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Effects of non-lethal concentrations of bioactive compounds on plant-related biofilms.

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Abstract

It has been estimated that at least 99 % of the world's microbial biomass exists in form of biofilm, a complex differentiated surface-associated community embedded in a self-produced polymeric matrix enabling microorganisms to develop coordinated and efficient survival strategies. Biofilm formation is a dynamic and cyclical process involving attachment, maturation and a final dispersal phase, and these steps are initiated by a variety of signals. Despite their positive effects in some cases, biofilms can be detrimental in different environmental domains since microorganisms are able to colonize almost all types of surfaces both abiotic and biotic, leading to consequences in terms of social and economic impact. These include human tissues, implantable medical devices, natural aquatic systems, plants, food and industrial lines. Once biofilm is formed, its eradication becomes difficult because its resilience to environmental stresses, disinfectants, and antimicrobial treatments.

Plants support a diverse array of microorganisms that exist in form of biofilms. Even if in some cases the association with plants leads to beneficial interactions promoting plant growth, inducing plant defense mechanisms and preventing the deleterious effects of pathogenic microorganisms, in other cases they have a significant negative impact. For instance, in agriculture, plant colonization of fungi and bacteria in form of biofilm is a cause of plant diseases, affecting crop quality and productivity. Indeed, despite the planktonic growth, biofilm lifestyle improves microbial resistance to antimicrobials up to several orders of magnitude, often reducing the possibility of treating biofilm effectively. In addition, due to the worrisome consequences related to the use of these substances on human health and on their persistence in the environment, increasingly regulations are arising to limit antimicrobial application. Furthermore, in addition to the principles of integrated pest management (IPM) embraced by the worldwide legislation aims to recommend alternative approaches to the application of pesticides, an innovative approach could be the use of biocide-free bioactive compounds characterized by novel targets, unique modes of action and properties that are separate from those currently highlighted in the use of antimicrobials. Indeed, the application of non-lethal doses of bio-inspired molecules able to interfere with specific key-steps involved in the biofilm formation process has been suggested as a complementary/alternative strategy to hinder biofilm formation. In addition, this approach also lead to deprive microorganisms of their virulence factors without affecting their viability and decreasing the selection pressure for biocides resistance. In this PhD thesis, the *in vitro* effects of non-lethal concentrations of several bioactive compounds were evaluated on the biofilm formation of different plant-associated microorganisms.

Specifically, the aim of this work was to provide new effective preventive or integrated solutions against bacterial and fungal biofilm formation.

In chapter III, the methanol extracts obtained by different plant portions of three seagrass species collected in Vietnam and in India (*Enhalus acoroides*, *Halophila ovalis* and *Halodule pinifolia*) were investigated for their effects in mediating non-lethal interactions on sessile *Escherichia coli* and *Candida albicans* cultures taken as models of bacterial and fungal biofilms respectively. The study was focused on anti-biofilm activities of seagrass extracts, without killing cells. Seagrass extracts appeared to be more effective in deterring microbial adhesion on hydrophobic surfaces than on hydrophilic. Results revealed that *E. acoroides* leaf extract proved to be the most promising extract among those tested. Indeed, the selected non-lethal concentrations of *E. acoroides* leaf extract were found to exert an anti-biofilm effect

on *C. albicans* and *E. coli* biofilm in the first phase of biofilm genesis, opening up the possibility of developing preventive strategies to hinder the adhesion of microbial cells to surfaces. The leaf extract also affected the dispersion and maturation steps in *C. albicans* and *E. coli* respectively, suggesting an important role in cell signaling processes. Methanolic extracts were characterized and major phenolic compounds were identified by MS/MS analysis, showing the unique profile of the *E. acoroides* leaf extract.

In chapter IV, two essential oils (PK and PK-IK) derived from two cultivars of *Perilla frutescens*, an annual short day plant widely used in therapeutics in the traditional medicine as well as in food preparations in Asian countries. Essential oils were extracted from the leaves and were characterized. Subsequeltly, their ability to affect biofilm formation of the phytopathogenic model fungi *Colletotrichum musae*, *Fusarium dimerum* and *F. oxysporum* have been studied. PK and PK-IK neither inhibited fungal growth nor were they utilized as a carbon energy source. In addition, PK and PK-IK essential oils showed excellent anti-biofilm performances inhibiting conidia germination and reducing conidia adhesion. Furthermore, they revealed a magnificent anti-biofilm effect even during biofilm maturation, affecting biofilm structural development, with a reduction of dried weight, extracellular polysaccharides and proteins. In all cases PK-IK displayed better activity than PK. Thus, the anti-biofilm effects were exploited with a non-lethal mechanism. This research supported the spreading of PK and PK-IK essential oils as biocide-free agents suitable for a preventive or integrative approach for sustainable crop protection.

Lastly, in chapter V, a non-lethal concentration of N-Acetylcysteine (NAC) was evaluated on the biofilm formation of *Xylella fastidiosa*, a phytopathogen bacterium that causes a range of economically important plant diseases worldwide and that has been recently found in Italy in olive plants, where it causes the olive quick decline syndrome (OQSD). NAC is a naturally occurring compound found in several vegetables (including garlic, onion, peppers and asparagus) and it is mostly known in clinical area, in which it is employed at lethal concentrations in the treatment of human diseases due to its ability to reduce bacterial adhesion, inhibit the production of extracellular polysaccharides and promote the dispersion of pre-formed mature biofilms. In this study, N-Acetylcysteine (NAC) was tested for its ability to affect biofilm response of *X. fastidiosa* CoDiRO strain, mimicking a preventive, a curative and a combination of both approaches. The not-lethal dose 0.08 mg/ml was chosen as representative of plant concentration after its application. NAC did not alter planktonic bacterial growth but promoted biofilm formation in terms of biofilm biomass (above 62 %) and matrix polysaccharides (above 53%) through a ROS-mediated mechanism. Additionally, NAC was not able to destroy *X. fastidiosa* biofilm when already established on the surface but rather, it was suitable to contain the biofilm infection limiting biofilm dispersal. On the contrary, a combination of both preventive and curative approach has been found promising in biofilm dissolving making it more vulnerable.

CHAPTER I

Introduction

Plant-associated biofilms

In nature, plants support a diverse array of microorganisms on their surfaces, within intercellular spaces of their tissues, including aerial plant portions and the vascular network and around their roots (Eberl et al., 2009). In addition to plant activity and phytoexudation phenomena, each plant portion presents a microhabitat characterized by different physical and chemical conditions that united to the environmental conditions (water availability, temperature and pH), leads to the colonization of a selected microbial taxa. Similarly, plants influence the microbial population density and promote the interaction between microorganisms and the plant through metabolic processes and cellular communication mechanisms (Eberl et al., 2009). These microorganisms may establish different plant-interactions as pathogens, commensals or mutualists or grow saprophitically on the nutrients released, affecting plant health and productivity. Biofilms constitute the assemblage-forms frequently observed in plant-associated microbes (Morris and Monier, 2003). Some of these aggregates seem to be the result of a microbial cell accumulation in the depressions formed at the junctions of epidermal cells since water moves along plant surfaces via capillarity (Foster, 1986). In other cases, microcolonies and biofilms formed on plant surfaces seems to be correlated with bacterial attachment and production of exopolymeric substances, active processes involved in biofilm formation (Morris and Monier, 2003). First observations of microcolonies on plants surfaces were reported on the leaves in the 1960s (Ruinen, 1961) and on the roots in the early 1970s (Rovira and Campbell, 1974). Although initially the plant-associated microcolonies were not seen as biofilms, recently the aggregation-phenomena has been correlated with biofilm formation (Morris et al., 2003). Several papers reported the importance of this growth strategy emphasizing the unique properties conferred by the sessile condition than the planktonic growth: an increase of the resistance to environmental stresses, including antimicrobial tolerance, more frequent horizontal gene transfer, the production of excreted signals that are only effective above a threshold concentration due to the high population density, the protection from predation and many others (Davey et al., 2000; Dunne, 2002). There are numerous direct and indirect mechanisms through which microorganisms can get into contact with plant tissues and subsequently start the biofilm formation processes. In soil, for example, the mechanisms of microbial mobility play an important role in the colonization of plants, driven by processes of chemotaxis regulated by nutrient gradient secreted by plant exudates (Morris and Monier, 2003). There are also passive mechanisms that can favor the meeting between the microorganisms and the various portions of the plant. Wind and rain can deposit microorganisms on the aerial parts of the plant while water flows around the root system can promote the adhesion of cells to radical tissues. Plant injuries also allow microorganisms to penetrate the tissues, including the vascular system. Finally, a common mechanism that facilitates microbial colonization of plant surfaces are lymph-feeding insects, which can become carriers of infections (Eberl et al., 2009). The microbial colonization of the phyllosphere seems to be the hardest process since the aerial parts of plants constitute a hostile environment to microorganisms, and this is due to the presence of light, heat and water scarcity that expose microorganisms to the risk of drying. Although the single cell or spore

can settle in any position on the air surface, the biofilms preferentially form where the stomata and trichomes are present, or along the foliar veins, in response to nutrient concentration and moisture, as in these positions cells survive better to drying stresses (Monier and Lindow, 2004). However, from 10^5 to 10^7 number of bacteria are recovered per leaf under optimal environmental conditions. *Pantoea agglomerans*, a non-pathogenic epiphyte, displays large aggregates of cells under favorable conditions that are similar to the microcolonies observed during biofilm formation on abiotic surfaces by different bacteria (Sabaratnam & Beattie 2003). On these exposed tissues, biofilm lifestyle plays an essential role to prevent desiccation, protecting the cells within the biofilm to adverse conditions in comparison with single cells (Monier and Lindow, 2005). On the other side, the xylem and the phloem of the plants represent a protected niche for the formation of biofilms. Generally, the microbial colonization of the vascular system takes place through an active invasion of insects that feed on vascular tissue. Mechanisms of colonization of xylem by pathogenic microorganisms have been extensively studied (Zaini et al., 2009; Koczan et al., 2011). These pathogens form the biofilm in the xylematic vessels producing copious doses of polysaccharides that block the vascular system, damaging the tissue. Moreover, degrading enzymes, secreted by pathogens, increase the damage and help the microbial dissemination in the vascular system. Some pathogens are even able to regulate the processes of colonization and diffusion, through cellular communication mechanisms (von Bodman et al., 2003). Lastly, regarding to the rizosphere context, the root cap and the area of active cell division are largely colonized in addition to the primary structure and root hairs. The root elongation zone, instead, immediately above the root cap, appears to be less subject to microbial colonization (Semenov et al., 1999; Watt et al., 2006). The foregoing examples suggest that plant-associated biofilms play an important role in plant health, providing benefits or, on the contrary, damaging them.

Positive and deleterious effects of plant-associated biofilms on plants

Plants live in close association with a huge diversity of microorganisms. A typical example of beneficial plant-microorganism interaction is represented by a group of bacteria that live in symbiosis with the roots and can promote the growth of the plants. These microorganisms belong to a wide variety of genera and are generally referred to Plant Growth Promoting Bacteria (PGPR). PGPR promote plant growth in two different ways, directly and indirectly. The promotion of plant growth directly occurs through the production by microorganisms of compounds, such as phytohormones, which facilitate the plant in the processes of absorption of some nutrients from the environment. In indirect promotion, PGPR prevent the deleterious effects of pathogenic microorganisms, producing antagonistic substances or inducing plant resistance to the pathogens (Beneduzi et al., 2012). Another example of beneficial interaction between plants and microorganisms is represented by some species of fungi that grow in a symbiotic relationship with the root cells of the higher plants; this lifestyle is called mycorrhiza. The roots of many plants such as corn, soybeans, cotton, tobacco, peas, apples, citrus, pines, poplars, birches, some species of turf, etc. have strong relationship with fungi within the soil (Ellis et al., 2008). Mycorrhizae appear to be extremely useful, and often necessary, for the optimal growth of many plants. Mycorrhizae in fact facilitate the plant in the processes of absorption of water and minerals. The relation plant-mycorrhizae helps the plant to be more competitive and more tolerant to environmental stresses such as water stress or the pathogens colonization. An example of the latter are fungi belonging to the genus *Trichoderma*

that are used as biocontrol agents against various phytopathogenic fungi, while others, such as *Arthrobotrys dactyloides* have shown the ability to trap and parasitize the pathogenic nematodes of some plants. Some fungi also produce useful antibiotics and enzymes that help the plant to defend against pathogens (Ellis et al., 2008).

Despite the positive effects that some microorganisms have on plants, plant colonization by fungi and bacteria in form of biofilm is also the cause of plant diseases, leading to an imbalance that determines a reduction or loss of production compared to a healthy plant (Agrios, 2012). Plant diseases caused by bacteria and fungi affect crops, and are responsible for significant losses or decrease the quality and safety of agricultural products. The type and the amount of losses caused by plant diseases vary depending on the plant and pathogen species, the geographical location and the environmental conditions (Agrios, 2012). It has been estimated that plant diseases, together with insects and weeds cause a plant decrease productivity between 20 and 40 % of global agricultural productivity (Oerke, 2006). In this scenario, pathogens still claim 10–16% of the global harvest (Chakraborty and Newton, 2011). In addition, some filamentous fungi can produce mycotoxins in foods as a result of fungal growth under favorable environmental conditions. Mycotoxins are secondary metabolites that cause a toxic response, termed as mycotoxicosis, after the ingestion of plant-based food by higher vertebrates and other animals, leading to the deterioration of liver and kidney function due to the induction of cell death. Some species of mycotoxigenic fungi produce these compounds before or immediately post harvesting (e.g. *Fusarium* species) or during storage processes (*Penicillium* and *Aspergillus* species) (Sweeney and Dobson, 1998; Doehlemann et al., 2017).

Phytopathogenic bacteria and biofilm formation

Although the number of bacterial species worldwide vary from tens of thousands to billions (Schloss and Handelsman 2004), only a very restricted number of them cause deleterious effects to the agricultural crops (Kado, 2010). In crop production, phytopathogenic bacteria are recently gaining the attention due to their difficult control through agrochemicals application and their undetected diffusion on plant propagation material (Lugtenberg, 2015). Nowadays, due to the latter aspect, many phytopathogenic bacteria are quarantine organisms. Phytopathogen bacteria occur mainly in climatic areas, their relevance decreases in zones characterized by arid climates since humidity plays an important role in disease development and pathogen dissemination (Lugtenberg, 2015). Some species infect only a specific single crop (e.g. *Clavibacter michiganensis* subsp. *sepedonicus*, the causal agent of the potato bacterial ring rot disease), others are polyphagous and are pathogenic to multiple host plants, as reported for *Ralstonia solanacearum* the causal agent of wilting diseases. Moreover, some phytopathogenic bacteria species can infect both plants and animals. *Dickeya dadantii* shows a phytopathogenic and a entomopathogenic lifestyle on ornamental plants and insects respectively (Lugtenberg, 2015). Several symptoms such as necrosis, wilting, tissues maceration and hyperplasia are highlighted in bacterial plant diseases. The host infection process is realized through the injection of effector molecules directly into plant cells to suppress the host response. In addition, virulence factors such as the production of toxins, hormones or plant cell wall-degrading enzymes promote the infection process (Lugtenberg, 2015). Bacteria can colonize intercellular spaces of the host plant or systematically the vascular system. Symptoms caused

by plant pathogenic bacteria are usually multiples on host, as reported for *Xanthomonas* and *Pseudomonas* spp. that display initially water-soaked lesions followed by necrosis and chlorosis of the tissues (Kado 2010).

In contrast to the active penetration in the host plants displayed by phytopathogenic fungi, bacteria are not able to penetrate the plant cuticle but require openings to enter the host. Bacterial accesses to host are correlated to the presence of wounds caused by severe weather conditions, cultural practices, insects and fungal growth. Moreover, lateral root formation, stomata and lenticels represent natural entry points (Zeng et al., 2010). Some plants species (tomato, pea and grape) oppose the bacterial infection inducing the closure of stomata, even if some bacterial species (*Pseudomonas syringae* and *Xanthomonas campestris*) possess several genes that encode different compounds able to reverse stomatal closure (Lugtenberg, 2015). Others phytopatogenic bacteria that cause infection in the vascular system of the host, such as *Liberibacter* spp. and *Xylella fastidiosa,* require to be vectored by insects to reach the vascular tissues of the host. Bacterial colonization of host plants is characterized by the formation of biofilm: this articulated process can be described in different development stages (fig. 1).

(I) Adhesion: The mechanism by which the bacterial cell "perceives" the presence of a solid surface (surface sensing process) is still under study. However, microorganisms are endowed with sensory structures able to measure the activity of water, the pH and the concentration of nutrients present on a surface. As soon as the microorganism reaches close to the surface, about 1 nm, the adhesion is influenced by the sum of attractive and repulsive forces between the two contact surfaces. These forces include electrostatic and hydrophobic interactions, Van der Waals forces and hydrodynamic forces. With the strengthening of specific interactions between microorganism and surface, adhesion becomes irreversible (Costerton, 1999). The organisms consolidate the adhesion process by means of specific ligands and producing the extracellular matrix.

(II) Development and maturation phase: Once completed the adhesion phase, it becomes irreversible and the biofilm increases in size, since the microorganisms begin to actively replicate and to secrete the extracellular compounds. Within biofilms, microorganisms are able to coordinate their activities through signal molecules, capable of modulating simultaneously the gene expression of an entire population (quorum sensing) (Costerton, 2007).

(III) Dispersion phase: The last stage of biofilm life, with the detachment of cells from the mature biofilm. The detachment consists of the return of part of the microbial community to the planktonic state, to colonize new habitats. Recent studies have highlighted the fact that this final stage is also regulated by signals produced by the community itself (cellular communication), which promote the breakdown of the biofilm. The dissemination of phytopathogenic bacteria involves plant propagation material, meteorological condition (wind, rain) and insects (Nadarasah and Stavrinides, 2011)

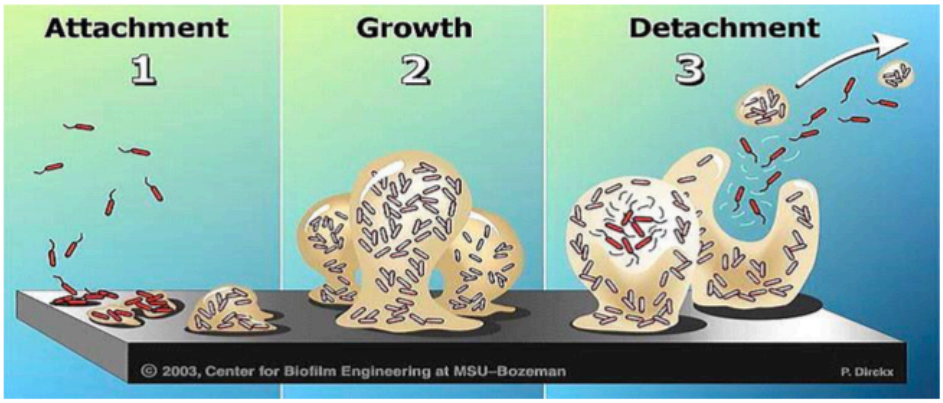

Fig. 1: Bacterial biofilm formation process at the solid/air interface (Dircky, CBE)

For instance, *Xylophilus ampelinus*, a Gram-negative bacterium that is the causal agent of bacterial necrosis of grapevine, survive long periods of time in the vascular tissues of the host plant forming thick biofilm and it spreads from there throughout the whole plant (Grall and Marceau, 2003). In another study, Monier and Lindow (2004) observed that on leaf surface *Pseudomonas syringae* pv. *syringae*, the causal agent of brown spot disease on bean, occurs as adherent biomass containing large aggregates composed of thousands of cells. Another plant-pathogen bacterium that is recently gaining the attention of the scientific community is *Xylella fastidiosa,* due to the serious and worrisome disease that causes in several economically important crops. *X. fastidiosa* evinces its pathogenicity in a cell density-dependent manner, in which biofilm formation represents a fundamental step for host colonization (insect and plant species) and disease development. Insect colonization starts with the bacterial cell adhesion to the insect foregut cuticle: initially cells are attached laterally but they become polarly forming a monolayer in fully colonized insects, probably to increase the cell surface exposure to optimize nutrient uptake (Chatterjee et al., 2008; Killiny and Almeida, 2009). During transmission to the plant, the attachment of single cells attach to xylem vessels elements is followed by the production of exopolysaccharide required for disease, highlighting areas with dense biofilms and high levels of exopolysaccharide that cause the water transport in the xylem vessels (Newman et al., 2003). Interestingly, for the success of bacterial adhesion and biofilm formation, type I adhesive pilus structure are required and earlier evidence suggests that this may involve interaction with xylem elements via active thiol groups on the pilus or another cell surface structure (Leite et al., 2004). The control of bacterial plant diseases is difficult because few options are available. Since a low number of pesticides is effective, the main strategies are focused on the prevention or on the exclusion of the pathogen, avoiding the contamination of crop plants.

Phytopathogenic fungi and biofilm formation

It has been estimated that most of the plant-related diseases are caused by fungi that develop on different portions of the plant in the form of biofilm (Agrios, 2012). All plant species can be affected, and each of the pathogenic fungal species can attack multiple plant species. Phytopathogenic fungi are a heterogeneous group due to their large number and diversity. However, phytopathogenic fungi are able to colonize the plant tissues and cause disease in several ways, also leading to death of the host. Some of them prefer feeding on dead material throught the extraction of nutrients after host-killing due to the production of toxins (necrotrophs). Others can persist in plants and utilize compounds produced by living tissues to manipulate plant metabolism (biotrophs) (Doehlemann et al., 2017). Another group of phytopatogenic fungi, named hemibiotrophic pathogens, start as biotrophs and then become necrotrophs. Phytopathogenic fungi cause localized or generalized symptoms on their hosts, and these symptoms may occur separately or simultaneously. In general, fungi cause local or general necrosis of plant tissues, and often result in reduced growth or stunted growth of organs or the entire plant (Agrios, 2012). Nevertheless, in biotrophic interactions, these fungi lead to the formation of pseudo-flowers (rust) or plant tumors (smuts) with the aim to promote fungal propagation (Doehlemann et al., 2017). Phytopathogenic fungi get inside plants through natural openings (e.g. stomata) or penetrate directly producing a structure called appressorium on a host cell (Lugtenberg, 2015). The major obstacle for plant pathogens is constituted by the cell wall, which represent a physical and chemical barrier. Nevertheless, the genome of many fungi contains several genes that encode different classes of cell-wall degrading enzymes. In necrotrophic fungi, a highly up-regulated expression of these genes occurs during the infection process (Zhao et al., 2013). In addition, they produce also secondary metabolites with toxic properties to promote their necrotrophic lifestyle (Lugtenberg, 2015). On the other side, biotrophic fungi have in their genome the same genes but they are lowly expressed during plant infection. For instance, Okmen et al (2013) reported the presence of detoxifying enzymes produced by *Cladosporium fulvum* to counteract the antifungal effects of saponin and α-tomatine produced at high concentrations in tomato plants during the fungal infection.

In contrast to the extensive literature describing the formation, the architecture, the chemical composition, the genetic regulation and antimicrobial resistance mechanisms of bacterial biofilms, limited knowledges are available regarding fungal biofilms. Moreover, most of this knowledge concerns yeasts of medical or commercial interest such as *Candida* spp., *Saccharomyces* spp. and *Aerobasidium neoformans* and not filamentous fungi like those involved in plant diseases (Harding et al., 2009). The reasons probably lie in the fact that filamentous fungi develop biofilms with characteristics that do not coincide exactly with those of bacterial biofilms or lievitiform fungi and therefore in the past have not been considered able to develop biofilms (Peiquian et al., 2014). For example, many filamentous fungi produce hyphae and/or invasive structures that extend beyond the liquid-air interface, or specialized structures, necessary for host penetration, sporulation and acquisition of nutrients from the host. These differences in morphology and growth complicate the understanding of filamentous fungal biofilms (Harding et al., 2009). The little knowledge about biofilms responsible for plant diseases is also since they mostly develop at the solid-air interface while most of the knowledge available is biofilms at the solid-liquid interface (Gorbushina, 2007; Gorbushina et al., 2009). In fact, the scientific literature several studies are dedicated to the study of underwater biofilms (solid-liquid interface), also thanks to the

development of standardized methods for reproducing these types of biofilms on a laboratory scale (Gorbushina et al., 2009). On the other hand, there seem to be few scientific studies reporting biofilm subaerous (solid-air interface). The only information available concerns the development of subaerial biofilms of epilithic microorganisms able to colonize stone substrates. Recently, some studies have been published regarding the development of filamentous fungal biofilms, but these are in any case of fungi of medical interest (Harding et al., 2009). The only research concerning biofilms produced by filamentous fungi and not associated with the medical field is that of Peiquian and collaborators (2014) which focuses on the study of the biofilm produced by the phytopathogenic fungus *Fusarium oxysporum*. Studies using microscopy techniques have revealed that this fungus is characterized by a highly heterogeneous architecture composed of numerous hyphae and abundant extracellular matrix rich in polysaccharides. Moreover, these researchers have shown that this biofilm is not very susceptible to heat, cold and UV rays compared to the corresponding fungal cells in planktonic form. Harding and colleagues (2009) proposed a model regarding the dynamics of formation of a filamentous fungal biofilm. This model is characterized by the presence of six phases (fig. 2):

(I) Adsorption of propagules. This phase involves the physical contact of the organism with a surface. It consists essentially in the deposition of spores or other propagules, such as hyphal or sporangia fragments, on a surface.

(II) Active adhesion. At this stage, most of the cells in the form of blastospores adhere to the surface. The adhesion of the spores is essential for the formation of the biofilm. In this process, in addition to non-specific forces such as hydrophobic and electrostatic interactions, adhesive substances are involved that are actively secreted by germinating spores, such as small proteins called hydrophobins. These proteins are exclusive to filamentous fungi and are involved in the adhesion of hyphae to hydrophobic surfaces. Hydrophobins can self-assemble at the hydrophilic-hydrophobic interface and modify the surface properties of the hyphae in response to environmental factors and development. It is also possible that hydrophobins form layers to seal hyphal aggregations in more complex structures.

(III) Microcolonies formation. This phase involves the apical elongation and the branching of the hyphae on the surfaces giving rise to a monolayer of hyphae grouped in microcolonies. This phase involves the production of the polymeric extracellular matrix which allows the growing colony to adhere tenaciously to the substrate. The formation of the extracellular matrix makes the adhesion phase irreversible.

(IV) Initial maturation. This phase involves the formation of compact networks of hyphae. This process includes stratification, the formation of hyphal bundles joined by the extrapolymer matrix and the formation of water channels. In this stage, quorum sensing plays a fundamental role in the specific physiology of the fungal biofilm.

(V) Development of mature biofilm. At this stage, the production of the matrix increases considerably, until the fungal community is completely absorbed. The fungal microcolonies and the extracellular matrix in which they are enclosed, build up the mature biofilm. In this step, the growth of hyphae increases considerably, especially at the superficial level, with the formation of fruiting bodies, sporangia, sclerotia and other structures. Aerial growth is often

a key feature of fruiting and scattering fungal structures.

(VI) Dispersion. This phase consists in the release and dispersion of the spores and/or in the diffusion of biofilm fragments. The detached fungal portions can act as new propagules and restart the cycle. This happens when the biofilm depletes the nutrients of a microenvironment, then produces and releases spores capable of dispersing and colonizing new surfaces and new ecological niches.

For instance, Harding and collaborators (2010) observed that *Botrytis cinerea*, a necrotrophic fungus that affects many plant species, was present on tomato stem as highly layered, with extensive hyphal networks embedded in an exopolymeric matrix. Li et al. (2014) reported the appearance of phenotypic changes of *Fusarium oxysporum* f. sp. *cucumerinum,* a soil-borne vascular fungal pathogen that causes *Fusarium* wilt, one of the most worrisome threats to cucumber (*Cucumis sativus* L.), affecting quality and productivity worldwide. They observed a highly heterogeneous architecture composed of robust hyphae and extracellular polysaccharidic materials, correlated to biofilm structures. The authors also reported that *F. oxysporum* biofilms were significantly less susceptible to stress conditions (heat, cold, UV light and fungicides) than the planktonic counterpart. Fungal biofilm formation process remains an extremely complex process that requires the coordination of many factors, some still unknown, despite the important implications of this phenomenon on social and economic life (Blankenship et al., 2006).

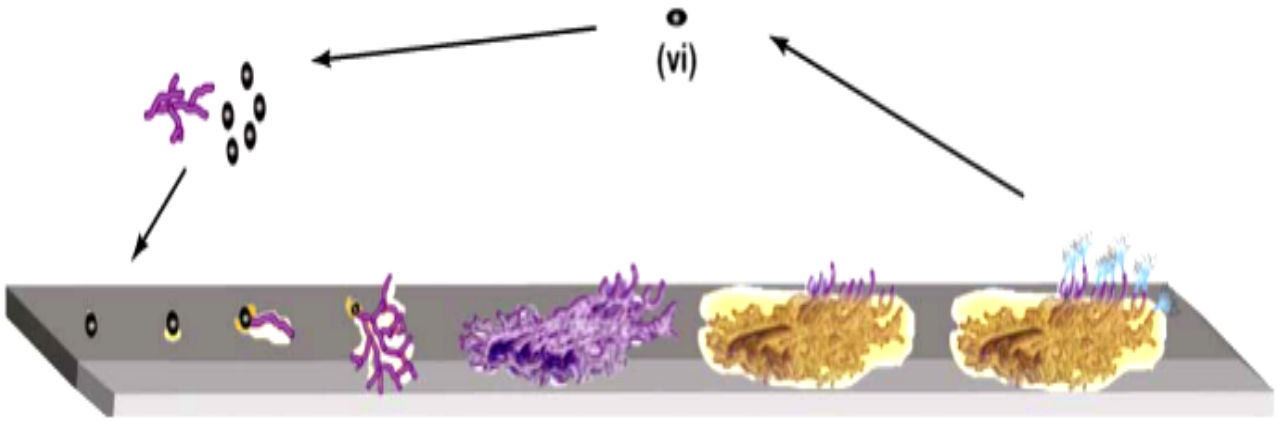

Fig. 2: Biofilm formation process in filamentous fungi (Harding et al., 2009).

Traditional strategies to counteract microbial biofilm formation: the use of pesticides

The traditional approaches for the control of plant diseases consist in the treatment of crops with pesticides. Liu et al. (2015) reported an annual amount of about 1,5 million tons of pesticides applied worldwide, highlighting an average of about 353 thousand tons of fungicides and bactericides consumed. In fact, in addition to their increased used in the past decades, the application of these chemicals led to safeguard and maintain crop yield and quality (Liu et al., 2015; Pretty et al., 2015). It has also predicted their massive application in the next few decades (Carvalho et al., 2017) arising several issues related to their persistence in the environment with harmful impact on human health via the food chain (Sousa et

al., 2014). In fact, their persistence is correlated with their intact presence in the environment over prolonged period of time, since they are not degraded into safer compounds (Sousa et al., 2014). An example was reported by a study published by Daghrir et al. (2013) highlighting that the massive use of tetracycline, a class of antibiotics mainly used for human therapy, veterinary and agricultural purposes led to their presence in several ecological compartments. Tetracycline are characterized by highly hydrophilic and low volatility properties that make this compound able to persist in the environment. The results of the presence of antibiotic residues have been translated in the appearance of antibiotic resistant microorganisms, with subsequent several problems to human health related to the induction of adverse effects that increase the risk to certain infections. It has been shown that microorganisms in form of biofilm can resist to the action of the antimicrobial agents up to a thousand times more than the counterpart in planktonic form (Davey et al., 2000). Moreover, the effectiveness of almost all the pesticides available on the market is tested on microbial cells or spores in planktonic form, more sensitive to the action of the biocide, and not on cells in the form of more resistant biofilms (Cappitelli et al., 2011). This last feature makes biofilms difficult to eradicate. The biofilm lifestyle confers to the microorganism several advantages in terms of antimicrobial tolerance and resistance. These aspects can be summarized as:

(I) A slow penetration of the antimicrobial agent through the biofilm thanks to the presence of the EPS, that acts as a physical barrier. Furthermore, some components of the matrix may limit the transport of the antimicrobial agent within the biofilm (Thien-Fah et al., 2001), or inactivate it by enzymatic activity (Stewart et al., 2001). Thus, the matrix limits the spread of unwanted substances within the biofilm, blocking and inactivating the antimicrobial agent (Stoodley et al., 2004).

(II) The physiological state of the biofilm. Although many antimicrobials can freely penetrate the matrix, the presence of areas with reduced metabolic or dormant activity appears to be a significant resistance factor. In fact, antimicrobials need to act as active cells, since the mechanism of action of almost everyone is based on the interruption of cellular processes. As a result, the presence of dormant cell groups could represent a general mechanism of antibiotic resistance (Stoodley et al., 2004).

(III) The existence in the biofilm of resistant phenotype subpopulations known as persister cells. These specialized survivor cells can tolerate certain antimicrobial agents and they are not killed by their lethal action (Lewis, 2010).

(IV) The transfer of antimicrobial resistance on mobile genetic elements trough horizontal gene transfer (Savage et al., 2013)

Prior to 1970, almost all fungicides used to control plant pathogens acted on multiple target sites of the fungus and, despite their widespread use, resistance to these compounds was a rare occurrence. After the introduction of specific site-action fungicides, at the end of the sixties, the resistance to fungicides by phytopathogenic fungi became, instead, a serious problem for crop protection (Regueiro et al., 2015). This phenomenon has led to an ever-increasing use in the doses of fungicides used to counteract fungal diseases and to maintain crop productivity, with serious repercussions. In addition, the use of excessive doses of fungicides may cause physiological and or morphological changes to the plant portions, as a

reduction in development, fall of flowers, leaf deformations, burns (Gullino et al., 2000; Gisi et al., 2002). The induction of microbial resistance phenomena to many of the most effective pesticides due to their abundant application in the past has led to a decrease of the availability of active substances (Sousa et al., 2014). Patel et al (2013) reported the results obtained from a survey made by the World Health Organization related to the risk of human health after pesticides exposure: it is described that each year in developing countries more than 50,000 people are poisoned and 5,000 die due to the agricultural exposure. This critical situation related to the impact of pesticides on human and animal health and environment led to the discourage of the use of biocides. Recently, a massive number of policies, technical reports, regulatory decisions and directives arisen, encouraging the use of new strategies important for human and animal health preservation and respectful for the environment, leading to a more sustainable agriculture (Directive 98/8/EC; Recommendation 2002/77/EC; SCENIHR report 2009; EFSA Summary Report 2009). For instance, in the European Community Directive 98/8/EC was reported a list of the approved biocidal compounds authorized for the distribution and the use in the commercial formulation within the European member states. Moreover, such compounds that manifested in the past sever risks for human health and environment were banned.

New strategies for controlling biofilm formation in plant diseases

In addition to the safety issues related to the use of pesticides in agricultural field already discussed, the high mutation rate of microorganisms that brings to the development and selection of resistant mutants constitutes a considerable problem for chemical control strategies. To this purpose, the worldwide legislation has embraced the principles of integrated pest management (IPM), recommending alternative approaches to the application of pesticides (EU Directive 2009/128/EC; Barzman et al., 2015). All these principles are characterized by the elimination of the inoculum sources or to minimize the pathogen diffusion. For instance, the planting of material derived by certified seed represents an effective strategy. It is also possible to reduce disease diffusion even destroying diseased or suspect plant material, eliminating the inoculum sources. In addition, another strategy is to minimize damages to plant tissues can prevent the creation of entry points for pathogens. Interestingly, to reduce the risk of airborne inoculum, seed production is performed in geographic areas characterized by an arid and semi-arid climate. The use of resistant crops is another good practice but must be avoided that tolerant plants are involved in the maintenance and in the dispersion of inoculum sources as latent infections (Lugtenberg, 2015). Even the treatment of propagation materials (seeds, bulbs and tubers) with warm water, hot air or aerated steam to seeds, bulbs and tubers can reduce populations of pathogens. Lastly, biological control represents an alternative way for plant protection and positive results have been obtained in the treatment of phytopathogen diseases. For instance, the combination of *Trichoderma asperellum* with composts derived from agricultural wastes, was used to suppress *Rhizoctonia solani* in cucumber seedlings (Trillas et al., 2006). Another example is reported by Wright et al (2001), that demonstrated that *Pantoea herbicola* is a biocontrol agent used against the fireblight caused by *Erwinia amylovora*.

In addition to these practices, an alternative way to the currently use of antimicrobials could be the use of strategies focused on innovative approaches that consist in disarming microorganisms without killing them (Flemming, 2005). Depriving the microorganisms of their virulence properties without compromising their existence leads to a decrease in the selective pressure and therefore to the mutations that induce resistance to pesticides. All these aspects could restore the efficacy of the currently antimicrobials, preserving their efficacy and avoiding the resistance phenomena (Rasko et al., 2010). Since the biofilm lifestyle enhances the virulence of the pathogen, the best strategy is to prevent its formation, using substances for the environment at non-lethal concentrations that are capable of interfering with the key-steps involved in the biofilm formation processes (Villa et al., 2012). To this purpose, it is possible manipulate the expression of specific phenotypes involved in microbial virulence traits affecting the multicellular behavior (e.g. binding ability, motility, cell-to-cell communication, yeast–hyphae-dimorphism) (Rasko et al., 2010; Villa and Cappitelli, 2013). In contrast with the use of antimicrobials, these compounds are characterized by different properties, targets and mode of action (Villa et al., 2012). These strategies might bring new products to the market and cover methodologies and novel approaches, making significant contributions to innovation and economic productivity (Villa et al., 2013). According to the biofilm formation cycle, it is possible to act at the level of the detection processes of the surface by the pathogen, avoiding microbial adhesion and keeping the pathogen in a planktonic form. Moreover, instead of destroying the organization of biofilm, it is possible to interfere with the cellular communication processes leading to a damage of the biofilm matrix, destabilizing its physical integrity. Lastly, a further possibility is to act on the final phase of biofilm formation, encouraging its detachment from surfaces (Villa et al., 2013; Cappitelli et al., 2014). All these aspects make these promising strategies to be potentially used in combination with conventional pesticides to eradicate the biofilm-related diseases (Villa et al., 2013). Nature offers a consistent number of new potential bioactive substances with unequaled complexity and structural diversity (Wong et al., 2008). Over millions of years, plants and animals have developed sophisticated strategies to prevent the colonization on their living tissues by harmful microbial species. Both aquatic and terrestrial plants offer very interesting classes of biologically active, low- molecular-mass (< 5 kDa) compounds ("parvome", parv=small, -ome= group), like alkaloids, terpenoids, flavonoids and coumarins, peptides, glycosides, nucleosides and polyphenols. They may act in a variety of ways: antibiotics, allosteric regulators, catalysis, catalytic cofactors, regulatory activities at level of DNA, RNA and protein, pigments, mutagens, antimutagens, receptor agonists, antagonists, signal molecules, siderophores, detergents, metal complexing/transporting agents, pheromones, toxins and other interesting activities (Davies and Ryan, 2012). However, during the intensive half-century of drug discovery, available natural compounds found in the plant parvome were screened mainly for their lethal effects, disregarding concentrations and ecologically relevant functions of these molecules in the natural environments. All that mattered were compounds effective in killing target microorganisms (Puglisi et al., 2007; Artini et al., 2012). To this

purpose, a non-toxic alternative to traditional antimicrobial agents is represented by natural substances or analogous that prevent microbial adhesion and the development of biofilms on surfaces at non-lethal concentrations (Cuzman et al., 2008). An example of this innovative technology derives from marine plants that possess interesting compounds with the ability to defend their plant tissue against colonization by harmful microorganisms (Barrios et al., 2005). In fact, aquatic organisms produce secondary metabolites able to prevent the microbial colonization on their surfaces (De Nys et al., 2002; Cloete, 2005). For instance, zosteric acid (ZA), a phenolic compound from the seagrass *Zostera marina*, is able to reduce both bacterial and fungal adhesion, and plays a pivotal role in shaping biofilm architecture at non-lethal concentrations. This can be seen through the reduction of biofilm biomass and thickness, and the thwarting of budded-to-hyphal-form transition. Additionally, ZA extends the performance of antimicrobial agents, and shows cytocompatibility towards soft and hard tissue (Villa et al., 2010; Villa et., 2011). A comparative proteomic study on *Escherichia coli* cells exposed to ZA has shown that, in bacterial cells, ZA acts as an environmental cue to global stress, promoting the expression of various protective proteins involved in reactive oxygen species (ROS) scavenging, the production of the signal molecule autoinducer-2, and the synthesis of flagella to escape from adverse conditions (Villa et al., 2012; Cattò et al., 2015). Some flavonoids found in citrus species were able to inhibit biofilm formation by interfering with the processes of cellular communication of *Vibrio harveyi* BB120 and *E. coli* O157:H7 (Vikram et al., 2012). In the recent years, due to the lack of information about the chemical determinants responsible for the inhibition/dispersion of microbial biofilms, the scientific community developed new scientific approaches aimed to quickly analyze and identify a huge number of molecules to discover new active compounds (Whortington et al., 2012; Khan et al., 2014). In 2005, Ren and collaborators published the first detailed report related to the screening of 13,000 compounds belonging to 176 plant families. For instance, they reported the identification of ursolic acid as new biofilm inhibitor from the tree *Diospyros dendo*. This molecule is a triterpenoid that has been subsequently demonstrated unable to exert a biocide effect on several bacteria (*E. coli*, *P. aeruginosa*, *V. harveyi*) but interestingly, it affects their biofilm formation. Subsequently, transcriptome analysis revealed that in the treatment of *E. coli*, ursolic acid acts as a signal that highly promotes cell movement, hindering the biofilm adhesion or destabilizing pre-formed biofilm (Ren et al., 2005). In a report by Panmanee et al. (2013), the screening of 42,865 compounds focused in the discover of molecules able to inhibit biofilm formation of *Staphylococcus epidermidis* was performed. In this study, 16 compounds possessed anti-biofilm properties at non-lethal concentration. These studies allowed to isolate several compounds deriving from nature with anti-biofilm properties characterized by diverse molecular scaffolds and biological activities. Starting from these knowledge, synthetic manipulation of these natural compounds allowed to develop medicinal chemistry programs designing more effective compounds with anti-biofilm properties (Worthington et al., 2012).

Bibliography

Agrios, G., 2012. Plant Pathology. Academic Press.

Artini, M., Papa, R., Barbato, G., Scoarughi, G.L., Cellini, A., Morazzoni, P., Bombardelli, E., Selan, L., 2012. Bacterial biofilm formation inhibitory activity revealed for plant derived natural compounds. Bioorganic & Medicinal Chemistry 20, 920-926.

Barrios, C.A., Xu, Q.W., Cutright, T., Newby, B.M.Z., 2005. Incorporating zosteric acid into silicone coatings to achieve its slow release while reducing fresh water bacterial attachment. Colloids and Surfaces B-Biointerfaces 41, 83-93.

Barzman, M., Barberi, P., Birch, A.N.E., Boonekamp, P., Dachbrodt-Saaydeh, S., Graf, B., Hommel, B., Jensen, J.E., Kiss, J., Kudsk, P., Lamichhane, J.R., Messean, A., Moonen, A.C., Ratnadass, A., Ricci, P., Sarah, J.L., Sattin, M., 2015. Eight principles of integrated pest management. Agronomy for Sustainable Development 35, 1199-1215.

Beneduzi, A., Ambrosini, A., Passaglia, L.M.P., 2012. Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. Genetics and Molecular Biology 35, 1044-1051.

Blankenship, J.R., Mitchell, A.P., 2006. How to build a biofilm: a fungal perspective. Current Opinion in Microbiology 9, 588-594.

Cappitelli, F., Polo, A., Villa, F., 2014. Biofilm Formation in Food Processing Environments is Still Poorly Understood and Controlled. Food Engineering Reviews 6, 29-42.

Cappitelli, F., Villa, F., Sorlini, C., 2011. New Environmentally Friendly Approaches against Biodeterioration of Outdoor Cultural Heritage. In Biocolonization of Stone: Control and Preventive Methods, Proceedings from the MCI Workshop Series, ed. Charola A. E., Mc. Namara C., Koestler R. J., pp. 51-58. Smithsonian Institution Scholarly Press.

Carvalho, F.P., 2017. Pesticides, environment, and food safety. Food and Energy Security 6, 48-60.

Catto, C., Dell'Orto, S., Villa, F., Villa, S., Gelain, A., Vitali, A., 2015. Unravelling the Structural and Molecular Basis Responsible for the Anti-Biofilm Activity of Zosteric Acid (vol 10, e0131519, 2015). Plos One 10.

Chakraborty, S., Newton, A.C., 2011. Climate change, plant diseases and food security: an overview. Plant Pathology 60, 2-14.

Chatterjee, S., Almeida, R.P.P., Lindow, S., 2008. Living in two worlds: The plant and insect lifestyles of *Xylella fastidiosa*. Annual Review of Phytopathology 46, 243-271.

Cloete, E.T., 2005. Alternative and conventional anti-fouling strategies - Abstracts. International Biodeterioration & Biodegradation 56, 121-134.

Costerton, J.W., 1999. Introduction to biofilm. International Journal of Antimicrobial Agents 11, 217-221.

Costerton, J.W., Stewart, P.S., Greenberg, E.P., 1999. Bacterial biofilms: A common cause of persistent infections. Science 284, 1318-1322.

Cuzman, O.A., Tiano, P., Ventura S., 2008. New Control Methods against Biofilms formation on the monumental stones. In 11th International Congress on Deterioration and Conservation of Stone, ed. Lukaszewicz J. W. and Niemcewicz P.; pp. 837–846. Torun, Poland: Nicolaus Copernicus University.

Daghrir, R., Drogui, P., 2013. Tetracycline antibiotics in the environment: a review. Environmental Chemistry Letters 11, 209-227.

Danhorn, T., Fuqua, C., 2007. Biofilm formation by plant-associated bacteria. Annual Review of Microbiology 61, 401-422.

Davey, M.E., O'Toole, G.A., 2000. Microbial biofilms: from ecology to molecular genetics. Microbiology and Molecular Biology Reviews 64, 847-+.

Davies, J., Ryan, K.S., 2012. Introducing the Parvome: Bioactive Compounds in the Microbial World. Acs Chemical Biology 7, 252-259.

de Nys, R., Steinberg, P.D., 2002. Linking marine biology and biotechnology. Current Opinion in Biotechnology 13, 244-248.

Doehlemann, G., Okmen, B., Zhu, W.J., Sharon, A., 2017. Plant Pathogenic Fungi. Microbiology Spectrum 5.

Dunne, W.M., 2002. Bacterial adhesion: Seen any good biofilms lately? Clinical Microbiology Reviews 15, 155-+.

Eberl, L., von Bodman, S.B., Fuqua, C., 2007. Biofilms on plant surfaces. In the Biofilm Mode of Life: Mechanisms and Adaptation. Kjelleberg, S., and Givskov, M. (eds). Norfolk, UK: Horizon Bioscience, pp. 215–234.

European Commission (2009). EU Action on Pesticides: "Our Food has Become Greener." Available at:http://ec.europa.eu/food/plant/plant_protection_products/eu_policy/docs/factsheet_pesticides_en.pdf [accessed February 10, 2014].

Gisi, U., Sierotzki, H., Cook, A., McCaffery, A., 2002. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. Pest Management Science 58, 859-867.

Gorbushina, A.A., 2007. Life on the rocks. Environmental Microbiology 9, 1613-1631.

Gorbushina, A.A., Broughton, W.J., 2009. Microbiology of the atmosphere-rock interface: how biological interactions and physical stresses modulate a sophisticated microbial ecosystem. Annual Review of Microbiology 63, 431-450.

Grall, S., Manceau, C., 2003. Colonization of *Vitis vinifera* by a green fluorescence protein-labeled, gfp-marked strain of *Xylophilus ampelinus*, the causal agent of bacterial necrosis of grapevine. Applied and Environmental Microbiology 69, 1904-1912.

Gullino, M.L., Leroux, P., Smith, C.M., 2000. Uses and challenges of novel compounds for plant disease control. Crop Protection 19, 1-11.

Hall-Stoodley, L., Costerton, J.W., Stoodley, P., 2004. Bacterial biofilms: From the natural environment to infectious diseases. Nature Reviews Microbiology 2, 95-108.

Harding, M.W., Marques, L.L.R., Howard, R.J., Olson, M.E., 2010. Biofilm morphologies of plant pathogenic fungi. The Americas Journal of Plant Science and Biotechnology; 4, 43–47.

Harding, M.W., Marques, L.L.R., Howard, R.J., Olson, M.E., 2009. Can filamentous fungi form biofilms? Trends in Microbiology 17, 475-480.

Kado, C.I., 2010. Plant bacteriology. APS Press, St. Paul, p 336.

Khan, M.S.A., Ahmad, I., Sajid, M., Cameotra, S.S., 2014. "Current and emergent control strategies for medical biofilms," in Antibiofilm Agents Springer Series on Biofilms, eds K. P. Rumbaugh and I. Ahmad (Berlin: Springer), 117– 159.

Killiny, N., Almeida, R.P.P., 2009. *Xylella fastidiosa* afimbrial adhesins mediate cell transmission to plants by leafhopper vectors. Applied and Environmental Microbiology 75, 521-528.

Koczan, J.M., Lenneman, B.R., McGrath, M.J., Sundin, G.W., 2011. Cell surface attachment structures contribute to biofilm formation and xylem colonization by *Erwinia amylovora*. Applied and Environmental Microbiology 77, 7031-7039.

Leite, B., Andersen, P.C., Ishida, M.L., 2004. Colony aggregation and b1ofilm formation in xylem chemistry-based media for *Xylella fastidiosa*. Fems Microbiology Letters 230, 283-290.

Lewis, K., 2010. Persister Cells. Annual Review of Microbiology, Vol 64, 2010 64, 357-372.

Li, P.Q., Pu, X.M., Shen, H.F., Zhang, J.X., Huang, N., Lin, B.R., 2014. Biofilm formation by *Fusarium oxysporum* f. sp *cucumerinum* and susceptibility to environmental stress. Fems Microbiology Letters 350, 138-145.

Liu, Y.B., Pan, X.B., Li, J.S., 2015. A 1961-2010 record of fertilizer use, pesticide application and cereal yields: a review. Agronomy for Sustainable Development 35, 83-93.

Lugtenberg, B.J.J., 2015. "Introduction to plant-microbe-interactions," in Principles of Plant-Microbe Interactions. Microbes for Sustainable Agriculture, ed. B. Lugtenberg (Berlin: Springer), 1–2.

Mah, T.F., Pitts, B., Pellock, B., Walker, G.C., Stewart, P.S., O'Toole, G.A., 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. Nature 426, 306-310.

Monier, J.M., Lindow, S.E., 2004. Frequency, size, and localization of bacterial aggregates on bean leaf surfaces. Applied and Environmental Microbiology 70, 346-355.

Monier, J.M., Lindow, S.E., 2005. Aggregates of resident bacteria facilitate survival of immigrant bacteria on leaf surfaces. Microbial Ecology 49, 343-352.

Morris, C.E., Monier, J.M., 2003. The ecological significance of biofilm formation by plant-associated bacteria. Annual Review of Phytopathology 41, 429-453.

Nadarasah, G., Stavrinides, J., 2011. Insects as alternative hosts for phytopathogenic bacteria. Fems Microbiology Reviews 35, 555-575.

Oerke, E.C., 2006. Crop losses to pests. Journal of Agricultural Science 144, 31-43.

Okmen, B., Etalo, D.W., Joosten, M., Bouwmeester, H.J., de Vos, R.C.H., Collemare, J., de Wit, P., 2013. Detoxification of -tomatine by *Cladosporium fulvum* is required for full virulence on tomato. New Phytologist 198, 1203-1214.

Panmanee, W., Taylor, D., Shea, C.J.A., Tang, H., Nelson, S., Seibel, W., Papoian, R., Kramer, R., Hassett, D.J., Lamkin, T.J., 2013. High-Throughput screening for small-molecule inhibitors of *Staphylococcus epidermidis* RP62a biofilms. Journal of Biomolecular Screening 18, 820-829.

Patel, I., Patel, V., Thakkar, A., Kothari, V., 2013. Microbial biofilms: microbes in social mode. International Journal of Advanced Biotechnology and Research; 1, 19-34.

Peiquian, L., Xiaoming, P., Hulifang, S., Jingxing, Z., Ning, H., Birun, L., 2013. Biofilm formation by *Fusarium oxysporum* f. sp. *cucumerinum* and susceptibility to environmental stress. FEMS Microbiol. Lett; 350:138–145.

Pretty, J., Bharucha, Z.P., 2015. Integrated Pest Management for Sustainable Intensification of Agriculture in Asia and Africa. Insects 6, 152-182.

Puglisi, M.P., Engel, S., Jensen, P.R., Fenical, W., 2007. Antimicrobial activities of extracts from Indo-Pacific marine plants against marine pathogens and saprophytes. Marine Biology 150, 531-540.

Rasko, D.A., Sperandio, V., 2010. Anti-virulence strategies to combat bacteria-mediated disease. Nature Reviews Drug Discovery 9, 117-128.

Regueiro, J., Lopez-Fernandez, O., Rial-Otero, R., Cancho-Grande, B., Simal-Gandara, J., 2015. A review on the fermentation of foods and the residues of pesticides-biotransformation of pesticides and effects on fermentation and food quality. Critical Reviews in Food Science and Nutrition 55, 839-863.

Ren, D.C., Zuo, R.J., Barrios, A.F.G., Bedzyk, L.A., Eldridge, G.R., Pasmore, M.E., Wood, T.K., 2005. Differential gene expression for investigation of *Escherichia coli* biofilm inhibition by plant extract ursolic acid. Applied and Environmental Microbiology 71, 4022-4034.

Rovira, A.D., Campbell, R., 1974. Scanning electron microscopy of micro-organisms on the roots of wheat. Microbial Ecolology 1:15–23.

Ruinen, J., 1961. The phyllosphere. I. An ecologically neglected milieu. Plant Soil 15:81–109.

Sabaratnam, S., Beattie, G.A., 2003. Differences between *Pseudomonas syringae* pv. *syringae* B728a and *Pantoea agglomerans* BRT98 in epiphytic and endophytic colonization of leaves. Applied and Environmental Microbiology 69, 1220-1228.

Savage, V.J., Chopra, I., O'Neill, A.J., 2013. *Staphylococcus aureus* biofilms promote horizontal transfer of antibiotic resistance. Antimicrobial Agents and Chemotherapy 57, 1968-1970.

Schloss, P.D., Handelsman, J., 2004. Status of the microbial census. Microbiology and Molecular Biology Reviews 68:686– 691.

Semenov, A.M., van Bruggen, A.H.C., Zelenev, V.V., 1999. Moving waves of bacterial populations and total organic carbon along roots of wheat. Microbial Ecology 37, 116-128.

Sousa, A.C.A., Pastorinho, M.R., Takahashi, S., Tanabe, S., 2014. History on organotin compounds, from snails to humans. Environmental Chemistry Letters 12, 117-137.

Stewart, P.S., Costerton, J.W., 2001. Antibiotic resistance of bacteria in biofilms. Lancet 358, 135-138.

Sweeney, M.J., Dobson, A.D.W., 1998. Mycotoxin production by Aspergillus, Fusarium and Penicillium species. International Journal of Food Microbiology 43, 141-158.

Trillas, M.I., Casanova, E., Cotxarrera, L., Ordovas, J., Borrero, C., Aviles, M., 2006. Composts from agricultural waste and the *Trichoderma asperellum* strain T-34 suppress *Rhizoctonia solani* in cucumber seedlings. Biological Control 39, 32-38.

Vikram, A., Jayaprakasha, G.K., Jesudhasan, P.R., Pillai, S.D., Patil, B.S., 2010. Suppression of bacterial cell-cell signalling, biofilm formation and type III secretion system by citrus flavonoids. Journal of Applied Microbiology 109, 515-527.

Villa, F., Albanese, D., Giussani, B., Stewart, P.S., Daffonchio, D., Cappitelli, F., 2010. Hindering biofilm formation with zosteric acid. Biofouling 26, 739-752.

Villa, F., Cappitelli, F., 2013. Plant-derived bioactive compounds at sub-lethal concentrations: towards smart biocide-free antibiofilm strategies. Phytochemistry Reviews 12, 245-254.

Villa, F., Pitts, B., Stewart, P.S., Giussani, B., Roncoroni, S., Albanese, D., Giordano, C., Tunesi, M., Cappitelli, F., 2011. Efficacy of Zosteric Acid Sodium Salt on the Yeast Biofilm Model *Candida albicans*. Microbial Ecology 62, 584-598.

Villa, F., Remelli, W., Forlani, F., Vitali, A., Cappitelli, F., 2012. Altered expression level of *Escherichia coli* proteins in response to treatment with the antifouling agent zosteric acid sodium salt. Environmental Microbiology 14, 1753-1761.

von Bodman, S.B., Bauer, W.D., Coplin, D.L., 2003. Quorum sensing in plant-pathogenic bacteria. Annual Review of Phytopathology 41, 455-482.

Watt, M., Hugenholtz, P., White, R., Vinall, K., 2006. Numbers and locations of native bacteria on field-grown wheat roots quantified by fluorescence in situ hybridization (FISH). Environmental Microbiology 8, 871-884.

Wong, C.C., Cheng, K.W., He, Q.Y., Chen, F., 2008. Unraveling the molecular targets of natural products: Insights from genomic and proteomic analyses. Proteomics Clinical Applications 2, 338-354.

Worthington, R.J., Richards, J.J., Melander, C., 2012. Small molecule control of bacterial biofilms. Organic & Biomolecular Chemistry 10, 7457-7474.

Wright, S.A.I., Zumoff, C.H., Schneider, L., Beer, S.V., 2001. *Pantoea agglomerans* strain EH318 produces two antibiotics that inhibit *Erwinia amylovora* in vitro. Applied and Environmental Microbiology 67, 284-292.

Zaini, P.A., De La Fuente, L., Hoch, H.C., Burr, T.J., 2009. Grapevine xylem sap enhances biofilm development by *Xylella fastidiosa*. Fems Microbiology Letters 295, 129-134.

Zeng, W.Q., Melotto, M., He, S.Y., 2010. Plant stomata: a checkpoint of host immunity and pathogen virulence. Current Opinion in Biotechnology 21, 599-603.

Zhao, Z.T., Liu, H.Q., Wang, C.F., Xu, J.R., 2013. Comparative analysis of fungal genomes reveals different plant cell wall degrading capacity in fungi. Bmc Genomics 14.

CHAPTER II

Aim of the research

The colonization of pathogenic microorganisms in form of biofilm on plant tissues leads annually to significant losses in terms of crop yields and a decrease of the quality and safety of agricultural products worldwide. Their recalcitrance to control practices, the arise of resistance phenomena and the continuous abolishment of some pesticides pose a hard challenge in the treatment of unwanted biofilms.

The aim of the present PhD thesis was to evaluate the non-lethal concentration effects of several natural compounds (plant methanolic extracts, essential oils and NAC, an analogue of cysteine) derived from different plant species on the biofilm formation of bacterial and fungal plant-associated biofilms.

CHAPTER III

Hindering the formation and promoting the dispersion of biofilms: non-lethal effects of seagrass extracts

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Abstract

Biofilms have great significance in several domains owing to their inherent tolerance and resistance to antimicrobial compounds. Thus, new approaches to prevent and treat unwanted biofilms are urgently required. To this end, three seagrass species collected in Vietnam and in India (*Enhalus acoroides*, *Halophila ovalis* and *Halodule pinifolia*) were investigated for their effects in mediating non-lethal interactions on sessile bacterial (*Escherichia coli*) and fungal (*Candida albicans*) cultures. The present study is focused on anti-biofilm activities of seagrass extracts, without killing cells. It was shown that up to 100 ppm of crude extracts did not adversely affect microbial growth, nor do they act as a carbon and energy source for the selected microorganisms. Seagrass extracts appear to be more effective in deterring microbial adhesion on hydrophobic surfaces than on hydrophilic. The results revealed that non-lethal concentrations of *E. acoroides* leaf extract: i) reduce bacterial and fungal coverage by 60.9% and 73.9%, respectively; ii) affect bacterial biofilm maturation and promote dispersion, up to 70%, in fungal biofilm; iii) increase luminescence in *Vibrio harveyi* by 25.8%. Methanolic extracts were characterized and major phenolic compounds were identified by MS/MS analysis, showing the unique profile of the *E. acoroides* leaf extract. *E. acoroides* leaf extract proved to be the most promising extract among those tested. Indeed, the selected non-lethal concentrations of *E. acoroides* leaf extract were found to exert an antibiofilm effect on *C. albicans* and *E. coli* biofilm in the first phase of biofilm genesis, opening up the possibility of developing preventive strategies to hinder the adhesion of microbial cells to surfaces. The leaf extract also affected the dispersion and maturation steps in *C. albicans* and *E. coli* respectively, suggesting an important role in cell signaling processes.

Introduction

The ability of microorganisms to colonize surfaces and develop into highly organized communities enclosed in a self-produced polymeric matrix is the predominant growth modality in both nature and artificial systems. Such lifestyle is called biofilm and it is characterized by alterations in microbial phenotypes with respect to growth rates and gene transcriptions (Costerton, 1999; Hall-Stoodley et al., 2004; Rayner et al., 2004).

Biofilms have great significance in different environmental domains since microorganisms are able to colonize almost all types of surfaces both abiotic and biotic, leading to consequences in terms of social and economic impact. These include human tissues, implantable medical devices, natural aquatic systems, plants, food and industrial lines. For instance, in public health, clinical-biofilms represent 65-80 % of microbial diseases currently treated by physicians in the developed world (Villa et al., 2013;

Macià et al., 2014). The presence of indwelling medical devices further increases the risk for biofilm formation and subsequent infection (Percival et al., 2015). The bacterium *Escherichia coli* and the polymorphic fungus *Candida albicans* are among the most frequent cause of bloodstream infections, and the predominant microorganisms isolated from infected medical devices (Nobile et al., 2015; Sharma et al., 2016). In another case, plant diseases caused by bacteria and fungi in form of biofilms affect crops, and are responsible for significant losses or decrease the quality and safety of agricultural products. The type and the amount of losses caused by plant diseases vary depending on the plant and pathogen species, the geographical location and the environmental conditions. It has been estimated that plant diseases, together with insects and weeds cause a plant decrease productivity between 20 and 40 % of global agricultural productivity. In this scenario, pathogens still claim 10–16% of the global harvest. *E.coli* was found on lettuce leaves (Mshar et al., 1999; Seo and Frank, 1999). Solomon et al (2002) revealed that *E. coli* O157:H7 can enter the lettuce plant through the root system and migrate throughout the edible portion of the plant. The migration of the bacterium within the plant tissues protected it by the application the action of sanitizing agents. These biofilms, as any other biofilm, exhibit dramatically decreased susceptibility to antimicrobial agents and resistant to the host immune clearance, which increases the difficulties for the clinical treatment of infections (Smith et al., 2008; Lebeaux et al.,2014). Furthermore, the antimicrobial arena is experiencing a shortage of lead compounds, and growing negative consumer perception against synthetic products has led to the search for more natural solutions (da Silva et al., 2017).

In this context, it has been reported that plant-derived extracts exhibit good antibiofilm properties against a range of microorganisms (Choi et al., 2017; da Silav et al., 2017; Teanpaisan et al., 2017). However, in the past, these extracts were mainly screened by focusing on their lethal effects (Abiala et al., 2016; Bisi-Jhonson et al., 2017; Elisha et al., 2017) disregarding their activity at non-lethal concentrations. At these concentrations, plant-derived extracts may reveal elegant mechanisms to sabotage the sessile lifestyle, manipulating the expression of stage-specific biofilm phenotypes (Villa et al., 2013). For instance, by affecting the cellular ability to attach to surfaces and by mystifying intercellular signals, the biofilm cascade might be hampered. Thus, non-lethal concentrations of plant-derived extracts can inspire innovative, eco-friendly and safe strategies aim at treating deleterious biofilms. Interfering with specific key steps that orchestrate biofilm genesis might offer new ways to disarm microorganisms without killing them, sidestepping drug resistance (Villa et al., 2013b).

Seagrasses, which belong to the halophytes, represent a functional group of underwater marine flowering plants that have developed several strategies to survive and reproduce in environments where the salt concentration is around 200 mM NaCl or more (Flower et al., 2008). As these plants grow in very high saline conditions, it is predicted that they could possess rare and new activities not reported for their terrestrial relatives (Joshi et al., 2015). Indeed, metabolomic studies have shown that increased salinity leads to changes in conserved and divergent metabolic responses in halophytes (Boestfleisch et al., 2014). Moreover, interesting activities of seagrass extracts, including antibacterial, antifungal, antialgal, antioxidant, anti-inflammatory, insecticidal, antimalarial and vasoprotective properties, have been reported (Hua et al., 2006; Gokce et al., 2008; Kannan et al., 2012).

Thus, the well described properties of seagrasses extracts offer a promising framework for investigating novel antibiofilm activities at non-lethal concentrations.

The present study explores, for the first time, the effect of extracts from different seagrasses (namely, leaves and roots from *Enhalus acoroides* Rich. ex Steud., Hydrocharitaceae, leaves of *Halophila ovalis* (R.Br.) Hook.f., Hydrocharitaceae, and leaves of *Halodule pinifolia* (Miki) Hartog, Cymodaceaceae) in mediating non-lethal interactions on sessile *Candida albicans* and *Escherichia coli* cultures, selected as model systems for fungal and bacterial biofilm infections, respectively. The work focuses on investigating the antibiofilm performance of seagrass extracts at sub-inhibitory concentrations, studying how they affect biofilm functional traits (such as adhesion, biofilm maturation, dispersal and quorum sensing), and induce cellular responses other than those associated with antimicrobial activities.

Methods

Plant material and extraction. Three species of seagrasses were collected in Vietnam and India and air-dried in a dark place (Table 1). The plants were separated into different organs (leaves and roots), and samples were cooled with liquid nitrogen and ground to a fine powder using a bead mill (Retsch), three times for 10 s at a frequency of 30/s. The samples were stored at -80°C prior to analysis. Crude extracts were obtained using 80% methanol (MeOH) as solvent. Around 50 mg of powdered seagrass material was weighed in a reaction tube and extracted with 800 μl 80% MeOH for 10 min with regular shaking. Then the extract was centrifuged for 5 min at 18000 x g and the supernatant transferred into a new reaction tube. These steps were repeated three times with 400 μl 80% MeOH each. The supernatants were collected in the same reaction tube and stored at -20°C. Phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride 0.137 M, sodium chloride, Fisher Scientific) was used to obtain several concentrations of each crude extract: 100, 10, 1, 0.1, 0.01 and 0.001 mg/l.

Microbial strains and growth media. The microbial strains *Candida albicans* SC5314 (ATCC MYA-2876) and *Escherichia coli* K-12 wild-type strain (ATCC 25404) were selected as model systems for fungal and bacterial biofilms respectively. *C. albicans* and *E. coli* strains were stored at –80°C in suspensions containing 50% glycerol and 2% peptone, and were routinely grown in amino acid-free yeast nitrogen base (YNB, Sigma-Aldrich) supplemented with 0.5% glucose (Conda) (YNBG) and Luria-Bertani broth (LB, Sigma-Aldrich), respectively, for 16 h at 30°C.

Planktonic growth in the presence of seagrass extracts as the sole source of carbon and energy. The ability of *C. albicans* and *E. coli* planktonic cells to grow in the presence of each extract as the sole carbon and energy source was tested using YNB and M9 (Sigma-Aldrich) mineral medium, respectively, supplemented with the highest working extract concentration: 100 mg/l. Then a 100 μl mix of mineral medium together with 45 μl (3% v/v) of the overnight culture (final concentration 10^8 cells/ml) and the highest concentration of each marine plant extract were used to fill each well of 96-well plates (Thermo Fisher Scientific) and incubated for 48 h at 30°C. A medium complemented with cells and glucose (5 g/l), and medium without cells, were used as positive and negative controls, respectively. Microbial growth was monitored using the PowerWave XS2 microplate reader (Biotek) measuring the absorbance at 600 nm (A_{600}) every 10 min. Six biological replicates of each treatment were performed. The obtained data were normalized to the negative control and reported as the mean of these.

Toxicity assay in the presence of seagrass extracts. Toxicity assays were carried out by evaluating the ability of the seagrass extracts to inhibit the planktonic growth of the selected microorganisms. For this, *C. albicans* and *E. coli* were grown YNBG and LB broth respectively supplemented with the highest working concentrations (10 and 100 mg/l) in 96-well plates (Thermo Fisher Scientific). Growth curves at 30°C were generated using Infinite® F200 PRO microplate reader (TECAN, Mannedorf, Switzerland) by measuring the optical density at 600 nm (OD_{600}) every 60 min for 30 h in wells inoculated with 45 μl (3% vol/vol) of an overnight culture (approximately 10^8 cells/ml). The positive control was represented by PBS supplemented with 45 μl (3% vol/vol) of the overnight culture. The polynomial Gompertz model was used to fit the growth curves to calculate the maximum specific growth rate (A600/min), using GraphPad Prism software (version 5.0, San Diego, CA, USA). Five biological replicates of each treatment were performed.

Microplate-based biofilm assay. The antibiofilm activity of seagrass extracts was assessed quantitatively as previously reported by Villa et al. (2010). Briefly, 200 μl of PBS containing 10^8 cells/ml supplemented with 0 (negative control), 100, 10, 1, 0.1, 0.01, and 0.001 mg/l of each crude extract were placed in hydrophobic and hydrophilic 96-well polystyrene-based microtiter plates (Thermo Fisher Scientific). After an incubation time of 24 h at 20 °C, *C. albicans* and *E. coli* planktonic cells were removed and adhered cells were stained using 0.1 mg/mlof Fluorescent Brightener 28 vital dye (Sigma-Aldrich) or 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) in PBS, respectively. After 20 min staining in the dark at room temperature the microtiter plates were washed twice with 200 µl PBS and the fluorescence intensity due to adhered cells was measured using a fluorescence microplate reader (TECAN, Manneford, Switzerland) at excitation wavelength of 335 nm and emission wavelength of 433 nm. A standard curve of fluorescence intensity versus cell number was determined and used to quantify the antibiofilm performance of the crude extracts. Cattò et al. (2015) proposed the following anti-adhesion ranges computing the percentage reduction in comparison to the negative control: ≤20% without anti-adhesion activity; between 20 and 30% and 30 and 40% low anti-adhesion activity and with moderate anti-adhesion activity respectively; ≥40% adhered cells with excellent anti-adhesion activity. Five biological replicates were performed for each condition and a percentage reduction in comparison to the negative control was calculated as (treated data – negative control data) × 100 / negative control data. The experiment was repeated three times.

Biofilm growth at the solid/liquid interface. The most promising plant extracts were screened for their effects on biofilm development. *C. albicans* biofilm was grown in the CDC biofilm reactor (Biosurface Technologies, Bozeman, MT, USA) as previously described by Villa et al. (2011). Briefly, two bioreactors hosting 24 polycarbonate coupons (to simulate a hydrophobic surface) were filled with YNBG and 1 ml of overnight planktonic culture (approximately 10^8 cells/ml) and, in one of them, 0.01 mg/l of *E. acoroides* leaf extract was added. Bioreactors were maintained under static conditions (no flow) for 24 h under mild stirring at 37 °C, promoting fungal adhesion to the surface of the removable polycarbonate coupons. After that, the dynamic phase was initiated and diluted YNGB was fluxed for 48 h at flow rate of 250 ml/h. Biofilm growth in the absence and presence of the extract was evaluated by

quantification of the biomass. At different time steps (24, 48 and 72 h) some polycarbonate coupons were collected in aseptic conditions and resuspended in 3 ml of PBS each. Subsequently, serial dilutions were carried out, and 10 µl were inoculated in petri dishes containing Tryptic Soy Broth medium (TSB, Sigma-Aldrich) complemented with agar (Merck) following the drop counting method. After 12 h at 30°C, *C. albicans* colonies were counted and the data obtained were normalized to the coupon area, and means were reported. The same protocol was used to obtain mature biofilm of *E. coli,* using LB as a medium, and evaluating 10 mg/l of *E. acoroides* leaf extract. Each experiment was repeated three times.

Biofilm dispersion assay. Mature *C. albicans* biofilm was grown in the CDC reactor in the presence and absence of 0.01 mg/l of *E. acoroides* leaf extract as reported below. As previously described by Cattò et al. (2017) [31], after 72 h polycarbonate coupons were collected, immersed in 27 ml of PBS for one minute at room temperature, serial dilutions were carried out and 10 µl were inoculated in petri dishes containing TSB supplemented with agar (Merck) following the drop counting method. After 12 h at 30°C, *C. albicans* colonies were counted and the percentage of biofilm dispersion was calculated as (number of viable cells from bulk PBS × 100) / (number of viable cells from bulk PBS + number of viable cells from the coupon biofilm) and means were reported. Three biological replicates were performed for each treatment and six technical replicates were performed for each experiment. The experiment was performed three times.

Biofilm growth at the solid/air interface. *E. coli* biofilm was grown on a sterile polycarbonate membrane (PC, Whatman Nucleopore, diameter 2.5 cm, pore diameter 0.2 μm) as previously described by Garuglieri et al. (2018). Briefly, 0.05 ml of an overnight culture (approximately 10^6 cells/ml) were inoculated at the center of a sterile polycarbonate membrane and, when the inoculum was completely dried, the membrane was carefully put inside a transwell structure (ThinCert™ Cell Culture Inserts with translucent PET membrane – Greiner bio-one) inlaid in a 6 well culture plate (Greiner bio-one). One ml of LB medium was inoculated in the basolateral compartment (plate well). Biofilm formation was performed at 37°C in aerobic conditions for 16 h. At different time points (0, 4, 6, 8, 16 h) some membranes were removed, biofilm was scraped off using a sterile loop, put inside a tube containing 1 ml of PBS and then homogenized twice using a homogenizer (IKA T10 basic Ultra-Turrax – Cole-Parmer Instrument Company). Then serial dilutions were prepared and 10 µl were inoculated in petri dishes containing LB with agar following the drop counting method. After 12 h at 37°C, *E. coli* colonies were counted and the biomass was quantified. This assay was assessed under three experimental conditions: i) treatment 1: growth in contact with 1 ml of LB with 10 mg/l of *E. acoroides* leaf extract for 16 h; ii) treatment 2: overnight culture grown with 10 mg/l of *E. acoroides* leaf extract, and then growth in contact with 1 ml of LB for 16 h; iii) treatment 3: overnight culture grown with 10 mg/l of *E. acoroides* leaf extract, and then growth in contact with 1 ml of LB with 10 mg/l of *E. acoroides* leaf extract for 16 h. In the control, the microorganisms grew in 1 ml LB inside a basolateral well for 16 h. The data obtained were divided by the area of the membrane, and the means were reported. The experiment was repeated three times.

Bioluminescence assay using *Vibrio harveyi*. Two hundred μl of autoinducer bioassay (AB) mineral medium (0.3 M NaCl, 0.05 M $MgSO_4$, 0.5% casein hydrolysate, 10 µM KH_2PO_4, 1 µM L-arginine, 50% glycerol, 0.01 µg/ml riboflavin, 1 µg/ml thiamine. pH 7. Sigma-Aldrich) containing 10% (V/V) of a tenfold dilution of an overnight culture of *Vibrio harveyi* BB170 (ATCC BAA-1117) grown in AB medium were supplemented with 10 mg/l of *E. acoroides* leaf extract respectively, and were placed in hydrophobic 96-well polystyrene-based microtiter plates (Thermo Fisher Scientific) with transparent bottom. The positive control was an AB mineral medium supplemented with 10% (V/V) tenfold dilution of the overnight culture. Absorbance (OD_{600nm}) and luminescence were measured using a microplate reader (VICTOR^{TM}X, Perkin Elmer, USA) every 8 h for 24 h, incubating the microtiter plate at 30°C during the experiment. The data obtained were normalized to the number of viable cells, divided by the area of the membrane, and the means reported. The experiment was repeated three times.

Quantification of total flavonoid contents (TFC). The total flavonoid content of the seagrass extracts was measured in 96-well plate according to a modified protocol from Dudonné et al. (2009). The wells were filled with 150 μl H_2O each. Dilutions of the methanolic seagrass extracts (1:2) were prepared and 25 μl of sample were filled in one well, with four replicates. A calibration curve with catechin hydrate with the following concentrations was prepared in 80% MeOH: 0, 10, 25, 50, 100, 125, 250 and 400 μg/ml. The calibration curve was placed on the plate in triplicate. In the next step, 10 μl $NaNO_2$ 3.75% were added into each well and incubated for 6 min. Afterwards, 15 μl of $AlCl_3$ 10% were added and incubated for 10 min. In the last step, 50 μl of NaOH 1 M were added and the absorption was measured at 510 nm in a microplate reader (Biotek, Winooski, USA). The slope of the calibration curve was used to calculate the total flavonoid content in mg catechin equivalent.

Quantification of total phenolic contents (TPC). To measure the total phenolic acid content, a modified protocol after Dewanto et al. (2002) was used with the same extracts described above. 96-well microtiter plate were filled with 100 μl H_2O each. From each sample, 10 μl were added; seagrass extracts were diluted 1:2. A gallic acid calibration curve with the following concentration was used: 0, 5, 10, 25, 50, 75, 100, 125 and 250 μg/ml. Next, 100 μl Na_2CO_3 7% were added and the plate was incubated for 100 min in the dark. The absorption was measured at 765 nm in a microplate reader. With the slope of the gallic acid calibration curve, the concentration of phenolic acids was calculated in mg gallic acid equivalent.

Determination of the oxygen radical absorbance capacity (ORAC). The analysis of the oxygen radical absorbance capacity (ORAC) was conducted according to a protocol based on Huang et al. (2002) and Gillespie et al. (2007) [36] with the same extracts. A black 96-well microtiter was used and the wells were filled with 120 μl fluorescein (112 nM) in phosphate buffer (75 mM, pH 7.4). Of each sample and the standard curve, 20 μl were added in each well. The standard curve of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was prepared in phosphate buffer with the following concentrations: 6.25, 12.5, 25 and 50 μM. Seagrass extracts were diluted 1:200 with methanol 80%. The microtiter plate was incubated for 15 min at 37°C. The fluorescence was then measured at 485/520 nm as time point zero. Next, 80 μl of 2,2′-azobis(2-amidino-propane) dihydrochloride (62 mM) were added

and the fluorescence was measured every minute for 80 min. The ORAC value was calculated as the difference between time point zero and 80 min and quantified with the Trolox standard curve.

LC-MS analysis. LCMS analysis was performed on a Shimadzu HPLC system (controller CBM-20A, two pumps LC-20AD, a column oven CTO-20AC and a photo diode array detector SPD-M20A; Shimadzu, Darmstadt, Germany) coupled to a Triple Tof 4600 mass spectrometer (AB Sciex, Canby, USA). The separation of extracted compounds was realised on a Knauer Vertex Plus column (250 x 4 mm, 5 μm particle size, packing material ProntoSIL 120-5 C18-H) with precolumn (Knauer, Berlin, Germany). The column oven temperature was set to 30°C and 25 μl of undiluted methanolic seagrass extract prepared as described above was injected. The solvent flow rate was 0.8 ml/min. In this time, a gradient was run from 10% to 90% B from minute 0 to 35, 2 min of 90% B, switch to 10% B in 1 min and subsequent equilibration at 10% B for 2 min. Solvent A (water) and B (methanol) were both supplemented with 2 mM ammonium acetate and 0.01% acetic acid. Mass spectra were monitored between 100 and 800 Da in negative ionisation mode. In addition, MS/MS spectra were generated with a collision energy of -30 eV and measured between 50 and 800 Da. Spectra for the most prominent peaks were compared to database entries in MassBank [37] (Horai et al. 2010) and ReSpect [38] (Sawada et al. 2012) for identification.

Statistical analysis. To evaluate statistically significant differences among samples, analysis of variance (ANOVA) via MATLAB software (Version 7.0, The MathWorks Inc, Natick, USA) was applied. Tukey's honestly significant different test (HSD) was applied for pairwise comparison to establish the significance of the data. Statistically significant results were represented by P values ≤ 0.05.

Results and discussion

Biofilm resistance to antimicrobial agents is a major worldwide issue. Therefore, a successful reduction of surface colonization can be a potential strategy for the management of unwanted biofilms, especially on medical devices, work surfaces, industrial lines and agricultural domain.
In these contexts, the use of plant-derived extracts to modulate biofilm genesis and dispersion may be a viable alternative. The present study is the first report describing the antibiofilm efficacy of non-lethal concentrations of *E. acoroides*, *H. pinifolia* and *H. ovalis* methanol extracts in counteracting microbial biofilms, highlighting the possibility that the selected seagrass species act as an extracellular signal mediating their biofilm activities.
E. coli and *C. albicans* were chosen as model systems for bacterial and fungal infections, respectively. *E. coli* biofilms are found to be the major causative agent of many intestinal infections, for recurrent urinary tract infections, and it also responsible for indwelling medical device-related infectivity Sharma et al., 2016). In addition, *E. coli* contamination was also found on lettuce leaves, leading to several issues even in agricultural domain (Stewart and Costerton, 2001). *C. albicans* is one of the very few fungal species causing disease in humans. These infections range from superficial mucosal and dermal infections, such as thrush, vaginal yeast infections, and diaper rash, to vascular catheters and dental implants infections (Nobile and Jhonson, 2015).

The bioactive properties of the seagrass species selected in this work are well known, and have been reported in detail by several authors (Kumar et al., 2008; Umamaheshwari et al., 2009; Natrah et al., 2015). However, until now attention has mainly focused on the antimicrobial activity of seagrass extracts, which, through disk diffusion assays, were investigated not in their capacity as biofilm-forming microorganisms but in their planktonic state. Using lethal concentrations, Umamaheshwari et al. (2009) reported the antibacterial activity of *H. ovalis* and *H. pinifolia* extracts, obtained using different solvents, against different microbial strains, recording maximum antibacterial activity by the ethanol extract of *H. pinifolia*. Instead, Choi et al. (2009) reported the antimicrobial properties of *Zostera marina* methanol extract and its organic solvent fractions on three human skin pathogens (*Staphylococcus aureus*, *S. epidermidis* and *C. albicans*), and Natrah et al. (2015) reported the antibacterial properties of methanol extracts of *E. acoroides* and other seagrass and seaweed species on different aquaculture pathogens (*Aeromonas hydrophila*, *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. anguillarum* and others).

In contrast, to the best of our knowledge, no papers have investigated the antibiofilm activity of *Enhalus acoroides, Halodule pinifolia* and *Halophila ovalis* at non-lethal concentrations against bacterial (*E. coli*) and fungal (*C. albicans*) biofilms. To this end, methanol extracts, obtained from different organs of three seagrass species (namely, *Enhalus acoroides* leaves and roots, *Halophila ovalis* leaves and *Halodule pinifolia* leaves) were screened for their ability to modulate biofilm genesis without killing cells. Methanol was used as the extraction solvent, having been previously reported as the most effective solvent to obtain high concentrations of bioactive compounds with antibacterial activity from seagrasses, compared to other extraction solvents (Lustigman and Brown., 1991; Sastry and Rao., 1994; Kumar et al., 2008).

Before evaluating the antibiofilm activity, the extracts, at concentrations of 100 mg/l, were first proved to not act as a carbon and energy source (Fig. 1) nor to affect the cellular growth of *C. albicans* and *E. coli* (Fig. 2 and Fig. 3). Therefore, concentrations ≤100 mg/l plant extract were used in the subsequent studies.

With the aim of investigating the effects of seagrass extracts on cell adhesion to surfaces, the first step of biofilm formation, microtiter based assays were performed (Fig. 4). The results revealed that *E. acoroides* and *H. ovalis* were the most promising extracts for *C. albicans*, with excellent anti-adhesion activity, reducing fungal coverage up to 73.89 ± 1.01% and 68.37 ± 2.49% at 0.01 and 1 mg/l, respectively. For *E. coli*, 10 mg/l of *E. acoroides* leaf extract was found to be the concentration with the highest reduction in cell adhesion (reduction of bacterial coverage by 60.86 ± 8.85%). Therefore, 0.01 mg/l and 10 mg/l *E. acoroides* leaf extract were chosen as the best non-biocidal concentrations for *C. albicans* and *E. coli* respectively, and were used in the subsequent studies. These concentrations significantly decreased the number of adhered cells on a hydrophobic surface, more so than on the hydrophilic one. Previous studies had highlighted the preference for hydrophobic surfaces, these reporting a decreased adhesion on the hydrophobic surface compared to the hydrophilic (Doss et al., 1993; Amiri et al., 2005). This is probably due to the hydrophobic nature of the aerial surfaces of plants (Koch et al., 2009).

In the present study the anti-adhesion activity of the seagrass extracts was dose-dependent, but the highest concentrations did not correspond to those with the best performance. Indeed, several studies have reported a weak activity of the compounds at low and high concentrations, and excellent activity at

intermediate concentrations (Rickard et al., 2006). Such a response, widely known in literature, is defined as hormesis, an adaptive behavior of microorganisms to provide resistance to environmental stress and improve the allocation of resources to ensure cell stability (Calabrese et al., 2003; Villa et al., 2013).
To further explore the effect of the most promising seagrass extract on biofilm development and detachment, CDC reactors were employed to reproduce biofilm at the solid/liquid interface, while for the assessment of the antibiofilm effect in the adhesion phase microplate-based biofilm assays are the most suitable (Williams et al., 2011; Coffey and Anderson, 2014; Cattò et al., 2017). In this study, a significant reduction in fungal coverage (up to 26.77 ± 9.01%) after 24 h (static adhesion phase) was observed in presence of 0.01 mg/l *E. acoroides* leaf extract (Figure 5a). This result confirms the anti-adhesion activity observed in microtiter assays. Coupons collected after 48 and 72 h showed no significant differences between treated and control samples.
In order to assess the possibility of 0.01 mg/l *E. acoroides* leaf extract to promote *C. albicans* biofilm-detachment from the surface of coupons, a biofilm dispersion assay was performed. Fig. 5b shows a significant increase in the number of dispersed cells in the treated biofilm, compared with the untreated (70 ± 6.83%), suggesting a further mechanism of action for the seagrass extract as biofilm dispersing agent. In fact, the phase of biofilm dispersion could be an interesting target for the development of new antibiofilm strategies, forcing the planktonic state and reestablishing the efficacy of traditional antimicrobial agents (Davies and Marques, 2009; Villa et al., 2013). Literature with information related to *C. albicans* biofilm dispersion is scarce. Farnesol and cis-2-decenoic acid showed dispersion-promotion of microcolonies of *C. albicans* biofilm (Davies and Marques, 2009; Uppuluri et al., 2010). In addition, Villa et al. (2012) reported that non-lethal concentrations of *Muscari comosum* ethanol bulb extract can modulate yeast adhesion and subsequent biofilm development on abiotic surfaces, and such concentrations could provide an extracellular signal responsible for biofilm dispersion.
For *E. coli*, the CDC reactor was not suitable to evaluate the possible effects of the extracts on the biofilm stages. Also other authors have reported the poor biofilm formation exhibited by *E. coli* K-12 strain under hydrodynamic conditions (Ghigo, 2001; Reisner et al., 2003; Reisner et al., 2006). The effect of 10 mg/l of *E. acoroides* leaf extract on *E. coli* biofilm formation was then evaluated using a membrane-supporting biofilm reactor, which allowed the formation of a biofilm at the solid/air interface. This technique forced the cells to attach to a surface, a feature that allowed direct investigation of the effect of the selected extract on the development of the biofilm, whilst bypassing the effect on the adhesion phase.
Fig. 6 shows no significant reduction in the number of viable cells during biofilm formation on the membrane treated with the extract, compared to the untreated, after 18 h in all the experimental conditions. Treatment 3 showed a growth rate slowdown in the interval 6 – 8 h, in which *E. coli* cells were in contact with the extract during both overnight growth and biofilm formation (reduction of cellular growth, compared to the control, up to 48.64 ± 4.02%). Interestingly, treatment 3 showed a biphasic growth curve compared with the growth curves of the other treatments, a trend that could be explained by the bioluminescence produced by *V. harvey*. As signaling molecules play an important role in biofilm development and detachment, the effects of 10 mg/l of *E. acoroides* leaf extract were investigated using *Vibrio harveyi,* suggesting other possible antibiofilm mechanisms of action of compounds in the chosen seagrass extract (Figure 7). The results revealed that at time 8 h, the samples treated with the leaf extract showed a significant increase in the relative luminescence emitted, compared to the control (25.75 ±

7.49). Villa et al. (2012) reported an increase of autoinducer-2 (AI-2) activity and a reduction in biofilm formation in *E. coli* cells treated with zosteric acid, a phenolic compound occurring in the seagrass *Zostera marina*. In fact, it has been hypothesized that the accumulation of AI-2 above a threshold level leads to reduced biofilm formation due to the induction of a hypermotile phenotype that is unable to adhere to the surface. Huber et al. (2003) demonstrated that some polyphenolic compounds containing a gallic acid residue commonly produced by some plant species inhibited intercellular communication in bacteria. Truchado et al. (2009) reported the ability of some phytochemical compounds (cinnamaldehyde, ellagic acid, resveratrol, rutin and pomegranate extract) to interfere with the quorum sensing system of *Yersinia enterocolitica* and *Erwinia cartovora*.

It has been well known that the antibiofilm activity of plant extracts is closely linked with the content of secondary metabolites, such as phenols and/or flavonoids, which represent the total amount of phenolic compounds in a plant extract (Choi et al., 2017). The phenolic compound content is also deeply associated with the antioxidant activity of plant extracts (Giada, 2013). Therefore, we determined the total phenolic acid (TPC) and flavonoid (TFC) content and the antioxidant activity (ORAC) of methanolic extracts in order to highlight features of the most promising antibiofilm extract, the *E. acaroides* leaf extract (Fig. 8). Results show that *E. acaroides* leaf extract presents the lower TPC and TFC values compared to other seagrasse extracts. Although the low content of phenolic compounds, the *E. acaroides* leaf extract displays a higher ORAC value compared to the root extract. This indicates the abundance of other, non-phenolic compounds with antioxidant capacity in the leaves of *E. acoroides*. Cattò et al. (2015) suggested the importance of antioxidant compounds in hindering biofilm formation. The researcher discovered that the mechanism of action behind the antibiofilm performance of zosteric acid, a secondary metabolite of the seagrass *Zostera marina*, is related to the antioxidant activity of the molecule, and its interaction with the WrbA protein responsible maintaining cellular homeostasis and defense against oxidative stress.

To gain more insight into possible antibiofilm compounds in the seagrass extracts, individual substances in the methanolic extract were analyzed by LC-MS (Fig. 9). Using two databases, MS/MS spectra were compared to identify compounds based on common MS/MS fragmentation patterns and masses. In the analyzed seagrass samples, a number of flavonoids, sugars, phenolic acids and a dicarboxylic acid were assigned to the peaks in the chromatogram (Table 2).

Preliminary analysis shows that the phytochemical profile of the *E. acaroides* leaf extract is mainly characterized by the presence of the flavones apigenin and luteolin, three kaempferol derivates and the carboxylic acids benzoic and azelaic acid. This unique quantitative and qualitative chemical composition confers antibiofilm properties to the *E. acaroides* leaf extract.

Some of these compounds have shown to exhibit antibiofilm properties at non-lethal concentrations. Kaempferol, apigenin and luteolin from red wine reduced biofilm formation of methicillin-sensitive *S. aureus* significantly (Cho et al., 2015). Sánchez et colleagues (2015) reported that sub-lethal concentrations of plant extracts inhibit *E. coli* and *S. aureus* biofilms. The antibiofilm properties of the extracts were associated to the presence of flavonoids, such as kaempferol and apigenin, which modulate bacterial cell-cell communication by suppressing the activity of the autoinducer-2 (Vikram et al., 2010). However, we should keep in mind that the antibiofilm effects of plant extracts could be the result of

interactions among different components of the extract at specific concentrations, and not only due to the effects of a single, predominant compound (Villa et al., 2013; Bazargani et al., 2015).

Conclusions

In conclusion, the *E. acoroides* leaf extract proved to be the most promising extract among those tested. Indeed, the selected non-lethal concentrations of *E. acoroides* leaf extract were found to exert an antibiofilm effect on *C. albicans* and *E. coli* biofilm in the first phase of biofilm genesis, opening up the possibility of developing preventive strategies to hinder the adhesion of microbial cells to surfaces. The leaf extract also affected the dispersion and maturation steps in *C. albicans* and *E. coli* respectively, suggesting an important role in cell signaling processes. These effects could be explained by the presence of active compounds like kaempferol and apigenin at specific concentrations in the extracts of *E. acoroides*, which are known to possess biofilm inhibiting properties. Furthermore, there could be a synergistic action of these flavonoids with other compounds occurring in the plant, enhancing the global antibiofilm effect. Currently, the leaf extract is being investigated with the objective of testing fractions for identifying the active compounds and to better understand the mechanisms of action of this seagrass species.

References

Abiala, M., Olayiwola, J., Babatunde, O., Aiyelaagbe, O., Akinyemi, S., 2016. Evaluation of therapeutic potentials of plant extracts against poultry bacteria threatening public health. Bmc Complementary and Alternative Medicine 16.

Amiri, A., Cholodowski, D., Bompeix, G., 2005. Adhesion and germination of waterborne and airborne conidia of *Penicillium expansum* to apple and inert surfaces. Physiological and Molecular Plant Pathology 67, 40-48.

Bazargani, M.M., Rohloff, J., 2016. Antibiofilm activity of essential oils and plant extracts against Staphylococcus aureus and *Escherichia coli* biofilms. Food Control 61, 156-164.

Bisi-Johnson, M.A., Obi, C.L., Samuel, B.B., Eloff, J.N., Okoh, A.I., 2017. Antibacterial activity of crude extracts of some South African medicinal plants against multidrug resistant etiological agents of diarrhoea. Bmc Complementary and Alternative Medicine 17.

Boestfleisch, C., Wagenseil, N.B., Buhmann, A.K., Seal, C.E., Wade, E.M., Muscolo, A., Papenbrock, J., 2014. Manipulating the antioxidant capacity of halophytes to increase their cultural and economic value through saline cultivation. Aob Plants 6.

Calabrese, E.J., Baldwin, L.A., 2003. Hormesis: The dose-response revolution. Annual Review of Pharmacology and Toxicology 43, 175-197.

Catto, C., Grazioso, G., Dell'Orto, S., Gelain, A., Villa, S., Marzano, V., Vitali, A., Villa, F., Cappitelli, F., Forlani, F., 2017. The response of *Escherichia coli* biofilm to salicylic acid. Biofouling 33, 235-251.

Catto, C., Dell'Orto, S., Villa, F., Villa, S., Gelain, A., Vitali, A., 2015. Unravelling the Structural and Molecular Basis Responsible for the Anti-Biofilm Activity of Zosteric Acid (vol 10, e0131519, 2015). Plos One 10.

Cho, H.S., Lee, J.H., Cho, M.H., Lee, J., 2015. Red wines and flavonoids diminish *Staphylococcus aureus* virulence with anti-biofilm and anti-hemolytic activities. Biofouling 31, 1-11.

Choi, H.G., Lee, J.H., Park, H.H., Sayegh, F.A.Q., 2009. Antioxidant and antimicrobial activity of *Zostera marina* L. extract. Algae 24(3):179-184.

Choi, H.A., Cheong, D.E., Lim, H.D., Kim, W.H., Ham, M.H., Oh, M.H., Wu, Y., Shin, H.J., Kim, G.J., 2017. Antimicrobial and Anti-Biofilm Activities of the Methanol Extracts of Medicinal Plants against Dental Pathogens *Streptococcus mutans* and *Candida albicans*. Journal of Microbiology and Biotechnology 27, 1242-1248.

Chung, P.Y., 2017. Plant-derived Compounds as Potential Source of Novel Anti-Biofilm Agents Against *Pseudomonas aeruginosa*. Current Drug Targets 18, 414-420.

Coffey, B.M., Anderson, G.G., 2014. Biofilm Formation in the 96-Well Microtiter Plate. Methods and Protocols 1149, 631-641.

Costerton, J.W., 1999. Introduction to biofilm. International Journal of Antimicrobial Agents 11, 217-221.

da Silva, D.T., Herrera, R., Batista, B.F., Heinzmann, B.M., Labidi, J., 2017. Physicochemical characterization of leaf extracts from *Ocotea lancifolia* and its effect against wood-rot fungi. International Biodeterioration & Biodegradation 117, 158-170.

Davies, D.G., Marques, C.N.H., 2009. A Fatty Acid Messenger Is Responsible for Inducing Dispersion in Microbial Biofilms. Journal of Bacteriology 191, 1393-1403.

Dewanto, V., Wu, X.Z., Adom, K.K., Liu, R.H., 2002. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. Journal of Agricultural and Food Chemistry 50, 3010-3014.

Doss, R.P., Potter, S.W., Chastagner, G.A., Christian, J.K., 1993. Adhesion of non-germinated Botrytis cinerea conidia to several substrata. Applied and Environmental Microbiology 59, 1786-1791.

Dudonne, S., Vitrac, X., Coutiere, P., Woillez, M., Merillon, J.M., 2009. Comparative Study of Antioxidant Properties and Total Phenolic Content of 30 Plant Extracts of Industrial Interest Using DPPH, ABTS, FRAP, SOD, and ORAC Assays. Journal of Agricultural and Food Chemistry 57, 1768-1774.

Elisha, I.L., Botha, F.S., McGaw, L.J., Eloff, J.N., 2017. The antibacterial activity of extracts of nine plant species with good activity against *Escherichia coli* against five other bacteria and cytotoxicity of extracts. Bmc Complementary and Alternative Medicine 17.

Flowers, T.J., Colmer, T.D., 2008. Salinity tolerance in halophytes. New Phytologist 179, 945-963.

Garuglieri, E., Meroni, E., Catto, C., Villa, F., Cappitelli, F., Erba, D., 2018. Effects of Sub-lethal Concentrations of Silver Nanoparticles on a Simulated Intestinal Prokaryotic-Eukaryotic Interface. Frontiers in Microbiology 8.

Giada, M.L.R., 2013. Food phenolic compounds: main classes, sources and their antioxidant power. (ed:Dr. Jose Antonio Morales-Gonzalez, ISBN:978-953-51-1123-8, InTech) 87-1.

Ghigo, J.M., 2001. Natural conjugative plasmids induce bacterial biofilm development. Nature 412, 442-445.

Gillespie, K.M., Chae, J.M., Ainsworth, E.A., 2007. Rapid measurement of total antioxidant capacity in plants. Nature Protocols 2, 867-870.

Gokce, G., Haznedaroglu, M.Z., 2008. Evaluation of antidiabetic, antioxidant and vasoprotective effects of *Posidonia oceanica* extract. Journal of Ethnopharmacology 115, 122-130.

Hall-Stoodley, L., Costerton, J.W., Stoodley, P., 2004. Bacterial biofilms: From the natural environment to infectious diseases. Nature Reviews Microbiology 2, 95-108.

Hilborn, E.D., Mermin, J.H., Mshar, P.A., Hadler, J.L., Voetsch, A., Wojtkunski, C., Swartz, M., Mshar, R., Lambert-Fair, M.A., Farrar, J.A., Glynn, M.K., Slutsker, L., 1999. A multistate outbreak of *Escherichia coli* O157 : H7 infections associated with consumption of mesclun lettuce. Archives of Internal Medicine 159, 1758-1764.

Horai, H., Arita, M., Kanaya, S., Nihei, Y., Ikeda, T., Suwa, K., Ojima, Y., Tanaka, K., Tanaka, S., Aoshima, K., Oda, Y., Kakazu, Y., Kusano, M., Tohge, T., Matsuda, F., Sawada, Y., Hirai, M.Y., Nakanishi, H., Ikeda, K., Akimoto, N., Maoka, T., Takahashi, H., Ara, T., Sakurai, N., Suzuki, H., Shibata, D., Neumann, S., Iida, T., Funatsu, K., Matsuura, F., Soga, T., Taguchi, R., Saito, K., Nishioka, T., 2010. MassBank: a public repository for sharing mass spectral data for life sciences. Journal of Mass Spectrometry 45, 703-714.

Hua, K.F., Hsu, H.Y., Su, Y.C., Lin, I.F., Yang, S.S., Chen, Y.M., Chao, L.K.P., 2006. Study on the antiinflammatory activity of methanol extract from seagrass *Zostera japonica*. Journal of Agricultural and Food Chemistry 54, 306-311.

Huang, D.J., Ou, B.X., Hampsch-Woodill, M., Flanagan, J.A., Prior, R.L., 2002. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate flourescence reader in 96-well format. Journal of Agricultural and Food Chemistry 50, 4437-4444.

Huber, A.B., Kolodkin, A.L., Ginty, D.D., Cloutier, J.F., 2003. Signaling at the growth cone: Ligand-receptor complexes and the control of axon growth and guidance. Annual Review of Neuroscience 26, 509-563.

Joshi, R., Ramanarao, M.V., Bedre, R., Sanchez, L., Pilcher, W., Zandkarimi, H., Baisakh, N., 2015. Salt adaptation mechanisms of halophytes: improvement of salt tolerance in crop plants, in Elucidation of Abiotic Stress Signaling in Plants, ed Pandey G. K., editor. (New York, NY: Springer). 243–279.

Kannan, R.R.R., Arumugam, R., Anantharaman, P., 2012. Chemical composition and antibacterial activity of Indian seagrasses against urinary tract pathogens. Food Chemistry 135, 2470-2473.

Koch, K., Bhushan, B., Barthlott, W., 2009. Multifunctional surface structures of plants: An inspiration for biomimetics. Progress in Materials Science 54, 137-178.

Kumar, C.S., Sarada, D.V.L., Gideon, T.P., Rengasamy, R., 2008. Antibacterial activity of three South Indian seagrasses, Cymodocea serrulata, Halophila ovalis and Zostera capensis. World Journal of Microbiology & Biotechnology 24, 1989-1992.

Lebeaux, D., Ghigo, J.M., Beloin, C., 2014. Biofilm-Related Infections: Bridging the Gap between Clinical Management and Fundamental Aspects of Recalcitrance toward Antibiotics. Microbiology and Molecular Biology Reviews 78, 510-543.

Lustigman, B., Brown, C., 1991. Antibiotic production by marine-algae isolated from the New York/ New Jersey Coast. Bulletin of Environmental Contamination and Toxicology 46, 329-335.

Macia, M.D., Rojo-Molinero, E., Oliver, A., 2014. Antimicrobial susceptibility testing in biofilm-growing bacteria. Clinical Microbiology and Infection 20, 981-990.

Natrah, F.M.I., Harah, Z.M., Sidik, B.J., Izzatul, N.M.S., Syahidah, A., 2015. Antibacterial Activities of Selected Seaweed and Seagrass from Port Dickson Coastal Water against different Aquaculture Pathogens. Sains Malaysiana 44, 1269-1273.

Nobile, C.J., Johnson, A.D., 2015b. *Candida albicans* Biofilms and Human Disease. In: Gottesman, S. (Ed.), Annual Review of Microbiology, Vol 69, pp. 71-92.

Percival, S.L., Suleman, L., Vuotto, C., Donelli, G., 2015. Healthcare-associated infections, medical devices and biofilms: risk, tolerance and control. Journal of Medical Microbiology 64, 323-334.

Rayner, J., Veeh, R., Flood, J., 2004. Prevalence of microbial biofilms on selected fresh produce and household surfaces. International Journal of Food Microbiology 95, 29-39.

Reisner, A., Haagensen, J.A.J., Schembri, M.A., Zechner, E.L., Molin, S., 2003. Development and maturation of *Escherichia coli* K-12 biofilms. Molecular Microbiology 48, 933-946.

Reisner, A., Krogfelt, K.A., Klein, B.M., Zechner, E.L., Molin, S., 2006. In vitro biofilm formation of commensal and pathogenic *Escherichia coli* strains: impact of environmental and genetic factors. J Bacteriol 188, 3572-3581.

Rickard, A.H., Palmer, R.J., Blehert, D.S., Campagna, S.R., Semmelhack, M.F., Egland, P.G., Bassler, B.L., Kolenbrander, P.E., 2006. Autoinducer 2: a concentration-dependent signal for mutualistic bacterial biofilm growth. Molecular Microbiology 60, 1446-1456.

Sanchez, E., Morales, C.R., Castillo, S., Leos-Rivas, C., Garcia-Becerra, L., Martinez, D.M.O., 2016. Antibacterial and Antibiofilm Activity of Methanolic Plant Extracts against Nosocomial Microorganisms. Evidence-Based Complementary and Alternative Medicine.

Sastry, V., Rao, G.R.K., 1994. Antibacterial substances from marine-algae – successive extraction using benzene, chloroform and methanol. Botanica Marina 37, 357-360.

Sawada, Y., Nakabayashi, R., Yamada, Y., Suzuki, M., Sato, M., Sakata, A., Akiyama, K., Sakurai, T., Matsuda, F., Aoki, T., Hirai, M.Y., Saito, K., 2012. RIKEN tandem mass spectral database (ReSpect) for phytochemicals: A plant-specific MS/MS-based data resource and database. Phytochemistry 82, 38-45.

Seo, K.H., Frank, J.F., 1999. Attachment of *Escherichia coli* O157 : H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. Journal of Food Protection 62, 3-9.

Sharma, G., Sharma, S., Sharma, P., Chandola, D., Dang, S., Gupta, S., Gabrani, R., 2016a. *Escherichia coli* biofilm: development and therapeutic strategies. Journal of Applied Microbiology 121, 309-319.

Smith, K., Hunter, I.S., 2008. Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates. Journal of Medical Microbiology 57, 966-973.

Solomon, E.B., Yaron, S., Matthews, K.R., 2002. Transmission of *Escherichia coli* O157 : H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Applied and Environmental Microbiology 68, 397-400.

Teanpaisan, R., Kawsud, P., Pahumunto, N., Puripattanavong, J., 2017. Screening for antibacterial and antibiofilm activity in Thai medicinal plant extracts against oral microorganisms. Journal of Traditional and Complementary Medicine 7(2): 172–177.

Truchado, P., Lopez-Galvez, F., Gil, M.I., Tomas-Barberan, F.A., Allende, A., 2009. Quorum sensing inhibitory and antimicrobial activities of honeys and the relationship with individual phenolics. Food Chemistry 115, 1337-1344.

Uppuluri, P., Chaturvedi, A.K., Srinivasan, A., Banerjee, M., Ramasubramaniam, A.K., Kohler, J.R., Kadosh, D., Lopez-Ribot, J.L., 2010. Dispersion as an important step in the *Candida albicans* biofilm developmental cycle. Plos Pathogens 6.

Vikram, A., Jayaprakasha, G.K., Jesudhasan, P.R., Pillai, S.D., Patil, B.S., 2010. Suppression of bacterial cell-cell signalling, biofilm formation and type III secretion system by citrus flavonoids. Journal of Applied Microbiology 109, 515-527.

Villa, F., Albanese, D., Giussani, B., Stewart, P.S., Daffonchio, D., Cappitelli, F., 2010. Hindering biofilm formation with zosteric acid. Biofouling 26, 739–752.

Villa, F., Borgonovo, G., Cappitelli, F., Giussani, B., Bassoli, A., 2012a. Sub-lethal concentrations of *Muscari comosum* bulb extract suppress adhesion and induce detachment of sessile yeast cells. Biofouling 28, 1107–1117.

Villa, F., Cappitelli, F., 2013. Plant-derived bioactive compounds at sub-lethal concentrations: towards smart biocide-free antibiofilm strategies. Phytochem. Rev. 12, 245–254.

Villa, F., Cappitelli, F., Cortesi, P., Kunova, A., 2017. Fungal Biofilms: Targets for the development of novel strategies in plant disease management. Front. Microbiol. 8, 654.

Villa, F., Remelli, W., Forlani, F., Vitali, A., Cappitelli, F., 2012b. Altered expression level of *Escherichia coli* proteins in response to treatment with the antifouling agent zosteric acid sodium salt. Environ. Microbiol. 14, 1753–1761.

Villa, F., Villa, S., Gelain, A., Cappitelli, F., 2013. Sub-lethal activity of small molecules from natural sources and their synthetic derivatives against biofilm forming nosocomial pathogens. Curr. Top. Med. Chem. 13, 3184–3204.

Williams, D.L., Woodbury, K.L., Haymond, B.S., Parker, A.E., Bloebaum, R.D., 2011. A modified CDC biofilm reactor to produce mature biofilms on the Surface of PEEK membranes for an in vivo animal model application. Current Microbiology 62, 1657-1663.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M., Vantriet, K., 1990. modeling of bacterial-growth curve. Applied and Environmental Microbiology 56, 1875-1881.

Table and figure captions

Tab. 1. Seagrass species and information about collection sites.

Species	Plant organ	Collection site	GPS	Collection date
Enhalus acoroides	Leaf	Nha Trang Bay, Vietnam	109.209208°E 12.158073°N	19.04.2011
Enhalus acoroides	Root	Nha Trang Bay, Vietnam	109.209208°E 12.158073°N	19.04.2011
Halophila ovalis	Leaf	Nha Trang Bay, Vietnam	109.209208°E 12.158073°N	19.04.2011
Halodule pinifolia	Leaf	Chilika Lagoon, India	85.418015°E 19.775105°N	16.02.2010

Tab. 2. Individual compounds identified by comparison of MS/MS spectra with database entries in *Enhalus acoroides* leaf extract (A), *E. acoroides* root extract (B), *Halophila ovalis* leaf extract (C) and *Halodule pinifolia* leaf extract (D). No = number of peak in Figure 9, RT = retention time, Mass = mass of precursor ion, MS/MS= fragment spectra obtained at -30 eV, Accession = accession number in database, Source = database used, n. i. = not identified, mod. = modified.

A - *E. acoroides* leaf extract						
No	**RT**	**Mass**	**MS/MS**	**Name**	**Accession**	**Source**
1	2.5	343.03	201.02, 157.03, 59.01	n. i.	-	-
2	2.7	312.12	179.05, 132.06, 89.02	n. i.	-	-
3	3.3	367.1	277.07, 187.04, 157.03	n. i.	**-**	**-**
4	7.2	134.04	107.03, 92.02	Adenine	PT200393	ReSpect
5	13.7	637.1	461.07, 285.04	Kaempferol-3-glucuronide, mod.	PT209240	ReSpect
6	14.8	275.15	233.12, 119.05	n. i.	-	-
7	15.2	121.03	92.02, 77.03	Benzoic acid	KO000321	MassBank
8	18.6	527.02	285.04, 241.00, 96.96	n. i.	**-**	**-**
9	20.1	511.05	269.04, 241.00, 96.96	n. i.	**-**	**-**
10	20.8	187.09	169.08, 125.09, 97.06	Azelaic acid	KO000124	MassBank
11	21.3	447.09	285.04	Kaempferol-3-O-glucoside	PS042209	ReSpect
12	22.5	461.07	285.04	Kaempferol-3-glucuronide	PS092408	ReSpect
13	27.5	285.04	151.00, 133.03	Luteolin	PS040410	ReSpect
14	29.5	269.04	225.05, 151.00, 117.03	Apigenin	PT203930	ReSpect
B - *E. acoroides* root extract						
No	**RT**	**Mass**	**MS/MS**	**Name**	**Accession**	**Source**
1	2.4	343.03	201.02, 157.03, 59.01	n. i.	-	-
2	2.7	312.12	179.05, 132.06, 89.02	n. i.	-	-
3	2.9	377.08	341.11, 179.05, 119.03, 89.02	Galactinol dihydrate, mod.	PT211910	ReSpect
4	4.3	216.98	173.02, 156.98, 136.94, 59.01	n. i.	-	-

5	7.2	134.04	107.03, 92.02	Adenine	PT200393	ReSpect
6	9.6	577.12	451.10, 425.08, 407.07, 289.07, 125.02	Procyanidin B2	PT204580	ReSpect
7	12.3	289.07	245.07, 203.07, 151.04, 109.03	+(-) Epicatechin	PT204560	ReSpect
8	13.8	637.1	461.07, 285.04	Kaempferol-3-glucuronide, mod.	PT209240	ReSpect
9	14.0	469.08	275.02, 193.05, 178.02, 149.06, 96.96	n. i.	-	-
10	14.8	275.15	233.12, 119.05	n. i.	-	-
11	15.3	121.03	92.02, 77.03	Benzoic acid	KO000321	MassBank
12	20.8	187.09	169.08, 125.09, 97.06	Azelaic acid	KO000124	MassBank
13	22.6	461.07	285.04	Kaempferol-3-glucuronide	PS092408	ReSpect
14	24.1	299.05	284.03, 256.03, 133.03	Kaempferide	PT204030	ReSpect
15	27.5	285.04	151.00, 133.03	Luteolin	PS040410	ReSpect
16	29.5	269.04	225.05, 151.00, 117.03	Apigenin	PT203930	ReSpect
17	31.2	329.23	229.14, 211.13, 171.10	n. i.	-	-
C - *H. ovalis* leaf extract						
No	**RT**	**Mass**	**MS/MS**	**Name**	**Accession**	**Source**
1	2.4	343.03	201.02, 157.03, 59.01	n. i.	-	-
2	2.9	377.08	341.11, 179.05, 119.03, 89.02	Galactinol dihydrate, mod.	PT211910	ReSpect
3	4.3	216.98	173.02, 156.98, 136.94, 59.01	n. i.	-	-
4	13.3	261.04	217.05, 189.05, 133.02	n. i.	-	-
5	15.5	121.03	92.02, 77.03	Benzoic acid	KO000321	MassBank
6	16.3	306.17	288.16	n. i.	-	-
7	17.5	479.08	316,02	Myricetin-3-galactoside	PS092809	ReSpect
8	19.5	463.09	301,03	Quercetin-3-O-beta-D-galactoside	PS046509	ReSpect
9	20.8	187.09	169.08, 125.09, 97.06	Azelaic acid	KO000124	MassBank

10	21.1	317.02	271.02, 149.02	n.i.	-	-
11	21.3	447.09	285.04	Kaempferol-3-O-glucoside	PS042209	ReSpect
12	23.5	301.03	255.03, 165.02, 133.03	n.i.	-	-
13	24.1	299.05	284.03, 256.03, 133.03	Kaempferide	PS040309	ReSpect
14	25.7	285.04	239.03, 185.06, 143.05, 117.03	Kaempferol	PR040027	MassBank
15	27.5	285.04	285.04, 151.00,133.02	Luteolin	PT204043	ReSpect
16	29.4	269.04	225.05, 151.00, 117.03	Apigenin	PT203930	ReSpect
D - *H. pinifolia* leaf extract						
No	**RT**	**Mass**	**MS/MS**	**Name**	**Accession**	**Source**
1	2.4	343.03	201.02, 157.03, 59.01	n. i.	-	-
2	2.9	377.08	341.11, 179.05, 119.03, 89.02	Galactinol dihydrate, mod.	PT211910	ReSpect
3	4.3	216.98	173.02, 156.98, 136.94, 93.03, 59.01	n. i.	-	-
4	6.6	473.07	311.04, 293.03, 179.03, 149.01	n. i.	-	-
5	9.6	577.12	451.10, 425.08, 407.07, 289.07, 125.02	Procyanidin B2	PT204580	ReSpect
6	12.1	289.07	245.07, 203.07, 151.04, 109.03	+(-) Epicatechin	PT204560	ReSpect
7	14.0	469.08	275.02, 193.05, 178.02, 149.06, 96.96	n. i.	-	-
8	19.1	641.17	473.13, 311.07, 167.03	n. i.	-	-
9	19.7	549.09	505.10, 463.09, 300.02, 271.02, 255.02	Quercetin-3-(6-malonyl)-glucoside	PT209340	ReSpect
10	20.8	187.09	169.08, 125.09, 97.06	Azelaic acid	KO000124	MassBank
11	21.1	505.09	463.08, 300.02, 271.02	Quercetin-3-O-beta-D-galactoside, mod.	PT204650	ReSpect
12	21.8	463.08	300.03, 271.02	Quercetin-3-O-beta-D-galactoside	PT204650	ReSpect
13	22.4	433.07	300.02, 271.02, 255.03, 179.00	Quercetin-3-arabinoside	PT209320	ReSpect
14	23.4	447.09	284.03, 255.03, 227.03	Kaempferol-3-glucoside	PT209270	ReSpect
15	24.6	417.08	284.03, 255.03, 227.03	Kaempferol-3-O-alpha-L-arabinoside	PT209220	ReSpect

16	26.3	301.03	178.99, 151.00, 121.03, 107.01	Quercetin	PT204090	ReSpect
17	27.4	285.04	199.03, 175.04, 151.00, 133.02	Luteolin	PT204043	ReSpect
18	27.7	315.05	300.02, 271.02, 255.03	Isorhamnetin	PM007432	ReSpect
19	29.5	269.04	225.05, 151.00, 117.03	Apigenin	PT203930	ReSpect

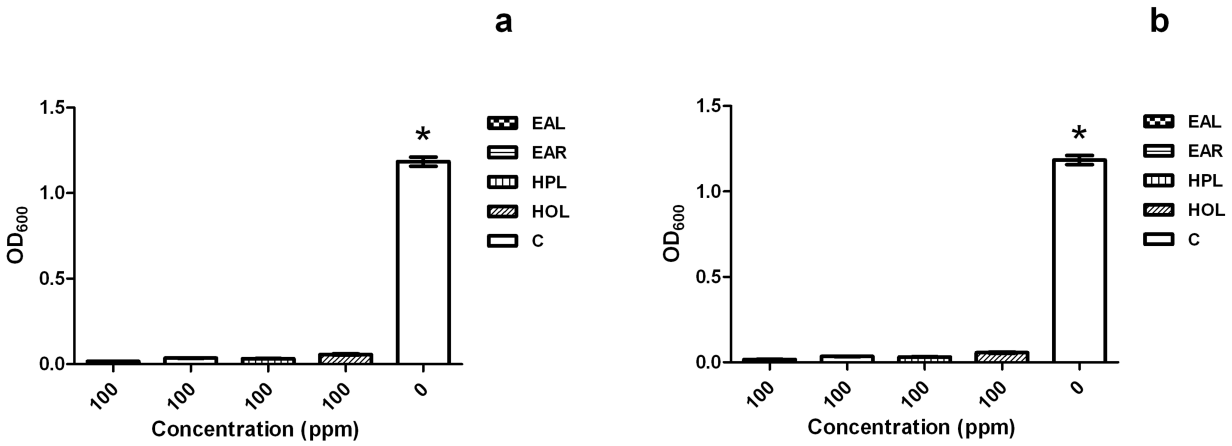
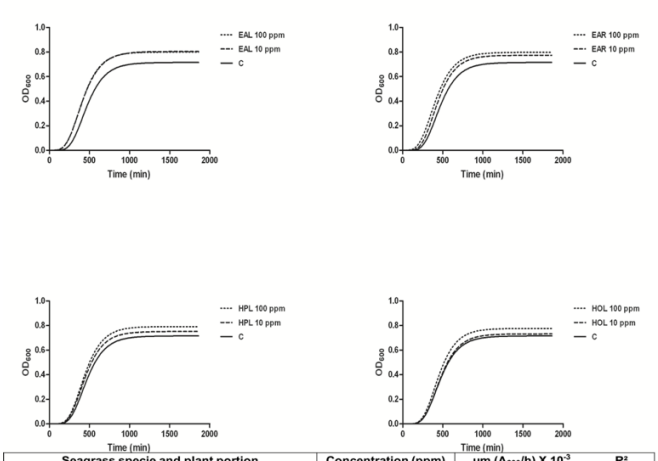
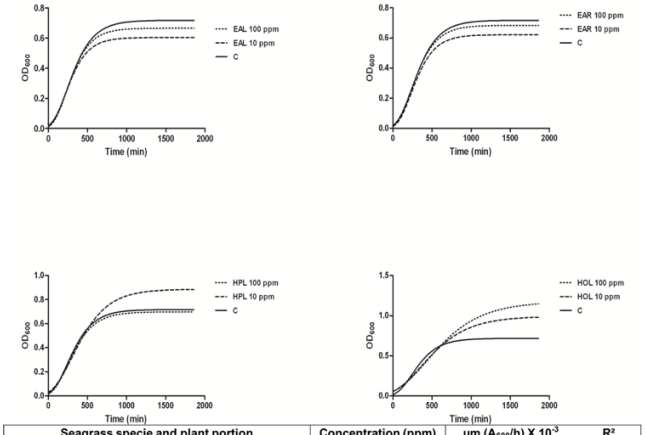


Fig. 1. *E. coli* (**a**) and *C. albicans* (**b**) planktonic growth with and without each seagrass extract at 100 mg/l. The positive control was set up with mineral medium supplemented with glucose at 5 g/l. Stars indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between the means of three independent replicates. (EAL= *Enhalus acoroides* leaf; EAR= *Enhalus acoroides* root; HPL= *Halodule pinifolia* leaf; HOL= *Halophila ovalis* leaf; C= Control).



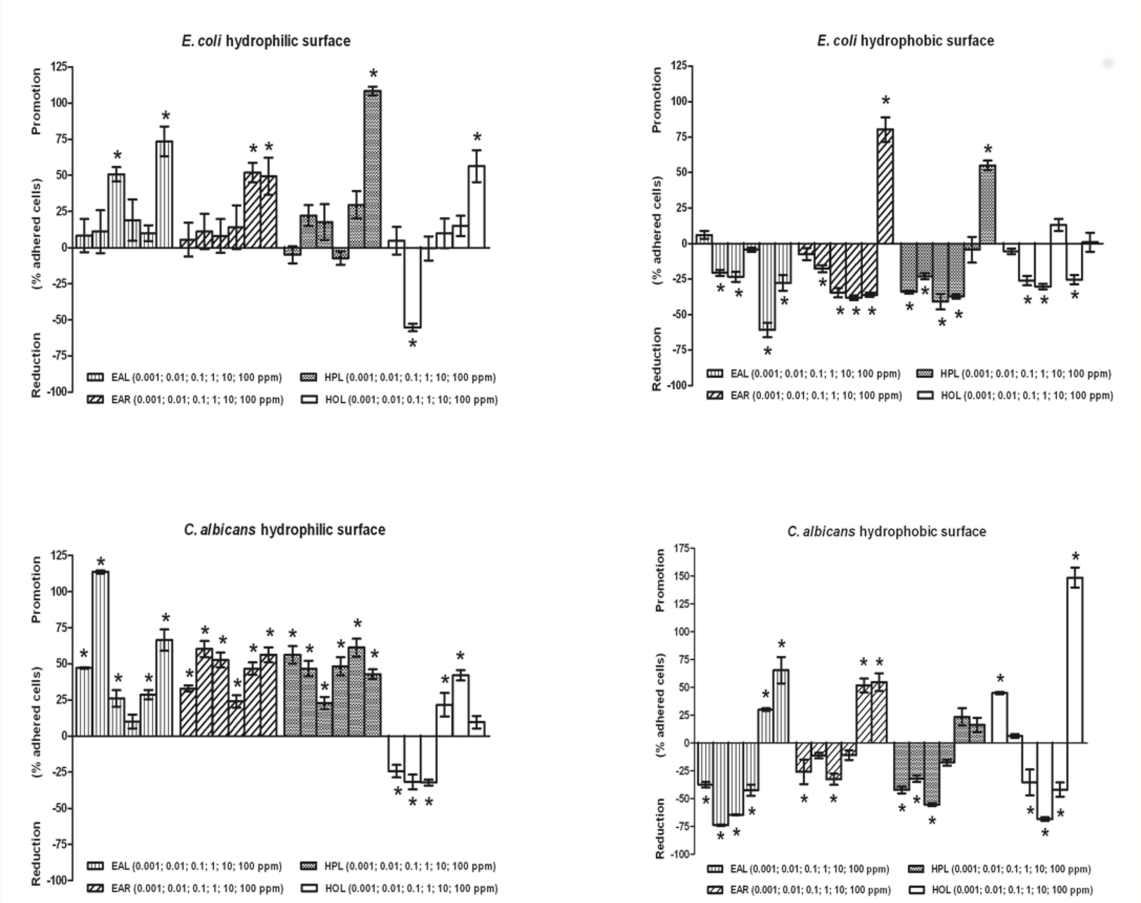
Seagrass specie and plant portion	Concentration (ppm)	μm (A_{600}/h) X 10^{-3}	R^2
Control (YNBG)	0	1.95±0.03	0.9943
Enhalus acoroides leaf	100	1.97±0.04	0.9930
	10	1.93±0.02	0.9936
Enhalus acoroides root	100	1.91±0.05	0.9919
	10	1.96±0.03	0.9921
Halodule pinifolia leaf	100	1.95±0.04	0.9938
	10	1.84±0.08	0.9929
Halophila ovalis leaf	100	1.93±0.05	0.9924
	10	1.96±0.05	0.9940

Fig. 2. OD-based growth curves of *C. albicans* in absence (control) and in presence of each seagrass extract at 10 and 100 ppm. Maximum specific growth rate (μ_m) and the goodness of fit (R^2) obtained by the Gompertz model. Data represent the mean ± SDs of three independent measurements. Means reported showed no statistically significant differences between control and treated samples (Tukey's HSD, $p \geq 0.05$). (EAL= *Enhalus acoroides* leaf; EAR= *Enhalus acoroides* root; HPL= *Halodule pinifolia* leaf; HOL= *Halophila ovalis* leaf; C= Control).



Seagrass specie and plant portion	Concentration (ppm)	μm (A_{600}/h) X 10^{-3}	R^2
Control (LB)	0	1.42±0.04	0.9945
Enhalus acoroides leaf	100	1.47±0.03	0.9959
	10	1.40±0.04	0.9946
Enhalus acoroides root	100	1.40±0.06	0.9953
	10	1.37±0.03	0.9948
Halodule pinifolia leaf	100	1.37±0.02	0.9949
	10	1.38±0.06	0.9937
Halophila ovalis leaf	100	1.42±0.01	0.9881
	10	1.45±0.09	0.9919

Fig. 3. OD-based growth curves of *E. coli* in absence (control) and in presence of each seagrass extract at 10 and 100 mg/l. Maximum specific growth rate (μ_m) and the goodness of fit (R^2) obtained by the Gompertz model. Data represent the mean ± SDs of three independent measurements. Means reported showed no statistically significant differences between control and treated samples (Tukey's HSD, $p \geq 0.05$). (EAL= *Enhalus acoroides* leaf; EAR= *Enhalus acoroides* root; HPL= *Halodule pinifolia* leaf; HOL= *Halophila ovalis* leaf; C= Control).



		0.001 ppm	0.01 ppm	0.1 ppm	1 ppm	10 ppm	100 ppm
E. coli hydrophilic surface	EAL	8.25±24.71%	11.07±25.61%	50.71±8.63%	19±28.76%	9.93±11.13%	73.51±17.95%
	EAR	5.51±20.27%	11.15±21.19%	8.11±20.25%	0.03±12.43%	51.92±13.64%	49.35±22.32%
	HPL	-4.89±10.31%	22.16±14.26%	17.65±24.88%	-7.24±8.25%	29.54±18.86%	108.34± 5.51%
	HOL	4.77±16.49%	-55.37±4.64%	-0.54±16.59%	15.34±17.96%	15.00±12.16%	56.36±22.18%
E. coli hydrophobic surface	EAL	6.05±4.59%	-20.60±3.72%	-23.35±6.07%	-4.36±3.09%	-60.76±8.86%	-27.66±9.95%
	EAR	-7.41±8.43%	-17.65±5.19%	-34.45±5.91%	-38.04±2.93%	-36.02±2.44%	80.29±14.74%
	HPL	-33.95±2.07%	-22.98±3.83%	-40.82±9.47%	-37.03±2.74%	-4.43±15.65%	54.92±5.95%
	HOL	-5.37±3.45%	-26.03±5.82%	-30.22±3.10%	13.14±8.63%	-25.31±6.81%	1.01±11.48%
C. albicans hydrophilic surface	EAL	47.13±12.91%	113.65±2.12%	26.07±9.83%	10.15±8.43%	28.65±5.79%	66.40±12.83%
	EAR	32.97±3.35%	60.28±9.53%	52.67±8.83%	24.03±7.27%	46.72±7.50%	56.25±8.98%
	HPL	56.21±10.47%	46.75±9.32%	22.97±7.16%	48.29±10.86%	61.23±12.34%	42.87±5.81%
	HOL	-24.32±7.48%	-31.76±8.75%	-32.23±4.20%	21.71±13.99%	42.03±6.00%	9.63±7.53%
C. albicans hydrophobic surface	EAL	-37.50±4.03%	-73.89±1.01%	-64.74±0.69%	-42.57±8.69%	29.90±2.07%	65.32±20.58%
	EAR	-25.81±19.29%	-11.14±4.99%	-32.69±8.81%	-10.91±7.64%	51.68±12.59%	54.69±13.88%
	HPL	-42.33±5.15%	-32.18±4.95%	-55.39±2.09%	-17.54±4.82%	23.59±13.40%	16.17±11.43%
	HOL	44.86±1.47%	6.23±3.02%	-35.67±20.22%	-68.36±2.99%	-41.95±10.92%	148.60±15.39%

Fig. 4. Microplate-based biofilm assay. Number of adhered cells of *E. coli* and *C. albicans* on hydrophilic and hydrophobic surface in absence (control) and in presence of non-lethal concentrations of seagrass extracts. According to post hoc analysis (Tukey's HSD, $p \leq 0.05$), stars indicate statistically significant differences between the means of three independent replicates. In addition, the mean ± SDs of the number of adhered cells with seagrass extracts at non-lethal concentrations on hydrophilic and hydrophobic surface are reported. Percentage reduction with respect to the control is calculated as (treated data – control data) × 100 / control data. The higher anti-adhesion effect for each microorganism was highlighted. (EAL= *Enhalus acoroides* leaf; EAR= *Enhalus acoroides* root; HPL= *Halodule pinifolia* leaf; HOL= *Halophila ovalis* leaf).

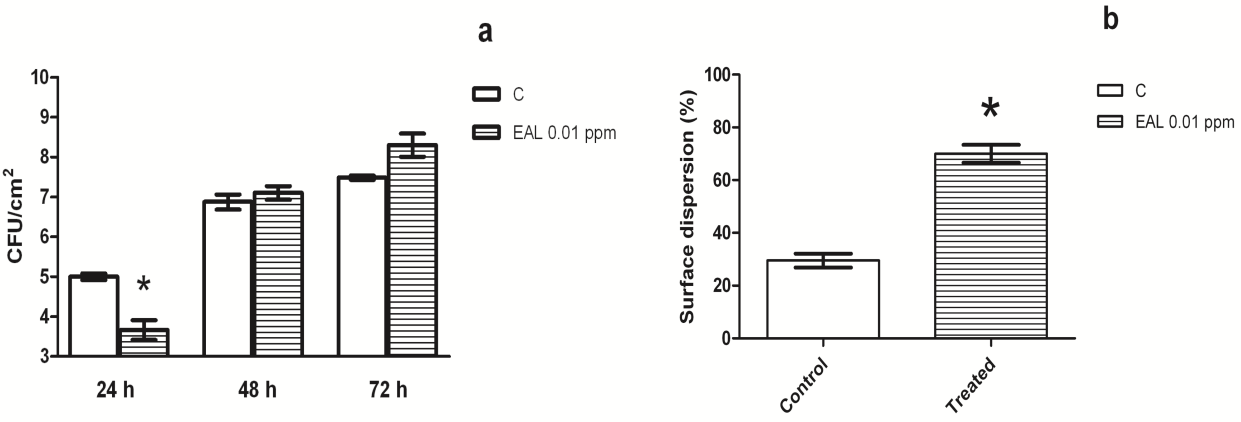


Fig. 5. CDC biofilm growth on polycarbonate coupons (**a**) and biofilm dispersion rate (**b**) of *C. albicans* in presence (treated) and in absence (control) of 0.01 ppm of *Enhalus acoroides* leaf extract. Stars indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between the means of three independent replicates. (C= control; EAL= *Enhalus acoroides* leaf).

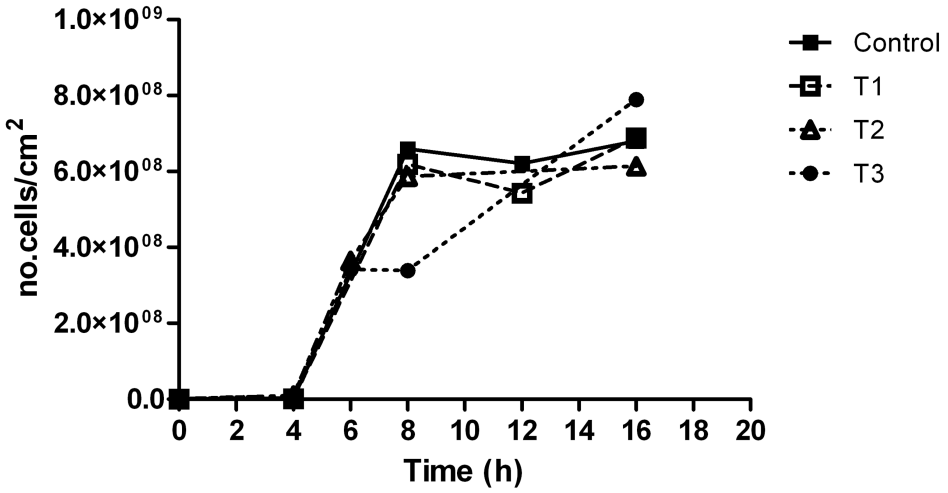


Fig. 6. Biofilm growth at the solid/air interface. *E. coli* biofilm grown on polycarbonate membrane under three experimental conditions: i) treatment 1: growth in contact with 1 ml of LB with 10 ppm of *E. acoroides* leaf extract; ii) treatment 2: overnight culture grown with 10 ppm of *E. acoroides* leaf extract and then growth in contact with 1 ml of LB; iii) treatment 3: overnight culture grown with 10 ppm of *E. acoroides* leaf extract and growth in contact with 1 ml of LB with 10 ppm of *E. acoroides* leaf extract. In the control, microorganisms grew in 1 ml LB inside a basolateral well. Data obtained were divided by the area of the membrane, and means were reported. The experiment was repeated three times. (T1= treatment 1; T2= treatment 2; T3= treatment 3).

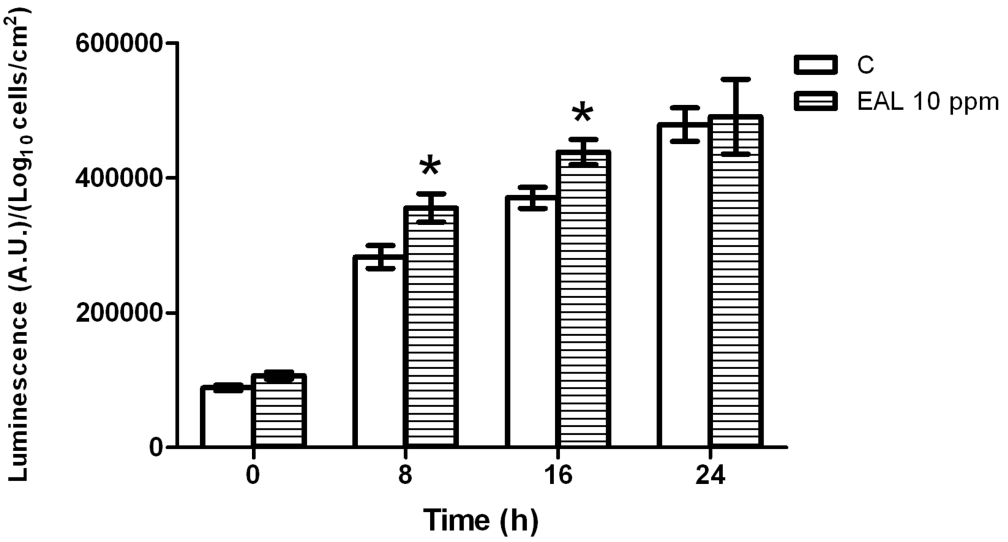


Fig. 7. Relative luminescence emitted by *Vibrio harveyi* in absence (control) and in presence of 10 ppm of *E. acoroides* leaf extract for 24 h. The relative luminescence has been calculated by normalizing luminescence by the number of adhered cells. Stars indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between the means of three independent replicates. (C= control; EAL= *Enhalus acoroides* leaf).

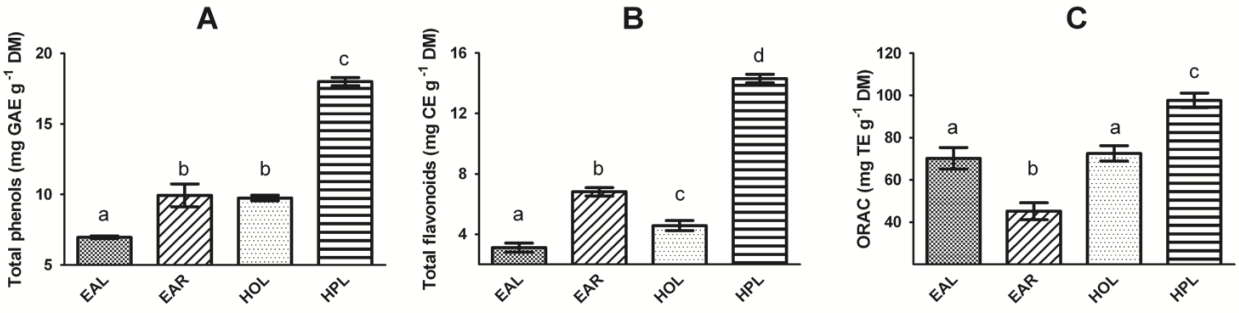


Fig. 8. Crude methanolic extracts were analyzed for (**A**) Total phenols in mg gallic acid equivalent (GAE) per g dry mass (DM), (**B**) Total flavonoids in mg catechin equivalent (CE) per g DM, and (**C**) ORAC in mg Trolox equivalents (TE) per g DM. Data represent the mean ± SDs and different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between the means of three independent measurements. (EAL= *Enhalus acoroides* leaf; EAR= *Enhalus acoroides* root; HPL= *Halodule pinifolia* leaf; HOL= *Halophila ovalis* leaf; C= Control).

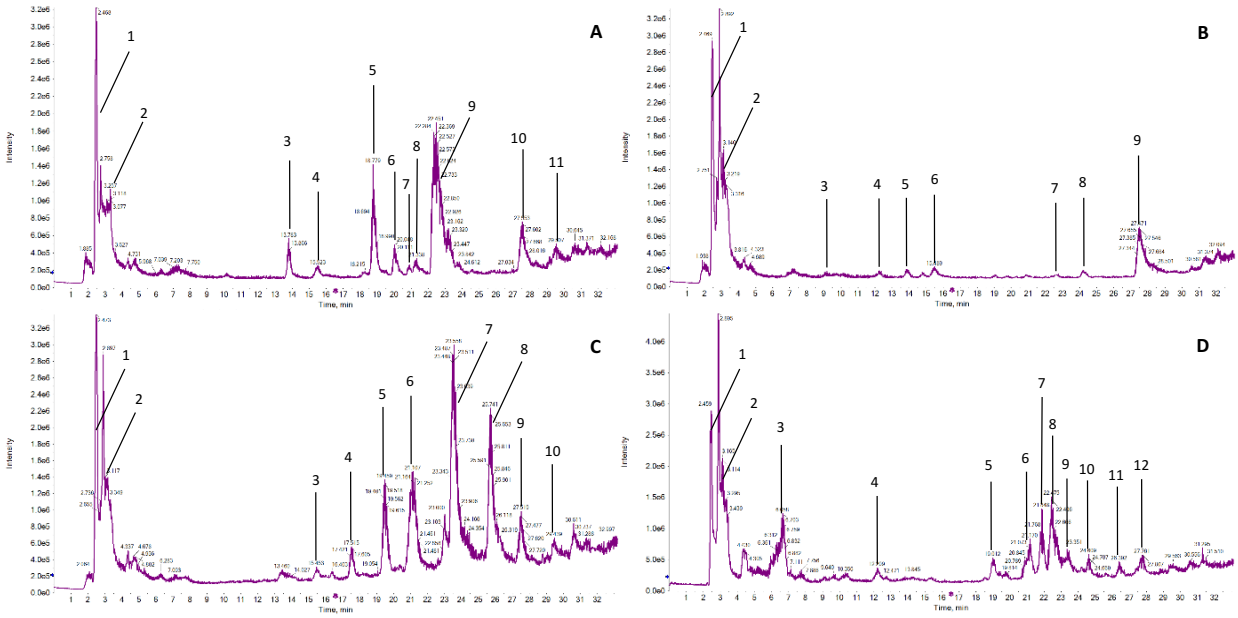


Fig. 9. Chromatograms from *E. acoroides* leaf extract (**A**), *E. acoroides* root extract (**B**), *H. ovalis* leaf extract (**C**) and *H. pinifolia* leaf extract (**D**) from minute 0-33. The relative intensity of mass between 100 and 800 Da is shown.

CHAPTER IV

Sub-lethal concentrations of *Perilla frutescens* essential oils affect phytopathogenic fungal biofilms

Article submitted to a peer-reviewed international journal

Abstract

The lack of deep knowledge of plant pathogenic fungal biofilms is reflected in the few existing environmental-friendly options for controlling fungal plant disease. Indeed, chemical fungicides still dominate the market but present-day concerns about their real efficacy, increasing awareness of the risk they pose to human health and the environment, and the incidence of fungicide resistance have all led to the current trend of near zero-market-tolerance for pesticide residues in fruit and vegetables. Here, essential oils (PK and PK-IK) from edible leaves of two cultivars of *Perilla frutescens* are proposed as new, effective, non-toxic, eco-friendly pesticide-free options suitable for a preventive or integrative approach for sustainable crop protection. PK and PK-IK were extracted and characterized, and their ability to affect biofilm formation of the phytopathogenic model fungi *Colletotrichum musae*, *Fusarium dimerum* and *Fusarium oxysporum* was studied at non-lethal doses. Both essential oils at 1000 and 2000 mg l^{-1} showed excellent anti-biofilm performance: i) reducing conidia adhesion up to 80.3±16.2%; ii) inhibiting conidia germination up to 100.0±0.0%; iii) affecting biofilm structural development, with a reduction in dry weight of up to 100.0±0.0% and extracellular polysaccharides and proteins up to 81.4±8.0% and 51.0±6.1% respectively. In all cases PK-IK showed better activity than PK.

Introduction

The application of pesticides in agricultural systems to control plant disease and safeguard crop yield and quality has increased in past decades (Liu et al., 2015; Pretty et al., 2015). Moreover, it has been predicted that pesticide application will substantially increase in the next few decades (Carvalho et al., 2017). However, the massive use of such chemicals is questionable because of draw-backs that include environmental persistence over a prolonged period of time and harmful impact on human health via the food chain (Sousa et al., 2014). According to a survey made by the Human Rights Council of the United Nations, about 200,000 people are poisoned annually and many across the world die because of toxic exposure to pesticides (HRCUN, 2017). Additionally, in recent years resistance to many of the most effective pesticides has emerged and spread in pathogen communities, lowering the availability of active substances (Luca et al., 2015).

Worldwide legislation has now adopted principles of integrated pest control to manage a more sustainable agriculture less dependent on pesticides (Barzman et al., 2015; EU, 2009). Specific measures include the use of disease- and pest-resistant crops, crop rotation, application of non-chemical control practices (thermic, mechanical, biological) and, as a last resort, the tactical use of agrochemicals (EU, 2009). However, local governments still struggle to put these principles into practice and chemical

control often remains the simplest and most cost-effective way for controlling the presence of phytopathogens (Ehler and Bottrell, 2010; Parsa et al., 2014).

Phytopathogenic fungi account for most pest disease around *the* world, seriously affecting agricultural production and food availability. Recently, it has become evident that fungal phytopathogens do not interact with the plant as individual entities, but rather at the community level in the form of biofilm (Pandin et al., 2011; Villa et al., 2017). This seriously compromises the picture as microorganisms in the biofilm lifestyle display increased tolerance to conventional biocides, and a capacity to evade the host's defences, compromising disease control (Stewart, 2002).

The concept of biofilm in plant pathology offers the opportunity to exploit new effective non-toxic eco-friendly pesticide-free agricultural practices. Interference with the key-steps orchestrating the genesis of virtually every biofilm (e.g., attachment, cell-to-cell communication, dispersion) could provide a way for new preventive strategies that do not necessarily exert lethal effects on cells, but rather sabotage the propensity for a biofilm lifestyle (Villa et al., 2017). As these substances do not act by killing the cells, they should not impose a selective pressure that would cause the onset of resistance (Villa and Cappitelli, 2013).

Terrestrial plants used as food offer a rich source of bioactive compounds with potential applications in the agri-food sector, as they are generally recognized as safe (Lucera et al., 2012).

Perilla frutescens is an annual short-day plant widely used in the therapeutics of traditional medicine and in food preparations in Asian countries (Laureati et al., 2010). It was recently found that some compounds of *P. frutescens* essential oil, namely perillaldehyde (PA), perillaketone (PK) and isoegomaketone (IK), activate the Transient Receptor Potential A1 ion channels involved in the perception of somatosensory sensations (Bassoli et al., 2009, Bassoli et al., 2013). Interestingly, compounds active on chemesthetic sensations, such as hot, cold and pungency, have promising anti-biofilm activity (Villa et al., 2012a). In this paper sub-lethal concentrations of two essential oils from *P. frutescens* leaves were tested for their anti-biofilm activity against the phytopathogenic fungi *Colletotrichum musae, Fusarium dimerum* and *F. oxysporum* included in the Top 10 fungal plant pathogen list (Dean et al., 2012). Moreover, *C. musae* has been found to develop resistance against benzimidazoles, the most commonly used fungicides (Khan et al., 2001). Compared to the large amount of work concerning the use of natural products as fungicides (Al-Samarrai et al., 2012, El-Hossary et al., 2017), only a few papers have dealt with molecules with anti-biofilm activity against phytopathogenic fungi at non-biocidal concentrations. Here, the ability of *P. frutescens* oils to prevent biofilm formation by interfering with conidia adhesion, germinative tube development and biofilm maturation was thoroughly investigated.

Methods

***P. frutescens* plant material and extraction.** Two green-leaf cultivars of *Perilla frutescens* (L.) Britt. ('Shiso Green' Koppert Cress and 'Korean perilla' Kitazawa Seed) were grown at the Fondazione Minoprio (Vertemate, Como, Italy, 45° 43'31''08 N and 09°4'20''40 E) as previously described (Martinetti et al., 2017). At the end of the flowering season the leaves were washed with water in the field the day before harvest, the following day they were cut and freeze-stored at -20 °C until distillation.

***P. frutescens* essential oil extraction.** Extraction and distillation was done in a pilot plant consisting of an essential oil extractor (Albrigi 120 l), heated by a 2000 W electric plate, and a water chiller, to recover the aromatic water (Fig. S1). The frozen leaves, in slices about 4 mm high, were placed on 3 perforated stainless steel AISI 304 plates, the perforations favoring the diffusion of the steam. The plates were set about 30 cm apart to simulate the normal conditions of fresh or dried vegetal material inside a distiller, and the leaves were arranged on the plates in such a way as to optimize their contact with the steam that extracts the essential oil. Under the bottom extractor plate we added 20 l of hot water, and the first aromatic water dropped about 45 min after starting the heating. The whole distillation lasted about 1.5 h. After distillation, the aromatic water was extracted with dichloromethane and the organic phase was dried under vacuum to give the essential oils.

***P. frutescens* essential oil characterization.** Essential oils were analysed on a Varian SD 200 liquid chromatograph with RP-column Alltima C18 (250 mm length, 4.6 mm ID, 5µ, Alltech). Twenty μl were injected and separated using methanol and water at 1 ml min^{-1} flow rate. Linear gradient elution was used for 15 min with methanol/water 70/30, followed by a gradient to 100 % methanol over 10 min. Signals were recorded with UV detention at 254 nm; Varian Galaxy software was used for the data processing. Pure PK was previously prepared by synthesis and characterized for its structure; pure IK was purified from 'Shiso Green' (Bassoli et al., 2013). In isocratic conditions the two compounds have retention times of 8.21 min (IK) and 10.42 min (PK). ^{1}H NMR spectra were recorded with respectively Bruker AMX - 300 (300 MHz) and Avance (600 MHz) instruments, using TMS as internal standard.

Fungal pathogens. *Colletotrichum musae* D128, *Fusarium dimerum* F30 and *Fusarium oxysporum* D221 (Department of Food, Environmental and Nutritional Sciences, University of Milan collection) were used as model systems for fungal biofilm. *C. musae* and *F. oxysporum* were grown on Potato Dextrose Agar (PDA, Difco Laboratories, USA) while *F. dimerum* was cultured on Czapek Agar (CA, Sigma Aldrich, USA). Fungi were maintained at 21 °C for 15 days until conidia collection. Conidia were collected in water and filtered through two layers of sterile gauze according to Kunova et al. (2016). Conidial concentrations were determined by conidia counting using a light microscope (Leica DM4000 M, Leica Microsystems, Germany) and a Thoma counting chamber.

Planktonic fungal growth in the presence of *P. frutescens* essential oils. The ability of PK and PK-IK *P. frutescens* oils to affect fungal growth was assessed by disk diffusion assay (López-Oviedo et al., 2006). Indeed, a planktonic assay was preferred over a biofilm assay, in accordance with the evidence that planktonic cells are more sensitive to biocides than their corresponding sessile counterpart (Stewart, 2002). Thus, any concentration that is not sub-lethal for planktonic cells is also not lethal for biofilms. Two hundred μL of 10^{6} ml^{-1} conidia were distributed uniformly on Petri plates containing either PDA for *C. musae* or CA for *F. oxysporum* and *F. dimerum*. Filter-paper discs (6 mm diameter, Oxoid, United Kingdom) were imbibed with concentrations of 0.001, 0.01, 0.1, 1, 10, 100, 1000, 2000 mg l^{-1} of PK or PK-IK *P. frutescens* oil with 3 % methanol (MetOH), and the discs were then placed at the center of the Petri plates. Filter paper discs were also prepared with Phosphate Buffer Saline (PBS, Sigma Aldrich, USA) or 3 % MetOH as negative controls, and the antifungal Procloraz (0.01 %, Sportak, BASF,

Germany) as positive control. The physiological pH value of each medium supplemented with *P. frutescens* oil was verified using a Jenway 3510 pH meter (pH 7.5). Fungi were maintained at 21 °C and, after 7 days, the diameter of inhibited fungal growth around each disc was measured. Three biological replicates were performed for each treatment and three technical replicates for each experiment.

***P. frutescens* essential oils as carbon source for fungal models.** The ability of fungi to grow with PK and PK-IK *P. frutescens* oils was evaluated by inoculating 200 μl of 10^6 ml^{-1} conidia on Petri plates containing a mineral medium agar (3.0 g l^{-1} sodium nitrate, 1.0 g l^{-1} di-potassium hydrogen phosphate, 0.5 g l^{-1} magnesium sulfate heptahydrate, 0.5 g l^{-1} potassium chloride, 0.01 g l^{-1} iron(II) sulfate heptahydrate, 15 g l^{-1} agar) with the addition of 0.001, 0.01, 0.1, 1, 10, 100, 1000, 2000 mg l^{-1} of PK or PK-IK *P. frutescens* oils supplemented with 3 % MetOH. Petri dishes were also prepared with PBS or MetOH as negative controls and with the addition of 30 g l^{-1} sucrose (Sigma Aldrich, USA) as a positive control. Fungi were maintained at 21 °C and, after 7 days the fungal growth in the presence of essential oils was evaluated by comparison with the positive control. Three biological replicates were performed for each treatment and three technical replicates for each experiment.

Conidia adhesion: adhesion kinetics. Conidia adhesion curves for each fungal strain were assessed in hydrophobic black-sided plates, as previously reported by Villa et al. (2010). Briefly, 200 µl of PBS containing 5 x 10^5 conidia were placed in microtiter plate wells and incubated at 21°C. After 0.25, 1 and then at 6 hourly intervals the microtiter plate wells were washed twice with 200 µl PBS, and the adhered conidia were stained using Fluorescent Brightener 28 (Sigma Aldrich, USA) in PBS for 20 min in the dark at room temperature. Fluorescence intensity was measured using the Infinite 200 PRO Microplate Reader (Tecan) at excitation wavelength of 335 nm and emission wavelength of 433 nm. A standard curve of fluorescence intensity was used to quantify the number of adhered conidia. Obtained data were normalized to the area and means were reported. Three biological replicates were performed for each treatment and three technical replicates for each experiment.
The kinetics of conidia adhesion were determined according to Jopia et al. (2010) using the Gompertz growth model adapted for microbial growth by Zwietering (1990). The Neperian logarithm of relative growth ($N\ N_0^{-1}$) was used as the growth variable, where N_0 is the adhered conidia at the earliest time point and N is the adhered conidia at time t (in hours). The data were fitted to the model by non-linear regression using the software GraphPad Prism software (version 5.0, San Diego, CA, USA). Growth kinetic parameters were obtained: lag phase duration (λ; h), maximum specific growth rate (μ_m; conidia cm^{-2} h^{-1}) and the maximum number of adhered conidia in the stationary growth phase (A; conidia cm^{-2}).

Adhesion assay. Two hundred µl of PBS containing 5 x 10^5 conidia with the addition of 0.001, 0.01, 0.1, 1, 10, 100, 1000, 2000 mg l^{-1} of PK or PK-IK *P. frutescens* oil supplemented with 3 % MetOH were placed in hydrophobic black-sided microtiter plate wells and incubated at 21°C. Experiments were also performed with PBS and 3 % MetOH as negative controls. After 24 h, wells were washed twice with 200 µl PBS, and adhered conidia were stained using Fluorescent Brightener 28 (Sigma Aldrich, USA) in PBS for 20 min in the dark at room temperature and processed as reported in the section 'Adhesion kinetics'.

The obtained data were normalized to the area, and the means were reported. Three biological replicates were performed for each treatment and six technical replicates for each experiment.

Fungal germination. To assess the influence of PK and PK-IK *P. frutescens* oil against fungal conidia germination, 30 μL of 10^6 ml^{-1} conidia were plated on PDA (*C. musae* and *F. oxysporum*) or CA (*F. dimerum*) with the addition of 0.001, 0.01, 0.1, 1, 10, 100, 1000, 2000 mg l^{-1} of PK or PK-IK *P. frutescens* oil and 3 % MetOH. Conidia were also plated with PBS and 3 % MetOH as negative controls. Plates were maintained at 21 °C and the proportion of germinated conidia was assessed by direct microscopic counts after 21 h of incubation. Counts were made in 10 random fields of view on each replicate. Three biological replicates were performed for each treatment and three technical replicates for each experiment.

Fungicidal versus fungistatic activity. Concentrations of PK and PK-IK *P. frutescens* oils that inhibited fungal conidial germination were tested for their fungicidal rather than fungistatic activity. Petri dishes were prepared with PDA (*C. musae* and *F. oxysporum*) or CA (*F. dimerum*) and the addition of PK and PK-IK *P. frutescens* oil at concentrations found inhibiting conidia germination supplemented with 3% MetOH. A Cellophane© membrane was placed in the middle of each plate and uniformly inoculated with 30 μL of 10^6 ml^{-1}. After 21 h of incubation at 21 °C, the proportion of germinated conidia was assessed by direct microscopic counting (Leica DM4000 M, Leica Microsystems, Germany). Later each membrane was transferred onto a new plate prepared with only PDA (*F. oxysporum* and *C. musae*) or CA (*F. dimerum*). The plates were maintained at 21 °C and after other 21 h of incubation the proportion of germinated conidia was assessed a second time by direct microscopic counts (Leica DM4000 M, Leica Microsystems, Germany). When the number of germinated conidia was found to have increased with respect to the first count, the PK and PK-IK effect on fungal model was considered fungistatic. Microscopic counts were made in 10 random fields of view on each replicate. Three biological replicates were performed for each treatment and three technical replicates for each experiment.

Biofilm structural development: fungal biofilm growth. Two mL of 10^6/mL conidia in 10 % diluted Potato Dextrose Broth (PDB, Sigma Aldrich, USA; *C. musae* and *F. oxysporum*) or 10 % diluted Czapek Broth (CB, Sigma Aldrich, USA; *F. dimerum*) were inoculated in Petri dishes (35 mm diameter) with the addition of 0.001, 0.01, 0.1, 1, 10, 100, 1000, 2000 mg l^{-1} of PK or PK-IK *P. frutescens* oil supplemented with 3 % MetOH. Biofilms were also grown with only PBS or 3 % MetOH as negative controls. Petri dishes were incubated at 21°C for 10 days allowing the development of mature biofilm. To avoid the negative effect of fungal metabolites, every 48 h the media were replaced with fresh ones composed by 10 % diluted media with the addition of the corresponding concentrations of *P. frutescens* oil supplemented with 3 % MetOH. The obtained biofilms were recovered and analysed for their biomass abundance and extracellular polymeric substances (EPS) composition.

Biomass and extracellular polymeric substances (EPS) extraction. After 10 days of incubation, biomass and EPS within the biofilm were divided. The media were removed from the Petri dishes and the biofilm resuspended in 2 ml of 2 % ethylenediaminetetraacetic acid (EDTA) and transferred into new

tubes. To remove biofilm aggregates, fungal suspensions were homogenized by a 30 s cycle at 14,500 rpm (T 10 basic Ultra-Turrax) followed by 15 s sonication (15 % amplitude, in water-bath; Branson 3510, Branson Ultrasonic Corporation, Dunburry, CT) and 30 s vortex mixing. Biofilm suspensions were incubated for 3 h at 4°C with gentle shaking (300 rpm) to extract EPS. Fungal suspensions were then centrifuged (11,000 g) for 30 min at 4°C. The supernatants containing soluble EPS were filtered through a 0.2 mm filter while the pellets were recovered for the biomass abundance assay.

Biomass abundance. Pellets obtained from the previous section were washed twice with distilled water in order to remove all EDTA traces and then incubated at 90 °C until complete exsiccation. Biomass abundance was obtained gravimetrically by fungal dry weight determination as previously described (Kunova et al., 2016). Obtained data were normalized to the area and the means are reported. Three biological replicates were performed for each treatment and three technical replicates for each experiment.

EPS composition. Filtered soluble EPS were analysed for their polysaccharides and protein content. The phenol-sulfuric acid assay was applied for polysaccharide determination using glucose as the standard (Masuko et al., 2005) whereas the Bradford method (1976) was applied to analyse the protein concentrations. Absorbance was measured using a UV/VIS 7315 Spectrophotometer (Jenway). Obtained data were normalized to the area and the means are reported. Three biological replicates were performed for each treatment and three technical replicates for each experiment.

Statistical analysis. The reduction percentage of conidia adhesion/conidia germination/biofilm structural parameters in comparison to the negative control prepared with 3 % MetOH was calculated as (PK and PK-IK *P. frutescens* oil data – negative control data) x 100 / negative control data. PK and PK-IK *P. frutescens* oil concentrations able to affect the above biofilm parameters by less than 20 %, with respect to the negative control, were considered to be without activity, between 20 % and 30 % with low activity, between 30 % and 40 % with moderate activity, and more than 40 % with excellent activity (Cattò et al., 2015).
Analysis of variance (ANOVA) via a software run in MATLAB (Version 7.0, The MathWorks Inc., Natick, USA) was applied to statistically evaluate any significant differences among the samples. The ANOVA analysis was carried out after verifying whether the data satisfied the assumptions of i) independence, ii) normal distribution and iii) homogeneity of variance. Tukey's honestly significant different test (HSD) was used for pairwise comparison to determine the significance of the data. Differences were considered significant for $p < 0.05$.

Results

***P. frutescens* essential oils extraction and composition.** The extraction procedure by the pilot plant gave essential oils in the following yields: 'Korean perilla' cultivar: 0.16%; 'Shiso Green' cultivar: 0.17 %. The HPLC analysis revealed that the essential oil from 'Korean perilla' cultivar contained PK (1-(3-furyl)-4-methyl-1-pentanone) as the main component (> 97 %), confirming it to be a pure PK-chemotype.

The essential oil from 'Shiso Green' cultivar contained a mixture of PK and IK (1-(3-furyl)-4-methyl-2-penten-1-one) in the proportion of 90 % and 10 % respectively. Both essential oils were also analysed by NMR to exclude the presence of other minor components not revealed by UV detection. (Fig. 1).

***P. frutescens* essential oils do not affect planktonic fungal growth.** Experiments showed that both PK and PK-IK at all the tested concentrations did not affect C. *musae*, *F. dimerum* and *F. oxysporum* as no growth inhibition was appreciable around the disk. The same result was obtained for both the negative controls, indicating that the addition of 3 % MetOH to each essential oil did not disturb fungal growth. On the contrary, a significant growth inhibition diameter was found around the disk imbibed with the antifungal Procloraz used as positive control (Fig. 2).

***P. frutescens* essential oils are not a carbon source for fungal models.** Experiments showed that both PK and PK-IK were not a carbon source for C. *musae*, *F. dimerum* and *F. oxysporum* as no fungal growth was visible when both essential oils were added to the mineral medium as sole carbon source. No fungal growth was obtained in the negative control plates. On the contrary, in the positive control prepared with the addition of sucrose to the mineral medium fungal growth was appreciable (Fig. 3).

***P. frutescens* essential oils affect conidia adhesion: adhesion kinetics.** Figure 4 shows the adhesion curves for each fungal strain. The obtained data show that the number of adhered conidia of all the fungal strains increased until 18 h of incubation, after which the number of adhered conidia remained constant over time (Fig. 1A). Therefore, a 18 h-incubation time was used for the subsequent conidia adhesion assay in the presence of *P. frutescens* essential oils.
The interpolation of obtained data with the Gompertz growth model provided conidia adhesion parameters (Fig. 1A). *F. dimerum* displayed the longer lag phase, followed by *C. musae* and *F. oxysporum*. *F. dimerum* and *C. musae* showed the same maximum specific growth rate followed by *F. oxysporum* whereas *C. musae* and *F. oxysporum* reached the major number of adhered conidia during the stationary phase.

Adhesion assay. The number of adhered conidia after 18 h of incubation in the presence of PK and PK-IK essential oils are reported in Figure 5.
F. oxysporum was the strain most affected by both essentials oils, followed by *F. dimerum*. Indeed, PK showed only a slightly anti-adhesion effect against *C. musae* at the maximum concentration tested. On the contrary, the same oil displayed an excellent anti-adhesion performance against both *F. dimerum* and *F. oxysporum*, at concentrations above 1000 ppm and 10 ppm respectively, with a reduction in the number of adhered conidia up to 82.3±9.7 % for *F. dimerum* and 73.4±16.9 % for *F. oxysporum*.
As regards PK-IK, concentrations above 0.1 ppm significantly reduced the number of adhered conidia in all the fungal strains. Indeed, the PK-IK effect was maximum against *F. oxysporum*, conidia adhesion being slightly affected by PK-IK at 0.01 ppm, moderately affected by PK-IK at 0.1 ppm and strongly affected by PK-IK at concentration above 1 ppm, with a maximum effect at 1000 ppm with a reduction in the number of adhered conidia of 61.6±15.2 %. Against *F. dimerum*, the same oil displayed low anti-adhesion performance between 0.1 and 10 ppm and maximum effect at 1000 and 2000 ppm with a

maximum reduction in the number of adhered conidia up to 53.1±13.5 %. When tested against *C. musae*, the anti-adhesion performance of PK-IK was lower in comparison to both *Fusarium* strains. The optimal effect was reached only at the maximum concentration tested with a reduction in the number of adhered conidia up to the 44.4±6.2 %, whereas the anti-adhesion effect was low from 0.01 to 10 ppm and moderate at 100 and 1000 ppm.
As no significant differences were found between the negative controls prepared with PBS and 3 % MetOH, the anti-adhesion effect against all fungal strains was totally attributable to the essential oils.

***P. frutescens* essential oils affect conidia germination.** The percentage of conidia germinated in the presence of each essential oil is shown in Figure 6. The data show that both essential oils significantly affect conidia germination at concentrations above 1000 ppm. Indeed, germination was completely inhibited by both PK and PK-IK at 2000 ppm, with a reduction of up to 100.0±0.0 % compared to the negative control. At 1000 ppm, PK significantly decreased the number of germinated conidia but the percentage reduction of germinated conidia in comparison to the negative control was always lower than 20 %. On the contrary, at the same concentration, PK-IK massively reduced conidia germination in all the fungal strains, with a reduction of 84.5±3.1 % in *C. musae*, of 84.5±2.0 % *F. dimerum* and of 99.3±1.2 % in *F. oxysporum*.
Samples treated with PK and PK-IK at 1000 and 2000 ppm were transferred onto a new medium without the addition of essential oils. After 21 h, all the fungal strains displayed 100 % of germinated conidia.
As no significant differences were found between the negative controls prepared with PBS and 3 % MetOH, the inhibition of germination was totally attributable to the essential oils.

***P. frutescens* essential oils affect biofilm structural development**
Biomass abundance. As shown in Figure 7A, C, E, visible inhibition of biofilm growth was found for all the fungal strains in the presence of PK and PK-IK at 1000 and 2000 mg l^{-1}. The biofilm was collected and analysed for biomass and EPS polysaccharide and protein content.
The dry weight of biofilms grown in the presence of PK and PK-IK at the different concentrations tested is reported in Figure 7B, D, F, G. The experiments revealed that both essential oils significantly reduced biofilm biomass at the maximum concentration tested, with a reduction of up to 100.0±0.0 % compared to the negative control. The effects of PK and PK-IK at the same concentrations were comparable in *C. musae* and *F. oxysporum* whereas PK-IK displayed a more major effect than PK against *F. dimerum*. At 1000 mg l^{-1} PK-IK reduced biomass massively in *C. musae* and *F. dimerum* and moderately in *F. oxysporum*. At the same concentration, PK displayed a similar effect against *F. dimerum* and *F. oxysporum*, but showed no effect on *C. musae* at the same concentration. Overall, *F. dimerum* was the strain most affected by both essential oils, followed by *C. musae* and *F. oxysporum*.
No differences were found between the negative controls prepared with PBS and 3 % MetOH.

EPS polysaccharide and protein contents. Experiments showed that both essential oils significantly reduced the polysaccharides content in the EPS matrix at the maximum concentration tested, with a reduction of up to the 81.4±8.0 % compared to the negative control, and comparable values between PK and PK-IK for all fungal strains (Figure 8). At 1000 mg l^{-1}, PK slightly reduced the polysaccharide

content in the biofilm matrix of *C. musae* and *F. dimerum*, whereas no effect was recorded in *F. oxysporum*. On the contrary, at 1000 mg l^{-1} PK-IK displayed an optimal performance against *C. musae*, a moderate effect toward *F. dimerum* and low effect in *F. oxysporum*. Comparing the effects against the different strains, *C. musae* resulted the most affected by the presence of both essential oils, followed by *F. dimerum* and *F. oxysporum*. No differences were found between the negative controls prepared with PBS and 3 % MetOH.

The experiments showed that essential oils affect the EPS protein content depending on the fungal strains (Figure 6). Indeed, *F. oxysporum* resulted the most affected, followed by *F. dimerum* and *C. musae*. The data showed no effect of either essential oil toward *C. musae* (Figure 9A, D). On the contrary, PK-IK significantly decreased the protein content in *F. dimerum* matrix up to the 40.7±3.4 % at 2000 mg l^{-1} (Figure 6B, D), whereas PK showed no activity against the same strain. Both essential oils affected the EPS protein content in *F. oxysporum*: PK displayed a moderate effect at 100 and 1000 mg l^{-1} and excellent activity at 2000 mg l^{-1}, while PK-IK resulted active at 1000 and 2000 mg l^{-1} with a reduction in the EPS protein content of up to the 51.0±6.1 % compared to the negative control (Figure 6C, D). At the same concentration, PK and PK-IK showed comparable activity in all the fungal strains, except for the effect at 1000 mg l^{-1} toward *F. dimerum*. In this case, PK-IK had better activity compared to PK. No differences were found between the negative controls prepared with PBS and 3 % MetOH.

Discussion

While much is known about biofilm formed by yeast species in medical and industrial settings, few descriptions of biofilm formed by filamentous plant pathogenic fungi are available (Harding et al., 2009; Pandin et al., 2017). This study investigated some features of three filamentous phytopathogenic fungi, i.e. *C. musae, F. dimerum* and *F. oxysporum*, contributing partially to fill the gap currently present in the literature. For the first time, a kinetic mathematical modelling of spore adhesion has been proposed, providing interesting details useful to accurately manage crop treatments for pest prevention (Fang and Ramasamy, 2015). The kinetic parameters highlight that fungal conidia adhesion is a rapid process that occurs irreversibly after a few hours of contact with a suitable surface. Indeed, a small number of conidia was found irreversibly attached after 3 hours contact with the surface, while 18 hours was sufficient time for the complete adhesion of all the inoculated conidia. Once irreversibly adhered to a surface, the conidia took a maximum period of 15 hours to germinate. Furthermore, the investigation of mature biofilms highlighted that all the fungal strains were characterized by an extracellular matrix with a large amount of polysaccharides and a low quantity of proteins.

The lack of a deep knowledge of plant pathogenic fungal biofilm is reflected in the few existing safe options for controlling fungal plant disease, therefore chemical fungicides still dominate the market (Villa et al., 2017). However, the current trend is to achieve near zero market-tolerance for pesticide residues in fruit and vegetables (Lamichhane et al., 2016).

In this research, chemical characterization allowed the identification of the main components of both *P. frutescens* essential oils, confirming the presence of PK as the main component of oil from the 'Shiso Green' cultivar, whereas a mixture of PK and IK was found in the oil from the 'Korean type' cultivar. Both PK and IK are two furyl ketone derivatives that had already been identified as major secondary

metabolites in *P. frutescens* plants (Bassoli et al., 2009; Bassoli et al., 2013). The achievement of a detailed quali- and quantitative analysis of the mixture composition is an important step forward in the development of alternative natural-based treatments. In the past, a number of essential oils have been proposed as anti-biofilm agents against both bacteria and fungi (Carezzano et al., 2010; Kocic-Tanackov et al., 2017; Manganyi et al., 2015; Nazzaro et al., 2017; Sarkhosh et al., 2018). However, in most of cases the active component in the oils was not identified due to the large number of different chemical compounds that interact with each other, making the antimicrobial action less reproducible.

The diffusion of essential oil compounds into the market requires a large amount of natural materials. Extraction facilities are now able to provide a constant supply and batch recovery of essential oils for organic agriculture. However, pilot scale plants involve a notably high capital investment that small growers and farmers can generally ill-afford (Babu et al., 2002). Moreover, the building up and maintenance of a high-quality collection of such plants is not only expensive but also practically impossible for industrial scale quantities. In the case of *P. frutescens*, many chemotypes are described in the literature (Yu et al., 1997). However, this paper shows that the very simple composition of the two selected essential oils, which are essentially mono- and bi-component oils, is an extremely rare and useful condition that makes recovery and analysis very simple and, most of all, allows reproducibility of the extracted material, an essential feature for developing industrial applications. Moreover, the use of a pilot plant and a full year's harvest allowed us to obtain an average but stable content of each active principle, also in the case of the PK-IK chemotype. Another important feature of *P. frutescent* essential oil's active components is their simple chemical structure, which offers the option of producing these natural drugs by chemical synthesis, with a satisfying level of purification and controlled concentrations (Bassoli et al., 2013). Additionally, their relatively simple structure and the lack of stereogenic centers make them a good starting point to derive new synthetic analogues, many of which have already been synthesised and demonstrated to be also potent agonists of the TRPA1 channel (Bassoli et al., 2013). Compounds with improved anti-biofilm activity, and new derivatives, could be used to derive structure-activity relationships (Villa et al., 2013). Thus, the peculiar properties of both *P. frutescent* essential oils offer an advantage over other essential oils, making their real application more feasible.

P. frutescens was previously investigated for its antimicrobial properties against both bacteria (Choi et al., 2010; Kim et al., 2011; Yamamoto and Ogawa, 2002) and fungi. Tian et al. (2014) proved that essential oil from *P. frutescens* (L.) Britt leaves exhibited antifungal activities against *Aspergillus flavus*, *A. oryzae*, *A. niger* and *Alternaria alternata*, involved in the spoilage of postharvest cherry tomatoes and grapes. The same authors further proved that 0.5 mL/L perillaldehyde completely inhibited mycelial growth of the same fungi. Additionally, a study by Ansari et al. (2016) demonstrated that planktonic growth of *Candida albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei* and C. *parapsilosis was affected by the presence of* perillyl alcohol, an active compound found in some cultivar of *P. frutescens*. However, these studies dealt with lethal concentrations, and experiments were carried out using shaken-liquid or solid media that favors fungal planktonic growth. Nowadays we know that biofilms are the major mode of microbial life in a natural environment (Costerton, 1999). Therefore, biofilm lifestyle should be considered a relevant feature when studying microbial processes *in vitro* (Lerch et al., 2017; Pandin et al., 2017).

In this research the activity of PK and PK-IK essential oils was tested at sub-lethal concentrations, also taking into account that phytopathogenic fungi interact with plants in the form of biofilms. Indeed, a preventive approach was considered, following the general principle of organic agriculture that dealing with the cause of a pest outbreak is preferable to treating the symptoms (Barzaman et al., 2015). Thus, each step essential to the development of fungal biofilm is a suitable target for intervention (De Vincenti et al., 2018). In line with the formation process of filamentous fungal biofilm (Ramage et al., 2012), PK and PK-IK oils were tested for their ability to modulate conidial adhesion, germline formation and biofilm maturation.

The present results showed that PK and PK-IK essential oils are good candidates as anti-biofilm agents against phytopathogenic fungi, with an activity depending on both fungal strain and concentration. Indeed, PK and PK-IK essential oils neither inhibited fungal growth nor were utilized as a carbon energy source at the concentrations tested. Thus, the above anti-biofilm effects were exploited with a non-lethal mechanism, a must in the challenge to contrast the development of resistant fungal strains.

Essential oils proved to exert an optimal anti-adhesion performance. Indeed, both PK and PK-IK essential oils were very active against *F. oxysporum* followed by F. *dimerum*. A minor effect was recorded against *C. musae*, where conidia adhesion was slightly affected in the presence of PK oil and reduced by up to 44.4±6.2 % in the presence of PK-IK oil at the maximum concentration tested. Notably, the PK-IK oil always displayed better activity than pure PK oil, becoming active at concentrations at least two orders of magnitude less than PK.

The trend showing PK-IK essential oil to be more active than PK oil also recurred in the germination experiments. At 1000 mg l^{-1} only PK-IK oil massively affected conidia germination. On the contrary, germ tube formation was completely inhibited by both essential oils at 2000 mg l^{-1}. Interestingly, once transferred to a medium lacking in oils, all the conidia reversed their status of inhibited germination, indicating that the PK and PK-IK oils did not kill the spores but rather induced a transitory protection mechanism. Indeed, evidence has shown that pathogenic fungi are able to perceive multiple cues from the environment, and use these to influence the development of infection structures, e.g. germ tubes and appressoria (Bahn et al., 2007). In line with a multitude of other anti-biofilm natural compounds, it is possible to speculate that PK and PK-IK essential oils could act as a negative environmental signal, increasing the ability of fungal stains to express various protective mechanisms, like the ability to form infectious structures, to escape from adverse conditions (Villa et al., 2012b; Villa and Cappitelli, 2013).

PK and PK-IK essential oils at 1000 and 2000 mg l^{-1} were also able to modulate the biofilm maturation of all the fungal strains, PK-IK showing greater activity than PK. The dry weight of all the fungal strains was reduced by up to 100 % after treatment with the oils, and the effects were also visible to the naked eye. Additionally, changes in the extracellular polysaccharide and protein contents were found in all the treated strains. The polysaccharide content decreased significantly in comparison to the non-treated control, with a maximum reduction of up to 81.4±8.0 % in *C. musae*, followed by *F. dimerum* (up to 79.4±7.8 %) and *F. oxysporum* (up to 70.5±4.4 %) in the presence of both PK and PK-IK at 1000 and 2000 mg l^{-1}. The protein amount was less affected than the polysaccharide content: no effect was found in *C. musae* whereas in *F. dimerum* only PK-IK decreased the EPS proteins by 40.7±3.4 %. Instead, both oils affected the F. *oxysporum* EPS protein content, with a maximum decrease of 51.0±6.1 % with PK-IK at 1000 mg l^{-1}.

It is reported that EPS is a defining characteristic of filamentous fungal biofilms, providing the cells protection from hostile factors such as antifungal agents (Ramage et al., 2012). Treatments that affect biofilm matrix formation, i.e. PK and PK-IK essential oils, greatly enhance the efficacy of some biocidal treatments as the antimicrobial agents could penetrate the biofilm more easily, exerting their effect even at concentrations below those normally used in traditional applications and thus providing a more potent control against the development of drug-resistant strains. Indeed, treatments based on co-dosed PK and PK-IK essential oils and conventional pesticides might be potentially used to maximize the anti-biofilm effect on plants (Villa et al., 2012a).

An *in vitro* study in humans and various animals revealed that PK and IK activate the Transient Receptor Potential A1 (TRP A1) (Bassoli et al., 2013, Cattaneo et al., 2014). TRP channels allow individual cells and entire organisms to detect changes in the external environment, e.g. temperature, osmolarity and chemesthetic sensations (Clapham, 2003; Vrins et al., 2008; Wang and Siemens, 2015). Interestingly, several fungal genomes present genes encoding TRP homologue structures, which share a high degree of sequence similarity and topology to those of humans (Kaleta and Palmer, 2011; Lange et al., 2016; Palmer et al., 2001; Prole and Taylor, 2012; Zhou et al., 2005). It has been speculated that modulators of the human TRP channel, e.g. PK and IK, may also have anti-fungal activity via effects on fungal TRP channel homologues (Prole and Taylor, 2012). Accordingly, in *Fusarium graminearum* and *Colletotrichum gramicola*, TRP homologues have been found to play an important role for hyphal growth and/or plant infection, being involved in the perception of, and response to, certain features of the host surface, e.g. the surface osmotic potential that needs to be sensed and tightly controlled to ensure successful colonization, appressorium development and penetration (Ihara et al., 2013; Lange et al., 2016). In this study, a massive reduction of adhered and germinated conidia was found after the treatment with both PK and PK-IK essential oils. Thus, in line with the previous considerations, these authors do not exclude an anti-biofilm mechanism by which PK and IK target and modulate the expression of TRP homologues, affecting the spore surface sensing mechanism and leading to a significant reduction of biofilm formation. Consistent with this chemo-sensory strategy, a number of food-derived molecules that are agonistic to TRP channels have also been found to display optimal anti-biofilm performance, e.g. cinnamaldehyde from cinnamon (Khan et al., 2017) and eugenol from clove oil (Khan and Ahmad, 2012). *In vitro* studies found that, in humans, IK is a more potent agonist of the TRP1 channel than PK, as the presence of a double bond in the IK chemical structure increases its ability to target TRP1 (Bassoli et al., 2013). Notably, in our fungal strains, PK-IK essential oil displayed better activity than pure PK essential oil, starting to be active at concentrations at least one order of magnitude less than PK, highlighting a major anti-biofilm activity of IK with respect to PK. These findings may further corroborate the hypothesis of a possible involvement of a TRP homologue channel in the anti-biofilm activity of *P. frutescens* essential oils. A possible reason for the major efficacy could be the presence of an extra double bond, conjugated to the carbonyl moiety in the IK structure. This hypothesis is consistent with the proposed mechanism for TRPA1 activation, where the increase in the molecule electrophilic properties makes it potentially more susceptible to undergo the attack of nucleophilic cellular sites, such as the thiol group of protein, and inhibiting activities within cells that are vital for the biofilm process, e.g. inactivating some enzymes important for biofilm formation. Accordingly, Worthington et al. (2012) designed a series of molecules bearing electrophilic functional groups to efficiently inhibit biofilm

formation through their nucleophilic reaction with a cysteine residue present in the quorum sensing receptor LuxR.

Conclusion

This research supports the incorporation of PK and PK-IK as alternative new, safe and eco-friendly products suitable for sustainable pest management applications, as well as being an attractive method for controlling postharvest fungal decay in fruit and vegetables. Indeed, being natural ingredients of dietary plants, these compounds should be well-tolerated by humans. This is especially true in the light of the major challenges of efficiently increasing and protecting crop yield, maintaining economic profits but at the same time preserving human health and the environment. The simple PK and IK chemical structure also provides a positive foundation for their synthetic production at an industrial level with affordable costs. The challenge now is to apply this knowledge in the agricultural context.

References

Al-Samarrai, G., Singh, H., Syarhabil, M., 2012. Evaluating eco-friendly botanicals (natural plant extracts) as alternatives to synthetic fungicides. Ann. Agric. Environ. Med. 19, 673–676.

Ansari, M.A., Fatima, Z., Hameed, S., 2016. Anticandidal effect and mechanisms of monoterpenoid, perillyl alcohol against *Candida albicans*. PLoS One 11, e0162465.

Babu, K.G.D., Kaul, V.K., Ahuja, P.S., 2002. Portable mini essential oil distillation apparatus. J. Sci. Ind. Res. 61, 952–960.

Bahn, Y.S., Xue, C., Idnurm, A., Rutherford, J.C., Heitman, J., Cardenas, M.E., 2007. Sensing the environment: lessons from fungi. Nat. Rev. Microbiol. 5, 57–69.

Barzman, M., Barberi, P., Birch, A.N.E., Boonekamp, P., Dachbrodt-Saaydeh, S., Graf, B., Hommel, B., Jensen, J.E., Kiss, J., Kudsk, P., Lamichhane, J.R., Messean, A., Moonen, A.C., Ratnadass, A., Ricci, P., Sarah, J.L., Sattin, M., 2015a. Eight principles of integrated pest management. Agron. Sustain. Dev. 35, 1199–1215.

Bassoli, A., Borgonovo, G., Caimi, S., Scaglioni, L., Morini, G., Moriello, A.S., Di Marzo, V., De Petrocellis, L., 2009. Taste-guided identification of high potency TRPA1 agonists from *Perilla frutescens*. Bioorg. Med. Chem. 17, 1636–1639.

Bassoli, A., Borgonovo, G., Morini, G., De Petrocellis, L., Moriello, A.S., Di Marzo, V., 2013. Analogues of perillaketone as highly potent agonists of TRPA1 channel. Food Chem. 141, 2044–2051.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Carezzano, M.E., Sotelo, J.P., Primo, E., Reinoso, E.B., Rovey, M.F.P., Demo, M.S., Giordano, W.F., Oliva, M.D., 2017. Inhibitory effect of *Thymus vulgaris* and *Origanum vulgare* essential oils on virulence factors of phytopathogenic *Pseudomonas syringae* strains. Plant Biol. 19, 599–607.

Carvalho, F.P., 2017. Pesticides, environment, and food safety. Food Energy Secur. 6, 48–60.

Cattaneo, A.M., Bengtsson, J.M., Borgonovo, G., Bassoli, A., Anfora, G., 2014. Response of the European grapevine moth *Lobesia botrana* to somatosensory-active volatiles emitted by the non-host plant *Perilla frutescens*. Physiol. Entomol. 39, 229–236.

Cattò, C., Dell'Orto, S., Villa, F., Villa, S., Gelain, A., Vitali, A., Marzano, V., Baroni, S., Forlani, F., Cappitelli, F., 2015. Unravelling the structural and molecular basis responsible for the anti-biofilm activity of zosteric acid. PLoS One 10, e0131519.

Choi, U.K., Lee, O.H., Lim, S.I., Kim, Y.C., 2010. Optimization of antibacterial activity of *Perilla frutescens* var. acuta leaf against *Pseudomonas aeruginosa* using the evolutionary operation-factorial design technique. Int. J. Mol. Sci. 11, 3922–3932.

Clapham, D.E., 2003. TRP channels as cellular sensors. Nature 426, 517–524.

Costerton, J.W., 1999. Introduction to biofilm. Int. J. Antimicrob. Agents 11, 217–221.

Dean, R., Van Kan, J.A.L., Pretorius, Z.A., Hammond-Kosack, K.E., Di Pietro, A., Spanu, P.D., Rudd, J.J., Dickman, M., Kahmann, R., Ellis, J., Foster, G.D., 2012. The Top 10 fungal pathogens in molecular plant pathology. Mol. Plant. Pathol. 13, 804–804.

De Vincenti, L., Glasenapp, Y., Cattò, C., Villa, F., Cappitelli, F., Papenbrock, J., 2018. Hindering the formation and promoting the dispersion of medical biofilms: non-lethal effects of seagrass extracts. BMC Complement. Altern. Med. 18, 168.

Ehler, L.E., Bottrell, D.G., 2000. The illusion of integrated pest management. Issues Sci. Technol. 16, 61–64.

El-Hossary, E.M., Cheng, C., Hamed, M.M., Hamed, A.N.E., Ohlsen, K., Hentschel, U., Abdelmohsen, U.R., 2017. Antifungal potential of marine natural products. Eur. J. Med. Chem. 126, 631–651.

European Commission, 2009. EU Action on Pesticides: "Our Food has Become Greener." Available at:http://ec.europa.eu/food/plant/plant_protection_products/eu_policy/docs/factsheet_pesticides_en.pdf.

Fang, Y., Ramasamy, R.P., 2015. Current and prospective methods for plant disease detection. Biosensors (Basel) 5, 537–561.

Harding, M.W., Marques, L.L.R., Howard, R.J., Olson, M.E., 2009. Can filamentous fungi form biofilms? Trends Microbiol. 17, 475–480.

Human Rights Council of United Nation, 2017. Report of the special rapporteur on effects of pesticides on the right to food A/HRC/34/48. Available at: http://www.ohchr.org/EN/Issues/Food/Pages/Annual.aspx.

Ihara, M., Hamamoto, S., Miyanoiri, Y., Takeda, M., Kainosho, M., Yabe, I., Uozumi, N., Yamashita, A., 2013. Molecular bases of multimodal regulation of a fungal Transient Receptor Potential (TRP) channel. J. Biol. Chem. 288, 15303–15317.

Jopia, P., Ruiz-Tagle, N., Villagran, M., Sossa, K., Pantoja, S., Rueda, L., Urrutia-Briones, H., 2010. Biofilm growth kinetics of a monomethylamine producing Alphaproteobacteria strain isolated from an anaerobic reactor. Anaerobe 16, 19–26.

Kaleta, M., Palmer, C., 2011. TRP Channels in yeast. Adv. Exp. Med. Biol. 704, 315–321.

Khan, M.S.A., Ahmad, I., 2012. Antibiofilm activity of certain phytocompounds and their synergy with fluconazole against *Candida albicans* biofilms. J. Antimicrob. Chemother. 67, 618–621.

Khan, S.H., Aked, J., Magan, N., 2001. Control of the anthracnose pathogen of banana (*Colletotrichum musae*) using antioxidants alone and in combination with thiabendazole or imazalil. Plant Pathol. 50, 601–608.

Khan, S.N., Khan, S., Iqbal, J., Khan, R., Khan, A.U., 2017. Enhanced killing and antibiofilm activity of encapsulated cinnamaldehyde against *Candida albicans*. Front. Microbiol. 8, 1641.

Kim, D.H., Kim, Y.C., Choi, U.K., 2011. Optimization of antibacterial activity of *Perilla frutescens* var. acuta leaf against *Staphylococcus aureus* using evolutionary operation factorial design technique. Int. J. Mol. Sci. 12, 2395–2407.

Kocic-Tanackov, S., Dimic, G., Mojovic, L., Gvozdanovic-Varga, J., Djukic-Vukovic, A., Tomovic, V., Sojic, B., Pejin, J., 2017. Antifungal activity of the onion (*Allium cepa* l.) essential oil against *Aspergillus*, *Fusarium* and *Penicillium* species isolated from food. J. Food Process. Preserv. 41, e13050.

Kunova, A., Bonaldi, M., Saracchi, M., Pizzatti, C., Chen, X.L., Cortesi, P., 2016. Selection of *Streptomyces* against soil borne fungal pathogens by a standardized dual culture assay and evaluation of their effects on seed germination and plant growth. BMC Microbiol. 16, 272.

Lamichhane, J.R., Dachbrodt-Saaydeh, S., Kudsk, P., Messean, A., 2016. Toward a reduced reliance on conventional pesticides in european agriculture. Plant Dis. 100, 10–24.

Lange, M., Weihmann, F., Schliebner, I., Horbach, R., Deising, H.B., Wirsel, S.G.R., Peiter, E., 2016. The Transient Receptor Potential (TRP) channel family in *Colletotrichum graminicola*: a molecular and physiological analysis. Plos One 11, e0158561.

Laureati, M., Buratti, S., Bassoli, A., Borgonovo, G., Pagliarini, E., 2010. Discrimination and characterisation of three cultivars of *Perilla frutescens* by means of sensory descriptors and electronic nose and tongue analysis. Food Res. Int. 43, 959–964.

Lerch, T.Z., Chenu, C., Dignac, M.F., Barriuso, E., Mariotti, A., 2017. Biofilm vs. planktonic lifestyle: consequences for pesticide 2,4-D Metabolism by *Cupriavidus necator* JMP134. Front. Microbiol. 8, 904.

Liu, Y.B., Pan, X.B., Li, J.S., 2015. A 1961-2010 record of fertilizer use, pesticide application and cereal yields: a review. Agron. Sustain. Dev. 35, 83–93.

López-Oviedo, E., Aller, A.I., Martín, C., Castro, C., Ramirez, M., Pemán, J.M., Cantón, E., Almeida, C., Martín-Mazuelos, E., 2006. Evaluation of disk diffusion method for determining posaconazole susceptibility of filamentous fungi: comparison with CLSI broth microdilution method. Antimicrob. Agents Chemother. 50, 1108–1111.

Lucas, J.A., Hawkins, N.J., Fraaije, B.A., 2015. The evolution of fungicide resistance. Adv. Appl. Microbiol. 90, 29–92.

Lucera, A., Costa, C., Conte, A., Del Nobile, M.A., 2012. Food applications of natural antimicrobial compounds. Front. Microbiol. 3, 287.

Manganyi, M.C., Regnier, T., Olivier, E.I., 2015. Antimicrobial activities, of selected essential oils against *Fusarium oxysporum* isolates and their biofilms. S. Afr. J. Bot. 99, 115–121.

Martinetti, L., Ferrante, A., Podetta, N., Bassoli, A., Borgonovo, G., Tosca, A., Spoleto, P., 2017. Effect of storage on the qualitative characteristics of perilla, a potential new minimally processed leafy vegetable. J. Food Process. Preserv. 41, e13214.

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.I., Lee, Y.C., 2005. Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Anal. Biochem. 339, 69–72.

Nazzaro, F., Fratianni, F., Coppola, R., De Feo, V., 2017. Essential oils and antifungal activity. Pharmaceuticals (Basel) 10, 86.

Palmer, C.P., Zhou, X.L., Lin, J.Y., Loukin, S.H., Kung, C., Saimi, Y., 2001. A TRP homolog in *Saccharomyces cerevisiae* forms an intracellular Ca2+-permeable channel in the yeast vacuolar membrane. Proc. Natl. Acad. Sci. *U. S. A.* 98, 7801–7805.

Pandin, C., Le Coq, D., Canette, A., Aymerich, S., Briandet, R., 2017. Should the biofilm mode of life be taken into consideration for microbial biocontrol agents? Microb. Biotechnol. 10, 719–734.

Parsa, S., Morse, S., Bonifacio, A., Chancellor, T.C.B., Condori, B., Crespo-Perez, V., Hobbs, S.L.A., Kroschel, J., Ba, M.N., Rebaudo, F., Sherwood, S.G., Vanek, S.J., Faye, E., Herrera, M.A., Dangles, O., 2014. Obstacles to integrated pest management adoption in developing countries. Proc. Natl. Acad. Sci. *U. S. A.* 111, 3889–3894.

Pretty, J., Bharucha, Z.P., 2015. Integrated pest management for sustainable intensification of agriculture in Asia and Africa. Insects 6, 152–182.

Prole, D.L., Taylor, C.W., 2012. Identification and analysis of cation channel homologues in human pathogenic fungi. PLoS One 7: e42404.

Ramage, G., Rajendran, R., Sherry, L., Williams, C., 2012. Fungal biofilm resistance. Int. J. Microbiol. 2012, 528521.

Sarkhosh, A., Schaffer, B., Vargas, A.I., Palmateer, A.J., Lopez, P., Soleymani, A., Farzaneh, M., 2018. Antifungal activity of five plant-extracted essential oils against anthracnose in papaya fruit. Biol. Agric. Hortic. 34, 18–26.

Sousa, A.C.A., Pastorinho, M.R., Takahashi, S., Tanabe, S., 2014. History on organotin compounds, from snails to humans. Environ. Chem. Lett. 12, 117–137.

Stewart, P.S., 2002. Mechanisms of antibiotic resistance in bacterial biofilms. Int. J. Med. Microbiol. 292, 107–113.

Tian, J., Zeng, X.B., Zhang, S., Wang, Y.Z., Zhang, P., Lu, A.J., Peng, X., 2014. Regional variation in components and antioxidant and antifungal activities of *Perilla frutescens* essential oils in China. Ind. Crops Prod. 59, 69–79.

Villa, F., Albanese, D., Giussani, B., Stewart, P.S., Daffonchio, D., Cappitelli, F., 2010. Hindering biofilm formation with zosteric acid. Biofouling 26, 739–752.

Villa, F., Borgonovo, G., Cappitelli, F., Giussani, B., Bassoli, A., 2012a. Sub-lethal concentrations of *Muscari comosum* bulb extract suppress adhesion and induce detachment of sessile yeast cells. Biofouling 28, 1107–1117.

Villa, F., Cappitelli, F., 2013. Plant-derived bioactive compounds at sub-lethal concentrations: towards smart biocide-free antibiofilm strategies. Phytochem. Rev. 12, 245–254.

Villa, F., Cappitelli, F., Cortesi, P., Kunova, A., 2017. Fungal Biofilms: Targets for the development of novel strategies in plant disease management. Front. Microbiol. 8, 654.

Villa, F., Remelli, W., Forlani, F., Vitali, A., Cappitelli, F., 2012b. Altered expression level of *Escherichia coli* proteins in response to treatment with the antifouling agent zosteric acid sodium salt. Environ. Microbiol. 14, 1753–1761.

Villa, F., Villa, S., Gelain, A., Cappitelli, F., 2013. Sub-lethal activity of small molecules from natural sources and their synthetic derivatives against biofilm forming nosocomial pathogens. Curr. Top. Med. Chem. 13, 3184–3204.

Vriens, J., Nilius, B., Vennekens, R., 2008. Herbal compounds and toxins modulating TRP channels. Curr. Neuropharmacol. 6, 79–96.

Wang, H., Siemens, J., 2015. TRP ion channels in thermosensation, thermoregulation and metabolism. Temperature (Austin) 2, 178–187.

Worthington, R.J., Richards, J.J., Melander, C., 2012. Small molecule control of bacterial biofilms. Org. Biomol. Chem. 10, 7457–7474.

Yamamoto, H., Ogawa, T., 2002. Antimicrobial activity of perilla seed polyphenols against oral pathogenic bacteria. Biosci. Biotechnol. Biochem. 66, 921–924.

Yu, H. C., Kosuna, K., & Haga, M., 1997. Perilla. The genus Perilla, Taylor and Francis Group, New York.

Zhou, X.L., Loukin, S.H., Coria, R., Kung, C., Saimi, Y., 2005. Heterologously expressed fungal transient receptor potential channels retain mechanosensitivity in vitro and osmotic response in vivo. Eur. Biophys. J. 34, 413–422.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M., Vantriet, K., 1990. Modeling of the bacterial-growth curve. Appl. Environ. Microbiol. 56, 1875–1881.

Figures

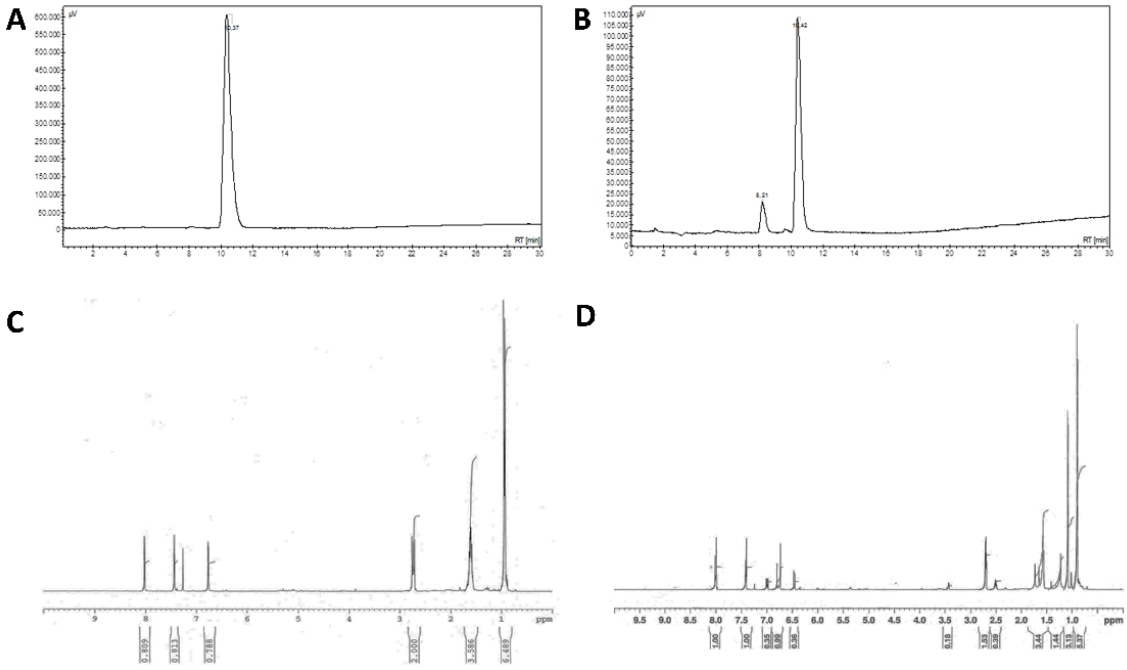


Fig. 1. HPLC chromatograms of PK (A) and PK-IK (B) *P.frutescens* essential oils; x axis: absorbance (μV), y axis: retention time (min). ^{1}H NMR of PK (C) and PK-IK (D) *P.frutescens* essential oils in $CDCl_3$.

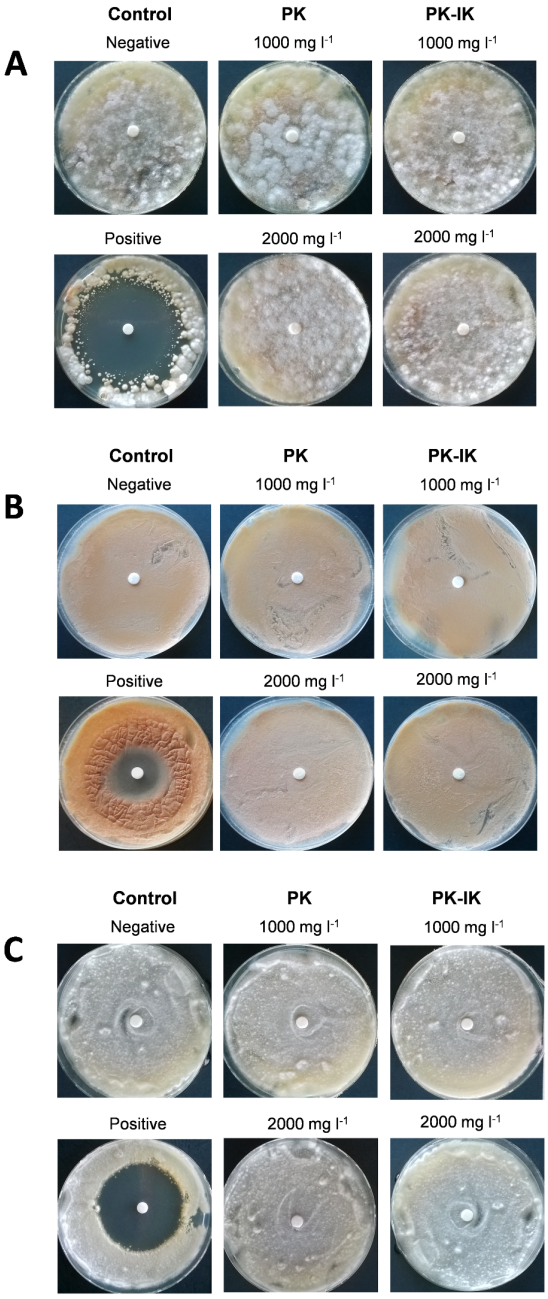


Fig. 2. Fungal growth in the presence of *P. frutescens* essential oils. Representative pictures of *C. musae* (A), *F. dimerum* (B) and *F. oxysprum* (C) grown in a Petri plates with filter-paper discs at the center imbibed with PK or PK-IK *P. frutescens* oil. Filter paper discs were also prepared with PBS or 3 % MetOH as negative controls, and the antifungal Procloraz as positive control.

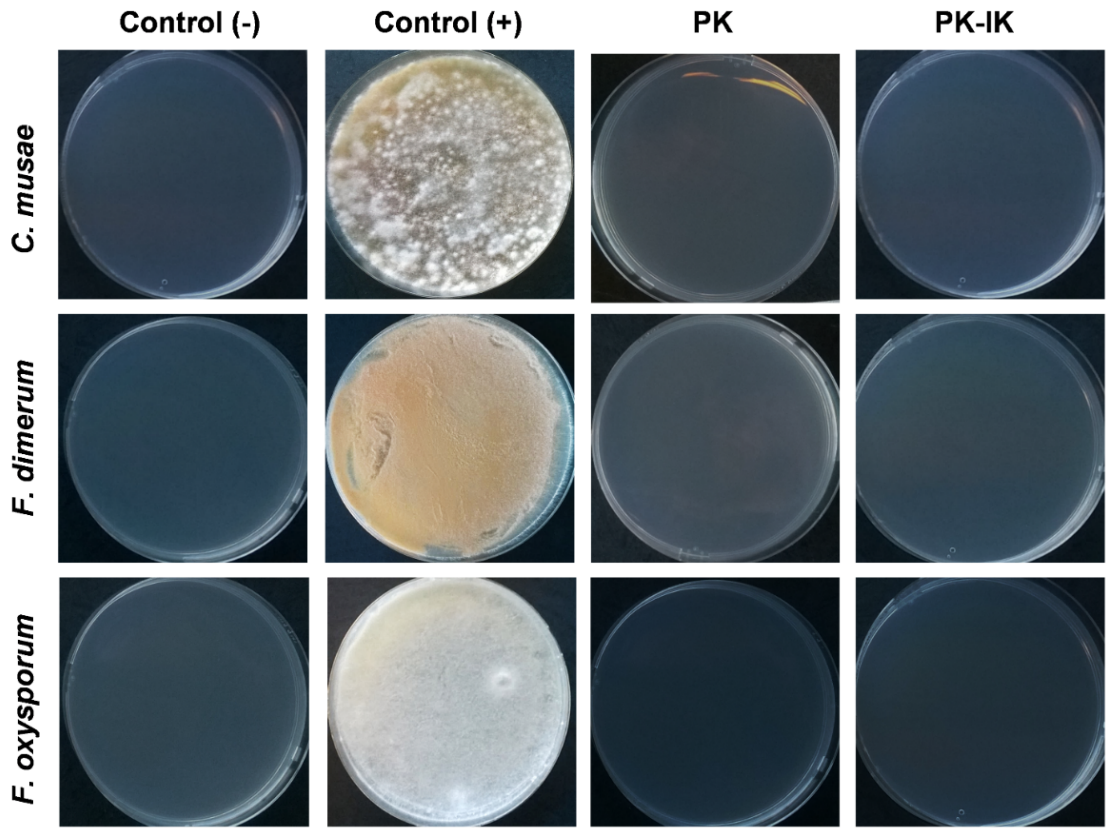
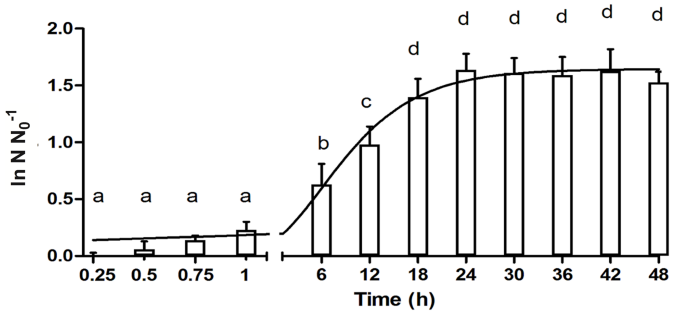


Fig. 3. Fungal growth in the presence of *P. frutescens* essential oils as sole carbon source. Representative pictures of *C. musae*, *F. dimerum* and *F. oxysprum* grown in mineral medium agar with the addition of 0.001, 0.01, 0.1, 1, 10, 100, 1000, 2000 mg l^{-1} of PK and PK-IK as sole carbon source. Petri dishes were also prepared with PBS or MetOH as negative controls and with the addition of sucrose as a positive control.

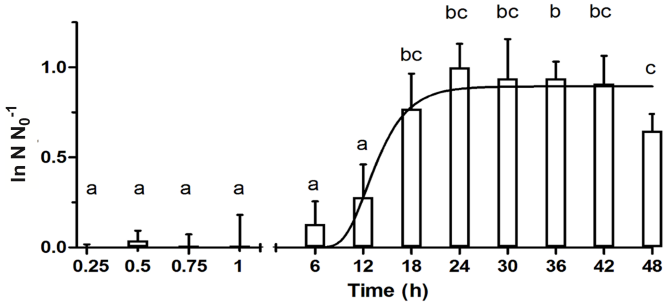
A

			C. musae	*F. dimerum*	*F. oxysporum*
Adhered conidia (conidia cm^{-2}) ($\times 10^5$)	Time (h)	0	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]
		0.25	0.78±0.11 [ab]	0.84±0.15 [b]	0.15±0.02 [a]
		0.50	0.83±0.14 [ab]	0.88±0.17 [b]	0.14±0.01 [a]
		0.75	0.90±0.25 [ab]	0.84±0.23 [b]	0.14±0.02 [a]
		1	0.98±0.18 [b]	0.85±0.16 [b]	0.15±0.01 [a]
		6	1.46±0.92 [c]	0.96±0.38 [b]	0.52±0.09 [b]
		12	2.09±0.35 [d]	1.11±0.33 [bc]	2.00±0.37 [c]
		18	3.15±0.50 [e]	1.83±0.36 [d]	2.32±0.44 [cd]
		24	4.00±0.48 [e]	2.29±0.35 [e]	2.81±0.19 [d]
		30	3.89±0.45 [e]	2.16±0.46 [de]	3.04±0.25 [d]
		36	3.83±0.53 [e]	2.15±0.20 [de]	3.08±0.34 [d]
		42	3.96±0.49 [e]	2.11±0.33 [de]	3.15±0.53 [d]
		48	3.60±0.32 [e]	1.62±0.18 [cd]	3.00±0.14 [d]
λ (h)			4.01±0.20 [A]	6.20±0.21 [B]	3.29±0.33 [C]
μ_m (conidia cm^{-2} h^{-1}) ($\times 10^4$)			8.56±0.78 [AB]	9.43±0.85 [A]	7.24±0.15 [B]
A (conidia cm^{-2}) ($\times 10^5$)			4.04±0.80 [A]	2.02±0.94 [B]	2.94±0.61 [AB]
R^2			0.94±0.14	0.90±0.10	0.99±0.11

B



C



D

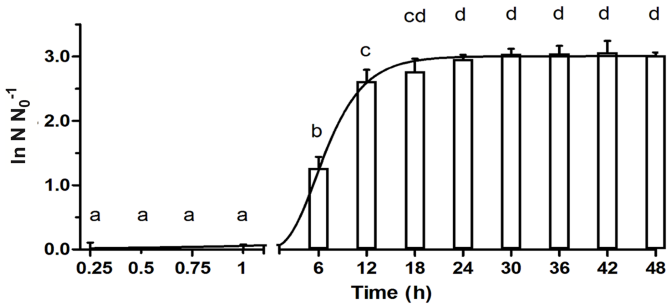
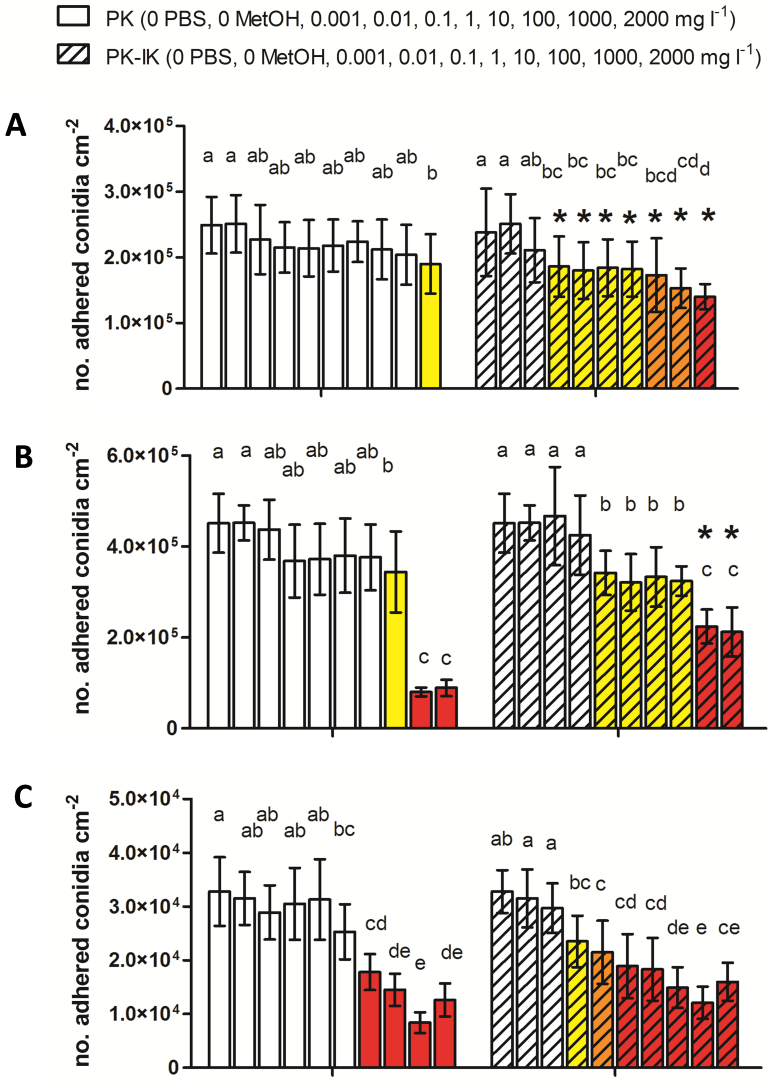


Fig. 4. Conidia kinetic adhesion. A: Number of adhered conidia at different times and adhesion kinetic parameters obtained by the Gompertz model. λ: lag phase duration, μ_m: maximum specific growth rate, A: maximum number of adhered conidia in the stationary growth phase, R^2: goodness of fit. B, C, D: Adhesion curve of *C. musae* (B), *F. oxysporum* (C) and *F. dimerum* (D) obtained by fitting the relative number of adhered conidia (log N N_0^{-1}) with the Gompertz model. Data represent the mean ± standard deviation of three independent measurements. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.01$) between the time steps (lower letter) or fungal strains (capital letter).



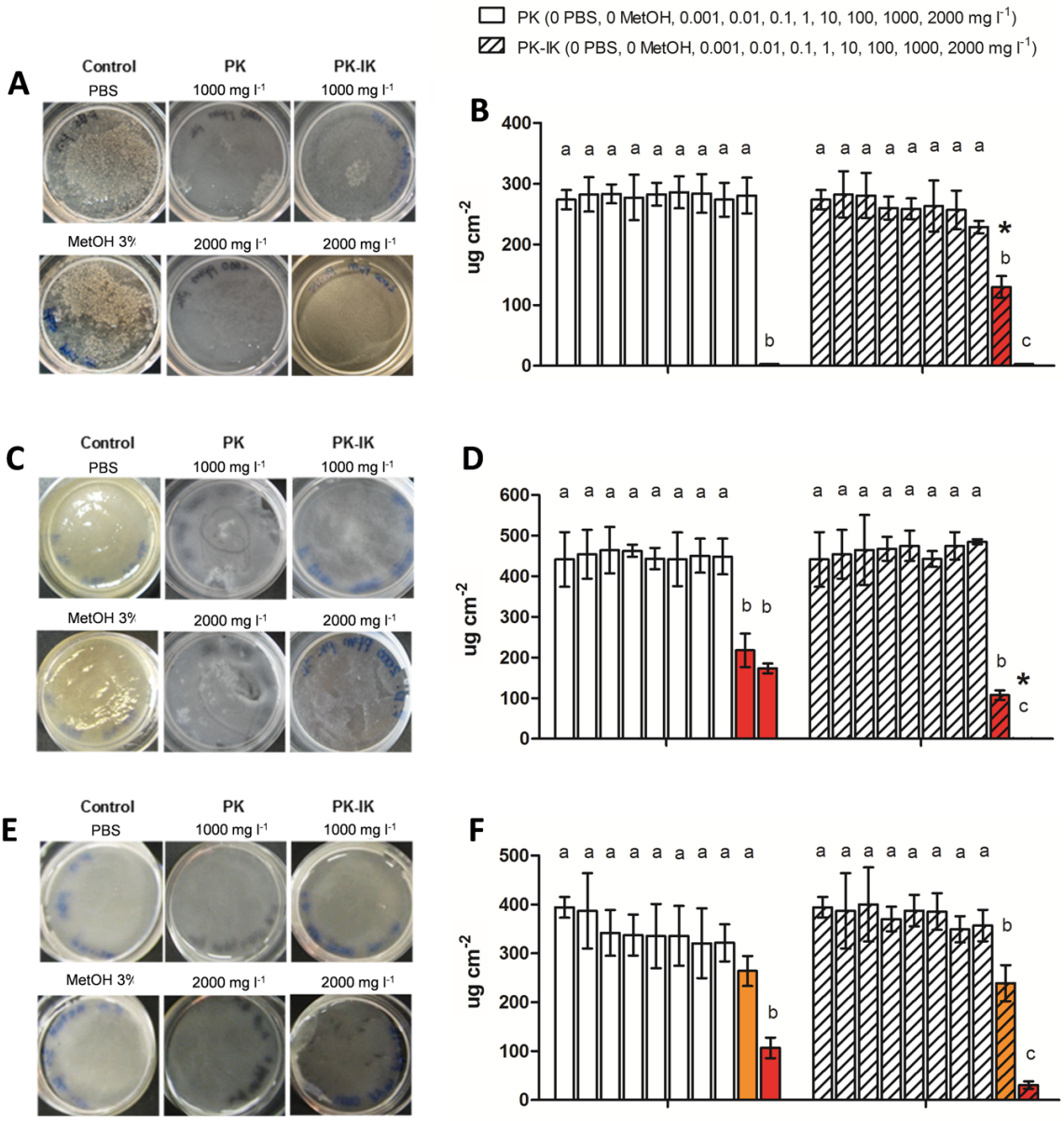
D

Conc. (mg l^{-1})	*C. musae*		*F. dimerum*		*F. oxysporum*	
	PK	PK-IK	PK	PK-IK	PK	PK-IK
0 (PBS)	-1.1±0.2 ^{a}	-1.1±1.5 ^{a}	-0.3±0.0 ^{a}	-0.3±0.0 ^{a}	4.1±0.8 ^{a}	4.1±0.8 ^{ab}
0 (MetOH)	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{ab}	0.0±0.0 ^{a}
0.001	-9.9±2.3 ^{ab}	-16.1±3.7 ^{ab}	-3.2±0.5 ^{ab}	3.4±0.8 ^{a}	-8.2±1.4 ^{ab}	-5.9±0.9 ^{a}
0.01	-14.3±2.6 ^{ab}	-26.0±6.4 ^{bc} *	-18.7±4.1 ^{ab}	-6.0±1.2 ^{a}	-3.4±0.7 ^{ab}	-25.5±5.2 ^{bc} *
0.1	-14.9±3.0 ^{ab}	-28.4±6.8 ^{bc} *	-15.8±3.4 ^{ab}	-24.3±3.4 ^{b}	-0.7±0.2 ^{ab}	-31.9±8.7 ^{c} *
1	-13.2±2.4 ^{ab}	-26.7±6.3 ^{bc} *	-17.8±3.7 ^{ab}	-29.0±5.6 ^{b}	-19.9±4.0 ^{bc}	-40.2±12.8 ^{cd} *
10	-10.8±1.5 ^{ab}	-27.4±6.3 ^{bc} *	-16.9±3.3 ^{ab}	-26.3±5.2 ^{b}	-43.4±8.1 ^{cd}	-42.0±13.4 ^{cd}
100	-15.7±3.4 ^{ab}	-31.2±8.2 ^{bcd} *	-23.9±6.2 ^{b}	-28.4±2.8 ^{b}	-54.1±11.2 ^{de}	-52.7±13.4 ^{de}
1000	-18.9±4.2 ^{ab}	-39.1±7.7 ^{cd} *	-82.3±9.7 ^{c}	-50.3±8.4 ^{c} *	-73.4±16.9 ^{e}	-61.6±15.2 ^{e} *
2000	-24.3±5.8 ^{b}	-44.4±6.2 ^{d} *	-80.3±16.2 ^{c}	-53.1±13.5 ^{c} *	-60.1±14.7 ^{de}	-49.2±10.9 ^{ce} *

Fig. 5. Conidia adhesion in the presence of *P. frutescens* essential oils. Number of adhered conidia of *C. musae* (A), *F. dimerum* (B) and *F. oxysporum* (C) in the presence of PK and PK-IK essential oils at different concentrations. Panel D shows the percentage reduction in comparison to the negative control prepared with the addition of 3 % MetOH. Data represent the mean ± standard deviation of at least four independent measurements. Different superscript letters statistically indicate significant differences (Tukey's HSD, $p \leq 0.01$) between the different concentrations, whereas an asterisk indicates a significant difference in the number of adhered conidia between PK and PK-IK at the same concentration. White: no effect; Yellow: low effect; Orange: moderate effect; Red: Excellent effect.

	Germinated conidia (%)					
Conc. (mg l^{-1})	*C. musae*		*F. dimerum*		*F. oxysporum*	
	PK	PK-IK	PK	PK-IK	PK	PK-IK
0 (PBS)	100.0±0.0 ^{a} (1.0±0.0)	100.0±0.0 ^{a} (1.0±0.0)	100.0±0.0 ^{a} (0.0±0.0)	100.0±0.0 ^{a} (0.0±0.0)	99.7±0.8 ^{a} (1.4±0.8)	99.7±0.8 ^{a} (1.4±0.8)
0 (3% MetOH)	99.0±1.7 ^{a} (0.0±1.7)	99.0±1.7 ^{ab} (0.0±1.7)	100.0±0.0 ^{a} (0.0±0.0)	100.0±0.0 ^{a} (0.0±0.0)	98.3±2.7 ^{a} (0.0±2.7)	98.3±2.7 ^{ab} (0.0±2.7)
0.001	100.0±0.0 ^{a} (1.0±0.0)	95.3±1.2 ^{ab} (-3.7±1.2)	100.0±0.0 ^{a} (0.0±0.0)	98.0±2.0 ^{a} (-2.0±2.0)	100.0±0.0 ^{a} (1.7±0.0)	99.3±1.2 ^{ab} (1.0±1.2)
0.01	100.0±0.0 ^{a} (1.0±0.0)	100.0±0.0 ^{ab} (1.0±0.0)	100.0±0.0 ^{a} (0.0±0.0)	100.0±0.0 ^{a} (0.0±0.0)	100.0±0.0 ^{a} (1.7±0.0)	100.0±0.0 ^{ab} (1.7±0.0)
0.1	91.3±6.1 ^{a} (-7.7±6.1)	98.7±1.2 ^{ab} (-0.3±1.2)	96.7±3.1 ^{a} (-3.3±3.1)	96.7±3.1 ^{a} (-3.3±3.1)	100.0±0.0 ^{a} (1.7±0.0)	98.0±2.0 ^{ab} (-0.3±2.0)
1	90.0±5.3 ^{ab} (-9.1±5.3)	99.3±1.2 ^{ab} (0.3±1.2)	95.3±2.3 ^{a} (-4.7±2.3)	98.7±1.2 ^{a} (-1.3±1.2)	100.0±0.0 ^{a} (1.7±0.0)	100.0±0.0 ^{ab} (1.7±0.0)
10	91.3±3.1 ^{ab} (-7.7±3.1)	95.3±3.1 ^{b} (-3.7±3.1)	94.0±4.0 ^{a} (-6.0±4.0)	96.0±2.0 ^{a} (-4.0±2.0)	98.0±2.0 ^{a} (-0.3±2.0)	95.3±4.2 ^{ab} (-3.1±4.2)
100	90.7±5.0 ^{ab} (-8.4±5.0)	88.0±2.0 ^{c} (-11.1±2.0)	96.0±4.0 ^{a} (-4.0±4.0)	98.7±1.2 ^{a} (-1.3±1.2)	96.7±1.2 ^{a} (-1.7±1.2)	94.0±2.0 ^{b} (-4.4±2.0)
1000	88.0±2.0 ^{b} (-11.1±2.0)	15.3±3.1 ^{d} * (-84.5±3.1)	81.0±2.2 ^{b} (-19.0±2.2)	15.3±2.0 ^{b} * (-84.5±2.0)	81.0±1.4 ^{b} (-17.6±1.4)	0.7±1.2 ^{c} * (-99.3±1.2)
2000	0.0±0.0 ^{c} (-100.0±0.0)	0.0±0.0 ^{e} (-100.0±0.0)	0.0±0.0 ^{b} (-100.0±0.0)	0.0±0.0 ^{c} (-100.0±0.0)	10.0±4.0 ^{c} (-89.8±4.0)	0.0±0.0 ^{d} * (-100.0±0.0)

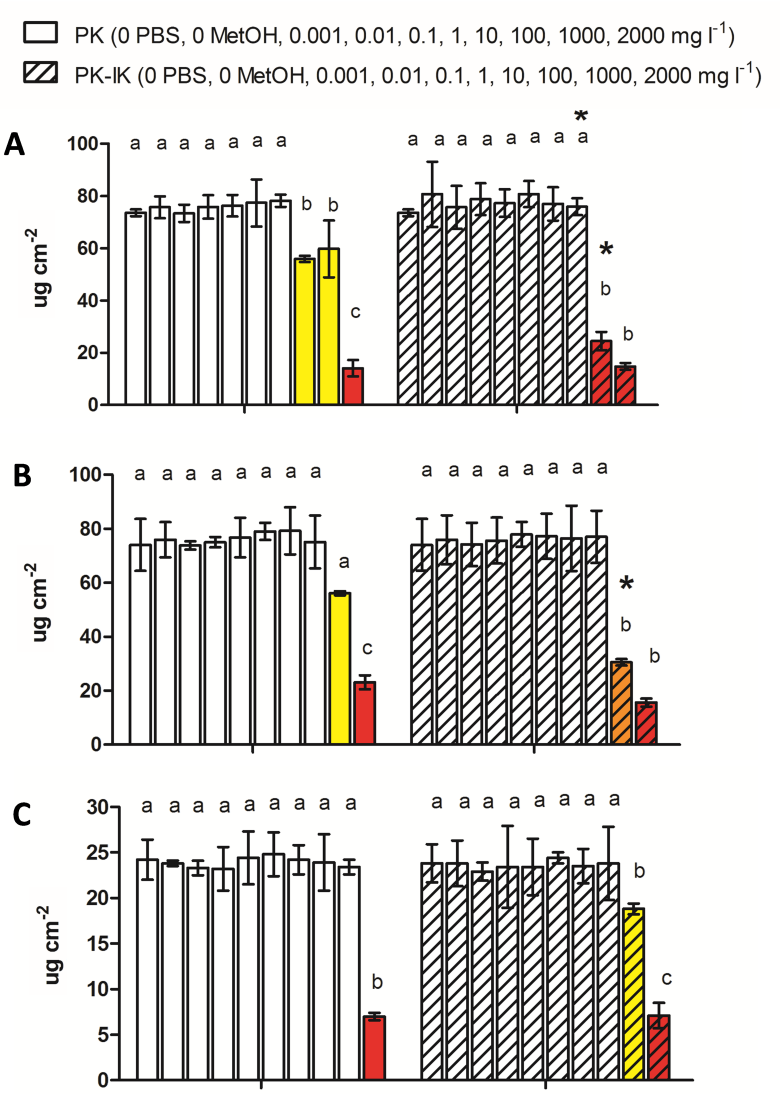
Fig. 6. Percentage of germinated conidia in the presence of *P. frutescens* essential oils. The percentage reduction, compared to the negative control prepared with the addition of 3 % MetOH, is reported in brackets. Data represent the mean ± standard deviation of at least three independent measurements. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.01$) between different concentrations, whereas an asterisk indicates a significative difference in the number of germinated conidia between PK and PK-IK at the same concentration. White: no effect; Yellow: low effect; Orange: moderate effect; Red: Excellent effect.



G

Conc. (mg l^{-1})	*C. musae*		*F. dimerum*		*F. oxysporum*	
	PK	PK-IK	PK	PK-IK	PK	PK-IK
0 (PBS)	-3.1±0.2 ^{a}	-3.1±0.2 ^{a}	-2.8±0.4 ^{a}	-2.8±0.4 ^{a}	1.9±0.1 ^{a}	1.9±0.1 ^{a}
0 (MetOH)	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}
0.001	0.3±0.0 ^{a}	-0.6±0.1 ^{a}	2.2±0.3 ^{a}	2.3±0.4 ^{a}	-11.7±1.6 ^{a}	3.4±0.7 ^{a}
0.01	-1.8±-0.2 ^{a}	-8.0±0.6 ^{a}	1.9±0.1 ^{a}	3.0±0.2 ^{a}	-12.8±1.6 ^{a}	-4.3±0.3 ^{a}
0.1	0.1±0.0 ^{a}	-8.4±0.6 ^{a}	-2.7±0.4 ^{a}	4.6±0.3 ^{a}	-13.3±2.6 ^{a}	0.1±0.0 ^{a}
1	1.2±0.1 ^{a}	-6.7±1.1 ^{a}	-2.3±0.1 ^{a}	-2.5±0.1 ^{a}	-13.2±2.4 ^{a}	-0.4±0.0 ^{a}
10	0.6±0.1 ^{a}	-9.1±1.1 ^{a}	-0.8±0.1 ^{a}	4.6±0.3 ^{a}	-17.1±3.8 ^{a}	-9.7±0.7 ^{a}
100	-3.1±0.3 ^{a}	-19.0±0.9 ^{a}	-1.2±0.1 ^{a}	6.8±0.1 ^{a}	-16.9±2.0 ^{a}	-7.8±0.7 ^{a}
1000	-0.6±0.1 ^{a}	-54.0±7.5 ^{b} *	-52.1±5.3 ^{b}	-76.4±4.4 ^{b}	-31.8±3.7 ^{a}	-38.2±5.9 ^{b}
2000	-100.0±0.0 ^{b}	-100.0±0.0 ^{c}	-61.9±4.3 ^{b}	-100.0±0.0 ^{c} *	-72.4±14.1 ^{b}	-92.1±13.4 ^{c}

Fig. 7. Biofilm biomass in the presence of *P. frutescens* essential oils. Biofilm grown in semi-batch conditions in the presence of essential oils. A naked-eye visible difference is shown between the control and treated samples: A: *C. musae*; C: *F. dimerum*; E: *F. oxysprum*. Dry weight of biofilm of *C. musae* (B), *F. dimerum* (D), *F. oxysprum* (F) and their percentage reduction in comparison to the negative control prepared with the addition of 3 % MetOH (G). Data represent the mean ± standard deviation of at least three independent measurements. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.01$) between different concentrations, whereas an asterisk indicates a significative difference in the dry biomass between PK and PK-IK at the same concentration. White: no effect; Yellow: low effect; Orange: moderate effect; Red: Excellent effect.

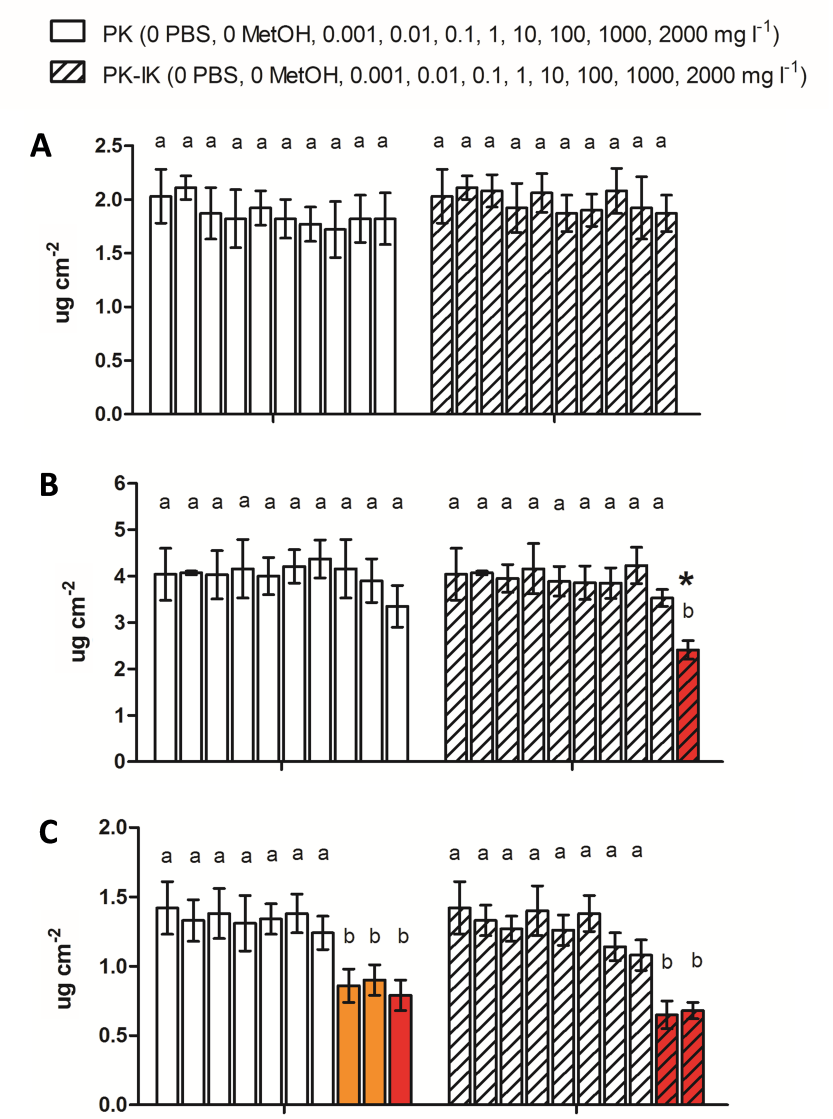


D

Conc. (mg l^{-1})	*C. musae*		*F. dimerum*		*F. oxysporum*	
	PK	PK-IK	PK	PK-IK	PK	PK-IK
0 (PBS)	-2.8±0.0 ^{a}	-2.8±0.0 ^{a}	-2.5±0.3 ^{a}	-2.5±0.3 ^{a}	1.8±0.2 ^{a}	0.1±0.0 ^{a}
0 (MetOH)	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}
0.001	-3.1±0.1 ^{a}	4.0±0.4 ^{a}	-2.7±0.1 ^{a}	-2.1±0.2 ^{a}	-2.1±0.1 ^{a}	-3.7±0.2 ^{a}
0.01	0.1±0.0 ^{a}	2.1±0.2 ^{a}	-1.2±0.0 ^{a}	-0.4±0.0 ^{a}	-2.6±0.3 ^{a}	-1.4±0.3 ^{a}
0.1	0.8±0.0 ^{a}	2.1±-0.1 ^{a}	4.1±0.2 ^{a}	2.7±0.2 ^{a}	2.6±0.3 ^{a}	-1.8±0.2 ^{a}
1	2.3±0.3 ^{a}	6.6±-0.4 ^{a}	1.1±0.1 ^{a}	1.8±0.2 ^{a}	4.0±0.4 ^{a}	2.5±0.1 ^{a}
10	3.2±0.1 ^{a}	1.6±0.1 ^{a}	4.4±0.5 ^{a}	0.7±0.1 ^{a}	1.6±0.1 ^{a}	-1.2±0.1 ^{a}
100	-26.1±0.6 ^{b}	0.3±0.0 ^{a} *	-1.0±0.1 ^{a}	1.5±0.2 ^{a}	0.7±0.1 ^{a}	-0.1±0.0 ^{a}
1000	-21.1±3.8 ^{b}	-67.6±9.7 ^{b} *	-26.0±0.3 ^{b}	-59.8±2.4 ^{b} *	-1.6±0.1 ^{a}	-20.8±0.7 ^{b}
2000	-81.4±8.0 ^{c}	-80.4±7.0 ^{b}	-69.5±8.0 ^{c}	-79.4±7.8 ^{b}	-70.5±4.4 ^{b}	-70.0±3.7 ^{c}

Fig. 8. EPS polysaccharide content in the presence of *P. frutescens* essential oils. Polysaccharides in the matrix of *C. musae* (A), *F. dimerum* (B) and *F. oxysporum* (C) biofilms grown in the presence of PK and PK-IK essential oils at different concentrations. Panel D shows the percentage reduction in comparison to the negative control prepared with the addition of 3 % MetOH. Data represent the mean ± standard deviation of at least three independent measurements. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.01$) between different concentrations, whereas

an asterisk indicates a significative difference in the dry biomass between PK and PK-IK at the same concentration. White: no effect; Yellow: low effect; Orange: moderate effect; Red: Excellent effect.



D

Conc. (mg l^{-1})	*C. musae*		*F. dimerum*		*F. oxysporum*	
	PK	PK-IK	PK	PK-IK	PK	PK-IK
0 (PBS)	-3.7±0.2 ^{a}	-3.7±0.5 ^{a}	-0.8±0.1 ^{a}	-0.8±0.1 ^{a}	6.7±0.8 ^{a}	6.7±0.8 ^{a}
0 (MetOH)	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}
0.001	-11.1±1.4 ^{a}	-1.2±0.1 ^{a}	-1.0±0.1 ^{a}	-2.9±0.2 ^{a}	3.9±0.3 ^{a}	-3.9±0.5 ^{a}
0.01	-13.6±2.1 ^{a}	-8.8±1.1 ^{a}	2.2±0.3 ^{a}	2.2±0.3 ^{a}	-1.3±0.2 ^{a}	5.9±0.9 ^{a}
0.1	-8.6±0.7 ^{a}	-2.3±0.2 ^{a}	3.5±0.3 ^{a}	-4.4±0.4 ^{a}	1.3±0.1 ^{a}	-5.3±0.4 ^{a}
1	-13.6±1.4 ^{a}	-11.1±1.0 ^{a}	-1.6±0.2 ^{a}	-5.1±0.5 ^{a}	3.9±0.4 ^{a}	3.9±0.4 ^{a}
10	-16.0±1.5 ^{a}	-9.9±0.8 ^{a}	7.3±0.7 ^{a}	-5.3±0.5 ^{a}	-6.5±0.6 ^{a}	-13.7±1.3 ^{a}
100	-18.5±2.8 ^{a}	-1.2±0.1 ^{a}	2.2±0.3 ^{a}	4.0±0.4 ^{a}	-35.3±3.2 ^{b}	-18.6±2.8 ^{a}
1000	-13.6±1.6 ^{a}	-8.6±1.3 ^{a}	-4.2±0.5 ^{a}	-13.3±0.7 ^{a}	-32.5±1.7 ^{b}	-51.0±6.1 ^{b}
2000	-13.6±1.8 ^{a}	-11.1±1.0 ^{a}	-17.6±2.4 ^{a}	-40.7±3.4 ^{b} *	-40.3±3.3 ^{b}	-49.0±6.6 ^{b}

Fig. 9. EPS protein content in the presence of *P. frutescens* essential oils. Proteins in the matrix of *C. musae* (A), *F. dimerum* (B) and *F. oxysporum* (C) biofilms grown in the presence of PK and PK-IK essential oils at different concentrations. Panel D shows the percentage reduction in comparison to the negative control prepared with the addition of 3 % MetOH. Data represent the mean ± standard deviation of at least three independent measurements. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.01$) between different concentrations, whereas an asterisk indicates a significative difference in the dry biomass between PK and PK-IK at the same concentration. White: no effect; Yellow: low effect; Orange: moderate effect; Red: Excellent effect.

CHAPTER V

N-Acetylcysteine promotes in vitro biofilm formation but limits the spread of infection of the phytopathogen *Xylella fastidiosa*

Article submitted to a peer-reviewed international journal

Abstract

Xylella fastidiosa is a phytopathogen bacterium that causes a range of economically important plant diseases. Recently it has been found in Italy in olive plants, where it causes the olive quick decline syndrome (OQSD). In this study, N-Acetylcysteine (NAC) was tested for its ability to affect biofilm response of *X. fastidiosa* CoDiRO strain, mimicking a preventive, a curative and a combination of both approaches. The not-lethal dose 0.08 mg/ml was chosen as representative of plant concentration after its application. NAC did not alter planktonic bacterial growth but promoted biofilm formation in terms of biofilm biomass (above 62 %) and matrix polysaccharides (above 53%) through a ROS-mediated mechanism. Additionally, NAC was not able to destroy *X. fastidiosa* biofilm when already established on the surface but rather, it was suitable to contain the biofilm infection limiting biofilm dispersal.

Introduction

Starting from 2010, the olive-growing area of the Apulia region, a South-Est district of the Italian country producing roughly 40% of the national olive oil, has been seriously affected by a phytodisease named olive quick decline syndrome (OQSD) (Martelli et al., 2016). OQDS disease is characterized by the appearance of severe leaf scorch and desiccation of branches and twigs, initially localized, but which rapidly extend to the entire crown, leading to the death of the olive plants (Saponari et al., 2013; Martelli et al., 2016). Millions of dollars in damage to the olive industry has been estimated due to the loss of approximately 10,000 ha of olive trees (Martelli et al., 2016; Strona et al., 2017). Unfortunately, with the available control strategies, OQSD infected plants eradication seems not achievable in the Apulia region due to the geographical distribution of the olive orchards and the widespread outbreak (Strona et al., 2017). Analysis on several symptomatic plant samples (olives, oleanders and almonds) has led to the association of OQDS with *Xylella fastidiosa* (Saponari et al., 2013; Cariddi et al., 2014). This bacterium is known worldwide due to the serious and worrisome diseases that causes in several economically important crops (Wells et al., 1987; Chang et al., 2009; Martelli et al., 2016) and therefore it is listed in the EPPO A1 quarantine pathogens (EPPO/OEPP 1992). Multilocus sequence analysis revealed that the Italian strain (referred to as CoDiRO or ST53) is genetically related to the *pauca* subspecies (Nunney et al., 2014; Marcelletti et al., 2016; Giampetruzzi et al., 2017), a variant apparently identical to the strain found in Costa Rica (Loconsole et al., 2014). Indeed, it was probably introduced in Italy with imported plant material. This is also supported by the interception in Netherlands (October 2014, Netherlands Food and Consumer Product Safety Authority) of ornamental coffle plants coming from Costa Rica and containing *X. fastidiosa* (Martelli et al., 2016; Bergsma-Vlami et al., 2017). Recently, olive orchids located in Argentina and in Brazil have displayed identical symptoms to those observed in Southern Italy.

Further analysis on infected plant samples revealed the presence of *X. fastidiosa* strains belonging to subp. *pauca*, but they are genetically different from the Italian one (Haelterman et al., 2015; Coletta-Filho et al., 2016). *X. fastidiosa* symptoms are associated with the extensive bacterial colonization of the xylem vessels through the formation of biofilm, clusters of surface-associated cells embedded in a self-produced polymeric matrix (Costerton., 2007). These aggregates cause the occlusion of xylem sap transport leading to water and mineral nutrients deficits to the plant canopy (Krivanek and Walker, 2005; De La Fuente et al., 2013). Biofilm condition is mandatory for the acquisition, the multiplication, the host (plant and insect) colonization and the disease development (Brlansky et al., 1983; Hill and Purchell, 1997; Chatterjee et al., 2008). So far, several strategies to control *X. fastidiosa* disease have been proposed to reduce the spread of the pathogen. Some of them have been directed on the reduction of vector population through insecticide application into plant nurseries or vineyard (Purchell, 1979; Krewer et al., 2012). Others have been focused on the production of transgenic plants (Aguero et al., 2005) or on the induction of a plant defense response (Dandekar et al., 2012). Further studies have been assessed directly on the pathogen, trying to reduce its movement within the plant (Lindow et al., 2014) or evaluating the *in vitro* antibacterial activity of different molecules (Kuzina et al., 2006; Amanifar et al., 2016; Bleve et al., 2018). Strona et al. (2017) reported that OQSD containing measures based mainly on host plant and vector control are doubtful due to the copiousness of *Philaenus spumarius,* the main vector related to *X. fastidiosa* transmission (Saponari et al., 2014), and other potential vectors in the area. Indeed, even if the problem could be rapidly solved using huge amount of insecticides, the massive spread of these substances would lead to unpredictable and critic environmental impacts, beside the risk to jeopardize the quality of olive crops (strona et al., 2017). Muranaka et al. (2013) and, more recently, Picchi et al. (2016) reported promising results in the treatment of *X. fastidiosa* and other phytopathogen bacteria with N-Acetylcysteine (NAC). NAC is a thiol compound commonly found in *Allium* plants and considered responsible, at least in part, for onion and garlic beneficial effects in warding off illnesses and cardiovascular protection (Souza et al., 2011; Nissar et al., 2013; Korou et al., 2014). Approved by FDA and with an excellent safety profile, it is largely used in clinical treatments against a broad range of pathologies, including pulmonary diseases, hepatic disorders and drug overdose treatments (Costa et al., 2017). Indeed, in the treatment of human diseases, NAC has shown antibiofilm activity, reducing bacterial adhesion, inhibiting the production of extracellular polysaccharides and promoting the dispersion of mature biofilms (Dinicola et al., 2014; Blasi et al., 2016). In Muranaka et al. (2013) lethal doses of NAC were tested *in vitro* and *in planta* against the *X. fastidiosa* subsp. *pauca* strain 9a5c that is the etiological agent of the Citrus Variegated Chlorosis (CVC) in Brasil. The Brazilian group reported that 6 mg/ml of NAC was the minimal inhibitory concentration (MIC) and starting from 1 mg/ml there was a reduction on bacterial adhesion, biofilm formation and exopolysaccharides production. Pesticides are generally employed at high concentrations to be capable of killing cells, but, downstream of the treated application point, there is a decrease in the concentration of the biocidal compound that goes from the applied concentration to zero, leading to sub-inhibitory levels Gilbert and McBain, 2003). Indeed, several factors such as the environmental conditions, the plant species and the physicochemical properties of the chemicals influence the plant uptake, making difficult the prediction of the uptake rate of a compound Behrendt and Brüggeman, 1993; Wang and Liu, 2006). For instance, Pimentel (1995) reported that only 0.1% of the total amount of pesticides applied as spray application for the control of

weed and pest reached the sites of action, losing the larger portion due to off-target deposition, photodegradation etc.

In this study, for the first time, NAC was tested *in vitro* on *X. fastidiosa* CoDiRO strain in order to evaluate its effect on biofilm formation at a non-lethal concentration. This paper aimed to advance the current state of art mainly related to the lethal effect of this compound, suggesting a possible situation within the plant where NAC could be present at a non-biocidal concentration. In the design of the experimental plan, NAC was tested by simulating different conditions (preventive, curative or both approaches) with the aim of evaluating its effects on different steps involved in the biofilm formation of *X. fastidiosa* CoDiRO strain.

Methods

Bacterial strain and culture conditions. *X. fastidiosa* CoDiRO strain subsp. *pauca* was originally isolated from olive plants (*Olea europea var. olgliarola*) (Saponari et al., 2013) affected by the olive quick decline syndrome (OQDS) grown in Agro di Gallipoli (Apulia region, Lecce, Italy). Bacteria were grown in Buffered Charcoal Yeast Extract (BCYE) agar medium (Wells et al. 1981) and maintained at 28°C. The strain was stored at -80°C in 25% glycerol and was routinely grown on PD3 medium for 8-10 days at 28°C (Davies et al. 1980) [2].

Chemical compound. N-acetylcysteine (NAC) was purchased from Sigma-Aldrich (A7250, ≥99% purity grade) and was dissolved in deionized water at room temperature to a concentration of 0.8 mg/ml. After adjusting pH to 6.8 with sodium hydroxide (1M, NaOH), the stock solution was sterilized by filtration through a 0.22 μm filter and diluted in sterile deionized water to a final concentration of 0.08 mg/ml.

Toxicity assay in presence of NAC. The ability of *0.08 mg/ml of NAC to* inhibit the planktonic growth of *X. fastidiosa* was investigated as previously reported by Kandel et al. (2017) with some modification. Briefly, *X. fastidiosa* colonies grown on PD3 agar plates for 8-10 days at 28°C were scraped and resuspended in one mL of PD3 broth. Ten μL of cell suspension were diluted in 200 μl of PD3 (initial optical density at 600 nm of 0.04) supplemented with 0 (negative control) and 0.08 mg/ml of NAC and were placed in transparent 96-well polystyrene-based microtiter plates (Thermo Fisher Scientific). The plates were incubated at 28 °C, with shaking at 150 rpm/min. Growth curves were generated by measuring the optical density at 600 nm (OD_{600}) every 24 h for 7 days using an Infinite F200 PRO microplate reader (TECAN, Mannedorf, Switzerland). The polynomial Gompertz model [3] was used to fit the growth curves and the maximum specific growth (OD_{600}/day) was calculated using the GraphPad Prism software (version 5.0, San Diego, CA, USA). Experiments were repeated independently three times with at least six technical replicates per time.

Solid/air interface biofilm growth. *X. fastidiosa* biofilm was grown on a transwell device as previously described by Garuglieri et al. (2018) with some modification. Briefly, *X. fastidiosa* colonies grown on PD3 agar plates were scraped and resuspended in 2 mL of PD3 broth without (-) and with the addition

of of 0.08 mg/ml of NAC (NAC). After 24 h of incubation at 28°C under agitation at 140 rpm, 50 μl of bacterial broth culture (0.5 optical density at 600 nm) were inoculated at the center of a sterile polycarbonate membrane (PC, Whatman Nucleopore, diameter 2.5 cm, pore diameter 0.2 μm) and, once the inoculum was completely dried, the membrane was carefully transferred inside a transwell device (ThinCertTM Cell Culture Inserts with translucent PET membrane – Greiner bio-one) inlaid in a 6 well culture plate (Greiner bio-one). One ml of PD3 medium without (-) and with the addition of 0.5 mM of NAC was added to inoculated the plate well. Mediums were replaced every 24 h. Biofilms were grown at 28°C for 7 days.
X. fastidiosa biofilm was growth in four different conditions: 1) planktonic cell pre-treatment and biofilm growth without NAC (named **-/-**), negative control; 2) planktonic cell pre-treatment with 0.08 mg/ml NAC and biofilm growth without NAC (named **NAC/-**), preventive treatment; 3) no planktonic cell pre-treatment and biofilm growth with 0.08 mg/ml NAC (named **-/NAC**), curative treatment 4) planktonic cell pre-treatment and biofilm growth with 0.08 mg/ml of NAC (named **NAC/NAC**), combined treatment. After 7 days, biofilms obtained from each treatment were analyzed for their biomass, extracellular polymeric substances (EPS) composition and intracellular and extracellular reactive oxygen species (ROS) and propensity to be detached.

Biofilm biomass quantification. Biofilm biomass was evaluated measuring the amount of cellular proteins (Zubkov et al. 1999; Azeredo et al. 2016). Briefly, two membranes for each treatment were transferred in 1 ml of PBS and biofilm was removed from the membrane surface by 1 min vortex mixing, 2 min sonication (50% amplitude, in waterbath; Branson 3510, Branson Ultrasonic Corporation, Dunburry, CT) followed by another 1 min of vortex mixing. Membranes were subsequently removed from the suspension and cells were broken by sonication (three cycles of 30 s at 40% power sonication with 15 s intervals; Branson 3510, Branson Ultrasonic Corporation, Dunburry, CT) followed by centrifugation (15 min at 4 °C at 8,000 rpm). The supernatant was collected and the amount of proteins was quantified by the Bradford assay (Bradford,1976) using bovine serum albumin as a standard. Obtained data were normalized against the membrane area, and the means were reported. The experiments were repeated three times with at least three technical replicates.

Live/dead biofilm assay. Seven-day biofilms grown on the membrane in the transwell devices were transferred in 1 ml of milli-Q water. Biofilms were dislodged from the membranes and cells were broken as reported in the section 'Biofilm biomass quantification'. The Live/Dead BacLight viability kit (Molecular Probes–Life Technologies, Carlsbad, CA, USA) was used to detect live and dead cells according to the manufacturer's instructions. The fluorescence intensity was measured using the Infinite 200 PRO Microplate Reader (Tecan) with excitation at 480 nm and emission at 516 nm for the live green cells, and excitation at 581 nm and emission at 644 nm for the red dead cells. Fluorescence intensity was normalized by the proteins within the biofilm, divided for the area of the membrane, and the means were reported. Relative viability within the biofilms was calculated by dividing the fluorescent intensity of normalized live cells by the fluorescent intensity of normalized dead cells in each sample. Experiments were repeated three times with at least six technical replicates per time.

Extraction and characterization of the extracellular polymeric substances (EPS). Seven day-biofilms from each treatment were analyzed for their extracellular proteins and polysaccharides content. EPS extraction was performed as reported by Villa et al. (2012). Briefly, three membranes for each treatment were transferred in 2 ml of 2% ethylenediaminetetraacetic acid (EDTA) and biofilm was dislodged from membranes by vortex mixing and sonication as described above in the section 'Biofilm biomass quantification'. After 3 h of incubation at 4°C under agitation of 300 rpm, biofilm cell suspensions were centrifuged for 20 min at 13.000 rpm at 4°C to separate EPS from cells and the supernatant was filtered (0.22 um pores size). The Bradford (1976) and the phenol– sulfuric acid (Masuko et al. 2005) methods were applied for quantification of proteins and polysaccharides using bovine serum albumin and glucose as standard respectivly. Obtained data were normalized by the proteins within the biofilm, divided for the area, and means were reported. Experiments were repeated three times with at least six technical replicates per time.

Oxidative stress assay. Seven day-biofilms from each treatment were transferred in 1 ml of 50 mM PBS and biofilm was detached from the membranes by vortex mixing and sonication as described above in the section 'Biofilm biomass quantification'. The suspension was centrifuged for 20 min at 13,000 rpm. The supernatant was transferred in another tube, filtered (0.22 pores size) and analyzed for the extra-cellular ROS content. The pellet was resuspended in 50 mM PBS, cells were broken by sonication as described previously in the section 'Biofilm biomass quantification', and the intra-cellular ROS content was quantified. Intra- and extra-cellular oxidative stresses were quantified using the ROS sensitive probe 2,7-dichlorofluorescein-diacetate according to Jakubowski et al. (2000). Fluorescence was measured using a microplate reader (TECAN, Manneford, Switzerland) at excitation wavelength of 485 nm and emission wavelength of 535 nm. Obtained data were normalized by the proteins within the biofilm, divided for the area, and means were reported. Experiments were repeated three times with at least five technical replicates per time.

Biofilm dispersion. Membranes with biofilms pre-grown for 7 days without or with NAC were transferred in petri dishes (ø 60 mm) and incubated with 2 ml of Phosphate buffered saline solution (PBS, Sigma-Aldrich, pH 6.8 adjusted by potassium hydroxide) or 0.08 mg/ml NAC for further 24 h at 28°C. Subsequently, biofilms on the membranes and those dislodged in the bulk liquid were analyzed for their biomass and the level of ROS.

Membranes with biofilms for each treatment were transferred in 1 ml of PBS. Biofilms were detached from the membranes by vortex mixing and sonication, cells were broken and centrifuged as described above in the section 'Biofilm biomass quantification'. Proteins amount was quantified by Bradford assay as described in the section 'Biofilm biomass quantification' and data were normalized by the membrane area. The amount of ROS within the biofilm was performed as reported in the section 'Oxidative stress assay'. ROS data were normalized by the proteins within the biofilm, divided for the area of the membrane, and means were reported. Experiments were repeated independently three times with at least four technical replicates per time.

Planktonic cells and the bulk media were divided by centrifugation at 8,000 rpm for 20 min. The cellular component in the pellet was resuspended in 1 ml of PBS and then analyzed for the biomass and ROS

content as for the biofilm on the membrane. The supernatant was filtered by 0.22 μm pores filter and the ROS content was performed to analyze the outer ROS content as reported in the section 'Oxidative stress assay'. ROS data were normalized by the proteins within the biofilm, divided for the area, and means were reported.
The detachment index was calculated as: (protein amount in the detached biofilm x 100)/ (protein amount detached biofilm + protein amount in the biofilm on the membrane). Experiments were repeated independently three times with at least four technical replicates per time.

Statistical analysis.
Two-tailed ANOVA and Student's t-test analysis, via a software run in MATLAB environment (Version 7.0, The MathWorks Inc., Natick, USA), were applied to statistically evaluate any statistically significant differences among the samples. ANOVA and Student's t-test analysis were carried out after verifying data independence (Pearson's chi-square test), normal distribution (D'Agostino-Pearson normality test) and homogeneity of variance (Bartlett's test). Tukey's honestly significant different test (HSD) was used for pairwise comparison to determine the significance of the data. Statistically significant results were depicted by p-values ≤ 0.05.

Results

NAC does not affect *X. fastidiosa* planktonic growth
Figure 1 shows the 7 day-growth curves of *X. fastidiosa* in presence and absence of 0.08 mg/ml NAC. Results indicate no statistically significant differences on the maximum specific growth rate (OD_{600}/day) between the NAC treated sample (0.031 ± 0.006; R^2= 0.964 ± 0.026) and the control sample (0.032 ± 0.002; R^2= 0.981 ± 0.010). Therefore, 0.08 mg/ml of NAC was considered a non-lethal concentration and used in the subsequent studies.

NAC does not affect viability of cells within the biofilm
The Live/Dead viability assay has been performed to verify the ability of NAC to affect biofilm viability. Relative viability calculated for each condition revealed no statistically significant differences among samples (Fig. 2).

NAC treatment increases biofilm biomass
NAC effects on *X. fastidiosa* biofilm biomass were assessed by quantifying the amount of proteins within the biofilm. Data showed that NAC significantly increased the amount of biofilm proteins in all the treatments in comparison to the control (Fig. 3), highlighting the maximum increase for the treatment NAC/NAC (78.1 ± 8.7%) and -/NAC (62.3 ± 5.9%), followed by NAC/- (22.3 ± 2.6%).

NAC affects the composition of the extracellular polymeric substances (EPS)
In figure 4a quantification of matrix polysaccharides is reported. Experiments revealed that NAC/- and -/NAC were statistically significantly different compared to the control -/-, with the maximum increase

for the treatment NAC/- (87.1 ± 21.6%), followed by -/NAC (53.1 ± 8.1%). With regard of the treatment NAC/NAC no statistically significant changes were detected in comparison to the control.
Figure 4b shows matrix protein amount. Graphs show a statistically significant reduction in the amount of proteins in all the treatments, with the highest reduction for the treatment NAC/NAC (91.4 ± 5.3%), followed by -/NAC (73.7 ± 15.1%) and NAC/- (31.4 ± 13%).

NAC affects the oxidative stress within the biofilm

Fig. 5 shows that biofilms exposed to NAC were more prone to accumulate ROS, in both intracellular and EPS matrix.
Intracellular levels of ROS (Fig. 5a) showed the highest statistically significant increase compared to the control -/- in the treatment NAC/NAC (3.6 ± 0.1-fold), followed by NAC/- (2.9 ± 0.7-fold) and -/NAC (2.6 ± 0.8-fold).
Inside the EPS (Fig. 5b), a statistically significant increase of ROS level compared with the control was observed in all treatments, with the highest value reported for NAC/- (1.5 ± 0.5-fold) and NAC/NAC (1.4± 0.5-fold), followed by -/NAC (1.4 ± 0.6-fold). No statistically significant differences were highlighted between the treatments.

NAC affects biofilm detachment

With the aim to evaluate the biofilm detachment by NAC, biofilm biomass on the membranes and those dislodged in the bulk liquid after a 24-h further treatment with PBS or NAC were analyzed in terms of protein quantification.
Biofilms remained on the membrane and pre-grown in condition -/- and -/NAC displayed a statistically significant increase in the protein amount when further treated for 24 h with NAC in comparison to the 24 h-treatment with PBS (3.1 ± 0.04-fold and 1.6 ± 0.08-fold respectively) (Fig. 6a). On the contrary, biofilm pre-grown in condition NAC/NAC displayed an increase of proteins when further treated for 24 h with PBS instead of NAC (-1.5 ± 0.2-fold) (Fig. 6a). With regard to biofilm pre-grown in condition NAC/-, no statistically significant changes were detected after a further treatment with PBS or NAC (Fig. 6a).
Biofilm in the bulk liquid revealed significant higher amount of proteins when pre-grown under conditions -/-, NAC/- and -/NAC and further treated with PBS instead of NAC (-1.9 ± 0.2-fold, -1.5 ± 0.1-fold and -1.3 ± 0.1-fold respectively) (Fig. 6b). On the contrary, when biofilm was pre-grown in condition NAC/NAC, proteins in the bulk liquid were significant higher when biofilm was further treated with NAC than after the treatment with PBS (1.9±0.1-fold).
When biofilm was pre-grown under condition -/- the further 24 h-treatment with NAC decreased the detachment index in comparison to the treatment with PBS (-3.0 ± 0.004-fold) (Fig. 7). No statistically differences were found in the detachment index when biofilm was pre-grown under condition -/NAC and further treated with PBS or NAC. On the contrary, when biofilm was pre-grown with -/NAC and NAC/NAC and further exposed to NAC for 24 h, the detachment index increased in comparison to the treatment with PBS alone (1.2 ± 0.006-fold and 2.2 ± 0.001-fold respectively). No statistical differences

were found in the detachment index when biofilm was pre-grown under condition -/NAC and further treated with PBS or NAC.

NAC affects the level of ROS after biofilm detachment

The level of ROS was quantified in the biofilm remained on the membrane, in the biofilm detached from the membrane and in the bulk liquid medium. Data revealed that mature biofilms remained on the membrane (Fig. 8a) and exposed to NAC were more prone to accumulate ROS in comparison to the corresponding ones treated with PBS, with the maximum increase in the treatment -/- (3.3 ± 0.7-fold), followed by -/NAC (1.6 ± 0.2-fold), NAC/NAC (1.6 ± 0.2-fold) and NAC/-(1.2 ± 0.2-fold). Biofilm in the recovered bulk displayed the same trend, but with an increase much higher in comparison to the negative controls treated with PBS (Fig. 8b). Indeed, the highest result was obtained for the treatment -/NAC (30.1 ± 5.8-fold), followed by NAC/NAC (14.5± 0.4-fold), **-/-** (9.0 ± 1.1-fold) and NAC/- (8.0 ± 1.5-fold). Data of level of ROS in the bulk liquid reported the highest statistically significant increase in comparison to the negative controls for the treatment **-/-** (67.2 ± 2.2-fold), followed by NAC/- (44.5 ± 8.6-fold), -/NAC (43.1 ± 5.9-fold) and NAC/NAC (28.8 ± 0.8-fold) (Fig. 8c).

Discussion

In the past, NAC has been proposed as a promising treatment against *X. fastidiosa* infections. Muranaka et al., (2013) proved that concentrations of NAC higher than 1.0 mg/mL significantly decreased *X. fastidiosa* biofilm formation and the amount of EPS with a significant increase in the planktonic biomass fraction. Indeed, the majority of the cells in the biomass were dead, and this response was dose-dependent with no viable cells being observed with 6 mg/mL of NAC. However, concentrations reported in these studies are much higher than those effectively achievable in plants where exposure is likely at lower concentrations.

Here the anti-biofilm effect of NAC was evaluated at a non-lethal concentration, mimicking the real plant exposure. Indeed, it is reported that plants absorbed NAC when added in the soil only at concentration between 0.48 and 2.4 mg/ml and that the maximal drug uptake by plant ranged between the 13 and 33 % (Muranaka et al., 2013). Therefore, 0.08 mg/ml NAC has been chosen in this work as representative of plant concentration after its application. Notably, concentrations of NAC lower than 1 mg/ml have been previously considered non-lethal for *X. fastidiosa* (Muranaka et al., 2013).

Considering the sudden outbreak of OQSD in the Southern Italy, in this research a preventive, a curative and a combination of both approaches by NAC has been mimicked against the *X. fastidiosa* CoDiRO strain. Indeed, NAC has been used to both treat planktonic cells upon their attachment (NAC/-) as well as cells during biofilm formation (-/NAC) or a combination of both (NAC/NAC). Moreover, a transwell membrane-supporting biofilm reactor was employed to simulate in vivo conditions and grow a mature biofilm at the solid/liquid/air interface (Kim et al., 2012).

Experiments showed that NAC significantly increased biofilm biomass above 62 % when added during biofilm formation whereas a weak increase up to 22 % was found when it was used as preventive approach to pre-treat planktonic growth upon cell attachment onto the surface. Moreover, biofilms

exposed to NAC were more prone to accumulate ROS, in both intracellular and extracellular biofilm portions.

Beside NAC has been used in antioxidant therapies, there is evidence that it may also have oxidant effects. In vitro study revealed that the oxidative metabolism of NAC can generate thiyl free radicals and can increase hydroxyl/free radical production via the Fenton reaction by reducing Fe^{+3} ions to Fe^{+2} (Sagristà et al., 2002; Abu-Kishk et al., 2010). Therefore, NAC lead cells to an oxidative imbalance also altering the Fe^{+3}/Fe^{+2} ratio. Notably, in bacteria, DNA damage from hydroxyl radicals generated by the Fenton reaction is a primary mechanism of cytotoxicity (Gusarov et al., 2005).

In *X. fastidiosa*, ROS act as an environmental cue that stimulates biofilm development during the early stages of plant colonization (Wang et al., 2016). Indeed, the experience of the oxidative stress is required by *X. fastidiosa* to achieve maximal xylem colonization (Wang et al., 2016). The *X. fastidiosa* entry in the biofilm mode is linked to the sensing of oxidative stress via OxyR, a redox-sensing transcription factor which is involved in xylem adhesion during early bacterial establishment in planta (Federici et al., 2012; Wang et al., 2016). Notably, in *X. fastidiosa*, biofilm formation is an inducible oxidative stress defence response that allow bacterial survival in the xylem environment, where there is an acute and prolonged production of ROS from a variety of sources, including the plant innate immune system and plant developmental processes (Gansert, 2003; Toledo et al., 2011; Villa et al., 2012). In line with the previous literature, it seems likely that NAC, increasing the level of ROS, provides the pressure conditions to promote biofilm formation. Indeed, we suggest the increase of biofilm biomass by NAC is an adaptive mechanism adopted by bacteria to protect themselves from the deleterious effect of ROS. Accordingly, previous studies have shown that several bacteria respond to sub-lethal doses of ROS producing antibiotics by increasing biofilm formation (Rachid et al. 2000; Hoffman et al. 2005).

As NAC mainly generate ROS via the Fenton reaction, an iron imbalance can not be excluded (Sagristà et al., 2002; Abu-Kishk et al., 2010). Notably, a link between iron availability and *X. fastidiosa* biofilm formation has been reported. Changes in biofilm architecture and metabolism have been described when *X. fastidiosa* was exposed to extreme variations in iron concentration. Interestingly, iron modulates the expression of genes involved in the secretion and function of some pili and fimbrie important for *X. fastidiosa* biofilm formation (Zaini et al., 2008). Moreover, in *X. fastidiosa* the production of some quorum sensing signals was found decreased in response to both excess iron and iron-limiting conditions (Fones et al., 2013). Other observations support the hypothesis that the iron concentration is sensed as an environmental signal to successfully establish a colonization process (Zaini et al., 2008) and stimulates biofilm formation (Kately et al., 2018).

The ability of NAC to promote biofilm formation has been previously described. Yi et al. (2018) showed that the combination of NAC and serum transferrin increases biofilm formation by *Staphylococcus aureus*, *Enterococcus faecalis*, and *Pseaudomonas aeruginosa*. The authors demonstrated that biofilm potentiation occurred primarily due to the perturbance of the redox status in the bacterial cells and the bioavailability of iron in the culture medium. Eroshenko et al. (2017) also found that *S. aureus* and *E. faecalis* biofilms were increased upon NAC treatments.

The increased amounts of polysaccharides in matrix may also be part of a stress response (Gambino et al., 2016). Here, matrix polysaccharides were found increased up to 53 % when NAC was added during biofilm formation and up to 87 % when only planktonic cells were treated. Interestingly, the role of the

redox-sensing transcription factor OxyR in the regulation of EPS exopolysaccharide production has been reported (Burbank and Roper, 2014; Jang et al., 2016). Indeed, some polysaccharides are able to scavenge hydroxyl radicals, in order to inhibit lipid and protein peroxidation (Tomida et al. 2010). Therefore, the increase of polysaccharides within *X. fastidiosa* biofilm upon NAC treatment could be considered an adaptive response that protects bacteria from the deleterious effect of ROS. Accordingly, Olofsson et al. (2003) reported a change in terms of polysaccharidic composition of *Klebsiella pneumoniae* biofilm after exposure to NAC. In addition, Yi et al. (2018) showed that the expression of some genes encoding for the production of extracellular polysaccharides was increased by NAC in *S. aureus*. Interestingly, aconitase, one of the 12 genes that regulate exopolysaccharides in *X. fastidiosa*, responds to intracellular levels of iron (Toney et al., 2006).

In contrast to polysaccharides, in our study, a significant decrease (up to 91 %) in the amount of EPS proteins was found in all treated biofilms, especially when NAC was applied only during biofilm formation (-/NAC) or upon both cell attachment and biofilm formation (NAC/NAC). One of the suggested mechanisms of action of NAC is correlated to the presence of the thiol site, which is involved in the destruction of intermolecular or intramolecular disulfide bonds in proteins (Aldini et al., 2018).

With the idea to mimic a curative approach, the ability of NAC to affect a mature biofilm was evaluated. When biofilm was pre-grown without treatments (-/-), a further exposure to NAC made biofilm stronger and more resistant to the dislodgement. The level of ROS after NAC treatment was also found increased in comparison to the corresponding counterpart treated with PBS, suggesting that the further treatment with NAC induces a protective response to enhance biofilm survival (Villa et al., 2012). Thus, results indicate that a curative approach is not suitable to destroy *X. fastidiosa* biofilm upon the development on the surface. However, in the lacking of an effective strategy to effectively counteract the plant infection, a way that contain biofilm-mediated infections within the plant could be a straight forward in the challenge against *X. fastidiosa* biofilm formation (Uppuluri and Lopez-Ribot, 2016). While biofilms create confined areas of persistent infections, dispersed cells are instrumental in the spreading of the diseases (Kaplan et al., 2010; Chua et al., 2014). Therefore, the decrease of cell dispersion may limit bacterial colonization of other sites within the plant and thus the spread of infection might be contained.

A combination of both preventive and curative approach has been also considered. When biofilm was pre-grown with cells treated with NAC since before their adhesion (NAC/NAC) a further treatment with NAC increases the detachment index, indicating that NAC made biofilm more prone to be detached. An increase of ROS was also found. It is likely that, the further treatment with NAC creates too strong adverse conditions, to which the best microbial strategy is to escape rather than activate drug resistance sessile mechanisms. Molecules that trigger biofilm dispersal have been seen as promising as once biofilm is dispersed into a population of planktonic cells it would immediately be physiologically more vulnerable (Kaplan et al., 2010). However, when biofilm was pre-grown with NAC only during its formation, no effect on detachment was recorded whereas when biofilm was pre-grown with planktonic cells treated with NAC only upon their attachment (NAC/-) the detachment index slightly decreased. Therefore, data highlight the importance of a continuous and well planned therapy if the biofilm dislodgment is wanted.

Conclusion

Taken together, results suggest that not-lethal low doses of NAC may be not suitable as an anti-biofilm agent as, under NAC treatment, biofilm was found increased by a ROS-mediated mechanism. Moreover, NAC is not suitable to destroy *X. fastidiosa* biofilm upon its establishment on the surface. However, NAC could be alternatively used to contain the biofilm-mediated infections in plant as it limits dispersal of phytopathogenic cells from the biofilms. On the contrary, a combination of both preventive and curative approach has been found promising in dissolving biofilm by triggering its dispersal. However, to be effective, NAC needs to be applied at the very early stages of infection, before cells start to adhere. Therefore, data highlight the importance of finding the right modality of treatments. Indeed, a wrong approach based on NAC-application of infected olive plants, might contribute to a low consistent success of the treatment. In future, this knowledge could be considered to correctly apply this compound in the agricultural context, trying to find a solution in the olive orchard affected by the OQSD.

References

Abu-Kishk, I., Kozer, E., Goldstein, L.H., Weinbaum, S., Bar-Haim, A., Alkan, Y., Petrov, I., Evans, S., Siman-Tov, Y., Berkovitch, M., 2010. Oral N-acetylcysteine has a deleterious effect in acute iron intoxication in rats. American Journal of Emergency Medicine 28, 8-12.

Aguero, C., Dandekar, A., Meredith, C., 2003. Evaluation of tolerance to Pierce's disease and botrytis in transgenic plants of *Vitis vinifera* L. expressing the pear pgip gene. In: Hajdu, E., Borbas, E. (Eds.), Proceedings of the 8th International Conference on Grape Genetics and Breeding, Vols 1 and 2, pp. 473-478.

Aguero, C.B., Uratsu, S.L., Greve, C., Powell, A.L.T., Labavitch, J.M., Meredith, C.P., Dandekar, A.M., 2005. Evaluation of tolerance to Pierce's disease and Botrytis in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene. Molecular Plant Pathology 6, 43-51.

Aldini, G., Altomare, A., Baron, G., Vistoli, G., Carini, M., Borsani, L., Sergio, F., 2018. N-Acetylcysteine as an antioxidant and disulphide breaking agent: the reasons why. Free Radical Research 52, 751-762.

Azeredo, J., Azevedo, N.F., Briandet, R., Cerca, N., Coenye, T., Costa, A.R., Desvaux, M., Di Bonaventura, G., Hebraud, M., Jaglic, Z., Kacaniova, M., Knochel, S., Lourenco, A., Mergulhao, F., Meyer, R.L., Nychas, G., Simoes, M., Tresse, O., Sternberg, C., 2017. Critical review on biofilm methods. Critical Reviews in Microbiology 43, 313-351.

Behrendt, H., Bruggemann, R., 1993. Modeling the fate of organic-chemicals in the soil-planet environment – model study of root uptake of pesticides. Chemosphere 27, 2325-2332.

Blasi, F., Page, C., Rossolini, G.M., Pallecchi, L., Matera, M.G., Rogliani, P., Cazzola, M., 2016. The effect of N-acetylcysteine on biofilms: Implications for the treatment of respiratory tract infections. Respiratory Medicine 117, 190-197.

Burbank, L., Roper, M.C., 2014. OxyR and SoxR Modulate the Inducible Oxidative Stress Response and Are Implicated During Different Stages of Infection for the Bacterial Phytopathogen *Pantoea stewartii* subsp *stewartii*. Molecular Plant-Microbe Interactions 27, 479-490.

Cariddi, C., Saponari, M., Boscia, D., De Stradis, A., Loconsole, G., Nigro, F., Porcelli, F., Potere, O., Martelli, G.P., 2014. Isolation of *Xylella fastidiosa* strain infecting olive and oleander in Apulia, Italy. Journal of Plant Pathology 96, 425-429.

Chang, C.J., Donaldson, R., Brannen, P., Krewer, G., Boland, R., 2009. Bacterial Leaf Scorch, a New Blueberry Disease Caused by *Xylella fastidiosa*. Hortscience 44, 413-417.

Chatterjee, S., Almeida, R.P.P., Lindow, S., 2008. Living in two worlds: The plant and insect lifestyles of *Xylella fastidiosa*. Annual Review of Phytopathology, pp. 243-271.

Chua, S.L., Liu, Y., Yam, J.K.H., Chen, Y.C., Vejborg, R.M., Tan, B.G.C., Kjelleberg, S., Tolker-Nielsen, T., Givskov, M., Yang, L., 2014. Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles. Nature Communications 5.

Costa, F., Sousa, D.M., Parreira, P., Lamghari, M., Gomes, P., Martins, M.C.L., 2017. N-acetylcysteine-functionalized coating avoids bacterial adhesion and biofilm formation. Scientific Reports 7.

Costerton, J.W., 2007. Introduction to biofilm. International Journal of Antimicrobial Agents 11, 217-221.

Dandekar, A.M., Gouran, H., Ibanez, A.M., Uratsu, S.L., Aguero, C.B., McFarland, S., Borhani, Y., Feldstein, P.A., Bruening, G., Nascimento, R., Goulart, L.R., Pardington, P.E., Chaudhary, A., Norvell, M., Civerolo, E., Gupta, G., 2012. An engineered innate immune defense protects grapevines from Pierce disease. Proceedings of the National Academy of Sciences of the United States of America 109, 3721-3725.

De La Fuente, L., Parker, J.K., Oliver, J.E., Granger, S., Brannen, P.M., van Santen, E., Cobine, P.A., 2013. The Bacterial Pathogen *Xylella fastidiosa* Affects the Leaf Ionome of Plant Hosts during Infection. Plos One 8.

Della Coletta, H., Francisco, C.S., Lopes, J.R.S., De Oliveira, A.F., Da Silva, L.F.D., 2016. First report of olive leaf scorch in Brazil, associated with *Xylella fastidiosa* subsp *pauca*. Phytopathologia Mediterranea 55, 130-135.

Dinicola, S., De Grazia, S., Carlomagno, G., Pintucci, J.P., 2014. N-acetylcysteine as powerful molecule to destroy bacterial biofilms. A systematic review. European Review for Medical and Pharmacological Sciences 18, 2942-2948.

Eroshenko, D., Polyudova, T., Korobov, V., 2017. N-acetylcysteine inhibits growth, adhesion and biofilm formation of Gram-positive skin pathogens. Microbial Pathogenesis 105, 145-152.

Federici, M.T., Marcondes, J.A., Picchi, S.C., Stuchi, E.S., Fadel, A.L., Laia, M.L., Lemos, M.V.F., Lemos, E.G.M., 2012. *Xylella fastidiosa*: An in vivo system to study possible survival strategies within citrus xylem vessels based on global gene expression analysis. Electronic Journal of Biotechnology 15.

Fones, H., Preston, G.M., 2013. The impact of transition metals on bacterial plant disease. Fems Microbiology Reviews 37, 495-519.

Gambino, M., Cappitelli, F., 2016. Mini-review: Biofilm responses to oxidative stress. Biofouling 32, 167-178.

Gansert, D., 2003. Xylem sap flow as a major pathway for oxygen supply to the sapwood of birch (*Betula pubescens* Ehr.). Plant Cell and Environment 26, 1803-1814.

Garuglieri, E., Meroni, E., Catto, C., Villa, F., Cappitelli, F., Erba, D., 2018. Effects of Sub-lethal Concentrations of Silver Nanoparticles on a Simulated Intestinal Prokaryotic-Eukaryotic Interface. Frontiers in Microbiology 8.

Giampetruzzi, A., Saponari, M., Loconsole, G., Boscia, D., Savino, V.N., Almeida, R.P.P., Zicca, S., Landa, B.B., Chacon-Diaz, C., Saldarelli, P., 2017. Genome-Wide Analysis Provides Evidence on the Genetic Relatedness of the Emergent *Xylella fastidiosa* Genotype in Italy to Isolates from Central America. Phytopathology 107, 816-827.

Gusarov, I., Nudler, E., 2005. NO-mediated cytoprotection: Instant adaptation to oxidative stress in bacteria. Proceedings of the National Academy of Sciences of the United States of America 102, 13855-13860.

Haelterman, R.M., Tolocka, P.A., Roca, M.E., Guzman, F.A., Fernandez, F.D., Otero, M.L., 2015. First presumptive diagnosis of *Xylella fastidiosa* causing Olive Scorch in Argentina.. Journal of Plant Pathology 97, 393-393.

Hill, B.L., Purcell, A.H., 1997. Populations of *Xylella fastidiosa* in plants required for transmission by an efficient vector. Phytopathology 87, 1197-1201.

Hoffman, L.R., D'Argenio, D.A., MacCoss, M.J., Zhang, Z.Y., Jones, R.A., Miller, S.I., 2005. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature 436, 1171-1175.

Jang, I.A., Kim, J., Park, W., 2016. Endogenous hydrogen peroxide increases biofilm formation by inducing exopolysaccharide production in *Acinetobacter oleivorans* DR1. Scientific Reports 6.

Kandel, P.P., Almeida, R.P.P., Cobine, P.A., De La Fuente, L., 2017. Natural Competence Rates Are Variable Among *Xylella fastidiosa* Strains and Homologous Recombination Occurs In Vitro Between Subspecies *fastidiosa* and *multiplex*. Mol Plant Microbe Interact 30, 589-600.

Kaplan, J.B., 2010. Biofilm Dispersal: Mechanisms, Clinical Implications, and Potential Therapeutic Uses. Journal of Dental Research 89, 205-218.

Korou, L.M., Agrogiannis, G., Pantopoulou, A., Vlachos, I.S., Iliopoulos, D., Karatzas, T., Perrea, D.N., 2010. Comparative antilipidemic effect of N-acetylcysteine and sesame oil administration in diet-induced hypercholesterolemic mice. Lipids in Health and Disease 9.

Krewer, G., Dutcher, J.D., Chang, C.J., 2002. Imidacloprid insecticide slows development of Pierce's disease in bunch grapes. Journal of Entomological Science 37, 101-112.

Krivanek, A.F., Walker, M.A., 2005. Vitis resistance to Pierce's disease is characterized by differential *Xylella fastidiosa* populations in stems and leaves. Phytopathology 95, 44-52.

Loconsole, G., Potere, O., Boscia, D., Altamura, G., Djelouah, K., Elbeaino, T., Frasheri, D., Lorusso, D., Palmisano, F., Pollastro, P., Silletti, M.R., Trisciuzzi, N., Valentini, F., Savino, V., Saponari, M., 2014. Detection of *Xylella fastidiosa* in Olive trees by molecular and serological methods. Journal of Plant Pathology 96, 7-14.

Maddox, C.E., Laur, L.M., Tian, L., 2010. Antibacterial Activity of Phenolic Compounds Against the Phytopathogen *Xylella fastidiosa*. Current Microbiology 60, 53-58.

Marcelletti, S., Scortichini, M., 2016. *Xylella fastidiosa* CoDiRO strain associated with the olive quick decline syndrome in southern Italy belongs to a clonal complex of the subspecies pauca that evolved in Central America. Microbiology-Sgm 162, 2087-2098.

Martelli, G.P., Boscia, D., Porcelli, F., Saponari, M., 2016. The olive quick decline syndrome in south-east Italy: a threatening phytosanitary emergency. European Journal of Plant Pathology 144, 235-243.

Muranaka, L.S., Giorgiano, T.E., Takita, M.A., Forim, M.R., Silva, L.F.C., Coletta, H.D., Machado, M.A., de Souza, A.A., 2013. N-Acetylcysteine in Agriculture, a Novel Use for an Old Molecule: Focus on Controlling the Plant-Pathogen *Xylella fastidiosa*. Plos One 8.

Nissar, A.U., Farrukh, M.R., Kaiser, P.J., Rafiq, R.A., Afnan, Q., Bhushan, S., Adil, H.S., Subhash, B.C., Tasduq, S.A., 2013. Effect of N-acetyl cysteine (NAC), an organosulfur compound from Allium plants, on experimentally induced hepatic prefibrogenic events in wistar rat. Phytomedicine 20, 828-833.

Nunney, L., Ortiz, B., Russell, S.A., Sanchez, R.R., Stouthamer, R., 2014. The Complex Biogeography of the Plant Pathogen Xylella fastidiosa: Genetic Evidence of Introductions and Subspecific Introgression in Central America. Plos One 9.

Oh, E., Andrews, K.J., Jeon, B., 2018. Enhanced Biofilm Formation by Ferrous and Ferric Iron Through Oxidative Stress in *Campylobacter jejuni*. Frontiers in Microbiology 9.

Picchi, S.C., Takita, M.A., Coletta, H.D., Machado, M.A., de Souza, A.A., 2016. N-acetylcysteine interferes with the biofilm formation, motility and epiphytic behaviour of *Xanthomonas citri* subsp *citri*. Plant Pathology 65, 561-569.

Pimentel, D., 1995. Amounts of pesticides reaching target pets – Environmental impacts and ethics. Journal of Agricultural & Environmental Ethics 8, 17-29.

Rachid, S., Ohlsen, K., Wallner, U., Hacker, J., Hecker, M., Ziebuhr, W., 2000. Alternative transcription factor sigma(B) is involved in regulation of biofilm expression in a *Staphylococcus aureus* mucosal isolate. Journal of Bacteriology 182, 6824-6826.

Sagrista, M.L., Garcia, A.F., De Madariaga, M.A., Mora, M., 2002. Antioxidant and pro-oxidant effect of the thiolic compounds N-acetyl-L-cysteine and glutathione against free radical-induced lipid peroxidation. Free Radical Research 36, 329-340.

Saponari, M., Boscia, D., Nigro, F., Martelli, G.P., 2013. Identification of DNA sequences related to *Xylella fastidiosa* in Oleander, Almond and Olive trees exhibiting leaf scorch symptoms in Apulia (Southern Italy). Journal of Plant Pathology 95, 668-668.

Saponari, M., Loconsole, G., Cornara, D., Yokomi, R.K., De Stradis, A., Boscia, D., Bosco, D., Martelli, G.P., Krugner, R., Porcelli, F., 2014. Infectivity and Transmission of *Xylella fastidiosa* by *Philaenus spumarius* (Hemiptera: Aphrophoridae) in Apulia, Italy. Journal of Economic Entomology 107, 1316-1319.

Souza, G.A., Ebaid, G.X., Seiva, F.R.F., Rocha, K.H.R., Galhardi, C.M., Mani, F., Novelli, E.L.B., 2011. N-Acetylcysteine an Allium Plant Compound Improves High-Sucrose Diet-Induced Obesity and Related Effects. Evidence-Based Complementary and Alternative Medicine, 1-7.

Strona, G., Carstens, C.J., Beck, P.S.A., 2017. Network analysis reveals why *Xylella fastidiosa* will persist in Europe. Scientific Reports 7.

Toledo, M.A.S., Schneider, D.R., Azzoni, A.R., Favaro, M.T.P., Pelloso, A.C., Santos, C.A., Saraiva, A.M., Souza, A.P., 2011. Characterization of an oxidative stress response regulator, homologous to Escherichia coli OxyR, from the phytopathogen *Xylella fastidiosa*. Protein Expression and Purification 75, 204-210.

Tomida, H., Yasufuku, T., Fujii, T., Kondo, Y., Kai, T., Anraku, M., 2010. Polysaccharides as potential antioxidative compounds for extended-release matrix tablets. Carbohydrate Research 345, 82-86.

Uppuluri, P., Lopez-Ribot, J.L., 2016. Go Forth and Colonize: Dispersal from Clinically Important Microbial Biofilms. Plos Pathogens 12.

Villa, F., Remelli, W., Forlani, F., Gambino, M., Landini, P., Cappitelli, F., 2012. Effects of chronic sub-lethal oxidative stress on biofilm formation by *Azotobacter vinelandii*. Biofouling 28, 823-833.

Wang, C.J., Liu, Z.Q., 2007. Foliar uptake of pesticides - Present status and future challenge. Pesticide Biochemistry and Physiology 87, 1-8.

Wang, P., Lee, Y., Igo, M.M., Roper, M.C., 2017. Tolerance to oxidative stress is required for maximal xylem colonization by the xylem-limited bacterial phytopathogen, *Xylella fastidiosa*. Molecular Plant Pathology 18, 990-1000.

Wells, J.M., Raju, B.C., Hung, H.Y., Weisburg, W.G., Mandelcopaul, L., Brenner, D.J., 1987. *Xylella fastidiosa* Gen-Nov, Sp-Nov, Gram-Negative, Xylem-limited, Fastidious plant bacteria related to Xantjomonas spp. International Journal of Systematic Bacteriology 37, 136-143.

Yin, S.P., Jiang, B., Huang, G.T., Zhang, Y.L., You, B., Chen, Y., Gong, Y.L., Chen, J., Yuan, Z.Q., Zhao, Y., Li, M., Hu, F.Q., Yang, Z.C., Peng, Y.Z., 2018. The Interaction of N-Acetylcysteine and Serum Transferrin Promotes Bacterial Biofilm Formation. Cellular Physiology and Biochemistry 45, 1399-1409.

Zaini, P.A., Fogaca, A.C., Lupo, F.G.N., Nakaya, H.I., Vencio, R.Z.N., da Silva, A.M., 2008. The iron stimulon of *Xylella fastidiosa* includes genes for type IV pilus and colicin V-like bacteriocins. Journal of Bacteriology 190, 2368-2378.

Zubkov, M.V., Fuchs, B.M., Eilers, H., Burkill, P.H., Amann, R., 1999. Determination of total protein content of bacterial cells by SYPRO staining and flow cytometry. Applied and Environmental Microbiology 65, 3251-3257.

Figures

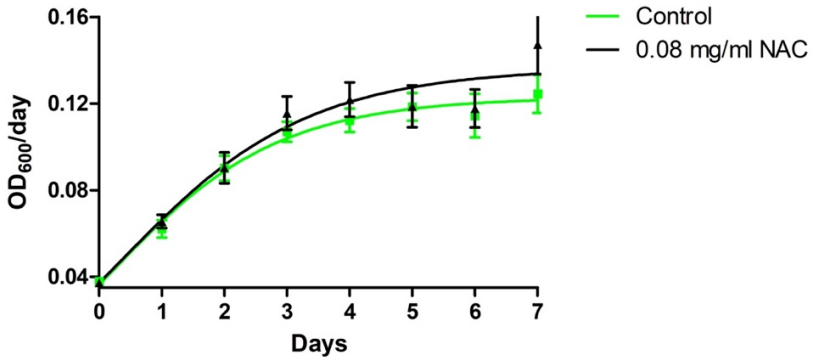


Fig. 1. OD_{600} values and Gompertz growth curves of *X. fastidiosa* without and with 0.08 mg/ml NAC. Data represent the mean ± standard deviation of three independent measurements.

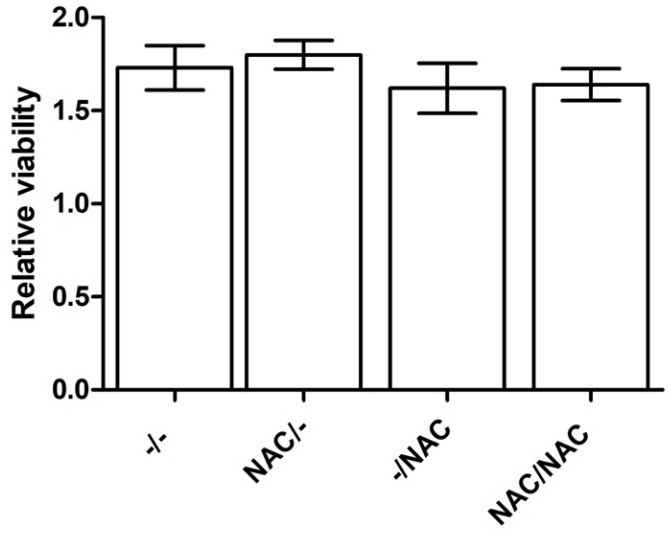


Fig. 2. Relative viability of X. *fastidiosa* biofilm grown under different treatments. Data represent the mean ± standard deviation of three independent measurements. No statistically significant differences (Tukey's HSD, $p \leq 0.05$) were found among treatments.

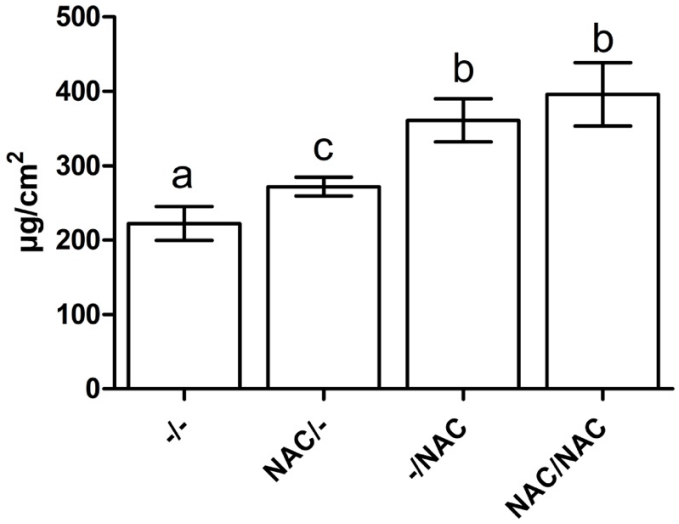


Fig. 3. Biofilm biomass expressed as protein amount of *X. fastidiosa* biofilm grown for 7 days under different conditions. Data represent the mean ± SDs of three independent replicates. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between conditions.

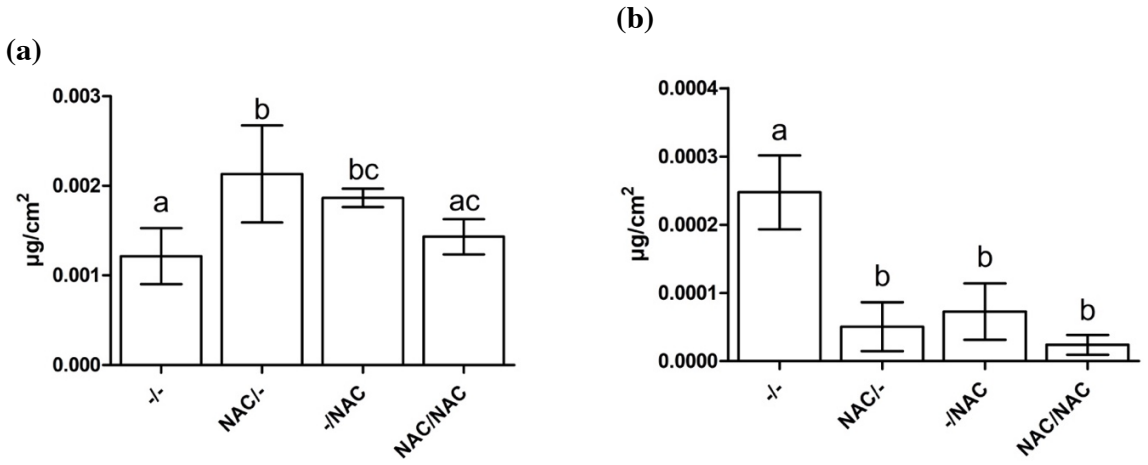


Fig. 4. Polysaccharides (**a**) and proteins (**b**) amount in the EPS of *X. fastidiosa* biofilm grown for 7 days under different conditions. Data represent the mean ± SDs of three independent biological replicates. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between conditions.

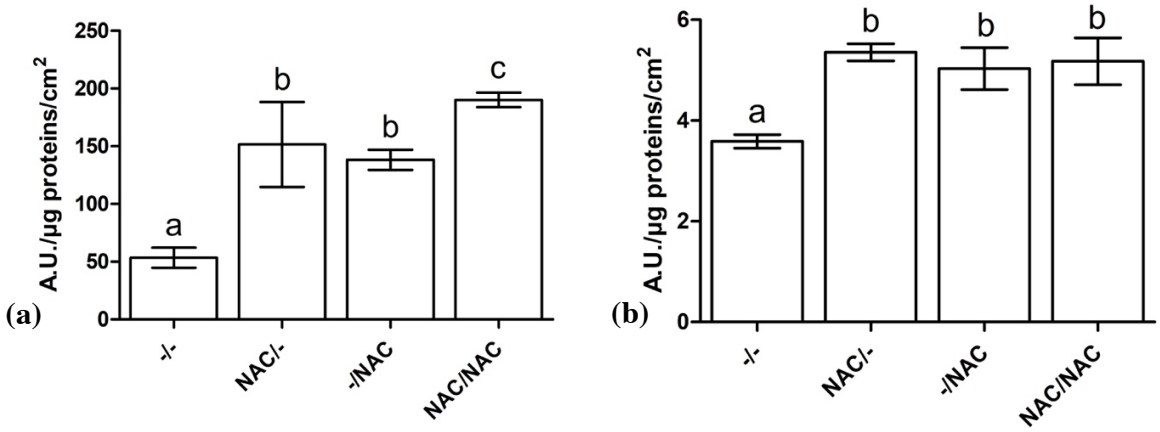


Fig. 5. Intracellular (**a**) and extracellular (**b**) level of ROS within *X. fastidiosa* biofilm grown for 7 days under different conditions. Data represent the mean ± SDs of three independent biological replicates. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between different conditions.

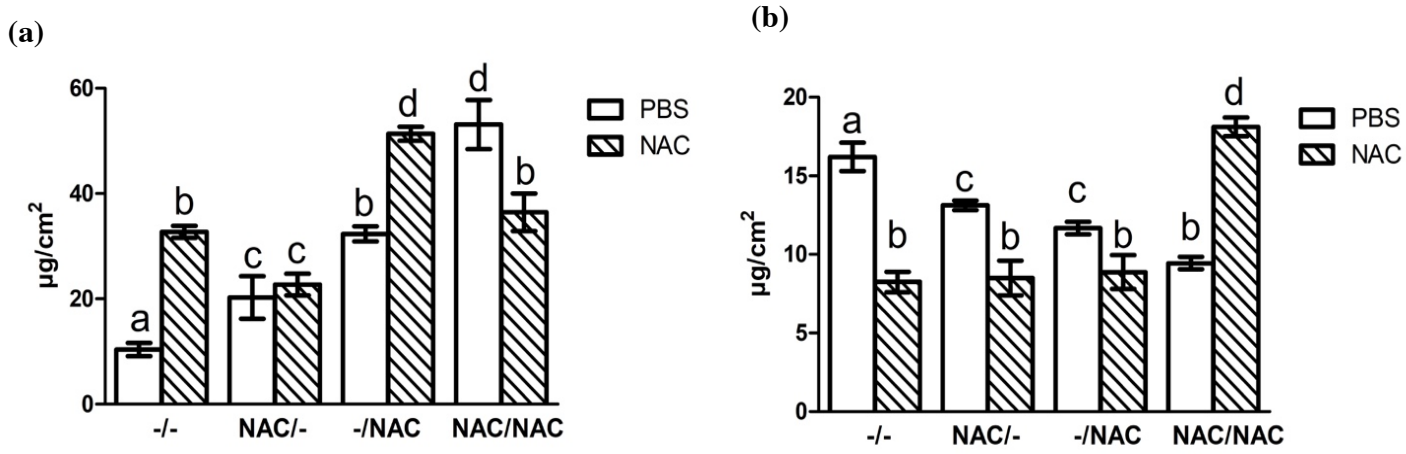


Fig. 6. Protein amount within biofilm biomass remained on the membrane (**a**) and those dislodged in the bulk liquid (**b**) after 24 h of treatment with PBS or NAC. Data represent the mean ± SDs of three independent biological replicates. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between different conditions.

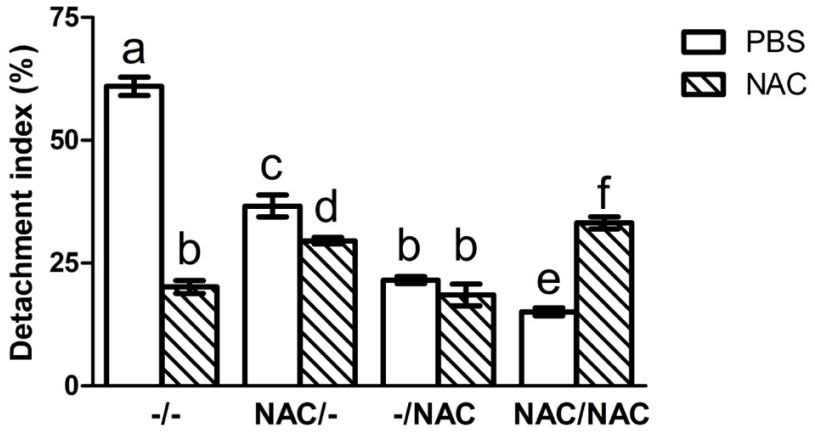


Fig. 7. Detachment index of *X. fastidiosa* biofilm grown under different conditions after 24 h PBS or NAC treatment. Data represent the mean ± SDs of three independent biological replicates. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between different conditions.

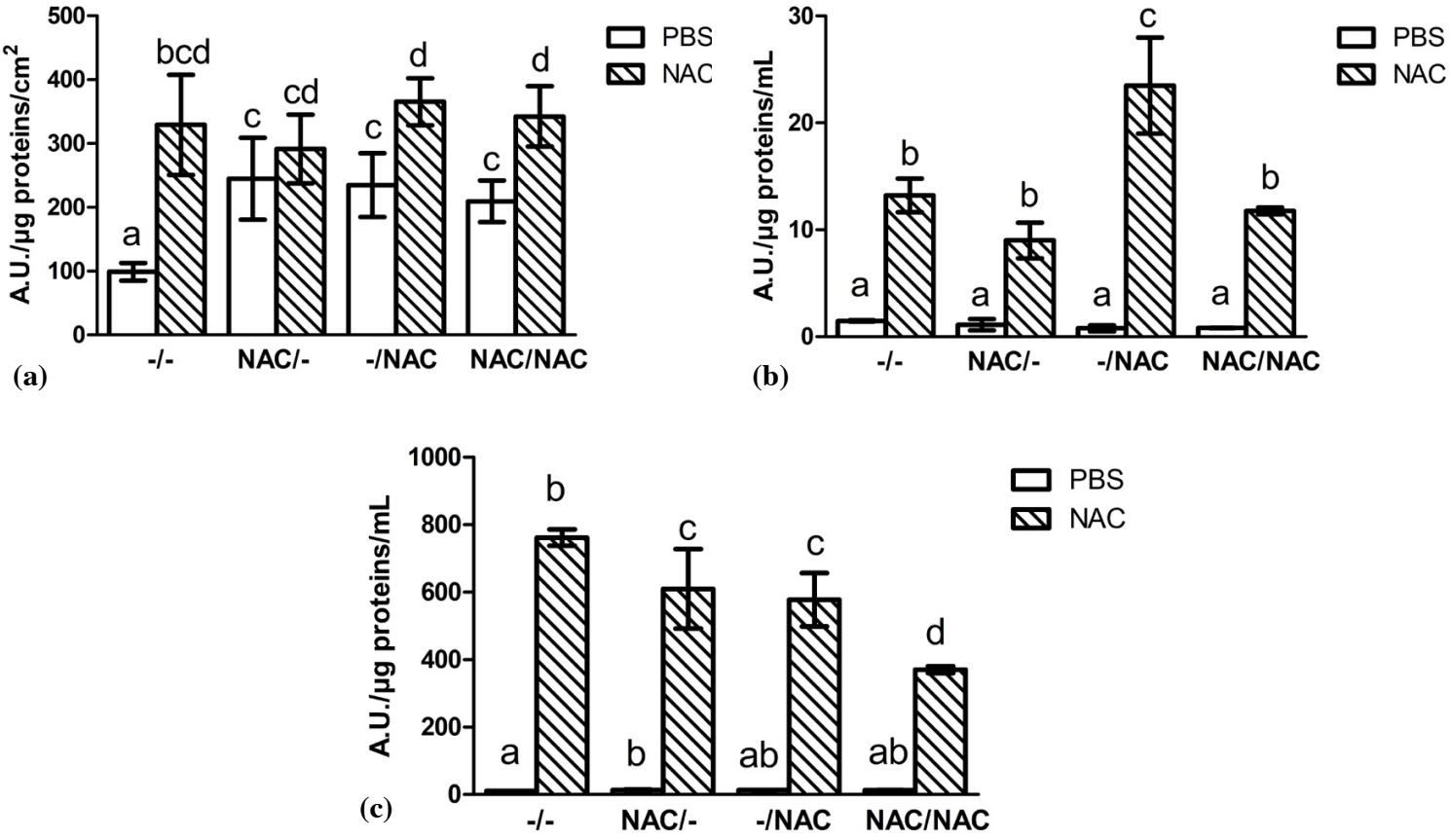


Figure 8. ROS levels within the biofilm remained on the membrane (**a**), detached from the membrane (**b**) and that in the bulk liquid (**c**) after 24 h in presence of PBS or NAC. Data represent the mean ± SDs of three independent biological replicates. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between different conditions.

CHAPTER VI

Conclusions

The colonization of pathogenic microorganisms in form of biofilm on plant tissues annually leads to significant losses in terms of crop yields and a decrease of the quality and safety of agricultural products worldwide. Their recalcitrance to control practices, the arise of resistance phenomena and the continuous abolishment of some pesticides has put a tremendous pressure on the scientific community to find alternative approaches for the treatment of deleterious plant-associated biofilms. In addition, the principles of integrated pest management (IPM) embraced by the worldwide legislation aims to recommend alternative approaches to the application of pesticides. To this purpose, the use of natural compounds derived from plants at non-lethal concentrations were previously reported as biocide-free agents able to affect bacterial and fungal biofilm formation cycle. In fact, they could avoid microbial adhesion or interfere with the cellular communication processes destabilizing its physical integrity or acting on the final phase of biofilm formation, encouraging cell detachment from the surface.

In this work, non-lethal concentrations of several natural compounds (plant methanolic extracts, essential oils and NAC, an analogue of cysteine) derived from different plant species were evaluated for their effects on formation of bacterial and fungal plant-associated biofilms.

In chapter III, we proved that:

- Methanolic extracts obtained from three seagrass species collected in Vietnam and in India (*Enhalus acoroides*, *Halophila ovalis* and *Halodule pinifolia*) did not adversely affect microbial growth, nor did they act as a carbon and energy source for the *Escherichia coli* and *Candida albicans*.
- *Enhalus acoroides* leaf extract was the most promising extract among those tested. Indeed, the selected non-lethal concentrations of *E. acoroides* leaf extract were found to exert an anti-biofilm effect on *C. albicans* and *E. coli* biofilm in the first phase of biofilm genesis. This result could open up the possibility to develop preventive strategies to hinder the adhesion of microbial cells to surfaces.
- *Enhalus acoroides* leaf extract also affected the dispersion and maturation steps in *C. albicans* and *E. coli* respectively, suggesting an important role in cell signaling processes.
- *E. acoroides* leaf extract was characterized and major phenolic compounds were identified by MS/MS analysis.

In chapter IV we proved that:

- Two essential oils (PK and PK-IK) extracted and characterized from the leaves of two cultivars of *Perilla frutescens* neither inhibited the growth nor were they utilized as a carbon energy source by three different phytopathogenic model fungi (*Colletotrichum musae*, *Fusarium dimerum* and *Fusarium oxysporum)*.
- PK and PK-IK essential oils showed excellent anti-biofilm performances inhibiting conidium

germination and reducing conidium adhesion.

- PK and PK-IK essential oils revealed a notably high anti-biofilm effect even during biofilm maturation. They affected the biofilm structural development, with a reduction of dried weight, extracellular polysaccharides and proteins.
- In all cases the anti-biofilm effects were exploited with a non-lethal mechanism but PK-IK displayed better activity than PK.

This research supported the spreading of PK and PK-IK essential oils as alternative biocide-free agents suitable for a preventive or integrative approach for sustainable crop protection.

In chapter V we proved that:

- A sub-lethal concentration of 0.08 mg/ml N-Acetylcysteine (NAC), a natural compound found in several plant species, did not affect the planktonic growth of *Xylella fastidiosa* as NAC did not change neither the growth rate of the bacterium nor its viability.
- 0.08 mg/ml NAC promoted *X. fastidiosa* biofilm formation by increasing the biofilm biomass (up to 62 %) and the amount of polysaccharides (up to 53%) in the EPS matrix through a ROS-mediated mechanism.
- 0.08 mg/ml NAC enhanced cell-surface and intercellular adhesiveness by decreasing the biofilm dispersion rates in all treated samples, while affecting the intracellular redox homeostasis.

Results obtained suggested that non-lethal concentrations of NAC increased the intercellular oxidative state in *X. fastidiosa*, which enhanced: i) biofilm formation and biomass, ii) the polysaccharide fraction of the extracted polymeric matrix and iii) the cell-substratum and cell-cell adhesion, reducing the biofilm detachment. Together, these findings suggest that non-lethal concentrations of NAC can act as a stress factor with pleiotrophic effects on *X. fastidiosa*, and it could trigger the undesired effect of stronger biofilm formation.
However, Chatterjee et al. (2008) sustained that the adhesive and cohesive properties of the polysaccharides present in the EPS matrix attenuate the pathogen's virulence by reducing cell detachment, inhibiting both the acquisition and transmission of this pathogen by sharpshooter vectors and the fast colonization of the plant through the vessels. Thus, an inefficient biofilm dislodgment reduces the number of cells that could be transmitted to healthy plant.
This study provides new information about the roles of NAC in *X. fastidiosa* aggregation and biofilm formation, suggesting novel strategies based on reducing the migration of the bacterium inside the host plant, and impairing the transmission by the insect vector.

In conclusion, this work reported the non-lethal concentrations effects of several natural compounds (plant methanolic extracts, essential oils and NAC, an analogue of cysteine) derived from different plant species on the biofilm formation of bacterial and fungal plant-associated biofilms. Further studies are needed to increase the knowledge about the modes of action of these bioactive compounds against microbial plant-associated biofilms.

Acknowledgement

I would like to express my sincere gratitude to Prof.ssa Francesca Cappitelli and Dr. Fabio Forlani. They provided me with many helpful suggestions, important advice and constant encouragement during the course of this work.

There are many people that have contributed to the implementation of this work. They gave me interesting feedback, suggestions and technical support. It was an honor to work with them. I would like to say thank to: Dr.ssa Federica Villa, Dr.ssa Federica Troiano and Dr. Daniel Vàzquez Nion.

I would like to thank Prof.ssa Jutta Papenbrock for her help and suggestions during my short experience in her lab at Leibniz University (Hannover, Germany).

Lastly, due to my last American period, I would like to thank Prof. Leonardo De La Fuente, his family and his laboratory group for the amazing experience I lived during my PhD period at Auburn University (Alabama). I totally fallen in love with the American culture.

Furthermore, I am deeply indebted to my collegue Dr.ssa Cristina Cattò for her understandings, endless patience and encouragement during these last years started with my master degree.

In the end, I would like to thank Erika and Chiara for their constant encouragement not only as PhD colleagues but also as friends.

Scientific products, research experiences and grant

Poster presentation at National and International Conferences

De Vincenti L. (2017) Unravelling the antibiofilm effects of plant derived compounds at non-lethal concentrations. 22nd Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 20-22 September, Bolzano, Italy. ISBN 978-88-98416-97-4.
Abstract - The aim of this project is to use non-lethal doses of bio-inspired molecules to interfere with specific key-steps that orchestrate biofilm formation. Thus, we disarm microorganisms without affecting their existence, sidestepping drug resistance and extending the efficacy of the current arsenal of antimicrobial agents. This PhD project is carried out in collaboration with the Institute of Botany of the Leibniz University of Hannover (Germany).

De Vincenti L. (2016) Unravelling the antibiofilm effects of plant derived compounds and nanoparticles at sub-lethal concentrations. 21th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 14 -16 September, Portici, Italy. ISBN 978-88-99648-06-0.
Abstract - The principal aim of this project is to unravel the effects of bioactive-rich fractions obtained from halophytes and nanoparticles (NPs) for the development of innovative biocide-free, eco-sustainable strategies to counteract unwanted biofilms. Using sub-lethal doses of bio-inspired molecules and NPs we offer an elegant way to interfere with specific key-steps that orchestrate biofilm formation, disarming microorganisms without affecting their existence, sidestepping drug resistance and extending the efficacy of the current arsenal of antimicrobial agents. This PhD project will be carried out in collaboration with the Institute of Botany of the Leibniz University of Hannover (Germany).

De Vincenti L., Cattò C., Borgonovo G., Bassoli A., Saracchi M., Villa F., Cappitelli F. "Evaluation of antibiofilm property of *Perilla frutescens* essential oils against plant pathogenic fungi". Abstract submitted for poster session at IV International Conference on Antimicrobial Research - ICAR2016, 29 June-1 July 2016, Torremolinos-Malaga (Spain).
Abstract - More than 10,000 species of fungi are responsible for extensive damage to plant. These microorganisms grow on plants in the form of biofilms, communities of microorganisms adhering to a surface and embedded in a self-produced polymeric matrix giving origin, in most cases, to diseases. The traditional approach used for the control of biofilm consists in the use of antimicrobials. However, issues such as the risk to develop resistance, the toxicity to human health, the impact on the environment as well as the EU restrictive directives and regulations have discouraged the use of antimicrobials in the agricultural field. An alternative strategy to biocides consists in using sub-lethal doses of non-toxic natural substances involving mechanisms subtler than the simple killing activities, like those influencing the multicellular behaviour of a phytopathogen (e.g., adhesion, cell-to-cell communication, EPS production, motility). In addition, as these substances do not exert their biocidal action, they do not impose a selective pressure causing the development of resistance.

In this work, the efficacy of two essential oils extracted from the leaves of the Asiatic plant *Perilla frutescens* has been evaluated for their anti-biofilm activities at sub-lethal concentrations against the model plant pathogenic fungi *Fusarium oxysporum*, *Fusarium dimerum* and *Colletotrichum musae*.
The obtained results showed that both essential oils (at concentrations ranging from 0,001 to 2000 ppm) i) were not a nutritional source for the fungal models; ii) did not affect the fungal grow; iii) showed a good anti-biofilm activity against *F. oxysporum*, *F. dimerum* and *C. musae* reducing spore adhesion up to 70%, 80% and 40 % respectively.
In conclusion, P. frutescens essential oils could be proposed as a good candidate as an innovative anti-biofilm agents against plant pathogenic fungi.

Garuglieri E., Zanchi R., Cattò C., Troiano F., **De Vincenti L.**, Cappitelli F. "Unraveling the effects of food-related engineered NANOparticles on the GUT interactive ecosystem (NanoGut)" - Abstract submitted for poster session at MD-2015 Microbial diversity: the challenge of complexity, Congress of Società Italiana di Microbiologia Agraria, Alimentare e Ambientale (SIMTREA), Perugia (Italy), 27th-29th October 2015.

National and International research experiences and projects

2017 - Research experience at the Institute of sustainable protection of plants (IPSP) at National Research Council (CNR) of Bari (Italy). Project title: "Susceptibility to Xylella fastidiosa infections and study of plant-pathogen interactions for the development of bacterial control strategies" (STIPXYT), Regione Puglia, ID- 35646.

2016 - Research experience at the Institute of Botany at Leibniz University of Hannover (Germany). German-Italian bilateral project title: "Bioactive secondary compounds from halophyte species inhibit biofilm formation of plant-pathogenic microorganisms on plant surfaces" (SAB-HAL), MIUR-DAAD Joint Mobility Program, ID-57265315.

Ph.D Traineeship abroad
Host University: Auburn University, Alabama, USA.
January – July 2018.
Traineeship title: "Unravelling the antibiofilm effects of plant derived compounds at non-lethal concentrations against the phytopathogenic bacteria *Xylella fastidiosa*"
PI: Prof. Leonardo De La Fuente

Work plan description: The research activities will be organized as follows:
1. In vitro assays
During this first part, it will be evaluated in vitro the ability of the selected compounds to interfere with specific key-steps that orchestrate biofilm formation (adhesion, maturation and dispersion phase). To further investigate the effect of the most promising compounds on biofilm development, microfluidic chambers will be employed to reproduce mature biofilm.

2. In planta assay

With the aim to evaluate in vivo the anti-biofilm performances of the most promising compounds, greenhouse experiments will be performed using model plants (e.g. tobacco). For assessment of the anti-biofilm compounds two treatments will be used: leaf spray and drench irrigation. After 3 weeks, all the leaves will be collected a quantitative polymerase chain reaction (qPCR) will be performed to highlight *X. fastidiosa* bacterial population differences in treated and untreated tobacco plants.

Grant

09/2017 - COST EU-funded programme: Short Term Scientific Mission (STSM) of the COST Action EuroXanth: Integrating science on *Xanthomonadaceae* for integrated plant disease management in Europe. Project title: "Unravelling the antibiofilm effects of plant derived compounds at non-lethal concentrations against the phytopathogenic bacteria *Xylella fastidiosa*".

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RESEARCH ARTICLE Open Access

Hindering the formation and promoting the dispersion of medical biofilms: non-lethal effects of seagrass extracts

Luca De Vincenti[1], Yvana Glasenapp[2], Cristina Cattò[1], Federica Villa[1*], Francesca Cappitelli[1] and Jutta Papenbrock[2]

Abstract

Background: Biofilms have great significance in healthcare-associated infections owing to their inherent tolerance and resistance to antimicrobial therapies. New approaches to prevent and treat unwanted biofilms are urgently required. To this end, three seagrass species (*Enhalus acoroides*, *Halophila ovalis* and *Halodule pinifolia*) collected in Vietnam and in India were investigated for their effects in mediating non-lethal interactions on sessile bacterial (*Escherichia coli*) and fungal (*Candida albicans*) cultures. The present study was focused on anti-biofilm activities of seagrass extracts, without killing cells.

Methods: Methanolic extracts were characterized, and major compounds were identified by MS/MS analysis. The antibiofilm properties of the seagrass extracts were tested at sub-lethal concentrations by using microtiter plate adhesion assay. The performance of the most promising extract was further investigated in elegant bioreactors to reproduce mature biofilms both at the solid/liquid and the solid/air interfaces. Dispersion and bioluminescent assays were carried out to decipher the mode of action of the bioactive extract.

Results: It was shown that up to 100 ppm of crude extracts did not adversely affect microbial growth, nor do they act as a carbon and energy source for the selected microorganisms. Seagrass extracts appear to be more effective in deterring microbial adhesion on hydrophobic surfaces than on hydrophilic. The results revealed that non-lethal concentrations of *E. acoroides* leaf extract: i) reduce bacterial and fungal coverage by 60.9 and 73.9%, respectively; ii) affect bacterial biofilm maturation and promote dispersion, up to 70%, in fungal biofilm; iii) increase luminescence in *Vibrio harveyi* by 25.8%. The characterization of methanolic extracts showed the unique profile of the *E. acoroides* leaf extract.

Conclusions: *E. acoroides* leaf extract proved to be the most promising extract among those tested. Indeed, the selected non-lethal concentrations of *E. acoroides* leaf extract were found to exert an antibiofilm effect on *C. albicans* and *E. coli* biofilm in the first phase of biofilm genesis, opening up the possibility of developing preventive strategies to hinder the adhesion of microbial cells to surfaces. The leaf extract also affected the dispersion and maturation steps in *C. albicans* and *E. coli* respectively, suggesting an important role in cell signaling processes.

Keywords: Seagrass extracts, Non-lethal concentrations, Antibiofilm activity, *Escherichia coli*, *Candida albicans*

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Background

The ability of microorganisms to colonize surfaces and develop into highly organized communities enclosed in a self-produced polymeric matrix is the predominant growth modality in both nature and artificial systems. Such lifestyle is called biofilm and it is characterized by alterations in microbial phenotypes with respect to growth rates and gene transcriptions [1–3].

Biofilms have great significance for public health, representing 65–80% of microbial diseases currently treated by physicians in the developed world [4, 5]. The presence of indwelling medical devices further increases the risk for biofilm formation and subsequent infection [6]. The bacterium *Escherichia coli* and the polymorphic fungus *Candida albicans* are among the most frequent cause of bloodstream infections, and the predominant microorganisms isolated from infected medical devices [7, 8]. These biofilms, as any other biofilm, exhibit dramatically decreased susceptibility to antimicrobial agents and resistant to the host immune clearance, which increases the difficulties for the clinical treatment of infections [9–11]. Furthermore, the antimicrobial arena is experiencing a shortage of lead compounds, and growing negative consumer perception against synthetic products has led to the search for more natural solutions [12].

In this context, it has been reported that plant-derived extracts exhibit good antibiofilm properties against a range of microorganisms [13–15]. However, in the past, these extracts were mainly screened by focusing on their lethal effects [16–18] disregarding their activity at non-lethal concentrations. At these concentrations, plant-derived extracts may reveal elegant mechanisms to sabotage the sessile lifestyle, manipulating the expression of stage-specific biofilm phenotypes [19]. For instance, by affecting the cellular ability to attach to surfaces and by mystifying intercellular signals, the biofilm cascade might be hampered. Thus, non-lethal concentrations of plant-derived extracts can inspire innovative, eco-friendly and safe strategies aim at treating deleterious biofilms. Interfering with specific key steps that orchestrate biofilm genesis might offer new ways to disarm microorganisms without killing them, sidestepping drug resistance [4].

Seagrasses, which belong to the halophytes, represent a functional group of underwater marine flowering plants that have developed several strategies to survive and reproduce in environments where the salt concentration is around 200 mM NaCl or more [20]. As these plants grow in very high saline conditions, it is predicted that they could possess rare and new activities not reported for their terrestrial relatives [21, 22]. Indeed, metabolomic studies have shown that increased salinity leads to changes in conserved and divergent metabolic responses in halophytes [23–25]. Moreover, interesting activities of seagrass extracts, including antibacterial, antifungal, antialgal, antioxidant, anti-inflammatory, insecticidal, antimalarial and vasoprotective properties, have been reported [26–28].

Thus, the well described properties of seagrasses extracts offer a promising framework for investigating novel antibiofilm activities at non-lethal concentrations.

The present study explores, for the first time, the effect of extracts from different seagrasses (namely, leaves and roots from *Enhalus acoroides* Rich. ex Steud., Hydrocharitaceae, leaves of *Halophila ovalis* (R.Br.) Hook.f., Hydrocharitaceae, and leaves of *Halodule pinifolia* (Miki) Hartog, Cymodaceaceae) in mediating non-lethal interactions on sessile *Candida albicans* and *Escherichia coli* cultures, selected as model systems for fungal and bacterial biofilm infections, respectively. The work focuses on investigating the antibiofilm performance of seagrass extracts at sub-inhibitory concentrations, studying how they affect biofilm functional traits (such as adhesion, biofilm maturation, dispersal and quorum sensing), and induce cellular responses other than those associated with antimicrobial activities.

Methods

Plant material and extraction

Three species of seagrasses (leaves and roots from *Enhalus acoroides* Rich. ex Steud., Hydrocharitaceae, leaves of *Halophila ovalis* (R.Br.) Hook.f., Hydrocharitaceae, and leaves of *Halodule pinifolia* (Miki) Hartog, Cymodaceaceae) were collected in Vietnam and India and air-dried in a dark place (Table 1). *Enhalus acoroides* and *Halophila ovalis* were collected and identified by Xuan-Vy Nguyen, Department of Marine Botany, Institute of Oceanography, Vietnam Academy of Science and Technology, Nha Trang City, Vietnam, based on morphological characters and controlled by ITS molecular marker analysis [29]. Specimens of *Enhalus acoroides* are stored in the herbarium of the Institute of Botany, Hannover, Germany (Specimen number: EA20130301). *Halodule pinifolia* was collected by Jutta Papenbrock and further identified by Thirunavakkarasu Thangaradjou, Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai, Tamilnadu, India, based on morphological characters and controlled by ITS molecular marker analysis [30]. Specimens are stored in the herbarium of the Annamalai University, Parangipettai, Tamilnadu, India.

The plants were separated into different organs (leaves and roots), and samples were cooled with liquid nitrogen and ground to a fine powder using a bead mill (Retsch), three times for 10 s at a frequency of 30/s. The samples were stored at − 80 °C prior to analysis. Crude extracts were obtained using 80% methanol (MeOH) as solvent. Around 50 mg of powdered seagrass material was weighed in a reaction tube and extracted with 800 μl 80% MeOH

Table 1 Seagrass species and information about collection sites

Species	Plant organ	Collection site	GPS	Collection date
Enhalus acoroides	Leaf	Nha Trang Bay, Vietnam	109.209208°E 12.158073°N	19.04.2011
Enhalus acoroides	Root	Nha Trang Bay, Vietnam	109.209208°E 12.158073°N	19.04.2011
Halophila ovalis	Leaf	Nha Trang Bay, Vietnam	109.209208°E 12.158073°N	19.04.2011
Halodule pinifolia	Leaf	Chilika Lagoon, India	85.418015°E 19.775105°N	16.02.2010

for 10 min with regular shaking. Then the extract was centrifuged for 5 min at 18000 x g and the supernatant transferred into a new reaction tube. These steps were repeated three times with 400 μl 80% MeOH each. The supernatants were collected in the same reaction tube and stored at − 20 °C. Phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride 0.137 M, sodium chloride, Fisher Scientific) was used to obtain several concentrations of each crude extract: 100, 10, 1, 0.1, 0.01 and 0.001 mg/l.

Microbial strains and growth media

The microbial strains *Candida albicans* SC5314 (ATCC MYA-2876) and *Escherichia coli* K-12 wild-type strain (ATCC 25404) were selected as model systems for fungal and bacterial biofilms respectively. *C. albicans* and *E. coli* strains were stored at − 80 °C in suspensions containing 50% glycerol and 2% peptone, and were routinely grown in amino acid-free yeast nitrogen base (YNB, Sigma-Aldrich) supplemented with 0.5% glucose (YNBG, Conda) and Luria-Bertani broth (LB, Sigma-Aldrich), respectively, for 16 h at 30 °C.

Quantification of total flavonoid contents (TFC)

The total flavonoid content of the seagrass extracts was measured in 96-well plate according to a modified protocol from Dudonné et al. [31]. The wells were filled with 150 μl H_2O each. Dilutions of the methanolic seagrass extracts (1:2) were prepared and 25 μl of sample were filled in one well, with four replicates. A calibration curve with catechin hydrate with the following concentrations was prepared in 80% MeOH: 0, 10, 25, 50, 100, 125, 250 and 400 μg/ml. The calibration curve was placed on the plate in triplicate. In the next step, 10 μl $NaNO_2$ 3.75% were added into each well and incubated for 6 min. Afterwards, 15 μl of $AlCl_3$ 10% were added and incubated for 10 min. In the last step, 50 μl of NaOH 1 M were added and the absorption was measured at 510 nm in a microplate reader (Biotek, Winooski, USA). The slope of the calibration curve was used to calculate the total flavonoid content in mg catechin equivalent.

Quantification of total phenolic contents (TPC)

To measure the total phenolic acid content, a modified protocol after Dewanto et al. [32] was used with the same extracts described above. 96-well microtiter plate were filled with 100 μl H_2O each. From each sample, 10 μl were added; seagrass extracts were diluted 1:2. A gallic acid calibration curve with the following concentration was used: 0, 5, 10, 25, 50, 75, 100, 125 and 250 μg/ml. Next, 100 μl Na_2CO_3 7% were added and the plate was incubated for 100 min in the dark. The absorption was measured at 765 nm in a microplate reader. With the slope of the gallic acid calibration curve, the concentration of phenolic acids was calculated in mg gallic acid equivalent.

Determination if the oxygen radical absorbance capacity (ORAC)

The analysis of the oxygen radical absorbance capacity (ORAC) was conducted according to a protocol based on Huang et al. (2002) [33] and Gillespie et al. [34] with the same extracts. A black 96-well microtiter was used and the wells were filled with 120 μl fluorescein (112 nM) in phosphate buffer (75 mM, pH 7.4). Of each sample and the standard curve, 20 μl were added in each well. The standard curve of 6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was prepared in phosphate buffer with the following concentrations: 6.25, 12.5, 25 and 50 μM. Seagrass extracts were diluted 1:200 with methanol 80%. The microtiter plate was incubated for 15 min at 37 °C. The fluorescence was then measured at 485/520 nm as time point zero. Next, 80 μl of 2,2′-azobis(2-amidino-propane) dihydrochloride (62 mM) were added and the fluorescence was measured every minute for 80 min. The ORAC value was calculated as the difference between time point zero and 80 min and quantified with the Trolox standard curve.

LC-MS analysis

LC-MS analysis was performed on a Shimadzu HPLC system (controller CBM-20A, two pumps LC-20 AD, a column oven CTO-20 AC and a photo diode array detector SPD-M20A; Shimadzu, Darmstadt, Germany)

coupled to a Triple Tof 4600 mass spectrometer (AB Sciex, Canby, USA). The separation of extracted compounds was realised on a Knauer Vertex Plus column (250 × 4 mm, 5 μm particle size, packing material ProntoSIL 120–5 C18-H) with precolumn (Knauer, Berlin, Germany). The column oven temperature was set to 30 °C and 25 μl of undiluted methanolic seagrass extract prepared as described above was injected. The solvent flow rate was 0.8 ml/min. In this time, a gradient was run from 10 to 90% B from minute 0 to 35, 2 min of 90% B, switch to 10% B in 1 min and subsequent equilibration at 10% B for 2 min. Solvent A (water) and B (methanol) were both supplemented with 2 mM ammonium acetate and 0.01% acetic acid. Mass spectra were monitored between 100 and 800 Da in negative ionisation mode. In addition, MS/MS spectra were generated with a collision energy of − 30 eV and measured between 50 and 800 Da. Spectra for the most prominent peaks were compared to database entries in MassBank [35] and ReSpect [36] for identification.

Planktonic growth in the presence of seagrass extracts as the sole source of carbon and energy

The ability of *C. albicans* and *E. coli* planktonic cells to grow in the presence of each extract as the sole carbon and energy source was tested using YNB and M9 (Sigma-Aldrich) mineral medium, respectively, supplemented with the highest working extract concentration: 100 mg/l. Then a 100 μl mix of mineral medium together with 45 μl (3% *v*/v) of the overnight culture (final concentration 10^8 cells/ml) and the highest concentration of each marine plant extract were used to fill each well of 96-well plates (Thermo Fisher Scientific) and incubated for 48 h at 30 °C. A medium complemented with cells and glucose (5 g/l), and medium without cells, were used as positive and negative controls, respectively. Microbial growth was monitored using the PowerWave XS2 microplate reader (Biotek) measuring the absorbance at 600 nm (A_{600}) every 10 min. Six biological replicates of each treatment were performed. The obtained data were normalized to the negative control and reported as the mean of these.

Growth inhibition assay in the presence of seagrass extracts

The ability of the seagrass extracts to inhibit the planktonic growth of the selected microorganisms was investigated. For this, *C. albicans* and *E. coli* were grown YNBG and LB broth respectively without (positive control) and with the highest working concentrations (10 and 100 mg/l) in 96-well plates (Thermo Fisher Scientific). Growth curves at 30 °C were generated using Infinite® F200 PRO microplate reader (TECAN, Mannedorf, Switzerland) by measuring the optical density at 600 nm (OD_{600}) every 60 min for 30 h in wells inoculated with 45 μl (3% vol/vol) of an overnight culture (approximately 10^8 cells/ml). The negative control was represented by PBS supplemented with 45 μl (3% vol/vol) of the overnight culture. The polynomial Gompertz model [37] was used to fit the growth curves to calculate the maximum specific growth rate (A_{600}/min), using GraphPad Prism software (version 5.0, San Diego, CA, USA). Five biological replicates of each treatment were performed.

Microplate-based biofilm assay

The antibiofilm activity of seagrass extracts was assessed quantitatively as previously reported by Villa et al. [38]. Briefly, 200 μl of PBS containing 10^8 cells/ml supplemented with 0 (positive control), 100, 10, 1, 0.1, 0.01, and 0.001 mg/l of each crude extract were placed in hydrophobic and hydrophilic 96-well polystyrene-based microtiter plates (Thermo Fisher Scientific). After an incubation time of 24 h at 20 °C, *C. albicans* and *E. coli* planktonic cells were removed and adhered cells were stained using 0.1 mg/ml of Fluorescent Brightener 28 vital dye (Sigma-Aldrich) or 4′, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) in PBS, respectively. After 20 min staining in the dark at room temperature the microtiter plates were washed twice with 200 μl PBS and the fluorescence intensity due to adhered cells was measured using a fluorescence microplate reader (TECAN, Manneford, Switzerland) at excitation wavelength of 335 nm and emission wavelength of 433 nm. A standard curve of fluorescence intensity versus cell number was determined and used to quantify the antibiofilm performance of the crude extracts. Percentage reduction with respect to the positive control is calculated as (treated data −control data) × 100 / control data. Cattò et al. [39] proposed the following anti-adhesion ranges computing the percentage reduction in comparison to the negative control: ≤20% without anti-adhesion activity; between 20 and 30% and 30 and 40% low anti-adhesion activity and with moderate anti-adhesion activity respectively; ≥40% adhered cells with excellent anti-adhesion activity. Five biological replicates were performed for each condition and a percentage reduction in comparison to the negative control was calculated as (treated data − positive control data) × 100/ positive control data. The experiment was repeated three times.

Biofilm growth at the solid/liquid interface

The most promising plant extracts were screened for their effects on biofilm development. *C. albicans* biofilm was grown in the CDC biofilm reactor (Biosurface Technologies, Bozeman, MT, USA) as previously described by Villa et al. [40]. Briefly, two bioreactors hosting 24 polycarbonate coupons (to simulate a hydrophobic surface) were filled with YNBG and 1 ml of overnight

planktonic culture (approximately 10^8 cells/ml) and, in one of them, 0.01 mg/l of *E. acoroides* leaf extract was added. Bioreactors were maintained under static conditions (no flow) for 24 h under mild stirring at 37 °C, promoting fungal adhesion to the surface of the removable polycarbonate coupons. After that, the dynamic phase was initiated and diluted YNGB was fluxed for 48 h at flow rate of 250 ml/h. Biofilm growth in the absence (positive control) and presence of the extract was evaluated by quantification of the biomass. At different time steps (24, 48 and 72 h) some polycarbonate coupons were collected in aseptic conditions and resuspended in 3 ml of PBS each. Subsequently, serial dilutions were carried out, and 10 μl were inoculated in petri dishes containing Tryptic Soy Broth medium (TSB, Sigma-Aldrich) complemented with agar (Merck) following the drop counting method. After 12 h at 30 °C, *C. albicans* colonies were counted and the data obtained were normalized to the coupon area, and means were reported. The same protocol was used to obtain mature biofilm of *E. coli,* using LB as a medium, and evaluating 10 mg/l of *E. acoroides* leaf extract. Each experiment was repeated three times.

Biofilm dispersion assay

Mature *C. albicans* biofilm was grown in the CDC reactor in the absence (positive control) and presence and of 0.01 mg/l of *E. acoroides* leaf extract as reported below. As previously described by Cattò et al. [41], after 72 h polycarbonate coupons were collected, immersed in 27 ml of PBS for one minute at room temperature, serial dilutions were carried out and 10 μl were inoculated in petri dishes containing TSB supplemented with agar (Merck) following the drop counting method. After 12 h at 30 °C, *C. albicans* colonies were counted and the percentage of biofilm dispersion was calculated as (number of viable cells from bulk PBS × 100) / (number of viable cells from bulk PBS + number of viable cells from the coupon biofilm) and means were reported. Three biological replicates were performed for each treatment and six technical replicates were performed for each experiment. The experiment was performed three times.

Biofilm growth at the solid/air interface

E. coli biofilm was grown on a sterile polycarbonate membrane (PC, Whatman Nucleopore, diameter 2.5 cm, pore diameter 0.2 μm) as previously described by Garuglieri et al. [42]. Briefly, 0.05 ml of an overnight culture (approximately 10^6 cells/ml) were inoculated at the center of a sterile polycarbonate membrane and, when the inoculum was completely dried, the membrane was carefully put inside a transwell structure (ThinCert™ Cell Culture Inserts with translucent PET membrane – Greiner bio-one) inlaid in a 6 well culture plate (Greiner bio-one). One ml of LB medium was inoculated in the basolateral compartment (plate well). Biofilm formation was performed at 37 °C in aerobic conditions for 16 h. At different time points (0, 4, 6, 8, 16 h) some membranes were removed, biofilm was scraped off using a sterile loop, put inside a tube containing 1 ml of PBS and then homogenized twice using a homogenizer (IKA T10 basic Ultra-Turrax – Cole-Parmer Instrument Company). Then serial dilutions were prepared and 10 μl were inoculated in petri dishes containing LB with agar following the drop counting method. After 12 h at 37 °C, *E. coli* colonies were counted and the biomass was quantified. This assay was assessed under three experimental conditions: i) treatment 1: growth in contact with 1 ml of LB with 10 mg/l of *E. acoroides* leaf extract for 16 h; ii) treatment 2: overnight culture grown with 10 mg/l of *E. acoroides* leaf extract, and then growth in contact with 1 ml of LB for 16 h; iii) treatment 3: overnight culture grown with 10 mg/l of *E. acoroides* leaf extract, and then growth in contact with 1 ml of LB with 10 mg/l of *E. acoroides* leaf extract for 16 h. In the positive control, the microorganisms grew in 1 ml LB inside a basolateral well for 16 h without the extract. The data obtained were divided by the area of the membrane, and the means were reported. The experiment was repeated three times.

B2ioluminescence assay using *Vibrio harveyi*

Two hundred μl of autoinducer bioassay (AB) mineral medium (0.3 M NaCl, 0.05 M $MgSO_4$, 0.5% casein hydrolysate, 10 μM KH_2PO_4, 1 μM L-arginine, 50% glycerol, 0.01 μg/ml riboflavin, 1 μg/ml thiamine. pH 7. Sigma-Aldrich) containing 10% (*V*/V) of a tenfold dilution of an overnight culture of *Vibrio harveyi* BB170 (ATCC BAA-1117) grown in AB medium were supplemented with 10 mg/l of *E. acoroides* leaf extract respectively, and were placed in hydrophobic 96-well polystyrene-based microtiter plates (Thermo Fisher Scientific) with transparent bottom. The positive control was an AB mineral medium supplemented with 10% (V/V) tenfold dilution of the overnight culture. Absorbance (OD_{600nm}) and luminescence were measured using a microplate reader (VICTOR™X, Perkin Elmer, USA) every 8 h for 24 h, incubating the microtiter plate at 30 °C during the experiment. The data obtained were normalized to the number of viable cells, divided by the area of the membrane, and the means reported. The experiment was repeated three times.

Statistical analysis

To evaluate statistically significant differences among samples, analysis of variance (ANOVA) via MATLAB software (Version 7.0, The MathWorks Inc., Natick, USA) was applied. Tukey's honestly significant different test (HSD) was applied for pairwise comparison to

establish the significance of the data. Statistically significant results were represented by P values ≤0.05.

Results

Seagrass extracts contain phenolic compounds and show antioxidant capacities

The methanolic extracts from the seagrass material contained phenolic acids as well as flavonoids (Fig. 1a-b). The content of phenols and flavonoids was highest in *H. pinifolia* leaf extracts with 18.0 ± 0.25 and 14.3 ± 0.25 mg/g dry mass (DM), respectively. In *E. acoroides*, the root material showed higher amounts of total flavonoids and phenols than the leaf material. For all seagrass species, the content of phenolic acids was higher than the flavonoid content with respect to the DM.

Methanolic extracts from the four seagrass species were analyzed for their antioxidant capacity (Fig. 1c). All tested extracts had the ability to absorb oxygen radicals. *H. pinifolia* showed the highest activity with 97.7 ± 2.7 mg Trolox equivalents (TE)/g DM. *E. acoroides* and *H. ovalis* leaf extracts showed similar antioxidant capacities with 70.2 ± 4.1 and 72.5 ± 2.9 mg TE/g DM, respectively. The root extract from *E. acoroides* displayed a lower ORAC value than the extract from the leaves (45.1 ± 3.2 mg TE/g DM).

LC-MS analysis of secondary metabolites

E. acoroides, *H. ovalis* and *H. pinifolia* show different compositions of secondary metabolites (Fig. 2). The identification of individual compounds in the methanolic extracts was done via the comparison of MS/MS spectra with database entries. The three seagrass species showed different profiles of secondary metabolites, in this case mainly flavonoids and phenolic acids (Table 2). In *E. acoroides* leaves, three flavonoles based on kaempferol were found. In addition, two flavones (apigenin and luteolin), one phenolic acid (benzoic acid) and the saturated dicarboxylic acid azelaic acid were identified. The root extract of *E. acoroides* also contained two kaempferol-based flavonoles and luteolin and also a procyanidin and a flavanole (epicatechin). In *H. ovalis* three flavonoids and one phenolic acid was found. *H. pinifolia* contained several flavonoles, either based on kaempferol or quercetin and also epicatechin.

Seagrass extracts are not used as carbon and energy source by *C. albicans* and *E. coli* and do not affect their planktonic growth

C. albicans and *E. coli* planktonic cells grown only in the presence of medium supplemented with glucose were used as the positive control of the experiment (Fig. 3). Note that the mineral medium supplemented with the highest concentration of tested plant extracts did not promote the growth of the selected microorganisms.

The response of the planktonic growth of the selected microorganisms in the presence of the seagrass extracts at the highest concentrations (10 and 100 mg/l) is reported in Figs. 4 and 5. *C. albicans* and *E. coli* growth rates (table in Figs. 4 and 5) showed that there are no statistically significant differences between the presence and the absence of the extracts obtained from every plant portion at any tested concentration. Therefore, concentrations ≤100 mg/l plant extract were used in the subsequent studies.

E. acoroides leaf extract inhibits cell adhesion on a hydrophobic surface

The percentage reduction of the number of adhered cells of *E. coli* and *C. albicans* on hydrophilic and hydrophobic surface in presence of non-lethal concentrations of seagrass extracts is showed in Fig. 6. The results revealed that *E. acoroides* and *H. ovalis* were the most promising extracts for *C. albicans*, with excellent anti-adhesion activity, reducing fungal coverage up to 73.89 ± 1.01% and 68.37 ± 2.49% at 0.01 and 1 mg/l, respectively. For *E. coli*, 10 mg/l of *E. acoroides* leaf extract was found to be the concentration with the highest reduction in cell adhesion (reduction

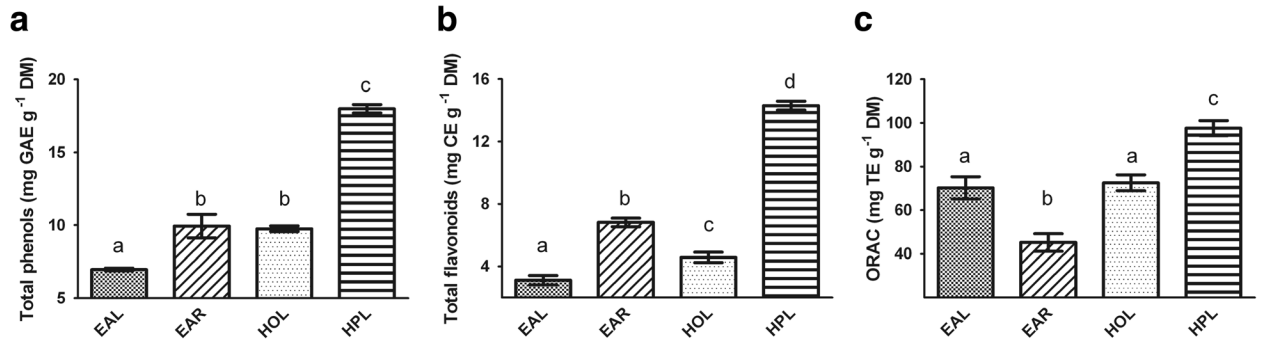


Fig. 1 Crude methanolic extracts were analyzed for (**a**) Total phenols in mg gallic acid equivalent (GAE) per g dry mass (DM), (**b**) Total flavonoids in mg catechin equivalent (CE) per g DM, and (**c**) ORAC in mg Trolox equivalents (TE) per g DM. Data represent the mean ± SDs and different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between the means of three independent measurements. (EAL = *Enhalus acoroides* leaf; EAR = *Enhalus acoroides* root; HPL = *Halodule pinifolia* leaf; HOL = *Halophila ovalis* leaf)

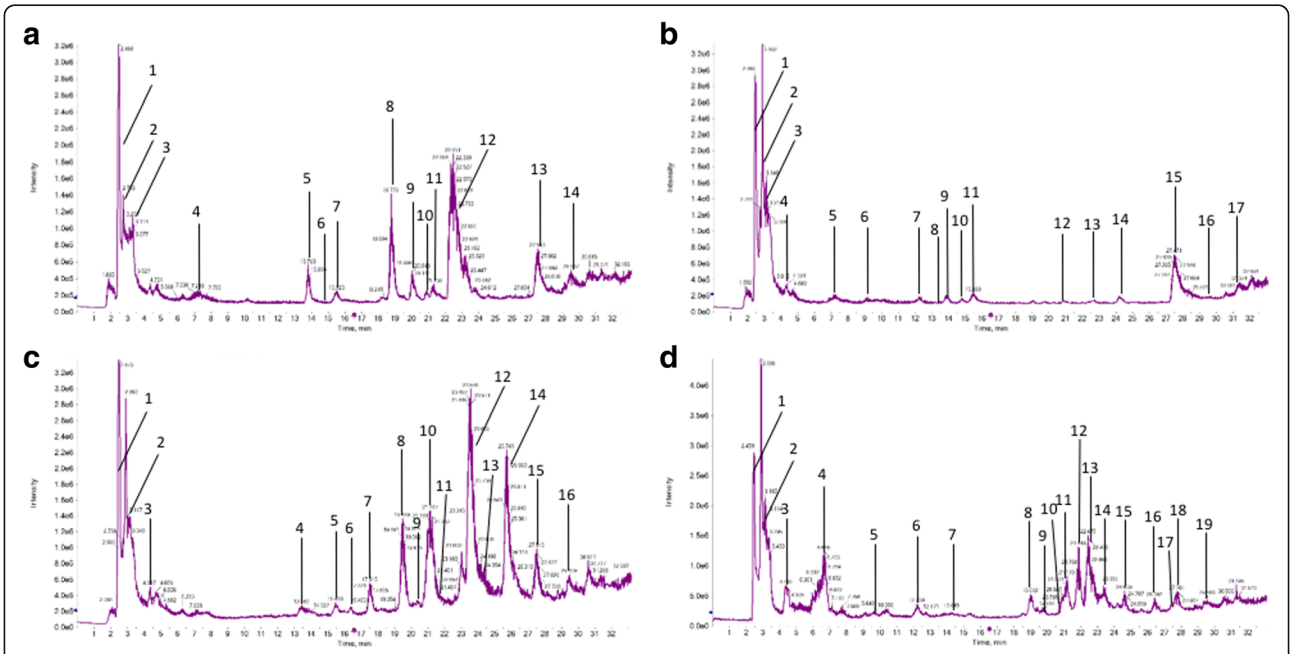


Fig. 2 Chromatograms from *E. acoroides* leaf extract (**a**), *E. acoroides* root extract (**b**), *H. ovalis* leaf extract (**c**) and *H. pinifolia* leaf extract (**d**) from minute 0–33. The relative intensity of mass between 100 and 800 Da is shown. Numbers indicate putatively identified substances in Table 2

of bacterial coverage by 60.86 ± 8.85%). Therefore, 0.01 mg/l and 10 mg/l *E. acoroides* leaf extract were chosen as the best non-biocidal concentrations for *C. albicans* and *E. coli* respectively, and were used in the subsequent studies.

E. acoroides leaf extract does not impact on biofilm growth curves, but does induce biofilm dispersion in *C. albicans* and interfere with AI2

A CDC reactor was used as the laboratory scale model system to grow a complex and mature *C. albicans* biofilm in the absence and presence of 0.01 mg/l *E. acoroides* leaf extract, the most effective concentration obtained from the adhesion assay.

Results in Fig. 7a indicated a significant reduction in the number of viable cells adhered on coupon surfaces treated with the extract, compared to the untreated ones, after 24 h (reduction of fungal coverage up to 26.77 ± 9.01%). Coupons collected after 48 and 72 h showed no significant differences between the treated biofilm and the control.

A significant increase in the number of dispersed cells in the treated biofilm (70 ± 6.83%) was observed (Fig. 7b).

A colony biofilm assay was used to grow a complex and mature *E. coli* biofilm in the presence and absence of 10 mg/l *E. acoroides*. Results in Fig. 8 showed no significant reduction in the number of viable cells during biofilm formation on the membrane treated with the extract, compared to the untreated, after 18 h in all the experimental conditions. Treatment 3 showed a growth rate slowdown in the interval 6–8, in which *E. coli* cells were in contact with the extract during both overnight growth and biofilm formation (reduction of cellular growth, compared to the control, up to 48.64 ± 4.02%). This growth curve was characterized by two exponential phases separated distinctly by an intermediate phase where the growth rate is very low. After that, at 16 h the number of viable cells was similar to the other treatments.

The effects of 10 mg/l of *E. acoroides* leaf extract on the cellular communication of *V. harveyi* were reported in Fig. 9. The results highlighted a significant increase in the relative luminescence emitted at time 8 h compared to the control (25.75 ± 7.49%).

Discussion

Biofilm resistance to antimicrobial agents is a major worldwide health care issue. Therefore, a successful reduction of surface colonization can be a potential strategy for the management of unwanted biofilms, especially on medical devices and work surfaces.

In this context, the use of plant-derived extracts to modulate biofilm genesis and dispersion may be a viable alternative. The present study is the first report describing the antibiofilm efficacy of non-lethal concentrations of *E. acoroides*, *H. pinifolia* and *H. ovalis* methanol extracts in counteracting microbial biofilms, highlighting the possibility that the selected seagrass species act as an extracellular signal mediating their biofilm activities.

E. coli and *C. albicans* were chosen as model systems for bacterial and fungal infections, respectively. *E. coli*

Table 2 Individual compounds identified by comparison of MS/MS spectra with database entries in *Enhalus acoroides* leaf extract (A), *E. acoroides* root extract (B), *Halophila ovalis* leaf extract (C) and *Halodule pinifolia* leaf extract (D)

No	RT	Mass	MS/MS	Name	Accession	Source
A - *E. acoroides* leaf extract						
1	2.5	343.03	201.02, 157.03, 59.01	n. i.	–	–
2	2.7	312.12	179.05, 132.06, 89.02	n. i.	–	–
3	3.3	367.1	277.07, 187.04, 157.03	n. i.	–	–
4	7.2	134.04	107.03, 92.02	Adenine	PT200393	ReSpect
5	13.7	637.1	461.07, 285.04	Kaempferol-3-glucuronide, mod.	PT209240	ReSpect
6	14.8	275.15	233.12, 119.05	n. i.	–	–
7	15.2	121.03	92.02, 77.03	Benzoic acid	KO000321	MassBank
8	18.6	527.02	285.04, 241.00, 96.96	n. i.	–	–
9	20.1	511.05	269.04, 241.00, 96.96	n. i.	–	–
10	20.8	187.09	169.08, 125.09, 97.06	Azelaic acid	KO000124	MassBank
11	21.3	447.09	285.04	Kaempferol-3-O-glucoside	PS042209	ReSpect
12	22.5	461.07	285.04	Kaempferol-3-glucuronide	PS092408	ReSpect
13	27.5	285.04	151.00, 133.03	Luteolin	PS040410	ReSpect
14	29.5	269.04	225.05, 151.00, 117.03	Apigenin	PT203930	ReSpect
B - *E. acoroides* root extract						
1	2.4	343.03	201.02, 157.03, 59.01	n. i.	–	–
2	2.7	312.12	179.05, 132.06, 89.02	n. i.	–	–
3	2.9	377.08	341.11, 179.05, 119.03, 89.02	Galactinol dihydrate, mod.	PT211910	ReSpect
4	4.3	216.98	173.02, 156.98, 136.94, 59.01	n. i.	–	–
5	7.2	134.04	107.03, 92.02	Adenine	PT200393	ReSpect
6	9.6	577.12	451.10, 425.08, 407.07, 289.07, 125.02	Procyanidin B2	PT204580	ReSpect
7	12.3	289.07	245.07, 203.07, 151.04, 109.03	+(–) Epicatechin	PT204560	ReSpect
8	13.8	637.1	461.07, 285.04	Kaempferol-3-glucuronide, mod.	PT209240	ReSpect
9	14.0	469.08	275.02, 193.05, 178.02, 149.06, 96.96	n. i.	–	–
10	14.8	275.15	233.12, 119.05	n. i.	–	–
11	15.3	121.03	92.02, 77.03	Benzoic acid	KO000321	MassBank
12	20.8	187.09	169.08, 125.09, 97.06	Azelaic acid	KO000124	MassBank
13	22.6	461.07	285.04	Kaempferol-3-glucuronide	PS092408	ReSpect
14	24.1	299.05	284.03, 256.03, 133.03	Kaempferide	PT204030	ReSpect
15	27.5	285.04	151.00, 133.03	Luteolin	PS040410	ReSpect
16	29.5	269.04	225.05, 151.00, 117.03	Apigenin	PT203930	ReSpect
17	31.2	329.23	229.14, 211.13, 171.10	n. i.	–	–
C - *H. ovalis* leaf extract						
1	2.4	343.03	201.02, 157.03, 59.01	n. i.	–	–
2	2.9	377.08	341.11, 179.05, 119.03, 89.02	Galactinol dihydrate, mod.	PT211910	ReSpect
3	4.3	216.98	173.02, 156.98, 136.94, 59.01	n. i.	–	–
4	13.3	261.04	217.05, 189.05, 133.02	n. i.	–	–
5	15.5	121.03	92.02, 77.03	Benzoic acid	KO000321	MassBank
6	16.3	306.17	288.16	n. i.	–	–
7	17.5	479.08	316,02	Myricetin-3-galactoside	PS092809	ReSpect
8	19.5	463.09	301,03	Quercetin-3-O-beta-D-galactoside	PS046509	ReSpect
9	20.8	187.09	169.08, 125.09, 97.06	Azelaic acid	KO000124	MassBank

Table 2 Individual compounds identified by comparison of MS/MS spectra with database entries in *Enhalus acoroides* leaf extract (A), *E. acoroides* root extract (B), *Halophila ovalis* leaf extract (C) and *Halodule pinifolia* leaf extract (D) *(Continued)*

No	RT	Mass	MS/MS	Name	Accession	Source
10	21.1	317.02	271.02, 149.02	n.i.	–	–
11	21.3	447.09	285.04	Kaempferol-3-O-glucoside	PS042209	ReSpect
12	23.5	301.03	255.03, 165.02, 133.03	n.i.	–	–
13	24.1	299.05	284.03, 256.03, 133.03	Kaempferide	PS040309	ReSpect
14	25.7	285.04	239.03, 185.06, 143.05, 117.03	Kaempferol	PR040027	MassBank
15	27.5	285.04	285.04, 151.00,133.02	Luteolin	PT204043	ReSpect
16	29.4	269.04	225.05, 151.00, 117.03	Apigenin	PT203930	ReSpect
D - *H. pinifolia* leaf extract						
1	2.4	343.03	201.02, 157.03, 59.01	n. i.	–	–
2	2.9	377.08	341.11, 179.05, 119.03, 89.02	Galactinol dihydrate, mod.	PT211910	ReSpect
3	4.3	216.98	173.02, 156.98, 136.94, 93.03, 59.01	n. i.	–	–
4	6.6	473.07	311.04, 293.03, 179.03, 149.01	n. i.	–	–
5	9.6	577.12	451.10, 425.08, 407.07, 289.07, 125.02	Procyanidin B2	PT204580	ReSpect
6	12.1	289.07	245.07, 203.07, 151.04, 109.03	+(–) Epicatechin	PT204560	ReSpect
7	14.0	469.08	275.02, 193.05, 178.02, 149.06, 96.96	n. i.	–	–
8	19.1	641.17	473.13, 311.07, 167.03	n. i.	–	–
9	19.7	549.09	505.10, 463.09, 300.02, 271.02, 255.02	Quercetin-3-(6-malonyl)-glucoside	PT209340	ReSpect
10	20.8	187.09	169.08, 125.09, 97.06	Azelaic acid	KO000124	MassBank
11	21.1	505.09	463.08, 300.02, 271.02	Quercetin-3-O-beta-D-galactoside, mod.	PT204650	ReSpect
12	21.8	463.08	300.03, 271.02	Quercetin-3-O-beta-D-galactoside	PT204650	ReSpect
13	22.4	433.07	300.02, 271.02, 255.03, 179.00	Quercetin-3-arabinoside	PT209320	ReSpect
14	23.4	447.09	284.03, 255.03, 227.03	Kaempferol-3-glucoside	PT209270	ReSpect
15	24.6	417.08	284.03, 255.03, 227.03	Kaempferol-3-O-alpha-L-arabinoside	PT209220	ReSpect
16	26.3	301.03	178.99, 151.00, 121.03, 107.01	Quercetin	PT204090	ReSpect
17	27.4	285.04	199.03, 175.04, 151.00, 133.02	Luteolin	PT204043	ReSpect
18	27.7	315.05	300.02, 271.02, 255.03	Isorhamnetin	PM007432	ReSpect
19	29.5	269.04	225.05, 151.00, 117.03	Apigenin	PT203930	ReSpect

No = number of peak in Fig. 9, RT = retention time, Mass = mass of precursor ion, MS/MS = fragment spectra obtained at − 30 eV, Accession = accession number in database, Source = database used, n. i. = not identified, mod. = modified

biofilms are found to be the major causative agent of many intestinal infections, for recurrent urinary tract infections, and it also responsible for indwelling medical device-related infectivity [43]. *C. albicans* is one of the very few fungal species causing disease in humans. These infections range from superficial mucosal and dermal infections, such as thrush, vaginal yeast infections, and diaper rash, to vascular catheters and dental implants infections [44].

The bioactive properties of the seagrass species selected in this work are well known, and have been reported in detail by several authors [45–47]. However, until now attention has mainly focused on the antimicrobial activity of seagrass extracts, which, through disk diffusion assays, were investigated not in their capacity as biofilm-forming microorganisms but in their planktonic state. Using lethal concentrations, Umamaheshwari et al. [46] reported the antibacterial activity of *H. ovalis* and *H. pinifolia* extracts, obtained using different solvents, against different microbial strains, recording maximum antibacterial activity by the ethanol extract of *H. pinifolia*. Instead, Choi et al. [48] reported the antimicrobial properties of *Zostera marina* methanol extract and its organic solvent fractions on three human skin pathogens (*Staphylococcus aureus*, *S. epidermidis* and *C. albicans*), and Natrah et al. [47] reported the antibacterial properties of methanol extracts of *E. acoroides* and other seagrass and seaweed species on different aquaculture pathogens (*Aeromonas hydrophila*, *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. anguillarum* and others).

In contrast, to the best of our knowledge, no papers have investigated the antibiofilm activity of *Enhalus acoroides, Halodule pinifolia* and *Halophila ovalis* at non-lethal concentrations against bacterial (*E. coli*) and

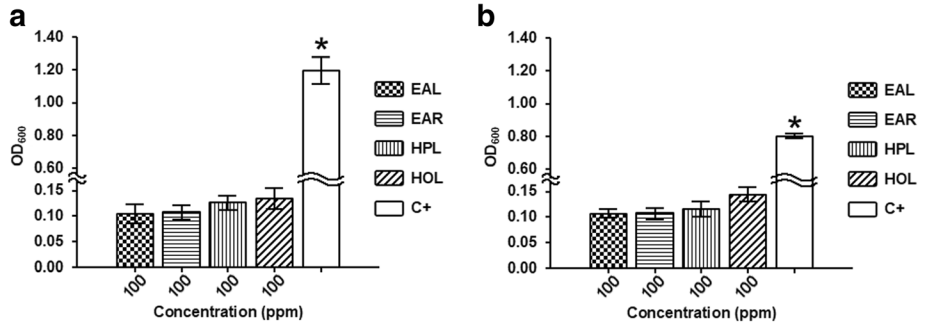
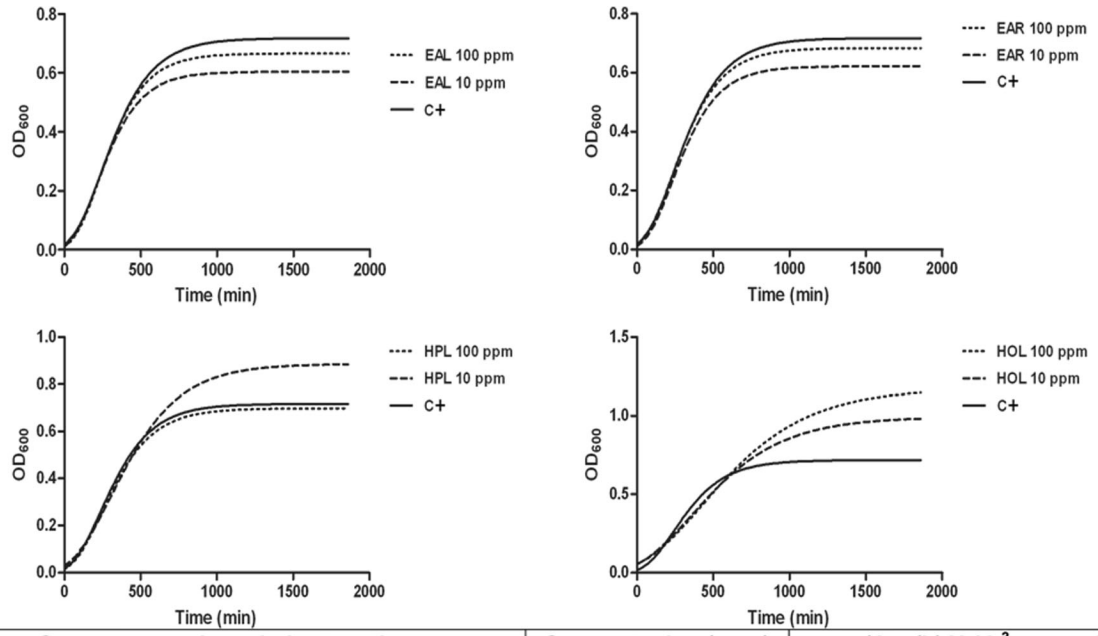
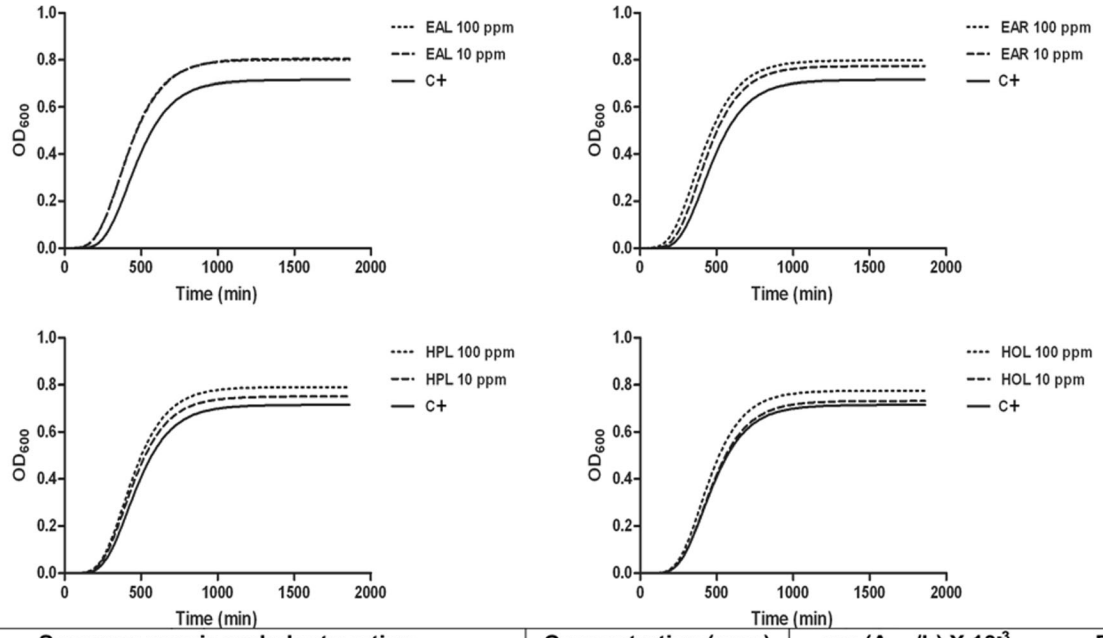


Fig. 3 *E. coli* (**a**) and *C. albicans* (**b**) planktonic growth without (positive control) and with each seagrass extract at 100 ppm. The positive control was set up with mineral medium supplemented with glucose at 5 g/l. Stars indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between the means of three independent replicates. (EAL = *Enhalus acoroides* leaf; EAR = *Enhalus acoroides* root; HPL = *Halodule pinifolia* leaf; HOL = *Halophila ovalis* leaf; C + = Positive control)



Seagrass specie and plant portion	Concentration (ppm)	μm (A_{600}/h) X 10^{-3}	R^2
Control (LB)	0	1.42±0.04	0.9945
Enhalus acoroides leaf	100	1.47±0.03	0.9959
	10	1.40±0.04	0.9946
Enhalus acoroides root	100	1.40±0.06	0.9953
	10	1.37±0.03	0.9948
Halodule pinifolia leaf	100	1.37±0.02	0.9949
	10	1.38±0.06	0.9937
Halophila ovalis leaf	100	1.42±0.01	0.9881
	10	1.45±0.09	0.9919

Fig. 4 OD-based growth curves of *C. albicans* in absence (positive control) and in presence of each seagrass extract at 10 and 100 ppm. Maximum specific growth rate (μ_m) and the goodness of fit (R^2) obtained by the Gompertz model. Data represent the mean ± SDs of three independent measurements. Means reported showed no statistically significant differences between the positive control and treated samples (Tukey's HSD, $p \geq 0.05$). (EAL = *Enhalus acoroides* leaf; EAR = *Enhalus acoroides* root; HPL = *Halodule pinifolia* leaf; HOL = *Halophila ovalis* leaf; C + = Positive control)



Seagrass specie and plant portion	Concentration (ppm)	μm (A_{600}/h) X 10^{-3}	R^2
Control (YNBG)	0	1.95±0.03	0.9943
Enhalus acoroides leaf	100	1.97±0.04	0.9930
	10	1.93±0.02	0.9936
Enhalus acoroides root	100	1.91±0.05	0.9919
	10	1.96±0.03	0.9921
Halodule pinifolia leaf	100	1.95±0.04	0.9938
	10	1.84±0.08	0.9929
Halophila ovalis leaf	100	1.93±0.05	0.9924
	10	1.96±0.05	0.9940

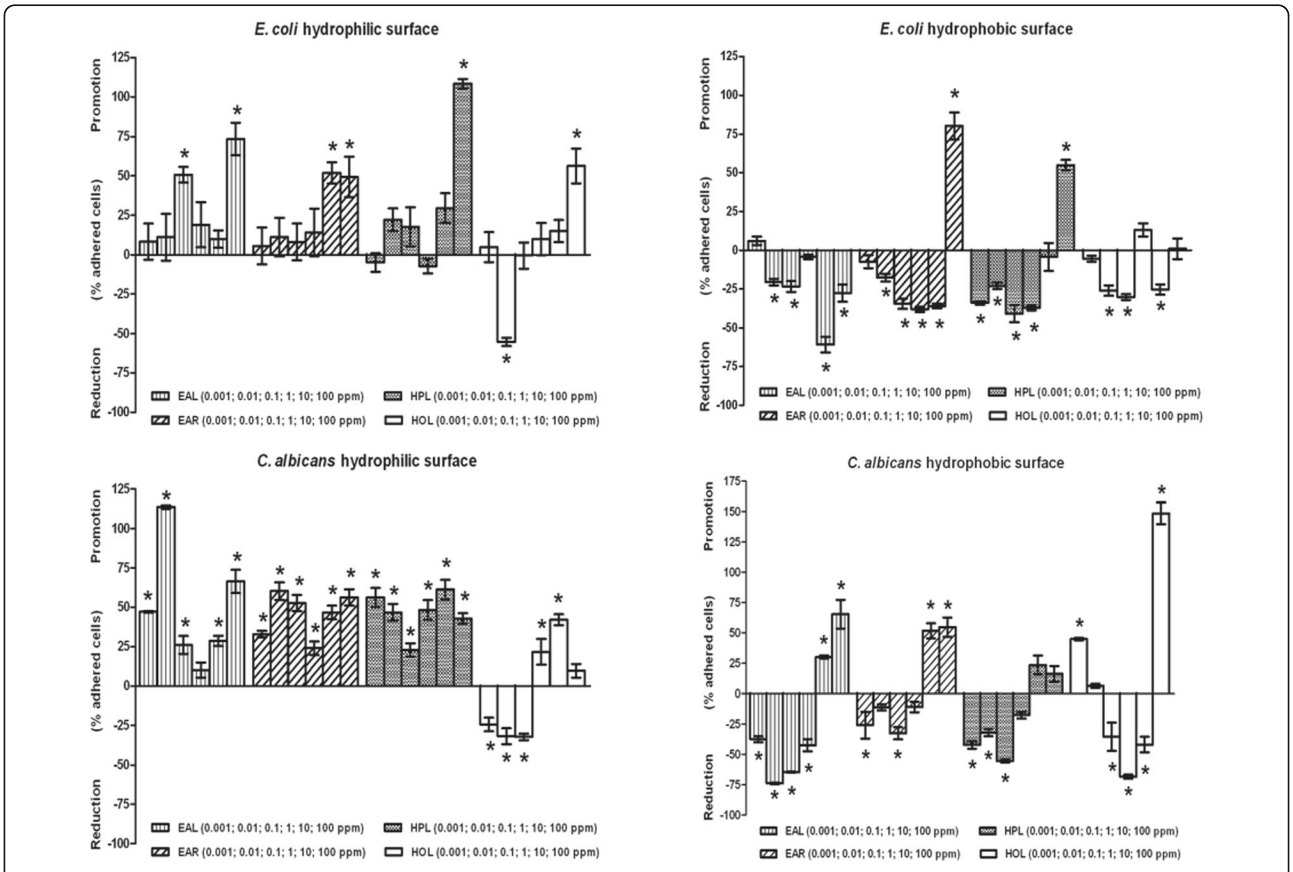
Fig. 5 OD-based growth curves of *E. coli* in absence (positive control) and in presence of each seagrass extract at 10 and 100 ppm. Maximum specific growth rate (μ_m) and the goodness of fit (R^2) obtained by the Gompertz model. Data represent the mean ± SDs of three independent measurements. Means reported showed no statistically significant differences between the positive control and treated samples (Tukey's HSD, $p \geq 0.05$). (EAL = *Enhalus acoroides* leaf; EAR = *Enhalus acoroides* root; HPL = *Halodule pinifolia* leaf; HOL = *Halophila ovalis* leaf; C + = Positive control)

fungal (*C. albicans*) biofilms. To this end, methanol extracts, obtained from different organs of three seagrass species (namely, *Enhalus acoroides* leaves and roots, *Halophila ovalis* leaves and *Halodule pinifolia* leaves) were screened for their ability to modulate biofilm genesis without killing cells. Methanol was used as the extraction solvent, having been previously reported as the most effective solvent to obtain high concentrations of bioactive compounds with antibacterial activity from seagrasses, compared to other extraction solvents [45, 49, 50].

Before evaluating the antibiofilm activity, the extracts, at concentrations of 100 mg/l, were first proved to not act as a carbon and energy source nor to affect the cellular growth of *C. albicans* and *E. coli*. Therefore, concentrations ≤100 mg/l plant extract were used in the subsequent studies.

With the aim of investigating the effects of seagrass extracts on cell adhesion to surfaces, the first step of biofilm formation, microtiter based assays were performed. The results revealed excellent anti-adhesion activity for *E. acoroides* leaf extract, reducing fungal coverage up to 74% and bacterial coverage up to 61% at 0.01 and 10 mg/l, respectively. Therefore, 0.01 mg/l and 10 mg/l *E. acoroides* leaf extract were chosen as the best non-biocidal concentrations for *C. albicans* and *E. coli* respectively, and were used in the subsequent studies. These concentrations significantly decreased the number of adhered cells on a hydrophobic surface, more so than on the hydrophilic one. Previous studies had highlighted the preference for hydrophobic surfaces, these reporting a decreased adhesion on the hydrophobic surface compared to the hydrophilic [51, 52]. This is probably due to the hydrophobic nature of the aerial surfaces of plants [53].

In the present study the anti-adhesion activity of the seagrass extracts was dose-dependent, but the highest concentrations did not correspond to those with the



		0.001 ppm	0.01 ppm	0.1 ppm	1 ppm	10 ppm	100 ppm
E. coli hydrophilic surface	EAL	8.25±24.71%	11.07±25.61%	50.71±8.63%	19±28.76%	9.93±11.13%	73.51±17.95%
	EAR	5.51±20.27%	11.15±21.19%	8.11±20.25%	0.03±12.43%	51.92±13.64%	49.35±22.32%
	HPL	-4.89±10.31%	22.16±14.26%	17.65±24.88%	-7.24±8.25%	29.54±18.86%	108.34± 5.51%
	HOL	4.77±16.49%	-55.37±4.64%	-0.54±16.59%	15.34±17.96%	15.00±12.16%	56.36±22.18%
E. coli hydrophobic surface	EAL	6.05±4.59%	-20.60±3.72%	-23.35±6.07%	-4.36±3.09%	-60.76±8.86%	-27.66±9.95%
	EAR	-7.41±8.43%	-17.65±5.19%	-34.45±5.91%	-38.04±2.93%	-36.02±2.44%	80.29±14.74%
	HPL	-33.95±2.07%	-22.98±3.83%	-40.82±9.47%	-37.03±2.74%	-4.43±15.65%	54.92±5.95%
	HOL	-5.37±3.45%	-26.03±5.82%	-30.22±3.10%	13.14±8.63%	-25.31±6.81%	1.01±11.48%
C. albicans hydrophilic surface	EAL	47.13±12.91%	113.65±2.12%	26.07±9.83%	10.15±8.43%	28.65±5.79%	66.40±12.83%
	EAR	32.97±3.35%	60.28±9.53%	52.67±8.83%	24.03±7.27%	46.72±7.50%	56.25±8.98%
	HPL	56.21±10.47%	46.75±9.32%	22.97±7.16%	48.29±10.86%	61.23±12.34%	42.87±5.81%
	HOL	-24.32±7.48%	-31.76±8.75%	-32.23±4.20%	21.71±13.99%	42.03±6.00%	9.63±7.53%
C. albicans hydrophobic surface	EAL	-37.50±4.03%	-73.89±1.01%	-64.74±0.69%	-42.57±8.69%	29.90±2.07%	65.32±20.58%
	EAR	-25.81±19.29%	-11.14±4.99%	-32.69±8.81%	-10.91±7.64%	51.68±12.59%	54.69±13.88%
	HPL	-42.33±5.15%	-32.18±4.95%	-55.39±2.09%	-17.54±4.82%	23.59±13.40%	16.17±11.43%
	HOL	44.86±1.47%	6.23±3.02%	-35.67±20.22%	-68.36±2.99%	-41.95±10.92%	148.60±15.39%

Fig. 6 Microplate-based biofilm assay. Percentage reduction of the number of adhered cells of *E. coli* and *C. albicans* on hydrophilic and hydrophobic surface in presence of non-lethal concentrations of seagrass extracts. According to post hoc analysis (Tukey's HSD, $p \leq 0.05$), stars indicate statistically significant differences between the means of three independent replicates. In addition, the mean ± SDs of the percentage reduction of the number of adhered cells with seagrass extracts at non-lethal concentrations on hydrophilic and hydrophobic surface are reported in the table. The higher anti-adhesion effect for each microorganism was highlighted. (EAL = *Enhalus acoroides* leaf; EAR = *Enhalus acoroides* root; HPL = *Halodule pinifolia* leaf; HOL = *Halophila ovalis* leaf)

best performance. Indeed, several studies have reported a weak activity of the compounds at low and high concentrations, and excellent activity at intermediate concentrations [54]. Such a response, widely known in literature, is defined as hormesis, an adaptive behavior of microorganisms to provide resistance to environmental stress and improve the allocation of resources to ensure cell stability [19, 55].

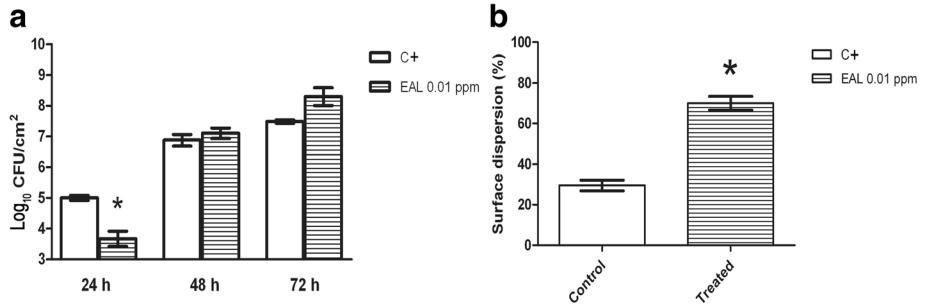


Fig. 7 CDC biofilm growth on polycarbonate coupons (**a**) and biofilm dispersion rate (**b**) of *C. albicans* in absence (positive control) and in presence (treated) of 0.01 ppm of *Enhalus acoroides* leaf extract. Stars indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between the means of three independent replicates. (C + = Positive control; EAL = *Enhalus acoroides* leaf)

To further explore the effect of the most promising seagrass extract on biofilm development and detachment, CDC reactors were employed to reproduce biofilm at the solid/liquid interface, while for the assessment of the anti-biofilm effect in the adhesion phase microplate-based biofilm assays are the most suitable [41, 56, 57]. In this study, a significant reduction in fungal coverage (up to 26.77 ± 9.01%) after 24 h (static adhesion phase) was observed in presence of 0.01 mg/l *E. acoroides* leaf extract. This result confirms the anti-adhesion activity observed in microtiter assays. Coupons collected after 48 and 72 h showed no significant differences between treated and control samples.

In order to assess the possibility of 0.01 mg/l *E. acoroides* leaf extract to promote *C. albicans* biofilm-detachment from the surface of coupons, a biofilm dispersion assay was performed. Results showed a significant increase in the number of dispersed cells in the treated biofilm, compared with the untreated (70 ± 6.83%), suggesting a further mechanism of action for the seagrass extract as biofilm dispersing agent. In fact, the phase of biofilm dispersion could be an interesting target for the development of new antibiofilm strategies, forcing the planktonic state and reestablishing the efficacy of traditional antimicrobial agents [4, 58]. Literature with information related to *C. albicans* biofilm dispersion is scarce. Farnesol and cis-2-decenoic acid showed dispersion-promotion of microcolonies of *C. albicans* biofilm [58, 59]. In addition, Villa et al. [60] reported that non-lethal concentrations of *Muscari comosum* ethanol bulb extract can modulate yeast adhesion and subsequent biofilm development on abiotic surfaces, and such concentrations could provide an extracellular signal responsible for biofilm dispersion.

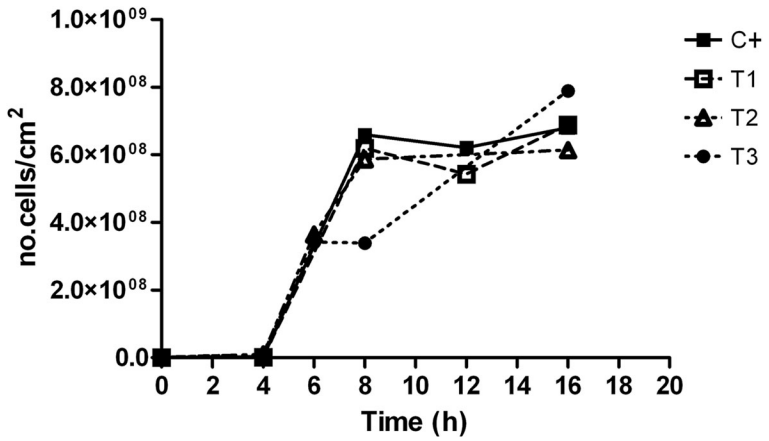


Fig. 8 Biofilm growth at the solid/air interface. *E. coli* biofilm grown on polycarbonate membrane under three experimental conditions: i) treatment 1: growth in contact with 1 ml of LB with 10 ppm of *E. acoroides* leaf extract; ii) treatment 2: overnight culture grown with 10 ppm of *E. acoroides* leaf extract and then growth in contact with 1 ml of LB; iii) treatment 3: overnight culture grown with 10 ppm of *E. acoroides* leaf extract and growth in contact with 1 ml of LB with 10 ppm of *E. acoroides* leaf extract. In the positive control, microorganisms grew in 1 ml LB inside a basolateral well without the extract. Data obtained were divided by the area of the membrane, and means were reported. The experiment was repeated three times. (T1 = treatment 1; T2 = treatment 2; T3 = treatment 3; C + = Positive control)

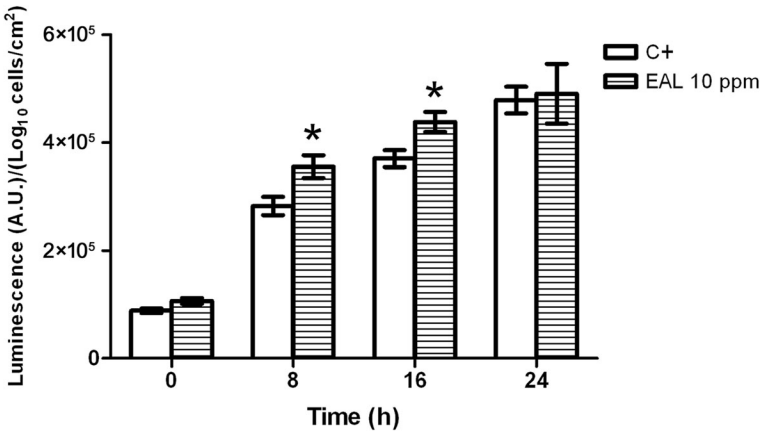


Fig. 9 Relative luminescence emitted by *Vibrio harveyi* in absence (positive control) and in presence of 10 ppm of *E. acoroides* leaf extract for 24 h. The relative luminescence has been calculated by normalizing luminescence by the number of adhered cells. Stars indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between the means of three independent replicates. (C + = Positive control; EAL = *Enhalus acoroides* leaf)

For *E. coli*, the CDC reactor was not suitable to evaluate the possible effects of the extracts on the biofilm stages. Also other authors have reported the poor biofilm formation exhibited by *E. coli* K-12 strain under hydrodynamic conditions [61–63]. The effect of 10 mg/l of *E. acoroides* leaf extract on *E. coli* biofilm formation was then evaluated using a membrane-supporting biofilm reactor, which allowed the formation of a biofilm at the solid/air interface. This technique forced the cells to attach to a surface, a feature that allowed direct investigation of the effect of the selected extract on the development of the biofilm, whilst bypassing the effect on the adhesion phase.

No significant reduction in the number of viable cells during biofilm formation on the membrane treated with the extract, compared to the untreated, after 18 h in all the experimental conditions was observed. Treatment 3 showed a growth rate slowdown in the interval 6–8 h, in which *E. coli* cells were in contact with the extract during both overnight growth and biofilm formation (reduction of cellular growth, compared to the control, up to 48.64 ± 4.02%). Interestingly, treatment 3 showed a biphasic growth curve compared with the growth curves of the other treatments, a trend that could be explained by the bioluminescence produced by *V. harvey*. As signaling molecules play an important role in biofilm development and detachment, the effects of 10 mg/l of *E. acoroides* leaf extract were investigated using *V. harveyi*, suggesting other possible antibiofilm mechanisms of action of compounds in the chosen seagrass extract. The results revealed that at time 8 h, the samples treated with the leaf extract showed a significant increase in the relative luminescence emitted, compared to the control (25.75 ± 7.49). Villa et al. [64] reported an increase of autoinducer-2 (AI-2) activity and a reduction in biofilm formation in *E. coli* cells treated with zosteric acid, a phenolic compound occurring in the seagrass *Zostera marina*. In fact, it has been hypothesized that the accumulation of AI-2 above a threshold level leads to reduced biofilm formation due to the induction of a hypermotile phenotype that is unable to adhere to the surface [64]. Huber et al. [65] demonstrated that some polyphenolic compounds containing a gallic acid residue commonly produced by some plant species inhibited intercellular communication in bacteria. Truchado et al. [66] reported the ability of some phytochemical compounds (cinnamaldehyde, ellagic acid, resveratrol, rutin and pomegranate extract) to interfere with the quorum sensing system of *Yersinia enterocolitica* and *Erwinia cartovora*.

It has been well known that the antibiofilm activity of plant extracts is closely linked with the content of secondary metabolites, such as phenols and/or flavonoids, which represent the total amount of phenolic compounds in a plant extract [13]. The phenolic compound content is also deeply associated with the antioxidant activity of plant extracts [67]. Therefore, we determined the total phenolic acid (TPC) and flavonoid (TFC) content and the antioxidant activity (ORAC) of methanolic extracts in order to highlight features of the most promising antibiofilm extract, the *E. acaroides* leaf extract. Results show that *E. acaroides* leaf extract presents the lower TPC and TFC values compared to other seagrasse extracts. Although the low content of phenolic compounds, the *E. acaroides* leaf extract displays a higher ORAC value compared to the root extract. This indicates the abundance of other, non-phenolic compounds with antioxidant capacity in the leaves of *E. acoroides*. Cattò et al. [39] suggested the importance of antioxidant compounds in hindering biofilm formation. The researcher discovered that the mechanism of action behind the antibiofilm performance

of zosteric acid, a secondary metabolite of the seagrass *Zostera marina*, is related to the antioxidant activity of the molecule, and its interaction with the WrbA protein responsible maintaining cellular homeostasis and defense against oxidative stress.

To gain more insight into possible antibiofilm compounds in the seagrass extracts, individual substances in the methanolic extract were analyzed by LC-MS. Preliminary analysis shows that the phytochemical profile of the *E. acaroides* leaf extract is mainly characterized by the presence of the flavones apigenin and luteolin, three kaempferol derivates and the carboxylic acids benzoic and azelaic acid. This unique quantitative and qualitative chemical composition confers antibiofilm properties to the *E. acaroides* leaf extract.

Some of these compounds have shown to exhibit antibiofilm properties at non-lethal concentrations. Kaempferol, apigenin and luteolin from red wine reduced biofilm formation of methicillin-sensitive *S. aureus* significantly [68]. Sánchez et colleagues [69] reported that sub-lethal concentrations of plant extracts inhibit *E. coli* and *S. aureus* biofilms. The antibiofilm properties of the extracts were associated to the presence of flavonoids, such as kaempferol and apigenin, which modulate bacterial cell-cell communication by suppressing the activity of the autoinducer-2 [70]. However, we should keep in mind that the antibiofilm effects of plant extracts could be the result of interactions among different components of the extract at specific concentrations, and not only due to the effects of a single, predominant compound [4, 71].

Conclusions

In conclusion, the *E. acoroides* leaf extract proved to be the most promising extract among those tested. Indeed, the selected non-lethal concentrations of *E. acoroides* leaf extract were found to exert an antibiofilm effect on *C. albicans* and *E. coli* biofilm in the first phase of biofilm genesis, opening up the possibility of developing preventive strategies to hinder the adhesion of microbial cells to surfaces. The leaf extract also affected the dispersion and maturation steps in *C. albicans* and *E. coli* respectively, suggesting an important role in cell signaling processes. These effects could be explained by the presence of active compounds like kaempferol and apigenin at specific concentrations in the extracts of *E. acoroides*, which are known to possess biofilm inhibiting properties. Furthermore, there could be a synergistic action of these flavonoids with other compounds occurring in the plant, enhancing the global antibiofilm effect. Currently, the leaf extract is being investigated with the objective of testing fractions for identifying the active compounds and to better understand the mechanisms of action of this seagrass species.

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Availability of data and materials
The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Authors' contributions
DVL performed the biological experiments and wrote the manuscript. GY extracted and analyzed the crude extracts from the plants by MS. CC participated in the design of the study and provided technical advices and lab supports. VF conceived, designed and coordinated the study. VF contributed substantially to the writing and revising of the manuscript. CF and PJ participated in the design of the study, in discussions and reviewed the manuscript. All authors read and approved the final manuscript.

Competing interest
The authors declare that they have no competing interests.

Ethics approval and consent to participate
Not applicable.

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References
1. Costerton JW. Introduction to biofilm. Int J Antimicrob Agents. 1999;11:217–21.
2. Hall-Stoodley L, Costerton WJ, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol. 2004;2:95–108. https://doi.org/10.1038/nrmicro821.
3. Rayner J, Veeh R, Flood J. Prevalence of microbial biofilms on selected fresh produce and household surfaces. Int J Food Microbiol. 2004;95:29–39.
4. Villa F, Villa S, Gelain A, Cappitelli F. Sub-lethal activity of small molecules from natural sources and their synthetic derivatives against biofilm forming nosocomial pathogens. Curr Top Med Chem. 2013;13(24):3184–204.
5. Macià MD, Rojo-Molinero E, Oliver A. Antimicrobial susceptibility testing in biofilm-growing bacteria. Clin Microbiol Infect. 2014;20(10):981–90. https://doi.org/10.1111/1469-0691.12651.
6. Percival SL, Suleman L, Vuotto C, Donelli G. Healthcare-associated infections, medical devices and biofilms: risk. tolerance and control J Med Microbiol. 2015;64(Pt 4):323–34. https://doi.org/10.1099/jmm.0.000032.
7. Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC. Hidden killers: human fungal infections. Sci Transl Med. 2012;4:165rv113. https://doi.org/10.1126/scitranslmed.3004404.
8. Akbari F, Kjellerup BV. Elimination of bloodstream infections associated with Candida albicans biofilm in intravascular catheters. Pathogens 2015;4(3):457–69. https://doi.org/10.3390/pathogens4030457.
9. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet. 2001;358:135–8. https://doi.org/10.1016/S0140-6736(01)05321-1.
10. Smith K, Hunter IS. Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates. J Med Microbiol. 2008;57:966–73. https://doi.org/10.1099/jmm.0.47668-0.
11. Lebeaux D, Ghigo JM, Beloin C. Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance

toward antibiotics. Microbiol Mol Biol Rev. 2014;78(3):510–43. https://doi.org/10.1128/MMBR.00013-14.
12. Chung PY. Plant-derived compounds as potential source of novel anti-biofilm agents against *Pseudomonas aeruginosa*. Curr Drug Targets. 2017; 18(4):414–20. https://doi.org/10.2174/1389450117666161019102025.
13. Choi HA, Cheong DE, Lim HD, Kim WH, Ham MH, Oh MH, Wu Y, Shin HJ, Kim GJ. Antimicrobial and anti-biofilm activities of the methanol extracts of medicinal plants against dental pathogens *Streptococcus mutans* and *Candida albicans*. J Microbiol Biotechnol. 2017;27(7):1242–8. https://doi.org/10.4014/jmb.1701.01026.
14. da Silva DT, Herrera R, Batista BF, Heinzmann BM, Labidi J. Physicochemical characterization of leaf extracts from *Ocotea lancifolia* and its effect against wood-rot fungi. Int Biodeterior Biodegradation. 2017;117:158–70. doi.org/10.1016/j.ibiod.2016.12.007
15. Teanpaisan R, Kawsud P, Pahumunto N, Puripattanavong J. Screening for antibacterial and antibiofilm activity in Thai medicinal plant extracts against oral microorganisms. J Tradit Complement Med. 2017;7(2):172–7. doi.org/10.1016/j.jtcme.2016.06.007
16. Abiala M, Olayiwola J, Babatunde O, Aiyelaagbe O, Akinyemi S. Evaluation of therapeutic potentials of plant extracts against poultry bacteria threatening public health. BMC Complement Altern Med. 2016;16(1):417.
17. Bisi-Johnson MA, Obi CL, Samuel BB, Eloff JN, Okoh AI. Antibacterial activity of crude extracts of some south African medicinal plants against multidrug resistant etiological agents of diarrhoea. BMC Complement Altern Med. 2017;17(1):321. https://doi.org/10.1186/s12906-017-1802-4.
18. Elisha IL, Botha FS, McGaw LJ, Eloff JN. The antibacterial activity of extracts of nine plant species with good activity against *Escherichia coli* against five other bacteria and cytotoxicity of extracts. BMC Complement Altern Med. 2017;17(1):133. https://doi.org/10.1186/s12906-017-1645-z.
19. Villa F, Cappitelli F. Plant-derived bioactive compounds at sub-lethal concentrations: towards smart biocide-free antibiofilm strategies. Phytochem Rev. 2013;12:245–54. https://doi.org/10.1007/s11101-013-9286-4.
20. Flowers TJ, Colmer TD. Salinity tolerance in halophytes. New Phytol. 2008; 179:945–63. https://doi.org/10.1111/j.1469-8137.2008.02531.x.
21. Flowers TJ, Colmer TD. Plant salt tolerance: adaptations in halophytes. Ann Bot. 2015:115327–31. https://doi.org/10.1093/aob/mcu267.
22. Joshi R, Ramanarao MV, Bedre R, Sanchez L, Pilcher W, Zandkarimi H, Baisakh N. Salt adaptation mechanisms of halophytes: improvement of salt tolerance in crop plants. In: Pandey GK, editor. Elucidation of abiotic stress signaling in plants. New York, NY: Springer; 2015. p. 243–79. https://doi.org/10.1007/978-1-4939-2540-7_9.
23. Selmar D. Potential of salt and drought stress to increase pharmaceutical significant secondary compounds in plants. Landbauforschung. 2008;58: 139–44.
24. Ksouri R, Ksouri WM, Jallali I, Debez A, Magné C, Hiroko I, Abdelly C. Medicinal halophytes: potent source of health promoting biomolecules with medical, nutraceutical and food applications. Crit Rev Biotechnol. 2012; 32:289–326. https://doi.org/10.3109/07388551.2011.630647.
25. Boestfleisch C, Wagenseil NB, Buhmann AK, Seal CE, Wade EM, Muscolo A, Papenbrock J. Manipulating the antioxidant capacity of halophytes to increase their cultural and economic value through saline cultivation. AoB Plants. 2014;6:plu046. https://doi.org/10.1093/aobpla/plu046.
26. Hua KF, Hsu HY, Su YC, Lin IF, Yang SS, Chen YM. Study on the antinflammatory activity of methanol extract from seagrass *Zostera japonica*. J Agric Food Chem. 2006;54:306–11. https://doi.org/10.1021/jf0509658.
27. Gokce G, Haznedaroglu MZ. Evaluation of antidiabetic, antioxidant and vasoprotective effects of *Posidonia oceanica* extract. J Ethnopharmacol. 2008;115:122–30.
28. Kannan RRR, Arumugam R, Anantharaman P. Chemical composition and antibacterial activity of Indian seagrasses against urinary tract pathogens. Food Chem. 2012;135:2470–3. https://doi.org/10.1016/j.foodchem.2012.07.070.
29. Nguyen XV, Höfler S, Glasenapp Y, Thangaradjou T, Lucas C, Papenbrock J. New insights into the DNA barcoding of seagrasses. Syst Biodivers. 2015;13: 496–508. https://doi.org/10.1080/14772000.2015.1046408.
30. Lucas C, Thangaradjou T, Papenbrock J. Development of a DNA barcoding system for seagrasses: successful but not simple. PLoS One. 2012;7:e29987. https://doi.org/10.1371/journal.pone.0029987.
31. Dudonné S, Vitrac X, Coutière P, Woillez M, Mérillon JM. Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. J Agric Food Chem. 2009;57:1768–74. https://doi.org/10.1021/jf803011r.
32. Dewanto V, Wu X, Adom KK, Liu RH. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J Agric Food Chem. 2002;50:3010–4. https://doi.org/10.1021/jf0115589.
33. Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J Agric Food Chem. 2002;50(16): 4437–44. https://doi.org/10.1021/jf0201529.
34. Gillespie KM, Chae JM, Ainsworth EA. Rapid measurement of total antioxidant capacity in plants. Nat Protoc. 2007;2(4):867–70. https://doi.org/10.1038/nprot.2007.100.
35. Horai H, Arita M, Kanaya S, Nihei Y, Ikeda T, Suwa K, Ojima Y, Tanaka K, Tanaka S, Aoshima K, Oda Y, Kakazu Y, Kusano M, Tohge T, Matsuda F, Sawada Y, Yokota Hirai M, Nakanishi H, Ikeda K, Akimoto N, Maoka T, Takahashi H, Ara T, Sakurai N, Suzuki H, Shibata D, Neumann S, Iida T, Tanaka K, Funatsu K, Matsuura F, Soga T, Taguchi R, Saito K, Nishioka T. MassBank: a public repository for sharing mass spectral data for life sciences. J Mass Spectrom. 2010;45:703–14. https://doi.org/10.1002/jms.1777.
36. Sawada Y, Nakabayashi R, Yamada Y, Suzuki M, Sato M, Sakata A, Akiyama K, Sakurai T, Matsuda F, Aoki T, Hirai MY, Saito K. RIKEN tandem mass spectral database (ReSpect) for phytochemicals: a plant-specific MS/MS-based data resource and database. Phytochemistry. 2012;82:38–45. https://doi.org/10.1016/j.phytochem.2012.07.007.
37. Zwietering MH, Jongenburger I, Rombouts FM, van't Riet K. Modeling of the bacterial growth curve. Appl Environ Microbiol 1990;56:1875–1881.
38. Villa F, Albanese D, Giussani B, Stewart P, Daffonchio D, Cappitelli F. Hindering biofilm formation with zosteric acid. Biofouling. 2010;26:739–52. https://doi.org/10.1080/08927014.2010.511197.
39. Cattò C, Dell'Orto S, Villa F, Villa S, Gelain A, Vitali A, Marzano V, Baroni S, Forlani F, Cappitelli F. Unraveling the structural and molecular basis responsible for the anti-biofilm activity of zosteric acid. PLoS One. 2015; 10(7):e0131519. https://doi.org/10.1371/journal.pone.0131519.
40. Villa F, Pitts B, Stewart PS, Giussani B, Roncoroni S, Albanese D. Efficacy of zosteric acid sodium salt on the yeast biofilm model *Candida albicans*. Microb Ecol. 2011;62:584–98. https://doi.org/10.1007/s00248-011-9876-x.
41. Cattò C, Grazioso G, Dell'Orto S, Gelain A, Villa S, Marzano V, Vitali A, Villa F, Cappitelli F, Forlani F. The response of *Escherichia coli* biofilm to salicylic acid. Biofouling. 2017;33:235–51. https://doi.org/10.1080/08927014.2017.1286649.
42. Garuglieri E, Meroni E, Cattò C, Villa F, Cappitelli F, Erba D. Effects of sub-lethal concentrations of silver nanoparticles on a simulated intestinal prokaryotic-eukaryotic interface. Front Microbiol. 2018;8:2698. https://doi.org/10.3389/fmicb.2017.02698.
43. Sharma G, Sharma S, Sharma P, Chandola D, Dang S, Gupta S, Gabrani R. *Escherichia coli* biofilm: development and therapeutic strategies. J Appl Microbiol. 2016;121(2):309–19. https://doi.org/10.1111/jam.13078. Epub 2016 Mar 11
44. Nobile CJ, Johnson AD. *Candida albicans* biofilms and human disease. Annu Rev Microbiol. 2015;69:71–92. https://doi.org/10.1146/annurev-micro-091014-104330.
45. Kumar SC, Sarada DVL, Gideon TP, Rengasamy R. Antibacterial activity of three south Indian seagrasses, *Cymodocea serrulata*, *Halophila ovalis* and *Zostera capensis*. World J Microbiol Biotechnol. 2008;24(9):1989–92. https://doi.org/10.1007/s11274-008-9695-5.
46. Umamaheshwari R, Thirumaran G, Anantharaman P. Potential antibacterial activities from vellar estuary; south east coast of India. Adv Biomed Res. 2009;3:140–3.
47. Natrah FMI, Harah ZM, Japar Sidik NI, Syahidah A. Antibacterial activities of selected seaweed and seagrass from port Dickson coastal water against different aquaculture pathogens. Sains Malays. 2015; 44(9):1269–73.
48. Choi HG, Lee JH, Park HH, Sayegh FAQ. Antioxidant and antimicrobial activity of *Zostera marina* L. extract. Algae. 2009;24(3):179–84. https://doi.org/10.4490/algae.2009.24.3.179.
49. Lustigman B, Brown C. Antibiotic production by marine algae isolated from the New York/New Jersey coast. Bull Environ Contam Toxicol. 1991;46:329–35. https://doi.org/10.1007/BF01688928.
50. Sastry VMVS, Rao GRK. Antibacterial substances from marine algae: successive extraction using benzene, chloroform and methanol. Bot Mar. 1994;37:357–60. https://doi.org/10.1515/botm.1994.37.4.357.

51. Doss RP, Potter SW, Chastagner GA, Christian JK. Adhesion of nongerminated *Botrytis cinerea* conidia to several substrata. Appl Environ Microbiol. 1993;59:1786–91.
52. Amiri A, Cholodowski D, Bompeix G. Adhesion and germination of waterborne and airborne conidia of *Penicillium expansum* to apple and inert surfaces. Physiol Mol Plant Pathol. 2005;67:40–8. https://doi.org/10.1016/j.pmpp.2005.07.003.
53. Koch K, Bhushan B, Barthlott W. Multifunctional surface structures of plants: an inspiration of biomimetics. Prog Mater Sci. 2009;54:137–78. https://doi.org/10.1016/j.pmatsci.2008.07.003.
54. Rickard AH, Palmer RJ, Blehert DS, Campagna SR, Semmelhack MF, Egland PG, Bassler BL, Kolenbrander PE. Autoinducer 2: a concentration-dependent signal for mutualistic bacterial biofilm growth. Mol Microbiol. 2006;60:1446–56. https://doi.org/10.1111/j.1365-2958.2006.05202.x.
55. Calabrese EJ, Baldwin LA. Hormesis: the dose-response revolution. Annu Rev Pharmacol Toxicol. 2003;43:175–97. https://doi.org/10.1146/annurev.pharmtox.43.100901.140223.
56. Williams DL, Woodbury KL, Haymond BS, Parker AE, Bloebaum RD. A modified CDC biofilm reactor to produce mature biofilms on the surface of PEEK membranes for an in vivo animal model application. Curr Microbiol. 2011;62:1657–63. https://doi.org/10.1007/s00284-011-9908-2.
57. Coffey BM, Anderson GG. Biofilm formation in the 96-well microtiter plate. Methods Mol Biol. 2014;1149:631–41. https://doi.org/10.1007/978-1-4939-0473-0_48.
58. Davies DG, Marques CN. A fatty acid messenger is responsible for inducing dispersion in microbial biofilms. J Bacteriol. 2009;191:1393–403. https://doi.org/10.1128/JB.01214-08.
59. Uppuluri P, Chaturvedi AK, Srinivasan A, Banerjee M, Ramasubramaniam AK, Köhler JR, Kadosh D, Lopez-Ribot JL. Dispersion as an important step in the *Candida albicans* biofilm developmental cycle. PLoS Pathog. 2010;6:e1000828. https://doi.org/10.1371/journal.ppat.1000828.
60. Villa F, Borgonovo G, Cappitelli F, Giussani B, Bassoli A. Sublethal concentrations of *Muscari comosum* bulb extract suppress adhesion and induce detachment of sessile yeast cells. Biofouling. 2012;28:1107–17. https://doi.org/10.1080/08927014.2012.734811.
61. Ghigo JM. Natural conjugative plasmids induce bacterial biofilm development. Nature. 2001;412:442–5. https://doi.org/10.1038/35086581.
62. Reisner A, Haagensen JA, Schembri MA, Zechner EL, Mølin S. Development and maturation of *Escherichia coli* K-12 biofilms. Mol Microbiol. 2003;48:933–46. https://doi.org/10.1046/j.1365-2958.2003.03490.x.
63. Reisner A, Krogfelt KA, Klein BM, Zechner EL, Mølin S. 2006. In vitro biofilm formation of commensal and pathogenic *Escherichia coli* strains: impact of environmental and genetic factors. J Bacteriol. 2006;188:3572–81. https://doi.org/10.1128/JB.188.10.3572-3581.2006.
64. Villa F, Remelli W, Forlani F, Vitali A, Cappitelli F. Altered expression level of *Escherichia coli* proteins in response to treatment with the antifouling agent zosteric acid sodium salt. Environ Microbiol. 2012;14:1753–61.
65. Huber AB, Kolodkin AL, Ginty DD, Cloutier JF. Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. Annu Rev Neurosci. 2003;26:509–63. https://doi.org/10.1146/annurev.neuro.26.010302.081139.
66. Truchado P, Lopez-Galvez F, Gil MI, Tomas-Barberan FA, Allende A. Quorum sensing inhibitory and antimicrobial activities of honeys and the relationship with individual phenolics. Food Chem. 2009;115:1337–44. https://doi.org/10.1016/j.foodchem.2009.01.065.
67. Reis Giada ML. Food phenolic compounds: main classes, sources and their antioxidant power, oxidative stress and chronic degenerative diseases Jose Antonio Morales-Gonzalez, IntechOpen. 2013. https://doi.org/10.5772/51687. Available from: https://www.intechopen.com/books/oxidative-stress-and-chronic-degenerative-diseases-a-role-for-antioxidants/food-phenolic-compounds-main-classes-sources-and-their-antioxidant-power.
68. Cho HS, Lee JH, Cho MH, Lee J. Red wines and flavonoids diminish *Staphylococcus aureus* virulence with anti-biofilm and anti-hemolytic activities. Biofouling. 2015;31(1):1–11. https://doi.org/10.1080/08927014.2014.991319.
69. Sánchez E, Rivas Morales C, Castillo S, Leos-Rivas C, García-Becerra L, Mizael Ortiz Martínez D. Antibacterial and antibiofilm activity of methanolic plant extracts against nosocomial microorganisms. J Evid Based Complementary Altern Med. 2016, Article ID 1572697;2016:8. https://doi.org/10.1155/2016/1572697.
70. Vikram A, Jayaprakasha GK, Jesudhasan PR, Pillai SD, Patil BS. Suppression of bacterial cell-cell signalling, biofilm formation and type III secretion system by citrus flavonoids. J Appl Microbiol. 2010;109(2):515–27. https://doi.org/10.1111/j.1365-2672.2010.04677.x.
71. Bazargani MM, Rohloff J. Antibiofilm activity of essential oils and plant extracts against *Staphylococcus aureus* and *Escherichia coli* biofilms. Food Control. 2016;61:156–64. https://doi.org/10.1016/j.foodcont.2015.09.036.