-Montes countryside regions. Indeed, these two regions account for 80% of olive and 83% of olive oil production in Portugal (INE, 2020). Olive crop is therefore of great economic importance in these rural, depressed regions, playing a major role in the development of local populations, while helping to prevent rural exodus (Besnard, Cornille and Terral, 2018b). Yet, besides these socio-economic benefits, there are also economic and environmental impacts that should be stressed, some of them linked to pest and disease management.

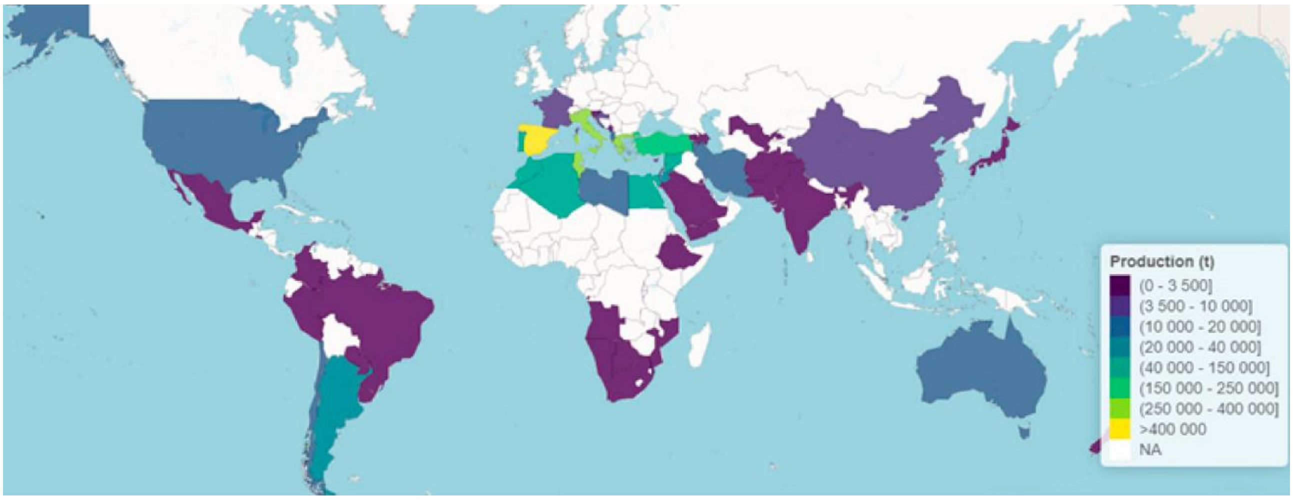


Figure 1. World and Europe Union production of olive oil for the 2019-2020 crop year. (Rocha et al, 2022).

Pest and diseases are main constraints in olive production, causing high yield losses (Férnandez-Escobar, 2013). It has been estimated that approximately 30% of the olives in the Mediterranean region are lost to pests and diseases, with annual control costs surpassing 200 million euros (Férnandez-Escobar, 2013). Their control is mostly based on the use of chemical pesticides, very often with limited efficacy (e.g., Cacciola et al. 2012; Daane et al. 2010; Férnandez-Escobar, 2013), and not compatible with sustainable production systems (i.e., organic and integrated production). Thus, in recent years, several efforts have been made to develop novel and environmental-friendly control strategies for management of olive tree pests and diseases, namely the use of biocontrol agents (Buonaurio et al., 2015; Fernández- Escobar et al., 2013; Rabiey et al., 2019; Preto et al, 2017).

2.2. The olive knot disease

Olive knot (OK) is a pathology caused by Gram-negative bacterium *Pseudomonas savastanoi* pv. *savastanoi* (*Pss)* to olive trees and considered as one of the most damages diseases in all olive growing areas in the world (Quesada et al., 2010). This bacterium produces tumors and galls (knots), especially in the stem and branches of olive tree (Fig. 2), through the production of indoleacetic acid (IAA), a plant growth hormone (Ouzari et al., 2008). Occasionally the bacteria can also infect leaves and fruits (Quesada et al., 2010). *Pss* is usually present in the phyllosphere, the above-ground portion of the plant, where it can colonize both the surface (i.e. epiphytic colonization) and the interior (endophytic) of olive tree tissues (Ramos et al., 2012). The infection is thought to be caused by epiphytic bacteria, which have shown their ability to survive for many years in the olive tree (Quesada et al., 2010). Wounds caused by harvesting, pruning, hail, frost, and leaf scars, permit epiphytic *Pss* to enter in the plant tissues and to generate knots (Quesada et al., 2010). Analogously to the tumor-inducing bacterium *Agrobacterium tumefaciens*, *Pss* may need plant-released signals from the wounds to activate the tumor formation, as when it enters through stomata it is not able to generate any visible symptoms (Lavermicocca et al., 2002). In fresh wounds of olive trees, the pathogen initially colonizes the tissues around the infection point and, through pectolytic and hemicellulolytic enzymes, disrupts the integrity of the host cells, producing cavities that are filled with the bacterium; alternatively, it can directly invade the xylem vessels (Buonaurio et al., 2015). In knots, *Pss* is normally found associated with other bacterial species, mainly belonging to the genus *Pantoea*, *Pectobacterium*, *Erwinia* and *Curtobacterium* (Buonaurio et al., 2015; Ouzari et al., 2008). By inducing an increase in the *Pss* population and consequently promoting the development of symptomatology, this multispecies consortium shown to play an important role in OK disease incidence and severity (Buonaurio et al., 2015).

The pathogen is usually introduced to new areas through infected plant material. The bacterium can survive and multiply as a saprophyte on plant surfaces (Ercolani, 1978, Quesada et al., 2010), as well as inside knots, from where it could be disseminated by rain, windblown aerosols, insects, and cultural practices, such as pruning.

Losses directly ascribed to OK are difficult to assess, but this disease is well recognized as one of the most damaging diseases of the olive crop (Ramos et al., 2012; Young, 2017). Typically, the OK disease causes (1) reduced tree vigor, (2) tree defoliation and consequently loss of photosynthetic area, (3) branches atrophy or dieback, which in severe cases can lead to tree death (Adaskaveg, Förster and Nguyen, 2017). Fruits from trees with olive knot symptoms can lead to olive oils with poor quality, rancid and salty with an unpleasant odor (De Vrieze et al., 2018; Quesada et al., 2010).

Figure 2. Olive knot disease observed in olive tree located in an orchard in Mirandela (Portugal). Gall formation on branches (**a**), twigs (**b**) and stems (**c**). Although rare, infection of fruit may also occur preventing fruit development and causing deformation (**c**). Credits: Paula Baptista, IPB-CIMO.

2.3. Olive knot management strategies

Current OK control methods are mainly preventive as it is quite difficult to eradicate the bacteria when it is already present in the olive grove (Quesada et al., 2010). In this context, it is recommended to use some cultural practices to prevent the disease, including: 1) plantations with tolerant cultivars; 2) cutting sick branches during pruning, taking care to disinfect wounds and cutting tools; 3) avoid cultural practices that may cause tree damage at periods of the year with high relative humidity, as it favors the infection; 4) efficient fertilization and watering to avoid wounds caused by defoliation; and 5) chemical control using copper-based fungicides, which is carried out to prevent infections. Indeed, chemical control of olive knot disease has given inconsistent results in fields experiments and even shows phytotoxicity to some tissues. This variability is due to several factors, such as timing of the treatments, climatic conditions, treatment application method, or physiological state of the host plant (Quesada et al., 2012). Therefore, the use of copper-based products to control olive knot, as in other bacterial plant diseases, is only effective when they are used in preventive strategies before the onset of infection or very early in the bacterial infection process (Quesada et al., 2012). Still, the effectiveness of these cultural practices and chemical treatments are usually low and the cost-benefit ratio is unfavorable to the olive grower (Quesada et al., 2010). Moreover, it was shown that some *Pss* strains are resistant to copper (Nguyen, Förster and Adaskaveg, 2017).

2.4. Biological control: a potential sustainable method for controlling olive knot

The lack of effective OK control methods, together with the growth of organic farming worldwide, has encouraged studies to identify biological control agents (BCA) that can be used against this disease (Quesada et al., 2010). Most of these studies revealed the potential of BCA for the control of *Pss* through *in vitro* or *in planta* assays performed under controlled conditions (Ghanney et al., 2016; Rokni- Zadeh et al., 2008; Krid et al., 2010; Lavermicocca et al., 2002; Maldonado-González et al., 2013). Both *Pseudomonas* and *Bacillus* strains are among the most exploited microorganisms as biocontrol agents against olive knot disease. For instances, several fluorescent *Pseudomonas* strains and *B. subtilis* were reported to inhibit significantly *Pss* under *in vitro* conditions (Rokni-Zadeh et al., 2013; Krid et al., 2010). Similarly, Lavermicocca et al. (2002) observed that the antimicrobial peptide bacteriocin produced by *P. syringae* pv. *ciccaronei* inhibited the multiplication of *Pss* and affected the epiphytic survival of the pathogen on the leaves and twigs of treated olive plants growing in controlled conditions. The inoculation of olive plants with *P. fluorescens* PICF7 was also showed the ability to decreased *Pss* population and restrained the pathogen to inner tissues of the tumors but was not able to suppress OK disease (Maldonado-González et al., 2013). Likewise, Ghanney et al. (2016) reported that the inoculation of olive plants with *B. mojavensis* ABC-7 can decrease knot weights and *Pss* population size, producing less necrotic tumors. More recently, Mina et al. (2020a) showed the ability of 27 bacterial strains to significantly inhibit *Pss* growth under *in vitro* conditions, being *Bacillus amyloliquefaciens* the most effective. This strain was subsequently tested in *in planta* experiments, showing the ability to significantly reduce OK disease severity (43.7%), knots weight (55.4%) and population size of *Pss* (26.8%) under greenhouse conditions (Mina et al., 2020a).

Although these works reveal encouraging results, the tested microorganisms did not meet the required level of biocontrol efficacy in terms of OK disease suppression. One possible factor underlying this lack of success might be related with the use of a single strain. It is expected that the use of different strains of biological control species, by combining various modes of action, will prevent failures and consequently improve biocontrol efficacy (Köhl et al., 2019). Such approach based on strain combinations could not only bring functional complementary, but it could also be useful in promoting the establishment and survival of the biocontrol agent in its host, which in turn is influenced by many factors including the resident microbiota (Ciancio, Pieterse and Mercado-Blanco, 2016). To date, few studies have tested the impact of mixed strains rather than single ones on plant protection against diseases. In maize, the application of a consortium of seven different bacterial species was showed to be effective in protect host plant against *Fusarium* (Niu et al., 2017). A better protection of tomato against Ralstonia-induced wilt was also observed when using a mixture of several *Pseudomonas* strains than when applying each strain individually (Hu et al., 2016). More recently, several *Pseudomonas* strains were showed to provided significantly better protection against *Phytophthora infestans* on leaf disks of potato when used in combination than when applied alone (De Vrieze et al., 2018).

The potentialities in using strain consortia for the control of OK disease have never been exploited. In this strategy both "natural" and "artificial" microbial consortia can be used. In the former case, it is expected that the microbial consortia will be easily established and survive in the host plant than "artificial" consortia. Indeed, the native microorganisms are already adapted to the crop, the resident microbiota and the environment in which they will be deployed. This aspect is of utmost importance for olive tree phyllosphere-associated microbial 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) (Gomes et al., 2019; Mina et al., 2020b). The presence and survival of a foreign microorganism in a particular microenvironment such as a plant tissue, or organ, result from beneficial (e.g. cooperation) and/or deleterious (e.g. competition) interactions exerted by other microorganisms present in the microbial community, and by the host plant (Jakuschkin et al., 2016). The additional advantage of exploiting biocontrol traits from indigenous olive-microorganisms is related with the compatibility of strains, since they are sharing the same niche. This strain's compatibility was showed to be important toward disease suppression (De Vrieze et al., 2018). It is then expected that the use of native, naturally collaborative microbial consortia should increase the biological control efficacy compared to the use of single-species microbial inoculants (Backman and Sikora, 2008). Hence, the use of endophytic microbial consortium obtain from olive tree could be a promising tool to suppress OK disease on olive crops. Such approach has been applied mostly in humans for treatment of several diseases (Van de Burgwal et al., 2018). The best example of the suppression of plant diseases through this approach is by using microbial consortia from suppressive soils. There are some studies showing that the transfer of natural microbial communities from disease suppressive soils to disease conducive ones, is effective in the management of several root plant diseases, including potato common scab, rhizoctonia root in sugar beet and tobacco black root rot (Gopal, Gupta and Thomas, 2013).

In particular, for the control of OK disease, the transplantation of endophytic microbiota from olive cultivars that show a certain degree of OK disease tolerance/resistance may be an interesting approach. It is expected that part of this tolerance/resistance comes from the endophytic microbial community that colonizes the host plant (Busby et al., 2016) due to the recognized impact of endophytes in improving host plant resistance to diseases (Latz et al., 2018).

Native plants and / or not subjected to an intensive breeding process tend to have a greater diversity of endophytic microorganisms as well as resistance to diseases than cultivated plants. Thus, it is expected that wild, unimproved (i.e., not subjected to breeding practices) or centennial plants, may have their resistance to certain diseases associated with this endophytic diversity (Zahn and Amend, 2017).

2.5. Endophytic microbiota and their mode of action in biological control

Endophytes are microorganisms that dwell within robust plant tissues by having a symbiotic association. They are associated with almost all plants studied till date. They also produce a wide range of compounds useful for plants for their growth, protection against environmental conditions (Nair and Padmavathy, 2014). However, the composition of the endophytic community can change depending on host genetics (Rybakova et al., 2016), geographic location of the plant (Martins et al., 2016), local environmental conditions (Gomes et al., 2018), pathogen infections (Gomes et al., 2019) and anthropogenic practices (Varanda et al., 2016). The plant-endophyte is seen as a mutualistic association, in which the microorganism receives nutrients and protection from the host, while the plant can see improved their resistance / tolerance to biotic (e.g., pathogenic, herbivorous, pest) and abiotic (e.g., drought, salinity, heavy metals) stresses (Suryanarayanan, 2013).

Endophytes can protect crops from damage by diseases via different modes of action, which have recently been reviewed by Fadiji et al. (2020). Plant defense via endophytes is mainly attributed to the production of endophyte secondary metabolites in the host plant (Suryanarayanan, 2013). These metabolites may directly inhibit phytopathogens growth through antibiosis and parasitism, or indirectly by inducing resistance or priming in the host plant (Latz et al., 2018). Another indirect way to antagonize pathogens is through competition for nutrients and space

(Backman and Sikora, 2008). Some of the compounds that inhibit pathogens include antibiotics (e.g., terpenoids, alkaloids and polypeptides), volatile organic compounds (e.g., acids, alcohols, alkyl pyrones, ammonia, esters, hydrogen cyanide, and ketones), iron- chelating compounds (e.g., siderophore), quorum sensing inhibitors and hydrolytic enzymes (Gao et al., 2010; Ownley et al., 2010).

3– Material and Methods

3. Material and methods

3.1. Isolation of endophytic bacteria from centenary olive tree

The endophytic bacteria was isolated from a centenary olive tree (Fig. 3), located in an olive grove over 250 years old in Sucçães (N 41º 29' 26.628"; W 7º 15' 31.219"), Mirandela (Northeast of Portugal). There are no olive cultivars resistant to olive knot, but some of them have been shown to be more tolerant to this disease than others (Young et al., 2004; Godena et al., 2012). The centenary olive tree used in the present study to isolate the endophytic microbiota was selected based on its high tolerance towards olive knot. Field observations over the last seven years, have been indicating that this centenary olive tree expressed lower incidence of infection rate and smaller galls than other trees present in the same orchard (Unpublished results). Asymptomatic branches were collected from the selected centenary olive tree on July 30, 2019, placed in sterile bags and transported at 4ºC to the laboratory.

The isolation of the endophytic bacteria from the harvested branches were performed using the methodology previously described by Ikeda et al. (2009), with some modifications. Accordingly, after washing the branches in running tap water, the leaves were removed, and the twigs (with 2 to 4 mm in diameter) were cut to about 5 cm length with a sterile scalpel. These twigs were then surface sterilized in order to eliminate the epiphytes, by using a procedure previously optimized. This consisted in the sequential immersion of twigs in 70% ehanol (v/v) for 2 min, sodium hypochlorite (5%, v/v chlorine) for 2 min, 70% ethanol (v/v) for 1 min, and three washes in sterile distilled water (2 min each). For microbiota extraction, 50 g of the sterilized twigs were weighed into 250 ml of bacterial cell extraction (BCE) buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 1% sodium metabisulfite and 4 g/L of polyvinylpolypyrrolidone - PVPP) and homogenized in T 25 ULTRA-TURRAX® (IKA) on high speed for six 1-min periods, under ice for cooling. The homogenate was filtered through a layer of sterilized Miracloth (CalBiochem, USA) and the filtrate was then centrifuged at 1,800 rpm (Eppendorf® Microcentrifuge model 5415R) for 5 min at 10°C. The supernatant was transferred to a clean tube and centrifuged at 6,000 rpm (Eppendorf® Microcentrifuge model 5415R) for 20 min at 10°C. The supernatant was discarded, and the pellet was suspended in 25 ml of BCE buffer (without sodium metabisulfite and PVPP). After filtration of the suspension through a layer of sterilized Kimwipe (Merck) to remove insoluble particles, the filtrate was centrifuged at 10,000 rpm (Eppendorf® Microcentrifuge model 5415R) for 10 min at

10°C. The supernatant was discarded, and the pellet was suspended again in 25 ml of BCE buffer (without sodium metabisulfite and PVPP). After suspension´s filtration on sterilized Kimwipe, the final filtrate was suspended in 3 ml of 50 mM Tris–HCl pH 7.5. This suspension was further overlaid on 2 ml of Histodenz (Nycodenz, Sigma-Aldrich) solution (8 g of Histodenz dissolved in 10 ml of 50 mM Tris–HCl pH 7.5), and centrifuged at 9,000 rpm (Eppendorf® Microcentrifuge model 5415R) for 40 min at 10°C. After centrifugation, the whitish band located at the interface of the upper and lower phases (Fig. 3b) was collected to a new tube, to which the same volume of sterile phosphate-buffered saline (PBS) solution at pH 7.0 was added. This solution, which contains the endophytic bacterial, was used for plant inoculation assays. Part of this solution was preserved in glycerol (30%, v/v) at -80ºC for metagenomic analysis of the bacterial communities.

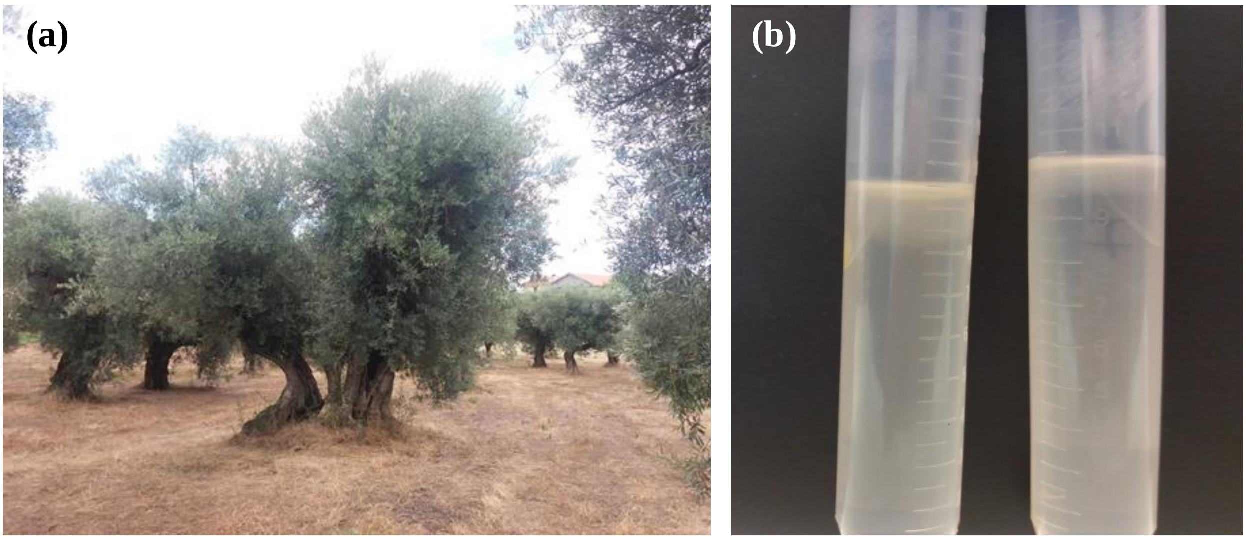


Figure 3. Isolation of endophytic bacteria from twigs of centenary olive tree. (**a**) Centenary olive tree used for the isolation of bacteria; (**b**) and endophytic bacteria (white band in the interface zone) extracted from its twigs. Credits: Paula Baptista, IPB-CIMO.

3.2. Pathogen inoculum production

Pseudomonas savastanoi pv. *savastanoi* strain EnVN39 was obtained from the microbial cultural collection of the Mountain Research Centre (CIMO-CC), Instituto Politécnico de Bragança (Portugal). This isolate was originally 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 et al., 2019) and V1–V4 regions from 16S rRNA gene, as described in Mina et al. (2020a). These sequences were deposited in GenBank. 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 the cells produced 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 sterile PBS buffer pH 7.0, and further used as an inoculum in plant assays.

3.3. Plant inoculation and experimental design

The experiments were conducted with one-year-old olive plantlets cv. *Cobrançosa* obtained from propagation of semi-woody cuttings as described by Mina et al. (2020a). Plantlets were grown in 500-mL pots filled with topsoil-perlite (3:1) mixture under controlled conditions (day/night thermal regime of 23º/18º ± 2ºC, 10 h light/14 h dark photoperiod and 70 ± 10% relative humidity), in the greenhouse of the Instituto Politécnico de Bragança, School of Agriculture. Four treatments were established: control plants mock-inoculated with `PBS pH 7.0; plants inoculated with the endophytic bacteria (bacteria); plants inoculated with the pathogen *P. savastanoi* pv. *savastanoi (Pss*); and plants inoculated with the endophytic bacteria and seven days later with the pathogen (bacteria+7d *Pss*). Inoculation of olive plantlets was performed according to Mina et al. (2020a). Briefly, 10 µl of inoculum was deposited in a V-shaped wound of 1 cm long (with about 2 mm deep by 5 mm wide) previously made on the main stem with a sterile scalpel. Each wound was wrapped with parafilm, and further removed one week later. A randomized complete block design with six replications, each consisting of 5 plantlets, was carried out per treatment (i.e, 30 olive plantlets per treatment). Plantlets were automatically sprayed for 10 seconds, every 40 minutes, during the first week after inoculation to maintain high humidity conditions. Afterwards, plantlets were watered when needed until the end of the assay (63 days post-inoculation, DPI); and were kept under the same greenhouse conditions mentioned above.

3.4. Disease assessment

Both the incidence and severity of olive knot was evaluated visually every 7 days, for 63 days after pathogen inoculation, in each treatment. Disease incidence was determined by dividing the number of plantlets that developed knots by the total number of plantlets assessed, in each treatment. Olive knot severity was evaluated by using the 1-6 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. The data of the incidence and severity was further used to calculate the areas under disease progress curves, using the following formula:

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

where the Yi represents the disease incidence or severity (1 to 6 scale) at ith observation, ti is the time (days) at the ith observation, and n is the total number of observations in which the olive knot disease development was recorded. Analysis of variance (ANOVA) was performed for both $\mathrm{AUDPC}_{\mathrm{incidence}}$ and $\mathrm{AUDPC}_{\mathrm{severity}}$ by multiple comparisons test (Tukey's method) to determine whether there exists a significant difference among treatment means at $p < 0.05$. Data were tested for homogeneity of variance before analyses, being transformed using log(x+1) in order to homogenize the variances.

3.5. Evaluation of endophytic bacterial community

At the end of the assay, plants were harvested and the endophytic bacterial community in all the treatments were also evaluated by 16SrRNA-based metagenomic analysis. This analysis also included the endophytic bacterial inoculum used to inoculated olive plantlets.

3.5.1. DNA extraction

Twigs segments from the inoculation site were surface disinfected using the same procedure previously described in section 3.1, and further grinding in liquid nitrogen with a mortar and pestle, before DNA extraction. The genomic DNA was extracted through an adaptation of the Healey et al. (2014) protocol. Around 0.1 g of grind twigs was suspended in 1.2 mL of pre-heated extraction buffer (0.1 M Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 2% (w/v) hexadecyltrimethylammonium bromide - CTAB) and kept at 70 °C, for 20 min, with intermittent mixing at every 5-min. Then, the tubes were centrifuged at 13,000 rpm for 5 min, at 4ºC, and the supernatant was collected to a new tube. To each tube, 1 volume of chloroform was added, and the solution was gently mix by inversion for 5 min. Aqueous phase was collected after centrifugation at 13,000 rpm for 10 min, at 4ºC. The extracted DNA was precipitated by adding 3 volumes of cold absolute ethanol and kept at -20 °C, for 1 hour. The final DNA precipitate was pelleted at 13,000 rpm, for 10 min, at 4ºC, and washed with 3 mL of 70% ethanol. After centrifugation at 13,000 rpm for 10 min, at 4ºC, the DNA pellet was air-dry and suspended in 40 µL of ultra-pure water. The same procedure was used to extract DNA from the bacterial inoculum, with exception of the first step where genomic DNA was extracted with the same volume of extraction buffer.

3.5.2. Bacterial 16S amplification and sequencing

In order to assess the integrity and suitability of the extracted DNA, the V3-V4 of 16S rRNA gene sequence was amplified, using the universal primer set of 341F (CCTACGGGNGGCWGCAG) and 785R (GACTACHVGGGTATCTAATCC) (Klindworth et al., 2013). PCR reactions were conducted in a total volume of 50 µL, containing 5 µL of 10x complete PCR buffer (pH 8.8, 0.1% tween 20, 25 mM $MgCl_2$), 1 µL dNTPs of 10 mM, 1 µL of each primer (10 µM), 4 µL of DNA extract, 0.2 µL of DFS-Taq DNA Polymerase (5 U/µL) (BIORON GmbH) and 37.8 µL of ultra-pure water. Negative controls comprised reactions with water replacing template DNA. Amplifications occurred in a MyCycler™ (Bio-Rad) thermocycler, with a program set for an initial denaturation step at 95ºC for 5 min, followed by 30 cycles of 94ºC for 40s, 48ºC – 56ºC for 50s, 72ºC for 45s, with a final extension step at 72ºC for 7 min. PCR products were run on a 1% (w/v) agarose gel, stained with GelRed® (Biotium) and visualized by fluorescence under ultraviolet radiation using a ChemiDocTMXRS transilluminator (BioRad). All the DNA samples resulted in a fragment of the expected size (≈450 bp). The quantity of purified DNA was determined in a micro-volume spectrophotometer, mySPEC (VWR, Portugal), and equimolar aliquots of 10 replicate plantlets samples were then combined to get three biological replicates per treatment.

Bacterial endophytic communities were assessed by metabarcoding (Illumina MiSeq system, Illumina), through paired-end sequencing (2 × 250 bp) for the V4 region of the 16S rRNA gene, using a sequencing service provider (Instituto Gulbenkian de Ciência - Genomics Unit, Portugal). The sequencing was performed with the primer set 515f (GTGYCAGCMGCCGCGGTAA) and 806r (GGACTACNVGGGTWTCTAAT), according to Walters et al. (2016).

3.5.3. Processing of sequencing data

The analyse of the 16S rRNA gene sequences from the studied microbial communities was performed in QIIME 1.9.1 (Kuczynski et al., 2011). Raw forward and reverse reads (from Illumina MiSeq.fastaq files) were merged, and subsequent quality control and demultiplexing steps were performed simultaneously by using the “multiple_split_libraries_fastq.py” script. Clustering of sequences into operational taxonomic units (OTUs) was performed using the "pick_open_reference_otus.py" script, with all default parameters. This script performs open reference clustering against the Greengenes v.13.8 database (OTUs clustered at 97%), using UCLUST v.1.2.22q, and allows to centroid-based clustering of unclassified reads to generate *de novo* references (Edgar, 2010; Rideout et al., 2014). Unassigned sequences and low abundance taxa (< 5 reads in all samples) were removed from the operational taxonomic unit (OTU) tables before downstream analysis.

3.5.4. Diversity and composition of bacterial community

Diversity of bacterial inhabiting olive plantlets from the different treatments was assessed by evaluating the richness (number of OTUs) and by computing Shannon–Wiener (H') diversity index with PAST3 (Harmmer & harper, 2001). The same software was used to generate box plots to depict diversity results and to performed one-way analysis of variance (ANOVA), with a Tukey's post-hoc comparison test ($p < 0.05$), to check differences among treatment diversity means. Before analysis, the normality of data was checked by using Shapiro-Wilk test.

To explore the similarity of bacterial community's composition between the different treatments, a non-metric multidimensional scaling (NMDS) was carried out with Bray-Curtis dissimilarity coefficients, that takes into consideration the presence/absence and abundance of bacterial taxa. Analysis of similarity (ANOSIM) was then conduct using Bray-Curtis dissimilarity matrices, to check for significant differences between bacterial communities. The R value generated by ANOSIM gives the degree of discrimination between groups and ranges from 0 (indistinguishable communities) to 1 (completely dissimilar) (Clarke & Gorley, 2015). NMDS and ANOSIM were performed using Community Analysis Package v. 4.0 (Seaby and Henderson, 2014). The differences in abundance of bacterial genera between plantlets inoculated with bacteria+7d *Pss* and *Pss* or bacteria was quantified as the log2-fold changes.

4– Results and Discussion

4.1. Effect of endophytic bacteria on olive knot development

The capacity of the endophytic bacteria isolated from centenary olive tree in reducing incidence (AUDPCi) and severity (AUDPCs) of olive knot, caused by the pathogen *P. savastanoi pv. savastanoi* (*Pss*), was evaluated by inoculation of olive plantlets in greenhouse conditions. Results showed that the inoculation of the endophytic bacteria reduced significantly ($p<0.05$) progress curve for incidence (up to 1.6-fold) and severity (up to 2.1-fold) of olive knot in comparison with plantlets inoculated solely with *Pss* (Fig. 4). On day 21, 67% incidence was reached on olives inoculated solely with *Pss*, while in bacteria+7d *Pss* treatment the incidence was 43% and remained stable until the end of the assay. Similarly, plantlets from the treatment bacteria+7d *Pss* plantlets did not exceed in average the severity index 2 (mild thickening of the wound) until the end of the assay, while olives inoculated with *Pss* have reached the severity index 4 (small knots at both the base and the top of the wound) at day 42 post-inoculation. Overall, this result showed that the consortium of the native bacteria retrieved from centenary olive tree was effective in reducing OK disease development. Thus, it is likely that the presence of these endophytes benefits the host plant by protecting it against *Pss* infection. We hypothesized that this beneficial effect might be associated with diverse protective mechanisms displayed by the endophytes, including direct inhibition of the *Pss* through antibiosis, mycoparasitism and competition, or indirect inhibition through induction of systemic resistance (ISR) or systemic acquired resistance (SAR) in the host plant (Fadiji et al., 2020). Moreover, our results showed that the centenary olive tree used in this study could be an important source of inoculum with valuable biocontrol proprieties for application in agriculture. The olive tree is known to host a great number of endophytic species, being some of them rare and unidentified (Mina et al., 2020b; Nicoletti et al., 2020; Costa et al., 2021) and, in particular the centenary olive tree used in the present work, seems to represent a repository of potential biocontrol agents against olive diseases. Although no specific works are available on olive tree, previous studies on *Vitis vinifera* L. reported that the endophytes colonizing resistant cultivars could contribute to high stress resistance of host plant towards pathogens (Kernaghan et al., 2017; Li et al., 2020). Thus, we hypothesized that the same could happen for the centenary olive tree used in the present work.

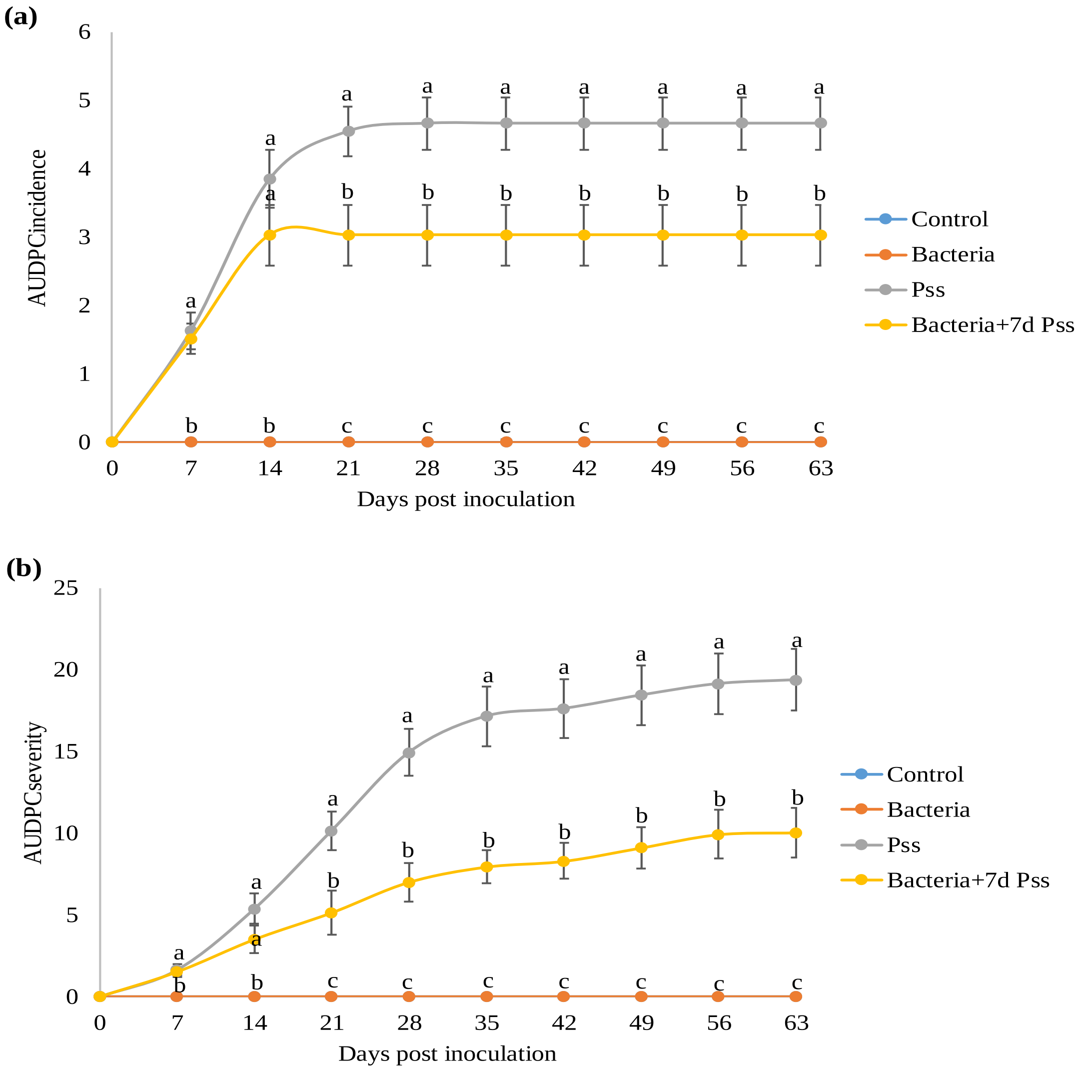


Figure 4 - Area under disease progress curve – AUDPC of incidence (**a**) and severity (**b**) of olive knot in olive plantlets inoculated with buffer (control), the endophytic bacteria (bacteria), the pathogen *Pseudomonas savastanoi pv. savastanoi* (Pss), the endophytic bacteria and seven days later with pathogen (bacteria+7d Pss). Data is presented as means ± SE (n=6). Statistically significant (p<0.05) differences between the four treatments, in each day, are indicated by different letters.

4.2. Effect of endophytic bacteria on native bacterial communities of olive plantlets

After 63 days post-inoculation, the olive plantlets from the four treatments (i.e., plants mock-inoculated with buffer – control, inoculated with endophytic bacteria, *P. savastanoi pv. savastanoi* (*Pss*), or with bacteria and 7 days later with *Pss*) were collected, and the endophytic bacteria communities inhabiting the portions of the stems inoculated were evaluated by metabarcoding. Overall, a set of 33,485 paired-end Illumina MiSeq V4 raw reads was generated, ranging from 3,971 to 393 raw reads per sample. After quality evaluations, a total of 1,705 high-quality sequences could be recovered (i.e. reads not corresponding to unassigned or plant organellar genome sequences).

Thus, almost 5,09% of total processed reads were classified as bacterial taxa from the Greengene reference collection (140 OTUs) or were unclassified, new reference taxa (24 OTUs). From those taxa, 55 OTUs comprised more than 5 reads and were used for further analysis. Most of these OTUs were identified up to genus level (71%), being only few bacterial OTUs (7%) identified to species level. This could be related with the limited genetic variation within V4 regions among closely related species (Johnson et al., 2019).

The overall bacterial communities inhabiting the olive plantlets were predominantly composed of Proteobacteria (87% of total reads) phylum, being the most represented genera *Pseudomonas* (68%) (Fig. 5a). The inoculum isolated from the centenary olive tree was composed mostly by bacteria from Proteobacteria (49% of total reads) and Tenericutes (25%) phyla, and *Mycoplasma* genus (25%) (Fig. 5b). Our results are in accordance to previous studies, being Proteobacteria reported to be the main phylum of olive tree endosphere of European olive cultivars (Müller et al., 2015; Giampetruzzi et al., 2020; Mina et al., 2020b; Federico et al., 2022). Similarly, *Pseudomonas* was often observed as one of the main inhabitants of the olive tree endosphere (Müller et al., 2015; Mina et al., 2020b; Federico et al., 2022). Therefore, these results suggest that the olive tree may possess a species-specific resident bacteria community in the endosphere, as previously suggested by other authors (Mina et al., 2020b; Federico et al., 2022). Interestingly, the bacterial inoculum from the centenary olive tree revels to be dominated by members of the *Mycoplasma* genus. As far as we known, this is the first report of the presence of this genus in the endosphere of olive tree, suggesting that centenary olive trees may represent a rich repository of rare and new endophytes. Although *Mycoplasma* species are mostly known to establish an endosymbiotic relationship with some red algae (Hollants et al., 2011), they have been also reported as plant endophytes (Bandra et al., 2006; Gouda et al., 2016).

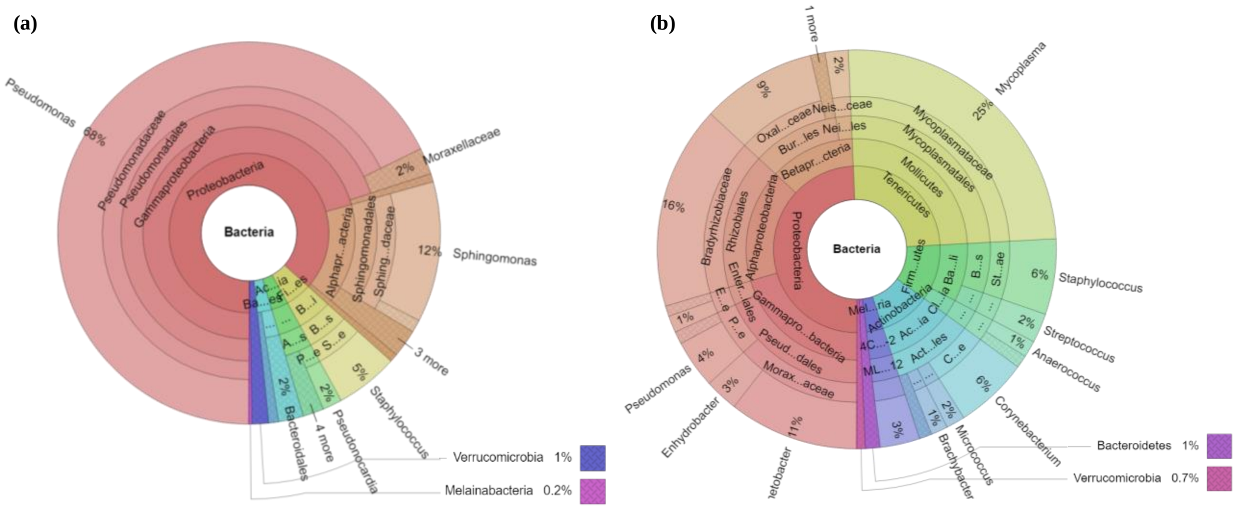


Figure 5 - Krona chart representing the relative abundance of operational taxonomic units (OTUs) of the whole endophytic bacterial communities in inoculated olive plantlets (**a**) and on the inoculum isolated from centenary olive tree (**b**). Figure was constructed using Krona tool (Ondov et al., 2011).

The inoculation of olive plantlets by the endophytic bacterial inoculum retrieved from the centenary olive tree did not significantly (p=0.334) affect the diversity of native bacterial communities, estimated by the richness and Shannon diversity index (H) (Fig. 6). However, both NMDS plots and ANOSIM analysis based on Bray-Curtis dissimilarity indexes (that considers the occurrence and abundance of species) showed that the native bacterial community composition varies significantly (global R=0.32, p=0.001) among the four treatments (Fig. 7). These differences were noticed mostly between plantlets inoculated with endophytic bacteria+7d *Pss* and control (R=0.67, p=0.05), suggesting a large impact of co-inoculation of bacteria and *Pss* on native bacteria community composition. In what concerns single inoculations, only plantlets inoculated with *Pss* showed a significantly different bacterial composition from the control (R=0.52, p=0.05), while plantlets inoculated with bacteria displayed a similar microbial composition to control plantlets (R=0.11, p > 0.05). This result suggests a greater impact of *Pss* on native bacterial community composition when compared to bacteria, which has a negligible effect. The comparison of bacterial composition between the treatment “bacteria+7d *Pss*” and *Pss* or bacteria, also reflect the greater effect of *Pss*, as only significant differences were found on native bacteria composition between “bacteria+7d *Pss*” and bacteria treatments (R=0.56, p=0.05). Previous studies have similarly found a change on the endophytic microbial community composition upon pathogen invasion (Gao et al., 2021; Jiemeng et al., 2022), including of *Pss* (Gomes et al., 2019). Endophytic microbial community assembly is mainly influenced by the host plant, environmental factors, and interactions among microbial members (van der Heijden and Hartmann, 2016; Durán et al., 2018). Therefore, it is likely that the *Pss* invasion might cause a disruption/change of the interactions, either cooperative or competitive, establish among the different microbes of the endosphere resulting in a shift of the endophytic composition. Likewise, the host plant itself may contribute for the change of the endophytic community, by recruiting different beneficial bacterial taxa to relieve stress from *Pss* invasion. This recruitment upon biotic or abiotic stresses was previously suggested to be a survival strategy conserved across the plant kingdom, called as the “cry for help” strategy (Gao et al., 2021; Liu et al., 2021). These hypothesis needs to be confirmed in future works.

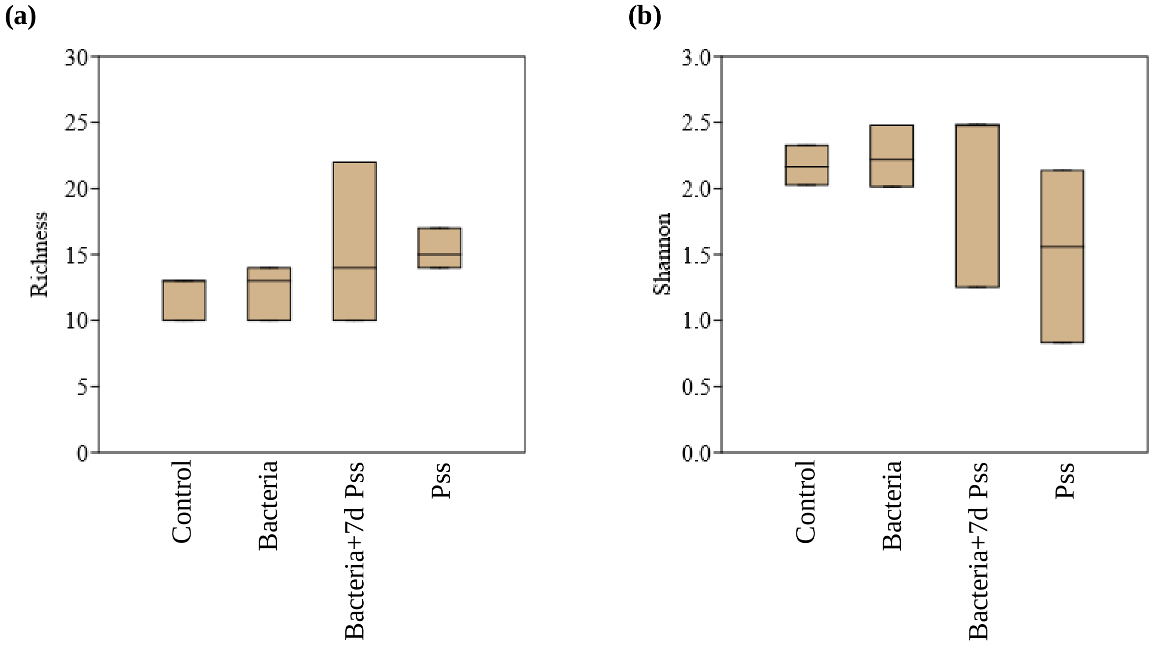
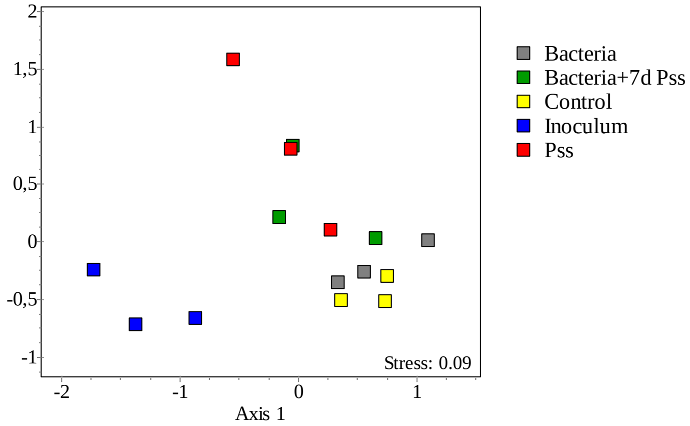


Figure 6 - Richness (**a**) and diversity (Shannon index, **b**) of the bacteria community in the olive plantlets mock-inoculated with buffer (Control), inoculated with endophytic bacteria (Bacteria), *Pseudomonas savastanoi pv. savastanoi* (*Pss*), or with endophytic bacteria and 7 days later with *Pss* (Bacteria+7d *Pss*). Box plots depict medians (central horizontal lines) and the inter-quartile ranges (boxes). Results are presented as mean ± SE (N = 3). No statistically significant differences (at $p<0.05$; one-way ANOVA, Tukey test) were found among the different treatments.



Pair comparison	**ANOSIM**
Control vs. Bacteria	0.11
Control vs. Pss	0.52*
Control vs. Bacteria+7d Pss	0.67*
Bacteria+7d Pss vs. Bacteria	0.56*
Bacteria+7d Pss vs. Pss	-0.11
Pss vs. Bacteria	0.48*

Figure 7 – Non-metric multidimensional scale (NMDS) plots corresponding to the clustering of bacterial endophytic communities inhabiting olive plantlets mock-inoculated with buffer (Control), inoculated with endophytic bacteria (Bacteria), *Pseudomonas savastanoi pv. savastanoi* (*Pss*), or with endophytic bacteria and 7 days later with *Pss* (Bacteria+7d *Pss*), as well as bacterial community present in the inoculum isolated from the centenary olive tree. Analysis of similarity (ANOSIM) showing the differences of the bacterial community composition between the several treatments is depicted on the right table. Asterisks indicate statistically significant differences (*$p < 0.05$). Cluster analysis and ANOSIM were performed with Bray-Curtis coefficient. Kruskal's stress values less than 0.2 represent good ordination plots.

The taxonomic differences of endophytes among treatments were evaluated by comparing differences on the relative abundances at family level (Fig. 8). The results showed that the endophytic community of plantlets inoculated with bacteria and control displayed a similar microbial composition, being dominated by members of the family Sphingomonadaceae. Members of this family are widespread in nature, occurring in soils, corals, eutrophic waters, clinical samples, and also in plants as endophyte or epiphyte (Balkwill et al., 2006). They are also recognized to display a variety of ecological roles, including plant pathogenic species (Grace et al. 2005), as well as species with beneficial effects such as plant growth promotion (Asaf et al., 2020) or disease reduction (Wachowska et al., 2013; Kämpfer et al., 2014).

Plantlets inoculated with *Pss* or with bacteria+7d *Pss* showed a similar bacteria endophytic composition. These two treatments were well-distinguished from the others, mainly due to the high abundance of Pseudomonadaceae members, that include the genus *Pseudomonas* (Fig. 8). This result corroborates the greater impact of *Pss* when compared to the bacteria inoculum, on the native bacteria composition, as previously reported. Members of the Pseudomonadaceae family are widely distributed, and includes pathogenic species of several crops (Xin et al., 2018; Buonaurio et al., 2015), including olive tree (Quesada et al., 2010), as well as species with plant growth promotor abilities or biocontrol proprieties against pathogens (Panpatte et al., 2016), including of olive tree (Gómez-Lama et al., 2018).

The composition of the inoculum is the most diverse, being dominated by members of the families Mycoplasmataceae and Nocardiodaceae (Fig. 8). Members of the family Mycoplasmataceae are distinguished phenotypically from other bacteria by their minute size and total absence of a cell wall. They are widespread in nature as parasites of humans, mammals, and plants (Bennett et al, 2019). Members of Nocardiodaceae family are known to be a gram-positive bacterium, some species are nonpathogenic while others are responsible for Nocardiosis disease. They tend to be found in soil rich in organic matter (Ryan et al., 2004).

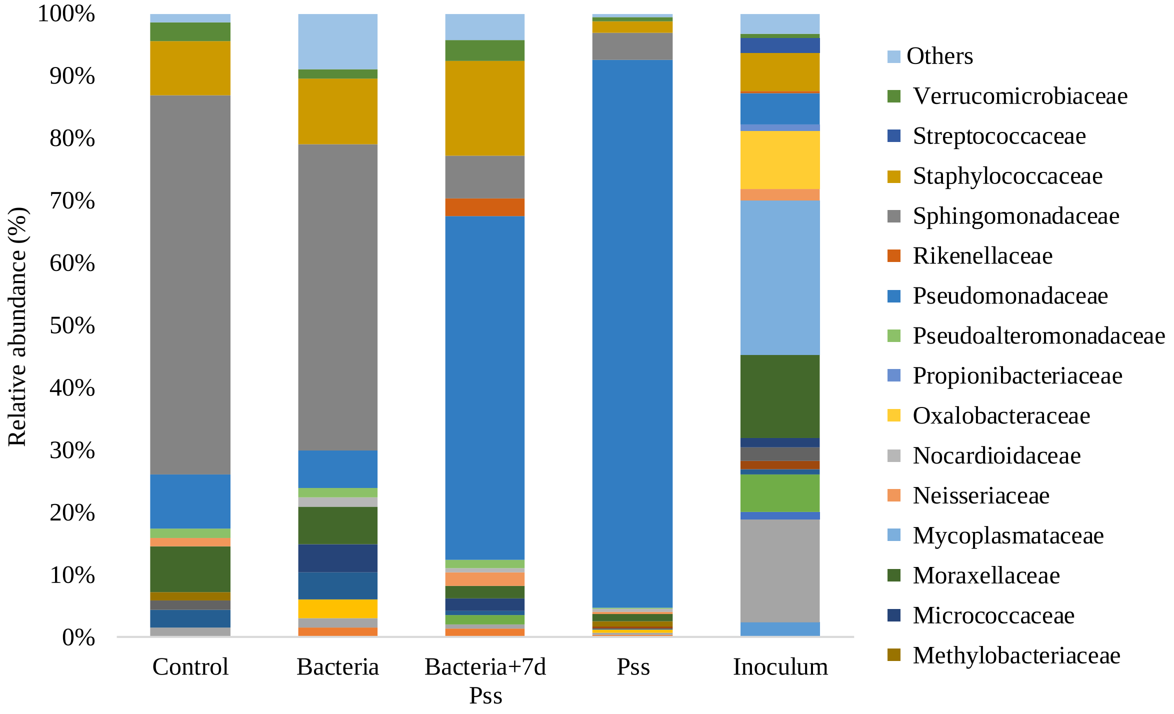


Figure 8 – Relative abundance of bacterial (at family level) inhabiting olive plantlets mock-inoculated with buffer (Control), inoculated with endophytic bacteria (Bacteria), *Pseudomonas savastanoi pv. savastanoi* (Pss), or with endophytic bacteria and 7 days later with Pss (Bacteria+7d Pss), as well as bacterial community present in the inoculum isolated from the centenary olive tree.

The bacterial genera/families with significantly differential relative abundance between olive plantlets inoculated with bacteria+7d *Pss* and plantlets inoculated with *Pss* or bacteria are depicted in

Figure 9. Interestingly, the results showed a decrease in abundance of *Pseudomonas* spp. in plantlets inoculated with bacteria+7d *Pss* in relation to plantlets solely inoculated with *Pss*, suggesting a reduction of pathogen abundance due to bacteria inoculation. Besides *Pseudomonas*, members of *Methylobacterium* and *Mycoplasma* genera also reduced significantly their abundance in bacteria+7d *Pss* plantlets in relation to *Pss* inoculated plantlets. *Methylobacterium* spp. includes species with beneficial effects on plants, including promotion of plant growth (Dourado et al., 2015) and antagonism of plant pathogens *in vitro* and *in vivo* (Grossi et al., 2020). In contrast, other genera/family increase significantly in abundance, namely Micrococcaceae, Rikenellaceae and *Corynebacterium*, in bacteria+7d *Pss* plantlets in relation to *Pss* inoculated plantlets, suggesting to play an important role on reducing the development of olive knot disease. Indeed, members of these Micrococcaceae (Uhlig et al., 2021) and Rikenellaceae (Zhang et al., 2022) families have been already reported to displayed antagonistic activity against several plant pathogens. Likewise, some members of the genus *Corynebacterium* were used to control different plant diseases, including olive leaf spot, which is a disease caused by the fungus *Spilocaea oleaginea* in olive tree (Al-Khatib et al., 2010).

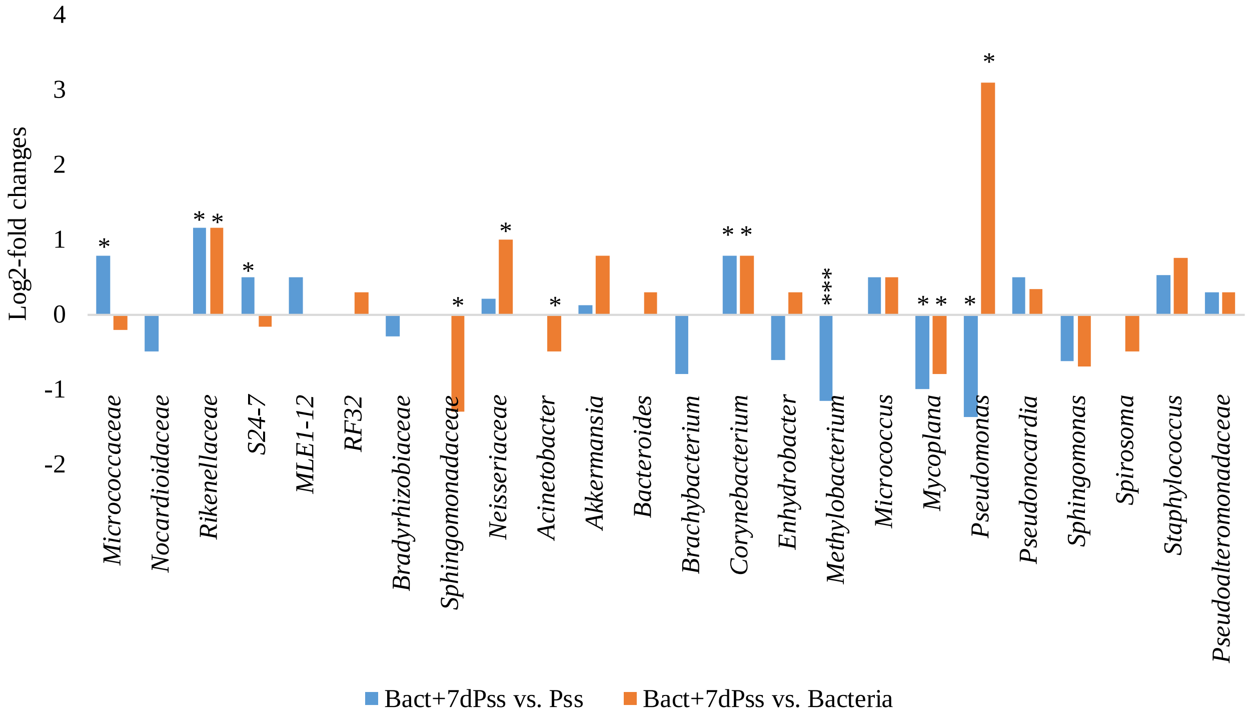


Figure 9 - Log2-fold changes in the abundance of bacterial genera/family in olive plantlets inoculated with bacteria and 7 days later with *Pseudomonas savastanoi pv. savastanoi* (Bact+7d *Pss*) in relation to plantlets inoculated with *P. savastanoi pv. savastanoi* (*Pss*) or bacteria (Bacteria).

Asterisks indicate statistically significant differences between pair comparison (*$p < 0.05$; ***$p < 0.001$).

In comparison to bacteria treatment, the olive plantlets inoculated with bacteria+7d *Pss* showed a significant increased on *Pseudomonas* abundance, followed by Rikenellaceae, Neisseriaceae and *Corynebacterium*, and a decreased on Sphingomonadaceae, followed by *Acinetobacter* and *Mycoplasma*. The observed increased on *Pseudomonas* was expected, being might be due to the inoculation of plants with *Pss*.

5 – Conclusions and future perspectives

5. Conclusions and future perspectives

The olive knot (OK) is among the diseases affecting olive trees, the most serious constrains to worldwide olive crop production. Crop losses caused by OK produces tumorous galls or knots, mostly on the stems and branches of infected trees. The main aim of this work was to elucidate the potential effect of using a consortium of endophytes, retrieved from centennial olive trees, on olive knot disease, caused by the bacterium *Pseudomonas savastanoi* pv. *savastanoi* (*Pss*). Based on the results obtained, it was possible to conclude that:

1) The inoculation of olive plantlets with endophytic bacteria reduced significantly both the incidence (up to 1.6-fold) and severity (up to 2.1-fold) of OK disease;

2) The inoculum used seems to modulate the endospheric bacterial community of the olive plantlets, by decreasing (*Pseudomonas*, *Methylobacterium* and *Mycoplasma*) and increasing (Micrococcaceae, Rikenellaceae and *Corynebacterium*) some bacterial members, that it is likely to play an important role in reducing the development of OK disease

3) Centenary olive trees may represent a rich repository of rare and new endophytes, that can be explore in agriculture as biological control agents. Indeed, the predominant bacterial genus found on the inoculum retrieved from the centenary olive tree was *Mycoplasma*, which is a recognized symbiont that was never reported before on olive tree. Therefore, studying endophytic communities of very old cultivars is of considerable interest.

Overall, these promising results indicate that it may be possible to reduce the effects of serious bacterial infestations by promoting a beneficial microbiome through the application of inoculum retrieved from resistant or tolerant plants. Despite the contribution of these results to decipher the potential of endophytes from centenarian olive trees of being a novel strategy to manage the OK disease, the full understanding of the diverse interactions that can take place in the olive endosphere and their mechanism of action are still unknown. Future research needs to decipher these complex and dynamic microbial interaction networks and assess their role in plant health.

6 - References

References

Ab Rahman, S. F. S., Singh, E., Pieterse, C. M., & Schenk, P. M. (2018). Emerging microbial biocontrol strategies for plant pathogens. *Plant Science*, *267*, 102-111.

Al-Khatib, M., Alhussaen, K., El-Banna, N., Zyadeh, M. 2010. Biological control of olive leaf spot (peacock spot disease) caused by Cycloconium oleaginum (Spilocea oleaginea). Journal of Microbiology and Antimicrobials Vol. 2(6), pp. 64-67.

Aranda, S., Montes-Borrego, M., Jiménez-Díaz, R. M., & Landa, B. B. (2011). Microbial communities associated with the root system of wild olives (Olea europaea L. subsp. europaea var. sylvestris) are good reservoirs of bacteria with antagonistic potential against Verticillium dahliae. *Plant and Soil*, *343*(1), 329-345.

Asaf, S., Numan, M., Khan, A. L., & Al-Harrasi, A. (2020). Sphingomonas: from diversity and genomics to functional role in environmental remediation and plant growth. Critical Reviews in Biotechnology, 40(2), 138-152.

Backman, P. A., & Sikora, R. A. (2008). Endophytes: an emerging tool for biological control. *Biological control*, *46*(1), 1-3.

Balkwill, F. (2006). TNF-α in promotion and progression of cancer. Cancer and Metastasis Reviews, 25(3), 409-416

Bandra, W. M. S., Seneviratne, G., & Kulasooriya, S. A. (2006). Interactions among endophytic bacteria and fungi: effects and potentials. Journal of biosciences, 31(5), 645-650.

Basim, H., Basim, E., & Ersoy, A. (2019). Phenotypic and genotypic characterization of

Pseudomonas savastanoi pv. savastanoi causing olive knot disease in Turkey. *Applied Ecology and Environmental Research*, *17*(6).

Besnard, G., Terral, J. F., & Cornille, A. (2018). On the origins and domestication of the olive: a review and perspectives. *Annals of botany*, *121*(3), 385-403.

Boussadia, O., Mariem, F. B., Mechri, B., Boussetta, W., Braham, M., & El Hadj, S. B. (2008). Response to drought of two olive tree cultivars (cv Koroneki and Meski). *Scientia Horticulturae*, *116*(4), 388-393.

Buonaurio, R., Moretti, C., da Silva, D. P., Cortese, C., Ramos, C., & Venturi, V. (2015). The olive knot disease as a model to study the role of interspecies bacterial communities in plant disease. *Frontiers in plant science*, *6*, 434.

Busby, P. E., Peay, K. G., & Newcombe, G. (2016). Common foliar fungi of Populus trichocarpa modify Melampsora rust disease severity. New Phytologist, 209(4), 1681-1692. doi: 10.1111/nph.13742.

Busby, P. E., Ridout, M., & Newcombe, G. (2016). Fungal endophytes: modifiers of plant disease. *Plant molecular biology*, *90*(6), 645-655.

Cacciola, S. O., Faedda, R., Sinatra, F., Agosteo, G. E., Schena, L., Frisullo, S., & di San Lio, G. M. (2012). Olive anthracnose. *Journal of Plant Pathology*, 29-44.

Cazorla, F. M., & Mercado-Blanco, J. (2016). Biological control of tree and woody plant diseases: an impossible task?. *BioControl*, *61*(3), 233-242.

Clarke, K. R., & Gorley, R. N. (2015). Getting started with PRIMER v7. *PRIMER-E: Plymouth, Plymouth Marine Laboratory*, *20*(1).

Conte, P., Fadda, C., Del Caro, A., Urgeghe, P. P., & Piga, A. (2020). Table olives: an overview on effects of processing on nutritional and sensory quality. *Foods*, *9*(4), 514.

Costa, D., Fernandes, T., Martins, F., Pereira, J. A., Tavares, R. M., Santos, P. M., ... & Lino-Neto, T. (2021). Illuminating Olea europaea L. endophyte fungal community. *Microbiological Research*, *245*, 126693.

Daane, K. M., & Johnson, M. W. (2010). Olive fruit fly: managing an ancient pest in modern times. *Annual review of entomology*, *55*(1), 151-169.

Datlof, E. M., Amend, A. S., Earl, K., Hayward, J., Morden, C. W., Wade, R., ... & Hynson, N. A. (2017). Uncovering unseen fungal diversity from plant DNA banks. *PeerJ*, *5*, e3730.

De Vrieze, M., Germanier, F., Vuille, N., & Weisskopf, L. (2018). Combining different potato-associated Pseudomonas strains for improved biocontrol of Phytophthora infestans. *Frontiers in microbiology*, *9*, 2573.

Dourado, M. N. (2015). Aparecida Camargo Neves A, Santos DS, Araújo WL (2015) Biotechnological and agronomic potential of endophytic pink-pigmented methylotrophic Methylobacterium spp. Biomed Res Int, 909016.

Durán, P., Thiergart, T., Garrido-Oter, R., Agler, M., Kemen, E., Schulze-Lefert, P., et al. (2018). Microbial interkingdom interactions in roots promote Arabidopsis survival. Cell 175 (4), 973–983.e914. doi: 10.1016/j.cell.2018.10.020

Dutta, K., Tsai, C. Y., Chen, W. H., & Lin, J. G. (2014). Effect of carriers on the performance of anaerobic sequencing batch biofilm reactor treating synthetic municipal wastewater. *International Biodeterioration & Biodegradation*, *95*, 84-88.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460-2461.

Ercolani, G. L. (1978). Pseudomonas savastanoi and other bacteria colonizing the surface of olive leaves in the field. *Microbiology*, *109*(2), 245-257.

Fadiji, A. E., & Babalola, O. O. (2020). Elucidating mechanisms of endophytes used in plant protection and other bioactivities with multifunctional prospects. Frontiers in Bioengineering and Biotechnology, 8, 467

Federico, V,. Leonardo, S,. Fabiano,S,. Stefano,G,. Marzia, V,. W Nissim G,. Fortunato et al. "Salt stress in olive tree shapes resident endophytic microbiota." Frontiers in plant science 13 (2022)

Fernández-Escobar, R., De la Rosa, R., Leon, L., Gomez, J. A., Testi, L., Orgaz, F., ... & Masallem, M. (2013). Evolution and sustainability of the olive production systems. *Options Mediterraneennes*, *106*, 11-42.

Gao, M., Xiong, C., Gao, C., Tsui, C. K., Wang, M. M., Zhou, X., ... & Cai, L. (2021). Diseaseinduced changes in plant microbiome assembly and functional adaptation. Microbiome, 9(1), 1-18

Ghanney, N., Locantore, P., Nahdi, S., Ferchichi, A., & Iacobellis, N. S. (2016). *Potential Biocontrol Effect of the Phylloplane Bacterium Bacillus mojavensis* (Vol. 7, No. 3, p. 1000337). A-BC-7 on the Olive Knot Disease. J Plant Pathol Microbiol 7: 337. doi: 10.4172/2157-7471.1000337 Page 2 of 5 J Plant Pathol Microbiol ISSN: 2157-7471 JPPM, an open access.

Giampetruzzi, A., Baptista, P., Morelli, M., Cameirão, C., Lino Neto, T., Costa, D., ... & Saldarelli, P. (2020). Differences in the endophytic microbiome of olive cultivars infected by Xylella fastidiosa across seasons. Pathogens, 9(9), 723.

Godena, S., Dminić, I., & Đermić, E. (2012). Differential susceptibility of olive varieties to olive knot disease in Istria. *Journal of Central European Agriculture*, *13*(1), 0-0.

Gomes, T. M. D. C. (2018). Role of olive tree phyllosphere microorganisms in the biological control of olive leaf spot and olive knot.

Gomes, T., Pereira, J. A., Lino-Neto, T., Bennett, A. E., & Baptista, P. (2019). Bacterial disease induced changes in fungal communities of olive tree twigs depend on host genotype. Scientific reports, 9(1), 1-10

Gómez-Lama Cabanas, C., Legarda, G., Ruano-Rosa, D., Pizarro-Tobias, P., Valverde-Corredor, A., Niqui, J. L., ... & Mercado-Blanco, J. (2018). Indigenous Pseudomonas spp. strains from the olive (Olea europaea L.) rhizosphere as effective biocontrol agents against Verticillium dahliae: From the host roots to the bacterial genomes. Frontiers in microbiology, 9, 277

Gopal, M., Gupta, A., & Thomas, G. V. (2013). Bespoke microbiome therapy to manage plant diseases. *Frontiers in microbiology*, *4*, 355.

Gouda, S., Das, G., Sen, S. K., Shin, H. S., & Patra, J. K. (2016). Endophytes: a treasure house of bioactive compounds of medicinal importance. Frontiers in microbiology, 7, 1538.

Grace, L. J., Charity, J. A., Gresham, B., Kay, N., & Walter, C. (2005). Insect-resistant transgenic Pinus radiata. Plant Cell Reports

Gros-Balthazard, M., Besnard, G., Sarah, G., Holtz, Y., Leclercq, J., Santoni, S., ... & Khadari, B. (2019). Evolutionary transcriptomics reveals the origins of olives and the genomic changes associated with their domestication. *The Plant Journal*, *100*(1), 143-157.

Grossi, CEM, Fantino, E., Serral, F., et al. 2020. Methylobacterium sp. 2A is a plant growthpromoting rhizobacteria that has the potential to improve potato crop yield under adverse conditions. Front Plant Sci 11. DOI: 10.3389/fpls.2020.00071

Hammer, Ø., Harper, D. A. T., & Ryan, P. D. (2001). PAST-palaeontological statistics, ver. 1.89. *Palaeontol. electron*, *4*(1), 1-9.

Healey, A., Furtado, A., Cooper, T., & Henry, R. J. (2014). Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species. *Plant methods*, *10*(1), 1-

Hollants, J., Leroux, O., Leliaert, F., Decleyre, H., De Clerck, O., & Willems, A. (2011). Who is in there? Exploration of endophytic bacteria within the siphonous green seaweed Bryopsis (Bryopsidales, Chlorophyta). PLoS One, 6(10), e26458.

https://www.fao.org/faostat/en/#home

Ikeda, S., Kaneko, T., Okubo, T., Rallos, L. E., Eda, S., Mitsui, H., ... & Minamisawa, K. (2009). Development of a bacterial cell enrichment method and its application to the community analysis in soybean stems. *Microbial Ecology*, *58*(4), 703-714

Jakuschkin, B., Fievet, V., Schwaller, L., Fort, T., Robin, C., & Vacher, C. (2016). Deciphering the pathobiome: intra-and interkingdom interactions involving the pathogen Erysiphe alphitoides. *Microbial ecology*, *72*(4), 870-880.

Johnson, J. S., Spakowicz, D. J., Hong, B. Y., Petersen, L. M., Demkowicz, P., Chen, L., ... & Weinstock, G. M. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nature communications, 10(1), 1-11.

Kernaghan, G., Mayerhofer, M., & Griffin, A. (2017). Fungal endophytes of wild and hybrid Vitis leaves and their potential for vineyard biocontrol. *Canadian Journal of Microbiology*, *63*(7), 583-595.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic acids research*, *41*(1), e1-e1.

Köhl, J., Kolnaar, R., & Ravensberg, W. J. (2019). Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. *Frontiers in plant science*, 845.

Krid, S., Rhouma, A., Mogou, I., Quesada, J. M., Nesme, X., & Gargouri, A. (2010). Pseudomonas savastanoi endophytic bacteria in olive tree knots and antagonistic potential of strains of Pseudomonas fluorescens and Bacillus subtilis. *Journal of plant pathology*, 335-341.

Kuczynski, J., Stombaugh, J., Walters, W. A., González, A., Caporaso, J. G., & Knight, R. (2012). Using QIIME to analyze 16S rRNA gene sequences from microbial communities. *Current protocols in microbiology*, *27*(1), 1E-5.

Latz, M. A., Jensen, B., Collinge, D. B., & Jørgensen, H. J. (2018). Endophytic fungi as biocontrol agents: elucidating mechanisms in disease suppression. *Plant Ecology &*

Diversity, *11*(5-6), 555-567.

Lavermicocca, P., Lonigro, S. L., Valerio, F., Evidente, A., & Visconti, A. (2002). Reduction of olive knot disease by a bacteriocin from Pseudomonas syringae pv. ciccaronei. *Applied and Environmental Microbiology*, *68*(3), 1403-1407.

Li, Z.; Chang, P.; Gao, L.; Wang, X. The Endophytic Fungus Albifimbria verrucaria from Wild Grape as an Antagonist of Botrytis cinerea and Other Grape Pathogens. Phytopathology 2020, 110, 843–850. https://doi.org/10.1094/PHYTO-09-19-0347-R

Maldonado-González, M. M., Prieto, P., Ramos, C., & Mercado-Blanco, J. (2013). From the root to the stem: interaction between the biocontrol root endophyte P seudomonas fluorescens PICF 7 and the pathogen P seudomonas savastanoi NCPPB 3335 in olive knots. *Microbial biotechnology*, *6*(3), 275-287.

Martins, F., Pereira, J. A., Bota, P., Bento, A., & Baptista, P. (2016). Fungal endophyte communities in above-and belowground olive tree organs and the effect of season and geographic location on their structures. *Fungal ecology*, *20*, 193-201.

Matas, I. M., Lambertsen, L., Rodríguez-Moreno, L., & Ramos, C. (2012). Identification of novel virulence genes and metabolic pathways required for full fitness of P seudomonas savastanoi pv. savastanoi in olive (O lea europaea) knots. *New Phytologist*, *196*(4), 1182-1196.

Mercado-Blanco, J., Alós, E., Rey, M. D., & Prieto, P. (2016). Pseudomonas fluorescens PICF7 displays an endophytic lifestyle in cultivated cereals and enhances yield in barley. *FEMS* *microbiology ecology*, *92*(8), fiw092.

Mina, D., Pereira, J. A., Lino-Neto, T., & Baptista, P. (2020). Screening the olive tree phyllosphere: search and find potential antagonists against Pseudomonas savastanoi pv. savastanoi. *Frontiers in microbiology*, *11*, 2051.

Mina, D., Pereira, J. A., Lino-Neto, T., & Baptista, P. (2020b). Epiphytic and endophytic bacteria on olive tree phyllosphere: exploring tissue and cultivar effect. Microbial ecology, 80(1), 145-157. doi.org/10.1007/s00248-020-01488-8

Müller, H., Berg, C., Landa, B. B., Auerbach, A., Moissl-Eichinger, C., & Berg, G. (2015). Plant genotype-specific archaeal and bacterial endophytes but similar Bacillus antagonists colonize Mediterranean olive trees. Frontiers in microbiology, 6, 138

Nair, D. N., & Padmavathy, S. (2014). Impact of endophytic microorganisms on plants, environment and humans. *The Scientific World Journal*, *2014*.

Nguyen, K. A., Förster, H., & Adaskaveg, J. E. (2017). Quaternary ammonium compounds as new sanitizers for reducing the spread of the olive knot pathogen on orchard equipment. *Plant Disease*, *101*(7), 1188-1193.

Nicoletti, R., Andolfi, A., & Salvatore, M. M. (2023). Endophytic fungi of the genus Talaromyces and plant health. In *Microbial Endophytes and Plant Growth* (pp. 183-213). Academic Press

Niu, B., Paulson, J. N., Zheng, X., & Kolter, R. (2017). Simplified and representative bacterial community of maize roots. *Proceedings of the National Academy of Sciences*, *114*(12), E2450-E2459.

Ouzari, H., Khsairi, A., Raddadi, N., Jaoua, L., Hassen, A., Zarrouk, M., ... & Boudabous, A. (2008). Diversity of auxin-producing bacteria associated to Pseudomonas savastanoi-induced olive knots. *Journal of Basic Microbiology*, *48*(5), 370-377.

Ownley, B. H., Gwinn, K. D., & Vega, F. E. (2010). Endophytic fungal entomopathogens with activity against plant pathogens: ecology and evolution. *BioControl*, *55*(1), 113-128.

Panpatte, D. G., Jhala, Y. K., Shelat, H. N., & Vyas, R. V. (2016). Pseudomonas fluorescens: a promising biocontrol agent and PGPR for sustainable agriculture. In Microbial inoculants in sustainable agricultural productivity (pp. 257-270). Springer, New Delhi

Preto, G., Martins, F., Pereira, J. A., & Baptista, P. (2017). Fungal community in olive fruits of cultivars with different susceptibilities to anthracnose and selection of isolates to be used as biocontrol agents. *Biological Control*, *110*, 1-9.

Quesada, C. A., Phillips, O. L., Schwarz, M., Czimczik, C. I., Baker, T. R., Patiño, S., ... & Lloyd, J. (2012). Basin-wide variations in Amazon forest structure and function are mediated by both soils and climate. *Biogeosciences*, *9*(6), 2203-2246.

Quesada, J. M., Penyalver, R., Pérez-Panadés, J., Salcedo, C. I., Carbonell, E. A., & López, M. M. (2010). Dissemination of Pseudomonas savastanoi pv. savastanoi populations and subsequent appearance of olive knot disease. *Plant Pathology*, *59*(2), 262-269.

Rabiey, M., Hailey, L. E., Roy, S. R., Grenz, K., Al-Zadjali, M. A., Barrett, G. A., & Jackson, R. W. (2019). Endophytes vs tree pathogens and pests: can they be used as biological control agents to improve tree health?. *European Journal of Plant Pathology*, *155*(3), 711-729.

Ramos, C., Matas, I. M., Bardaji, L., Aragón, I. M., & Murillo, J. (2012). P seudomonas savastanoi pv. savastanoi: some like it knot. *Molecular plant pathology*, *13*(9), 998-1009.

Rideout, J. R., He, Y., Navas-Molina, J. A., Walters, W. A., Ursell, L. K., Gibbons, S. M., ... & Caporaso, J. G. (2014). Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. *PeerJ*, *2*, e545.

Rodrigues, N. M. S. (2018). *Characterization of wild and centenarian olive trees for their valorization* (Doctoral dissertation, Universidad de León).

Rokni-Zadeh, H., Li, W., Yilma, E., Sanchez-Rodriguez, A., & De Mot, R. (2013). Distinct lipopeptide production systems for WLIP (white line-inducing principle) in P seudomonas fluorescens and P seudomonas putida. *Environmental microbiology reports*, *5*(1), 160-169.

Ryan, K. J., & Ray, C. G. (2004). Medical microbiology. McGraw Hill, 4(370)

Rybakova, D., Cernava, T., Köberl, M., Liebminger, S., Etemadi, M., & Berg, G. (2016). Endophytes-assisted biocontrol: novel insights in ecology and the mode of action of Paenibacillus. *Plant and soil*, *405*(1), 125-140.

Suryanarayanan, T. S. (2013). Endophyte research: going beyond isolation and metabolite documentation. *Fungal ecology*, *6*(6), 561-568.

Uhlig, E., Kjellström, A., Nurminen, N., Olsson, C., Oscarsson, E., Canaviri-Paz, P., ... & Håkansson, Å. (2021). Use of bacterial strains antagonistic to Escherichia coli for biocontrol of spinach: A field trial. Innovative Food Science & Emerging Technologies, 74, 102862.

Van de Burgwal, L. H. M., Van der Waal, M. B., & Claassen, E. (2018). Accelerating microbiota product development: The Societal Impact Value Cycle as a conceptual model to shape and improve public-private valorization processes. *PharmaNutrition*, *6*(4), 157-168.

van der Heijden, M. G., Hartmann, M. (2016). Networking in the plant microbiome. PloS Biol. 14 (2), e1002378. doi: 10.1371/journal.pbio.1002378

Varanda, C., Portugal, I., Ribeiro, J., Silva, A., & Silva, C. M. (2016). Influence of polyphosphoric acid on the consistency and composition of formulated bitumen: standard characterization and NMR insights. *Journal of analytical methods in chemistry*, *2016*.

Wachowska, U., Irzykowski, W., Jędryczka, M., Stasiulewicz-Paluch, A. D., & Głowacka, K. (2013). Biological control of winter wheat pathogens with the use of antagonistic Sphingomonas bacteria under greenhouse conditions. Biocontrol Science and Technology, 23(10), 1110-1122.

Walters, W., Hyde, E. R., Berg-Lyons, D., Ackermann, G., Humphrey, G., Parada, A., ... & Knight, R. (2016). Improved bacterial 16S rRNA gene (V4 and V4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. *Msystems*, *1*(1), e00009-15.

Xin, X. F., Kvitko, B., & He, S. Y. (2018). Pseudomonas syringae: what it takes to be a pathogen. Nature Reviews Microbiology, 16(5), 316-328.

Young, J. M. (2004). Olive knot and its pathogens. *Australasian Plant Pathology*, *33*(1), 33-39.

Young, V. B. (2017). The role of the microbiome in human health and disease: an introduction for clinicians. Bmj, 356.

Zahn, G., & Amend, A. S. (2017). Foliar microbiome transplants confer disease resistance in a critically-endangered plant. PeerJ, 5, e4020

Zhang, J., Ahmed, W., Dai, Z., Zhou, X., He, Z., Wei, L., & Ji, G. (2022). Microbial consortia: An engineering tool to suppress clubroot of Chinese cabbage by changing the rhizosphere bacterial community composition. Biology, 11(6), 918.