UNIVERSITÀ DEGLI STUDI DEL MOLISE

Department of Agricultural, Environmental and Food Sciences

PhD Course in:

AGRICULTURE TECHNOLOGIES AND BIOTECHNOLOGIES
(CURRICULUM: SUSTAINABLE PLANT PRODUCTION AND PROTECION)

CYCLE (XXXIV)

Related Disciplinary Scientific Sector:
AGR/12 (Plant Pathology)

PhD Thesis

Selection, evaluation, and characterization of bioactive compounds for their activity against *Xylella fastidiosa* and other phytopathogenic bacteria

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Academic Year 2020/2021

Riassunto

L'interesse per la ricerca di nuove sostanze attive utilizzabili per il controllo dei batteri fitopatogeni è una delle più grandi sfide che riguardano le tendenze moderne in materia di protezione delle piante giacché contro questi agenti patogeni mancano mezzi di controllo efficaci. In particolare, contro *Xylella fastidiosa pauca*, l'agente causale del disseccamento rapido dell'olivo (OQDS), il cui primo focolaio in Europa si è verificato nel 2013 sugli ulivi della Penisola Salentina, è urgente trovare misure di controllo efficaci. Le misure di quarantena e sanificazione, il controllo degli insetti vettori insieme alle azioni politiche non sono state in grado di arrestare la diffusione del batterio e dei suoi insetti vettori. In questo scenario risulta oggi importante trovare una strategia per controllare il patogeno e salvare il patrimonio olivicolo. Nel presente lavoro nuove sostanze bioattive e sostenibili sono state valutate e selezionate per la loro attività antimicrobica contro *X. fastidiosa* pauca e altri batteri patogeni delle piante. Nella prima parte della ricerca, i prodotti selezionati sono stati saggiati contro 13 dei più importanti e diffusi batteri fitopatogeni, determinando la concentrazione minima inibitoria (MIC) per ciascun batterio e per ciascun prodotto. Molti dei prodotti testati hanno evidenziato una forte attività antibatterica contro la maggior parte dei batteri fitopatogeni, mostrando un'attività simile e talvolta anche migliore rispetto agli antibiotici usati come controllo. La seconda parte dell'attività si è concentrata sulla valutazione dei prodotti selezionati nei confronti di *X. fastidiosa* subsp. *pauca* (*Xfp*). A partire dai precedenti esperimenti, i prodotti sono stati saggiati *in vitro* per valutare l'attività battericida contro *Xfp* utilizzando il time-kill assay. Successivi esperimenti sono stati condotti su piante di olivo in vaso mantenute in serra in condizioni controllate per valutare la capacità dei prodotti più promettenti di contrastare *Xfp*. La maggior parte dei prodotti saggiati ha mostrato un elevato effetto battericida *in vitro* e nelle prove *in planta*, alcuni prodotti somministrati per via fogliare hanno significativamente ridotto la presenza i sintomi sulle piante trattate. Inoltre, alla fine degli esperimenti, sulle piante trattate, è stata anche osservata una significativa riduzione della popolazione batterica rispetto alle condizioni di partenza. Tuttavia, a seguito dell'interruzione dei trattamenti, le piante hanno subito un rapido decadimento arrivando successivamente a morte. Nel complesso, i risultati ottenuti nella presente ricerca sono positivi e attestano l'elevata efficacia e l'ampio spettro di attività della maggior parte dei prodotti saggiati, in quanto capaci di inibire la crescita di diverse specie di batteri fitopatogeni, ivi inclusi diversi ceppi e sottospecie di *X. fastidiosa* (*Xf*). Essendo la maggior parte dei prodotti testati sistemici o citotropici, essi risultano molto

interessanti in quanto oltre ad una inibizione diretta dei patogeni batterici, tali prodotti possono indurre risposte di difesa nella pianta e controllare anche batteri endofiti, come ad esempio *Xf*, che colonizzano i tessuti vascolari delle piante ospiti. Ulteriori esperimenti *in vivo*, su piante in vaso o meglio in campo saranno necessari per confermare i risultati positivi ottenuti nella presente ricerca.

Summary

The interest for the search of new active compounds for controlling plant pathogenic bacteria is a big challenge in a modern trend concerning plant protection since against these pathogens there is a lack of effective control means. In particular, against *Xylella fastidiosa pauca,* the causal agent of the Olive Quick Decline Syndrome (OQDS), whose first outbreak in Europe occurred in 2013 on the olive trees of the Salento Peninsula, there is an urgent need to find effective control measures. Against this bacterium, quarantine and sanitation measures, control of insect vectors as well as political actions are not able to arrest the spread of the disease(Morelli et al., 2021). Therefore, it is important to find a strategy to control the pathogen and to save the olive-growing heritage. In this work, new bioactive and sustainable substances were evaluated and selected for their antimicrobial activity against *X. fastidiosa pauca* as well as other plant pathogenic bacteria. In the first part of the research, selected products were tested against 13 widespread and important phytopathogenic bacteria determining the minimum inhibitory concentration (MIC) of each product against each bacterium. Many of the tested products evidenced a strong antibacterial activity against most of the tested phytopathogenic bacteria, and the activity was often similar and sometimes even better than standard antibiotics used as control. The second part of the work focused on the evaluation of the selected products against *X. fastidiosa* subsp. *pauca* (*Xfp*), strain ST53 responsible of OQDS. Starting from previous experiments, *in vitro* assays were assessed to evaluate bactericidal activity against *Xfp* by the time-kill assay. Furthermore, experiments on pot grown olive plants kept in green-house conditions were carried out to evaluate the capability of the most promising compounds to control the disease caused by *Xfp*. Most of the tested products showed bactericidal effect on the bacterium *in vitro* and *in planta* experiments, some of them applied by foliar treatments were able to significantly reduce the presence of *Xfp* symptoms. In addition, on treated plants a significant reduction of the bacterial population was observed at the end of the experiments compared to the starting conditions. However, once the treatments were stopped, the plants underwent a rapid decay that led to their death. Collectively, results of experiments reported in the present thesis are positive and attested the large spectrum capability of most of the tested compounds to inhibit the growth of different species of widespread and dangerous phytopathogenic bacteria, even including different strains and subspecies of *X. fastidiosa* (*Xf*). Since most of the tested products are systemic or cytotropic, they are very interesting being able to directly inhibit endophytic bacteria, such as *Xf* which colonizes the vascular tissues of host plants, as

well as inducing defence responses in plant. Further experiments *in vivo*, on pot grown plants or rather in the field are necessary to confirm the positive result obtained in the present research.

Contents

1. INTRODUCTION

1.1. Biological Control of Plant Pathogenic Bacteria

Biological control of plant pathogens is a reduction of inoculum or disease producing activity of a pathogen accomplished by one or more organisms (Cook & Baker, 1983). Here, the term biological control is used in the broader sense. In the twenty-first century, high crop yields are achieved through heavy use of synthetic pesticides and fertilizers, and through new cultivars grown in monoculture. Conventional control methods for plant pathogenic bacteria contain avoidant, exclusive, eradicative, protective, resistance, and therapeutic applications (Bastas & Kannan, 2015). All these methods can prevent disease according to i) the time, the year and the location of an area; ii) the preventive measures (attempt on the introduction of inoculum, elimination, destruction, or inactivation of the inoculum); iii) utilization of cultivars resistant or tolerant to the bacteria; iv) the cure of already infected plants. The success of plant disease control by synthetic chemicals created a general perception that chemical control could provide a permanent solution to disease problems in modern agriculture (Huang & Wu, 2009). The term "sustainable agriculture" has become a norm in modern agriculture (Thind, 2019). Environmental and ecological issues continue to have an impact on agriculture, and, for this, all technologies developed for agricultural production must be economically and ecologically sustainable, and safe for the environment, farmers and consumers. Numerous non-chemical methods for the control of crop diseases, such as healthy plants propagation materials, disease-resistant varieties, crop rotation, application of naturals antimicrobials (plant extracts, microbial extracts, minerals organic amendments, and biocontrol agents) are considered less harmful than synthetic chemical pesticides and, therefore, offer great potential for application in agriculture. No method alone can provide complete control of crop diseases. The integration of all effective and eco-compatible measures with a dynamic management of the agro-ecosystem is the best strategy for the control of diseases in crops. In this era of measures aimed at the sustainability of agriculture and the environment, research on energy saving and environmentally friendly methods for the sustainable management of crop diseases is a priority and a great challenge. In the sustainability era, all practical solutions to plant disease control must be based on environmental safety, natural resource conservation, and biodiversity maintenance (Bastas & Kannan, 2015).

1.2. *Xylella fastidiosa* subsp. *pauca* strain ST53

Xylella fastidiosa Wells et al., (1987) is a Gram-negative bacterium whose are rodshaped cells, 1.0-4.0 x 0.25-0.50 μm, with a characteristic rippled cell wall, and without flagella (Wells et al., 1987). The bacterium, belonging to the *Xanthomonadaceae* family, is the only species of the genus. It has been well documented for its worldwide spread and infection of a broad range of plant species (infecting more than 300 plant species), and in several cases without causing symptoms (EFSA, 2021). Different subspecies of *X. fastidiosa* are known, which are described mainly based on the phylogenetic relationship and specificity host plants (Nunney et al., 2014). The current classification of the bacterium species is based on three taxonomically valid *X. fastidiosa* subspecies (*fastidiosa*, *multiplex*, and *pauca*), that are generally accepted by the scientific community to be the main grouping, although there is no type strain available for subspecies *pauca* in the public databases (Marcelletti & Scortichini, 2016). Proposals also exist for the establishment of the subspecies *sandyii*, *morus*, and *tashke* (Burbank & Ortega, 2018), and the taxonomy of these bacteria is continuously evolving due to the fast acquisition of new genomic information and strain distinction by multilocus sequence typing (Maiden et al., 1998).

X. fastidiosa, is a xylem-limited bacterium, lives and reproduces in the xylem vessels of the host plants and also in the mouth of its insect vectors (Agrios, 2005). It is a very slow-growing bacterium, difficult to isolate from xylem vessels and to be cultivated *in vitro* due to its non-uniform distribution within the plant, and the natural tendency to form biofilms and cell aggregates (Campanharo et al., 2003). This bacterium infects numerous host plant species, on which it can be latent or induce from mild to severe symptoms. The main diseases caused include Pierce's disease of grapevine (*Vitis vinifera* L.) (Davis et al., 1978; Su et al., 2013), citrus variegated chlorosis (Chang et al., 1993), coffee leaf scorch (Li et al., 2001), pecan leaf scorch (Sanderlin & Heyderich-Alger, 2000), phony peach (Wells, 1983), plum leaf scald (Raju, 1982), and almond leaf scorch (Mircetich et al., 1976). *X. fastidiosa* has also been known to be the causal agent of leaf scorch diseases in landscape plants such as oleander (Purcell et al., 1999), mulberry (Hernandez-Martinez et al., 2006) and oak (Barnard et al., 1998).

Among the new *X. fastidiosa* outbreak during recent years a devastating disease infecting olive plants, with severe symptoms was first described in 2013 (Saponari et al., 2013 and 2017), in the Salento aerea of Apulia, Italy. The causal agent, *X. fastidiosa* subsp. *pauca*

strain “De Donno” ST53 (Giampetruzzi et al., 2017; D’Attoma et al., 2020), spread rapidly since its main vector *Philaenus spumarius* L. (*Hemiptera*: *Aphrophoridae*) is a widespread and very efficient xylem sap-feeding insect (Cornara et al., 2017). The conditions that favoured the spread of *X. fastidiosa* in Italy are favourable to the vector, the extensive monocultures of two susceptible native olive cultivars (Cellina di Nardò and Ogliarola salentina) and the number of infected plants *X. fastidiosa* (Luvisi et al., 2017). Since its first detection, *X. fastidiosa* subspecies *pauca* (*Xfp*) has infected about 4 million trees in the outbreak area (Schneider et al., 2020), causing huge economic losses in olive growing, as well as dramatic changes in the Mediterranean landscape, where olive trees play a key role in cultural heritage and an important element in the tourism sector.

Genetic analysis of the *Xfp* “De Donno” strain suggest that this pathogenic strain has been introduced and it is not native from the Mediterranean region. In fact, the phylogenetic analysis indicates that the strain is closely related to a strain of *X. fastidiosa* from Costa Rica, which is capable of infecting oleander (*Nerium oleander* L.) and coffee (*Coffea* spp.). Based on this information, it is suspected that the introduction of *X. fastidiosa* in the Mediterranean region is due to the import of ornamental plants (Giampetruzzi et al., 2017; Loconsole et al., 2016). Furthermore, according to some studies conducted on the genomic diversity of different subspecies of *X. fastidiosa*, the introduction of the olive strain ST53 in Puglia was dated to 2008 (Vanhove et al., 2022).

As in other outbreaks of *X. fastidiosa* (such as Pierce's disease and citrus variegated chlorosis), the current approach to manage the disease is based on control activities that integrate agronomic interventions (tillage and elimination of weeds), chemicals, and measures aimed at reducing vector populations, including also the elimination of the inoculum sources; however, such measures to date have not proved sufficient to control the spread of the disease. The search for a cure for plants infected with *X. fastidiosa* is a continuous process to which not only academics, professionals, and growers, but also ordinary citizens contribute continuously (for example by searching for spontaneous olive seedlings that have survived to infection with *Xfp* in the infected area). Due to the severity of the disease and the economic importance of susceptible crops, finding a solution is more than necessary to revive the olive sector, which is strongly rooted in the Apulian culture (Morelli et al., 2021).

1.3. Perspective of control strategies against *X. fastisiosa pauca*

The risk of outbreaks of bacterial plant diseases is constantly increasing due to global trade in plant materials; but unfortunately, current control strategies are often unsatisfactory. Development of new control strategies is of the utmost urgency; basic research on plant pathogenic bacterium interactions, especially at a molecular level, can substantially contribute to the attainment of this goal (Buonaurio, 2008). As just mentioned for the control of the plant pathogenic bacteria, there are not many weapons to counteract these pathogens. Gilbert et al. (2010) reported that the lack of chemicals for restraining bacterial diseases in orchards creates a need for alternative methods such as approaches based on prevention, biological control, and plant resistance. Disadvantages of chemical applications include accumulation of chemical residues in vegetal products and high costs; furthermore, emergence of resistant bacterial strains have been reported, for example to antibiotics and copper (Jones & Jones, 1985; Ritchie & Dittapongpitch, 1991; Stall et al., 1986). In this scenario, concerning biocontrol tentative of *Xylella fastidiosa*, Morelli et al., (2021) reviewed an extensive literature of the approaches that have been taken, including *in vitro* studies and the application *in vivo* of potential treatment solutions directly to affected olive trees. From this relevant review, that completely describe the scenario and the status of research activities to control *X. fastidiosa,* researchers have produced a growing body of literature on the attempts to control the pathogen, in addition to government measures, through the application of different treatments (Saponari & Boscia, 2019).

The different studies analysed are based on different control approaches, which in turn involve mineral formulations, chemical compounds, natural products, and microbial antagonists. These control strategies, considered in a context of integrated management of the bacterium and the insect vectors, combined with the capability of some compounds to inducing resistance in plants, could prove to be effective in controlling the pathogen, or at least allowing coexistence, with less economic impact and landscape losses. Most of these studies are at the preliminary stage, although in the absence of an effective strategy, some of these attempts appear to be at least promising.

1.3.1. Minerals and other compounds

Regarding minerals and other compounds some *in vitro* studies (Cruz et al., 2012; Cobine et al., 2013; Navarrete & De La Fuente, 2015) showed that alterations of mineral homeostasis (e.g., zinc, copper, and calcium ion) may have a significant effects on *X. fastidiosa,* affecting relevant biological features (such as biofilm formation, growth rate, and possibly interfering with the expression of its virulence traits in the host tissues). Recently, in this scenario, plant ionome investigation were carried out to investigate how the ionome, i.e., the relative content of mineral elements found in a specific tissue (Salt et al., 2008), could interfere with the expression of symptoms caused by *X. fastidiosa*. D'Attoma, et al. (2019a; 2019b), for example, provided evidence that higher contents of calcium and manganese may contribute to traits of resistance in olive plants cultivar Leccino. Del Cocco et al. (2020), by data collected from a parallel approach that relied on a metabolomic analysis to reveal substantial changes in the metabolic profiles of olive cultivars sensitive to *X. fastidiosa* (Girelli et al., 2017), studied the perturbation of the ionomic profile of the leaves of *X. fastidiosa* infected trees treated (via foliar spray) with a zinc-copper-citric acid biocomplex (Dentamet®). Scortichini et al. (2018) reported that Dentamet® foliar spray treatments, on the olive canopy, were able to reduce the severity of the disease associated with *X. fastidiosa*; however, the narrow time frame of the application and the limited number of observations did not allow conclusive evidence of the complete eradication of the pathogen. Tatulli et al. (2021) in a field mid-term evaluation of the control capability of Dentamet® foliar treatments, veiled that the bacterial concentration tended to decrease in trees regularly sprayed with the biocomplex over 3–4 years.

Other mineral solution were also tested in Italy for evaluating their potentially activity against *X. fastidiosa* aside zinc and copper. Dongiovanni et al. (2021) sprayed OQDS-affected trees with ammonium chloride, detecting clear symptom reductions after treatments, although no significant differences in the bacterial populations were observed. Recently, metal nanooxides have also been explored as carriers for the direct release of phytodrugs targeting *X. fastidiosa* in olive plants. Baldassarre et al. (2020) evidenced an alteration of the bacterial cell wall, by transmission electron microscopy observation, following the interactions with calcium carbonate nanocarriers, which were absorbed by the olive roots and successfully translocated to conductive tissues.

Among the most well-studied control strategies for *X. fastidiosa* the mucolytic cysteine analogue, the N-acetylcysteine (NAC), used mainly to treat human diseases (Hafez et al., 2009), had shown promising inhibitory effects on *X. fastidiosa* strain 9a5c and its associated disease in sweet orange plants (Muranaka et al., 2013). On the way of this experience, field trials were performed in Apulia to verify the NAC effect on OQDS (de Souza et al, 2019). In general, treatment with NAC seems to decrease disease progression, especially using NAC endotherapy; however, qPCR assays did not show any significant reduction in the bacterial population size. In *in vitro* experiments it was observed that sub-lethal concentrations of NAC had a significant effect on *X. fastidiosa* biofilm formation, inducing a hyper-attaching phenotype, with potential impacts on strain virulence and vector acquisition (Cattò et al., 2019).

Other experimental approaches used to evaluate the antibacterial activity against *X. fastidiosa* are those adopted by Baldassarre et al. (2020) who tested chitosan-coated fosethyl-aluminum nanocrystals and Moll et al. (2021) and Baró et al. (2020) who tested some antimicrobial peptides.

So far none of the mineral-based approaches have led to an effective control of *X. fastidiosa* and further studies are still needed to validate the effects obtained by the most promising products in reducing symptoms.

Consequently, no data exist regarding the development of *X. fastidiosa* resistance to the applied minerals or on potential effects on the olive microbiota. As many of these minerals or compounds have significant *in vitro* effects on the bacterium lifestyle or survival, future research trends should consider to optimize their delivery to better target *X. fastidiosa* in the xylem network (Morelli et al., 2021).

1.3.2. Plant- and microbial-derived compounds

Medicinal plants and microorganisms are rich sources of bioactive compounds. Although these sources are mainly used to discover medicines for humans, but their use in agriculture in recent years is playing an important role in the fight against plant pathogens (Bastas & Kannan, 2015).

Regarding *Xylella fastidiosa pauca* (*Xfp*) (Bleve et al., 2018), explored the use of natural products from plants or microorganisms, evaluating *in vitro* the antimicrobial activities of

different plant-derived phenolics compounds (4-methylcathecol, cathecol, veratric acid, caffeic acid, and oleuropein), of a filtered fractions of olive mill wastewaters (OMW), a crude extract of *Trichoderma* spp., and some fungal toxins. All phenolic compounds and fungal toxins tested showed inhibitory activity against *Xfp*, although limited to reversible bacteriostatic effects. The crude extract from a *Trichoderma citrinoviridae* strain culture showed bactericidal properties. It is also interesting to note that the addition of microfiltered OMW fractions in the growth medium affected the growth of *Xfp*.

Several other phenolic compounds have been evaluated *in vitro* for their potential use against *X. fastidiosa* some of these phenolic compounds have been found to be effective in inhibiting the growth of the bacterium. In particular, catechol, caffeic acid and resveratrol showed the highest inhibitory potential against the pathogen (Maddox et al., 2010). Similarly Lee et al. (2020) investigated the effect of biologically relevant concentrations of the phenolic compounds coumaric acid, gallic acid, epicatechin, and resveratrol on growth of *X. fastidiosa in vitro*. The authors reported that none of these compounds inhibited bacterial growth, but epicatechin and gallic acid reduced cell-surface adhesion. Cell-cell aggregation decreased with resveratrol treatment, but the other phenolic compounds tested had minimal effect on aggregation.

Regarding microbial derived compounds, another way that has aroused much interest concerns the biocontrol studies of *X. fastidiosa* and its plant symptoms associated with the complex mechanisms of quorum sensing. This aspect has been extensively investigated in grapevine and citrus plants (Lindow et al., 2014; Caserta et al., 2017). The lifecycle of *X. fastidiosa* proved to be finely regulated by a complex metabolic pathway regulated by a family of short-chain fatty acid molecules known as diffusible signal factors (DSF) (Beaulieu et al., 2013; Chatterjee et al., 2008). Regarding the OQDS Vona et al. (2019) reported preliminary studies aiming to identify DSF molecules of *Xfp* and exploit strategies for modulating its biofilm formation.

Also, important to mention is the work of Clavijo-Coppens et al. (2021) that isolated from various sources (with a preliminary characterization) several phages active on different *Xylella fastidiosa* strains.

1.3.3. Biocontrol agents of *X. fastidiosa*

Non-pathogenic bacteria can improve plant performance, for example plant growth and development through various mechanisms such as improvement of plant nutrition, protection of plants from various pathogens, induction of plant host-defense mechanisms, and others (Panneerselvam et al., 2015). Among the modern trend available for disease-control strategies, the use of microbes is considered one of the best promising strategies.

In this context, it is well known that wide range of beneficial microorganisms are correlated to plants rhizosphere and phyllosphere (such as endophytes and ectophytes plant associate bacteria, plant growth promoting rhizobacteria, etc.). Some studies, thanks to the continuous evolution of molecular tools in plant pathology, reported that in some instances differences between susceptible and resistant/tolerant varieties for a disease have been correlated to the plant microbiome (Compant et al., 2021; Mitter et al., 2019).

Recently, Vergine et al. (2019) explored the microbiome associated with *X. fastidiosa*-infected and -uninfected olive trees in Salento and results show that the susceptible cultivar "Cellina di Nardò" showed a drastic dysbiosis after *X. fastidiosa* infection, while "Leccino" (both infected and uninfected) maintained a similar microbiota. Giampetruzzi et al. (2020) analysed the dynamics of endophytic microbiome during the infection of *Xylella fastidiosa* in field-grown plants, of the susceptible and resistant olive cultivars Kalamata and FS17. It was observed that the progression of the infections, revealed that *Xylella* tends to occupy the whole ecological niche suppressing the diversity of the endophytic microbiome. However, this trend was found to be reduced in the resistant cultivar FS17. Although differences in the microbiomes of susceptible versus resistant cultivars were observed, Zicca et al. (2020) did not find no one bacteria isolated from olive trees located in the area affected by *X. fastidiosa*, "De Donno" and showing effectiveness in inhibiting the pathogen.

Among other relevant studies, Baccari et al. (2018) reported that the known beneficial endophyte *Paraburkholderia phytofirmans* PsJN, isolated from onion roots (Compant et al., 2008; Sessitsch et al., 2005) was found to be effective in reducing severity of Pierce's disease symptoms and populations of *X. fastidiosa* Temecula1 in grapevine. The bacterium is known for its ability to colonize different plants (Compant et al., 2008; Mitter et al., 2013), and to stimulate their growth, protecting them from biotic and abiotic stresses (Mitter et al., 2013). On this way Morelli et al. (2019) in a preliminary trial, tested the effectiveness of PsJN as a biocontrol agent against *Xylella fastidiosa pauca* "De Donno" strain and the results not

revealed significant differences in the reduction of OQDS symptoms in therapeutic treatments, nor reduction of the new infections upon preventive applications. Many researches aimed to investigate the plant microbiome and microbial biocontrol of *Xylella* under field conditions are ongoing (Morelli et al., 2021), however, currently no validated microbiological formulations are available to combat the pathogen.

2. AIM OF THE RESEARCH

Since the first discovered outbreak of *Xylella fastidiosa* in Europe on olive trees (*Olea europaea*), in the Salento Peninsula (Saponari et al., 2013), the lack of effective control measures against the pathogen as well as in generally to control plant pathogenic bacteria, causes a rapid spread of the pathogen. Quarantine and sanitation measures, the control of the insect vectors and also political and scientific action are not able to arrest the spread of the bacterium and his insect vector (Morelli et al., 2021). In this scenario it is important to find a strategy to control the pathogen and to save the olive-growing heritage, that is the main objective of my thesis.

The aim of my work was to select and evaluate new sustainable bioactive substances for their antimicrobial activity against important and widespread plant pathogenic bacteria. The first step of the work was to choose the substances and the respective commercial formulations that in most of the cases were provided by private companies. As all the products were available in the laboratory, I started to evaluate their antimicrobial activities using firstly as model systems two bacterial strains stored in the microbial collection of the Plant Pathology laboratory, at the University of Molise.

Following, other objectives of my research were to enlarge the experiments by evaluating the selected products for their broad-spectrum antibacterial activities against 7 important phytopathogenic bacteria, and to assay the most promising products against 4 strains and subspecies of *Xylella fastidiosa*, with particular attention to *Xylella fastidiosa* subsp. *pauca* ST53 the strain responsible of the Olive Quick Decline Syndrome (OQDS) in Apulia. For all the tested products the minimum inhibitory concentration (MIC) was assessed in order to determine the dosage of use of each product to kill the bacterium *in vitro* and potentially control the pathogen *in vivo*. Furthermore, *in planta* experiments in green-house conditions were carried out to evaluate the capability of the most promising compounds to control the disease caused by *Xfp* on pot grown olive plants.

3. SCREENING OF BIOFERTILIZERS AND OTHER BIOACTIVE COMPOUNDS FOR THEIR ANTIMICROBIAL ACTIVITY

3.1. Materials and Methods

3.1.1. Bacterial strains and growth condition

Antibacterial activity: the antibacterial activity was preliminary analysed on non-quarantine plant pathogenic bacteria routinely used in our laboratory using two bacterial strains: i) *Pseudomonas syringae* pv. *tomato* strain DC3000 and ii) *Xanthomonas campestris* pv. *pelargoni.*

The strains were kindly provided from Dr Stefania Loreti (CRA-PAV, Rome) and were cultured on King's B Agar and YDC plates, respectively for 2 days at 28°C. Then, a single colony was sub-cultured in Nutrient Broth (NB) and incubate overnight at 28°C in a rotatory shaker incubator. The adjusted bacterial suspension (OD_{600}=0.5) was used as inoculum for all the experiments using Nutrient Agar (NA) as medium.

Antibacterial activity against some important quarantine and non-quarantine plant pathogenic bacteria: these assays were conducted at the Research Institute for Horticulture and Seeds (INRAE, Angers, France) laboratory's, using the following plants pathogenic bacteria: i) *Erwinia amylovora* strain CFBP 1430, ii) *Xanthomonas arboricola* pv. *pruni* strain CFBP 3903, iii) *Ralstonia solanacearum* strain CFBP 3671, iv) *Clavibacter michiganensis* subsp. *michiganensis* strain CFBP 6885, v) *Pseudomonas syringae* pv. *actinidiae* strain CFBP 8288, vi) *X. euvesicatoria* strain 9.2 CFBP 3274 and vii) *X. campestris* pv. *campestris* strain 8004.

The strains were taken from the bacterial collection of CIRM-Plant Associated Bacteria / CIRM-CFBP of the EmerSys research team (Emergence Systematics and Ecology of Plant-Associated Bacteria) of IRHS (Research Institute for Horticulture and Seeds), INRAE, Angers (France), and the experiments were also performed in the institute's laboratories. The strains were all cultured on Tryptone Soy Agar (TSA) for 2 days at 28°C, a single colony were sub-cultured in Tryptone Soy Broth (TSB) and incubated overnight at 28°C in a rotatory shaker incubator. For each strain, an adjusted bacterial suspension (OD_{600}=0.5) was used as inoculum for the experiments using Tryptone Soy Agar (TSA) as medium.

Antibacterial activity against quarantine *Xylella fastidiosa* subspecies and strains: these assays were performed at the Institute for Sustainable Plant Protection (IPSP-CNR) Unit of Bari, using the following strains and subspecies of *X. fastidiosa*: *i) X. fastidiosa* subsp. *pauca* strain ST53 (*Xfp*), ii) *X. fastidiosa* strain CO33 (*XfCO33*), iii) *X. fastidiosa* subsp. *multiplex* strain TOS1 (*XfmTOS1*), and iv) *X. fastidiosa* subsp. *multiplex* strain ESVL (*XfmESVL*).

The strains were taken from the bacterial collection of the institute, and all the experiments were performed in the institute's laboratories. The bacterial strains were taken from -80 cryovials, cultured on Buffered Charcoal Yeast Extract agar medium (BCYE) for 7 days at 28°C. Then, strains were sub-cultured for 10 days at 28°C, on Periwinkle wilt medium (PW) for *XfCO33* and *XfmESVL* and on Pierce's Disease no. 3 agar medium (PD3) for *Xfp* and *XfmTOS1*. The bacterial biomass was kept with a sterile 10 µL loop and suspended in 2 mL of 1% Phosphate-buffered saline (PBS) buffer, adjusting bacterial suspension to 0.5 OD_{600}, used as inoculum for all the experiments on the appropriate medium.

3.1.2. Media and buffer solutions

The liquid and agar media used in this study were prepared using deionized water and sterilized in autoclave at 1.2 atmospheres and 120°C for 20 minutes, or by filtration using filters with a porosity of 0.22µm in diameter. Solid culture media were poured into Petri dishes before solidification. Buffer solutions were prepared with RO ultrapure water and sterilized by filtration (0.22µm pore size, Millipore). Below are listed the media composition and recipe step by step.

King's B Agar (KBA): bacteriological peptone, 20 g; dipotassium phosphate (K_2HPO_4), 1.5g; magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), 1.5g; glycerol, 10mL; bacteriological agar, 16g; distilled water, 1L. The medium was stirred for 5 minutes on a magnetic stirrer and autoclaved at 1.2 atmospheres, 120°C for 20 minutes.

Yeast Dextrose Calcium Carbonate Agar (YDC): dextrose, 20g; yeast extract, 10g; calcium carbonate ($CaCO_3$), 20g; bacteriological agar, 20g; distilled water, 1L. The medium was stirred for 5 minutes on a magnetic stirrer and autoclaved at 1.2 atmospheres, 120°C for 20 minutes.

Nutrient Broth (NB): beef extract, 1g; yeast extract, 2g; peptone, 5g; sodium chloride (NaCl), 5g; distilled water, 1L. The medium was stirred for 5 minutes on a magnetic stirrer and autoclaved at 1.2 atmospheres, 120°C for 20 minutes.

Nutrient Agar (NA): beef extract, 1g; yeast extract, 2g; peptone, 5g; sodium chloride (NaCl), 5g; bacteriological agar, 16g; distilled water, 1L. The medium was stirred for 5 minutes on a magnetic stirrer and autoclaved at 1.2 atmospheres, 120°C for 20 minutes.

Tryptone Soy Broth (TSB): casein (pancreatic digest), 17g; soya peptone (papaic digest), 3g; sodium chloride (NaCl), 5g; dipotassium phosphate (K_2HPO_4), 2.5g; dextrose 2.5g; distilled water, 1L. The medium was stirred for 5 minutes on a magnetic stirrer and autoclaved at 1.2 atmospheres, 120°C for 20 minutes.

Tryptone Soy Agar (TSA): casein (pancreatic digest), 17g; soya peptone (papaic digest), 3g; sodium chloride (NaCl), 5g; dipotassium phosphate (K_2HPO_4), 2.5g; dextrose 2.5g; bacteriological agar 16g; distilled water, 1L. The medium was stirred for 5 minutes on a magnetic stirrer and autoclaved at 1.2 atmospheres, 120°C for 20 minutes.

Pierce's Disease no. 3 agar medium (PD3): pancreatic digest of casein (tryptone [Difco] or trypticase peptone [BBL]), 4g; papaic digest of soy meal (soytone [Difco] or phytone peptone [BBL]), 2g; trisodium citrate ($Na_3C_6H_5O_7$), 1g; disodium succinate, 1g; hemin chloride stock (0.1 % bovine hemin chloride dissolved in 0.05 N NaOH), 10 ml; magnesium sulphate heptahydrate ($MgSO•7H_2O$), 1g; dipotassium phosphate (K_2HPO_4), 1.5g; potassium dihydrogen phosphate (KH_2PO_4), 1g; potato starch, 2g; BactoAgar, 18g; distilled water, 1L. The medium was stirred for 5 minutes on a magnetic stirrer and autoclaved at 1.2 atmospheres, 120°C for 20 minutes.

Buffered Charcoal Yeast Extract agar medium (BCYE): yeast extract, 10g; activated charcoal, 2.0g; L-cysteine $HCl•H_2O$, 0.4g; ferric pyrophosphate, soluble, 0.25g; agar, 17.0g; and ACES buffer (Sigma), 10g in 1L distilled water.

ACES buffer was first hydrated in 500ml of distilled water at 50°C. The buffer was then mixed with a solution containing ca. 40ml of 1.0 N KOH in 440mL of distilled water. This mixture was used to hydrate the charcoal, yeast extract, and agar and was then autoclaved at 1.2 atmospheres, 120°C for 20 minutes; finally, the mixture was cooled and equilibrated at 50°C. L-Cysteine hydrochloride and soluble ferric pyrophosphate were dissolved together in 20 ml of water, sterilized through a 0.25 µm-pore size membrane filter, and then added to the mixture. The pH of the final solid medium was 6.9 at 25°C. The amount of KOH used was varied to adjust or change pH (Wells et al., 1981).

Periwinkle wilt medium (PW): phytone peptone, 4.0g; trypticase peptone, 1.0g; dipotassium phosphate (K_2HPO_4), 1.2g; hemin chloride stock (0.1% bovine heroin chloride [Sigma Chemical Co., St. Louis, Missouri] in 0.05 N NaOH), 10ml; potassium dihydrogen phosphate (KH_2PO_4), 1.0g; BactoAgar, 16.0g; magnesium sulphate heptahydrate ($MgSO_4•7H_2O$), 0.4g; phenol red stock (0.2% phenol red in distilled water), 10ml; glutamine stock (8.0% glutamine-free base, Sigma; in distilled water), 50ml; bovine serum albumin fraction-five (BSA) stock (20% bovine albumin, Sigma, no. A4503; in distilled water), 30ml; distilled water, total volume 1,000ml. All ingredients except BSA and glutamine were added, mixed, and dissolved in the order given. The basal medium was autoclaved at 1.2 atmospheres, 120°C for 20 minutes and finally cooled at 50°C. The glutamine stock was gently heated to dissolve the glutamine, and both the glutamine and BSA stocks were filter-sterilized (0.2-1µm membrane filter). The filter-sterilized stocks were added to the autoclaved basal medium at 50°C (Davis et al., 1981).

3.1.3. Antimicrobial products: formulation and chemical characteristics

AlgatanGea®: is an organic fertilizer, provided by the company LT Natural Group srl (Casalbuttano ed Uniti, CR - IT), with bio-stimulating properties made up of different species of marine algae and polyphenols of vegetal origin that provide precious nutritional elements to plants. The use of this fertilizer is allowed in organic agriculture.

Tannins U1, U2, U3 and U4: experimental tannins in different formulations.

- U1 is a sweet chestnut (*Castanea sativa*) hydrolysable tannins water extract.
- U2 is sulfited quebracho (*Schinopsis lorentzii*) condensed tannins water extract.
- U3 a mixture (1:9) of tara (*Casealpina spinosa*) pods solvent extract of hydrolysable tannins and sweet chestnut hydrolysable tannins water extract.
- U4 a mixture (1:1) of sweet chestnut hydrolysable tannins water extract and sulfited quebracho condensed tannins water extract.

Each product assayed was a liquid formulation, containing 40% tannins and 60% water (Canzoniere et al., 2021), and was provided by Silvateam S.p.A. (San Michele Mondovì, Italy). The chemical structures of the tannins assayed in this study were previously characterized by Pizzi et al. (2009), Giovando et al. (2013), Radebe et al. (2013). Turkey gall, and chestnut woods were analyzed and compared using matrix-assisted laser desorption/ionization time-of-flight, MALDI-TOF and Molino et al. (2018).

SILIFORCE ®: a fluid mixture of micro-elements containing silicic acid, molybdenum and zinc distributed ad provided by ILSA S.p.A.. The ortho-silicic acid is totally bioavailable for the plant. This formulation allows elemental silicon to penetrate tissues and exert a remarkable biological activity improving nutrient assimilation and transfer into plants.

Kalex Zn® and Kalex Cu®: the Kalex Line is a range of bio-stimulants and fertilizer that increases the natural resistance of plants against biotic and abiotic agents distributed by Alba Milagro International S.p.A:

- Kalex Zn is a liquid fertilizer containing zinc phosphite. It prevents zinc deficiency. It enhances the natural resistance of plants and increases vegetation, blooming and fruit quality.
- Kalex Cu is a liquid fertilizer containing copper phosphite. It contributes to enhancing crop health.

Bioflav: is a new experimental flavonoid-based glycolic extract bioformulation provided by AgroVentures LLC, USA.

Kiforce®: is a liquid formulation distributed by Alba Milagro International S.p.A. Its active ingredients (chitosan and derivatives) act as elicitors, i.e., inducers of the plant's response mechanisms to negative factors such as parasitic attacks or stressful conditions.

3.1.4. Disk diffusion assay and MIC determination

The inhibitory activity and the Minimum Inhibitory Concentration (MIC) were determined in triplicate by using the disk diffusion assay method (Bauer et al., 1966) with some modifications. The method consists in spreading the bacterial suspension on the agar surface (or including it inside the agar) and after drying for some minutes, placing 9 filter paper disk (6 mm ø) on the agar surface of each plate: one filter at the centre and 8 in radial pattern equidistant from each other, at 1.5 cm from the edge of the plate (**Figure 1**). On each disk 15 µL of the respective concentration of a tested product or a positive/negative control were spotted. In all the experiments the solutions of each product were prepared as following described. The maximum starting concentration was determined based on the origin of the product (mineral/synthetic or natural extract), indication of dosage for field application and/or some results obtained in assays with other pathosystem. To obtain the staring concentration as stock concentration were proceed as show, for example, to prepare a 4% v/v stock solution, 40 µL of the product, as it is, were added at 960 µL of H_2O; instead for 8% v/v solution, 80 µL of the product, as it is, were added at 920 µL of H_2O. The only exception was for Algatan Gea (AG) aqueous extract that was freshly prepared from the powder solution in H_2O, suspending 8 g of AG fine powder in 100 mL H_2O, shaking for 1 h, filtrated with sterile gauze, centrifugated at 14000 RPM for 10 min, collecting only the supernatant and sterilizing the solution with 0.22 µn sterile filter in 1.5 mL sterile tubes stocks. From the starting concentration (stock solution) were make up some serial dilution 1:1 of the substances obtaining seven serial dilutions (e.g., 500µL of starting concentration in 500µL of H_2O solution and so on).

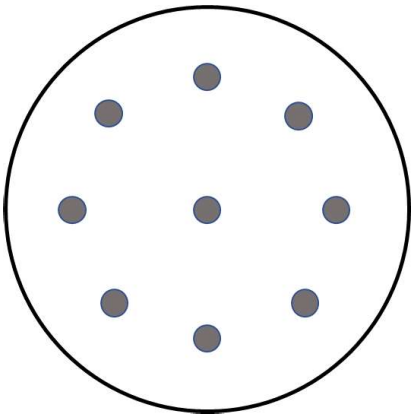

Figure 1. Scheme adapted from Kirby-Bauer Disk Diffusion Susceptibility Test Protocol (Bauer et al., 1966).

Bacterial inoculation for all the bacterial strain the inoculum was taken from a fresh solid culture 3-day old (except for *Xylella* strains) on appropriate medium. With a sterile inoculating loops a single colony of each bacterium were taken and suspended in 6 mL of NB (for *Pst* and *Xcp*) or in 6 mL of TSB for all the other bacteria (except for *Xylella fastidiosa* strains) and cultured over night at 28°C on orbital shaker at 140 RPM. After each bacterial culture was centrifugated at 4000 RPM for 10 min, resuspended in 10 mL of SDW

and adjusted to obtain a cell suspension with an absorbance near to 0.5 OD_{600} (around $1x10^9$ CFU/mL). The bacteria were incorporated 1:100 (to obtain a bacterial suspension around $1x10^8$ CFU/mL) in TSA maintained at 40°C and placed into Petri dish (around 15 mL for each plate).

For *Xf* strains the inoculum was taken from a fresh solid culture of 5/6-day old (PD3 medium for *Xfp* and *Xfm*TOS1; PW medium for *Xf*CO33 and *Xfm*ESVL). With a 10 µL sterile inoculating loops two or three drop-strips of *Xf* on solid culture were taken and suspended in 2 mL of PBS 1x solution to obtain a bacterial suspension with an absorbance near to 0.5 OD_{600} (around $4x10^8$ CFU/mL). For each bacterial concentration, 100 µL were plated in 90 mm Petri dish and spread by the glass beads spreading technique using the same medium as mentioned before (PD3 medium for *Xfp* and *Xfm*TOS1; PW medium for *Xf*CO33 and *Xfm*ESVL).

After, on dried agar, 9 filter paper disks (6 mm ø) for each plate were placed one at the centre of the plate as positive control (antibiotics mixture: Ampicillin 100 µg/mL and Streptomycin 250 µg/mL) and 8 arranged equidistant in a radial pattern at 1.5 cm from the edge of the plate (marking the concentration from 0 to 4 or 8 %). In the plate, on each disk 15 µL of the respective concentration of the tested product or of the positive/negative control were spotted (only for AGETOH, for the negative control 15 µL of 8% ethanol were spotted to exclude some effect of the residual ethanol in the extracted product).

Plates were incubated at 28°C for 2 days (7-10 days for *Xf* strains), daily monitored for observation and descriptive analysis before of the last survey when diameter of the inhibition halo (HI) in mm (**Figure 2**) and acquisition of the most representative image were performed.

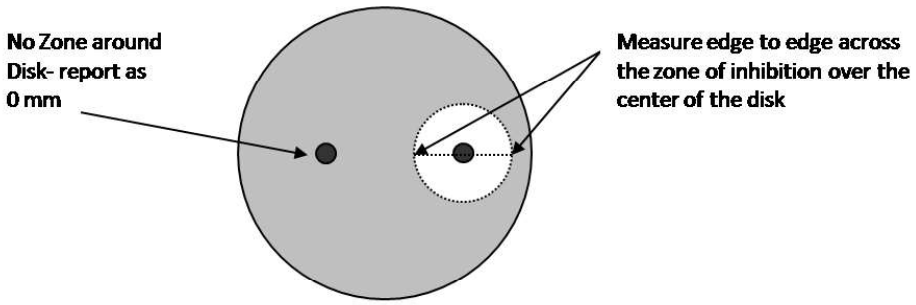

Figure 2. Schematic representation of the inhibition halo diameter detection (Bauer et al., 1966).

3.1.5. Statistical Analyses

All the data were subjected to variance analysis (ANOVA) using the statistical analysis software GraphPad PRISM 9. Significant ($P < 0.05$) values are considered according to the one-way analysis of variance (ANOVA) using the Fisher's Least Significant Difference (LSD) test.

3.2. Results

3.2.1. Evaluation of antibacterial activity

A preliminary evaluation of the antimicrobial activity of the selected products were tested against *Pseudomonas syringae* pv. *tomato* (*Pst*) and *Xanthomonas campestris* pv. *pelargoni* (*Xcp*) using the disk diffusion assay with some modifications (Bauer et al., 1966).

The evaluation of the antibacterial activity show that all the tested products were able to inhibit the growth of both bacteria, showing different level of sensitivity to the tested compounds, only AG resulted less effective against *Xcp* (**Figure 3**).

The results of the experiments were showed in graphics in the section "Table and Figure". In which each histogram and bar represents the mean and the standard deviation of the diameter (mm) of the inhibition halo (IH) for each product considering as limit of detection 20 mm (Ø of the plate). POS_C represent the positive control, the drug reference (ampicillin 100 ppm + streptomycin 250 ppm); and NEG_C the negative (untreated) control (only water).

As showed the **Figure 4**, all the products significantly reduced the growth of *Pst*. Kalex Zn inhibited the growth from the lower concentration, 0.062 % v/v; Kalex Cu inhibited the growth at 1% v/v, and the mixture of the two compounds (Kalex Zn + Kalex Cu, 3:1) inhibited the growth at 0.125% v/v; Siliforce and AlgatanGea inhibited the growth of the bacterium at 0.5% v/v. The tannin-based product U2 inhibited the growth at 0.5 % v/v; the other three U1, U3 and U4 at 0.125 % v/v.

Regarding *Xcp* (**Figure 5**), Kalex Zn and the mixture Kalex Zn+Kalex Cu inhibited the growth of the bacterium at 0.125 % v/v; Kalex Cu inhibited the growth at 0 .5% v/v; Siliforce

inhibited the growth of the bacterium at 0.5% v/v. Algatan Gea was less effective against *Xcp* as well as the tannin U2, that inhibited the bacterial growth at 8 and 2 % v/v, respectively. The other three tannin-based products U1, U3 and U4 inhibited the bacterial growth at 0.5, 0.25, 0.1 % v/v, respectively.

In all cases the increase of the concentration corresponded to an increase of HI.

3.2.2. Antibacterial activity against some important and dangerous plant pathogenic bacteria

Results on the evaluation of antibacterial activity carried out against the selected important and dangerous plants pathogenic bacteria (*Erwinia amylovora, Xanthomonas arboricola* pv. *pruni*, *Ralstonia solanacearum*, *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas syringae* pv. *actinidiae*, *X. euvesicatoria* and *X. campestris* pv. *campestris*), although with different degree, demonstrated the large spectrum of antibacterial activity of all the tested product. For example, it was observed that the woody plants phytopathogenic bacteria *E. amylovora* and *X. arboricola* pv. *pruni* were resistant or in some instance less sensitive to tannins (U1, U2, U3 and U4) and Algatan Gea than the other bacteria. Also, *Pseudomonas syringae* pv. *actinidiae* showed an intermediate resistance to tannin U3 (**Figure 6**).

The result of the evaluation of each product against the selected phytopathogenic bacteria were reported and summarized in **Table 1-9**. For each of the seven tested bacteria the tables report the mean and standard deviation (mean±SD) of the diameter in mm of the inhibition halo (IH) for each concentration of the tested products considering as limit of detection 20 mm diameter. POS_C represent the positive control, the drug reference (ampicillin 100 ppm + streptomycin 250 ppm) and NEG_C the negative control, the water control (untreated).

Algatan Gea resulted ineffective against *Erwinia amylovora* and *X. arboricola* pv. *pruni*, but it was able to inhibit the growth of all the other bacteria at the higher concentration reducing the inhibition diameter size as the concentration decreases (**Table 1**). The lower concentration able to reduce the growth of *X. euvesicatoria* and *C. michiganensis* subsp. *michiganensis* was 2% v/v with IH of 8.2±0.3 and 7.5±0.7 mm, respectively. *P. syringae* pv. *actinidiae* was inhibited at 1% v/v with IH of 7.5±0.6 mm. *Ralstonia solanacearum* and *X.*

campestris pv. *campestris* were inhibited from 0.25% v/v to higher concentrations, with IH ranging from 8.3±0.9 to 11.2±0.7 mm.

Kalex Zn, Kalex Cu and Kalex Zn+Kalex Cu and Si were able to inhibit the growth of all the bacterial strains at the higher concentration, reducing the inhibition diameter size as the concentration decreased.

The lower concentration of Kalex Zn (0.062% v/v) was able to inhibit the bacterial growth of *Erwinia amylovora*, *Xanthomonas euvesicatoria*, *C. michiganensis* subsp. *michiganensis*, *P. syringae* pv. *actinidiae* and *X. campestris* pv. *campestris* with inhibition halos (IHs) of 7.5±0.3, 7.3±0.3, 9.5±1.3, 7.3±0.1 and 8.5±0.3 mm, respectively (**Table 2**). *Ralstonia solanacearum* and *X. arboricola* pv. *pruni* were inhibited from 0.250 and 0.125 % v/v, with IHs of 9.3±1 and 7.8±0.5 mm, respectively.

Kalex Cu (**Table 3**) inhibited the growth of *Erwinia amylovora*, *X. arboricola* pv. *pruni* and *X. campestris* pv. *campestris* from 0.25% v/v, with IHs of 7.8±0.3, 9.1±0.2, and 8.9±0.8 mm, respectively; *Xanthomonas euvesicatoria* was inhibited from 0.125% v/v, with IH of 7.6±0.4 mm; C. *michiganensis* subsp. *michiganensis*, *P. syringae* pv. *actinidiae*, and *Ralstonia solanacearum* were inhibited from the concentration 0.5% v/v with IHs of 7.4±0.5, 7.9±0.4 and 7.2±1.1 mm, respectively.

Kalex Zn+Kalex Cu (**Table 4**) inhibited the growth of *P. syringae* pv. *actinidiae* from 0.062 % v/v with an IH of 7.3±0.1mm. All the other bacteria were inhibited from 0.125% v/v with IHs of 7.7±0.3, 7.8±0.2, 8.8±0.4, 7.8±0.3, 8.1±0.6 and 9.1±0.9 mm for *Erwinia amylovora*, *Xanthomonas euvesicatoria*, *C. michiganensis* subsp. *michiganensis*, *Ralstonia solanacearum*, *X. arboricola* pv. *pruni* and *X. campestris* pv. *campestris*, respectively.

Siliforce (**Table 5**) inhibited the growth of *Erwinia amylovora*, *Xanthomonas euvesicatoria* and *X. campestris* pv. *campestris* at 0.5% v/v with IHs of 8.3±0.3, 7.8±0.3 and 8.8±0.6, respectively. *C. michiganensis* subsp. *michiganensis* and *P. syringae* pv. *actinidiae* were inhibited at 0.250% v/v with IHs of 9±0.5 and 6.5±0.9, respectively. *Ralstonia solanacearum* and *X. arboricola* pv. *pruni* at 1% v/v were inhibited whit IHs of 7.5±0.2 and 8.5±0.3, respectively.

U1, U2 and U3 were ineffective against *Erwinia amylovora* and *X. arboricola* pv. *pruni*; for all the others bacterial strains all the products were able to inhibit the growth of the bacteria.

U1 (**Table 6**) inhibited the growth of *Xanthomonas euvesicatoria* from 0.5% with an IH of 6.9±0.8 mm; *C. michiganensis* subsp. *michiganensis* and *P. syringae* pv. *actinidiae* were inhibited from 1% v/v with IHs of 6.8±0.7 and 6.9±0.3, respectively. *Ralstonia solanacearum* and *X. campestris* pv. *campestris* were inhibited at 0.125% v/v with IHs of 8.1±0.4and 13.5±0.6 mm, respectively.

U2 (**Table 7**) inhibited the growth of *Xanthomonas euvesicatoria*, *P. syringae* pv. *actinidiae* and *X. campestris* pv. *campestris* from 2% v/v (IH 7.8±0.9, 7.4±0.2, 9±0 mm, respectively); *C. michiganensis* subsp. *michiganensis* and *Ralstonia solanacearum* from 1% v/v (IH 7.9±0.1, 7.8±0.5 mm, respectively).

U3 (**Table 8**) inhibited the growth of *Ralstonia solanacearum* and *X. campestris* pv. *campestris* from 0.125 % v/v (IH 9.2±0.6 0, 15.1±0.5 mm Ø, respectively); *Xanthomonas euvesicatoria* 2% v/v (IH 8.1±0.2 mm); *C. michiganensis* subsp. *michiganensis* 1% v/v (6.6±0.6); and *P. syringae* pv. *actinidiae* 8% v/v (IH 9.3±0.6)

U4 (**Table 9**) inhibited the growth of *Xanthomonas euvesicatoria* at the major concentration (8% v/v) with an IH of 9.7±0.5 mm; *Xanthomonas euvesicatoria* and *P. syringae* pv. *actinidiae* at 1% v/v (IH 8±0.7, 8±0.1 mm, respectively); *C. michiganensis* subsp. *michiganensis* at 0.5 % v/v (IH 7.3±0.2 mm); *Ralstonia solanacearum* and *X. arboricola* pv. *pruni* at 0.25% v/v (IH 8.5±0.5, 6.8±1.4 mm, respectively) *X. campestris* pv. *campestris* at 0.125% v/v (IH 11±0.6 mm).

3.2.3. Antibacterial activity against *Xylella fastidiosa*

Xylella fastidiosa is the main target pathogen of the present research. Due the evidence of the large spectrum antibacterial activities of the selected products evidenced against all the tested phytopathogenic bacteria, the next step was to evaluate if these products had the same activities also against different strains and subspecies of *Xylella fastidiosa*: *X. fastidiosa* subsp. *pauca* strain ST53 (*Xfp*), ii) *X. fastidiosa* strain CO33 (*XfCO33*), iii) *X. fastidiosa* subsp. *multiplex* strain TOS1 (*XfmTOS1*), and iv) *X. fastidiosa* subsp. *multiplex* strain ESVL (*XfmESVL*). Data on the sensitivity to the tested products of the four *X. fastidiosa* subspecies and strains are resumed in the heatmap of **Figure 7**.

The disk diffusion assay against *X. fastidiosa* subsp. *pauca* strain ST53 (*Xfp*) (**Figure 8**) showed that this strain was resistant to Kalex Zn, the mixture of Kalex Zn + Kalex Cu and Kiforce, whereas it was inhibited significantly ($P < 0.05$) at 1% v/v by Kalex Cu, U1, U2, U3 and 2% v/v by AG, AGEtoh and U4; Siliforce and BioFlav resulting less efficiently, the growth was inhibited at 4 % an 8 % v/v, respectively.

The strain CO33 of *X. fastidiosa* (*XfCO33*) (**Figure 9**) resulted sensible to Kalex Zinc, Kalex Cu, the mixture of Kalex Zinc and Kalex Cu, U1 and U2, whereas it was inhibited significantly ($P <0.05$) at 0.5 % v/v; Siliforce inhibit significantly ($P < 0.05$) the bacterium at 1%; AGEtoh and U3 inhibits the growth at 4 and 2% v/v, respectively, whereas AG, U4, BioFlav and Kiforce resulted ineffective at the tested concentrations.

X. fastidiosa subsp. *multiplex* strain TOS1(*XfmTOS1*) (**Figure 10**), resulted sensible to Kalex Cu, AG, AGEtoh, U1, U3. It was significantly inhibited ($P <0.05$) respectively at 1 % v/v for Kalex Cu and U3; 2 % v/v for AG and AGEtoh, 0,5 % v/v by U1; the strain showed less sensibility to Kalex Zn, U4 (4% v/v) and U2 (8% v/v) and it was resistant to Kalex Zn + Kalex Cu mixture, Siliforce, BioFlav and Kiforce.

X. fastidiosa subsp. *multiplex* strain ESVL (*XfmESVL*) (**Figure 11**), was significantly inhibited ($P <0.05$) at 2% v/v by Kalex Zn, Kalex Cu, U1, U3., whereas it was less sensitive to AG, AGEtoh, U2 and U4 (4 % v/v) and resulted resistant to the Kalex Zn + Kalex Cu mixture, Siliforce, BioFlav and Kiforce.

3.2.4. MIC determination

The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of an antimicrobial ingredient or agent that is bacteriostatic (prevents the visible growth of bacteria). MICs are used to evaluate the antimicrobial efficacy of various compounds by measuring the effect of decreasing concentration of antimicrobial compounds over a defined period in terms of inhibition of microbial population growth (Andrews, 2001).

The MIC concentrations of all the bacterial strains are reported in **Table 10** for *P. syringae* pv. *tomato* and *X. campestris* pv. *pelargonic*, in **Table 11** for *Erwinia amylovora, X. euvesicatoria*, *C. michiganensis* subsp. *michiganensis*, *P. syringae* pv. *actinidiae*, *Ralstonia solanacearum*, *X. arboricola* pv. *pruni*, *X. campestris* pv. *campestris*, and in **Table 12** for *X. fastidiosa* subsp. *pauca* strain ST53, *X. fastidiosa* strain CO33, *X. fastidiosa* subsp. *multiplex* strain TOS1, *X. fastidiosa* subsp. *multiplex* strain ESVL.

4. ANTIBACTERIAL ACTIVITY AGAINST *XYLELLA FASTIDIOSA* SUBSP. *PAUCA* STRAIN ST53

4.1. Materials and Methods

4.1.1. Bacterial strains and growth condition

X. fastidiosa subsp. *pauca* strain ST53 (*Xfp*) was taken from the bacterial collection of Institute for Sustainable Plant Protection of the National Research Council of Italy (IPSP-CNR) unit of Bari. The experiments with this bacterial strain were performed in the institute's laboratories, accredited to manipulate quarantine *Xylella fastidiosa* strains in Italy. The bacterium was cultured on Buffered Charcoal Yeast Extract agar medium (BCYE) for 7 days at 28°C, and then it was subcultured for 10 days at 28°C, on Pierce's Disease no. 3 agar medium (PD3). The bacterial biomass was kept with a sterile 10 µL loop and suspended in sterile distilled water (SDW), adjusting bacterial suspension to 0.5 OD_{600}, and used as inoculum for all the experiments *in vitro*.

4.1.2. Media and buffer solution

The liquid and agar media used in this study were prepared using deionized water and sterilized in an autoclave at 1.2 atmospheres and 120°C for 20 minutes, or by filtration using filters with a porosity of 0.22µm in diameter. Solid culture media were poured into Petri dishes before solidification. Buffer solutions were prepared with deionized water and sterilized by filtration (0.22µm pore size, Millipore). Media composition and recipe step by step are listed below.

Pierce's Disease no. 3 agar medium (PD3): pancreatic digest of casein (tryptone [Difco] or trypticase peptone [BBL]), 4g; papaic digest of soy meal (soytone [Difco] or phytone peptone [BBL]), 2g; trisodium citrate ($Na_3C_6H_5O_7$), 1g; disodium succinate, 1g; hemin chloride stock (0.1 % bovine hemin chloride dissolved in 0.05 N NaOH), 10 ml; magnesium sulphate heptahydrate ($MgSO•7H_2O$), 1g; dipotassium phosphate (K_2HPO_4), 1.5g; potassium dihydrogen phosphate (KH_2PO_4), 1g; potato starch, 2g; BactoAgar, 18g; distilled water, 1L. The medium was stirred for 5 minutes on a magnetic stirrer and autoclaved at 1.2 atmospheres, 120°C for 20 minutes.

Buffered Charcoal Yeast Extrat agar medium (BCYE): yeast extract, 10g; activated charcoal, 2.0g; L-cysteine $HCl•H_2O$, 0.4g; ferric pyrophosphate, soluble, 0.25g; agar, 17.0g; and ACES buffer (Sigma), 10g in 1L distilled water.

ACES buffer was first hydrated in 500ml of distilled water at 50°C. The buffer was then mixed with a solution containing ca. 40ml of 1.0 N KOH in 440mL of distilled water. This mixture was used to hydrate the charcoal, yeast extract, and agar and was then autoclaved at 1.2 atmospheres, 120°C for 20 minutes; finally, the mixture was cooled and equilibrated at 50°C. L-Cysteine hydrochloride and soluble ferric pyrophosphate were dissolved together in 20 ml of water, sterilized through a 0.25 µm-pore size membrane filter, and then added to the mixture. The pH of the final solid medium was 6.9 at 25°C. The amount of KOH used was varied to adjust or change pH (Wells et al., 1981).

4.1.3. Time-kill assay of *Xylella fastidiosa pauca*

To assess if the MIC concentration (or in some instances the field dose concentration) for each product was able to kill bacteria cell we used the time-kill assay reported by Osburne et al., 2006 improved by applying some modifications required for optimize the experiment due to the fastidious growth of *Xfp*. The concentration used was: 1% v/v for the four tannins products (U1, U2, U3 and U4) and Siliforce (Si), 2% v/v for Algatan Gea water and ethanolic extracts (AG and AGEtoh), and 1.2 % v/v for Kalex Zn and Kalex Cu (K-Zn and K-Cu). Each product was included in PD3 medium to reach the respective concentration, after the medium was poured in sterile petri dish plate (9mm Ø). Only PD3 medium was used as negative control and PD3 with 50 µg/mL of kanamycin as positive control.

Three 20µL spots, of a 0.5 OD_{600} *Xfp* bacterial cell suspension was spotted for each time point (1, 2, 4, 6, 8, 12, 24 hours) on each plate containing the respective product's concentration. For each time point, each spot was recovered and resuspended in 5mL of SDW separately, making three serial decimal dilutions. For each dilution, 10 µL of cell suspension were spotted and plated on BCYE medium and incubated at 28°C for 20 days. The experiment was repeated 2 times with three replicates.

4.1.4. Green house *in vivo* biocontrol assays

4.1.4.1. Bacterium inoculation and plants material used for the inoculation

In November 2019 olive rootstocks (from 3 years old olive plants cv. Ogliarola) were inoculated by grafting *Xfp* infected olive scions harvested from positive olive plants (cv. Ogliarola) taken from a naturally infected field as described by Saponari et al. (2017). Infected scions were collected/harvested from the infected area of the Salento Peninsula; healthy scions (used as negative control grafted plants) were collected from negative olive plants (cv. Ogliarola) in a free *Xfp* area. Each scion was analysed by qPCR for assessing the presence or absence of the bacterium. Small pieces of midveins and petioles (~ 1 g) were collected for each scion and processed for DNA extraction.

The samples were crushed with a hammer in extraction bags and homogenized using the semi-automated homogenizer Homex 6 (Bioreba, Switzerland) with 5 ml of CTAB buffer (2% Hexadecyl trimethyl-ammonium bromide, 0.1 M Tris-HCl pH 8, 20 mM EDTA and 1.4 M NaCl). Extract aliquots (1 ml) were transferred into a 2 ml microcentrifuge tube, incubated at 65°C for 30 min and processed for DNA extraction using Maxwell® RSC PureFood GMO and Authentication Kit protocol (Promega) and Maxwell RSC automated platform (Promega). The qPCR must be performed using the primers and the TaqMan probe designed by Harper et al. (2010) as described by (Saponari & Loconsole, 2021).

Final reaction conditions were as follows: real-time PCR reactions were done in 20µL reaction volumes containing 6.48µL of molecular-grade water, 10µL of 2× qPCR TaqMan™ Fast Universal PCR Master Mix (AB) (Cod. 4352042), 0.6 µL of 10 µM stock solution of *X. fastidiosa* sense (XF-F) and antisense (XF-R) primers, 0.2 µL of 10 µM stock solution of 6′FAM/BHQ-1- labelled XF-P probe, 12 µL of 50 µg/uL of molecular grade bovine serum albumin (BSA) (non-acetylated) (UltraPure™ BSA (50 mg/mL), Invitrogen), and 2 µl of total DNA template. The primers and probes mentioned before are: XF-F (forward) 5'-CAC GGC TGG TAA CGG AAG A-3', XF-R (reverse) 5'-GGG TTG CGT GGT GAA ATC AAG-3', XF-P (probe) 5' 6FAM -TCG CAT CCC GTG GCT CAG TCC-BHQ-1- 3'. PCR conditions: pre-incubation at 50°C for 2 minutes followed by 95°C for 10 minutes, followed by 40 cycles of (94°C for 10 seconds and 62°C for 40 seconds).

The 2 years cutting clones used as rootstock were cut at 30cm from the crown, cutting out all the rootstocks shoot above the graft. The twigs were cut in 10-15cm scions and clef grafted on the rootstock biding the graft union and the scion with plastic film (**Figure 12**). After 2 weeks all the grafted alive twigs on the rootstocks were selected for the experiments, the dead twigs were already grafted with new scions using fresh plant material.

4.1.4.2. Host plant, growth conditions and *Xfp* detection

The host plant used in this experiment are the well-known *Xfp* susceptible olive cultivar Ogliarola. Nursery's healthy cutting clones' plants (~ 200 plants, 3 years old) are used as rootstock and inoculated as described previously. Plants were made to grow in greenhouse conditions at a temperature of 20–24°C (winter) and 25–30°C (summer), irrigating regularly two or three times a week at the Institute for Sustainable Plant Protection (IPSP), unit of Bari. The experiments started in December 2019 and ended in January 2021 when all the positive control plants are dead. *Xfp* were monitored by qPCR during the experiments by sampling 4-5 leaf randomly harvested for each plant. Petioles (~ 0,5 g) were collected for each scion and were crushed with a hammer in extraction bags and homogenized using the semi-automated homogenizer Homex 6 (Bioreba, Switzerland) with 5 ml of CTAB buffer. Extract aliquots (1 ml) were transferred into a 2 ml microcentrifuge tube, incubated at 65°C for 30 min and processed for the DNA extraction and qPCR analysis as described in the previous paragraph.

4.1.4.3. Treatments and experimental design

The following experimental schedule was adopted: 2 soil treatments (preventive and curative) and 6 foliar-spray treatments; untreated and uninoculated (*Xfp* negative control), and untreated and inoculated plants (*Xfp* positive control) were used as control for both soil and foliar-spray treatments. For each treatment 3 replicates of 3 plants (9 plants total) were used; only for the soil treatments, because of the possibility of non-engraftment of some grafts in the preventive treatments, 4 replicates of 3 plants (12 plats total) were used.

Treatments were made up starting from the information obtained by the *in vitro* experiments. The best 7 products were selected based on the MIC concentration and their potential application to induce plant defences. The products used, the modality of their application and the concentration applied were described in **Table 13**.

The number and the time of treatments and each sampling for monitoring bacterial population by qPCR, were summarized in the gnat chart (**Figure 13** and **14**) starting from the inoculation time.

4.1.5. Statistical Analyses

All the data were subjected to variance analyses (ANOVA) using the statistical analysis software GraphPad PRISM 9 and in some instance by multiple t test analysis. Significant ($P < 0.05$) values are considered according to the one-way and two-way analysis of variance (ANOVA) and two-way using the Fisher's Least Significant Difference (LSD) test. Statistical significance in multiple t test analysis was determined using the Holm-Sidak method ($P<0.05$).

4.2. Results

4.2.1. Time-kill assay

Xylella fastidiosa subsp. *pauca* was exposed over time to the selected concentration of each product. The results of this experiment, clearly show that all the products, except Algatan Gea ethanolic extract (AGEtoh) and Siliforce (Si) that resulted less effective, were able to kill the bacterial cells.

The results of these experiments are showed in **Figure 15-24**. In each graph, bars represent the mean and standard deviation (6 pooled replicates, of 2 independent experiments of 3 replicas) of bacterial growth (Log_{10}CFU/mL) at different time point including the negative control (PD3) only PD3 medium, and the positive control (Kn), the medium with 50 µg/mL of kanamycin.

Algatan Gea water extract (AG) was able to significantly kill the bacterium after 24 hours of contact compared to the negative control (PD3) ($P <0.001$); the same results were obtained with the positive control (Kn), in which the bactericidal trend is very similar to AG. As shown in **Figure 15** bactericidal activities increased by increasing the exposure time to Kn and AG.

Algatan Gea ethanolic extract (AGEtoh) was able to reduce significantly ($P <0.001$) the bacterium after 24 hours of contact compared to the negative control (PD3) but it was not able to kill completely the bacterium, as the positive control (Kn) did at 24 h (**Figure 16**).

The Tannin U1 (U1) was able to kill completely the bacterium after 12 hours of contact compared to the negative control (PD3), comparable results were obtained with the positive control (Kn). As shown in **Figure 17**, bactericidal activities increased by increasing the time exposure to Kn and U1. During the experiments U1 result more effective than Kn over time, starting from 2 hours of exposure the bactericidal activity resulted tendentially stronger than Kn.

Tannin U2 (U2) was able to significantly kill the bacterium after 24 hours of contact compared to the negative control (PD3), the same results were obtained with the positive control (Kn). As shown in **Figure 18** bactericidal activities increased by increasing the exposure time to Kn and U2, for both, the bactericidal trend was very similar.

Tannin U3 (U3) was able to completely kill the bacterium at 12 hours of contact compared to the negative control (PD3), the same results were obtained with the positive control (Kn). As shown in **Figure 19** the bactericidal activity increased by increasing the exposure time to Kn and U3. During the experiments U3 resulted more effective than Kn over time, starting from 2 hours of exposure the bactericidal activities result tendentially stronger than that exerted by Kn.

Tannin U4 (U4) was able to completely kill the bacterium after 24 hours of contact compared to the negative control (PD3), the same results were obtained with the positive control (Kn). As shown in **Figure 20**, bactericidal activities increase by increasing the exposure time to Kn and U4. U4 resulted more effective than Kn over time, starting from 2 hours of exposure the bactericidal activities resulted tendentially stronger than that exerted by Kn.

Siliforce (Si) was able to reduce significantly ($P < 0.001$) the bacterium at 24 hours of contact compared to the negative control (PD3) but it was not able to kill completely the bacterium, as the positive control (Kn) did at 24 h (**Figure 21**).

Kalex Zn (K-Zn) was able to reduce significantly ($P < 0.001$) the bacterium after 2 hours of contact and this product was able to kill completely the bacterium after 4 hours of contact, compared to the negative control (PD3) and the positive control (Kn). As shown in **Figure 22**. Kn's bactericidal activities increased by increasing the exposure time to Kn.

Similar results were obtained with Kalex Cu (K-Cu) (**Figure 23**) and the mixture of Kalex Zn and Kalex Cu (K-Zn+K-Cu) (**Figure 24**) since both products were able to completely kill ($P < 0.001$) the bacterium after 4 hours of contact compared to the negative control (PD3) and the positive control (Kn). As shown in the figures, K-Cu did not significantly reduce the bacterium after 2 h, while the mixture of K-Zn+K-Cu was able to significantly reduce the bacterium after 2 hours ($P<0.03$).

4.2.2. Effect of treatments on plant growth and disease index rate

The experimental schedule adopted included 2 soil treatments (AG_Soil 10= curative; and AG_Soil 5= preventive) and 6 foliar-spray treatments (Tannins U1, U2, U3, U4; Kalex Zn= K-Zn; and Kalex Cu= K-Cu). Untreated and uninoculated (O_C_Neg), and untreated and inoculated olive plants (O_C_Pos) were used as negative and positive control, respectively, for both soil and foliar-spray treatments. During the experiments, periodically, from the inoculation time to the end of the experiment, some physiological parameters, such as plant height and shoots numbers, were assessed. Plant height and shoot number were taken from the graft point (for plant height the graft point is considered as 0; for shoot number only the new shoots from the engrafted scions were considered.

Regarding plant height and shoot number in plants treated on the soil with Algatan Gea at the end of the experiments there was no difference between treated and the untreated (positive control) plants. Instead, the uninoculated and untreated plants (negative control) showed a significantly higher number of shoot than inoculated treated and untreated plants (**Figure 25** and **Figure 26**).

In foliar treatments, plant height was very similar between treated plants (44.17cm for U1, 35.83 for U2, 33.67 for U3, 44.17 for U4, 38.61 K-Zn, and 35.00 K-Cu); only U1 and U4 showed statistically different ($P<0.05$) among them, the other treatments were not significantly different from U1 and U4 in pairwise comparisons. U1 was the only treatment resulting statistically different from O_C_Pos (27.43 cm). O_C_Pos resulted significantly higher than all the other treatments ($P<0.05$). Comparing the two control O_C_Neg (66.00cm) and O_C_Pos a significative difference, $P<0.001$, was observed (**Figure 27**).

Concerning the increment (%) of shoot number, in plants treated with U1 and U2 was observed an increment of 12.42% and 8.10%, respectively, that was statistically significative ($P<0.05$) to U3, U4 and the negative control (O_C_Neg) (35.19%, 32.99%, 41.82% respectively); Not significance was observed between U1 and U2, and the other treatments in multiple comparisons. U3 (35.19%) and U4 (32.99%) results statistically different ($P<0.05$) from U1 (12.42%), U2 (8.10%) and O_C_Pos (2.78%); Not significance were observed between U3 and U4 and the other treatments in multiple comparisons.

K-Zn (28.21%) was statistically different ($P<0.05$) from O_C_Pos (2.78%); and K-Cu (17.47%) from O_C_Neg (41.82%). Comparing the two controls, the increment of shoots of O_C_Neg (41.82%) is significantly higher than O_C_Pos (2.78%) ($P<0.001$) (**Figure 28**).

In addition to the assessment of physiological parameters, in each survey the disease index of each treatment was recorded by using an appropriate disease scale.

In soil applications, no treatment (preventive or curative) was able to reduce the disease severity on treated plants (**Figure 29**).

A different scenario was observed for foliar treatments (**Figure 30)**. In particular, U1, U2, U3 and K-Zn significantly reduced the disease severity compared to O_C_Pos ($P<0.05$) showing a diseases index (%) of 22.22%, 11.11%, 22.22% and 37.78%, respectively. No significant differences were observed comparing these treatments with the O_C_Neg (0%) (only K-Zn resulted significantly different from the O_C_Neg). U4 (80.00%) and K-Cu (75.56%) were not statistical significative respect to O_C_Pos (75.00%), but results statistically different from O_C_Neg.

4.2.3. Effect of treatments on bacterial population assessed by qPCR

During the *in vivo* experiments, a qPCR approach was used for evaluating and monitoring the bacterial population in treated and untreated plants.

Results reported in **Figure 31** evidence that all foliar treatments (U1, U2, U3, U4, K-Zn and K-Cu) significantly reduced the bacterial population at T1 (the end of the experiments) compared to the bacterial population detected at T0 (soon before application of treatments).

Regarding soil treatments, AG curative (AG_Soil 10) and AG preventive (AG_Soil 5) were observed to significantly increase the bacterial population. Regarding the positive control (O_C_Pos), although the mean of bacterial population at T1 resulted tendentially higher than T0, no significative increase was detected, and this was probably due to the death of many plants.

To quantify bacterial population reduction or increment data were analysed calculating the Δ (%) Normalized Log_{10}CFU/mL.

As showed in **Figure 32** for all the foliar treatments *Xylella* was significantly lower ($P<0.05$), compared to the O_C_Pos and no significative difference was observed between treatments in pairwise comparison. The bacterium was reduced of a Δ (%) of -22.05%, -14.65%, -23.32%, -35.97%, -18.71% and -18.34%, respectively in plant treated with U1, U2, U3, U4, K-Zn and K-Cu. In the positive control Xylella was increased of the 31.88%.

Soil treatments (**Figure 33**) showed an increase of *Xylella* concentration, like the positive control; plant treated with AG preventive showed a significantly increase of bacterial population compared to the O_C_Pos and AG curative.

5. DISCUSSION

The management of bacterial diseases in plants can be very difficult due to i) high variability of pathogens, ii) gene transfer between pathogens (which lead to the development of resistance) and, especially, iii) lack of effective chemicals (e.g. bactericides) that are also harmless to the environment (Obradovic et al., 2004). For example, traditional bactericides and antibiotics (e.g., kasugamycin, nitrapyrin, octhilinone, oxolinicacid, oxytetracycline, probenazole, streptomycin, tecloftalam, and thiomersal) are not allowed in EU, while the use of copper derivatives (e.g., copper hydroxide, copper sulphate, copper oxychloride), is increasingly limited, because of their negative impact on the environment and natural ecosystems. Furthermore, it is well known that many plant pathogenic bacteria have acquired resistance to synthetic pesticides, copper and antibiotics (White et al., 2002). All these constraints and limitations fuelling the need for a new generation of highly effective but also environmentally friendly pesticides. In a such context, the importance of natural derivatives (e.g. vegetal extracts, essential oils, decoctions, hydrosols, etc.) and the use of fertilizers in crop protection is increasingly being recognized under the concept of organic and integrated pest and disease management (IPDM) (Ragsdale, 2000). Under this concept, all possible strategies of plant pests and disease control methods are integrated to minimize the excessive use of synthetic pesticides (Beg & Ahmad, 2002). Exploitation of naturally available compounds delaying growth and activity of undesirable microorganisms, in a more realistic and ecologically sound way for plant protection, will be prominent in the future development of commercial pesticides, with emphasis on the management of plant diseases in general, but particularly on bacterial diseases (Gottlieb et al., 2002). Strategies may include antimicrobials compounds (plant derived or microbial derived) and the use of bioactive products, also called plant activators, inducing resistance in plants to many pathogens, including bacteria (Sticher et al., 1997). Plant activators have no direct antimicrobial activity but can elicit plants to initiate preinfectional defense reactions such as the accumulation of pathogenesis-related proteins (PR) and ultrastructural changes (Benhamou & Belanger, 1998; Inbar et al., 1998; Louws et al., 2001).

In the present Ph.D. thesis, the following new and eco-friendly commercial and experimental products were selected and evaluated for their antimicrobial activity again different genera and species of plant pathogenic bacteria.

AlgatanGea® (LT Natural Group srl, Italy): is an organic fertilizer with bio-stimulating properties made up of different species of marine algae and polyphenols of vegetal origin that provide precious nutritional elements to plants. The product can stimulate the

metabolism and activate the vegetative development by improving the physiological state of the plants, triggering germination and root development, increasing production yields, height, and plant biomass; it strengthens the plant's natural defenses against biotic and abiotic stresses, increases photosynthetic efficiency improving adsorbing power (e.g. by stimulating root development and plant biomass), and modulates phytopathogens (e.g. by direct antimicrobial activity, triggering of plant defences, etc.). The use of this fertilizer is allowed in organic agriculture on several crops.

Tannins U1, U2, U3 and U4 (Silvateam S.p.A. Italy): these are four experimental products in different formulations:

- U1, sweet chestnut (*Castanea sativa*) hydrolys-able tannins water extract;
- U2, sulfited quebracho (*Schinopsis lorentzii*) condensed tannins water extract;
- U3, mixture (1:9) of tara (*Casealpina spinosa*) pods solvent extract of hydrolysable tannins and sweet chestnut hydrolysable tannins water extract;
- U4, mixture (1:1) of sweet chestnut hydrolysable tannins water extract and sulfited quebracho condensed tannins water extract.

Tannins, have several effects as antioxidant, antimicrobial, and metal complexing agents. Some patents described their use as nitrogen release modulators and iron complexing agent to fight plant chlorosis and to control seed-borne disease and nitrosamines and mycotoxins during plant and food processing. Their biostimulating activity was assessed on early plant growth (starter effect) and found related to earlier production of a larger plant fine root mass, with greater P early uptake. Furthermore, they increased resistance to nematodes, and a protective effect on some bacterial diseases was also disclosed, such as bacterial speck of tomato (Canzoniere et al., 2021). Environmental and soil toxicities of tannins were also investigated finding a very low impact of these products and their possible use to reduce Cu application in agriculture (Miele et al., 2019).

SILIFORCE ® (ILSA S.p.A., Italy): it is a fluid mixture of micro-elements containing silicic acid, molybdenum and zinc, when distributed by the ortho-silicic acid is totally bioavailable for the plant. This formulation allows elemental silicon to penetrate into tissues and exerts a remarkable biological activity by improving the assimilation and transfer of nutrients in plants. The product can improve lymphatic circulation and stimulates the growth of the root system while reducing the evapotranspiration rate. It also induces resistance to fungal

diseases and makes foliar surfaces inhospitable to parasites. The efficacy is strictly dependent on the bioavailability of the ortho-silicic acid contained in it.

Kalex Zn® and Kalex Cu® (Alba Milagro International S.p.A., Italy): Kalex Line includes a range of bio-stimulants and fertilizers increasing the natural resistance of plants against biotic and abiotic agents. The presence of a particular form of phosphorus (phosphite) promotes the production of phytoalexines, which are natural self-defence agents useful against diseases caused by fungi and, particularly, oomycetes (e.g., *Phytophthora* spp., *P. viticola*, *B. lactucae*, *Peronospora* spp., *P. cubensis*). The Kalex line has high nutritional value for plants, containing different available elements. These products improve vegetative development, flowering, fruit setting and ripening, giving higher yields, enhancing translocation of nutrients through the conducting vessels.

Kalex Zn is a liquid fertilizer containing zinc phosphite. It prevents zinc deficiency. It enhances the natural resistance of plants and increases vegetation, blooming and fruit quality.

Kalex Cu is a liquid fertilizer containing copper phosphite. It contributes to enhance crop health. In the form of phosphite, copper has a high biological activity. It can help the control of a wide range of diseases caused by oomycetes (e.g., *Phytophtora* spp, *P. viticola*, *B. lactucae*, *Peronospora* spp., *P. cubensis*) and bacteria as well (e.g., *Erwinia* spp., *Pseudomonas* spp., *Xanthomonas* spp.).

Bioflav (AgroVentures LLC, USA): it is a flavonoid-based glycolic extract that performs physiological activity on the plant, improves photosynthesis, growth, germination, and rooting, improving the quality of the vegetal products. It strengthens growth processes, increases the ability to adapt to biotic and abiotic stress factors, stimulates the defense mechanisms of plants by increasing the availability of phenolic and antioxidant compounds and strengthening cell walls. It also has a chelating, antioxidant, anti-enzymatic and UV protection action.

Kiforce® (Alba Milagro International S.p.A., Italy): It is a liquid formulation with active ingredients (chitosan and derivatives) acting as elicitors, i.e., inducers of the plant's response mechanisms to negative factors such as parasitic attacks or stressful conditions. The natural extracts of Kiforce favour the production of defensive substances with an excellent residual effect in plant organisms (such as phytoalexins, chitinases and specific defense proteins), which can act locally or systemically. Furthermore, the presence of microelements in the

formulation prevents the onset of deficiencies and strengthens the defense reactions of plants, combining the nutritional functions with the eliciting effect of natural extracts.

The antimicrobial activity was firstly evaluated against *Pseudomonas syringae* pv. *tomato* (*Pst*) and *Xanthomonas campestris* pv. *pelargoni* (*Xcp*) using the disk diffusion assay that resulting the best method to screen rapidly many products against many phytopathogenic bacteria (Bauer et al., 1966).

Pst, is the causal agent of bacterial speck of tomato, and was first described by Okabe (1933) on tomato in Taiwan. The economic importance of the disease caused by this phytopathogenic bacteria was realized in the late 1970s when it caused serious losses to winter tomato crop in USA (Thind, 2019).

Xcp is the causal agent of bacterial blight in plants of the genera *Pelargonium* and *Geranium.*

The management of both disease is based on preventive measures also involving the use of copper-based products, but through the years cases of resistance of this bacteria to copper-based products and antibiotics were observed (Cazorla et al., 2002; Sundin & Bender, 1993). Accordingly, as evidenced by research of the present thesis, *Pst* resulted less sensitive to Kalex Cu compared to the other products.

For this purpose, new antibacterial compounds and formulates compatible with organic agriculture and IPM and alternative to copper products are needed. All the tested products were able to inhibit the growth of both bacteria, showing different degree of sensitivity at the tested concentration. In all cases the size of the Inhibition Halo (IH) was directly dependent to the concentration. The results underline the potentially use of these compounds as bactericides for the control of these plant pathogenic bacteria.

The second step of the research was focused on the evaluation of the useful potential of these compounds for the management of numerous plant bacterial diseases. Then, for this purpose, the selected antimicrobials compounds were tested against the following seven important phytopathogenic bacteria, causing severe crop losses worldwide:

i) *Erwinia amylovora* (*Ew*), ii) *Xanthomonas arboricola* pv. *pruni* (*Xap*), iii) *Ralstonia solanacearum* (*Rs*), iv) *Clavibacter michiganensis* subsp. *michiganensis* (*Clm*), v) *Pseudomonas syringae* pv. *actinidiae* (*Psa*), vi) *X. euvesicatoria* (*Xe*) and vii) *X. campestris* pv. *campestris* (*Xcc*).

- *Ew* is the causal agent of the fire blight, that is probably the most devastating bacterial disease of some pome fruits and also the most feared by growers (Thomson, 2001).

The pathogen is capable of completely destroying pome fruit orchards in a single growing season (Vanneste, 2000). The bacterium infects more than 180 plant species including fruit trees, wild and ornamental plants, most of them belonging to the *Rosaceae* family (Kim et al., 2001). No single control measure is effective for its management but an integrated approach involving sanitation, horticultural practices, biocontrol agents and chemicals, is suggested to minimize disease severity (Thind, 2019).

- *Xe* is the causal agent of the bacterial spot of tomato and pepper, a serious disease of both crops. The pathogen causes severe crop losses, yield reduction and qualitative deterioration of the fruits because of its defoliation effects and severe spotting of fruits (Jones 2001). The management of the disease includes sanitary and preventive measures able to decrease its incidence. Use of copper compounds and other antimicrobial compounds (streptomycin) has created a serious problem of developing pathogenic strains resistant to these chemicals (Thind, 2019). To control copper resistant strains, a combination of copper and mancozeb applied preventively as sprays after transplanting, was suggested. However, since mancozeb in Europe was recently withdrawn from the market for its potential toxicity to consumers new alternative products need to be found.
- *Clm* is the causal agent of bacterial canker of tomato. It was one of the most dangerous phytopathogenic bacteria, the occurrence of the disease was reported worldwide wherever tomatoes are grown. Although outbreaks of this bacterium are now sporadic, it is very destructive both for field and greenhouse crops. It is a quarantine pathogen and many countries (Canada, USA, European Union, and some other countries) enforce zero tolerance for import and export of plant material infected with the pathogen. The management of bacterial canker of tomato poses a significant challenge due to the lack of effective chemicals and resistant varieties as well as to the systemic nature of the pathogen (Thind, 2019).
- *Psa* is the causal agent of the bacterial canker of kiwi and was first reported in California, USA in 1980. In the EPPO region, the disease was first noticed in northern Italy in 1992 where it remained sporadic and with a low incidence for 15 years. However, in 2007 and 2008 economic losses occurred, particularly in the Lazio region, and the possible spread of the disease to other kiwifruit producing regions in Italy began to raise concerns. Due to the emerging problem in the Mediterranean region, the EPPO Secretariat decided to add it to the EPPO Alert List.

In Italy, it is estimated that the economic losses, including impact on trade, due to *Psa* have reached 2 million euros (Balestra et al., 2009). The disease has also been reported in China, New Zealand, Australia, Spain, Chile, and Korea. The management of the disease includes sanitation and preventive measures that can be helpful in minimizing the disease incidence. The treatment with antimicrobial compounds (copper and zinc-based products, biostimulants, fungicides, antibiotics) is one of the most used control measures, but bacterial resistance to these antimicrobials was detected in natural population of *Psa* (Goto et al., 1994; Lee et al., 2005; Vanneste and Voyl, 2003; Han et al., 2004)

- *Rs* causes bacterial wilt of solanaceous plants. This pathogen causes a highly destructive disease of many solanaceous and some non-solanaceous crops. It is considered the world's single most destructive bacterial plant disease. The bacterium infects many solanaceous plants (potato, tomato, eggplant, tobacco, and pepper), and some non-solanaceous hosts like banana, peanut, and heliconia as well as geranium and other ornamental plants and weeds. More than 200 plant species belonging to 50 families are attacked by the bacterium. The management of the disease is very difficult due to the pathogen soil-borne nature, wide host range and high pathogenic variability as well as the limited host resistance.
- *Xap* causes bacterial spot of stone fruits. The disease was first described by E.F. Smith in 1903 on plums in Michigan, USA. Although it was primarily a problem for peach and nectarine, the disease also occurs on apricots, plums and, to a lesser extent on cherries and other stone fruits. Besides cultivated species, all the ornamental *Prunus* species and hybrids can be affected by this bacterium (Stefani, 2010). In a review article, Lamichhane (2014) provided an overview of *X. arboricola* diseases of stone fruit, almond, and walnut trees and discussed the current and future management strategies. It is very difficult to control the disease on highly susceptible and on moderately susceptible varieties under optimal environmental conditions for disease development. All the preventive and sanitation measures should be taken to minimize the losses. A major problem to control *Xap* is the lack of antimicrobial compounds and products to counteract the disease, due to the sensitivity of host plants to copper that cause phytotoxicity on plant.
- *Xcc* is the causal agent of black rot (bacterial wilt) of crucifer, one of the most destructive diseases of cruciferous crops. The disease was first reported by Garman in 1891 from Lexington Kentucky, USA on cabbage. The pathogen was one of the

first bacteria showing a seed-borne nature (Harding et al., 1904). It is considered the most important worldwide disease of crucifers, attacking all cultivated brassicas, radishes, and numerous cruciferous weeds. It affects many cultivated and wild crucifers. In warm and wet climate, the losses due to black rot may exceed 50% on some crops. The disease occurs wherever the cabbage is grown. Management of the disease is based mainly on eradicative and preventive measures (e.g., spraying the crop with copper-based products at 10-day intervals).

Collectively, the results obtained in the assays conducted with the above reported phytopathogenic bacteria confirm the large spectrum antibacterial activities of most of the tested products and their high potential to be used in integrated approaches to control these pathogens on different crops.

Only against woody plant pathogenic bacteria *(i.e, Ew, Xap,* and *Psa*) among the tested products tannins-derivatives and Algatan Gea (AG) were ineffective or less effective. It is possible that the resistance to tannins and AG (which also includes tannins in its formulation) of these bacteria is due to their natural lifestyle characterized by their ability to adapt, survive and spread in the woody tissues of plants where polyphenolic molecules (such as phenolic acids, flavonoids, etc.) are very abundant. For example Pletzer & Weingart (2014) analysed the function of some multidrug efflux pumps of *Erwinia amylovora*, and their role in resistance towards antimicrobial plant compounds, such as flavonoids.

Another work of the present research was the evaluation of the selected compounds for their activity *in vitro* and *in vivo* against strains and subspecies of the quarantine bacteria *Xylella fastidiosa*.

Xylella fastidiosa is a fastidious xylem-inhabiting bacterium causing severe plant diseases worldwide, such as Pierce's disease of grape in California USA, Citrus Variegated Chlorosis in Brazil (Agrios, 2005) and Olive Quick Decline Syndrome (OQDS) in Italy (Saponari et al., 2017). Furthermore the different subspecies can be pathogenic on more than 300 plant species (Morelli et al., 2021). This fastidious bacterium is unable to grow on conventional bacteriological media, requiring special media for its cultivation, on which it still grows very slowly. All gram-negative xylem-inhabiting fastidious bacteria are transmitted by xylem-feeding insects, such as sharpshooter, leafhoppers (*Cicadellinae*) and spittlebugs (*Cercopidae*) (Cornara et al., 2017). The vectors can acquire and transmit the bacteria in less than two hours. Viruliferous adult insects can transmit the bacteria for all their life but do not transfer them on to progeny.

The antimicrobial activity against *Xylella fastidiosa* was evaluated against the following 4 strains or subspecies of *Xylella fastidiosa*:

- *X. fastidiosa* subsp. *pauca* ST53 (*Xfp*): it is a strain associated with the quick decline syndrome of olive (OQDS), discovered in 2013 in Apulia (Salento peninsula, southern Italy) (Saponari et al., 2017);
- *X. fastidiosa* CO33 (*XfCO33*): it is a strain isolated from a coffee plant intercepted in northern Italy, representing a novel multilocus sequence typing profile, ST72 (G. Loconsole, personal communication). Genome sequence analysis and phylogenetic studies revealed that CO33 is related to isolates of subsp. *sandyi* or of subsp. *morus*, corroborating the genetic complexity of this plant pathogen bacterium and the role of homologous recombination on *X. fastidiosa* diversity (Giampetruzzi et al., 2015);
- *X. fastidiosa* subsp. *multiplex* TOS1 (*XfmTOS1*): it is a strain discovered in, an outbreak reported in the Italian region of Tuscany; with infections were identified in seven different plant species (*Rhamnus alaternus*, *Polygala myrtifolia, Rosmarinus officinalis, P. amygdalus, Cistus* spp., *Lavandula* spp., *Spartium junceum*) (Saponari et al., 2019).
- *X. fastidiosa* subsp. *multiplex* strain ESVL (*XfmESVL*): it is a strain discovered in 2017 in mainland Spain affecting almond trees (Giampetruzzi et al., 2018).

The results of the present study revealed that sensitivities of the strains and subspecies of *Xf* to the tested antimicrobial products were different.

Xfp was resistant to Kalex Zn, Kalex Zn + Kalex Cu and Kiforce, while it showed intermediate sensitivity to Bioflav, and highly sensitive to all other products.

Xf_CO33 was resistant to AG, U4, Bioflav and Kiforce and sensitive to all the other products.

The two tested strains of *X. fastidiosa multiplex* (*Xfm_TOS1* and *Xfm_ESVL*) showed a similar response. They were resistant to Kalex Zn and Kalex Cu, Siliforce, Bioflav and Kiforce, weakly susceptible to U2, and susceptible to all the other products.

To study the bactericidal effect *in vitro* of selected compounds against *X. fastidiosa* subsp. *pauca* strain ST53, the agent of olive quick decline syndrome (OQDS), the concentrations were selected according i) the MIC concentration evaluated in previous experiments using the disk diffusion assay, ii) the field recommended doses and iii) the potential use of some products as plant defence activators. The selected products were tested against *Xfp* using the

time kill assay method that is usually used to study the activity of antibiotics against bacteria since this assay can determine both bactericidal or bacteriostatic activity of an antimicrobial compound over time (Osburne et al., 2006).

Xfp was exposed over time to the selected concentration of each product. The results of this experiment, clearly show that all the products, except Algatan Gea ethanolic extract (AGEtoh) and Siliforce (Si) that resulted less effective, were able to kill the bacterial cells after 24 h of contact.

Tannins (U1, U2, U3, U4) and Algatan Gea showed a very interesting bactericidal activity, resulting so effective as the antibiotic control (kanamycin), and at some concentrations U1, U3 and U4, they resulted even tendentially more efficient than kanamycin, from a statistical point of view.

As evidenced in the experiments, Kalex Zn and Kalex Cu and their mixture were the treatments with the best bactericidal activity.

However, for Kalex Zn the results obtained by the time kill assay are in contrast with the disk diffusion assay by which Kalex Zn not clearly inhibited the bacterial growth. These discordant results are probably due to *Xylella fastidiosa* aggregates that are little sensitive to the zinc. Furthermore, as evidenced in the experiments the disk diffusion assay show criticisms only against *X. fastidiosa* and not with other phytopathogenic bacteria tested produce aggregates in *in vitro* condition. Therefore, the disk diffusion assay is probably not appropriate to assay Zinc compounds against *Xylella fastidiosa*.

Some of the best antimicrobial products *in vitro* were chosen for the following greenhouse experiments on olive plants. In these trials, the experimental schedule adopted included 2 soil treatments (curative and preventive) and 6 foliar-spray treatments. Untreated and uninoculated control, and untreated and inoculated control were used as negative and positive control, respectively, for both soil and foliar-spray treatments. During the experiments, periodically, from the inoculation time to the end of the experiment, some physiological parameters, such as plant height and shoots numbers, were monitored. Moreover, in each survey for all treatments the disease index was recorded by using an appropriate disease scale. In addition, a qPCR approach was used for monitoring the bacterial population in treated plants over the time.

At the end of the experiments, plant height and shoot number of plants treated on the soil with Algatan Gea (AG) were not different from untreated-inoculated plants. However, this result is affected by the phytotoxic effect observed on plant treated with AG, probably due to an excess of product accumulated in the soil of plants kept under greenhouse condition and not exposed to atmospheric precipitation and/or frequent irrigations. Indeed in a previous trial in open air (nursery, potted young plants and field adult plants) plants treated with the same dosage of AG did not show sign of phytotoxicy and had a consistent increase of growth with respect to untreated plants (Del Grosso & Lima, 2021). In the present greenhouse experiments, although plants were irrigated two or three times a week, due the hight humidity of the controlled condition in such protected environment, the soil electrical conductivity (EC) measurements ("total salts" concentration in the soil) was very high: more than 4500 $\mu S/cm^2$ on treated plants with AG compared to~ 700-1000 $\mu S/cm^2$ of the 2 untreated control.

In foliar treatments, the average plant height was very similar between treated and untreated plants (control). Only plants treated with Tannin U1 were statistically higher than those of untreated controls. In general, plants of the untreated-uninoculated control were richer of shoots than all treated plants.

Concerning the shoot number, plant treated with Tannin U3, U4 and K-Zn showed a significant increase with respect to untreated-inoculated plants, but anyway not statistically different. Comparing the two controls, the increment of shoots of untreated-uninoculated plants was significantly higher than untreated-inoculated ones.

In addition to the assessment of physiological parameters, in each survey the disease index in plants of each treatment was recorded by using an appropriate disease scale.

In soil applications, no treatment (preventive or curative) was able to reduce the disease severity on plants.

A different scenario was found in foliar treatments, in which Tannins U1, U2 and U3 as well as K-Zn significantly reduced the disease severity compared to the untreated-inoculated control. No significant differences were observed comparing these treatments with the untreated-uninoculated control, only K-Zn show a slightly higher disease rate resulting significantly different compared to the untreated-uninoculated control, but no difference was observed comparing K-Zn with U1, U2 and U3.

As observed in a parallel experiment in open field, in which Kalex Zn and Kalex Cu were tested, the results were positive and tendentially similar. However, when after a vegetative season the treatments were stopped, the previous treated plants evidenced a progressive increase of symptoms over time.

To evaluate the effect of treatments on bacterial population inside the plants a qPCR approach was used for monitoring the bacterial population in treated plants at two times: i) soon before starting of treatments (T0); and ii) the end of the experiments (T1).

Results evidenced that all foliar treatments significantly reduced the bacterial population at the end of the experiments (T1) compared to the bacterial population detected at T0.

In soil treatments, in the untreated-inoculated control, the mean of the bacterial population at T1 resulted tendentially higher than at T0.
AG curative and preventive treatments did not reduce the bacterial population, but on the contrary, these seem to increase it. However, in these two trials we have to consider that many soil treated plants dead because of phytotoxic effects of the products and then at the last sampling a very low and unrepresentative number of plants remained for each thesis.

6. CONCLUSION

The interest for the search of new active compounds for controlling plant pathogenic bacteria is a big challenge in a modern plant protection strategy of management.

In this scenario, the aim of this PhD thesis was to find new sustainable antimicrobial products (mineral and vegetal derived compounds) effective against important phytopathogenic bacteria and particularly against *Xylella fastidiosa.*

Nowadays, no chemical curative disease control mean is available to counteract *Xylella fastidiosa*. Preventive measures, such as rapid identification of new outbreaks, eradication and quarantine, to the adoption of resistant varieties, cultural and sanitation measures (including protection of nursery stock from infection and nursery certification), and insect vector control are the only available weapons to counteract the bacterium. However, these methods are not sufficient due the complexity of the disease epidemiology, because the bacterium has many symptomless hosts, including weeds, ornamentals, and other crops. Over the years, however, the new infections tend to increase, becoming endemic in many areas, as in Apulia for *X. fastidiosa pauca* on olive plants. In this area it is necessary to create conditions of coexistence with the pathogen. Under this scenario, much research is underway to find solutions to better and directly fight the bacterium and its main vector insect, *Philaenus spumarius* L. (Morelli et al., 2021). The main objective was to find a strategy to control the pathogen and save the olive heritage.

The research was focused on the testing of new and innovative products and substances, evaluating their usefulness as antimicrobials against this pathogen as well as other phytopathogenic bacteria. Firstly, the most effective method for testing the antibacterial activity of selected products was set up.

In the first part of the research the chosen antimicrobial products were tested against 13 widespread and important phytopathogenic bacteria, whereas the second part of the activity focused on the evaluation of the selected products against *Xylella fastidiosa* subsp. *pauca* (*Xfp*), the causal agent of the Olive Quick Decline Syndrome (OQDS).

Results of assays showed that many of the tested products had a strong bactericidal activity against most of the tested bacteria, and the activity was often similar and sometimes even better than the standard antibiotics used as a control.

Against *Xfp*, after the *in vitro* investigation, an *in vivo* biocontrol assay was carried out on young olive plants maintained in greenhouse conditions. The results showed that some of the foliar treatments were able to significantly reduce the presence of symptoms on the treated plants. In addition, a significant reduction of the bacterial population was observed

at the end of the experiments compared to the starting conditions. However, once the treatments were suspended, the plants underwent a rapid decay that led to their death.

Collectively, results of experiments reported in the present thesis are positive and confirmed the large spectrum capability of most of the tested compounds to inhibit the growth of different species of widespread and dangerous phytopatogenic bacteria, even includeing different strains and subspecies of *X. fastidiosa,* among them *X. fastidiosa pauca* strain ST53 responsible for Olive Quick Decline on olive plants. Since most of the tested products are systemic or cytotropic, they are very interesting due to their ability of inducing a defence response in plant and control endophytic bacteria, such as *Xf*, which colonize the internal or vascular tissues of the host plant.

Further experiments *in vivo*, on pot grown plants or in the field are necessary to confirm the positive result obtained in the present research.

Tables and Figures

Table 1. Large spectrum antimicrobial activity *in vitro* of Algatan Gea (AG) against nine important plant pathogenic bacteria by the disk diffusion assay. For each concentration of product, the mean (± SD) of the inhibition halo diameter (in mm) against each bacterial strain is reported. The max detection limit, according to the plate width, is of 20 mm (Ø). POS_C represents the positive control: drug reference (ampicillin 100 ppm + streptomycin 250 ppm); NEG_C represents the negative control: water control (untreated).

Inhibition zone diameter (mm)							
Concentration (v/v)	***Erwinia amylovora***	***Xanthomonas euvesicatoria***	***C. michiganensis* subsp. *michiganensis***	***Pseudomonas syringae* pv. *actinidiae***	***Ralstonia solanacearum***	***Xanthomonas arboricola* pv. *pruni***	***Xanthomonas campestris* pv. *campestris***
AG 8%	0	12.4±0.5	11.2±0.9	12.7±0.2	>20	0	>20
AG 4%	0	9.6±0.3	9.5±0.8	10.1±0.8	19.9±1.7	0	>20
AG 2%	0	8.2±0.3	7.5±0.7	9.4±0.9	16±1.1	0	>20
AG 1%	0	0	0	7.5±0.6	12.4±1	0	16.7±0.2
AG 0.5%	0	0	0	0	10.2±0.6	0	13.8±0.6
AG 0.25%	0	0	0	0	8.3±0.9	0	11.2±0.7
AG 0.12%	0	0	0	0	0	0	0
NEG_C	0	0	0	0	0	0	0
POS_C	17.3±0.6	12.1±0.5	18.7±0.8	13.9±1.5	11.6±0.4	22.1±1.2	15.3±0.6

Table 2. Large spectrum antimicrobial activity *in vitro* of Kalex Zn (K-Zn) against nine important plant pathogenic bacteria by the disk diffusion assay. For each concentration of product, the mean (± SD) of the inhibition halo diameter (in mm) against each bacterial strain is reported. The max detection limit, according to the plate width, is of 20 mm (Ø). POS_C represents the positive control: drug reference (ampicillin 100 ppm + streptomycin 250 ppm); NEG_C represents the negative control: water control (untreated).

Inhibition zone diameter (mm)							
Concentration (v/v)	***Erwinia amylovora***	***Xanthomonas euvesicatoria***	***C. michiganensis* subsp. *michiganensis***	***Pseudomonas syringae* pv. *actinidiae***	***Ralstonia solanacearum***	***Xanthomonas arboricola* pv. *pruni***	***Xanthomonas campestris* pv. *campestris***
K-Zn 4%	>20	>20	>20	18.6±0.7	>20	>20	>20
K-Zn 2%	>20	>20	>20	14.6±0.7	18.1±0.4	19±2	>20
K-Zn 1%	>20	16.9±0.3	>20	17.2±1	14±0.5	14.9±1.2	17.6±0.8
K-Zn 0.5%	17.3±0.2	14.3±0.4	>20	13.4±0.6	10.6±0.7	12.1±0.1	14±0.7
K-Zn 0.25%	12.6±0.2	12.4±0.2	19.6±0.3	10.6±0.1	9.3±1	9.9±0.8	11.4±0.7
K-Zn 0.12%	8.8±0.7	9±0.1	15.7±2.6	9.5±0.5	0	7.8±0.5	10.1±0.2
K-Zn 0.06%	7.5±0.3	7.3±0.3	9.5±1.3	7.3±0.1	0	0	8.5±0.3
NEG_C	0	0	0	0	0	0	0
POS_C	17.3±0	11.8±0.4	17.9±0.7	15.7±0.6	11.9±0.2	18.8±1.6	14.1±0.4

Table 3 Large spectrum antimicrobial activity *in vitro* of Kalex Cu (K-Cu) against nine important plant pathogenic bacteria by the disk diffusion assay. For each concentration of product, the mean (± SD) of the inhibition halo diameter (in mm) against each bacterial strain is reported. The max detection limit, according to the plate width, is of 20 mm (Ø). POS_C represents the positive control: drug reference (ampicillin 100 ppm + streptomycin 250 ppm); NEG_C represents the negative control: water control (untreated).

Inhibition zone diameter (mm)							
Concentration (v/v)	***Erwinia amylovora***	***Xanthomonas euvesicatoria***	***C. michiganensis* subsp. *michiganensis***	***Pseudomonas syringae* pv. *actinidiae***	***Ralstonia solanacearum***	***Xanthomonas arboricola* pv. *pruni***	***Xanthomonas campestris* pv. *campestris***
K-Cu 4%	>20	13.2±0.9	>20	17.4±0.7	16.6±1.4	17.6±2.3	>20
K-Cu 2%	>20	12.4±0.9	13.8±0.7	12.2±1	13.2±1.3	13.9±1.4	14.5±1.8
K-Cu 1%	14.3±1.3	10.6±0.4	10.5±0.4	9.5±0.5	9.9±0.6	12±0.8	12±0.5
K-Cu 0.5%	9.2±0.3	9.7±0.5	7.4±0.5	7.9±0.4	7.2±1.1	10.8±0.4	10.7±0.3
K-Cu 0.25%	7.8±0.3	8.7±0.9	0	0	0	9.1±0.2	8.9±0.8
K-Cu 0.12%	0	7.6±0.4	0	0	0	0	0
K-Cu 0.06%	0	0	0	0	0	0	0
NEG_C	0	0	0	0	0	0	0
POS_C	19.4±0.4	13.9±1.6	20.1±0.7	14±0.8	12.7±0.6	20.8±2.1	14.6±0.7

Table 4. Large spectrum antimicrobial activity *in vitro* of the mixture of Kalex Zn + Kalex Cu (K-Zn+K-Cu) against nine important plant pathogenic bacteria by the disk diffusion assay. For each concentration of product, the mean (± SD) of the inhibition halo diameter (in mm) against each bacterial strain is reported. The max detection limit, according to the plate width, is of 20 mm (Ø). POS_C represents the positive control: drug reference (ampicillin 100 ppm + streptomycin 250 ppm); NEG_C represents the negative control: water control (untreated).

Inhibition zone diameter (mm)							
Concentration (v/v)	***Erwinia amylovora***	***Xanthomonas euvesicatoria***	***C. michiganensis* subsp. *michiganensis***	***Pseudomonas syringae* pv. *actinidiae***	***Ralstonia solanacearum***	***Xanthomonas arboricola* pv. *pruni***	***Xanthomonas campestris* pv. *campestris***
K-Zn+K-Cu 4%	>20	>20	>20	>20	19±0.5	>20	>20
K-Zn+K-Cu 2%	>20	18.4±1.3	>20	>20	15±1.2	17.2±0.7	>20
K-Zn+K-Cu 1%	19.2±0.6	15.1±0.6	18.3±1.2	14.3±1.3	12.3±0.4	14.1±0.4	16.2±0.5
K-Zn+K-Cu 0.5%	16.3±1.1	12±0.5	13.9±1.7	11.4±0.9	10.5±0.6	11.7±0.3	13.3±0.4
K-Zn+K-Cu 0.25%	10.3±0.8	9.7±0.5	12.2±1	10.4±0.7	8.5±0.4	10.1±0.5	11.7±0.1
K-Zn+K-Cu 0.12%	7.7±0.3	7.8±0.2	8.8±0.4	8.9±0.5	7.8±0.3	8.1±0.6	9.1±0.9
K-Zn+K-Cu 0.06%	0	0	0	7.3±0.1	0	0	0
NEG_C	0	0	0	0	0	0	0
POS_C	16.4±0.2	13.4±0.8	20.4±1.2	14.4±0.8	11.7±0.2	20.6±1.3	15.2±0.4

Table 5. Large spectrum antimicrobial activity *in vitro* of Siliforce (Si) against nine important plant pathogenic bacteria by the disk diffusion assay. For each concentration of product, the mean (± SD) of the inhibition halo diameter (in mm) against each bacterial strain is reported. The max detection limit, according to the plate width, is of 20 mm (Ø). POS_C represents the positive control: drug reference (ampicillin 100 ppm + streptomycin 250 ppm); NEG_C represents the negative control: water control (untreated).

Inhibition zone diameter (mm)							
Concentration (v/v)	***Erwinia amylovora***	***Xanthomonas euvesicatoria***	***C. michiganensis* subsp. *michiganensis***	***Pseudomonas syringae* pv. *actinidiae***	***Ralstonia solanacearum***	***Xanthomonas arboricola* pv. *pruni***	***Xanthomonasc ampestris* pv. *campestris***
Si 8%	>20	14.5±1	>20	17±1.8	13.9±1.4	14.2±0.4	17.5±0.6
Si 4%	18.9±1.1	11.9±0.3	>20	12.5±1.4	11.2±1.3	12.2±0.4	13.5±0.6
Si 2%	15.2±0.3	10.3±0.5	18±2.2	10.3±0.7	8.9±0.3	10.5±0.4	11.9±0.3
Si 1%	10.4±0.4	8.9±0.2	15.5±0.8	9.2±1	7.5±0.2	8.5±0.3	10.4±0.9
Si 0.5%	8.3±0.3	7.8±0.3	13.2±0.3	8.3±1.2	0	0	8.8±0.6
Si 0.25%	0	0	9±0.5	6.5±0.9	0	0	0
Si 0.12%	0	0	0	0	0	0	0
NEG_C	0	0	0	0	0	0	0
POS_C	17.3±0.4	13.6±1	17.2±1.4	14.7±0.5	11.8±0.6	18.8±1.6	15±1.3

Table 6. Large spectrum antimicrobial activity *in vitro* of Tannin U1 (U1) against nine important plant pathogenic bacteria by the disk diffusion assay. For each concentration of product, the mean (± SD) of the inhibition halo diameter (in mm) against each bacterial strain is reported. The max detection limit, according to the plate width, is of 20 mm (Ø). POS_C represents the positive control: drug reference (ampicillin 100 ppm + streptomycin 250 ppm); NEG_C represents the negative control: water control (untreated).

Inhibition zone diameter (mm)							
Concentration (v/v)	***Erwinia amylovora***	***Xanthomonas euvesicatoria***	***C. michiganensis* subsp. *michiganensis***	***Pseudomonas syringae* pv. *actinidiae***	***Ralstonia solanacearum***	***Xanthomonas arboricola* pv. *pruni***	***Xanthomonas campestris* pv. *campestris***
U1 8%	0	14.1±0.1	10.8±1.1	12.3±0.8	>20	0	>20
U1 4%	0	11.6±1	8.7±0.7	10.5±0.9	>20	0	>20
U1 2%	0	9.6±0.3	7.5±0.2	8.4±0.5	19.9±1	0	>20
U1 1%	0	8.4±0.4	6.8±0.7	6.9±0.3	18.3±1.7	0	>20
U1 0.5%	0	6.9±0.8	0	0	14.9±0.3	0	18.5±1
U1 0.25%	0	0	0	0	11.6±1	0	16±1.1
U1 0.12%	0	0	0	0	8.1±0.4	0	13.5±0.6
NEG_C	0	0	0	0	0	0	0
POS_C	15.2±0.9	12.3±0.9	13.7±0.4	12.1±0.8	11.7±0.3	19.9±0.8	13.9±0.5

Table 7 Large spectrum antimicrobial activity *in vitro* of Tannin U2 (U2) against nine important plant pathogenic bacteria by the disk diffusion assay. For each concentration of product, the mean (± SD) of the inhibition halo diameter (in mm) against each bacterial strain is reported. The max detection limit, according to the plate width, is of 20 mm (Ø). POS_C represents the positive control: drug reference (ampicillin 100 ppm + streptomycin 250 ppm); NEG_C represents the negative control: water control (untreated).

Inhibition zone diameter (mm)							
Concentration (v/v)	***Erwinia amylovora***	***Xanthomonas euvesicatoria***	***C. michiganensis* subsp. *michiganensis***	***Pseudomonas syringae* pv. *actinidiae***	***Ralstonia solanacearum***	***Xanthomonas arboricola* pv. *pruni***	***Xanthomonas campestris* pv. *campestris***
U2 8%	0	11.6±0.6	16.1±0.3	12±0.7	13.4±0.8	0	14.1±0.5
U2 4%	0	9.3±0.8	12.9±0.6	8.8±0.5	11.2±0.5	0	10.8±0.4
U2 2%	0	7.8±0.9	10.7±0.6	7.4±0.2	8.7±0.3	0	9±0
U2 1%	0	0	7.9±0.1	0	7.8±0.5	0	6±0.1
U2 0.5%	0	0	0	0	0	0	0
U2 0.25%	0	0	0	0	0	0	0
U2 0.12%	0	0	0	0	0	0	0
NEG_C	0	0	0	0	0	0	0
POS_C	15.9±1.8	11.5±0.8	12.9±0.6	13.1±1.3	11.4±0.5	18±0.6	14.7±0.1

Table 8. Large spectrum antimicrobial activity *in vitro* of Tannin U3 (U3) against nine important plant pathogenic bacteria by the disk diffusion assay. For each concentration of product, the mean (± SD) of the inhibition halo diameter (in mm) against each bacterial strain is reported. The max detection limit, according to the plate width, is of 20 mm (Ø). POS_C represents the positive control: drug reference (ampicillin 100 ppm + streptomycin 250 ppm); NEG_C represents the negative control: water control (untreated).

Inhibition zone diameter (mm)							
Concentration (v/v)	***Erwinia amylovora***	***Xanthomonas euvesicatoria***	***C. michiganensis* subsp. *michiganensis***	***Pseudomonas syringae* pv. *actinidiae***	***Ralstonia solanacearum***	***Xanthomonas arboricola* pv. *pruni***	***Xanthomonas campestris* pv. *campestris***
U3 8%	0	13.7±0.4	12.7±0.7	11.3±0.7	>20	0	>20
U3 4%	0	9.7±0.5	9.5±0	9.3±0.6	>20	0	>20
U3 2%	0	8.1±0.2	8.3±0.1	0	>20	0	>20
U3 1%	0	0	6.6±0.6	0	18.5±0.7	0	>20
U3 0.5%	0	0	0	0	15.6±1.3	0	>20
U3 0.25%	0	0	0	0	12.4±0.8	0	18.4±0.3
U3 0.12%	0	0	0	0	9.2±0.6	0	15.1±0.5
NEG_C	0	0	0	0	0	0	0
POS_C	15.3±0.3	11±0.8	13.5±1.1	12.3±0.8	12±0.6	19.2±0.6	14.9±0.2

Table 9. Large spectrum antimicrobial activity *in vitro* of Tannin U4 (U4) against nine important plant pathogenic bacteria by the disk diffusion assay. For each concentration of product, the mean (± SD) of the inhibition halo diameter (in mm) against each bacterial strain is reported. The max detection limit, according to the plate width, is of 20 mm (Ø). POS_C represents the positive control: drug reference (ampicillin 100 ppm + streptomycin 250 ppm); NEG_C represents the negative control: water control (untreated).

Inhibition zone diameter (mm)							
Concentration (v/v)	***Erwinia amylovora***	***Xanthomonas euvesicatoria***	***C. michiganensis* subsp. *michiganensis***	***Pseudomonas syringae* pv. *actinidiae***	***Ralstonia solanacearum***	***Xanthomonas arboricola* pv. *pruni***	***Xanthomonas campestris* pv. *campestris***
U4 8%	9.7±0.5	14±0.4	14.6±0.9	14.3±1.8	>20	16.6±1.9	>20
U4 4%	0	12.1±0.7	11.8±0.4	9.9±0.9	16.1±2.6	14.1±1	>20
U4 2%	0	9.8±0.6	10±0.4	8±0.1	14.5±2.1	12.4±1.6	>20
U4 1%	0	8±0.7	8.1±0.5	6.9±0.1	12.7±1.7	11±1.7	>20
U4 0.5%	0	0	7.3±0.2	0	10.3±0.6	8.9±1.4	16.6±0.6
U4 0.25%	0	0	0	0	8.5±0.5	6.8±1.4	14.2±0.5
U4 0.12%	0	0	0	0	0	0	11±0.6
NEG_C	0	0	0	0	0	0	0
POS_C	15.5±0.2	12.2±1.3	13.4±1	12.4±1.4	12.1±0.4	22.3±2.9	12.6±0.7

Table 10. Descriptive statistics (mean±SD of 3 replicates) of the minimum inhibitory concentration (MIC) values of *in vitro* tested products against *Pseudomonas syringae* pv. *tomato* (*Pst*) and *Xanthomonas campestris* pv. *pelargoni* (*Xcp*). Only significant ($P < 0.05$) MIC values are considered according to the one-way analysis of variance (ANOVA) using the Fisher's Least Significant Difference (LSD) test.

	P. syringae* pv. *tomato		***X. campestris* pv. *pelargoni***	
	Inhibition zone (mm)	MIC % v/v	Inhibition zone (mm)	MIC % v/v
SI	9.7±0.6	0.5	10±0	1
AG	7.7±0.6	0.5	8±0	8
U1	9±0	0.12	8±0	1
U2	8±1	0.5	8.7±1.2	4
U3	9±1	0.12	7.3±0.6	0.5
U4	10.7±0.6	0.25	7.7±1.2	1
K-Zn	8.7±0.6	0.06	8.7±0.6	0.12
K-Cu	11.7±1.2	2	8±0	0.5
K-Zn+K-Cu	8.3±1.2	0.12	7.7±0.6	0.12

Table 11. Descriptive statistics (mean±SD of 3 replicates) of the minimum inhibitory concentration (MIC) values of *in vitro* tested products against *Erwinia amylovora (Ew), X. euvesicatoria (Xe), C. michiganensis subsp. michiganensis (Clm), P. syringae pv. actinidiae (Psa), Ralstonia solanacearum (Rs), X. arboricola pv. pruni (Xap), X. campestris pv. campestris (Xcc).* Only significant ($P < 0.05$) MIC values are considered according to the one-way analysis of variance (ANOVA) using the Fisher's Least Significant Difference (LSD) test. Note: (ni) = non inhibited.

	Erwinia amylovora		***X. euvesicatoria***		***C. michiganensis* subsp. *michiganensis***		***P. syringae* pv. *actinidiae***		***Ralstonia solanacearum***		***X. arboricola* pv. *pruni***		***X. campestris* pv. *campestris***	
Tested product	Inhibition zone (mm)	**MIC (% v/v)**	Inhibition zone (mm)	**MIC (% v/v)**	Inhibition zone (mm)	**MIC (% v/v)**	Inhibition zone (mm)	**MIC (% v/v)**	Inhibition zone (mm)	**MIC (% v/v)**	Inhibition zone (mm)	**MIC (% v/v)**	Inhibition zone (mm)	**MIC (% v/v)**
SI	8.3±0.3	0.5	7.8±0.3	0.5	9±0.5	0.25	8.3±1.2	0.5	7.5±0.2	1	8.5±0.3	1	8.8±0.6	0.5
AG	ni	ni	8.2±0.3	2	7.5±0.7	2	7.5±0.6	1	8.3±0.9	0.25	ni	ni	11.2±0.7	0.25
U1	ni	ni	8.4±0.4	1	7.5±0.2	2	8.4±0.5	2	8.1±0.4	0.12	ni	ni	13.5±0.6	0.12
U2	ni	ni	7.8±0.9	2	7.9±0.1	1	7.4±0.2	2	7.8±0.5	1	ni	ni	9±0	2
U3	ni	ni	8.1±0.2	2	8.3±0.1	2	9.3±0.6	4	9.2±0.6	0.12	ni	ni	15.1±0.5	0.12
U4	9.7±0.5	8	8±0.7	1	7.3±0.2	0.5	8±0.1	2	8.5±0.5	0.25	8.9±1.4	0.5	11±0.6	0.12
K-Zn	7.5±0.3	0.06	7.3±0.3	0.06	9.5±1.3	0.06	7.3±0.1	0.06	9.3±1	0.25	7.8±0.5	0.12	8.5±0.3	0.06
K-Cu	7.8±0.3	0.25	7.6±0.4	0.12	7.4±0.5	0.5	7.9±0.4	0.5	7.2±1.1	0.5	9.1±0.2	0.25	8.9±0.8	0.25
K-Zn+K-Cu	7.7±0.3	0.12	7.8±0.2	0.12	8.8±0.4	0.12	7.3±0.1	0.06	7.8±0.3	0.12	8.1±0.6	0.12	9.1±0.9	0.12

Table 12. Descriptive statistics (mean±SD of 3 replicates) of the minimum inhibitory concentration (MIC) values of *in vitro* tested products against *X. fastidiosa subsp. pauca strain ST53 (Xfp), X. fastidiosa strain CO33 (XfCO33), X. fastidiosa subsp. multiplex strain TOS1 (XfmTOS1), and X. fastidiosa subsp. multiplex strain ESVL (XfmESVL).* Only significant ($P < 0.05$) MIC values are considered according to the one-way analysis of variance (ANOVA) using the Fisher's Least Significant Difference (LSD) test. Note: (ni) = non inhibited.

	X. fastidiosa* subsp. *pauca* strain *ST53		***X. fastidiosa* strainCO33**		***X. fastidiosa* subsp. *multiplex* strain TOS1**		***X. fastidiosa* subsp. *multiplex* strain ESVL**	
Tested product	Inhibition zone (mm)	**MIC (% v/v)**	Inhibition zone (mm)	**MIC (% v/v)**	Inhibition zone (mm)	**MIC (% v/v)**	Inhibition zone (mm)	**MIC (% v/v)**
AG	7.3±0.6	2	ni	ni	10.7±0.6	2.0	10.7±1.2	4.0
AGEtoh	8±1.7	1	7±1	2	8.3±0.6	2.0	18±0	4.0
U1	9.7±1.5	1	8.3±0.6	0.5	8±1	0.5	12±1	2
U2	9±1	1	8±0	0.5	20.3±0.4	8	9±0	4
U3	11.7±2.9	1	18±1	4	12.7±2.1	1	12.3±4.2	2
U4	15.3±1.5	2	ni	ni	18.7±3.8	4	12±0	4
SI	7±0	4	7.3±0.6	1	ni	ni	ni	ni
K-Zn	ni	ni	9±0	0.5	8±0	4	9.7±6	2
K-Cu	10±0	1	7.3±1.2	0.5	8±0	1	11.7±0.6	2
K-Zn+K-Cu	ni	ni	8±0	0.5	ni	ni	ni	ni
Bioflav	14.3±2.9	8	ni	ni	ni	ni	ni	ni
Ki	ni	ni	ni	ni	ni	ni	ni	ni

Table 13. *In vivo* experimental schedule. Treatment's description, product concentration, modality of application and number of plants.

***IN VIVO* EXPERIMENT- SUSCEPTIBLE HOST (CV. OGLIAROLA)**

SAMPLE	TREATMENT	DOSE	APPLICATION	N. OF PLANTS
C-	NEGATIVE CONTROL (Uninoculated- untreated plants)	/	/	3X3
C+	POSITIVE CONTROL (Inoculated- untreated plants)	/	/	3X3
U1	TANNIN U1 (Inoculated- treated plants)	1 % v/v	FOLIAR SPRAY	3X3
U2	TANNIN U2 (Inoculated- treated plants)	1 % v/v	FOLIAR SPRAY	3X3
U3	TANNIN U3 (Inoculated- treated plants)	1 % v/v	FOLIAR SPRAY	3X3
U4	TANNIN U4 (Inoculated- treated plants)	1 % v/v	FOLIAR SPRAY	3X3
K-Zn	KALEX Zn (Inoculated- treated plants)	1.2 % v/v	FOLIAR SPRAY	3X3
K-Cu	KALEX Cu (Inoculated- treated plants)	1.2 % v/v	FOLIAR SPRAY	3X3
AG_P	ALGATAN GEA PREVENTIVE (Inoculated- treated plants)	2 % v/v	SOIL TREATMENT	3X4
AG_C	ALGATAN GEA CURAIVE (Inoculated- treated plants)	2 % v/v	SOIL TREATMENT	3X4

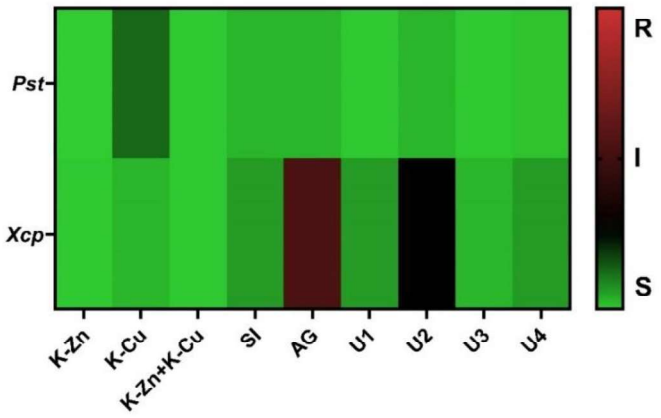

Figure 3. Heat map representing susceptibility of *Pseudomonas syringae* pv. *tomato* (*Pst*) and *Xanthomonas campestris* pv. *pelargoni* (*Xcp*) to tested products. The susceptibility for the two strains were tested by the disk diffusion assay method, against mentioned products and the heat map was constructed to compare the susceptibility among the strains considering the lower concentration able to significantly inhibit ($P < 0.05$) the growth of the bacterium. (S) denotes high susceptibility, (I) denote intermediate behaviour, and (R) denoted resistance to the different products. Significant ($P < 0.05$) value are considered according to the one-way analysis of variance (ANOVA) using the Fisher's Least Significant Difference (LSD) test.

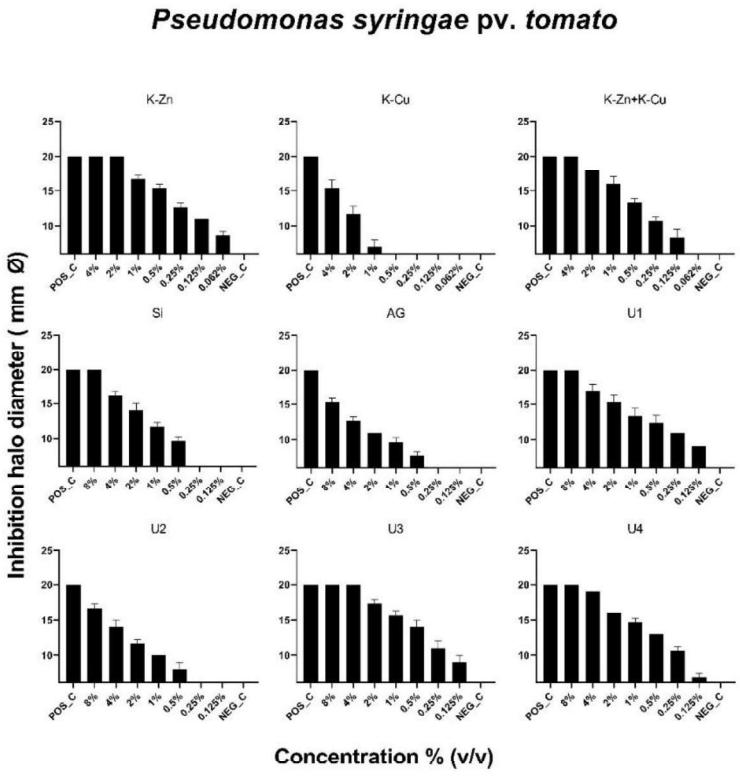

Figure 4. Dose dependent inhibition of *Pseudomonas syringae* pv. *tomato* (*Pst*) by the disk diffusion assay. In each histogram, bars represent the mean and the standard deviation of the diameter (in mm) of the inhibition halo for each product and each concentration, considering as limit of detection 20 mm (Ø). Graphs represent different products: Kalex Zn (K-Zn), Kalex Cu (K-Cu), Kalex Zn+Kalex Cu (K-Zn+K-Cu), Siliforce (Si), Algatan Gea (AG), Tannins (U1, U2, U3, U4). In each graph (POS_C) represent the positive control, the drug reference (ampicillin 100 ppm + streptomycin 250 ppm); and (NEG_C) the negative control, the water control (untreated).

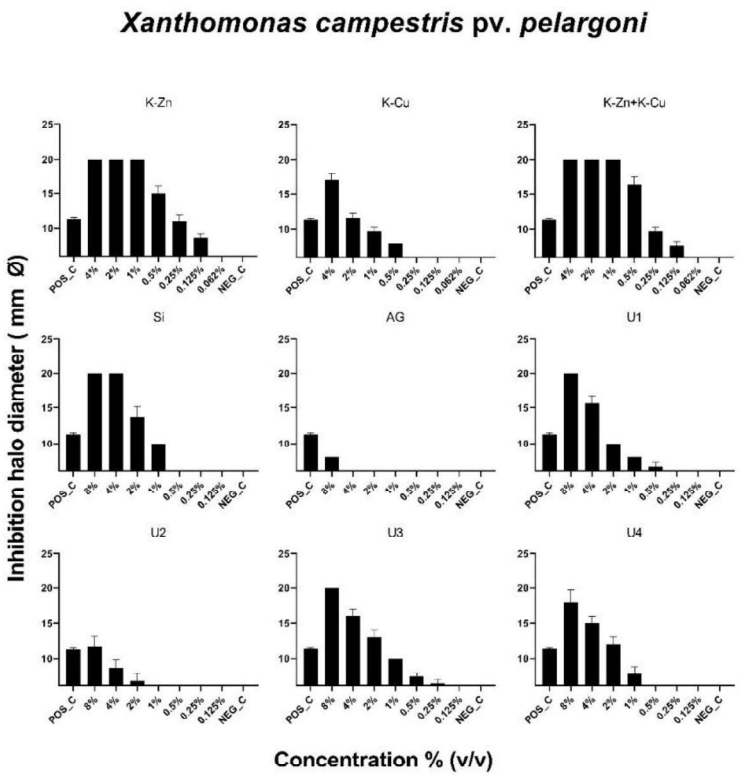

Figure 5. Dose dependent inhibition of *Xanthomonas campestris* pv. *pelargoni* (*Xcp*) by the disk diffusion assay. In each histogram, bars represent the mean and the standard deviation of the diameter (in mm) of the inhibition halo for each product and each concentration, considering as limit of detection 20 mm (Ø). Graphs represent different products: Kalex Zn (K-Zn), Kalex Cu (K-Cu), Kalex Zn+Kalex Cu (K-Zn+K-Cu), Siliforce (Si), Algatan Gea (AG), Tannins (U1, U2, U3, U4). In each graph (POS_C) represent the positive control, the drug reference (ampicillin 100 ppm + streptomycin 250 ppm); and (NEG_C) the negative control, the water control (untreated)

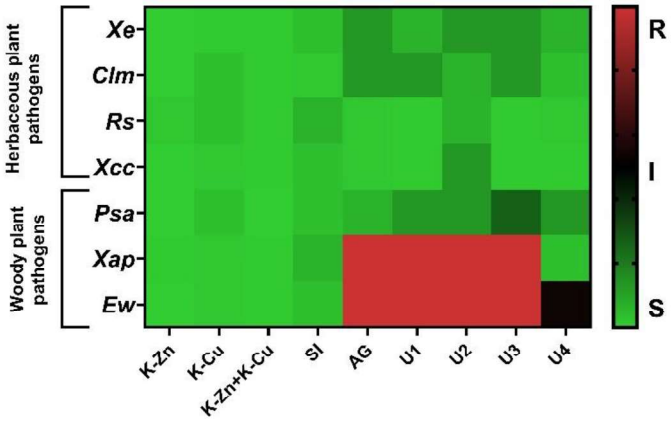

Figure 6. Heat map representing susceptibility of *Erwinia amylovora* (*Ew*), *X. euvesicatoria* (*Xe*), *C. michiganensis subsp. michiganensis* (*Clm*), *P. syringae pv. actinidiae* (*Psa*), *Ralstonia solanacearum* (*Rs*), *X. arboricola pv. pruni* (*Xap*), *X. campestris pv. campestris* (*Xcc*) to tested products. The susceptibility for the seven strains were tested by the disk diffusion assay method, against mentioned products and the heat map was constructed to compare the susceptibility among the strains considering the lower concentration able to significantly inhibit ($P < 0.05$) the growth of the bacterium. (S) denotes high susceptibility, (I) denote intermediate behaviour, and (R) denoted resistance to the different products. Significant ($P < 0.05$) value are considered according to the one-way analysis of variance (ANOVA) using the Fisher's Least Significant Difference (LSD) test.

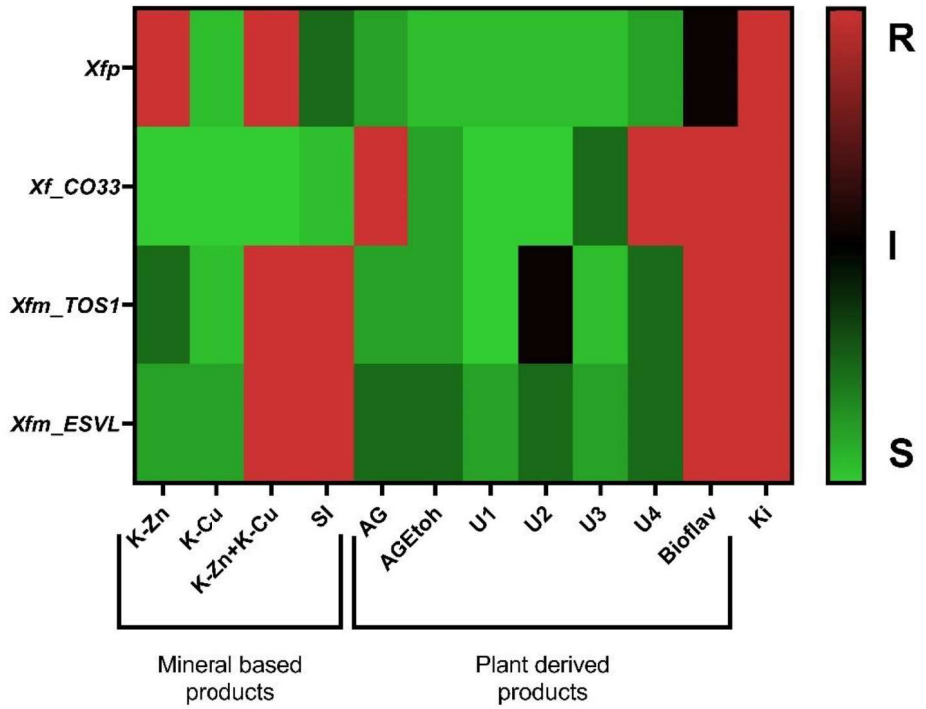

Figure 7. Heat map representing susceptibility of *X. fastidiosa* subsp. *pauca* strain *ST53 (Xfp), X. fastidiosa* strain *CO33 (XfCO33), X. fastidiosa* subsp. *multiplex* strain *TOS1 (XfmTOS1),* and *X. fastidiosa* subsp. *multiplex* strain *ESVL (XfmESVL)* to tested product. The susceptibility for the *X. fastidiosa* strains and subspecies were tested by the disk diffusion assay method, against mentioned products and the heat map was constructed to compare the susceptibility among the strains considering the lower concentration able to significantly inhibit ($P < 0.05$) the growth of the bacterium. (S) denotes high susceptibility, (I) denote intermediate behaviour, and (R) denoted resistance to the different products. Significant ($P < 0.05$) value are considered according to the one-way analysis of variance (ANOVA) using the Fisher's Least Significant Difference (LSD) test.

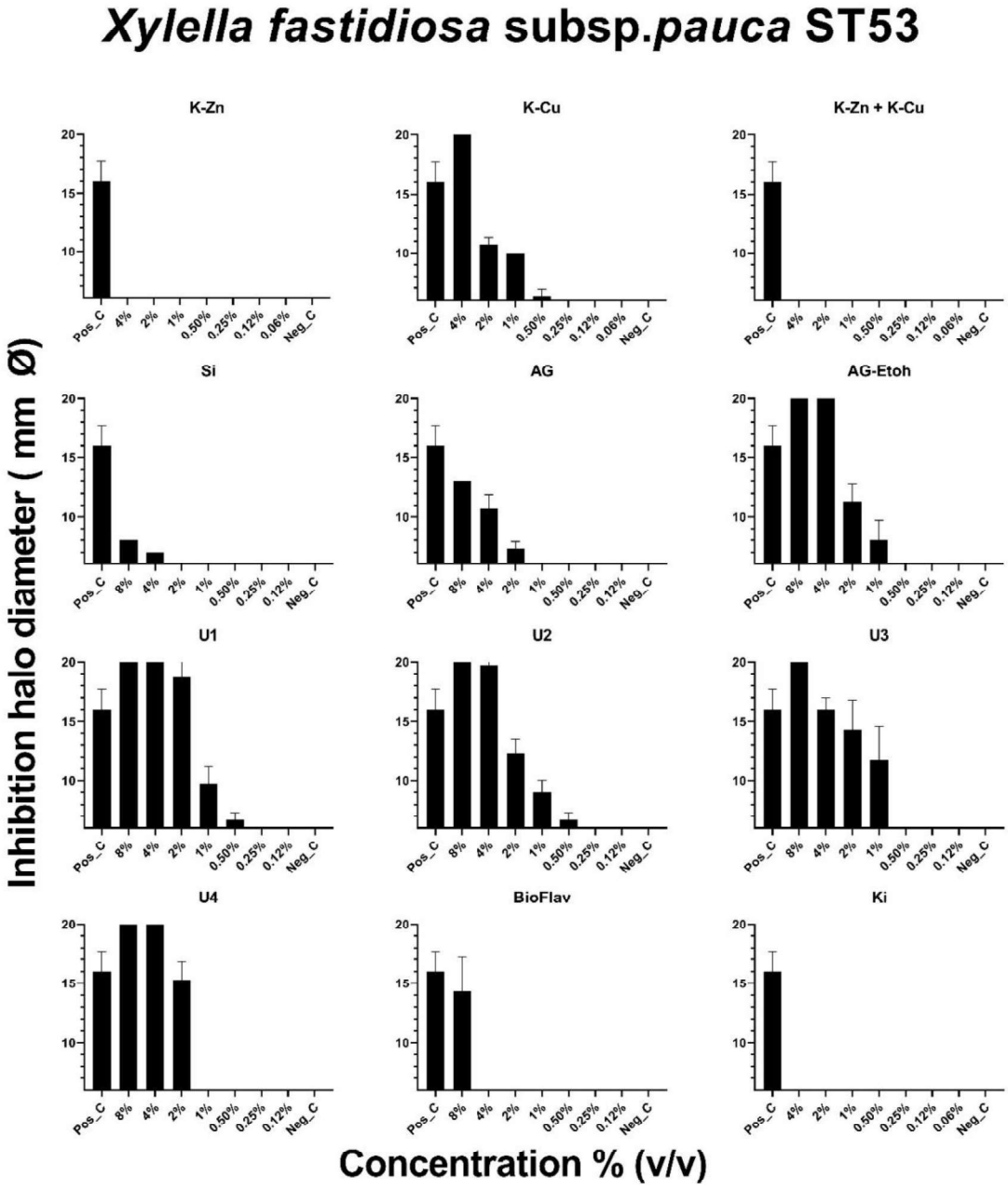

Figure 8. Dose dependent inhibition of *Xylella fastidiosa* subsp. *pauca* (*Xfp*) by the disk diffusion assay. In each histogram, bars represent the mean and the standard deviation of the diameter (in mm) of the inhibition halo for each product and each concentration, considering as limit of detection 20 mm (Ø). Graphs represent different products: Kalex Zn (K-Zn), Kalex Cu (K-Cu), Kalex Zn+Kalex Cu (K-Zn+K-Cu), Siliforce (Si), Algatan Gea water extract (AG), Algatan Gea ethanolic extract (AG-Etoh), Tannins (U1, U2, U3, U4), flavonoid-based glycolic extract (BioFlav) and Kiforce (Ki). In each graph (POS_C) represent the positive control, the drug reference (ampicillin 100 ppm + streptomycin 250 ppm); and (NEG_C) the negative control, the water control (untreated).

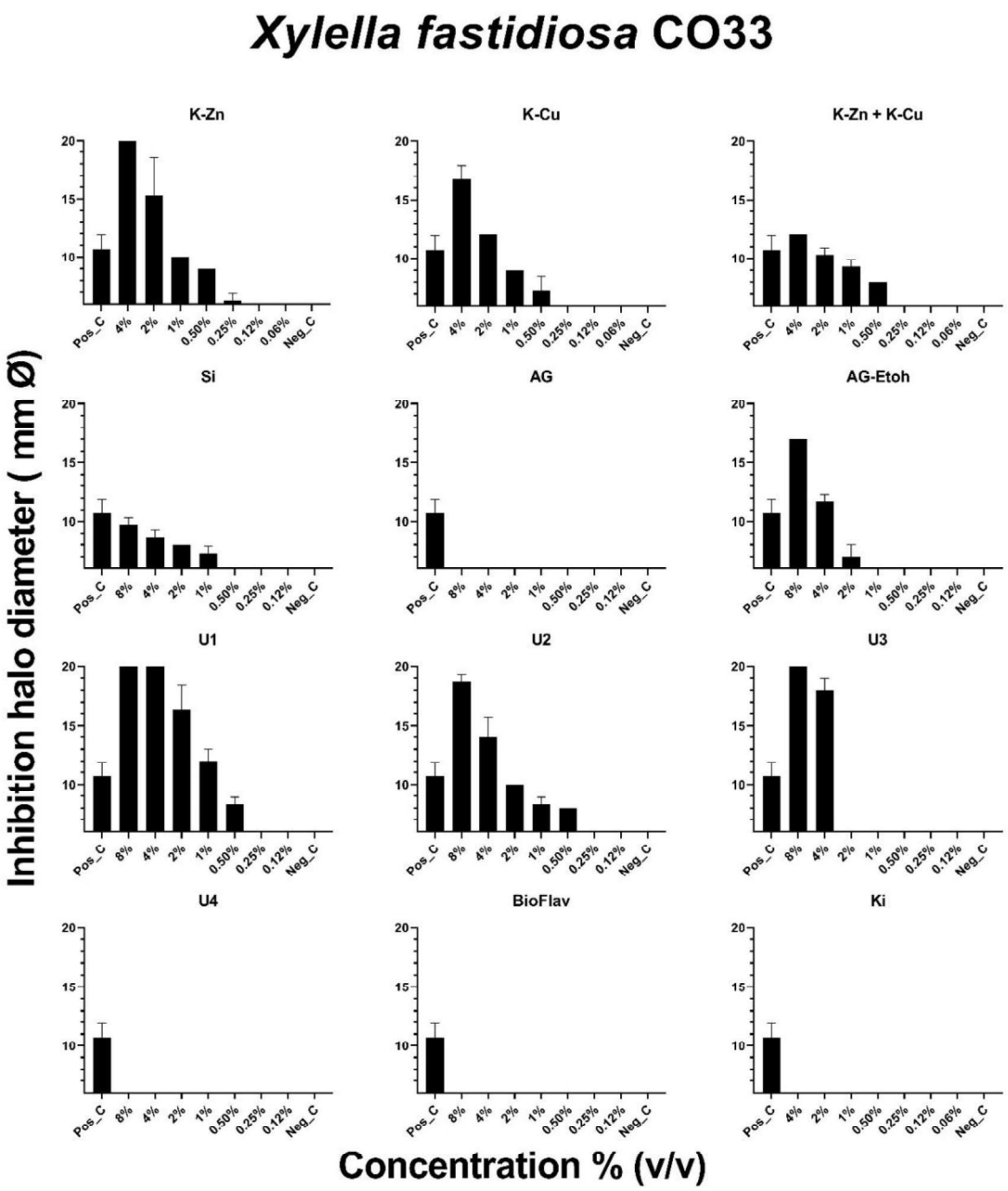

Figure 9. Dose dependent inhibition of *Xylella fastidiosa* strain CO33 (*Xf_CO33*) by the disk diffusion assay. In each histogram, bars represent the mean and the standard deviation of the diameter (in mm) of the inhibition halo for each product and each concentration, considering as limit of detection 20 mm (Ø). Graphs represent different products: Kalex Zn (K-Zn), Kalex Cu (K-Cu), Kalex Zn+Kalex Cu (K-Zn+K-Cu), Siliforce (Si), Algatan Gea water extract (AG), Algatan Gea ethanolic extract (AG-Etoh), Tannins (U1, U2, U3, U4), flavonoid-based glycolic extract (BioFlav) and Kiforce (Ki). In each graph (POS_C) represent the positive control, the drug reference (ampicillin 100 ppm + streptomycin 250 ppm); and (NEG_C) the negative control, the water control (untreated).

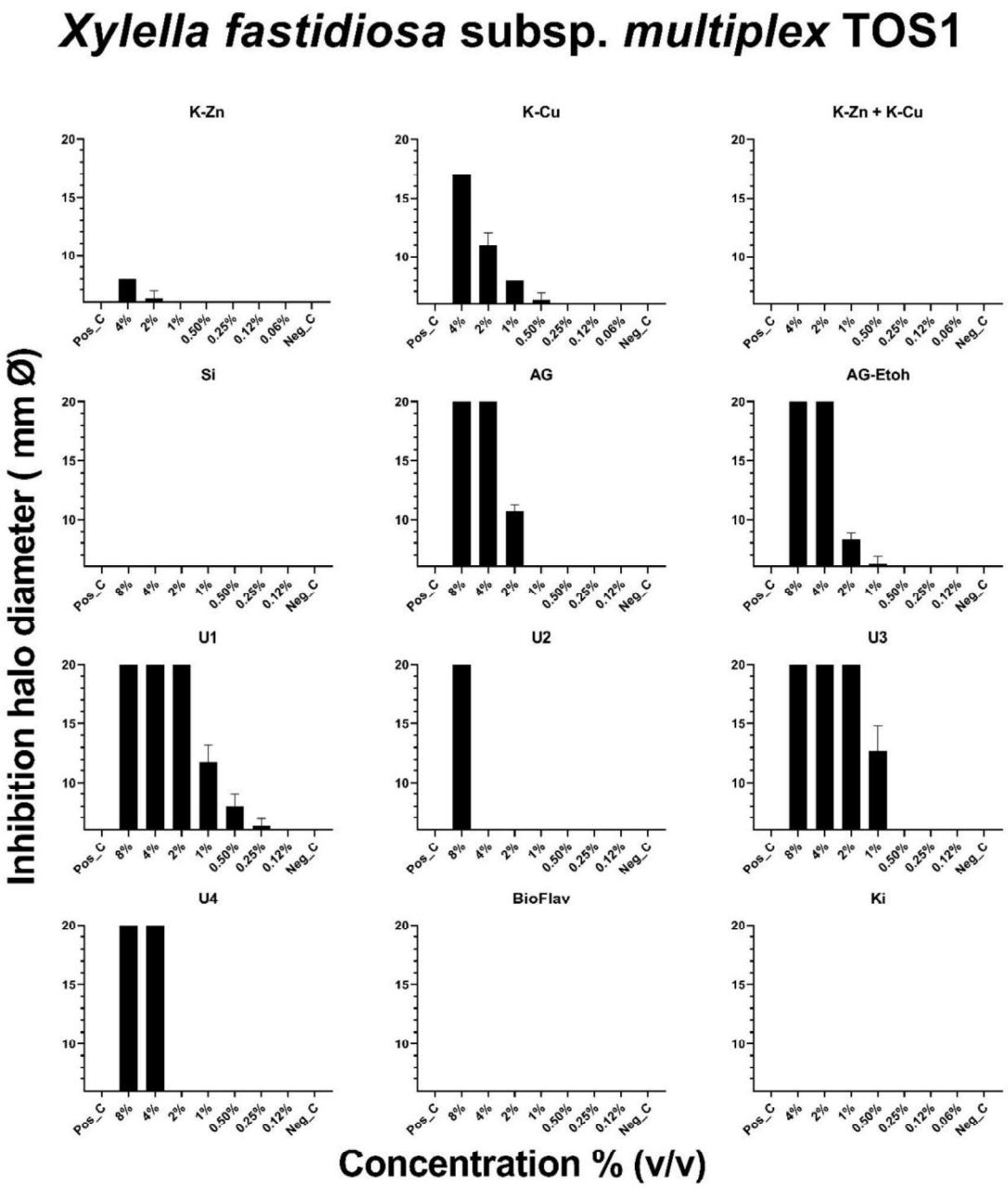

Figure 10. Dose dependent inhibition of *Xylella fastidiosa* subsp. *multiplex* strain TOS1 (*Xfm_TOS1*) by the disk diffusion assay. In each histogram, bars represent the mean and the standard deviation of the diameter (in mm) of the inhibition halo for each product and each concentration, considering as limit of detection 20 mm (Ø). Graphs represent different products: Kalex Zn (K-Zn), Kalex Cu (K-Cu), Kalex Zn+Kalex Cu (K-Zn+K-Cu), Siliforce (Si), Algatan Gea water extract (AG), Algatan Gea ethanolic extract (AG-Etoh), Tannins (U1, U2, U3, U4), flavonoid-based glycolic extract (BioFlav) and Kiforce (Ki). In each graph (POS_C) represent the positive control, the drug reference (ampicillin 100 ppm + streptomycin 250 ppm); and (NEG_C) the negative control, the water control (untreated).

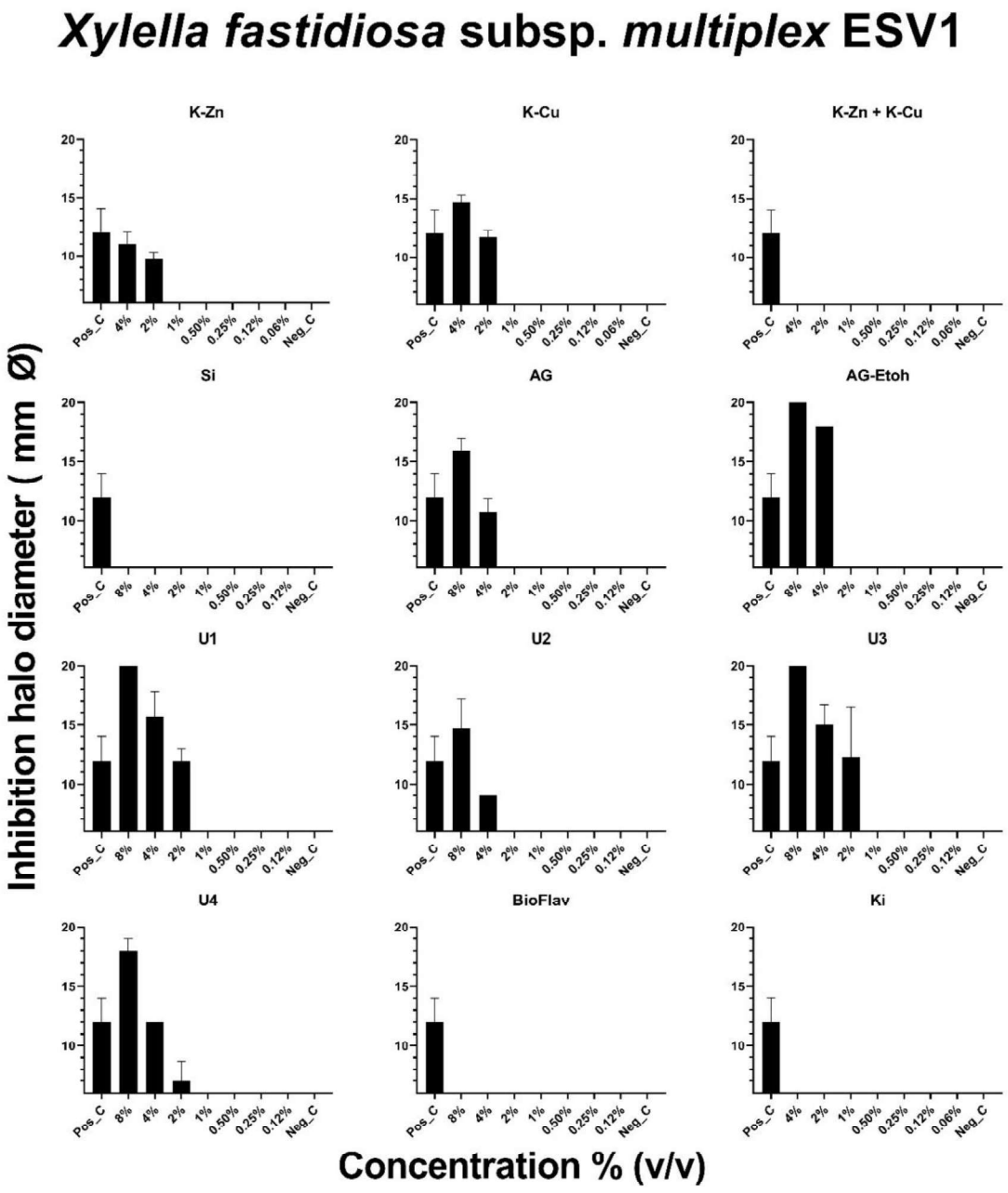

Figure 11. Dose dependent inhibition of *Xylella fastidiosa* subsp. *multiplex* strain ESVL (*Xfm_ESVL*) by the disk diffusion assay. In each histogram, bars represent the mean and the standard deviation of the diameter (in mm) of the inhibition halo for each product and each concentration, considering as limit of detection 20 mm (Ø). Graphs represent different products: Kalex Zn (K-Zn), Kalex Cu (K-Cu), Kalex Zn+Kalex Cu (K-Zn+K-Cu), Siliforce (Si), Algatan Gea water extract (AG), Algatan Gea ethanolic extract (AG-Etoh), Tannins (U1, U2, U3, U4), flavonoid-based glycolic extract (BioFlav) and Kiforce (Ki). In each graph (POS_C) represent the positive control, the drug reference (ampicillin 100 ppm + streptomycin 250 ppm); and (NEG_C) the negative control, the water control (untreated).

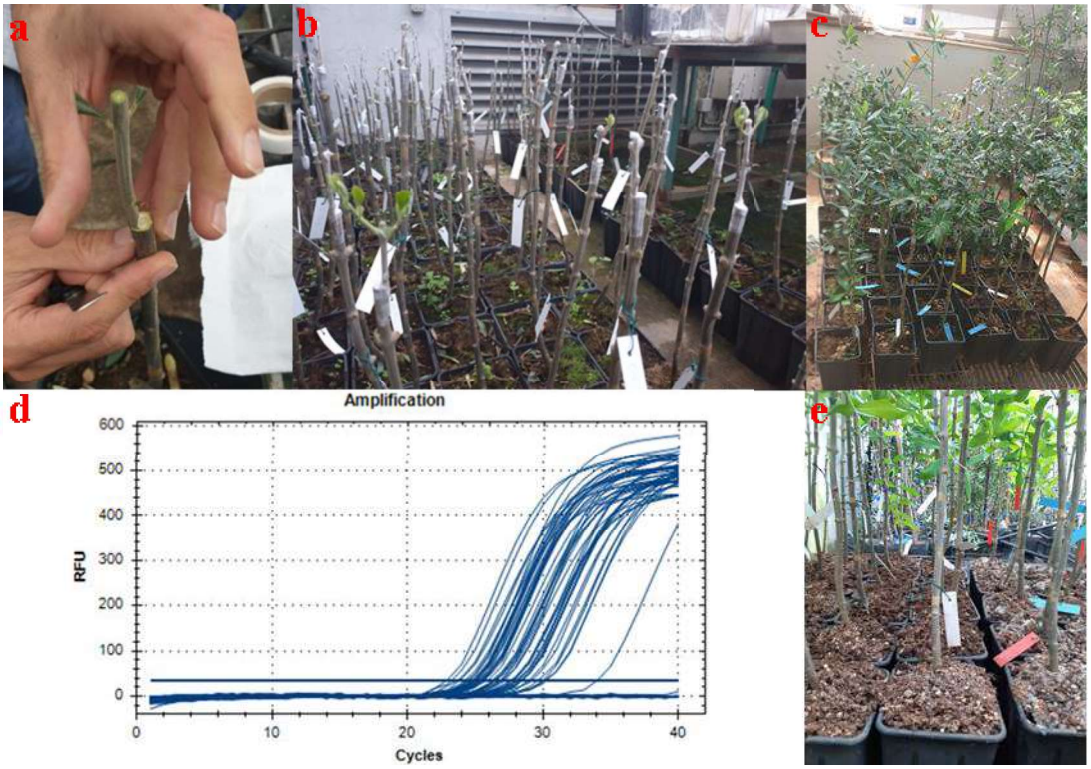

Figure 12. Grafting operation (a), engraftment (b), grafted plants for foliar treatments (c), scions qPCR results (d) and soil treatments (e).

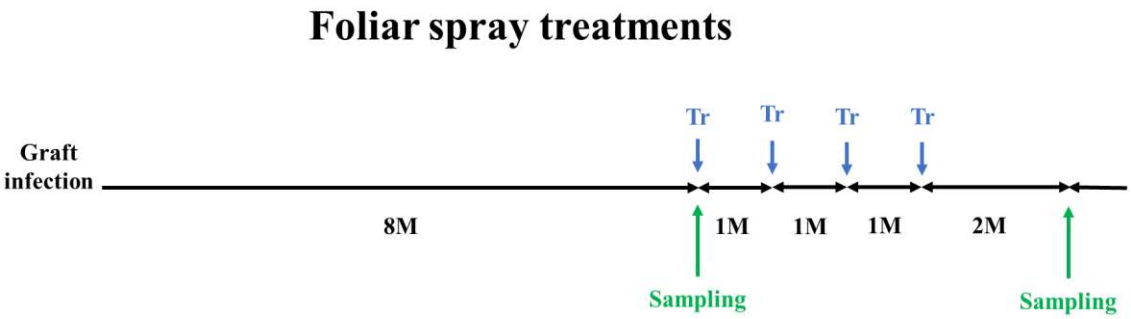

Figure 13. Gant Chart of foliar treatments, from the inoculation time. Months (M preceded by numbers), treatments (Tr) and sampling for *X. fastidiosa* detection by qPCR.

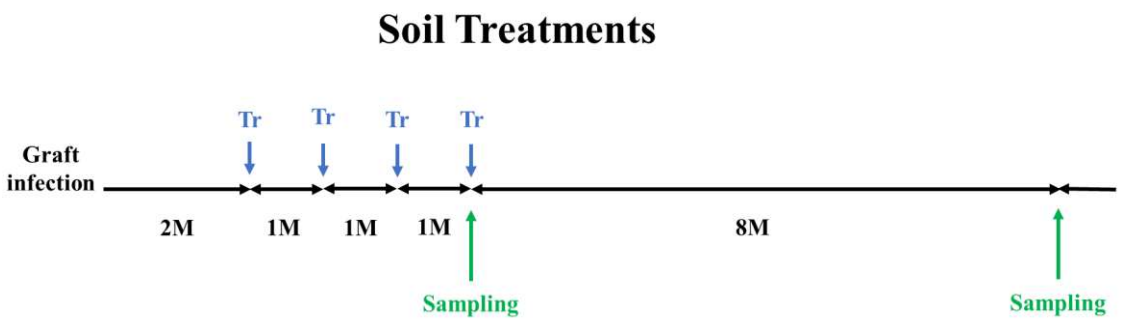

Figure 14. Gant Chart of soil treatments from the inoculation time. Months (M preceded by numbers), treatments (Tr) and sampling for *X. fastidiosa* detection by qPCR.

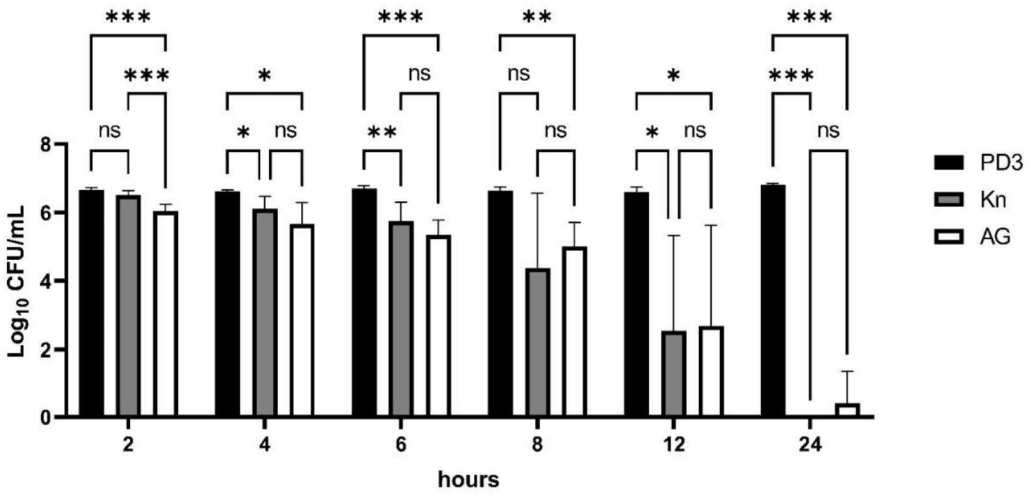

Figure 15. Time-Kill assay of Algatan Gea aqueous extracts (AG) compared to the two control PD3 (negative control) and Kn (positive control, 50µg/mL of Kanamycin). Each bar represents the mean and standard deviation of pooled data from two independent experiments (6 replications in total). Statistical significance was determined by two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. ns (non-significant); * (P <0.05); ** (P<0.03); ***(P<0.001).

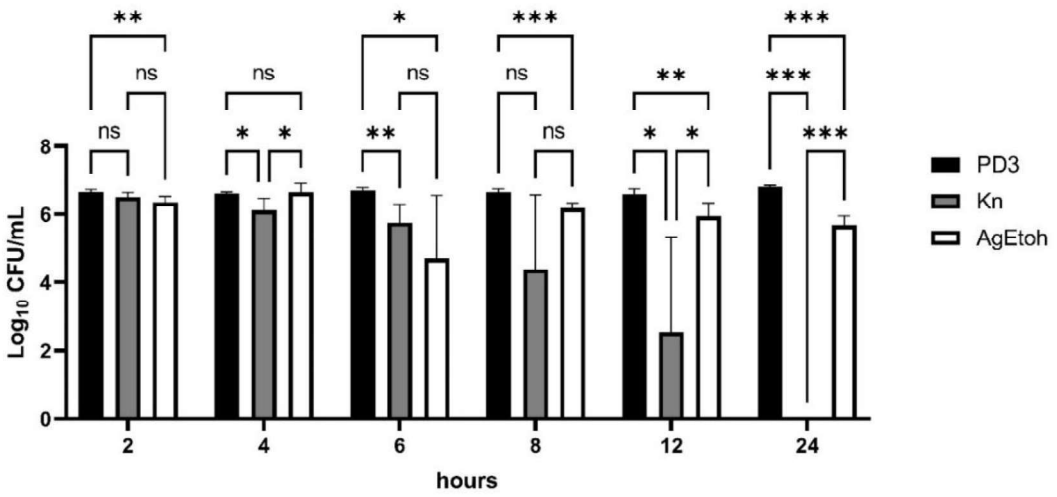

Figure 16. Time-Kill assay of Algatan Gea ethanol extracts (AGEtoh) compared to the two control PD3 (negative control) and Kn (positive control, 50µg/mL of Kanamycin). Each bar represents the mean and standard deviation of pooled data from two independent experiments (6 replications in total). Statistical significance was determined by two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. ns (non-significant); * (P <0.05); ** (P<0.03); ***(P<0.001).

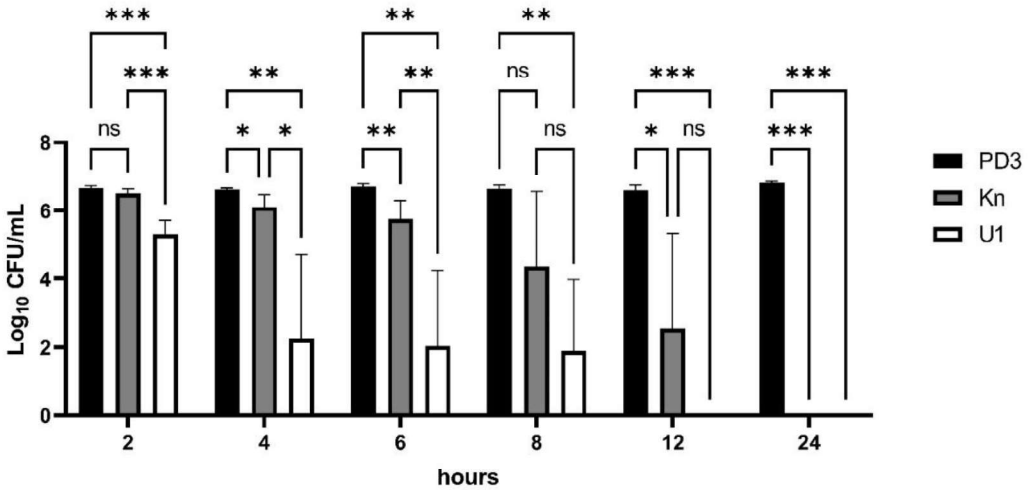

Figure 17. Time-Kill assay of Tannin U1 compared to the two control PD3 (negative control) and Kn (positive control, 50µg/mL of Kanamycin). Each bar represents the mean and standard deviation of pooled data from two independent experiments (6 replications in total). Statistical significance was determined by two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. ns (non-significant); * (P <0.05); ** (P<0.03); ***(P<0.001).

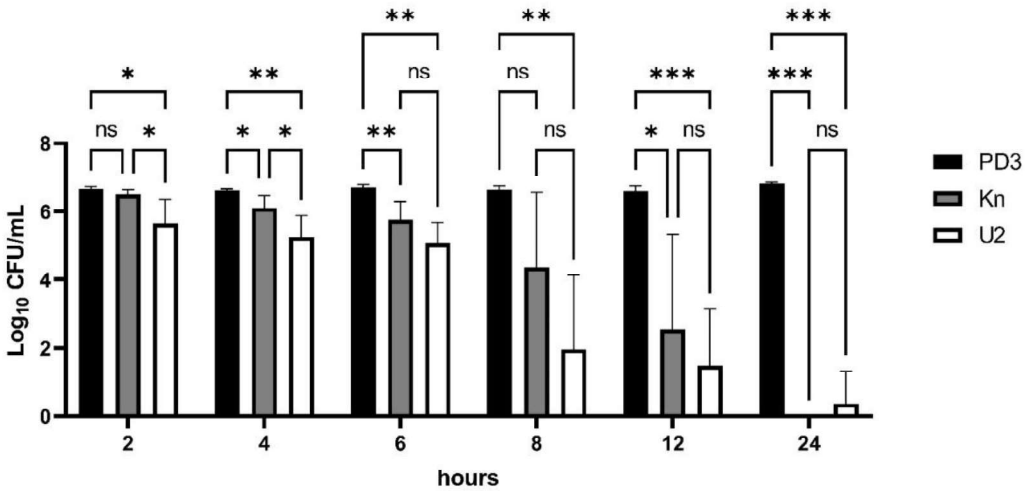

Figure 18. Time-Kill assay of Tannin U2 compared to the two control PD3 (negative control) and Kn (positive control, 50µg/mL of Kanamycin). Each bar represents the mean and standard deviation of pooled data from two independent experiments (6 replications in total). Statistical significance was determined by two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. ns (non-significant); * (P <0.05); ** (P<0.03); ***(P<0.001).

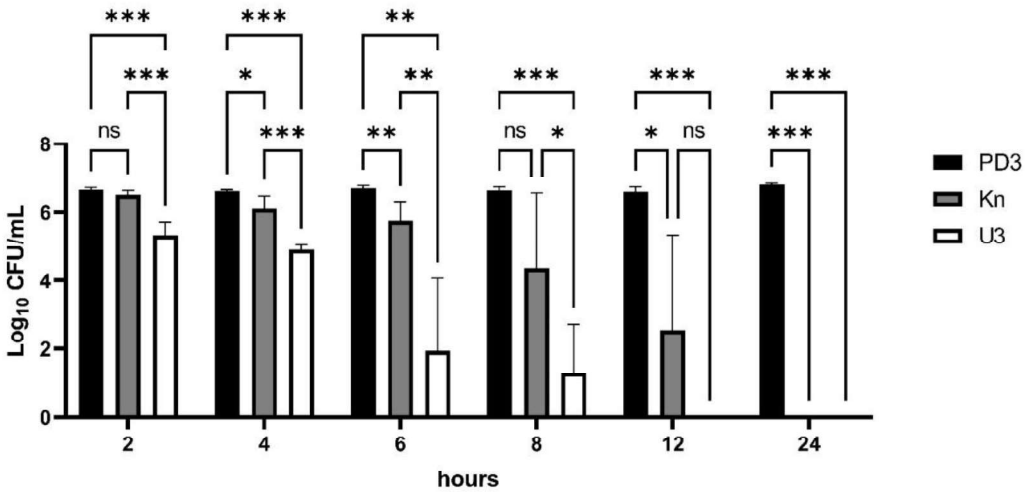

Figure 19. Time-Kill assay of Tannin U3 compared to the two control PD3 (negative control) and Kn (positive control, 50µg/mL of Kanamycin). Each bar represents the mean and standard deviation of pooled data from two independent experiments (6 replications in total). Statistical significance was determined by two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. ns (non-significant); * (P <0.05); ** (P<0.03); ***(P<0.001).

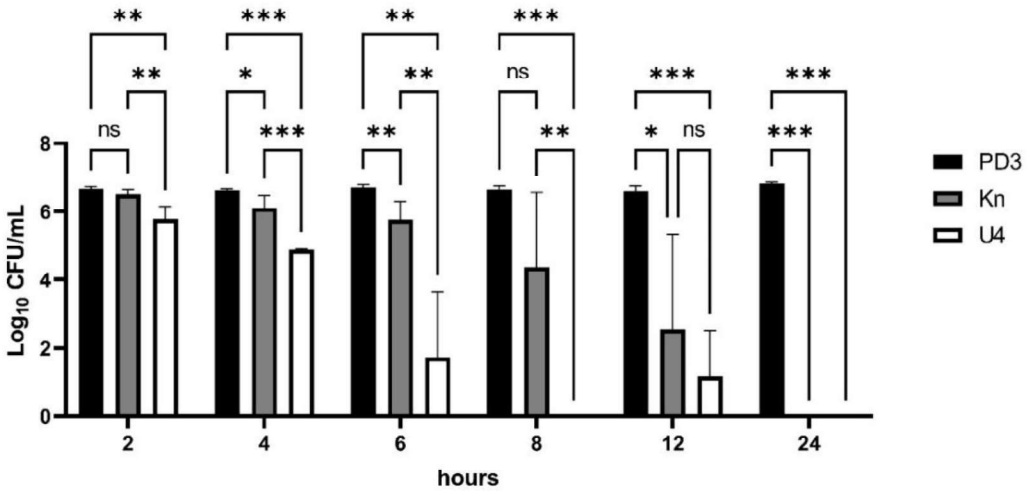

Figure 20. Time-Kill assay of Tannin U4 compared to the two control PD3 (negative control) and Kn (positive control, 50µg/mL of Kanamycin). Each bar represents the mean and standard deviation of pooled data from two independent experiments (6 replications in total). Statistical significance was determined by two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. ns (non-significant); * (P <0.05); ** (P<0.03); ***(P<0.001).

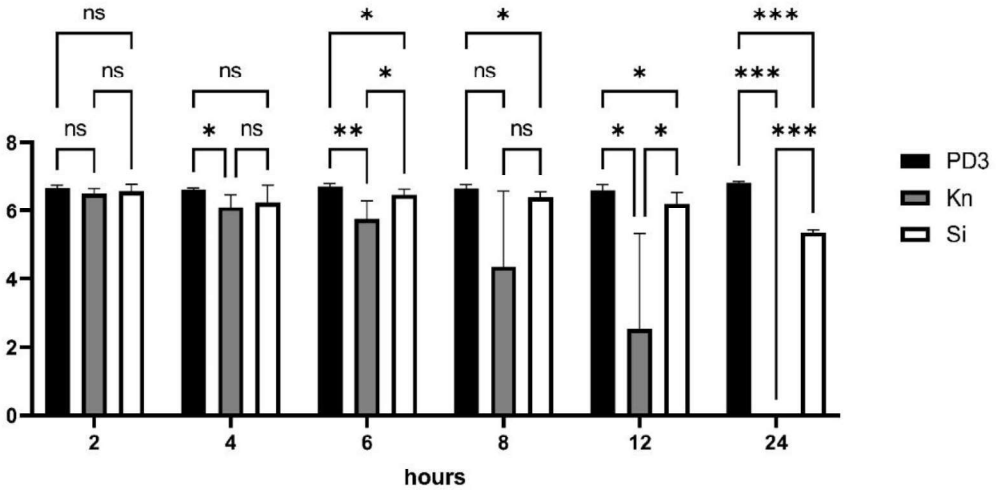

Figure 21. Time-Kill assay of Siliforce (Si) compared to the two control PD3 (negative control) and Kn (positive control, 50µg/mL of Kanamycin). Each bar represents the mean and standard deviation of pooled data from two independent experiments (6 replications in total). Statistical significance was determined by two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. ns (non-significant); * (P <0.05); ** (P<0.03); ***(P<0.001).

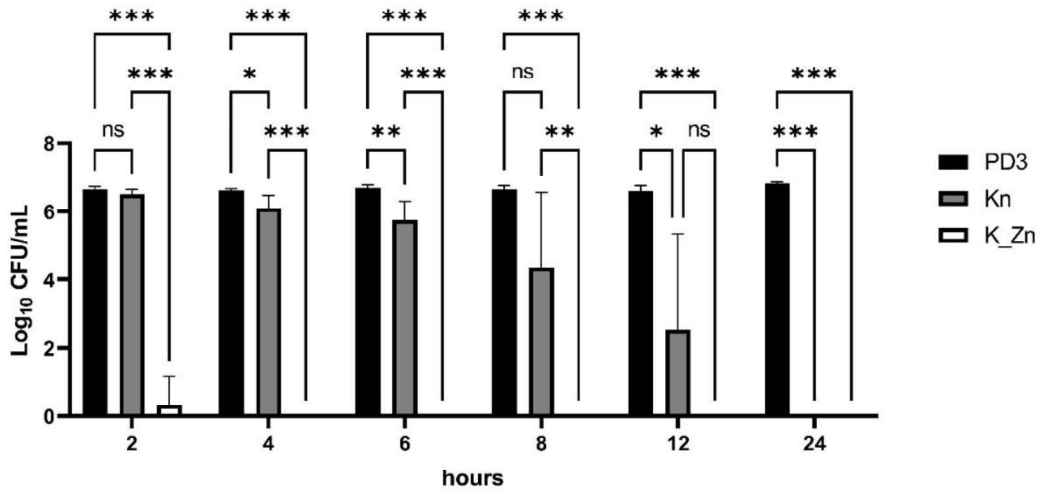

Figure 22. Time-Kill assay of Kalex Zn compared to the two control PD3 (negative control) and Kn (positive control, 50µg/mL of Kanamycin). Each bar represents the mean and standard deviation of pooled data from two independent experiments (6 replications in total). Statistical significance was determined by two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. ns (non-significant); * (P <0.05); ** (P<0.03); ***(P<0.001).

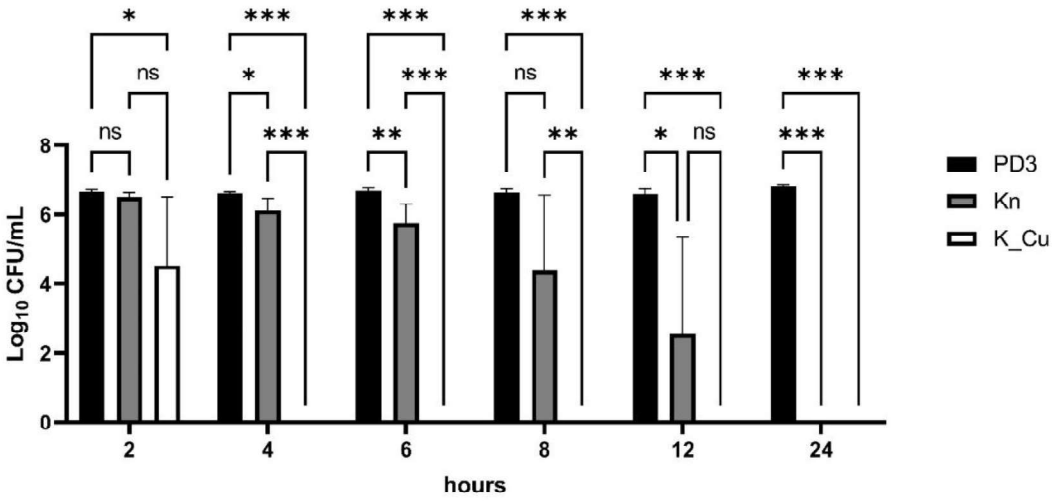

Figure 23. Time-Kill assay of Kalex Cu compared to the two control PD3 (negative control) and Kn (positive control, 50µg/mL of Kanamycin). Each bar represents the mean and standard deviation of pooled data from two independent experiments (6 replications in total). Statistical significance was determined by two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. ns (non-significant); * (P <0.05); ** (P<0.03); ***(P<0.001).

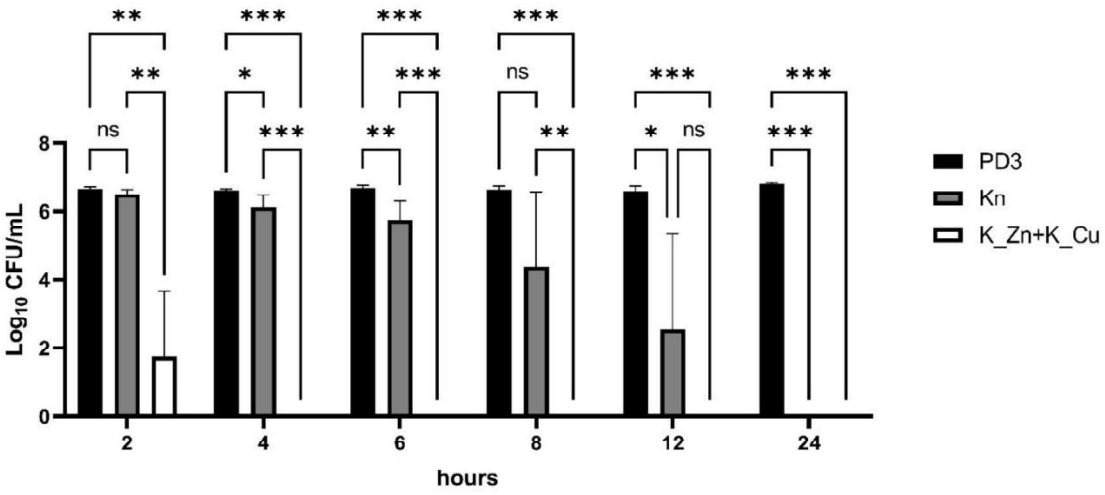

Figure 24. Time-Kill assay of Kalex Zn + Kalex Cu compared to the two control PD3 (negative control) and Kn (positive control, 50µg/mL of Kanamycin). Each bar represents the mean and standard deviation of pooled data from two independent experiments (6 replications in total). Statistical significance was determined by two-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. ns (non-significant); * (P <0.05); ** (P<0.03); ***(P<0.001).

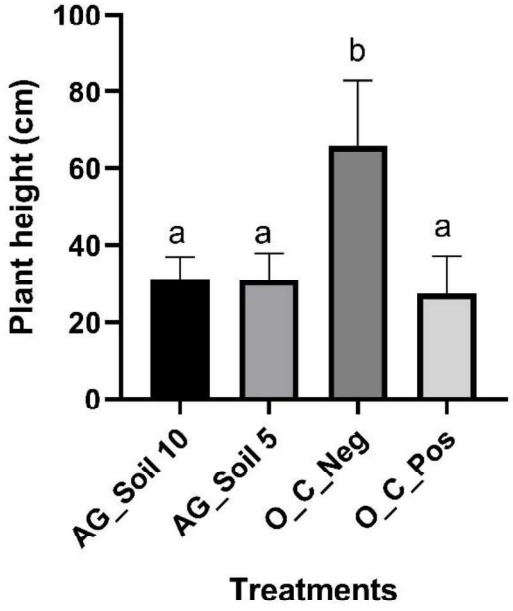

Figure 25. Mean of plant height (cm) of olive plants treated by soil application. O_C_Neg = Negative Control (uninoculated - untreated plants); O_C_Pos = Positive Control (inoculated - untreated plants); AG_Soil 10 and AG_Soil 5 = Algatan Gea curative and preventive soil treatments, respectively, on inoculated plants with *Xfp*. Bars indicate the mean and standard deviation of three replicates of three plants per replication. Notes: Differences were analysed by one-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. Values on histograms marked with different letters are statistically different at P<0.05.

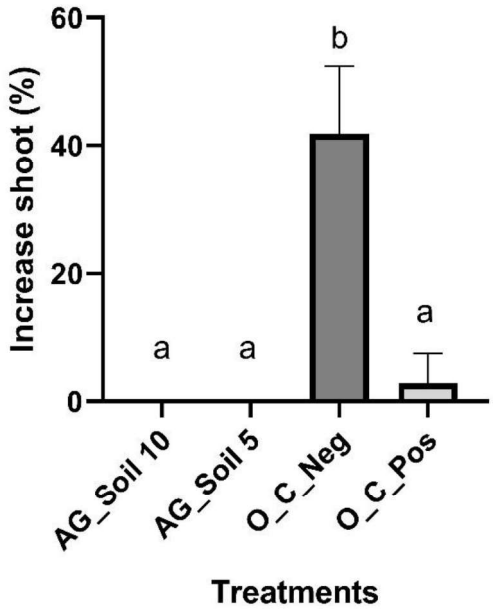

Figure 26. Increment of number of shoots (%) of olive plants treated by soil application. O_C_Neg = Negative Control (uninoculated - untreated plants); O_C_Pos = Positive Control (inoculated - untreated plants); AG_Soil 10 and AG_Soil 5 = Algatan Gea curative and preventive soil treatments, respectively, on inoculated plants with *Xfp*. Bars indicate the mean and standard deviation of three replicates of three plants per replication. Notes: Differences were analysed by one-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. Values on histograms marked with different letters are statistically different at P<0.05.

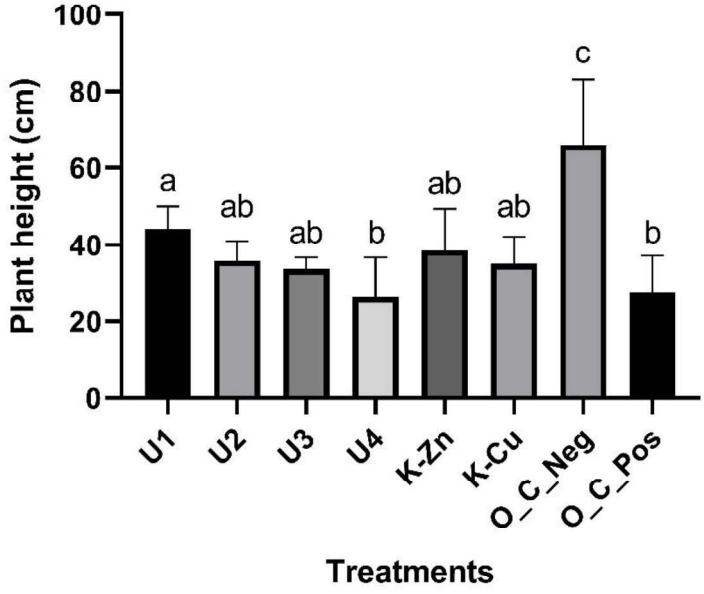

Figure 27. Mean of plant height (cm) of olive plants treated by foliar spray. O_C_Neg = Negative Control (uninoculated - untreated plants); O_C_Pos = Positive Control (inoculated - untreated plants); U1, U2, U3 and U4=plants inoculated with *Xfp* and treated with different tannins by foliar spraying; K-Zn, K-Cu= plant inoculated with *Xfp* and treated with Kalex Zn and Kalex Cu by foliar spraying. Bars indicate the mean and standard deviation of three replicates of three plants per replication. Notes: Differences were analysed by one-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. Values on histograms marked with different letters are statistically different at P<0.05.

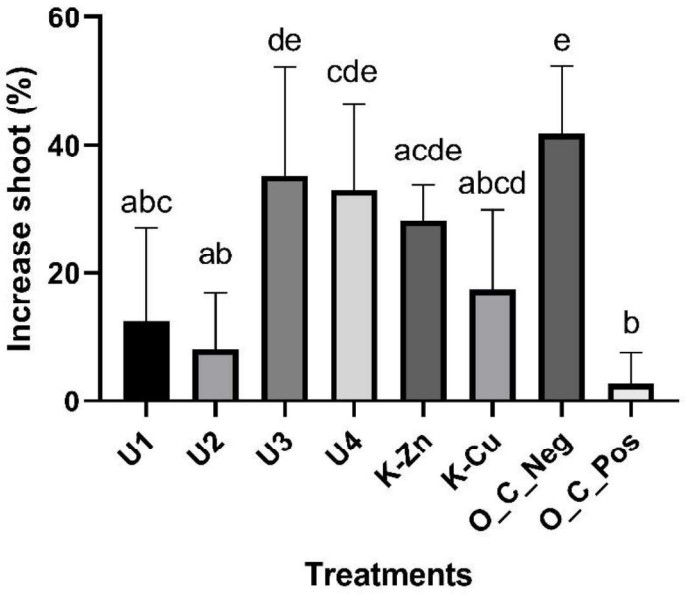

Figure 28. Increment of number of shoot (%) of olive plants treated by foliar spray. O_C_Neg = Negative Control (uninoculated - untreated plants); O_C_Pos = Positive Control (inoculated - untreated plants); U1, U2, U3 and U4=plant inoculated with *Xfp* and treated with different tannins by foliar spraying; K-Zn, K-Cu= plant inoculated with *Xfp* and treated with Kalex Zn and Kalex Cu by foliar spraying. Bars indicate the mean and standard deviation of three replicates of three plants per replication. Notes: Differences were analysed by one-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. Values on histograms marked with different letters are statistically different at P<0.05.

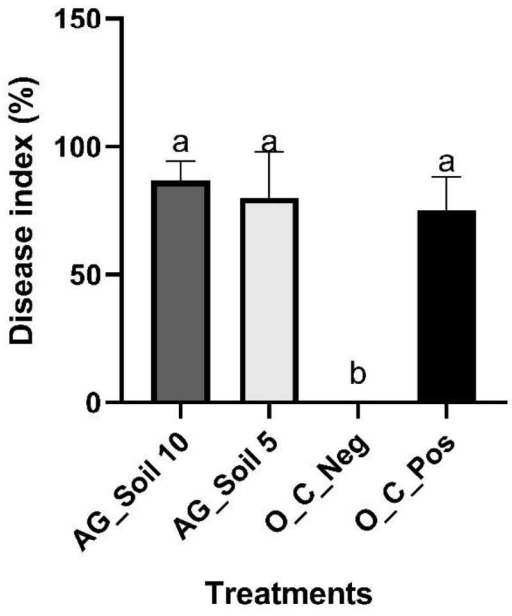

Figure 29. Disease index, DI (%), calculated by using an empirical scale from 0 (no symptoms) to 5 (100% symptoms or dead plant), of foliar treatments on pot grown olive plants inoculated with *Xylella fastidiosa* subsp. *pauca* ST53. O_C_Neg = Negative Control (uninoculated - untreated plants); O_C_Pos = Positive Control (inoculated - untreated plants); AG_Soil 10 and AG_Soil 5 = Algatan Gea curative and preventive soil treatments, respectively, on inoculated plants with *Xfp*. Bars indicate the mean and standard deviation of the DI (%) calculated on three replicates of three plants per replication. Notes: One-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. Values on histograms marked with different letters are statistically different at P<0.05.

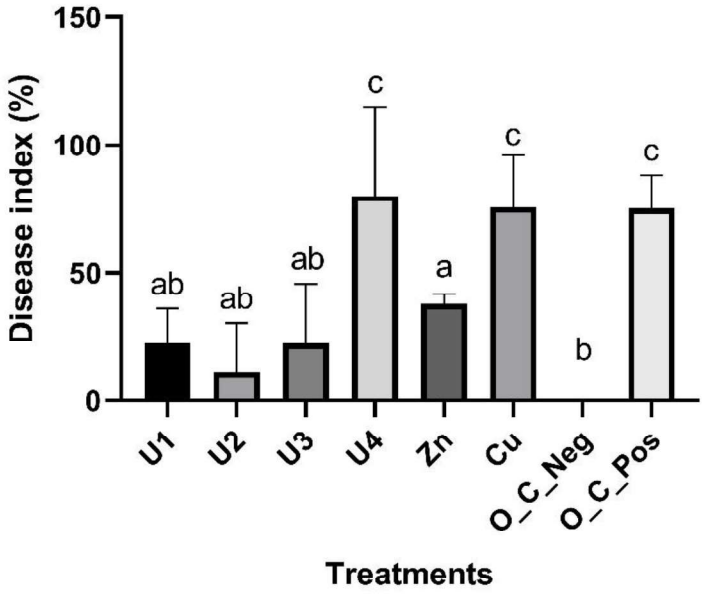

Figure 30. Disease index, DI (%), calculated by using an empirical scale from 0 (no symptoms) to 5 (100% symptoms or dead plant), of foliar treatments on pot grown olive plants inoculated with *Xylella fastidiosa* subsp. *pauca* ST53. O_C_Neg = Negative Control (uninoculated - untreated plants); O_C_Pos = Positive Control (inoculated - untreated plants); U1, U2, U3 and U4=plants inoculated with *Xfp* and treated with different tannins by foliar spraying; K-Zn, K-Cu= plants inoculated with *Xfp* and treated with Kalex Zn and Kalex Cu by foliar spraying. Bars indicate the mean and standard deviation of the DI (%) calculated on three replicates of three plants per replication. Notes: One-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. Values on histograms marked with different letters are statistically different at P<0.05.

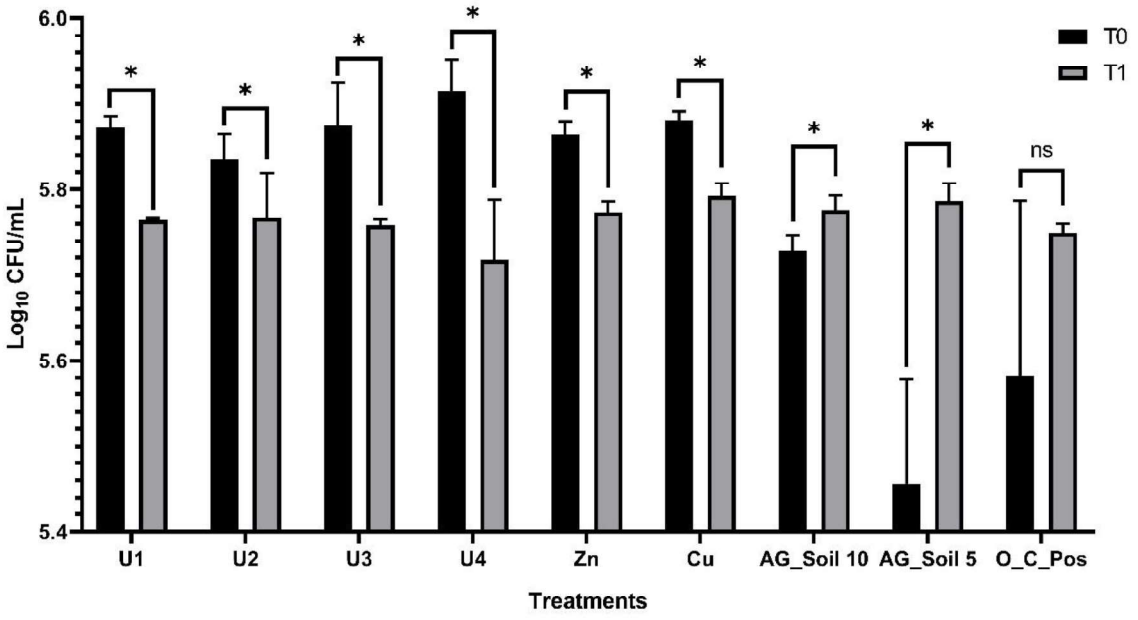

Figure 31. Differences of bacterial population in inoculated and treated plants from T0 (before treatments) and T1 (last survey). O_C_Pos = Positive Control (inoculated - untreated plants); U1, U2, U3 and U4=plant inoculated with *Xfp* and treated with different tannins by foliar spraying; K-Zn, K-Cu= plant inoculated with *Xfp* and treated with Kalex Zn and Kalex Cu by foliar spraying); AG_Soil 10 and AG_Soil 5 = Algatan Gea curative and preventive soil treatments, respectively, on inoculated plants with *Xfp*. Bars indicate the mean and standard deviation of the Log_{10}CFU/mL calculated on three replicates of three plants per replication. Notes: Statistical significance was determined by a multiple t test analysis using the Holm-Sidak method (P<0.05).

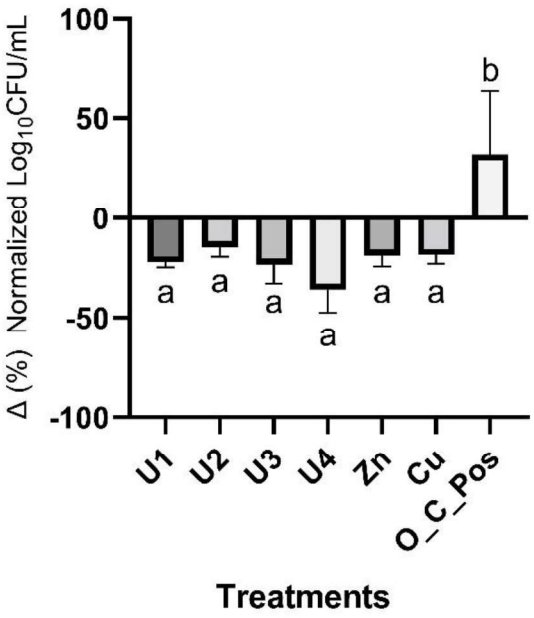

Figure 32. Delta (%) of the bacterial reduction in plants treated by foliar spray. O_C_Pos = Positive Control (inoculated - untreated plants); U1, U2, U3 and U4=plant inoculated with *Xfp* and treated with different tannin products by foliar spraying; K-Zn, K-Cu= plant inoculated with *Xfp* and treated with Kalex Zn and Kalex Cu by foliar spraying. Bars indicate the mean and standard deviation of delta (%) calculated by normalized Log_{10} CFU/mL value of three replicates of three plants per replication. Notes: Differences were analysed by one-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. Values on histograms marked with different letters are statistically different at $P<0.05$.

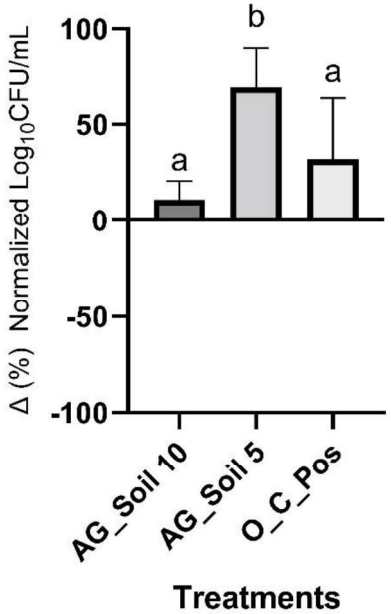

Figure 33. Delta (%) of the bacterial reduction in plants treated by soil treatments. O_C_Pos = Positive Control (inoculated - untreated plants); AG_Soil 10 and AG_Soil 5 = Algatan Gea curative and preventive soil treatments, respectively, on inoculated plants with *Xfp*. Bars indicate the mean and standard deviation of delta (%) calculated by normalized Log_{10} CFU/mL value of three replicates of three plants per replication. Notes: Differences were analysed by one-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. Values on histograms marked with different letters are statistically different at $P<0.05$.

Figure 34. Representative photos of different treatments on pot grown olive plants inoculated with *Xylella fastidiosa* subsp. *pauca* ST53. U1, U2, U3 and U4 = plant inoculated with *Xfp* and treated with different tannin product by foliar spraying; K-Zn, K-Cu = plant inoculated with *Xfp* and treated with Kalex Zn and Kalex Cu, respectively, by foliar spraying; AG_Soil 10 and Soil 5= curative and preventive Algatan Gea soil treatments, respectively, on *Xfp* inoculated plants. C- = Negative Control (uninoculated - untreated plants); C+ = Positive Control (inoculated - untreated plants). The images were taken on January 2021.

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