Universidade de Aveiro Departamento de Química
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Diana Martins Correia

A formulation based on cationic porphyrins in the photoinactivation of *Pseudomonas syringae* pv. *actinidiae*

Fotoinativação da *Pseudomonas syringae* pv. *actinidiae* por uma formulação baseada em porfirinas catiónicas

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Professora Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e co-orientação da Professora Doutora Maria da Graça de Pinho Morgado Silva Neves, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro.

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palavras-chave

Terapia fotodinâmica antimicrobiana, *Pseudomonas syringae*, porfirinas catiónicas, kiwi, desenvolvimento de resistências

resumo

A *Pseudomonas syringae* pv. *actinidiae* (Psa) é uma bactéria fitopatogénica Gram negativa responsável por causar cancro bacteriano na planta do kiwi. Na última década, esta bactéria afetou fortemente a produção deste fruto a nível mundial, levando a perdas económicas significativas. Geralmente, o controlo da Psa baseia-se no uso de pulverização à base de cobre que apresenta elevada ecotoxicidade e persistência ambiental.

A aplicação da terapia fotodinâmica antimicrobiana como uma alternativa promissora na inativação da Psa já foi demonstrada em estudos recentes tendo-se alcançado reduções do teor de Psa de 4 log, usando como fotossensibilizador o derivado porfirínico tetracatiónico **Tetra-Py^{+}-Me** em 3 ciclos de tratamento com irradiância de 150 mW cm^{-2}. Neste tipo de terapia é necessária a ação conjunta de um fotossensibilizador (PS), oxigénio molecular e luz visível que conduz à produção de espécies reativas de oxigénio (ROS), capazes de comprometer a integridade da célula microbiana. Dada a inespecificidade da ação fotodinâmica, as espécies reativas de oxigénio produzidas podem reagir com diferentes alvos celulares, tornando-se improvável o desenvolvimento de resistências por parte dos microrganismos.

O presente trabalho teve como objetivo avaliar a eficiência fotodinâmica de uma formulação constituída por cinco porfirinas catiónicas na inativação da Psa. Esta formulação foi preparada de modo a ter como principal componente a porfirina tri-catiónica considerada um dos fotossensibilizadores mais eficientes na fotoinativaçao de diversos microrganismos. Os estudos *in vitro* realizados na concentração de 5.0 µM e uma irradiância de 4.0 mW cm^{-2}, permitiram obter uma fotoinativação de 7,4 log ao fim de 60 min de irradiação. Dada a eficiência fotodinâmica observada foram realizados diversos ensaios com o fotossensibilizador numa concentração de 50 µM em folhas de kiwi (*ex vivo*), sob diferentes condições de irradiação e de contaminação: 1) luz artificial de baixa irradiância (4.0 mW cm^{-2}) com inoculação artificial, 2) radiação solar (23 e 60 mW cm^{-2}) com inoculação artificial e 3) radiação solar (23 mW cm^{-2}) com folhas naturalmente contaminadas. Foram ainda avaliadas neste estudo a recuperação de viabilidade da Psa e o desenvolvimento de resistências após sucessivos tratamentos fotodinâmicos sub-letais.

Os ensaios *ex vivo* com folhas artificialmente contaminadas com Psa permitiram observar uma fotoinativação de 2,8 e 4,5 log em condições de iluminação artificial e radiação solar, respetivamente, após 90 min de irradiação. Após um segundo tratamento com radiação solar a fotoinativação foi de 6,2 log. A fotoinativação da Psa nas folhas de kiwi naturalmente contaminadas foi de cerca de 2,3 log após 90 min de irradiação ao sol.

Ao fim de 10 ciclos de tratamento em condições sub-letais de fotoinativação da Psa não se observou desenvolvimento de resistências (manteve-se o perfil de inativação fotodinâmico) nem ocorreu recuperação de viabilidade por parte da Psa. Os resultados deste estudo sugerem que a terapia fotodinâmica antimicrobiana poderá ser uma alternativa aos métodos atuais no controlo da Psa, uma vez que a sua inativação foi demonstrada inclusive sob radiação solar, sem se terem observado efeitos negativos sobre as folhas de kiwi.

keywords **Antimicrobial photodynamic therapy, *Pseudomonas syringae*, cationic porphyrins, kiwi, resistance development**

abstract

Pseudomonas syringae pv. *actinidiae* (Psa) is a Gram negative phytopathogenic bacterium responsible for bacterial canker on kiwifruit plant. Over the last decade, this bacterium dramatically affected the production of this fruit worldwide, causing significant economic losses. Generally, Psa control consists in the application of copper based sprays which are toxic and persist in the environment.

The application of antimicrobial photodynamic therapy (aPDT) as an alternative to inactivate Psa has already been demonstrated in recent studies that showed a 4 log Psa reduction using the cationic porphyrin **Tetra-Py^{+}-Me** as photosensitizer (PS) and 3 consecutive cycles of treatment with a light irradiance of 150 mW cm^{-2}. In this type of therapy occurs the interaction of a photosensitizer (PS), molecular oxygen and visible light, that leads to the generation of reactive oxygen species (ROS), capable to comprise the integrity of microbial cells. Given the non-specificity of the photodynamic action, these reactive species can interact with different cell targets, making resistance development an improbable event.

The present work aimed to evaluate the photodynamic efficiency of a formulation constituted with 5 cationic porphyrin derivatives, as photosensitizer, in Psa inactivation. This formulation was prepared to have as main component the tri-cationic porphyrin which is considered one of the most efficient photosensitizers in the photoinactivation of microorganisms.

The *in vitro* study with a PS concentration of 5.0 µM and low radiance (4.0 mW cm^{-2}), showed a 7.4 log photoinactivation after 60 min. Due to the observed efficiency in the *in vitro* tests, several assays were performed with the PS at 50 µM on kiwifruit leaves (*ex vivo*), under different conditions of light and inoculation: 1) low radiance (4.0 mW cm^{-2}) with artificial inoculation; 2) sunlight (60 and 23mW cm^{-2}) with artificial contamination; 3) sunlight (23 mW cm^{-2}) with naturally contaminated leaves. aPDT viability recovery and resistance development assays were also assessed.

In the *ex vivo* assays with artificially contaminated leaves were observed a 2.8 and 4.5 log inactivation with low radiance and sunlight, respectively, after 90 min irradiation. After a second treatment with sunlight a 6.2 log inactivation was achieved. The photoinactivation on naturally contaminated leaves was about 2.3 log after 90 min sunlight irradiation.

Ten consecutive cycles of phototreatment in sub-lethal conditions, showed that Psa does not develop resistance, nor recover viability.

The results of this study suggest that aPDT can be an alternative to the current methods used to control Psa, since it was possible to inactivate this bacterium under sunlight, without damaging the leaves.

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List of acronyms and abbreviations

PDT	Photodynamic Therapy
PS	Photosensitizer
aPDT	Antimicrobial photodynamic therapy
ROS	Reactive oxygen species
Gram (+)	Gram-positive bacteria
Gram (-)	Gram-negative bacteria
RH	Relative humidity
MIC	Minimum inhibitory concentration
Psa	*Pseudomonas syringae* pv. *actinidiae*
Tetra-Py^{+}-Me	5,10,15,20-tetrakis(1-methylpyridinium-4-yl) porphyrin tetra-iodide
Cu_2O	Cuprous(I) oxide
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
PBS	Phosphate Buffered Saline
DC	Dark control
LC	Light control
CFU	Colony Forming Units
ANOVA	Analysis of variance

CHAPTER I – Introduction

1.1 Photodynamic therapy

The first references showing the potentiality of the approach known today as photodynamic therapy (PDT) started to appear more than 100 years ago [1]. On that period, it was reported the death of some microorganisms after being exposed to dyes and visible light. Later, it was found that the presence of molecular oxygen was also crucial for the cell death due to the toxicity of the oxygen species generated [2]. This therapy can be direct or indirect. When the radiation is applied and it is absorbed by biological molecules, PDT is considered direct. On the other hand, when a chemical compound – known as photosensitizer (PS) – is added before the irradiation, PDT is classified as indirect. So, photodynamic therapy is a technology that requires the interaction of three components: light, a photosensitizer (PS) and molecular oxygen.

The undue and excessive use of antibiotics lead to the spread of multi-resistant bacteria [3]. Actually, the problem regarding antimicrobial drug resistance has already been officially recognized in statements from governmental and health entities [4,5]. Fortunately, this acknowledgment raised awareness for the need to develop alternatives to antimicrobial control and during the past 20 years research in this field has expanded significantly [6]. Over the years PDT was mainly used to treat tumors. Nevertheless, in addiction to cancer treatment, this technology is also applied against infectious agents in other fields, being specifically designated as antimicrobial photodynamic therapy (aPDT). Some medical fields where it is considered easy to implement aPDT are dermatology and dentistry, since in both cases the treatments only require the expose of the infected tissue to the PS and then to light [7,8]. In the food industry the approach can be used to remove contaminants such as bacteria or fungi during food processing [9]. In veterinary medicine good results were achieved when aPDT was used to treat sheep with lymphadenitis abscesses [10]. This therapy is also applied in the fish farming field [11,12]. Other application is the control of plant pathogens, which could be an ecologic alternative to fungicides and sprays based on heavy metals like copper, frequently used for this purpose [13,14]. Besides that PDT can also be used to improve security in medical devices such as catheters [15].

Among others therapies, like phage therapy, aPDT is being revived due to its potential on treating multi-drug resistant infections. Besides, aPDT shows many other strengths comparing to conventional antimicrobial treatment [6,16,17]:

- Less probability of resistance development. Since aPDT is not specific and it involves *in situ* production of ROS that can affect several biomolecular sites, this therapy bypasses the mechanisms of resistance. Unfortunately microorganisms can easily develop resistance against many of the available antibiotics due to their single mode of action, so this is a major advantage of aPDT comparing to conventional antibiotic treatment.
- Low risk to induce mutagenic effects.
- Multitarget and broad-spectrum of action. Unlike antibiotics, photoantimicrobials can act, not only in the inactivation of bacteria (both Gram positive and Gram negative), but also of viruses, protozoa, and fungi.
- Safeness and nontoxicity. It is known that at the normally used concentrations (μM range) photoantimicrobials are harmless to the tissues, whether they are excited by light or not.
- Easy to implement. This therapy only requires a light source, the presence of molecular oxygen and a suitable photosensitizer.
- Quick lethal effects. Although conventional treatments take hours or even days and several doses to induce effects, PDT demonstrates rapid killing effect. It is estimated that a single PS molecule can generate 10,000 molecules of singlet oxygen.
- Appropriate to empiric treatment. When there is a lack of diagnosis and the microorganism causing the infection is not identified, this broad-spectrum and non-specific therapy could be a realistic alternative.

1.1.1 Mechanism of action

After PS activation by light there are two main type of pathways - type I and type II - that can occur to generate reactive oxygen species (ROS) (Figure 1) [2,6,18]. However, due to the available cellular substrates, the characteristics of the PS and other factors, one of these two pathways is usually favored.

Type I reaction involves the transfer of a hydrogen atom or an electron from an excited triplet state to cellular substrates, leading to the formation of free radicals. The free radicals then interact with oxygen molecules and ROS are generated. Usually, the superoxide anions ($O_2^{\bullet -}$) produced react with water, producing hydrogen peroxide (H_2O_2). Also, at high concentrations of H_2O_2, hydroxyl radicals are present ($OH^{\bullet}$). Type

II reaction is considered the most relevant for PDT because is the major pathway in the photooxidative cell damage and it involves the direct energy transfer between the PS and molecular oxygen. This energy exchange between the PS and molecular oxygen generates singlet oxygen ($^1O_2$) which is highly reactive, has a short life span and a limited action space, so only the closest molecules will react. In spite of that, the PS has a catalytic and regenerative ability, producing numerous singlet oxygen molecules over time, as long as light and molecular oxygen are present.

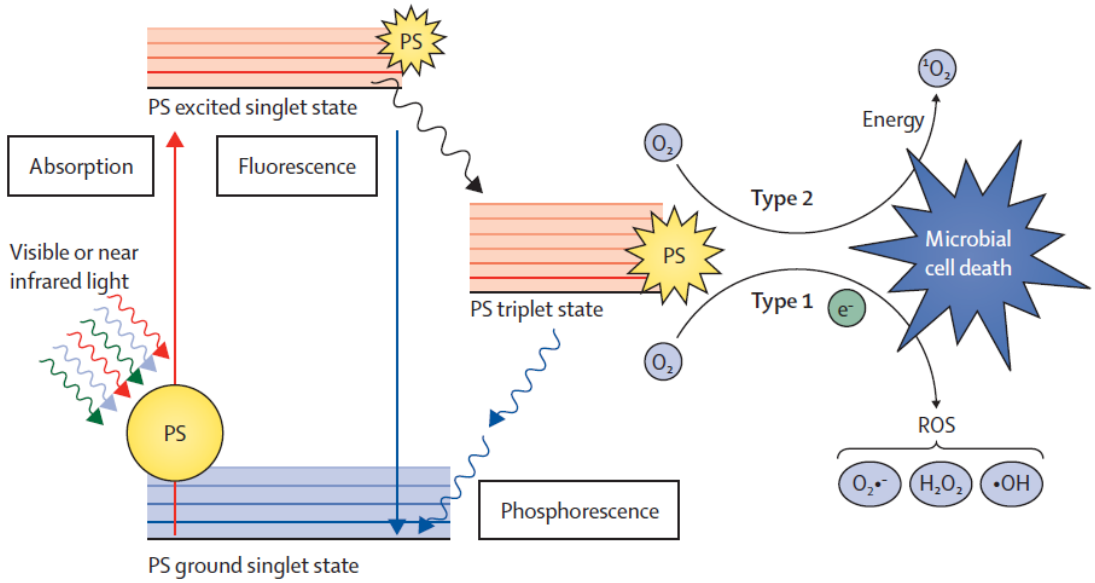

Figure 1 – Representation of the two pathways (type 1 and 2) of the mechanism of photodynamic action [6].

Both reactions originate ROS that instantly interact with biological components of the cell wall, like proteins, lipids (unsaturated fatty acids of membranes), amino acid residues (cysteine, histidine, and tryptophan), nucleic acid bases (guanine and thymine) and also the pigments in certain cells [16,19]. Then, the organic and metabolic functionality of the cell is lost, leading to its inactivation.

1.1.2 Photosensitizer

The photosensitizer (PS) has a special role in PDT because it is after its activation by light that the ROS are able to be generated [1,20,21]. Thus, the first law of photochemistry says that in the photodynamic process it is necessary that the photosensitizer molecule absorbs light at a certain wavelength. The capacity of the PS to absorb light at this certain wavelength depends on its structure and on the electronic absorption spectrum.

Porphyrins represent an important class of PS and were the first type of compounds to be used in PDT against tumors and to be accepted in clinic (e.g. Photofrin®) [1,26]. The first PSs were obtained from hematoporphyrin (Figure 2) and showed some limitations like long-lasting skin sensitivity on patients, the lack of reasonably-sized absorption band (> 650 nm), large dose necessary to produce consistent PS uptake and poor tumor localization. Motivated by this, several research groups all over the world were able to develop molecules with better and more adequate features to be used as PS for cancer and also to inactivate microorganisms. Nowadays, a series of efficient PS based on natural or synthetic porphyrins or in their analogues (*e.g.* chlorins, corroles, phthalocyanines) either as free-bases or coordinated with metal in the inner core were developed [22–24].

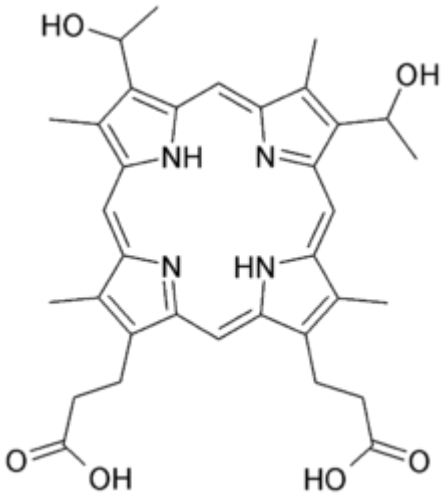

Figure 2 – Chemical structure of hematoporphyrin [22].

In the literature, are mentioned some characteristics that a good PS should have considering their application in aPDT [6,25,26]:

- Photostability, so that it can be used in aPDT assays and not being quickly degraded.
- Solubility, so it does not aggregate or precipitate. If the PS aggregate it is no longer available to bind to cells, thus there is a decrease in its capacity to photoinactivate.
- Positive charge principally for Gram negative bacteria. Photoinactivation is higher with positively charged PS because the positive charge stimulates a tight electrostatic interaction with the negative charges on the surface of bacteria.
- Amphiphilic properties. Studies showed that a PS with amphiphilic character has more affinity to the bacteria.

1.1.3 Light

Nowadays, a vast type of light sources had already been tested to inactivate microorganisms, but the conventional lamps, also designated as non-coherent light sources, were the first to be used in PDT assays [1,6,20,21,27]. The reason why conventional lamps were tested first is the fact that these lamps are cheap, accessible and easy to use; however, they lack on other features, like the ability to control the light dose applied. So, in order to overcome such limitations, the lasers, also known as coherent light sources, started to be used in aPDT and became commonly used due to their ability to produce a monochromatic light (with an exact wavelength) and to control light dose. Other important factor is to match the wavelength with the chosen PS, in order to maximize the yield of produced ROS [20,21]. Regarding the influence of the tissue, it is important to refer that the direction of the light is also affected by the inhomogeneity of the cells, namely the presence of organelles, macromolecules, also interstitial layers in fungi.

Regarding antimicrobial photodynamic therapy the greater the intensity and the irradiation time, the better efficiency of inactivation [20,21,26,28]. Using sunlight as light source in aPDT is a clever choice, especially for environmental applications, because porphyrins absorb in the visible range, making the process cheaper and easy to

implement. Sunlight has already been used to inactivate microorganisms, due to the synergistic effect of the ultraviolet and infrared regions.

1.1.4 Molecular oxygen

The principle of PDT inactivation is the production of reactive oxygen species that will affect several cellular targets, leading to cell dead [6,20,21]. Thus, oxygen is definitely a fundamental element in this therapy. More specifically, singlet oxygen ($^1O_2$) has to be generated in order to achieve inactivation [16,20,29,30]. This reactive oxygen species has a very short life time that is approximately 600 ns and 3 μs and a radius of action near 100 nm. Although generation of ROS, and thus, singlet oxygen production depends on the PS performance, the target cell and the environment in which they produced are also important.

1.1.5 aPDT in the inactivation of Gram negative bacteria

Another relevant aspect for the success of aPDT is taking in consideration the microorganism we are aiming to inactivate, because, for instance, Gram negative (-) or Gram positive (+) bacteria have some differences regarding their cell structure that will condition the efficiency of inactivation [16,19]. As is illustrated on Figure 3 Gram (+) bacteria has a cell wall composed by lipoteichoic and teichoic acids that are organized in numerous layers of peptidoglycan (30-100 nm) and only one phospholipid bilayer. This peptidoglycan layer in Gram (+) is porous, so it allows the penetration of the PS into the cell. On the contrary, Gram (-) has a thinner layer of peptidoglycan but it has an intricate outer membrane that disables antimicrobial agents to penetrate the cell. This outer membrane in Gram (-) is composed by glycolipids (lipopolysaccharides, lipoproteins and β-barrel proteins) and by phospholipid bilayer that acts as an anchor for the glycolipids and de peptidoglycan. So, it is obvious that it is more difficult to obtain a PS that can mediate the photoinactivation of Gram (-).

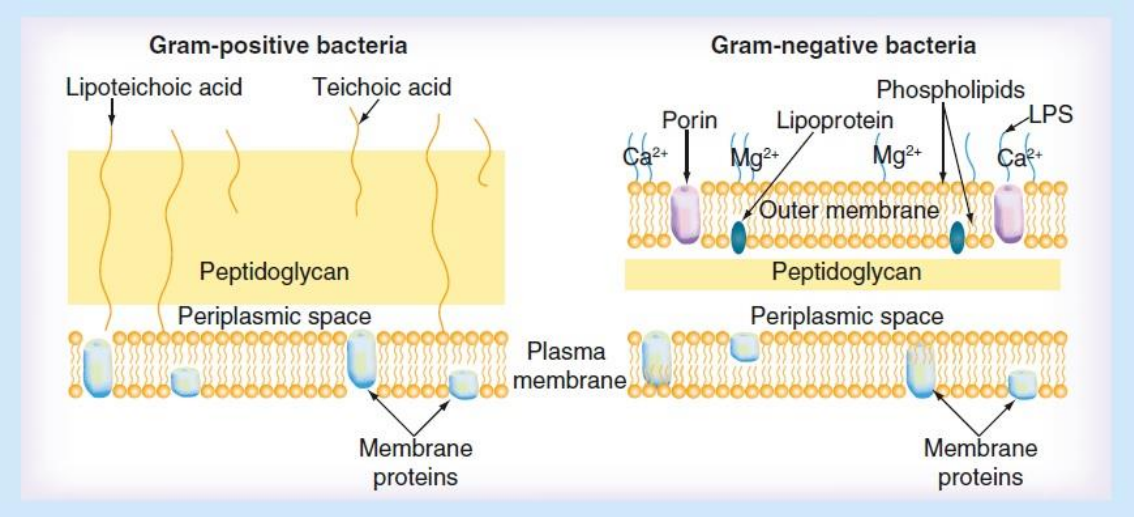

Figure 3 - Bacterial cell structure of Gram (-) and Gram (+) bacteria [16].

1.2 *Pseudomonas syringae* infections on plants

Pseudomonas syringae is an aerobic Gram negative, rod-shaped bacterium, which infects several plants, including the peach tree, the tomato plant and the kiwifruit plant [31,32]. One of its characteristics that makes possible to distinguish this species from other *Pseudomonas* species is the fact that *P. syringae* is not able to emit fluorescence [33]. The strains of this bacterium species are divided in groups named *pathovars*, accordingly to their differences in what concerns the plants they infect - their hosts. Currently, 60 *pathovars* are identified, and each one infects normally only one plant species; however, some *pathovars* are able to infect a restrict group of similar plants.

Since this species is pathogenic to many plants, it is important to understand how its transmission occurs. *Pseudomonas syringae* can be transmitted through different vectors: 1) aphids or plant lice; 2) water, since the bacterial life cycle might be related to the water cycle; 3) seeds and vegetative material; 4) pollen; 5) plantation workers and/or the equipment that contact with the plant [34–36].

Pseudomonas syringae pv. *actinidiae* (Psa) represents one of the *P. syringae pathovars*, it infects *Actinidea* genus plants and it was first isolated from a kiwifruit plant in 1989, in Japan [37]. Psa is more invasive at temperatures between 10 and 20 °C, with an optimal growing temperature of 15 °C (±3 °C) [38]. Even though this bacterium can grow at temperatures up to 25 °C, as it was shown in recent studies that, its capacity of infection is reduced in these conditions. Therefore, the Psa appears mostly on spring,

affecting the development of the plant and, on autumn/winter it can cause severe damage. Besides that, it is resistant to antibiotics and vegetable nitric oxide, having the capacity to capture iron and catabolize aromatic compounds on plants. Psa can be distinguished from other strains through biochemical tests. On Table 1 are the results of the tests that can be used to identify this bacterium.

Table 1 - *R*esults of the biochemical tests to identify Psa [37,39].

Negative tests	**Positive tests**
Oxidase	Catalase
Tyrosinase and the characteristic fluorescent pigment of the *Pseudomonas* genus (poly-β-hydroxybutyrate)	Urease
Liquefaction of gelly	Tobacco hypersensitivity reaction
Soft rot of potato and activity dihidrolase of arginine	Levan production
Esculin	
Starch	

Psa strains can be divided in 5 groups named *biovars* accordingly with their virulence (Table 2) [33,40]. The *biovar* 1 produces and secretes phaseolotoxin and it was first found on Japan. The *biovar* 2 was found on Korea and secretes the coronatine toxin. The *biovar* 3 is the most virulent one, it includes the pathogenic strains discovered after 2008 in Italy and later in other countries, thus is also the one that has led to economic losses. The *biovar* 4 first appeared in New Zealand and it is not virulent. Lastly, the *biovar* 5 was recently located in Japan and does not produce toxins like *biovars* 1 and 2. In 2009, the European and Mediterranean Plant Protection Organization (EPPO) included Psa disease in A2 alert list, a list that identifies the pests recommended for regulation as quarantine [41]. Presently, Psa has already been found in various countries, for instance Italy, Portugal and New Zealand, being considered the responsible agent of bacterial canker on kiwifruit plant [42–44].

Table 2 - Geographic distribution and virulence of the five Psa *biovar* [45].

Biovar	**Countries**	**Virulence**
1	Japan, Italy	High virulence, especially to Hayward cultivars
2	Korea	High virulence, especially to Hayward cultivars
3	Italy, Chile, China, New Zealand, France, Portugal, Japan, Korea	High virulence, especially Hort16A cultivar Less virulent on Hayward than Psa1 and Psa2
4	New Zealand, Australia	Low virulence
5	Japan	Low virulence

1.2.1 *Pseudomonas syringae* pv. *actinidiae* and the kiwifruit plant

Psa is the pathogenic agent responsible for bacterial canker on kiwifruit plant [46]. In 2008 happened the first outbreak of this disease in Italy, caused by *biovar* 3 of this species. On the following years the occurrence of this bacterium was registered in orchards in many European countries, like France, Italy, Spain, Portugal but also in New Zealand, Japan and Korea [37,43,44,46–49]. This disease has already caused economic losses in these countries, especially in Italy and New Zealand, which are the world leader producers of this fruit. Zespri®, the major kiwifruit producing company in New Zealand, registered a 7.7 million Euros decrease of net profit between 2010 and 2011, due to this disease [50]. Consequently, from 2008 to the present day the scientific research in this field increased significantly, also motivated by the lack of efficient and eco-friendly treatments available to control this bacterium.

1.2.2 Kiwifruit production and market

Annually 1.4 million tons of kiwifruit are produced, making the market of this fruit economically relevant worldwide [51]. The major producers are Italy, New Zealand and, recently, China (Figure 4).

1993 - 1995			2003 - 2005			2008-2011 (p)		
Rank	Country	Production	Rank	Country	Production	Rank	Country	Production
1	Italy	322 730	1	Italy	401 622	1	China	491 667
2	New Zealand	224 000	2	China	341 000	2	Italy	429 885
3	Chile	125 333	3	New Zealand	303 000	3	New Zealand	385 049
4	France	77 570	4	Chile	151 667	4	Chile	186 667
5	Japan	51 267	5	France	76 157	5	Greece	79 433
6	Greece	41 681	6	Greece	50 000	6	France	66 890
7	United States	38 213	7	Japan	38 100	7	Japan	37 467
8	China	23 167	8	United States	28 335	8	Iran	30 000
9	Portugal	9 394	9	Iran	20 333	9	United States	25 371
10	South Korea	8 787	10	South Korea	12 000	10	South Korea	15 833

Figure 4 - Top 10 kiwifruit producing (in tons) countries between 1993 and 2011 [52].

The kiwifruit industry exists practically around the *Hayward* cultivar, to the point that most consumers only know this variety. In 1904 a seed from China was introduced in New Zealand and almost all the kiwifruit cultivars grown in commercial orchards are descended from it. In spite of such limited sampling, the first plantations in New Zealand showed a considerable variation in the fruits derived from different plants. An example of another cultivar is the *Koryoku* that was first generated from an open pollination from the *Hayward* cultivar and which has a fruit that is sweeter than that. Other is *Hort 16A*, commercially known by "gold" kiwifruit that belongs to *Actinidea chinensis* species, it has the second most appealing fruit besides *Hayward* cultivar because of the almost absence of hair and its appealing flavor.

In Portugal, during the 1980s the kiwifruit production increased exponentially, having reached a 2000 ha area of plantation in 1992 [52,53]. In Figure 5 is illustrated the production evolution from 1987 to 2013. The main regions of production are Entre Douro and Minho and Beira Litoral, since they have the appropriate conditions for the plant development. In general, the strengths of Portugal for the cultivation of this fruit are the almost absence of frost in the autumn, which allows a late harvest, giving time for the fruit to sweeten. Thus, Portuguese kiwifruit can reach a place in the international market, as long as the production is sufficient.

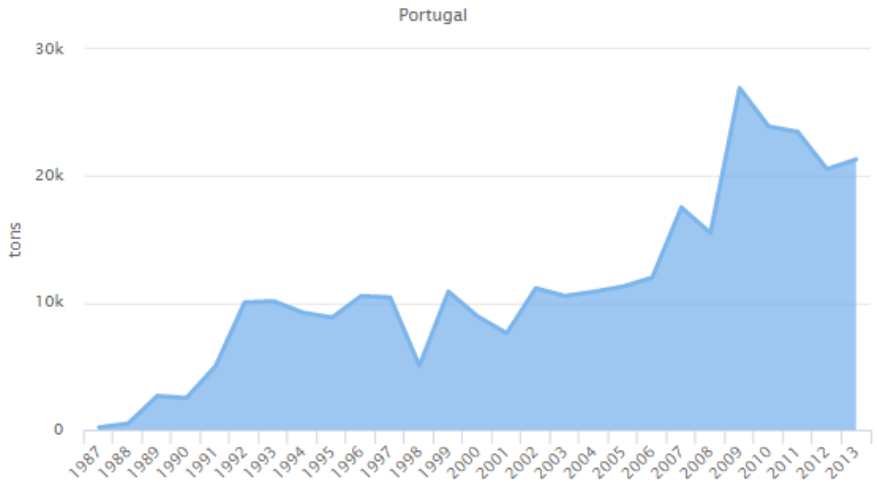

Figure 5 - Kiwifruit production growth in Portugal between 1987 and 2013 (x-axis) in tones of fruit (y-axis*)* [54].

Data from the National Institute of Statistics (INE) show that kiwifruit production decreased consecutively between 2009 and 2014 [55]. Nevertheless, in 2013 were produced 21 306 tons of kiwifruit in Portugal, that represented more than 11 million Euros in exports [56]. Besides sanitary, physiological and environmental problems, one of the major contributions for this decrease was the occurrence of bacterial canker caused by Psa. On March 2010, this bacterium was first detected in Entre Douro and Minho region. In the subsequent year new outbreaks were identified in Lousada, Amarante and other regions in the north of the country. Later, in 2013, Psa was found in Santa Maria da Feira, Valença, Vila do Conde, Marco de Canaveses, Felgueiras e Oliveira do Bairro [57]. It is important to refer that in Portugal there is a national plan for the control of this disease and that its fulfillment is essential in the prevention. In Figure 6 are represent the Portuguese regions affected by Psa in 2015.

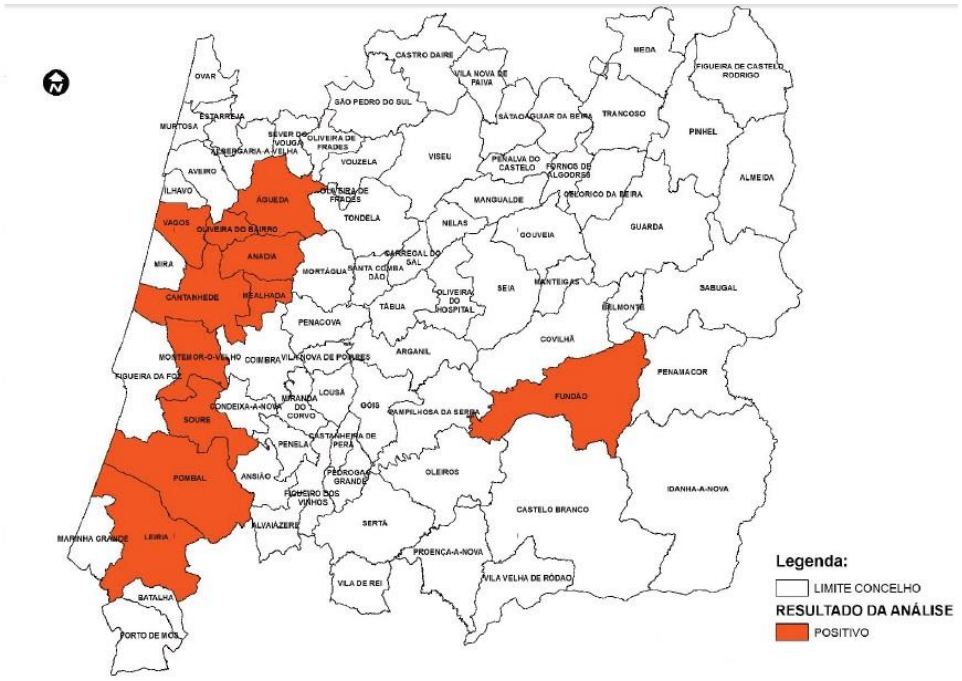

Figure 6 - Portuguese regions affected with Psa by 2015. Orange colored: positive test for Psa. (Adapted from [57]).

1.2.3 Psa infection on kiwifruit

The cultivation of kiwifruit is severely affected by the presence of Psa because, on the proper conditions, this bacterium reduces significantly the harvest, leading to economic losses [50,57]. The mostly affected species are *Actinidea chinensis* and *Actinidea deliciosa*, but the wild species *Actinidea arguta* and *Actinidea kolomikta* can also be infected [53].

Rated as the responsible for the bacterial canker on kiwifruit, Psa evidences a great ability to grow in the various steps of the plant growth [38,42]. Initially, this bacterium has an epiphytic behavior, laying on the surface of the plant without taking any nutrients from it. So, Psa can be in contact with the plant for some time without symptoms appear, until its growing conditions reach an optimal state. After that, the bacterium enters in the vascular system of the plant, through natural or caused (by birds, insects and/or human contact) openings, colonizing the pollen and leaves or even setting in the roots, leading to the systemic infection. Psa has an optimal growing temperature of 15 °C (±3 °C), thus spring and autumn are the most critical seasons for the infection development. In terms of gender, male vines reveal infections symptoms first, but

female vines are more severely affected. On other hand, younger plants are the most sensitive to infection.

Genetic studies revealed that Psa strains found in Europe are very similar, suggesting that they have a common infection outbreak and the plant trading market allowed the dissemination [58]. Moreover, the environmental conditions like frost, hail and winds may have been in favor of Psa spread.

Although there is some information about it, the transmission of Psa is still hard to clarify. On the literature are mentioned the main routes of transmission, which are the air (especially strong winds); the water, not only from the rain, but also from de irrigation systems; and the soil which can also be infected [35,38]. Additionally, the transmission can occur through contact with workers and agro equipment; with infected vegetal material from other plants; with animals like insects and/or lice; and also through the pollen. Another risk factor is the trading of infected seedlings for cultivation, since it allows the infection dissipation between distant places. On the other hand, when seeds are used to grow a new plant, even if Psa reaches the surface of the fruit, because of their small size, the probability of infection is low, but it can happen if the orchard is extremely infected. The environmental factors also can promote and even aggravate the infection because they can favor de growth of Psa. Beyond that, the release of exudates in the orchards - particularly when they have high concentration of inoculum – and plant lesions, are also points of entrance for the bacterium.

The first symptoms, named primary, arise during the spring in the leaves, stems and shoots and, less frequently, small tumors can appear. In this season, the leaves develop brown points, normally surrounded by yellow shiny halos (Figure 7A) [58]. In the *Actinidea* genus plants, like kiwifruit plant, these kinds of halos are due to the activity of a phytotoxin similar to phaseolotoxin [59,60].

The heaviest shoots can fall and release exudates, the others shrink and lead to the atrophy of the fruits (Figure 7B) [58]. The flower sepals become darker and, lastly, the trunks also release white and red exudates (Figure 7C). When the infections have high bacterial concentration the exudate has white color.

Secondary symptoms emerge in the middle of the winter, especially in the tree branches and trunks, in which is observed the release of exudates. By the end of this season, this release is accentuated, leading to the formation of “ring” in the trunk (Figure 7D). Besides, the degradation of lignin and phenolic compounds occurs, which lastly conducts to the plant death.

Figure 7 - Examples of characteristic symptoms caused by Psa infection on kiwifruit plant. (A) Brown points with yellow halos on the leaves; (B) Atrophy of the fruits; (C) Release of red exudate [61]; (D) Formation of a "ring" in the trunk after exudate release.

1.2.4 Preventive measures, available treatments and alternatives to control Psa

Since Psa represents an emergent problem, it is necessary to evaluate the available treatments for its prevention and control [42]. The prevention plays a very important role because it is the primary measure used to control this disease, since it includes the basic hygiene rules of maintenance in the orchards [38]. Some of these practices are: the disinfection of agro tools; being aware of grafting process because it can be a font of infection; to have protection against winds, rain and frost; to give the plants the water and nutrients in the right amounts for them to grow; and the removal of animals that can cause infection like lice, birds, slugs, snails, beetles and cicadas. On the prevention context, in Portugal exists a national plan to control Psa on kiwifruit plants [62]. This plan comprises, for instance, a prospection formulary that has to be filled every time there is an inspection. Furthermore, it is mandatory to register the acquired plants and to have an historic of ins and outs of vegetable material, like fruits or wood. In order to raise awareness among the producers, the national plan also includes actions of learning

for them, approaching various points, since the symptoms of the disease to laboratorial analyses. Additionally, the localization of the plant can prevent potential infections because a stable plant, far from stress conditions is less susceptible to an infection.

Another important aspect regarding the prevention topic is the monitoring the orchards, having in consideration the materials and the workers, in order to track back the origin of the infection and evaluate possible risks [45]. For instance, if a producer suspects that certain plant is infected and considers it is a risk for the plantation, he must identify the plant and take samples for laboratorial analyses. Once Psa has negative results for many laboratorial tests, two important ones to identify are API test and sucrose transformation into acid [38,63]. So, the bacteria must be isolated for further morphologic and molecular tests, especially Polymerase Chain Reaction (PCR) because it is the most successful in Psa identification [64]. PCR kits are now available in the market, and they are easy to use by the producers, giving results at real time. Hypersensitivity tests can also be made for a quick Psa detection, since a positive test means a high probability of phytophatogenic *Pseudomonas* species detection. However, these procedures are not enough to spot this disease, thus it is necessary to take additional measures [45].

The available treatments are essentially based on heavy metal compounds - mostly copper(I) - or antibiotics. Regarding the different countries, there are some differences in their treatment approach but the copper based sprays prevail as the most efficient one [65]. Copper(I) ions travel through the cell walls of the bacteria and disrupt the cellular enzyme activity, which lastly leads to cell death [66].

Although copper is an essential metal for normal plant growth and metabolism, since it participates in physiological processes like photosynthetic electron transport and as cofactor for some metalloproteins, its excess in cells represents a problem to the plant [67]. At concentrations above those needed for normal plant growth, copper can not only inhibit plant development, but also interfere with cell processes like photosynthesis. Studies indicated that plants which grow at high levels of this metal showed reduced biomass, reduced content of chlorophyll, chlorotic symptoms, oxidative stress and alterations in the composition of both chloroplasts and thylakoid membrane [68]. Besides, soil with toxic levels of copper can also be toxic to grazing animals [69]. After copper based spays are applied, copper accumulates and cannot be degraded [70,71].

The use of antibiotics, like Streptomycin, is legal in Asiatic countries, being illegal or very restricted in New Zealand. In this last one, copper based products and a commercial product named Actigard™ are the principal approaches to combat Psa [45]. This commercial product contains a compound named acibenzolar S-methyl, derived from salicylic acid which reduces the disease incidence. Comparatively, in Portugal, Italy and the further European countries, the use of antibiotics is forbidden, thus the copper sprays are the most common treatment and, in a minor scale, are also used products based on antagonist bacteria, like *Bacillus subtilis*, or a product named Bion® which is the equivalent to Actigard™ [72]. It is known that heavy metals accumulate, being toxic for the environment, and that the antibiotic use for this purpose is not allowed in several countries, since it can leave residues on the fruits and increase the development of multi-resistant bacteria [73]. Consequently, it is imperative to search for viable and ecologic alternatives to control Psa on kiwifruit plant.

In the literature, there are some possible alternatives to copper products and antibiotics [65]. One example is the use of elicitors, which are compounds that stimulate the immune system of the plant, reducing bacterial activity. However, the use of elicitors is not efficient by itself, so it has to be combined with other methods. Another alternative could be the use of heat to kill Psa in the pollen, one of its transmission vehicles [74]. Everett *et al.* (2012) studied different combinations of temperature, time and relative humidity (RH) with the objective of testing which combination was more efficient to kill Psa in artificially contaminated pollen. The authors observed that the most promising treatment combined 35 °C, with RH at 50% or less for a period of at least 20 hours.

Phage therapy had also been considered an alternative to control Psa on kiwifruit plants and it could actually be a possible reality since this therapy has already been used to treat bacterial diseases in plants before, for instance in tomato and pepper plants [75,76]. Besides there are recent studies about isolation and characterization of bacteriophages against Psa for further application on phage therapy assays [77,78].

1.2.5 aPDT as an alternative in the control of bacterial infections in plants

In spite of PDT being a widely study therapy, most of the studies are centered on the clinical field, whether is applied on cancer treatment or skin infections. However, its potential should not be limited by that and some recent studies showed a new interest on using aPDT in the inactivation of plant pathogens.

In a previous study aPDT was tested *in vitro* to assess the inactivation of three plant-pathogenic fungi – *Colletotrichum acutatum, Colletotrichum gloeosporioides* and *Aspergillus nidulans* - which normally are treated with intense use of fungicides [13]. Common hosts of these fungi include many eudicotyledonous plants like citrus, strawberry and apple plants. Initially the efficacies of the PS were established by determining the minimum inhibitory concentration (MIC) of each. After that, since the three fungi produce conidia in their life cycle which are responsible for fungi dispersion, environmental persistence and host infection, the effects of aPDT with four phenothiazinium PS on the conidia was also tested. The authors observed that derivative **S137** (Figure 8) was the most efficient because it showed inactivation effects at the lowest MICs and it was also effective in killing fungi conidia, enabling a 5 log reduction. Similar results were obtained with the PS new methylene blue N (**NMBN**). One important point about these results is that conventional fungicides only have the ability to act on metabolically active cells, but the assays with photosensitizers **S137** and **NMBN** killed the conidia. Also, it was verified that the aPDT did not cause any sort of damage to the leaves or other structures of the plant, whether the plants were young or adult.

As far as we know, there is only one study about the application of this approach in the inactivation of Psa [14]. Jesus *et al.* (2017) studied the application of aPDT to control Psa *versus* the application of copper(I) sprays, which represent the conventional treatment against this bacterium. For that, the porphyrin **Tetra-Py^{+}-Me** was used as PS, and the *ex vivo* assays performed on leaves showed a 4 log inactivation after 3 consecutive treatments under white light with an irradiance of 150 mW cm^{-2}. Also, the results with cuprous(I) oxide suggest that the recommend concentration of copper(I) (50 g hL^{-1}) could be reduced, because Psa inactivation was observed with a concentration 10 times lower.

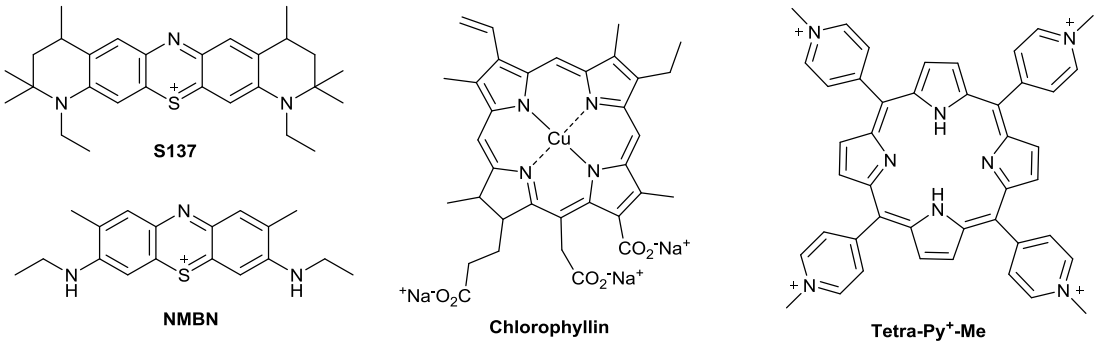

Figure 8 - Photosensitizers tested to the control of bacterial infections in plants [13].

Data about aPDT applied to agricultural field, more specifically against plant-pathogens is scarce, but these results are very promising regarding aPDT application in agricultural context as an alternative to conventional fungicides or other treatments like copper sprays.

1.2.6 Legislation

According to the Decision of the Commission's implementing No 2012/756/EU of 5 December 2012, which stipulates emergency measures to prevent the introduction and spread in the European Union of the bacterium *Pseudomonas syringae* pv. *actinidiae* (Psa), the Kiwi propagating material, excluding the seeds, but including live pollen, may be moved within the Community accompanied by plant passport certifying compliance with the phytosanitary requirements stipulated.

On point paragraph 2 of Article No.7 of Decree-Law No. 154/2005 of 6 September, the Direção Geral de Alimentação e Veterinária (DGAV), as Autoridade Fitossanitária Nacional, issued the Circular No.01/DSFMMP/2012 of 05 March 2012 on phytosanitary control measures to limit the spread of areas infected by bacteria.

1.3 Objectives and approaches

This work aimed to evaluate the efficiency of a new PS formulation constituted by a mixture of cationic porphyrin derivatives in Psa inactivation, for posterior application in kiwi plantations to prevent/control Psa infections. This new mixture contains a high percentage (44%) of a tricationic porphyrin (**Tri-Py$^+$-Me-Mono-PF**) bearing a pentafluorophenyl group that is a very efficient PS in the photoinactivation of

microorganisms. In spite of being efficient, to obtain this tricationic porphyrin in a pure form a laborious chromatography work-up is needed. By using this mixture as PS, the separation step is eliminated, so time and resources are saved, yet a good percentage of **Tri-Py^{+}-Me-Mono-PF** is still present. First, the PS formulation was tested *in vitro* at 5.0 μM under low intensity white light (4.0 mWcm^{-2}), to verify if the mixture was suitable for this application. The second phase was testing the formulation *ex vivo* at 50 μM under low white light irradiance or directly under sunlight, with naturally or artificially contaminated leaves. Lastly, resistance development and viability recovery after consecutive aPDT cycles were evaluated.

CHAPTER II – A formulation based on cationic porphyrins in the photoinactivation of *Pseudomonas syringae* pv. *actinidiae*

2.1 Introduction

Pseudomonas syringae pv. *actinidiae* (**Psa**) is the Gram negative phytopathogenic bacterium responsible for bacterial canker on kiwifruit plant. This bacterium, under proper conditions is reducing significantly the harvest and consequently the kiwifruit market is being severely affected in several countries worldwide, namely big producers like Italy, New Zealand and Portugal [43,45,79,80]. The mostly affected species are *Actinidea chinensis* and *Actinidea deliciosa*, but the wild species *Actinidea arguta* and *Actinidea kolomikta* can also be infected [53]. Initially this bacterium has an epiphytic behavior, laying on the surface of the plant without taking any nutrients from it. So, Psa can be in contact with the plant for some time without inducing external symptoms, until an optimal state on its development is reached. After that, the bacterium enters in the vascular system of the plant, through natural or non-natural openings (caused by birds, insects and/or human contact), colonizing the pollen and leaves or even setting in the roots, leading to the systemic infection. **Psa** has an optimal growing temperature of 15 °C (±3 °C), thus spring and autumn are the most critical seasons for the infection development.

Psa is in general controlled with the application of copper compounds which can be toxic to the plant at the applied concentrations (35-50 g hL^{-1}) [65]. Most of the sprays are based on cuprous oxide (Cu_2O) at 83.9%, equivalent to a metallic copper content of 75% [81]. At concentrations above average in plant tissues (10 μg g^{-1} dry weight [82]), copper can not only inhibit plant development, but can also interfere with cell processes like photosynthesis [67,68,73]. Thus, in spite of being efficient, copper formulations should be avoided. The antibiotic Streptomycin can also be applied in some countries, however, its use is restricted because the risk of resistance development. Besides, some other augmentative products are being used to strength the plant's immune system, but only in complement to the copper sprays [65].

So, it is recognized that the search of efficient and eco-friendly protocols to control Psa merits the attention of the scientific community. A previous study from our group showed that antimicrobial photodynamic therapy (aPDT) can be a potential alternative to Psa photoinactivation [14]. aPDT is an approach that requires the combination of a photosensitizer (PS), light and molecular oxygen. The photosensitizer after being activated by light is able to trigger a series of events affording reactive oxygen species (ROS); these cytotoxic species can then interact with biological

components of the cell wall, like proteins, lipids (unsaturated fatty acids of membranes), amino acid residues (cysteine, histidine, and tryptophan), nucleic acid bases (guanine and thymine) and, in certain cells, also with pigments [16,19]. After these events, the organic and metabolic functionality of the cell is lost, leading to its inactivation.

It is well-known that the efficiency of aPDT is strongly related with the structural features of the PS. From the plethora of available PS (e.g. phenothiazinium dyes like methylene blue, TBO among other) tetrapyrrolic macrocycles like porphyrins and analogues are meriting a special attention due to their effectiveness to photoinactivate a wide range of microorganisms like bacteria, fungi, viruses, yeasts and protozoa [11,83–85].

In particular, the studies show that the presence of positive charges in the PS core is an important feature to photoinduce the direct inactivation of Gram negative bacteria without the presence of a permeabilazing agent [16,19,25,27]. Cationic PS can also photoinactivate efficiently Gram positive bacteria, but in these cases anionic or neutral ones can also be considered. Gram negative bacteria are usually less efficiently inactivated by negatively charged or neutral agents due to their highly organized outer membrane. However, the presence of positive charges in the PS seems to facilitate its electrostatic interaction with the outer cell surface of Gram negative bacteria, inducing an initial limited damage, which favors the penetration of the PS [86]. The number of positive charge and their distribution in the PS core have also an important role on PDT efficiency [87]. Additionally, the affinity of the PS for the bacteria increases with the amphiphilic character of the PS, which is usually accompanied by an increase in the photocytotoxic activity [25,87].

Considering the PS structural features required by Gram negative bacteria, in the first study where the potentiality of aPDT was considered to control Psa, the photosensitizer selected was the widely studied and easily accessible tetracationic porphyrin 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (**Tetra-Py^{+}-Me**) (Figure 9) [14]**.** The results obtained in *ex vivo* were really promising since after 3 consecutive cycles at 150 mW cm^{-2} a Psa inactivation of 4 log was attained. It is important to refer that data about aPDT applied to agricultural field, more specifically against plant-pathogens is scarce, but our results seems to indicate that this approach merits special attention as an alternative to conventional antibiotics, fungicides or other treatments like copper sprays [13,14,88].

In most of the studies performed in our laboratory and others concerning aPDT, the PS are based on symmetric or asymmetric meso-tetraarylporphyrins [85,89]. These synthetic porphyrins can be easily prepared from commercial available aldehydes and pyrrole and are excellent templates for further functionalization [87]. Depending on the aldehydes and on the further functionalization of the macrocycle, a high number of efficient PS derivatives were developed with unique chemical, physical and electronic properties.

During our studies concerning aPDT we found that the tricationic porphyrin (**Tri-Py^{+}-Me-Mono-PF**) bearing a pentafluorophenyl group is a highly efficient PS being able to photoinactivate a wide range of microorganisms under less severe conditions than other PS namely **Tetra-Py^{+}-Me** [26,83,87]. However the access to this porphyrin in a pure form requires a laborious chromatographic work-up. Although the neutral precursor of this asymmetric porphyrin is prepared under adequate stoichiometric conditions to favor its formation, the condensation of pyrrole with pentafluorobenzaldehyde and 4-pyridinecarbaldyhde leads also to the obtainment of the neutral precursors of the mono-, di- and tetra- substituted cationic porphyrins (Figure 10).

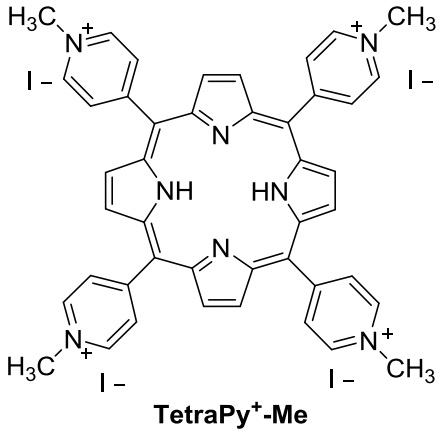

Figure 9 - Structure of the cationic porphyrin Tetra-Py^{+}-Me used in previous studies as PS [14].

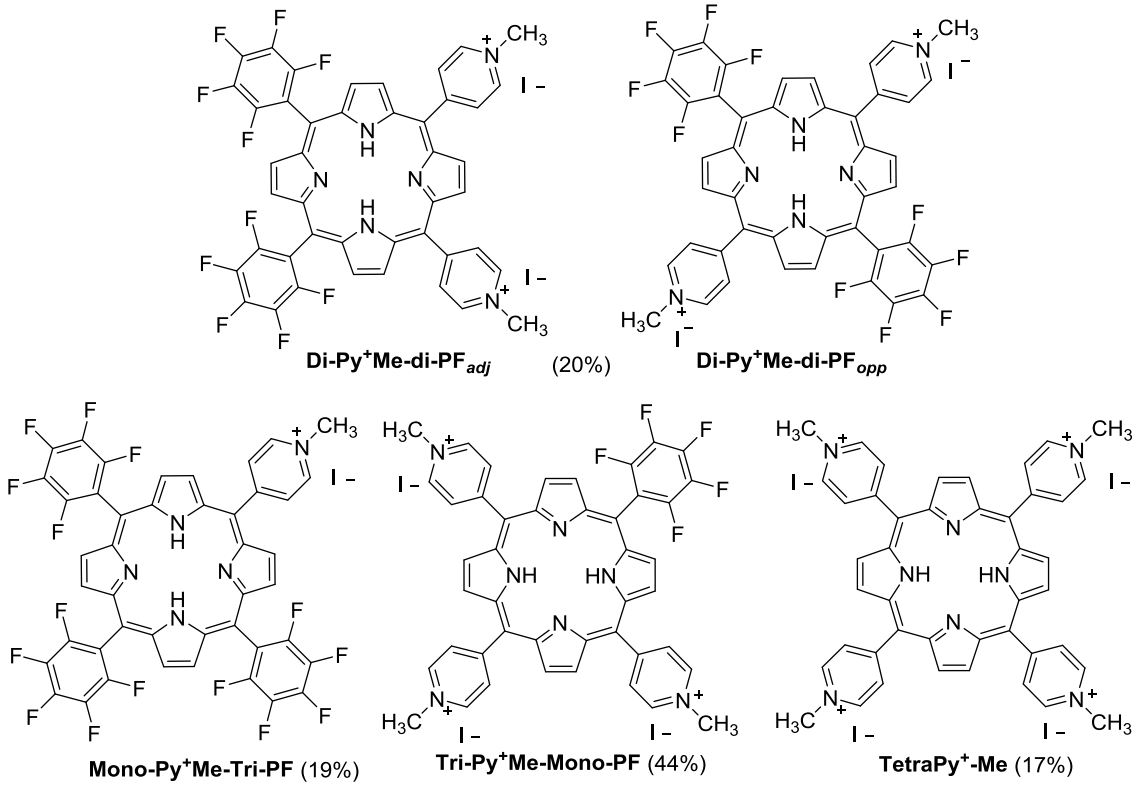

Figure 10 – Structures of the cationic porphyrins in the combined mixture used in this study as PS [90].

In order to overcome this limitation, we envisage that the non-separated mixture of these neutral porphyrins after being quaternized with methyl iodide could be also efficient in photoinactivation processes. In a parallel study it was demonstrated that the efficiency of this blend of cationic porphyrins to photoinactivate Gram positive and Gram negative bacteria is similar to the one obtained by the tricationic porphyrin [90].

Taken in account that the use of the non-separated cationic porphyrins mixture reduces significantly the production costs and also the production time, the possibility to use this efficient mixture to control bacterial diseases namely in field applications, like in kiwi plantations seems reasonable since a high quantity of PS is necessary.

So, in the present work, the aim was to evaluate if the efficacy of the combined mixture of cationic porphyrins shown in Figure 10 as PS is superior to the one obtained with **Tetra-Py$^+$-Me** in the photoinactivation of Psa. The assays were performed in *in*

vitro and in *ex vivo* using kiwi leaves artificially and natural contaminated with Psa. The possibility of this Gram negative bacterium after PDT treatment to develop resistance or to recover viability was also analyzed.

2.2 Methods

To evaluate the potentiality of the PS in the inactivation of *P. syringae* and to select the best inactivation conditions, different experimental assays were performed. The first experiments were performed *in vitro* using the non-separated mixture of porphyrins. PBS solutions containing the bacterium and a low concentration of PS (5.0 μM) were irradiated for different periods under artificial PAR light (380-700 nm) provided by fluorescent lamps at an irradiance of 4.0 mW cm^{-2}. In a second phase, the developed protocol was tested in *ex vivo*, using kiwi leaves artificially and naturally (provided by Portuguese Association of Kiwifruit Producers) contaminated with *P. syringae*, using the PS at 50 μM under artificial PAR light and sunlight (23 and 60 mW cm^{-2}). The PS concentration was increased in the ex vivo assays accordingly to previous studies [14]. In a third phase, bacterial resistance development and viability recovery after consecutive aPDT cycles were evaluated. Lastly, the potential negative effects of aPDT on kiwi leaf cells were also evaluated in *ex vivo* experiments at different concentrations of PS and under sunlight radiation.

2.2.1 Bacterial strain and growth conditions

Pseudomonas syringae pv. *actinidiae* (DSMZ, Germany), a colony obtained from a fresh cultured plate of Tryptic Soy Agar (TSA, Liofilchem), was inoculated in Tryptic Soy Broth (TSB, Liofilchem) and grew aerobically at 25 °C under 130 rpm for 20 h. Afterwards, an aliquot was transferred into fresh TSB incubated overnight at the same growth conditions to reach the early stationary phase (optical density at 600 nm of 1.3 ± 0.1 corresponded to ≈ 10^8 colony forming units (CFU/mL).

2.2.2 Photosensitizer

The mixture of PSs 5-(1-methylpyridinium-4-yl)-10,15,20-tris(pentafluorophenyl)porphyrin mono-iodide (**Mono-Py^+-Me-Tri-PF**), 5,15-bis(1-methylpyridinium-4-yl)-10,20-bis(pentafluorophenyl)porphyrin di-iodide (**Di-Py^+-Me-**

di-PF$_{opp}$), 5,10-bis(1-methylpyridinium-4-yl)-15,20-bis(pentafluoro-phenyl)porphyrin di-iodide (**Di-Py^{+}Me-di-PF$_{adj}$**), 5,10,15-tris(1-methylpyridi-nium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide (**Tri-Py^{+}-Me-Mono-PF**) and 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (**Tetra-Py^{+}Me**) used in this study was prepared according to the literature [87]. In summary, the non-separate mixture of these PS derivatives was obtained from a crossed Rothemund reaction of pyrrole and the adequate aromatic aldehydes (pyridine-4-carbaldehyde and pentafluorobenzaldehyde) at reflux in acetic acid and nitrobenzene. The nitrobenzene and acetic acid were then distilled under reduced pressure. The crude material was taken in dichloromethane and subjected to a pre-chromatographic separation using (silica gel) and eluted with a gradient from dichloromethane to dichloromethane/methanol (95:5). The porphyrinic mixture obtained was then precipitated in dichloromethane/acetone mixture. The cationization of all compounds presented in the neutral mixture was carried out using a large excess of methyl iodide and dry dimethylformamide (DMF) as solvent at 40 °C, overnight, in a closed flask. The reactional mixture was cooled to room temperature and diethyl ether was added to precipitate the methylated derivatives. The solid obtained was filtered, washed with diethyl ether and redissolved in methanol/water being after concentration, reprecipitated in methanol/acetone. The porphyrinic compostiton of the non-separate mixture assessed by chromatographic analysis was 19% (**Mono-Py^{+}Me-Tri-PF**), 20% (**Di-Py^{+}Me-Di-PF**), 44%(**Tri-Py^{+}Me-Mono-PF**) and 17% (**TetraPy^{+}Me**). A stock solution of this PS at 500 µM, was prepared in dimethyl sulfoxide (DMSO) and stored in the dark at room temperature. Before each assay the stock solution was sonicated during 30 min at room temperature (ultrasonic bath, Nahita 0.6 L).

2.2.3 Irradiation conditions

The effect of PS were evaluated by exposing the samples and controls to artificial white light (PAR radiation, 13 lamps OSRAM 21 of 18 W each one, 380–700 nm) at an irradiance of 4.0 mW cm^{-2} at defined times.

The photoinactivation experiments were also carried out with solar light outside of the laboratory. Samples were exposed to solar light on sunny spring/summer days, in the Littoral Centre of Portugal, with irradiance variances between 23 and 60 mW cm-^{2}. All the irradiances were measured with a Power Meter Coherent FieldMaxII-Top combined with a Coherent PowerSens PS19Q energy sensor.

2.2.4 *In vitro* aPDT assays

Bacterial suspensions (≈ 10^8 CFU mL^{-1}) were prepared, with PS at 5.0 µM, in phosphate buffered saline (PBS: 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per liter; pH 7.4) from the early stationary phase cultures, distributed in 100 mL beakers (final volume of 10 mL per beaker). The beakers (for the sample and the controls) were incubated in the dark for 10 min at 25 °C under 100 rpm stirring to promote the porphyrin binding to the cells, and then irradiated by artificial PAR white light. Light and dark controls were carried out simultaneously to the sample procedure: light control (LC) comprised a bacterial suspension exposed to light; and dark control (DC) comprised a bacterial suspension incubated with the PS at the studied concentrations but protected from light. Also, two more controls were used to check if the medium (TSA-C) and the buffer (PBS-C) were not contaminated. Sample and LC were irradiated for 60 min and DC was kept in the dark. Aliquots were collected before irradiation and after 10, 15, 20, 30 and 60 min of light exposure. After each photosensitization period, the suspensions were serially diluted in PBS, plated in TSA and incubated at 25 °C for 48 h. The colony forming units (CFU) were determined on the most appropriate dilution on the agar plates. Three independent experiments were performed and, for each, two replicates were plated.

2.2.5 *Ex vivo* aPDT assays

Ex vivo aPDT assays were performed on kiwifruit leaves provided by Portuguese Association of Kiwifruit Producers (Associação Portuguesa de Kiwicultores), with PS at 50 µM, under different radiation conditions: artificial PAR white light at low irradiance (4.0 mW cm^{-2}), the same used in the *in vitro* assays and sunlight.

The leaves portions (with an area of 16 cm^2) were placed on watch glasses and each was first sprayed with 50 µL of fresh overnight bacterial suspension diluted in 450 µL of PBS. After, the leave portions were sprayed with 50 µL of PS also diluted in 450 µL of PBS, to achieve a final concentration of 50 µM of PS. There was a leave portion for each time. The leaves were kept in the dark 30 min for pre-incubation. Light and dark controls were carried out simultaneously to the sample procedure. Also, an

additional control was used to evaluate the natural microbiota of the leaves used.

Sample and LC were irradiated for 90 min and DC was kept in the dark. Three leave portions (sample, LC and DC) were collected before irradiation and after 30, 60 and 90 min of light exposure, then putted in Erlenmeyer flasks with 20 mL of PBS and stirred for 30 min at 130 rpm. After this 30 min stirring, serial dilutions were made from this suspensions and the CFU were determined as described above. Three independent experiments were performed and, for each, two replicates were plated.

Another *ex vivo* assay was performed under sunlight as described above, but with naturally contaminated leaves brought directly from orchards affected by Psa and provided by the Portuguese Association of Kiwifruit Producers. In these experiments, no additional bacterial contamination of the leaves was done. Given the different level of contamination found in which leaf (leaves collected in different dates), the results of the two assays were treated independently. For each assay, only one leaf was used and, because of their reduce dimension, only times 0 and 90 min were tested.

2.2.6 *Ex vivo* aPDT inactivation cycles

The *ex vivo* inactivation with leaves artificially contaminated did not permitted a total inactivation. In order to achieve that, an additional assay was performed, in which the leaves were under sunlight for 2 cycles of 90 min, and PS at 50 µM was sprayed in the beginning of each new cycle.

2.2.7 Evaluation of resistance development and viability recovery after aPDT

To check if Psa are able to develop resistance after aPDT, new bacterial suspensions were produced after each cycle of Psa bacteria exposure to photodynamic treatment, according to Tavares *et al.* (2010) [91]. In order to obtain a modest bacterial inactivation, the bacterial suspension in the presence of the PS (5.0 µM) was exposed to PAR white light in cycles of 40 min irradiation (4.0 mW cm^{-2}) in the same conditions of the aforementioned photoinactivation assays. This will allow to test if the bacteria affected by the PS, though not in such a drastic way as it occurs when they are irradiated for a long period, are able to develop resistance to aPDT. After each cycle of 40 min, aliquots from sample and controls were aseptically taken and plated by pour plating on TSA (in duplicate). The plates were incubated at 25 °C for 48 h. After that, three

remaining colonies were picked up with an inoculation loop and added to TSB medium and grew aerobically at 25 °C under 130 rpm for 20 h. With this fresh culture the inactivation procedure was repeated. This procedure was repeated consecutively ten times.

To evaluate viability recovery, in the end of the irradiation procedure, aliquots of the sample and controls were taken and plated on TSA by the pour plate technic (in duplicates). The plates were incubated at 25 °C, in the dark, and the number of bacteria counts was assessed after 48, 96 and 120 h, to detect the delayed development of bacteria. Three independent experiments were performed and, for each, two replicates were plated.

2.2.8 Evaluation of possible side effects in kiwi leaves after photosensitization

In order to check the possible side effects in kiwi leaves, a test was performed with different PS concentrations (5.0 μM, 20 μM, 50 μM and 100 μM) based on previous work by Menezes *et al.* (2014) [13]. On the leaf surface were dropped 10 μL of each PS concentration and 10 μL of PBS as control. The drops were added, as described, every day at the same hour, on the exact same spot, for five days. Pictures were taken in the first and fifth day of test.

2.2.9 Statistical analyses

Statistical analyses were done with GraphPad Prism 6.01. Normal distributions were assessed by the Kolmogorov-Smirnov test. To assess the significance of the differences between the tested conditions (both irradiation time and porphyrin derivative on bacterial inactivation) a one-way univariate analysis of variance (ANOVA) and a *t*-test were performed. A value of $p < 0.05$ was considered significant.

2.3. Results

2.3.1 *In vitro* Psa inactivation

The results obtained from the Psa inactivation experiments performed *in vitro* at an irradiance of 4.0 mW cm^{-2} (Figure 11) show that the combined action of cationic porphyrins (formulation used as PS) at 5.0 µM was able to cause a bacterial decrease of 7.4 log (ANOVA, $p < 0.05$) after 60 min of irradiation. An inactivation of 3.2 log (ANOVA, $p < 0.05$) was achieved after only 10 min of irradiation.

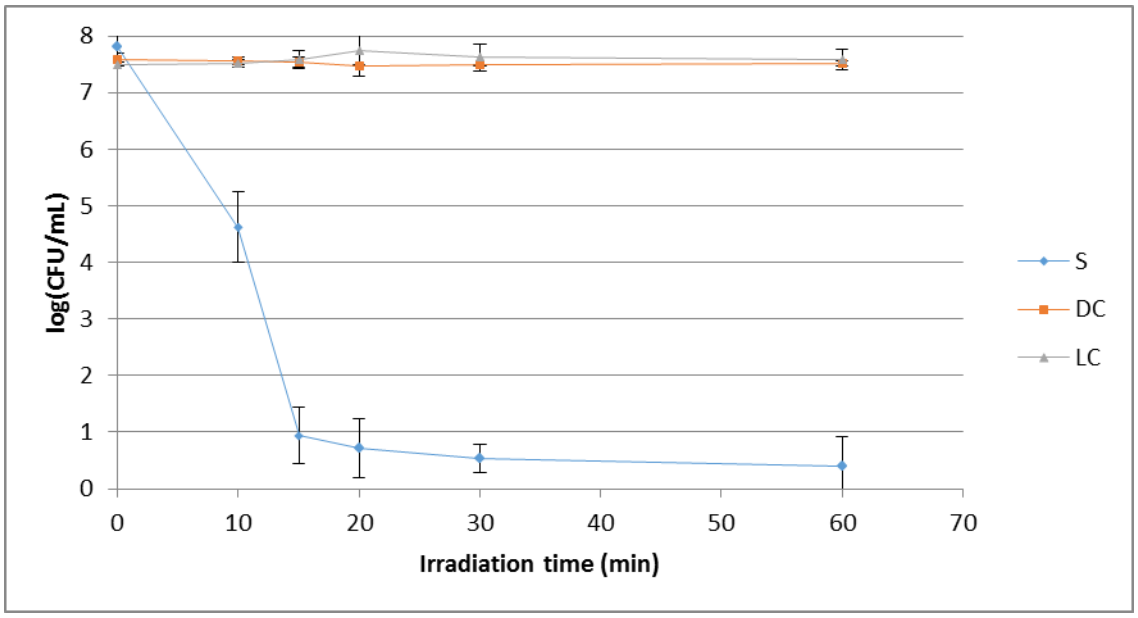

Figure 11 - *In vitro* Psa inactivation in PBS with PS at 5.0 µM under artificial white light at an irradiance of 4.0 mW cm^{-2}. S: Sample; LC: Light Control; DC: Dark Control.

2.3.2 *Ex vivo* Psa inactivation with artificial white light at low irradiance in artificially contaminated leaves

The results in Figure 12 show the surviving profile of Psa present in artificially contaminated leaves after being irradiated with artificial white light at an irradiance of 4.0 mW cm^{-2} in the presence of the PS at 50 µM. Under these conditions a bacterial reduction of 1.9 log (ANOVA, $p < 0.05$) was observed in the first 30 min of irradiation and of 2.8 log (ANOVA, $p < 0.05$) after 90 min of treatment.

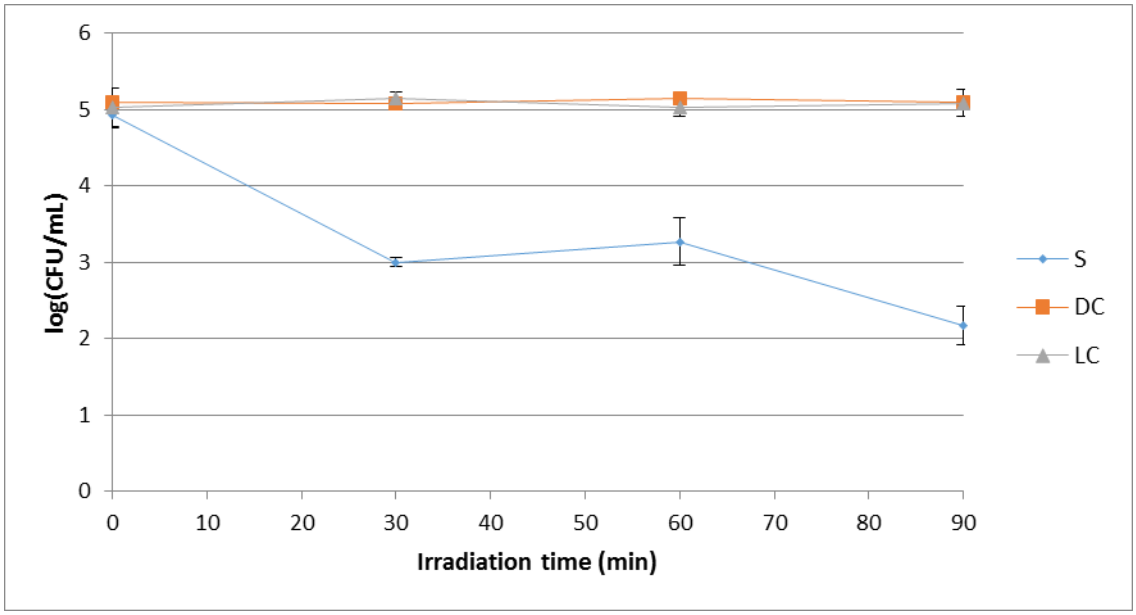

Figure 12 - *Ex vivo* inactivation of Psa with PS at 50 µM under artificial white light at an irradiance of 4.0 mW cm^{-2}. S: Sample; LC: Light Control; DC: Dark Control.

2.3.3 *Ex vivo* Psa inactivation with sunlight in artificially contaminated leaves

The assays performed under sunlight irradiation (23 mW cm^{-2}) with the PS at 50 µM in leaves artificially contaminated with Psa (Figure 13), showed a 3.0 log (ANOVA, $p < 0.05$) reduction in the bacterial concentration after 30 min of treatment and of 4.5 log after 90 min (ANOVA, $p < 0.05$).

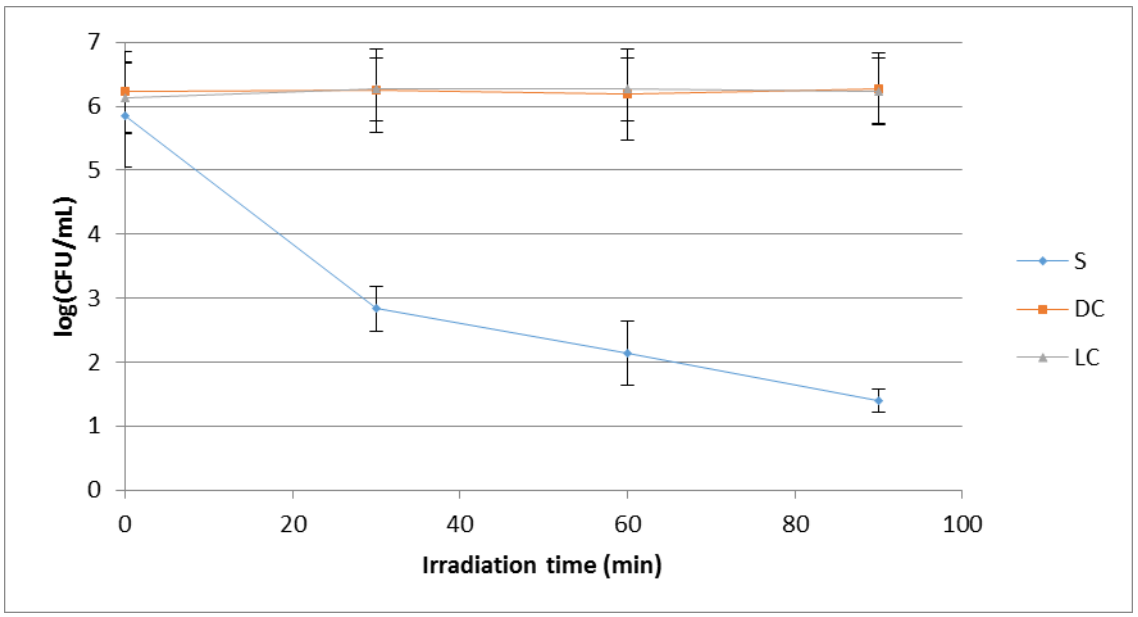

Figure 13 - *Ex vivo* Psa inactivation with PS at 50 µM under sunlight (23 mW cm^{-2}). S: Sample; LC: Light Control; DC: Dark Control.

2.3.4 *Ex vivo* Psa inactivation with sunlight in naturally contaminated leaves

In Figure 14 are summarized the results obtained in the assays performed with leaves naturally contaminated with Psa. After 90 min under sunlight irradiation at 23 mW cm^{-2} with the PS at 50 µM, the less contaminated leave suffers a bacterial reduction of 2.9 log ($p < 0.05$) (Figure 14A) while for the more contaminated one a reduction of 2.5 log ($p < 0.05$) (Figure 14B).

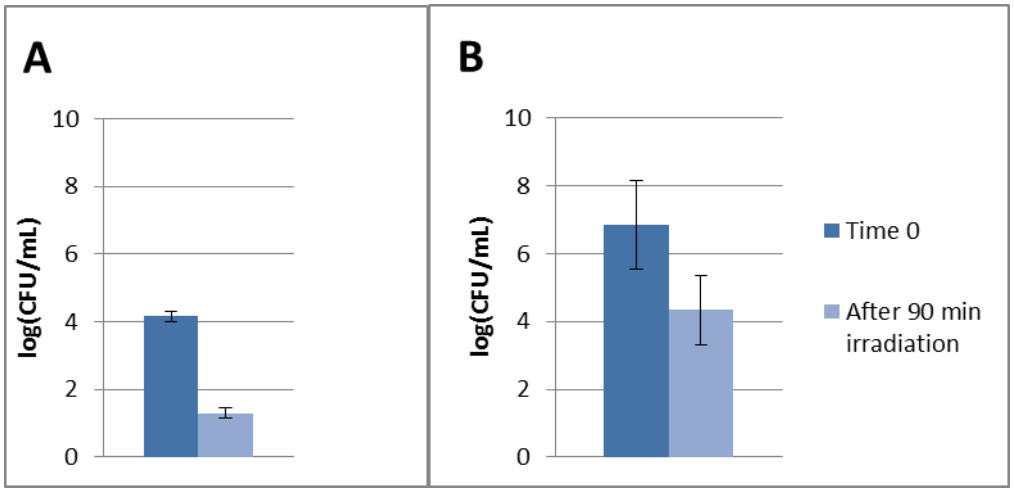

Figure 14 - *Ex vivo* inactivation of Psa on naturally contaminated leaves with PS at 50 µM and sunlight (23 mW cm^{-2}) using different naturally contaminated leaves. A) Leaf with an innitial contamination of 4.2 log; B) Leaf with an innitial contamination of 6.8 log.Dark blue: time 0. Light blue: after 90 min of irradiation.

2.3.5 *Ex vivo* Psa inactivation with two consecutive cycles of sunlight in artificially contaminated leaves

The results obtained when two consecutive treatments of 90 min each were performed in the presence of the PS at 50 µM under sunlight irradiation at 60 mW cm^{-2} are depicted in Figure 15. Under these conditions a final reduction of 6.2 log ($p < 0.05$) was achieved. Spraying the leaves after the 1st cycle with another dose of the PS at 50 µM followed by 90 min of sunlight irradiation allowed a further bacteria reduction of 1.0 log ($p < 0.05$) remaining just ≈ 0.5 log to inactivate.

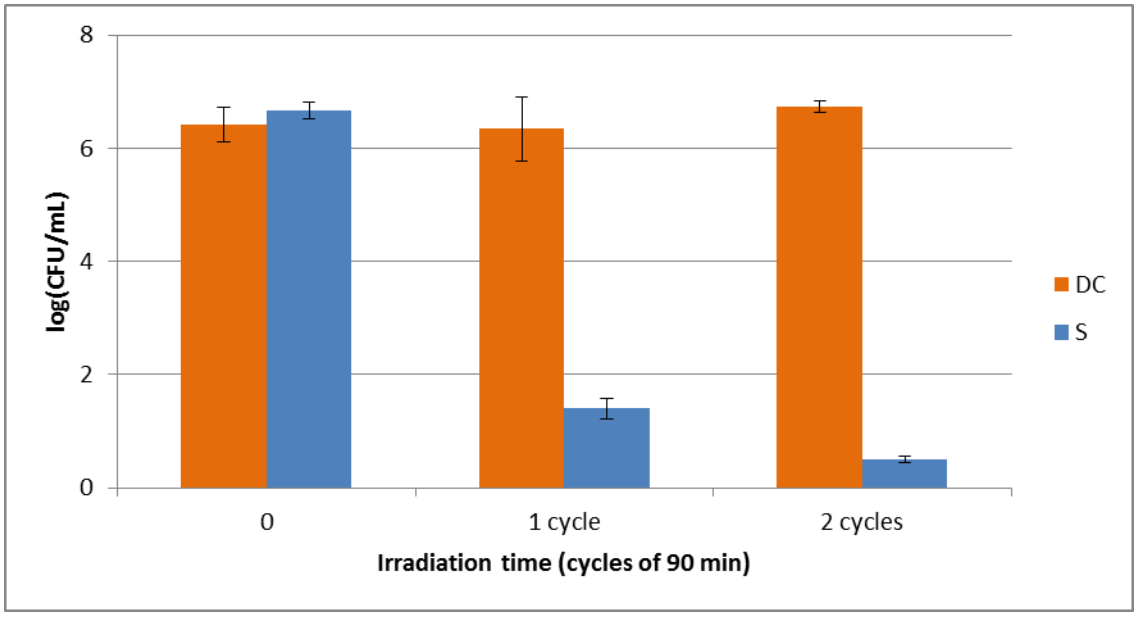

Figure 15 – Ex vivo Psa inactivation with PS at 50 µM and two cycles of sunlight (60 mW cm^{-2}) exposure. S: Sample; DC: Dark Control.

2.3.6 aPDT resistance development study

In Figure 16 are summarized the results obtained from the assays where it was evaluated the possibility of Psa to gain resistance after ten consecutive treatments. These treatments were performed under white light (4.0 mW cm^{-2}) in cycles of 40 min of irradiation in the presence of the PS at 5.0 µM in order obtain a modest bacterial inactivation. In the yy-axis are presented the values of log (N_0/N), in which N_0 represents the number of initial bacteria determined by bacterial plaque counts before irradiation, and N represents the number of surviving bacteria determined by the same method. In the xx-axis are presented the number of treatments. The rate of Psa inactivation in ten consecutive treatments at 5.0 µM was almost the same, so no statistical differences were observed in the sample and controls (data not shown) between the 10 treatments ($p > 0.05$).

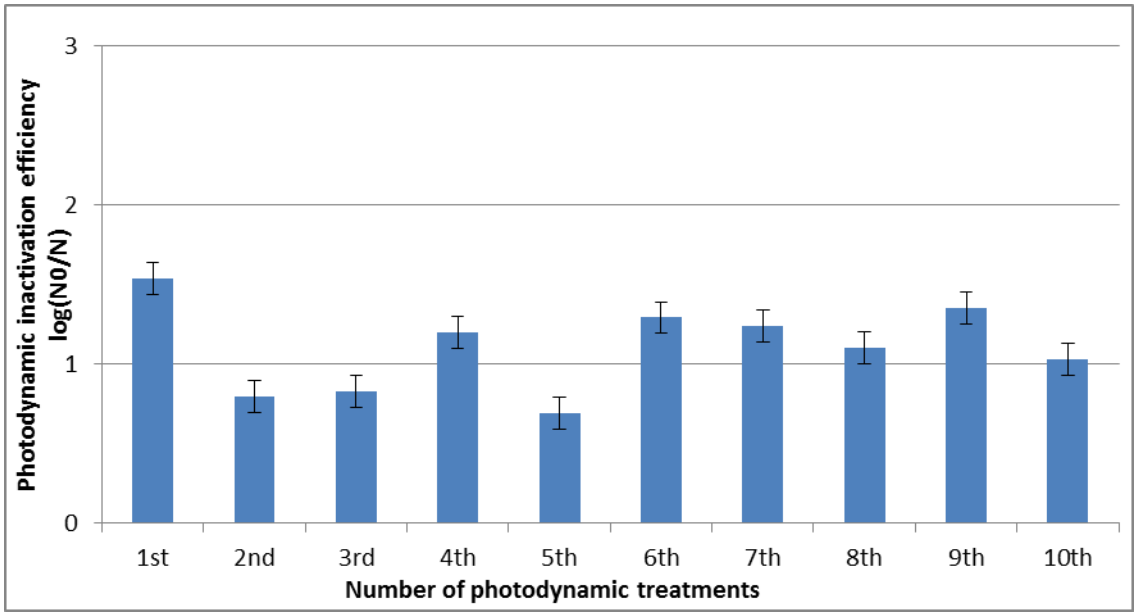

Figure 16 - Photodynamic inactivation efficiency of ten consecutive cycles of Psa, with PS 5.0 µM and 40 min irradiation with white light (4.0 mW cm^{-2}). S: Sample; log (N0/N) – N0: the number of initial bacteria determined by bacterial plaque counts before irradiation; N: represents the number of surviving bacteria determined by bacterial plaque counts.

2.3.7 aPDT viability recovery

In Figure 17 are summarized the results obtained from the assays where it was evaluated a possible recover of viability after a treatment with PS at 5.0 μM and after irradiation with white light (4.0 mW cm^{-2}) during 40 min. The number of bacteria was assessed after 48, 96 and 120 hours and it was observed that the initial reduction of ≈ 8 log did not vary significantly ($p > 0.05$). In these experiments after the initial treatment each sample was incubated at 25 °C in the dark. The number of bacteria was similar in both controls during the 120 h of incubation at 25 °C ($p > 0.05$).

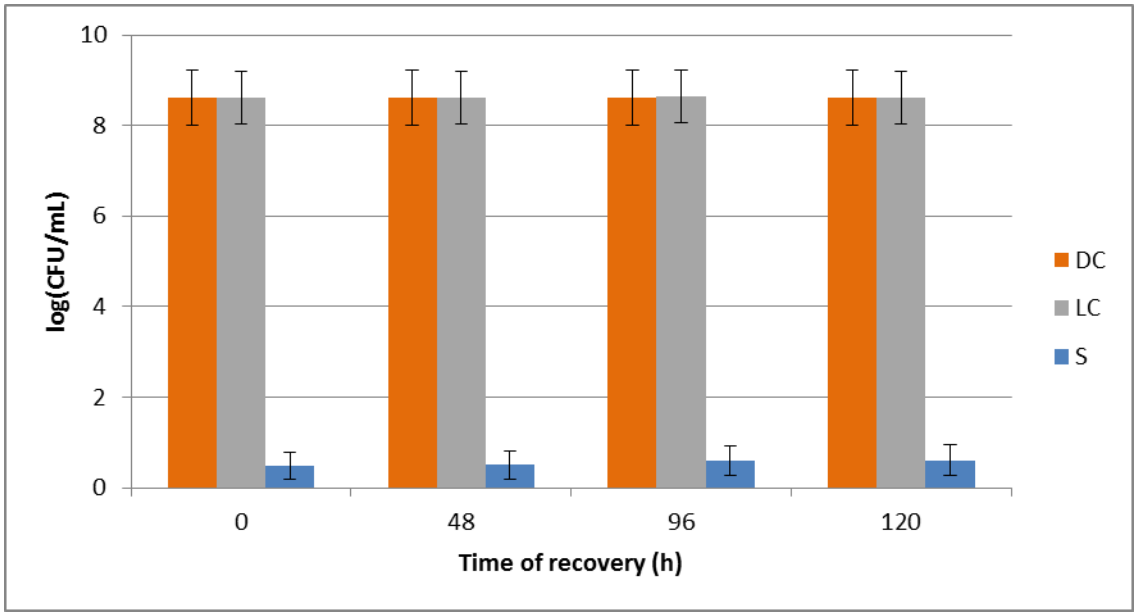

Figure 17 - Variation of Psa concentration with PS at 5.0 μM and after irradiation with white light (4.0 mW cm^{-2}) during 40 min, after 48, 96 and 120 h.

2.3.8 Evaluation of possible side effects in kiwi leaves after sensitization

Possible alterations in the color and/or texture of the kiwi leave after the aPDT treatment under sunlight was evaluated for five consecutive days by adding each day 10 µL of the combined mixture of cationic porphyrins at concentrations 5.0 µM, 20 µM, 50 µM and 100 µM (Figure 18). From the pictures it is patent that the leaves did not suffer any visual alteration after these daily applications of PS and exposure to sunlight (Figure 18A *versus* 18B).

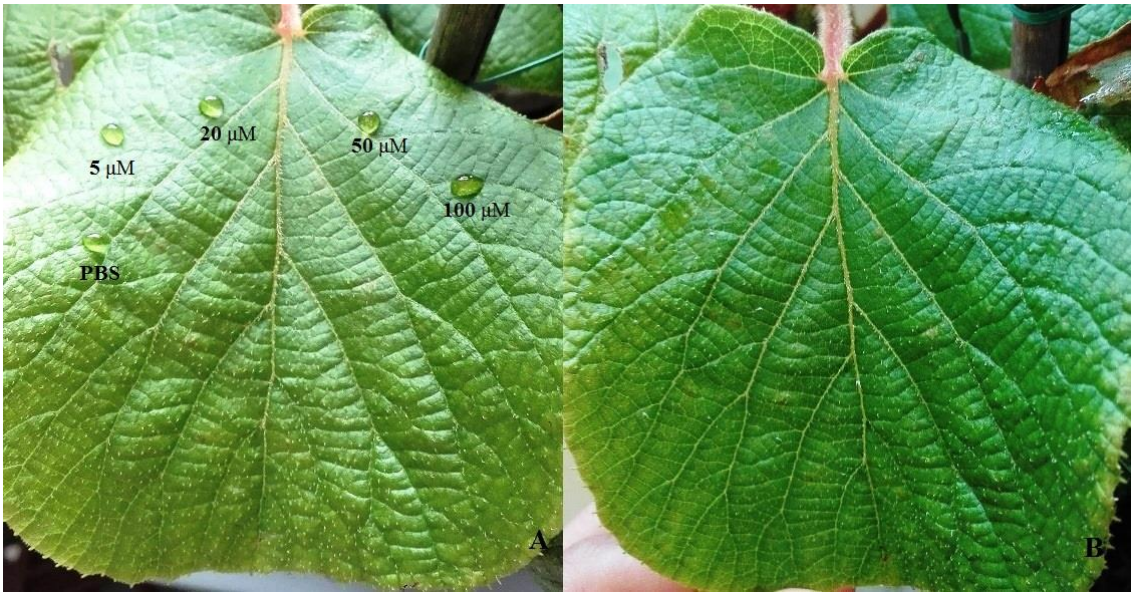

Figure 18 - Sensitization test images in kiwi leaves after one day of treatment with PS at different concentrations. On the left (A) are the drops of the four concentrations (5, 20, 50 and 100 µM) and the control (PBS). On the right (B) is the leaf aspect after 5 days of daily application and exposure to sunlight.

2.4 Discussion

Since the outbreak of Psa in Italy in 2008, approaches to control this bacterium have been considered of high priority by kiwifruit producers worldwide [47,58]. To control Psa, alongside with preventive care, copper sprays are often used, causing an environmental problem due to copper accumulation [67]. Besides, this bacterium is rapidly spread and it is becoming more virulent comparing to 2010, so these sprays alone cannot prevent another outbreak [80]. In order to overcome this problem and find an alternative way of treatment that is both efficient and eco-friendly, aPDT was tested to control this pathogenic agent that affects kiwifruit. Previous studies have already showed that the application of aPDT to control pathogenic microorganisms in the environmental, for instance to control plant pathogens such as fungi and more specifically to control Psa, can be an effective alternative treatment [13,14].

The principal aim of this work was to evaluate if a better performance in the photoinactivation of Psa could be attained when the non-separated mixture of the five cationic porphyrins showed in Figure 10 is used as PS, alternatively to **Tetra-Py$^+$-Me**. In the composition of this blend of cationic porphyrins is present in higher percentage the highly efficient tricationic porphyrin bearing a pentafluorophenyl group (**Tri-Py$^+$-Me-Mono-PF**) accompanied by the expected mono-, di- and tetra-cationic porphyrins. This mixture had already been tested against a *Staphylococcus aureus* strain and a bioluminescent *Escherichia coli* strain, showing effective inactivation of both bacteria [90]. In fact, the sensitizing efficiency of this mixture is not too different from the one observed for **Tri-Py$^+$-Me-Mono-PF**, but the cost and the time during its preparation were highly diminished when compared with the obtainment of the pure form.

The efficiency of this mixture was first evaluated *in vitro* at 5.0 μM, with artificial PAR white light at an irradiance of 4.0 mW cm^{-2}, showing a Psa reduction of around 7 log after only 15 min of treatment (Figure 11). Comparing to the previous study, in the same conditions, but with the tetracationic porphyrin **Tetra-Py$^+$-Me**, only after 60 min was achieved such inactivation (Figure 19) [14]. Besides, after 270 min irradiation, there was still around 1 log left to inactivate.

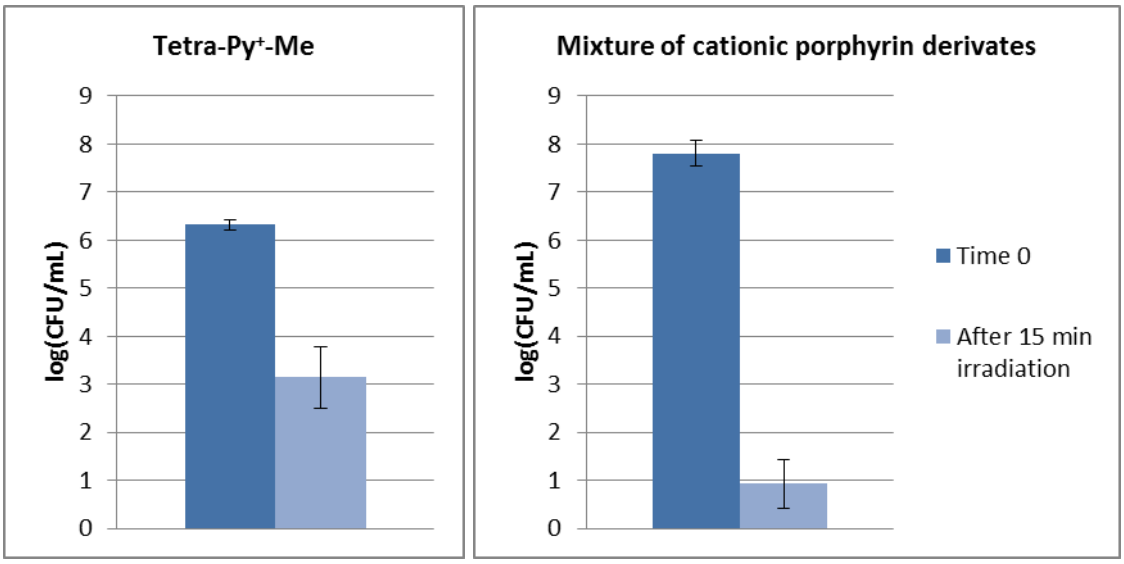

Figure 19 – Comparison between *in vitro* Psa photoinactivation with **Tetra-Py^{+}-Me** (left) [14] and the mixture of cationic porphyrin derivatives (right), after 15 min irradiation with white light at an irradiance of 4.0 mW cm^{-2}. Dark blue: Time 0; Light blue: After 15 min irradiation.

These results prompt us to evaluate how the efficiency of the combined mixture of cationic porphyrins is affected by natural environmental conditions more close to the ones of kiwifruit production. In these assays the aPDT tests were performed on kiwifruit leaves (*ex vivo*), artificially or naturally contaminated with Psa, and sprayed with PS at 50 µM and photoactivated by artificial white light at low irradiance (4.0 mW cm^{-2}) and under sunlight. Since the assays under sunlight were performed on different dates, the light intensity was different (23 mW cm^{-2} in the 90 min assays and 60 mW cm^{-2} in the consecutive cycles assays). However, similar situations occur during the year in kiwi plantations. The results showed that at an irradiance of 4.0 mW cm^{-2} an inactivation of around 3.0 log is attained after 90 min of treatment at a PS concentration of 50 µM (Figure 12). The major period of decrease was observed after the first 30 min of irradiation, in which a 1.9 log reduction was achieved. Comparing these results with the ones obtained in the exact same conditions but under sunlight (23 mW cm^{-2}) (Figure 13), which its irradiance is approximately 6 times higher, it was corroborated that the intensity of the light is an important feature to be taken into account in PDT protocols. The inactivation under sunlight was 4.4 log, thus was 1.6 log greater than that achieved with artificial white light. Also, the mixture formulation under test was again more effective than the **Tetra-Py^{+}-Me** alone, allowing a 3.2 log superior inactivation (Figure 20) [14]. Thus, these experiments reinforce the idea that this approach could possibly be applied in nature on kiwifruit leaves to control Psa.

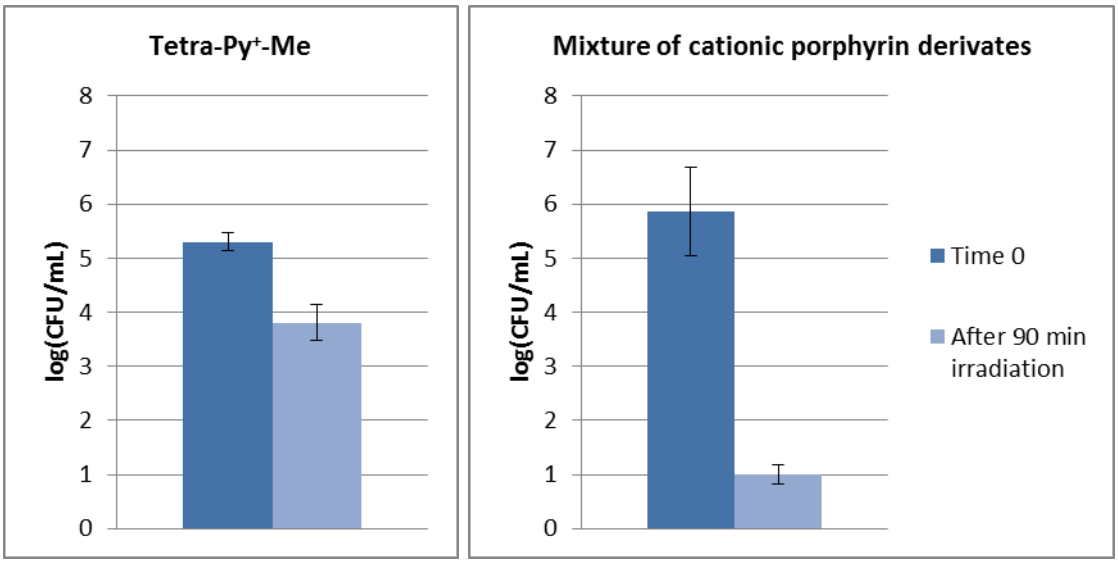

Figure 20 – Comparison between *ex vivo* Psa photoinactivation with **Tetra-Py$^+$-Me** (left) [14] and the mixture of cationic porphyrin derivates (right), after 90 minutes irradiation with sunlight. Dark blue: Time 0; Light blue: After 90 min irradiation.

Knowing that a better efficiency to photoinactivate Psa could be attained by repeating the treatment cycle, news assays were performed after the first cycle of treatment (Figure 15). The PS was again applied on the leaves and, after 30 min of the pre-incubation period; the leaves were exposed to another cycle of 90 min of sunlight irradiation. After this 2nd cycle of treatment, a total decrease of bacterial concentration from 6.6 to 0.5 log was obtained. Previously, with **Tetra-Py$^+$-Me**, there was still 2.5 log left to inactivate by the end of the 3rd cycle (Figure 21). These results show that the total Psa inactivation on plantations can be attained with more than one aPDT cycle of treatment. In fact, even the protocols based on copper sprays which are the main treatment to control Psa, require more than one application, because of the loss of copper by natural factors like wind and/or rain and also because the new tissue needs to be protected [92,93]. Other treatments in the plant pathogen control also require several applications, for instance in the case of fungicides during the growing season to keep coverage as new growth emerges and weathering removes previous coverage, multiple doses are applied in different periods [94].

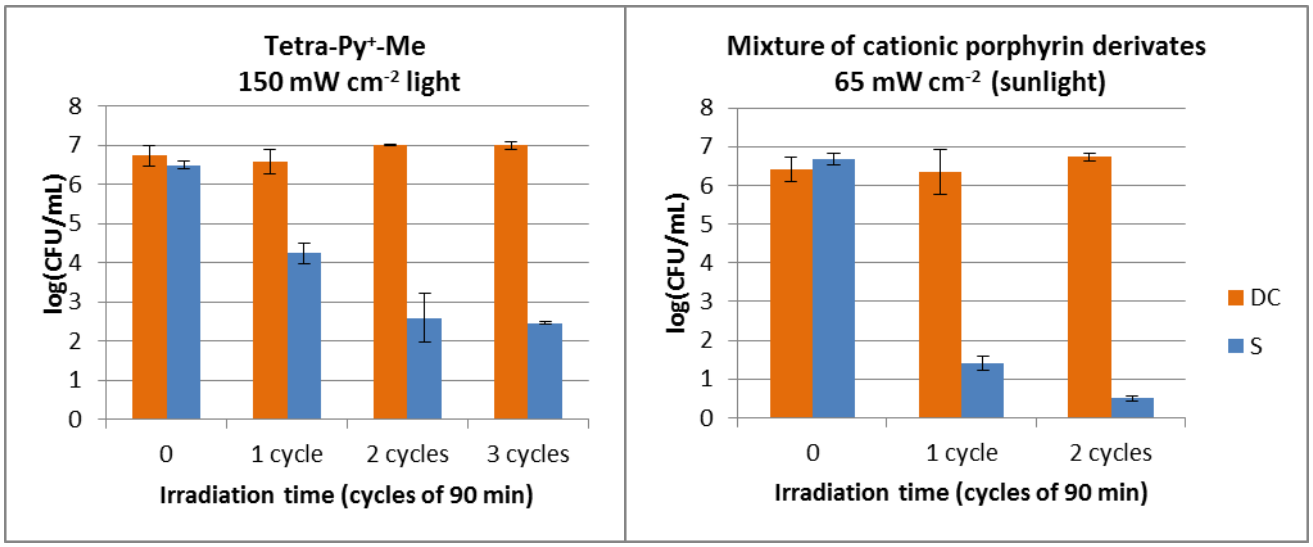

Figure 21 – Comparison between *ex vivo* Psa photoinactivation cycles with **Tetra-Py^{+}-Me** (left) [14] and the mixture of cationic porphyrin derivates, with light at an irradiance of 150 mW cm^{-2} and 60 mW cm^{-2}, respectively. DC: Dark control; S: Sample.

The results obtained when aPDT assays were extended to leaves naturally contaminated with Psa confirm the effectiveness of the results obtained in artificially contaminated leaves (Figure 14). These leaves were brought to the lab directly from a severally contaminated plantation in Portugal. Since the leaves were collected in different dates, its level of contamination was different, so the results of the two assays were treated independently. By applying PS at 50 µM and exposing the leaves to 90 min of sunlight (23 mW cm^{-2}) was achieved a 2.9 log (Figure 14A) and a 2.5 log inactivation (Figure 14B). Further studies applying several cycles to naturally contaminated leaves should be done since as observed with artificially contaminated leaves, after consecutive cycles a greater inactivation is achieved.

Although the development of resistance after aPDT is considered an improbable event due to the multi-target mechanism of photoinactivation, this aspect must be confirmed with Psa a much less studied bacteria [6,20,21]. The mechanism involving the photodynamic process consist in reactions mediated by ROS that will interact in a non-specific way with different biological components of the cell, namely proteins, lipids, amino acid residues, nucleic acid bases and cell pigments [6,16,19]. The results obtained in this work (Figure 16) are in agreement with the ones previously reported [95–97]. The number of surviving bacteria was approximately the same in all cycles of irradiation. That means no appreciable development of resistance in partially inactivated bacteria was observed. In fact, the efficiency of photoinactivation underwent no significant change in ten subsequent irradiation cycles. If resistance to aPDT would

occur, significant reductions in bacterial photoinactivation efficiency would be detected between the 10 experiments. This would be interpreted as an indication of enhanced resistance of the bacteria of later generations in relation to the ones in the initial stocks. Nevertheless, this was not observed, thus invalidating the hypothesis of Psa resistance development to aPDT.

Like resistance development, viability recovery should be considered an unlikely event, for the same reason - the multi-target mechanism of aPDT. In fact, in this study, during 120 h of incubation after phototreatment, the bacteria had all the necessary conditions to recover from the photodynamic treatment, but no recovery was observed (Figure 17). After 40 min of treatment, period not long enough to achieve total bacterial inactivation, the surviving bacteria did not recover their viability. Again, these results corroborate the literature data [95,96]. In conclusion, besides being efficient to photoinactivate the Psa, this PS does not promote or allow resistance development at least after 10 consecutive treatments or viability recovery of the surviving bacteria after aPDT.

The sensitization test showed that this PS does not cause any damage to the leaves when exposed to sunlight (Figure 18). These results corroborate previous ones by Jesus *et al.* (2017) [14]. The absence of damage could be due to the relatively low concentrations used and to the short dark preincubation period. Also, the leave's cuticle can act as barrier, making the PS crossing harder. During aPDT procedure, the reactive species generated have short half-lives and their diffusion is limited, so the damage is restricted to structures close to the PS. Therefore, as the PS stayed outside the cuticle, the internal leaf structure was not damaged by the aPDT and Psa inactivation is inactivated without damaging the tissue. On the contrary, as demonstrated in previous studies, copper sprays cause severe damage to the leaves, showing phytotoxic symptoms, like silver-brown leaves, discoloration, cracking and appearance of spots [98]. Again, aPDT seem to be a safer alternative to control Psa when compared to copper treatment.

The results of this study show that the combination of cationic porphyrin derivatives (formulation tested) is an excellent alternative to the tetracationic porphyrin to inactivate Psa and the protocol is viable in leaves naturally contaminated with Psa. The results show also that the development of resistance and the viability recovery after aPDT are improbable events. Additionally and contrary to copper(I) applications the

aPDT protocol did not cause any damage to the leaves when exposed to sunlight. Overall, the mixture of cationic porphyrins can be an adequate PS to control the Psa in kiwi plantations, being more effective than **Tetra-Py^{+}-Me** with a substantial decrease on costs and production time.

2.5 Conclusions and future perspectives

The non-separated mixture of porphyrins was more effective than the tetracationic on the inactivation of the bacterium. Overall, the new formulation can be an adequate PS to control the Psa in kiwi plantations, since it was able to photoinactivate Psa under sunlight, without causing damage to the leaves.

Future work should be focused on test the formulation *in vivo*, to evaluate further side effects of the therapy to the plant and more studies should be performed on naturally contaminated leaves. Also, it is important to test other less expensive photosensitizers with recognized efficiency to photoinactivate other Gram negative bacteria and already accepted in clinical field.

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CHAPTER III - Appendix

Annex I – Applicable legislation in Portugal

Além do disposto no **Decreto-Lei n.º 154/2005,** e suas alterações, relativo às medidas de proteção fitossanitária destinadas a evitar a introdução e dispersão no território nacional e comunitário, de organismos prejudiciais aos vegetais e produtos vegetais qualquer que seja a sua origem ou proveniência, Decreto-Lei que transpõe a Diretiva n.º 2000/29/CE, do Conselho, importa para efeitos de aplicação de algumas das medidas mencionadas neste plano de ação, ter ainda em conta os seguintes diplomas:

Decreto-Lei n.º 329/2007, que regula a produção, controlo, certificação e comercialização de materiais de propagação e de plantação de espécies hortícolas, com exceção das sementes, e de materiais de propagação de fruteiras e de fruteiras destinadas à produção de frutos;

Decreto-Lei n.º 124/2006, alterado e republicado pelo Decreto-lei n.º 17/2009, de 14 de janeiro, que estabelece as medidas e ações a desenvolver no âmbito do Sistema Nacional de Defesa da Floresta contra Incêndios.

Annex II – Submitted paper