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Apulian olive sapwood endophytes to cope with *Xylella fastidiosa*: community analysis, antagonists screening, colonization efficiency, and genes expression

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Extended abstract

This dissertation comprises a series of studies aimed at exploring the cultivable endophytic communities indigenous to the sapwood of Apulian olive cultivars, identifying potential antagonists against *Xylella fastidiosa*, *pauca* ST53, and demonstrating their colonization efficiency and associated effects on olive defence-related genes. This work starts with an extensive analysis of cultivable sapwood endophytes from resistant and susceptible cultivars distributed across phytosanitary zones in Apulia. After multiple isolation of endophyte colonies, the results were translated into quantitative indicators. It was found that the resistant cultivar 'Leccino' showed high stability and diversity of the endophyte unit compared to the susceptible cultivar 'Ogliarola salentina', whether infected or not by *Xf*. Moreover, this variation was observed at the genus level, where several bacterial (such as *Bacillus* and *Pantoea*) and fungal (*Pithomyces* and *Paraconiothyrium*) genera with commonly known antagonistic potency were isolated more frequently from the resistant cultivar. Later, several isolates of bacterial and fungal species were subjected to *in vitro* screening for antagonistic activity against *Xf* ST53. Isolates of *Bacillus subtilis*, *Bacillus pumilus* and *Pantoea agglomerans* exhibited significant potency of growth inhibition of *Xf*. A similar effect was shown by the fungal isolates of *Paraconiothyrium brasiliense*. Meanwhile, SYBR-Green real-time primers with high specificity and sensitivity to the target species of direct antagonists (*B. subtilis*, *P. agglomerans* and *P. brasiliense*), indirect antagonists (*Curotbacterium flaccumfaciens*) and symbionts (*Methylobacterium mesophilicum*) were developed to follow the establishment of the inoculum in resistant and susceptible olive cultivars. After inoculation of the endophytes, the regulation of defence-related genes in the above-ground tissues of the olive was studied. Both real-time PCR and plate counting revealed the successful establishment of the endophytic inoculum in the cultivars 'Leccino' and 'Cima di Mola'. Among the bacterial direct antagonists, *B. sublitis* showed high stability and persistence within the internal tissues of both olive cultivars. Similarly, the fungal isolate of *P. brasiliense* was consistently recovered with a high colonization rate in the stem segment of both cultivars. Induction of defence-related gene expression was found almost exclusively in olives inoculated with *B. sublitis*. Overall, our results demonstrate the efficacy and suitability of direct/indirect antagonists such as *B. sublitis* as a potential control strategy of *Xf* ST53 in Apulian olives.

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CHAPTER I

General introduction, hypotheses, and objectives

Summary

In this chapter, a general introduction is devoted to the definition of endophytes, their association with the plant, and their exploitation in agriculture, especially in the management of plant diseases. Meanwhile, a section is presented to consider the emergency of Olive Quick Decline Syndrome as a case study. In this context, we reviewed extensively the control attempts of this syndrome including the researched bases of olive resistance mechanisms. Finally we proposed several hypotheses concerning the potential control of Olive Quick Decline Syndrome by the exploitation of olive endophytes. The objectives are described and divided into research sections.

1.1 General introduction

1.1.1 Endophytes definitions and prospective

The term endophyte (*endon*; within and *phyton;* plant) was first coined to describe the presence of microbial cells in plant tissues examined microscopically [1]. Following that, several endophytic microorganisms were identified colonizing leaves, stems, and roots of apparently healthy plants [2-4]. Therefore, the definition of endophytes has passed through changes along with the advancements in the research field. This concept continued to remain unexplored until the definition of endophytes came into existence, when [5-9] simply redefined it based on the location of microbial colonization in the plant and its interaction effect on plant health; endophyte is an endosymbiotic microorganism that temporarily or permanently occupies the inter- and/or intracellular healthy tissue of the plant without causing visible disease symptoms. However, the above definition could not be applied to non-cultivable endophytes, therefore Bulgarelli [10] proposed endophytes as a set of microbial genomes located within plant organs. Overall, it is believed that the existence of an endophytic relationship with the plant existed on Earth hundreds of millions of years ago, which has been demonstrated in fossilized tissues of stems and leaves [11].

1.1.2 Ecological perspective of endophytes biodiversity

Endophytes colonize the plant horizontally through the environment or vertically from parent to progeny or in a mixed manner [12, 13]. Rhizospheric and phyllospheric microbial communities play a significant role in environmentally transcended endophytes, which are among the most complex, diverse and well-adapted assemblages in the biosphere. Once recruited from a large pool of phyllospheric or rhizospheric species and clones, endophytes can occupy plants locally or systemically and both inter- and intracellularly [14]. Endophytes occur in all tissue types within many plant species, suggesting a ubiquitous existence in most, if not all, higher plants. Moreover, some endophytic assemblages consisting of rare or singleton species have been found once or few times dominating the same host.

Variation in climatic conditions, geographical coordinates and plant species are important factors affecting the diversity of fungal and bacterial endophytes [15]. In the context of geographical variations, endophytes are more abundant in high temperature zones than in cold zones [16-19]. Plant age shows a non-negligible effect on the richness and diversity of endophytes, and with increasing duration of exposure to endophyte inoculum, the density of endophytes in the plant also increases. Therefore, endophytes are usually harbored more by

older plants than by younger ones [20-22]. Within the same host, the distribution and diversity of endophytes in different plant tissues are influenced by their ability to utilize nutrients, resulting in various modes of association with their host, which include symbiotic, mutualistic and antagonistic [23].

1.1.3 The significance of endophytes-plant interactions

Endophyte-plant interactions can be beneficial or neutral for the host plant [24]. Beneficial interactions include interactions responsible for mineral and nutrient uptake and supply to the plant (such as free-living or nitrogen-fixing bacteria) [25], interactions that inhibit the growth or activity of plant pathogens and thus indirectly stimulate plant health (such as. biocontrol agents) [26], and interactions that directly affect plant growth through the production of phytohormones (such as plant growth-promoting rhizobacteria or mycorrhizal fungi) [27]. Neutral interactions refer to interactions in which the presence of microorganisms in the rhizosphere or plant tissue neither benefits nor harms the plant. Such interactions are a major reason for the increasing interest in endophyte applications in sustainable agriculture, environment (e.g. detoxification) [23], industry (e.g. production of biocatalysts) [28], medicine (antibiotics production) [29].

In sustainable agriculture, endophytes are mostly employed as biocontrol agents against plant pathogens. The advantage of these endophytic agents, which return to the endophytic stage after application, is that they are better protected against biotic and abiotic threats coming from outside the plant. Moreover, they are ecologically adapted to the target niche, which means that they can overcome defence reactions. Many studies tended to use such endophytic bioagents in controlling local or systemic plant diseases. In local pathogenic infections, integrated formulations of endophyte species such as *Bacillus* and *Pseudomonas* have been used to control fungal diseases (such as powdery and downy mildew) and bacterial cankers (such as citrus and poplar cankers) [30-35]. Similarly, wilt diseases caused by systemic pathogenic infections have found the greatest interest in endophytic biocontrol treatments in various fruit and vegetables crops (tomato [36], cotton [37], eggplant [38], olive [39] and many others).

1.2 Case study

Olive quick decline syndrome (OQDS) is a destructive vascular disease that appeared suddenly at the end of the first decade of the XX century in the Apulian groves of Lecce province [40]. The incidence of the disease has been increasing rapidly in the affected area, devastating 6.5 million olive trees by 2019 [41]. The syndrome is caused by *Xylella fastidiosa pauca* ST53, a known xylem-limited, vector-borne, polyphagous and fastidious Gram-negative bacterium [42, 43]. The bacterium shares many features with vascular pathogens in the perspective of symptom development, which ends dramatically with total decay. The syndrome represents an urgent crisis calls a necessity to develop viable control approaches to cope with the progressive demarcation of the olive sector in Apulia.

1.2.1 Control attempts of olive quick decline syndrome

The management of *Xf* in Italy has been defined, as for any quarantine pathogen, by exclusion or containment, eradication of infected hosts, sustained control measures and finally implantation of resistant cultivar substitutes. In 2015, the European Commission implemented the containment plan of OQDS in Apulia. The Salento peninsula was divided into three zones (Apulian regional law, 195/2015): the infected, the buffer and the containment zone, where the following measures were implemented (UE, 2015/789). Movement of plant material (*Xf* hosts) is prohibited. Comprehensive and continuous monitoring of vectors, olives and alternative hosts for the presence of *Xf* is a mandatory measure to assess the spread of infection and intervene with immediate eradication. In order to maintain the health status of olive groves, the Commission has also recommended good agricultural practices: Soil fertilization and tillage, irrigation and pruning [44-46].

The use of chemical applications has been employed in a very dramatic direct control of the *Xf* vectors designed to reduce the population in the affected area. Dongiovanni [47] treated *Philaenus spumarius* with different insecticides; imidacloprid, organophosphorus, neonicotinoids and pyrethroids. Imidacloprid showed the highest mortality rates from 76.7% to 100% 3 days after treatment (DAT) and persistence up to 15 DAT with mortality rates of more than 40%. In addition, they studied the treatment effect of natural oils on *Xf* vectors, which showed no significant effect on mortality and/or persistence. Other chemical approaches remained under assessment, such as the systemic treatment of affected trees by copper based products. Dentamet® is a zinc, copper and citric acid biocomplex introduced by Scortichini

[48]; the product was evaluated to reduce the severity of *Xf* symptoms on olives. It also reduced bacterial cell density in the leaves of treated trees, as demonstrated by quantitative real-time PCR. The results of the referred are based on a limited sample size and additional data are thus needed to verify the effectiveness of this approach to manage the disease. Also, this contradicts the finding that copper-based products significantly enhance virulence characteristics of *Xf* strains [49].

Biological control of *Xf* on olives is limited by the lack of data on olive-inhabiting microorganisms. Consequently, the diversity of the microbiome and the role of endophytes are future research areas that should be explored. This approach was indirectly initiated when Fausto [50] identified the major bacterial endophytic taxa within *Olea europaea* (L.) cv. 'Maiatica'. However, the experiment sought to evaluate the differences in bacterial richness and diversity indices between two management systems, revealing important bacterial taxa such as *Curtobacterium* and *Pseudomonas* with physiological and protective functions for olive health. At the same time, the microbiome of resistant and susceptible olives was thoroughly investigated for ecological stability by advanced genomic metabarcoding [51]; the referenced study showed a high stability of the microbiome of the resistant cultivar under infection of *Xf* with a certain species richness as *Pseudomonas*, compared to the microbiome of the susceptible cultivar after infection. Finally, an in vitro antagonistic activity evaluation of strains belonging to different species of the genus *Bacillus* was performed by Zicca [52]. *Bacillus velezensis* strains (namely strains D747 and QST713) are already registered and commercially available as biocontrol agents and can produce several antimicrobials. Remarkable antagonistic activities against *Xf* were recorded for some *B. velezensis* strains in both dual culture and well diffusion methods. While none of the endophytes isolated natively from olive tissue showed any signs of inhibition

1.2.2 Review of olives resistance to *Xylella fastidiosa*

In Apulia, different varieties of olives are grown and the resulting high genetic diversity brings advantages, such as disease resistance. During the outbreak of *Xf* on olives, the predominant cultivars 'Ogliarola salentina' and 'Cellina di Nardò' have shown high disease susceptibility. In contrast, plants of *Olea europaea*; 'Leccino' and 'Favolosa', although infected, appear asymptomatic and show better vegetative growth with less aggressive and sporadic desiccation around the canopy [53]. Experimentally, the resistant cultivars were found to have lower bacterial concentration during infection [54].

In order to investigate the mechanism of 'Leccino' resistance at the molecular level, a comparison of the transcriptomes of olive cultivars under *Xf* infection was carried out by Giampetruzzi [55]. Their experiment was dedicated to the identification of the transcriptomic results of the resistant olive 'Leccino' and the susceptible 'O. salentina' in the healthy and infected states. The transcriptomes of healthy 'Leccino' and susceptible 'O. salentina' showed that the two cultivars did not differ significantly in the expressed genes, with no clear dominance of protein class function observed among the altered transcripts. Conversely, differential gene expression between *Xf*-infected plants of 'Leccino' and 'O. salentina' showed clearly cut incompatible profiles. In particular, a lower number of up-regulated genes was found in cultivar ' Leccino' compared to 'O. salentina', and one gene was exclusively shared between the two cultivars, which is discussed as a response to biotic stress. Down-regulated genes were predominant in cvs Leccino compared to O. salentina, while 75 were common. Overall, a solid mechanism involving cell wall properties by upregulating and encoding receptor-like kinases (RLK) and receptor-like proteins (RLP) dominated the response of 'Leccino', which was absent in 'O. salentina'.

Later, it was suggested that olive resistance was related to the activity of phenolic compounds, which was recognized as a promoter of plant resistance to bacterial diseases [44]. The mentioned study found similar biochemical presentations between resistant (Leccino & Favolosa) and susceptible (Cellina di Nardo & O. salentina) cultivars; quinic acid was noticed as the only phenolic compound for which the concentration was higher in Xf-infected olive trees. Afterward, Sabella [56] investigated the seasonal analysis of phenolic compounds in healthy and infected 'Leccino' and 'Cellina di Nardo'. A reduction of hydroxytyrosol glucoside was observed in both infected cultivars. However, 'Leccino' showed a significant increase in quinic acid, which is a precursor of lignin. Therefore, the study hypothesized that lignin

biosynthesis is involved in the defence response of 'Leccino' against *Xf* infection. The analysis thoroughly revealed the up- and down-regulated genes encoding enzymes that function in the biosynthesis of lignin and hydroxytyrosol glucoside. Overall, quantification of the enzymes studied indicated that lignin was significantly increased in the infected cultivar 'Leccino' compared to the susceptible cultivar, suggesting a critical role for lignin in olive resistance to *Xf*.

In the same context, Novelli [57] applied spectrophotometric and chromatographic techniques to measure the content of secondary metabolites and a molecular quantification technique to monitor the variation in gene expression between resistant and susceptible olive cultivars. The study proposed that the defensive dilemma of 'Leccino' against *Xf* is related to the presence of reactive oxygen species (ROS), which act both directly as oxidants and indirectly as secondary cell messengers for the biosynthesis of antimicrobial and antioxidant compounds in olive tissues. Their experiments showed a higher accumulation of ROS in 'Leccino' samples compared to 'Cellina di Nardò' under the infection of *Xf*. Consequently, a marked up-regulation of defence-related genes, such as NADPH oxidase, some protein kinases, pathogen-plant response factors and metabolic enzymes, as well as a marked production of specific antioxidant and antimicrobial molecules was observed in infected 'Leccino' plant.

Xylem cavitation and embolism is the phenomenon of blockage of xylem vessels by biotic and abiotic stress. It affects the capacity of water transport from soil to leaves, however, plants use different mechanisms to minimize the effects of cavitation and embolism and these vary among plants at cultivar level. To cope with cavitation, some plants initiate starch hydrolysis to refill the xylem vessels affected by cavitation. Sabella [58] suggested that the loss of hydraulic conductivity of the vessel by *Xf* could trigger embolism. The ability of infected plants to detect and respond to embolism by activating mechanisms to restore hydraulic conductivity may influence the severity of the disease. In their experiment, infected 'Leccino' stem cross-sections showed dense accumulation and aggregation of starch granules in the xylem vessels. Therefore, it was suggested that 'Leccino' is constitutively less susceptible to cavitation than the susceptible cultivar. To verify this hypothesis, they performed an analysis of gene expression in both cultivars under healthy and infected conditions. It was found that genes belonging to families involved in embolism recognition and replenishment mechanisms: Aquaporins, sucrose transporters, carbohydrate metabolism and enzymes related to starch degradation, alpha and beta amylase, are strongly modulated in 'Leccino' to overcome embolism.

1.2.3 Hypothesis

- The diversity of Apulian olive cultivars, which showed different susceptibilities to *Xf* infection, could have exceptional stability of cultivable endophytes in the sapwood. Moreover, the long life span of olive tree is known to increase the total endophyte population, and therefore, it may harbor several antagonistic endophytes that could contribute to promising biocontrol

- The resistance mechanism of olive cultivars (cv. Leccino and cv. Favolosa) continues to be intensively studied, but, as with any plant, it is suspected that their baseline resistance is systematic and/or acquired resistance. In this context, there might be olive endophytes capable of triggering olive plant defence mechanisms against pathogenic infections.

1.2.4 Objective

The objective of the study was to explore the core of culturable endophytes found in the sapwood of Apulian olives under different variation factors, to evaluate the antagonistic activity of the collected endophytes against *Xf* ST53 and to demonstrate the colonization efficiency and mediated health traits of selected endophytes in inoculated Apulian olive cultivars.

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CHAPTER II

Community analysis of cultivable sapwood endophytes from Apulian olive varieties with different susceptibility to *Xylella fastidiosa*

Summary

Endophytes are symptomless fungal and/or bacterial microorganisms found in almost all living plant species. The symbiotic association with their host plants by colonizing the internal tissues has endowed them as a valuable tool to suppress diseases, to stimulate growth, and to promote stress resistance. In this context, the identification of cultivable endophytes residing the sapwood of Apulian olives might be a promising control strategy for xylem colonizing pathogens as *Xylella fatidiosa*. To date, olive's sapwood cultivable endophytes are still under exploration; therefore, this work pursues a study of diversity and occurrence variation of cultivable endophytes in the sapwood of different olive varieties under the effect seasonality, geographical coordinates, and *Xf* infection status. Briefly, our study confirms the stability of sapwood cultivable endophytic communities in the resistant olive variety, presents the seasonal and geographical fluctuation of olive's sapwood endophytes, describes the diversity and occurrence frequency of fungal and bacterial genera, and finally retrieves some of sapwood-inhabiting fungal and bacterial isolates are known as biocontrol agents of plant pathogens. Thus, the potential role of these bacterial and fungal isolates in conferring olive tree protection against *Xf* should be further investigated.

Keywords; Endophytes, Sapwood, cultivable, diversity, *Xylella fastidiosa*

2.1 Introduction

In the last decade, olive groves in Apulia have been devastated by the arrival of the xylem-limited bacterium *Xylella fastidiosa* subspecies *pauca* (ST53), which causes a complex of severe symptoms called olive quick decline syndrome (OQDS) [1]. The severity of the syndrome symptoms depends on the age and health status of the infected tree, with initially infected plants showing leaf scorch and twig desiccation, eventually the infection prevails the canopy and reaches the skeletal looking trees [2]. The incidence of the disease has increased rapidly in the olive groves of the peninsula since the first outbreak in southern Apulia [3, 4]. By 2019, 6.5 million olive trees on 715,000 ha were severely damaged by the disease [5]. Due to its wide host range and transmission potency, this bacterium is considered a globally emerging plant threat by the European Commission [6]. Although tremendous scientific efforts have been made, effective control of *Xf* is still lacking. The discovery of resistant olive cultivars such as 'Leccino' represents the hope of obtaining indirect environmentally friendly control of the disease [7, 8]. The study of 'Leccino' resistance involved several research topics, including the genes conferring complete resistance to the bacterium and the physiological, physical and biochemical interactions of the cultivar with *Xf* during infection [9-12].

Endophytes are well-known beneficial microorganisms found in almost all living plant species and are perceived as new approaches to control plant pathogens [13]. In this context, their symbiotic association with the plant by colonizing internal tissues is utilized for suppressing diseases, stimulate growth and promote stress resistance [14, 15]. Although endophytes have been successfully applied as biocontrol agents [16-18], the potential mechanism of plant pathogen inhibition by endophytes also depends on various biotic or abiotic factors. Based on numerous reports, seasonality, soil and atmospheric composition, plant variety, and health status are the main factors affecting the variability and functions of endophytic communities [19-23]. To date, a single 16S rRNA metabarcoding study has assessed the overall stability of the olive microbiome under *Xf* infection [24]. However, knowledge about the culturable endophytic community living in olive trees is still very scarce. Therefore, we believe that the structure and dynamics of the olive cultivable endophytic community, including *Xf,* may be shaped by complex multilateral interactions between the abiotic environment and its biotic inhabitants. Understanding the endophytic composition of the lymph of Apulian olive trees with different susceptibility, seasonality and geographical location could provide a beneficial context for establishing efficient biocontrol tools to manage *Xf* infection.

2.2 Materials and methods

2.2.1 Samples collection and surface sterilization

The sampling program was designed to target three representative olive-growing sites in the demarcated area of Apulia with different phytosanitary status determined by regional law DDS (203/2016) and EU in May 2018 (*Xf*-free, contaminated and infected): site I (Valenzano, province of Bari), site II (Locorotondo, province of Bari) and site III (province of Lecce). Olive groves (25-50 years old) were selected based on similar agronomic practices carried out in the last 5 years (e.g. winter pruning of trees). As shown in (Table 1), 30 trees were considered: 15 from the cv. Leccino (5 for each site), which are resistant to infection, and 15 from the susceptible cvs. Ogliarola salentina and Oliva rossa, which are susceptible to infection and are genetically closely related [25]. Eight representative twigs (15-20 cm) per tree were collected, transferred to the laboratory under refrigerated conditions, and treated with a 2% sodium hypochlorite solution for 5 min. After rinsing in distilled water, they were cut into ~ 9 cm long sections. Surface disinfection was performed under aseptic conditions by washing in 70% ethanol for 2 min, sodium hypochlorite solution (10% available Cl) for 2 min and 70% ethanol for 30 s, followed by two rinses in sterile distilled water to remove bleach residues [26, 27].

Table 1. Seasonal twig sampling from different olive cultivars with different susceptibility to *Xf* infection located in different phytosanitary zones.

Sampling sites	Health status	Years	Sampling seasons			Location	Varieties	Nº
Site I	*X.f*-free	2018 - 19	Fall	Summer	Winter	Valenzano BA	Leccino	5
							Ogliarola salentina	5
Site II	Contaminated	2018 - 19	Fall	Summer	Winter	Locorotondo BA	Leccino	5
							Oliva rossa	5
Site III	Infected	2018 - 19	Fall	Summer	Winter	Lecce	Leccino	5
							Ogliarola salentina	5

2.2.2 Bacterial endophytes characterization

Sap extraction from the twigs for isolation of endophytes was performed using the patented syringe method (CIHEAM - IAMB, WO2017017555A1). The method consists of injecting 2 ml of sterile PBS (pH 7) from one end of the twig through the vessels and collecting the sap from the other end. The collected sap was concentrated by low speed centrifugation (4000 rpm, 2 min) and serially diluted suspensions were plated in 5 replicates of the media nutrient agar (NA, OXOID - IT) and King B (KB) [28, 29]. The Petri dishes were sealed and incubated at 25°C for 12 days. The bacterial colonies were purified and transferred to the Plant Bacterial Collection of CIHEAM-Bari at -80°C. Subsequently, the most frequently obtained colonies were categorized and subjected to morphological and biochemical characterization according to classical differentiation tests: colony structure and texture, cell shape and motility, Gram, catalase, oxidase, indoleacetic acid (IAA) and phosphate solubility tests [30-32].

Bacterial DNA was extracted following the classical phenol-chloroform methodology [33, 34]. Genomic DNA was used as a template in a PCR reaction with the primers 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and reverse primer 1387R (5′-GGGCGGWGTG-TACAAGGC-3′) allowing amplification of a fragment of approximately 1.3 Kbp of the 5' end of the 16S rRNA gene [35, 36]. The PCR mixtures contained 2 µL of 50 ng/µL template DNA, 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).

2.2.3 Fungal endophytes characterization

Fungal isolation from olive sap was carried out following the methodology of twig printing [37, 38]. Sterile pliers were used to loading a light pressure on the sterilized surface of twigs; then, the sap was printed ten times per plate of Potato Dextrose Agar (PDA, OXOID. Milan-IT) and four replicate plates were prepared. The unsterilized twigs were printed as control. Plates were incubated at 25°C for 5-14 days depending on fungi growth rates. The colonies obtained were purified through several inoculations on 1.5% water agar. The final pure cultures

were transferred in PDA slant tubes and stored at 4°C in the Plant Microbiology Collection of CIHEAM-Bari.

Fungal colonies were grouped according to their macro and micromorphological characteristics following [39]. Subsequently, isolates were grown on Potato Dextrose Broth (PDB, Difco™ - IT) to enrich the mycelium and then extract DNA material following [40]. The ITS region was selectively amplified by PCR using the universal primers ITS1 (5´-TCCGTAGGTGAACCT TGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) according to White [41], which hybridize on rDNA. 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 55°C for 1 min, and an extension step at 72°C for 1 min, followed by 1 cycle at 72°C for 6 min.

2.2.4 Molecular identification

The amplification products were sequenced in both directions using Eurofins Genomics (https://www.eurofinsgenomics.eu/). The accuracy of the sequences obtained was evaluated using FinchTV 1.4 software (https://finchtv.software.informer.com/). The taxonomy of 16S rRNA sequences was examined at the phylum and genus levels based on the RDP Bayesian Classifier [42]. ITS sequences were submitted to the online search engine BLAST of National Centre for Biotechnology Information (NCBI). Considering the morphological analyses, each isolate was assigned to a genus if the sequence had ≥ 98% identity to a valid sequence deposited in Genbank. For genera having ITS as barcoding region, the isolate was assigned to a species if the identity was ≥ 98 %. Consistently, the freely available software MEGA.X was used to confirm the similarity of sequence profiles by constructing a phylogenetic tree using the Tamura-Nei model [43]. Analyses were performed using 500 bootstrap replicates. Finally, representative sequences that showed pronounced similarities to different species of a genus were deposited in NCBI under specific accession numbers.

2.2.5 Statistical analysis

The statistical approach was carried out using univariate and multivariate descriptive analyses, parametric and non-parametric, which were conducted on the concentrations and specific counts of endophytic isolates extracted from the sapwood of different olive cultivars. To assess the bacterial richness, a quantification of the colony forming unit was determined for bacterial colonies in the sapwood of olive twigs, noting the concentration in logarithm to base 10 (Log CFU / ml [44]. On the other hand, the quantification of endophytic fungal isolates was evaluated by the colonisation rate (CR) and isolation rate (IR), which are presented as percentages and are preferably used as an indication of fungal abundance when there is a high incidence of multiple infections as in our study [45, 46]. The relative abundance of classified endophytic morphotypes was estimated by the relative frequency of each specific microorganism (at the level of phyla & genera for bacteria and order & genera for fungi) relative to the total number of communities detected [47].

To investigate the differences in the defined response variables (Log CFU / ml, CR, IR and the number of isolates) in correspondence with abiotic and biotic factors defined in four explanatory variables: the variety susceptibility (levels: More, Less), sampling sites (levels: site I, site II and site III), seasonality (levels: summer, winter and Fall) and *Xf* infection, (levels: *Xf*-pos and *Xf*-neg). The univariate parametric test (factorial ANOVA) was applied to test the separability between levels of the defined explanatory variables. For the data with a "slight" or "significant" deviation from normality (especially for bacterial concentration), a non-parametric univariate model similar to ANOVA was applied (Kruskal-Wallis test) to avoid reducing power and increasing the probability of type I error (typical of parametric analyses) [48]. Since common non-parametric tests are not suitable to evaluate the interactions between multiple factors [49], the aligned rank transformation method (ART) was applied to address this condition [50, 51]. Finally, a multivariate approach (discriminant analysis) was applied to the variables CR and IR to understand which variable has the greatest influence on the overall endophytic fungal community of olive cultivars (Leccino, O.salentina and O.Rossa) with different susceptibility to *Xf* infection. Statistical analyses were performed using the SPSS software package (version 12.0), and the ART methodology was implemented through the freely downloadable ARTools software (http://depts.washington.edu/ilab/proj/art/index.html).

2.3 Results

2.3.1 Bacterial morphological, biochemical, and molecular characterization

Seasonally, an approximate average of 3400 colonies was obtained belonging to the sampled olive varieties. Among those, 142 isolates were selected as most frequently isolated, and based on morphological properties, they were clustered into 16 groups. The colonial morphology within the groups varied from small to large, flat to raised, transparent to heavily pigmented, with circular to irregular edges. Considering the cell morphology, most of the isolates were motile rods, which presented individually or in short chains. The KOH test showed that 64% of the selected bacterial isolates were gram-positive; 74% and 54% of the isolates showed a positive reaction to oxidase and catalase tests, respectively. Concerning biochemical characteristics, 76.6% and 54.8% of the tested isolates presented a positive reaction to IAA production and P-solubilization tests, respectively (Table 2). Few clusters of bacterial isolates were found to dominate the selected collection by corresponding to the same biochemical features; therefore, the scope of the molecular study was reduced to 73 isolates.

Table 2. Clustering of the obtained bacterial isolates from olive varieties based on common morphological and biochemical features.

Group code	No. of Iso-lates [124]	Colony morphology	Consistency	Texture	Gram	Shape	Motility	Catalase	Oxidase	IAA test	P- solubilization test
OSB 1	6	Yellow, Large and Circular	Slimy	Flat	+ve	Rods	+	+	+	-	+
OSB 2	10	Yellow, Small and Irregular	Creamy	Flat	+ve	Rods	+	+	-	+	+
OSB 3	7	Yellow, Small and Irregular	Slimy	Flat	+ve	Rods	+	+	-	+	+
OSB 4	9	Pink, Large and Circular	Creamy	Raised	-ve	Rods	-	-	+	+	+
OSB 5	13	White, Small and Irregular	Slimy	Flat	+ve	Rods	-	+	+	+	-
OSB 6	14	Yellow, Large and Circular	Mucoid	Raised	-ve	Rods	+	+	-	+	-
OSB 7	9	White, Large and Circular	Creamy	Flat	+ve	Rods	+	+	+	+	-
OSB 8	10	White, Small and Circular	Creamy	Flat	-ve	Rods	+	+	-	+	+
OSB 9	4	White, Small and Circular	Slimy	Flat	-ve	Rods	+	+	+	+	+
OSB 10	5	Yellow, Large and Circular	Creamy	Convex	-ve	Rods	+	+	+	+	-
OSB 11	11	Brown, large and Circular	Mucoid	Raised	+ve	Rods	+	-	+	-	-
OSB 12	7	Orange, small and Circular	Slimy	Flat	-ve	Rods	+	+	+	-	+
OSB 13	5	White, large and Irregular	Slimy	Flat	-ve	Rods	+	-	-	-	+
OSB 14	10	Red, Small, and Circular	Creamy	Raised	+ve	Rods	-	-	+	+	+
OSB 15	7	Yellow, Large and Circular	Mucoid	Flat	+ve	Rods	+	-	-	+	-
OSB 16	3	White, Small and Circular	Slimy	Flat	-ve	Rods	+	+	+	+	-

Based on the Bayesian RDP classifier, the taxonomy of the 16S rRNA sequences was examined at the phylum level and the most sequenced phyla associated with the sapwood of all olive varieties were *Proteobacteria*, *Actinobacteria* and *Firmicutes*. Sequences assigned to *Proteobacteria* and *Firmicutes* were more abundant in the bacterial community of 'Leccino' sap (52.4 and 34.2%, respectively) than in the bacterial community of 'O. salentina' and 'O. rossa' sap. Conversely, sequences assigned to the phylum *Actinobacteria* were more frequent in the sap of 'O.Salentina' (36%) and 'O. Rossa' (28%) than in the community of 'Leccino' sap (14%) (Fig 1).

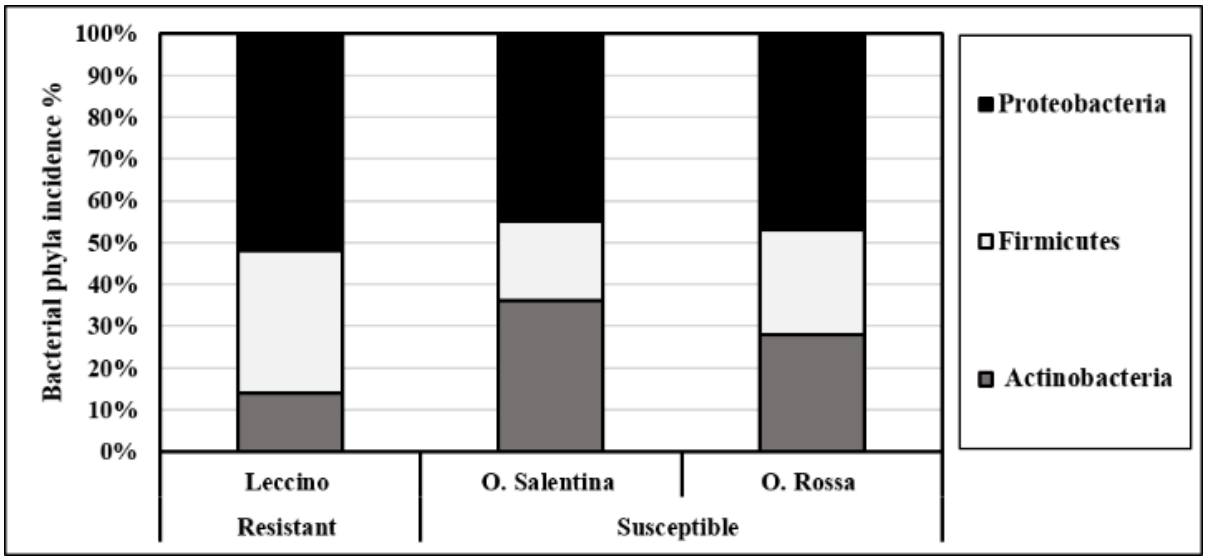

Figure 1. Overall incidence and taxonomic diversity of endophytic bacteria phyla in the sapwood of the studied olive varieties.

During the sampling seasons, the bacterial community showed a significant variability with the sapwood of all olive varieties, which harbored different bacterial profiles but not all taxa. Sequencing analysis showed that the core of cultivable endophytic bacterial community in the sapwood corresponded to 25 different bacterial taxa belonging to 7 families and 10 genera: *Bacillus, Mehtylobacterium, Frigoribacterium, Curtobacterium, Okibacterium, Pantoea, Paenibacillus, Pseudomonas, Sphingomonas,* and *Sphingobium* (Fig 2). The dominant genera common in all olive varieties were *Bacillus, Methylobacterium,* and *Paenibacillus*. These three genera accounted for approximately half of the isolates, which belonged to at least three different species.

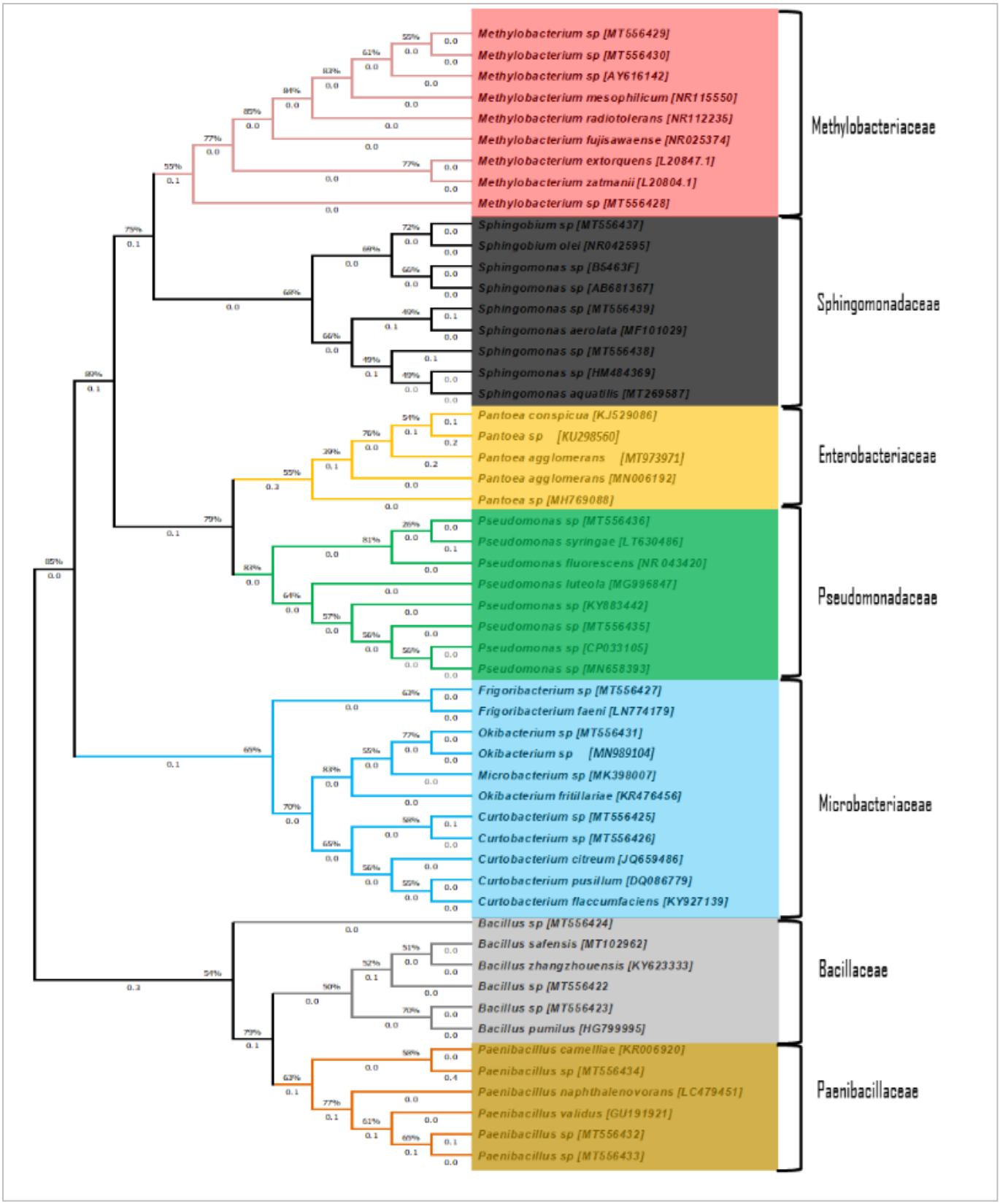

Figure 2. Phylogenetic tree based on 16S rDNA gene analysis of the most frequently identified cultivable bacterial endophytes from olive sapwood, in reference to closest type strains obtained from the Ribosomal Database in NCBI. The accession numbers (MT556422 - MT55639, MT973971) refer to our annexed bacterial sequences in NCBI.

2.3.2 Bacterial occurrence and frequency variability

The total number of colonies that appeared on both NA and KB increased during the incubation period. Most colonies of endophytic bacteria appeared within 10 days of incubation. This tendency was observed on both KB and NA plates regardless of olive varieties. The presence of culturable bacteria in the sapwood was assessed using different sets of the extracted sap, which covered a broad overview of the endophytic preferential organisms. In general, the endophytic bacterial community detected in the sap ranged from 3.59 ± 0.52 log CFU ml^{-1} to 8.94 ± 0.37 log CFU ml^{-1} in most cases.

The statistical approach was employed to investigate the influence of the explanatory variables (sampling sites, *Xf* infection and seasonality) on the response variable (CFU/ml). Primarily, the univariate analysis of the interactions of (site*season) (site*variety) showed no significant effect on the plate count indicator ($P > 0.05$), while the interaction of (season*variety) exclusively revealed a significant effect on the plate count indicator ($P < 0.001$). Considering the variety variable, CFU average revealed a higher bacterial count in sapwood of 'Leccino' compared to 'O. salentina' and 'O. rossa' at all assessed sampling sites studied ($P = 0.006$, $P = 0.004$, respectively). In addition, the bacterial occurrence in samples of 'Leccino' and 'O. salentina' varied slightly within sampling sites, and there was a consistent pattern in terms of zone effect producing more colonies than other zones (Fig 3A).

The hypothesis of seasonal variability in bacterial richness was statistically supported, as the summer samples of 'Leccino' exhibited a difference ($P < 0.001$) compared to the fall and winter samples, whereas in the samples of 'O. salentina' and 'O. rossa', only the summer bacterial community differed from the winter community ($P = 0.003$) from the winter one, while the fall bacterial community was indistinguishable from the winter samples (Fig 3B). As for the effect of *Xf* infection on the variation of bacterial richness in the sap of the 'Leccino' and 'O. salentina' twigs, the non-parametric analysis (Kruskal Wallace) showed a significant difference only between the healthy and the infected 'O. salentina' tree, with the analysis indicating a decrease in the endophytic bacterial community in the diseased plants (2-sided test <0.001) (Fig. 3C). On the other hand, the infected 'Leccino' showed a high stability of the bacterial community (2-sided test = 1.000).

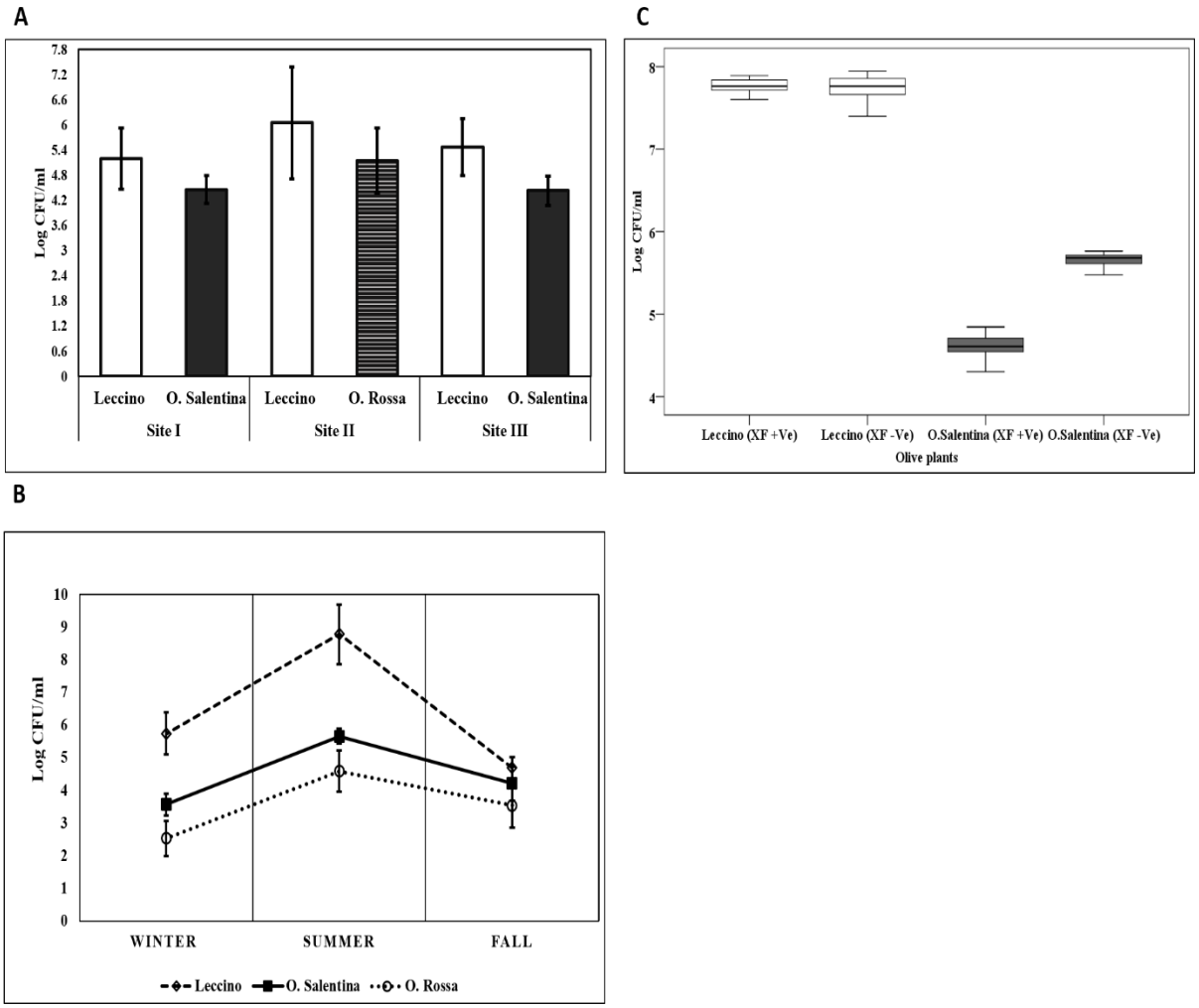

Figure 3. Illustrations of bacterial endophytes occurrence and variation. (A) The annual average of CFU/ml obtained from the twig's sap of different olive varieties within different sampling sites. Data represent mean ± Std. (B) Seasonal variation of bacterial occurrence in different olive varieties. The occurrence is based on the number of colonies that appeared on NA and King B media. Bars indicate a significant difference between means by one-way analysis of variance (ANOVA) and the least significant difference (LSD) tests ($p < 0.05$). (C) Boxplot diagram showing a variation of the bacterial community occurrence between infected and non-infected olive varieties in the sampling site III.

Further analysis examined how bacterial populations of the ten major genera varied by sampling site and varieties. In general, the endophytes studied showed significant variation between and within the sampled sites. At all sampling sites, the genera *Bacillus*, *Pantoea* and *Curtobacterium* were most frequently isolated from the sap of 'Leccino', while *Pseudomonas* was predominant in the sap of 'O. salentina' and 'O. rossa'. At sampling site I, *Bacillus*, *Curtobacterium* and *Pantoea* genera showed higher isolation frequency from the sap of 'Leccino' than from the sap of 'O. salentina'. On the other hand, *Paenibacillus* and *Pseudomonas* species were more abundant in 'O. salentina' sap than in 'Leccino' sap. Although species sharing was still observed at site I, there were also solid differences in frequency

between the two varieties: the genera *Okibacterium* and *Sphingomonas* genera were found exclusively in the twigs of 'Leccino' cultivar. A similar representation was shown when comparing genera from the sap of 'Leccino' and 'O. rossa' at the sampling site II, as *Frigoribacterium* and *Sphingomonas* genera were never found in the sap 'O. rossa'.As the sampling site III represented olive trees under the infection pressure of *Xf*, the sap of 'O. salentina' showed the lowest level of bacterial diversity and isolation frequency except for Pseudomonas sp (Fig 4).

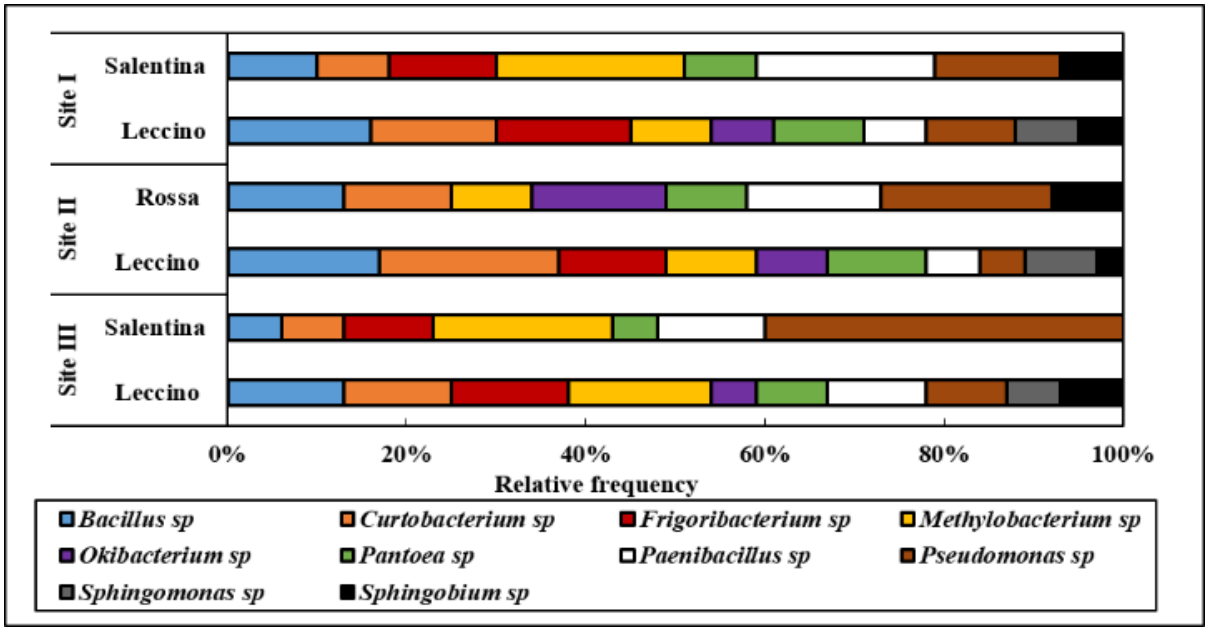

Figure 4. Relative frequency of identified endophytic bacteria colonizing olive varieties in different sampling sites.

2.3.3 Fungal morphological and molecular characterization

Fungal endophytes were isolated from all processed plants. The imprint tests indicated that surface disinfection procedures efficiently eliminated epiphytic fungi (data not shown). A total of 2273 fungal colonies were obtained from 240 twigs collected from 30 olive trees. However, only in 77.5% (186/240) of the collected twigs from different sampling sites and olive tree varieties were found emerging fungal colonies. The fungal isolates obtained showed distinct features regarding colony color, shape and growth rate of mycelium. Based on the obtained characteristics, the isolates were assigned to different morphological groups, of which 60 representative isolates were molecularly identified by sequencing the ITS region. As a result, 33 taxa were found belonging to 8 orders representing the clustered groups (Fig 5). *Pleosporales*, *Eurotiales* and *Phaeomoniellales* resulted to be the most abundant orders and accounted for

more than half of the assigned isolates in all olive varieties. In contrast, the orders *Hypocreales*, *Mycocaliciales* and *Stigmatodiscales* were the least abundant. The diversity of orders among endophytic fungi was higher in the twigs of 'O. salentina' than in those of 'Leccino' and 'O. Rossa'. Isolates of 'O. rossa' were never assigned to the order *Diaporthales*, and none of the isolates of 'Leccino' were assigned to the order *Stigmatodiscales* (Fig 5).

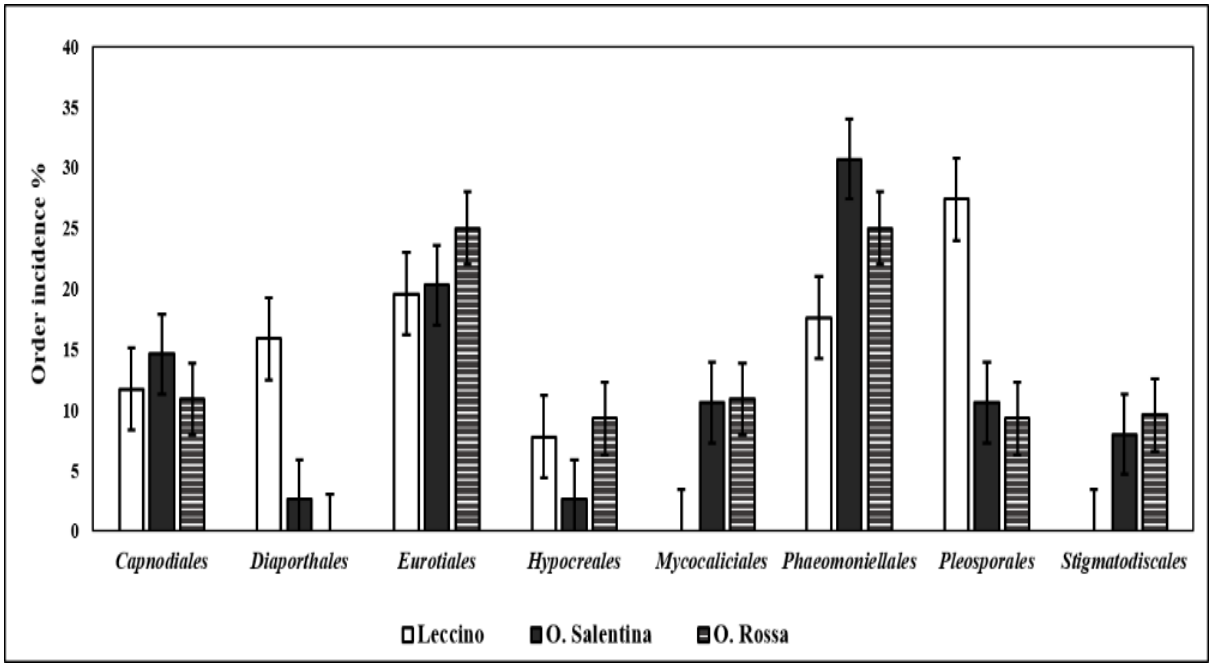

Figure 5. Taxonomic distribution of the endophytic fungi, which were isolated from the twigs of 'Leccino', 'O. salentina', and 'O. rossa' cultivars identified by ITS sequencing.

The ITS sequences assigned the 60 fungal isolates to 14 genera, with representative isolates deposited under specific accession numbers (Table 3). Relative fungal density showed that the genera *Cladosporium*, *Penicillium*, *Neophaeomoniella*, and *Pseudophaeomoniella* were the most abundant endophytic fungi colonizing the olive varieties. Although a partitioning of genera among olive cultivars was observed, some isolates were found colonizing single and/or double varieties, as in the case of *Paraconiothyrium brasiliense*, which exclusively inhabited the twigs of 'Leccino'. Moreover, *Phoma* and *Cytospora* genera were found exclusively in the twigs of 'Leccino' and 'O. Salentina'. Similarly, *Mycocalicium* and *Stigmatodiscus* were found in the twigs of 'O. salentina' and 'O. rossa', while the genus *Libertasomyces* was associated with the twigs of 'Leccino' and 'O. rossa' (Table 3).

Table 3. Molecular characterization and relative density of fungal endophytes colonizing the twigs of different olive varieties in Apulia.

Groups	Identity	Accession #N	Reference	Blast ID	Source	Total Relative Density %		
						Leccino	Salentina	Rossa
MF1	*Aspergillus* spp.	MT558577-78	MH398045.1	99%	L, S & R	9.8	1.77	4.69
MF2	*Cladosporium* spp.	MT558579-80	LN834380.1	98%	L, S & R	15.7	11.50	10.94
MF3	*Cytospora* spp.	MT558581	KY496629.1	98%	L & S	11.8	2.65	0.00
MF4	*Fusarium* spp.	MT558582	KT004553.1	99%	L, S & R	7.84	2.65	7.81
MF5	*Libertasomyces platani*	MT558583	KY173416.1	99%	L & R	3.92	0.00	4.69
MF6	*Mycocalicium* spp.	MT558584	AJ972853.1	98%	S & R	0.00	10.62	12.50
MF7	*Neophaeomoniella* spp.	MT558585	NR138001.1	99%	L, S & R	5.88	15.05	14.06
MF8	*Paraconiothyrium brasiliense*	MT558586-87	KR909140.1	99%	L	7.84	0.00	0.00
MF9	*Paraphaeosphaeria* sp	MT558588	GU985234.1	99%	L, S & R	3.92	4.42	6.25
MF10	*Penicillium* sp	MT558589-90	MK102703.1	99%	L, S & R	9.80	14.16	10.94
MF11	*Phoma* sp	MT558593	GU183116.1	99%	L & S	3.92	8.85	0.00
MF12	*Pithomyces chartarum*	MT558591	MH860227.1	99%	L, S & R	7.84	0.88	4.69
MF13	*Pseudophaeomoniella oleae*	MT558592	NR_137966.1	99%	L, S & R	3.92	14.16	7.81
MF14	*Stigmatodiscus oculatus*	MT558594	MH756071.1	99%	S & R	0.00	7.96	9.38

L: Leccino, S:Ogliarola salentina, R: Oliva rossa

2.3.4 Fungal occurrence and variability

The assemblages of fungal endophytes recovered at each site were statistically analyzed to assess the effect of the site, season, *X.f* infection, and olive varieties on fungal colonization and isolation rates. Overall, the comparative analysis (MANOVA) showed no significant interaction effect between (sampling sites * varieties) on fungal colonization and isolation rates (P = 0.915). However, separately studied both factors revealed significant effects on fungal colonization and isolation rates (P_{sites} = 0.001, $P_{varieties}$ = 0.03). At the varieties level, 'Leccino' olives presented lower colonization rates compared to 'O. rossa' (P_{CR} < 0.001) and 'O. salentina' (P_{IR} =0.002), whereas no significant effect was found on isolation rates. At the sampling site level, a much-noticed elevation of fungal isolation rates was found in the healthy site (I) compared to the infected site (III) (P_{IR} = 0.024), whereas the healthy site reflected a trending significance on colonization rates (P_{CR} = 0.045). Finally, the comparison of both variables within varieties revealed a high fungal content within the twigs of 'O. salentina' and 'O. rossa' compared to 'Leccino' at specific sites (Fig 6A, B).

The hypothesis of seasonality was implied to compare the quantitative variation of fungal isolates within 'Leccino' at all sites, 'O. salentina' (the site I and site III), and 'O. Rossa' (site II). Generally, the fall season showed a positive effect on the number of fungal isolates in all evaluated olive varieties and sampling sites (P = 0.044). Therefore, the abundance of summer fungal isolates was constantly found to be decreasing compared to winter and fall (Fig 6C). At the seasonal level, fungal communities (N of fungal isolates) in 'Leccino' in site I (37 ± 2.27) and site III (34 ± 7.23) was found less abundant than that of 'O. salentina' in site I (66 ± 8.99) and site III (57.8 ± 6.09) (P = 0.027) (Fig 6C). A similar scenario was noticed at sampling site II, the comparison between 'O. rossa' and 'Leccino' isolates showed that 'O. Rossa' seasonally revealed a higher mean of isolates (51.6 ± 4.64) than 'Leccino' (42.6 ± 5.04) (P = 0.001) (Fig 6C). Lastly, the study of *Xf* infection effect on the fungal abundance at sampling site III showed no significant effect among infected and non-infected 'Leccino' and 'O. salentina' trees (P = 0.761, P =0.130) (Fig 6D).

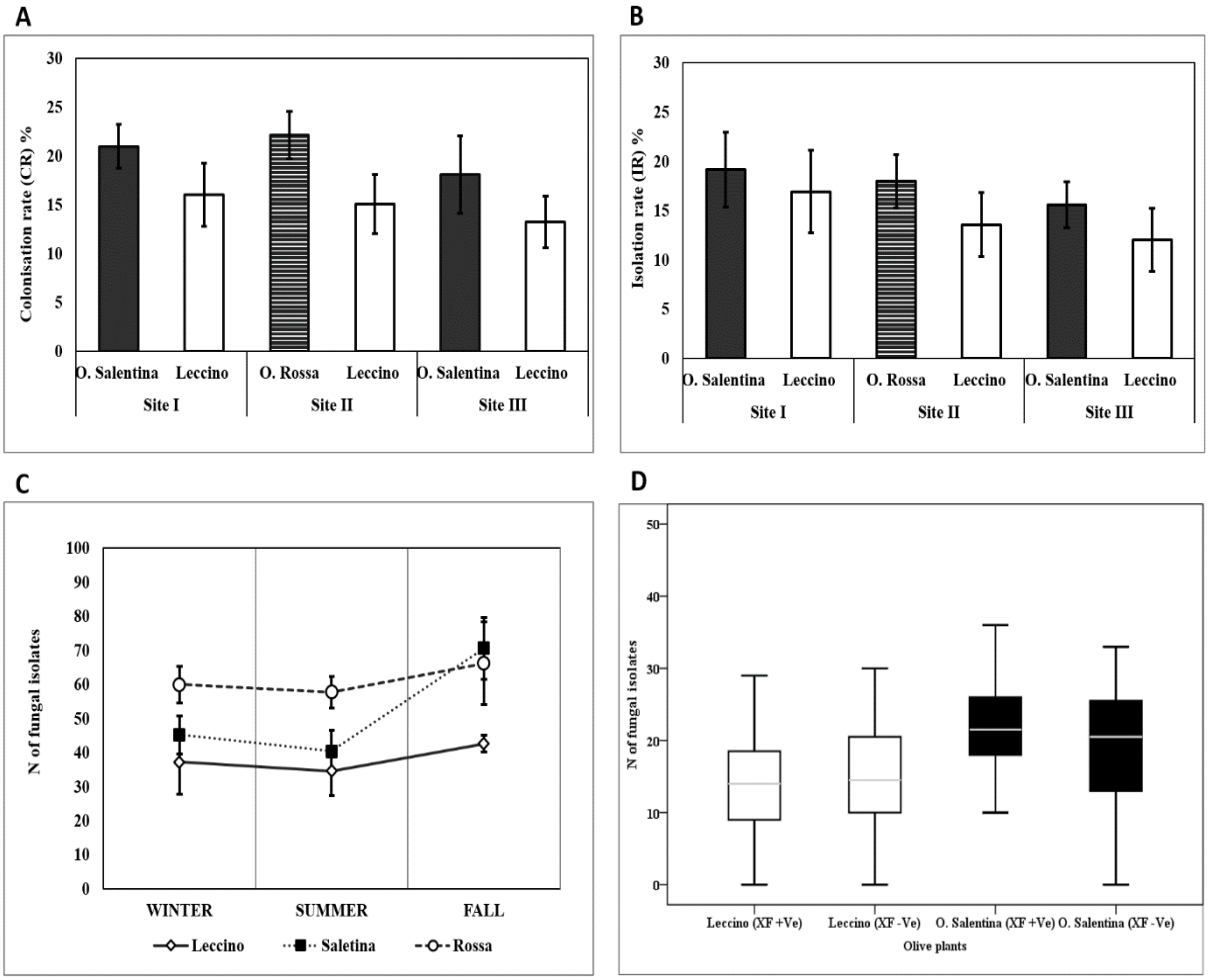

Figure 6. Illustrations of fungal endophytes occurrence and variations. (A) The histogram illustrates the variation of fungal colonization rates between different olive varieties belonging to different sampling sites. Bars represent SEM. (B) The histogram shows the variation of fungal isolation rates between different olive varieties belonging to different sampling sites. Bars represent Std. (C) The curves illustrate the representative mean of fungal isolates ± Std, which represents the seasonal dynamic of fungal endophytes occurrence within different olive varieties. (D) Boxplot depicts the interactive effect of 'Leccino' and 'O. salentina' health status * varieties on the mean number of isolates.

2.4 Discussion

Endophytic microorganisms colonizing sapwood are perceived at a low population level compared to rhizospheric ones [19, 52, 53]. Nevertheless, they are more specific than rhizospheric microorganisms as they are well adapted and able to form multilateral interactions within the plant leading to health promotion and resistance [54]. The use of sapwood endophytic microorganisms has been implemented as an attractive approach to manage plant vascular diseases in different hosts [55-58]. In this context, the serious uncontrolled vascular pathogen *Xf* has been ravaging the Apulian olive cultivars, among which 'Leccino' proved to be a highly resistant cultivar and, as such, could represent the core of potential control strategies [7, 59]. As such, the microbiome of Apulian olive varieties especially ‘Leccino’ has acquired a crucial significance that might be linked to the resistance mechanisms [24, 60]. Apart from the metabarcoding of 16S rRNA of olive microbiome and activity of some bacterial isolates against *Xf* studies [24, 61], there is a lack of data in the literature about the cultivable endophytic communities in the sapwood of ‘Leccino’, ‘O. rossa’, and ‘O. salentina’ olives. Therefore, this study intended to assess the endophytic communities residing in the sapwood of susceptible and resistant olives, with consideration of several determining factors of endophytes diversity and richness.

Taken together, our analyses on the cultivable bacterial endophytes in olives suggest that bacterial richness in sapwood is mainly influenced by olive varieties, seasonality and sampling site. In this regard, the resistant cultivar 'Leccino' revealed a high bacterial population, indicating a great stability of its cultivable endophytic population in agreement with the microbiome study conducted [24]. Since high temperature is a determinant of bacterial richness in a tree [62-64], our study confirmed that olive sapwood has the highest bacterial population in summer. Although our sampling pattern included fields with the same cultural practices, the same olive cultivar in different fields showed differences in bacterial composition in the sapwood. This phenomenon could be related to the bacterial richness in the soil between field [60].

Our results showed that the phyla *Proteobacteria* and *Actinobacteria* were commonly found prevalent in the sapwood of olive cultivars, confirming previous study [60], in which both phyla were associated with plant growth promoting properties and resistance induction [65-67]. Interestingly, the sapwood of 'Leccino' exhibited a distinct elevation in *Firmicute*s phylum, which is known to encompass a wide range of potential antagonists such as the genus *Bacillus* [68]. In general, sapwood showed lower bacterial diversity and richness compared to other organs of olive [69], which was confirmed in our study on olive sapwood by repeated colonization with 10 genera. Some genera such as *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Curtobacterium*, *Methylobacterium*, *Sphingomonas* and *Pantoea* have been reported to colonize the sapwood of olive trees and other hosts [58, 60, 70-72], while genera such as *Microbacterium*, *Frigoribacterium* and *Sphingobium* have not been reported as sapwood endophytes in olives. Although there were limited differences among the genera colonizing the sapwood of olives, the relative frequency indicated a distinct quantitative variation among the common genera in the sapwood of 'Leccino', 'O. salentina' and 'O. rossa'. We stated a high frequency of *Curtobacterium* and *Bacillus* and an exclusive isolation of *Sphingomonas* from the sapwood of 'Leccino'. It is worth noting that *Curtobacterium* was similarly prevalent in the scape plants, which were associated with Citrus Variegated Chlorosis disease (CVC) [73, 74]. Consequently, the prevalence of *Curtobacterium* in 'Leccino' is extremely important to investigate as it is a candidate for *Xf* antagonism in olive trees and has been validated to inhibit *Xf* growth and reduce CVC symptoms on citrus trees In contrast, lower levels of *Methylobacterium* and *Pseudomonas* genera were found in the sapwood of 'Leccino'. However, the prevalence of *Pseudomonas* in 'O. salentina' might be related to its susceptibility to olive knot disease [75]. Finally, the genus *Methylobacterium* was found to encode a positive association with the intensity of CVC symptoms caused by the siderophore symbiosis of *Xf* growth [76]. Therefore, we can recommend to investigate the synergistic effect of *Methylobacterium* on *Xf* (ST53) growth in olive trees.

Primarily, our study suggests that olive twigs have low abundance and diversity of fungal endophytes compared to the previously studied endophytic community in olives root and leaves [77, 78]. Variation factors such as location and seasonality represented a valid hypothesis that showed differential counts for fungal endophyte coloniasation and isolation rates among the studied sites and sampling seasons. Overall, the increase in isolation and occurrence rates in the fall season and between sites could be related to high humidity and

variability in soil characters, which was in accordance with the spatial and temporal variation of olive endophytes observed in Portugal [19, 79].

In agreement with previous data [80, 81], 87.5% of the retrieved isolates were assigned to the phylum Ascomycota. At the order level, fungal endophytes in olive sap were predominantly defined as belonging to *Pleosporales*, *Eurotiales* and *Phaeomoniellales*, which is relatively common in the sapwood of various plants [82-86]. In relation to the relative density of endophytic fungi, only a few genera were found to colonize the sapwood of 'Leccino' to a greater extent compared to other olive cultivars. Among these, *Cladosporium* spp, *Paraconiothyrium brasiliens* and *Pithomyces chartarum* are particularly interesting, being reviewed to possess biocontrol activity against pests and pathogens. During the last decade, *Cladosporium* species have been considered as biological control agents with considerable potential. Torres [87] reported some *Cladosporium* strains as successful candidates for the treatment of white rust disease on chrysanthemum plant. Severe diseases such as apple scab were effectively controlled by the integrated use of *Cladosporium cladosporioides* H39 to control *Venturia inaequalis* [88]. The successful use of such isolates has also been associated with the ability to produce volatiles, which encode highly regarded plant growth promoting properties [89]. Most recently, *Cladosporium* has been considered as an active entomopathogenic genus, with these isolates showing promising control against pests such as moths, aphids and whitefly [90, 91].

Interestingly, this study represents the first report of *P. brasiliens* as an endophytic component of the olive sap population. However, it has been considered as a new biocontrol agent against various phytopathogens due to its production of antifungal metabolites [92, 93]. Similarly, *P. chartarum* has been isolated exclusively from the sapwood of 'leccino' and its occurrence represents an attractive finding for its antimicrobial and enzymatic activity [94]. Our study has most probably drawn attention to the occurrence of the genus *Paraphaeosphaeria* in olive sapwood, indicating their use as antifungal and antibacterial agents widely used worldwide to manage pathogenic vegetable diseases [95, 96].

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CHAPTER III

In vitro evaluation of antagonistic activity of olives endophytes against *Xylella fastidiosa*; development of SYBR Green real time primers for potential antagonists

Summary

Various endophytes possess plant health importance as growth promoters or biocontrol agents (BCAs). In addition to these beneficial properties, endophytes are considered allies of their host plant to prevent and/or control economically important pathogens. The endophyte collections consisted of 124 bacterial and 63 fungal isolates obtained from the sapwood of different Apulian olive cultivars and tested for their *in vitro* antagonistic activity against *X. fastidiosa* subsp. *pauca*. ST53. At the bacterial level, three isolates belonging to *Bacillus subtilis*, *Bacillus pumilus* and *Pantoea agglomerans* showed a significant potency of *Xf* growth inhibition by dual culture and disc diffusion methods. A similar effect was found when fungal isolates of *Paraconiothyrium brasiliense* inhibited *Xf* growth. Our results suggested that direct antagonists plus potential indirect antagonists or symbionts should undergo through several assays of colonization efficiency in olives. Thus, we successfully designed SYBR Green real-time PCR for *B. subtilis*, *P. agglomerans*, *P. brasiliense*, *Methylobacterium mesophilicum*, and *Curtobacterium flaccumfaciens*. We believe that the designed primers with high specificity and sensitivity for the targeted species may assist in tracking the establishment of the inoculum and the effects on *Xf* in olives.

Keywords; ST53, endophytes, *in vitro* antagonists, colonisation, real-time PCR.

3.1 Introduction

Microbial biological control is the utilization of living microorganisms, usually bacteria or fungi, to suppress plant diseases via different modes of action [1]. As biocontrol agents, their functional bioactivities against diseases are defined as indirect or direct antagonism. Indirect antagonists can control diseases by inducing or enhancing plant resistance to pathogen infections or by competing for nutrients and space [2]. n the other hand, some biocontrol agents may possess direct antagonistic interaction against pathogens through hyperparasitism or antibiosis, which refers to direct invasion of pathogen mycelium or cell structure, or by producing antimicrobial secondary metabolites with active inhibitory effects on pathogens [3, 4]. Direct antagonists may be rhizospheric or endophytic microorganisms, subdivided according to their inhibiting environment, which is known to determine the efficacy of selected bioagents for disease control. The relative stability of bioactivity of endophytes in the internal environment of the plant makes them more associated with the control of plant vascular pathogens In a pure culture (solid or liquid), direct antagonists can be identified by their apparent inhibition of pathogen growth in simplified bioassays such as dual culture and disc diffusion [5].

In the literature, many studies have described hyperparasitic fungal and bacterial endophytes as potential antagonists to economically important pathogens [6]. At the bacterial level, *Bdellovibrio bacteriovorus* is a known predatory endophytic bacterium that has the unusual property of using cytoplasm of other Gram-negative bacteria as nutrients. In initial research on biological control, specific strains of *B. bacteriovorus* have controlled a wide range of plant pathogenic bacteria such as *Agrobacterium tumefaciens*, *Xanthomonas vesicatoria*, *Erwinia carotovora* pv. *carotovora* and *Pseudomonas syringae* pv. *glycinea* [7]. At the fungal level, the most studied mycoparasites belong to the genus *Trichoderma* [8]. The observation of such antagonistic mycoparasitism has been noted against various plant fungal pathogens where they produce structures for attachment and infection and kill their hosts through cell wall degrading enzymes (CWDEs). For example, *Trichoderma atroviride* may have effective control through its mycoparasitic interaction with *Botrytis cinerea* and *Phytophthora capsici* [9].

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 [10]. 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 [11], while *Pseudomonas* genera have been more associated with antibiotic metabolites such as pyrrolnitrine and phenazine [12]. Fungal antagonists can also produce antimicrobial compounds. For *Trichoderma*, 6- PAP, gliovirin, gliotoxin, viridin and many other compounds with antimicrobial activity have been studied [13].

To obtain endophytic direct antagonists as microbial biocontrol agents for plant pathogens, fundamental procedures must be followed. First and foremost, a collection of endophytes with a range of different isolates is crucial to identify organisms as potential antagonists against the targeted pathogen [14, 15]. Then, various tests are performed to screen their antagonistic activity *in vitro*, in which the mode of action can be defined. At last, the selected antagonists are inoculated into the plant mainly together with the pathogen; the effect on the inoculants is generally monitored by the efficiency of plant colonization and reduction of disease severity. In our study, we targeted a collection of olive endophytes in various *in vitro* screening assays for their direct antagonistic activity against *Xf* ST53. This is important because few studies have been conducted to retrieve direct antagonists for a better biocontrol strategy of *Xf*; radicinin from *Cochliobolus* sp. has been found to be able to inhibit *Xf* from grapevine [16], while recently published studies have stated the absence of antagonists natively isolated from Apulian olives to inhibit *Xf* ST53 growth *in vitro* [17]. Moreover, we selected highly antagonistic isolates from this study to evaluate their efficiency in colonizing Apulian olive cultivars. Thus, we developed a molecular monitoring tool based on SYBR Green real-time PCR to quantify the inoculated antagonists and evaluate their establishment progress.

3.2 Materials and methods

3.2.1 Olive endophytes collection

The endophytes collection was obtained through an extensive two-year program of seasonal isolation and characterization of the Apulian olive sapwood endophytic community. Sampling was focused on three sites: Site I (Valenzano), Site II (Locorotondo) and Site III (Lecce), corresponding to native olive cultivars with different phytosanitary status and susceptibilities to *Xf* infections [resistant; cv. Leccino] [susceptible; cv. O. salentina and cv. O. rossa]. In this context, representative bacterial (10 genera and 18 species) and fungal (13 genera and 18 species) isolates were deposited respectively under specific NCBI accession numbers (MT556422 - MT556438 & MT973971, MT558577- MT558594). Bacterial colonies and fungal hyphae were stored in 20% glycerol on the Key Laboratory of IAMB microbiological collections at -20°C. The activity of bacterial and fungal isolates was routinely monitored by culturing on nutrient agar (NA, OXOID, Milan - IT) and potato dextrose agar (PDA, OXOID, Milan - IT) growth media.

3.2.2 *In vitro* antagonistic activity of endophytes against *Xylella fastidiosa*

Initially, *Xf* ST53 colonies were freshly isolated and molecularly characterized from infected olive plants 'O. salentina' located in the studied field of the infected zone (Lecce, 40.454748/18.153636). The bacterium was routinely cultured on buffered charcoal yeast extract (BYCE) medium and incubated at 28°C, which was later used for the following antagonist screening tests. 124 bacterial and 63 fungal isolates were subjected to rapid evaluation of potential antagonistic activity against *Xf* ST53 using the rapid stab dual culture method [18-20]. The growth medium of BYCE was prepared following the indicated instructions [21], and cultured by 150 µl of *Xf* suspension ($10^{\wedge 8}$ CFU/ml) and incubated under 28°C. After 24 h, bacterial colonies freshly grown in NA for 48 h were picked with sterile toothpicks and inoculated onto the BYCE plates previously cultured with *Xf* by stabbing vertically downwards through the agar (4 stabs/plate). To assess the antagonistic activity of the fungal isolates, a sterile toothpick was immersed in 500 µl of mycelial suspension (0.025 g/ ml potassium phosphate buffer, PBS), which was simultaneously stabbed into a BYCE plate previously cultured with *Xf* (4 stabs/plate). Untreated toothpicks were used as negative controls, and the zone of inhibition was measured after 8-12 days of incubation at 28°C.

Following the result of the rapid stab dual culture method, a secondary diffusion method based on paper discs was used to quantify the variation in zone of inhibition between antagonists [22-24]. Bacterial antagonists were cultured in 30 ml nutrient broth (OXOID, Milan - IT), and the growth rate and corresponding (CFU) value were constantly monitored by spectrophotometry (Lambda 365, IAMB). After 36 and 72 h of incubation, the broth was centrifuged at 12,000 rpm for 3 min, cell-free supernatants were obtained by filtration (0.2 µm) and pellets were suspended in (10^8 CFU ml-1) (1X PBS, pH 7.4). Sterilized 6-mm paper discs were treated with 100 µl of the cell-free supernatant, cell suspension and spore solution, which were simultaneously transferred to *Xf* - cultured BYCE as described above. PBS-treated discs served as negative control and the measurement of inhibition zones was taken during 8-12 days of incubation [25]. The taken measurements were subjected to the statistical analysis of parametric mean comparison (one-way ANOVA) performed using SPSS software package (version 12.0).

3.2.3 Development of SYBR Green Real-time PCR primers

SYBR Green-based primer pair for each endophyte was designed based on species-specific gene sequences deposited in NCBI (http://www.ncbi.nlm.nih.gov/) as shown in (Table 2). An extensive BLAST search was performed and gene sequences were aligned using the BioEdit sequence alignment editor. Within these sequences, unique regions specific to the organism were identified and primers were designed using Primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). All primers were synthesized by (Eurofins Genomics. Ebersberg, Germany). To evaluate the specificity of the primers, conventional PCR assays were performed using a panel of endophytes commonly associated with olives as PCR templates. Briefly, DNA was extracted from the intended and unintended bacterial and fungal colonies using the CTAB extraction method [26]. 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), also they were sequenced by Eurofins Genomics (https://www.eurofinsgenomics.eu/), blasted in the NCBI, and deposited under specific accession numbers.

The sensitivity, standard curve and melting curve of each primer set were evaluated in real-time PCR performed in a CFX96™ (IAMB, Bari) using CFX Manager™ version 1.0 software (Bio-Rad Laboratories) for experimental setup and data analysis. The qPCR protocols were performed with a 10-fold dilution of endophyte DNA [5: 5, $4:5*10^{-1}$, $3:5*10^{-2}$, $2:5*10^{-3}$, $1:5*10^{-4}$] ng μl^{-1} duplicated to generate the standard curve. Further dilutions [$5*10^{-5}$, $5*10^{-6}$, $5*10^{-7}$, and $5*10^{-8}$] ng μl^{-1} were used to determine the sensitivity of the primers. The amplification mixture (25 µl) consisted of 12.5 µl of SYBR® Select Master Mix (Thermofisher Scientific), 0.5 µM of each primer, and 1 µl of DNA, and no DNA samples serving as negative controls. Thermal cycling was performed according to the manufacturer's instructions, varying the annealing temperature depending on the primer pair (Table 1). Melting analysis was performed under the following conditions: 1 min at 95 °C, from 55 °C to 95 °C (40 cycles with 0.5 °C increments per 5 s). Similarly, olives from which targeted endophytes were originally isolated were tested by qPCR, using 50 ng ml^{-1} of DNA material extracted from 0.5 g of stem segments according to the instructions of DNeasy Plant Mini Kit (QIAGEN).

Table 1. SYBR Green-based primer sets designed on species-specific genes of bacterial and fungal sequences deposited in the NCBI gene bank.

Endophyte	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
M. mesophilicum	mxa F	MSM1: AGATCGACGACACCGTCAAC	183 bp	57 °C	MN530982.1
					KF650708.1
		MSM2: TGCCCATGTAGAAGGCTTGG			FJ157953.1
P. agglomerans	16S rRNA	PAG1: CCTGGACAAAGACTGACGCT	171 bp	59 °C	CP046722.1
					MT803622.1
		PAG2: TTTAACCTTGCGGCCGTACT			MN943534.1
P. brasiliense	ITS	PBFW: TGAAGAACGCAGCGAAATGC	198 bp	60°C	MT626608.1
					MT558587.1
		PBRV: CGCTGCCAATGAATTTGGGG			MT230470.1

3.3 Results

3.3.1 *In vitro* activity of endophytes against *Xylella fastidiosa* [ST53]

Different endophytes exhibited different levels of antagonistic activity against *Xf* ST53 in rapid stab dual cultures methodology. Out of 124 different isolates belonging to 18 bacterial species, three bacterial species (*B. subtilis*, *B. pumilus*, and *P. agglomerans*) were found to possess *in vitro* inhibition halo ranging from 8 to 15 mm on *Xf*-cultured BYCE media. After 10 days of incubation, *B. subtilis* endophyte was the most effective in inhibiting the growth of *Xf* up to 15.2 ± 1.69 mm, whereas *B. pumilus* and *P. agglomerans* exhibited inhibition halo up to 8.7 ± 2.47 and 11.82 ± 1.42 mm, respectively (Table 2). Concerning the fungal *in vitro* bioactivity against *Xf*, two fungal isolates belonging to *P. brasiliense* [MT86 and MT87] displayed a very clear inhibition on BYCE plates. However, an evident difference was found between both isolates, where MT86 isolate measured an inhibition halo up to 19.28 ± 0.86 mm and 17.8 ± 0.75 for MT87 after 8 days of incubation (Table 2).

Table 2. Multiple isolates belonging to the bacterial and fungal endophytes collection used in rapid stab *in vitro* screening of antagonistic activity against *X. fastidiosa*. ST53

Bacterial isolates	Accession N°	Inhibition zone (mm)
Bacillus subilits	MT556422	15.2 ± 0.69
Bacillus pumilus	MT556423	8.7 ± 0.47
Bacillus thermoamylovorans	MT556424	NA
Curtobacterium flaccumfaciens	MT556425	NA
Curtobacterium pusillum	MT556426	NA
Frigoribacterium faeni	MT556427	NA
Methylobacterium adhaesivum	MT556428	NA
Methylobacterium radiotolerans	MT556429	NA
Methylobacterium mesophilicum	MT556430	NA
Okibacterium fritillariae	MT556431	NA
Pantoea agglomerans	MT973971	11.82 ± 0.42
Paenibacillus validus	MT556432	NA
Paenibacillus camelliae	MT556433	NA
Paenibacillus naphthalenovorans	MT556434	NA
Pseudomonas sp.	MT556435	NA

Pseudomonas syringae	MT556436	NA
Sphingobium olei	MT556437	NA
Sphingomonas sp.	MT556438	NA
Fungal isolates	**Accession N°**	**Inhibition zone (mm)**
Aspergillus versicolor	MT558577	NA
Aspergillus sydowii	MT558578	NA
Cladosporium perangustum	MT558579	NA
Cladosporium ramotenellum	MT558580	NA
Cytospora punicae	MT558581	NA
Fusarium lateritium	MT558582	NA
Libertasomyces platani	MT558583	NA
Mycocalicium victoriae	MT558584	NA
Neophaeomoniella eucalypti	MT558585	NA
Paraconiothyrium brasiliense	MT558586	19.28 ± 0.86
Paraconiothyrium brasiliense	MT558587	17.3 ± 0.52
Paraphaeosphaeria sp	MT558588	NA
Penicillium chrysogenum	MT558589	NA
Penicillium chrysogenum	MT558589	NA
Phoma sp	MT558593	NA
Pithomyces chartarum	MT558591	NA
Pseudophaeomoniella oleae	MT558592	NA
Stigmatodiscus oculatus	MT558594	NA

To validate the obtained results, a comparison of the activity of the bacterial antagonists was performed by using cell-free supernatant and pellet suspension of each isolate at similar incubation times. In general, all bacterial isolates chosen showed inhibition of *Xf* growth by cell-free supernatant (CFS) or pellet suspension (PS). Comparison of inhibition zones at the species level showed significant agreement with the stab method, with the highest inhibition of *Xf* culture associated with *B. subtilis* discs, followed by *P. agglomerans* and *B. pumilus* (P <0.01) (Fig. 1). Regarding the inhibition measurements of CFS & PS discs, high inhibition activity of CFS discs was observed, especially for those collected after 72 hours of incubation (P <0.05). On the other hand, the highest activity of PS discs was associated with those collected after 36h of incubation (P <0.05). In detail, CFS /72h and PS /36h discs of *B. subtilis* and *P. agglomerans* exclusively showed significant differences in inhibition measurements (P <0.01), while no significant differences were found in *B. pumilus* discs (P >0.05) (Fig 1). At the fungal level, discs with *P. brasiliense* spore solution successfully exhibited *Xf* growth inhibition as found in the rapid stab dual culture methodology. However, comparison of *P. brasiliense* [MT86 & MT87] isolates showed slight significance in promoting *Xf* growth inhibition, with the halo produced by the MT86 isolate being larger than that of MT87 (P = 0.035) (Fig 2).

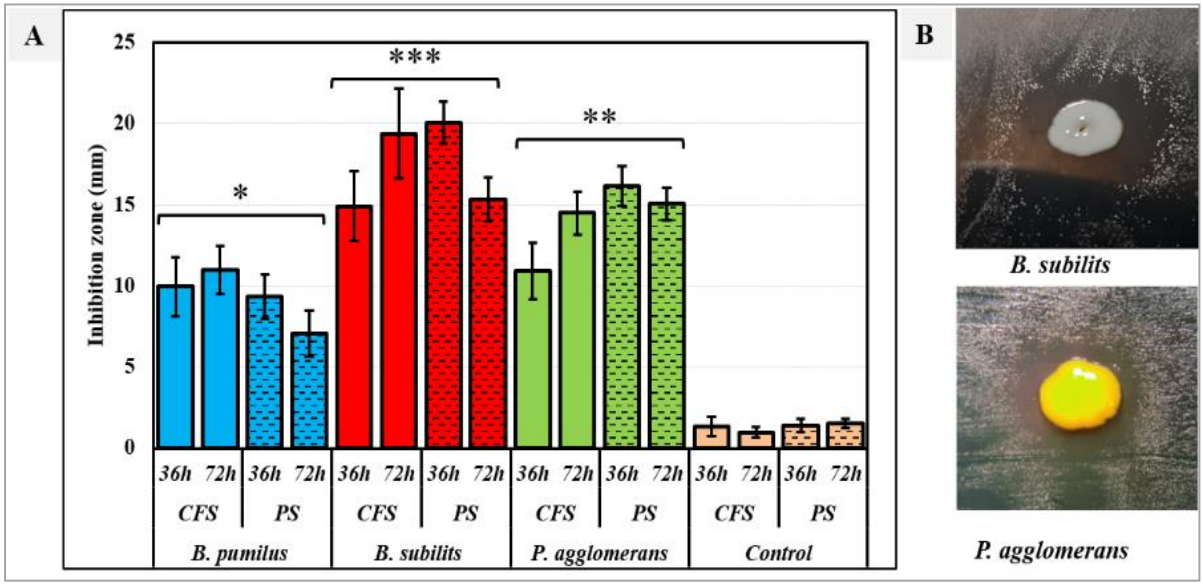

Figure 1. (A) Histogram illustration of *Xf* growth Inhibition exhibited by different bacterial endophytes; *B. Pumilus*, *B. subtilis*, and *P.agglomerans*. The measurement in (mm) represents the inhibition zone of cell-free supernatant (CFS) and pellet suspension (SS) harvested at 36h and 72h of incubation. Bars represent the standard deviation of measurement replicates. P-value are shown as follow (***; P ≤ 0.001, **; P ≤ 0.01, *; P ≤ 0.05). (B) *Xf* growth inhibition exhibited by *B. subtilis*, and *P.agglomerans.*

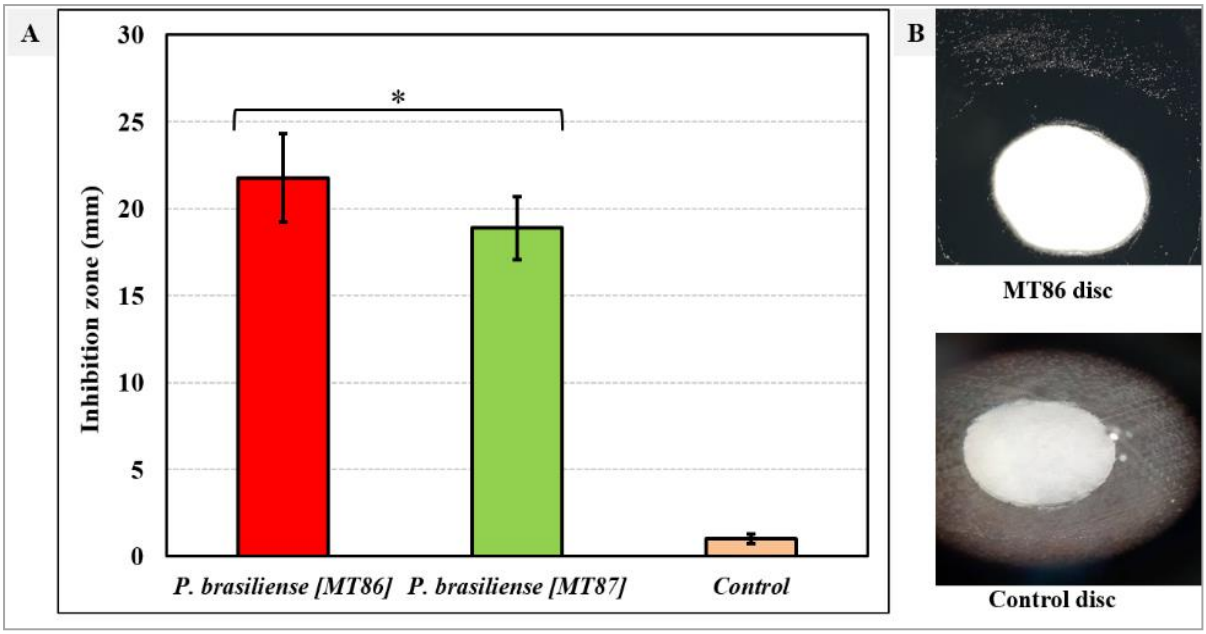

Figure 2. (A) Histogram illustration of *P. brasiliense* inhibition of *Xf* growth. Bars represent the standard deviation of measurement replicates and the P-value is shown as (*; $P \leq 0.05$). (B) The inhibition zone exhibited by the mycelium disc of *P. brasiliense* [MT86], negative control disc.

3.3.2 Development of SYBR Green Real-time PCR primers

The specificity of primers in Conventional PCR

Figure 3 shows that the designed primer set specific for each target endophyte was successfully amplified in conventional PCR assays. When the agarose gel was analyzed, the obtained amplicons of the primer sets corresponded exactly to the indicated amplification size (HAN2/HAN5: 175 bp, CSF1/CSF2: 181 bp, MSM1/MSM2: 183 bp, PAG1/PAG2: 171 and PBFW/PBRF: 198 bp), and no amplification was observed in the negative control. Extracted DNA from the control panel of bacterial and fungal endophytes of olive listed in Table 2 were found to be negative once they served as non-target templates. In addition, no visibility of nonspecific bands or primer dimers was detected. The revealed amplicon of each primer set was subjected to sequencing for identity confirmation; the resulting sequence of each primer set confirmed specificity for each isolate with BLASTN nucleotide identity (≥ 99%). The sequences were deposited in NCBI under specific accession numbers: *B. subtilis* [MW118674], *C. flaccumfaciens* [MW118675], *M. mesophilicum* [MW118676], *P. agglomerans* [MW130955], and *P. brasiliense* [MW090798].

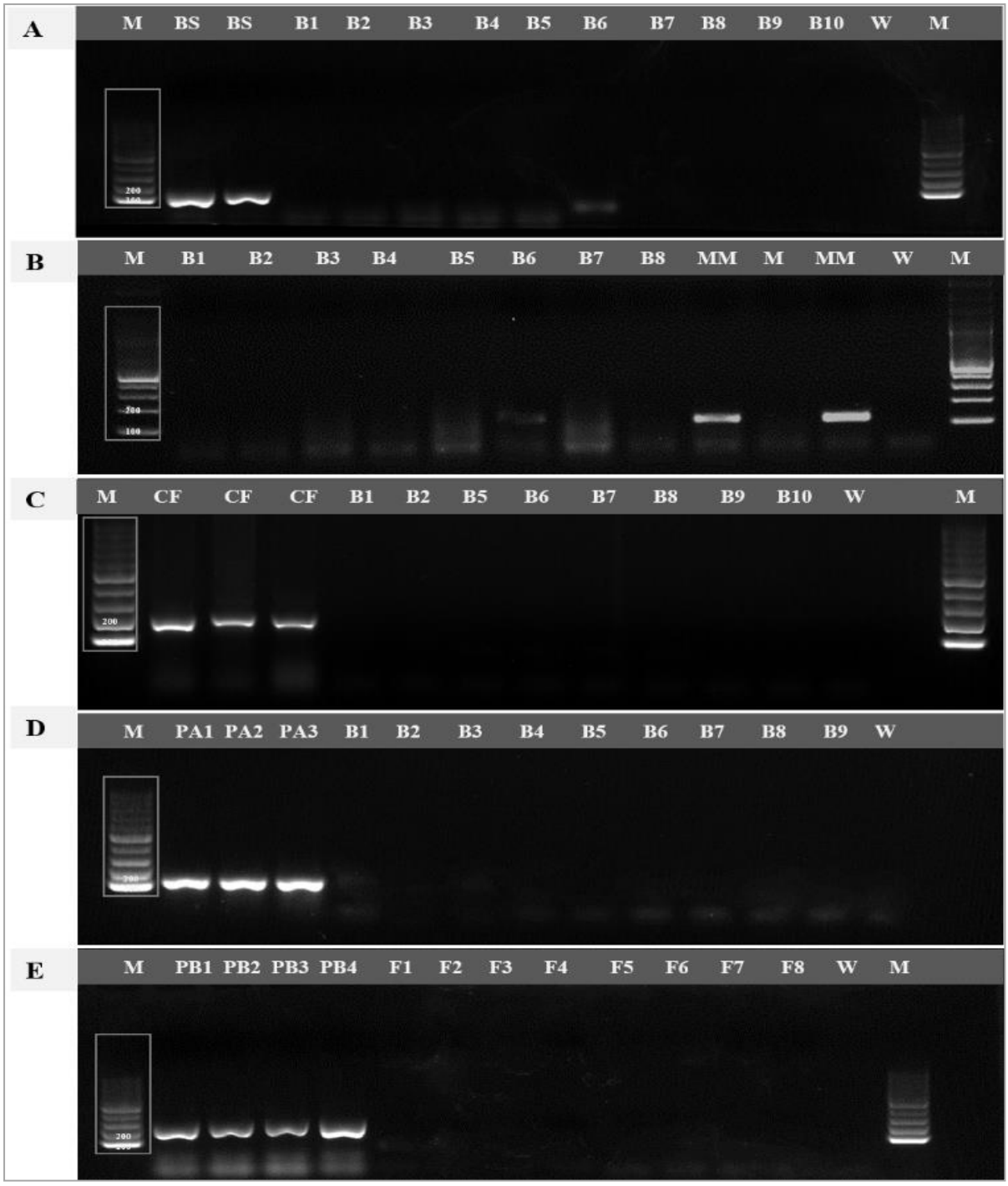

Figure 3. Agarose gel electrophoresis analysis of the PCR products obtained from cell cultures DNA. (A) PCR product *B. subtilis* with an amplicon size of 175 bp. (B) PCR product of *M. mesophilicum* (MM) with an amplicon size of 183 bp. (C) PCR product of *C. flaccumfaciens* (CF) with an amplicon size of 181 bp. (D) PCR product of DNA *P. agglomerans* (PA)with amplicon size of 171. (E) PCR product of *P. brasiliense* (PB) with amplicon size of 198 bp. B1-B10 are olives non targeted bacterial panel used as the negative control. F1-F8 are olives non targeted fungal panel used as the negative control. 100 bp marker is the lane (M), and lane (W) refers to no DNA template control.

Real-time PCR of designed primers and calibration curves

Real-time PCR reactions were performed using a 10-fold dilution of target genomic DNA based on a specific gene, yielding corresponding Ct values within the respected amplicon size of each primer set and negative control de-voiding of template DNA. Thus, 10-fold dilution in the range of $5*10^{-1}$ to $5*10^{-4}$ ng μL^{-1} successfully generated valid standard and melting curves specific to each primer set. Detection and quantification of *B. subtilis* DNA (cell cultures/olive tissue) were successful when the HAN2/HAN5 primer set was used (Fig 4A&B). The specificity of the reaction was also confirmed by melting temperature analysis, which was constant for the amplicon obtained (79.1 ± 0.3 °C) (Fig 4C). By using a 10-fold dilution of B. subtilis DNA based on the gyr A target gene, the Ct values ranged from 11.51 ± 0.52 to 23.45 ± 0.39, where the Ct values and the dilutions of the target DNA were linearly correlated with high coefficient of determination (Y= -3.216X + 27.11, R^2= 0.996, E= 105%) (Fig 4D). The sensitivity of the primer was determined by the successful quantification reactions, which ranged from $5*10^{-1}$ to $5*10^{-6}$ ng μL^{-1}.

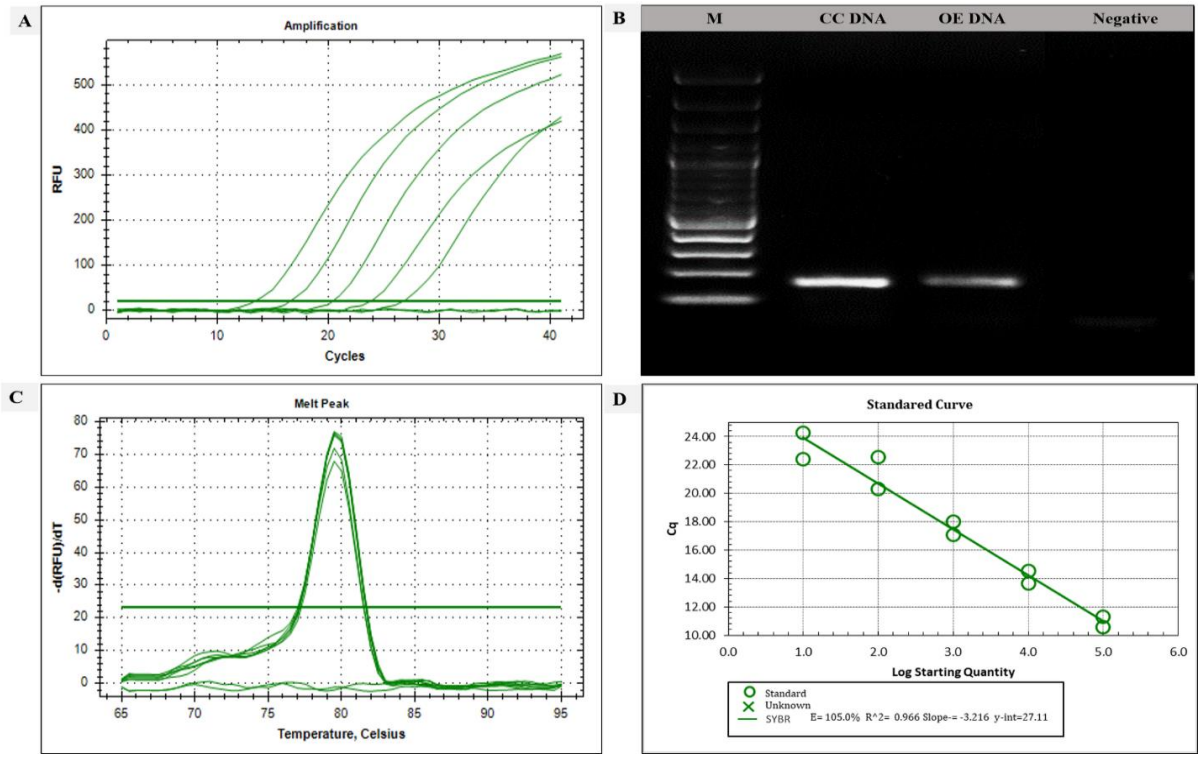

Figure 4. (A) *B. subtilis* typical amplification plot. (B) Agarose gel analysis of DNA from cell culture (CC) and olives tissue (OE). (C) Representative melting curves using SYBR Green for the detection of *B. subtilis*. (D) Standard curve of real-time PCR optimized on 10-fold serially diluted DNA from a pure culture *B. subtilis*.

Validation of the CFS1/CFS2 primer set showed successful detection and quantification of *C. flaccumfaciens* extracted DNA from pure cell cultures and olive tissues (Fig 5A&B). In addition to the generation of a single clear band of the referred size of 181 bp, a constant melting temperature (82 ± 0.21°C) was found (Fig 5C). The amplification reaction of a 10-fold dilution of *C. flaccumfaciens* DNA based on the Rec A target gene generated Ct values ranging from 17.41 ± 0.12 to 31.75 ± 0.33. Thereby, the Ct values and target DNA dilutions were linearly correlated with high coefficient of determination (Y= -3.135X + 33.918, R^2= 0.945, E= 98%) (Fig 5D). The sensitivity of the primer was determined by the successful quantification reactions, which ranged from $5*10^{-1}$ to $5*10^{-5}$ ng μL^{-1}.

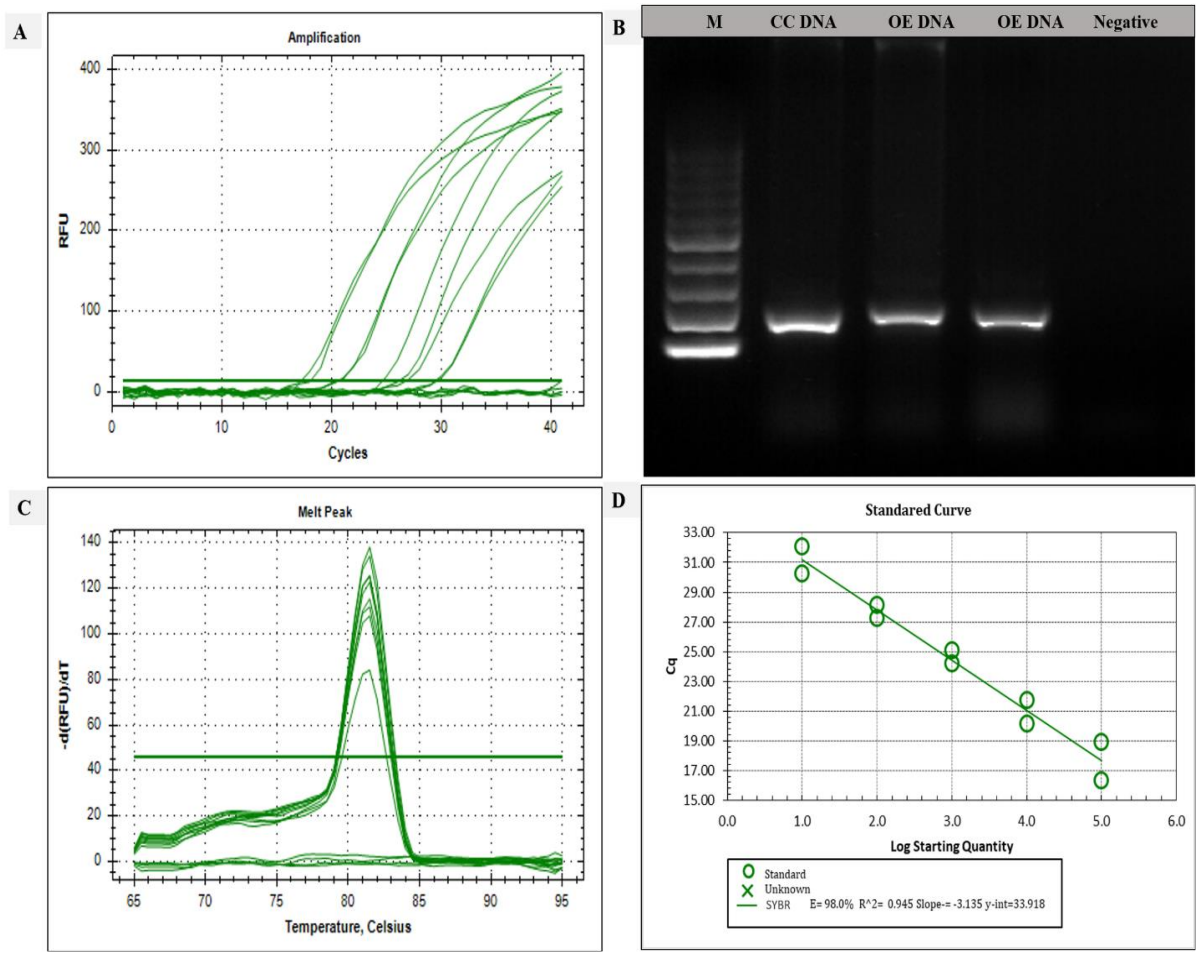

Figure 5. (A) *C. flaccumfaciens* typical amplification plot. (B) Agarose gel analysis of DNA from cell culture (CC) and olives tissue (OE). (C) Representative melting curves using SYBR Green for the detection of *C. flaccumfaciens*. (D) Standard curve of real-time PCR optimized on 10-fold serially diluted DNA from a pure culture *C. flaccumfaciens*.

Similarly, the MSM1/MSM2 primer set showed successful detection and quantification of *M. mesophilicum* extracted DNA from pure cell cultures and olive tissues (Fig 6A&B). The specificity of the primers was demonstrated by the generation of a single clear band with a size of 183 bp and a melting temperature of (78 ± 0.15°C) (Fig 6C). Amplification reaction of a 10-fold dilution of *M. mesophilicum* DNA based on the Mxa F target gene generated Ct values ranging from 21.41 ± 0.12 to 34.08 ± 0.25. Thereby, the Ct values and target DNA dilutions were linearly correlated with high coefficient of determination (Y= -3.322X + 37.427, R2= 0.962, E= 100%) (Fig. 6D). The sensitivity of the primer was determined by the successful quantification reactions, which ranged from $5*10^{-1}$ to $5*10^{-4}$ ng μL^{-1}.

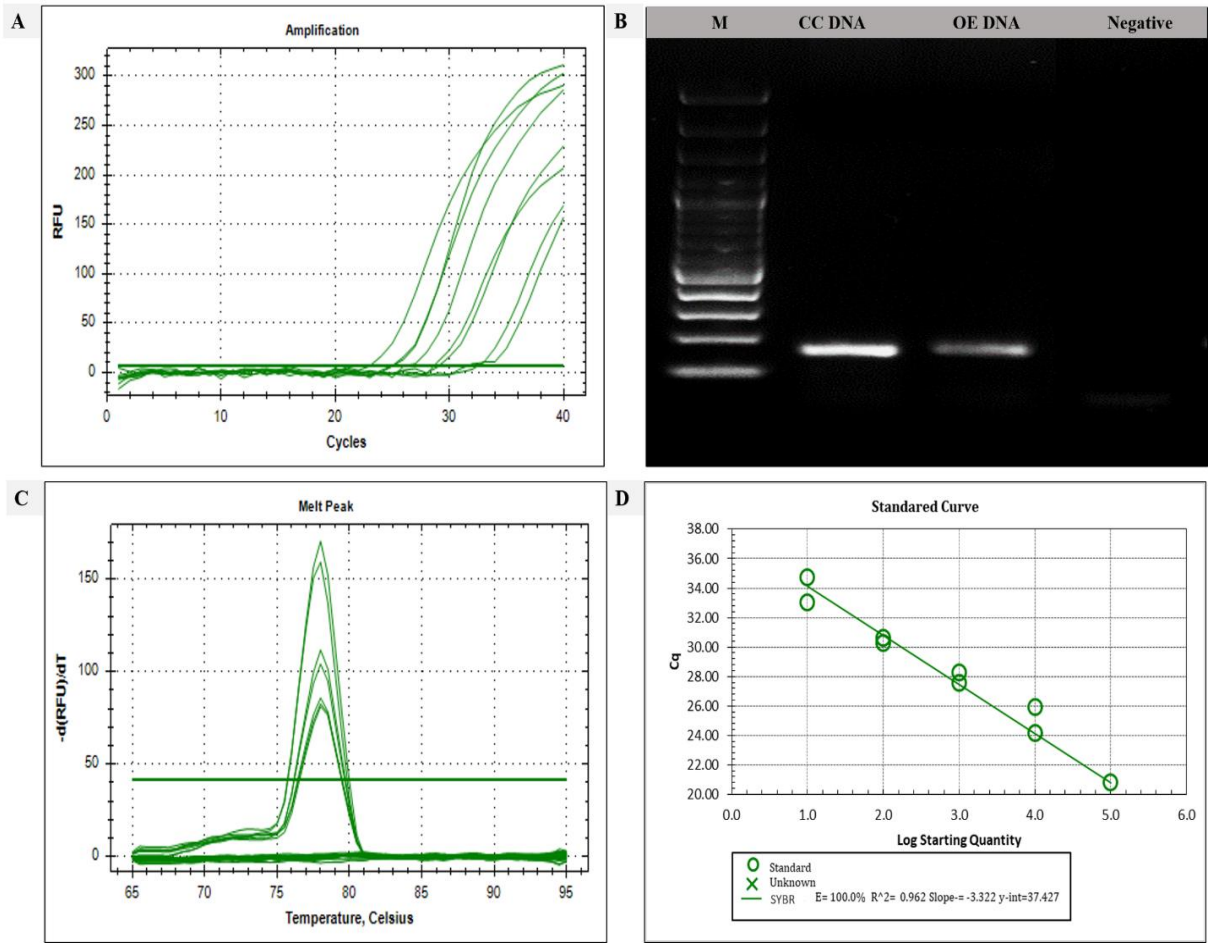

Figure 6. (A) *M. mesophilicum* typical amplification plot. (B) Agarose gel analysis of DNA from cell culture (CC) and olives tissue (OE). (C) Representative melting curves using SYBR Green for the detection of *M. mesophilicum*. (D) Standard curve of real-time PCR optimized on 10-fold serially diluted DNA from a pure culture *M. mesophilicum.*

As part of the validation of real-time PCR for the detection and quantification of *P. agglomerans*, primers PAG1/PAG2 proved effective when tested on extracted DNA from cell culture and olive tissue (Fig 7A&B). Moreover, the amplification reaction of the referred primers set specifically targeted the 16S rRNA to obtain a single band of size 171 bp at constant melting temperature (78.4 ± 0.17 °C) (Fig 7C). The amplification reaction of a 10-fold dilution of *P. agglomerans* DNA generated Ct values ranging from 18.36 ± 0.41 to 34.17 ± 0.15. Thereby, the Ct values and the dilutions of the target DNA were linearly correlated with high coefficient of determination (Y= -3.458X + 35.728, R^2= 0.948, E= 95%) (Fig 7D). The sensitivity of the primer was determined by the successful quantification reactions, which ranged from $5*10^{-1}$ to $5*10^{-4}$ ng $\mu L^{-1.}$

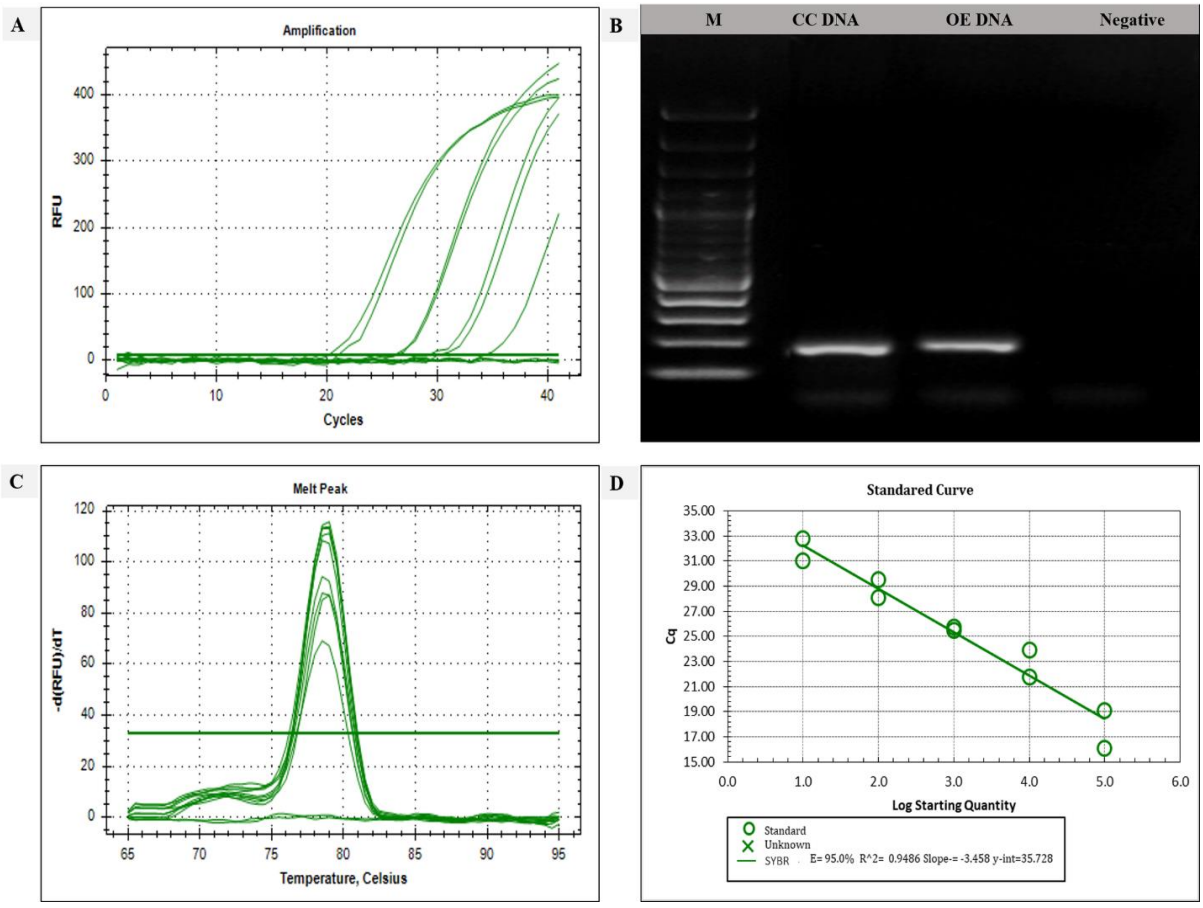

Figure 7. (A) *P. agglomerans* typical amplification plot. (B) Agarose gel analysis of DNA from cell culture (CC) and olives tissue (OE). (C) Representative melting curves using SYBR Green for the detection of *P. agglomerans*. (D) Standard curve of real-time PCR optimized on 10-fold serially diluted DNA from a pure culture *P. agglomerans*.

Finally, the primer set PBFW/PBRF designed for the ITS region of *P. brasiliense* proved to be effective for the detection and quantification of *P. brasiliense* DNA extracted from (mycelial culture/ olive tissue). This was established by the amplification plot obtained and clear band (198 bp) generated from positive samples (Fig 8A&B). The specificity of the reaction was also confirmed by melting temperature analysis, which was constant for the amplicon obtained (86.1 ± 0.2 °C) (Fig. 8C). By using a 10-fold dilution of *P. brasiliense* DNA based on the target gene ITS, the Ct values ranged from 24.11 ± 0.21 to 38.5 ± 0.35. Thereby, the Ct values and the dilutions of the target DNA were linearly correlated with high coefficient of determination (Y= -3.472X + 42.52, R^2= 0.9702, E= 94%) (Fig 8D). The sensitivity of the primer was determined by the successful quantification reactions, which ranged from $5*10^{-1}$ to $5*10^{-4}$ ng μL^{-1}.

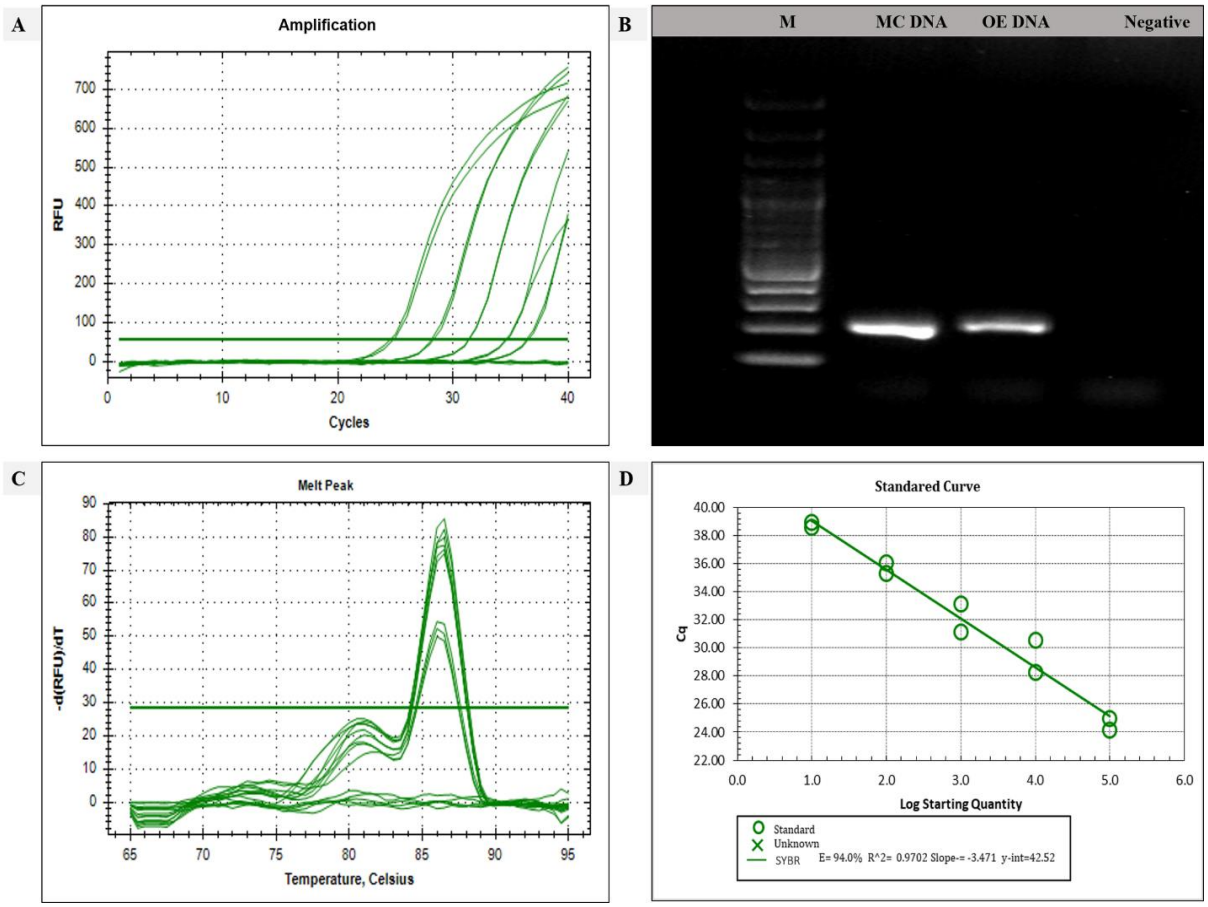

Figure 8. (A) *P. brasiliense* typical amplification plot. (B) Agarose gel analysis of DNA from cell culture (CC) and olives tissue (OE). (C) Representative melting curves using SYBR Green for the detection of *P. brasiliense.* (D) Standard curve of real-time PCR optimized on 10-fold serially diluted DNA from a pure culture *P. brasiliense.*

3.4 Discussion

In recent years, the endophytic communities of Apulian olives have been targeted in several studies within a journey of microbiome characterization [27], endophytes isolation upon seasons or varieties [28], and, most recently, *in vitro* antagonistic activity evaluation against *Xf* ST53 [17]. Knowing that none of the obtained isolates from Apulian olives was reported to inhibit *Xf* growth in vitro, this work was carried out as a continuation of an extensive characterization and variation of culturable bacterial and fungal endophytes from different olive cultivars with respect to their susceptibility to *Xf* [29]. Therefore, we hypothesized that our endophytic collection might contain isolates capable of inhibiting *Xf* growth as a first step towards selecting potential biocontrol agents.

The antagonistic activity of the endophytic bacterial and fungal isolates was investigated *in vitro* using the agar dual culture method and disc diffusion method. The results showed that three bacterial isolates (*B. subtilis*, *B. pumilus* and *P. agglomerans*) and one fungal isolate (*P. brasiliense*) from the sapwood of different olive varieties inhibited the growth of *Xf.* The bacterial isolates belonged to *Bacillus* and *Pantoea* species, which are commonly known biocontrol agents for various plant pathogens and have been previously reported to colonize Italian olive varieties with great antagonistic activities [30, 31]. On the other hand. *P. brasiliense* has never been reported to colonize olive plants. Comparison of the antagonistic activity of our bacterial isolates showed a major inhibition effect by cell-free filtrate and cell suspension, confirming that *B. subtilis*, *B. pumilus* and *P. agglomerans* produce active antimicrobial substances [32-35]. In fact, our *Bacillus* isolates showed much noted similarity in *Xf* growth inhibition activity to *Bacillus* strains from wheat grains used by Zicca [15]. Although there is no previous work confirming the antagonistic activity of *P. agglomerans* and *P. brasiliense* against *Xf,* these candidates remain interesting for further investigation and testing, and their colonization efficiency is still unclear. In particular, *P. brasiliense* is considered to be of great importance for the active production of broad-spectrum antimicrobial compounds such as danthron [36].

In the preliminary experimental procedures for validation of endophytic biocontrol agents, there is a necessity to monitor the establishment of the biocontrol agent in the host inner environment [37]. Therefore, a rapid diagnostic test to confirm the presence of targeted antagonists in the olive plant is an essential tracking methodology. This is important as if the antagonist of interest fails as a biological control agent in in vivo experiments, it can be

determined if the inoculum is still present but ineffective or has failed to establish. In the current study, we developed SYBR Green real-time PCR primers specific for the detection and quantification of the antagonistic candidates discovered in this study (*B. subtilis*, *P. agglomerans*, and *P. brasiliense*), including other important endophytes (*M. mesophilicum* and *C. flaccumfaciens*) for their significant interactions with *Xf* on citrus plants [38].

In our studies, evaluation of SYBR-green real-time PCR primers showed high specificity for the targeted endophyte. Products specific for the genes of the targeted endophyte were obtained and showed high concordance with the indicated band size and matching sequences. Moreover, we may say that the applied validation method was able to correctly identify the targeted endophytes both in cultures and in olive tissues with the presence of related and unrelated species as negative controls. This is in agreement with the same procedures adopted by several studies [39-41]. The validated primer set targeting the gyr A gene of *B. subtilis* showed very high efficiency, specificity and sensitivity in terms of detection and quantification, also proving the separability of gyr A for *B. subtilis* from related *Bacillus*, as indicated in previous works [42, 43]. Gonçalves [44] pointed out the housekeeping gene rec A of *C. flaccumfaciens* as the preferred reference for molecular makers, which was confirmed by the validation of our primers with very good efficiency, specificity and sensitivity. Although the designed primers targeting the mxa F gene of *M. mesophilicum* proved their specificity and efficiency of detection and quantification, the mxa F gene remains useful with great concerns about its expression variability, as described by Dourado [45], Lau [46]. *P. agglomerans* primers targeted the 16S rRNA and presented satisfying efficiency and detection sensitivity, nevertheless, there were no other related species to confirm the specificity. At last, designed primers of *P. brasiliense* were found adaptable to SYBR Green detection and quantification with high efficiency, specificity, and sensitivity.

In conclusion, our endophytic collection presented several endophytic isolates that showed antagonistic activity against *Xf*. However, several endophytes obtained did not show *in vitro* inhibition of *Xf*, which does not imply that their beneficial role is excluded. Endophytes are known to promote plant health by inducing hormone production and/or triggering Induced Systemic Resistance (ISR) [47, 48]. To validate the obtained antagonists, a study should be carried to determine their colonization efficiency in Apulian olives. Thus, we developed a rapid detection and quantification tool to monitor the presence of targeted antagonists and simultaneously evaluate their significance in coping with *Xf* pathogenicity in the olive plant.

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CHAPTER IV

Colonization efficiency of promising endophytes in Apulian olive cultivars and their modulation of defence related genes

Summary

Endophytes have been explored and found to be an effective approach to control plant vascular diseases, especially in the presence of suitable antagonists. However, this depends on their establishment efficiency within the inner sphere of plant and their significance in inducing plant health. The present study aimed to assess the potential effects of promising endophytes on various genes associated with systemic defence mechanisms, to be applied against *Xylella fastidiosa* in Apulian olive cultivars. Therefore, the establishment of endophytes inoculums was monitored by Real-time PCR and plat countering. In addition, we evaluated the regulation of defence related genes in the aerial tissues of olive upon endophytes inoculation. Primarily, real-time PCR and plate-counting methods revealed the successful establishment of endophytic inoculums in cultivars 'Leccino' and 'Cima di Mola'. Among the bacterial isolates, *Bacillus sublitis* and *Curtobacterium flaccumfaciens* have showed high stability and persistence within the inner tissues of both olive cultivars. Likewise, the fungal isolate of *Paraconiothyrium brasiliense* was constantly recovered with a high colonization rate in the stem segment of both cultivars. The relative expression of defence related genes was found limited to olives inoculated with *Bacillus sublitis* and *Curtobacterium flaccumfaciens*, while *Pantoea agglomeransa* and *Methylobacterium mesophilicum* did not impose significant modulation of the investigated genes. This proves the potency and suitability of direct antagonist as *Bacillus sublitis* to be considered as potential control strategy of *Xylella fastidiosa* ST53 in Apulian olives.

Keywords; Endophytes, antagonists, olives, defence genes, *Xylella fastidiosa* ST53,

4.1 Introduction

Antagonistic endophytic microbes are a promising group of microorganisms that can provide frontline resistance and growth to the plant by different modes of action [1]. This property makes endophytes an attractive tool to be harnessed in modern sustainable agricultural practices [2, 3]. How endophytes prosper in disease suppression depends on the endophyte mode of action (direct and indirect antagonism). Parasitism, antibiosis, and competition are known features of direct antagonistic endophytes, while indirect antagonistic endophytes are associated with mediated plant resistance [4]. The process of plant resistance induction by endophytes is complicated, in which still there is more to explore. In most cases, endophytes are capable of triggering an Induced Systemic Resistance (ISR) response [5]. The characteristic responses of systemic defense mechanisms activated in plants colonized by endophytes were described early [6] and include the induction activities of some enzymes related to phenylalanine ammonia lyase (PAL), Malate dehydrogenase (MDH), peroxidase (PO), polyphenol oxidase (PPO), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APx), guaiacol peroxidase (GPx) [7, 8]. Similarly, several defence-related transcription factor families such as WRKY, MYB, NAC and MYC have been upregulated in plants colonized by endophytes [9]. Although endophytes trigger ISR, many studies suggest a crosstalk between ISR-induced jasmonic acid (JA) and ethylene (ET) signaling pathways and systemic acquired resistance (SAR), which increases endogenously synthesized salicylic acid (SA) and coupled with coordinated expression of PR genes [10].

The use of endophytic antagonists is becoming an important approach for the control of vascular pathogens due to their ability to thrive within the plant and prime the plant resistance [11]. There are several examples of successful application of endophytes to control vascular diseases; *Pseudomonas* and *Bacillus* spp. have been described to induce ISR and consecutive enhanced pathogen defence in plants associated with wilt diseases. This has been noted in olive plants as *Pseudomonas fluorescens* PICF7 has been demonstrated to control *Verticillium* wilt by inducing systematic resistance in olives [12-14]. In mulberry trees, the use of *Bacillus subtilis* has managed bacterial wilt disease caused by *Ralstonia solanacearum* [15]. In this context, *Xylella 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 endophytic antagonists as biocontrol agents. Affected citrus with citrus variegated chlorosis (CVC) was studied

thoroughly for the diversity of endophytes and their significant impact on controlling the disease; repeatedly isolated *Curtobacterium flaccumfaciens* from asymptomatic plants has shown antagonistic activity with symptoms reduction of *Catharanthus roseus* infected by *Xf* [16, 17]. In the case of Pierce's disease, similar results were obtained by using *Paraburkholderia phytofirmans*, where inoculated grapes exhibited reduced incidence of *Xf* leaf scorch symptoms and induced plant defence response compared to non-treated plants [18]. Concerning Olive Quick Decline Syndrome (OQDS), the study of endophytes have received the attention of several researchers especially with the appearance of resistant plants (cv. Leccino), however, recently published research has stated the absence of antagonists natively isolated from Apulian olives to inhibit the growth of *Xf* ST53 *in vitro* [19]. Overall, our *in vitro* screening of endophytes inhibiting the growth of *Xf* ST53 has successfully defined antagonistic isolates. Thus, these isolates have received major attention to be studied at levels of establishment in the inner sphere of Apulian olives and their modulation of defence-related genes.

4.2 Material and Methods

4.2.1 Evaluation of endophytes colonization efficiency in olives

The experiment was designed to target different promising endophytes repeatedly isolated from Apulian olive cultivars. Among them were bacterial and fungal isolates defined as direct *Xf* ST53 antagonists (Chapter III): *Bacillus subtilis*, *Pantoea agglomerans* and *Paraconiothyrium brasiliense*. In addition, two other bacterial isolates natively obtained from Apulian olive cultivars (*Curtobacterium flaccumfaciens* & *Methylobacterium mesophilicum*) were also considered in this experiment due to their reported interactions (indirect antagonist and symbiont) with *Xf* on citrus plants [20-22]. Stem colonization by inoculated endophytes was assessed for two months by plat counting and molecular technique based on real-time PCR.

Inoculums preparation and plants inoculation

The experiment was performed on one-year-old olive plants belonging to cv. Leccino and cv. Cima di Mola (a well-known synonym of Ogliarola salentina [23]). 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, IAMB) and diluted to 10^8 CFU ml$^-$

[1] (1X PBS, pH 7.4). For *P. brasiliense*, a spore stock suspension was prepared in 0.1% Triton X-100 (BIORAD. IT), and the spore concentration was adjusted to $1.5*10^8$ spores ml^{-1}. Three replicates of each cultivar were mechanically inoculated following needle punctures methodology [24, 25]. In brief, five square-shaped slits (about 2 mm deep by 3 mm wide) were made aseptically in the stem base and upper parts of approximately 50 cm high plant. The slits were injected by 25 µl-droplets of bacterial or fungal suspension by Ultrafine insulin syringes (BD Veo™), and sealed with Parafilm. Negative controls were inoculated with 1X PBS or 0.1% Triton X-100. All plants were maintained under the controlled conditions mentioned above.

Endophytes 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 according to the instructions of DNeasy Plant Mini Kit (QIAGEN). The amplification mixture (25 µl) consisted of 12.5 µl of SYBR® Select Master Mix (Thermofisher Scientific), 0.5 µl (10 mM) of the previously validated primers (Table 1, Chapter III), 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, Chapter III). In addition, the developed real-time PCR assay was used to confirm the identity of morphologically similar colonies obtained by the plate counting method

Endophytes isolation and plate counting

The population of inoculated endophytes was determined in the twigs of both cultivars by excising 2-cm-long stem segments that homogeneously covered the experimental plants at 15, 30, 45, and 60 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 [26]. 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 [27]. On the other hand, the colonization efficiency of *P. brasiliense* was estimated by cultured stem segments (8 stem discs/3 replicates) on PDA, thus calculating the colonization rate within the biological replicates of both olive cultivars [28].

4.2.2 Reverse transcription qPCR analysis of defence-related genes

As the inoculated endophytes confirmed establishment in the experimental olive plants, RT-qPCR experiments were performed to evaluate the effect of endophyte inoculation on specific identified Expressed Sequence Tags (ESTs) previously used in different studies [29-33]. Validated primers with specificity of amplification at 55 °C were imported from the literature targeting several key transcripts responsible for induced resistance traits.

Total RNA extraction and quality control

To assess the variation in olive gene expression after inoculation, homogeneous aerial tissues (stem and leaves) were collected at different time points after treatment. Therefore, samples belonging to three biological replicates were taken at 4 and 8 days from endophyte-treated plants and controls. A total of 128 aboveground tissue samples were collected and immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total RNA was extracted separately from inoculated plants and control samples at each indicated time point to avoid contamination. Plant/ Fungi Total RNA Purification Kit (NORGEN, Canada) was used to extract total RNA from 100 mg of olive tissue according to the manufacturer's instructions, and genomic DNA was removed according to the supplemented RNase-Free DNase I kit. The RNA obtained was controlled for quantity and quality by both agarose gel electrophoresis and spectrophotometry using a NanoDrop™ One spectrophotometer (Thermo Scientific™, Italy).

RT-qPCR of genes expression in olives

Primers targeted specifically five genes with different functional roles in olive defence mechanisms were included, involving key biosynthetic and metabolic pathways of systematic acquired resistance (SAR) and Induced systematic resistance (ISR) (Table 1). All RT-qPCRs were performed in the CFX96™ (CIHEAM-Bari) with the CFX Manager™ version 1.0 software (Bio-Rad Laboratories) using SYBR® Green Quantitative RT-qPCR Kit (Sigma-Aldrich, Germany). The 20 µL PCR contained 12.5 µl of 2X SYBR Green Quantitative RT-PCR buffer, 0.4 µl of 10 mM of each primer, 0.2 µl of Reverse transcriptase MMLV, 2 uL of 12.5 ng RNA, and 4.5 µl of nuclease-free water. All reactions were performed in a one-step protocol; the cycling program was set to 30 min of cDNA synthesis at 50°C, denaturation for 3 min at 95°C, and 40 cycles of 5 s at 95°C, 30 s at 50°C, 30 s at 72°C. The melting curves of RT-qPCR products were validated to confirm the amplification of single PCR band. The reaction conditions were as follows: initial denaturation for 5 min at 95°C, cooling to 55°C and

melting from 55°C to 95°C at a transition rate of 0.5°C every 5 sec. Gene expression levels were normalized to the *O. europaea* β-actin gene, which was used as a housekeeping gene and amplified under the same conditions. Three biological replicates per treatment with four technical replicates were performed for each gene after 4 and 8 days of endophyte inoculation. Relative expression (RE) values were calculated according to the Pfaffl method [34], and statistically analyzed using one-way ANOVA (Dunnett's test) (GraphPad Prism version 8.00)

Table 1. Validated primers list imported from Schilirò [10] study, which targeted the olive genetic responses upon the inoculation of endophytic *Pseudomonas fluorescens* PICF7

Putative gene	**Function**	**Primers (5'-3')**	**Size**	**Linear equation**	**R2**	**Efficiency**
Phenylalanine ammonia-lyase	Phenylpropanoids biosynthesis	Fw:AGGATTTGCTTCGAGTGGTT	230 bp	y=−3.34x+28.115	0.99	99%
		RF: GGCAATTCTTGCACTCTCAA				
Malate dehydrogenase	Biosynthesis of plant hormones	Fw: AGAATTGGATTTGGGTGAGC	242 bp	y = 23.52x +22.2	0.98	92.3%
		Rv: TTAATGGGGTCCCAGATGTT				
Acetone cyanohydrinlyase	Salicylic acid-binding protein 2	Fw: GAAAGAGATGGAAGCGGAAA	246 bp	y = 23.40x+24.66	0.99	96.2%
		Rv: ACACAGGGAAATGCATCAAA				
WRKY 5	SA signal transduction	Fw: GCATGGTGCAAGAAGTAGGA	213 bp	y = 23.41x+30.13	0.99	96.6%
		Rv: CAGCAACAAACGCTACACCT				
GRAS1	Signal transduction defence response	Fw: CGGCGCTCTATATCTTGGAT	200 bp	y = 23.27x+32.51	1	102%
		Rv: ACGTCAAAACGATGGAGTCA				
Olea europaeabeta -actin (act1)	Citoskeletal integrity	Fw: GCTTGCTTATGTTGCTCTCGAC	308 bp	y = 23.39+29.99	0.99	97%
		Rv: TGATTTCCTTGCTCATACGGTC				

4.3 Results

4.3.1 Evaluation of endophytes colonization efficiency in olives

Endophytes establishment evaluation by real-time PCR

Figure 2 presents a summary of real-time PCR results that positively indicated successful establishment and colonization of both olive cultivars by inoculated endophytes; this was perceived by comparing Cq values between inoculated plants and controls. Certain increments in the endophyte population were detected at different times after inoculation. Before inoculation, *B. subtilis* LEC2 with a late amplification threshold was detectable in the experimental plants, but Cq values decreased, indicating high colonization of the inoculum after 30 and 45 days in 'Leccino' and 'Cima di Mola', respectively. Similarly, the inoculums of *C. flaccumfaciens* BF36AZ presented a high entity in the tissues of olives upon inoculation with relatively lower Cq values in ‘Leccino’ than in ‘Cima di Mola’. On the other hand, the isolates of *M. mesophilicum* and *P. agglomerans* showed high colonization entity exclusively at early stages (15 and 30 days) after inoculation, indicated by obtained low Cq values (< 25). At last, *P. brasiliense* MT86 was initially absent from experimental plants, however, the isolate was detected and quantified after inoculation within the inner sphere of the plant by low Cq values.

Endophytes establishment evaluation by plates counting

As the real-time PCR technique has been successfully used to monitor endophyte biomass in many studies [35-37], conventional techniques (isolation and plate counting) have also been used to assess population size, growth and movement of inoculum in the plant. Therefore, we measured the population size of each endophyte within the surface sterilized stem segments. After inoculation, the bacterial endophytes showed variation in stability and persistence within the inner tissues of the two olive cultivars. Both isolates of *B. subtilis* and *C. flaccumfaciens* were found to be stable in the inner tissues of both cultivars with a slight elevation in population size, consistently recovering over 10^4 CFU/ml from the stem segments during the inoculation period (Fig 1A&B). Meanwhile, the isolates of *M. mesophilicum* and *P. agglomerans* were recovered in relatively small population sizes ($<10^3$ CFU/ml) after 30 days of inoculation, indicating that these inoculums did not persist effectively in the experimental plants (Fig 2 C&D).

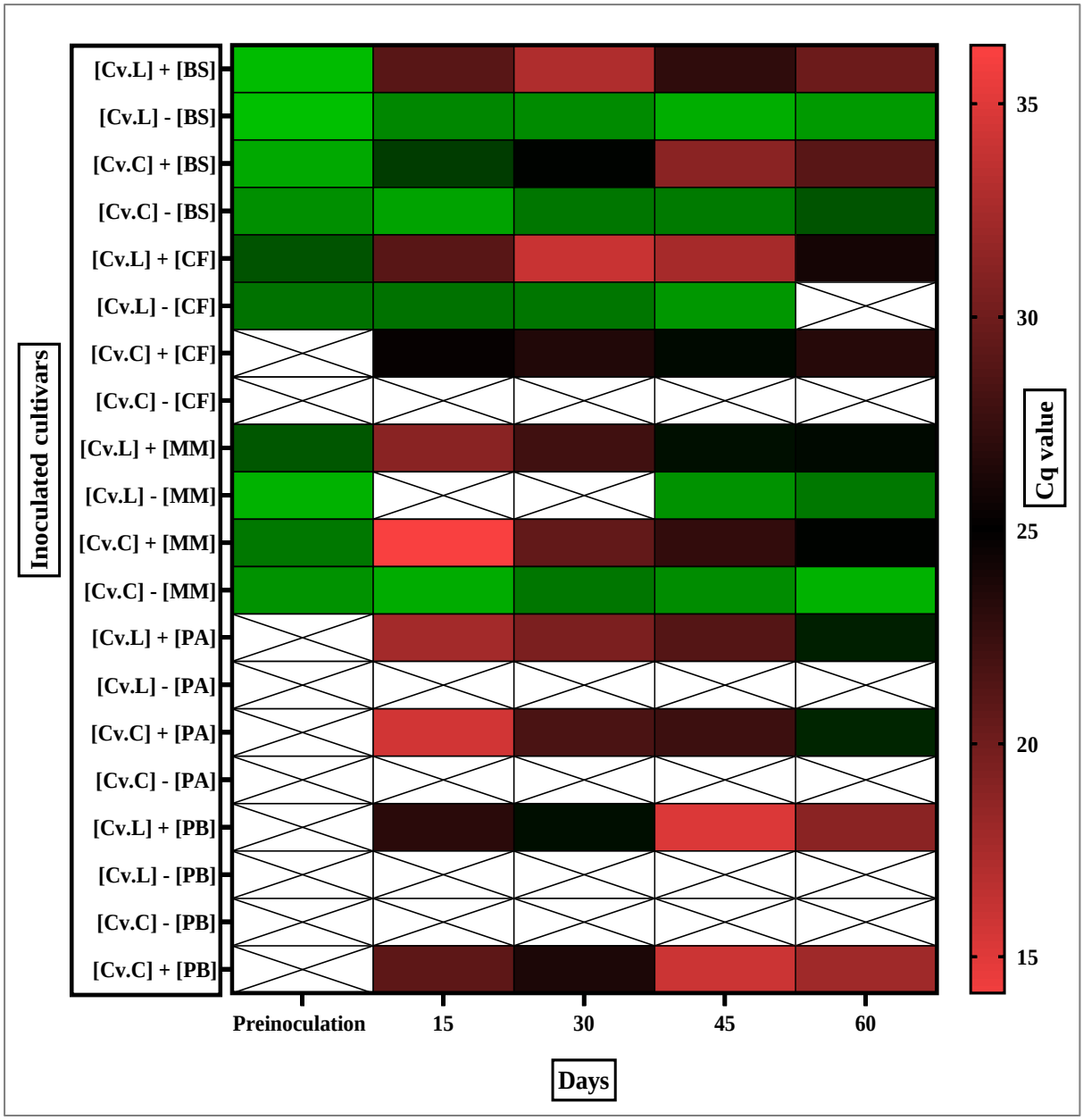

Figure 1. Heat map showing the real-time PCR quantification of inoculated endophytes: *B. subtilis* [BS], *C. flaccumfaciens* [CF], *M. mesophilicum* [MM], *P. agglomerans* [PA], and *P. brasiliense* [PB] in the cultivars of 'Leccino' [Cv.L] and 'Cima di Mola' [Cv.C]. The data is presented as an average of cq values obtained from inoculated and non-inoculated plants during two months of assessment.

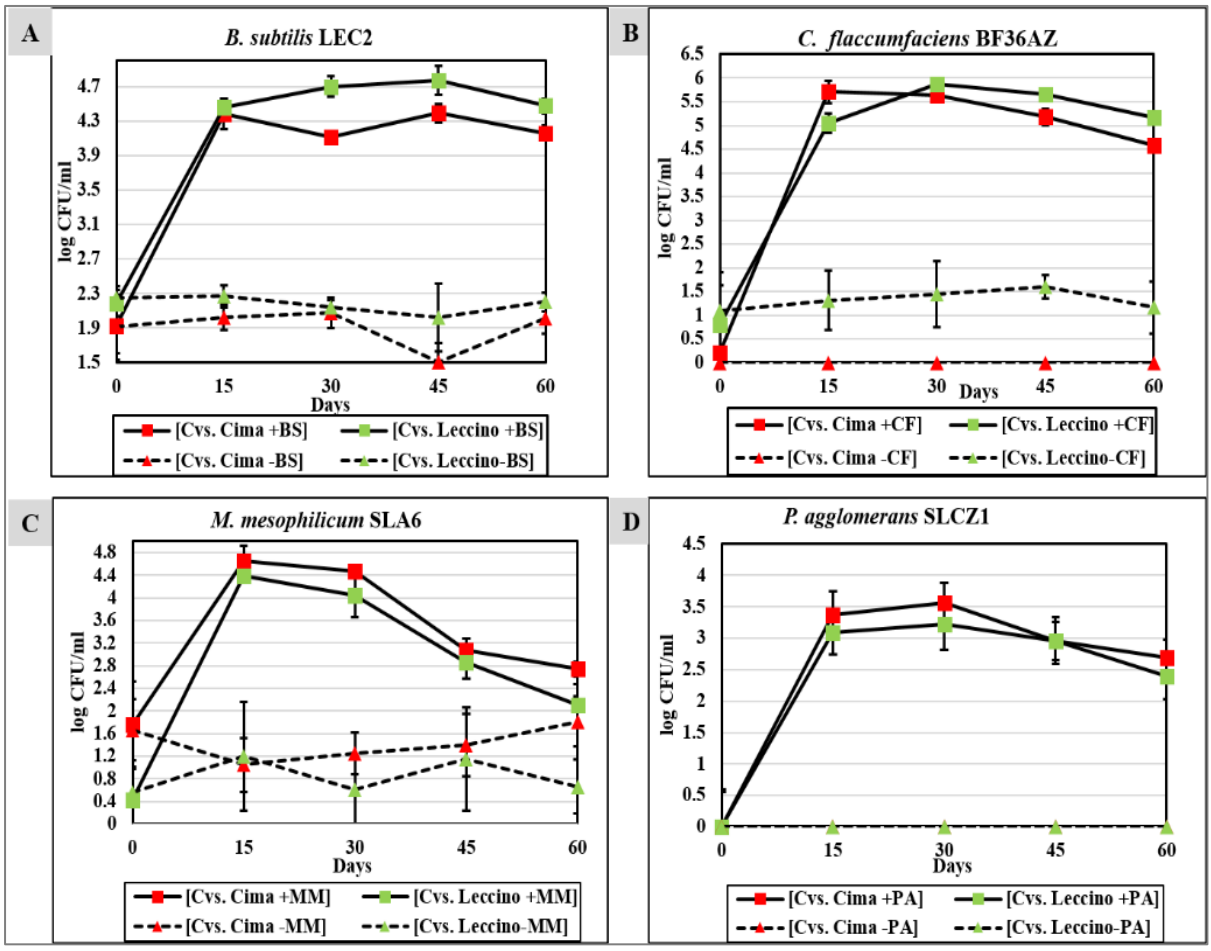

Figure 2. Monitoring the population size (CFU/ml) of endophytic bacterial inoculums in 'Leccino' and 'Cima di Mola' during two months after inoculation. **A**. represents the population size of *B. subtilis* (BS). **B**. represents the population size of *C. flaccumfaciens* (CF). **C**. represents the population size of *M. mesophilicum*. **D**. represents the population size of *P. agglomerans*. All bacterial inoculums were measured in three biological replicates of inoculated olive varieties and controls.

Considering the monitoring of *P. brasiliense* MT86 colonization in the inner sphere of both olive varieties, cultured stem segments constantly revealed high colonization rates of *P. brasiliense* after inoculation. The colonization rate of the fungal inoculum ranged from 62% to 83.3% in the segments of 'Cima di Mola', and 66.67% to 70.83% in the segments of 'Leccino' (Fig 3). Overall, control plants remained none colonized by *P. brasiliense* during the evaluation period.

Plants	Colonization rate %				
	Preinoculation	15 days	30 days	45 days	60 days
Cima di mola (IN)	0.00%	62.50%	79.17%	87.50%	83.33%
Cima di mola (C)	0.00%	0.00%	0.00%	0.00%	0.00%
Leccino (IN)	0.00%	70.83%	66.67%	75.00%	70.83%
Leccino (C)	0.00%	0.00%	0.00%	0.00%	0.00%

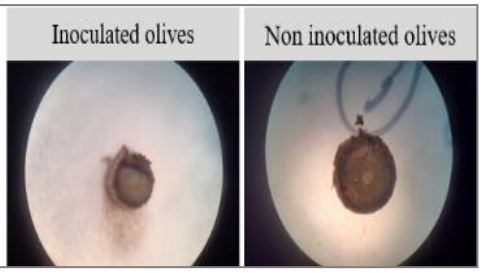

Figure 3. Colonization rates of *P. brasiliense* MT86 was obtained after the inoculation of 'Leccino' and 'Cima di Mola' olive varieties. Colonized segments (IN) showed evident growth of the referred inoculum, while no growth was noticed in control plants (C).

4.3.2 RT-qPCR analysis of defence-related genes response

To understand the beneficial effect of olive colonization by inoculated endophytes, specific genes involved in olive defence mechanisms were tested. First, total RNA extracted from 100mg of olives aerial tissues were successfully obtained with high purity (A260/280 > 1.8) and quantity (˜50ng) following the adopted procedures. The imported primers were validated and confirmed their specificity and efficiency of defence-related gene quantification (data not shown). Furthermore, the modulation of gene expression was preliminarily confirmed as some inoculated olives presented a much-noticed upregulation of defence-related genes, which was observed by RT-qPCR results and agarose gel electrophoresis (Fig 4).

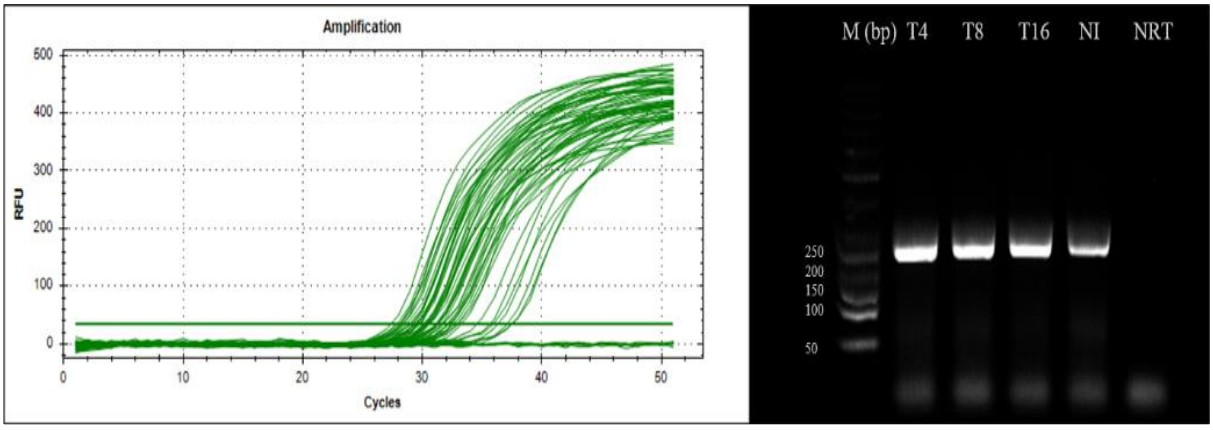

Figure 4. RT-qPCR amplification plot and agarose gel electrophoresis showing clear variation in the expression of GRAS1 gene between colonized olives and controls. T4 and T8 present GRAS1 gene amplifications after 4, 8 days of endophyte inoculation, while NI is referred to untreated olive plants and NRT is a reaction control with no reverse transcription.

Gene expression RT-qPCR assays showed that inoculated endophytes enable the two Apulian olive cultivars to elicit several defence features associated with health promotion and counteracting pathogenic infections. Our findings suggest these evident features were mostly related to plants inoculated with *B. subtilis* LEC2 and *C. flaccumfaciens* BF26AZ. Although the relative expression analysis of olive plants inoculated with *M. mesophilicum* SLA6 and *P. agglomerans* SLCZ1 revealed few trending changes, still these were found statistically unsupported when compared to control plants. Similarly, no changes were observed in the regulation of defence-related genes in both olive cultivars inoculated with *P. brasiliense* MT86.

Olive genes response to B. subtilis LEC2 inoculation

B. subtilis inoculation relatively instituted similar gene expression between both olive cultivars, with MDH, GRAS1, and WRYK5 up-regulated to similar levels in the aerial tissues of inoculated plants (Fig 5). Meanwhile, there were slight uncommon variation in the expression of SA-BP and PAL at cultivar level and days after treatment. Malate dehydrogenase (MDH), which is thought to be associated with plant hormone biosynthesis and stress tolerance [38], was significantly induced in both inoculated cultivars and reached the maximum expression at the 8^{th} day postinoculation ($P < 0.0002$) (Fig 5). Salicylic acid-binding protein 2 (SA-BP), like other SA binding proteins, is known to play an important role in SA signal transduction pathways that positively influence plant innate immunity and induced resistance [39]. In this regard, SA -BP was found to be significantly up-regulated in 'Leccino' at the 4^{th}-day postinoculation ($P< 0.002$), while it was significantly induced in 'Cima di Mola' at the 8^{th}-day postinoculation ($P< 0.002$) (Fig 5). The expression of GRAS1, the key transduction protein of plant defence was found alike between both varieties, where it was exclusively found up-regulated at the 8^{th}-day postinoculation ($P<0.033$) (Fig 5). The precursor of phenylpropanoids biosynthesis ,phenylalanine ammonia-lyase (PAL) [40] was found upregulated in both varieties at both 4^{th} and 8^{th} days postinoculation, however, a slight variation of upregulation levels were noticed between the days after inoculation (Fig 5). At last, the expression of WRKY5 was highly upregulated in both varieties at the 8^{th}-day postinoculation compared to the 4^{th}-day postinoculation. Together, these data indicated that *B. subtilis* activated the expression of defence related genes in the aerial part of both olive cultivars, which was confirmed by the stability of housekeeping gene (ACTIN) expression in the experimental plants.

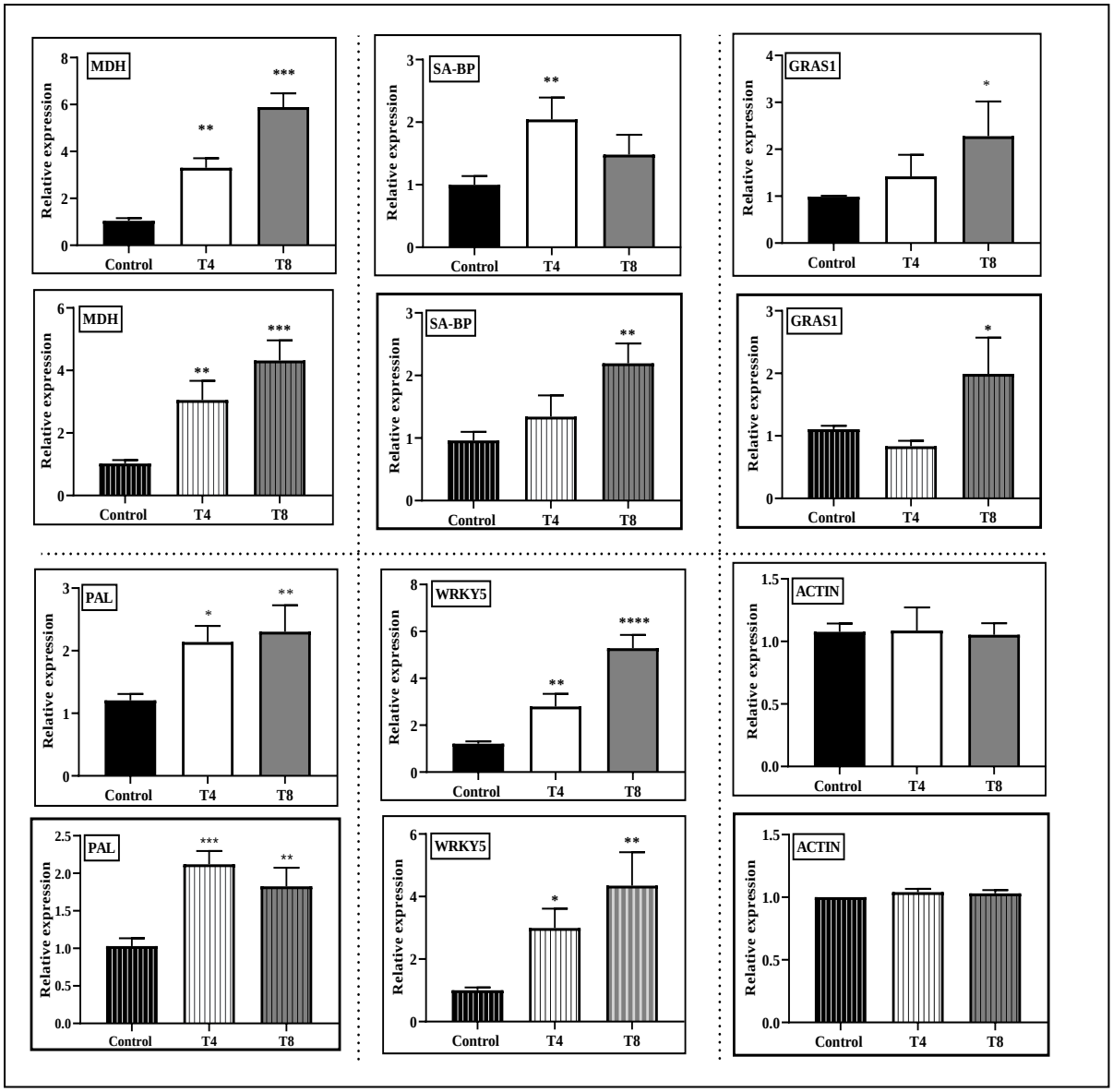

Figure 5. Relative expression analysis of defence-related genes in the aerial tissues of Apulian olive varieties [Leccino; plain colors] and [Cima di Mola; strapped pattern] inoculated with *B. subtilis* LEC2. Total RNA extracted after 4 and 8 days of inoculation was subjected to RT-qPCR to quantify five defence-related genes [MDH, SA-BP, GRAS1, PAL, and WRKY5] in reference to the housekeeping gene [ACTIN]. Bars are the mean of relative expression ± SD of 3 biological replicates of each variety with 4 technical replicates of each one. Statistically, significant differences are found according to one-way ANOVA and Dunnett's test and presented as *, $P \leq 0.033$; **, $P \leq 0.0021$; ***, $P \leq 0.0002$; ****, $P \leq 0.0001$.

Olive genes response to C. flaccumfaciens BF26AZ inoculation

The results obtained (Fig 5) indicate that the up-regulated expression of investigated genes was shared by olive cultivars subjected to *C. flaccumfaciens* BF26AZ. The inoculation of olive plants promoted efficient elevations in upregulating defence-related genes, which might confer some degree of resistance to pathogenic infection. Similar responses within SA-BP, WRKY5, and PAL genes of 'Leccino' and 'Cima di Mola' were found at late stage of postinoculation. However, both cultivars presented much-observed upregulation of MDH at early stage of postinoculation, and there was a different response between both cultivars in the context of GRAS1 gene regulation. The relative expression of MDH was revealed at higher levels of upregulation in both varieties at the 4^{th}-day postinoculation ($P<0.002$) (Fig 5). On the other hand, SA-BP was significantly upregulated at higher levels in both cultivars at the 8^{th}-day postinoculation compared to the 4^{th}-day postinoculation (Fig 5). Expression of GRAS1 was found significantly upregulated at the 8^{th}-day postinoculation in 'Leccino' plants ($P<0.033$), whereas 'Cima di Mola' presented a high level of GRAS1 upregulation at 4^{th}-day postinoculation ($P<0.002$) (Fig 5). At last, PAL and WRKY5 were upregulated in both varieties only at the 8^{th}-day postinoculation ($P<0.002$) (Fig 5)

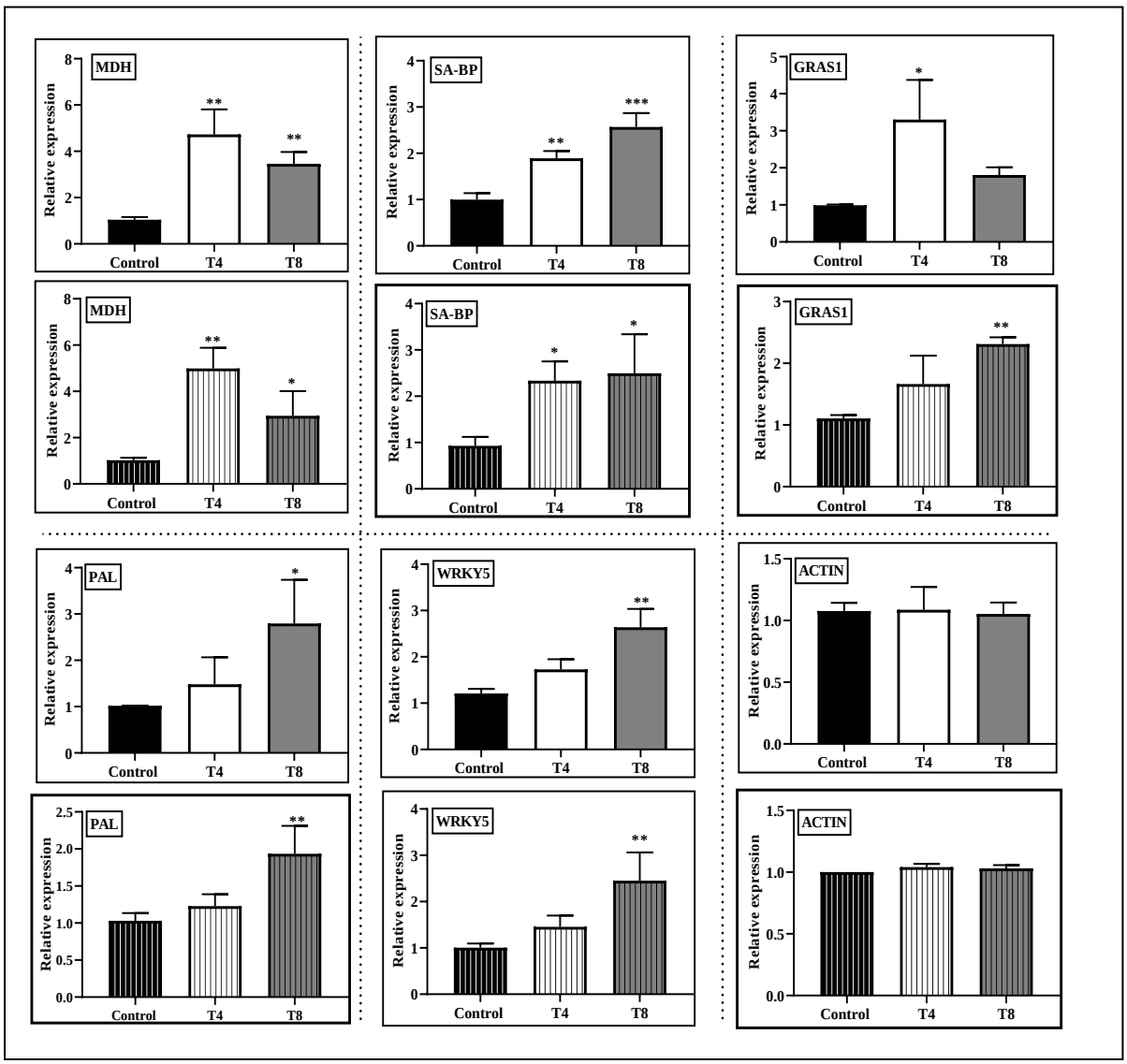

Figure 6. Relative expression analysis of defence-related genes in the aerial tissues of Apulian olive varieties [Leccino; plain colors] and [Cima di Mola; strapped pattern] inoculated with *C. flaccumfaciens* BF36AZ. Total RNA extracted after 4 and 8 days of inoculation was subjected to RT-qPCR to quantify 5 defence-related genes [MDH, SA-BP, GRAS1, PAL, and WRKY5] in reference to the housekeeping gene [ACTIN]. Bars are the mean of relative expression ± SD of 3 biological replicates of each variety with 4 technical replicates of each one. Statistically significant differences are determined according to one-way ANOVA and Dunnett's test and presented as *, $P \leq 0.033$; **, $P \leq 0.0021$; ***, $P \leq 0.0002$; ****, $P \leq 0.0001$.

4.4 Discussion

Key to the control of vascular pathogens by the endophytic antagonist is the evaluation of the ability of the inoculum to systematically thrive in the target plant [41]. In addition to its role of pathogen inhibition, the plant/antagonist interaction can enhance host defence performance. This is often referred to as the priming of induced systematic resistance (ISR) [42], but recent studies have considered the terms ISR and systemic acquired resistance (SAR) to be synonymous due to the crosstalk between ET, JA and SA pathways [10]. Although priming of plant defence has been associated with beneficial rhizobacteria, endophytes that actively live in the aboveground part of the plant are also capable of priming plant resistance to vascular pathogens [24]. In this case study, we tested *Xf* ST53 antagonists, indirect antagonists and symbionts isolated from olive sapwood for their colonisation and establishment in different Apulian cultivars. This allowed the observation of their activity in modulating olive defence and the selection of isolates that could represent a potential candidate for strategic biocontrol application.

Evaluation of inoculum establishment inside olive plants revealed variation in stability and persistence among our tested isolates. In other words, although our tested inoculum isolates were isolated from the xylem sap, they does not seem to have similar stability of colonizing olive plants. This phenomenon could be related to a complex of plant-endophyte interactions as described by several studies [43-46]. The adopted methods (real-time PCR and plate counting) have successfully determined which endophyte is highly capable of colonizing the inner sphere of Apulian olive cultivars, however, some results were presented conversely by both approaches. This is a well-known experimental issue to be considered; 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 [47]. In our study, the inoculum of *B. subtilis* LEC2 and *C. flaccumfaciens* BF26AZ established in olive plants, which was confirmed by both approaches. Thus, our results indicated that both bacterial isolates can harness olives innate as a habitat for their growth, which is expected since both isolates belong to two genera associated with the olive endophytic microbiome and have been repeatedly isolated from the olive plants [48, 49]. Similarly, our study showed that *P. brasiliense* MT86 is capable of establishing in both olive cultivars, where this fungus remains an exclusive endophyte to olive plants. On the other hand, *M. mesophilicum* SLA6 and *P. agglomerans* SLCZ1 were less efficient in establishing in both cultivars. This might be

explained since both bacteria have been known as facultative endophytes and associated with the rhizosphere of the different hosts [50-52].

Our study also showed that inoculation of potentially antagonistic isolates can induce the defence mechanism of olive. This was confirmed by the up-regulation of the defence-related genes studied in plants inoculated with the isolates of *B. subtilis* LEC2 (*Xf* ST53-direct antagonist) and *C. flaccumfaciens* BF26AZ (*Xf*-Indirect antagonist). Meanwhile, other inoculum of *M. mesophilicum* (*Xf* symbiont), *P. agglomerans*, and *P. brasiliense (Xf* ST53-direct antagonist) had no effect on olive plant defence during the evaluation period. Regardless of the low stability of *M. mesophilicum* in olives, the beneficial properties of the bacterium are more functionally related to nitrogen fixation and antioxidant metabolism in the host [53, 54]. Meanwhile, *P. agglomerans* have been extensively found along with *Pseudomonas savastanoi* pv. *Savastanoi* in olive knots [55], which was recently described to possess several contributions to the pathogen virulence [56]. Concerning *P. brasiliense*, our finding that it has no impact on olive defence is usually the rule as fungal endophytes are not commonly associated with modulating the ISR [57].

The defence-related genes studied (MDH, SA - BP, PAL, GRAS1 and WRKY5) were similarly modulated in aerial tissues of olive plants inoculated with *Pseudomonas fluorescens* PICF7 [56]. However, the up-regulation of these genes was more conspicuous in plants treated with *B. subtilis* LEC2 than in those treated with *C. flaccumfaciens* BF26AZ. Overall, *B. subtilis* is remarkably known to control diseases in olives and other hosts [57-61], and its presence in olive tissues had positive effects on defence responses. In this regard, direct antagonist that can induce systemic resistance could be one of the most important operating mechanisms when it comes to biocontrol of plant vascular pathogens. In conclusion, we can recommend *B. subtilis* LEC2 as a potential agent for a better biocontrol strategy of *Xf* ST53 in Apulian olive. To our knowledge, this is the first detailed study of the colonisation pattern and defence response for potential *Xf* ST53-antagonistic endophytes in the aerial tissues of Apulian olive cultivars.

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CHAPTER V

Conclusion remarks, future applications & recommendations, and appendices

Summary

This chapter presents affirmative conclusions, obtained through the conducted studies on cultivable sapwood endophytes of Apulian olives. Within that, the main highlights of the study are presented and evidenced by data analyzed in the previous chapters. Besides, some limitations and their effect on the research strategy have been identified. Later, a devoted section presents the research main outputs, of which possibly will be applied in the field of endophytes beneficial utilization to manage such vascular pathogens in olive plant. Still, we recommend further topics to be studied. Finally, appendixes of the research topics will be supplemented.

5.1 Conclusion remarks

My dissertation research aimed to investigate the core of cultivable endophytes residing in the sapwood of Apulian olives under different variation factors, to explore the antagonistic activity of endophytes collection against *X. fastidiosa* ST53, and to demonstrate the colonization efficiency and mediated health features of mainly obtained antagonists in inoculated Apulian olive cultivars.

In the second chapter, I used culture-based techniques to maximize the recovery of bacterial and fungal endophytes from the sapwood of resistant and susceptible olive cultivars. Based on the extensive statistical analysis of colony indicators, the stability of the endophytic community in the resistant cultivar 'Leccino' was confirmed. Meanwhile, several variation factors were found affecting the diversity and richness of olives sapwood endophytes. Among those, the factor of *Xf*- infection, which revealed lower levels of diversity and stability of bacterial endophytes in susceptible olive plants. Due to the huge number of isolates, several categorization tests were adopted. However, the most frequently obtained groups formed the core of cultivable endophytes in the sapwood olives. With the correspondence to defined genera, relative frequency and density showed which plant is rich with the targeted genera. Thereby, several endophytes with previously demonstrated antagonistic potency against plant pathogens were found. Some of those were even richer in resistant cultivars across the investigated sites; bacterial genera as *Bacillus*, *Curobacterium*, and *Pantoea* and fungal genera as *Pithomyces* and *Paraconiothyrium* were more frequently isolated from 'Leccino' sapwood. To our knowledge, this part included the first report of different bacterial and fungal species occurrence in investigated olives. Finally, there are some concerns in the culture-based method that impose a noticeable bias toward fast-growing endophytes. Although that was considered by using rich and low nutrient media, still several types of media should also be used with a longer period of incubation.

In the third chapter, I described in detail the *in vitro* antagonistic activity of fungal and bacterial endophytic collection against *Xf* ST53. The rapid stab method was adopted in this study to conduct mass screening of potential antagonists. As result, several bacterial and fungal isolates were distinguished by inhibiting the growth of *Xf* ST53. It was necessary to evaluate which isolate possess better inhibition using the disc diffusion method. Our findings suggested that *B. subtilis* was the most effective bacterial antagonists under different conditions. However, *B.*

pumilus and *P. agglomerans* were also valid antagonists at a lower level of inhibition. In the context of fungal antagonists, the inhibition of *Xf* ST53 growth was found limited to *P. brasiliense*.

This section received major attention for proving the existence of endophytic antagonists of *Xf* ST53, however, we believed those potential antagonists collectively should undergo through colonization efficiency in olive plants. Thus, we developed successfully primers pair for each targeted isolate. We faced certain difficulties that should be taken into consideration; the growth and manipulation of *Xf* ST53 remain extremely difficult and time-consuming, where the measurement of the halo zone is restricted on the appearance of *Xf* colonies.

In the fourth chapter, I validated the colonization efficiency of several endophytes in the aerial tissues of Apulian olive varieties. Those endophytes were chosen upon their antibiosis activity or being reported as symbiont or indirect antagonists in previous studies. Upon inoculation, we determined which endophyte can mediate the response of defence-related genes. We found out that *B. subtilis* LEC2 and *C. flaccumfaciens* BF26AZ have excellent ability to thrive in the inner sphere of both olive cultivars, and their inoculums were tracked and recovered successfully for two months. Besides, those isolates induced the expression of several defence-related genes, however, the isolate LEC2 mediated higher levels of upregulation compared to the isolate BF26AZ. Other potential endophytes as *M. mesophilicum* SLA6 (symbiont) and *P. agglomerans* SLCZ1 (direct antagonist) revealed low stability in the inoculated olive plants, and they had no impact on the defence response. Concerning *P. brasiliense* MT86 (direct antagonist), was successfully found colonizing the stem segments of olive plants, with no activity toward the defence response. We believe those antagonistic candidates could be integrated into a biocontrol strategy of *Xf* ST53, however, that may require a further experimental understanding of their effect on counteracting olives bacterium infection. During this section, there were difficulties in using the developed real-time PCR primers to quantify the inoculum. Consequently, the method was preferably used to track the establishment of the inoculum.

5.2 Future applications and recommendations

- This work has created solid database for cultivable endophytes obtained from different Apulian olive cultivars. In this context, studying the endophytic community serves as a gateway to developing ways to utilize the capabilities of endophytes such as in phytoremediation and further understanding the roles endophytes play within olive plants.

- By our findings of different antagonists to *Xf* ST53, the opportunity of developing successful and applicable biocontrol agents seems feasible. In fact, most of our work enabled future studies to focus on limited candidates. At bacterial level, *B. subtilis* seems to be highly antagonistic, excellent colonizer, and mediator of olive defence related genes. While the fungal isolate of *P. brasiliense* remains also good direct antagonist that needed to be fully explored.

- Trials must confirm the candidate endophyte is antagonistic *in vivo* and improves health or survivability of *Xf*-infected olive plants. In order to carry out *in vivo* trials, inoculation methods must include our adopted method (stem punctures) and spraying young plant with a solution of bacterial and or fungal suspension. Beyond that, mixtures of potential antagonists could form better health promotion of infected plants.

There is still a lot more research that needs to be conducted on the endophytes that are beyond the scope of this study

- What are the metabolic capabilities of the identified antagonists, as well do any of the endophytes produce secondary metabolites?

- Given that the developed Real time PCR primers were validated on DNA serially diluted, what are the expectations from validating the primers on DNA form serially diluted colonies or mycelium suspension? Could that indicate the inoculum population density with a minimum error?

5.3 Appendices

5.3.1 Appendices (Chapter II)

Data analysis of figure 3A

The purpose of the analysis was to study the variation of bacterial log CFU/ml levels within different olive varieties locating within different zones. To carry that, the Univariate Analysis of Variance was used.

- To calculate log CFU/ ml = [Number of colonies counted] × 10 × [how many times the sample must be multiplied to get to the original concentration: for example, 10^5] = Number of colony forming units (**CFU**) per milliliter of starting culture.
- The obtained data was assigned to varieties and zones, which were test for normality and homogeneity of variances.
- Transformation of data (ranktcfu) was obtained through the following equation = Id.normal (Rank fractional, Mean, Standard deviation).

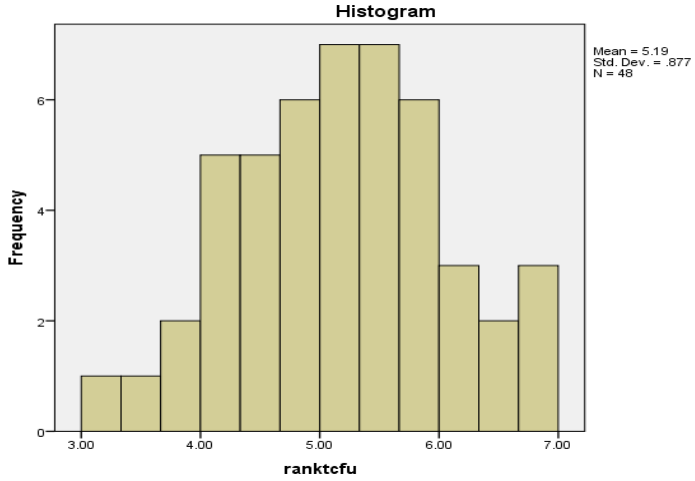
To study the main effect and interaction between factors and dependent variable (CFU), the Univariate analysis showed P values < 0.05

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
CFU	.138	48	.022	.935	48	.011
ranktcfu	.033	48	.200*	.989	48	.940

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction



Levene's Test of Equality of Error Variances[a]

Dependent Variable: ranktcfu

F	df1	df2	Sig.
2.426	5	42	.051

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Zones * Varieties + Zones + Varieties

Between-Subjects Factors

		Value Label	N
Zones	CZ	CZ	16
	HZ	HZ	16
	IZ	IZ	16
Varieties	Leccino	Leccino	24
	Rossa	Rossa	8
	Salentina	Salentina	16

Tests of Between-Subjects Effects

Dependent Variable: ranktcfu

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	14.157[a]	5	2.831	5.413	.001
Intercept	1168.030	1	1168.030	2233.168	.000
Zones * Varieties	.074	1	.074	.141	.709
Zones	.995	2	.497	.951	.395
Varieties	11.182	2	5.591	10.690	.000
Error	21.968	42	.523		
Total	1329.200	48			
Corrected Total	36.124	47			

a. R Squared = .392 (Adjusted R Squared = .319)

Multiple Comparisons

Dependent Variable: ranktcfu

LSD

(I) Varieties	(J) Varieties	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Leccino	Rossa	.5542	.29525	.067	-.0416	1.1500
	Salentina	1.1653*	.23342	.000	.6943	1.6364
Rossa	Leccino	-.5542	.29525	.067	-1.1500	.0416
	Salentina	.6111	.31316	.058	-.0209	1.2431
Salentina	Leccino	-1.1653*	.23342	.000	-1.6364	-.6943
	Rossa	-.6111	.31316	.058	-1.2431	.0209

Based on observed means.

Multiple Comparisons

Dependent Variable: ranktcfu

LSD

(I) Zones	(J) Zones	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CZ	HZ	.5139	.25569	.051	-.0022	1.0299
	IZ	.5288*	.25569	.045	.0128	1.0448
HZ	CZ	-.5139	.25569	.051	-1.0299	.0022
	IZ	.0149	.25569	.954	-.5011	.5309
IZ	CZ	-.5288*	.25569	.045	-1.0448	-.0128
	HZ	-.0149	.25569	.954	-.5309	.5011

Based on observed means.

The error term is Mean Square(Error) = .523.

*. The mean difference is significant at the .05 level.

The error term is Mean Square(Error) = .523.

*. The mean difference is significant at the .05 level.

Data analysis of figure 3B

The analysis was conducted by similar methodology used in Fig 3A, with consideration seasonality & varieties VS CFU/ml

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Rankcfuseasonal	.023	180	.200*	.998	180	.998
CFU.ml	.184	180	.000	.877	180	.000

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Tests of Between-Subjects Effects

Dependent Variable: Rankcfuseasonal

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	472.604[a]	8	59.076	277.317	.000
Intercept	4234.030	1	4234.030	19875.698	.000
Seasons	181.800	2	90.900	426.710	.000
Varieties	236.843	2	118.421	555.902	.000
Seasons * Varieties	53.961	4	13.490	63.327	.000
Error	36.427	171	.213		
Total	4743.061	180			
Corrected Total	509.031	179			

a. R Squared = .928 (Adjusted R Squared = .925)

Multiple Comparisons

Dependent Variable: Rankcfuseasonal

LSD

(I) Seasons	(J) Seasons	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Fall	Summer	-1.8566*	.08427	.000	-2.0229	-1.6902
	Winter	.4717*	.08427	.000	.3053	.6380
Summer	Fall	1.8566*	.08427	.000	1.6902	2.0229
	Winter	2.3282*	.08427	.000	2.1619	2.4946
Winter	Fall	-.4717*	.08427	.000	-.6380	-.3053
	Summer	-2.3282*	.08427	.000	-2.4946	-2.1619

Based on observed means.

The error term is Mean Square(Error) = .213.

Multiple Comparisons

Dependent Variable: Rankcfuseasonal

LSD

(I) Varieties	(J) Varieties	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Leccino	Rossa	2.8097*	.08427	.000	2.6434	2.9761
	Salentina	1.3959*	.08427	.000	1.2295	1.5622
Rossa	Leccino	-2.8097*	.08427	.000	-2.9761	-2.6434
	Salentina	-1.4139*	.08427	.000	-1.5802	-1.2475
Salentina	Leccino	-1.3959*	.08427	.000	-1.5622	-1.2295
	Rossa	1.4139*	.08427	.000	1.2475	1.5802

Based on observed means.

The error term is Mean Square(Error) = .213.

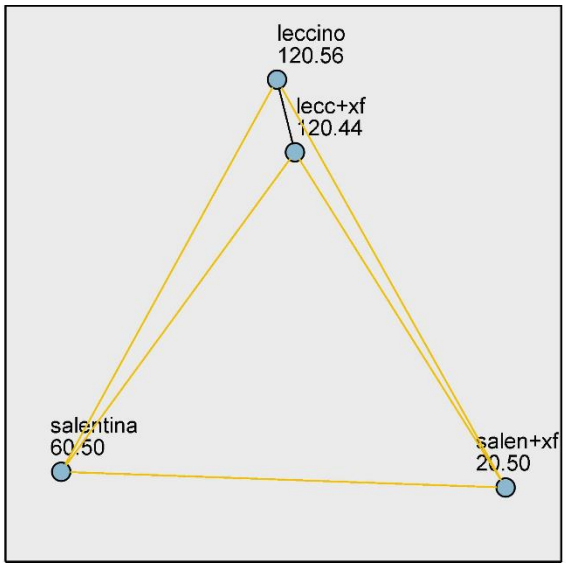
*. The mean difference is significant at the .05 level.

*. The mean difference is significant at the .05 level.

Data analysis of figure 3C

Fig 3D: The purpose of the analysis was to study the variation of bacterial log CFU/ml levels within infected and non-infected olive varieties. To carry that, nonparametric analysis was carried.

Pairwise Comparisons of Varieties



Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Each node shows the sample average rank of Varieties.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
salen+xf-salentina	-40.000	10.356	-3.863	.000	.001
salen+xf-lecc+xf	99.938	10.356	9.650	.000	.000
salen+xf-leccino	100.062	10.356	9.662	.000	.000
salentina-lecc+xf	59.938	10.356	5.788	.000	.000
salentina-leccino	60.062	10.356	5.800	.000	.000
lecc+xf-leccino	-.125	10.356	-.012	.990	1.000

Data analysis of figure 6A&B

Fig 6A, B: The purpose of the analysis was to study the variation of fungal colonization and isolation rates within different olive varieties located in healthy (I), contaminated (II) and infected sites (III). Thus, a multivariate analysis of variances was employed to study the effect of both independent variables (Varieties &Sites) on both dependent variables (CR, IR) and the interaction within different variables

- To calculate CR= (Total number of twig successfully colonized/total number of twigs) *100

- To calculate IR= (Avg number of isolates / number of twig prints) *100

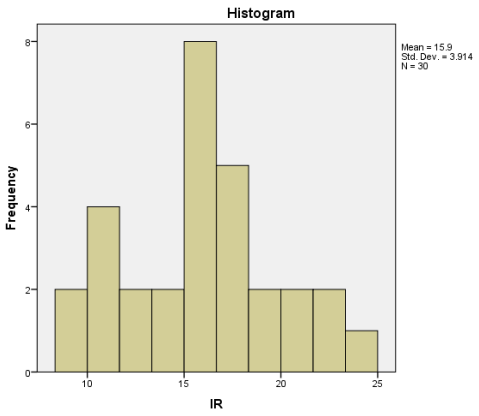
- The obtained data was assigned to varieties and zones, which were test for normality and homogeneity of variances.

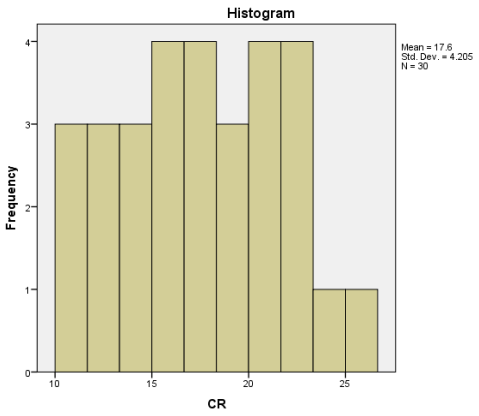
Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
CR	.103	30	.200*	.961	30	.330
IR	.115	30	.200*	.967	30	.457

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction





Levene's Test of Equality of Error Variances[a]

	F	df1	df2	Sig.
CR	.700	5	24	.629
IR	1.112	5	24	.380

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Zones + Varieties + Varieties * Zones

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[c]
Corrected Model	CR	299.295[a]	5	59.859	6.728	.000	.584	33.641	.990
	IR	183.394[b]	5	36.679	3.374	.019	.413	16.868	.822
Intercept	CR	9389.075	1	9389.075	1055.335	.000	.978	1055.335	1.000
	IR	7455.107	1	7455.107	685.698	.000	.966	685.698	1.000
Varieties * Zones	CR	.010	1	.010	.001	.974	.000	.001	.050
	IR	2.016	1	2.016	.185	.671	.008	.185	.070
Varieties	CR	243.993	2	121.997	13.712	.000	.533	27.425	.995
	IR	90.723	2	45.361	4.172	.028	.258	8.344	.678
Zones	CR	40.584	2	20.292	2.281	.124	.160	4.562	.418
	IR	93.304	2	46.652	4.291	.026	.263	8.582	.691
Error	CR	213.522	24	8.897					
	IR	260.935	24	10.872					
Total	CR	9804.103	30						
	IR	8029.583	30						
Corrected Total	CR	512.817	29						
	IR	444.329	29						

a. R Squared = .584 (Adjusted R Squared = .497)

b. R Squared = .413 (Adjusted R Squared = .290)

c. Computed using alpha = .05

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.987	881.467[b]	2.000	23.000	.000
	Wilks' Lambda	.013	881.467[b]	2.000	23.000	.000
	Hotelling's Trace	76.649	881.467[b]	2.000	23.000	.000
	Roy's Largest Root	76.649	881.467[b]	2.000	23.000	.000
Zones	Pillai's Trace	.385	2.862	4.000	48.000	.033
	Wilks' Lambda	.638	2.895[b]	4.000	46.000	.032
	Hotelling's Trace	.530	2.916	4.000	44.000	.032
	Roy's Largest Root	.449	5.386[c]	2.000	24.000	.012
Varieties	Pillai's Trace	.644	5.693	4.000	48.000	.001
	Wilks' Lambda	.401	6.666[b]	4.000	46.000	.000
	Hotelling's Trace	1.385	7.618	4.000	44.000	.000
	Roy's Largest Root	1.300	15.601[c]	2.000	24.000	.000
Varieties * Zones	Pillai's Trace	.062	.756[b]	2.000	23.000	.481
	Wilks' Lambda	.938	.756[b]	2.000	23.000	.481
	Hotelling's Trace	.066	.756[b]	2.000	23.000	.481
	Roy's Largest Root	.066	.756[b]	2.000	23.000	.481

a. Design: Intercept + Zones + Varieties + Varieties * Zones

b. Exact statistic

b. The statistic is an upper bound on F that yields a lower bound on the significance level

Multiple Comparisons

Dependent Variable		(I) Varieties	(J) Varieties	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
CR	LSD	Leccino	Rossa	-7.33*	1.540	.000	-10.51	-4.15
			Salentina	-4.76*	1.218	.001	-7.27	-2.24
		Rossa	Leccino	7.33*	1.540	.000	4.15	10.51
			Salentina	2.58	1.634	.128	-.80	5.95
		Salentina	Leccino	4.76*	1.218	.001	2.24	7.27
			Rossa	-2.58	1.634	.128	-5.95	.80
	Bonferroni	Leccino	Rossa	-7.33*	1.540	.000	-11.30	-3.37
			Salentina	-4.76*	1.218	.002	-7.89	-1.62
		Rossa	Leccino	7.33*	1.540	.000	3.37	11.30
			Salentina	2.58	1.634	.384	-1.63	6.78
		Salentina	Leccino	4.76*	1.218	.002	1.62	7.89
			Rossa	-2.58	1.634	.384	-6.78	1.63
IR	LSD	Rossa	Leccino	-2.59	1.836	.171	-6.38	1.20
			Salentina	-1.57	1.452	.291	-4.56	1.43
		Leccino	Rossa	2.59	1.836	.171	-1.20	6.38
			Salentina	1.02	1.947	.603	-2.99	5.04
		Salentina	Leccino	1.57	1.452	.291	-1.43	4.56
			Rossa	-1.02	1.947	.603	-5.04	2.99
	Bonferroni	Rossa	Leccino	-2.59	1.836	.513	-7.32	2.13
			Salentina	-1.57	1.452	.874	-5.30	2.17
		Leccino	Rossa	2.59	1.836	.513	-2.13	7.32
			Salentina	1.02	1.947	1.000	-3.99	6.04
		Salentina	Leccino	1.57	1.452	.874	-2.17	5.30
			Rossa	-1.02	1.947	1.000	-6.04	3.99

Based on observed means.
The error term is Mean Square(Error) = 12.642.

*. The mean difference is significant at the .05 level.

Multiple Comparisons

Dependent Variable		(I) Zones	(J) Zones	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
CR	LSD	HZ	CZ	-.11	1.334	.935	-2.86	2.64
			IZ	2.82*	1.334	.045	.07	5.58
		CZ	HZ	.11	1.334	.935	-2.64	2.86
			IZ	2.93*	1.334	.038	.18	5.69
		IZ	HZ	-2.82*	1.334	.045	-5.58	-.07
			CZ	-2.93*	1.334	.038	-5.69	-.18
	Bonferroni	HZ	CZ	-.11	1.334	1.000	-3.54	3.32
			IZ	2.82	1.334	.135	-.61	6.26
		CZ	HZ	.11	1.334	1.000	-3.32	3.54
			IZ	2.93	1.334	.113	-.50	6.37
		IZ	HZ	-2.82	1.334	.135	-6.26	.61
			CZ	-2.93	1.334	.113	-6.37	.50
IR	LSD	HZ	CZ	-1.74	1.590	.285	-5.02	1.54
			IZ	2.09	1.590	.202	-1.20	5.37
		CZ	HZ	1.74	1.590	.285	-1.54	5.02
			IZ	3.82*	1.590	.024	.54	7.11
		IZ	HZ	-2.09	1.590	.202	-5.37	1.20
			CZ	-3.82*	1.590	.024	-7.11	-.54
	Bonferroni	HZ	CZ	-1.74	1.590	.856	-5.83	2.35
			IZ	2.09	1.590	.606	-2.01	6.18
		CZ	HZ	1.74	1.590	.856	-2.35	5.83
			IZ	3.82	1.590	.073	-.27	7.92
		IZ	HZ	-2.09	1.590	.606	-6.18	2.01
			CZ	-3.82	1.590	.073	-7.92	.27

Based on observed means.
The error term is Mean Square(Error) = 12.642.

*. The mean difference is significant at the .05 level.

Data analysis of figure 6C

The analysis of seasonality effect on the number of fungal isolates by twig printing method.

Levene's Test of Equality of Error Variances[a]

Dependent Variable: AVG

F	df1	df2	Sig.
1.988	8	36	.076

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Season * Varieties + Season + Varieties

Tests of Between-Subjects Effects

Dependent Variable: AVG

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[b]
Corrected Model	7081.244[a]	8	885.156	3.225	.007	.417	25.797	.929
Intercept	114811.756	1	114811.756	418.258	.000	.921	418.258	1.000
Varieties	4091.244	2	2045.622	7.452	.002	.293	14.904	.922
Season	2018.178	2	1009.089	3.676	.035	.170	7.352	.639
Varieties * Season	971.822	4	242.956	.885	.483	.090	3.540	.253
Error	9882.000	36	274.500					
Total	131775.000	45						
Corrected Total	16963.244	44						

a. R Squared = .417 (Adjusted R Squared = .288)

b. Computed using alpha = .05

Multiple Comparisons

Dependent Variable: AVG

	(I) Season	(J) Season	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	Fall	Summer	15.53*	6.050	.015	3.26	27.80
		Winter	12.33*	6.050	.049	.06	24.60
	Summer	Fall	-15.53*	6.050	.015	-27.80	-3.26
		Winter	-3.20	6.050	.600	-15.47	9.07
	Winter	Fall	-12.33*	6.050	.049	-24.60	-.06
		Summer	3.20	6.050	.600	-9.07	15.47
Bonferroni	Fall	Summer	15.53*	6.050	.044	.34	30.72
		Winter	12.33	6.050	.147	-2.86	27.52
	Summer	Fall	-15.53*	6.050	.044	-30.72	-.34
		Winter	-3.20	6.050	1.000	-18.39	11.99
	Winter	Fall	-12.33	6.050	.147	-27.52	2.86
		Summer	3.20	6.050	1.000	-11.99	18.39

Based on observed means.

The error term is Mean Square(Error) = 274.500.

*. The mean difference is significant at the .05 level.

Multiple Comparisons

Dependent Variable: AVG

	(I) Varieties	(J) Varieties	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	Leccino	Rossa	-23.20*	6.050	.000	-35.47	-10.93
		Salentina	-13.93*	6.050	.027	-26.20	-1.66
	Rossa	Leccino	23.20*	6.050	.000	10.93	35.47
		Salentina	9.27	6.050	.134	-3.00	21.54
	Salentina	Leccino	13.93*	6.050	.027	1.66	26.20
		Rossa	-9.27	6.050	.134	-21.54	3.00
Bonferroni	Leccino	Rossa	-23.20*	6.050	.001	-38.39	-8.01
		Salentina	-13.93	6.050	.081	-29.12	1.26
	Rossa	Leccino	23.20*	6.050	.001	8.01	38.39
		Salentina	9.27	6.050	.403	-5.92	24.46
	Salentina	Leccino	13.93	6.050	.081	-1.26	29.12
		Rossa	-9.27	6.050	.403	-24.46	5.92

Data analysis of figure 6D

Fig 6D: The purpose of the analysis was to study the variation of fungal isolates average within infected and non-infected 'Leccino' and 'Salentina' olives located in infected sites (III). Thus, one way ANOVA was employed to study the effect of Xf infection o on number of fungal isolates form both varieties

To calculate N of isolates= accumulation of retrieved fungal isolates from each varieties according to seasons. The obtained data was assigned to varieties and seasons, which were test for normality and homogeneity of variances.

Test of Homogeneity of Variances

Tests of Normality

Descriptives

Fungalavg

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Leccino +XF	40	15.0500	7.69932	1.21737	12.5876	17.5124	.00	30.00
Leccino - XF	40	14.5500	7.36224	1.16407	12.1954	16.9046	.00	29.00
Saletina +XF	40	19.7250	7.84787	1.24086	17.2151	22.2349	.00	33.00
Salentina-XF	40	22.2250	6.37900	1.00861	20.1849	24.2651	10.00	36.00
Total	160	17.8875	7.95821	.62915	16.6449	19.1301	.00	36.00

Multiple Comparisons

Dependent Variable: Fungalavg

	(I) Varieties	(J) Varieties	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	Leccino +XF	Leccino - XF	.50000	1.64227	.761	-2.7439	3.7439
		Saletina +XF	-4.67500*	1.64227	.005	-7.9189	-1.4311

		Salentina-XF	-7.17500*	1.64227	.000	-10.4189	-3.9311
		Leccino +XF	-.50000	1.64227	.761	-3.7439	2.7439
	Leccino - XF	Saletina +XF	-5.17500*	1.64227	.002	-8.4189	-1.9311
		Salentina-XF	-7.67500*	1.64227	.000	-10.9189	-4.4311
		Leccino +XF	4.67500*	1.64227	.005	1.4311	7.9189
	Saletina +XF	Leccino - XF	5.17500*	1.64227	.002	1.9311	8.4189
		Salentina-XF	-2.50000	1.64227	.130	-5.7439	.7439
		Leccino +XF	7.17500*	1.64227	.000	3.9311	10.4189
	Salentina-XF	Leccino - XF	7.67500*	1.64227	.000	4.4311	10.9189
		Saletina +XF	2.50000	1.64227	.130	-.7439	5.7439
		Leccino - XF	.50000	1.64227	1.000	-3.8887	4.8887
	Leccino +XF	Saletina +XF	-4.67500*	1.64227	.030	-9.0637	-.2863
		Salentina-XF	-7.17500*	1.64227	.000	-11.5637	-2.7863
		Leccino +XF	-.50000	1.64227	1.000	-4.8887	3.8887
	Leccino - XF	Saletina +XF	-5.17500*	1.64227	.012	-9.5637	-.7863
		Salentina-XF	-7.67500*	1.64227	.000	-12.0637	-3.2863
Bonferroni		Leccino +XF	4.67500*	1.64227	.030	.2863	9.0637
	Saletina +XF	Leccino - XF	5.17500*	1.64227	.012	.7863	9.5637
		Salentina-XF	-2.50000	1.64227	.780	-6.8887	1.8887
		Leccino +XF	7.17500*	1.64227	.000	2.7863	11.5637
	Salentina-XF	Leccino - XF	7.67500*	1.64227	.000	3.2863	12.0637
		Saletina +XF	2.50000	1.64227	.780	-1.8887	6.8887

*. The mean difference is significant at the 0.05 level.

5.3.2 Appendices (Chapter III)

Data analysis of figure 1A

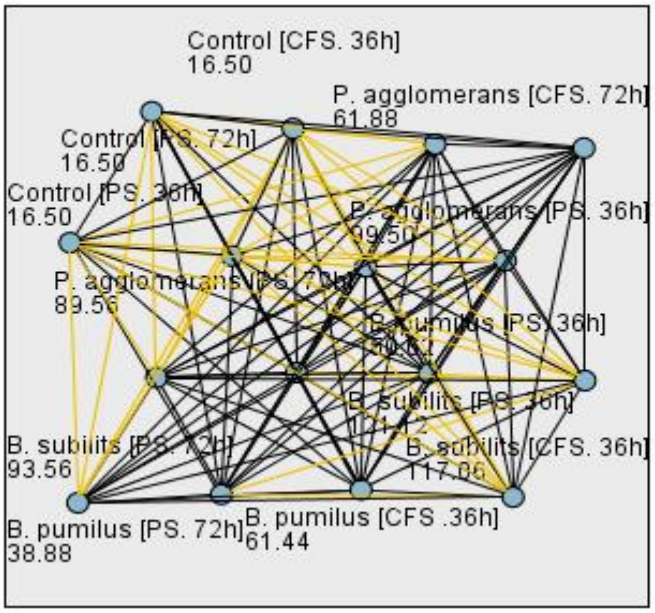
Non-parametric comparison of inhibition zone (mm) between bacterial antagonists groups

Hypothesis Test Summary

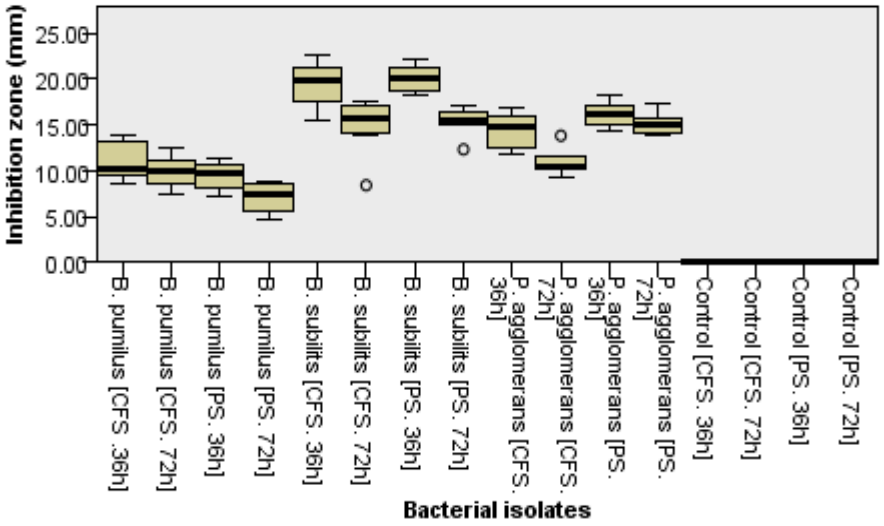
	Null Hypothesis	Test	Sig.	Decision
1	The medians of Inhibition zone (mm) are the same across categories of Bacterial isolates .	Independent-Samples Median Test	.000	Reject the null hypothesis.
2	The distribution of Inhibition zone (mm) is the same across categories of Bacterial isolates .	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Pairwise Comparisons of Bacterial isolates



Independent-Samples Kruskal-Wallis Test



Total N	128
Test Statistic	117.995
Degrees of Freedom	15
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

Data analysis of figure 2A

One way ANOVA comparison of inhibition zone (mm) between *P. brasiliense* isolates.

ANOVA

izmm

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2528.889	2	1264.444	343.388	.000
Within Groups	99.421	27	3.682		
Total	2628.310	29			

Multiple Comparisons

Dependent Variable: izmm

	(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Bonferroni	P. brasiliense [MT86]	P. brasiliense [MT87]	2.89000*	.85817	.007	.6996	5.0804
		Control	20.76000*	.85817	.000	18.5696	22.9504
	P. brasiliense [MT87]	P. brasiliense [MT86]	-2.89000*	.85817	.007	-5.0804	-.6996
		Control	17.87000*	.85817	.000	15.6796	20.0604
	Control	P. brasiliense [MT86]	-20.76000*	.85817	.000	-22.9504	-18.5696
		P. brasiliense [MT87]	-17.87000*	.85817	.000	-20.0604	-15.6796
Dunnett T3	P. brasiliense [MT86]	P. brasiliense [MT87]	2.89000*	1.04725	.040	.1206	5.6594
		Control	20.76000*	.85565	.000	18.3017	23.2183
	P. brasiliense [MT87]	P. brasiliense [MT86]	-2.89000*	1.04725	.040	-5.6594	-.1206
		Control	17.87000*	.61685	.000	16.1046	19.6354
	Control	P. brasiliense [MT86]	-20.76000*	.85565	.000	-23.2183	-18.3017
		P. brasiliense [MT87]	-17.87000*	.61685	.000	-19.6354	-16.1046
Games-Howell	P. brasiliense [MT86]	P. brasiliense [MT87]	2.89000*	1.04725	.035	.1930	5.5870
		Control	20.76000*	.85565	.000	18.3805	23.1395
	P. brasiliense [MT87]	P. brasiliense [MT86]	-2.89000*	1.04725	.035	-5.5870	-.1930
		Control	17.87000*	.61685	.000	16.1607	19.5793
	Control	P. brasiliense [MT86]	-20.76000*	.85565	.000	-23.1395	-18.3805
		P. brasiliense [MT87]	-17.87000*	.61685	.000	-19.5793	-16.1607

*. The mean difference is significant at the 0.05 level.

5.3.2 Appendices (Chapter IV)

Data analysis of figure 5

Data analysis of induced defence related genes in 'Cima di mola' by *B. subtilis*

MDH

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?			
Control vs. T4	-2.034	-3.246 to -0.8216	Yes	**	0.0054	B T4
Control vs. T8	-3.296	-4.509 to -2.084	Yes	***	0.0004	C T8

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. T4	1.020	3.054	-2.034	0.4235	3	3	4.803	6
Control vs. T8	1.020	4.316	-3.296	0.4235	3	3	7.784	6

.....

SA-BP

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?			
Control vs. T4	-0.3813	-1.038 to 0.2749	No	ns	0.2419	B T4
Control vs. T8	-1.234	-1.890 to -0.5775	Yes	**	0.0030	C T8

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. T4	0.9600	1.341	-0.3813	0.2292	3	3	1.664	6
Control vs. T8	0.9600	2.194	-1.234	0.2292	3	3	5.383	6

.....
..

GRAS1

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?			
Control vs. T4	0.2741	-0.5209 to 1.069	No	ns	0.5458	B T4
Control vs. T8	-0.8841	-1.679 to -0.08913	Yes	*	0.0333	C T8

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. T4	1.107	0.8326	0.2741	0.2777	3	3	0.9871	6
Control vs. T8	1.107	1.991	-0.8841	0.2777	3	3	3.184	6

.....
..

PAL									
Dunnett's multiple comparisons test			Mean Diff.	95.00% CI of diff.		Sig-nificant?			
Control	vs.	T4	-1.090	-1.525 to -0.6556		Yes	***	0.0007	B	T4
Control	vs.	T8	-0.7954	-1.230 to -0.3606		Yes	**	0.0035	C	T8
Test details			Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control	vs.	T4	1.030	2.120	-1.090	0.1518	3	3	7.181	6
Control	vs.	T8	1.030	1.825	-0.7954	0.1518	3	3	5.238	6
.....										
WRKY5										
Dunnett's multiple comparisons test			Mean Diff.	95.00% CI of diff.		Sig-nificant?				
Control	vs.	T4	-1.989	-3.659 to -0.3197		Yes	*	0.0252	B	T4
Control	vs.	T8	-3.346	-5.016 to -1.677		Yes	**	0.0022	C	T8
Test details			Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control	vs.	T4	1.003	2.993	-1.989	0.5832	3	3	3.411	6
Control	vs.	T8	1.003	4.350	-3.346	0.5832	3	3	5.738	6
.....										
ACTIN										
Dunnett's multiple comparisons test			Mean Diff.	95.00% CI of diff.		Sig-nificant?				
Control	vs.	T4	-0.04000	-0.09108 to 0.01108		No	ns	0.1127	B	
Control	vs.	T8	-0.03033	-0.08141 to 0.02075		No	ns	0.2308	C	
Test details			Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control	vs.	T41.000	1.040	-0.04000		0.01784	3	3	2.242	6
Control	vs.	T81.000	1.030	-0.03033		0.01784	3	3	1.700	6

Data analysis of induced defence related genes in 'Leccino' by *B. subtilis*								
.....								
MDH								
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Signifi-cant?					
Control vs. T4	-2.263	-3.248 to -1.277	Yes	**	0.0011	B	T4	
Control vs. T8	-4.846	-5.831 to -3.861	Yes	****	<0.0001	C	T8	
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. T4	1.041	3.303	-2.263	0.3441	3	3	6.576	6
Control vs. T8	1.041	5.887	-4.846	0.3441	3	3	14.08	6
.....								
SA-BP								
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Signifi-cant?					
Control vs. T4	-1.045	-1.710 to -0.3800	Yes	**	0.0073	B	T4	
Control vs. T8	-0.4818	-1.147 to 0.1831	No	ns	0.1408	C	T8	
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. T4	0.9992	2.044	-1.045	0.2322	3	3	4.500	6
Control vs. T8	0.9992	1.481	-0.4818	0.2322	3	3	2.075	6
.....								
GRAS1								
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Signifi-cant?					
Control vs. T4	-0.4353	-1.614 to 0.7432	No	ns	0.5057	B	T4	
Control vs. T8	-1.289	-2.467 to -0.1102	Yes	*	0.0356	C	T8	

Test details		Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control	vs. T4	0.9880	1.423	-0.4353	0.4116	3	3	1.058	6
Control	vs. T8	0.9880	2.277	-1.289	0.4116	3	3	3.131	6
.....									
PAL									
Dunnett's multiple comparisons test				Mean Diff.	95.00% CI of diff.		Signifi-cant?		
Control	vs. T4	-0.9316	-1.612 to -0.2510		Yes	*	0.0139	B	T4
Control	vs. T8	-1.098	-1.779 to -0.4173		Yes	**	0.0065	C	T8
Test details		Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control	vs. T4	1.207	2.138	-0.9316	0.2377	3	3	3.919	6
Control	vs. T8	1.207	2.305	-1.098	0.2377	3	3	4.619	6
..... **WRKY5**									
Dunnett's multiple comparisons test				Mean Diff.	95.00% CI of diff.		Signifi-cant?		
Control	vs. T4	-1.592	-2.652 to -0.5330		Yes	**	0.0091	B	T4
Control	vs. T8	-4.075	-5.135 to -3.016		Yes	****	<0.0001	C	T8
Test details		Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control	vs. T4	1.207	2.799	-1.592	0.3700	3	3	4.303	6
Control	vs. T8	1.207	5.282	-4.075	0.3700	3	3	11.01	6
.....									
ACTIN									
Dunnett's multiple comparisons test				Mean Diff.	95.00% CI of diff.		Signifi-cant?		
Control	vs. T4	-0.01000	-0.3042 to 0.2842		No	ns	0.9931	B	T4
Control	vs. T8	0.02333	-0.2708 to 0.3175		No	ns	0.9632	C	T8
Test details		Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF

Control	vs. T4	1.077	1.087	-0.01000	0.1027	3	3	0.09733	6	
Control	vs. T8	1.077	1.053	0.02333	0.1027	3	3	0.2271	6	

Data analysis of figure 6

One way ANOVA anaylis of induced defence related genes in 'Cima di mola' by *C. flaccumfaciens*									
.....									
MDH									
Dunnett's multiple comparisons test		Mean Diff.	95.00% CI of diff.		Signifi-cant?				
Control	vs. T4	-3.967	-5.854 to -2.079		Yes	**	0.0017	B	T4
Control	vs. T8	-1.925	-3.813 to -0.03752		Yes	*	0.0465	C	T8
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF	
Control	vs. T4	1.020	4.987	-3.967	0.6593	3	3	6.017	6
Control	vs. T8	1.020	2.945	-1.925	0.6593	3	3	2.920	6
.....									
SA-BP									
Dunnett's multiple comparisons test		Mean Diff.	95.00% CI of diff.		Signifi-cant?				
Control	vs. T4	-0.3813	-1.038 to 0.2749		No	ns	0.2419	B	T4
Control	vs. T8	-1.234	-1.890 to -0.5775		Yes	**	0.0030	C	T8
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF	
Control	vs. T4	0.9600	1.341	-0.3813	0.2292	3	3	1.664	6
Control	vs. T8	0.9600	2.194	-1.234	0.2292	3	3	5.383	6
.....									
GRAS1									
Dunnett's multiple comparisons test		Mean Diff.	95.00% CI of diff.		Signifi-cant?				
Control	vs. T4	-0.5568	-1.200 to 0.08684		No	ns	0.0826	B	T4
Control	vs. T8	-1.205	-1.848 to -0.5610		Yes	**	0.0031	C	T8

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. T4	1.107	1.663	-0.5568	0.2248	3	3	2.477	6
Control vs. T8	1.107	2.311	-1.205	0.2248	3	3	5.359	6

..... **PAL**

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?			
Control vs. T4	-0.1975	-0.7683 to 0.3733	No	ns	0.5438	B T4
Control vs. T8	-0.9045	-1.475 to -0.3336	Yes	**	0.0071	C T8

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. T4	1.030	1.227	-0.1975	0.1994	3	3	0.9905	6
Control vs. T8	1.030	1.934	-0.9045	0.1994	3	3	4.536	6

..... **WRKY5**

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?			
Control vs. T4	-0.4504	-1.346 to 0.4457	No	ns	0.3221	B T4
Control vs. T8	-1.448	-2.344 to -0.5521	Yes	**	0.0064	C T8

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. T4	1.003	1.454	-0.4504	0.3130	3	3	1.439	6
Control vs. T8	1.003	2.451	-1.448	0.3130	3	3	4.627	6

..... **ACTIN**

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?			
Control vs. T4	-0.04000	-0.09108 to 0.01108	No	ns	0.1127	B T4
Control vs. T8	-0.03033	-0.08141 to 0.02075	No	ns	0.2308	C T8

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. T4	1.000	1.040	-0.04000	0.01784	3	3	2.242	6
Control vs. T8	1.000	1.030	-0.03033	0.01784	3	3	1.700	6

One way ANOVA anaylis of induced defence related genes in ‘Leccino’ by *C. flaccumfaciens*

.....

....MDH

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Signifi-cant?			
Control vs. T4	-3.690	-5.311 to -2.068	Yes	**	0.0011	B T4
Control vs. T8	-2.419	-4.040 to -0.7974	Yes	**	0.0094	C T8

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. T4	1.041	4.730	-3.690	0.5663	3	3	6.515	6
Control vs. T8	1.041	3.460	-2.419	0.5663	3	3	4.271	6

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SA-BP

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Signifi-cant?			
Control vs. T4	-0.8911	-1.388 to -0.3937	Yes	**	0.0039	B T4
Control vs. T8	-1.568	-2.066 to -1.071	Yes	***	0.0002	C T8

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. T4	0.9992	1.890	-0.8911	0.1737	3	3	5.129	6
Control vs. T8	0.9992	2.567	-1.568	0.1737	3	3	9.027	6

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GRAS1

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Signifi-cant?			
Control vs. T4	-2.314	-3.789 to -0.8401	Yes	**	0.0074	B T4
Control vs. T8	-0.8191	-2.293 to 0.6553	No	ns	0.2658	C T8

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. T4	0.9880	3.302	-2.314	0.5150	3	3	4.494	6
Control vs. T8	0.9880	1.807	-0.8191	0.5150	3	3	1.591	6

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PAL

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Signifi-cant?			
Control vs. T4	-0.4633	-1.956 to 1.029	No	ns	0.6042	B T4
Control vs. T8	-1.777	-3.269 to -0.2845	Yes	*	0.0253	C T8

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. T4	1.019	1.483	-0.4633	0.5213	3	3	0.8888	6
Control vs. T8	1.019	2.796	-1.777	0.5213	3	3	3.409	6

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WRKY5

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Signifi-cant?			
Control vs. T4	-0.5209	-1.149 to 0.1077	No	ns	0.0948	B T4
Control vs. T8	-1.431	-2.059 to -0.8022	Yes	**	0.0011	C T8

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. T4	1.207	1.728	-0.5209	0.2195	3	3	2.373	6
Control vs. T8	1.207	2.637	-1.431	0.2195	3	3	6.517	6

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ACTIN

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Signifi-cant?			
Control vs. T4	-0.01000	-0.3042 to 0.2842	No	ns	0.9931	B T4
Control vs. T8	0.02333	-0.2708 to 0.3175	No	ns	0.9632	C T8

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. T4	1.077	1.087	-0.01000	0.1027	3	3	0.09733	6
Control vs. T8	1.077	1.053	0.02333	0.1027	3	3	0.2271	6