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Ph.D. Course

Plant and Animal Sciences **(XXXIV)**

Eco-sustainable strategies to control *Xylella fastidiosa* subsp. *pauca* Strain De Donno by novel biological control agents: screening and mode of action in Apulian olive cultivars and Oleander plants

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Extended Abstract

This dissertation comprises a series of studies aimed at exploring the cultivable bacterial communities inhabiting leaf surfaces of the main host species of *Xylella fastidiosa* ST53 present in the Apulia region, screening of potential antagonists, revealing their mechanism of action and identifying possible antimicrobial compounds secreted. Furthermore, evaluating their capability to colonize Apulian olive cultivars and *Nerium. oleander* host. This work started with the isolation of potential biological control agents (BCAs) from leaves of *Olea europaea, Polygala myrtifolia, Rosmarinus officinalis, Nerium oleander, Laurus nobilis, Myrtus communis, Prunus dulcis, Prunus avium* and *Cistus creticus* hosts present in various *X. fastidiosa* outbreaks occurred in the demarcated area of Apulia region. Later, several isolates were obtained and screened *in vitro* for antagonistic activity against *X. fastidiosa* ST53 and were subjected to morphological, biochemical, and molecular characterization. *In vitro* dual culture tests showed that 12 out of 89 isolates inhibited *X. fastidiosa* growth with an appearance of clear inhibition zones between 4.0 and 34 mm. Sequencing the 16S rRNA gene revealed different species belonged to the genera *Pantoea*, *Microbacterium*, *Stenotrophomonas*, *Delftia, Sphingomonas, Curtobacterium* and *Pseudomonas*. In the framework of the thesis, several tests were carried out to elucidate and understand the mode of action of different antagonists and their ability to produce culture filtrates (CFs) with antimicrobial compounds. Moreover, five bacterial endophytic strains natively obtained from Apulian olive cultivars were also included in the experiment of antimicrobial activity against *X. fastidiosa* ST53. Results showed that *Pseudomonas. graminis* BA2 and *Pantoea. agglomerans* BA69 isolates produced siderophores. However, *Stenotrophomonas. rhizophila* BA102 and *Microbacterium. oxydans* BA104 isolates produced protease and HCN respectively. Importantly, our results showed that *Bacillus subtilis, Bacillus pumilus, Peanbacillus rigui, S. rhizophila* and *M. oxydans* had the

highest antagonistic activity *in vitro* with the production of culture filtrates which inhibit the growth of the pathogen likely secreting active substances in the liquid medium. Bacterial isolates demonstrating the strongest antagonistic activity (*P. agglomerans*, *Curotbacterium flaccumfaciens, B. subtilis, M. oxydans* and *S. rhizophila*) were inoculated in Apulian olive cultivars and *N. oleander* hosts. The establishment of bacterial inoculums were monitored by Real-time PCR, conventional PCR and plat counting. The results obtained showed that the different isolates tested were able to colonize the stem of the two olive cultivars and *N. oleander* plant during the assessment period of about one month. In detail, *P. agglomerans* BA69 was recovered with relatively high population size (10^6 CFU/ml) from *N. Oleander* plant and was less efficient in establishing in the two olive cultivars. Both isolates of *B. subtilis* L39 and *C. flaccumfaciens* BA54 were found to be stable in the stem segments of both olive cultivars and *N. oleander* plant. They were able to reach approximately 10^6 CFU/ml population size in *N. oleander* and over 10^4 CFU/ml population in olive cultivars. Endophytic colonization of *M. oxydans* BA104 and *S. rhizophila* BA102 were found to require more time compared with other isolates tested based on the plate counting approach. On the other hand, preliminary promising results were obtained from HPLC-HRMS analysis in which we succeed to annotate 10 metabolites that were mainly antibiotics from CFS of *B. subtilis* and *M.oxydans*. Particularly, 3'-Deoxydihydrostreptomycin metabolite that was detected in CFS of *M. oxydans* BA104 seems to be the most interesting since we revealed also its fragmentation pattern compared to other metabolites that did not show enough high abundance to be fragmented. Overall, our results demonstrate the efficacy and suitability of several antagonists to be exploited in control strategy against *X. f*astidiosa ST53. Nevertheless, HPLC-HRMS results needs to be carried on to clearly identify the metabolites responsible for the antibacterial activity against *X. fastidiosa* ST53.

Table of content

List of figures

List of Tables

Introduction

Emerging infectious diseases and invasive species are major economic and public-health concerns worldwide, and they are on the rise as a result of socioeconomic, ecological, and environmental variables such as human population density and growth, drug resistance, and host diversity (Jones *et al*., 2008). In terms of plant health, diseases such as European ash dieback (Gross *et al*., 2014) and sudden oak death (Grünwald *et al*., 2019) have spread throughout broad swaths of the globe, killing millions of plants.

In autumn 2013, a disease outbreak affecting olive trees occurred in a restricted area of the Ionian coast of Salento (Apulia, southeastern Italy). It was the occurrence of the Olive Quick Decline Syndrome (OQDS) disease caused by *X. fastidiosa*, a xylem-limited phytopathogenic bacterium (Saponari *et al.*, 2013, 2016; Loconsole *et al.*, 2014). Genetic and genomic investigations revealed that infected olive trees harbored a previously undescribed genotype, categorized as sequence type ST53, related to strains of the subspecies *pauca* (Giampetruzzi *et al.*, 2017). *X. fastidiosa* is a Gram-negative, non-motile, fastidious bacterium that colonizes the xylem of different hosts with very peculiar biological and epidemiological behaviors (Chatterjee *et al.*, 2008). It has very high pathogenicity on the local olive cultivars 'Cellina di Nardò', 'Ogliarola salentina', and other ornamental and perennial hosts (Saponari *et al.*, 2017). The transmission of the bacterium from one plant to another is mainly attributed to different xylem sap-feeding insects. Sharpshooters are the most common vectors in the American continent, spittlebugs appear to have a major role in the outbreaks so far discovered in Europe (Cornara *et al.*, 2019), with *Philaenus spumarius* being the very active and widespread vector in the affected area of Apulia (Cornara *et al.*, 2017). In the plant, *X. fastidiosa* shares many characteristics with vascular pathogens in the perspective of symptom development, which starts with leaf scorching and ends dramatically with total decay (Martelli, 2015). The incidence of the disease has increased rapidly through the heavily olive-grown countryside of

the Lecce peninsula. From there, the affected area has expanded and the infection has spread over more areas, killing millions of olive trees (Maggiore *et al.*, 2019). Such rapid expansion of the front of the infections implied the adoption of containment measures, i.e., to put in place all available means to mitigate the impact of the bacterial infections entrenched in this territory and not eradicable. Thus, the disease has become an urgent crisis that needs the development of control strategies. Actually, due to a lack of therapy and progressive demarcation of the olive sector in Apulia, interest in biological control of *X. fastidiosa* through the use of natural occurring antagonistic microorganisms has increased (EFSA Panel on Plant Health (EFSA PLH Panel) *et al.*, 2019). The functional bioactivities of beneficial bacteria against diseases are defined as direct and indirect antagonism. Some bacteria may possess direct antagonistic activity against pathogens through hyperparasitism or antibiosis. Meanwhile, indirect antagonists can control diseases by inducing or enhancing plant resistance to pathogen infections or by competing for nutrients (Pal and McSpadden Gardener, 2006). Bacterial antagonists may be rhizospheric, phyllospheric, endophytic microorganisms, classified according to their inhibiting environment. Direct antagonists, which actively produce a broad spectrum of antimicrobial metabolites, are considered the most potent mode of action against competitors, allowing competitive advantages for antibiotic-producing microorganisms in resource-limited environments. They are a large number of known antibiotics produced in small amounts by many endophytic microorganisms and released into the environment (Martinez-Klimova *et al.*, 2017). The production of antimicrobial metabolites, mostly with broad-spectrum activity, has been reported for biocontrol bacteria belonging to *Agrobacterium*, *Bacillus*, *Pantoea*, *Pseudomonas*, and many other genera. *Bacillus* genera have been observed to produce several antimicrobial substances (Caulier *et al.*, 2019), while *Pseudomonas* genera have been more associated with antibiotic metabolites such as pyrrolnitrine and phenazine (Huang *et al.*, 2018). Indeed, the lack of any therapeutic formulation for curing infected olives

further emphasizes the need to develop effective and sustainable control strategies. So far, few studies have been conducted to retrieve direct antagonists for a better biocontrol strategy of *X. fastidiosa*. Particularly, the endophyte *Curtobacterium flaccumfaciens* has been found to limit the growth of *X. fastidiosa* in vitro and reduce the symptoms generated in Catharanthus roseus (Lacava *et al.*, 2007). (Baccari *et al.*, 2019) reported also that the endophytic bacterium *Paraburkholderia phytofirmans* strain PsJN controls *X. fastidiosa* infections in the grapevine. while recently published studies have stated the absence of antagonists natively isolated from Apulian olives to inhibit *X. fastidiosa ST53* growth in vitro (Zicca *et al.*, 2020).

To this aim, the main objectives of this thesis were: (i) to target epiphytes inhabiting leaf surfaces of different host species by *in vitro* screening assays for their direct antagonistic activity against *X. fastidiosa* ST53; (ii) to elucidate and understand the possible mode of action of different antagonists and their ability for the production in liquid culture of antimicrobial substances; (iii) to identify possible antibacterial metabolites produced and to select highly antagonistic isolates from this study to evaluate their efficiency in colonizing Apulian olive cultivars and *Nerium oleander* hosts.

CHAPTER I

Literature review

I.1. *Xylella fastidiosa* – Background

I.1.1. History

In the late 1800s, more than one century before a sudden decline of 14,000 ha of grapes (*Vitis* spp.), induced by a 'mysterious' disease had appeared in the Los Angeles area in California. This disease was described in detail in 1887 by N.B. Pierce and was therefore named "Pierce's disease" (PD) of grapevine. A similar disease was also observed in peach (*Prunus persica*) in 1890 in Georgia (USA). At that time no causal organism could be cultured from the infected vines. Moreover, the disease was graft transmissible and was first attributed to a viral infection (Hutchins *et al*., 1953).

Subsequently, a thin, rod-shaped, and Gram-negative bacterium was cultured for the first time from infected plants in 1978, and Koch's postulates were satisfied (Davis *et al*., 1978). Finally, in 1987, the causal agent for PD was revealed and classified as *X. fastidiosa* based on its fastidious growth. Vectors are mainly sharpshooters, froghoppers, and spittlebugs (*Cicadellidae*) with no latent period and have no transovarial transmission of the bacterium (Severin, 1949). In addition to PD, this bacterium has been implicated as the causal agent of many other significant plant diseases on citrus, peach, almond, and olive.

A severe citrus variegated chlorosis (CVC) epidemic in Brazil in 1987 was observed and *X. fastidiosa* was isolated from diseased trees (Chang *et al.*, 1993). Notably, the citrus strain of *X. fastidiosa* was actually the first plant-pathogenic bacterium to have its genome sequenced. The pathogen or associated diseases had been reported only on the territory of South, Central, and

North America till 1998. Nowadays, the bacterium is located in Europe and Asia continents, and it is one of the biggest challenges inside the plant protection sphere around the world.

I.1.2. Biology

X. fastidiosa is a Gram-negative bacterium with fastidious growth requirements. The bacterial cells are non-motile, non-flagellate, rod-shaped cells, strictly aerobic, with rounded or tapered ends, numerous irregular ridges or folds on the cell wall surface and with an optimum growth temperature at 26-28°C (Wells *et al.*, 1987).

X. fastidiosa colonizes the xylem, a water transport network of interconnected vessels composed of dead cells, forming an aggregated biofilm inside the plant. The formation of a biofilm plays an important role in the colonization of the insect vector and plant environments for *X. fastidiosa*. In heavily infected plants, *X. fastidiosa* biofilms are abundant (**Figure 1B**), which contributes to the blockage of sap flow, resulting in plant stress and disease. The bacterium can move downstream, but is also able to migrate upstream, via twitching motility, using type IV pili (Meng *et al.*, 2005). Twitching motility is a way of flagellar-independent bacterial movement over moist surfaces. Type I and type IV pili of *X. fastidiosa* play different roles in twitching motility, biofilm formation and cell-cell aggregation. Whereas type IV pili are essential for motility, but also biofilm formation (Li *et al.*, 2007). The sap blocking biofilm is formed in two distinct habitats, the host plant and the foregut of the vector (**Figure 1A)** (Chatterjee, Almeida, *et al.*, 2008).

X. fastidiosa proliferates only in xylem vessels, in roots, stems, leaves and the biofilm composition vary between plant host and vector, and it is actively produced by the pathogen. Bacterial colonization of plants is based on xylem vessels and movement through the xylem via pit membranes. The bacterium can colonize neighboring vessels through pits, after degradation of the pit membranes, which is also stimulated by a diffusible signal from the bacterium (Newman *et al*., 2004). Moreover, *X. fastidiosa* secretes exopolysaccharides (EPS)

in both plant vessels and insect vector foregut, which permit the formation of biofilms affirming that they are essential for plant virulence and vector transmission of *X. fastidiosa* (Killiny *et al*., 2013).

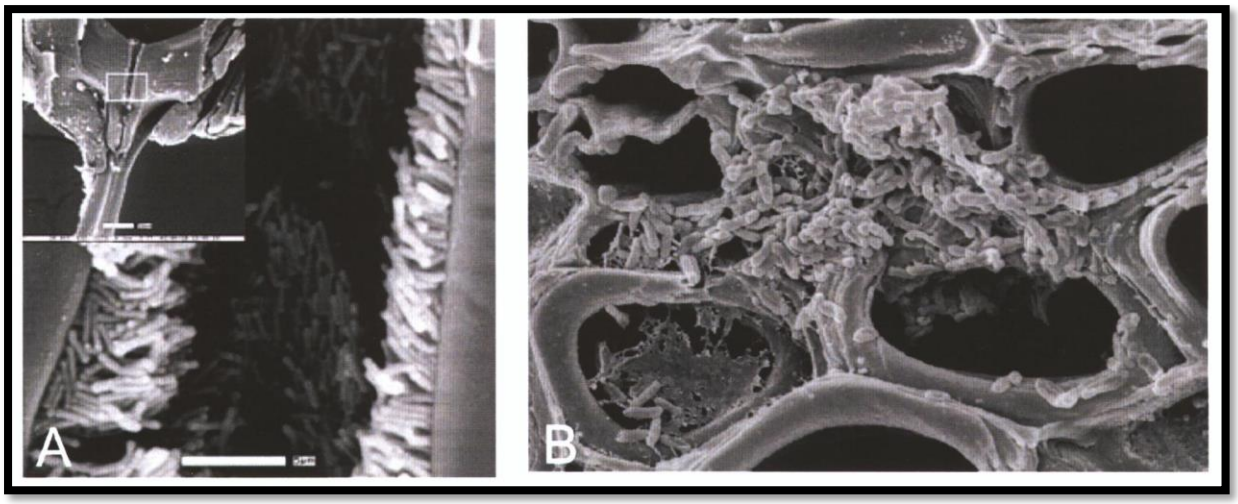

Figure 1: (A): Cells of *X .fastidiosa* in biofilms attached on the cuticle of foregut **(B):** Cells of *X. fastidiosa* in xylem vessels (source: Janse & Obradovic, 2010)

I.1.3. Symptoms

The presence of *X. fastidiosa* can have a broad effect on its host: from symptomless to plant death. The majority of host plants infected with *X. fastidiosa* do not show any symptoms, however, some display symptoms that include leaf scorching, defoliation, chlorosis or bronzing along the leaf margin, and dwarfing. The bronzing may intensify before browning and drying. Symptoms are usually more pronounced in stressed plants, and they can differ according to the plant species or cultivar and environmental conditions (Janse & Obradovic, 2010). Symptoms are more obvious in late spring, summer and early autumn and they are favored by high temperature (25-28°C) and stressed conditions for the plant (López *et al*., 2017). Interestingly, X. *fastidiosa* is the causal agent of several severe diseases, among which Pierce's disease of grapevine, leaf scorch of almond, leaf scorch of coffee, phony peach, plum leaf scald, citrus variegated chlorosis (CVC) and olive Quick Decline Syndrome (OQDS).

(Figure 2).

Figure 2: A. Typical leaf scorch symptoms, caused by *X. fastidiosa* subsp. *fastidiosa* on grapevine leaves; **B.** Typical symptoms on leaves (variegated spots) and fruits (dwarf growth) of Citrus caused by *X. fastidiosa* subsp. *pauca*; **C.** Severe symptoms of leaf scorch on *Platanus occidentalis* (sycamore) caused by *X. fastidiosa* subsp. *fastidiosa*; **D.** Reduced growth of peach (on the left) affected by the phony peach disease (Janse & Obradovic, 2010).

I.1.4. Taxonomy, host plants and classification

X. fastidiosa infects a wide host range that comprises ornamental, cultivated and wild plants and is responsible for plant diseases with great socioeconomic consequences. In 1995, Hill and Purcell concluded regarding the *X. fastidiosa* host range that plants in 29 families were hosts of this bacterium. Indeed, the bacterium is highly polyphagous, infecting 638 plant species, and in several cases without causing symptoms. These species cover hundreds of host plant genera

in 87 botanical families. The list of hosts in Europe is regularly updated with the results of surveys (Delbianco *et al.*, 2021).

Thus, there is high uncertainty with regard to the potential host range of *X. fastidiosa* in the European flora as a wide range of European wild plant species have never been exposed to the bacterium and it is not known whether they would be hosts, and, if so, whether they would develop symptoms. In 1987, based on 16S ribosomal ribonucleic acid Wells *et al*. discovered that different strains of the bacterium were related to Xanthomonads. These bacteria form a distinct group, for which the name *X. fastidiosa* was proposed and a new genus was established with one species in the gamma subgroup of the eubacteria (Wells *et al*., 1987).

Genetically diverse, this species is divided into subspecies but genetic traits governing this classification are poorly understood. Currently, the taxonomic classification of *X. fastidiosa* includes 6 subspecies reported in many hosts in different areas of the world (**Table 1**). The responsible strain of OQDS in the Apulia region in 2013, worthily referred to as the De Donno strain (ST53), is genetically linked to the *pauca* subspecies (Giampetruzzi *et al.*, 2015). Among the latest findings, a new outbreak has been reported in the Italian region of Tuscany, with infections identified in seven different plant species. Multilocus sequence typing approach revealed the occurrence of isolates harboring a new sequence type, denoted ST87, genetically related to strains of subsp. *multiplex*, but different from the genotypes of this subspecies previously characterized in Europe (Saponari *et al*., 2019). There is no type of strain available for subsp. *pauca* in the public databases. Currently, the three subspecies (*fastidiosa*, *multiplex*, and *pauca)* are generally accepted by the scientific community to be the main grouping (Marcelletti & Scortichini, 2016).

Table 1: Subspecies of *X. fastidiosa*, area of origin and main hosts

(Source: Almeida & Nunney, 2015)

Subspecies	Origin	Main hosts and area recorded
fastidiosa	Central America	Grapevine (North and Central America, Peru, Iran, Taiwan), Almond (USA, Argentina)
multiplex	Temperate and subtropical North America	Oleander, stone fruits, oaks, olive (USA), polygala (France)
sandyi	Southern regions of USA	Oleander (USA)
pauca	South America	Citrus, coffee, olive (Italy, Argentina, Brazil), oleander (Italy, Costa Rica)
morus	Eastern and western America	Mulberry
tashke	Southwestern of USA	*Chitalpa tashkentensis*

I.1.5. Geographical distribution

For several years, *X. fastidiosa* remained confined to the American continent, and was thought to be restricted to that region. The plant-associated bacterium *X. fastidiosa* has been blooming since 2013 in Europe and Asia. In 1994, it was first noticed in Taiwan causing leaf scorch on Asian pear (*Pyrus pyrifolia*). In the European and Mediterranean regions, the finding in Apulia (Southern Italy) represented the first confirmed detection in Europe (Saponari *et al*., 2013). In October 2015, the bacterium was discovered in France on the island of Corsica on *Polygala myrtifolia* plants, Provence (2019), and Occitanie (2020). Afterward, in October 2016, it was found for the first time in Islas Baleares on sweet cherry (*Prunus avium*) in the garden center of the island of Mallorca (EPPO, 2018). Besides, the National Plant Protection Organization

of Portugal recorded the detection for the first time of *X. fastidiosa* subsp. *multiplex* on lavender plants in December 2018. Recently, *X. fastidiosa* subsp. *fastidiosa* was detected in June 2019 on symptomatic almond trees in Israel. Thus, *X. fastidiosa* is located nowadays in different continents: North America (USA, Canada, and Mexico), South America (Argentina, Brazil, Costa Rica, Paraguay, and Venezuela), Asia (Iran, Taiwan, Israel) and Europe (Italy, France, Spain and Portugal) **(Figure 3)**.

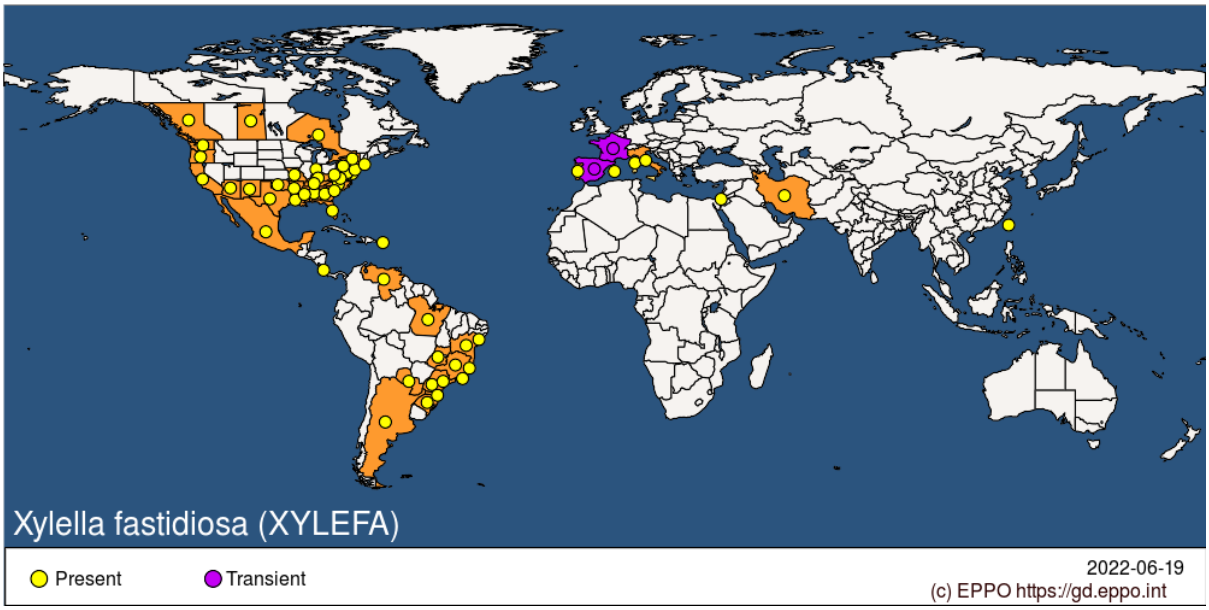

Figure 3: World distribution of *X. fastidiosa* (yellow: present – purple: transient) – map generated in 19/06/2022 (EPPO-2021)

I.1.6. Vectors

As a vector-borne pathogen, the plant-to-plant transmission of *X. fastidiosa* is directly related to the presence, abundance, and behavior of xylem-fluid feeding insects vectors in relation to infected and healthy plants. These insects belonging to the order *Hemiptera Auchenorryncha* species belonging to the superfamily *Cercopoidea* (froghoppers and spittlebugs), the superfamily *Cicadoidea* (cicadas), and the subfamily *Cicadellidae Cicadellinae* (sharpshooters) feed almost exclusively on xylem-sap (Novotny & Wilson, 1997), thus are all candidate vector of the bacterium. However, insects that feed on mesophyll or phloem are not

able to transmit *X. fastidiosa* (Almeida *et al.,* 2005) (Purcell 1980). The insect vector can acquire *X. fastidiosa* and directly transmit it to a new host plant without a latent period. It is persistent and propagative but not circulative within its insect vector (Hill & Purcell, 1995). There is no trans-stadial or transovarial transmission. Bacteria are restricted to the alimentary canal and do not infect the insect body. After the acquisition, the bacterium is kept on the cuticular lining of the insect foregut and multiply in the cibarium and precibarium (Almeida & Purcell, 2006). Vectors lose the infectivity with molting as the foregut is of ectodermal origin and is renewed with it. Thus, newly emerged adults must feed on an infected plant to become infectious. The meadow spittlebug *Philaenus spumarius* L. (*Hemiptera*: *Aphrophoridae*) is a polyphagous insect widespread throughout Europe (Cornara *et al.*, 2018). Later surveys for the identification of another potential insect vector of *Xylella* indicate that *P.spumarius* is the predominant xylem sap feeder in south Italy and has an important role in spreading the pathogen (Ben Moussa *et al.*, 2016; Elbeaino *et al*., 2014). In a recent report, *Neophilaenus campestris* and *P. italosignus* are two additional spittlebug species able to acquire the bacterium and transmit the pathogen to different hosts. However, their role in the current olive epidemic in the Apulia region in Italy remains marginal (Cavalieri *et al*., 2019).

I.1.7. Special case in Apulia region: Olive Quick Decline Syndrome

In late 2013, a disease outbreak affecting olive trees occurred in Gallipoli, a restricted area in the southern of Italy. It was the occurrence of the Olive Quick Decline Syndrome (OQDS) disease caused by *X. fastidiosa*, a devastating xylem-restricted quarantine bacterium (Saponari *et al*., 2013). This finding was the first confirmed detection of this exotic pathogen, which had never been detected in any European Union (EU) country. After the identification of *X. fastidiosa* in olive trees, surveys were immediately prolonged to surrounding plant species, searching firstly for plants showing typical symptoms of leaf scorch. Oleander (*Nerium oleander*) and almond (*Prunus dulcis*) were also found infected in the area, both showing

symptoms of leaf scorch and/or, in the case of oleander, shoot dieback. So far, the bacterium has been isolated and/ or detected in more than 30 host/species in Italy (Saponari, *et al.*, 2019), including many ornamentals, endemic species of the Mediterranean flora, and three crop species: olive, cherry (*Prunus avium*), and almond. Bacterial infections were found in the early foci, especially on ancient and century-old olive trees (**Figure 4B**), which also showed severe attacks of leopard moth, variously expanded browning of the sapwood of twigs, branches, and trunks linked with the presence of different wood- inhabiting fungal species (Nigro *et al.*, 2013). These consistent relationships lead to the notion that OQDS is produced by xylem tissue damage induced by the leopard moth, wood decay fungi, and *X. fastidiosa* colonization, which is ultimately responsible for the occlusion of the remaining active vessels. These consistent associations led to the hypothesis that OQDS could result from the impairment of the xylem tissues caused by the concomitant actions of the leopard moth, wood decay fungi (whose establishment is favored by the leopard moth galleries), and *X. fastidiosa* colonization, ultimately responsible for the occlusions of the remaining active vessels. As such, the disease was originally named in Italian "Complesso del Disseccamento Rapido dell'Olivo (CoDiRO)" to indicate its association with a complex of causal agents. The OQDS is defined by the progressive onset of symptoms, which begin with classic leaf scorch at the tip of the leaf and advance to the petiole with twig wilting. In fact, *X. fastidiosa* subspecies *pauca* ST53 shares many features with vascular pathogens in the perspective of symptom development, which ends dramatically with total decay (**Figure 4C**). These symptoms are initially present on the apical part of the canopy. However, with disease progression, the above-mentioned symptoms appear at the rest of the tree resulting in a 'burnt-like' appearance. The *X. fastidiosa* genotype proliferating in the Apulia Region is now unmistakably the cause of OQDS, as well as severe infections in other hosts. This scenario is consistent with reports of severe olive desiccations that have recently been reported in Brazil (Coletta-Filho *et al.*, 2016) and Argentina (Haelterman *et al.*, 2015) where olive is not a major

crop, but where epidemics of *X. fastidiosa* subsp. *pauca* are known to have been present for a long time.

In the case of Apulia, the wide distribution of olive trees, abundance of vector populations, and warm summers allow *X. fastidiosa* to grow and proliferate, complicating its control (Strona *et al*., 2017). The syndrome represents an urgent crisis that necessitates the development of viable control approaches to deal with the progressive demarcation of the olive sector in Apulia (Saponari *et al.*, 2019), including intensive surveys to delimit the infected area and rule out the presence of the bacterium in other parts of the Italian peninsula as well as in other Member States.

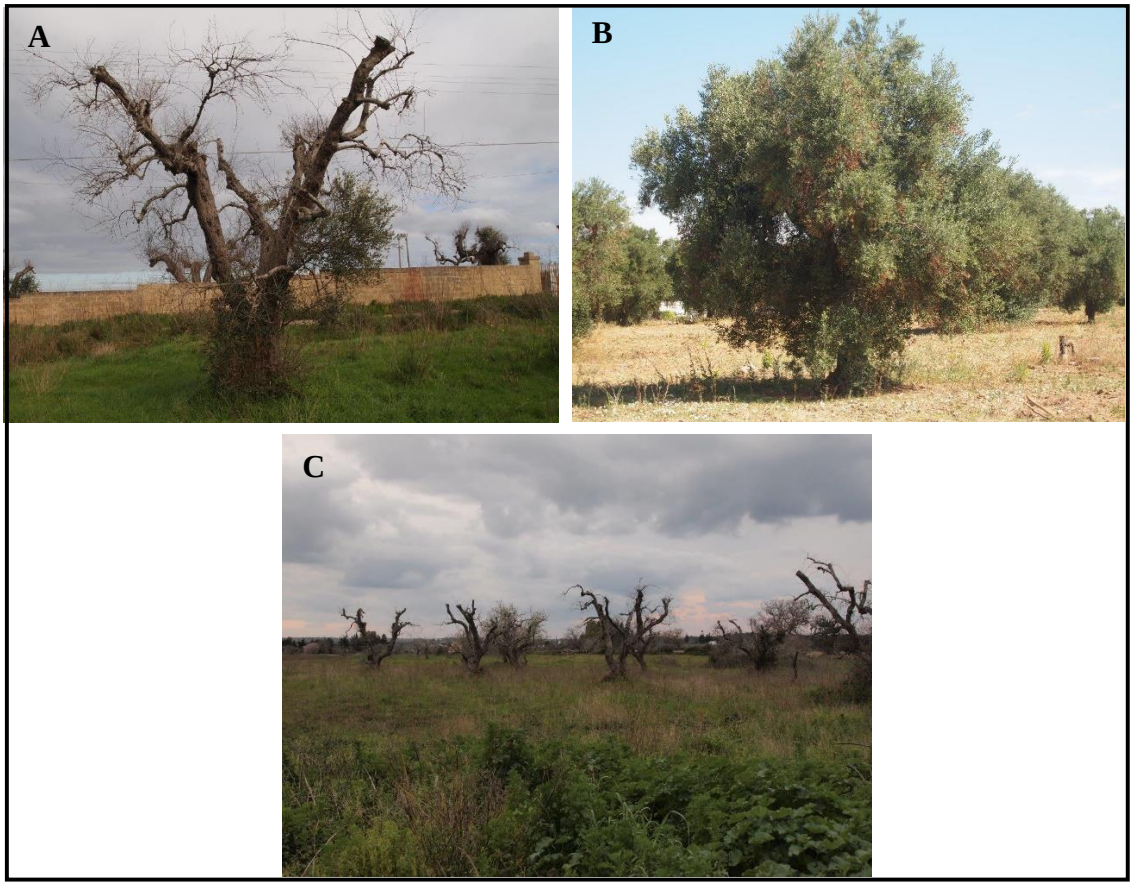

Figure 4: A, Olive tree at advanced stage of disease and **B,** centenary olive trees with canopies showing manifest branch desiccations induced by *X. fastidiosa* subsp. *pauca* ST53. **C,** Olive orchard at advanced stage of disease with whole trees dead (Valentini., 2015).

I.1.8. Economic and social impact

The insect-vectored bacterial plant disease *X. fastidiosa* has a wide host range and has a considerable economic and social impact in the agriculture and horticulture industries. Severe outbreaks have lately been identified in Italy, Spain, France, and Portugal, which were previously exclusive to the Americas. The Italian outbreak began in Apulia in 2013 and has already extended over 100 kilometers, devastating millions of olive trees, and is still expanding. Recent data indicate the occurrence of about 5 million unproductive, heavily infected or dead, olive trees in Salento, leading to a loss of about 10% of Italian olive oil (White *et al*., 2020). According to spatially explicit economic models, *X. fastidiosa* may impact Italian olive growers from 1.5 to 5.9 billion euros in the next 50 years and cause further economic losses by

affecting cultural heritage and landscape values (Schneider e*t al*., 2020). The disease induces enormous social and cultural influence in the region. It reshapes the landscape in Italy's olive-growing regions and has put into a difficult situation the growers who are against eradication measures to cut down centuries-old trees (Martelli, 2015)

In California alone, it has been estimated that Pierce's disease directly costs the grape-growing sector $104 million per year in reduced yield, management costs, and regulatory costs (Tumber *et al.*, 2014). Since the initial outbreak, the disease has spread through a large proportion of the olive trees (*Olea europaea*) in southern Apulia (Martelli, 2016). Furthermore, it has been estimated that olive-producing countries could lose billions of euros to the disease (Schneider *et al*., 2020) and considering the vast distribution of olive orchards in Apulia, further disease dissemination is expected.

I.2. Natural antagonists

Bacteria that reduce the incidence or severity of plant diseases are referred to as biocontrol agents and if they exhibit antagonistic activity against a pathogen, it is called an antagonist. (Arwiyanto, 2014). Biocontrol agents provide protection against an array of pathogens. The result of the BCA application is to decrease the incidence and severity of diseases. To suppress disease and promote plant health, a successful biocontrol agent requires a mixture of adaption and biocontrol factors. The screening methods for BCAs with direct biocontrol activity consists in general of co-cultivating the pathogen and the BCA on (semi-)solid medium and evaluating the antagonistic capacity of the BCA by measuring the zone of pathogen growth inhibition (Pliego *et al.*, 2011; Sales *et al.*, 2016). Direct or indirect microbial interactions can be linked to biocontrol factors. Direct interactions occur between the pathogen and the biocontrol agent. Indirect interactions are the interactions between the biocontrol agent and the host plant which improves the plant's health, like resistance to the disease **(Figure 5)**.

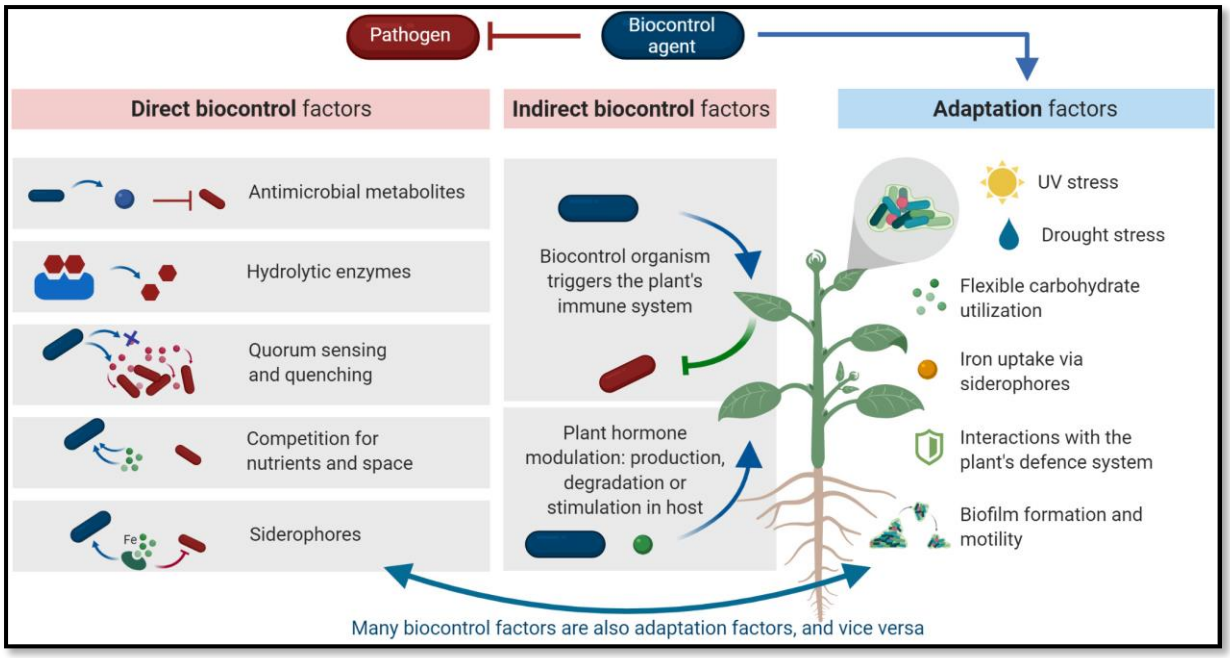

Figure 5: Direct interactions are interactions directly between biocontrol agent (blue rod) and pathogen (red rod). Indirect interactions are interactions between biocontrol agent and host plant that lead to an enhanced immunity of the host plant against pathogen infection (Source: Morelli *et al.*, 2021)

Bacteria as BCAs are widely reported, and in some cases, their modes of action against the plant pathogen have been elucidated. Antagonistic bacteria act via a range of modes of action. Some BCAs interact with plants by inducing resistance or priming plants without any direct interaction with the targeted pathogen (Conrath *et al.*, 2015; Pieterse *et al.*, 2014). Some antagonists may compete for space and nutrients or other mechanisms modulating the growth conditions for the pathogen (Spadaro & Droby, 2016). Antagonists acting through hyperparasitism and antibiosis are directly interfering with the pathogen. Production of antimicrobial secondary metabolites with inhibiting effects against pathogens is another direct mode of action (c). Antagonistic bacteria can produce several other metabolites including antibiotics, enzymes and volatiles, which may play important roles in the control of pathogens. Antibiotics produced by bacterial biocontrol agents comprise chemically heterogeneous groups of organic low molecular weight compounds. For instance, antibiotics produced by

Pseudomonas species include phenazines, phloroglucinols, oomycin A, pyoluteorin, pyrrolin, 2,3-de-epoxy-2,3-didehydro-rhizoxin (Raaijmakers *et al.*, 2002). Biological control agents used for bacterial plant pathogen include *Bacillus*, *Erwinia*, *Streptomyces*, *Pseudomonads* especially fluorescent Pseudomonad, an avirulent form of the pathogen, bacteriophage, protozoa, and Bdellovibrio. The potential of antagonists to produce such compounds *in vitro* does not necessarily correlate with their *in-situ* antagonism. Understanding the mode of action of BCAs is essential to achieve optimum disease control (Köhl *et al.*, 2019).

I.3. Use of epiphytes and endophytes as biological control agents

Biological control of plant diseases by endophytic and epiphytic microorganisms is a demanding future prospect for plant protection. In the literature, there are several examples of these organisms illustrating their potential as biocontrol agents in controlled or laboratory-scale environments. However, isolates that worked well in the laboratory did not always work in the field. The ability of potential antagonists to survive, grow and perform effectively under the often-hostile environment on or in aerial plant parts has not been considered sufficiently when screening for biocontrol agents. In general, several isolates with antagonistic activity have been isolated from the phyllosphere (Cook & Baker, 1983; Sahu *et al*., 2021), but only a few are able to execute biological control under natural conditions. Many studies have shown that the indigenous microflora in the phyllosphere protects against diseases (Cook & Baker, 1983; Reinecke, 1981). This is demonstrated by less severe diseases on leaves containing the normal leaf flora than on leaves where the microflora has removed or reduced by chemical treatments (Pusey *et al*., 2009). Endophytes are commonly used as biocontrol agents against plant infections in sustainable agriculture. The advantage of these endophytic agents is that they are better protected against biotic and abiotic challenges from outside the plant since they revert to the endophytic stage after application. They are also environmentally suited to the target niche, allowing them to overcome defense responses. In many research, endophytic bioagents were

used to control local or systemic plant diseases. Integrative formulations of endophyte species such as *Bacillus* and *Pseudomonas* have been utilized to manage fungal diseases in local pathogenic infections (such as powdery and downy mildew) and bacterial cankers (such as citrus and poplar cankers) (Daungfu *et al*., 2019; Gao *et al*., 2015). Similarly, endophytic biocontrol treatments for wilt diseases caused by systemic pathogenic infections have sparked the most attention in a variety of fruit and vegetable crops such as tomato, eggplant, olive and many others (Maldonado-González *et al*., 2015; Nawangsih *et al*., 2011; Ramesh *et al*., 2009).

I.4. Control of *X. fastidiosa*

At this time, it should be evident that *X. fastidiosa* is a pathogen that is rapidly growing as one of the world's most serious agricultural pathogens, with no known cure. Researchers have produced a growing body of literature on the attempts to control the pathogen, in addition to government measures, through the application of various treatments, that might be grouped into three classes, depending on the target of the treatment: the infected plant, the bacterium, or its vector. The different research studies have relied on several control approaches, which in turn involve mineral formulations, chemical compounds, natural products, and microbial antagonists.

I.4.1. Targeting the infected plants

The removal of infected plants had been sometimes successful in the containment of the early-phase epidemics. For instance, the eradication of a single infected spot in Germany (Baldi & La Porta, 2017) as well as the containment of the infected zone in Southern California, where an integrated strategy has been applied to reduce the spread of the Pierce's disease to the north (Bruening *et al.*, 2014).

In response to the outbreak of *X. fastidiosa* in Italy, the European Commission (EC) took immediate measures to prevent the introduction and spread of the disease by executing

Commission Implementing Decision (EU) 2015/789. This legislative outlined a detailed contingency plan, the establishment of demarcated areas, containment, eradication and, protection measures against *X. fastidiosa*. With the ceaseless spread of *X. fastidiosa* in Europe, on the 14th of August 2020, the EC decided to revoke (EU) 2015/789 by introducing the new Regulation (EU) 2020/1201, which included new and adapted control measures based on the latest findings from the European Food Safety Authority (EFSA). Briefly, Regulation (EU) 2020/1201 (EU, 2020) mandates that upon the detection of an outbreak due to *X. fastidiosa* in any EU member state, the relevant plant health authorities within that member state ought to, without delay, establish a demarcated area, which includes an infected zone and a buffer zone. In addition, pruning of infected plants has also been attempted to moderate symptoms or to recover plants from infection, knowing that the bacterium tends to move from terminal sprigs, which are usually targeted by infected insects, toward the trunk of the plant. Pruning against *X. fastidiosa*, alone or in combination with other treatments, was previously described in oleander (Grebus & Henry, 1999), Citrus (Beretta *et al.*, 1996), grapevine (Feil *et al*., 2003), almond (Haviland & Viveros, 2005), and olive trees (Martelli *et al*., 2016). Another plant targeting method is the so-called "cold therapy" that has been proposed as a means to control the spread of *X. fastidiosa*. Bacteria were found to be sensitive to cold (Feil & Purcell, 2001), and a treatment plan for affected grapes was proposed (Lieth *et al*., 2011; Meyer & Kirkpatrick, 2008). However, it is unclear whether the results obtained on vine grapes can be extended to other plants. Screening for resistant and tolerant cultivars is also another methodology used against *X. fastidiosa*. The basic idea is to exploit resistant crop varieties, similar to what has been successfully done in the past for different parasites. In Apulia, several field observations showed that although the more widely represented cultivars Ogliarola salentina and Cellina di Nardò are highly susceptible to *X. fastidiosa*, resistance traits were found in the cultivar Leccino. Studies of the plant response to the infection showed that in xylem tissues of field-

grown Ogliarola salentina and Leccino olives: (i) the bacterium population size of Leccino is 100 times lower ($4x10^4$ vs. $2x10^6$ CFU/mL) than that of Ogliarola salentina (D'Attoma *et al.*, 2020; Giampetruzzi *et al.*, 2016).

I.4.2. Targeting the bacterium

Several chemicals, including antibiotics, metal compounds, and natural substances have also been tested *in vitro* and in fields against *X. fastidiosa*. Among them, compounds, Dentamet®, a zinc-copper–citric acid bio complex, was used as treatment via foliar spray and had shown that this complex can reduce *X. fastidiosa*-associated disease severity. However, the restricted time range of the application and the limited number of observations did not allow for conclusive evidence of complete eradication of the pathogen (Scortichini *et al.*, 2018). Aside from the treatment with zinc and copper, other procedures to control *X. fastidiosa* in olive plants and employing mineral solutions have been attempted in Italy. OQDS-affected trees showed clear symptoms reductions when sprayed with ammonium chloride, but no significant alterations in bacterial populations were found (Crescenza *et al.*, 2021). Metal nanooxides have recently been investigated as carriers for the direct release of chemicals for the treatment of *X. fastidiosa* in olive trees. Following interactions with calcium carbonate nanocarriers, which were absorbed by the olive roots and successfully translocated to conductive tissues, transmission electron microscope investigations revealed a change of the bacterial cell wall (Baldassarre *et al.*, 2020). Among the most well-studied control strategies for *X. fastidiosa* is also N-acetylcysteine (NAC). To confirm the effect of NAC on OQDS, some field trials were conducted in Apulia. In general, NAC treatment appears to slow disease development, particularly when combined with NAC endotherapy; nonetheless, qPCR studies revealed no significant reduction in bacterial population size (Alessandra A de Souza, 2019). Other strategies was used also to reduce *X. fastidiosa* including fosetyl–aluminum nanocrystals coated with chitosan and antimicrobial peptides (AMPs) (Baró *et al.*, 2020), although, so far

such mineral solutions have not guide to efficient *X. fastidiosa* disease control and further products are still needed. *In vitro* antimicrobial activities of different classes of plant-derived phenolic compounds (4-methylcathecol, cathecol, veratric acid, caffeic acid, and oleuropein), filtered fractions of olive mill wastewaters (OMW), *Trichoderma* spp. culture extracts, and fungal toxins were investigated by Bleve *et al.*, 2018. All of the phenolic substances studied had some inhibitory effects against the *X. fastidiosa* strain "De Donno" isolated from olive plants, but only reversible bacteriostatic effects. Furthermore, ophiobolin A and gliotoxin inhibited bacterial growth, although a crude culture extract from a *Trichoderma* citrinoviridae strain was bactericidal. Interestingly, the addition of microfiltered OMW fractions in the growth medium impacted “De Donno” growth. Experiments to reduce *X. fastidiosa* and its associated plant symptoms were made based on its diffusible signal factors. *X. fastidiosa* lifecycle showed to be regulated by a complex metabolic pathway regulated by a family of short-chain fatty acid molecules known as diffusible signal factors (DSF) (Beaulieu *et al*., 2013; Chatterjee, Newman, *et al*., 2008). Endophytic microorganisms, competing with *X. fastidiosa* and able to alter its virulence, have also been exploited to control the diseases, in a strategy called symbiotic control. *Methylobacterium mesophilicum* and *Curtobacterium flaccumfaciens* in citrus, several fungi in vine grapes, *Paraburkholderia phytofirmans* in vine grapes and recently in olive trees are examples of microorganisms that are able to downregulate *X. fastidiosa* growth or plant symptoms, either because they compete with the pathogen or because they secrete compounds able to change its virulence (Azevedo *et al*., 2016; Dourado *et al*., 2015). For *Paraburkholderia phytofirmans*, Preliminary trials aimed at testing its effectiveness as a biocontrol agent in the “De Donno” olive pathosystem in Italy have not revealed significant differences in the reduction of OQDS symptoms in therapeutic treatments, nor reduction of the new infections upon preventive applications (Morelli *et al*., 2019). Recent research looked at the involvement of microbial endophytes residing the sapwood of Apulian

olives that might be a promising control strategy for xylem colonizing pathogens as *X. fastidiosa* and in the expression of resistance characteristics against OQDS, which has been identified in various olive cultivars (Hanani *et al*., 2021). The resistant cultivar Leccino showed a greater diversity of the microbial communities in comparison with the susceptible cultivar Cellina di Nardò (Vergine *et al.*, 2019).

In addition, *Methylobacterium mesophilicum* and *M. radiotolerans* strains were found to be able to produce extracellular siderophores. Microorganisms producing these Fe^{3+}-binding compounds can significantly enhance their biocontrol efficiency (Susi *et al.*, 2011). A different line of research involves biocontrol of the bacterium using specific phages, able to lysate *X. fastidiosa*. Various virulent phages, active also against the pathogen *Xanthomonas*, were shown to inhibit *X. fastidiosa in vitro* (Ahern *et al.*, 2014).

I.4.3. Targeting the insect vector

In terms of the various strategies employed to reduce the number of juveniles, several experiments have been carried out in Italy and Spain. Dongiovanni *et al*., 2018 compared over three years the efficacy of several applications targeting weeds and ground vegetation as a tool to decrease juveniles *P. spumarius* and *N. campestris*. The following trials were compared: no-tillage; soil tillage performed twice; soil tillage performed only during winter; sowing *Poaceae* crops; shallow ploughing; mulching; application of herbicides; pyroweeding. In these studies, the population densities (nymphs/m2) clearly revealed that all interventions were able to dramatically reduce the juveniles of both spittlebug species when compared to non-disturbed ground vegetation (no-tillage). The application of a systemic herbicide, pyroweeding, and double tillage, in particular, produced the best results for both spittlebugs. In addition to weed management, several insecticides, most notably the neonicotinoid Imidacloprid, have been tried with considerable success, particularly in California. Overall, pyrethroids were found to be the most effective class of insecticides in the testing of measures against nymphal

populations in Italy and Spain, confirming prior findings acquired for the sharpshooter leafhopper *Homalodisca vitripennis Germar* (Hemiptera: cicadellidaea) (Prabhaker *et al*., 2006). In Europe, surveys for natural enemies of spittlebugs have been intensified also in recent years. Egg parasitoids were found in Portugal, according to Reis et al. (Reis *et al*., 2018). The presence of the egg parasitoid *Ooctonus vulgatus Haliday* was reported recently in Corsica (Mesmin *et al*., 2019). Moreover, Liccardo et al. (Liccardo *et al*., 2020) studied the predation patterns of the generalist predator *Zelus renardii Kolenati* on *P. spumarius.*

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CHAPTER II

Screening, identification, and molecular characterization of a novel biological control agent targeting the phytopathogen *X. fastidiosa* ST53 in Apulia region

II.1. Background

Biological disease control is now widely recognized as an important approach in integrated pest management due to its significant potential as an alternative/complement to these pesticides (Integrated Pest Management). However, one of the obstacles to large-scale biocontrol deployment is the lack of effective, commercially available biocontrol agents (BCAs). The discovery of novel BCAs is thus a crucial stage in the development of commercial biocontrol products, and it necessitates the use of fast and reliable screening methods capable of evaluating large numbers of candidate BCAs (Raymaekers *et al.*, 2020).

Olive quick decline syndrome (OQDS) caused by *X. fastidiosa* subsp. *pauca* ST53 poses a severe threat to the agriculture of Mediterranean countries and causes intense damages to the olive trees in the Apulia region in Italy. So far, few studies have been conducted to retrieve direct antagonists for a sustainable strategy of *X. fastidiosa.* Particularly, the endophyte *Curtobacterium flaccumfaciens* has been found to limit the growth of *X. fastidiosa* in vitro and reduced the symptoms generated in Catharanthus roseus (Lacava *et al.*, 2007). Baccari *et al.*, 2019 reported also that the endophytic bacterium *Paraburkholderia phytofirmans* strain PsJN controls *X. fastidiosa* infections in the grapevine, while recently published studies have stated the absence of antagonists natively isolated from Apulian olives to inhibit *X. fastidiosa* ST53 growth *in vitro* (Zicca *et al.*, 2020). To date, recent research study was carried out to understand

the endophytic composition of the sapwood of Apulian olive trees with different susceptibility, seasonality, and geographical location that could create an advantageous context for the setting up of efficient biocontrol tools to cope with *X. fastidiosa* infection (Hanani *et al.*, 2021). Therefore, since no effective control measures are currently available to eliminate the bacteria from a diseased host plant. In this chapter, we targeted epiphytes inhabiting leaf surfaces of different host species by *in vitro* screening assays for their direct antagonistic activity against *X. fastidiosa* ST53.

II.2. Material and Methods

II.2.1. Isolation of potential biological control agent (BCAs):

Sampling of leaves from different hosts of the bacterium was carried out in various *X. fastidiosa* outbreaks occurred in the demarcated area in Apulia region defined in the Commission Implementing Decision (EU) 2018/927 and in the Decision of the Regional Phytosanitary Service. The sampled host species were *Olea europaea*, *Polygala myrtifolia*, *Rosmarinus officinalis*, *Nerium oleander*, *Laurus nobilis*, *Myrtus communis*, *Prunus Dulcis*, *Prunus avium* and *Cistus creticus*

Ten leaves were picked randomly from each plant species and the total surface area was estimated with "ImageJ 1.52v" software to determine leaf areas. Leaves from the different hosts were washed separately in flasks containing 50 ml of sterile distilled water and gently shacked for 2 hours at 150 rpm. A dilution series was made of each sample solution and 100 µl each of the 10^2, 10^3, 10^4 and 10^5 dilutions were spread onto King B and NA medium. After 48 h incubation at 27°C the total colonies growth on plates were counted. Representatives of each group with similar morphological characteristic were reisolated for further tests. Glycerol stocks were prepared in nutrient broth with final glycerol stocks concentration of 25% (v/v) and stored at (-80°C) (Balestra *et al.*, 2005; Ercolani, 1991).

II.2.2. Screening of potential antagonist strains against *X. fastidiosa* ST53:

The antagonistic activity of the isolated bacteria against *X. fastidiosa* ST53 was studied on BCYE solid nutrient media by the dual culture method previously described by (Zicca *et al*., 2020) with slight modification. To estimate the antagonistic activity, bacteria were co-plated in the following order: three drops of *X. fastidiosa* suspension (10^6 CFU/ml), each containing 20 µl, were placed at the top of the petri dish, 1 cm apart, and allowed to slowly flow down to the opposite side of the plate, resulting in three parallel rows of *X. fastidiosa* cultures. After 24 h of incubation at 28 ±2°C, an aliquot of 5 µl of a suspension of the potential BCA (10^8 CFU/ml) was placed on the top of each row of *X. fastidiosa* cultures and ampicillin (20 µg/ml) was used as a positive control.

After 7–12 days of incubation at 28 ±2°C, the antagonistic activity was determined as an area of *X. fastidiosa* growth inhibition, which was measured as the distance between the edges of *X. fastidiosa* growth and the growth of the tested strain. The tests were performed in triplicate.

II.2.3. Characterization and identification of potential antagonistic strains:

The potential BCAs selected were submitted to some morphologically and biochemically tests (e.g. fluorescence production test, levan production test, oxidase test) (Goszczynska *et al*., 2000; Klement *et al*., 1990) and were identified by analysis of DNA sequence of the 16S rDNA (Marchesi *et al*., 1998) .

II.2.3.1 Solubility assay in KOH

A good alternative to Gram staining is represented by the KOH solubility assay; for which a drop of 3% KOH solution in sterile distilled water is placed on a glass slide. Then, with a sterile loop, an aliquot of the antagonistic bacterial culture is streaked on the glass slide to obtain a homogeneous mixture. If the latter appears to be viscous and filamentous, bacterium under investigation are Gram-, otherwise they are Gram+. This is due to the ability of KOH to degrade

only the cell wall of Gram- bacteria but not the one of Gram+ bacteria (Goszczynska *et al*., 2000).

II.2.3.2 Production of fluorescent pigments on King's B

Fluorescein is a fluorescent pigment (from green to blue colours) visible under ultraviolet rays (λ365 nm). To perform this test, it is necessary to allow the growth of bacteria on agar medium King's B (KB) (King *et al*., 1954). A strain of *Pseudomonas syringae* (CFBP311) and *Pseudomonas viridiflava* (LMG 2352) were used as positive control respectively. The plates were observed 24, 48 and 72 hours after being placed in the incubator at 26±1°C, using a UV lamp.

II.2.3.3 Levan production

Due to the action of the enzyme Levan Saccharase, many bacterial species are able to split sucrose into two simple sugars that constitute it: glucose and fructose. While Glucose is used as carbon source, Fructose is polymerized in an extracellular polysaccharidic form, called Levan, which is a polymer of β-D-Fructose. Levan colonies are characterized by whitish dome-shaped mucous To verify this capacity for the selected natural bacterial antagonists aliquots of their culture were streaked, through a sterile loop for bacteriology, in Petri dishes with nutrient agar medium containing 5% Sucrose (NAS) (Klement *et al.*, 1990). The plates were observed after 24 hours of incubation at 26±1°C.

II.2.3.4. Oxidase production:

A few drops of a 1% NNN'N-tetramethyl-p-phenylene-diamino-dihydrochloride (TFDD) solution were placed on filter paper (Whatman No. 1) with a Pasteur pipette. Then, with the tip of a toothpick, a small number of bacterial colonies from 24 h cultures were transferred onto the filter paper. The color change of the filter paper to dark purple within 30 seconds indicated a positive response to oxidase production (Kovacs, 1956).

II.2.4. Molecular characterization and identification of antagonistic bacteria:

A molecular characterization of the antagonistic isolates was performed by ERIC-PCR. The genomic DNAs of each bacterial isolate were extracted from single colonies grown for 48h at 28°C on KB-agar (King B) plates. Cells were harvested and their DNA was extracted using the standard CTAB based procedure (PM 7/24 (4) *Xylella fastidiosa*, 2019). In detail, 1 ml of bacterial suspension was incubated for 30 min at 70°C. DNA was extracted by adding 1 ml of chloroform and centrifuging for 10 min at 13000 rpm. Then, 750 µl of supernatant were transferred to a new tube and diluted with cold isopropanol (450 µl). This dilution was gently mixed and stored at -20°C for minimum 2 hours. Next step requires centrifugation for 20 min at 13000 rpm. The supernatant was discarded, whereas the pellet was washed off with 1 ml of ethanol 70%. One more centrifugation was needed for 10 min at 13000 rpm. After removing ethanol, Eppendorf tubes were air-dried. Eventually, remained pellets were suspended by adding 100 µl of sterile water in each tube and mixing with vortex. DNA material prepared in this way can be used immediately, or stored at -20°C.

Genomic DNA was used as a template in a PCR reaction with the primer pair ERIC1R/ERIC2 (5′-ATGTAAGCTCCTGGGGATTCAC-3′/5′-AAGTAAGTGACTGGGGTGAGCG-3′) (Smith *et al*. 2001; Versalovic *et al.* 1994). The PCR reactions were carried out in a total volume of 25µl containing 12,5 µl of 2X Master Mix PCR (ThermoFisher Scientific, Milan, Italy), 0,5µl from each primer forward and reverse (10mM) and 2µl of genomic DNA. Amplifications were conducted starting with initial denaturation at 95°C 7 min followed by 30 cycles (94°C 1 min, 52°C 1 min, 65°C 8 min) and one final step at 65°C 16 min before cooling at 4°C. Amplicons were separated by electrophoresis in 2% TBE agarose gel and band patterns were visualized using Gel Doc EZ System (BIORAD. Milan-IT). For Bacterial identification, the 16S rDNA gene was amplified by Polymerase Chain Reaction (PCR) using approximately 50 ng of genomic DNA in a final volume of 25 µl. Almost all the gene (about 1300 bp) was

amplified using the primer pair 63f (5′-CAGGCCTAACACATGCAAGTC-3′) and reverse primer 1387r (5′-GGG CGGWGTGTACAAGGC-3′) (Marchesi *et al*., 1998), 5 µL of 5X Phusion Green HF buffer (ThermoFisher Scientific, Milan, Italy), 0.5 µL of 50 mM $MgCl_2$, 0.5 µL of 10 mM dNTP, 0.4 µL of 10 µM of each primer, 0.6 µL of DMSO, 0.25 µL of 2.0 U/µL of Phusion DNA polymerase (ThermoFisher Scientific) and nuclease-free water up to 25 µL reaction volume. PCR cycling parameters were as follows: 98°C for 30 s, 35 cycles of 98°C for 10 s, at 55°C for 30 s, and 72°C for 45 s and a final extension at 72 for 7 min. Reaction products were analyzed by electrophoresis in 1.2% TAE agarose gel and DNA bands were visualized at Gel Doc EZ System (BIORAD. Milan-IT).

The amplification products were sequenced in both directions using Eurofins Genomics (https://www.eurofinsgenomics.eu/). Sequences were submitted to the National Center for Biotechnology Information (NCBI) online search engine BLAST. The assigned sequences that had ≥ 98% identity to a valid sequence were deposited in NCBI under specific accession numbers. Consistently, the IQtree algorythm on Galaxy server was used to construct a phylogenetic tree generated with 1000 bootstrap repeats using Maximum likelihood method and was edited using Itols-v5.

II.2.5. Ability to colonize Olive leaves:

The best potential BCAs strains were assessed for their ability of colonizing olive leaves. A 2 years-old, potted olive plant cv. Ogliarola salentina were used. The plants were watered daily, and a mineral solution was distributed weekly into the pots to maintain optimum nutritional conditions. The experiments were carried out in a greenhouse where temperature was maintained at 25±2°C during the day and 15±2 °C during the night; the relative humidity was maintained between 70 and 80%, during the whole experiment, by using automatic cooling.

For inoculation, the strongest BCAs strains in vitro were streaked onto LB-agar plates and incubated at 28°C for 48 h. Cells were suspended in 100 ml of sterile distillate water (SDW) and the density of the suspension was adjusted to 1 x 10^8 CFU/ml using a spectrophotometer at 600 nm. Olive plants were sprayed with each bacterial suspension until leaves got homogeneously wet by spraying plants with a CO_2-pressurized handheld sprayer equipped with a large orifice (diameter 1.4 mm, Tee-Jet 8004) nozzle operating at a pressure of 28 g cm2 to produce fine spray droplets. Inside the greenhouse, 2 h before and 2 h after bacterial inoculation, the relative humidity was maintained at 90% to favor stomata opening. Each BCA strain was sprayed on 4 olive plants. For estimation of bacterial population sizes, 18 hours, 1, 7, 15 days after spraying, 10 leaves were harvested from each treatment (replicates) of plants treated with different BCAs. Leaves were immersed separately in flasks containing 10 ml of sterile distilled water (SDW) and shacked for 2 hours at 150 rpm. Serial dilutions of leaf washings were plated onto LB-agar plates amended with 100 µg of streptomycin per ml using spiral plate system (Don Whitley Scientific, mod. WA01GG). Plates were incubated 48 h at 27°C and the single colonies developed were counted to obtain the CFU/cm^2 of leaves (O'brien & Lindow, 1989).

II.2.6. Statistical analysis

In *in vitro* tests the mean, standard error and standard deviation were calculated.

In *in vivo* tests bacterial population dynamics were evaluated using the Log transformed population sizes. The population sizes were expressed per square centimeter of leaf, being estimated using ImageJ software to determine leaf areas. Statistical analysis was performed using SPSS software package (version 26.0). Data concerning antagonistic activity were compared by applying a one-way ANOVA followed by Tukey's test to determine significantly different values (P<0.05)

II.3. Results:

II.3.1. Determination of total bacterial population on leaves of different host plant species of *X. fastidiosa*:

A total of 89 bacterial isolates were obtained from 9 different host plant species sampled from various *X. fastidiosa* outbreaks occurred in the infected zone of the demarcated area in Apulia region (Table 2). Overall, differences were observed between bacterial population size of various leaves. The highest population was recorded on *Cistus criticus*, *Polygala myrtifolia* and *Olea europea* leaves with a mean of 5 x 10^5 CFU/cm^2 ,1,23 x 10^5 CFU/cm^2 and 4,67 x 10^4 CFU/cm^2 respectively. For *Prunus avium and Prunus dulcis,* population size obtained were approximately the same (2,20 x 10^4 CFU/cm^2 and 2 x 10^4 CFU/cm^2). Furthermore, there is a slight difference observed between *Nerium oleander* and *Rosmarinus officinalis* population size (6,80 x 10^3 CFU/cm^2 and 4,80 x 10^3 CFU/cm^2). However, *Laurus nobilis* and *Myrtus communis* showed the lowest population size (2,30 x 10^3 CFU/cm^2 and 2,40 x 10^3 CFU/cm^2) **(Figure 7)**.

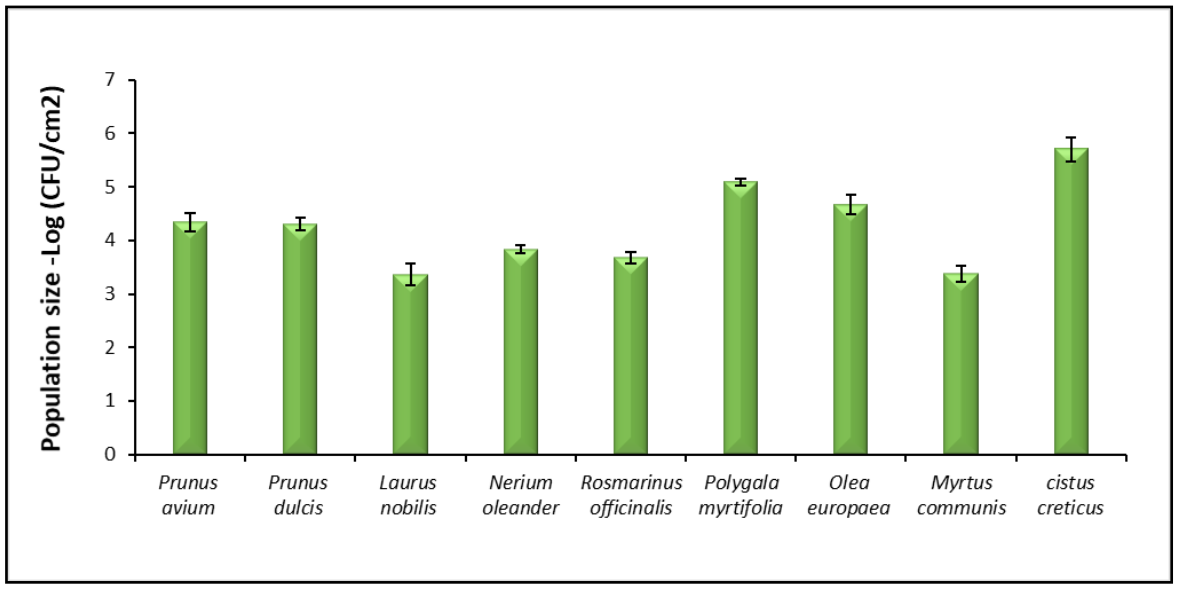

Figure 6: Total bacterial population on leaves of different host plant species of *X. fastidiosa*, Errors bars indicate the standard error

II.3.2. Antagonistic activity of different potential BCAs strains against *X. fastidiosa* strain ST53:

A total of 89 epiphytic bacteria were screened for their ability to inhibit *X. fastidiosa* (strain De Donno) by in vitro dual culture assay. From the results, we found 12 of the test strains isolated from different host plants had antagonistic activity to *X. fastidiosa* (Table 2). Isolates BA69 (isolated from *Nerium oleander*), BA104, and BA102 (isolated from *Polygala myrtifolia*) showed the highest activity causing very large inhibition halos (Table 2) with a mean growth zone of 34, 16.6, and 12.3 mm, respectively, suggesting that these strains could efficiently inhibit the growth of *X. fastidiosa* strain De Donno. No significant differences were observed between isolates BA95 (isolated from *Myrtus communis),* BA103 (isolated from *Polygala myrtifolia*) and BA110 (isolated from *Olea europaea*) with a mean inhibition halo of 10, 10.3 and 9.6 mm. Furthermore, results also indicates that BA43 (isolated from *Olea europaea*) and BA111(isolated from *Polygala myrtifolia*) isolates were able to curtail *X. fastidiosa* growth, as indicated by the clearing zone of 7 mm with no significant difference. BA91, BA54, and BA2 isolates isolated from (*Olea europaea and Rosmarinus officinalis*) were weak inhibitors of the pathogen with a mean inhibition zone of 4, 4.66, and 6 mm, respectively **(Figure 8, Table 2).**

Table 2: *In vitro* inhibition results of dual culture assays:

Code	Collected from: Host plant	BCAs strains Name	Antagonistic activity (+/-) *	Size (mm ± SD) of the growth inhibition halo
M4	*Rosmarinus officinalis*	BA1	-	0
M4	*Rosmarinus officinalis*	BA2	+	6,0 ± 1[de]
M4	*Rosmarinus officinalis*	BA3	-	0
M4	*Rosmarinus officinalis*	BA4	-	0
M4	*Rosmarinus officinalis*	BA5	-	0
M4	*Rosmarinus officinalis*	BA6	-	0
M4	*Rosmarinus officinalis*	BA7	-	0
M4	*Rosmarinus officinalis*	BA8	-	0
M4	*Rosmarinus officinalis*	BA10	-	0
M4	*Rosmarinus officinalis*	BA11	-	0

M4	*Rosmarinus officinalis*	BA12	-	0
M4	*Rosmarinus officinalis*	BA13	-	0
M3	*Nerium oleander*	BA14	-	0
M1	*Olea europaea*	BA15	-	0
M1	*Olea europaea*	BA20	-	0
M2	*Olea europaea*	BA21	-	0
M4	*Rosmarinus officinalis*	BA27	-	0
C5	*Cistus creticus*	BA28	-	0
C5	*Cistus creticus*	BA29	-	0
M4	*Rosmarinus officinalis*	BA30	+	8 ± 1^{cd}
C5	*Cistus creticus*	BA31	-	0
C5	*Cistus creticus*	BA32	-	0
C5	*Cistus creticus*	BA33	-	0
ID8	*Olea europaea*	BA34	-	0
Id8	*Olea europaea*	BA35	-	0
M1	*Olea europaea*	BA36	-	0
ID8	*Olea europaea*	BA37	-	0
ID8	*Olea europaea*	BA38	-	0
ID5	*Olea europaea*	BA39	-	0
ID5	*Olea europaea*	BA40	-	0
ID8	*Olea europaea*	BA41	-	0
ID5	*Olea europaea*	BA42	-	0
ID5	*Olea europaea*	BA43	+	7 ± 1^{de}
ID5	*Olea europaea*	BA44	-	0
C5	*Cistus creticus*	BA45	-	0
ID5	*Olea europaea*	BA46	-	0
ID9	*Nerium oleander*	BA47	-	0
ID9	*Nerium oleander*	BA48	-	0
M3	*Nerium oleander*	BA49	-	0
ID9	*Nerium oleander*	BA50	-	0
ID9	*Nerium oleander*	BA51	-	0
ID9	*Nerium oleander*	BA52	-	0
ID9	*Nerium oleander*	BA53	-	0
ID8	*Olea europaea*	BA54	+	$4{,}66 \pm 1{,}52^{e}$
ID8	*Olea europaea*	BA55	-	0
ID9	*Nerium oleander*	BA56	-	0
ID9	*Nerium oleander*	BA57	-	0
ID9	*Nerium oleander*	BA58	-	0
ID9	*Nerium oleander*	BA59	-	0
ID9	*Nerium oleander*	BA60	-	0
C5	*Cistus creticus*	BA61(1)	-	0
C5	*Cistus creticus*	BA61(2)	-	0
ID9	*Nerium oleander*	BA62	-	0
ID5	*Olea europaea*	BA63	-	0

ID5	*Olea europaea*	BA64	-	0
ID5	*Olea europaea*	BA65	-	0
ID8	*Olea europaea*	BA66	-	0
ID5	*Olea europaea*	BA67	-	0
ID9	*Nerium oleander*	BA69	+	34 ± 2^{a}
ID9	*Nerium oleander*	BA70	-	0
ID8	*Olea europaea*	BA71	-	0
C80	*Prunus avium*	BA72	-	0
C78	*Prunus dulcis*	BA73	-	0
C78	*Prunus dulcis*	BA74	-	0
C91	*Laurus nobilis*	BA76	-	0
C80	*Prunus avium*	BA77	-	0
C78	*Prunus dulcis*	BA78	-	0
C78	*Prunus dulcis*	BA79	-	0
C91	*Laurus nobilis*	BA81	-	0
C78	*Prunus dulcis*	BA83	-	0
AM2	*Olea europaea*	BA90	-	0
AM2	*Olea europaea*	BA91	+	4 ± 1^{e}
AM2	*Olea europaea*	BA92	-	0
AM2	*Olea europaea*	BA93	-	0
AM3	*Myrtus communis*	BA95	+	10 ± 1^{cd}
AM2	*Olea europaea*	BA97	-	0
AM1	*Polygala myrtifolia*	BA98	-	0
AM1	*Polygala myrtifolia*	BA99	-	0
AM1	*Polygala myrtifolia*	BA100	-	0
AM1	*Polygala myrtifolia*	BA101	-	0
AM1	*Polygala myrtifolia*	BA102	+	$12,3 \pm 2,08^{bc}$
AM1	*Polygala myrtifolia*	BA103	+	$10,3\pm 1,52^{b}$
AM1	*Polygala myrtifolia*	BA104	+	$16,6\pm 1,52^{cd}$
AM3	*Myrtus communis*	BA105	-	0
AM3	*Myrtus communis*	BA106	-	0
AM3	*Myrtus communis*	BA107	-	0
AM2	*Olea europaea*	BA109	-	0
AM2	*Olea europaea*	BA110	+	$9,6\pm 1,52^{cd}$
AM1	*Polygala myrtifolia*	BA111	+	$7\pm 1,52^{de}$

* (-): no inhibition of *X. fastidios*a growth; (+): a halo of *X. fastidiosa* growth inhibition was detected

Values with different letters are statistically different with $P<0.05$ as determined by one-way analysis of variance (ANOVA) followed by the Tukey test.

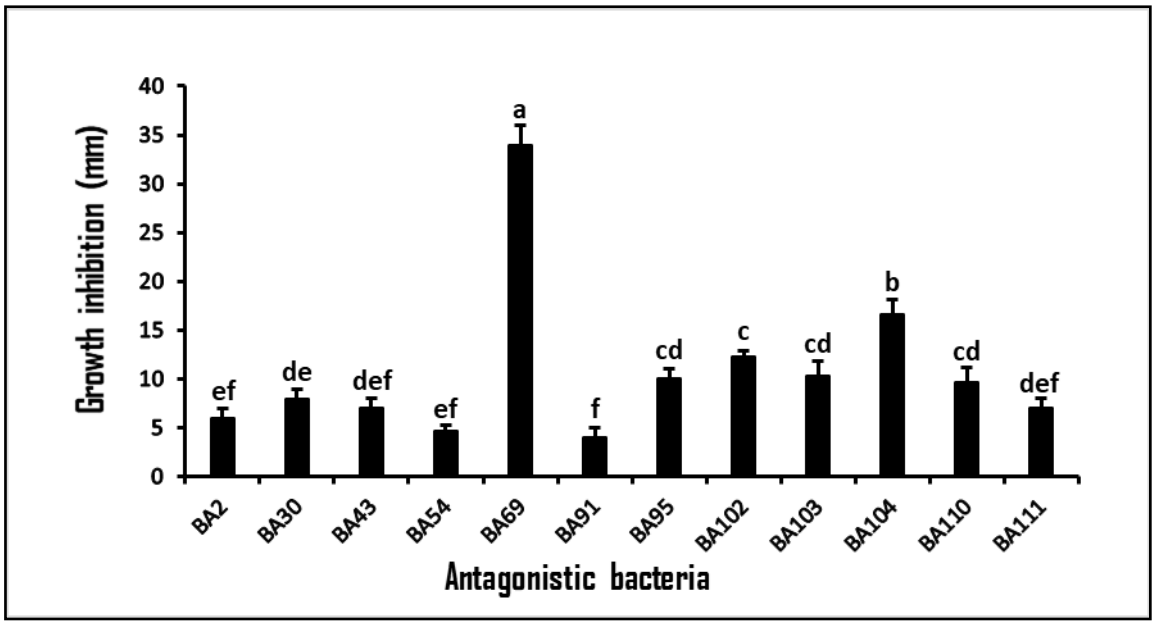

Figure 7: Graphical representation of the growth inhibition of *X. fastidiosa* (ST53) by antagonistic bacteria. Different letters indicate statistically different antagonistic activities with $P<0.05$ as determined by one-way analysis of variance (ANOVA) followed by the Tukey test.

II.3.3. Molecular characterization and ERIC results:

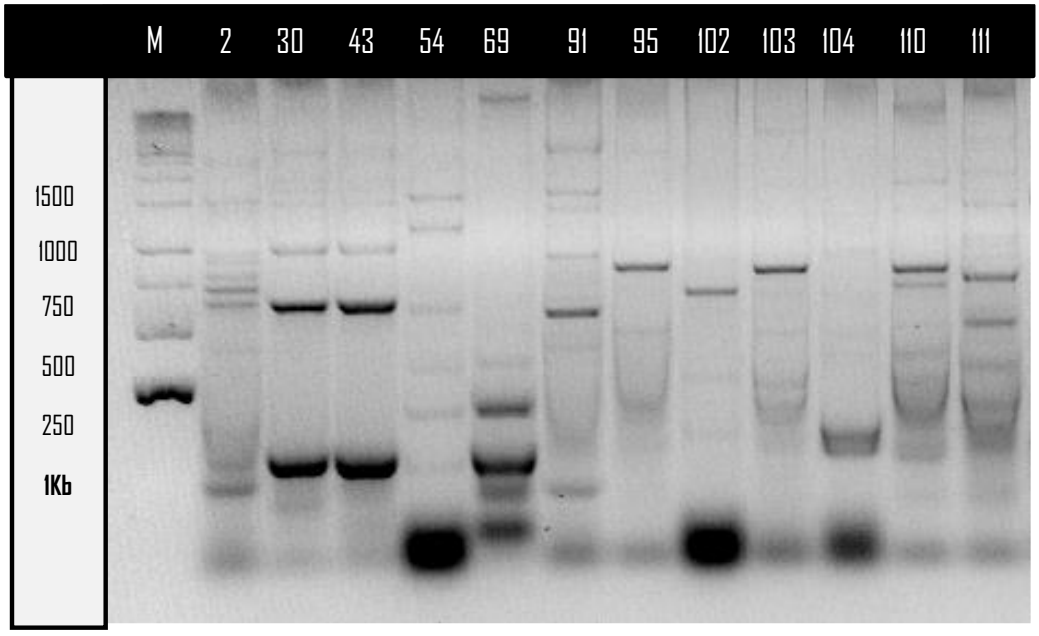

Figure 8: Patterns of ERIC-PCR fingerprint on agarose gel electrophoresis in 2% TBE agarose gel of antagonistic epiphytic bacteria isolated from different host plants. Lanes 2-111 corresponding to: BA2, BA30, BA43, BA54, BA69, BA91, BA95, BA102, BA103, BA104, BA110 and BA111 isolates, Lane M: 1Kb DNA marker.

An ERIC-PCR analysis was carried out to reveal clonal relationship between the twelve antagonistic isolates before identification with 16s rRNA identification. The isolates were analyzed for distinct DNA amplification patterns by ERIC-PCR that was able to discriminate all of the 12 isolates examined. The number and variety of DNA band profiles of the isolates obtained in the current study are presented in **(Figure 9)**. Results highlighted diverse ERIC-PCR profiles indicating the genetic diversity of the epiphytic bacterial antagonistic isolates even if obtained from the same host plant such as isolates BA2 and BA30 isolated from *Rosmarinus officinalis*. As seen, isolates BA30 and BA43 produced the same DNA profile and shared particularly the same bands. Similarly, BA103 and BA110 generated approximately similar DNA profile indicating a high degree of similarity despite of their isolation from different hosts.

II.3.4. Characterization and identification of BCAs:

Bacterial identity was determined using morphological and biochemical characteristics as levan production, fluorescence pigment production, Gram stain and oxidase, *etc*. Table 3. To confirm the identity of these antagonistic isolates 16s rDNA gene was sequenced and subjected to BLAST searches. The KOH test showed that seven out of the twelve antagonistic bacteria were gram-positive. Five isolates showed positive reaction to oxidase test. However, none of the antagonistic isolates was able to produce levan on nutrient agar and sucrose (NAS) medium or produce fluorescence pigment on King B medium (Table 3).

From the sequences and blast results, we identified epiphytic antagonistic bacteria, BA91, BA110, BA104, BA111 and BA103 as *Microbacterium oleivorans* (99,03%), *Microbacterium spp* (100%), *Microbacterium oxydans* (99,82%), *Microbacterium phyllosphaerae* (99,91%) *and Microbacterium spp* (99,91%) isolated from *Olea europea* and *Polygala myrtifolia* respectively. The genus *Pseudomonos* was also isolated from *Rosmarinus officinalis* with isolates belonged to the species *garminis.* Furthermore, other species assigned to the genera

Delftia, *Pantoea*, and *Stenotrophomonas* were isolated from different ornamental plants that were *Rosmarinus officinalis*, *Nerium oleander*, and *Polygala myrtifolia*. Isolates BA30 and BA43 showed a highest similarity to *Delftia acidovorans* species (100%). For isolate BA69 that showed the highest antagonistic activity causing very large inhibition halos (Figure 10) was assigned to *Pantoea agglomerans* species (99,91%) and isolate BA102 to *Stenotrophomonas rhizophila* species (100%) (Table 3).

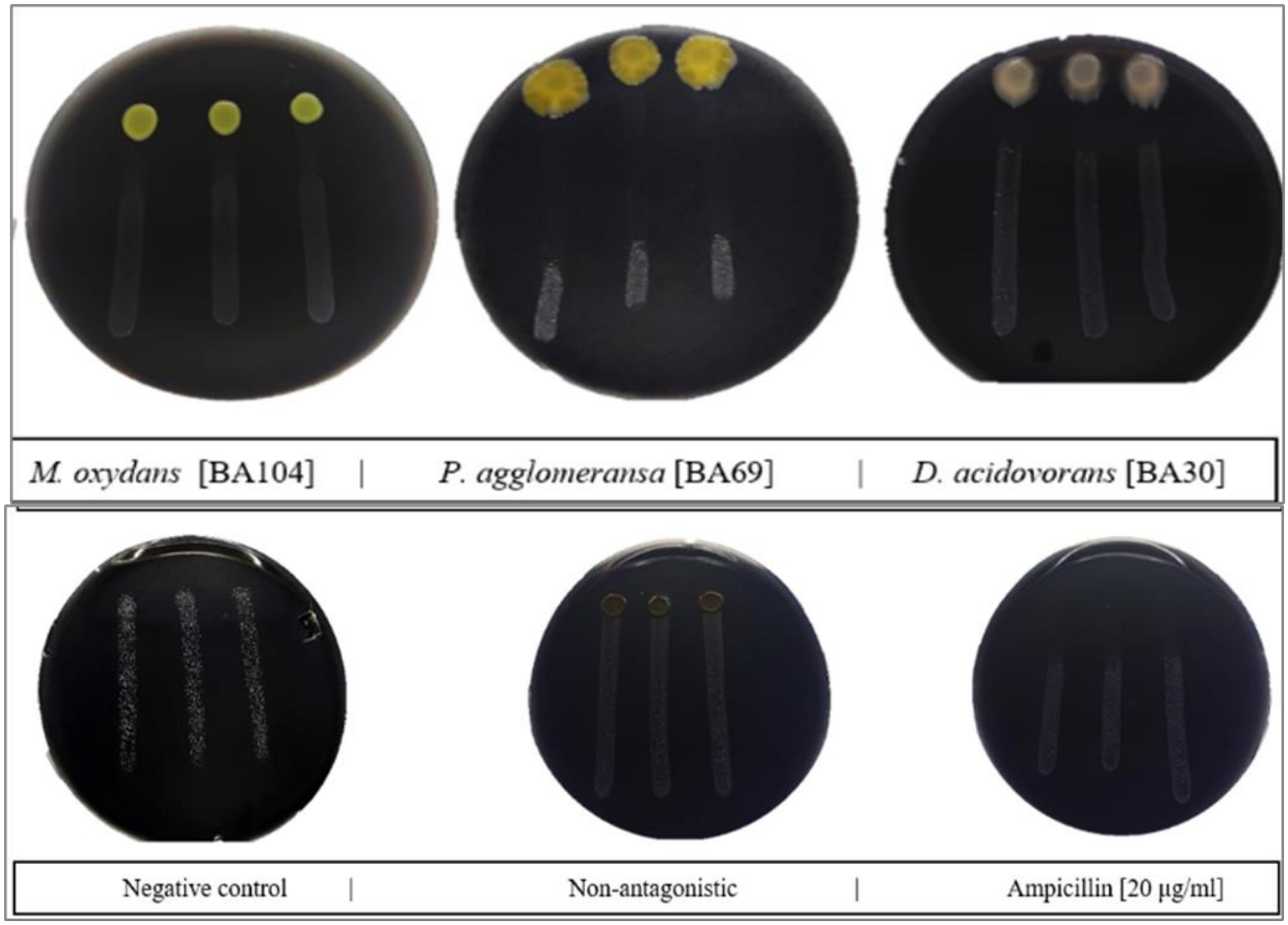

Figure 9: Dual-culture assay of antagonistic activity of potential epiphytic BCAs isolated from different host plant species against *X. fastidiosa* strain ST53**.**

Table 3: Main characteristics of the selected BCAs. Identification tests: OX (oxidase test); LE (levan production); FL (fluorescence pigment production on King B); GR (Gram Stain), - = negative, + = positive.

BCAs strains Name	Collected from: Host plant	GR	Ox	FL	LE	Belonging to	% Of 16S rRNA gene identity
BA2	*Rosmarinus officinalis*	-	-	-	-	*Pseudomonas graminis*	98,28%
BA30	*Rosmarinus officinalis*	-	+	-	-	*Delftia acidovorans*	100%
BA43	*Olea europaea*	-	+	-	-	*Delftia acidovorans*	100%
BA54	*Olea europaea*	+	+	-	-	*Curtobacterium flaccumfaciens*	100%
BA69	*Neirum oleander*	-	-	-	-	*Pantoea agglomerans*	99,91%
BA91	*Olea europaea*	+	-	-	-	*Microbacterium oleivorans*	99,03%
BA95	*Myrtus Communis*	+	+	-	-	*Sphingomonas molluscorum*	99,91%
BA102	*Polygala myrtifolia*	-	+	-	-	*Stenotrophomonas rhizophila*	100%
BA103	*Polygala myrtifolia*	+	-	-	-	*Microbacterium spp.*	99,91%
BA104	*Polygala myrtifolia*	+	-	-	-	*Microbacterium Oxydans*	99,82%
BA110	*Olea europaea*	+	-	-	-	*Microbacterium spp.*	100%
BA111	*Polygala myrtifolia*	+	-	-	-	*Microbacterium phyllosphaerae*	99,91%

II.3.5. Phylogenetic Tree

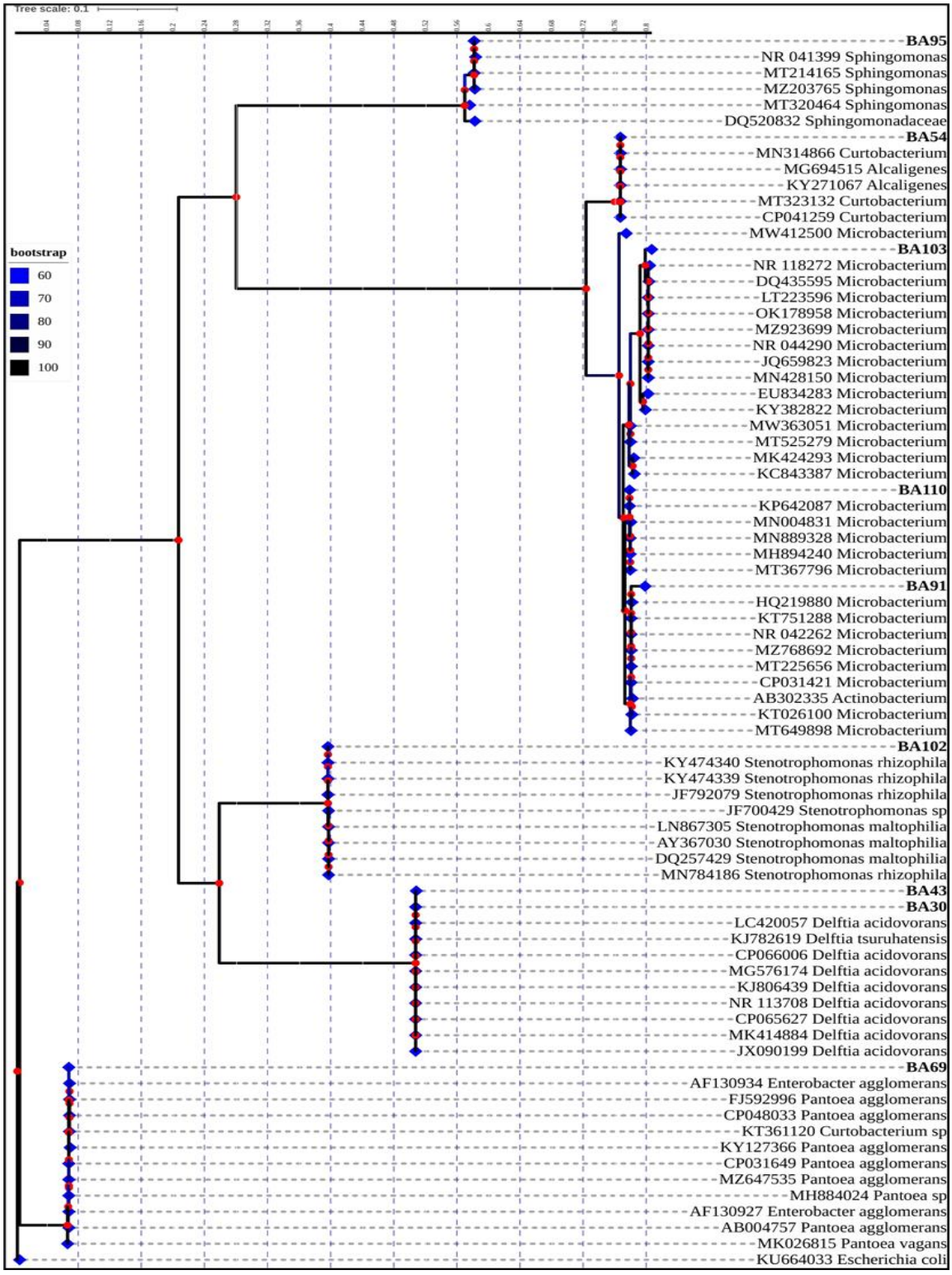

Figure 10: Phylogenetic tree based on 16S rDNA gene analysis of the antagonistic bacteria along with the sequences from selected references strains. The phylogeny analysis was constructed with the IQtree algorythm on Galaxy server and was generated using Maximum likelihood method (1000 bootstrap repeats) and edited using Itols-v5.

II.3.6. Ability to colonize Olive leaves:

After inoculation, the strongest bacterial antagonists BA102, BA69, BA103 and BA104 showed variation in stability and persistence on olive leaves of the cv. Ogliarola salentina. In particular, they were capable to decrease populations of 2-unit log in 15 days. Isolate BA103 showed the best characteristics reaching 1,67 x 10^3 CFU/cm2 at the end of the experiment (**Figure 11).** However, *S. rhizophila* BA102, *M. oxydans* BA104 and *P. agglomerans* BA69 were recovered in relatively small population sizes (<10^3 CFU/ml) after 15 days of inoculation indicating that the inoculum did not persist effectively on olive leaves of the cv. Ogliarola salentina.

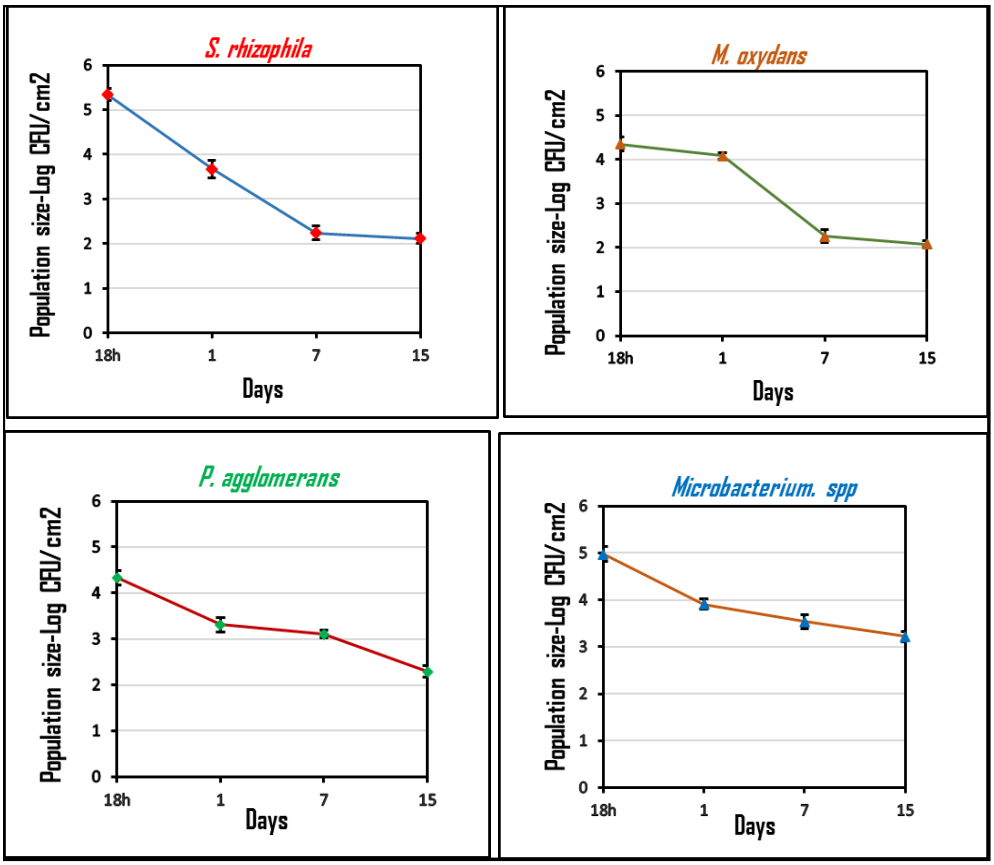

Figure 11: Population dynamics of BCAs: BA102 *S. rhizophila*, BA104 *M. oxydans*, BA69 *P. agglomerans* and BA103 *Microbacterium spp* on olive leaves, errors bars indicate the standard error.

II.4. Discussion:

The application of bacteria as biological control agents has been considered one of the most promising alternatives to protect plants against infection by phytopathogens and minimizing the use of synthetic pesticides (O'Brien, 2017). A fast-growing field of research focuses on microbial biocontrol. Generally, the phyllosphere harbors a diverse set of microbes. These microbes interact closely with each other and with the host plant. Among them are pathogens, causing disease in the host plant and reducing yields in agriculture, but also beneficial microbes which can be the key to environmentally friendly solutions to protect crops from diseases. Indeed, a high number of epiphytic bacteria have been already reported successfully as biocontrol agents (BCAs) against several phytopathogens (Legein *et al.*, 2020). Microbes arrive on the phyllosphere rather stochastically via the air, soil, rain, or insects. However, only selected taxa successfully colonize the phyllosphere (Maignien *et al.*, 2014). In the present findings, differences were observed between the amount of natural-occurring bacterial populations on leaves of different hosts. The highest population was recorded on *Cistus criticus, Polygala myrtifolia* and *Olea europea* leaves. Although, for *Prunus avium and Prunus dulcis* species, population size obtained were approximately the same. In fact, some studies have previously indicated that certain plants species and cultivar tend to support greater epiphytic bacterial population than others (Lindow *et al.*, 1978). The plant species that bacteria colonize influence their epiphytic population size for instance population of *Pseudomonas syringae* varied as much as 17-fold from plant to plant (O'brien & Lindow, 1989).

These results suggest that the variability in bacterial population size between the nine different host plant species used in the present study could be explained because of the differences between leaf characteristic of each plant species that could make leaf surface of *Cistus criticus* or *Polygala myrtifolia* for instance more favorable environment for living bacteria than the other species used. Previous studies have reported that the leaf surfaces host a dense population

of bacteria (i.e., epiphytes) estimated to reach 10^7 bacteria per cm^2 of leaf surface (Remus-Emsermann *et al*., 2014). Despite the high cell density, leaf surfaces also are a challenging ecosystem to colonize and grow on. Epiphytes must cope with constant ultraviolet (UV) radiation exposure, low water and nutrient availability and large temperature fluctuations throughout the day, making leaves an extreme environment (Schlechter *et al.*, 2019). The establishment of these microhabitats depends on the physicochemical properties of the leaf surface and the ability of microorganisms to adapt and modify this environment. Furthermore, these considerable variations in population sizes are caused in great part by the large fluctuations in the physical, nutritional conditions characteristic of the phyllosphere , leaf age and sampling time (Ercolani, 1991).

Biological control of various plant diseases using microorganisms is an effective approach to control diseases in an eco-friendly manner. The first step is to screen for potential biological control agents, and the main screening method under in vitro conditions is based on antagonistic activity. This is important because few studies have been conducted to retrieve direct antagonists for a better biocontrol strategy of *X. fastidiosa*; radicinin from *Cochliobolus* sp. has been found to be able to inhibit *X. fastidiosa* from grapevine (Aldrich *et al*., 2015), while recently published studies have stated the absence of antagonists natively isolated from Apulian olives to inhibit *X. fastidiosa* ST53 growth *in vitro* (Zicca *et al.*, 2020). In our study, the antagonistic activity of the epiphytic bacterial was investigated *in vitro* using the agar dual culture method. Our results clearly indicate antagonistic activity, in vitro, of 12 epiphytic bacteria belonging to different species of the genera Stenotrophomonas, *Pantoea*, *Microbacterium*, *Pseudomonas*, *Delftia*, *Curtobacterium* and *Sphingomonas*. It is interesting to note that these species were reported previously from diverse plants and microenvironments, they are also able to live in the endosphere of plants (Lumactud *et al.*, 2016; Schmidt *et al.*, 2021; Woźniak *et al.*, 2019) and in the case of *X. fastidiosa,* it is essential to select a potential

bio-control agent among epiphytic bacteria able to colonize the xylem. Therefore, further research is necessary to ascertain the endophytic efficiency colonization by the epiphytic antagonists founded in the present study. Frequently occurring genera in phyllosphere communities are *Methylobacterium*, *Sphingomonas*, *Pseudomonas*, and *Pantoea* (Delmotte *et al*., 2009; Lindow & Brandl, 2003; Vorholt, 2012). In present findings, *Stenotrophomonas, Microbacterium,* and *Delftia* genera were successfully isolated from the leaf surface and even though they were recovered with small population size after 15 days ($>10^3$ CFU/cm^2) from cv. Ogliarola salentina leaf surface, their efficiency to colonize epiphytically olive surface leaves has been ascertained. Among the different epiphytic antagonists observed in our results, *Pantoea agglomerans* BA69 demonstrated the strongest antagonistic activity against *X. fastidiosa* strain De Donno in Dual-culture bioassay. The genus *Pantoea* contains several plant pathogens, as well as biocontrol agents effective against a range of pathogens such as *B. cinerea, X. campestris*, and the most extensively studied, *E. amylovora* (Walterson & Stavrinides, 2015). Previous studies have reported the capacity of *S. rhizophila* to antagonize different pathogens in vitro, such as V*erticillium dahlia, Pythium ultimum Rhizoctonia solani, Colletotrichum gloeosporioides, Sclerotinia sclerotiorum*, among others (Kai *et al*., 2007; Reyes-Perez *et al.,* 2019). Moreover, the findings of this study provide evidence of the antagonistic capacity of the species *P. garminis* BA2, *M. oxydans* BA104, M. *oleivorans* BA91, *M. phyllospahrea* BA111, *D. acidovorans* BA30, *C. flaccumfaciens* BA54 and *S. molluscorum* BA95 against *X. fastidiosa* De Donno strain. In general, these species have been cited in previous studies for their potential antagonistic ability against other phytopathogens (Cho *et al*., 2007; Collazo *et al*., 2017; Han *et al*., 2005; Sahu et al., 2021; Szentes *et al*., 2013). To our knowledge, this is the first report about the potential antagonistic activity of epiphytes against the phytopathogen *X. fastidiosa* De Donno strain.

Bacterial species belonging to *Delftia* genera were described recently as soil bacteria with plant growth promoting and biocontrol properties (Han *et al*., 2005; Vacca *et al.*, 2005). Several beneficial properties (alkaline protease and phosphatase activity, phytase and lecithin degrading properties) of *Delftia lacustris* bacterial strains isolated from the same environment were also reported (Tamás *et al*., 2012). Furthermore, a few strains detected are known as pathogens of humans (Szentes *et al*., 2013). The endophyte *Curtobacterium flaccumfaciens* has been found previously to limit the growth of *X. fastidiosa* in vitro and reduced the symptoms generated in *Catharanthus roseus* (Lacava *et al*., 2007; Lacava *et al* 2004). *Curtobacterium flaccumfaciens* was also known as biological control agent against other pathogens such as *Agrobacterium tumefaciens* and *Acidovorax citrulli* (Sumer & Yesim, 2018; Tolba & Soliman, 2013). Furthermore, *P. graminis* was reported before as a highly protective against fire blight on different plant tissues and it could be a prospective candidate for future development as a biopesticide against fire blight (Mikiciński *et al.*, 2016).

In conclusion, our epiphytic collection presented several epiphytic isolates that showed antagonistic activity against *X. fastidiosa*. However, several epiphytes obtained did not show *in vitro* inhibition of *X. fastidiosa*, which does not imply that their beneficial role is excluded. Our results indicated antagonistic activity of epiphytic species belonging to different genera. Further research should be also carried out to evaluate the capability of those antagonists to inhibit *X. fastidiosa in vivo* and under natural field conditions and to validate their endophytic colonization efficiency in Apulian olives.

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CHAPTER III

Potential mode of action of the best biological control agent against *X. fastisiosa* ST53 and detection of possible secondary metabolites using HPLC-HRMS analysis

III.1. Background

The generation of antibiotics and competition for nutrients are the most prevalent mode of action of antagonistic bacteria, according to studies undertaken to elucidate the mechanisms of antagonistic microorganisms. Identifying several methods of action makes it easier to combine strains, such as bacteria with bacteria or bacteria with fungi, to attack diseases with a wider range of microbial weapons (Kilic-Ekici & Yuen, 2004; Schisler *et al.*, 1997). Along this same line, biotechnology can be applied to further improve strains that have prized qualities (e.g., formulation ease, stability, or otherwise exceptionally suited to plant colonization) by creating transgenic strains that combine multiple mechanisms of action (Chin-A-Woeng *et al*., 2001; Huang *et al*., 2004; Timms-Wilson *et al*., 2000). Biocontrol by beneficial bacteria might be achieved by direct antibiosis, competition for niches and nutrients, interference with pathogen signaling or by inducing plant resistance (Berg, 2009; Lugtenberg & Kamilova, 2009). Furthermore, biocontrol may be achieved through the breakdown of pathogen virulence factors or phytotoxins, resulting in a reduction in disease symptoms. Direct antagonists are the most effective method of action against competitors because they actively create a wide range of antimicrobial metabolites allowing competitive advantages for antibiotic-producing microorganisms in resource-limited environments (Compant *et al.*, 2005). They are a large number of known antibiotics produced in small amounts by many endophytic microorganisms and released into the environment (Hurst *et al*., 2007). The production of antimicrobial

metabolites, mostly with broad spectrum activity, has been reported for biocontrol bacteria belonging to *Agrobacterium, Bacillus, Pantoea, Pseudomonas* and many other genera. *Bacillus* genera have been observed to produce lipopeptides such as iturin, surfactin and fengycin (Ongena & Jacques, 2008), while *Pseudomonas* genera have been more associated with antibiotic metabolites such as pyrrolnitrine and phenazine (Raaijmakers & Mazzola, 2012). In this context, *X. fastidiosa* remains the most serious vascular pathogen affecting a wide range of crops with a lack of control. Indeed, since the bacterium is nowadays associated with economically important diseases, there are studies and attempts to use antagonists as biocontrol agents. Few studies have been conducted to retrieve direct antagonists for a better biocontrol strategy of *X. fastidiosa*; radicinin from *Cochliobolus* sp. has been found to be able to inhibit *X. fastidiosa* from grapevine (Aldrich *et al*., 2015), while recently published studies have stated the absence of antagonists natively isolated from Apulian olives to inhibit *X. fastidiosa* ST53 growth *in vitro* (Zicca *et al.,* 2020*).* In this chapter, several tests were carried out to elucidate and understand the mode of action of different antagonists found in chapter II.

III.2. Material and Methods

III.2.1. Screening for competition for nutrients as mechanism of action by siderophore production assay:

Siderophore production was carried out using CAS agar assay according to a method previously described by (Schwyn & Neilands, 1987) and O-CAS assay described by.Pérez-Miranda *et al.*, 2007. The blue CAS medium was prepared according to Schwyn and Neilands (1987). The medium for a liter of overlay was as follows: Chrome azurol S (CAS) 60.5 mg, hexadecyltrimetyl ammonium bromide (HDTMA) 72.9 mg, Piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES) 30.24 g, and 1 mM$FeCl_3 \cdot 6H2O$ in 10 mM HCl 10 mL. Agarose (0.9%, w/v) was used as gelling agent.

Table 4: Composition of CAS agar medium:

Ingredients	1 L
Chrome azurol S (CAS)	60.5 mg
Hexadecyltrimetyl ammonium bromide (HDTMA)	72.9 mg
Piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES)	30.24 g
$FeCl_3 \cdot 6H_2O$	0.0027 g in 10 ml of 10 mM HCl
Agar	15 g

The antagonistic strains were spot inoculated into NA, NSA or KB agar plates and incubated at 27°C for 48 hours. Siderophore detection was achieved after applying 10 mL or 30 mL overlays of CAS medium to agar plates containing cultured bacteria to be evaluated for siderophore synthesis. Positive results were recorded as change in color of the medium from blue to yellow, purple or orange, exclusively surrounding producer microorganisms.

III.2.2. Screening for secondary metabolites production such as Hydrogen cyanide HCN:

Production of Hydrogen cyanide (HCN) by antagonistic bacteria was detected using the method described by (Alström & Burns, 1989). Briefly, 100 µ L of bacterial suspension was spread on nutrient agar medium, sealed with parafilm and incubated in an inverted position at 26–28°C with picric acid indicator papers (5% picric acid /2% Na_2CO_3 solution) placed inside the lids. A color change of indicator from yellow to brown or reddish brown was recorded as HCN production.

Table 5: KBG medium composition:

Ingredients	1 L
Peptone	10g
K_2HPO_4	1.5g
Glycerol	10ml
$MgSO_4$	1.5g
Glycine	4.4 g
Agar	15g

III.2.3. Screening for production of lytic enzymes by the potential biocontrol agent (BCAs) such as Protease:

Bacterial isolates were tested for protease production on skim milk (SKM) agar medium according to method previously described (Smibert & Krieg, 1994). The isolates were spot inoculated into SKM medium and maintained at 28°C for 48 h. Extracellular protease production was determined by the presence of a clear halo zone around the bacterial colony.

Table 6: Composition of SKM medium:

Ingredients	1 L
$NH_4H_2PO_4$	1g
KCl	0.2g
$MgSO_4+7H_2O$	0.2g
Skim milk solution (7%)	100ml
Agar Technical	15 g

III.2.4. Screening for antimicrobial activity against *X. fastidiosa* ST53 (Strain De Donno):

III.2.4.1. Bacterial collection:

Bacterial isolates defined as direct *X. fastidiosa* ST53 antagonists (Chapter II) with five other bacterial endophytic strains deposited in the Culture Collection of CIHEAM-Bari (*Paenibacillus rigui* S55, *Bacillus subtilus* L39, *Bacillus pumilus* L36, *Paenibacillus naphthalenovorans* L49 *and Pseudomonas hibiscicola* L77*),* natively obtained from Apulian olive cultivars and their 16S rRNA gene region were previously submitted in the National Center for Biotechnology Information (NCBI) under specific accession numbers (Table 7). These isolates were included in this experiment due to their reported antimicrobial activity against other several pathogens among them (*Xf* ST53) (Zicca *et al*., 2020) and some of them have been already considered and registered as biocontrol agents against various plant pathogens (Fiedler & Heerklotz, 2015; Grady *et al*., 2016).

Table 7: Endophytic bacterial isolates

Bacteria	Isolate	Host Plant	Accession Number
Paenibacillus naphthalenovorans	L49	*Olea europaea* ^{L}	MZ047304
Paenibacillus rigui	S55	*Olea europaea* ^{S}	MZ047306
Pseudomonas hibiscicola	L77	*Olea europaea* ^{L}	MZ047305
Bacillus pumilus	L36	*Olea europaea* ^{L}	MZ047307
Bacillus subtilis	L39	*Olea europaea* ^{L}	MZ047308

^{L} : cv. Leccino. ^{S} : cv. Ogliarola salentina

III.2.4.2. Well diffusion test:

All the antagonistic strains used in this study were evaluated for antimicrobial compounds production in liquid culture against *X. fastidiosa* ST53 (strain De Donno) by well diffusion test and broth dilution test (Dagher *et al*., 2020; Zicca *et al*., 2020).

Bacterial isolates were 2% inoculated in 20 ml of PD3 broth and incubated under shaking (140 rpm) at 25°C. After 48 h of incubation, the cultures were sampled and cell-free culture filtrates were obtained by centrifugation (13,000 rpm, 4 °C, 10 min) and filtration through 0.22 μm filters. For agar well diffusion method, plates containing 20 ml of PD3 agar media were seeded with an *X. fastidiosa* suspension to obtain three rows, as previously described. Subsequently, at the end of the middle row, a well (8 mm diameter) was made and filled with 150 μL of culture filtrate. Tests were performed in triplicate. The plates were incubated at 28°C for at least 6 days and the antimicrobial activity was detected as an area of *X. fastidiosa* growth inhibition and measured as the distance between the well and the *X. fastidiosa* growth.

III.2.4.3. Broth dilution test:

For broth dilution method, PD3 broth was dispensed (10 ml final volume) into 50-ml sterile tubes and then inoculated with 1 ml 10^4 *X. fastidiosa* suspension and 1ml of cell-free culture filtrate. Tubes without any culture filtrates were used as control. After inoculation, the tubes were incubated at 28°C for 14 days and growth of *X. fastidiosa* was evaluated at 600 nm using an Ultrospec 3000 spectrophotometer (Amershan-Pharmacia Biotech, Cambridge, UK) (Lacava *et al*., 2004).

III.2.4. HPLC-HRMS Analysis:

The CFS of the two antagonistic bacteria *M. oxydans* BA104 and *B. subtilis* L39 were analyzed for the presence of secondary antimicrobial metabolites using high-resolution mass spectrometry technologies coupled with liquid chromatography (HPLC-HRMS). The analyses

were performed with a HPLC-HRMS apparatus composed of a Dionex 3000 HPLC (Thermo Fisher, Milan, Italy) and an Orbitrap Fusion HRMS (Thermo Fisher, Milan, Italy). Chromatography was performed on a reversed-phase column (Luna C18 (2) 150 x 2.1 mm 3um id, 100Å) (Phenomenex, Bologna, Italy). The run was carried out through an organic gradient of B formic acid 0.1% in Acetonitrile in A (formic acid 0.1% in Water). The gradient conditions was the following: initial parameters: 5% B, 100% B in 40 minutes, 100% B held for 2 minutes, 6 minutes reconditioning. The flow was kept at 0.2 ml/min for the entire chromatographic run. MS was operated both in positive and negative ions, source parameters were the following: Sheath gas: 35; Auxiliary gas: 21; Sweep gas: 2; Source voltage: 4000 V (+ ion) and 3200 V (- ion); Capillary temperature: 300°C; Vaporizer temperature: 275°C. Survey MS acquisition was set from 100 to 1000 m/z with a resolution of 30k. Dependent scan acquisition was performed on all the signals higher than 10000 counts, CE energy was set to 25 for positive and negative polarities. In particular, the untargeted antimicrobial metabolite screening approach was carried out to find all those antimicrobial compounds with a more or less significant abundance that were not present in the initial culture broth but were characteristic of a particular strain of bacteria. Through this approach, features (signals) were obtained, each corresponding to a molecule typical of bacterial fermentation, of primary or secondary metabolism. Particularly, thanks to the discriminating power of high-resolution mass spectrometry, coupled with the structural elucidation capabilities provided by the analysis in Tandem MS, a molecule was attributed to a feature through the process called "annotation". Regarding the annotation process, for each feature, the identification code was reported, the sample (s) where it appears, the calculated formula of the ion and its level of annotation (3: accurate mass, 2: accurate mass with fragmentation). If the annotation was not possible (no trace of fragmentation or no result), a list of possible formulas was calculated based on the high-resolution mass/charge value was proposed. For the annotation of the compounds of

interest, a mixed approach was used between manual annotation (extrapolation of the structure through exact mass) and, where possible, that assisted by software that allows simulating the structure as a function of fragmentation using the METFrag software. For the manual integration, the parameters that have been used were Delta PPM max: 4, Elements in use: 14 N, 16 O, 12 C, 1 H, 32 S and nitrogen Rule: E.E. Ions. However, for METFrag software, the following parameters were applied: PPM precursor search: 5, PPM fragment search: 5, Search database: Kegg, Pubchem, Chemspider and minimum score: 0.9.

III.3. Results:

III.3. 1. Siderophore, protease and HCN production by antagonistic bacteria:

Among isolates tested, *Pseudomonas graminis* BA2 and *Pantoea agglomerans* BA69 were able to produce siderophore in O-CAS agar medium. Also isolates *Stenotrophomonas rhizophila* BA102, *Microbacterium phyllosphaerae* BA111, *Microbacterium* spp. BA110 and *Sphigomonas molluscorum* BA95 produced a clear halo zone on SKM medium, indicating extracellular protease production. Among isolates tested for Hydrogen cyanide production, only *Microbacterium oxydans* BA104 revealed a positive reaction (**Table 8** and **Figure 12**).

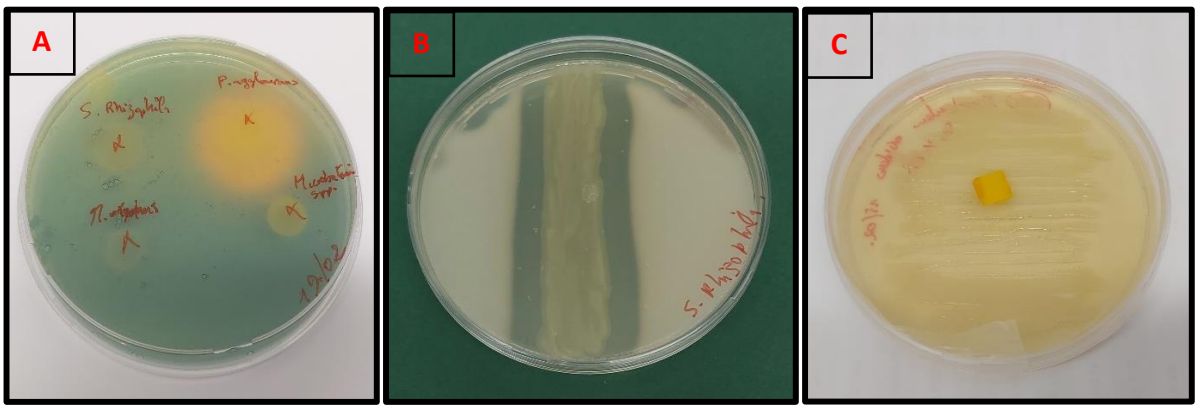


Figure 12: A) Production of siderophores by *P. agglomerans* BA69 in O-CAS assay, **B)** Protease production by *S. rhizophila* BA 102 in SKM medium, **C)** HCN production by *M. oxydans* BA104 in KBG medium

Table 8: Production of antibiotics, protease, hydrogen cyanide and siderophore by antagonistic bacteria

Bacteria	Isolate	Host Plant	Siderophore Production	Protease Production	HCN Production
Pseudomonas graminis	BA2	*Rosmarinus officinalis*	+	-	-
Delftia acidovorans	BA30	*Rosmarinus officinalis*	-	-	-
Pantoea agglomerans	BA69	*Neirum oleander*	+	-	-
Microbacterium oleivorans	BA91	*Olea europaea*	-	-	-
Stenotrophomonas rhizophila	BA102	*Polygala myrtifolia*	-	+	-
Microbacterium oxydans	BA104	*Polygala myrtifolia*	-	-	+
Microbacterium phyllosphaerae	BA111	*Polygala myrtifolia*	-	+	-
Sphingomonas molluscruom	BA95	*Myrtus communis*	-	+	-
Delftia acidovorans	BA43	*Olea europaea*	-	-	-
Microbacterium spp	BA110	*Olea europaea*	-	+	-
Curtobacterium flaccumfaciens	BA54	*Olea europaea*	-	-	-
Microbacterium spp	BA103	*Polygala myrtifolia*	-	-	-

III.3. 2. Antimicrobial results:

III.3. 2. 1. Result of well diffusion test:

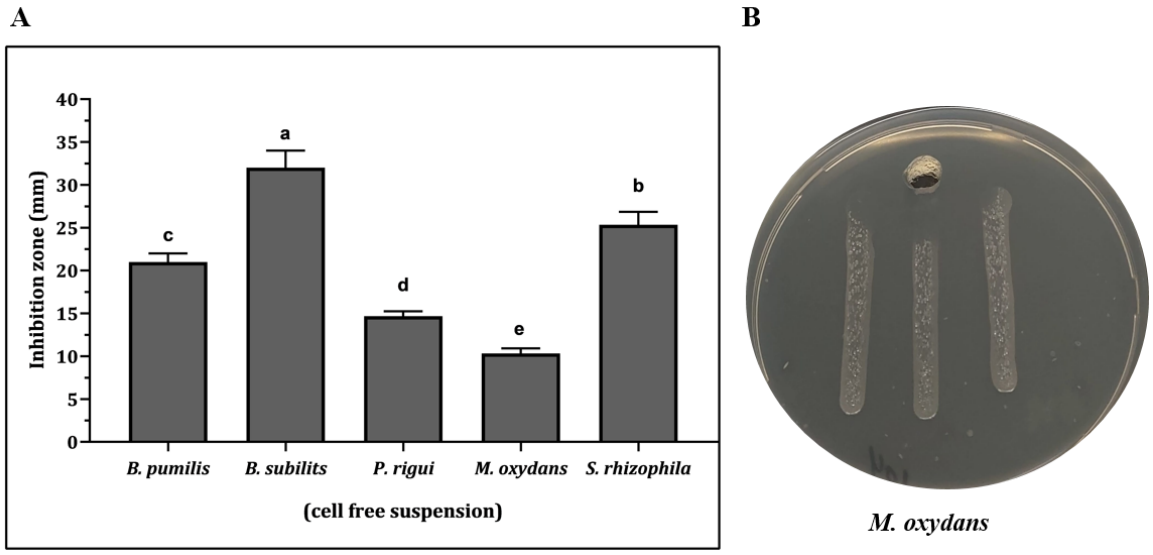


Figure 13: Antimicrobial activity against *X. fastidiosa* ST 53 (strain De Donno) of the culture filtrates of *B. pumilus* L36, *B. subtilis* L39, *P. rigui* S55, *M. oxydans* BA 104 and *S. rhizophila* BA 102. Different letters indicate statistically different antimicrobial activities with $P<0.05$ as determined one-way analysis of variance (ANOVA) followed by the Tukey test.

All the epiphytic antagonistic isolates including the five endophytic isolates used in this chapter were tested for the production in liquid culture of antimicrobial compounds against *X. fastidiosa* ST53 Our results indicated that cell-free supernatant of *B. subtilis* L39, *B. pumilus* L36, *P. rigui* S55, *S. rhizophila* BA102 and *M. oxydans* BA104 showed a relevant antimicrobial activity. These activities were statistically significant between the different species. The lowest antimicrobial activity was recorded from *M. oxydans* BA104 however, the highest inhibition of *X. fastidiosa* was observed in the presence of CFS of *B. subtilis* L39 with more than 30 mm of inhibition zone followed by *S. rhizophila* BA102, *B. pumilis* L36 and *P. rigui* S55, respectively. Cell-free supernatants from these three isolates formed inhibition halos between 15.0–25.0mm on *X. fastidiosa* culture. Interestingly, isolates determined to be strongly

inhibitory in the direct antagonism assay (*in vitro* antagonistic activity, Chapter II) produced CFS with no visible inhibitory activity against *X. fastidiosa* ST53

III.3. 2. 2. Result of broth dilution test:

In broth dilution assay, antibacterial activity against *X. fastidiosa* ST53 was detected in cells-free filtrates of *M. oxydans* BA104, *S. rhizophila* BA102, *B. pumilis* L36 and *P. rigui* S55, respectively. *X. fastidiosa* growth was reduced in the presence of their CFS, however *X. fastidiosa* growth appeared to be stimulated by the presence of cell-free suspension of *P. graminis* BA2. For this isolate, it is unlikely that the production of antimicrobial compounds is important because inhibition growth was only observed when there was a direct cell-to-cell interaction.

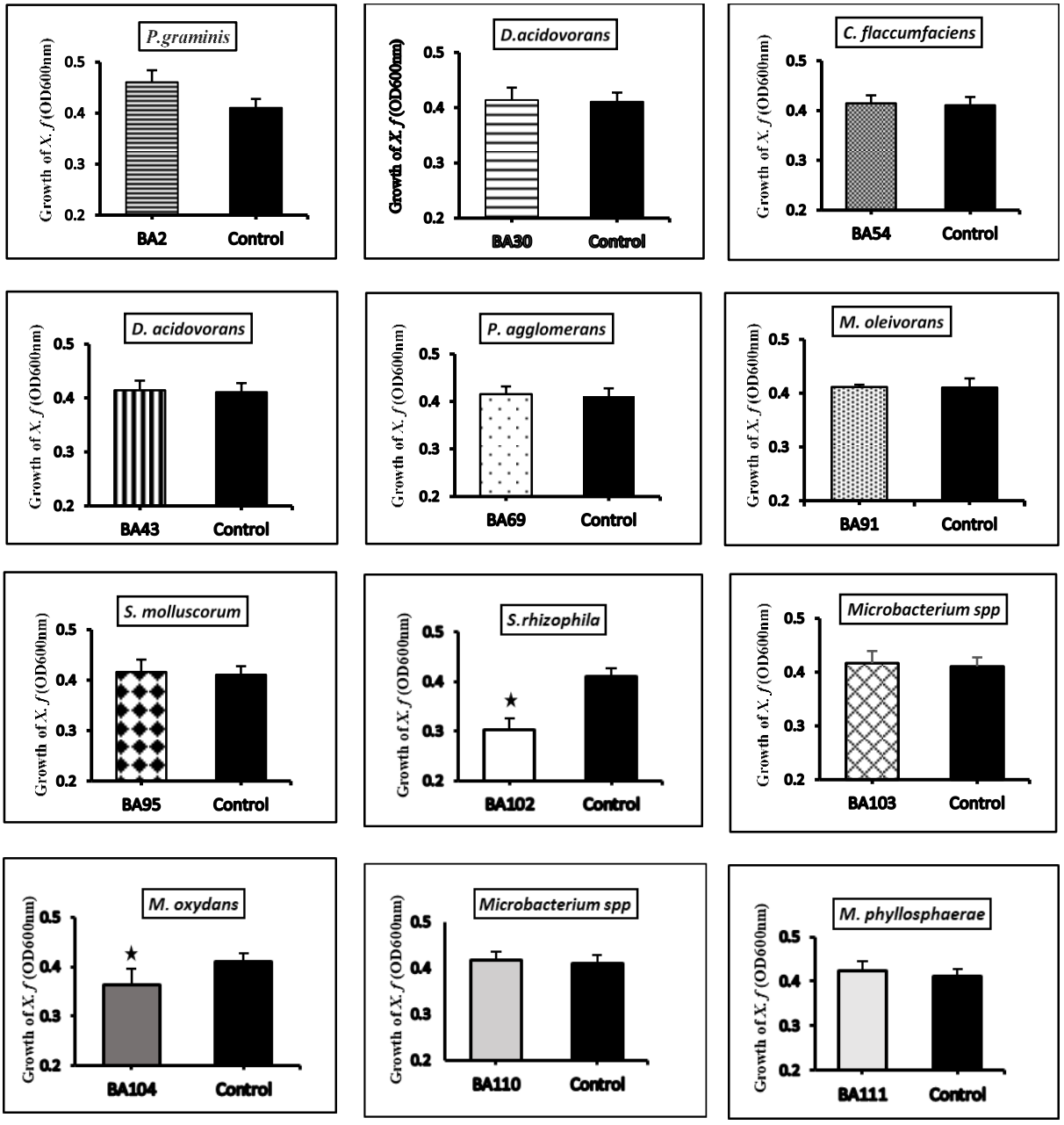


Figure 14: Effect of cell-free supernatants of the epiphytic bacteria on the growth of *X. fastidiosa* ST53. The supernatants were individually tested by adding them to PD3 broth, inoculating the supplemented PD3 broth with *X. fastidiosa* and evaluating growth spectrophotometrically at A600 nm after 14 days.

*****: the stars indicate statistically significant difference in comparison with the control with $P<0,05$ as determined by one-way analysis of variance (ANOVA) followed by the Dunnett test using SPSS software program

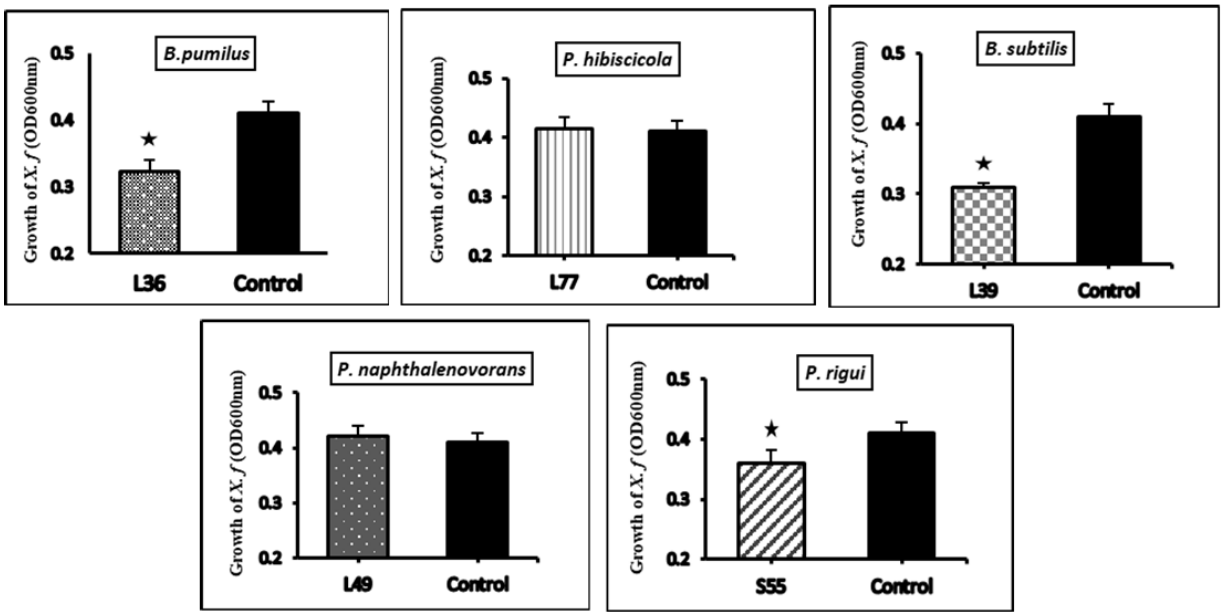


Figure 15: Effect of cell-free supernatants of the endophytic bacteria on the growth of *X. fastidiosa* ST53. The supernatants were individually tested by adding them to PD3 broth, inoculating the supplemented PD3 broth with *X. fastidiosa* and evaluating growth spectrophotometrically at A600 nm after 14 days.

*****: the stars indicate statistically significant difference in comparison with the control with $P<0,05$ as determined by one-way analysis of variance (ANOVA) followed by the Dunnett test using SPSS software program.

III.3. 3. HPLC-HRMS results:

Based on HPLC and mass spectrometry data, and by comparison with literature and searching with known database, a total of 125 discriminating features were detected in *M. oxydans* BA104 and 118 discriminating features in *B. subtilis* L39. Among those, some features were putatively annotated by means of the discriminating power of HRMS accurate mass detection. In the present study, we succeed to annotate 10 compounds from the cell-free suspension of the endophyte *B. subtilis* L39 and the epiphyte *M. oxydans* BA104 **(Table 9)**. Most of the metabolites annotated were mainly antibiotics such as Tetrangomycin, Clavamycin, Calcimycin A23187 and Geldanamycin and were secreted commonly from both isolates. However, Terpenoid was found only in the culture filtrate of *B. subtilis* L39, while N6 -

Acetylkanamycin-B, Isoflavonoids, Valclavam and 3'-Deoxydihydrostreptomycin were detected only in the CFS of *M. oxydans* BA104. Above all, 3'-Deoxydihydrostreptomycin seems to be the most interesting since we also found its MS2 fragmentation pattern. However, other molecules don't show enough high abundance to be fragmented. The chromatograms and spectra of some of those features are showed in the following figures. Features annotated only with accurate mass will be indexed as third-level annotated, while features annotated also by tandem mass fragmentation will be indexed as second-level annotated as specified by (Fiehn *et al*., 2007).

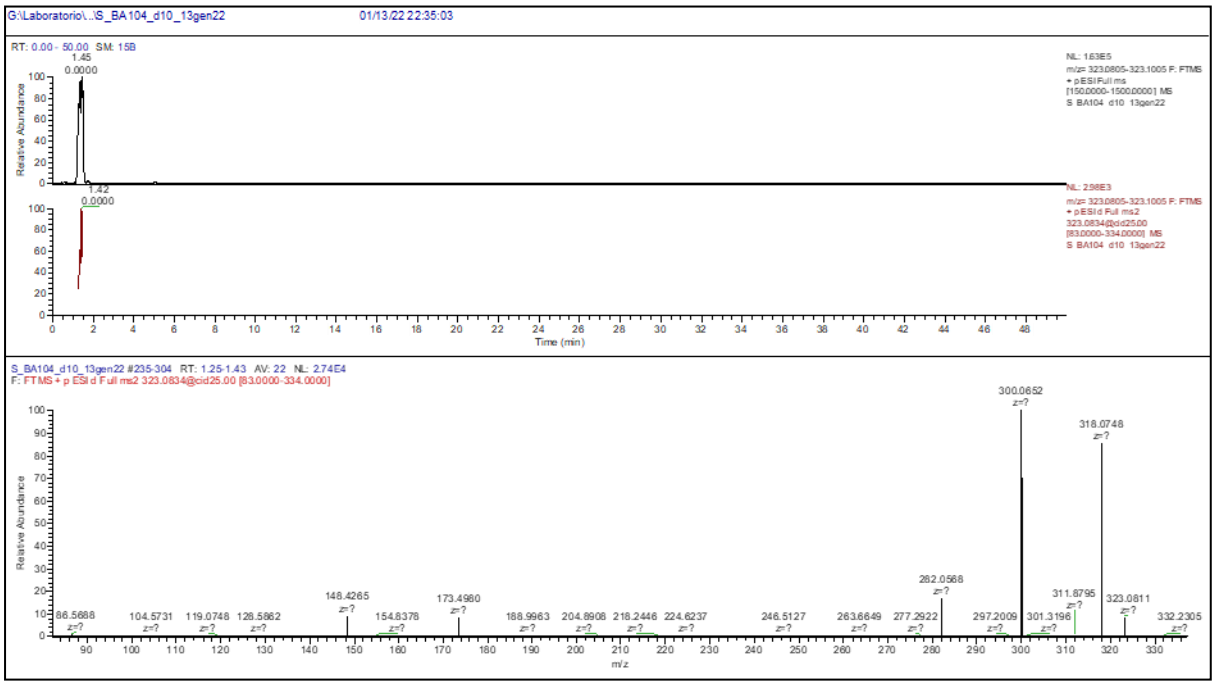


Figure 16: EIC (Extracted ion Chromatogram), and MS2 for Decarboxytetracenomycin F1 in BA104

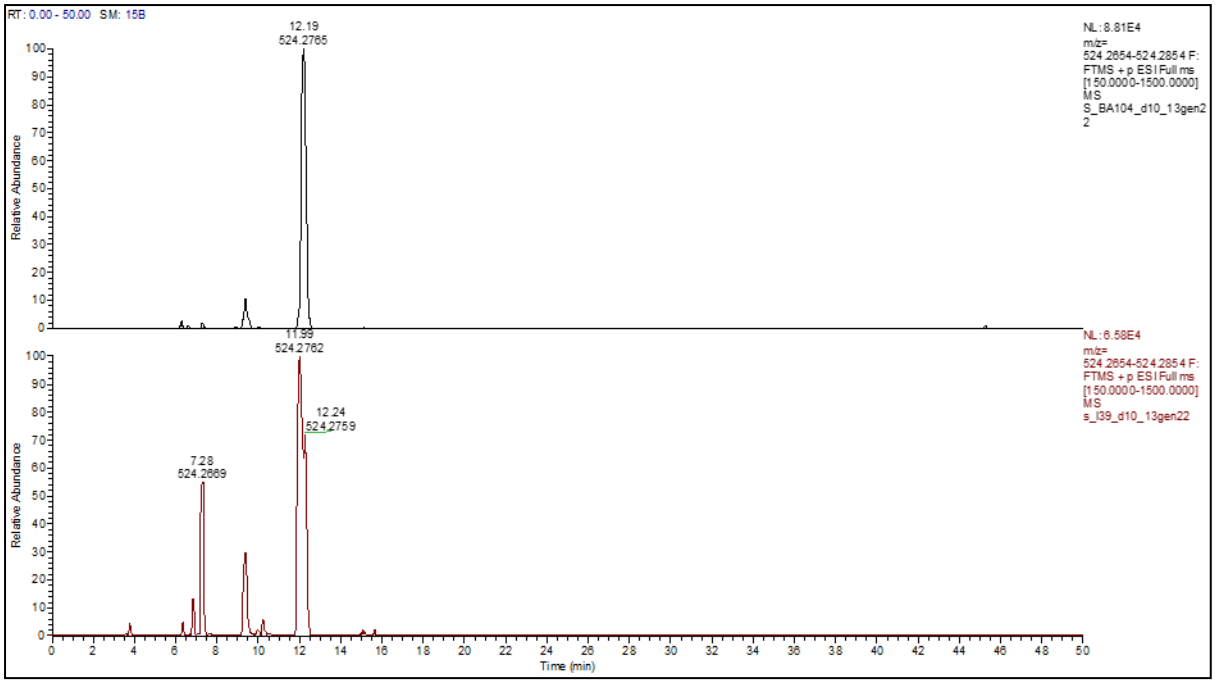


Figure 17: EIC (Extracted ion Chromatogram), for Calcimycin A23187 in BA104 (upper side) and L39 (lower part)

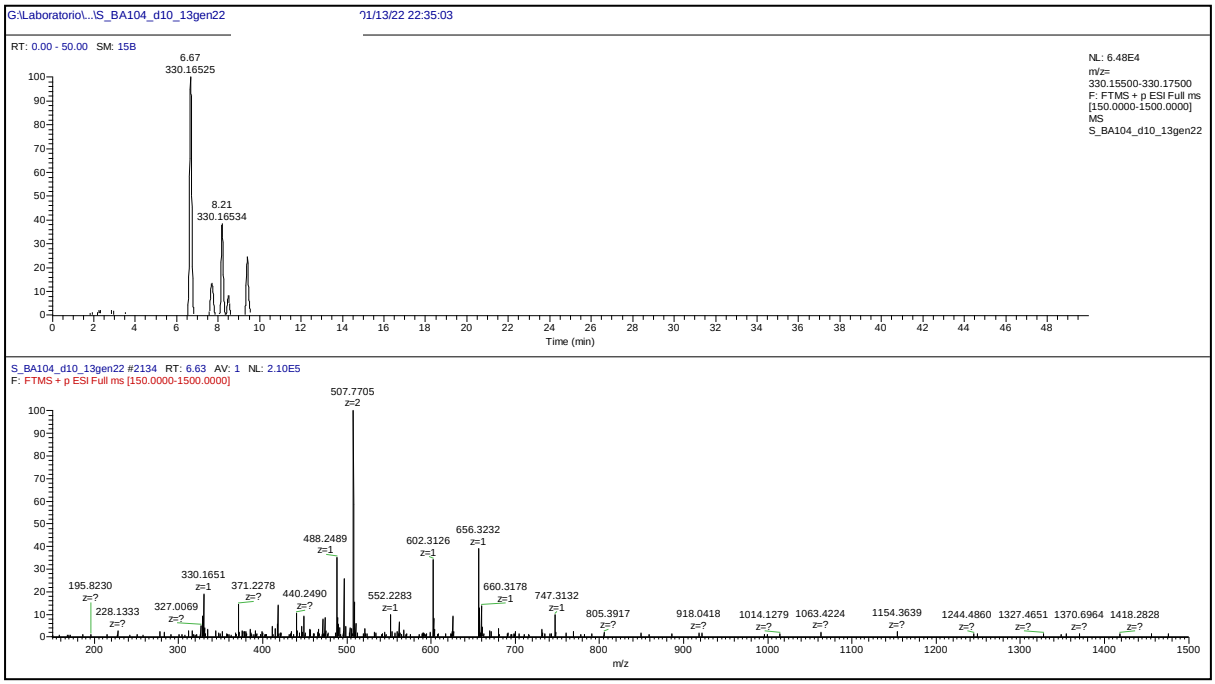


Figure 18: EIC (Extracted ion Chromatogram) and full mass spectrum, for 5-Hydroxy-3',4'-methylenedioxy-6",6"-dimethylpyrano [2",3":7,8] isoflavone in BA104

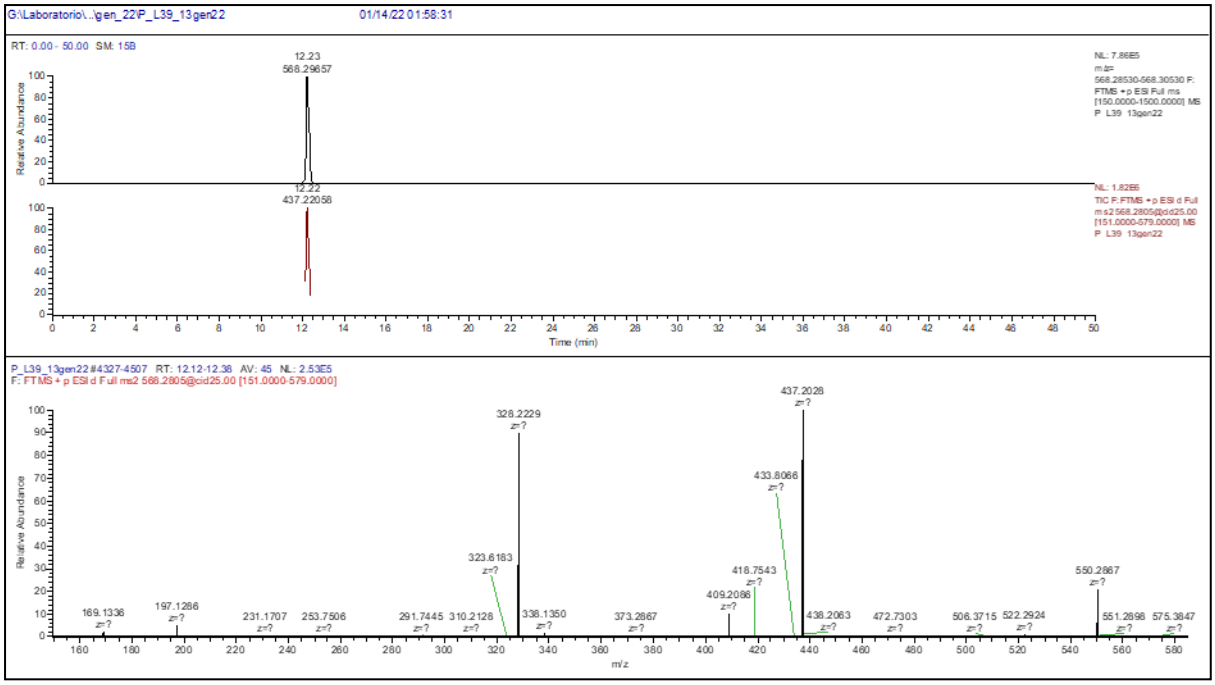


Figure 19: EIC (Extracted ion Chromatogram), and MS2 for 3'-Deoxydihydrostreptomycin in BA104

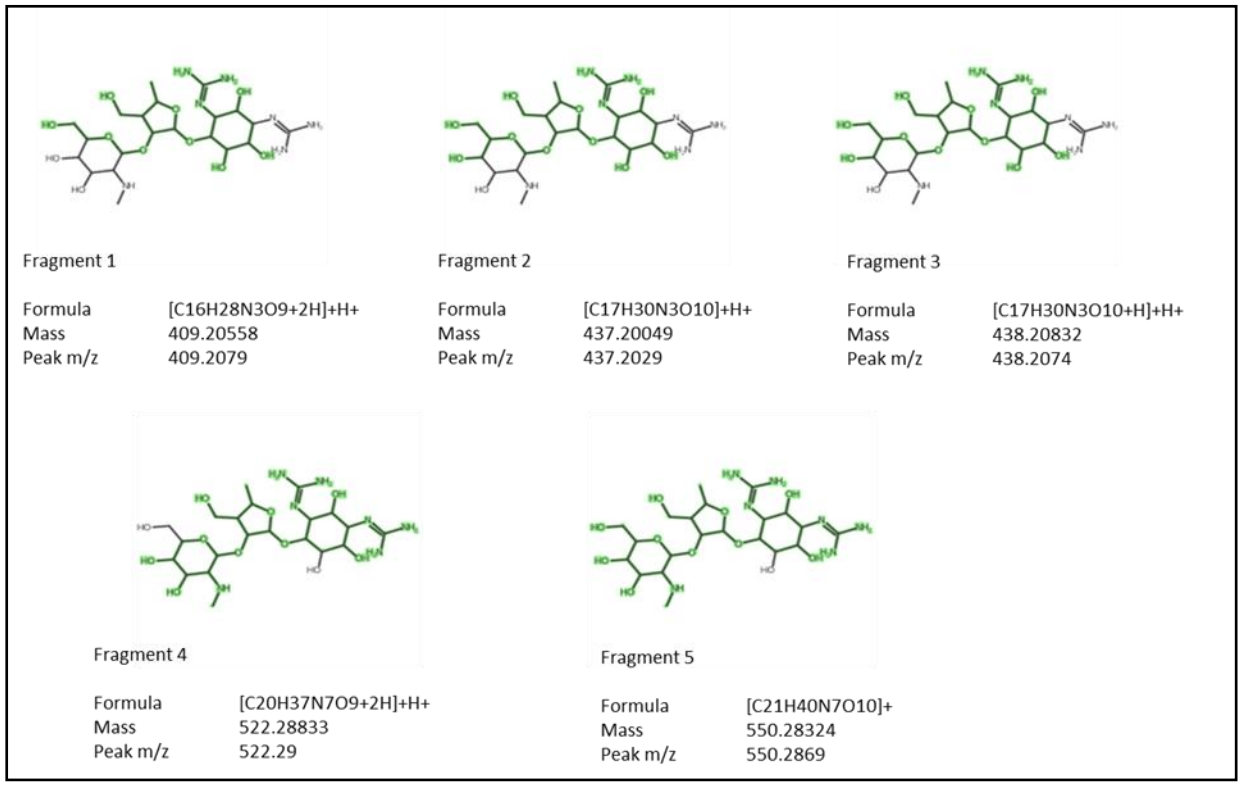
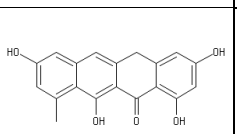
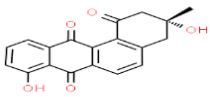
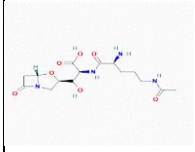
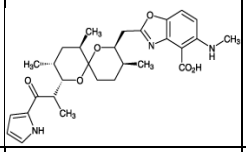
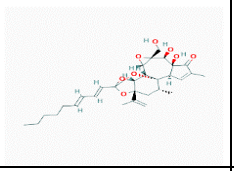
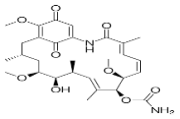
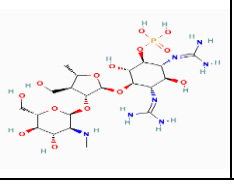
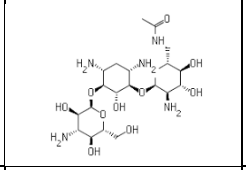
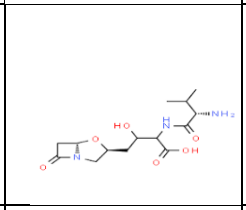
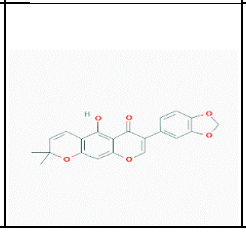


Figure 20: Fragmentation pattern for 3'-Deoxydihydrostreptomycin found in BA 104 (the structure of the main fragments obtained from the MSMS analysis of 3'-Deoxydihydrostreptomycin)

Table 9: Possible interesting compounds annotated with HPLC-HRMS

Id Kegg	Bacteria	MSI annotation level:	Compound Name	Chemical Structure	Chemical Formula	Delta ppm
C12374	BA104 L39	**2**	Decarboxytetracenomycin F1		C19H14O5	-2.72
C12396	BA104 L39	**3**	Tetrangomycin		C19H14O5	-2.72
C17395	BA104 L39	**3**	Clavamycin F		C15H24N4O7	-2.99
C11309	BA104 L39	**3**	Calcimycin A23187		C29H37N3O6	-0.10
C09198	L39	**3**	Terpenoid EA-I;Excoecariatoxin;Simplexin, 22,23,24,25-tetradehydro-		C30H40O8	-1.39
C11222	BA104 L39	**3**	Geldanamycin		C29H40N2O9	-0.77
C03755	BA104	**2**	3'-Deoxydihydrostreptomycin		C21H41N7O11	2.92

C03154	BA104	**3**	N6 -Acetylkanamycin-B		C20H39N5O11	2.56
C06668	BA104	**3**	Valclavam		C14H23N3O6	-2.65
LMPK1 2050249	BA104	**3**	5-Hydroxy-3',4'-methylenedioxy-6",6"-dimethylpyrano[2",3":7,8]isoflavone [Isoflavonoids [PK1205]]		C21H16O6	2.53

III.4. Discussion:

Finding out BCAs' mode of action is very important to select the best ones. To properly use biocontrol microorganisms, it is important to understand the mechanisms of action involved in biocontrol activity, to develop safe application processes; this is also an important background to select new and efficient strains. Basic information must be generated at both, the biochemical and the molecular level, contributing in this way, in the elucidation of effects such as antibiosis, competition for nutrients and induction of resistance (Nannipieri *et al*., 2017). According to studies conducted to reveal the mode of action of antagonistic microorganisms, the production of antibiotics and competition for nutrients are the most prominent (Benbow & Sugar, 1999). In this study, *P. graminis* BA2 and *P. agglomerans* BA69 isolates produced siderophores in in vitro experiments. These results are in agreement with previous studies about the production of siderophores by various strains of *P. agglomerans* and *Pseudomonas* species as one of their mode of action against various plant pathogenic bacteria (Mikiciński *et al*., 2016; Sharifazizi *et al*., 2017). However, *S. rhizophila* BA102 and *M. oxydans* BA104 isolates produced protease and HCN respectively and both were able to secrete CFS with antibacterial compounds in well and broth diffusion assays. These results showed that both strains with *P. graminis* BA2 and *P. agglomerans* BA69 isolates were able to use different mechanisms of action such as antibiosis and competition against *X. fastidiosa* ST53. Furthermore, our results point out the secretion of protease by *S. rhizophila, M. phyllosphaerae and S. molluscorum* isolates. In fact, previous studies have reported the detection of lytic enzymes by these genus (Vida *et al*., 2017). All that suggests that one of the mechanisms of these antagonistic bacteria against *X. fastidiosa* is the production of protease. In general, A variety of microorganisms also exhibit hyperparasitic activity, attacking pathogens by excreting cell wall hydrolases (Chernin & Chet, 2002). In our results, a high level of antimicrobial activity was observed in two epiphytic isolates. The isolates BA102 and BA104 that were closely related to *Stenotrophomonas*

rhizophila (100%) and *Microbacterium oxydans* (99%). This result is in agreement with previous studies about the potential to control pests and the antimicrobial activity of *M. oxydans* (Irshad *et al*., 2013; Ozsahin *et al*., 2014). Importantly, our results point out the highest antimicrobial activity *in vitro*, of *Bacillus* isolates and *Peanbacillus rigui* with the production of culture filtrates which inhibit the growth of the pathogen likely secreting active substances in the liquid medium. Those isolates together with *M. oxydans* and *S. rhizophila* above-mentioned and differently from the other isolates, showing antagonistic and antimicrobial capacity suggesting that the mode of action of these isolates is different from other bacteria used in this study. In general, *Bacillus* strains are well known for their ability to secrete a variety of antimicrobial compounds, and some of them have been already considered and registered as biocontrol agents against various plant pathogens. As an example, the strain Q713 of *Bacillus subtilus* produces lipopeptides belonging to the families of the surfactins, iturins, and fengycins (Fiedler & Heerklotz, 2015). Moreover, the genes responsible for the production of these substances as well as antibiotics, such as macrolactin, bacilysin, and difficidin, have been identified in its genome (Pandin *et al*., 2018). These findings coincide with recent results, which concluded that several Bacillus strains were able to inhibit in vitro the growth of *X. fastidiosa* strain De Donno (Zicca *et al*., 2020). Furthermore, the findings of this study provide evidence that *P. rigui* is able to produce in liquid culture substances with inhibitory effects. Many *Paenibacillus* species compete with other microorganisms through the production of a wide range of antimicrobial compounds such as peptides that are extremely significant for biocontrol in agriculture (Grady *et al*., 2016). Moreover, *P. agglomerans, P. graminis, M. oleivorans, M. phyllospharaea, D. acidovorans, S. molluscorum and Microbacterium.* spp. showed only antagonistic activity without inhibitory effect with cell-free suspension. For these isolates, it is unlikely that the production of antimicrobial compounds is important because inhibition growth was only observed when there was a direct cell-to-cell interaction. These

results suggest that the concentration of bactericidal compounds, if any, was insufficient to be detected using the methods that were applied in our assay, or that the inhibition of growth could be mediated by a contact- or proximity-dependent mechanism involving bactericidal compounds or quorum-sensing molecules which can achieve inhibition at very low concentrations. Although the number of putative mechanisms involved in the antagonistic activity of *Xf* that have been explored in this study, we were not able to clarify the possible mode of action of some of them. For instance, *D. acidovorans* BA30 and BA43, *C. flaccumfaciens* BA54 and *Microbacterium* spp BA103 and BA110 did not produce siderophore, HCN or protease. Moreover, no antimicrobial activity was detected in *in vitro* assays. All that suggests that other studies are also required to elucidate the modes of action of these isolates.

A possible explanation for this result is the fact that antagonism activity was mediated by other mechanisms and considering the fastidious growth of *X. fastidiosa*, their faster growth rate allows them to deplete nutrient availability and thus limit the pathogen growth. Nevertheless, other studies are also required to elucidate the modes of action of the strains whose intact cells are antagonistic but whose CFS are not. Further research should be also carried out to evaluate the capability of those antagonists to inhibit *X. fastidiosa in vivo* and under natural field conditions.

The use of microorganisms and their metabolites may represent an alternative to decrease the impacts of pesticides applications in the field. As result, several registrations of commercial biocontrol products for control leaf and fruit diseases have been deposited (Junaid *et al.*, 2013). Specially, endophytes have been demonstrated to possess the ability to survive in high diverse plants, thereby helping to reduce of herbivory, to control phytopathogens, to induce systemic resistance, and to promote plant growth (Farrar *et al*., 2014; Gao *et al*., 2010). In this way, in the present work, we assessed the potential of *M. oxydans* and *B. subtilis* for their secretion of

antibacterial substances to control the phytopathogen *X. fastidiosa* ST53. The genus *Microbacterium* has been reported as a producer of several metabolites with biological activity (Isik *et al*., 2014) however, there are no reports of activity against *X. fastidiosa* ST53. HPLC-HRMS results showed that N6 -Acetylkanamycin-B, Isoflavonoids, Valclavam and 3'-Deoxydihydrostreptomycin were detected in the strain BA104. Generally, the genus *Microbacterium*, actinomycetes, bacteria, and fungi were already described as producers of isoflavones (Chang *et al*., 2007; Hazato *et al*., 1979; Savi *et al*., 2019). This class of compounds has been associated with antifungal and antibacterial activity against *Staphylococcus aureus*, *Klebsiella pneumonia*, *Escherichia coli*, and vancomycin resistant *Entercoccus faecalis* (Azzouzi *et al*., 2014; Yu *et al*., 2016). In fact, most research on isoflavonoids and flavonoids has focused on plant sources, and relatively less attention has been paid on microorganisms as the potential sources of these compounds. Although, the presence of metabolic pathways of isoflavonoid and flavonoid biosynthesis in microorganisms has been confirmed. More importantly, isoflavonoids possess a wide range of biological activities, such as antibacterial, antifungal, antiviral (Dastidar *et al*., 2004; Orhan *et al*., 2010), antitumor *(Kopustinskiene et al*., 2020), anti-inflammatory (Sychrová *et al*., 2020), and antiaging activities (Gupta & Prakash, 2014). In the present findings, 3'-Deoxydihydrostreptomycin detected in CFS of *M. oxydans* BA104 showed a significant abondance compared to other molecules since we succeed also to obtain its fragmentation pattern. Interestingly, to our knowledge the 3'-Deoxydihydrostreptomycin which is a derivative of streptomycin, aminoglycoside antibiotic, with bactericidal property together with valclavam and acetyl kanamycine have only been shown to be produced by *Streptomyces* spp. (Baldwin *et al*., 1994; McGowan *et al*., 1998). Hence, their emergence in the CFS warrants further investigation to analyze if its biosynthesis was triggered by the BA104 strain. Indeed, antibiotics such as streptomycin and kanamycin have been shown to act in the 16S rRNA that is part of the 30S ribosome subunit

(Brötz-Oesterhelt & Brunner, 2008; Chopra et al., 2002; Nikaido, 2009) thereby causing the misreading of the genetic code resulting in the production of non-functional proteins, consequently leading to bacterial cell death. Streptomycin and its derivatives has been one of the major antibiotics used to control several bacterial plant pathogens such as *Erwinia amylovora*, (Slack *et al*., 2021), *Pseudomonas syringae* (Bashan & de-Bashan, 2002) and *Agrobacterium tumefaciens* (Saadoun & Al-Momani, 2008). Additionally, the antibacterial activity of antibiotics such as Tetrangomycin, Clavamycin, Calcimycin A23187, Geldanamycin and Valclavam were already described in previous reports against several plant pathogens (Boudjeko *et al*., 2017; Gou *et al*., 2017). It is noteworthy that lipopeptides such as surfactin, iturins and fengycins known to be secreted by the species *B. subtilis* (Fiedler & Heerklotz, 2015; Jha *et al*., 2016) were not detected among the molecules annotated in our study. This could be explained considering that in our experiment we have used an untargeted metabolite screening approach to find possible antimicrobial compounds together with annotation process. Therefore, our results of HPLC-HRMS are considered as preliminary results and further research using target approach and standards to clearly identify a specific molecule is still needed. In conclusion, antagonistic bacteria found in this study were able to use different mechanisms of action against *X. fastidiosa*. Moreover, our results showed antimicrobial effect of *Bacillus* isolates, *Peanbacillus rigui*, *S. rhizophila* and *M. oxydans*. Interestingly, results of HPLC-HRMS analysis are considered as promising preliminary results thus confirmation of the identification of the annotated antibiotics and other possible antimicrobial secondary metabolites is still needed as future work. Nevertheless, since there is no predominant abundance of antibiotic-like molecules thus, it is possible that the antimicrobial action against *X. fastidiosa* ST53 can be carried out by different classes of molecules, paving the way to new potential discoveries.

III. 5. References

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CHAPTER IV

Endophytic colonization efficiency of promising biological control agents in Apulian olive cultivars and *Nerium oleander* plant

IV.1. Background:

Antagonistic microbes are a potential group of microorganisms that, through a variety of mechanisms, can give frontline resistance and growth to plants (Bacon & White, 2000). The mode of action of antagonists determines how well they inhibit disease (direct and indirect antagonism). Direct antagonistic bacteria are known for parasitism, antibiosis, and competition, whereas indirect antagonistic bacteria are linked to mediated plant resistance (Fadiji & Babalola, 2020). Because of their ability to live within the plant and promote plant resistance, endophytic antagonists are becoming a popular method for controlling vascular pathogens (Kaouthar Eljounaidi *et al.*, 2016). There have been multiple reports of biological control agents successfully controlling vascular diseases; *Pseudomonas* and *Bacillus* spp. have been shown to produce ISR and subsequently increased pathogen defense in plants with wilt diseases. This has been observed in olive trees, where *Pseudomonas fluorescens* PICF7 has been shown to reduce *Verticillium* wilt in olives by establishing systemic resistance (Gómez-Lama Cabanás *et al*., 2014; Schilirò *et al.*, 2012). *Bacillus subtilis* has been utilized to treat bacterial wilt disease caused by *Ralstonia solanacearum* in mulberry trees (Ji *et al.*, 2008). In this context, *X. fastidiosa* remains the most dangerous vascular pathogen, affecting a wide range of crops with a lack of control. Indeed, as the bacteria is now linked to economically significant diseases, investigations and attempts to use endophytic antagonists as biocontrol agents are being conducted. Affected citrus with citrus variegated chlorosis (CVC) was

thoroughly investigated for the diversity of endophytes and their impact on disease control; *Curtobacterium flaccumfaciens* isolated from asymptomatic plants has shown antagonistic activity with symptom reduction in *Catharanthus roseus* infected by *X. fastidiosa* (Azevedo *et al.*, 2016; Hanani et *al.*, 2021; Lacava *et al.*, 2007).

Similar results were observed with *Paraburkholderia phytofirmans* in the case of Pierce's disease, where inoculated grapes had a lower incidence of *X. fastidiosa* leaf scorch symptoms and an induced plant defense response compared to non-treated plants (Baccari *et al.*, 2019). In the case of Olive Quick Decline Syndrome (OQDS), several researchers have focused on endophytes, especially since the appearance of resistant plants (cv. Leccino). However, a recently published study found that there are no antagonists natively isolated from Apulian olives that can inhibit the growth of *X. fastidiosa* ST53 in vitro (Zicca *et al.*, 2020). Overall, our *in vitro* screening of a novel biological control agent inhibiting the growth of *X. fastidiosa* ST53 has successfully defined antagonistic isolates (Chapter II and III). Thus, these isolates have received major attention to be studied at levels of establishment in the inner sphere of Apulian olives and *Nerium oleander* plant. Particularly, in the case of *X. fastidiosa*, it is essential to select a potential bio-control agent among epiphytic bacteria able to colonize the xylem. Therefore, in the present chapter, the establishment of bacterial inoculums was monitored by Real-time PCR, conventional PCR and plat counting.

IV.2. Material and methods

IV.2.1. Evaluation of colonization efficiency in olives and *Nerium oleander* plant:

Among the different epiphytic and endophytic antagonists observed in our results (Chapter II and III), *Pantoea agglomerans* BA69, *Bacillus subtilis* L39, *Microbacterium oxydans* BA104 and *Stenotrophomonas rhizophila* BA102 were considered in this experiment as they demonstrated the strongest antagonistic and antimicrobial activity against *X. fastidiosa* strain

De Donno in Dual-culture bioassay. In addition to *Curtobacterium flaccumfaciens* BA54 that was also included in this chapter due to their reported interactions (indirect antagonist and symbiont) with *X. fastidiosa* on citrus plants. Stem colonization by inoculated antagonistic bacteria selected was assessed for one month by plat counting, conventional PCR and molecular technique based on real-time PCR.

IV.2.1.1. Inoculum preparation and plants inoculation

The experiment was performed in November on one-year-old olive plants belonging to cv. Leccino, cv. Ogliarola Salentina and *Nerium oleander* host. Plants were incubated in a growth chamber (70% relative humidity, 16 h of light, 25-28°C: 9 h of dark). Bacterial isolates were freshly grown in nutrient broth at 28°C for 48 h, harvested by centrifugation at 6000 rpm for 3 min, and the concentration of the suspended pellet was determined by measuring the optical density (λ = 600 nm) (Lambda 365) and diluted to 10^8 CFU ml^{-1} (1X PBS, pH 7.4). Three replicates of each cultivar were mechanically inoculated following needle punctures methodology (Baccari *et al*., 2019; Ghanney *et al.*, 2016). In brief, ten droplets, each containing 10 ul of inoculum, were positioned separately on the stem of olives and *N. oleander* plants (at least 10 cm between each inoculation point), and a 21-gauge syringe needle was utilized to puncture the stem through the droplets. The plants were observed until the inoculum was taken up by the plants. Negative controls were inoculated with 1X PBS or 0.1% Triton X-100. All plants were maintained under the controlled conditions mentioned above. Only one round of inoculation was performed for each set of plants.

IV.2.1.2. Bacteria detection by conventional PCR:

To evaluate the endophytic colonization of the antagonistic bacteria used in this chapter, conventional PCR assays were performed after plant inoculation for *P. agglomerans* BA69, *B. subtilis* L39 and *C. flaccumfaciens* BA54. Briefly, DNA was extracted from the three biological

replicates of the two olive cultivars and *N. oleander* plant using the CTAB extraction method (Hallmann *et al.*, 2006). The 25-µl PCR mixture contained 1 µl of 50 ng/µL DNA template, 12.5 µl of 2× DreamTaq Hot Start Green PCR Master Mix (Thermofisher Scientific), 0.5 µl of 10 µM of each primer, and 10.5 µl of nuclease-free water. PCR cycling parameters were as follows: 1 cycle at 95°C for 3 min, followed by 35 cycles with a denaturation step at 95°C for 30 s, an annealing step at the appropriate temperature for 1 min (Table 1), and an extension step at 72°C for 1 min, followed by a cycle at 72°C for 6 min. Reaction products were analyzed by electrophoresis in 1.2% TAE agarose gel and DNA bands were visualized at Gel Doc EZ system (BIORAD).

Bacteria Isolate	Target gene	Sequence (5'-3')	Product size	Annealing Tm	References
B. subtilis	gyr A	HAN2: ACAAACATTCCTCCGCACCA	175 bp	55 °C	CP045818.1
					EU138629.1
		HAN5: CTCGGCCTGATTCGTATGCT			LK936504.1
C. flaccumfaciens	rec A	CSF1: TCTGGCTCCATCGACCTC	181 bp	56 °C	KX591826.1
					CP041259.1
		CSF2: CGCAGCTGGTTGATGAAGAT			MK167889.1
P. agglomerans	16S rRNA	PAG1: CCTGGACAAAGACTGACGCT	171 bp	59 °C	CP046722.1
					MT803622.1
		PAG2: TTTAACCTTGCGGCCGTACT			MN943534.1

Table 10: SYBR Green-based primer sets designed on species-specific genes of bacterial sequences deposited in the NCBI gene bank (PhD Thesis Hanani, 2020).

IV.2.1.3. Bacteria quantification by Real-time PCR

Inoculated plants were analyzed by real-time PCR using 50 ng ml^{-1} DNA material extracted from 0.5 g of surface-sterilized stem segments of both olive cultivars and *N. Oleander* plants according to the classical phenol-chloroform method (Minas *et al.*, 2011). The amplification mixture (25 µl) consisted of 12.5 µl of SYBR® Select Master Mix (Thermofisher Scientific), 0.5 µl (10 mM) of the designed and validated primers (Table 1), 1 µl of 50ng DNA, and no DNA samples served as negative controls. Thermal cycling was performed according to the manufacturer's instructions, varying the annealing temperature depending on the primer pair

(Table 1). In addition, the developed real-time PCR assay was used to confirm the identity of morphologically similar colonies obtained by the plate counting method.

IV.2.1.4. Bacteria isolation and plate counting

The population of inoculated bacteria was determined in the twigs of both cultivars of olives and *N. oleander* plant by excising 2-cm-long stem segments that homogeneously covered the experimental plants at 7, 15, 21, and 30 days after inoculation. Under aseptic conditions, stem segments were surface sterilized by washing in 70% ethanol for 2 min and sodium hypochlorite solution (10% available Cl) for 2 min, followed by two rinses in sterile distilled water (Hallmann *et al*., 2006). To quantify the bacterial colonization, the stem segments were macerated in sterile extraction bags (BIOREBA, IT), suspended in 2 ml PBS, subjected to a 10-fold serial dilution, and plated out on NA. The colonies obtained with identical morphological characteristics were confirmed and CFU ml^{-1} was calculated for referred isolates in both olive cultivars and *N. oleander* plant (Fung, 2009).

IV.3. Results:

IV.3.1. Bacteria establishment evaluation by conventional PCR

Figure 16 summarized the results of the conventional PCR for the isolates *B. subtilis* L39, *P. agglomerans* BA69 and *C. flaccumfaciens* BA54 in three biological replicates of inoculated olive varieties and *N. oleander* plants. After one week of inoculation the target bacteria were successfully amplified in conventional PCR assays. When the agarose gel was analyzed, single bands were visualized, and no amplification was observed in the negative control. The obtained amplicons observed seem to correspond exactly to the indicated amplification size (HAN2/HAN5: 175 bp, CSF1/CSF2: 181 bp and PAG1/PAG2: 171bp) expected. Therefore,

PCR results showed clearly that needle-inoculated bacterial cells were detectable in the stem of the three hosts, distal from the inoculation site.

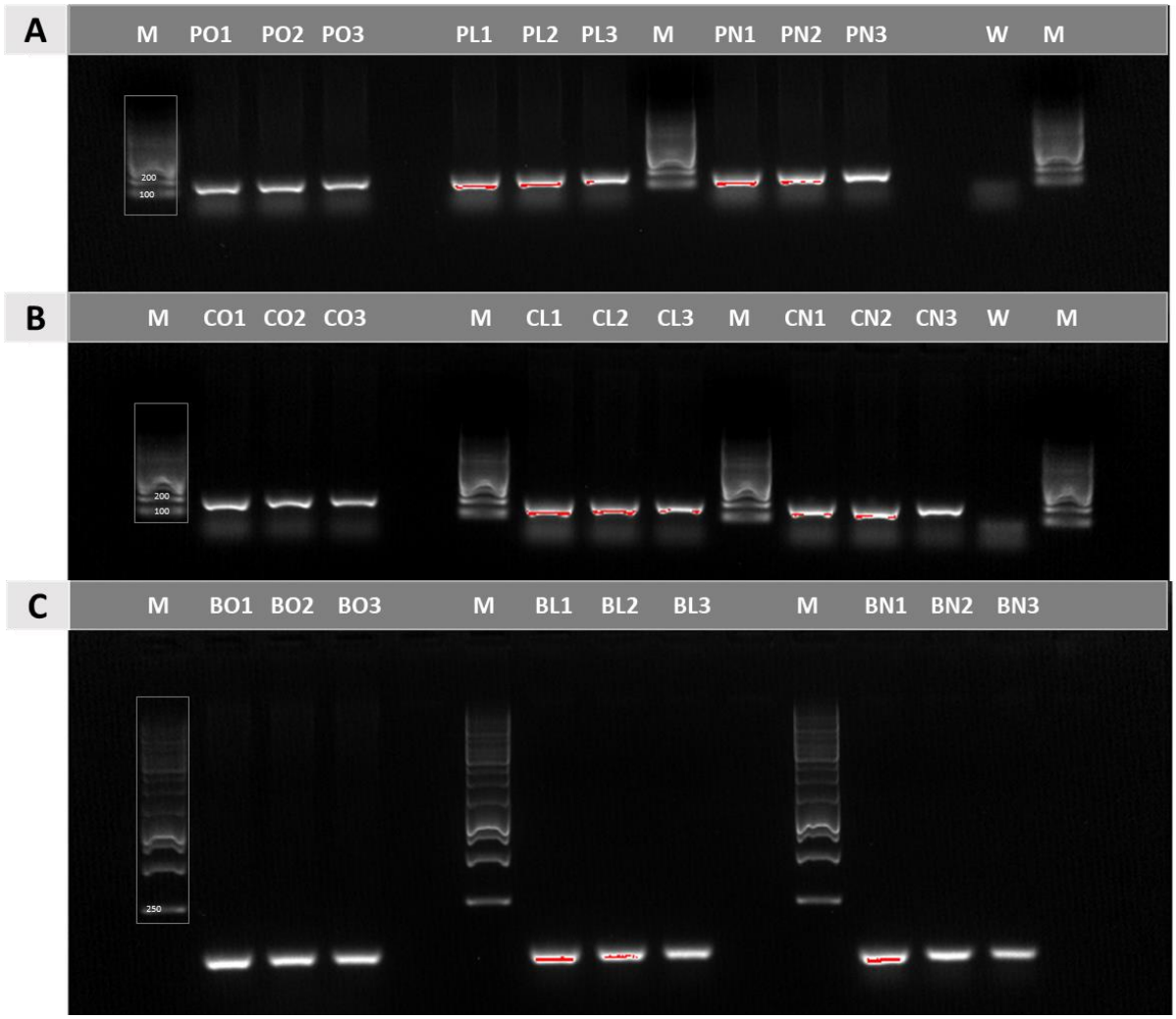

Figure 21: Agarose gel electrophoresis analysis of the PCR products obtained from **(A)** PCR product of DNA *P. agglomerans* BA69 from the 2 olive cultivars cv Ogliarola salentina, cv. Leccino and *Nerium oleander* (PO, PL and PN) with amplicon size of 171bp. (B) PCR product of DNA *C. flaccumfaciens* BA54 from the 2 olive cultivars cv Ogliarola salentina, cv. Leccino and *Nerium oleander* (CO, CL, and CN) with an amplicon size of 181 bp. (C) PCR product of DNA *B. subtilis* L39 from the 2 olive cultivars cv Ogliarola salentina, cv. Leccino and *Nerium oleander* (BO,BL and BN) with an amplicon size of 175 bp. 100 bp marker is the lane (M) for (A) and (B) and 1kb Marker for (C), and lane (W) refers to no DNA template control.

IV.3.2. Bacteria establishment evaluation by real-time PCR

Figure 17 and 18 presents a summary of real-time PCR results that positively indicated successful establishment and colonization of both olive cultivars and *N. Oleander* plant by inoculated bacteria, as determined by comparing *Cq* values between inoculated and control plants. Distinct increases of the inoculated bacteria population were detected at different times after inoculation. Before inoculation, *B. subtilis* L39 with a late amplification threshold was detectable in the experimental plants, but Cq values decreased, indicating high colonization of the inoculum after 15 and 21 days in 'Leccino', 'Ogliarola Salentina' and *'N. Oleander',* respectively. Similarly, the inoculums of *P. agglomerans BA69* presented a high entity in the tissues of experimental plants upon inoculation with relatively lower Cq values in *'N. oleander'* than in olive cultivars. On the other hand, the isolate *C. flaccumfaciens* BA54 showed high colonization entity exclusively at early stages (7 and 15 days) after inoculation, indicated by obtained low Cq values (< 25) in 'Leccino', 'Ogliarola Salentina' than *'N. oleander'* plant.

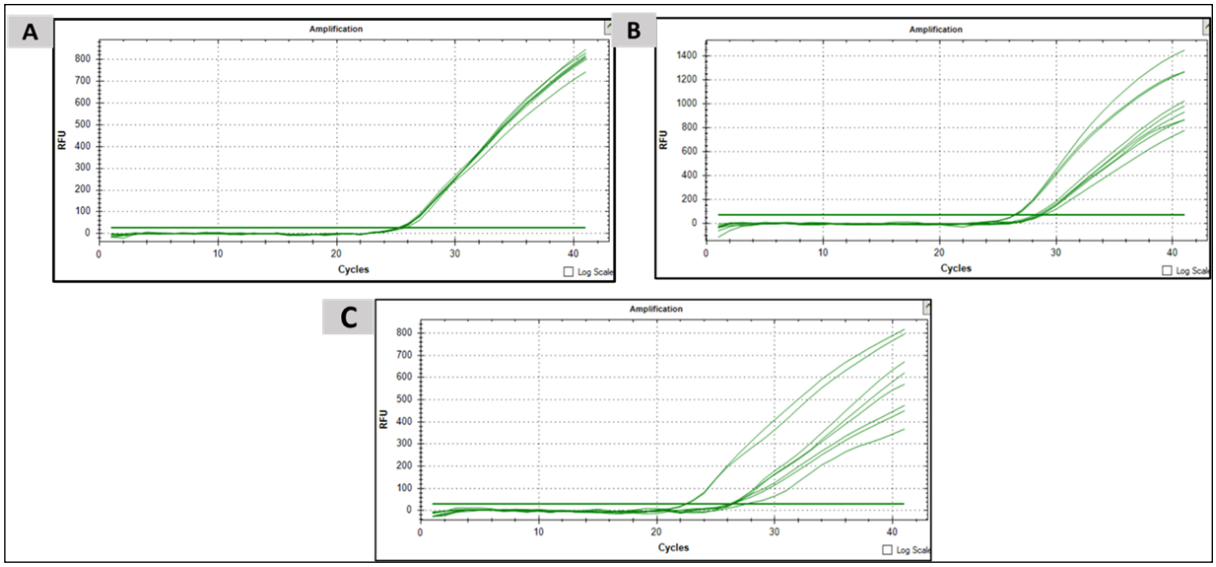

Figure 22: Real- Time amplification curves for inoculated bacteria after one month of incoculation: **(A)** *B. subtilis* L39 amplification plot. **(B)** Typical amplification plot for *C. flaccumfaciens* BA54. **(C)** Amplification curve for *P. agglomerans* BA69

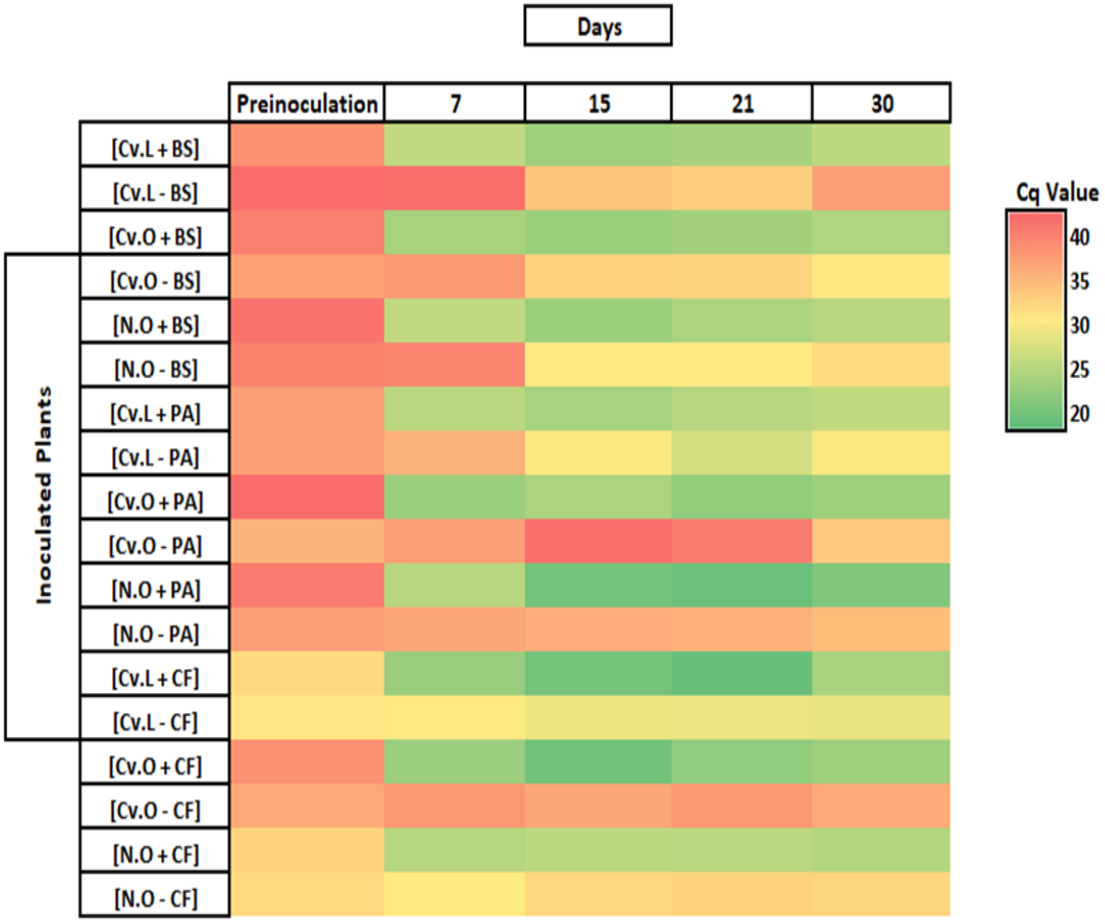

Figure 23: Heat map showing the real-time PCR quantification of inoculated bacteria: *B. subtilis* [BS], *C. flaccumfaciens* [CF] and *P. agglomerans* [PA] in the cultivars of 'Leccino' [Cv. L], 'Ogliarola salentina' [Cv.O] and *Nerium oleander* [N.O]. The data is presented as an average of Cq values obtained from inoculated and non-inoculated plants during one month of assessment

IV.3.3. Bacteria establishment evaluation by plates counting

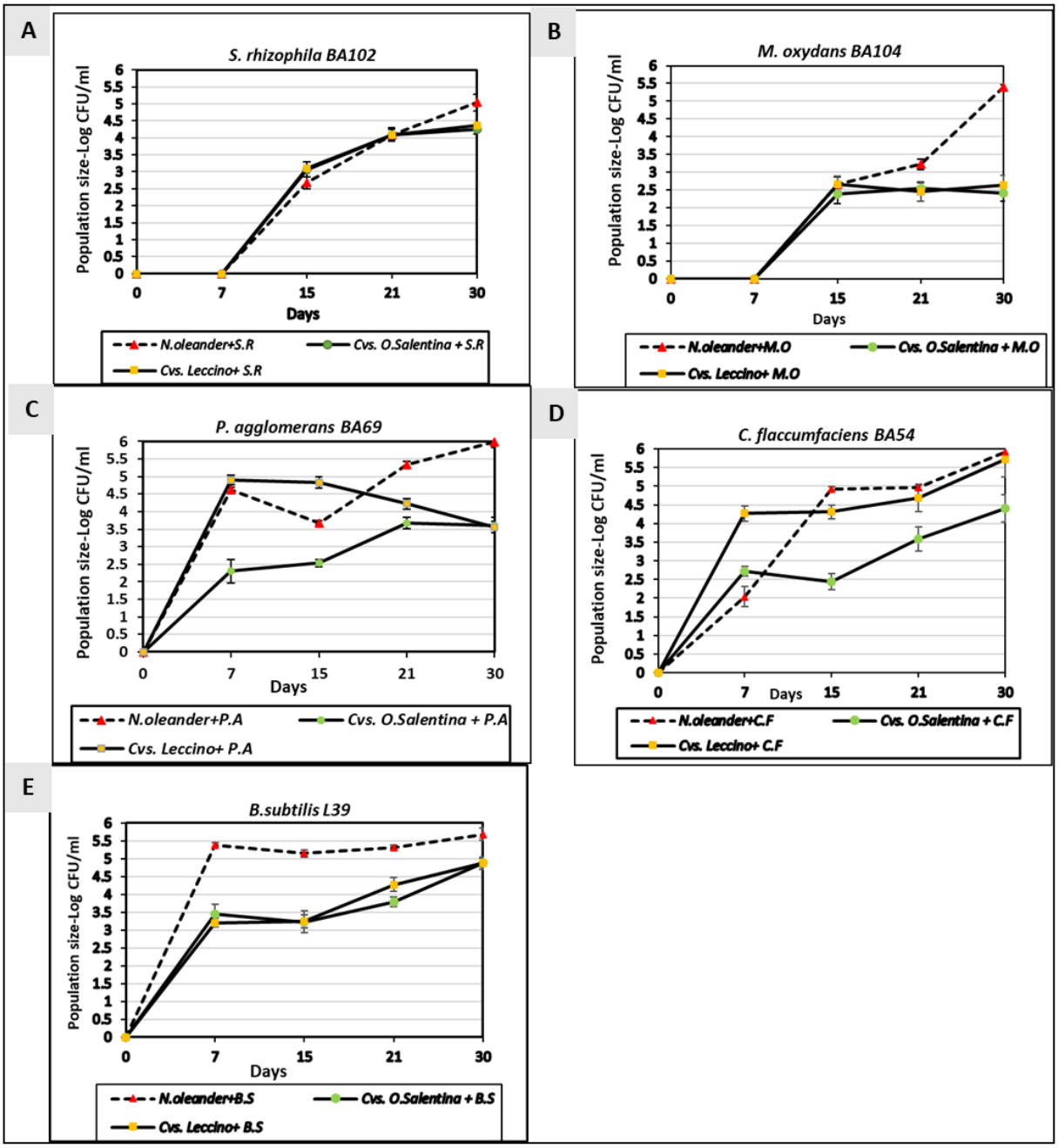

Figure 24: Monitoring the population size (CFU/ml) of bacterial inoculums in 'Leccino', 'Ogliarola Salaentina' and *N. oleander* during one month after inoculation. **A.** represents the population size of *S. rhizophila* BA102 (S.R). **B.** represents the population size of *M. oxydans* BA104 (M.O). **C.** represents the population size of *P. agglomerans* BA69 (P.A) **D.** represents the population size of *C. flaccumfaciens* BA54 (C.F). **E.** represents the population size of *B. subtilis* L39 (B.S). All bacterial inoculums were measured in three biological replicates of inoculated olive varieties and *N. oleander*.

The real-time PCR technique has been successfully used to monitor bacteria biomass in many studies (Chow et al., 2018; Landa et al., 2013), conventional techniques such as isolation and plate counting have also been used to assess population size, growth and movement of inoculum in the plant. Thus, we measured the population size of each inoculated bacteria within the surface-sterilized stem segments. After inoculation, our results indicate variation in stability and persistence within the inner tissues of the two olive cultivars and *N. oleander* plant. One week after inoculation of *M. oxydans* BA104 and *S. rhizophila* BA102, no bacterial colonies were recovered from all experimental plants. *S. rhizophila* BA102 isolate was found to be stable in the inner tissues of both olive cultivars and *N. oleander* plant recovering over 10^4 CFU/ml from the stem segments during the rest of the assessment period (Fig 17A). Meanwhile, *M. oxydans* BA104 isolate was successfully recovered from *N. oleander* plant starting from 15 days after inoculation (approximately > 10^5 CFU/ml), however, it was recovered in relatively small population sizes (<10^3 CFU/ml) from both olive cultivars after one month, indicating that this inoculum did not persist effectively in the Apulian olive cultivars (Fig 17 B). Both isolates of *B. subtilis* L39 and *C. flaccumfaciens* BA54 were found to be stable in the stem segments of olive cultivars and *N. oleander* plant. They were able to reach approximately 10^6 CFU/ml population size in *N. oleander* and over 10^4 CFU/ml population size from both olive cultivars (Fig 17 D&E). After 30 days of inoculation, *P. agglomerans* BA69 was recovered in relatively small population sizes (<10^3 CFU/ml) from both olive cultivars, indicating that these inoculums did not persist effectively in olives, however, it was recovered with relatively high population size (10^6 CFU/ml) from *N. oleander* plant.

IV.4. Discussion:

In the case of *X. fastidiosa,* it is essential to select a potential bio-control agent among epiphytic antagonistic bacteria found in the previous chapters (II and III) able to colonize the xylem. Therefore, we tested colonization and establishment of five promising antagonists in two different Apulian cultivars and *N. oleander* host.

Evaluation of inoculum establishment inside olive and *N. oleander* plants revealed variation in stability and persistence among our tested isolates. In other words, Plant defense mechanisms, competition with innate microorganisms, both abiotic parameters (temperature, moisture of the air and/or soil, etc.) and biotic factors (predation, antagonist, competition, etc.) are probable reasons for the failure of permanent endophytic colonization and variation in stability and persistence among the tested isolates (Raaijmakers *et al*., 2009).

Although some of our tested inoculum isolates were isolated from the phyllosphere, their bacterial cells were successfully detectable in the stem of the three different hosts after inoculation. Indeed, it is interesting to note that the genera Stenotrophomonas, *Pantoea*, *Microbacterium and Curtobacterium* were reported previously from diverse plants and microenvironments, they are also able to live in the endosphere of plants (Lumactud *et al*., 2016; Schmidt *et al*., 2021; Woźniak *et al.*, 2019)

The adopted methods (Conventional PCR, real-time PCR and plate counting) have successfully determined which isolate is highly capable of colonizing the inner sphere of Apulian olive cultivars and *N. oleander* plant. In general, the real-time PCR can quantitatively indicate the presence of inoculum with a minimum of time and high sensitivity, but, the method cannot distinguish between viable or dead cells of inoculum (Rilling *et al*., 2019). In our study, the inoculums of endophytic *B. subtilis* L39 and the epiphytic isolate *C. flaccumfaciens* BA54 were established in olive and *N. oleander* hosts and were confirmed by all approaches used. Thus, our results indicated that both bacterial isolates can harness olives and *N. oleander* innate as a

habitat for their growth, which is expected since the isolate *B. subtilis* L39 was already isolated from the xylem sap of olive and both isolates belong to two genera associated with the olive endophytic microbiome and have been repeatedly isolated from the olive plants (Fausto *et al*., 2018; Vergine *et al.*, 2019). On the other hand, *P. agglomerans* BA69 was recovered with a relatively high population size (10^6 CFU/ml) from *N. oleander* plant and was less efficient in establishing in both olive cultivars. This might be explained since this bacterium was initially isolated from *N. oleander* plant and has been known also as epiphytic or facultative endophytes of olives and associated with the rhizosphere of the different hosts (Egamberdieva *et al*., 2015; Gayder *et al*., 2020).

Results of the plate counting approach revealed that endophytic colonization of both olive cultivars and *N. Oleander* plant by the two isolates *S. rhizophila* BA102 and *M. oxydans* BA104 seems to require more time compared with the other isolates tested. In detail, after one week of inoculation, no bacterial colonies were visible on the plates. A possible explanation for this result is the fact that biocontrol strains have not usually been isolated from the plant organ to which they are applied (Bruisson *et al.*, 2019; Müller *et al*., 2016), thus the case of *S. rhizophila* and *M. oxydans* bacteria that were isolated in the present study from *Polygala myrtifolia* plant also some bacteria often have an epiphytic phase before entering the plant tissues (Pfeilmeier *et al*., 2016). Although, after two weeks, re-isolation of both isolates *M. oxydans* and *S. rhizophila* was successful. Those isolates can engage in beneficial interactions with plants, and *it is* often found in association with plants and are able to colonize more or less in all the microenvironments such as from internal plant tissues, particularly from the vascular tissues of the root, stem and rhizosphere (Arfaoui *et al*., 2019; Berg *et al*., 1996; Juhnke & des Jardin, 1989).

In conclusion, the results here obtained showed that the different isolates tested were able to colonize the stem of both olive cultivars and *N. oleander* plant during the assessment period of

about one month. Endophytic colonization of both isolates *M. oxydans* BA104 and *S. rhizophila* BA102 were found to require more time compared with other isolates tested based on re-isolation and plate counting approach. Although, assessment of their endophytic colonization efficiency using molecular tools is still needed to confirm the identity of morphologically similar colonies obtained by the plate counting method.

However, the plant defense mechanisms and the presence of potentially pathogenic bacterial strains in the olive stem may trigger a plant's defensive system and mechanisms, thereby affecting the persistence and stability of endophytic colonization of tested isolates induced by needle-inoculation of experimental plants. Moreover, further investigations are required to check whether the endophytic colonization of the tested isolates is stable in olives once planted in olive orchards.

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CHAPTER V

Overall Conclusions and recommendations

The present research aimed to investigate epiphytes inhabiting leaf surfaces of different *X. fastidiosa* hosts in the Apulia region under several variation factors, to screen their antagonistic activity against *X. fastidiosa* ST53, to reveal the mechanism of action of mainly obtained antagonists and to demonstrate the colonization efficiency in inoculated Apulian olive cultivars and *Nerium oleander* plant.

In the second chapter, culture-based techniques have been employed in order to isolate natural-occurring bacteria present on leaves of 9 different plant species. Overall, differences were observed between the bacterial population size of various leaves. These considerable variations in population sizes can be explained in great part by several various factors such as the plant species, leaf characteristics and fluctuations in the physical and nutritional, conditions of the phyllosphere.

Furthermore, the *in vitro* antagonistic activity of bacterial epiphytic isolates has been described in detail. The dual-culture method was adopted in this study to conduct mass screening of potential antagonists. As result, several bacterial isolates were distinguished by inhibiting the growth of *X. fastidiosa* ST53. It was necessary to evaluate which isolate possesses better inhibition. Our findings suggested that *P. agglomerans* demonstrated the strongest antagonistic activity against *X. fastidiosa* ST53 followed by *S. rhizophila* and *M. oxydans.* Meanwhile, *P. garminis, M. oleivorans, M. phyllospahrea, D. acidovorans, C. flaccumfaciens* and *S. molluscorum* were also valid antagonists at a lower level of inhibition.

This section received major attention for proving the existence of epiphytic antagonists of *X. fastidiosa* ST53, however, we believed those potential antagonists collectively should undergo through colonization efficiency in olive plants and for the case of *X. fastidiosa*, it is fundamental to select a potential bio-control agent among epiphytic bacteria able to colonize the xylem. To our knowledge, this is the first study about the potential antagonistic activity of epiphytes against the phytopathogen *X. fastidiosa* De Donno strain. In general, we faced certain difficulties that should be taken into consideration; the growth and manipulation of *X. fastidiosa* ST53 remain extremely difficult and time-consuming, where the measurement of the halo zone is restricted on the appearance of *X. fastidiosa* colonies.

In the third chapter, several tests were conducted to elucidate and comprehend the mechanism of various antagonists, as well as their ability to produce CFS with antimicrobial compounds. In fact, five bacterial endophytic strains natively obtained from Apulian olive cultivars and previously reported for their antimicrobial activity against other pathogens were also included in the screening of antimicrobial activity against *X. fastidiosa* ST53. Our results showed also that antagonistic isolates were able to use different mechanisms of action such as competition and secretion of antibacterial substances against *X. fastidiosa* ST53.

In the fourth chapter, we validated the colonization efficiency of selected antagonistic bacteria that were chosen upon their strongest antagonistic and antimicrobial activity in the previous chapter and their reported interactions (indirect antagonist and symbiont) with *X. fastidiosa* in previous studies. Upon inoculation, we found out that *B. subtilis* L39 and *C. flaccumfaciens* BA54 have excellent ability to thrive in the inner sphere of both olive cultivars and *N. oleander* plant, and their inoculums were tracked and recovered successfully for one month. Other potential antagonistic *P. agglomerans* BA69, was successfully found colonizing the stem segments of *N. oleander* plant and was less efficient in establishing in both olive cultivars. However, based on the plate counting approach, endophytic colonization of both isolates *M.*

oxydans BA104 and *S. rhizophila* BA102 required more time than the other isolates tested. We believe those antagonistic candidates could be integrated into a biocontrol strategy of *X. fastidiosa* ST53, however, that may require a further experimental understanding of their effect on counteracting olives bacterium infection. In the present study, we were able to annotate 10 antimicrobial compounds from the cell-free suspension of the endophyte *B. subtilis* L39 and the epiphyte *M. oxydans* BA104. Particularly, 3'-Deoxydihydrostreptomycin secreted by *M. oxydans* BA104 appears to be the most interesting among the ten compounds as its MS2 fragmentation pattern was revealed. Other molecules, on the other hand, do not have enough high abundance to be fragmented. Interestingly, results of HPLC-HRMS analysis are considered as promising preliminary results. However, further research is required to confirm the identification of the annotated compounds and other probable antibacterial secondary metabolites. Nonetheless, it is possible that the antibacterial action against *X. fastidiosa* ST53 was carried out by different classes of molecules, opening the door to new potential discoveries.

By our findings of different antagonists to *X. fastidiosa* ST53, the opportunity of developing successful and applicable biocontrol agents seems feasible. Most of our work enabled future studies to focus on limited candidates. Additional investigations on olive trees will be required to determine the ability of these antagonistic strains to inhibit *X. fastidiosa* in vivo and control OQDS. It may take years as the olive plants infected by *X. fastidiosa* may remain symptomless even for up to one year. Nonetheless, our findings pave the way for future research to develop long-term solution for OQDS control strategy involving bio-control agent(s) or their secreted metabolites.