Universidade de Aveiro Departamento de Biologia
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Terapia fotodinâmica antimicrobiana no controlo da transmissão de *Pseudomonas syringae* pv. *actinidiae* pelo pólen do kiwi

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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 coorientação 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 fotodinânica antimicrobiana, *Pseudomonas syringae* pv. *actinidiae,* infeção bacteriana, fotossensibilizadores, kiwi, pólen

resumo

Pseudomonas syringae pv. *actinidiae* (Psa) é uma bactéria fitopatogénica, responsável pelo cancro na planta do kiwi, o qual está a afetar as plantações e produção de kiwi mundialmente. Atualmente, os únicos tratamentos disponíveis baseiam-se no uso de compostos à base de cobre e/ou antibióticos sob a forma de sprays, compostos estes que são altamente tóxicos e podem inclusive levar ao desenvolvimento de resistência por parte das bactérias. Além disso, provou-se que o pólen é um dos vetores responsáveis pela disseminação da Psa entre plantações.

A terapia fotodinâmica antimicrobiana (aPDT) tem sido alvo de elevado interesse científico por diversos grupos de investigação a nível mundial e apresenta-se como alternativa eficaz para a inativação da Psa. A aPDT consiste no uso de um fotossensibilizador que, ao absorver luz na presença de oxigénio promove a formação de espécies reativas de oxigénio (ROS), as quais têm a capacidade de afetar de forma irreversível diversas estruturas celulares (como proteínas, lípidos e ácidos nucleicos).

Este estudo tem como principal objetivo avaliar a eficácia da aPDT na inativação da Psa no pólen do kiwi, usando como fotossensibilizadores o novo azul de metileno (NMB) e o azul de metileno (MB). Inicialmente, foi avaliada a capacidade de inativação da Psa *in vitro* com NMB a 1.0, 2.5 e a 5.0 μM, na ausência e na presença de KI (a 100 mM), com uma intensidade luminosa de 50 mW cm^{-2}. As melhores condições de inativação *in vitro* conseguidas com NMB foram replicadas com MB (a 5.0 μM) e com adição de KI. Posteriormente, foram realizados ensaios *ex vixo* no pólen artificialmente contaminado com Psa, com MB a 50 μM e a mesma intensidade de luz, durante 180 min. Por fim, averiguou-se se a capacidade de germinação do pólen era afetada pela aPDT.

Nos ensaios *in vitro* observou-se uma redução da concentração da Psa de cerca de 8 log UFC mL^{-1} após 45 min de irradiação, enquanto que nos ensaios *ex vivo* a concentração de Psa decresceu cerca de 3 log UFC mL^{-1} após 180 min. Não se observaram efeitos negativos na germinação do pólen após aPDT.

Os resultados obtidos neste estudo mostram que aPDT é um método seguro e eficaz para inativar a Psa no pólen do kiwi. Desta forma, a aPDT com um fotossensibilizador de custo reduzido como o MB e cujo uso já está aprovado para algumas aplicações, pode ser uma alternativa promissora para o controlo da transmissão da Psa pelo pólen do kiwi.

keywords

Antimicrobial photodynamic therapy, photosensitizers, bacterial infection, *Pseudomonas syringae* pv. *actinidiae,* kiwifruit, pollen

abstract

Pseudomonas syringae pv. *actinidiae* (Psa) is a phytopathogenic bacterium responsible for the bacterial canker in kiwifruit plants that is highly affecting kiwifruit production worldwide. The available treatments for this problem consist in spraying the orchards with copper derivates and/or antibiotics which are highly toxic to the environment and can lead to the development of bacterial resistance. Furthermore, pollen is proved to be responsible for the dissemination of Psa among orchards.

Antimicrobial photodynamic therapy (aPDT) has been the subject of extensive research by several research groups all over the world and may be an effective alternative in the inactivation of Psa. aPDT consists in the use of a photosensitizer (PS) molecule that absorbs light in the presence of oxygen and promotes the formation of highly reactive oxygen species (ROS) that irreversibly damage various cellular components such as proteins, lipids and nucleic acids.

The aim of this study was to evaluate the effectiveness of aPDT in the inactivation of Psa in kiwifruit pollen, using New Methylene Blue (NMB) and Methylene Blue (MB) as PSs. First, the degree of inactivation of Psa was assessed *in vitro* using the NMB at 1.0, 2.5 and 5.0 μM, in the presence and absence of KI (at 100 mM), under an irradiance of 50 mW cm^{-2}. Secondly, the best *in vitro* inactivation conditions were also tested with MB (at 5.0 μM) and KI. Subsequently, *ex vivo* experiments using artificially contaminated kiwifruit pollen were carried out with MB at 50 μM under irradiance of 50 mW cm^{-2} for 180 min. Lastly, pollen germination assays were performed to evaluate if pollen ability to germinate was affected by aPDT.

A reduction in Psa concentration of *ca.* 8 log CFU mL^{-1} in the *in vitro* assays after 45 min of irradiation was observed, whereas in the *ex vivo* assays the decrease in Psa concentration was *ca.* 3 log CFU mL^{-1} after 180 min. No negative effects were observed in the kiwifruit pollen germination after aPDT treatment.

The results obtained in this study showed that aPDT is an effective and safe method to inactivate the Psa in kiwifruit pollen, indicating that aPDT with a PS already approved and with low cost (MB), can be a promising alternative in the control of Psa transmission by the kiwifruit pollen.

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List of Abbreviations

aPDT, Antimicrobial Photodynamic Therapy

CFU, Colony Forming Units

DC, Dark Control

DMSO, Dimethyl Sulfoxide

LA, Luria Bertani Agar

LB, Luria Bertani

LC, Light Control

MB, Methylene Blue

NA, Nutrient Agar

NMB, New Methylene Blue

PBS, Phosphate-Buffered Saline

PDT, Photodynamic Therapy

PS, Photosensitizer

rpm, revolutions per minute

RBCA, Rose-Bengal Chloramphenicol Agar

ROS, reactive oxygen species

TSA, Tryptic Soy Agar

TSB, Tryptic Soy Broth

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Objectives and Thesis Outline

The main goal of this work was to evaluate the efficiency of photodynamic therapy to inactivate *Pseudomonas syringae* pv. *actinidiae* in kiwifruit pollen. For that, *in vitro* inactivation assays were performed using the New Methylene Blue (NMB) and Methylene Blue (MB) with and without the addiction of KI. *Ex vivo* experiments with kiwifruit pollen were then performed with MB also with and without KI. Pollen germination assays were also done to assess if aPDT affect the pollen viability.

The present document was divided in three main chapters:

CHAPTER 1 – Introduction

This chapter includes a general literature review on the context of the developed work. The state of the art about *Pseudomonas* genus and, more deeply, *Pseudomonas syringae* species and the plant pathogen *Pseudomonas syringae* pv. *actinidiae* are mentioned, as well as the adjacent problems caused to the *Actinidia* plant species, involving both environmental and economic issues. Available treatments are here presented and its limitations are exposed. At this point, photodynamic therapy is explored/presented as a possible alternative to the currently used treatments and previous studies showing its efficacy in the inactivation of microorganisms are referred as well.

CHAPTER 2 – Antimicrobial photodynamic therapy in the control of *Pseudomonas syringae* pv. *actinidiae* transmission by kiwifruit pollen

This chapter is structured as an original research manuscript. A brief introduction about the underlying concepts on the developed work is presented. The experimental design developed for this research work is presented in this chapter – the steps that were taken from the *in vitro* experiments to inactivate Psa to the *ex vivo* ones and the final evaluation of the pollen germination; the obtained results are here showed and discussed. Conclusions about the best experimental conditions are taken, as well as some considerations about the possible application of the tested treatment in real conditions are described in the form of future perspectives.

CHAPTER 3 – References

In this chapter are listed the references consulted to the present work, *i.e.* to the writing of both Chapter 1 and Chapter 2.

CHAPTER 1 – Introduction

1.1. *Pseudomonas* genus

Pseudomonas genus was first proposed by the Botanist Walter Migula in 1894. It initially comprised all bacteria that were Gram-negative rods, strictly aerobic, nonsporulating and motile by one or more polar flagella (Peix et al., 2009; Young, 2010). This not so restricted definition of the genus led to erroneous classification of a major number of species and by the middle of the XX century, more than 800 species had been included in the *Pseudomonas* genus (Andreani & Fasolato, 2017; Peix et al., 2009).

More recently, using molecular techniques (such as DNA/DNA hybridization and comparative analysis of 16S r-DNA) in addition to the classical microbiology approaches, the definition of bacteria from *Pseudomonas* genus was refined and distinct phylogenetic groups were identified (Andreani & Fasolato, 2017; Palleroni, 1993, 2003; Young, 2010).

The genus is currently characterized by being Gram-negative, straight or slightly curved rods, catalase positive, obligate aerobic and non-spore forming and by having one or more flagellum (that confers motility) (Andreani & Fasolato, 2017; Young, 2010). They are mesophilic (optimal growth at 25 - 35 ºC), but some of them are psychrotrophic (being able to grow at lower/refrigeration temperatures). The optimum pH is above 5.8 and they have the capability of using various organic compounds as energy and carbon sources (Andreani & Fasolato, 2017). *Pseudomonas* can be found in various habitats, from clinical to the environment (Grosso-Becerra et al., 2014).

1.1.1. *Pseudomonas syringae*

Pseudomonas syringae is the main phytopathogen of *Pseudomonas* genus and one of the best studied plant pathogens. More than 60 pathovars were already identified, being each pathovar's name given accordingly to the host from where it was first isolated (Xin et al., 2018).

P. syringae was originally isolated from diseased plants and it is related to devastation of various fruit orchards causing economical losses in important crop species. However, many isolates that belong to this specie may exist as commensals on plants and may be non-pathogenic (Xin et al., 2018). Also, the pathogenicity or the capability of infection of the bacteria may be affected by environmental conditions as humidity, temperature, nutrient availability, UV radiation, plant microbiota, etc. (Xin et al., 2018).

1.1.2. *Pseudomonas syringae* pv. *actinidiae*

Pseudomonas syringae pv. *actinidiae* (Psa) is a plant pathogen and the main causal agent of bacterial canker of green-fleshed kiwifruit *(Actinidia deliciosa)* and yellow-fleshed kiwifruit *(Actinidia chinensis),* causing severe economic losses worldwide (European and Mediterranean Plant Protection Organization - EPPO, 2014; Scortichini et al., 2012. It was also isolated from other species as *Actinidia arguta* and *Actinidia kolommikta* (Scortichini et al., 2012). Contrarily to the other *Pseudomonas*, Psa is oxidase-negative (Donati et al., 2014).

Psa was first isolated, identified and described in Japan in 1984 (Takikawa et al., 1989). Afterward, until 2008 Psa was also found in China, Italy and South Korea (Scortichini et al., 2012; Scortichini, 1994; Donati et al., 2014). However, more recently Psa has re-emerged causing destruction of the main areas of kiwifruit production worldwide, being considered as a pandemic disease (EPPO, 2014; Donati et al., 2014; Scortichini et al., 2012).

Some studies have determined that there are presently some genetically different populations of Psa spread over the world (Scortichini et al., 2012). These strains/biovars show genetic variability and may be differentiated according to the presence of certain genes that code for toxins as phaseolotoxin, coronatine and effector proteins and other DNA gene sequences (Young, 2010). These low molecular weight and diffusible toxins produced by Psa have the capacity to infect the host plant (Tamura, 2002). Depending on the toxins produced, these strains/biovars vary on their aggressiveness to the plant.

Biovar 1 was detected in Japan (1984-1989) (Takikawa et al., 1989) and later in Italy (1992) (Scortichini 1994; Scortichini et al., 2012;). Psa1 produces and secretes phaseolotoxin, whose gene cluster was acquired by horizontal gene transfer (Tamura, 2002). Biovar 2 has only been isolated in South Korea and is characterized by being capable of producing coronatine and by the absence of phaseolotoxin secretion (Chapman et al., 2012). Biovar 3 is a highly pathogenic strain and is affecting kiwifruit orchards in several countries worldwide (as New Zealand, Italy, France, Spain, Portugal and China) (Abelleira et al., 2014). Psa3 has no genes for the expression of phaseolotoxin neither coronatine, however, it was reported to have genes that encode for effector proteins (Scortichini et al., 2012). Psa3 was initially called “Italian” and then called “Psa-V” (from virulent) due to its high aggressiveness (Abelleira et al., 2014; Chapman et al., 2012). Biovar 4 was only reported in few orchards from New Zealand, Australia and France until date and its shown to be less aggressive, causing only leaf symptoms and not being responsible for significant economic losses. Psa4 was also referred to as

Psa-LV (meaning Less Virulent or Low Virulence) (Abelleira et al., 2014; Chapman et al., 2012). This biovar was recently proposed to be called as Psaf (*Pseudomonas syringae* pv. *actinidifoliorum*) (Cunty et al., 2015). More recently, Biovar 5 (2012) and Biovar 6 (2015) were found and identified in limited places in Japan, being endemic at present (Fujikawa & Sawada, 2016; Sawada et al., 2016).

1.2. *Actinidia* genus

Actinidia spp. are the only plant species that are known to host Psa (EPPO, 2014).

Kiwifruit is native from China and is the most common crop in the genus *Actinidia* (Actinidiaceae family), which is a deciduous woody fruiting vine (Nishiyama, 2007; Singletary, 2012). There are several *Actinidia* species cultivated worldwide that exhibit different physical characteristics, nutritional properties and flavours (Singletary, 2012). However, only 3 of these species have relevant commercial importance: *Actinidia deliciosa* (green kiwi), *Actinidia chinensis* (gold/yellow or red-fleshed kiwi) and *Actinidia arguta* (hardy kiwi) (Almeida et al., 2018).

A. deliciosa is the most commercialized kiwifruit, counting with about 85% of the kiwi commercial production worldwide and *A. chinensis* is the second one with about 15% (Hancock et al., 2008).

1.2.1. Types of kiwi (*Actinidia* species)

Actinidia deliciosa is the vulgar green kiwi and the most common one. It has a brown skin with dense brown hair and bright green flesh contrasting with the white core and black small edible seeds (Nishiyama, 2007).

A. deliciosa was first cultivated outside China at the beginning of the 20th century and by about 1930 the first commercial orchards were established in New Zealand. However, only in the 60s kiwifruit was launched to the world markets (Hancock et al., 2008; Nishiyama, 2007).

Figure 1. *Actinidia deliciosa* *(in: https://www.kiwicoop.com/api/images/48/600/0003E4E14A4BB1.png)*

Nowadays the cultivar “Hayward” is the most widely planted and the most commercially available one, representing about 90 to 95% of the international kiwifruit trade (Hancock et al., 2008; Nishiyama, 2007). This cultivar is also used as a standard (against which new cultivars are evaluated) and has a long storage life which is advantageous to export by ship to markets worldwide, even distant ones (Ferguson, 1991).

Actinidia chinensis is closely related to *A. deliciosa* but its characterized by having a less hairy skin (Nishiyama, 2007; Singletary, 2012). *A. chinensis* is usually known as the golden or yellow kiwi (due to its yellow-flesh color).

Figure 2. *Actinidia chinensis* *(in: https://www.kiwicoop.com/api/images/50/600/0003E64093D872.png)*

A. chinensis is more recent than *A. deliciosa* and significant quantities were only traded internationally at the end of the 20th century (Hancock et al., 2008).

The cultivar "Hort16A" was developed by HortResearch in New Zealand and is the most widely planted cultivar of *A. chinensis* (Hancock et al., 2008). Now it is on the markets by the name "ZESPRI™ GOLD Kiwifruit" and it is grown under license to ZESPRI International in countries as Italy, Japan, Korea, France, Portugal and US (Nishiyama, 2007).

There are also red-fleshed cultivars that belong to the *Actinidia chinensis* species.

"Hongyang" was selected by a red clone from *A. chinensis* in China and was the first red-fleshed kiwifruit cultivar in the world. This cultivar has a vivid red core and a yellowish-green flesh with a sweeter taste and higher vitamin C content that "Hayward" (Wang et al., 2003).

As "Hongyang" cultivar has limited tolerance to high temperatures, more recently another red-fleshed kiwifruit cultivar was selected. "Chuhong" was officially released in February of 2005 in China (Zhong et al., 2007).

Figure 3. *Actinidia chinensis* *(in: https://www.kiwicoop.com/api/images/52/600/0003E64089D923.png)*

Figure 4. *Actinidia arguta* *(in: https://www.kiwicoop.com/api/images/51/600/0003E63B551A36.png)*

Actinidia arguta, also known as kiwiberry, hardy or baby kiwi, is grape shaped, weighs around 5-15 g and can be eaten whole. It is greenish and is covered by a smooth, hairless and edible skin. *A. arguta* has a more intense and sweeter flavor than the other *Actinidia* species (Singletary, 2012; Williams et al., 2003).

1.2.2. Kiwifruit industry, trade and consumption worldwide

The world's largest producers of kiwifruit over the past years have been Italy, China and New Zealand, which represent about 90% of the international production. China has a major role on kiwi trade, as the major producing country with over 2 million tonnes only in 2017, Italy counts with almost 550 thousand tonnes and New Zealand produces more than 400 thousand tonnes, according to the FAO – Food and Agriculture Organization of the United Nations (**Figure 5**).

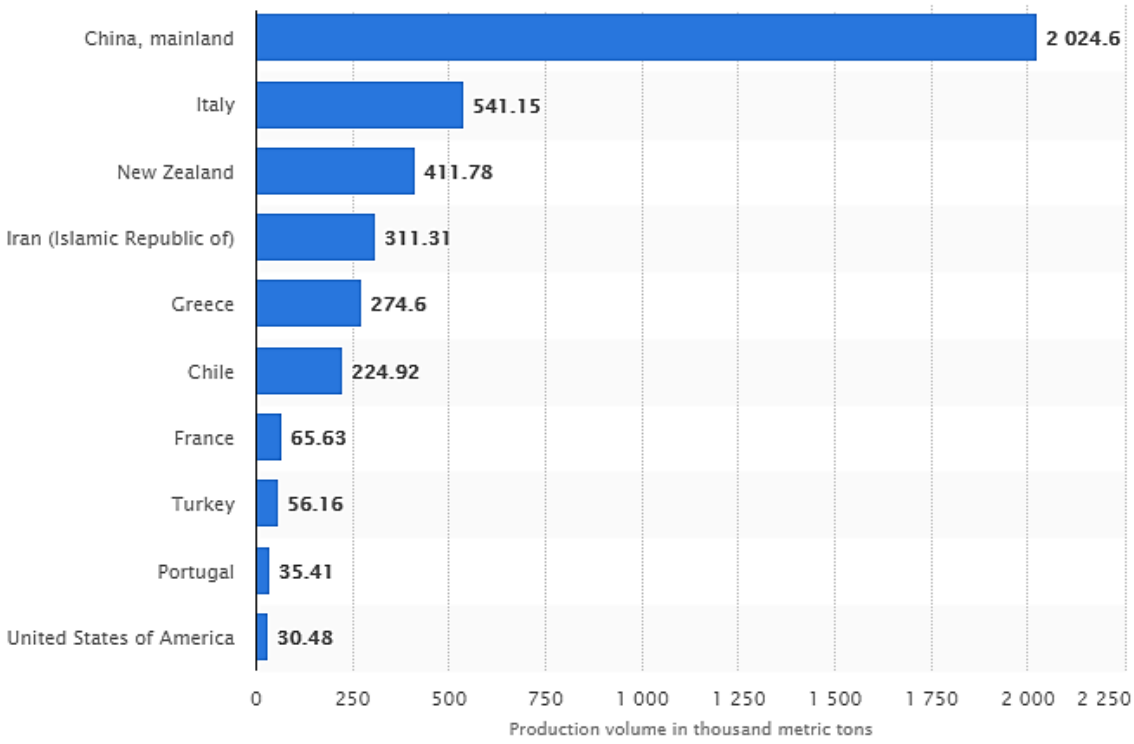

Figure 5. Production volume of kiwifruit worldwide in 2017 (in tonnes) (source: FAO) *(In: https://www.statista.com/statistics/812434/production-volume-of-leading-kiwi-producing-countries/, accessed in 23/02/2019)*

Despite being the main producer, China does not export much, not even being in the top ten exporters. New Zealand and Italy are the major exporters, with over 58 and 41 thousand tonnes, respectively (FAO, 2016).

However, kiwifruit orchards are being devastated globally due to bacterial canker caused by Psa outbreaks. This scenario results in major economic losses on kiwifruit industries worldwide, *i.e.* in Italy, China, New Zealand, France, Portugal among other countries (Cameron & Sarojini, 2014).

Currently, in Portugal, due to the lack of wintery cold, atypical springs and Psa, a fall in the kiwi production is being observed and the national average productivity is about 15 tonnes per hectare, according to Portuguese Association of Kiwifruit growers (APK - Associação Portuguesa de Kiwicultores). In addition, due to recent investment in new orchards production, the national productivity is expected to increase by 41 thousand tonnes by 2022 (APK). However, this estimate may be undermined due to the spread of the phytopathogen Psa.

1.2.3. Kiwifruit bacterial canker caused by Psa

Psa causes a bacterial canker disease in kiwifruit plants that results in massive damages in the orchards (Takikawa et al., 1989; Scortichini et al., 2012; EPPO, 2014).

The bacteria may have two phases of growth: the epiphytic phase and the endophytic phase. In the epiphytic phase the Psa lives on plant tissues' surfaces (as stems, leaves, flowers and fruits) and can remain latent without causing symptoms to the plant. The endophytic phase happens when the bacteria enters the plant (through openings or injuries caused by animals, human manipulation, the weather, etc) and is capable of colonize the intercellular apoplast space (Xin et al., 2018).

The most common symptoms of the disease are leaf spots, especially on younger leaves (Donati et al., 2014). Leaves in green-fleshed and yellow-fleshed kiwifruits, contaminated with Psa develop dark brown lesions surrounded by a yellowish halo (Scortichini et al., 2012).

Other symptoms may also occur, as appearance of small tumours, blossom necrosis, twig/sprouts fall, release of a brownish exudate (that may become whitish when the bacterial concentration is higher) (Scortichini et al., 2012). In extreme cases, Psa can lead to extensive vine death and devastation of whole orchards.

1.2.4. Psa transmission in kiwifruit plants and pollen

Psa was already found in leaves and pollen from orchards that present symptoms of bacterial canker, but was also found in asymptomatic vines (Gallelli et al., 2011; Vanneste, Giovanardi et al., 2011).

The pathogen growth and dispersion are favoured by high humidity conditions and temperatures ranging from 12 to 20 ºC that are frequent in the spring and autumn (Scortichini et al., 2012; EPPO, 2019). When Psa appears in the spring, it usually affects

the development of the plants; when in autumn/winter it causes plant damage (Scortichini et al., 2012).

Psa can also be spread by natural conditions (as wind, frost, rain, hail and snow storms), as well as by some techniques and equipment used in agriculture or through a vector (*i.e.* humans or honeybees). Additionally, it was also suggested that Psa may be spread through infected pollen, both naturally or through artificial pollination (Vanneste, Giovanardi et al., 2011). On the other hand, higher temperatures (*i.e.* temperatures above 25 ºC that are more common during summer) can reduce the multiplication and the spread of Psa, reducing the degree of infection of the plant and decreasing the exudate production (Scortichini et al., 2012; Vanneste, 2017; EPPO, 2019).

Recent studies have already shown that Psa can be transmitted from diseased plants to healthy ones through pollen, being this process considered as one of the main infection pathways in field condition, however, a lot is unknown about the dissemination of Psa from plant to plant (Donati et al., 2018).

The process of pollination, which can occur naturally or be artificially assisted, is strictly related to the quality and size of kiwifruit. For that reason, producers collect the pollen and have adopted an artificial pollination process to improve fruit size and production (APK; Donati et al., 2018).

Nearly 10 - 15% of this pollination is artificially performed with acquired pollen, which is also very expensive (about 2 000 to 3 500 €/kg) (APK). For this reason, it is of extreme importance to use pollen that is Psa free in order to control the dissemination of bacterial canker between plants or orchards (Donati et al., 2018).

1.2.5. Disease prevention and available treatments

In order to control the dissemination of Psa in kiwifruit orchards and to minimize economic losses, there are some preventive measures and strategies that are commonly adopted (Cameron & Sarojini, 2014). There are also some already approved treatments and new strategies have also been studied to control the dissemination of Psa in kiwifruit orchards (Jeyakumar et al., 2014).

Some preventive measures are: maintenance of hygiene conditions, disinfection of the agriculture tools/equipment used, protection from animals that may transmit the pathogen, monitorization of the orchards, elimination of the diseased parts or vines, to reduce the risk of spreading in the orchard (Cameron & Sarojini, 2014; Jeyakumar et al., 2014; APK).

In most cases, these preventive measures are not enough to eradicate Psa and other procedures may be needed, *i.e* chemical treatments, namely copper-based formulations or antibiotics (Cameron & Sarojini, 2014; Jeyakumar et al., 2014).

The copper-based bactericides, e.g. cuprous oxide (Cu_2O) are considered the most effective procedure and are currently the most used worldwide (Jesus et al., 2018). Copper-based sprays are usually applied on the surface of the plants providing a superficial protection to the leaves which avoids the bacteria from entering in the plant. However, if the plant is already infected, copper-based compounds have little or no effect. Despite being often used due to its low cost and being easy to apply, copper formulations may have severe toxic effects (as soil and water contamination, negative impact on microflora and eventually the development of copper-resistant bacteria) when applied in a continued way (Nakajima et al., 2002; Seiler & Berendonk, 2012; Jesus et al., 2018). For that reason, some too concentrated copper formulations (as $CuSO_4$, known as the Bordeaux mixture) are not recommended for use anymore due to its high toxicity to humans and environment. However, alternatives as copper hydroxide ($Cu(OH)_2$) and copper oxychloride ($Cu_3Cl_2(OH)_4$) are still applied in a diversity of crop fields, once they are less concentrated (Jeyakumar et al., 2014; Mackie et al., 2012).

Antibiotics (*i.e.* streptomycin) are used in some countries from Asia and New Zealand for controlling plant pathogens. However, they are also being avoided in other countries as a result of the increasing appearance of resistant bacteria (Jeyakumar et al., 2014). In Italy, Portugal and other countries from Europe, the use of antibiotics for plant pathogens control is not allowed being the copper-based compounds preferred (Cameron & Sarojini, 2014; Vanneste, Kay, et al., 2011).

Psa control and treatment are a challenge with most of the approved measures being only preventive or having a toxic or negative impact in humans and the environment or other issues related to bacterial resistance (Cameron & Sarojini, 2014). For those reasons, new cost-effective alternatives with little or none impact to the environment are required to control Psa in kiwifruit plants and pollen (Jesus et al., 2018).

New studies have been taking place in order to control the Psa on the kiwifruit orchards without comprising the quality of the fruits, being toxic for the environment and also avoiding the acquisition of resistance by the bacteria.

Phage therapy (which is based on the use of bacteriophages to selectively control bacteria) (Almeida et al., 2009) is one of these approaches already studied to biocontrol the kiwifruit canker disease (Pinheiro et al., 2019).

Antimicrobial photodynamic therapy is another promising alternative and previous studies have already demonstrated the efficiency of this approach to photoinactivate the Psa both *in vitro* and *ex vivo* (in kiwi leaves), using cationic porphyrins, and no negative impacts have been observed in leaves (Jesus et al., 2018; Martins et al., 2018).

1.3. Photodynamic Therapy

1.3.1. Discovery

The first reported of the now-called antimicrobial photodynamic therapy (aPDT) was published in 1900, by Raab. This report shows the inactivation of *Paramecium caudatum* when exposed to sunlight, in the presence of dyes (acridine or eosin) (Wainwright et al., 2017). In 1905, a similar approach was used to tumour cells and reported by Von Tappeiner and Jesionek, Raab's supervisors (Wainwright et al., 2017).

Photodynamic therapy is clinically used as a cancer treatment in hospitals and clinics worldwide for over 25 years (Cieplik et al., 2018; Wainwright, 1998; Wainwright et al., 2017). However, antimicrobial applications of this therapy are not yet valued by clinicians and other health-care responsibles (Wainwright et al., 2017).

The beginning of the so-called era of resistance triggered a more intensive and extensive focus on antimicrobial alternatives to the conventional agents. The broad spectrum of action and multi-target character of PDT lowers the risk of resistance development and presents as an advantage towards the conventional antimicrobial agents (*i.e.* antibiotics) and for that reason, antimicrobial applications of the photodynamic processes gained and are still getting special attention among medical and scientific communities (Cieplik et al., 2018; Wainwright et al., 2017).

1.3.2. Mechanisms of action

Photodynamic therapy requires the combination of 3 compounds: molecular oxygen, visible or near infrared light and a photosensitizer (PS) (Almeida et al., 2015; Alves et al., 2013, 2015; Costa et al., 2012; Wainwright et al., 2017).

This therapy requires that the PS after light irradiation has the capability to absorb a photon in order to transfer an electron from its ground state (S_0) to a short-lived excited singlet state (S_n). From here, the PS can return to the ground state (S_0) by emission of

fluorescence (F) or by dissipation of heat (H); alternatively, an inter-system crossing (ISC) process can occur giving rise to a longer-living triplet state (T_1) that may interact with substrates and/or molecular oxygen ($^3O_2$). This interaction can afford reactive oxygen species (ROS) by two type of mechanisms. In **Type I** mechanism**,** charge (electrons) from de PS are transferred to surrounding substrates, forming radical ions that react with oxygen and produce reactive species, such as superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and/or highly reactive free hydroxyl radicals (HO•). In **Type II** mechanism, energy is transferred directly from the excited PS to the triplet ground state molecular oxygen ($^3O_2$), forming excited singlet oxygen ($^1O_2$) that may oxidize biological molecules. At the end of each cycle, the PS in its ground state (S_0) has the capability to absorb a new photon and to generate more ROS (Alves et al., 2014; Cieplik et al., 2018; Hamblin & Hasan, 2004; Lichtman & Conchello, 2005; Wainwright et al., 2017).

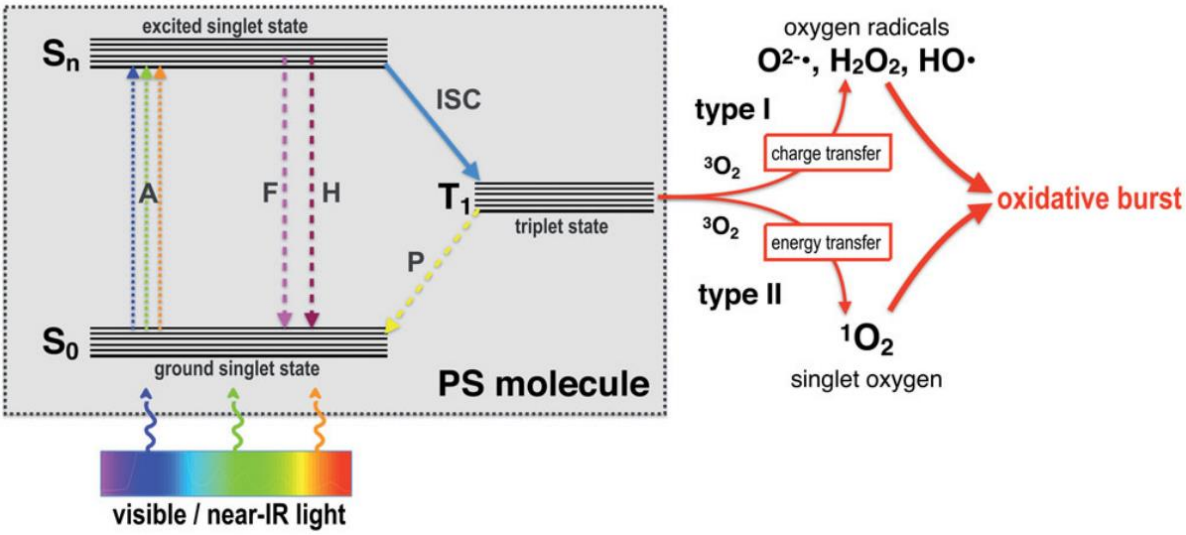

Figure 6. Photochemical and photophysical mechanisms of antimicrobial photodynamic therapy (aPDT) (Cieplik et al., 2018).

1.3.3. PDT components

1.3.3.1. Molecular Oxygen

The presence of molecular oxygen ($^3O_2$) is fundamental for this therapy, leading to the production of ROS through the two different mechanisms (Type I or Type II) already mentioned.

ROS have a broad spectrum of action without a specific cell target, being capable of act over various biomolecules such as proteins, lipids and nucleic acids (Almeida et al.,

2015; Alves et al., 2015). If ROS accumulate inside the cell it can lead to oxidative stress, which may then result in structural changes on the external structures, cytoplasmatic membrane and cell wall, resulting in loss of function (Almeida et al., 2015; Alves et al., 2014; Cieplik et al., 2018).

Singlet oxygen ($^1O_2$) generated via Type II mechanism of PDT is highly reactive and is characterized by having a very short lifetime as a result of its unstable electronic configuration (Alves et al., 2014).

1.3.3.2. Light sources

Another requirement for PDT is the presence of light that matches the activation spectrum of the PS (that corresponds to its peak/band of absorption), so that it can be excited (Wilson & Patterson, 2008).

Light can be natural (sunlight) or from an artificial white light source, being the three main light sources: lasers, LEDs and halogen lamps. Lasers are used due to their high efficiency and monochromaticity. Light emitting diodes (LEDs) are usually preferred over lasers because of their low cost and wider emission spectrum. Halogen lamps have the advantage of being cheaper, accessible and can be spectrally filtered to match the PS, but they cause more heating than lasers or LEDs (Cieplik et al., 2018; Wilson & Patterson, 2008).

Efficiency of PDT may vary with the type of light used, the intensity of light applied and the duration of the irradiation period. For aPDT, a longer irradiation period and/or bigger intensity of light may improve the efficiency of the inactivation, allowing the use of smaller quantities or concentrations of the PS (Alves et al., 2015).

For environmental *ex vivo* and *in vivo* applications, sunlight might be a great option making it easier and cheaper to apply. Previous aPDT studies have already demonstrated that is possible to reduce bacterial concentration of Psa *ex vivo* (in kiwifruit leaves) using sunlight with irradiance varying between 23 and 65 mW cm^{-2} (Jesus et al., 2018; Martins et al., 2018).

1.3.3.3. Photosensitizers

Photosensitizers are *per se* non-toxic molecules that can become reactive when irradiated with light with spectral range for its excitation (Wainwright et al., 2017). PDT's efficiency is highly related with the PS used and its characteristics.

PSs differ on their chemical, physical and electronic properties. The chemical structure of the PS may affect the type of mechanism (Type I or Type II) that is predominant during the PDT. Both mechanisms/pathways may occur simultaneously in some cases but usually one of them is preferred (Alves et al., 2014).

The net charge of the PS is also an important factor for its efficiency on photodynamic inactivation, in specific, cationic PSs are preferred on the photoinactivation of Gram-negative bacteria due to its structurally more complex composition (Alves et al., 2009, 2013).

In the last three decades, the discovery of new antimicrobial PSs (also known as photoantimicrobials) has been the subject of an extensive research as represented on **Figure 7** (Wainwright et al., 2017).

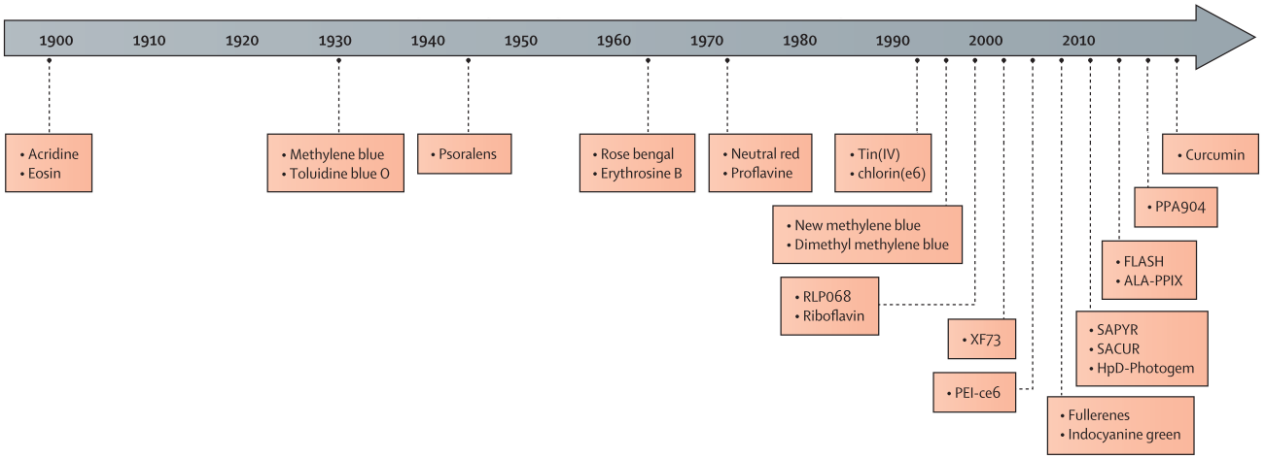

Figure 7. Timeline for photoantimicrobial discovery (*adapted from* Wainwright et al. 2017).

Among all the PS available, the most studied ones are: tetrapyrrolic macrocycles (e.g. porphyrins) and phenothiazinium (e.g. Methylene Blue) and xanthene (e.g. Rose Bengal) dyes (Alves et al., 2014, 2015).

Methylene Blue

Methylene Blue (MB) is one of the phenothiazinium dyes which is commonly used as a histological stain but also as a PS.

It is known that MB has the capability of binding to interfaces that are negatively charged. It is also known that MB has an absorbance band in the visible spectrum at 600-700 nm region and has little or none absorption on the remain of the visible spectrum, resulting in a blue appearance of this dye (Tardivo et al., 2005).

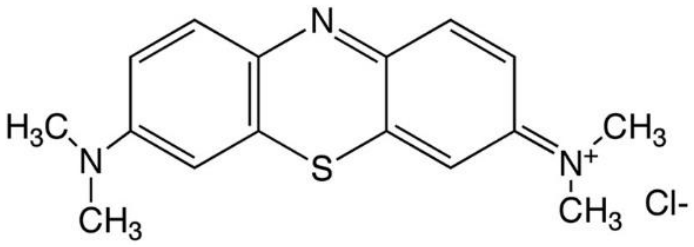

Figure 8. Methylene Blue molecular structure (Dai et al., 2011).

MB can be used as a photosensitizer for PDT and predominantly acts through Type I mechanism leading to the formation of ROS by electron transfer to oxygen, which can react with biomolecules (Alves et al., 2014). MB can as well act through Type II mechanism forming singlet oxygen that reacts with molecules from the cell wall/membrane (as peptides and phospholipids) (Huang et al., 2018; Wainwright & Crossley, 2002).

MB is clinically used and approved for treatment of some diseases and tumours (Tardivo et al., 2005), in dentistry (Teichert et al., 2002) and in the photodecontamination of blood plasma (Lozano et al., 2013; Sousa et al., 2019; Wainwright et al., 2007).

Previous studies already showed that MB was efficient in the inactivation of various microorganisms thought aPDT, namely Gram-positive bacteria such as *Staphylococcus aureus* and Gram-negative bacteria such as *Pseudomonas aeruginosa* (Pereira et al., 2018) and *Vibrio parahaemolyticus* (Deng et al., 2015). MB has also showed to have antimicrobial activity against fungi such as *Colletotricum abscissum* (Gonzales et al., 2017) and *Candida albicans* (Sousa et al., 2019; Teichert et al., 2002) and viruses (Floyd et al., 2004), when aPDT is applied.

New Methylene Blue

New Methylene Blue (NMB) is a derivative from Methylene Blue but with higher lipophilicity (Fekrazad et al., 2017). However, in terms of charge is also positively charged.

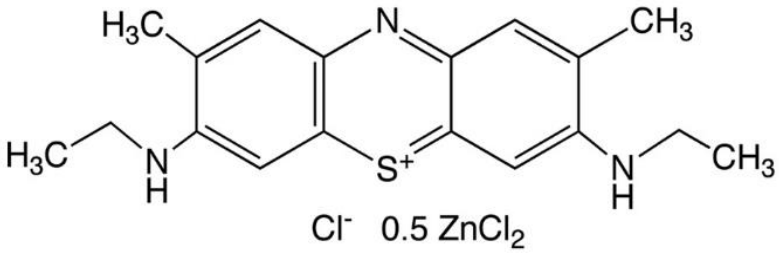

Figure 9. New Methylene Blue molecular structure (Dai et al., 2011).

NMB was also proved to be efficient in the inactivation of some microorganisms, *i.e.* some Gram-positive and Gram-negative bacteria (Phoenix et al., 2003), fungal species as *Candida albicans* (Fekrazad et al., 2015), *Trichophyton rubrum* (Fekrazad et al., 2017) and *Colletotrichum acutatum* (Menezes et al. 2014).

1.3.4. Inactivation of Gram-positive and Gram-negative bacteria by aPDT

Antimicrobial photodynamic therapy (aPDT) is used to kill microorganisms and it was already proved to be efficient against bacteria, viruses, fungi and protozoa (Almeida et al., 2015; Alves et al., 2013; Costa et al., 2012). However, the efficiency of this therapy is not the same for all the microorganisms (Almeida et al., 2015; Alves et al., 2009).

In specific for bacteria, Gram-positive and Gram-negative have different cell wall composition which determine the efficiency of photodynamic inactivation (Almeida et al., 2015; Alves et al., 2013).

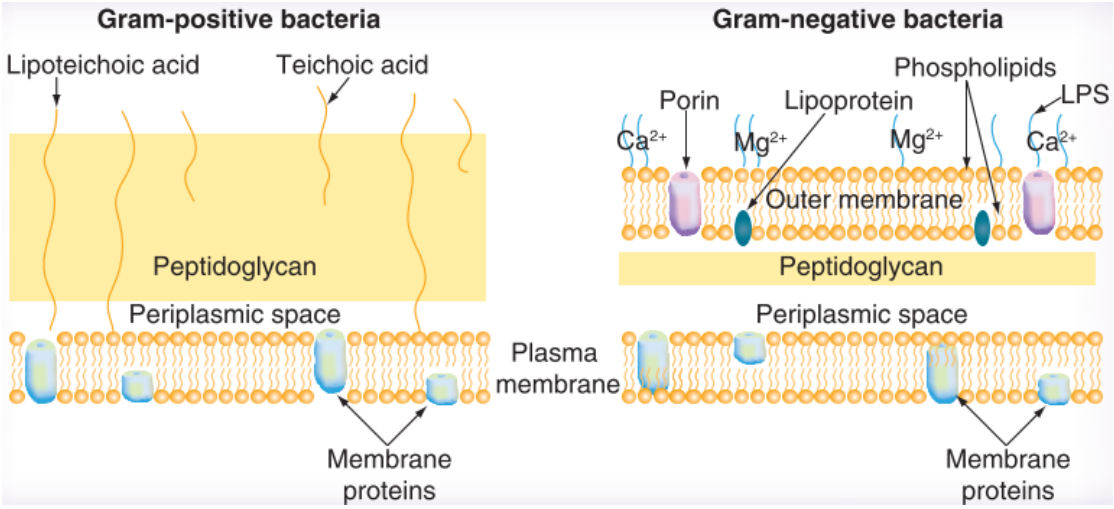

Figure 10. Gram-positive and Gram-negative cell wall composition (Alves et al., 2014).

Gram-positive cell wall is majorly composed by porous peptidoglycan layers with teichoic and lipoteichoic acids, that allows the penetration of PS into the cell (Alves et al., 2014). Anionic or neutral PS are able to destroy Gram-positive bacteria, but have less or none capability to inactivate Gram-negative bacteria (Alves et al., 2009).

Gram-negative bacteria have a highly organized and far more complex cell wall composition, constituted by an outer membrane (with a phospholipid bilayer, lipoproteins and lipopolysaccharides) which functions as an impermeable barrier and makes the inactivation trough aPDT more difficult (Alves et al., 2013, 2014).

In this case, the presence of positive charges has been showed to be an important factor to photo-inactivate Gram-negative bacteria. Cationic PSs can interact by electrostatic forces with Gram-negative's cell wall causing disruption of its organization, binding and consequently allowing the PS to enter into the cell (Alves et al., 2014; Kharkwal et al., 2011). Additionally, it was showed that membrane permeabilizing agents (as $CaCl_2$, EDTA, etc.) can help on the destabilization of the cell wall, facilitating the PS penetration into the cell. Also, the combination of aPDT with some organic or inorganic salts can improve its efficiency against Gram-negative bacteria, as is the case of potassium iodide (KI) which can increase the microbial inactivation rate in comparison with the use of PS alone (Hamblin, 2017; Vieira et al., 2018).

1.3.5. Potentiation of aPDT by potassium iodide

KI is an inert inorganic and non-toxic salt (Vecchio et al., 2015; Vieira et al., 2018) and was reported as a safe and effective drug (Food and Drug Administration, 2001). KI has a potentiator effect on aPDT, due to a sequence of parallel reactions (**Figure 11**). First, the $^1O_2$ reacts with KI (*i.e.* with I^-) producing peroxyiodide ($HOOI_2^-$), which can be decomposed by two different pathways, depending how the PS binds with the microbial cells. Free iodine (I_2/I_3^-) and hydrogen peroxide (H_2O_2) are stable species that can be formed through one of the pathways. The other pathway is based on the homolytic cleavage of $HOOI_2^-$, producing short-live and highly-reactive iodine radicals ($I_2^{\bullet-}$) that are highly toxic when generated next to the target cells (Hamblin, 2016; Santos et al., 2019; Vecchio et al., 2015; Vieira et al., 2018).

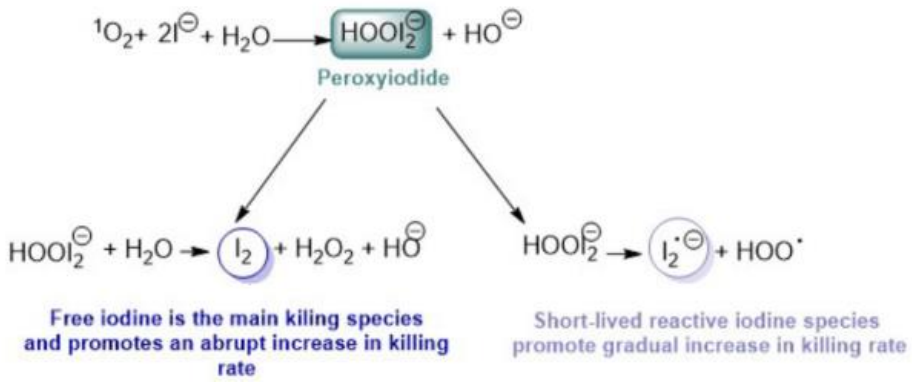

Figure 11. Schematic representation of peroxyiodide decomposition produced by the reaction of $^1O_2$ and KI (Vieira et al., 2018).

Various *in vitro* and *in vivo* studies have shown that the combination of KI with neutral porphyrins, fullerenes and dyes (e.g. Eosin, Rose Bengal and MB) increase the inactivation of several microorganisms such as *Acinetobacter baumannii*, *P. aeruginosa*, *Salmonella* Typhimurium, *Escherichia coli*, methicillin-resistant *S. aureus* and the fungal yeast *C. albicans* (Huang et al.,2017; Huang et al., 2018; Reynoso et al., 2017; Santos et al., 2019; Vecchio et al., 2015; Zhang et al., 2015).

1.3.6. aPDT in the control of Psa

Psa is a Gram-negative bacterium which may difficult its inactivation. However, previous studies have already successfully demonstrate that it is possible to photoinactivate Psa *in vitro* and in *ex vivo* (in kiwifruit leaves) with cationic porphyrins, *i.e.* with Tetra-Py(+)-Me (Jesus et al., 2018) and with a formulation of five cationic porphyrins (Martins et al., 2018).

In order to control Psa dissemination, it is of major importance to treat kiwifruit pollen. However, there is still no approved treatment to efficiently do it and few efforts are being done.

Heat was studied as a potential alternative to inactivate the Psa present in the pollen. The combination of 35 °C with relative humidity at 50% or less, during more than 20 h, was found to be the treatment with more potential to control the Psa without loss of pollen viability (Everett et al., 2012).

According to the APK, kiwifruit pollen can be stored at - 20 °C until 3 years maintaining its viability. This process is also thought to avoid pollen contamination and is currently the unique measure taken to control Psa in pollen. As kiwifruit pollination is frequently artificially performed with acquired pollen (which is also very expensive), it is of utter importance to develop an effective method to ensure the absence of Psa in pollen and at the same time to maintain its viability.

As antimicrobial photodynamic therapy was already proved to be efficient *in vivo* and *ex vivo* to control the Psa in buffer and in kiwifruit leaves, it is thought to be a promising alternative to inactivate this bacterium in contaminated pollen. In this context, the work **Antimicrobial photodynamic therapy in the control of *Pseudomonas syringae* pv. *actinidiae* transmission by kiwifruit pollen** was developed and presented.

CHAPTER 2 – Antimicrobial photodynamic therapy in the control of *Pseudomonas syringae* pv. *actinidiae* transmission by kiwifruit pollen

Abstract

Pseudomonas syringae pv. *actinidiae* (Psa) is a phytopathogen responsible for bacterial canker in kiwifruit plants, highly affecting kiwifruit production worldwide, which can be disseminated through pollen. The aim of this study was to evaluate the effectiveness of aPDT in the inactivation of Psa in kiwifruit pollen using New Methylene Blue (NMB) and Methylene Blue (MB) *in vitro* and in *ex vivo* in the presence or absence of potassium iodide (KI). Pollen germination assays were also performed to evaluate if it was affected by aPDT.

Higher reduction of Psa was achieved using NMB combined with KI. The NMB (5.0 μM) and KI (100 mM) allowed the total inactivation of the bacterium (*ca.* 8 log CFU mL^{-1}) after 90 min of irradiation. This PS (5.0 μM) alone promoted a significantly lower reduction (3.7 log CFU mL^{-1}). The most efficient NMB condition (5.0 μM of PS and 100 mM of KI) was used to study the photodynamic efficiency of MB. MB combined with KI photoinactivated more efficiently the Psa *in vitro* than NMB, causing the same bacterial reduction (*ca*. 8 log CFU mL^{-1}) in half of the irradiation time (45 min). Therefore, MB was selected for the subsequent *ex vivo* aPDT assays in pollen. Almost all the Psa cells added artificially to the pollen (3.2 log CFU mL^{-1}), were photoinactivated by aPDT (3.1 log CFU mL^{-1}), whereas aPDT had a low effect upon pollen natural microorganisms. When KI was combined with the MB, a significant increase in aPDT effectiveness was observed (reduction of 4.5 log CFU mL^{-1}) in the pollen artificially contaminated. No negative effects were observed in the pollen germination after aPDT.

The results showed that aPDT is an effective and safe method to inactivate the Psa in kiwifruit pollen, indicating that aPDT with a PS already approved and with low cost (MB), can be a promising alternative in the control of Psa transmission by the kiwifruit pollen.

Keywords: Antimicrobial photodynamic therapy, photosensitizers, bacterial infection, *Pseudomonas syringae* pv. *actinidiae,* kiwifruit pollen

2.1. Introduction

Pseudomonas syringae pv. *actinidiae* (Psa) is a phytopathogen and the causal agent of bacterial canker of both green-fleshed kiwifruit (*Actinidia deliciosa*) and yellow-fleshed kiwifruit (*Actinidia chinensis*), resulting in massive damages in the orchards (Takikawa et al., 1989) and provoking severe economic losses worldwide (EPPO, 2014; Scortichini et al., 2012). Psa was first isolated, identified and described in Japan in 1984 (Takikawa et al., 1989). Afterwards, until 2008, Psa was also found in China, Italy and South Korea (Donati et al., 2014; Scortichini, 1994; Scortichini et al., 2012). However, more recently Psa has re-emerged causing destruction of the main areas of kiwifruit production worldwide, being considered as a pandemic disease (Donati et al., 2014; EPPO, 2014; Scortichini et al., 2012).

Presently there are six genetically different biovars of Psa spread all over the world (Scortichini et al., 2012), varying on their aggressiveness to the plant. Biovar 3 is highly pathogenic and is affecting kiwifruit orchards in several countries worldwide (as New Zealand, Italy, France, Spain, Portugal and China) (Abelleira et al., 2014; Scortichini et al., 2012), being called “Psa-V” (from virulent) due to its high aggressiveness (Abelleira et al., 2014; Chapman et al., 2012).

Psa control and treatment is a challenge. The approved treatments for this disease consist of spraying the orchards with copper derivatives and/or antibiotics, but such treatments are highly toxic to both humans and the environment and may lead to the development of bacterial resistance (Cameron & Sarojini, 2014; Jesus et al., 2018). Antimicrobial photodynamic therapy (aPDT) can be a promising alternative, allowing to surpass the negative impacts of the current treatments.

aPDT requires the combination of three components: a photosensitizer (PS), visible light and molecular oxygen, resulting in the production of reactive oxygen species (ROS) which affect lipids, proteins and nucleic acids (Almeida et al., 2015; Alves et al., 2013, 2015; Costa et al., 2012), causing irreversible damages in microorganisms. The multitarget nature of aPDT minimizes the risk of bacterial resistance development, which provides an advantage over conventional treatments (Tavares et al., 2010; Tavares et al., 2011; Bartolomeu et al., 2016, 2017 , 2018; Jesus et al., 2018).

aPDT has been tested in the control of plant diseases caused mainly by fungi (Gonzales et al., 2010; Luksiene et al., 2004; Menezes, Pereira et al., 2014; Menezes, Rodrigues et al., 2014), but only very recently the aPDT application in the control of Psa-induced kiwifruit canker has been reported by our research group (Jesus et al., 2018; Martins et al., 2018), without negative effects on the leaves of kiwifruit plants. The results

showed that aPDT mediated by cationic porphyrins was able to inactivate successfully Psa both *in vitro* and in *ex vivo* (in kiwifruit leaves) conditions (Jesus et al., 2018; Martins et al., 2018).

Kiwifruit pollen has been proven to be responsible for the dissemination of Psa among orchards (Donati et al., 2018). However, few efforts have been taken to efficiently inactivate Psa in the pollen in order to control its dissemination. In fact, there is no approved treatment yet to efficiently inactivate Psa in the kiwifruit pollen. The use of heat as a potential method to inactivate the Psa present in the pollen was already studied (Everett et al., 2012). The authors found that the combination of 35 °C with relative humidity at 50% or less, during more than 20 h, was the treatment conditions with more potential, so far, to control the Psa without loss of pollen viability. According to the Portuguese Association of Kiwifruit growers (APK, Associação Portuguesa de Kiwicultores), kiwifruit pollen stored at -20 °C up to 3 years preserves its viability and avoids also its contamination, which is the only measure that is taken to control Psa in pollen. Moreover, assisted pollination is increasing worldwide. In Portugal, for example, assisted pollination currently already represents 10 – 15% of the kiwifruit production (APK). Once assisted pollination uses acquired pollen, which is very expensive (2,000 – 3,500 €/kg), it is of extreme importance to develop an effective method to ensure the absence of Psa in sold pollen and at the same time maintaining its viability.

In the present study, the inactivation of Psa was first studied in *vitro* using two well known phenothiazine derivatives; New Methylene Blue (NMB) and Methylene Blue (MB) in the presence and the absence of potassium iodide (KI) in order to find the best conditions to photoinactivate Psa. Then, the efficiency of aPDT to control Psa contamination in kiwifruit pollen was studied using the best conditions found. Pollen germination was also quantified in order to evaluate the pollen viability following the aPDT treatment. The *in vitro* assays were performed in phosphate buffer solution (PBS) and the *ex vivo* assays, using the trade kiwifruit pollen artificially contaminated with a Psa Biovar 3, the CRA-FRU 8.43 strain. In fact, Biovar 3 is highly aggressive and was the phytopathogen responsible for the global pandemic of kiwifruit firstly reported in Italy in 2008.

2.2. Materials and Methods

To evaluate the potential of aPDT with NMB and MB in the photoinactivation of Psa and to select the best photoinactivation conditions to be used in the pollen disinfection, *in vitro* assays were performed. The first experiments *in vitro* were performed in phosphate buffered saline (PBS), using NMB at different concentrations (1.0, 2.5 and 5.0 μM) without and with KI (an aPDT potentiator) (Santos et al., 2019; Vieira et al., 2018) at a concentration of 100 mM. In a second step, the best *in vitro* aPDT conditions established for NMB were applied to MB (5.0 μM of MB with and without the addition of 100 mM of KI). Then, the best tested PS (MB) was used in *ex vivo* assays, using trade kiwifruit pollen (containing its natural microorganisms) and trade kiwifruit pollen also artificially contaminated with Psa. In these *ex vivo* assays, the MB concentration was increased to 50 μM, accordingly to our previous studies (Jesus et al., 2018; Martins et al., 2018). The natural microorganisms present in the kiwifruit pollen (total viable count, fungi and bacterial endospores) were quantified in the trade pollen samples by viable plate count and the Schaeffer-Fulton endospore stain was also performed. To evaluate the efficiency of pollen contamination with PSA, which was used in *ex vivo* assays, the Psa concentration was determined in the pellet (obtained by centrifugation from a Psa overnight culture) used to contaminate the pollen sample and in the pollen samples before the aPDT experiments. Lastly, the pollen germination ability was evaluated following aPDT treatment.

2.2.1. Bacterial strain and growth conditions

The *Pseudomonas syringae* pv. *actinidiae* strain CRA-FRU 8.43 (Psa 3, also referred as Psa V or Biovar 3) isolated in Lazio, Italy, in 2008, and obtained from the Culture Collection of the Centro di Ricerca per la Frutticoltura (Rome, Italy) (Ferrante & Scortichini, 2010; Firrao et al., 2018) was used. The bacterial strain was grown in Luria Bertani Agar (LA, Liofilchem, Italy) at 25 °C for 48 h and then kept at 4 °C. Before each assay, a colony of the bacteria was aseptically inoculated in 30 mL of Tryptic Soy Broth (TSB, Liofilchem, Italy) and grown aerobically for 24 h at 25 °C under stirring (120 rpm). The viable cell density was approximately 10^8 colony-forming units per mL (CFU mL^{-1}).

2.2.2. Kiwifruit pollen

The kiwifruit trade pollen was kindly supplied by Portuguese Association of Kiwifruit growers (APK, Associação Portuguesa de Kiwicultores, Santa Maria da Feira, Portugal). The pollen, obtained in dried form, was stored at -20 °C in Falcon tubes duly sealed with Parafilm™.

2.2.2.1. Quantification of pollen natural microorganisms and endospore stain

The natural microorganisms of pollen – total viable count, fungi cells and bacterial endospores, were quantified by plating the pollen suspensions (prepared in PBS) in LA and Rose-Bengal Chloramphenicol Agar (RBCA) (Merck KGaA, Darmstadt, Germany). For that, two aqueous suspensions of 10 mg of pollen in 500 μL PBS were prepared. One of these suspensions was ten-folded diluted (10^0 to 10^{-3}) in PBS and 100 μL of each dilution were spread-plated in LA and RCBA media. The LA plates were incubated at 25 °C for 42 h and the RBCA plates at 25 °C for 5 days. The other suspension was heated at 80 °C for 10 min before plating in LA in order to determine the number of bacteria forming endospores. After the incubation periods, the number of CFU was counted on the most appropriate dilution. Three independent assays were performed for each different condition.

The presence of bacteria forming endospores was also evaluated by observing pollen samples under the optical microscope. 100 μL of the non-heated pollen suspension (non-diluted - 10^0) was spread-plated in Nutrient Agar (NA) (Merck KGaA, Darmstadt, Germany) enriched with 10 mg/L of Manganese Sulphate ($MnSO_4$) and the plate was incubated for 3 days at 25 ºC. The Schaeffer-Fulton endospore stain method (Schaeffer & Fulton, 1933) was used to distinguish between the vegetative cells and the endospores present in the pollen. The malachite green was used to stain the endospores and the safranin was used to stain the vegetative cells. The prepared slides were then observed under the optical microscope at 1000x total magnification (Upright Microscope - ECLIPSE 80i equipped with Digital Sight DS-U3 for data acquisition and Nikon Digital Sight DS-Ri1 camera (Tokyo, Japan)).

2.2.3. aPDT assays

2.2.3.1. Photosensitizers

A stock solution (500 μM) of New Methylene Blue (CAS 6586-05-6, Sigma-Aldrich®/Merck KGaA, Darmstadt, Germany) was prepared in dimethyl sulfoxide (DMSO). A stock solution (500 μM) of Methylene Blue (CAS 122965-43-9, Sigma-Aldrich®/Merck KGaA, Darmstadt, Germany) was prepared in PBS. Both stock solutions were wrapped in aluminium foil and stored in glass flasks in the dark, being sonicated in an ultrasonic bath for 15 min before each assay.

2.2.3.2. Light sources

The artificial white light was provided by a LED projector, (EL®MARK, power, voltage and frequency of 20 W, ~230 V and ~50 Hz, respectively). The light irradiance was measured and adjusted to 50 mW.cm^{-2} with the aid of a power and energy meter (model FieldMaxII-Top from Coherent, Santa Clara CA, USA) connected to a high-sensitivity sensor (model PS19Q, Coherent).

2.2.3.3. *In vitro* aPDT assays with NMB and MB

A bacterial suspension (overnight inoculum with 10^9 CFU mL^{-1} diluted tenfold in PBS to a final concentration of ≈10^8 CFU mL^{-1}) was prepared and 5.0 mL were distributed in Petri plates (ϕ = 40 mm) with the NMB added to a final concentration of 1.0, 2.5 or 5.0 μM. The Petri plates were incubated in the dark during 10 min at room temperature under magnetic stirring (100 rpm), to promote the PS binding to the cells. Light and dark controls were also prepared: the light control (LC) contained only the bacterial suspension; the dark control (DC) contained the bacterial suspension incubated with the PS at the studied concentration but was protected from light with aluminium foil. Sample and LC were irradiated under white light at an irradiance of 50 mW cm^{-2} for 90 min; the DC was kept in the dark for the same period of aPDT treatment. Aliquots of the sample, LC and DC were collected at time 0 (after the pre-irradiation period and immediately before irradiation) and after 5, 15, 30, 45, 60 and 90 min of irradiation, serially diluted in sterile PBS and 3 droplets of 5.0 μL of each dilution were plated in LA. The plates were then incubated at 25 °C for 48 h and the number of CFU was counted on the most appropriate dilution on the agar plates.

Similar assays were performed using the same NMB concentrations (1.0, 2.5 and 5.0 μM) with the addition of KI (at a concentration of 100 mM). In these experiments, besides the LC, two other controls were included: a LC+KI (with KI at 100 mM) and a DC(MB+KI) (with the NMB at the studied concentration and KI at 100 mM maintained in the dark).

The assays with MB were done only at the concentration of 5.0 μM with and without the addition of KI at a final concentration of 100 mM.

Three independent assays in triplicate were performed for each different condition.

2.2.3.4. *Ex vivo* aPDT assays with MB in kiwifruit pollen

aPDT assays were performed with kiwifruit pollen containing its natural microorganisms (not contaminated artificially with Psa, hereafter designated as non-contaminated pollen) and in kiwifruit pollen containing its natural microorganisms and artificially contaminated with Psa.

For the assays with non-contaminated pollen, 10 mg of pollen were weighed into an eppendorf, added with 500 μL of sterile PBS and serially diluted (10^0 to 10^{-5}) in PBS.

Petri plates (ϕ = 40 mm) containing 40 μL of MB at 50 μM, spread on top of LA were prepared in duplicate, one to be used at the beginning of the assay (T_0, not irradiated), and the other to be irradiated (T_F). Light and dark controls were prepared simultaneously: to the light control (LC), 40 μL of sterile PBS were spread on the surface of LA (without addition of MB); the dark control (DC) was prepared in the same way as the sample (with 40 μL of MB at 50 μM spread on top of LA plate) but was wrapped with aluminium foil to avoid the light incidence. These plates were divided into hexants (labeled 10^0 to 10^{-5}) and two 5.0 μL-droplets of each dilution of pollen suspension were plated into the corresponding hexant. A pre-incubation of 1 h in the dark was made, in order to allow the PS binding to the bacterial cells. The sample and LC plates were irradiated with white light at an irradiance of 50 mW cm^{-2} for 180 min, whereas DC plate was kept protected from light for the same period. The plates were then incubated at 25 °C for 48 h and the number of CFU was counted on the most appropriate dilution on the agar plates. Three independent assays in duplicate were performed.

Similar assays were performed using KI as a potentiator agent. A solution containing MB at the concentration of 50 μM and KI at 100 mM was prepared and 40 μL of this solution were spread on the top of two LA plates – one used to do the treatment (MB+KI) and the second one used as the dark control [DC(MB+KI)] which was protected from light during the irradiation period. Three independent assays in duplicate were performed.

The same procedure was used for the aPDT assays performed with the kiwifruit pollen artificially contaminated with Psa. The assays were also performed with MB in the absence and the presence of KI.

To contaminate the pollen with Psa, 5.0 mL of an overnight Psa culture were centrifuged (5 min at 5,000 rpm), the supernatant discarded, and the pellet resuspended in 1.0 mL of PBS. This bacterial suspension was added to 50 mg of pollen, gently homogenized via inverting and swirling, and incubated for 30 min at room temperature. The resulting mixture was then poured into sterilized standard filter paper and left to dry out in a laminar flow chamber for *ca.* 15 min. Subsequently, the pollen was scrapped off the filter paper, weighed again, and 10 mg were suspended in 500 μL of PBS, serial diluted (10^0 – 10^{-6}) and then plated using the procedure used in the aPDT experiments with the non-contaminated pollen.

The success of the artificial contamination of pollen with Psa was also evaluated before the aPDT experiments. A suspension of 10 mg of non-contaminated pollen and a suspension of 10 mg of artificially contaminated pollen were both prepared in 500 μL of PBS, serially diluted (10^0 – 10^{-8}), and three 5.0 μL-droplets of each dilution were plated in LA. The resuspended pellet resulting from the centrifugation of the overnight Psa inoculum that was used to artificially contaminate the pollen was similarly diluted and plated. All the LA plates were incubated at 25 °C for 48 h and the number of CFU was counted on the most appropriate dilution on the agar plates.

The schematic representation of the experimental procedure followed for the *ex vivo* aPDT assays is depicted in detail in **Figure 1**.

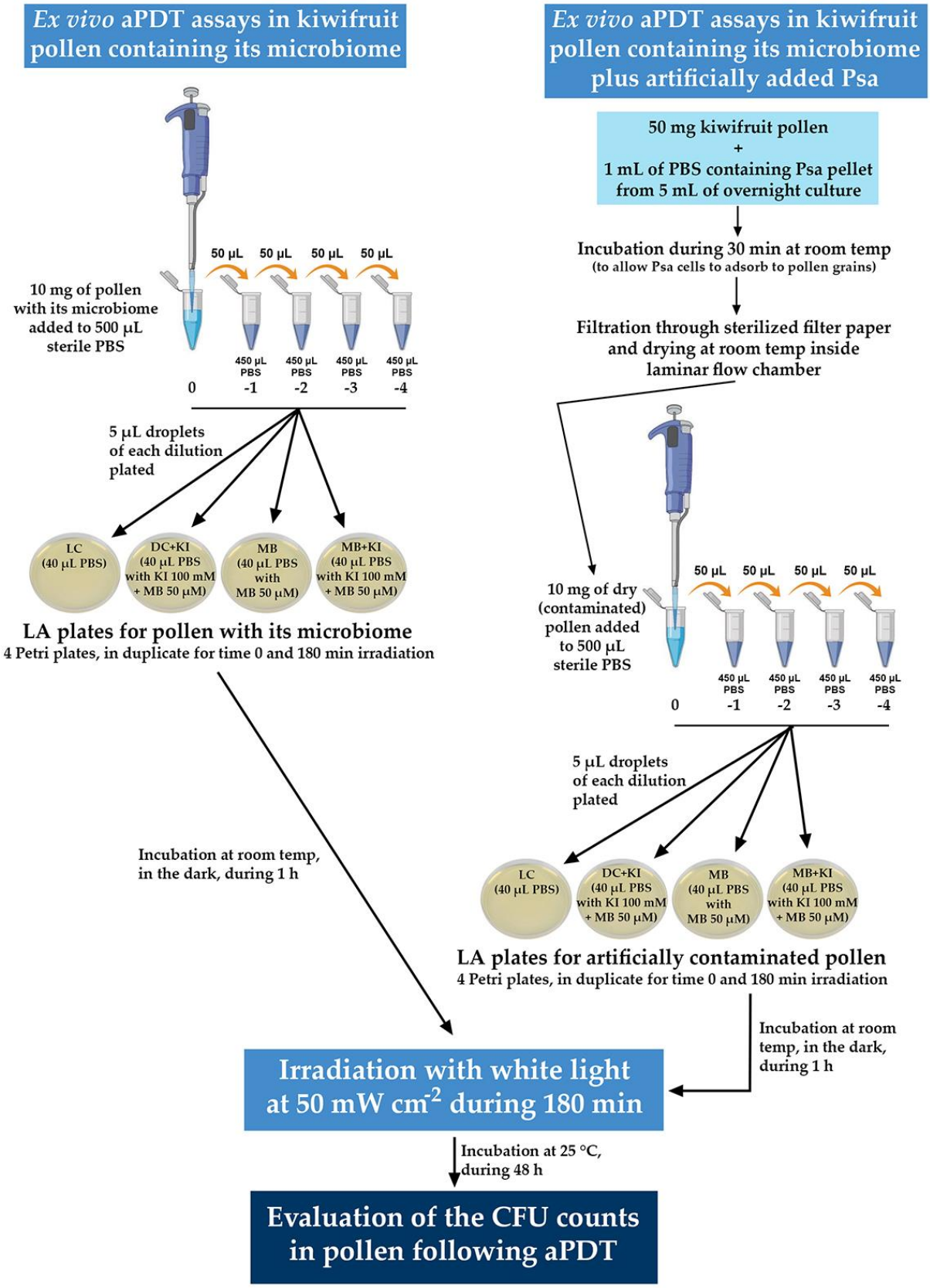

Figure 1. Schematic representation of the experimental procedure followed for the *ex vivo* aPDT assays.

2.2.4. Evaluation of pollen germination after aPDT

To assess if the pollen viability was affected after aPDT, the pollen germination ability was evaluated. For this, 5.0 mg pollen were weighed into eppendorf tubes and added with 200 μL PBS. To the pollen sample (MB) 40 μL of MB at 50 μM was added; to the sample added with KI (MB+KI), 40 μL of a solution containing MB at 50 μM and KI at 100 mM was added. Light and dark controls were also prepared: to the light control (LC) 40 μL of PBS was added; to the dark control [DC(MB+KI)] 40 μL of the same solution prepared with MB at 50 μM and KI at 100 μM was added. The resulting suspension mixtures were homogenized by gently inverting and swirling, after which they were transferred into the corresponding well on 12-well cell culture plates. All the samples were prepared in duplicate: one for T_0 (without irradiation) and the other to be irradiated (T_F). LC, MB and MB+KI were then irradiated with artificial white light at an irradiance of 50 mW cm^{-2} for 180 min. DC(MB+KI) was protected from light via wrapping with aluminum foil.

After aPDT treatment, to each well in the plates from the previous step were added 1.20 mL of pollen germination medium (KCl 100 mg/L, H_3BO_3 100 mg/L, $CaCl_2$ 300 mg/L, $MgSO_4$ 200 mg/L, sacarose 2.5%, PEG 10%, pH 7.5) and the plates were incubated at 25 °C during 24 h under gentle orbital shaking. After the incubation period, three microscope slides were prepared for each suspension using 100 μL aliquots of the suspension and observed under 100x total magnification (Upright Microscope - ECLIPSE Ni-U equipped with CoolLED pE-300-W, Digital Sight DS-U3 for data acquisition and Nikon Digital Sight DS-Qi1Mc camera (Tokyo, Japan)). For each slide, at least 25 photomicrographs were gathered, amounting to a total of 75 photomicrographs for each suspension/well. Pollen germination was evaluated by calculating the percentage of germinated pollen grains out of a total number of pollen grains in the 75 photomicrographs.

The schematic representation of the experimental procedure followed for evaluating the pollen germination is depicted in detail in **Figure 2**.

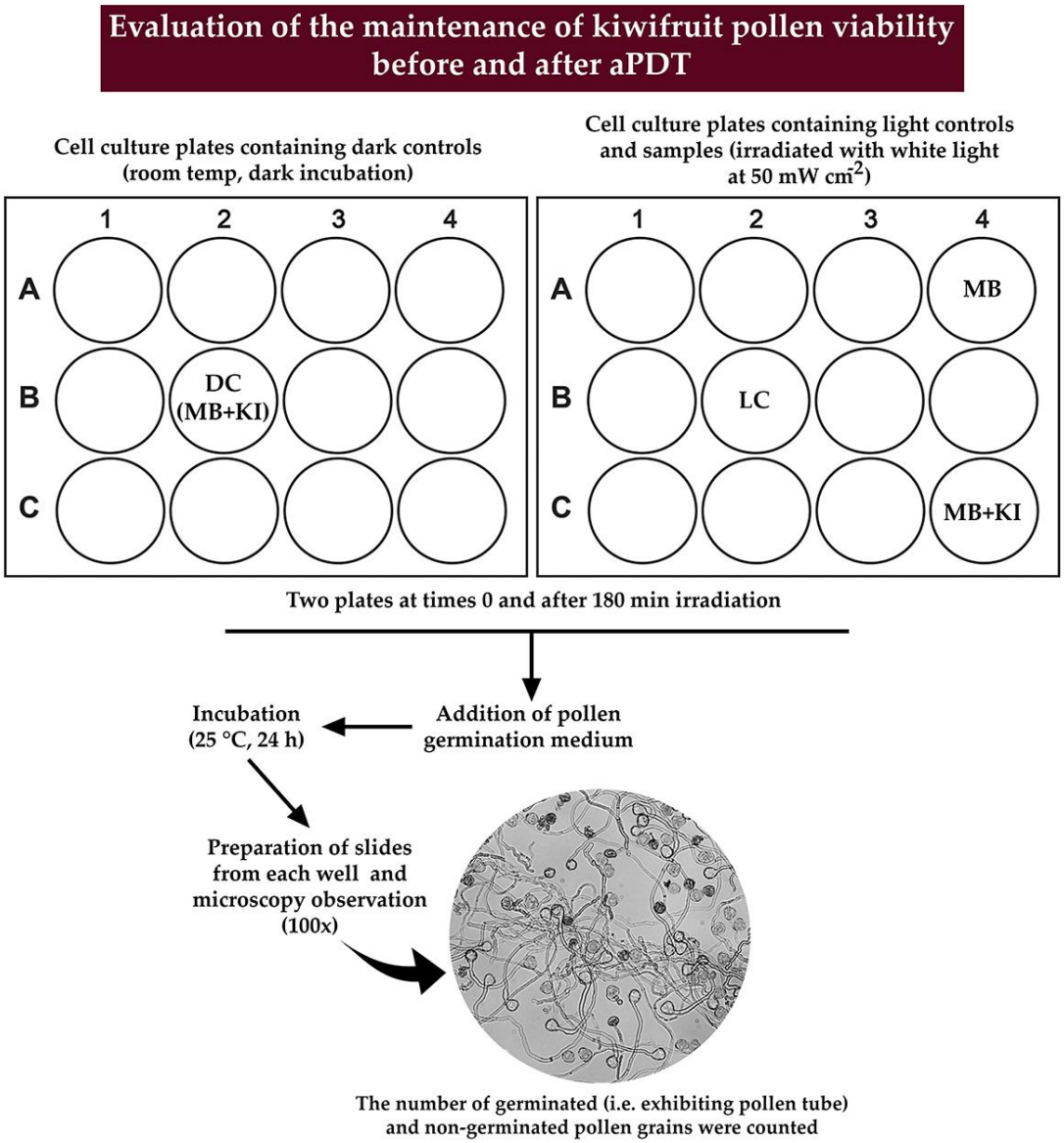

Figure 2. Schematic representation of the experimental procedure used to evaluate the pollen germination after aPDT.

2.2.5. Statistical analyses

Statistical analysis of the data was carried out using the software GraphPad Prism 7.04 (GraphPad Software, San Diego CA, USA). The normal distribution of the data was checked by a Kolmogorov-Smirnov test and the homogeneity of variance was assessed by the Brown Forsythe test. For a pairwise comparison of the means was used the Tukey's multiple comparison test. The significance of bacterial concentrations between treatments, and along the experiments, was tested using two-way ANOVA and Dunnet's multiple comparison tests were applied to assess the significance of the differences

between the tested conditions. For different treatments, the significance of differences was evaluated by comparing the results obtained in the test samples after treatment with the results obtained for the correspondent test samples before treatment. For all cases, at least three independent assays in duplicate were performed. In the case of *ex vivo* aPDT assays with MB in kiwifruit pollen, six independent assays were performed using MB alone. A $p<0.05$ value was considered to be statistically significant.

2.3. Results

2.3.1.Quantification of pollen natural microorganisms and endospore stain

The results obtained show that the concentration of viable microorganisms present on kiwifruit pollen counted in LA was 5.1 log CFU mL^{-1} (**Figure 3**). The number of fungi counted in RBCA was 4.7 log CFU mL^{-1}. The number of bacterial endospores counted in LA after heating the sample to 80 ºC for 10 min was 3.6 log CFU mL^{-1}.

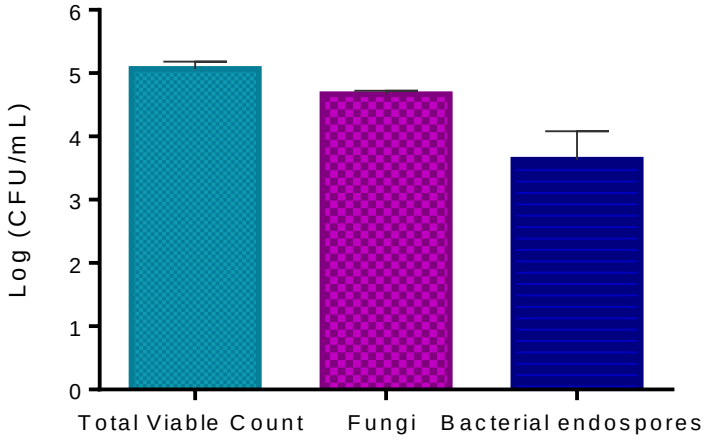

Figure 3. Quantification of natural microorganisms in trade pollen: total viable count, fungi and bacterial endospores. Values represent the mean of three experiments; error bars represent the standard deviation.

After the Schaeffer-Fulton endospore stain, the presence of endospores (as green ellipses) was visualized under microscopy (**Figure 4**).

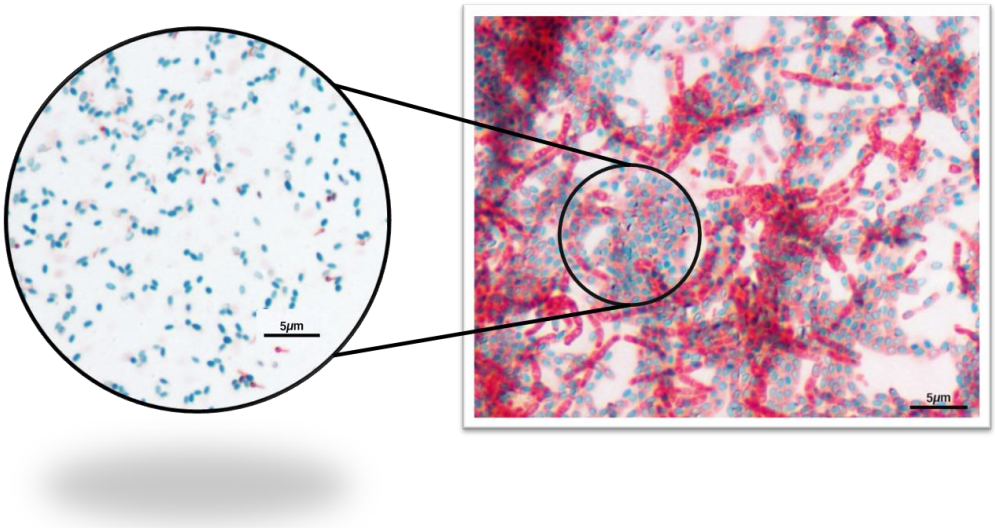

Figure 4. Photomicrographs of the pollen Schaeffer-Fulton endospore stain - 1000x total magnification.

2.3.2. *In vitro* aPDT assays with NMB and MB

The results obtained from the *in vitro* inactivation assays using NMB at 1.0, 2.5 and 5.0 μM with and without KI (**Figures 5a**, **5b** and **5c**) show that the highest reductions of Psa are achieved with NMB in the presence of KI comparing to the ones achieved with the NMB alone. The results also show that NMB at 5.0 μM with the presence of KI at 100 mM (**Figure 5c**) was the best tested condition to inactivate Psa, allowing the total inactivation of the bacteria (*ca.* 8 log CFU mL^{-1} (ANOVA, $p<0.05$)) after 90 min of white light irradiation at an irradiance of 50 mW cm^{-2}. This PS at 5.0 μM in the absence of KI had promoted a reduction of bacterial concentration significantly lower, with a maximum of *ca.* 3.7 log CFU mL^{-1} (ANOVA, $p<0.05$) reduction. When NMB was used at the concentration of 2.5 μM and combined with KI, the total photoinactivation of Psa was achieved after 120 min of irradiation (**Figure 5b**). With NMB at the lowest tested concentration (1.0 μM), a reduction of only *ca.* 3.6 log CFU mL^{-1} (ANOVA, $p<0.05$) in the bacterial concentration was achieved even in the presence of KI and after 240 min of light irradiation (**Figure 5a**).

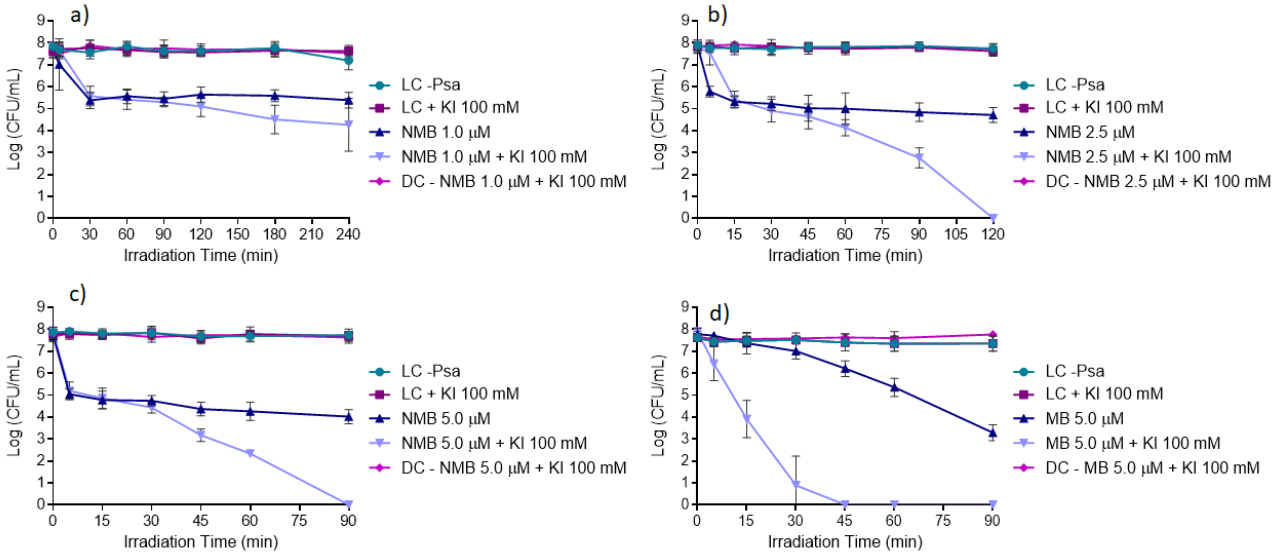

Figure 5. *In vitro* inactivation of Psa CRA-FRU 8.43 via aPDT, using NMB at **a)** 1.0 μM, **b)** 2.5 μM and **c)** 5.0 μM and **d)** MB at 5.0 μM as photosensitizers, in the absence and presence of KI (at 100 mM). Values represent the mean of three independent assays in triplicate; error bars represent the standard deviation. LC, light control; DC, dark control. Lines just combine the experimental points.

The photodynamic efficiency of MB was also studied using the most efficient NMB conditions (5.0 μM with the addition of KI at 100 mM) and the results represented in **Figure 5d**, show that the combination of MB with KI was more effective than NMB with KI, attaining the same reduction of *ca.* 8 log CFU mL^{-1} (ANOVA, $p<0.05$) in bacterial concentration (total inactivation) in just half of the irradiation time (45 min). Therefore, MB

was selected for the subsequent *ex vivo* aPDT assays in kiwifruit trade pollen.

It is also important to refer that all the controls performed, *i.e*. LC, light control; LC+KI, light control with KI and DC(PS+KI), dark control with PS+KI, remained stable during the whole experiment, indicating that Psa viability was not affected by light (LC) itself, neither by the PS alone nor PS plus KI in the absence of light [DC(PS+KI)].

2.3.3. *Ex vivo* aPDT assays in kiwifruit pollen with MB

2.3.3.1. Photoinactivation of pollen natural microorganisms

The results obtained in the *ex vivo* aPDT assays performed with MB at 50 μM with non-artificially contaminated pollen (containing only its natural microorganisms) and irradiated for 180 min with white light at an irradiance of 50 mW cm^{-2} showed that aPDT treatment induces a reduction of only 0.5 log CFU mL^{-1} (ANOVA, $p<0.05$) in the viability of the natural microorganisms after the irradiation protocol (**Figure 6**). When the MB combined with KI was used in the aPDT treatment of non-artificially contaminated pollen, a significant decrease of *ca.* 1.1 log CFU mL^{-1} (ANOVA, $p<0.05$) in the natural microorganisms survival (**Figure 6**) was obtained. Light (LC) and dark [DC(MB+KI)] controls do not show statistically significant reductions. Comparing the effect of MB with and without KI in the photoinactivation of natural microorganisms of trade pollen it was possible to see that MB+KI promotes a decreasing of more 0.6 log CFU mL^{-1} in the viability of the natural microorganisms than MB alone, showing the potentiator effect of KI.

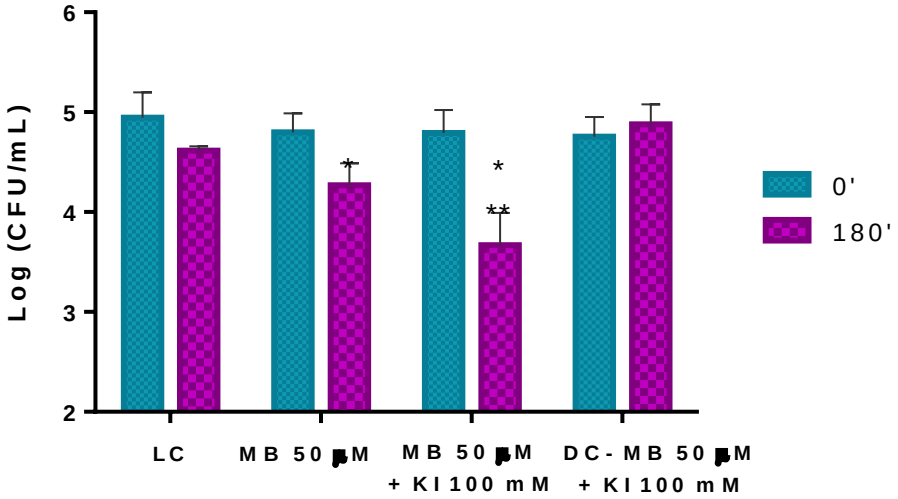

Figure 6. *Ex vivo* aPDT assay of pollen natural microorganisms, irradiated with 50 mW cm^{-2} for 180 min, using MB at 50 μM and MB at 50 μM with the addition of KI (at 100 mM). Values represent the mean of three independent assays in duplicate; error bars represent the standard deviation. *$p<0.05$ (relatively to each condition at time 0'); **$p<0.05$ (relatively to MB). LC, light control; DC, dark control.

2.3.3.2. Photoinactivation of artificial Psa contaminated pollen

To evaluate the aPDT action in Psa present in the kiwifruit pollen, the trade pollen was previously contaminated with Psa in order to be assured that this bacterium was also present in pollen besides its own natural microorganisms (**Figure 7**).

The Psa inoculum used to contaminate the pollen had a concentration of 9.7 log CFU mL^{-1}, leading to a total of 8.3 log CFU mL^{-1} on pollen (which corresponds to pollen natural microorganisms plus added Psa). This value is clearly above of pollen natural microorganisms concentration which is 5.1 log CFU mL^{-1}. These results indicate that *ca.* 3.2 log CFU mL^{-1} of Psa added to pollen.

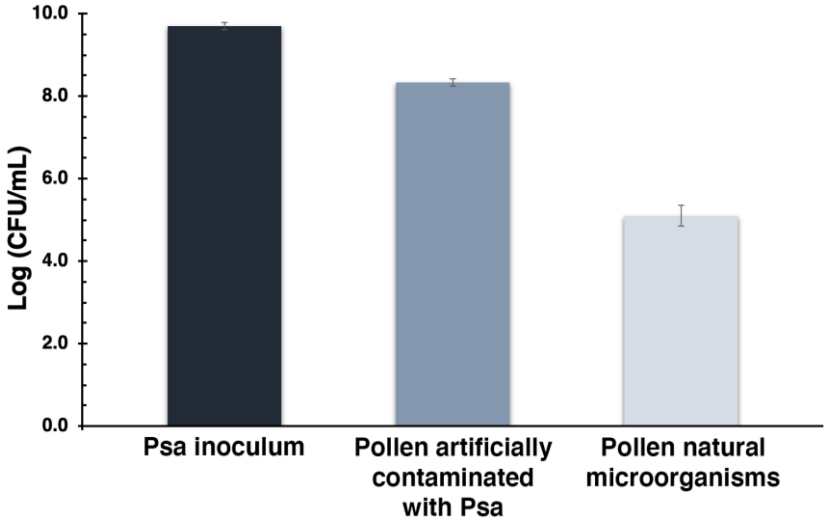

Figure 7. Efficiency of artificial pollen contamination with Psa. The first bar represents the Psa inoculum used to contaminate the pollen; the bar in the middle represents the total contamination achieved; the last bar corresponds to the pollen natural microorganisms. Values represent the mean of three independent assays in triplicate; error bars represent the standard deviation.

This pollen containing its natural microorganisms and artificially contaminated with Psa was then subjected to aPDT using MB at 50 µM alone and MB at the same concentration combined with KI at 100 mM and the results are presented in **Figure 8**. The results showed a remarkable aPDT effect performed by MB and MB+KI in the photoinactivation of Psa in kiwi pollen. When MB was used alone a reduction of *ca.* 3.1 log CFU mL^{-1} (ANOVA, $p<0.05$) of bacterial survival was achieved after the white light irradiation period of 180 min at an irradiance of 50 mW cm^{-2}.

When aPDT treatment was carried out with the combination of MB at 50 µM and KI at 100 mM a decrease of *ca.* 4.5 log CFU mL^{-1} (ANOVA, $p<0.05$) was observed under the same irradiation conditions. This corresponds to an additional significant decrease of

ca. 1.4 log CFU mL^{-1} (ANOVA, $p<0.05$) when comparing the results with the PS alone, showing, again, the potentiator effect of KI (**Figure 8**).

Also in this case, light and dark controls do not show statistically significant reductions.

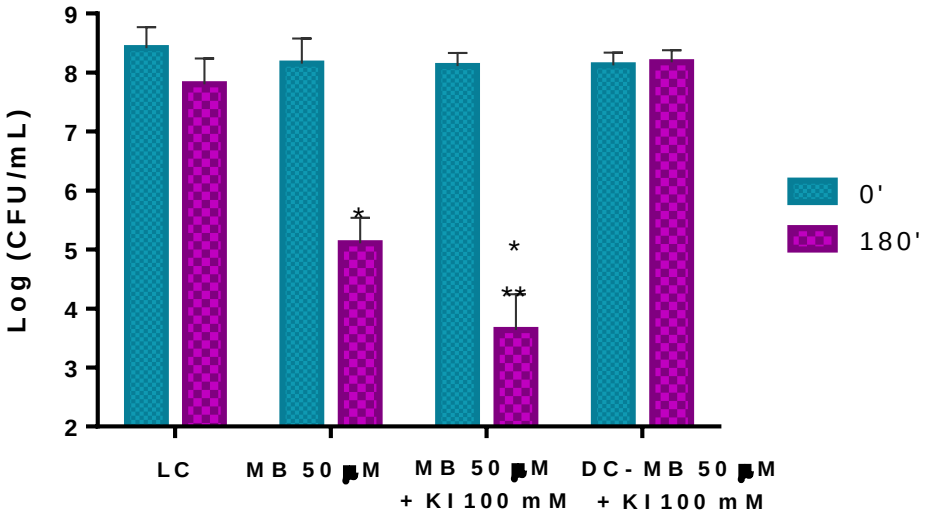

Figure 8. *Ex vivo* aPDT assay on pollen containing its natural microorganisms plus artificial Psa contamination, irradiated with 50 mW cm^{-2} for 180 min, using MB at 50 μM and MB at 50 μM with the addition of KI at 100 mM. Values represent the mean of three independent assays in duplicate; error bars represent the standard deviation. *$p<0.05$ (relatively to each condition at time 0'); **$p<0.05$ (relatively to MB). LC, light control; DC, dark control.

2.3.4. Evaluation of pollen germination after aPDT

The evaluation of pollen ability to germinate following aPDT assays was performed by assessing the capability of pollen grains to germinate before and after being subjected to aPDT. The results obtained and statistically analysed, clearly indicate that aPDT has no significant detrimental effect on pollen germination, with none of the samples reducing its germination percentage (**Figure 9**). The sample treated with MB exhibits a pollen germination of 44 and 51%, before and after aPDT respectively. The sample treated with MB combined with KI exhibits percentages of 46 and 60%, before and after aPDT respectively.

Light and dark controls also show that pollen percentages of germinations did not suffer reduction when submitted to aPDT.

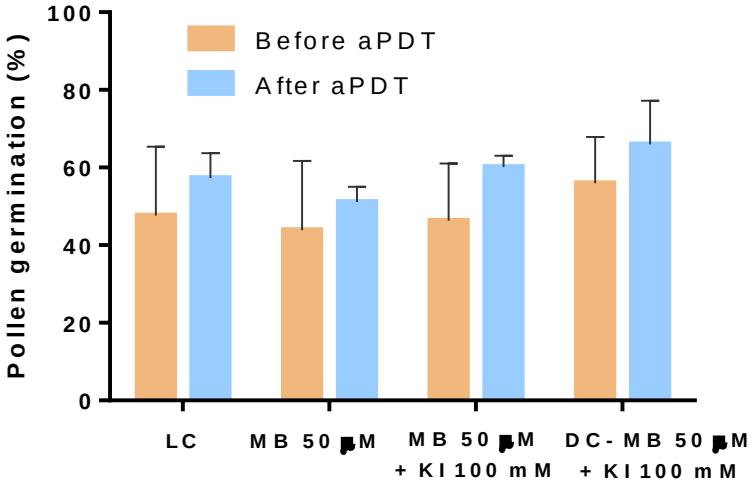

Figure 9. Evaluation of pollen germination before and after aPDT. Values represent the mean of pollen germination percentage resulting from three independent assays in triplicate; error bars represent the standard deviation.

2.4. Discussion

Kiwifruit pollen has been recognized as an important source and vector of Psa dissemination, hence elimination of Psa contamination in kiwifruit pollen is of utmost importance for the kiwifruit production sector. While there are not any antimicrobial treatments currently available for use in pollen, research is slowly progressing towards the development of potentially effective, while environmentally safe, antimicrobial approaches for this high-added value commodity. In the literature, only one study specifically related to the treatment of pollen using heat (Everett et al., 2012) was found. Two other recent studies have demonstrated that aPDT can be used to successfully control Psa in kiwifruit leaves (Jesus et al., 2018; Martins et al., 2018), which prompted to think that aPDT could be an efficient alternative to inactivate Psa from kiwifruit pollen.

Buffered solutions, such as PBS, are useful to evaluate the behaviour and efficacy of the PSs in a medium without organic matter and cell interference in order to select the best PS and the more appropriate aPDT conditions. However, as the composition of the test matrix is important in aPDT, in order to pave the real application, it is required to test the PSs in relevant settings, such as is the case of this study in pollen.

Firstly, *in vitro assays* were performed with NMB at three different concentrations (1.0, 2.5 and 5.0 μM) with and without KI (at 100 mM). These assays showed that when using KI as a potentiator agent, significant higher reductions of the bacteria survival are achieved when comparing with the ones obtained with the PS alone. It was also observed that the PS at 5.0 and 2.5 μM in the presence of KI allowed total inactivation (*ca.* 8 log CFU mL^{-1} reduction) of the bacteria, but the highest concentration led to a significantly higher reduction of the time needed to inactivate the Psa (90 min vs. 120 min of irradiation, respectively) (**Figure 5c** and **5b**).

As MB is a widely known PS due to its antimicrobial effects (Wainwright & Crossley, 2002), the best conditions of photoinactivation with NMB (PS at 5.0 μM and with the addition of KI at 100 mM) were used for the PS MB (at the same concentration). A Psa total inactivation was also obtained, but when KI was added, only half of the time was needed to inactivate the Psa (45 min) (**Figure 5d**).

Previous aPDT studies already showed that MB was an efficient PS in the inactivation of various microorganisms, namely other Gram-positive bacteria as *Staphylococcus aureus* and Gram-negative bacteria as *Pseudomonas aeruginosa* (Pereira et al., 2018) and *Vibrio parahaemolyticus* (Deng et al., 2015), and fungi as *Colletotricum abscissum* (Gonzales et al., 2017) and *Candida albicans* (Teichert et al., 2002).

Moreover, aPDT with other PS, *i.e.* Tetra-Py(+)-Me porphyrin lead to a Psa decrease of 6 log in the *in vitro* assays after 60 min under an irradiance of 4.0 mW cm^{-2} and a decrease of 1.8 log CFU mL^{-1} with an irradiance of 150 mW cm^{-2} in the *ex vivo* assays using kiwi leaves (Jesus et al., 2018). Other study, have also shown a 7.4 log CFU mL^{-1} Psa decrease *in vitro* after 60 min of irradiation (4.0 mW cm^{-2}) on the presence of a formulation constituted by five cationic porphyrin derivatives and a 2.8 log CFU mL^{-1} decrease on artificially contaminated leaves with the same formulation and light irradiance (Martins et al., 2018).

The potentiator effect of KI was extensively studied by several groups and was demonstrated that KI reacts with $^1O_2$, affording free iodine (I_2/I_3^-), hydrogen peroxide (H_2O_2) and iodine radicals ($I_2^{\cdot-}$), that are extremely bactericidal (Freire et al., 2016; Hamblin, 2017; L. Huang et al., 2017; Y. Huang et al., 2018; Reynoso et al., 2017; Vecchio et al., 2015; Vieira et al., 2018; Wen et al., 2017; Zhang et al., 2015). In fact, several *in vitro* and *in vivo* studies have shown the potentiation of fullerenes, Rose Bengal and MB by KI towards several microorganisms such as *Acinetobacter baumannii*, *P. aeruginosa*, *C. albicans* and methicillin-resistant *S. aureus* (L. Huang et al., 2017; Y. Huang et al., 2018; Reynoso et al., 2017; Vecchio et al., 2015; Zhang et al., 2015). The results obtained *in vitro* on the present study and comparing with previous ones prompted us to evaluate if MB was efficient for photoinactivation assays *ex vivo, i.e.* in kiwifruit pollen.

Since the *ex vivo* aPDT assays were performed in trade pollen that already contained its natural microorganisms, the pollen natural microorganisms were first quantified (**Figure 3**). As pollen is not a nutrient-rich matrix and water availability is low, suggesting the growth of some bacteria but also of some fungi and some sporulating bacteria, the bacterial, fungal and bacterial endospore concentrations in the pollen grains were determined. A microbial concentration of around 5 log CFU mL^1 was determined in LA (a non-selective medium) which allows mainly the growth of bacteria, but also of some fungi after 48 h of incubation (MacWilliams & Liao, 2016). The fungi concentration was also determined in RBCA (a fungi selective medium) after 5 days of incubation. A fungi concentration of 4.7 log CFU mL^{-1} was determined. A 3.6 log CFU mL^{-1} of bacterial endospores was observed in the heated pollen suspension incubated in the LA. The Schaeffer Fulton stain allowed to visualize the high number of endospores (**Figure 4**). The addition of the manganese sulphate to the culture medium promoted the bacterial sporulation, confirming the existence of endospore-forming bacteria in the pollen. These results (**Figure 3** and **4**) show that the microorganisms naturally present on pollen are

abundant and that some less aPDT susceptible microorganisms (bacterial endospores and fungi) relatively to Psa (a non-sporulating Gram-negative bacterium) are present in higher concentrations (Oliveira et al., 2009; Setlow, 2005). In fact, the aPDT treatments performed with MB at 50 μM with non-contaminated pollen were almost ineffective against the pollen natural microorganisms (**Figure 6**). Even with the addition of KI (at 100 mM) a decrease of only 1.1 log CFU mL^{-1} (ANOVA, $p<0.05$) was observed (**Figure 6**), which can be justified by the high concentration of fungi and endospores present in the pollen.

Since the main goal of this work was to evaluate the efficiency of aPDT to inactivate Psa in kiwifruit pollen, the non-Psa contaminated pollen was artificially contaminated with Psa. A protocol to artificially contaminate the provided pollen was efficiently developed allowing to contaminate the pollen with around 3.2 log CFU mL^{-1} of Psa. As the natural microorganisms content was about 5.1 log CFU mL^{-1}, a total of 8.3 log CFU mL^{-1} of microorganisms was present in the artificially contaminated pollen (**Figure 7**). When the artificially contaminated pollen was submitted to aPDT in the presence of MB alone a reduction of 3.1 log CFU mL^{-1} (ANOVA, $p<0.05$) was achieved after 180 min of white light irradiation (**Figure 8**). An additional decrease of 1.4 log CFU mL^{-1} (ANOVA, $p<0.05$) was observed when KI was used as a potentiator agent, with a total reduction of 4.5 log CFU mL^{-1}(ANOVA, $p<0.05$), which corresponds to an effective Psa inactivation. These results also demonstrate the potentiator effect of KI.

As the Psa concentration of the artificially contaminated pollen was *ca.* 3.1 log CFU mL^{-1}, it is possible to conclude that almost all the Psa cells added artificially (*ca.* 3.2 log CFU mL^{-1}) to the pollen were inactivated by aPDT, whereas aPDT had a low effect upon pollen natural microorganisms.

According to the American Society of Microbiology, the minimum reduction required for a new approach to be termed as an antimicrobial is at least 3 log CFU mL^{-1} which corresponds to a reduction of 99.9% of bacterial concentration (ASM, 2015), so the aPDT protocol used has proved to be an efficient approach, even without KI, to inactivate Psa in kiwifruit pollen.

Knowing that aPDT efficiency could not be dissociated from the maintenance of pollen ability to germinate, its ability to germinate was also evaluated under controlled conditions before and after the aPDT treatment. The results show that the aPDT treatment did not affect negatively the germination (**Figure 9**).

The results of this study show that the easily accessible photosensitizer used in this study (MB) is an excellent approach to photoinactivate Psa in pollen and the protocol designed and used may be feasible to be used in the treatment of kiwifruit pollen naturally

contaminated with Psa, which has been proven to be responsible for the dissemination of Psa among orchards (Donati et al., 2018). An additional positive outcome of this antimicrobial technology is the fact that a low effect in the pollen natural microorganisms was observed, which may be a positive aspect to pave aPDT application in the environment, since non-pathogenic microorganisms present in pollen may not be too affected.

The field application of aPDT can be similar to that already tested *ex vivo* for the kiwifruit leaves (Jesus et al., 2018; Martins et al., 2018), that is, by spraying the kiwifruit pollen with the PS and using white light LEDs as light source. However, to translate the application of this strategy into practice, more studies are needed, namely using sunlight irradiation after pollen application in the orchards.

Overall, the extensively studied PS, MB allows an effective photoinactivation of *Pseudomonas syringae* pv. *actinidiae* in kiwifruit pollen under white light irradiation without imparting any significative damage to the trade pollen features.

2.5. Future Perspectives

As a continuation of the present work, it would be interesting to perform the *ex vivo* aPDT protocol developed using natural sunlight instead of the artificial white light used. The use of sunlight presents as an important factor *i.e.* for the subsequent *in vivo* assays.

Therefore, it is also noteworthy to apply the *in vitro* and *ex vivo* methodology and conclusions obtained to develop in more detail an *in vivo* procedure, so that the developed protocol could be applied in a larger scale (*i.e*. in whole plants and in kiwifruit plantations).

It would also be interesting to test this methodology with other PS (*e.g.* chlorophyll), in order to evaluate efficacy of them on the inactivation of Psa both *in vitro* and in *ex vivo* assays.

CHAPTER 3 – References

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