Universidade de Aveiro Departamento de Biologia
Ano 2016

Vânia Isabel Rodrigues Jesus

Photodynamic therapy in the control of *Pseudomonas syringae* pv. *actinidiae* in kiwi plants

Terapia fotodinâmica no controlo da *Pseudomonas syringae* pv. *actinidiae* em plantas de kiwi

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Photodynamic therapy in the control of *Pseudomonas syringae* pv. *actinidiae* in kiwi plants

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, 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, *Pseudomonas syringae*, *Actinidia deliciosa*, fotossensibilizador, porfirina tetracatiónica

resumo

O kiwi é o fruto produzido pela *Actinidia deliciosa* e é mundialmente consumido e comercializado. O cultivo deste fruto, tem vindo a ser extremamente afetado pela *Pseudomonas syringae* pv. *actinidiae* (*Psa*), uma bactéria fitopatogénica cada vez mais dispersa. Deste modo, pode originar grandes perdas económicas, afetando gravemente o comércio de kiwi em alguns países, incluindo Portugal, que tem vindo a aumentar significativamente a sua produção e a afirmar-se no comércio internacional.

Os tratamentos disponíveis para esta doença ainda são escassos, sendo a pulverização dos pomares com compostos de cobre o mais usado, nomeadamente o óxido cuproso (Cu_2O). Contudo, estes compostos devem ser evitados devido à sua elevada toxicidade e, por isso, é essencial a procura de novas formas de controlo da *Psa* que assegurem também a integridade das plantas. A terapia fotodinâmica antimicrobiana (aPDT) pode ser uma abordagem alternativa para inativar a *Psa*. A aPDT consiste no uso de um fotossensibilizador (PS) que absorve radiação e transmite a energia ou eletrões adquiridos a moléculas de oxigénio, formando espécies de oxigénio altamente reativas que afetam diferentes alvos moleculares, o que torna muito improvável o desenvolvimento de resistência nos microrganismos. É ainda de salientar, que atualmente, a aplicação da aPDT não foi testada no controlo da *Psa* em plantações de kiwi.

Assim, o objetivo deste trabalho consistiu em avaliar a eficácia da aPDT para inativar ou reduzir a *Psa*, usando a porfirina Tetra-Py^+-Me como PS e diferentes intensidades de radiação, nomeadamente 40 W m^{-2}, 150 W m^{-2} e luz solar (por ser adequada para usar nas plantações). Inicialmente foi analizado o grau de inativação da *Psa in vitro* com 5µM de Tetra-Py^+-Me sob baixa radiação (40 W m^{-2}). Depois, os ensaios *ex vivo* usando folhas de kiwi artificialmente contaminadas, foram feitos com o PS a uma concentração dez vezes maior (50µM) sob 150 W m^{-2} e irradiação solar.

Nos ensaios *in vitro* foi observada uma redução de 6 logs após 90 min de irradiação. Nos ensaios *ex vivo* o decréscimo foi menor, redução de aproximadamente 1,8 log a 150 W m^{-2}, 1,2 log a 40 W m^{-2} e 1,5 log sob a radiação solar. Não foram observados efeitos negativos nas folhas após o tratamento. O óxido cuproso foi testado *in vitro* à concentração recomendada na legislação portuguesa (50 g hL^{-1}) e em concentrações 10 vezes mais baixas, que inativam a *Psa* de forma eficaz após cinco minutos de tratamento.

No geral, demonstrou-se que aPDT *in vitro* e *ex vivo*, usando um derivado de porfirina sob radiação solar natural, é um método eficaz para inativar a *Psa*, sendo que não danifica a planta e pode ser aplicada por pulverização. A fim de explorar o potencial real da aPDT como uma alternativa ao uso intensivo de tratamentos convencionais, são necessários mais estudos para determinar a eficácia da aPDT em condições de campo e também para avaliar o impacto ambiental desta nova abordagem. Também foi demonstrado que a concentração de cobre atualmente recomendada para tratar a *Psa* pode ser significativamente reduzida. Outros estudos *ex vivo* e *in vivo* são, contudo, necessários.

keywords

Photodynamic therapy, *Pseudomonas syringae*, *Actinidia deliciosa*, photosensitizer, tetracationic porphyrin

abstract

Kiwifruit is produced by *Actinidia deliciosa* and is consumed and marketed worldwide. The cultivation of this fruit has been greatly affected by *Pseudomonas syringae* pv. actinidiae (*Psa*), a phytopathogenic bacterium that is increasingly dispersed. This can lead to severe economic losses, seriously affecting kiwifruit trade in some countries, including Portugal, which has significantly increased its production and established itself in international trade. The available treatments for this disease are still scarce and spraying the orchards with copper compounds is the most used, in particular cuprous oxide (Cu_2O). However, these compounds should be avoided due to their high toxicity and, therefore, it is essential to search for new ways of controlling *Psa* which also ensure the integrity of the plants. Antimicrobial photodynamic therapy (aPDT) can be an alternative approach to inactivate *Psa*. aPDT consists in the use of a photosensitiser (PS) which absorbs radiation and transmits the acquired energy or electrons to oxygen molecules to form highly reactive oxygen species that affect different molecular targets which makes it very unlikely that the microbes can develop resistance. It should also be noted that, currently, the application of aPDT has not been tested for the control of *Psa* in kiwi plantations.

Thus, the objective of this work was to evaluate the effectiveness of aPDT to inactivate or reduce *Psa*, using the porphyrin Tetra-Py+-Me as a PS and different radiation intensities, namely 40 W m^{-2}, 150 W m^{-2} and sunlight (because it is suitable for use in plantations). Initially, the degree of inactivation of *Psa in vitro* with 5 μM Tetra-Py^+-Me under low radiation (40 W m^{-2}) was tested. After, *ex vivo* experiments usingartificially contaminated kiwi leaves, were done with PS concentration ten times higher (50 μM) under 150 W m^{-2} and sunlight irradiation.

In the *in vitro* assays, a reduction of 6 logs was observed after 90 min of irradiation. In the *ex vivo* tests, the decrease was lower, approximately 1.8 log reduction at 150 W m^{-2}, 1.2 log at 40 W m^{-2} and 1.5 log under solar radiation. No negative effects were observed on leaves after treatment. Cuprous oxide tested *in vitro* at the recommended concentration in Portuguese legislation (50 g hL^{-1}) and at 10 times lower concentrations, efficiently inactivated *Psa* (5 log inactivation) after a few minutes of treatment.

Overall it was demonstrated that *in vitro* and *ex vivo* aPDT with a porphyrin derivative under natural solar radiation is effective to inactivate *Psa*, does not damage the plant and can be applied by spraying. In order to explore the real potential of aPDT as an alternative to the intensive use of conventional treatments, further studies are necessary to determine the effectiveness of aPDT in field conditions and also to evaluate environmental impact of this new approach. It was also showed that copper concentration currently advised to treat *Psa* can be greatly reduced. Further *ex vivo* and *in vivo* studies are, however, required.

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List of acronims and abbreviations

PDT	Photodynamic Therapy
PS	Photosensitizer
aPDT	Antimicrobial photodynamic therapy
Gram (-)	Gram-negative
S_1	Singlet state
S_0	Ground state
T_1	Triplet state
ISC	Intersystem crossing
VR	Vibrational relaxation
ROS	Reactive oxygen species
$O_2^{-\bullet}$	Superoxide anions
H_2O_2	Hydrogen peroxide
$OH^{\bullet}$	Hydroxyl radicals
$^1O_2$	Singlet oxygen
Gram (+)	Gram-positive
CFU	Colony Forming Units
HpD	Hematoporphyrin
Psa	*Pseudomonas syringae* pv. *actinidiae*
Psa-V	Highly virulent *Psa*
Psa-LV	Low virulence *Psa*
PBS	Phosphate Buffered Saline
Tetra-Py^+-Me	5,10,15,20-tetrakis(1-methylpyridinium-4-yl) porphyrin tetra-iodide
OD	Optical Density
TSA	Tripticase Soy Agar
TSB	Tripticase Soy Broth
rpm	Rotations per minute
LC	Light control
DC	Dark control
APK	Associação Portuguesa de Kiwicultores
RLU	Relative Light Units

ANOVA	Analysis of variance
S	Sample

CHAPTER 1 – Introduction

1.1 Photodynamic therapy

Photodynamic therapy (PDT) is a therapy that requires the interaction of three components: light, photosensitizer (PS) and oxygen [1].

In order to explain the photodynamic process, the following topics will be approached: historical aspects, mechanism of action, PDT in the inactivation of pathogenic microorganisms (aPDT), inactivation of Gram-negative bactéria, photosensitisers, light sources, oxygen and benefits and limitations of aPDT.

1.1.1 Historical aspects

The concept of PDT has been used for about 100 years, but only in the last century, it became most widely used and studied especially in diseases control. During the past 20 years, research activity in the PDT field has expanded enormously [2-5].

The first PDT studies were performed in Europe and the results were mainly published in French, German and Danish, so this therapy only became better known after the World War II, when the articles began to be written in English. The first study cited was developed in Munich by a medical student and his teacher, Oscar Raab and Herman von Tappeiner, respectively. In this study, they found that the combination of red light with acridine causes the death of a specie of paramecium. Raab noted also that the combination acridine and light was more effective than the use of acridine or sunlight alone or acridine subjected to sunlight before being added to the paramecium. Then, he discovered that light was not toxic, and that this toxicity was due the interaction of the light with the chemical [1,2,6].

In 1907, it was first used the term "dynamic action" when Von Tappeiner and Jodlbauer demonstrated that photosensitivity reactions need oxygen. Finsen, Raab and Von Tappeiner can be highlighted as pioneers in this technology, when they developed photochemotherapy as a therapeutic way [1,2,6,7].

Porphyrins are one of the most studied groups of PS and the main photosensitizing molecules used in PDT because they have the ability to absorb at diferent wavelengths. These molecules were identified in the mid-nineteenth century and the first porphyrin to be developed was hematoporphyrin by Scherer in 1841 while studying the nature of blood [2,8].

1.1.2 Mechanism of action

An important component in PDT is the PS, a compound that can be photoactivated by visible or ultraviolet light whit in an appropriate wavelength that depends on the structure and electronic absorption spectrum of the PS [7,9-11,]. In the excited state, the PS can interact with oxyen producing highly cytotoxic species to the cells like singlet oxygen and/or free radicals. It is noteworthy that the cell density is an important parameter for defining the effectiveness of antimicrobial PDT (aPDT) [9,12-15].

In the photodynamic process, the PS absorbs energy from the light and tranfere to molecular oxygen. The absorption of a photon at an appropriate wavelength, initially leads to the production of an unstable electronically excited state (S_1) of the PS molecule, with a very short lifetime (Figure 1). The excited PS molecule can then decay to the ground state (S_0) providing the light emission, fluorescence (radiative pathway) or by intersystem crossing (ISC) that enables the excitation for a triplet state (T_1) in which a vibracional relaxation (VR) occurs. In the T_1 state molecules have a longer lifetime. At this point, the PS can return to state S_1 by spin inversion and phosphorescence, or by a non-radiative process. Since the T_1 state promotes a longer lifetime, PS molecule can follow two different reactions (Type I and Type II).

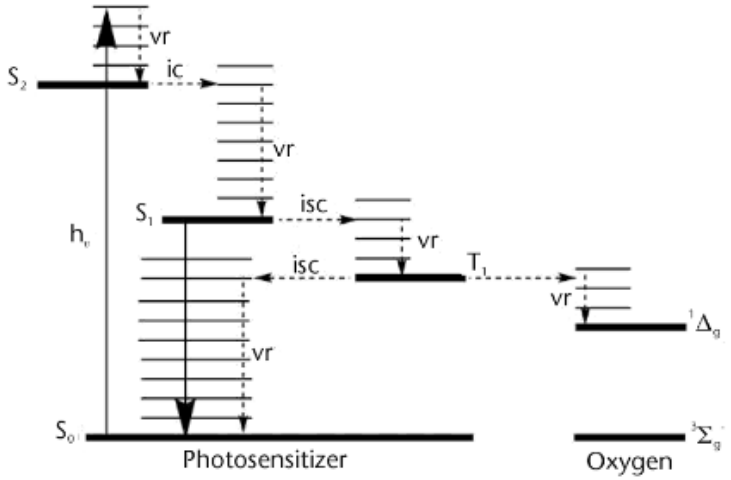


Figure 1 – Electronic transitions with photodynamic agentes according to Jablonski energy levels diagram [1].

In type I reaction, an electron or hydrogen atom is transferred from an excited triplet state to cellular substrates, forming free radicals that will interact with oxygen molecules, which in turn, originate reactive oxygen species (ROS) [1,3,5,12]. Generally, molecular oxygen

produces superoxide anions ($O_2^{-\bullet}$), which when reacting with water may produce hydrogen peroxide (H_2O_2) passing through the membranes and therefore can react with biological molecules and cause oxidative damage inside the cell. In addition, H_2O_2 at high concentrations affords hydroxyl radicals ($OH^{\bullet}$) which have high redox potential and for this reason readily reacts with any biological molecule, causing their oxidation (Fenton reaction) [3-5,7,14,16].

The reaction of type II is more relevant to the PDT, because occurs the direct transfer of energy between PS and molecular oxygen. This is generally accepted as the major pathway in the photooxidative cell damage [1,3,5,7,12]. This reaction occurs from the fundamental state and produces singlet oxygen ($^1O_2$) *in situ*, a highly reactive species. On the other hand, it has a short life span and a limited action space, so only the closest molecules will react. Nevertheless, the PS has catalytic and regenerative ability, producing numerous singlet oxygen molecules over time, as long as there is light and oxygen [3,5,7,12,17].

All these reactions may occur simultaneously, however, one will predominate according to the characteristics of PS and the available cellular substrates, among others. Even so, type II reaction is the predominant one [3,4,18]. Both reactions originate ROS that readily interact with biological componentes of cell wall, including proteins (cysteine, histidine, and tryptophan), lipids (unsaturated fatty acids of membranes), nucleic acid bases (guanine and thymine) and also cells pigments [1,3-7,12,15,18,19]. In this way the organic functionality is compromised and consequently leads to the inactivation of the cell. Damage caused by PDT in plasma membrane can be observed within minutes after light exposure [12,13].

The type of the PS and the range of radiation used should be chosen according to the target. An important aspect to evaluate when this therapy is used to inactivate microorganisms is to determine the possible side/toxic effects in the host [2,5,7]. These effects are due to apoptosis or necrosis induction, such as was first demonstrated in 1991 [2,5,16].

1.1.3 Photodynamic therapy in the inactivation of pathogenic microorganisms (aPDT)

The discovery of antibiotics has revolutionized the treatment of bacterial infections and PDT studies stagnated. However, overuse of antibiotics has led to increased resistance,

so in the last years PDT has been suggested as an alternative to these drugs. This technique has demonstrated good results in microbial inactivation (*in vitro*) besides being cost-effective and environmentally friendly. Thus, PDT has emerged as a new and effective technique for controlling pathogens [4,11,18,20].

Although antimicrobial PDT (aPDT) has been known for about a century, the underlying mechanisms of its action are not completely understood, however some aspects are already defined, namely, the fact of increasing cell wall permeability, which consequently allows the influx of sensitizer molecules, enhancing the photosensitizing effect. So the main prerequisite for bacterial photoinactivation is accumulation of the PS, which depends on bacteria growth state (larger in the exponential phase) [2,11,15,20]. The bacterial cytoplasmic membrane loses its integrity through a process called lipid peroxidation that leads to loss of fluidity and increased ion permeability. However, it can also occur damage of membrane enzymes and receptors [3,4,7,18].

PDT has shown to be a very promising alternative and an effective method to inactivate microorganisms (bacteria, including multidrug-resistant strains, microfungi, protozoa, viruses and yeasts). Additionally, it is also effective in inactivating spores and biofilms. In this way, microbial infections can be controlled and until now the developmente of resistance was not detected [4,9-12,15,18].

Gram-positive and Gram (-) bacteria are not affected in the same way by PDT, because they have different cell-wall chemistry. Some studies have found that Gram (+) are generally more susceptible to PDT than Gram (-), due the difficulties of the PS to enter in Gram (-) cells. This occurs due to the physiological and structural differences of their cell wall; the Gram (-) cells have an external protection constituted by a lipopolysaccharide layer strongly charged that impedes the penetration of $^1O_2$, a lipopolysaccharide layer strongly charged. Thus, what differs between both bacteria is the way that the PS crosses the membrane [1,4,7,9,10,15,17].

According to the American Society of Microbiology, any new approach must achieve a reduction of at least 3 $\log_{10}$ colony forming units (CFU) (killing efficiency of 99.99% or more) to be termed antimicrobial or antibacterialPrior to exposure to light, a dark preincubation period is required for the PS to interact with the cellular components of the

bacterial wall. So that the efficiency of the photosensitization process can be dependent on this preincubation [18].

1.1.3.1 Inativation of Gram-negative bacteria

Gram (-) bacteria show significant resistance, due to the complexity of the outer membrane. The difficulty of PS and other molecules to cross their membranes is due to the fact that are composed of a lipid bilayer sandwiching the peptidoglycan layer and an outer layer of lipolysaccharide (Figure 2). This creates an impermeable barrier that results in a low degree of permeability [4,8,18,21-23]. While a diferent situation occurs in Gram (+) bactéria, which have only one lipid membrane and a highly porous cell wall (multiple layers of peptidoglycan) (Figure 2). Therefore, PDT-killing of Gram (+) is definitely much easier to accomplish than that of Gram (-). Even so, membrane permeability of the Gram (-) differs from species to species, so the sensitivity pattern can be diferent [4,8,17,18,21-23].

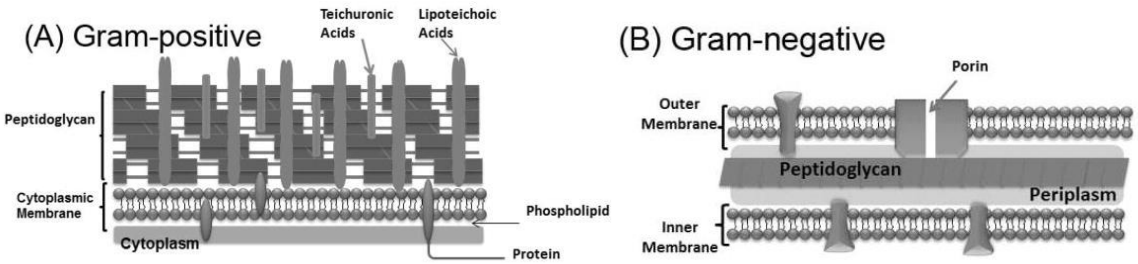


Figure 2 - Structures of the cell walls of Gram (+) (A) and Gram (-) (B) [8].

Several studies have been conducted in order to select the best PS for Gram (-) and these approaches involve the optimization of the chemical structure to achieve a good penetration into the bacterial cell wall. According to some studies in *Escherechia coli* and *Pseudomonas* aeruginosa, anionic compounds did not show positive results for PDT inactivation, even using high light intensities [8,11,18]. Low molecular weight, chain length and charge of the PS can determine the efficiency of the membranes permeability in different species or strains of Gram (-), because these parameters makes easier the input of the PS across the membrane. On the other hand, these characteristics may be unnecessary or even detrimental in Gram (+) [21,23-25].

Polycationic PS have a dual action: first can bind to the anionic regions of the lipopolysaccharides, destroying them and disorganizing the outer membrane structure resulting in the formation of channels which increases its permeability and then photosensitises cells, after cross the outer membrane of Gram (-), process known as self-promoted uptake pathway [4,15,17,18,21,23,25]. Thus, in the presence of this type of PS, Gram (-) do not require membrane permeabilizing agents (e.g. $CaCl_2$, EDTA or polymixin B nonapeptide) which facilitates the photosensitization process and fostered the PDT studies in these bacteria. On the contrary, some studies indicate that the PS does not need to interact with the membrane because the $^1O_2$ production in its vicinity allows the diffusion across the membrane into the cell [4,11,18,21].

1.1.4 Photosensitisers

The majority of the first PS used in cancer PDT are derivatives of hematoporphyrin, a synthetic porphyrin synthesized from heme. Much of PS evolution resulted from the clinical need to improve their action and to reduce side effects. As it was observed significant side effects in the tissues, it was necessary to develop new PS which absorb at higher wavelengths in order to reach the deepest parts of the tissue for greater PDT efficiency and selective localization [1,2,5,7].

Some of these new compounds are phthalocyanines, meso-tetra(hydroxyphenyl) porphyrins, 5-aminolevulinic acid (ALA, a prodrug), tin ethyl etiopurpurin, texaphyrins, benzoporphyrin derivative monoacid ring A and *N*-aspartyl chlorin e6 [2].

In addition to dose and intensity of light, the efficacy of PDT also depends on the chemical structure of PS, its binding ability and *in vitro* studies of target cells. An important aspect for the development of this therapy is to understand how PS operate in microbial cells in order to construct more suitable PS molecules [4,11,18].

PS molecular structure is an important factor for the success of PDT, as it uses the energy of radiation to yield useful energy [1,11,18,25]. Thus, some ideal features of these compounds can be highlighted in particular [3,5-7,10,11,15,18,20,25]:

- High chemical purity and easy synthesis
- Non toxic in absence of light

- Photostability
- Soluble (should not aggregate or precipitate in biological systems)
- Positive charge and symmetry
- Amphiphilic molecules to facilitate its penetration and distribution
- High yield production of ROS
- Not require high cost for its activation
- High absorption peak in the visible range
- Nonmutagenic and non-genotoxic
- Selectivity for the target cells
- Be easy to deliver into the specific infection site
- Broad spectrum of action

Therefore, a PS can be designed in accordance with certain biological parameters or to produce certain ROS in order to make the process more suitable, particularly to achieve more easily the microorganisms [7,12,26]. The kinetics of a PS depends on its water solubility and positive charge (cationic), because these features allow an easy interaction with Gram (+) and have a certain interaction with Gram (-) which have low affinity for anionic or neutral lipophilic molecules/PS [5,7,10,18,23.25,26,28]. Hydrophobic compounds usually require delivery vehicles to target cells. Although, the anionic PS are not used, because they have a low interaction with Gram (-), they may still be effective in higher concentrations or through increasing the permeability of the outer membrane [1,5,7,11,15,20,23,26].

Some PS can be easily prepared by partial syntheses from abundant natural materials, such as heme, chlorophyll and bacteriochlorophyll. This route leads to both economical and environmental advantages compared to complicated total chemical synthesis [3,4].

PS may bind to surface cell, but generally antibacterial photoinactivation occurs by permeabilization of the cell membrane, due the reactive species produced by unbound PS. Upon entering, the PS can accumulate and prevent penetration of light, which can lead to the self-shielding effect. Additionally, may occur its self-destruction due to reactions with other molecules, including ROS, which is called photobleaching [3,5,9].

The PS toxicity depends on its chemical properties, formulation, concentration, microenvironment of activity and also the phenotype of the target cells. In addition, the PS

molecule can be directed to certain membrane enzymes or even to specific cellular compartments within the cell [1,5,11,23].

Most PS are porphyrins and chlorins, these can be used as free-bases or coordinated with a metal in the inner core of the macrocycle; the structure of these macrocycles are similar to protoporphyrin IX, the ligand of the heme group and to chlorophylls [1,2,18]. The PS are in general classified as first, second or third generation. The first generation is related to derivatives of hematoporphyrin (HpD), namely Photofrin which was the first sensitizing drug molecules used in PDT. Additionally, this complex misture of porphyrins has been widely used to treat cancers on an investigational basis. In the 90s appears the so-called second generation of PS in order to overcome some of the limitations of the first generation. An important goal was to obtain pure compounds with optimized photophysical properties. The most widely studied compounds are porphyrins, in particular chlorins, bacteriochlorins and phthalocyanines [1,5,20,28,29]. Some of this second generation of PS are substituted in the *meso* positions (5, 10, 15 and 20) with phenyl groups bearing halogens or other bulky groups. The majority absorb light at the longer wavelengths and exhibit maximum absorption peak in the red wavelength, so may be used in lower concentrations [5,13,20,29,30]. One of the main features which make them good compounds for PDT is their high efficiency to produce $^1O_2$; in general this efficiency is accompained by high stability and no toxicity in the absence of light [4,5,18,29]. Then a third-generation of PS has been developed in order to direct and increase the PS affinity to specific targets and in particular to certain cellular compartments. For example, some of these PS may be coupled to antibodies to direct their action in cellular tumor tissues [29].

Some PS can be synthetized in order to be positively charged, as it is the case of some porphyrins allowing them to interact and inactivate both Gram (+) and Gram (-) [11,18,25,31].

As porphyrins have a reduced or lipophilic state, their passage through the barriers becomes easier, allowing a better antimicrobial action against Gram (-). So, positively charged porphyrins are generally more efficient and can act at lower concentrations than neutral and anionic PS [8,11,15,25]. The number and the arrangement of positive charges, and the *meso* substituent groups on the structure of porphyrin appear to have different effects on the photoinactivation of Gram (+) and Gram (-) bacteria. According to some studies, *meso*-substituted cationic porphyrins and phthalocyanines are effective in the photodynamic

inactivation of both bactéria [9,11,18,31]. Several studies demonstrate a high rate of bacterial inactivation with tri- and tetracationic porphyrinic PS compared with di- and monocationic molecules [9,25].

1.1.5 Light sources

A very wide variety of coherent and non-coherent light sources have been tested to inactivate microorganisms, ranging from basic tungsten-filament lamps to lasers. Conventional lamps (noncoherent lights) were the first light sources used in PDT and the use of filters allowed to select a specific wavelength. Though these lamps are easy to use and relatively cheap, do not allow control the light dose to be applied and causes a significant thermal increase. Due to these limitations, the lasers (coherent light) were developed and currently are the light sources commonly used in PDT because produce a monochromatic light (exact wavelength), the light dose (product of intensity and the duration of exposure) to be administered is easy to calculate and the light transmited by an optical fiber can be used for localized treatment [1-3,5-7,11,28].

The radiations used in PDT are from the visible and near-infrared regions, because have higher penetration potencial in tissues compared to blue light. The same light dose can be achieved by varying the light irradiance, the irradiation time or both. Even so, the emission spectrum of the light source must cover all the PS absorption spectrum or at least some absorption bands. It is also important to emphasize that the wavelength must match with the selected PS because the yield of ROS will be maximized. Thus, the efficacy of PDT depends on the type and duration of irradiation and the irradiated light dose. To avoid excess heating, the irradiation power should not exceed 200 mW cm^{-2}. PS mechanism of cell inactivation depends on the pre-illumination time. In the case of microorganisms, the greater the intensity and irradiation time, better efficacy is verified, allowing the use of less efficient PS or even lower concentrations [1,4,5,11].

An important factor in this therapy is to understand how the type of radiation can interact with tissues to determine possible side effects. Thus the most important components in the effectiveness of this process are the wavelength and the biological material. Additionally, the direction of the light is also affected by the inhomogeneity of the cells, in particular, the presence of organelles, macromolecules, also interstitial layers in fungi, etc.

This is very important because the directionality is a crucial factor in the ability of light penetration [1,3].

The use of sunlight during the photoinactivation process is a good choice, especially for environmental applications, because porphyrins absorb in the visible range and it is a way to make the protocol more easily applicable and to be a cheaper technique. Sunlight has been used in order to inactivate microorganisms, due to the synergistic effect of the ultravioleta and infrared parts of sunlight [4,11].

1.1.6 Oxygen

Oxygen is a key element in the PDT because it produces reactive species, particularly $^1O_2$ that is very important for the photodynamic process due to ability to oxidize many key organic functional groups of the cell [1,7,12,17,18]. $^1O_2$ has a very short life time in the order of 600 ns 3 μS (3 - 4 μs in water), corresponding to an action radius of approximately 100 nm (1 μm in water and 50 nm in protein-rich lipid layers). However, it is noteworthy that this occurs in proximity of the PS and its performance depends on the PS, target cell and environment in which it is produced [1,5,13,16,18].

1.1.7 Benefits and limitations of aPDT

Efficiency of aPDT is directly related with the ability of the PS to generate $^1O_2$ (type II mechanism) and/or free radicals (type I mechanism) during the photodynamic process. Therefore, the kind of PS (monomers, dimers or higher order aggregates) is an important factor for the success of PDT, as it ensures greater efficiency in the photoinactivation [12,25,26]. *In situ* and *in vivo* assays show that negative effects of PS on host cells do not occur when are used micromolar concentrations, moreover these concentrations are effective to microbial inactivation. Additionally, the recovery and reutilization of the sensitizer molecules is also an importante factor, because becomes the process cheaper, easy to apply and environmental-friendly [11,18,20,32].

Some PDT advantages that merit to be highlighted are: the possibility of being applicable to a wide range of microorganisms; to be an effective antimicrobial treatment where the efficiency is independent of the antibiotic resistance pattern; to have a short period

of inactivation and no photoresistance or mutagenicity is developed, even after multiple treatments [9,11,20].

It is believed that side effects and cytotoxicity of PDT should essentially be related with the amount and distribution of the PS (mainly found in the plasma membrane, mitochondria, nuclei and lysosomes), the intensity of light and oxygen availability. However, one of the limitations is the fact that it is a localized process and so this treatment can only be applied to surface areas. Additionally, it was found that the microbial photoinactivation is more effective in the absence of organic matter, but this problem can be overcomed by increasing moderately the PS concentrations [2,5,11,18].

The food industry stands out as a very important area where PDT can be used, because the number of microbial food-borne disease has been increasing which represents great losses of productivity and consequently high cost. The main inactivation techniques are based on traditional thermal therapies and although effective, the approach can trigger reactions in food matrix (texture, physical appearance, functionality and organoleptic properties) and can even lead to nutritional losses [4,20].

So, some properties and the the quality of food can be affected by the conventional inactivation. The photosensitization technique might open a new way for the development of nonthermal, effective and ecologically friendly antimicrobial technology, which might be applied for food safety. Since the photosensitization does not require high intensities, the preservation of sensory properties, functional and nutritional food is higher, however, the superficial action is the main limitation of this technique [4,20].

Additionally, the diversity of possible applications that have been proposed in recent years, as well as the progress made in this research area demonstrate that photoinativation is a promising method of disinfection/sterilization with promising practical application in the short term [4].

1.2 *Pseudomonas syringae*

Pseudomonas syringae is the main phytopathogenic species of *Pseudomonas* genus, while others are beneficial to plants because they can produce phytohormones, or do not have any interaction [36]. This species has very distinct populations due to selective pressure

and adaptations to various environments to which it is exposed, thus allowing the emergence of new strains or the increased virulence in some of them [33,34,35]. Contrary to most *Pseudomonas*, *P. syringae* are unable to produce fluorescence and this is an important feature that can distinguish *P. syringae* in this genus [34,36,37].

P. syringae was first identified in lilac plant, however, have been identified about 60 pathovars in different hosts. Generally, each host has a specific pathovar, but some pathovars have the ability to infect a restricted range of taxonomically related plants. Thus, the classification becomes difficult, but ordinarily the name of each patovar is given according to the host in which it was first isolated [33,34].

Although *P. syringae* is considered an ubiquitous bacterium, since intervenes in the water cycle and thus reaches different places through the rain and snow, agricultural environments are the most common habitat of this species. Thus, it can be said that *P. syringae* colonizes mainly high humidity locals. It is also noted that this bacterium seems to have an important role in the water cycle due to ice nucleation capability (Figure 3). Another important feature of this bacterium is the ability to modify and adapt the structure of the population in order to ensure its survival, especially in high-altitude environments and low temperatures [33,35].

1.2.1 *Pseudomonas syringae* pv. *actinidiae*

Pseudomonas syringae pv. actinidiae (*Psa*) is Gram (-), strictly aerobic, non-spore forming, presents pectolytic activity, may occur in a single cell, in pairs or short chains, has mobility due to the presence 1 to 3 polar flagella and has a DNA 58.5 -58.8 mol% GC [37,38]. *Psa* has the ability to resist to antibiotics, nitric oxide plant origin, and has high capacity to capture iron and catabolize aromatic compounds from plants, such as observed with other phytopathogenics [39,40]. One way to distinguish the *Psa* from other bacteria in the *Pseudomonas* genus is the evaluation of the biochemical characteristics and some metabolic reactions as it is described in Table 1 [34,36-38,41,42].

Table 1- Negative and positive reactions of *Psa*.

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

Psa is considered an emerging invasive pathogenic bacteria of the *Actinidia* genus plants and presents no risk to other plants, animals or even humans [38,43-47]. The disease caused by *Psa* is a bacterial canker of the kiwi plant. Initially, can colonize the surface of the plant without causing significant infections (latent infection), but upon entering the plant can cause severe damage and even death [38,44,47,48].

1.2.1.1 Structure and growth condictions

Lower temperatures (10 to 20 °C) increases the invasiveness of the bacteria that withstands up to about 25 °C, however, above this limit their growth is compromised. Its optimum temperature is at 15 °C (± 3 °C). However, recent studies in Europe (France, Portugal and Spain) show that *Psa* can grow above 25 °C, but their capacity of infection is lower [38,46,47]. *Psa* takes place mainly in two stages (spring and fall/winter). In the spring, affects the development of the flowers, leaves and stems, and fall/winter may damage trunks and stems hibernation. It is in this last stage that the disease tends to worsen due to cold and humid climate that provides ideal conditions for the growth of the bacteria [38,47,48].

Psa has a regulatory system called plant associated bacteria, its mechanisms are still unclear, but probably the plant signals are responsible for the virulence and consequently growth and persistence of the bacteria in the plant [43]. An important virulence factor of the *Psa* is phaseolotoxin, which seems to reduce or inhibit the growth of other microorganisms. It was also found that *Psa* has genes that allow higher robustness to antibiotics, siderophytes, multiple genes and genes involved in the degradation of lignin and other phenolic compounds. However, its virulence can be significantly changed by loss or gain of genetic

mobile elements, which promotes diversity thus ensuring adaptation to new environments [37,39].

In symbiosis with certain bacteria, *Psa* increases its infection capacity, particularly with *P. syringae pv. syringae* and *P. syringae pv. viridiflava* [49]. But recently it was discovered a natural pathogen, *Bacillus amyloliquefaciens*, especially D747 strain, which seem to be useful in the biological control biological control, when is applied by Bacstar™ spray. Using *B. amyloliquefaciens* for spraying *Actinidia* plantations infected with *Psa*-V (highly virulent strain) and analyzing its leaves and flowers, it was observed high concentrations of *B. amyloliquefaciens* D747 and a large *Psa* reduction [38,49,51].

1.2.1.2 Patogenicity

Plants tumors are different from those that occur in animal tissues, since excessive cell proliferation occurs by the bacterial effect and thus it is also called bacteriosis. These tummors usually occur on surface vegetable tissue, especially into the trunks or roots of some plants, including rhizocarpics [35,52].

Study of phytopathogenic species is very important because it allows to evaluate or even to prevent the virulence, controling potential epidemics. In agricultural context, study of phytopathogenic microorganisms is essential and must be made in detail to determine the mechanisms that affect immune competence of the plants and their survival. Therefore, it is necessary to develop appropriate cultivation practices [35].

In 2009, the European and Mediterranean Plant Protection Organization (EPPO) included *Psa* disease in A2 alert list and recognizes *Psa* as a harmful organism for *Actinidia* plants; a list of pest recommended for regulation as quarantine organisms [62,63].

Two *Psa* strains were identified in New Zealand, a highly virulent (*Psa*-V) and another low virulent (*Psa*-LV). However, it was agreed that *Psa* designates the most virulent strain, as it is the strain that has become a constant concern in the kiwis production. Nevertheless, it is believed that *Psa*-LV is common on orchards, but possibly symptoms do not affect plants significantly [44]. Additionally, from some genetic and molecular studies it was found that *Psa*-V interferes with nitric oxide metabolism, that is an important factor for the immune

system of plants against potencial diseases. Furthermore, *Psa* encodes proteins that act in the woody tissue of the host, causing wilting and even death of the plant [43,45].

Additionally, *Psa* may be classified in four populations (*Psa* 1, *Psa* 2, *Psa* 3 and *Psa* 4) and its prevalence can be observed in diferente producers kiwifruit countries (Table 2). It is important to note that each population originates different levels of infection and consequently leads to different economic losses. The *Psa* 3 population (*Psa*-V) is considered the most virulent and is established in several countries, mainly in Chile, China, Spain, France, Italy, Portugal and New Zealand [62].

Table 2 - Classification of *Psa* populations and their geographical distribution and virulence [62].

Biovar	Localization	Virulence
Psa1	Japan, Italy (1992)	Moderate High
Psa2	Korea	Moderate
Psa3	Chile, China, Spain, France, Italy, Portugal, New Zealand	High
Psa4	Australia, New Zealand, France	Low

1.3 *Actinidia deliciosa*

Actinidia derived from the term "Aktec" meaning radius. This designation is due to the fact that the plant has various divisions radiating the stylet and ends in the pistil, characteristic that persists during the formation of the fruit [55].

The *Actinidia* genus has about 60 species of high variability. The features that most characterize each species are appearance of buds, stems, leaves, flowers and fruits. Some species produce edible fruits of different colors and sizes, while others are used only as ornamental plants, since they have a visually attractive foliage. All species are perennial and most deciduous, except the plants of warm climates [53,54,55].

Actinidia deliciosa is a climbing plant, deciduous, can reach 9 metres high and produces fruits called kiwis. The optimal altitude for natural growth of the plant is around 600 to 2000 meters, particularly mountain areas, moist, shady, ditches, along the creeks on

the ends of forests and even gaps. It should be noted that these specific factors of each plant are crucial to determine their climatic requirements of cultivation [44,47,54,56].

Actinidia deliciosa has a period of dormancy during the winter, in which the plant stagnates, loses its leaves and kiwis are harvested. Plant only returns to its growth in the spring/summer and starts budding, flowering and pollination processes [57,58]. The ideal vine growth occurs in deep and firm soils, rich in organic matter content, slightly acidic (pH of 5 - 6.5) and with sandy and wet consistency. Sometimes it is necessary to adjust the nutrient levels and accordingly fertilizers are used to improve soil characteristics and also help in growth plant. So, during growth, *Actinidia deliciosa* requires large amouts of water, especially in the summer. Sometimes it may even be necessary to use irrigation systems, but the soil must be well drained because too much moisture can make them unsuitable for development of kiwi plants [55,56,59]. Young vines need high humidity and shade conditions and generally die at temperatures below -1°C. Additionally, their growth is easily affected by spring frosts, which combined with the low percentage of sunlight can reduce or even inhibit fruiting. On the other hand, the mature vines can tolerate temperatures below -12 °C and severe winter frosts. However, autumn frosts can substantially hamper development of the plant and the own fruit, since they can delay or even inhibit the growth of the flowers. If frosts occur after the blossoming, the fruit growth is affected [54,55,56,59].

A. deliciosa has female and male plants and both produce pollen, but only the male pollen is feasible. However, female plants have very attractive styles to facilitate pollination [53,56]. Additionally, studies at the Royal Horticultural Societ (1909), verified that the fruiting requires simultaneous cultivation of both sexes. In commercial plantations, males should be spread evenly across the ground in the ratio from 1 male/8-9 female (10 to 12% of males) and for the process of pollination to be successful it is necessary to combine the stages pollination of both sexes [54,56].

Generally, vines just bear fruit 3 to 4 years after planting, and these must be early harvested to allow ripening outside of the vine. Some strains produce small bunches 3 to 5 fruits while others originate larger clusters which can contain up to 30 fruits [44,53,55]. The most common fruits have an oval structure with brownish skin, covered in short and brown hair over a pulp that can assume different colors (green, yellow, red or even whitish) according to the strain. In turn, the pulp has small black seeds that are edible. The ripe fruit

are very juicy and have a characteristic flavor (acid or lightly acid), but some varieties can be fibrous in the middle. The kiwifruit has several benefits for health because is considered one of the most nutritious fruit (high vitamin C contente, twice more than the orange, and many other nutrients and antioxidants) and may even enhance the immune response [44,56,60].

A. deliciosa is a China native plant, where is called 'yao tao' (Chinese strawberry) and grew naturally. The cultivation for commercial purposes started only after the plant was brought to New Zealand, where came to be called the kiwi to facilitate trade. This name was made official in 1974 and to date has been used this designation. In 1986 were distinguished two strains according to the different characteristics of the fruit. So it was appointed *A. deliciosa* for the kiwifruit hair and *Actinidia chinensis* for smooth skin kiwi [53,55,56,60].

The kiwi seeds were brought from China to New Zealand in 1904, but only in 1910 vines produced fruit for the first time. From these first crossing plants, progeny resulted in a high variety of species, which were selected with the best characteristics in order to produce fruits with the desired and most appreciated features in the market. This way it began the spread and commercialization of this variety of kiwis [53,55,56,60].

In 1925, Hayward Wright, a New Zealander horticulturist produced a species of kiwi from *A. deliciosa* with green pulp and it was very successful, since it is considered by many the tastiest, besides being larger and look better in relation to other kiwis. This was designated kiwi 'Hayward' and even today is the best known and consumed throughout the world. In 1953, production increased significantly, began the first exports and thus New Zealand has become the world's leading producer [53,54,60].

1.3.1 Industry and internacional trade of kiwi

The overall production of kiwifruit exceeds the production of other kinds of fruit. The success of kiwifruit may have been driven largely by the insistence of New Zealand innovation in the marketing and fruit cultivation processes in the country. Over time, the kiwi stood out in the industry worldwide and in some countries is a very important crop in the international market level. New Zealand was the country most developed in the production and marketing of kiwi, revealing a great climate adaptation and more than 90% of kiwifruit production is for export. ZESPRI is the main company in New Zealand kiwifruit

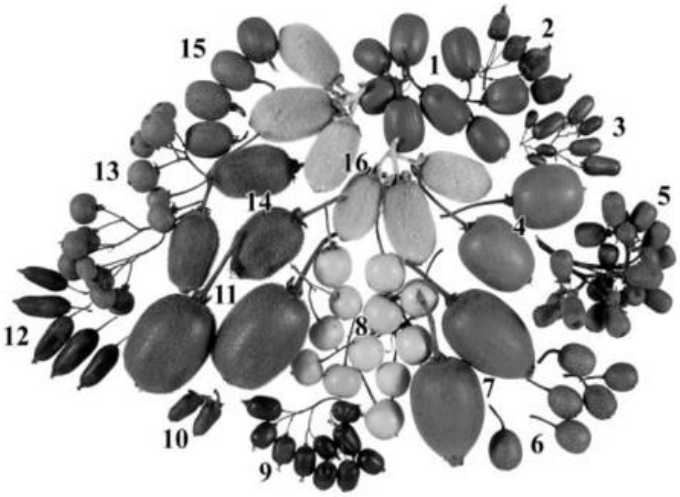
marketing [53,54,55,56,60]. Initially, Hayward was the main cultivated variety and its export has increased so much that it became one of the most important products in the New Zealand economy, accounting for 60% of total exports of fruits. Thus, other countries have also chosen to market this variety, to be the most popular among consumers due to its attractive features (large size and better appearance, and is considered by many the tastiest). Still it has a top quality conservation to other varieties, thus favoring exports over long distances. For these reasons, the introduction of new varieties on the market becomes more difficult [53,55,60].

Italy, New Zealand and Chile are among the leading producers of kiwifruit for many years. Between 1993 to 2011, the principal world producers do not change significantly, but can stand out the big ascent of China that became the leading trade in 2008 (Table 3). Although China is the origin country of kiwi, its commercial cultivation was uncommon and most of the consumed fruits came from wild vines. Its commercial production has been growing substantially, however, the most trade still takes place in the internal market [53,54,55,60].

Table 3 - Top 10 of the main kiwi-producing countries and their production volumes in metric tons. These values refer to different periods (1993 - 1995; 2003 - 2005; 2008 to 2010) [60].

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

Besides *A. deliciosa*, the most commercialized species, are *A. chinensis*, *Actinidia arguta* and *Actinidia kolomickta*, since they produce edible fruit (Figure 4). Currently there are several other species of distinct features, some do not produce edible fruits or have unpleasant taste and therefore can be used as ornamental plants or as rootstocks [53,55,56,64].



1 *A. rufa*	5 *A. latifolia*	9 *A. arguta*	13 *A. guilinensis*
2 *A. melanandra*	6 *A. indochinensis*	10 *A. fulvicoma*	14 *A. setosa*
3 *A. glaucophylla*	7 *A. chinensis* 'Hort16A'	11 *A. deliciosa* 'Hayward'	15 *A. chrysantha*
4 *A. chinensis*	8 *A. macrosperma*	12 *A. arguta* var. *purpurea*	16 *A. eriantha*

Figure 3 - Fruit diversity in *Actinidia* genus and comparasion between species [53].

Due to its characteristics and the high demand, kiwi Hayward became the standard of the kiwifruit, leading to an exponential increase in its production and marketing. Nevertheless, new varieties of fruits are being created, trough the selection and combination of different plants features. So currently fruits vary significantly in size, shape, color, flavor, aroma and even in the absence of hair. The color may vary both in the shell (green and brown), such as in pulp (yellow and green to red). *A. chinensis* is most similar to the kiwi Hayward variety and for a time were considered the same species, although botanically are different [54,56]. Besides the kiwi Hayard, other varieties of *A. deliciosa* are produced both pistillate (female or producers) or staminate (male or pollinators). Thus, female are Abbott, Allison, Bruno, Monty and male Matua, Tomuri and Chieftain [64].

In the late 70s, New Zealand stands out from its main market competitor (Chile) which offers lower prices, studied new ways to produce new varieties and this process resulted in the Kiwi 'Gold Hort16A' (commercial name: ZESPRI™ GOLD). This was registered in 1991 and has a beaked, many hair (very thin and easily removable), bright yellow squash and commercially very attractive flavor. In May 2014, it was discovered a new variety resulting from Hort16A variety, the kiwi 'Gold3', which is tolerant to *Psa* [54,61,62]. Nowadays, about 10% of the total area is reserved for new cultivar *A. chinensis* Hortl6A and

its orchards are increasingly diverse. The Hort16A is the second most internationally traded variety [54,64].

One of the most cultivation methods used in Kiwi culture is grafting and in order to increase its output must be made an annual pruning, especially in winter to remove the old shoots. Another essential factor is pollination, which must be enhanced through strategically placed beehives by plantations, because the natural pollination and wind are not sufficient for this purpose. Seeds cultivation produces highly variable offspring, so this kind of crossing should be avoided when it is intended ensure the same progeny characteristics, unless a selection is made of the kiwifruit [44,55,56].

1.3.2 *Psa* bacteriosis in kiwi

The kiwifruit cultivation is a difficult and lengthy process and in appropriate conditions, *Psa* can significantly reduce the yield and cause severe damage in plant, thus resulting in major economic losses [38,55,56,62]. *Psa* was first isolated and described in Asia (China, Japan, Korea) in 1980. Then, it was found in many other parts of the world, such as Italy, France, New Zealand, Chile (large producers of kiwis), in the most frequently cultivated species (*A. deliciosa* and *A. chinensis*) [65]. This disease has been responsible for significant economic losses in France, Spain, Portugal, Chile, South Korea and Japan, but mainly in Italy and New Zealand where the kiwi production is very importante [38,55,56,62].

Actinidia deliciosa have an intensive transport system that facilitates the movement of substrates and excretion products in and out of the cell, respectively, the *Psa* exhibits high growth ability during the various stages of growth plant [34,38]. *Psa* can be considered epiphytic, if grow in the plant surface, where shows little danger or endophyte when it reaches the internal tissues causing serious infections [46,58]. Once the optimal growth of bacteria occurs in cold and moist climates, autumn and spring are critical times for plants. Additionally, excessively low temperatures and wind factors can break the stems and branches of vines, causing stress in plants thus hindering its growth and immune response [47,60]. Although the plants are affected regardless of gender, female plant develops symptoms faster than males and infection is more severe. Relatively to age, the young plants are more susceptible to infections than mature plants, especially those with less than five years [35,39].

The main species affected are *A. chinensis* and *A. deliciosa*, but were also detected infections in wild species, *A. arguta* and *A. kolomikta*. However, Italian researchs verified that the species *A. chinensis* seems more susceptible than *A. delicious* cv. Hayward. In France, the two species were equally affected [35,37,38].

According to some studies, the European regions reveal high genetic homogeneity for *Psa*, which indicates that it has a common initial focus and probably was quickly disseminated by the marketing of infected plants. Moreover, improper pruning practice, eventually in combination with unfavorable environmental conditions (late frost, hail, high winds, etc.), probably played a central role in the outbreak of *Psa*. Similarly, the lack of control of this pathogen may also have been important in their dessiminação [65].

Psa generally enters in plant through natural openings or open injuries caused by insects or humans (e.g. pruning processes). Besides colonizing leaves and pollen, sometimes bacteria can settle in the rhizosphere. However, *Psa* has the ability to remain in a latent state until the conditions become optimal for their growth, in other words, *Psa* presence in the vines does not necessarily indicate development of symptoms. After this period, bacteria can enter in the vascular system and possibly develop a systemic infection [35,38,46,53,58,59]. *A. deliciosa* can also be affected by *Agrobacterium tumefaciens*, nematodes, worms, insects, moths and others, and in many different parts of plant. But generally, kiwi is not attacked by the fruit fly, because its hair skin act as a natural defense [56].

Psa transmission is still not fully clarified but it is thought that three main routes are: air (strong winds and heavy or persistent rain), and water (rain and irrigation) and soil. Other ways of transmission, includes contact with infected plant material (during grafting processes), possibly infected pollen, animals, humans (shoes) and equipment, vehicles and farming tools. The environmental factors may also promote infections, because can dispersing exudates by orchards, particularly in high concentrations of inoculum, or when create lesions in plants, that are a potential input focus of bacteria. Although the *Psa* can reach fruit surface, does not represent risk of infection when using seeds for cultivation, since the probability of bacteria achieving the seed is very small, however, can occur in highly contaminated orchards. Other risk factor is the commercialization of infected seedlings for cultivation, that can dissipate the infection between distant locations [35,38,46,53,58,59].

First symptoms are seen in the leaves, stems and buds, usually in the spring, and eventually may still arise small tumors. Winter and early spring are usually the most critical time, because that is when occur the most severe cancers in the trunks and branches, causing serious injuries and in more advanced infection cases some plants die after a few months. Generally female species have symptoms faster than male-plants and in some species leaves symptoms occur to a lesser extent [37,40,61]. The production of chlorotic lesions on leaves of genus *Actinidia* plants, can be the result of a phytotoxin similar to faseolotoxin, although this is not a requirement for the bacterium multiplication [66,67].

During the spring, leaves can develop small dark brown points that can have an angular form surrounded by a bright and yellow chlorotic halo not always visible. Sometimes, it can occur the coiling of the leaves and the release of colorless exudates through the stomata (Figure 4). Flower buds become brownish, shriveled and the heaviest buds fall, may even release colorless exudates (Figure 5). Infected sepals can darken and shoots usually shrivel, leading to fruits atrophy. The trunk of vines can release exudates white to dark red from lenticels (Figure 6) [35,37,38,44,46,58,59].

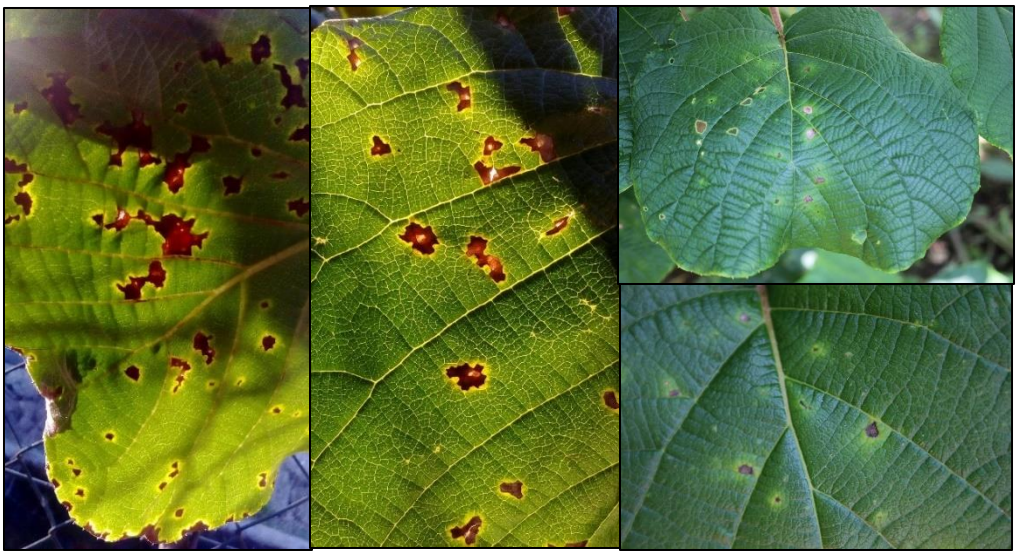


Figure 4 - Leaf dark spots with chlorotic halos in *Actinidia* plants (Images provided by APK).

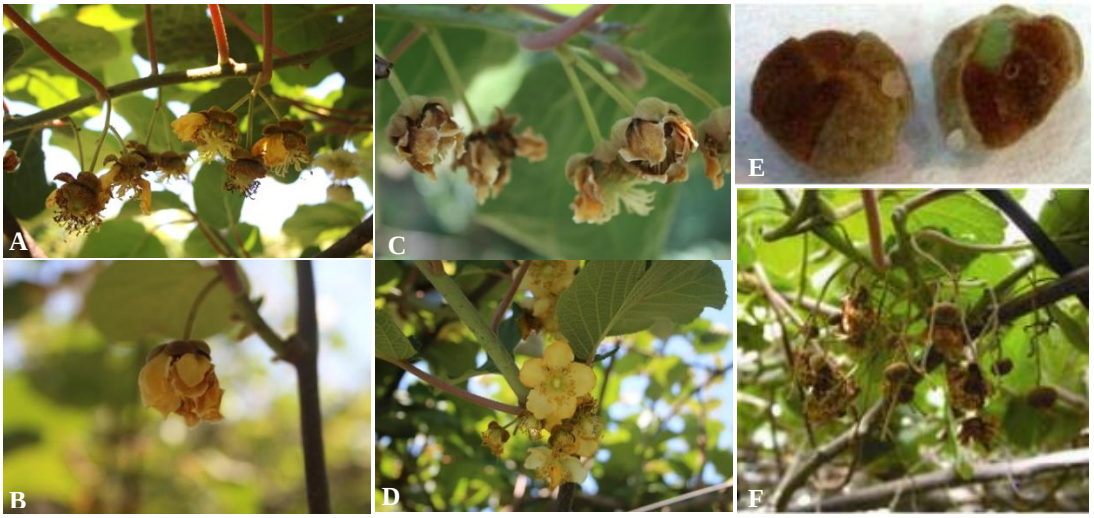


Figure 5 – *Psa* symptoms in vines. A, B, C e D) Browning of flower (Images provided by APK); E) Browning buds with exudate; F) Bud desiccation and necrosis [68].

By the middle of winter, it start secondary symptoms, which usually occur one year after leaves symptoms (primary symptoms). These appear on the trunk and branches, where we can observe the release of small droplets of exudate and at the end of the season, in more advanced stages of infection, the reddish-brown exudate is released in large quantities and can accumulate forming a ring (Figure 6). Also in this phase of infection, the exudate can destroy the woody tissue due to degradation of lignin and other phenolic compounds and thereby kill the vine. In infections with inoculum at high bacterial concentrations, exudates exhibit a whitish color [38,39,46,58,59,69].

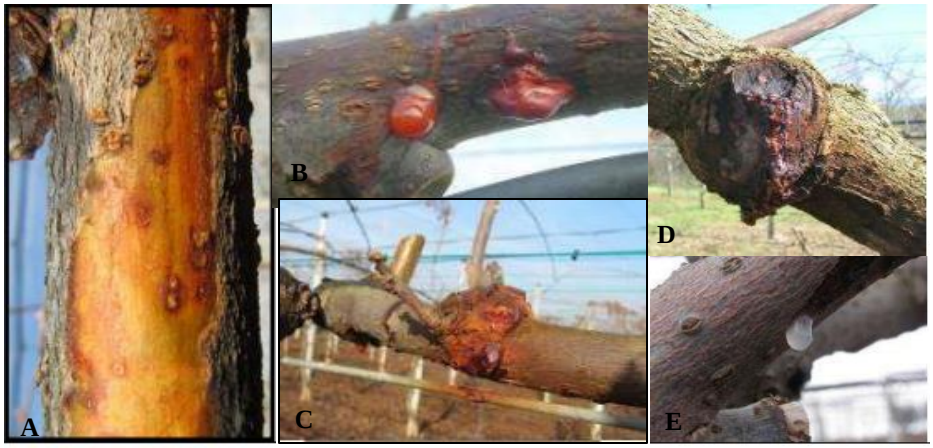


Figure 6 – Cancers and exudates released in infected vines. A) Reddening of lenticels; B) red exudate droplets; C and D) Truncks with accumulation of exudates released; E) White ooze exudating [67].

1.3.3 Preventive care of *Psa* in kiwi

Although the disease was already quite dissipated, the control means are still scarce, so preventive measures are the best way to prevent the development of the *Psa*. Thus, while there are no effective methods for treating *Psa* it is essential to identify the main foci of the disease and eliminate possible contaminations [70]. Some essential aspects for diagnosis and prevention of *Psa* are the monitoring of vines, working materials and implemented techniques, and this allows to identify and track potential risks. If the producer suspects that the plant is infected, he must identify it and take samples for laboratory studies. The results can be positive, undetectable or undefined. It is important to refer that a negative result does not mean that the bacterium is not present in plantation [59].

Protect the plant from a possible infection is essential because when it established a systemic infection, it is not possible to eliminate the bacteria. Thus, it is important to take some prophylactic measures to avoid or reduce the inoculum. Some of these measures include disinfection of agricultural tools (pruning and harvesting) and avoid grafting with possible contaminated material; maintaining hygiene from the orchard and equipments; wear protective systems against wind, rain and frosts; keep the water levels and proper nutrients for healthy plant growth and take care of the vines in order not to damage them. Another preventive measure may be the removal of potentially dangerous animals for the growth of young shoots, especially insects, birds, snails, slugs, lice, beetles and cicadas. Sometimes an early spraying with suitable compounds may be a good way to protect vines, however, should be avoided because it can be toxic for the plant [37,38,59].

The localization of the plant may be one way to prevent bacterial infection, if plants are stable and protected of stress conditions, because if are less susceptible to environmental factors, less damage occurs. On the other hand, plants can be replaced by more resistant strains. Moreover, it is necessary to provide a soil with a healthy biota, stimulating the beneficial microorganisms, providing appropriate proportions of water and if necessary, add suitable nutrients. It can still be used a pre-flowering trunk girdle, which consist in a deep cut from the bark to the phloem in order to avoid the bacteria dispersion throughout surface of the plant [59].

1.3.4 Available treatments

There are currently no curative treatments for *Psa*, and all existing treatments are preventive measures. These include common bactericides like copper-based agrochemicals, disinfectants or sterilants, and antibiotics. However, in European Union the use of antibiotics for control of plant pathogenic bacteria is restricted or ilegal [71-73]. For instance, streptomycin sprays were used in the past, but *Psa* resistance was already observed [37,71,74-76].

The spraying of copper-based bactericides is considered the most effective practice in protecting against *Psa* giving a cover on the surface of the plant, killing the bacteria on the surface of the leaf and preventing the bacteria from entering the vine. Copper compounds shoul be sprayed immediately after winter pruning, two and four weeks after bud break and in high risk situations like after a major wind, rain or hail event. Copper has minimal effect once the infection has occurred, so the emphasis is on obtaining uniform and complete coverage throughout the canopy. The efficacy of copper in plant protection can be considerably improved by the reducing the particle size of the spray, because more surface areas are available per gram of product to release copper ions when moisture is presente [71,72,74,77].

The effectiveness of the commercial copper compounds depends on the formulation and concentration of copper salts used. Ideally, copper on the leaf surface should be at a high enough concentration to kill the bacteria but low enough not to cause injury to the plant. Wettable powders and dispersible granules are considered to present less risk of phytotoxicity than liquid formulations and these would be the preferred copper product of choice. The aim is to keep the number of sprays to a minimum while giving the best cover possible, especially in times of high risk of infection [71,72,74].

Copper ions are essential for bacterial species, but can induce toxic cellular effects if levels of free ions are not controlled. The mechanisms up to now reported as at the basis of the antimicrobial properties of copper include its ability to perform oxidation-reduction reactions. Under aerobic conditions and via the Fenton and HaberWeiss reactions, this redox property enables copper to catalyze the production of $OH^{\bullet}$, originating damages to lipids, proteins and nucleic acids, enzyme catalytic sites, blocking of the energy transport system, disrupting the integrity of cell membranes [71,72].

Past experiences with copper-based sprays applied to kiwifruit orchards have shown occasional phytotoxic effects and the occurrence of copper-resistant bacterial, especially in asia. These plant injuries may arise due to time of application (cold and wet weather conditions), and the application of excessive rates of Cu (frequency of application). The repeated use of copper-based bactericides/fungicides to control horticulture plant diseases has led to long-term accumulation of Cu in the surface of some agricultural soils [71,74].

The cuprous oxide (Cu_2O) is the copper formulation more frequently used worldwide in kiwifruit cultivations. The main active compound of this copper-based product is Cu_2O (83.9%), equivalent to a metallic copper content of 75%, formulated in finely dispersible granules (WG), hollow, to increase the wetting, suspension, adhesion and persistence, allowing a more uniform distribution product formulated on the treated surfaces [77,78]. Its high efficacy is due to the adequate size of the particles, guarantee a greater uniformity in the distribution and cover of the plant surfaces and less quantity is needed to obtain the best results, besides it is the most economical [76,77]. However, the continued use of copper compounds, often several times throughout the year and also for many years, may result in many severe ecotoxicological effects, such as soil contamination, phytotoxicity, heavy negative impact on soil and epiphytic microflora, and in the development and spread of copper- and antibiotic-resistant bacteria in agroecosystems as well. In addition, heavy metals used in agriculture might promote the spread of antibiotic resistance via co-selection [71,80].

Some augmentative sprays may stimulate the immune system of the plant and consequently reduce *Psa* activity, however, it is necessary to maintain low inoculum levels, through of the orchards cleaning and use of other preventive sprays. The elimination of diseased vines or the affected part reduces the risk of spreading in the orchard. This is a good procedure to be adopt and should be performed as soon as possible to avoid the worsening of infection [38,59,67]. Since the most conventional techniques have not been successful with this bacterium, *Psa* has sparked too much interest. In this sense, numerous studies have emerged, essentially in the genetics and phylogenetics. As a result of these studies, the *Psa* taxonomy has been slightly amendments [38,41,69]. In future, the use of resistant plants and pollinators, genetic agents of biocontrol and compounds inducing the immune system of the plant are possible solutions for this infection [37].

1.4 Kiwi production in Portugal

The *Actinidia* plant was introduced in Portugal in 1973 by Ponciano Monteiro, a lawyer of Porto, which imported plants from France and cultivated them in his farm in Vilar de Andorinho, Vila Nova de Gaia, after tasting the fruit in a Paris restaurant. As the result was successful, new kiwifruit growers start to appear. During the 80's the production grew exponentially and in 1992 reached a planted area of 2,000 hectares [64].

Portugal was the 9th largest producer of kiwifruit between 1993 and 1995, but left the top 10 production shortly after, though internal production has increased slightly [62]. After 2002, the kiwifruit cultivation rose sharply, with a cultivation area that went from 700 to more than 1,300 hectars until 2012. Thus, Figure 7 shows the evolution of kiwifruit production in Portugal from 1987 to 2013, where some production losses during this period can be observed [65,81].

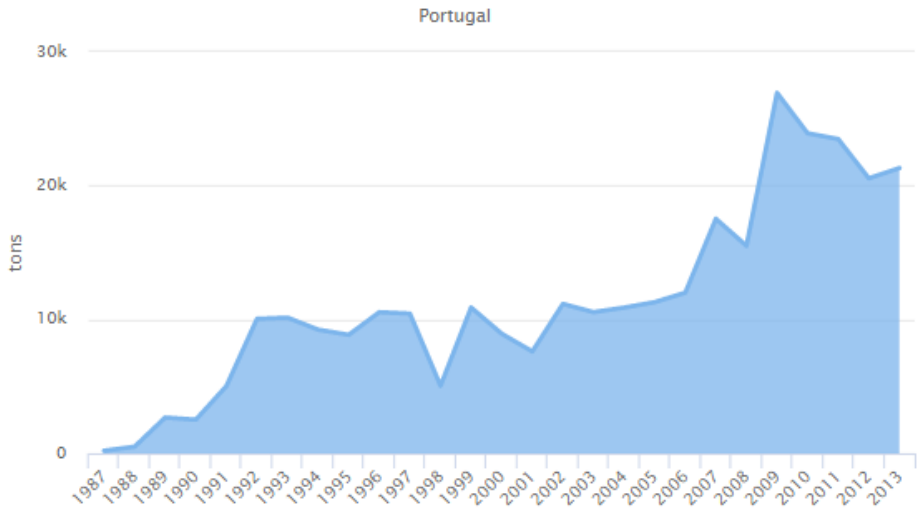


Figure 7 - Kiwifruit production growth in Portugal from 1987 to 2013 (x-axis) are quantity produced in tons (y-axis).

Regions with more impact on the kiwi production in Portugal are Entre Douro, Minho and Beira Litoral, as they have the right conditions for the proper development of the plant. This is an important alternative compared to other crops and a major source of income and regional development [62,64,82,83].

The main advantage of portuguese kiwi comes from the absence of autumn frosts, allowing a late harvest and consequently gives a sweeter taste. So, kiwi, can reach the international market successfully, although production is not sufficient to suppress the needs

of the internal market. It should be noted that the highest percentage of kiwi produced and commercialized in this country is the cultivar Hayward [64,82].

1.4.1 *Psa* in Portugal and adopted measures

The *Psa* was detected for the first time in Portugal in 2010, in the region of Entre Douro and Minho. According with the molecular studies it was found that the disease was due to *Psa*3, the most virulent strain. It is estimated that the incidence of the disease may have even reached 30% [42,62]. In 2012, it was confirmed that the *Psa* was present in the main areas of cultivation. In some cases, due to the high percentage of observed damage, destruction of orchards has been strongly advocated. Since the north of the country is the largest producer of kiwifuit, this is also the most affected by the *Psa*. However, the central zone has also revealed high levels of contamination in 2015, with the highest incidence in the coastal zone [42,63,65].

The main concern is the lack of ways to combat *Psa*, so producers are establishing preventive measures to avoid possible contamination. Therefore, it is necessary to supervise the orchards, to detect the first symptoms in order to identify the *Psa* as soon as possible and, finally, to take the appropriate procedures. In some cases of more serious infection, it may be necessary to destroy plants or plant material (burning or burial in deep trenches over 50 cm deep) [62].

For each inspection or harvest is necessary fill out a prospection form, which is included in Nacional Action Plan. Still, it is mandatory to make a record of the acquired plants and a history of the outputs of plant material (fruit and pruning wood). To alert and raise awareness for the National Action Plan, reporting actions were done, covering several themes, such as the symptoms of the disease, legislation, *Psa* laboratory analysis, the risk of infection and control strategies [63,83].

1.4.2 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 *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 [63,83].

In Annex 1, are listed some legislations applicable in Portugal [83].

1.5 Objectives and approaches

This work aims to develop a safe aPDT protocol for use in kiwi plantations to reduce the activity of the *Psa*.

In a first phase, a tetracationic PS was tested at different concentrations and light sources to check the best requirements to inactivate this bacterium in order to determine the appropriate conditions to apply the tecnhology *in vivo*. These experiments were done in buffered saline (PBS) under white light.

In a second phase, the developed protocol was tested in *ex vivo*, using kiwi leaves artificially contaminated with *P. syringae* under different light sources: low intensity white light (fluorescent lamps), high intensity white light (LumaCare lamp) and sunlight.

In a third phase, the efficacy of aPDT to inactivate *P. syringae* was compared to that of the tradicional approved method used in Portugal, Cu_2O at 50 g hL^{-1}. In addition, other lower Cu_2O concentrations were also tested in order to evaluate if the Cu_2O dose can be reduced. These experiments were done *in vitro*.

CHAPTER 2 – Photodynamic therapy in controlling the *Pseudomonas syringae* pv. *actinidiae* of kiwi

2.1 Introduction

Psa is the main phytopathogenic species of *Pseudomonas* genus, causing an infectious disease in *Actinidia* plants (kiwi plant), the *Psa* [36,37]. *Psa* is considered an emerging invasive pathogen of kiwi and presents no risk to other plants, animals or even humans. The *Psa* infection can cause cancer in kiwi plant. Initially, colonizes the surface of the plant without causing significant infections, but upon entering the plant can cause severe damage and even death [38,44,47,48].

The production of kiwifruit is an important industry worldwide and in some countries is a very important crop in the international market level, in particulary New Zeland, Italy and, Chile [53,54,55,56,60]. The kiwifruit cultivation is a lengthy process and in appropriate conditions, *Psa* can significantly reduce the yield and cause severe damage in plant, thus resulting in major economic losses around the world [38,55,56,62]. Autumn/winter and early spring are critical times for plants, because facilitates the growth of *Psa*, due the cool and moist climates and other climatic factors, like wind which can break stems and branches, hindering its growth and immune response, besides producing entry points for the bacteria [46,59]. During this time occur the most severe cancers, inicially with release of small droplets of exudate and at the end of the season, in more advanced stages of infection, the reddish-brown exudate is released in large quantities and can accumulate forming a ring. In these advanced infection cases, plants die after a few months [39,46,58,59,65].

Psa generally enters in plant through natural openings or open injuries caused by insects or humans (e.g. pruning processes), however has the ability to remain in a latent state until the conditions become optimal for their growth and consequently develop a systemic infection [35,38,54,58]. The first symptoms, usually occurs in leaves (small dark brown points surrounded by a bright and yellow chlorotic halo not always visible), stems and buds [39,46,58,59].

Some treatments have been used, in particular copper compounds which is the most frequently used, but has to be administered at the right time and in appropriate concentrations (50 and 35 g hL^{-1}), and is very toxic to the environment [58,71,72,73,84,85,86].

The Cu_2O is the copper formulation more frequently used worldwide in kiwifruit cultivations. The main active compound of this copper-based product is Cu_2O (83.9%),

equivalent to a metallic copper content of 75%, formulated in finely dispersible granules (WG), hollow, to increase the wetting, suspension, adhesion and persistence, allowing a more uniform distribution product formulated on the treated surfaces [77,78]. Its high efficacy is due to the adequate size of the particles, guarantee a greater uniformity in the distribution and cover of the plant surfaces and less quantity is needed to obtain the best results, besides it is the most economical [76,77]. However, the continued use of copper compounds, often several times throughout the year and also for many years, may result in many severe ecotoxicological effects, such as soil contamination, phytotoxicity, heavy negative impact on soil and epiphytic microflora, and in the development and spread of copper resistant bacteria in agroecosystems as well. In addition, heavy metals used in agriculture might promote the spread of antibiotic resistance via co-selection [71,80].

Streptomycin sprays can be also used, however, should be avoided because develops bacterial resistance [35,58,75]. Additionally, augmentative sprays (elicitor-based sprays, Spotless, Omiwett, BION®, SERENADE® MAX can also be used to stimulate the immune system of plant, but is necessary to maintain low inoculum levels, through of the orchards cleaning [35,57,87,88,89,90]. A good procedure to be adopted is the elimination of diseased vines or the affected part, that reduces the risk of spreading in the orchard [66,91].

As the use of antibiotics has led to increased resistance and the copper is highly toxic to the environment, new alternative approaches to control *Psa* in kiwifruit cultivation are necessary. aPDT has been shown to be an effective alternative cost-effective and environmentally friendly methodology to inactivate microorganisms [4,11,18,20]. This method shows to be effective against bacteria, including multidrug-resistant strains, fungi, protozoa, viruses, yeasts, spores and biofilms and until now the development of photoresistance or mutagenicity, even after multiple treatments was not detected [9,20].

aPDT uses a PS, a photoactivable compound by radiation (visible or ultraviolet light), in an appropriate wavelength that promotes electronic excitement of the oxygen molecules for an excited singlet state. Then, ROS are produced, especially singlet oxygen that has great cytotoxic to microorganisms [9,12,13,14,15]. These reactive molecules readily interact with biological componentes of external structures of microorganisms, proteins and lipids, and also with nucleic acid bases [1,3,4,5,7,18,19,98].

The objective of this work was to develop a safe protocol of aPDT to prevent or to treat the *Psa* in kiwi plantations.

2.2 Methods

To develop a safe an effective approach to inactivate the *Psa*, different conditions were tested. First, the tetracationic Tetra-Py^{+}-Me (5,10,15,20-tetrakis(1-methylpyridinium-4-yl) porphyrin tetra-iodide, a porphyrin already tested by the work group to inactivate efficiently Gram (-) bacteria) was tested *in vitro*, in PBS, at different intervals, at a low PS concentration, under low intensity artificial light (fluorescent lamps) to evaluate the best conditions to inactivate the bacterium [32]. In a second phase, the developed protocol was tested in *ex vivo*, using kiwi leaves artificially contaminated with *P. syringae*, using different concentrations of PS, under different light sources: low intensity white light (fluorescent lamps), high intensity white light (LumaCare lamp) and sunlight. The potential negative effects of aPDT on kiwi leave cells were also evaluated in ex vivo at different concentrations of PS and under different light sources.

The efficacy of aPDT inactivation was compared with that of copper *in vitro*. As in kiwifruit plantations copper is used at high concentrations (50 and 35 g hL^{-1}) and it is not possible to stop its action during bacterial incubation in culture media, the efficacy of copper inactivation of *Psa* was determined, the optical density (OD). As the color and tubidity of copper solution are high, the OD was only determined for a low concentration of a copper solution (0.5 g hL^{-1}). It was not possible to read the OD in more concentrated solutions of copper. In order to test the effect of copper at high concentrations other real time method was used, the bioluminescence. The bacterial bioluminescence method is considered to be a rapid, sensitive and cost-effective choice to monitor the antibacterial action of chemicalst/treatments, once the inactivation can be measured directly, continuously and in a nondestructively high-throughput screening. A strong correlation between bioluminescence and viable counts was demonstrated in experimental systems where the light output reflects the actual cells metabolic rate [12,25]. Thus, a recombinant bioluminescent *E. coli* transformed strain was used as a model of Gram (-) bacteria (e.g. Psa). The viability of *E. coli* in the presence of copper was done by measuring in real time the bioluminescence in a luminometer. Although *E. coli* as *Psa* is a Gram (-) and, consequently, its inactivation by

copper is similar to that of *Psa*, the inactivation of *E. coli* by copper was also evaluated by OD determination. Thus, the bacterial inactivation by higher concentration of copper solutions was evaluated using the bioluminescent *E. coli*. The color and turbidity of copper solutions do not affect the measurement of bioluminescence. The efficacy of copper to inactivate the *Psa* was also determine by a culture depend method, pour plating, for two low concentrations (5 and 15 g hL^{-1}).

The potential negative effects of aPDT and of copper on kiwi leave cells were also evaluated in *ex vivo* at different concentrations of PS and under different light sources.

2.2.1 Bacterial strain and growth conditions

A *Psa* strain (CRA-FRU 8:43) Lazio (2008) 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 24 h. Afterwards, an aliquot was transferred into fresh TSB incubated overnight at the same growth conditions to reach the early stationary phase (OD at 600 nm of 1.4 ± 0.1 corresponded to ≈ 10^8 CFU mL^{-1}.

2.2.2 Photosensitizer

The Tetra-Py^+-Me (Figure 8), a patented compound from our research group with a large spectrum of activity was used as PS [32]. The porphyrin purity was confirmed by thin layer chromatography and by ^1H NMR spectroscopy. A stock solution (500 µM) was prepared in dimethyl sulfoxide and sonicated for 30 min before use. Tetra-Py^+-Me in DMSO: λ_{max} (log ε) 425 (5.46), 516 (4.30), 550 (3.78), 588 (3.86), 644 (3.34) nm; Tri-Py^+-Me-PF in DMSO: λ_{max} (log ε) 422 (5.48), 485 (3.85), 513 (4.30), 545 (3.70), 640 (3.14) nm.

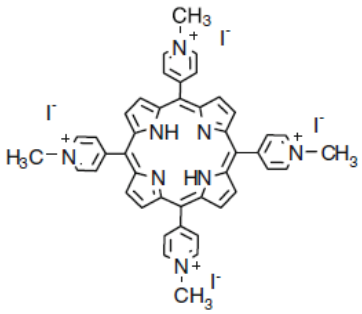


Figure 8 - Structural representation of the tetra cationic porphyrin (Tetra-Py^+-Me) used in the PDT assays [32].

2.2.2.1 *In vitro* photosensitization procedure

Bacterial suspensions (≈ 10^8 CFU mL^{-1}) were prepared, 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 litre; pH 7.4), from the early stationary phase cultures, distributed in 100 mL beakers (final volume of 10 mL per beaker), incubated in the dark with porphyrin for 10 min at 25 °C under 100 rpm stirring to promote the porphyrin binding to the cells, and then irradiated by artificial white light (PAR radiation, a system consisting in 13 parallel OSRAM 2' 18 W/840 lamps with an irradiance of 40 W m^{-2}, emitting in the range of 380–700 nm).

Bacterial suspensions, with 5.0 µM of PS, were irradiated up to 270 min (total light dose of 64.8 J cm^{-2}) and sub-samples of 1.0 mL were collected before irradiation and after 15, 30, 60, 90, 180 and 270 min of light exposure. After each photosensitization interval, the suspensions were serially diluted in PBS, plated in TSA and incubated at 25 °C for 48 h. The CFU were determined on the most appropriate dilution on the agar plates. 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. Three independent experiments were performed and, for each, two replicates were plated.

2.2.2.2 *Ex vivo* photosensitization procedure

The *ex vivo* assays were performed on kiwi leaves provided by Associação Portuguesa de Kiwicultores (APK), at 50 µM of PS, under diferent radiation conditions: low intensity white light, the same used *in vitro* assays (PAR radiation), high intensity white light, (with a fluence rate of 150 W m^{-2} from an interchangeable fiber optic probe (400–800 nm) coupled to a 250 W quartz/halogen lamp (LC-122; LumaCare, Newport Beach, CA, USA) and sunlight (650 W m^{-2}, sampling events were recorded at the meteorological station of the University of Aveiro, located near the laboratory where the experiments were done [93]. For PAR radiation two preincubation times in the dark were tested, 10 and 30 min. For the other two light sources, the dark preincubation was 30 min.

For each light intensity condition, twelve kiwi leaves were placed in petri dishes and an area of 16 cm^2 of each leaf was sprayed with 50 µl of fresh overnight bacterial suspension at ≈ 10^6 CFU mL^{-1} diluted in 450 µL of PBS. After, the contaminated area of each leave was sprayed with 50 µl of PS at 500 µM (final concentration 50 µM) diluted in 450 µl of PBS. Leaves were preincubated in the dark for 10 or 30 min and then subjected to irradiation. To maintain a humid atmosphere, plates with leaves were placed on a water bath closed with cling film. At time zero and after 30, 60 and 90 min. of irradiation, three leaves were withdrawn and each one was stirred in 20 ml of PBS for 30 min (130 rpm). Then serial dilutions were made from the suspention and the CFU were determined as described above. For each irradiation condition three independent experiments were performed (with three samples in each experiment) and, for each of the three samples, two replicates were plated.

Light and dark controls were carried out simultaneously to the sample procedure. The dark controls were prepared as described for the sample but were protected from light during irradiation. The light control was contamined with the bacterial suspention and after was spayed with 500 µL of PBS without the PS.

2.2.3 Inactivation of *Psa* by copper (cuprous oxide)

The Cu_2O tested in this work is the most concentrated copper fungicide product available (NORDOX Industries AS, Oslo). This copper-based product is formulated in finely dispersible granules (WG), hollow, to increase the wetting, suspension, adhesion and persistence, allowing a more uniform distribution product formulated on the treated surfaces [78,83]. The degree of fineness of the particles (1 to 5 µm, with 80% less than 1.8 ↕0m) causes greater adhesion, persistence and wash resistance by raining than other copper-based formulations. The adequate size of the particles, guarantee a greater uniformity in the distribution and cover of the plant surfaces and consequently a less quantity is needed to obtain the best results [70,76].

Cu_2O was prepared according to product instructions, in distilled water and under shaking. A stock solution of 500 g hL^{-1} was prepared and maintaine in the dark at room temperature.

2.2.3.1 Determination OD in *Psa* and *E. coli* cultures

Bacterial suspensions of *Psa* and of *E. coli* (≈ 10^8 CFU mL^{-1}) were prepared, in phosphate buffered saline, from the early stationary phase cultures, distributed in 100 mL beakers (final volume of 10 mL per beaker), incubated in the dark with Cu_2O solution at a final concentration of 0.5 g hL^{-1} at 25 °C under 100 rpm stirring. A control without Cu_2O was prepared and was incubated simultaneously to the samples. Aliquots of treated and control samples were collected at time 0, 5, 10, 15, 30, 60, 90, 150, 180, 240, 270, 300 and 330 and OD was read at 600 nm in UV-Visible spectrometer (Dynamica HALO DB - 20 UV-VIS). Three independent experiments were performed and for each sample three replicates were read.

2.2.3.2 Determination of bioluminescence in *E. coli* cultures

Bioluminescent *E. coli* suspensions (≈ 10^8 CFU mL^{-1}) were prepared from an overnight culture, diluted 1:10 in PBS and distributed in 100 mL beakers (final volume of 10 mL per beaker). Different concentrations of Cu_2O (5, 7.5, 10, 15, 20, 25, 35 and 50 g hl^{-1}) were added to the beakers which were incubated at 25 °C for 150 min under 100 rpm stirring. A control without Cu_2O was prepared and was incubated simultaneously to the samples. Aliquots of treated and control samples were collected at time 0, 5, 10, 15, 30 and from here, every 10 minutes and the bioluminescence was measured (relative light units (RLU) in the luminometer (GLOMAX 20/20 Luminometer, Promega). Three independent experiments were performed and for each sample three replicates were read.

2.2.3.3 Determination of colony forming units in *Psa*

Bacterial suspensions (≈10^8 CFU mL^{-1}) were prepared in PBS, from the early stationary phase cultures, distributed in 100 mL beakers (final volume of 10 mL per beaker), with Cu_2O at a final concentration of 5 and 15 g hL^{-1} under 100 rpm stirring at 25 °C. A control without Cu_2O was carried out simultaneously to the sample procedure. Sub-samples of 0.1 mL were collected at time 0 and after 10, 20 and 30 min of incubation. The suspensions were serially diluted in PBS, plated in TSA and incubated at 25 °C for 48 h. The CFU were

determined on the most appropriate dilution on the agar plates. Two independent experiments were performed and, for each, two replicates were plated.

2.2.4 Evaluation of the possible side effects in kiwi leaves after sensizitation procedure

In order to verify the possible side effects in kiwi leaves, a sensitization test was performed with different PS concentrations (5 μM, 20 μM, 50 μM and 100 μM) based in de Menezes et al (2014) [94]. In adaxial surface of each leaf four drops of 10 μl in the four aforementioned concentrations and a control drop (10 μl of PBS) were added. Leaves were placed under sunlight and observed for five days. The drops were added to mid-morning and in the next day at about the same time, new droplets with the same concentrations were placed on the location where the previous drop had been added. This procedure was carried out in this way for 5 days.

The same procedure was performed to verify the Cu_2O side effects on kiwi leaves. In this way, Cu_2O was added in adaxial surface of each leaf at concentrations 0.5 g hl^{-1}, 5 g hl^{-1}, 15 g hl^{-1}, 20 g hl^{-1}, 35 g hl^{-1} and 50 g hl^{-1}. A drop of PBS was added to leaf as a control.

2.2.5 Statisticals analysis

Statistical analyses were performed using GraphPad 6.01. Normal distributions were assessed by the Kolmogorov-Smirnov test. The significance of both irradiation time and porphyrin derivative on bacterial inactivation was assessed by two-way univariate analysis of variance (ANOVA) model with the Bonferroni *post-hoc* test. A value of $p < 0.05$ was considered significant.

2.3 Results

2.3.1 *In vitro* photosensitization

The resuts obtained from the *in vitro* PDT assays revealed that porphyrin Tetra-Py^{+}-Me at 5 µM was able to cause a bacterial decrease of about 6 log after 60 min of irradiation. A sharp decrease of *Psa*, from 7 to 4 log (≈ 3 log; ANOVA, $p < 0.05$) was observed, in the first 5 min of irradiation and the other 3 log (ANOVA, $p < 0.05$) decrease was observed after 60 mim of irradiation. After this period until 270 min of irradiation, the bacteria concentration remained constant (Figure 9).

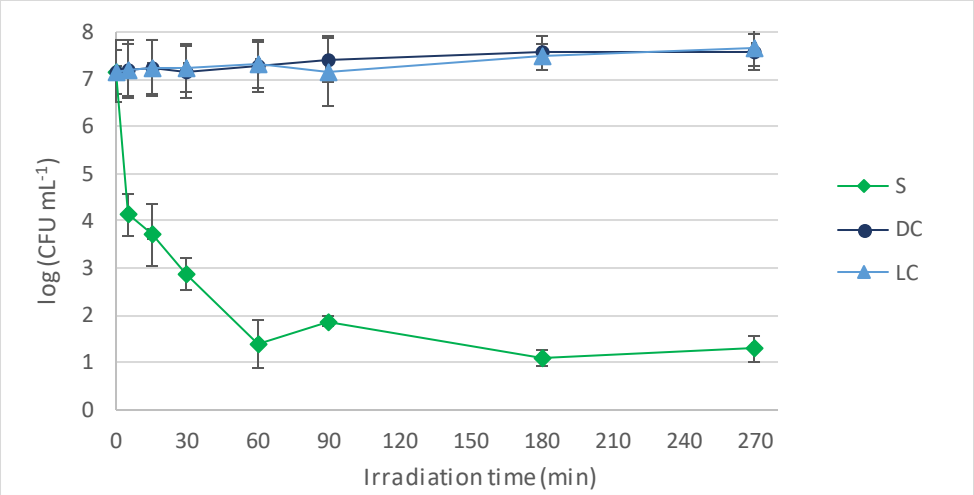


Figure 9 – In vitro assay for the *Psa* nactivation with Tetra-Py^{+}-Me at 5 µM and 40 W m^{-2} of irradiance (S: Sample; LC: Light Control; DC: Dark Control).

2.3.2 *Ex vivo* photosensitization

2.3.2.1 Inactivation with low light intensity (PAR radiation)

The *ex vivo* assays performed with the same radiation used *in vitro* (40 W m^{-2}) showed a lower rate of inactivation, even using a high porphyrin concentration of 50 µM (data not shown). In the experiments with 10 min of dark preincubation a reduction of bacterial concentration of 0.8 log was observed after 90 min of exposure to light. However, and contrary to what happen in the *in vitro* tests, the bacterial concentration in light and dark controls increased by almost 1 log (ANOVA, $p < 0.05$) from the beginning of the assay to the end (Figure 10).

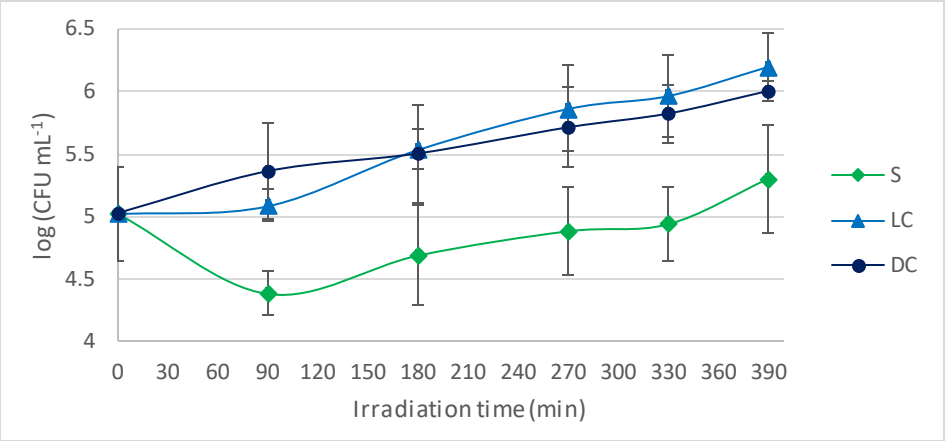


Figure 10 - Ex vivo assays for the *Psa* inactivation with Tetra-Py^{+}-Me at 50 µM and 40 W m^{-2} of irradiance with 10 minutes of dark preincubation (S: Sample; LC: Light Control; DC: Dark Control).

The irradiation in the same conditions but using a pre-incubation of 30 min caused a bacterial reduction of 0.9 log (ANOVA, $p < 0.05$) up to 90 min of radiation. The bacterial concentration in both controls increased slowly during the experiment, less than 0.5 log (ANOVA, $p < 0.05$). Having into account the increase of bacterial concentration in the control, the reduction in the sample after 90 min of treatment was around 1.2 log (ANOVA, $p < 0.05$) (Figure 11).

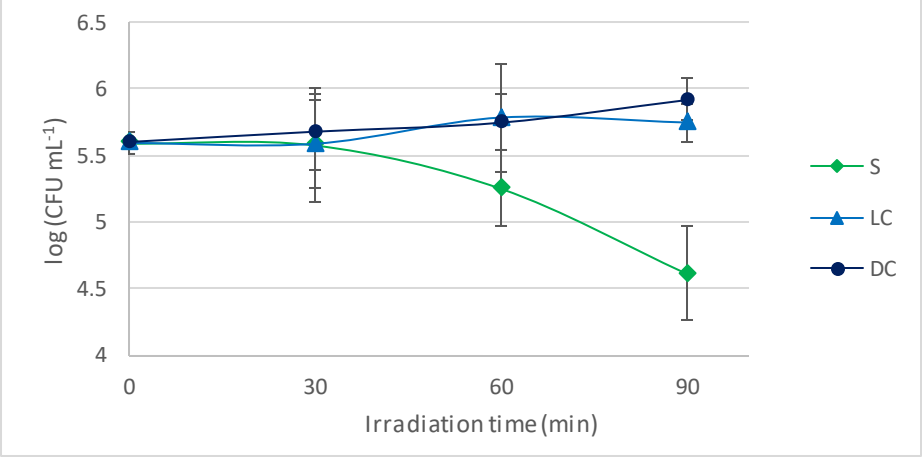


Figure 11 - Ex vivo assays for the *Psa* inactivation with Tetra-Py^{+}-Me at 50 µM and 40 W m^{-2} of irradiance with 30 minutes of dark preincubation (S: Sample; LC: Light Control; DC: Dark Control).

2.3.1.2 Inactivation with high light intensity (LumaCare)

The assays performed using a more intense radiation (150 W m^{-2}) and at the same concentration of porphyrin, 50 µM, showed a bacterial reduction of 1.8 log (ANOVA, p <

0.05) after 90 min of irradiation. However, and contrary the previous experiments when it was used irradiation with low light intensity, a reduction of about 1.2 log (ANOVA, $p < 0.05$) was already observed after 30 min of irradiation. A slight increase of bacterial concentration (≈ 0.5 logs; ANOVA, $p < 0.05$) in controls was observed (Figure 12).

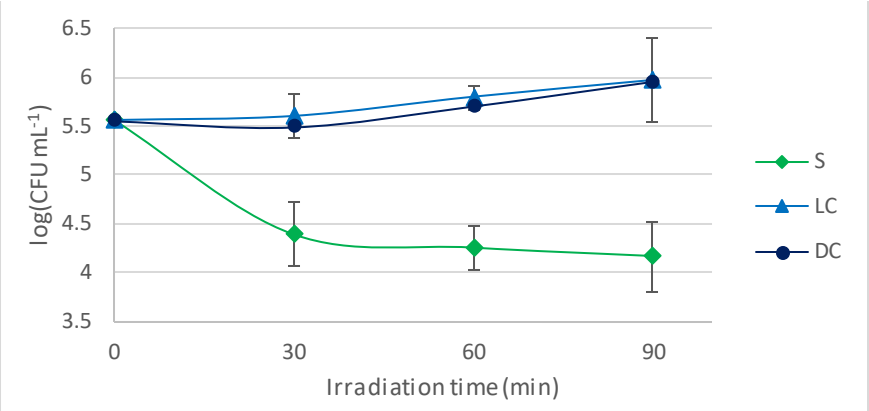


Figure 12 - Ex vivo assays for the *Psa* inactivation with Tetra-Py$^+$-Me at 50 µM and 150 W m^{-2} of irradiance with a dark preincubation of 30 min (S: Sample; LC: Light Control; DC: Dark Control).

2.3.1.3 Inactivation with sunlight

The assays performed under sunlight (650 W m^{-2}) in the presence of Tetra-Py$^+$-Me at 50 µM showed a decrease of bacterial concentration of about 1.5 log (ANOVA, $p < 0.05$) after 90 min of treatment. After 30 min of irradiation, the decrease was about 0.8 log (ANOVA, $p < 0.05$). The bacterial concentration in the controls was stable during the experiments (Figure 13).

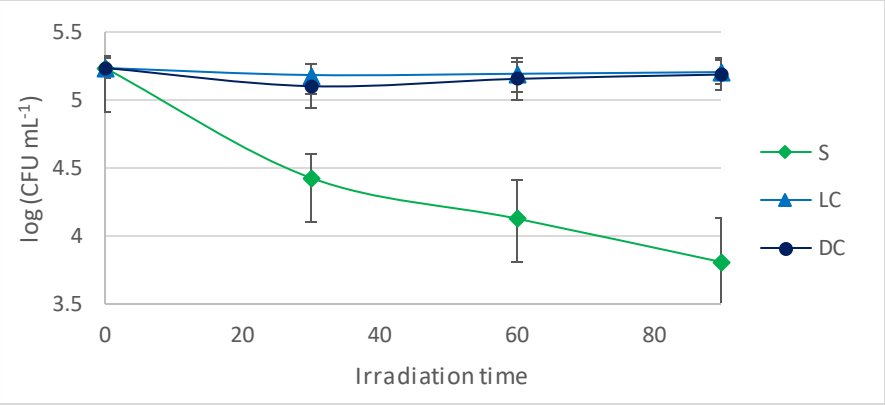


Figure 13 - Ex vivo assays for the *Psa* inactivation with Tetra-Py$^+$-Me at 50 µM and under sunlight irradiation (S: Sample; LC: Light Control; DC: Dark Control).

2.3.3 Inactivation of bacteria by cuprous oxid

2.3.3.1 Optical density

The OD measurements showed that both bioluminescent *E. coli* and *Psa* showed a similar pattern of inactivation during the 330 min of treatment with 0.5 g hl^{-1} of Cu_2O. The highest decrease was observed during the first 15 min for both bacteria. *E. coli* showed a decrease in OD of approximately 0.23 (ANOVA, $p < 0.05$) at the end of the 330 min (Figure 14A) and the *Psa* showed a OD reduction of 0.29 (ANOVA, $p < 0.05$) after 330 min (Figure 14B).

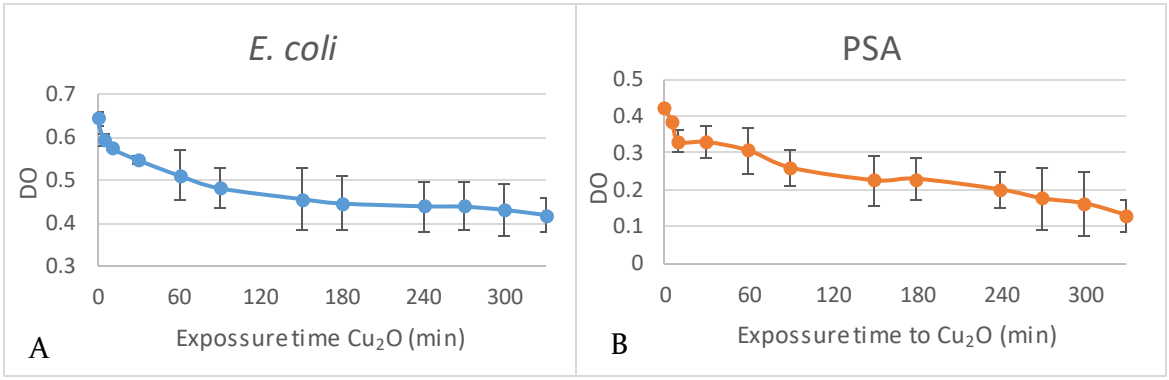


Figure 14 – Optical density inactivation of bioluminescent *E.coli* (A) and *Psa* (B) with Cu_2O at 0.5 g hL^{-1}.

2.3.3.2 Bioluminescent measures

The reduction of the bioluminescence of *E. coli* varied with the Cu_2O concentrations (5, 7.5, 10, 15, 20, 25, 35 and 50 g hL^{-1}). For the 35 and 50 g hl^{-1} concentrations, adecrease to the detection limit of the method (reduction of 4.9 log, ANOVA, $p < 0.05$) was observed after to 15 and 30 min, respectively. At 15, 20 and 25 g hL^{-1} Cu_2O a reduction to the detection limit was observed after 40 min of exposure (reduction of 4.9 log, ANOVA, $p < 0.05$). At 5, 7.5 and 10 g hl^{-1} Cu_2O a steady decrease of about 4.9 log (ANOVA, $p < 0.05$) was observed up to 50 min The bacterial concentration in the control was stable during the experiment (Figure 15).

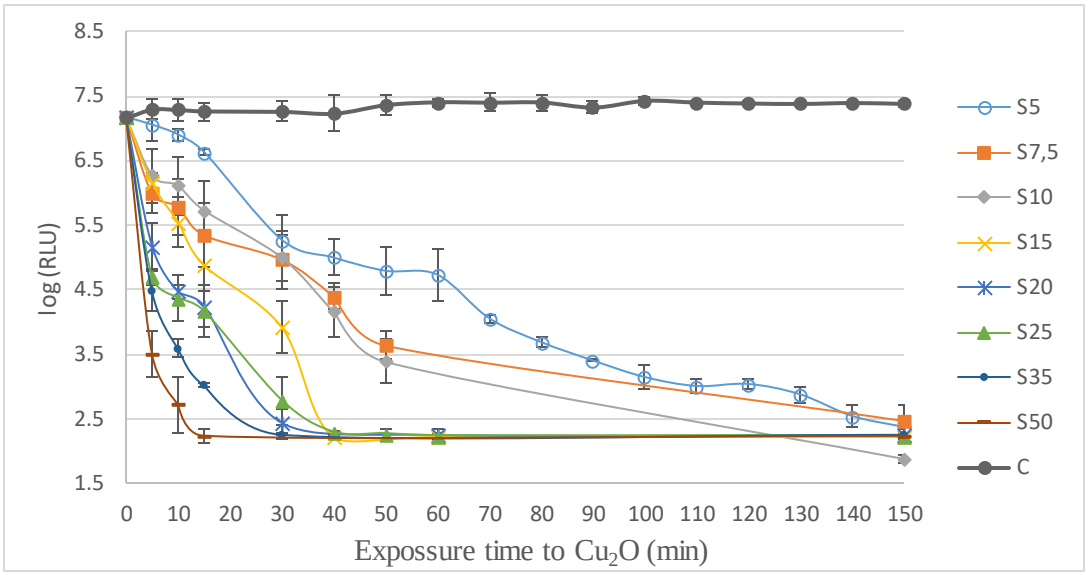


Figure 15 – Bioluminescent assays for E. coli inactivation with Cu_2O exposure (S5: Sample with 5.0 g hL^{-1}; S7.5: Sample with 7.5 g hL^{-1}; S10: Sample with 10.0 g hL^{-1}; S15: Sample with 15.0 g hL^{-1}; S20: Sample with 20.0 g hL^{-1}; S25: Sample with 25.0 g hL^{-1}; S35: Sample with 35.0 g hL^{-1}; S50: Sample with 50.0 g hL^{-1}; C: control without Cu_2O).

2.3.3.3 Colony forming units in *Psa* plating

The results obtained by pour plating at Cu_2O concentrations of 5 g hL^{-1} and 15 g hL^{-1} exposure, showed a reduction of about 5.5 log (ANOVA, $p < 0.05$) after 30 min (Figure 16A) and approximately 7.5 log (ANOVA, $p < 0.05$) after 20 min (Figure 16B), respectively. The control remained constant throughout the test.

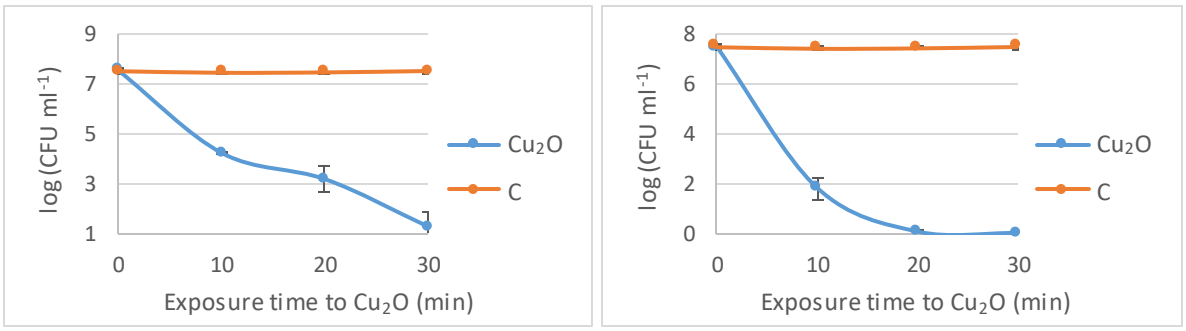


Figure 16 – *Psa* inactivation with 5 g hL^{-1} Cu_2O (A) and 15 g hL^{-1} (B) determined by cultivation on TSA plates. C: control without Cu_2O.

2.3.4 Evaluation of possible side effects in kiwi leaves after sensitization

The possible side effects in kiwi leaves due to porphyrin sensizitation were evaluated for five consecutive days by adding Tetra-Py^+-Me at four different concentrations

(5 µM, 20 µM, 50 µM and 100 µM). In these experiments no visual alteration was observed even at the highest PS concentration (100 µM) after five days. Figure 17 illustrates how the drops were placed on the leaves and how these were one day after, in which one can observe that the droplets evaporate and no color change or spot was detected. Similar results were obtained for the other four days.

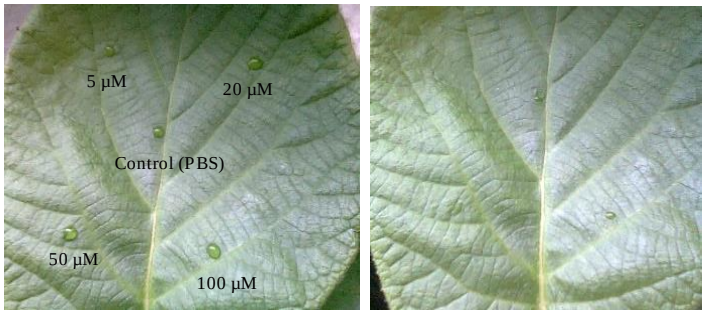


Figure 17 - Sensitization test images in kiwi leaves after one day of treatment with PS at different concentrations. In the left figure, is observe the drops of the four concentrations (periphery) and the control (center) and in the right image, the result obtained the following day.

The possible side effects on kiwi leaves were also evaluated for five consecutive days for Cu_2O at six different concentrations (0.5, 5, 15, 20, 35 and 50 g hL^{-1}). Figure 18A illustrates how the drops were placed on the leaves. After five days, all tested concentrations showed a discoloration at the exact location where the droplets were placed (Figure 18B). The effects were more visible after washing the copper residues that were accumulated on the leaves (Figure 18C).

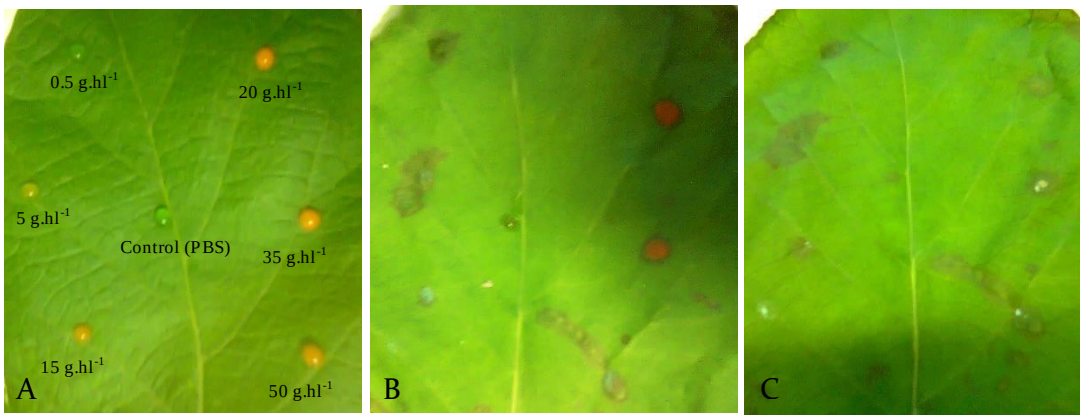


Figure 18 - Sensitization test images in kiwi leaves with Cu_2O at different concentrations. A) Six drops at diferent concentration (periphery) and a drop for the control (center) in the first day of treatment. B) Results after five days of treatment. C) Results after washing the leaf.

2.4 Discussion

The intensive cultivation of crop plants increases the potential for the emergence and rapid spread of new diseases. Although *Psa* was first reported as a canker-causing pathogen of kiwifruit in the mid-1980s, a new outbreak of the disease occurred in 2008. As kiwifruit is clonally propagated, other outbreak can happen due to the emergence of new strains that that can be rapidly spread throughout growing areas worldwide. However, the treatments of this disease are still scarce. Spraying orchards with high concentrations of copper compounds, particularly with cupric oxid, is regularly used, but it is often necessary to destroy crops by burning to control the disease.

To overcome deficiencies of conventional antimicrobial treatments, alternative approaches, such as aPDT, has been tested to control pathogenic microorganisms not only in the clinical but also in environmental areas, such as in agriculture [4,11,95,96]. aPDT has been tested to control plant pathogens caused mainly by fungi [96,97]. However, no aPDT application to control the *Psa* of kiwifruit plantations was yet done. Consequently, there are no data regarding the effects of aPDT on *Psa* on kiwifruit plant. In this study, we evaluated the efficacies of *in vitro* aPDT with porphyrin derivative in the inactivation of *Psa*. Porphyrins are among the most studied PS since their photochemical and photophysical characteristics can be modulated through adequate synthetic strategies. The tetracationic porphyrin Tetra-Py^+-Me was selected for the propsed study considering that it is a highly effective PS against bacteria, viruses and fungi [11,98].

The efficacy of Tetra-Py^+-Me to photoinactivate *Psa* was evaluated initially by determining the bacterial inactivation in a buffer solution (PBS). The results showed that *P. syringae* was efficiently inactivated (reduction of 6 log) using a low PS concentration (5 μM) under a low light intensity (40 W m^{-2}). Most of the *Psa* inactivation was observed in the first 30 min of treatment. As the experiments in solution were less laborious and less time-consuming, allowed the evaluation of several treatment times simultaneously, which was convenient to establish the conditions for aPDT in kiwi leaves.

aPDT to control *Psa* in the field would be applied under conditions very different from the controlled conditions used in the laboratory. Instead of artificial light sources, the sun would be the light source, allowing the use of the full-spectrum of natural solar radiation. The broad emission spectra and high irradiances in visible spectra enable solar radiation to

excite visible-light-activated PS such as porphyrins and UV-radiation would also contribute to direct *Psa* inactivation, allowing also the excitation of UV activated PS. Moreover, light cycles are long and repeated daily, which increase bacterial inactivation efficiency. In this study, in order to evaluate the efficiency of aPDT to control *Psa* in kiwifruit plantations, we tested the *Psa* inactivation in *ex vivo*, using kiwi leaves artificialy contamined with *P. syringae*, under solar radiation. The results show that aPDT with 50 µM of PS and 1.5 h of solar exposure reduce the bacterial concentration by 1.5 log. Although the concentration of the *Psa* was not reduced so drastically as *in vitro*, reduction or multiplying avoidance of the initial bacterial inoculum is among the strategies used to control plant diseases [100,101]. The *Psa* inactivation with solar radiation was, however, higher than that observed with the artificial low light intensity PAR radiation (40 W m^{-2}) and similar to that obtained using an artificial high light intensity (150 W m^{-2}). With PAR radiation source, at 50 µM of PS, bacterial concentration was reduced by 0.9 log after after 1.5 h of exposure. With LumaCare source, at 50 µM of PS, bacterial concentration was reduced by 1.8 log after after 1.5 h of exposure. With artificial light sources, PAR radiation and LumaCare, a slight increase in bacterial concentration (increase of 0.5-1.0 log) in controls was observed. However, no increase was observed when experiments were done under solar radiation. At a first glance this seems to indicate the UV radiation of solar light could be the cause of no increase of bacterial concentration in controls under sun irradiation. However, no difference was observed in bacterial concentration between light and dark controls when sun light was used. These differences can be due to natural variations in leaves sets used in the different experiments.

The results of these experiments suggest that, solar radiation does not affect the PS activity. PS was applied as spraying on leaves surface being exposed directly to solar radiation on clear days in a temperate region in early summer. The results of bacterial inactivation with LumaCare source were similar to those obtained with sun. The LumaCare spectrum does not include most of the UV radiation.

As the *Psa* was not so efficiently inactivated in *ex vivo* than *in vitro*, more than one applications of aPDT would be required to control *Psa* in the kiwifruit plantations. However, frequent applications are also required to control *Psa* with conventional treatments. Copper, the most frequently used treatment is not systemic, therefore reapplication is required to protect new tissue, especially when the copper layer is eroded by wind / heavy rain [83,101].

Several applications are not uncommon during the plant cycle to control the disease, depending upon its incidence and the rainfall. Copper compounds have been recommended for spraying immediately after winter pruning, at bud break, two and four weeks after bud break, and in hig risk situations like after a major wind, rain or hail event [71]. Further studies using several aPDT applications on kiwi leaves are necessary to evaluate the effectiveness of aPDT to control *Psa* in kiwifruit plantations. Moreover, studies using long dark preincubation periods with the PS are also needed. In this work the increase of 10 to 30 min of the dark preincubation period increase the efficiency of aPDT to control the *Psa* in *ex vivo* (increase of 0.5 log in bacterial reduction).

Beside effective, a aPDT ptotocol must be safe and no damage to host cells should be observed. However, both the microorganism and the host will be exposed to light and to the PS. In this study no damage was observed on kiwi leaves treated with the PS and exposed to solar radiation for five days. The lack of damage can be expalined by the fact that PS concentration and dark preincubation period were low to affect host cells. It is well known that aPDT can be specific to microorganisms when PS is applied at low concentrations (µM range) and for short periods (minutes) [4,8,11,18]. Moreover, leaf cuticle of the kiwi leaves can also act as barrier, avoiding the PS to cross the leaf cuticle. As the reactive species generated during aPDT have very short half-lives, their diffusion is limited, and therefore, damage is restricted to structures close to the PS [5,13,20,23,101]. Consequently, as the PS remained outside the cuticle, the internal leaf structure was not damaged by the aPDT. Thus, *Psa* on the leaf surface that are in contact with the PS may be inactivated without damaging the leaf tissues. Some studies have reported that the application of traditional Cu-based bactericides significantly affects the kiwifruit yield [102]. Copper, cause phytotoxic symptoms e.g. discolouration and cracking of stalks, silver-brown leaves, and the appearance of spots on the lower surfaces of the kiwifruit leaves. The application of aPDT to control *Psa* in leaves could be a safe alternative to copper application.

The use of aPDT in the environment to control pathogens, imply the application of the PS in large areas, which require the use of environmentally safe PS. The porphyrinic derivatives used in this study, has a suitable animal toxicity profile when used at low concentrations [11].

It is also well known that the repeated use of copper-based bactericides to control horticulture plant diseases has led to long-term accumulation of Cu in the surface of some agricultural soils throughout the world [72,84]. For example, the repeated spraying of Bordeaux mixture in France to control vinedowny mildew has resulted in a considerable build-up of total Cu concentrations in the top soil, reaching values commonly ranging from 100 up to 1500 mg/kg [103-106]. Althought KVH advice the use of a maximum of 8 kg of active copper per hectare per year for conventional growers and maximum 6 kg for organic growers [92,107]. The Cu dynamics and the long term effect of Cu residues and its accumulation in the kiwifruit orchard soil system have yet not been studied in detail. Guinto et al (2012) analysed the total Cu concentration of top soils collected from 20 kiwifruit orchards and found that the mean Cu concentration did not exceed 35 mg/kg soil. However, the Cu concentration was significantly increased when they compared the Cu levels with 2009 samples collected from the same area. The results of this study suggest, however, that the most frequently used copper-based bactericides to control *Psa* in kiwifruit plantations is applied in concentrations much higher than necessary (50 and 35 g hL^{-1}, in Europe and New Zeland, respectively, for Cu_2O). Ideally, Cu on the leaf surface should be at a high enough concentration to kill the bacteria but low enough not to cause injury to the plant. Our results suggest that Cu even when applied at low concentrations can damage the leaves. Plant injury may arise due to a lack of lime in the Cu mixture, cold and wet weather conditions (at time of application) but also due to the application of excessive rates of Cu [108,109]. However, according withour *in vitro* results, the Cu_2O concentration applied to control the *Psa* in kiwifruits plantations can be reduced at least by 10 times.

In addition, the efficacy of copper has been significantly reduced by the occurrence of copper-resistant strains of *Psa* [87]. Nevertheless, there have been no studies published on the occurrence of Cu resistant *Psa* in Kiwifruit vines, a research gap that needed to be addressed for the effective control of *Psa* in Kiwifruit vines. Nakajima et al (2002) found that the genetic and molecular basis of the copper resistance of *Pseudomonas syringae* pv. *tomato* in tomato was similar to copper resistance genes from *Psa* [110]. These authors demonstrated that all strains isolated at the beginning of bacterial canker outbreaks in Japan (in 1984) were copper sensitive with a minimum inhibitory concentration (MIC) of 0.75 mM $CuSO_4$. However, in 1987 and 1988 some strains isolated were copper resistant, with the MIC ranging from 2.25 to 3.0 mM. They also concluded that, with the repeated spraying of

copper-based bactericides, the *Psa* showed the development of additional genes responsible for maximum resistance to copper. Masami et al (2004), identified the mechanism of copper resistance in *Psa* [87]. However, some studies observed that there was no development of Cu resistant *Psa* strains [111].

As all studies that tested the possible development of microbial resistance to aPDT proved that microorganisms are not able to develop resistance, aPDT can be a safe alternative to copper for the effective control of *Psa* in kiwifruit plantations [11,12,15,20,98,112,113]. The potential lack of development of resistance mechanisms is due to the mode of action and type of biochemical targets (multi-target process) of aPDT [11,18].

Overall we demonstrated that *in vitro* and *ex vivo* aPDT with a porphyrin derivative with natural solar radiation is effective to inactivate *Psa*, does not damage the plant host and can be applied by spraying. In order to explore the real potential of aPDT as an alternative to the intensive use of conventional treatments, further studies are necessary to determine the effectiveness of aPDT in planta under field conditions and also to evaluate environmental impact of this new approach.

2.5 Future perspectives

After this work, it would be interesting to evaluate:

- the effect of other PS on *Psa* inactivation, especially natural compounds (e.g. chlorophyll) and even PS mixtures with different ROS production pathways, for example the conjugation of tetracationic porphyrin, which produce mainly singlet oxygen to methylene blue that, contrary to the porphyrin, produces essentially free radicals;
- the efficacy of aPDT to inactivate *Psa* isolates from contaminated kiwifruit plantations;
- the efficacy of successive aPDT cycles to inactivate *Psa*;
- the possibility of development of aPDT-resistant mutants after successive cycles of treatment;
- the efficacy of aPDT to inactivate *Psa in vivo* (using the all plant) and in the field (in kiwifruit plantations).

CHAPTER 3 – References

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CHAPTER 4 – Appendix

Annex 1 – Legislations applicable in Portugal [83].

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 na comunitário, de organismos prejudiciais aos vegetais e produtos vegetais qualquer 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.

Regulamento (CE) n.º 1107/2009, do Parlamento Europeu e do Conselho, de 21 de outubro, relativo à colocação dos produtos fitofarmacêuticos no mercado.