Universidade de Aveiro
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Phage therapy in the inactivation of *Pseudomonas syringae* pv. *actinidiae* in kiwifruit plants

Terapia fágica na inativação de *Pseudomonas syringae* pv. *actinidiae* em plantas de 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 da Doutora Carla Sofia Gomes Pereira (coorientadora), investigadora do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave

Terapia fágica, bacteriófago, *Pseudomonas syringae* pv.*actinidiae*, cancro de kiwi.

resumo

Pseudomonas syringae é um agente causador de doenças numa ampla variedade de plantas e inclui mais de 60 pathovars. *Pseudomonas syringae* pv. *actinidiae* (Psa) é um dos pathovars desta espécie e o agente causal de cancro bacteriano em plantas de kiwi. A Psa é responsável por grandes perdas económicas, afetando seriamente a produção global de kiwis em muitos países, incluindo Portugal. Os tratamentos mais comuns para o biocontrolo de infeções causadas pelos diversos pathovars de *Pseudomonas syringae* em plantas envolvem derivados de cobre e / ou antibióticos. No entanto, estes tratamentos devem ser evitados devido à sua elevada toxicidade e ao desenvolvimento de resistências a estes químicos nas bactérias. Uma alternativa promissora aos tratamentos convencionais é o uso da terapia fágica. Os fagos são vírus que infetam bactérias, sendo considerados antibacterianos por serem capazes de causar lise bacteriana, apresentar especificidade e replicação rápida. O uso de fagos para controlar infeções bacterianas tem sido relatado por vários investigadores em diversas áreas. No entanto, não há nenhum estudo sobre o uso de fagos para eliminar Psa em plantas de kiwi. Assim, o objetivo deste trabalho foi avaliar a eficácia da terapia fágica para controlar infeções causadas por *P. syringae* em plantas. O fago ϕ6 (fago seguro e disponível comercialmente) foi caracterizado de forma a avaliar a sua potencial aplicação no controlo de doenças causadas por *P. syringae*, nomeadamente as causadas por Psa, e também causadas pelo seu hospedeiro natural *P. syringae* pv. *syringa*e. Inicialmente, o fago ϕ6 foi caracterizado em termos de gama de hospedeiros, período latente, número de explosão, adsorção ao hospedeiro e desenvolvimento de mutantes resistentes aos fagos utilizando o seu hospedeiro natural *P. syringae* pv. *syringae*. Tendo em conta que, as plantas de kiwi estão expostas à variação natural dos fatores ambientais, a influência do pH, temperatura, radiação solar e radiação UV na viabilidade do fago ϕ6 também foi avaliada. Inicialmente, a interação fago-bactéria foi caracterizada *in vitro,* utilizando meio de cultura líquido, à MOI de 1 e 100. Os resultados monstraram que o fago exibe um amplo espectro lítico, infectando além do hospedeiro *P. syringae,* as estirpes de Psa CRA-FRU 12.54 e CRA-FRU 14.10. Os ensaios *in vitro* de mostraram que o uso do fago ϕ6 na MOI de 1 e 100, pode ser uma alternativa eficaz ao controle de *P. syringae.* No entanto, a MOI de 1 (redução máxima de 3,9 log UFC / mL) foi mais eficaz que a MOI de 10 e 100 (redução máxima de 2,6 log UFC / mL). A viabilidade do fago ϕ6 foi principalmente afetada pela exposição à radiação UV-B (diminuição de 7,3 log UFP / mL após 8 horas), exposição à radiação solar (diminuição de 2,1 PFU / mL após 6 horas) e altas temperaturas (diminuição de 8,5 UFP / mL após 6 dias a 37 °C e decréscimo de apenas 2,0 log UFP / mL após 67 dias a 15 °C e 25 °C). A viabilidade do fago não foi significativamente afetada a temperaturas mais baixas (diminuição de 2,0 log PFU / mL após 67 dias a 15 °C e 25 °C) e pH na gama 6,5-7,0 (diminuição de 2,3 log PFU / mL em pH 7 e 7,5 e 2,7 log PFU / mL a pH 6,5). Numa segunda fase, para confirmar que este fago pode ser utilizado para controlar as estirpes de Psa CRA-FRU 12.54 e CRA-FRU 14.10, realizaram-se ensaios *in vitro*, em meio de cultura líquido, e *ex vivo*, utilizando folhas de kiwis artificialmente contaminadas. Nos ensaios *in vitro*, foi observada uma redução de aproximadamente 2,0 log UFC/ mL para as duas estirpes de Psa após 24 h de incubação. Nos testes *ex vivo*, a redução foi menor após 24 h de incubação (1,1 log UFC/mL no caso da estirpe Psa CRA-FRUA 12.54 e 1,8 log UFC/mL no caso da estirpe Psa CRA-FRU 14.10). A terapia fágica mostrou ser um método eficaz e seguro para inativar a Psa nas plantas de kiwi. A fim de explorar todo o potencial desta terapia, são necessários mais estudos, nomeadamente estudos no campo, em pomares de kiwis, aplicando os fagos no final do dia ou durante o período noturno para evitar a inativação do fago pela radiação UV e pela temperatura alta.

keywords

Phage therapy, bacteriophage, *Pseudomonas syringae* pv. *actinidiae*, kiwifruit canker.

abstract

Pseudomonas syringae (*P. syringae*) is the causative agent of diseases in a wide variety of plants and includes more than 60 pathovars. *Pseudomonas syringae* pv. *actinidiae* (Psa) is one of the pathovars of this species and the causative agent of bacterial cancer in kiwifruit plants. Psa is responsible to high economic losses, seriously affecting the global production of kiwifruit in many countries, including Portugal. The most common treatments for biocontrol of Psa and other infections caused by *Pseudomonas syringae* pathovars in plants involve copper derivatives and /or antibiotics. However, these treatments should be avoided due to both their high toxicity and development of bacterial resistance. One promising alternative to conventional treatments is the use of phage therapy to control Psa infections in plants. Phages are bacterial viruses and their antibacterial nature through induction of bacterial lysis, their high host specificity and rapid reproduction enable them to control bacterial populations. The use of phages to control bacterial infections has been reported across numerous fields by many researchers. However, there is no report regarding the use of phages to eliminate Psa in kiwifruit plants. Thus, the objective of this work was to evaluate the efficacy of phage therapy to inactivate or reduce Psa in kiwifruit plants. Phage Φ6 (a commercially available and safe phage) was characterized in order to evaluate its potential application in the control of diseases caused by *P. syringae*, namely caused by Psa. Initially, phage ϕ6 was characterized in terms of host range, latent period, burst size, adsorption to the host and development phage-resistant mutants using its natural host *P. syringae* pv. *syringae*. As the kiwifruit plants are exposed to the natural variability of environmental factors, the influence of pH, temperature, solar radiation and UV radiation on phage ϕ6 viability was also evaluated. First, the phage-bacteria interaction was characterized *in vitro*, using liquid culture medium at MOI of 1 and 100. The results revealed that the phage exhibited a broad lytic spectrum against the tested bacteria, infecting, besides the host *P. syringae*, the Psa strains CRA-FRU 12.54 and CRA-FRU 14.10. The phage at MOI 1 and 100, can be an effective alternative to control the species *P. syringae*. However, the MOI of 1 (maximum reduction of 3.9 log CFU/mL) was more effective than MOI of 100 (maximum reduction of 2.6 log CFU/mL). The viability of phage ϕ 6 in PBS was primarily affected by UV-B radiation exposure (decrease of 7.3 log PFU/mL after 8 h), solar radiation exposure (decrease of 2.1 PFU/mL after 6 h) and high temperatures (decrease of 8.5 PFU/mL after 6 days at 37 °C). The viability of the phage was not significantly affected by conditions as lower temperatures (decrease 2.0 log PFU/mL after 67 days at 15 °C and 25 °C) and pH (decrease 2.3 log PFU/mL at pH 7 and 7.5 and 2.7 log PFU/mL at pH 6.5). Second, to confirm if this phage can be used to control the Psa strains CRA-FRU 12.54 and CRA-FRU 14.10*, in vitro*, using liquid culture medium, and *ex vivo* experiments, using artificially contaminated kiwifruit leaves, were done. In the *in vitro* assays, a reduction of approximately 2.0 log CFU/mL of both Psa strains was observed after 24 h of incubation. In the *ex vivo* tests, the decrease was lower after 24 h of incubation, 1.1 log CFU/mL of reduction for Psa CRA-FRU 12.54 and 1.8 log CFU/mL of reduction for Psa CRA-FRU 14.10. Overall, phage therapy showed to be an effective and safe method to inactivate the Psa in kiwifruit plants. In order to exploit the full potential of this therapy, further studies are needed, namely field studies in kiwifruit orchards, applying the phages at the end of the day or during night period in order to avoid phage inactivation by UV radiation and high temperature.

Table of Contents

List of acronyms and abbreviation

ANOVA	Analysis of variance
CFU	Colony Forming Unit
EOP	Efficiency of Plating
MOI	Multiplicity of Infection
PBS	Phosphate Buffered Saline
PFU	Plaque Forming Unit
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth

List of tables

List of figures

Objectives and thesis outline

Objectives

The main objective of this study was to evaluate the efficacy of phage therapy to control infections caused bu *Pseudomonas syringae* pv. *actinidiae* (Psa) in kiwifruit plants. For this, a well characterized and commercially available phage, phage ϕ6, was used. The experiments were done *in vitro*, in liquid culture medium, and *ex vivo*, in artificially contaminated kiwifruit leaves, using two strains of Psa, but also the natural host of the phage ϕ6, *Pseudomonas syringae* pv. *syringae*.

The specific objectives can be summarized as:

- Preparation and characterization of phage ϕ6 in the host *P. syringae* pv. *syringae.*
- Evaluation of the potential effect of phage ϕ6 in the inactivation of *P. syringae* pv. *syringae*.
- Selection of the optimal protocol for viral inactivation of bacteria under different conditions (different concentrations and environmental factors).
- Evaluation of the potential of phage ϕ6 in the inactivation of Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10 strains.
- Development of a protocol for controlling Psa in artificially contaminated leaves of kiwi plants, using phage ϕ6.

Thesis Outline

This document is divided into five chapters. Chapter 1 includes a literature review, serving as a basis for the experimental work carried out in Chapters 2 and 3. In chapter 2 it is evaluated the efficiency of phage therapy in the control of *P. syringae* pv. *syringae*. Describes the preparation and characterization in terms of host range, latent period, burst size, adsorption to the host and viability of the phage to different environmental factors. The development of phage-resistant mutants was also evaluated after exposition to phage ϕ6. In chapter 3 it is evaluated the efficiency of phage ϕ6 to control Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10 *in vitro* and *ex vivo* assays using artificially contaminated leaves of kiwifruit plants. The characterization, in terms of latent period, burst size and adsorption to the host, was also performed. In chapter 4 it is enumerated the main conclusions obtained in the experimental work and the perspectives for future work. Chapter 5 includes the references used in the present document.

Chapter 1. Bacteriophages in the control of *Pseudomonas syringae* pv. *actinidiae* in kiwifruit plants: an integrative view

1.1. Abstract

In the last decade, the worldwide production of kiwifruit has been highly affected by *Pseudomonas syringae* pv. *actinidiae* (Psa). Psa has led to severe economic losses that have been seriously affecting the kiwi fruit trade. The available treatments for this disease are still scarce, with the most common involving frequent spraying of the orchards with copper derivatives, in particular cuprous oxide (Cu_2O). However, these copper formulations should be avoided due to both their high toxicity and development of bacterial resistance to this metal. Antibiotics are also used in some countries, but the development of bacterial resistance to the antibiotics has also been a reality. Therefore, it is essential to search for new approaches that should allow a sustainable agriculture production, avoiding the apparition of resistant Psa bacterial strains. Attempts to develop and establish highly accurate approaches to combat and prevent the occurrence of the bacterial canker in kiwifruit plants are currently under study, using specific viruses of bacteria (bacteriophages or phages) to eliminate the Psa. The characteristics of kiwifruit canker promoted by Psa, pathways of Psa transmission, prevention and control of Psa, phage therapy (PT) in kiwifruit orchards as a new approach to control Psa, together with potential ways to circumvent the inactivation of phage particles by abiotic factors via their structural and functional stabilization, are discussed in an integrated fashion, together with the advantages of PT over other therapies.

Keywords: *Pseudomonas syringae* pv. *actinidiae;* kiwi canker; phage therapy

1.2. Introduction

Kiwifruit is native from China and has become popular worldwide due to its sensory and nutritional properties. Kiwifruit belongs to family *Actinidiaceae* and genus *Actinidia*. The genus *Actinidia* has 76 species (Huang and Ferguson, 2007) but among these only two species *Actinidia deliciosa* (fuzzy kiwifruit) and *Actinidia chinensis* (golden kiwifruit) have been produced commercially (Zhang et al., 2010). Currently about 1.5-1.6 million tonnes of kiwifruit are produced each year. China, Italy and New Zealand together represent about

90% of the international kiwifruit production. In 2013, China produced about 1.765.847 million tonnes, Italy 447.560 million tonnes and New Zeland 382.337 million tonnes kiwifruit (Guroo et al., 2017). However, since the emergence of an economically devastating Psa outbreak in Japan in the 1980s on green-fleshed kiwifruit cultivar (*A. deliciosa*), the disease took a contagious turn causing severe economic loss to kiwifruit industries in Italy, China, South Korea, Spain, New Zealand and other countries (Cameron and Sarojini, 2014). In the last decade, bacterial canker in kiwifruit orchards caused by Psa has become quite problematic worldwide (Fujikawa and Sawada, 2016; Poulter et al., 2018; Wilstermann et al., 2017), affecting the global production of kiwifruit and leading to severe economic losses.

The treatments currently available for this bacterial disease are still scarce, with the most common involving frequent spraying of the orchards with copper derivatives or use of streptomycin. However, such copper formulations and antibiotics should be avoided due to both their high toxicity and development of bacterial resistance to both these chemicals (Cameron and Sarojini, 2014; Marcelletti et al., 2011). Consequently, alternative methods need to be developed to control Psa. New safe alternatives to metals and antibiotics that would reduce the appearance of antibiotic-resistant and metal-resistant bacteria and, at the same time, would allow a sustainable agriculture production without the use of the traditional environmentally toxic products, are currently the subject of research all over the world.

New attempts to develop highly targeted approaches to combat and prevent Psa-driven bacterial canker in kiwifruit plants are currently under study. Phage therapy can be an eco-friendly alternative approach to prevent and control Psa in kiwifruit plants, using specific viruses as potential biological control agents to eliminate the causative agent of this disease, based on their specificity and safety to the environment (Almeida et al., 2009; Ormälä and Jalasvuori, 2013; Sillankorva et al., 2012). Bacteriophages (or phages) are viruses that specifically infect bacteria, causing the lysis of their bacterial host and the release of newly formed viral particles.

This method of treatment was used to treat and prevent bacterial infection diseases in the former Soviet Union and Eastern Europe, however, was abandoned by the West in

the 1940 with the appearance of the antibiotics. The emergence of pathogenic bacteria resistant to antibiotics has recently motivated the western scientific community to reevaluate phage therapy as a valid option for the treatment of bacterial infections. Recent studies testify the use of phages as biocontrol agents in food, agriculture, aquaculture, veterinary science and to control food-borne diseases is a great potential new tool (Endersen et al., 2014; Gutiérrez et al., 2016; Jones et al., 2012; Loc-Carrillo and Abedon, 2011; Lu and Koeris, 2011; Zaczek et al., 2015). There are already on the market several phage-based products approved for the food area (ListShield, SalmoLyse®, Ecoshield™ and ShigaShield™ from Intralytix Inc. (Baltimore MD, U.S.A.) and PhageGuard Listex™ from Micreos Food Safety (Wageningen, Netherlands)) and agriculture area (EcoShield™ from Intralytix Inc. (Baltimore MD, U.S.A.) and Agriphage™ from Omnilyticus (Salt Lake City UT, USA) and Phagelux Inc. (Sanghai, China)) (Abuladze et al., 2008; Balogh et al., 2003; Moye et al., 2018; Soffer et al., 2017). However, there are not yet phages aproved for the phage-based biocontrol of Psa-induced kiwifruit canker.

This review discusses the characteristics of kiwifruit canker promoted by Psa, pathways of Psa transmission, Psa prevention and control, potential of PT in kiwifruit orchards to eradicate Psa, advantages of PT over other therapies, together with potential ways to circumvent the inactivation of phage particles by abiotic factors via their structural and functional stabilization.

1.3. Infection of plants by *Pseudomonas syringae*

Pseudomonas syringae is a fluorescence producing aerobic Gram-negative bacillus that infects several plants (Arnold and Preston, 2019; Bultreys and Kałużna, 2010; Xin et al., 2018; Young and Syringae, 2010). The strains of this bacterial species are divided into groups, designated pathovars, according to their differences with respect to the plants that they infect, such as Psa which infects the kiwifruit plant (Dye et al., 1980).

Currently, 60 pathovars have been identified which usually infect a single host (Xin et al., 2018). However, some pathovars have the ability to infect a restricted group of similar plants (Arnold and Preston, 2019; Xin et al., 2018).

The symptoms and symptom development depend on the plant species infected, the plant part infected, the strain of *P. syringae* and the environmental conditions. More than one symptom can be observed simultaneously on a single plant. Generally, flowers and/or flower buds may turn brown to black, dormant buds die (particularly common on cherries and apricots), necrotic leaf spots appear, appearance of discolored and/or blackened leaf veins and petioles (arising from systemic bacterial invasion and infection), appearance of spots and blisters on fruits, shoot-tips appears as dead, blackened twig tissues (quite common on maples), stem cankers (characterized by depressed areas in the bark, which darken with age), exsudation of a gummy substance from cankers on fruiting and flowering stone fruits (referred to as "gummosis") (Pscheidt and Ocamb, 2018). Shoot tip dieback was the most common symptom observed on 40 woody deciduous plants collected from Pacific Northwest nurseries (Canfield et al., 1986; Pscheidt and Ocamb, 2018).

P. syringae can be isolated from all plants having the tip dieback symptom, with plants most commonly and most severely affected being maple, dogwood, filbert, blueberry, magnolia, lilac, oriental pear, aspen, linden and kiwifruit plants. This clearly shows the widespread nature of the disease. *P. syringae* produces four toxins: coronatine (a polyketide molecule), phaseolotoxin (a sulfodiaminophosphinyl peptide), syringomycin (a lipodepsinonapeptide) and tabtoxin (a β-lactam) (Bender et al., 1999; Hwang et al., 2005), all of which contribute to chlorosis (yellowing of the leaf tissues, resulting typically from chloroplast disruption) or necrosis. In addition, phaseolotoxin has also been implicated in pathogen growth and spread in plants (Hwang et al., 2005).

Although no consensus exists among researchers about the severity of plant diseases caused by *P. syringae*, most researchers consider this bacterium a weak and opportunistic pathogen that capitalizes on a host weakened by some predisposing condition. Freezing damage make plants more susceptible to infection by *P. syringae* (Ferrante and Scortichini, 2014; Morris and Lamichhane, 2015).

Freezing due to cold temperatures wounds the plant, allowing the bacterium to enter and destroy plant cells. Interestingly, many strains of *P. syringae* catalyze the formation of ice crystals both on and in plant tissues (Ferrante and Scortichini, 2014; Lindow et al., 1982), being generally referred to as ice nucleation-active (INA) bacteria (Hwang et al., 2005).

Their presence on the plant raises the freezing temperature above that at which sensitive plant tissues would normally freeze (Ferrante and Scortichini, 2014). Since most frost-sensitive plants have no significant mechanism of frost tolerance, they must be protected from ice formation to avoid frost injury. Ice nucleation activity of *P. syringae* is conferred by a single gene that encodes an outer membrane protein (Li et al., 2012).

Individual ice-nucleation proteins do not serve as ice nuclei, but they form large, homogeneous aggregates that collectively orient water molecules into a configuration mimicking the crystalline structure of ice, thereby catalyzing ice formation (Borkar and Yumlembam, 2017). Oriented water molecules freeze at temperatures slightly below zero (-2 °C - -10 °C) instead of supercooling.

1.4. Infection of kiwifruit plants by Psa

The Psa pathovar was isolated for the first time in 1989 from a kiwifruit plant - *A. deliciosa* - in Japan (Takikawa et al., 1989).

Psa is more invasive at temperatures between 10 °C and 20 °C, thriving at an optimum temperature of 15 ± 3 °C. Although this bacterium can grow at temperatures above 25 °C, as has been shown in recent studies, at these temperatures its ability to infect is reduced (CABI, 2015; Andrea Fox, 2011; Kiwifruit vine health, 2019). Thus, Psa appears in plantations mainly in the spring, affecting the development of the plants, and in autumn/winter, causing plant damages. In addition, this bacterium can be resistant to antibiotics (Jeyakumar et al., 2014; Marcelletti et al., 2011; Scortichini et al., 2014), and has the ability to trap iron and catabolize aromatic compounds from plants (Marcelletti et al., 2011).

Psa can be distinguished from other strains of the same genus by biochemical testing, with some of the tests that can be used to identify Psa being displayed in Table 1.1.

Table 1.1. Biochemical characterization of Psa.

Biochemical test	Typical result
Oxidase	-
Tirosinase and fluorescent pigment characteristic of the genus *Pseudomonas* (poly-*p*-hydroxybutyrate)	-
Liquefaction of gelatina	-
Potato rot and arginine dihydrolase activity	-
Esculin	-
Starch	-
Urease	+
Catalase	+
Tobacco hypersensitivity reaction	+
Levan production	+
Sorbitol	+

Psa can be divided into 6 groups designated biovars, according to their virulence (Chapman et al., 2012; Fujikawa and Sawada, 2019). Biovar 1 produces and secretes a toxin called phaseolotoxin and was first found in Japan. Biovar 2 was discovered in Korea and secretes the coronatin toxin (Bender et al., 1999). On the other hand, biovar 3 corresponds to the pathogenic strains discovered after 2008 in Italy (Ferrante and Scortichini, 2010) and later in several European countries and in Japan (Sawada et al., 2015), being the one that has led to more economic losses because it is the most virulent (Chapman et al., 2012). Psa biovar 3 is a pandemic virulent group found worldwide that does not produce any known toxins (Fujikawa and Sawada, 2016), but produces fluorescent compounds, i.e., pyoverdine, when grown on King's B medium (McAtee et al., 2018). Biovar 4 has been discovered in New Zealand and is not virulent. Biovar 5 was recently located in Japan and does not produce the toxins produced by biovars 1 and 2 (Fujikawa and Sawada, 2016). The biovar 6 was also discovered in Japan, beign most likely an endemic lineage similar to biovar 5, but in contrast to biovar 5, biovar 6 can produce two phytotoxins (phaseolotoxin and coronatine) (Fujikawa and Sawada, 2019).

Currently, Psa has been found in several countries, namely Italy, Portugal and New Zealand, being considered the responsible for the bacterial cancer in the kiwifruit plants (Balestra et al., 2010; Everett et al., 2011; Scortichini, 1994). Kiwi cultivation is severely affected by the presence of Psa because, under appropriate conditions, this bacterium

significantly reduces yields, leading to major economic losses (CABI, 2015; Scortichini et al., 2012).

The main kiwifruit species affected are *Actinidea chinensis* and *A deliciosa*. Infections have also been detected in the wild species *Actinidea arguta* and *Actinidea kolomikta* (Kim et al., 2016; Marcelletti et al., 2011; Ushiyama et al., 1992).

Considered the responsible for bacterial canker disease in kiwi, Psa shows a high capacity for growth in several phases of the plant's own growth (CABI, 2015). Initially, Psa behaves as an epiphytic bacterium because it remains supported on the surface of the plant without removing nutrients, that is, this bacterium can remain in a latent state for a long time without symptoms occurring in the plant, until the conditions of growth reach the optimum point. After this latency phase, Psa enters the vascular system of the plant through openings caused by some animals, such as birds or insects, or by human manipulation, colonizing pollen (Donati et al., 2018) and leaves, sometimes fixing to the roots, leading to the development of a systemic infection (CABI, 2015; Ferguson, 1999; NZKGI, 2016).

Female plants show faster symptoms of infection, whereas male plants exhibit more pronounced symptoms. On the other hand, younger plants are the most sensitive ones to infection (Abelleira et al., 2011; Kiwifruit vine health, 2019).

The first symptoms appear during spring in the leaves, stems, shoots and, less commonly, small tumors may appear (Renzi et al., 2012). In the spring period, the leaves develop brown dots often surrounded by bright yellow halos (Figure 1.1a-b).

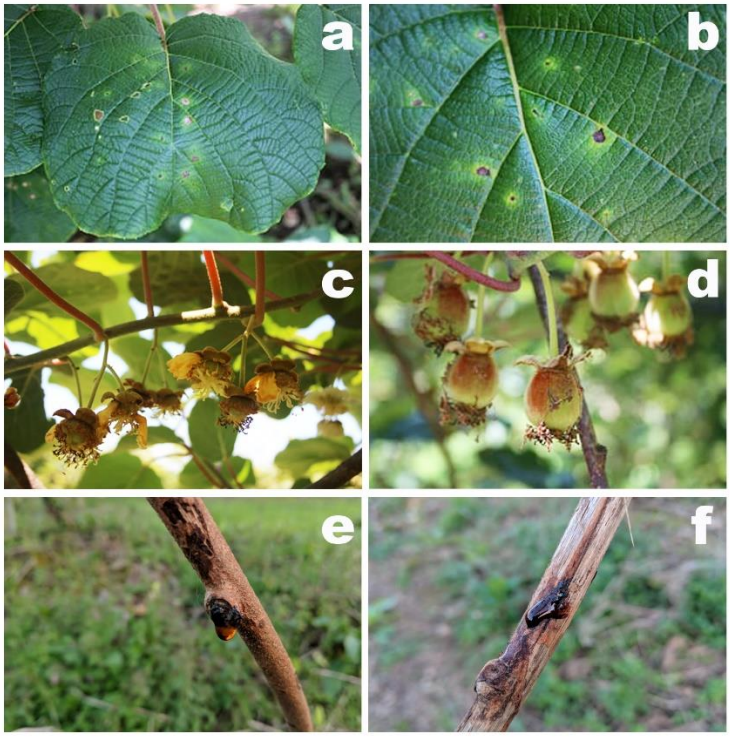

Figure 1.1. Leaves of kiwifruit plant infected with Psa, evidencing brown spots surrounded by characteristic chlorotic halos (a, b), atrophy of kiwifruits due to infection by Psa (c, d), and release of red exsudate (e, f) in a kiwifruit plant infected by Psa.

The appearance of these chlorotic halos on the otherwise green leaves may be due to the activity of the plant toxin phaseolotoxin (Sawada et al., 1997; Tamura et al., 2002). In the same way, the sepals of the flowers darken and the shoots of the leaves acquire a brownish coloration and wither (Prencipe et al., 2017; Renzi et al., 2012). Heavier sprouts may even fall and release exudates (Renzi et al., 2012), while others shrink leading to fruit atrophy (Figure 1.1c-d), as the bacteria invades and blocks vascular tissues.

Secondary symptoms appear in the middle of winter mainly on the branches and trunks, in which the release of exudates of reddish/brownish color (Figure 1.1e-f) is observed (Balestra et al., 2010; Prencipe et al., 2017; Renzi et al., 2012). At the end of this season, the release of exsudate is accentuated and, in addition, causes the degradation of lignin and phenolic compounds, ultimately leading to the death of the plant in worst case scenarios. When the infections present high bacterial concentration, the released exudate becomes white (Balestra et al., 2010).

The severity of Psa impact differs between species of kiwifruit. The damage is more severe on yellow-fleshed kiwifruits (*A. chinensis*) than on the green-fleshed cultivar (viz. *A. deliciosa* cv. "Hayward"), which is commonly grown in New Zealand (Buriani et al., 2014; Ferrante and Scortichini, 2009; Froud et al., 2018).

Psa significantly impacts the kiwifruit industry from the economic point of view. In 1992, Psa was discovered in northern Italy, but it remained sporadic with few cases for 15 years. But, in 2007/2008, the environmental conditions were ideal for spread of the disease, having occurred the first outbreak of this disease in Italy, caused by the biovar 3 of this bacterial species, and kiwifruit orchards in the region of Lazio were virtually decimated, costing Italy ca. 2 million euros (including loss of trade as well as physical damages). In the following years, it occurred in plantations in several European countries, namely France, Switzerland, Spain and Portugal, in New Zealand, Chile, Japan, Australia and Korea (Balestra et al., 2011, 2010; Everett et al., 2011; Koh et al., 1996; Scortichini et al., 2012; Vanneste et al., 2011; Vanneste et al., 2012; Vanneste et al., 2011; Vanneste, 2017; Yu et al., 2016). Psa was discovered in November 2010, being present in around 92% of the New Zealand regions where kiwifruit is grown. In Japan, control using copper compounds and antibiotics has led to the development of resistant Psa strains (Cameron and Sarojini, 2014). In 2019, Psa has been added to the European and Mediterranean Plant Protection Organisation (EPPO) Alert List.

Despite ongoing control measures, Psa-driven kiwifruit canker continues to occur in Japan, Korea and in several European countries (McCann et al., 2017, 2013).

Psa share common (potentially) important vectors of transmission with other pathovars: (i) aphids or plant-lice; (ii) contaminated water (not only from rain, but also from irrigation systems) and soil; (iii) seeds and organic material (infected budwood and transportation of infested nursery stock); (iv) pollen (spread by strong winds, rain, birds and insects, moving easily between plants and orchards under the right environmental conditions); and (v) human operators (inadvertently carrying the bacteria on their clothing or shoes) and kiwifruit orchard handling equipment (both mechanical equipments and pruning tools) that contact with the plant (Borkar and Yumlembam, 2017; Jones et al., 1983; Morris et al., 2008; NZKGI, 2016; Pscheidt and Ocamb, 2018; Stavrinides et al., 2009).

Genetic studies reveal that the strains of Psa found in Europe are quite similar, suggesting that the focus of infection is common and that the spread occurred with the sale of infected plants (Renzi et al., 2012). This dissemination was probably favored by environmental conditions such as frost, hail and winds and by the lack of care in relation to this bacterium (Reglinski et al., 2013; Scortichini, 2018).

It is known that environmental factors promote and aggravate the infection because they can favor the growth of Psa. In addition, dispersal of exudates in orchards, particularly when they have high inoculum concentrations, or lesions in plants, are a potential source for the entry of the bacteria.

Another risk factor is the commercialization of infected seedlings for cultivation, which can disseminate the infection between distant sites (Gao et al., 2016; Kim et al., 2016; Patel et al., 2014).

Kiwifruit as a commodity do not represent a biosecurity threat, and its surfaces do not appear to be a suitable niche for a long term survival of Psa as an epiphyte (Stefani and Giovanardi, 2011) and, therefore, kiwifruits do not appear to represent a pathway for Psa dissemination.

Kiwifruit plant flowers can provide a protected and nutrient-rich environment to the epiphytic microflora, thus representing a sensible entry point for Psa (Donati et al., 2018, 2014), causing flower browning and fall and systemic invasion of the host plant. Infected male flowers produce contaminated pollen, which can transmit Psa to healthy plants (Donati et al., 2018; Wilstermann et al., 2017).

The role of pollen in disseminating Psa in kiwifruit orchards was investigated by Vanneste et al. (2011), Stefani and Giovanardi (2011), Donati et al. (2018), together with the survival of the pathogen as an epiphyte on leaves and fruits. Psa was found as an epiphyte for several weeks after pollination (Purahong et al., 2018; Stefani and Giovanardi, 2011). Hence, the role of pollen in disseminating Psa associated to the long epiphytic survival of the pathogen on kiwifruit plant leaves was established by those researchers.

Understanding the importance of pollen and pollination for the dissemination of Psa from plant to plant is of utmost importance for the control of kiwifruit bacterial canker

(Vanneste et al., 2011). Hence, Psa may migrate to developing flowers in infected kiwifruit plants, contaminating the anthers and resulting in the production of contaminated pollen.

Donati and collaborators also found that Psa was more frequently found in asymptomatic flowers and on pollen derived from asymptomatic flowers, than on symptomatic ones, suggesting that symptomatic flowers probably drop from the vine as their metabolism is no longer effective (Donati et al., 2018). For instance, infected pollen has been implicated as a source of Psa introduction in New Zealand (Scortichini et al., 2012; Vanneste et al., 2011), a major producer and exporter of kiwifruit.

In recent reports by Pattemore et al. (2014) and Woodcock and Hons (2016), honeybees were established as vectors of Psa dissemination, since they collect pollen from flowers and distribute it to different kiwifruit vines. Hence, there is a concern that these bees can either become contaminated with Psa from infected vines or transfer infected pollen from one vine to another, facilitating the spread of Psa infection. In experiments in which honeybees were exposed to pollen contaminated with Psa, it was found that the bacteria could be recovered from the bees for up to 2 weeks after the initial contact (Woodcock and Hons, 2016). Therefore, hives and honeybees may be implicated as viable sources of Psa infection and can facilitate the spread of Psa.

1.5. Control of Psa-induced bacterial canker

Besides prevention (red heptamer), Figure 1.2 depicts all the currently used and potential new strategies for controlling Psa in kiwifruit orchards (pink heptamers) along with the latest (promising) state-of-the-art strategies, viz. antimicrobial photodynamic and phage therapies (blue heptamers).

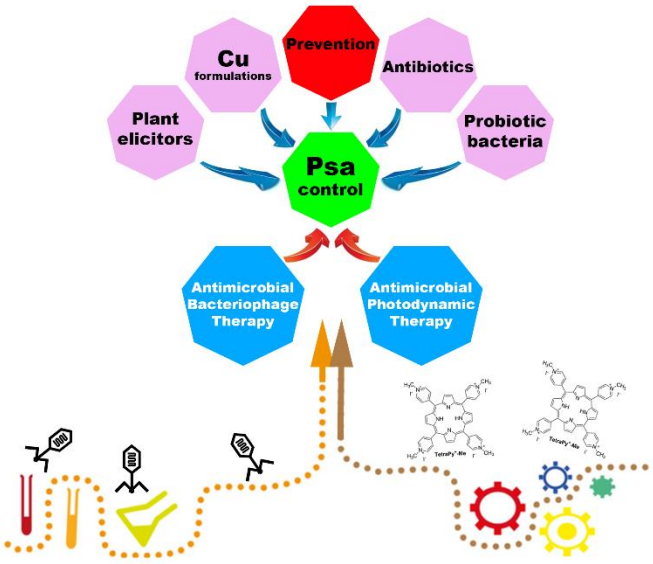

Figure 1.2. Relationship between currently available strategies for controlling Psa in kiwifruit orchards (red and pink heptamers), and potential new alternatives (blue heptamers) based on antimicrobial phage therapy and photodynamic therapy approaches.

1.5.1. Preventive measures

The control of Psa-induced canker of kiwifruit plants has been relying mainly on preventive measures, since there are no Psa eradication treatments (Borkar and Yumlembam, 2017; Buriani et al., 2014; Monchiero and Gullino, 2015; NZKGI, 2016; Ward, 2014). These preventive measures include disinfecting agricultural tools (mainly pruning equipment), maintaining hygienic conditions in orchards, restriction of unnecessary access to the orchard, paying attention to the grafting process that can be a source of contamination, having protection systems against wind, frost and rain, providing water and nutrients in quantities suitable for a healthy plant growth, and removal of animals that may cause infection such as lice, birds, slugs, snails, beetles and cicadas.

In this context, in Portugal there is the National Plan of Action for the Control of Psa in kiwi (MAMAOT, 2012). This plan includes, for example, a prospectus form that has to be completed at each inspection. In addition, it is mandatory to make a record of the plants purchased and a history of the exits of plant material (fruits and pruning wood) (Rios et al., 2016). In order to alert and sensitize producers about this plan, information actions are

carried out, covering various topics ranging from symptoms and control strategies to laboratory analysis.

Another important aspect of prevention is the monitoring of orchards, taking into account the materials and operators, in order to trace the origin of the infection and to evaluate possible risks (NZKGI, 2016). For example, if a producer suspects that a given plant is infected and considers that it might jeopardize the remaining planting, samples from this plant must be collected for laboratory analysis.

Since Psa shows negative results for many laboratory tests, two important tests to identify Psa are the analytical profile index (API®) test and the transformation of sucrose into acid (CABI, 2015; Ferguson, 1999). Still, bacterial strain must be isolated for morphological and molecular tests, especially via PCR (Polymerase chain reaction)-based assays, once that is the most successful test for its identification.

Currently, there are already some PCR kits available on the market that are easy to use by farmers and thus provide immediate tracking. These kits are based in specific primers that targets a specific fragment of the hrpW gene of the pathogenicity (hrp) region of Psa and involved in the hypersensitive response. Hypersensitivity tests on tobacco leafs can also be performed for rapid detection of Psa, since a positive result means a high probability of detection of phytopathogenic species of *Pseudomonas* (Lopez et al., 2009; Loreti et al., 2018).

In addition, the location of the plant can also prevent possible infections because a stable plant and far from stress conditions is less susceptible to infection (NZKGI, 2016).

These preventive procedures are not enough to halt the disease, and additional preventive measures are needed (Pandey et al., 2017; Velásquez et al., 2018). Although such preventive measures have been implemented in the kiwifruit orchards, frequent outbreaks in different countries have been observed, which imply the use of different treatments to control Psa.

1.5.2. Approved treatments

Among the products approved and currently used for treatment one can find essentially those based on heavy metals (especially copper), antibiotics and elicitors (Table 1.2).

1.5.2.1. Bactericidal and/or bacteriostatic approaches

Copper-based products

Spraying copper-based bactericides in orchards is considered the most effective practice in protecting against Psa, providing a cover on the surface of the plant, killing the bacteria on the surface of the leafs and preventing the bacteria from entering the vines. Copper-based compounds should be sprayed immediately after winter pruning, two and four weeks after bud break and in high-risk situations such as after a major wind, rain or hail event. Copper has only a minimal effect once the infection has occurred, so the emphasis is on obtaining uniform and complete plant coverage throughout the canopy. The efficacy of copper in plant protection can be considerably enhanced by reducing the droplet particle size of the spray, since more surface area will be available per gram of product to release copper ions when moisture is present (Holmes and Jeyakumar, 2014; Jeyakumar et al., 2014; Life project after Cu, 2015; Nordox, 2013; Parker, B.; Scarrow, 2011).

The effectiveness of commercial copper compounds depends on both the formulation and the 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. Copper forms complexes within phytopathogens, destroying cell proteins and disrupting all enzyme activity (Jeyakumar et al., 2014).

Table 1.2. Currently approved treatments for controlling Psa in kiwifruit plants.

Treatment type	Host plant / Part of plant	Pathovar / strain name	Type of assay	Control agent	Reduction of bacterial load	References
Copper-based bactericidal/bacteriostatic	Kiwifruit plant (*Actinidiae chinensis* var. *chinensis* Hort16A)	Psa	*In planta*	Copper oxide (NORDOX™ 75WG at 37.5g/100L). Approved by the IMO (Institute for Marketecology - Switzerland) for use in organic farming under Regulations (EC) 889/2008 and 834/2007 and in accordance with the NOP / USDA (National Organic Program of USA).	18% - 100%	(Tyson et al., 2017)
	Kiwifruit plant	Psa	*In planta*	COPTYZIN™ (95 g/L copper (Cu), chelated by tetraethylene pentamine (CuTEPA chelate) in the form of a soluble concentrate); Approved pursuant to the HSNO Act 1996, Approval Ref. HSR100658.	n.a.	(Micro-Nutrients NZ, 2019)
	Kiwifruit plant	Psa8, Psa9, Psa10, Psa11, Psa CRA-FRU 8.43	*In vitro*	BIOBACTER® (zinc sulfate and peroxide acid). In accordance with the NOP / USDA (National Organic	Inhibition after 5 days	(Corrado et al., 2018)

				NORDOX® (copper (II) oxide, 75% Cu)	No inhibition	
				COPPER-LUX50® (copper (II) oxychloride, 50% Cu)	No inhibition	
				FOSETYL-Al® (aluminum ethylphosphonate). United States Environmental Protection Agency Registration standard #PB84206564, EPA MRID Number 47370502.	Inhibition after 8 days	
				YETI-R® (Ag, Cu and Fe)	No inhibition	
				YETI-F® (Mn and B)	No inhibition	
Antibiotic bactericidal/bacteriostatic	Kiwifruit plant	Psa	*In planta*	Streptomycin (used only in Asian countries and New Zealand). Approved by the Agricultural Compounds and Veterinary Medicines Group (ACVM - New Zealand).	n.a.	(NZKGI, 2016)
	Kiwifruit plant	Psa strain NZ V-13	*In planta*	Kasugamycin (used only in New Zealand). Approved by the Agricultural Compounds and Veterinary Medicines Group (ACVM - New Zealand).	n.a.	(Ghods et al., 2015)
Resistance inducer approaches	Kiwifruit plant	Psa	*In planta*	Actigard™ 50WG (acibenzolar S-methyl). EPA Reg. No. 100-922.	n.a.	(NZKGI, 2016; Reglinski et al., 2013; Vanneste, 2012)
Biological approaches	Kiwifruit plant	Psa	*In vitro* and greenhouse *in planta*	*Bacillus subtilis* QST713 (Serenade Max™). EPA	9.1% - 36.1% (in reducing fireblight in	(Stewart, A.; Hill, R.; Stark, 2011)

				Registration # 069592-00011.	apple, promising for suppressing Psa)	
				Bacillus subtilis var. *amyloliquefaciens* D747 (BacStar™)	n.a. (promising for suppressing Psa)	
				Pseudomonas fluorescens A506 (BlightBan® A506). EPA Registration # 228-710.	9.1% - 36.1% (in reducing fireblight in apple, promising for suppressing Psa)	
	Kiwifruit plant	Psa	Commercial product, AureoGold™ (Arysta)	*Aureobasidium pullulans* (≥840g/kg of $4x10^9$ CFU/g *Aureobasidium pullulans*). Registered pursuant to the ACVM Act 1997 No. P009589, HSNO Approval Code: HSR101029.	30% reduction in buds	(Arysta - LifeScience, 2018)
					87.5% reduction in leafs; >90% global reduction	
	Kiwifruit plant (*Actinidiae deliciosa* var. *deliciosa*)	Psa	Greenhouse, *in planta*	Endophytes from *Leptospermum scoparium* (*Pseudomonas* sp. T1R21, *Pseudomonas* sp. T4MS32A and *Pseudomonas* sp. T4MS33)	100% reduction 5 cm above or below wound site; all combined, reduced Psa by 1000-fold 1 cm below the wound site	(Wicaksono et al., 2018)
	Kiwifruit plant (*Actinidiae chinensis* var. *deliciosa* cv. Hayward)	Psa CFBP7286-GFPuv, Psa NCPPB3739, Psa IVIA3700-1	Greenhouse, *in planta*	*Lactobacillus plantarum* CC100	No inhibition (Psa resistant to kanamycin)	(Daranas et al., 2019)
				Lactobacillus plantarum PM411	70.0% - 75.4% (0.6 log reduction in leaves)	

				Lactobacillus plantarum TC92	84.5% - 96.3%	
				Leuconostoc mesenteroides CM160	No inhibition	
				Leuconostoc mesenteroides CM209	No inhibition	

Legend: n.a.: not available.

Treatments based on copper (copper sulphate and/or copper oxychloride) have until now played a vital role in the control of bacterial canker in kiwifruit plants (Carvalho et al., 2017; Gould et al., 2015; Jeyakumar et al., 2014; Vanneste et al., 2012). In Italy, Portugal and other European countries, copper sulphate is the major allowed compound used by organic growers for chemical control of Psa (Vanneste et al., 2011). Currently, in New Zealand, COPTYZIN™ (from Micro-Nutrients NZ Ltd), a copper bactericide with copper chelated by tetraethylene pentamine in the form of a soluble concentrate (Table 1.2), is used for the control of Psa in kiwifruit. Psa is in general controlled with the application of copper compounds at 35-50 g h L^{-1} (Cameron and Sarojini, 2014). Cuprous oxide (Cu_2O) is the copper formulation more frequently used worldwide in kiwifruit orchards (viz. NORDOX™ 75WG) (Table 1.2). 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 hollow granules, to increase wetting, suspension, adhesion and persistence, hence allowing a more uniform product distribution on the treated surfaces (Cropsol, 2019; Nordox, 2013). Its high efficacy is due to the small size of the particles, providing a greater uniformity in the distribution and coverage of the plant surfaces, and to the lower amounts needed to obtain the best results (Lee, J. H.; Kim, J. H.; Kim, G. H.; Jung, J. S.; Hur, J. S.; Koh, 2005; Nordox, 2013). At concentrations above average in plant tissues (10 µg $g^{-1}_{dry\ weight}$) (Schulin, R.; Johnson, A; Frossard, 1995), copper not only inhibits plant development but also interferes with cell processes like photosynthesis (Jesus et al., 2018; Mackie et al., 2012; Yruela, 2005).

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, heavy negative impacts on both soil and epiphytic microflora, as well as in the development and spread of copper-resistant bacteria in agroecosystems. In addition, heavy metals used in agriculture might promote the spread of antibiotic resistance via co-selection (Cazorla et al., 2002; Jeyakumar et al., 2014; Parker, B.; Scarrow, 2011; Seiler and Berendonk, 2012).

The use of copper sprays in kiwifruit orchards has been the subject of extensive reviewing (Jeyakumar et al., 2014).

$CuSO_4$ is no longer recommended for use, since it is highly soluble and toxic to both humans and the environment (Jeyakumar et al., 2014; Mackie et al., 2012). Other copper formulations such as copper hydroxide ($Cu(OH)_2$) and copper oxychloride ($Cu_3Cl_2(OH)_4$) are less soluble, being now preferentially used in most countries. The great majority of copper products used to date in kiwifruit orchards in New Zealand are in the form of $Cu(OH)_2$ and copper oxide (CuO) (Jeyakumar et al., 2014; Mackie et al., 2012). Although Cu_2O is highly unstable and toxic to bacterial cells, it is far more soluble in water than CuO (virtually insoluble in water), and therefore the later is preferably used (NCBI, 2019). The use of Bordeaux mixture is restricted, being only used during the dormant seasons (Jeyakumar et al., 2014; Parker, B.; Scarrow, 2011).

Wettable powders and dispersible granules are considered to present less risk of phytotoxicity than liquid formulations and hence should be the copper product of choice. The aim is to keep the number of sprays to a minimum while providing the best coverage possible, especially in times of high risk of infection (Jeyakumar et al., 2014; Kiwifruit Vine Health Inc., 2017; LPAC (Life project after Cu), 2015; Parker, B.; Scarrow, 2011).

Antibiotics

In Asian countries and New Zealand, the use of the antibiotic streptomycin (Table 1.2) is allowed for controlling plant pathogens (Buriani et al., 2014; Cameron and Sarojini, 2014; Vanneste et al., 2011; Young, 2012) and is one of the main ways of fighting Psa (NZKGI, 2016). Comparatively, in Italy, Portugal and other European countries, the use of antibiotics is not allowed (Cameron and Sarojini, 2014). More than two decades ago, streptomycin injection in kiwifruit plant trunks also started to be used in Korea, appearing to effectively cure infected vines (Koh et al., 1996). However, streptomycin induces the surge of bacterial resistance and leaves residues on kiwifruits. In 2013, New Zealand's Environmental Protection Authority approved the use of the antibiotic kasugamycin (an aminoglycoside antibiotic isolated from *Streptomyces kasugaensis*) for controlling kiwifruit canker caused by Psa strain NZ V-13 (Table 1.2) (Ghods et al., 2015).

In a very recent report by Flores et al. (2018), 18 Chilean Psa isolates exhibited resistance to the antibiotics rifamycin SV, lincomycin and vancomycin, being sensitive to

minocycline and troleandomycin and showing variable sensitivity to aztreonam, nalidixic acid, and fusidic acid. These worrying results put an emphasis on the need to develop greener and safer alternatives to antibiotics, in the fight against Psa-induced kiwifruit canker.

1.5.2.2. Resistance inducer approaches

According to Reglinski et al. (2013), the integration of plant-induced resistance to Psa could provide systemic protection of kiwifruit plants before the onset of infection.

In New Zealand, the commercial product Actigard™ (Table 1.2) is also one of the main ways of fighting Psa (NZKGI, 2016). This commercial product contains a compound named acibenzolar S-methyl (ASM, a fungicide), derived from salicylic acid, which reduces the incidence of the disease (Reglinski et al., 2013; Vanneste, 2012). Specifically, ASM has been shown to be one of the most effective plant elicitors to improve the tolerance of kiwifruit plants to Psa, activating the plant's own defense system by increasing the transcription of W-box controlled genes. However, due to the risk of fruit-persistent residues, the foliar application of ASM is severely restricted (Reglinski et al., 2013).

The use of elicitors (compounds that stimulate the immune system of plants thus reducing bacterial activity in them) represents an alternative for copper-based products and antibiotics and is already mentioned in the specialty literature (Cameron and Sarojini, 2014). However, the use of these compounds alone is not effective and they have to be combined with other methods.

Although the use of elicitors and heat to control Psa have already been tested (K. Wurms et al., 2017; K. V Wurms et al., 2017), further studies are needed for these to be truly viable alternatives.

1.5.2.3. Biological approaches

In Italy, Portugal and other European countries, other products based on antagonistic bacteria such as *Bacillus subtilis* (Scortichini, 2013) are used, viz. Serenade Max™ and BacStar™ (Table 1.2). Biological approaches may contribute to an integrated control of Psa, but until now the knowledge about their efficacy and reliability under different environmental conditions is scarce. This type of approach to control plant diseases is based on the use of a viable organism to control the infectious agent via antagonistic interactions. Biocontrol agents are usually predators, pathogens or parasites of the bacterium to be controlled (Stewart, A.; Hill, R.; Stark, 2011).

In the specific case of Psa, its biological control might be attained via three different mechanisms, viz. (i) amensalism [i.e. antibiosis (the biocontrol organism produces metabolic substances that inhibit growth)], (ii) competition (between the biocontrol agent and the pathogen, for space and resources) and (iii) parasitism and predation (the biocontrol organism lives off the pathogen, killing it) (Stewart, A.; Hill, R.; Stark, 2011).

In a study by Stewart and collegues (2011), showed that the most effective microbial candidates for the biocontrol of Psa were *Bacillus subtilis* strain QST 713 (Serenade Max™), *Bacillus subtilis* var. *amyloliquefaciens* D747 (BacStar™) and *Pseudomonas fluorescens* A506 (BlightBan®A506) (Table 1.2), due to both their wide range of target pathogens and/or diseases, target crops and different modes of action (Stewart, A.; Hill, R.; Stark, 2011) (Table 1.2).

Quite recently, Wicaksono et al. (2018) reported a biocontrol strategy using *Pseudomonas* sp. endophytic bacteria from *Leptospermum scoparium* as an alternative strategy to manage Psa (Table 1.2). Endophytic bacteria are able to produce antimicrobial compounds, siderophores and induce systemic resistance, to inhibit disease development by plant pathogens. These authors successfully transferred endophytes from *Leptospermum scoparium* to kiwifruit plants *(A. deliciosa)* and succeeded in maintaining their biocontrol of Psa. They found out that the three endophytic *Pseudomonas* evaluated (and transmitted to *A. deliciosa* by wound inoculation) possessed multiple antibiotic producing genes (viz. phenazine, 2,4-diacetylphloroglucinol (2,4-DAPG), HCN), and were able to inhibit Psa growth *in vitro* by an antibiosis mechanism via production of diffusible compounds. Hence, the endophytic bacteria *Leptospermum scoparium* were demonstrated

to suppress Psa *in vitro* being able to colonise kiwifruit and exert biocontrol of Psa (Wicaksono et al., 2018).

Very recently, a new biological control for kiwifruit disease was developed by Plant & Food Research Mt Albert (Auckland, New Zealand) in collaboration with Arysta LifeScience, and registered as Aureo Gold™ (Ref. P009589). Aureo Gold™ (Table 1.2) is a formulation that contains ca. 840g/kg of 4×10^9 cfu/g *Aureobasidium pullulans* (Arysta, 2018), a natural yeast strain that globally reduces growth and spread of Psa bacteria by more than 90% (Arysta - LifeScience, 2018) (Table 1.2).

The use of lactic acid bacteria (LAB) to control multiple pathogens that affect plants, in particular Psa in kiwifruit plants, *Xanthomonas arboricola* pv. *pruni* in *Prunus* and *Xanthomonas fragariae* in strawberry, was also recently approached by several researchers (Daranas et al., 2019). The antagonistic activity of *Lactobacillus plantarum* CC100, *Lactobacillus plantarum* PM411, *Lactobacillus plantarum* TC92, *Leuconostoc mesenteroides* CM160 and *Leuconostoc mesenteroides* CM209 against the aforementioned plant pathogens was duly established by Daranas et al. (2019), due to their broad-spectrum activities (Table 1.2).

Although a variety of biological controls are now available for use, their effective adoption will require a deeper understanding of the highly complex interactions among plants, people and environment.

While most bacterial pathogens will be susceptible to one or more biological control strategies, their practical implementation on a commercial scale is constrained by several factors in relation to the alternative disease control strategies including (but not limited to) cost, convenience, efficacy and reliability. However, biological control should be applied only when such agronomic practices as good sanitation, soil preparation, water management and host resistance are insufficient to attain an effective disease control.

1.5.3. Potential new treatments

Among the potential new treatments, are new copper complexes, thermal treatment, photodynamic therapy (PDT) and other new compounds enumerated in Table 1.3, and also phage therapy which is one of the most promising potential new treatments with generalized worldwide growing acceptance (Table 1.4), and which is detailed in this review.

1.5.3.1. Bactericidal / bacteriostatic new copper complexes

Corrado et al. (2018) synthesized new copper (I) complexes and tested them in several strains of Psa, succeeding in inhibiting their growth *in vitro* (Table 1.3). The authors did not report, however, the reduction achieved in bacterial loads.

1.5.3.2. Thermal treatment

One example of a possible treatment may be the use of heat to kill the Psa present in the pollen (Table 1.3) as it may be a vehicle for transmission of the bacteria (Everett et al., 2012). Everett et al. *(*2012) studied different combinations of temperature, time and relative humidity (RH) in order to test the most effective combination to kill Psa in artificially contaminated pollen. These authors found that the treatment with more potential leading to the death of Psa without loss of pollen viability was the combination of 35 °C with RH at 50% or less, during more than 20 h.

Table 1.3. New approaches with major potential for controlling Psa in kiwifruit plants.

Treatment type	Host plant / Part of plant	Pathovar / strain name	Type of assay	Control agent	Reduction of bacterial load	References
Copper-based bactericidal/ bacteriostatic	Kiwifruit plant	Psa8, Psa9, Psa10, Psa11, Psa CRA-FRU 8.43	*In vitro*	$[Cu(Cl)(HL)_2]$, copper (I) complex, ($C_{26}H_{28}ClCuN_4O_6S_2$)	Inhibition	(Corrado et al., 2018)
				$[Cu(I)(HL)_2]$, copper (I) complex ($C_{26}H_{28}ICuN_4O_6S_2$)	Inhibition	
Thermal treatment	Pollen of kiwifruit plant	Psa	*Ex vivo*	Combination of 35 °C with RH at 50% or less, during more than 20 h	100%	(Everett et al., 2012)
Photodynamic therapy (PDT)	Kiwifruit plant leaves	Psa	*In vitro*	Porphyrin Tetra-Py^+-Me	6 log after 90 min of irradiation (5.0 µM and irradiance of 4.0 mW cm^{-2})	(Jesus et al., 2018)
			Ex vivo	Porphyrin Tetra-Py^+-Me (50 µM)	4 log with 3 cycles of 150 mW cm^{-2}	
					1.8 log at 150 mW cm^{-2}	
					1.2 log at 4.0 mW cm^{-2}	
					1.5 log at solar radiation	
	Kiwifruit plant leaves	Psa	*In vitro*	Tri-cationic porphyrin (Tri-Py^+Me-Mono-PF)	7.4 log after 60 min (5.0 µM and low irradiance (4.0 mW cm^{-2}))	(Martins et al., 2018)
			Ex vivo (artificially contaminated kiwi leaves)	Tri-cationic porphyrin (Tri-Py^+Me-Mono-PF)	2.8 log (50 µM and low irradiance (4.0 mW cm^{-2}))	

					4.5 log (50 µM and sunlight)	
			In planta (naturally contaminated kiwi leaves)	Tri-cationic porphyrin (Tri-Py^{+}Me-Mono-PF)	2.3 log after 90 min (50 µM and sunlight)	
Other compounds	Kiwifruit plant	Psa	*In vitro*	Geraniol and citronellol	Inhibit Psa growth	(Buriani et al., 2014)
	Kiwifruit plant	Psa	*In vitro*	Antimicrobial peptides	n.a.	(Cameron et al., 2014; Choi et al., 2017)
	Kiwifruit plant	Psa	*In vitro*	Clove bud, cinnamon, oregano, thyme, cumin, fennel, basil and garlic essential oils	≥ 40% Psa growth inhibition with respect to the control (300 ppm)	(Pucci et al., 2018)

Legend: n.a.: not available.

Table 1.4. Commercially approved and treatment approaches under development of phage uses in agriculture.

Treatment status		Host plant	Species / pathovar / strain name	Type of investigation	(Bio)control agent	Reduction of bacterial load	Comments	References
Approved	Commercially available product	Tomato	*Xanthomonas campestris* pv. *vesicatoria*	Greenhouse and *in field*	Agriphage™	45% - 79%	Skim milk-formulated phage mixtures.	Omnilytics (Salt Lake City UT, U.S.A.); (Balogh et al., 2003)
		Peppers and tomatoes	*Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato*	*In field*		n.a.	Phage cocktail. EPA approved, Reg. No. 67986-1.	Phagelux Inc. (Shanghai, China, www.phagelux.com).
		Tomatoes, broccoli, spinach	*Excherichia coli* O157:H7	*In planta*	EcoShield™	1-3 log	Phage cocktail. FDA clearance FCN No. 1018.	Intralytix Inc. (Baltimore MD, U.S.A., www.intralytix.com); (Abuladze et al., 2008; Moye et al., 2018)
Under development	*In vivo* / *ex vivo* tests	Peach trees and peach leaves	*Xanthomonas campestris* pv. *pruni*	*In planta*	Phage F_8 suspension (10^8 PFU/mL)	n.a.	Weekly phage treatment significantly reduced the number of diseased fruits as well as the area and the percentage of infected surface	(Saccardi et al., 1993)

							per fruit in one of three orchards studied.	
		Tobacco	*Ralstonia solanacearum*	*In planta*	Phage P4282	n.a.	-----	(Tanaka et al., 1990)
		Tomato	*Ralstonia solanacearum*	*In planta*	Phage ϕRSL1	n.a.	Phage treatment successfully inhibited the growth of bacteria, but did not completely kill it.	(Fujiwara et al., 2011)
			Xanthomonas campestris	*In planta*	Cocktail of four phages	n.a.	Phages were only slightly efficient in inactivating the bacterium	(Flaherty et al., 2000; Kalpage and Costa, 2014)
		Leek (*Allium porrum*) leaves	*Pseudomonas syringae* pv. *porri* strains CFBP1687 and CFBP1770	*Ex vivo*	Cocktail of phages vB_PsyM_KIL1, vB_PsyM_KIL2, vB_PsyM_KIL3, vB_PsyM_KIL4, vB_PsyM_KIL5, vB_PsyM_KIL3b	n.a.	Phages KIL2 and KIL3b decreased lesion length in leaves. Only phage KIL3b showed significant reduction, demonstrating its *in planta* antibacterial effect.	(Rombouts et al., 2016)
		Mushrooms (*Agaricus bisporus*)	*Pseudomonas tolaasii* 6264	*Ex vivo*	Phage hb1a	67% after 5 h up to 15 h of incubation;	Lytic phage	(Min-Hee Kim et al., 2011)

						100% after 13h.		
					Phage hb2d	74% after 7h up to 15 h of incubation; 100% after 15h.	Lytic phage.	
					Phage bp5e	31% - 52% after 7 h - 9h, with no complete lysis after 15 h of incubation, regardless of size of incubation.	Mildly virulent phage.	
			Pseudomonas tolaasii	Greenhouse, *in planta*	Phage ϕPto-bp6g	100% when phage was inoculated at 0 h	Used as prophylaxis.	(Nguyen et al., 2012)
						40% when phage was inoculated at 10 h	-----	
		Rice	*Xanthomonas oryzae* pv. *oryzae*	*Ex vivo*	Phages P4L, P43M, P23M1, P37L, P37M and P37M1	100%, 96% and 86%, when phages were applied 1, 3 and 7 d prior to the inoculation with *X. oryzae*, respectively	Used as prophylaxis.	(Chae et al., 2014; Jones et al., 2007)

		Bean (*Phaseolus valgarius* L.)	*Pseudomonas syringae* pv. *phaseolicola*	Greenhouse, *in planta*	Phages Ph1, Ph2	58.6% - 61.1%	Phage cocktail was more effective than single phages.	(Eman and Afaf, 2014)
					Cocktail of phages Ph1 + Ph2	70.8%		
	In vitro tests	Banana	*Ralstonia solanacearum*	*In vitro*	Phages ϕRSSKD1, ϕRSSKD2	n.a.	Phages were able to infect all *R. solanacearum* isolates.	(Addy et al., 2016)
		Soybean	*Pseudomonas syringae* pv. *glycinea*	*In vitro*	Phages ϕGH1, ϕGH2, ϕGH3	n.a.	Phages were able to infect all *Pseudomonas syringae* pv. *glycinea* isolates.	(Susianto et al., 2014)
			Pseudomonas syringae pv. *glycinea* strain H3	*In vitro*	Phage ϕSK2a	n.a.	Isolation and characterization of phages.	(Addy and Wahyuni, 2016)
					Phage ϕSK2b			
					Phage ϕSK2c			
					Phage ϕMGX1			
		Bean grains	*Pseudomonas syringae*	*In vitro*	Phage h-mutants	n.a.	Brown spot, bean and pea blight and ice nucleation completely eliminated. US Patent number 4,828,999 (May 9, 1989).	(Jackson, 1989)
		Tomato	*Ralstonia solanacearum* MAFF211514	*In vitro*	Phage ϕRSB2	1.24 log	Phage cocktail was more effective than single phages.	(Bhunchoth et al., 2015)
					Phage ϕRSJ2	1.00 log		
					Cocktail of phages ϕRSB2 + ϕRSJ2	2.00 log		

			Ralstonia solanacearum M4S	*In vitro*	Phages ϕRSA1, ϕRSB1, ϕRSL1	Growth inhibition	Coexistence of bacterial cells and phage effectively prevented wilting.	(Fujiwara et al., 2011)
		Elberta peach leaves	*Xanthomonas campestris* pv. *pruni*	*In vitro*	Lytic phage for *Xanthomonas campestris* pv. *pruni*	48% and 42% when phage suspension (10^9-10^{10} PFU/mL) was applied on the leaf surface 1 h or 24 h before pathogen inoculation, respectively.	There was no significant effect when phages were applied after bacterial inoculation, although phage lysate was stable for at least 24 h after application.	(Civerolo and Keil, 1969)

Legend: n.a.: not available.

1.5.3.3. Photodynamic therapy (PDT)

In the search for alternative approaches aiming at overcoming the deficiencies encountered in conventional antimicrobial treatments in several areas, PDT (Table 1.3) has been tested by several researchers in the control of pathogenic microorganisms (Alves et al., 2014b, 2014a; Luksiene et al., 2004; Simões et al., 2016; Tavares et al., 2011). (Alves et al., 2015) extensively reviewed the potential applications of porphyrin-based photosensitizers in PDT beyond the medical scope, viz. applications on the environment, water and foodstuff (including insect pest elimination, water disinfection and elimination of food-borne pathogens), and applications for domestic, industrial and healthcare settings (including porphyrin-embedded fabrics and paper).

PDT has been tested in the control of plant pathogens caused mainly by fungi (Luksiene, Z.; Peciulyte, D.; Lugauskas, 2004; Menezes et al., 2014a, 2014b), but only very recently the PDT application in the control of Psa-induced kiwifruit canker has been reported (Table 1.3) (Jesus et al., 2018; Martins et al., 2018), without any detrimental impacts on the leaves of kiwifruit plants. The PDT may thus be an effective and alternative approach to inactivate Psa in kiwifruit orchards (Jesus et al., 2018; Martins et al., 2018). The rationale underlying antimicrobial PDT involves the presence of a photosensitizer molecule, light and molecular oxygen, which combined are responsible for the formation of highly cytotoxic reactive oxygen species (ROS)(Alves et al., 2014) that readily interact with biological components of the external structures of microorganisms. Due to the functioning rationale of this antimicrobial approach, it is virtually impossible for microrganisms to develop resistance against this treatment (Bartolomeu et al., 2018, 2017, 2016b, 2016a; Jesus et al., 2018; Tavares et al., 2010).

In their research, Jesus et al. (2018) evaluated the effectiveness of a cationic porphyrin (5,10,15,20-tetrakis (1-methylpyridinium-4-yl) porphyrin tetra-iodide (Tetra-Py+-Me) as photosensitizer and different light intensities for the photoinactivation of Psa3 (PsaV or Biovar 3, isolated in Lazio, Italy, in 2008), a highly aggressive phytopathogen responsible for the global pandemic of kiwifruit canker). In *ex vivo* experiments, using kiwifruit plant leaves artificially contaminated with the Psa strain, a reduction of 1.8 log in

the bacterial load at an irradiance of 150 mW cm^{-2}, 1.2 log at 4.0 mW cm^{-2} and 1.5 log under solar radiation were obtained (Jesus et al., 2018). After three successive cycles of treatment under 150 mW cm^{-2}, a 4 log inactivation of Psa was achieved, with no negative effects whatsoever observed on kiwifruit plant leaves following treatment (Jesus et al., 2018).

In a second study of the same research group (Martins et al., 2018), a formulation constituted by a mixture of porphyrins (19% of 5-(1-methylpyridinium-4-yl)-10,15,20-tris(pentafluorophenyl)porphyrin mono-iodide (Mono-Py+-Me-Tri-PF), 20% of 5,15-bis(1-methylpyridinium-4-yl)-10,20-bis(pentafluorophenyl) porphyrin di-iodide (Di-Py+-Me-di-PF), 44% of 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentaf luorophenyl) porphyrin tri-iodide (Tri-Py+-Me-Mono-PF) and 17% of 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py+Me)) was used to evaluate the efficiency of PDT in the inactivation of the same Psa strain. The new formulation was designed to have as main component a tri-cationic porphyrin, which is considered one of the most efficient photosensitizers in the photoinactivation of microorganisms. *In vitro*, the mixture under low irradiance promoted a 7.4 log photoinactivation of Psa. *Ex vivo* assays carried out on artificially contaminated kiwifruit plant leaves, promoted a 2.8 and 4.5 log inactivation with low irradiance and sunlight, respectively. The photoinactivation on naturally contaminated kiwifruit plant leaves was ca. 2.3 log under sunlight irradiation. After ten consecutive cycles of phototreatment in sub-lethal conditions no bacterial resistance or viability recovery was observed, hence suggesting that PDT under sunlight may be a viable alternative to the current methods used in the control of Psa-induced canker without imparting any damage to the leaves.

In spite of the efficacy of the antimicrobial photodynamic approach, care should be exercized in the application of this antimicrobial therapy in the agricultural field, since the highly reactive oxygen species produced during its application do not discriminate between harmful and beneficial microentities (either bacteria, viruses or fungi). Hence, more research is needed in order to shed light on the alterations to the natural non-pathogenic microflora.

1.5.3.4. Other compounds

Several other compounds (Table 1.3) for controlling the epiphytic populations of Psa have been evaluated by industry and companies in New Zealand (Reglinski et al., 2013). In addition, the monoterpenes geraniol and citronellol have showed an inhibitory effect *in vitro* against Psa (Buriani et al., 2014). Regreatably, the authors did not report the reduction achieved in bacterial loads.

Several researchers reported recently on the efficacy of antimicrobial peptides against the Gram (-) Psa (Cameron and Sarojini, 2014; Choi et al., 2017) (Table 1.3), which supposedly target the Psa bacterial membrane *in vitro*, leading to its disaggregation. However, *ex vivo* and *in vivo* studies are required to address their efficacy in kiwifruit plants.

Very recently, Pucci et al. (2018) evaluated several essential oils with potential inhibitory capabilities against Psa (Table 1.3). The essential oils tested showed minimum inhibitory concentration values of 300 ppm (oregano), 600 ppm (cumin, fennel, clove bud, thyme) and 1200 ppm (garlic, dill weed, cinnamon, coriander, eucalyptus, tea tree, spearmint, basil). Oregano and clove bud oils had a minimum bactericidal concentration value of 600 ppm; dill weed, coriander, tea tree, basil and thyme oils had a minimum bactericidal concentration value of 1200 ppm. The essential oils of clove bud, cinnamon, oregano, thyme, cumin, fennel, basil and garlic led to at least a 40% Psa growth inhibition with respect to the control already at 300 ppm, thus showing a relatively high bacteriostatic activity, and were thus considered as the most promising for inhibiting Psa.

Due to the current lack of control measures and the risks of the current approved treatments, the development of new greener and viable alternatives is urgently needed to halt Psa-induced canker in the kiwifruit plants. A targeted and tailored approach to treat infections in plants caused by phytopathogens is phage therapy. Due to the highly specific nature of phage particles, phage therapy of plant infections can be a potential approach with the promise of controlling and/or eradicating infections of kiwifruit plants caused by Psa. This type of therapy will be discussed in detail in the following sections.

1.6. Phage therapy in the control of phytopathogens

Phages are entities devoid of metabolic machinery, requiring a bacterium host to take over its biochemical machinery and replicate (Belay et al., 2018; Harada et al., 2018; Rios et al., 2016). Most phage particles isolated to date interact with bacterial cells expressing specific receptors at the surface of their external structures.

Phage-derived polysaccharide depolymerases are enzymes used by phage particles to hydrolyse the capsular or structural polysaccharides of their specific bacterial hosts, at the beginning of the phage infection process (Figure 1.3) (Drulis-Kawa et al., 2015; Harada et al., 2018; Rios et al., 2016). The phage ability to enzymatically overcome these structures is vital for them to gain access to the appropriate receptors responsible for irreversible phage attachment to the host cell.

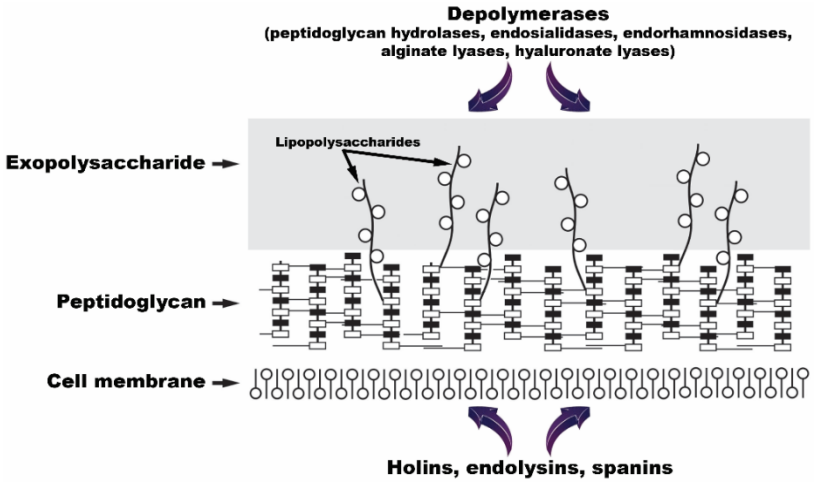

Figure 1.3. Putative sites of attack of phage-derived hydrolytic enzymes on (Gram-negative) cells of Psa (adapted from Drulis-Kawa, Majkowska-Skrobek and Maciejewska (2015)).

Bacterial lysis is the means by which adjacent bacterial hosts are infected. Importantly, differences in the consequences of infection are distinguished on the basis of the phage's life cycle as lysogenic or lytic. Lysogenic phages become integrated into the host genome, may confer additional virulence factors to or prevent subsequent phage infection of its host, and delay bacteriolysis, while lytic phages are not subject to host genome integration and infection results in bacterial lysis (El-Shibiny and El-Sahhar, 2017; Riley, 2006). In the lytic cycle, endowed by strictly lytic phage particles, the metabolism of the bacterial host is overtaken and re-targeted to the production of new phage virions in

time cycles that depend on a particular bacterial host, via replication of the phages' genome in the bacterial cytoplasm which, helped by phage-derived holin, lysin and spanin enzymes, causes an abrupt disintegration of the bacterium and expells the virion progeny to the extracellular space (Drulis-Kawa et al., 2015; Fernandes and São-José, 2018; Harada et al., 2018; Rios et al., 2016). The hydrolytic enzymes, viz. holin, endolysin and spanin are encoded by the phage particles. Holins and endolysins are used to destroy the inner membrane and the murein sacculus, respectively, whereas spanins (characteristic of highly specialized phages for Gram-negative bacteria) are responsible for crossing the outer membrane. By perforating the inner cell membrane, holin hydrolytic enzymes control the access of phage muramidases to the peptidoglycan layer and thus determine the exact moment of bacterial lysis (Drulis-Kawa et al., 2015). For these reasons, lytic phages are highly favored over their lysogenic counterparts in therapeutic settings.

The advantages of phage particles when compared to copper-based chemicals and antibiotics include (but are not limited to): (i) high specificity to a given bacterial species or strain, depending on the kind of receptor that recognizes them; (ii) naturally ubiquitous in the biosphere; (iii) plant communities are constantly exposed to these biological (although metabolically inert) entities; (iv) fairly ease of isolation; (v) exhibit an exponential growth and replicate inside the bacteria, hence accumulating in extremely high concentrations where they are needed the most, while there are still viable bacterial host cells; (vi) successive administrations of phage preparations are not necessary, since as long as the target bacterium is present phage particles will be amplified until the bacterial host virtually disappears; (vii) possess a high ability to penetrate bacterial biofilms; (viii) they can mutate and surpass bacterial resistance; and (ix) the use of only a low concentration may allow an effective antimicrobial treatment (Almeida et al., 2009; Drulis-Kawa et al., 2012; Duarte et al., 2018; El-Shibiny and El-Sahhar, 2017; Harada et al., 2018; Hill et al., 2018; Lima et al., 2019; Rios et al., 2016).

Phage-based plant antimicrobial therapy is, however, not devoid of disadvantages, including: (i) the bacteria responsible for the infection needs to be firstly isolated and identified, after which a specific phage can be isolated from an environmental sample; (ii) only strictly lytic phage particles may be used in phage therapy, which reduces the number

of available phages eligible to integrate this therapy; (iii) bacteria may develop resistance to phage infection (which can be overcome by using a cocktail of different lytic phage particles (Casey et al., 2018)), but resistant bacteria are not as virulent and do not grow as fast as their phage-susceptible counterparts (Almeida et al., 2009; León and Bastías, 2015; Moreirinha et al., 2018; Oechslin, 2018); and (iv) plain (non-protected) phage particles are sensitive to abiotic factors, mainly to ultraviolet radiation (Chan and Abedon, 2012; Duarte et al., 2018; Harada et al., 2018; Rios et al., 2016; Wittebole et al., 2014).

To prevent bacteria from evolving resistance to their natural predators, researchers are engineering phages so as to turn the bacterial immune system known as CRISPR against itself (Barrangou, 2015; Bikard and Barrangou, 2017; Doss et al., 2017; Hatoum-Aslan, 2018; Lima et al., 2019; Pursey et al., 2018). This is the approach followed by two biotech companies, viz. Locus Biosciences (Morrisville NC, U.S.A.) and Eligo Bioscience (Paris, France) (Reardon, 2017). CRISPR-Cas9 engineered phages allow to circumvent all the potential limitations and/or disadvantages of wild phage particles (Lima et al., 2019). By targeting the bacterial cell genome, CRISPR-Cas9 engineered phages will likely be the next step in the evolution of targeted antimicrobials (Bikard and Barrangou, 2017; Hatoum-Aslan, 2018; Lima et al., 2019; Pursey et al., 2018).

1.6.1. Applications of phages in agriculture

Some studies have investigated the efficacy of phage therapy in the agrofood industries, and some phage suspensions are already approved to be used in agriculture either to control infections or as a preventive measure (Table 1.4), namely AgriPhage™ from Phagelux (Shanghai, China) (approved by the Organic Materials Review Institute (OMRI) as a product compatible with organic food production (Monk et al., 2010) and EcoShield™ from Intralytix Inc. (Baltimore MD, U.S.A.). There have been few attempts to use phages towards preventing bacterial infections in agriculture using *in vivo* assays, most of the studies report only *in vitro* and *ex vivo* tests, isolation and characterization of new phages and their efficacy to destroy the phytopathogens in laboratory conditions (Table 1.4).

In vivo and *ex vivo* uses of phages in agriculture have previously been described in the treatment of bacterial infections in peach trees (Saccardi et al., 1993). A phage was selected for its ability to infect various strains of *Xanthomonas campestris* pv. *pruni* (Table 1.4) and for surviving 10 days on the leaves of the plant. After, the phage suspension (10^8 PFU/mL) was applied to the plants on a weekly basis, every 10 days, and every 15 days (the authors did not mention, though, how the phages were applied). The phage application prevented disease symptoms in more than 92% of the cases, one week after inoculation (Saccardi et al., 1993). In one of the orchards studied, the weekly application of the phage suspension reduced the number of damaged peaches by 55%, the area of the infected plant by 34.5% and the percentage of fruit damaged surface by 35.2%.

The use of phages was also studied in the control of the bacterium *Ralstonia solanacearum* in the tobacco plant (Table 1.4) and in this study the virus application slightly reduced the percentage of wilted plants when compared to the control (Buttimer et al., 2017; Fujiwara et al., 2011; Tanaka et al., 1990), indicating some enhancement of control of bacterial wilt of tobacco. The authors did not report, though, the values attained in bacterial load reduction and how the phages were applied in the tobacco leaves.

In a greenhouse trial aiming at pretreating tomato seedlings with phage ϕRSL1 (Table 1.4), bacterial wilt caused by adventitious *Ralstonia solanacearum* was prevented in all plants, whereas untreated plants all wilted. In spite of successfully inhibiting the growth of bacteria, phage ϕRSL1 did not completely kill it though (Fujiwara et al., 2011). The authors did not mention, though, the bacterial loads involved in the trials. Flaherty *et al.* (2000) and Kalpage and Costa (2014) used a cocktail of four phages to control *Xanthomonas campestris*, the bacteria responsible for the spot in the tomato plant, in the field, verifying that the phages were only slightly efficient in inactivating the bacterium. In their field trials, weekly (early morning) spray applications of the phage cocktail suspension were found to reduce the disease severity of bacterial spot on tomato plants by an average of 17%. The low percentage of phage-induced bacterial inactivation was probably due to a reduced phage endurance on plant foliage which could be ascribed to harmful environmental factors such as rain and sunlight UV radiation (Tewfike and Desoky, 2015).

Evidence on the use of phage therapy in the treatment of contaminated mushrooms also exists (Min-Hee Kim et al., 2011; Munsch and Olivier, 1995; Nguyen et al., 2012). Nguyen et al. (2012) isolated, characterized and applied a phage ϕPto-bp6g for inhibiting *Pseudomonas tolaasii* causing brown blotch disease in mushrooms, strongly inhibiting (decreasing bacterial growth) the necrotic activity of this bacterium by 100% when phage was inoculated at 0 h and by 40% when phage was inoculated at 10 h (Table 1.4), showing that phage ϕPto-bp6g may be applied for the effective protection of mushrooms against *P. tolaasii* infection. Similarly, Kim et al. (2011) isolated and characterized three phages of *P. tolaasii* 6264 (viz. phages hb1a, hb2d and bp5e) and succeeded in controlling the same disease in mushrooms (Table 1.4). When phages hb1a (1×10^5 PFU/mL) and hb2d (1×10^5 PFU/mL) were added at a bacteria:phage ratio of 100:1, the absorbance decreased 67% after 5 h up to 15 h of incubation and after 13 h of incubation complete bacterial lysis was observed for phage hb1a, the absorbance decreased by 74% after 7 h and complete lysis was observed after 15 h for phage hb2d. In the case of phage bp5e (7×10^5 PFU/mL), the bacteria:phage ratios corresponding to 5000:7, 1000:7 and 100:7 showed decreases in absorbance of 31% - 52% after 7-9 h and no complete bacterial lysis was observed after 15 h of incubation regardless of the size of inoculation (Min-Hee Kim et al., 2011).

Pseudomonas syringae pv. *phaseolicola*, the phytopathogen infecting bean (*Phaseolus valgarius* L.) was, in a greenhouse (*in planta*, artificially contaminated) study (Table 1.3), treated with phages Ph1 and Ph2. Isolated, these phages succeeded in killing between 58.6% - 61.1% of the bacterial load. However, when added together as a cocktail, the reduction in bacterial load was of 70.8% (Eman and Afaf, 2014).

1.6.2. Phage therapy to control Psa

Several research groups have reported on the isolation, morphologic characterization, genome sequence and host-range determination of phages that could be used for the biocontrol of the kiwifruit canker disease (Di Lallo et al., 2014; Frampton et al., 2014, 2015; Yin et al., 2019; Yu et al., 2016), however, there are only two *in vitro* studies about the use of phages to control the Psa and no *ex vivo*, *in vivo* or field study has been reported so far (Dy et al., 2018). These will be the subject of further discussion below.

1.6.2.1. Available lytic phages against Psa

There are some studies reported on the isolation and characterization of phage particles targeting Psa, and their main features are displayed in Table 1.5. Notwithstanding the relatively large number of isolated phages against different pathovars of *P. syringae*, ca. half of those were tested against Psa (Figure 1.4), which is not surprising, owing to the problem represented by this phytopathogen.

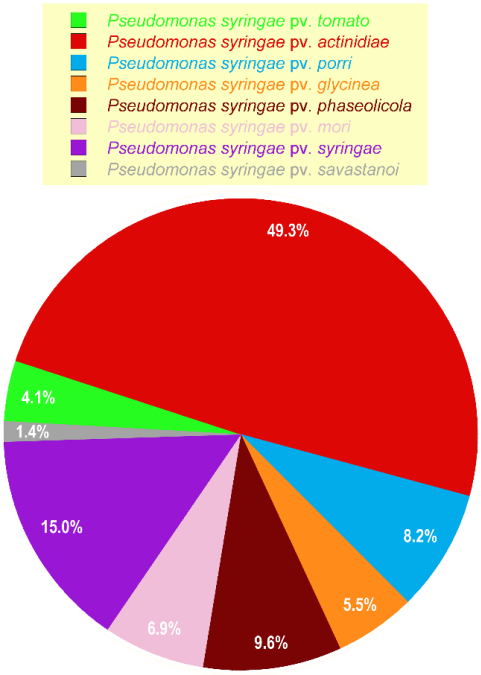

Figure 1.4. Isolated lytic phage particles described in the literature for different *P. syringae* pathovars.

From the isolated phages, most of them belong to the family *Myoviridae* (69.4 %) and *Podoviridae* (22.2 %), but some are classified as *Siphoviridae* (8.4 %), as shown in Table 1.5.

Most of these phages survive for long periods at temperatures between 4 °C and 25 °C (Frampton et al., 2014), with some of them surviving at higher temperatures 40-60 °C (Yin et al., 2019; Yu et al., 2016), tolerate a wide range of pH values (3 to 11) and UV-B radiation during 1 h (Yu et al., 2016). Some of these isolated phages infected different strains of Psa (Frampton et al., 2014; Yin et al., 2019; Yu et al., 2016), revealing potential to be used in phage therapy to control the kiwifruit canker.

Table 1.5. Main features of isolated phages targeting Psa.

Strain (biovar)	Origin of strain	Phage	Taxonomy	Full size (nm)	Nucleic acid type	Infectivity / host range	Survival of phage	Phage genome size (kb)	Reference
CRA-FRU 8.43	Italy	ϕPSA1	*Siphoviridae*	260	linear dsDNA	Temperate, narrow host range	n.a.	51.090	(Di Lallo et al., 2014)
		ϕPSA2	*Podoviridae*	n.a.	linear dsDNA	Lytic, broad host range	n.a.	40.472	
ICMP 18800	New Zealand	ϕPSA1	*Myoviridae*	206.9	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	(Frampton et al., 2014)
		ϕPSA17	*Podoviridae*	55.9	n.a.	++, virulent	4 °C, 25 °C, 6 months	30.000	
		ϕPSA21	*Myoviridae*	293.1	n.a.	++, virulent	4 °C, 25 °C, 6 months	>300.000	
		ϕPSA173	*Siphoviridae*	252.8	n.a.	Temperate	4 °C, 25 °C, 6 months	110.000	
		ϕPSA267	*Myoviridae*	186.6	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA268	*Myoviridae*	181.4	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA281	*Myoviridae*	179.2	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	

		ϕPSA292	*Myoviridae*	204.7	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA300	*Myoviridae*	201.7	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA315	*Myoviridae*	181.0	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA316	*Myoviridae*	184.3	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA317	*Myoviridae*	173.5	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA331	*Myoviridae*	202.6	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA343	*Myoviridae*	194.1	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA347	*Myoviridae*	202.3	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA374	*Myoviridae*	204.6	n.a.	++, virulent	4 °C, 25 °C, 6 months	97.800	
		ϕPSA375	*Myoviridae*	167.7	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA381	*Myoviridae*	194.2	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	

		ϕPSA386	*Myoviridae*	168.2	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA393	*Myoviridae*	202.2	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA394	*Myoviridae*	198.1	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA397	*Myoviridae*	169.8	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA410	*Myoviridae*	208.4	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
		ϕPSA440	*Myoviridae*	208.2	n.a.	++, virulent	4 °C, 25 °C, 6 months	95.000	
ICMP 18800	Paengaroa, New Zealand	vB_PsyP_phiPsa17	*Podoviridae*	60	linear dsDNA	++, lytic	n.a.	40.525	(Frampton et al., 2015)
KBE9 (2); KGY4 (2); PJC7 (2); YCS3 (2); JJ18 (2); JYS5 (2); SYS1 (3); SYS2 (3); SYS3 (3); SYS4 (3); KACC10584 (2); KACC10587 (2);	Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); KACC; KACC; KACC; KACC; KACC; KACC; KACC; KACC	KHUφ34	*Myoviridae*	231	DNA	++; ++; ++; ++; ++; +; +; +; ++; +; ++; ++; -; ++; ++; ++; ++; ++	pH 3-11; 40 °C, 1 h; UV-A light @ 365 nm, 1 h (320 mW/m^2)	74.000	(Yu *et al.*, 2016)

KACC10592 (2); KACC10593 (2); KACC10595 (2); KACC16847 (2); KACC16848 (2); KACC16849 (2)									
KBE9 (2); KGY4 (2); PJC7 (2); YCS3 (2); JJ18 (2); JYS5 (2); SYS1 (3); SYS2 (3); SYS3 (3); SYS4 (3); KACC10584 (2); KACC10587 (2); KACC10592 (2); KACC10593 (2); KACC10595 (2); KACC16847 (2); KACC16848	Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); KACC; KACC; KACC; KACC; KACC; KACC; KACC; KACC	KHUφ38	*Podoviridae*	90	DNA	++; -; ++; ++; ++; ++; +; +; -; +; ++; -; -; ++; ++; ++; ++; ++	pH 3-11; 40 °C, 1 h; UV-A light @ 365 nm, 1 h (320 mW/m^2)	58.000	

(2); KACC16849 (2)									
KBE9 (2); KGY4 (2); PJC7 (2); YCS3 (2); JJ18 (2); JYS5 (2); SYS1 (3); SYS2 (3); SYS3 (3); SYS4 (3); KACC10584 (2); KACC10587 (2); KACC10592 (2); KACC10593 (2); KACC10595 (2); KACC16847 (2); KACC16848 (2); KACC16849 (2)	Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); KACC; KACC; KACC; KACC; KACC; KACC; KACC; KACC	KHUφ44	*Myoviridae*	220	DNA	++; ++; ++; ++; ++; ++; ++; +; +; ++; ++; ++; ++; ++; ++; ++; ++	pH 3-11; 40 °C, 1 h; UV-A light @ 365 nm, 1 h (320 mW/m^2)	60.000	
KBE9 (2); KGY4 (2); PJC7 (2); YCS3 (2); JJ18 (2); JYS5 (2);	Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea);	KHUφ59	*Podoviridae*	87	DNA	++; -; ++; ++; ++; ++; -; ++; -; -; -; ++; ++; ++; ++; ++; ++	pH 3-11; 40 °C, 1 h; UV-A light @ 365 nm, 1 h	41.500	

SYS1 (3); SYS2 (3); SYS3 (3); SYS4 (3); KACC10584 (2); KACC10587 (2); KACC10592 (2); KACC10593 (2); KACC10595 (2); KACC16847 (2); KACC16848 (2); KACC16849 (2)	Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); KACC; KACC; KACC; KACC; KACC; KACC; KACC; KACC						(320 mW/m^2)		
KBE9 (2); KGY4 (2); PJC7 (2); YCS3 (2); JJ18 (2); JYS5 (2); SYS1 (3); SYS2 (3); SYS3 (3); SYS4 (3); KACC10584 (2); KACC10587 (2); KACC10592	Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); Jeju (Korea); KACC; KACC; KACC; KACC; KACC; KACC; KACC; KACC	KHUφ74	*Podoviridae*	88	DNA	++; -; ++; ++; ++; ++; ++; ++; -; -; ++; ++; ++; ++; ++; ++	pH 3-11; 40 °C, 1 h; UV-A light @ 365 nm, 1 h (320 mW/m^2)	32.000	

(2); KACC10593 (2); KACC10595 (2); KACC16847 (2); KACC16848 (2); KACC16849 (2)									
KBE9 (2); KGY4 (2); PJC7 (2); YCS3 (2); JJ18 (2); JYS5 (2); SYS1 (3); SYS2 (3); SYS3 (3); SYS4 (3); KACC10584 (2); KACC10587 (2); KACC10592 (2); KACC10593 (2); KACC10595 (2); KACC16847 (2); KACC16848 (2);	Wando (Korea)	PPPL-1	*Podoviridae*	100	DNA	++; -; ++; ++; ++; ++; ++; ++; -; ++; ++; +; ++; ++; ++; ++; ++; ++	pH 3-11; 40 °C; UV-A light @ 365 nm	41.149	(Park et al., 2018)

KACC16849 (2)									
Biovar 3	China	φXWY0013	*Siphoviridae*	252	n.a.	Broad host range	pH 2-12; 25-60 °C	n.a.	(Yin et al., 2019)
		φXWY0014	*Myoviridae*	193	n.a.	Broad host range	pH 2-12; 25-60 °C	n.a.	
		φXWY0026	*Podoviridae*	102	n.a.	Broad host range	pH 2-12; 25-60 °C	n.a.	

Legend: n.a.: not available; ++: clear plaques; +: turbid plaques; -: no plaques; KACC: Korean Agricultural Culture Collection.

1.6.2.2. *In vitro* studies of phage therapy

The few phage therapy studies, only two, reported in the literature to control the Psa were all carried out *in vitro* conditions and are described in Table 1.6.

From the 70 Psa phages isolated from the soil in kiwifruit orchards in Korea (Table 1.5) by Yu et al. (2016), 5 of the most effective ones were selected for further experiments (Table 1.6). The 5 lytic phages were, in general, stable under different conditions, remaining viable when exposed at 40 °C for 1 hour and at pH values from 3 to 11 during the same period, being inactivated at 60 °C and pH 12 (Yu et al., 2016). In addition, with only one exception, the phages were able to maintain their lytic activity when subjected to UV-A radiation (365 nm) for at least 20 minutes (Table 1.4). The 5 phages were tested on Psa strain KBE9 (Table 1.6) and exhibited different efficacies to reduce the Psa (Table 1.6). Phage KHUϕ34 promoting a 81.5% reduction in optical density at 600 nm (OD 600 nm) over a period of 80 h, phage KHUϕ38 a 63% reduction in OD 600 nm over a period of 80 h and phage KHUϕ44 61.1% reduction in OD 600 nm over a period of 80 h. Phages KHUϕ74 and KHUϕ59 were innefective due to bacterial resistance emergence (Table 1.6).

Park et al. (2018) tested the phage PPPL-1 against the Psa strain KBE9 and observed a Psa reduction of 50% after 12 h of treatment (Table 1.6).

Although the results already obtained in these two studies are promising, more *in vitro* studies using more phages and phage cocktails, evaluating at the same time their efficiency to inactivate the Psa under different physico-chemical conditions, as well as *ex vivo* and field studies are urgently needed in order to transpose this approach to the industry.

1.6.2.3. Overcoming phage inactivation by abiotic factors

There are yet some issues that should be considered when using phages in agricultural applications. These issues are related to the fact that the agricultural fields where phages will be applied are open fields and there is little control over environmental

conditions such as high temperature, high exposition to ultraviolet radiation, ion contents, desiccation, soil moisture and high and low pH, which can cause phage decline on the phyllosphere (Iriarte *et al.*, 2007; Balogh *et al.*, 2010; Silva *et al.*, 2013; Y. J. Silva *et al.*, 2014; Buttimer *et al.*, 2017; Duarte *et al.*, 2018).

Such abiotic factors can inactivate phage particles by imparting damage to their structural elements (capsid, sheath, tail) and/or promoting DNA structural changes (Bae et al., 2012; Chan et al., 2013; Chan and Abedon, 2012; Duarte et al., 2018; Melo et al., 2014; Wittebole et al., 2014).

Ackermann et al. (2004) showed that tailed phages are the most stable in adverse conditions, irrespective of being contractile, non-contractile, or short tails, although phages with a large capsid survive better than phages with a smaller head.

1.6.2.3.1. Abiotic factors that cause phage decline in the phyllosphere

Temperature. Temperature is a crucial factor for lytic phage survival (Hudson et al., 2005; Mojica and Brussaard, 2014; Yolanda J Silva et al., 2014), since it plays a fundamental role in attachment to, penetration of, and multiplication within, bacterial host (Bae et al., 2012). At lower than optimal temperatures, fewer phage genetic material penetrate into bacterial host cells; therefore, fewer of them can be involved in the multiplication phase. Higher temperatures can prolong the length of the latent stage (Tey et al., 2009). From observation of the data displayed in Table 1.5, one can see that most of the lytic phages isolated for Psa survive for long periods at temperatures between 4 °C and 25 °C (Frampton et al., 2014), which correspond to the optimum growth temperature range of Psa.

Table 1.6. *In vitro* studies of phage therapy for controlling Psa in kiwifruit plants.

Host plant	Species / pathovar / strain name	Type of assay	(Bio)control agent	Reduction of bacterial load	References
Kiwifruit plant	Psa (New Zealand virulent strains (ICMP18800, ICMP18801, ICMP18885, ICMP18886, ICMP19101, NZ2V, NZ7V, NZ10V, ABAC9, ABAC79A, ABAC79B), Italy (ICMP18743, ICMP18744, ICMP18745, ICMP18746, ICMP19079), Japan (ICMP9853, ICMP19104, ICMP9617, ICMP9855), South Korea (ICMP19072, ICMP19071))	*In vitro*	Phage ϕPsa17	n.a.	(Frampton et al., 2015)
	Psa strain KBE9	*In vitro*	Phage KHUφ34	50%, after 75h	(Yu et al., 2016)
			Phage KHUφ38	64%, after 25h	
			Phage KHUφ44	33%, after 50h	
			Phage KHUφ59	Innefective (emergence of bacterial resistance)	
			Phage KHUφ74	Innefective (emergence of bacterial resistance)	
	Psa strain KBE9	*In vitro*	Phage PPPL-1	50% in 12 h (MOI=0.01 in a Psa bacterial suspension with 10^8 CFU/mL)	(Park et al., 2018)
	Psa XWY0007	*In vitro*	Phages φXWY0013, φXWY0014, φXWY0026	Lytic phages with high burst sizes	(Yin et al., 2019)

Legend: n.a.: not available

pH. The acidity of the environment is another important factor that directly influences phage particle stability and activity, due to their irreversible coagulation and precipitation (Bae et al., 2012). Langlet et al. (2007) referred in their studies that the concentration of hydrogen ions influences phage aggregation, demonstrating for example that MS2 phages showed significant ability to aggregate when the pH was lower than or equal to their isoelectric point, forming aggregates as large as 6 µm in diameter. This may cause a reduction in phage counts and an easier elimination of phage aggregates via their adsorption on membranes than their single virion counterparts. From the data included in Table 1.5, one can see that some of the phages isolated for Psa tolerate a wide range of pH values (2 to 12) (Park et al., 2018; Yin et al., 2019; Yu et al., 2016).

Ion contents. Osmotic shock due to the presence of ions has also been shown to inactivate phages, since a rapid change in osmotic pressure due to salt may lead to phage DNA extruding from the tail or to break their heads (Bae et al., 2012; Fister et al., 2016; Hudson et al., 2005). There is, however, no specific information in the literature on the effect of ion contents pertaining to phages isolated for Psa.

Ultraviolet radiation. One major factor affecting phage longevity in the phyllosphere is UV light. Biologically harmful ultraviolet radiation (100-400 nm) has been found to be a principal factor contributing to the decline of viral infectivity of phages (Buerger et al., 2019; Duarte et al., 2018; Jacquemot et al., 2018; Mojica and Brussaard, 2014; Park et al., 2018; Shaffer et al., 1999; Yagura et al., 2011; Yu et al., 2016). Solar radiation can directly affect free viruses by degrading proteins, altering nucleic acid structure and resulting in decreasing infectivity. However, viral particles appear more vulnerable to inactivation than to destruction (Wommack et al., 1996). Shorter wavelengths (290 nm - 320 nm) can result in the modification of viral proteins and formation of photoproducts such as cyclobutane pyrimidine dimers (Hotze et al., 2009; Rule Wigginton et al., 2010). Since common lethal photoproducts of UV radiation are thymine dimers, DNA phages (containing thymine) are generally more sensitive to damage by UV radiation than RNA phages (not containing thymine) (Mojica and Brussaard, 2014). Furthermore, dsDNA or dsRNA phages are more resistant to UV radiation than ssDNA or ssRNA phages (Lytle and Sagripanti, 2005; Tseng

and Li, 2005; Turgeon et al., 2014; Verreault et al., 2015). There are, however, phages isolated against Psa able to tolerate extended UV-B doses (Yu et al., 2016).

1.6.2.3.2. Strategies for the structural and functional stabilization of phage particles

The application of phage particles in the environment (such as in kiwifruit orchards) at high titers at the end of the day or during the night period (in which UV radiation is limited), either in free form (as a spray of concentrated phage particles or cocktail of phage particles) or trapped within micro- or nanocarriers (endowing them with enhanced rigidity, promoted by increased viscosity (Balcao and Vila, 2015), may bypass the harmful damages caused by solar radiation (Duarte et al., 2018).

Moreover, the use of a living bacterial cell delivery system can ensure survival and amplification of the phage particles prior to reach the pathogen (Svircev et al., 2018), improving the persistence of phage populations in the soil (Jones et al., 2012). Frampton *et al.* (2014) identified a potential carrier bacterium to improve viral survival, but additional tests will be necessary to test if such bacterial strain is completely nonpathogenic and if it will influence the phage treatment. These authors reported that phage ϕPsa17 was able to infect a non-pathogenic strain of *Pseudomonas*, viz. *P. fluorescens* ABAC62, at the expense of a high decrease in titer relative to Psa, and hence the potential exists to use *P. fluorescens* ABAC62 with phage ϕPsa17 as a phage-carrier combination for phage biocontrol of Psa.

Additionally, preventive cuts in the kiwifruit vines may be treated with plastic paint or resin impregnated with Psa lytic phage particles, aiming at sealing the cut and at the same time preventing the entry of Psa. Plastic paint and resin possess a great durability, as they are not so prone to be leached by rainwater or irrigation via sprinkling (as countermeasures for frost).

Phage particles are apparently innert proteinaceous constructs possessing cavities and crevices endowed with adsorption capabilities and hyper-reactivity characteristics (Balcao and Vila, 2015; Harada et al., 2018). Enhanced stability in protein entities results from changes in the protein core, mostly due to increased burial of hydrophobic groups away from aqueous solvent, providing greater solvation energy and thus favoring folding.

However, proteinaceous constructs such as phage particles are prone to denaturation via conformational changes that can be either reversible or irreversible (Balcão and Vila, 2015). The solution lies in protecting phage particles, either via encapsulation within nanocarriers or via binding to macroscopic supports, thus rendering them insoluble (Duarte et al., 2018; Hosseinidoust et al., 2011; Malik et al., 2017; Nogueira et al., 2017; Sharma et al., 2019; Wang et al., 2016). Combined, these strategies promote their structural and functional stabilization (Balcão et al., 2014, 2013; Balcão and Vila, 2015). Due to being imprisoned in very confined environments, the molecular motions of water molecules slow down. Hence, entrappment in nanoporous matrices or nanocapsules has the same effect as the osmolytes with respect to changing the water activity. Due to both increased (translational, vibrational and rotational) viscosity and entropic (physical) confinement, phage particles can be fully stabilized (Balcão et al., 2014; Balcão and Vila, 2015; Rios et al., 2016) against abiotic factors if environmental applications such as dispersal in crops are sought.

1.7. Conclusions and future trends

Due to the acquisition of phytopathogen resistance to conventional chemical treatments and to the highly deleterious effects of such chemicals upon the environment, the search for more biocompatible and biodegradable treatments is being the subject of extensive scientific research all over the world. The already approved and currently used copper and antibiotic products have proven to be effective in killing the pathogen responsible for kiwifruit canker but are not selective, killing indiscriminately all plant microbiome and do not eradicate the Psa. Phage therapy seems to be a promising and highly effective strategy to control Psa.

Lytic phages against Psa have been easily isolated from environmental sources, and some of those are already characterized and *in vitro* tested in two phage therapy studies involving Psa, showing promise for controlling Psa and not posing the environmental threats as do their chemical counterparts, viz. antibiotics and copper-based compounds. In addition, phage particles against Psa show stability under different temperature and pH values encompassing the optimum growth conditions of kiwifruit plants (viz. pH between

5.5 and 6 and temperatures between 10 °C and 20 °C). In spite of the fact that long UV radiation exposure is believed to affect the longevity of phage particles, there are already some isolated lytic phages for Psa that tolerate UV-B radiation for 1 h or more. However, in practice this will be a no-issue, since phage preparations can be applied at the end of the day or at night, where solar radiation is negligible or virtually null. Additionally, protection of Psa-specific phages within micro- and/or nanocarriers will likely improve their structural and functional stability for field applications.

Despite all the information already existing in the literature mainly pertaining to isolation and characterization of lytic phages against Psa, there are yet few studies of phage therapy against Psa. Moreover, *ex vivo* and *in vivo* field phage therapy experiments are also imperative in order to attain a deeper knowledge of the phage-host interactions, coupled with isolation of different lytic phages for different biovars of the pathogen aiming at production of an effective phage cocktail, in order to transpose this technology to the kiwifruit industry.

Chapter 2. Efficiency of phage ϕ6 for biocontrol of *Pseudomonas syringae* pv. *syringae* infections in plants: an *in vitro* preliminary study

2.1 Abstract

Pseudomonas syringae is a plant-associated bacterial species which has been divided into more than 60 pathovars, being the *Pseudomonas syringae* pv. *syringae* the main causative agent of diseases in a wide variety of fruit trees. The most common treatments for biocontrol of *P. syringae* pv. *syringae* infections has involved copper derivatives and/or antibiotics. However, these treatments should be avoided due to their high toxicity to the environment and promotion of bacterial resistance. Therefore, it is essential to search for new approaches for controlling *P. syringae* pv. *syringae*. Phage therapy can be a useful alternative tool to the conventional treatments to control *P. syringae* pv. *syringae* infections in plants. In the present study, the efficacy of bacteriophage (or phage) ϕ6 (a commercially available phage) was evaluated in the control of *P. syringae* pv. *syringae*. As the plants are exposed to the natural variability of physical and chemical parameters, the influence of pH, temperature, solar radiation and UV-B irradiation on phage ϕ6 viability was also evaluated in order to develop an effective phage therapy protocol. The host range analysis revealed that the phage, besides its host (*P. syringae* pv. *syringae*), infects also the *Pseudomonas syringae* pv. *actinidiae* CRA-FRU 12.54 and CRA-FRU 14.10 strains, not infecting strains from the other tested species. The both multiplicity of infection (MOI) tested, 1 and 100, were effective to inactivate the bacterium, but the MOI 1 (maximum reduction of 3.9 log CFU/mL) was more effective than MOI 100 (maximum reduction of 2.6 log CFU/mL). The viability of phage ϕ6 was mostly affected by exposure to UV-B irradiation (decrease of 7.3 log PFU/mL after 8 h), exposure to solar radiation (maximum reduction of 2.1 PFU/mL after 6 h) and high temperatures (decrease of 8.5 PFU/mL after 6 days at 37 °C , but decrease of only 2.0 log PFU/mL after 67 days at 15 °C and 25 °C). The host range, high bacterial control and low rates of development of phage-resistant bacterial clones (1.20×10^{-3}) suggest that this phage can be used to control *P. syringae* pv. *syringae* infections in plants, but also to control infections by *Pseudomonas syringae* pv. *actinidiae*, the causal agent of bacterial canker of kiwifruit. Although the stability of phage ϕ6 was affected by UV-B and solar radiation, this can be overcome by the application of phage suspensions at the end of the day or at night.

Keywords: Phage treatment, phytopathogenic bacteria, multidrug resistant bacteria, physico-chemical factors

2.2 Introduction

Pseudomonas syringae pv. *syringae* is a phytopathogenic bacterium responsible for bacterial canker and blast (stone and pome fruits). This bacterium infects more than 180 species of plants, among them *Citrus* sp. and *Prunus* sp. (Bradbury, 1986). It affects all commercially grown *Prunus* species including peach (*Prunus persica*), European plum and French prune (*P. domestica*), Japanese plum (*P. salicina*), sweet cherry (*P. avium*), apricot (*P. armeniaca*), tart cherry (*P. cerasus*), almond (*P. dulcis*) and other stone fruits (Kennelly et al., 2007). *P. syringae* pv. *syringae* is also responsable by bacterial blast of orange (*Citrus sinensis*) and mandarin (*Citrus rediculate*) and black pit of orange fruits, causing economic losses worldwide (Ivanović et al., 2017).

P. syringae pv. *syringae* causes damage especially under cold and humid conditions in spring when the development and spread of the bacterial blast happens quicker and more easily, and when the shoots or fruits are damaged by wind, hail or thorns (Ivanović et al., 2017; Pscheidt and Ocamb, 2018). The disease symptoms appear as water-soaked lesions extended to the mid-vein and to the twigs surrounding the base of the petiole. Ultimately, the leaves dry and curl, while still firmly attached, and eventually fall without petioles. The necrotic areas on twigs further enlarge and the twigs are eventually killed within three to four weeks (Ivanović et al., 2017; Pscheidt and Ocamb, 2018). This pathovar can be disseminated by wind, rain, insects, or propagated via either infested wood or nursery stock. Equipments such as pruning tools and mechanical harvesters may also carry the phytopathogen from plant to plant in addition to causing entry wounds.

Currently, the management of most fruit tree diseases caused by *P. syringae* pv. *syringae* is almost unattainable, due to the lack of effective chemical or biological control measures, little available knowledge of host resistance, and the endophytic nature of the pathogen during some phases of the disease cycle (Kennelly et al., 2007). The available treatments for this disease are still scarce, with the most common involving frequent

spraying the orchards with copper derivatives in particular cuprous oxide (Cu_2O) and/or antibiotics. However, these strategies are not completely effective. Moreover, the massive use of copper and antibiotics can promote the development of resistance in the pathogen and changes in the structure of bacterial communities (Altimira et al., 2012). One of the most promising alternatives to the use of these strategies is the use of phages.

The use of phages (viruses that infect bacteria) as biocontrol agents was hindered by the advent of antibiotics, but, in recent years, the continuous selection of bacteria resistant to antibiotics or other antimicrobial agents has led to a new emphasis on phage therapy (Almeida et al., 2009; Lima et al., 2019; Rios et al., 2016). Phage therapy has several potential advantages over the use of copper compounds and antibiotics. Phages are usually highly specific to a single species or even strain of bacteria and therefore cause less damage to the normal microflora. Phages are self-replicating as well as self-limiting, replicate exponentially as bacteria and decline when bacterial number decreases (Hawkins et al., 2010; Park and Nakai, 2003; Pereira et al., 2011).

Phages have been used as therapeutic or prophylactic agents to control bacterial diseases in plants and there are already some approved applications of phages in the agriculture sector (Czajkowski et al., 2014; Bae et al., 2012; Frampton et al., 2012). Moreover, the isolation and characterization of new phages has been described for several pathovars of *P. syringae* such as *P. syringae* pv. *tomato* (Prior et al., 2007, Cuppels, 1983), *P. syringae* pv. *phaseolicola* (Mindich et al., 1999; Qiao et al., 2010; Vidaver et al., 1973), *P. syringae* pv. *syringae* (Nordeen et al., 1983), *P. syringae* pv. *morsprunorum* (Smith et al., 1994), *Pseudomonas syringae* pv. *porri* (Rombouts et al., 2016) and *P. syringae* pv. *actinidiae* (Di Lallo et al., 2014; Frampton et al., 2014, 2015; Park et al., 2018; Yin et al., 2019; Yu et al., 2016). Although Nordeen et al. (1983) isolated and characterized phages of the phytopathogen *P. syringae* pv. *syringae*, they did not evaluate the application of these phages to inactivate this bacterial pathovar. Moreover, to the best of our knowledge, no studies so far evaluated the therapeutic potential of other phages to control *P. syringae* pv. *syringae.*

The aim of this study was to evaluate the efficiency of one of the best-studied and already commercially available phages, the phage ϕ6, to control infections by *Pseudomonas*

syringae pv syringae. To our best knowledge, phage ϕ6 was initially characterized with the plant-pathogenic *P. syringae* pv. *phaseolicola* HB10Y (Mindich et al., 1988; Vidaver et al., 1973) but no inactivation studies were performed by those authors, and was not yet tested to inactivate *Pseudomonas syringae* pv. *syringae*. The phage ϕ6 is an already genomically characterized phage, that neither codifies for integrase genes, which prevents lysogeny (Wei et al., 2009; Wickner, 1993; Yang et al., 2016), nor for virulent factors and antibiotic/metals resistance (Li and Dennehy, 2011), can be thus considered as safe to be used to control bacterial infections. As one of the major concerns regarding the use of phages to control infections is the emergency of phage-resistant mutants after treatment, the development of resistant mutants of *P. syringae* pv. *syringae* to phage ϕ6 was also evaluated in this study. Additionally, considering that plants are exposed to environmental factors and that the viability of phages can be affected by physico-chemical factors, in this study the influence of pH, temperature, solar radiation and UV-B irradiation on phage ϕ6 stability was also evaluated in order to develop an effective phage therapy strategy.

2.3. Materials and methods

2.3.1. Bacterial strains and growth conditions

The bacterial strain *Pseudomonas syringae* pv. *syringae* (DSM 21482) was purchased from Leibniz-Institute DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmmH (Braunschweig, Germany). *P. syringae* pv. *actinidiae* strains (CRA-FRU 8.43, 12.54 and 14.10) were purchased from Culture Collection of C.R.A. - Centro di Ricerca per la Frutticoltura (Roma, Italy). *Pseudomonas aeruginosa* (ATCC 27853), *Aeromonas hydrophila* (ATCC 7966), *Salmonella enterica* serovar Typhimurium (ATCC 13311 and ATCC 14028), *Escherichia coli* (ATCC 25922 and ATCC 13706) and *Vibrio parahaemolyticus* (DSM 27657), were purchased from ATCC and DSM culture collections, respectively. The other bacterial strains used in this study were isolated in previous research works from water samples collected in Ria de Aveiro (Aveiro, Portugal) (Louvado et al., 2012; Oliveira et al., 2014).

All bacteria were grown in Tryptic Soy Broth (TSB, Roseto degli Abruzzi (Te), Italia). The bacterial strains were stored at -80 °C in 10% glycerol. Before each assay, a stock culture of each bacteria was aseptically inoculated in 30 mL of TSB and was grown during 18 h at 25 °C with orbital shaking set at 120 rpm stirring. Then, an aliquot (300 µL) of each bacterial culture was transferred to 30 mL of fresh TSB and grown during 18 h at 25 °C under orbital shaking (120 rpm). The viable cell density was approximately 10^9 colony-forming units (CFU) /mL.

2.3.2. Preparation of phage ϕ6 and enrichment

Phage ϕ6 (DSM 21518) was purchased from Leibniz-Institute DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmmH (Braunschweig, Germany). Phage ϕ6 is a double-stranded RNA phage and belongs to the family *Cystoviridae* (Callanan et al., 2018; Mäntynen et al., 2018). Phage suspensions were prepared departing from the phage stock previously prepared in SM buffer [0.1 M NaCl (Sigma, Molsheim MO, USA), 8 mM $MgSO_4$ (Sigma), 20 mM Tris-HCl (Sigma), 2% (w/v) gelatin, pH 7.5] using *P. syringae* pv. *syringae* as host. Three hundred microliters of the phage stock were added to thirty millilitres of SM buffer and one millilitre of *P. syringae* pv. *syringae* in the exponential growth phase. The suspension was grown overnight and incubated at 25 °C under orbital shaking set at 50 rpm. The lysate was centrifuged at 13000 rpm for 10 min at 4 °C and the supernatant was filtered through a polyethersulphate membrane with 0.22 µm pore-size (Merck-Millipore, Darmstadt, Germany), to remove intact bacteria or bacterial debris. The phage suspension was stored at 4 °C until use was in order and the titre was determined via the double-layer agar method (Adams, 1959). Successive dilutions of the phage suspension were performed in phosphate buffered saline [PBS; 137 $mmol^{-1}$ NaCl (Sigma), 2.7 $mmol^{-1}$ KCl (Sigma), 8.1 $mmol^{-1}$ $Na_2HPO_4 \cdot 2H_2O$, 1.76 $mmol^{-1}$ KH_2PO_4 (Sigma), pH 7.4] and 500 µL of each dilution were added to 200 µL of fresh *P. syringae* pv. syringae culture, mixed with 5 mL of TSB 0.6% top agar layer [30 g/L TSB (Liofilchem), 6 g/L agar (Liofilchem), 0.05 g/L $CaCl_2$ (Sigma), 0.12 g/L $MgSO_4$ (Sigma), pH 7.4] and placed over a Petri plate containing solid Tryptic Soy Agar (TSA, Roseto degli Abruzzi (Te), Italia). The plates were

incubated at 25 °C for 18 h. The results were expressed as plaque forming units per milliliter (PFU/mL).

2.3.3. Determination of the molar extinction coefficient of the isolated phage ϕ6 particles

The molar extinction coefficient of phage ϕ6 was determined according to Rios et al. (2018). Sucessive dilutions of phage ϕ6 (10^8 PFU/mL), using different volumes of the phage suspension, were prepared. After, the absorbance was determined using a spectrophotometer (model Halo DB-20, Livingston, United Kingdom) at 265 nm (wavelength producing the maximum absorption of the phage suspension) and 320 nm (wavelength where there is little light absorption from phage chromophores).

2.3.4. Phage ϕ6 host range: spot test and efficiency of plating (EOP) assays

Phage host range was determine using the bacterial strains listed in Table 2.2. Phage host range was determined by spot testing according to the procedure described by Pereira et al. (2016). Briefly, three milliliters of TSB 0.6% agar, previously inoculated with 300 µL of bacterial culture (Table 2.2) were overlaid on solid TSA and spotted with 10 µL of the phage suspension. The plates were incubated at 25 °C and examined for the presence of lysis plaques after 18 h. Bacterial sensitivity to the phage was established by a clear lysis zone at the spot. Depending on the clarity of the spot, bacteria were differentiated according to either clear lysis zone (+) or no lysis zone (−). The EOP was determined for those bacteria with positive spot tests (occurrence of a clear lysis zone), using the double-layer agar method (Adams, 1959). The plates were incubated at 25 °C and examined for the presence of plaques after 18 h. The EOP for each bacterial host was calculated by comparison with an efficacy of *P. syringae* pv. *syringae* (host). The EOP was calculated as [average PFU on target bacteria / average PFU on host bacteria] x 100 (Melo et al., 2014; Kutter, 2009). The EOP value obtained with the host strain was considered as EOP = 100%. EOP values are presented in the manuscript as the mean of three independent measurements followed by their standard deviation.

2.3.5. One-step growth curve analysis

Phage ϕ6 suspension (final concentration of 10^6 PFU/mL) was added to 10 mL of the bacterial culture of *P. syringae* pv. *syringae* (cell density of 10^9 CFU/mL) in order to have a MOI of 0.001 and the resulting suspension incubated without shaking during 5 min at 25 °C (Mateus et al., 2014). The mixture was centrifuged at 10000 rpm for 5 min, the pellet was re-suspended in 10 mL of TSB and incubated at 25 °C. Samples (1 mL) were collected at time 0 and at time intervals of 10 min up to 150 min of incubation and immediately tittered by the double-layer agar method (Adams, 1959). The plates were incubated at 25 °C and examined for the presence of plaques after 18 h. Three independent assays were performed. The results were subsequently plotted to determine the phage eclipse period, latent period, intracellular accumulation period and burst size. The one-step growth curves data produced was better adjusted via nonlinear fitting the data to a typical sigmoidal curve (or 4-parameter logistic regression model) (Equation (1)):

$$Log\ (P_t) = m_1 + \frac{m_2 - m_1}{1 + \left(\frac{t}{m_3}\right)^{m_4}} \quad (1)$$

where P_t is the phage concentration (PFU/mL) at time *t,* m_1 is the response at t = 0, m_2 is the response at t = ∞, m_3 is the curve inflection point, m_4 is the slope that defines the steepness of the curve, and *t* is the time (min). Nonlinear fitting of the phage growth data to the model in Equation (2) was performed using the software KaleidaGraph v. 4.5.2 for MacOS X.

2.3.6. Adsorption curve

Phage ϕ6 suspension (final concentration of 10^6 PFU/mL) was added to 10 mL of the bacterial culture (final concentration of 10^9 CFU/mL) to obtain a MOI of 0.001 (Stuer-Lauridsen et al., 2003) and the resulting suspension was incubated at 25 °C. The mixture

was centrifuged at 10000 rpm for 5 min and supernatants were immediately filtered through 0.2 µm pore-size filters (Millipore Bedford, MA, USA). The filtrates containing unadsorbed or reversibly adsorbed phage particles were diluted and titrated. The plates were incubated at 25 °C and examined for plaques after 18 h. Adsorption was expressed as the percentage decrease of phage titre in the supernatant, as compared to the time zero. Suspensions of phage without any bacterial cells were used as no-adsorption standard for calculations (Stuer-Lauridsen et al., 2003). Three independent assays were performed. The adsorption rate was estimated via nonlinear fitting the experimental data to the model in Equation (2)(García et al., 2019; Santos et al., 2014; Shao and Wang, 2008):

$$Ln\left(\frac{P_t}{P_0}\right) = -\delta\left(\frac{X_0}{\mu(t)}\right)\left(e^{\mu(t)\cdot t} - 1\right) \quad (2)$$

where P_t and P_0 are phage concentrations at times *t* and *0*, respectively, □ is the adsorption rate to be estimated, X_0 is the concentration of (susceptible, uninfected) bacterial cells at time *0*, $\mu(t)$ is the bacteria multiplication rate and *t* is the infection time. Nonlinear fitting of the adsorption data to the model in Equation (2) was performed using the software KaleidaGraph v. 4.5.2 for MacOS X.

2.3.7. Bacterial kill curves

P. syringae pv. *syringae* (final concentration of 10^5 CFU/mL) inactivation by the phage ϕ6 (final concentrations of 10^5 and 10^7 PFU/mL) was evaluated at MOI 1 and MOI 100. For each assay, two control samples were included: the bacterial control (BC) and the phage control (PC). The bacterial controls were inoculated with *P. syringae* pv. *syringae* but not with phage ϕ6 and the phage controls were inoculated with phage ϕ6 but not with bacterial cells. Controls and test samples were incubated exactly under the same conditions. Aliquots of test samples (BP, bacteria plus phage) and of the bacterial and phage controls were collected at time 0 and after 2, 4, 6, 8, 10, 12, 14, 18 and 24 h of incubation. In all assays, the phage titre was determined in triplicate by the double-layer agar method (Adams, 1959) after an incubation period of 18 h at 25 °C. Bacterial concentration was

determined in triplicate in solid TSA medium via the drop-plate method after an incubation period of 48 h at 25 °C. Three independent experiments were performed for each condition.

2.3.8. Detection of host sensitivity to phage ϕ6 during bacterial kill curve assays

The bacterial colonies of the bacterial control (BC) and test samples (BP) of the bacterial kill curve assays were used (as described in section 2.7.). The MOI of 1 was selected for these experiments because it was the best condition to control *P. syringae* pv. *syringae*. To check whether the bacterial strain remained sensitive to the phage ϕ6 during the bacterial kill assays, ten isolated colonies (randomly picked) of the bacterial control and test samples at time 6, 12, 18 and 24 h were inoculated separately into TSB and grown at 25 °C during 18 h at 120 rpm and tested using the spot test procedure. Three hundred microliters of bacterial culture previously inoculated with TSB 0.6% agar were overlaid on solid TSA and spotted with 10 µL of the phage ϕ6 suspension. The plates were incubated at 25 °C and examined for the presence of lysis plaques after 18 h. Bacterial sensitivity to the phage was established by a clear lysis zone at the spot test. The frequency of sensitive *P. syringae* pv. *syringae* to the phage ϕ6 was calculated as the number of spot tests positive in [test sample (BP) / the number of spot test positive in bacterial control (BC)] x 100.

2.3.9. Determination of the rate of emergence of bacterial mutants

The development of resistant mutants of *P. syringae* pv. *syringae* to phage ϕ6 was evaluated according to the procedure described by Filippov *et al.* (2011). To determinate the frequency of phage-resistant bacteria, ten isolated colonies from a plate with sensitive bacteria were selected and inoculated into ten test tubes with 5 mL of TSB, grown at 25 °C for 18 h at 120 rpm stirring. Aliquots of 100 µL from the 10^{-1} to 10^{-3} dilutions of the bacterial culture and aliquots of 100 µL of the phage from a stock solution of 10^{8} PFU/mL were inoculated in test tubes containing TSB 0.6% agar, plated on TSA plates and incubated at 25 °C for 48 h. Simultaneously, 100 µL-aliquots of 10^{-5} to 10^{-7} dilutions of the bacterial

culture were plated by incorporation on TSA plates without phage and incubated at 25 °C for 48 h. The averaged number of colonies of mutants (obtained from the ten isolated colonies) in 1 mL of culture (prepared from the culture with phages) was divided by the averaged number of colonies of the control (prepared from the culture without phages) (Filippov et al., 2011). Three independent assays were performed.

2.3.10. Fitness of phage-resistant bacterial mutants

The growth of the sensitive and resistant bacterial cell populations was quantified in the presence and in the absence of the phage ϕ6, in order to evaluate the cost ("the fitness") that the bacteria suffers to develop resistance to the phage. The MOI of 1 was selected for these experiments because it was the best condition to control *P. syringae* pv. *syringae*. Sensitive *P. syringae* pv. *syringae* was added to 2 out of 4 samples in order to obtain a final concentration of 10^5 CFU/mL. One of the samples inoculated with sensitive *P. syringae* pv. *syringae* was inoculated with phage ϕ6 to obtain a final concentration of 10^5 PFU/mL (sensitive with phage ϕ6) and the remaining infected sample was not added with phage (sensitive bacteria without phage). Mutants resistant to phage ϕ6 were added to 2 out of 4 samples to obtain a final concentration of 10^5 CFU/mL. One of these samples was inoculated with phage ϕ6 (resistant bacteria with phage ϕ6) to obtain a final concentration of 10^5 PFU/mL and the remaining infected sample was not added with phage (resistant bacteria without phage). Samples were incubated at 25 °C and bacterial concentration (CFU/mL) was determined in triplicate via the drop plate method in solid TSA medium at time 0 and after 6, 12, 18 and 24 h of incubation. The plates were incubated at 25 °C for 48 h. Three independent experiments were performed.

2.3.11. Assessment of the effect of environmental factors upon phage viability

The effects of temperature, pH and radiation (sunlight and UV-B light) upon the viability of phage ϕ6 (final concentration of 10^7 PFU/mL) was tested in 30 mL of phosphate buffered saline (PBS). In the experiments to evaluate the effect of pH and temperature, aliquots were collected after 0, 4, 6, 12, 18, 24, 30, 36, 42, 54 and 67 days of incubation. To evaluate the effect of UV-B irradiation, aliquots were collected after 0, 2, 4, 6, 8, 10 and 12

of incubation. To assess the effect of solar radiation, aliquots were collected after 0, 2, 4 and 6 h of exposure. Phage titre was determined in triplicate via the double-layer agar method and plates were incubated at 25 °C for 18 h. Three independent experiments were performed for each condition.

2.3.11.1. Temperature experiments

To evaluate the effect of temperature upon phage viability, the samples were maintained at a constant temperature (15, 25 and 37 °C) in an incubating chamber. The experiments were performed in sterile PBS at pH 7.0.

2.3.11.2. pH experiments

In order to evaluate the effect of pH upon phage viability, suspensions of phage ϕ6 were added to sterile PBS with pH values of 6.5, 7.0 and 7.5. During these experiments, the temperature of the samples was kept at 25 °C**.**

2.3.11.3. UV-B irradiation experiments

In order to evaluate the effect of UV-B irradiation (290-320 nm), one used an ultra-violet type B lamp TL 20 W/12 RS (Philips, Holland) placed at a distance of 25 cm from the samples. The experiments were performed in sterile PBS at pH 7.0 and at ambient temperature. The control sample (UV-B C) was incubated in the same conditions as the test sample (UV-B), but was not exposed to UV-B radiation.

2.3.11.4. Solar radiation experiments

To evaluate the effect of solar radiation, a suspension of phage ϕ6 was added to sterile PBS at pH 7.0 and exposed to natural solar radiation. The control sample (SR C) was incubated in the same conditions as the test sample (S), but was not exposed to solar radiation. The experiments were performed under a solar irradiance of 2.82 kWh/m^2 in a day with ambient temperature ranging from 14 °C - 24 °C.

2.3.12. Statistical analysis

Statistical analysis of the data was performed using the software GraphPad Prism 7.04 (GraphPad Software, San Diego CA, USA). Normal distribution of the data was checked by a Kolmogorov-Smirnov test and the homogeneity of variance was assessed by the Levene's test. Significance was accepted at $p < 0.05$. Tukey's multiple comparison test was used for a pairwise comparison of the means. The significance of bacterial and viral concentrations between treatments, and along the experiments, was tested using two-way ANOVA and the Bonferroni post-hoc test (Section 2.4.6). For different treatments, the significance of differences was evaluated by comparing the result obtained in the test samples with the results obtained for the correspondent control samples, for the different times. Two-way ANOVA was used to examine differences between the concentration of resistant bacteria and sensitive bacteria in the presence/absence of the phage after 6, 12, 18 and 24 h of incubation (Section 2.4.9). The significance of the effect of physico-chemical factors on phage ϕ6 viability and incubation time was assessed by one-way analysis of variance (Section 2.4.10). A value of $p < 0.05$ was considered to be statistically significant.

2.4. Results

2.4.1. Phage preparation and enrichment

Phage ϕ6 formed clear plaques on the *P. syringae* pv. *syringae*with a diameter of 1 - 2 mm (Figure 2.1). Phage plaques exhibit a secondary halo in the frontier of the lysis plaque of phage (Figure 2.1). High titre suspensions (10^8 - 10^9 PFU/mL) were obtained for the phage ϕ6.

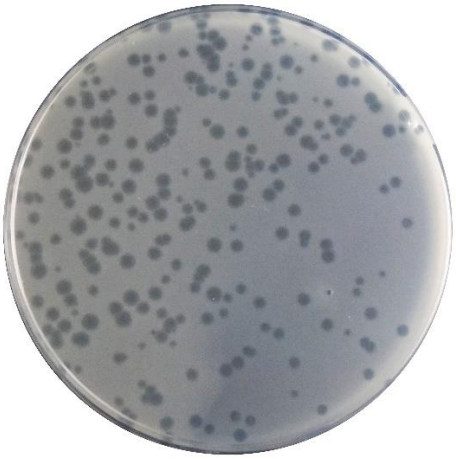

Figure 2.1. Morphology of the ϕ6 lysis plaques.

2.4.2. Analysis of the concentrated phage ϕ6 suspension via UV-Vis spectrophotometry

The results obtained from the UV-Vis scanning performed to the concentrated phage ϕ6 suspension is displayed in Figure 2.2a, whereas the data utilized to prepare the calibration curve relating the phage particle concentration and its corrected absorbance (Figure 2.2b) is displayed in Table 2.1.

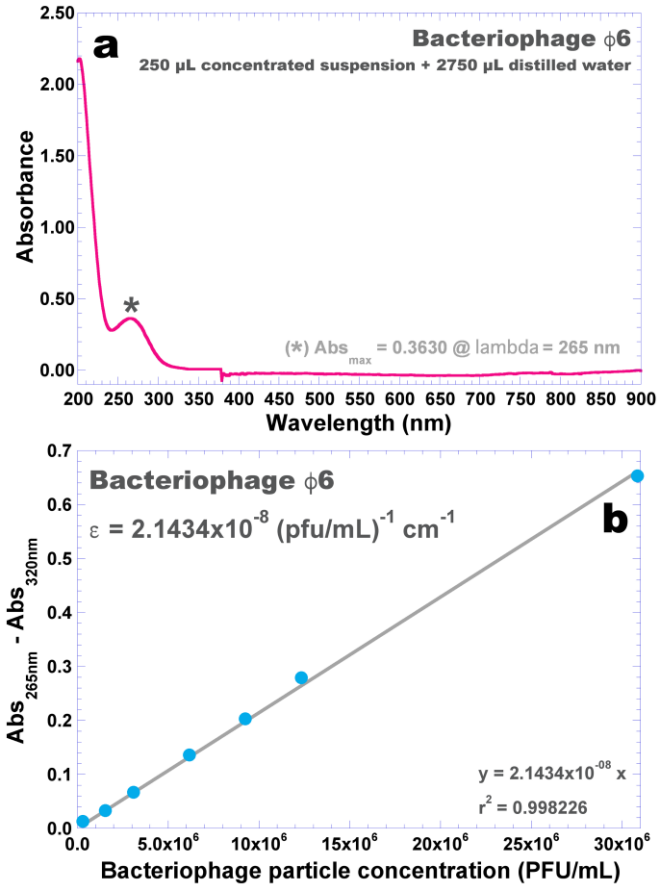

Figure 2.2. Wavelength screening of phage ϕ6 suspension allowing observation of the wavelength producing maximum absorption of phage ϕ6 particles (a) and calibration curve produced for the relationship between the concentration of (whole) phage particles in suspension and the absorption of the suspension at 265 nm corrected for cell debris and other intracytoplasmatic proteins at a wavelength of 320 nm (b).

A pronounced minimum absorption can be observed around 245 nm (indicative of the absence of bacterial cell debris and any other intracytoplasmatic proteins and presence of high concentration of virions) (Figure 2.2a).

Table 2.1. Data utilized to prepare a calibration curve aiming at determining the molar extinction coefficient of phage ϕ6 (whole) particles.

Sample volume of concentrated phage suspension (µL)	Final volume of dilution (µL)	Number of phage particles in the sample volume of concentrated phage suspension	Phage particle concentration (PFUs/mL)	$Abs_{265\ nm}$	$Abs_{320\ nm}$	Abs_{265nm}-Abs_{320nm}
5	3000	$9.2500x10^5$	$3.08333x10^5$	0.0130	0.0000	0.0130
10	3000	$1.8500x10^6$	$6.16667x10^5$	0.0110	0.0110	0.0000
25	3000	$4.6250x10^6$	$1.54167x10^6$	0.0340	0.0010	0.0330
50	3000	$9.2500x10^6$	$3.08333x10^6$	0.0710	0.0040	0.0670
100	3000	$1.8500x10^7$	$6.16667x10^6$	0.1510	0.0150	0.1360
150	3000	$2.7750x10^7$	$9.25000x10^6$	0.2300	0.0270	0.2030
200	3000	$3.7000x10^7$	$1.23333x10^7$	0.3180	0.0390	0.2790
500	3000	$9.2500x10^7$	$3.08333x10^7$	0.7640	0.1110	0.6530

A linear fitting of the Beer-Lambert equation was then performed to the experimental data (Abs_{265nm}-Abs_{320nm}=ƒ[phage particle concentration, PFU/mL]), allowing determination of the molar extinction coefficient of phage ϕ6 as $\varepsilon = 2.1434x10^{-8}$ $(pfu/mL)^{-1}$ cm^{-1}.

2.4.3. Host range and efficiency of plating (EOP) assays

The spot tests and EOP results indicated that phage ϕ6, in addition to *P. syringae* pv. *syringae*, formed phage lysis plaques on 2 of the 25 strains tested (Table 2.2). Phage ϕ6 infected *P. syringae* pv. *actinidiae* CRA-FRU 12.54 and *P. syringae* pv. *actinidiae* CRA-FRU 14.10, with an efficiency of 101.3% and 96.8%, respectively.

Table 2.2. range of phage ϕ6 determined on 25 bacterial strains. Clear lysis zone (+) and not lysis zone (-). The plating with the host strain was considered as EOP = 100%.

Strains	Spot test	EOP (%)
Pseudomonas syringae pv. *syringae* DSM 21482	+	100 (host)
Pseudomonas syringae pv. *actinidiae* CRA-FRU 8.43	-	0
Pseudomonassyringae pv. *actinidiae* CRA-FRU 12.54	+	101.3
Pseudomonas syringae pv. *actinidiae* CRA-FRU 14.10	+	96.8
Pseudomonas aeruginosa ATCC 27853	-	0
Pseudomonas aeruginosa	-	0
Pseudomonas gingeri	-	0
Pseudomonas putida JQ619028	-	0
Pseudomonas putida JQ824856	-	0
Pseudomonas sp. JX047434	-	0
*Pseudomonas*sp. EF627998	-	0
Pseudomonas sp. AF411853	-	0
Pseudomonas sp. HF679142	-	0
Pseudomonas sp. AB772943	-	0
Pseudomonas sp. EU306338	-	0
Pseudomonas sp. AY332207	-	0
Pseudomonas sp. JN033360	-	0
Pseudomonas stutzeri EU167940	-	0
Pseudomonas rhodesiae JX994152	-	0
Escherichia coli ATCC 13706	-	0
Escherichia coli ATCC 25922	-	0
Salmonella typhimurium ATCC 13311	-	0
Salmonella typhimurium ATCC 14028	-	0
Aeromonas hydrophila ATCC 7966	-	0
Vibrio parahaemolyticus DSM 27657	-	0

2.4.4. One-step growth curve analysis

Non-linear fitting the one-step growth data to a 4-parameter logistic model resulted in a good correlation coefficient (viz. 0.9997) and showed that the eclipse period, latent period and intracellular accumulation period lasts 80 min, 100 min and 20 min, respectively (Figure 2.3). The burst size of phage ϕ6 was 60 ± 1 PFU/ host cell (Figure 2.3).

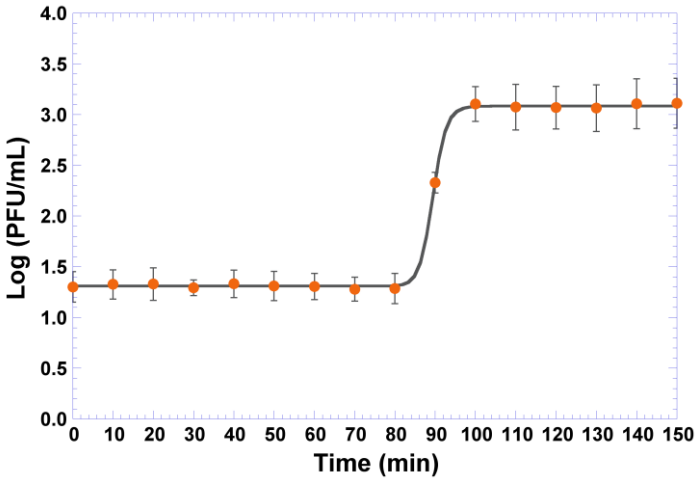

Figure 2.3. One-step growth curve of phage ϕ6 in the presence of *P. syringae* pv. *syringae* as host. Values represent the mean of three experiments; error bars represent the standard deviation.

2.4.5. Adsorption curve

Phage ϕ6 adsorption assays showed that approximately 50% of the phage particles adsorb to *P. syringae* pv. *syringae* after 30 min, 75% adsorbed after 60 min and 95% adsorbed after 120 min (Figure 2.4). Nonlinear fitting the experimental data to the model depicted in Equation (2) resulted in a good correlation coefficient (viz. 0.9951) and allowed determination of the adsorption rate of phage ϕ6 onto *P. syringae* pv. *syringae* cells as δ = (9.495 ± 0.660) x 10^{-12} PFU^{-1} CFU^{-1} mL^{-1} hr^{-1} and a bacteria multiplication rate $\mu(t)$ = (2.489 ± 2.055) x 10^{-3} hr^{-1}.

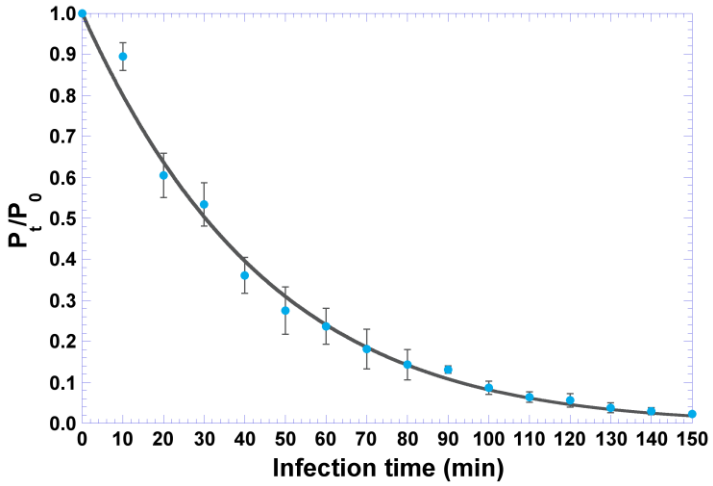

Figure 2.4. Adsorption curve of phage ϕ6 particles onto *P. syringae* pv. *syringae* host cells, allowing to calculate the phage particles adsorption rate following non-linear fitting of a logarithmic function to the experimental data.

2.4.6. Bacterial kill curves

At a MOI of 1 and 100, the maximum of *P. syringae* pv. *syringae* inactivation with phage ϕ6 was 3.9 and 2.6 log CFU/mL (Figure 2.5a, ANOVA, $p < 0.05$), achieved after 12 h and 24 h of incubation, when compared with those of the bacterial control (BC). During the first 10 h of incubation, the inactivation factor was similar for MOI 1 and 100 (ANOVA, $p > 0.05$). At a MOI of 1, after 12 h, 14 h and 18 h of incubation, the decrease in *P. syringae* pv. *syringae* counts (3.9, 3.7 and 3.6 CFU/mL, respectively) was significantly higher (ANOVA, $p < 0.05$) than that obtained with the MOI of 100 (1.8, 2.1 and 2,6 CFU/mL, respectively). The rates of bacterial reduction at the end of treatment were statistically similar (ANOVA, $p > 0.05$) for the two MOI values (Figure 2.5a).

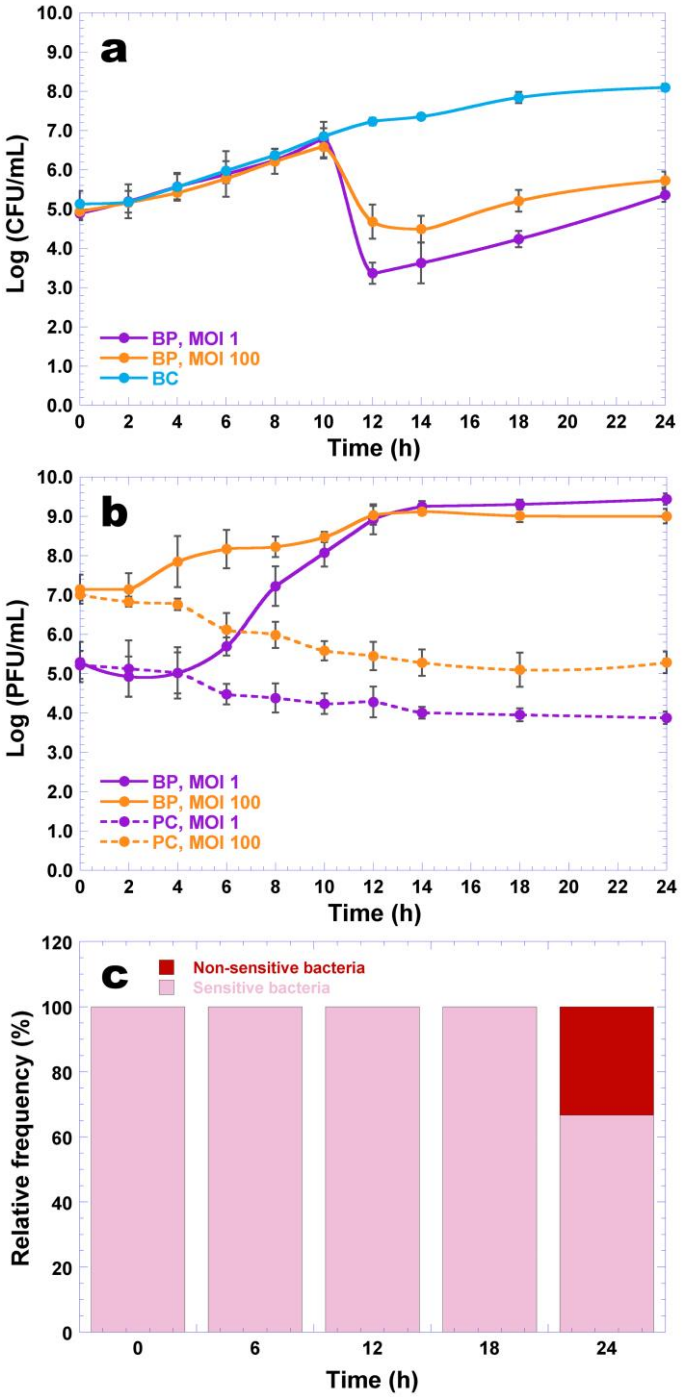

Figure 2.5. Inactivation of *P. syringae* pv. *syringae* by phage ϕ6 at a MOI of 1 and 100 during 24 h. (a) Bacterial concentration: BC, bacteria control; BP, bacteria plus phage. (b) Phage concentration: PC, phage control; BP, bacteria plus phage. (c) Relative frequency of the sensitivity of *P. syringae* pv. *syringae* to phage ϕ6 at a MOI of 1 after 6, 12, 18 and 24 h of incubation, following spot testing the randomly chosen bacterial colonies. Values represent the mean of three independent assays; error bars represent the standard deviation.

Bacterial density in the BC increased by 3.0 log CFU/mL (ANOVA, $p < 0.05$) during the 24 h of incubation (Figure 2.5a). During the 24 h timeframe of the experiments, phage concentration in the controls (PC) decreased (1.3 log PFU/mL and 1.7 log PFU/mL, ANOVA, $p < 0.05$) for the MOI of 1 and 100, respectively (Figure 2.5b). When phage ϕ6 was incubated in the presence of its host, a significant increase in phage particle concentration (4.1 and 1.9 log PFU/mL, ANOVA, $p < 0.05$) was observed for the MOI of 1 and 100 (Figure 2.5b).

At time zero and after 6,12, 18 and 24 h of incubation, ten colonies of the bacterial control and test sample were isolated, to check whether the *P. syringae* pv. *syringae* remained sensitive to the phage ϕ6 during the bacterial kill assays. During the first 18 h of incubation, *P. syringae* pv. *syringae* was sensitive to phage ϕ6, only after 24 h of incubation was observed the appearance of non-sensitive *P. syringae* pv. *syringae* cells (33.3%) to phage ϕ6 (Figure 2.5c).

2.4.7. Determination of the rate of emergence of bacterial mutants

The frequency of *P. syringae* pv. *syringae* mutants resistant to phage ϕ6 was $(1.20 \pm 0.62) \times 10^{-3}$ after 24 h of treatment (Table 2.3).

Table 2.3. Frequency of *P. syringae* pv *syringae* spontaneous phage-resistant mutants.

Control sample (CFU/mL)	**Sample treated with phage ϕ6 (CFU/mL)**	**Frequency of phage-resistant bacterial mutants**
$(1.47 \pm 0.19) \times 10^{8}$	$(1.75 \pm 0.12) \times 10^{5}$	$(1.20 \pm 0.62) \times 10^{-3}$

2.4.8. Fitness of phage-resistant mutants

In the presence of phage ϕ6, after 6 h of incubation, no differences were found between the growth of resistant bacteria and the growth of sensitive bacteria (Figure 2.6, ANOVA, $p < 0.05$). However, after 12, 18 and 24 h of incubation differences were observed between the growth of resistant bacteria and the growth of sensitive bacteria. Resistant

bacteria reached a higher concentration at 12, 18 and 24 h of incubation when compared to its sensitive counterpart. In the absence of phage ϕ6, no differences (Figure 2.6, ANOVA, $p > 0.05$) were found between the concentration of resistant bacteria and the concentration of sensitive bacteria.

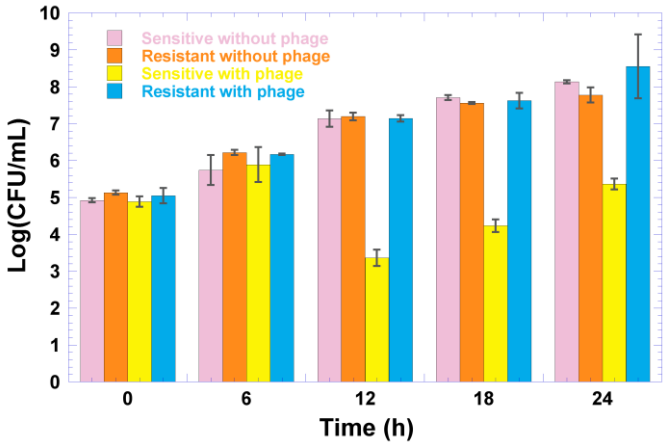

Figure 2.6. *In vitro P. syringae* pv. *syringae* concentration of resistant mutants *versus* their sensitive cells in the presence or absence of phage ϕ6 at MOI of 1 after 6, 12, 18 and 24 h of incubation.

2.4.9. Assessment of the effect of environmental factors in the phage ϕ6 viability

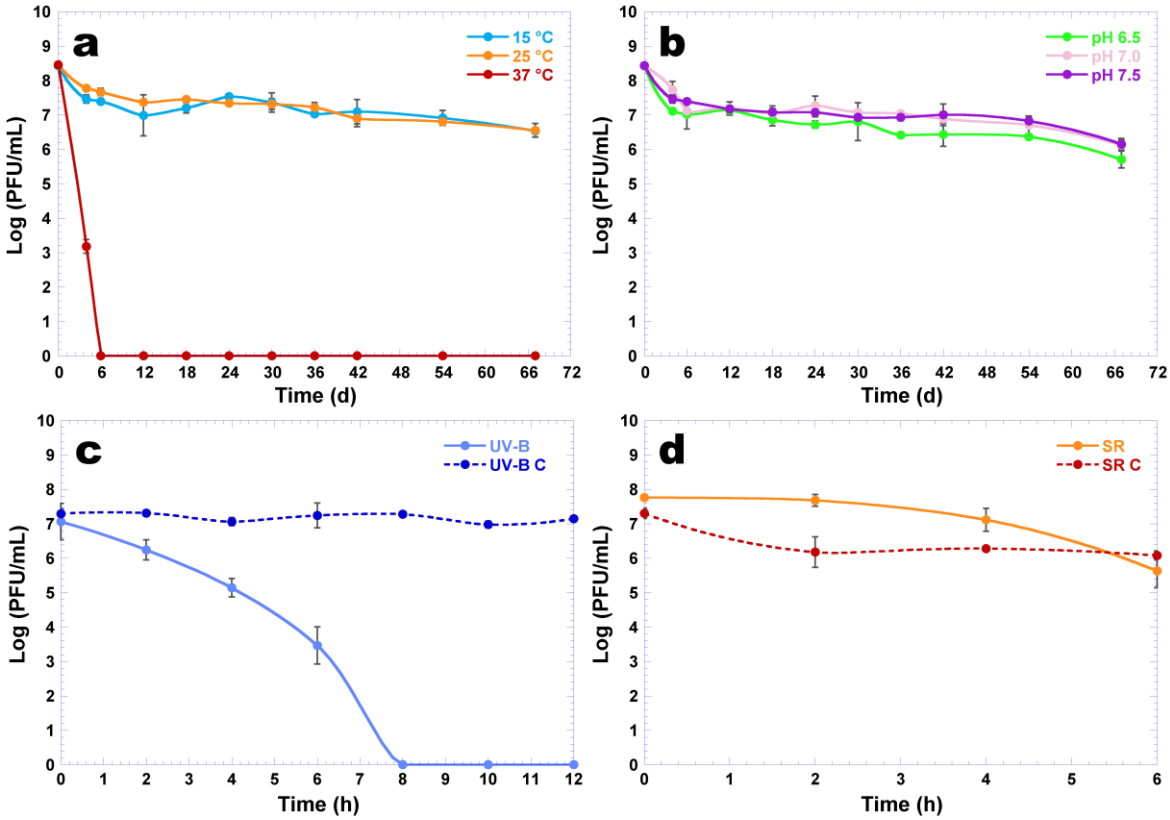

Figure 2.7. Survival of phage ϕ6 following exposure to different temperature values (a), different pH values (b), UV-B irradiation (c) and solar radiation (d). Values represent the mean of three independent experiments; error bars represent the standard deviation. UV-B: phage exposed to UV-B irradiation; UV-B C: phage control - phage not exposed to UV-B irradiation; SR: phage exposed to solar radiation; SR C: phage control - phage not exposed to solar radiation.

2.4.9.1. Temperature experiments

The reduction in the concentration of viable phage ϕ6 particles was higher at 37 °C than at 15 °C and 25 °C (Figure 7a, ANOVA, $p < 0.05$). A maximum decrease of 8.5 log PFU/mL was observed after 6 days when the samples were kept at a temperature of 37 °C, but after only 4 days at this temperature the decrease was of 5.3 log PFU/mL. When the temperature was decreased to 15 °C and 25 °C, the rate of maximum reduction slightly

decreased to 2.0 log PFU/mL after 67 days of incubation. The difference between these two temperatures was not statistically significant (Figure 2.7a, ANOVA, $p > 0.05$).

2.4.9.2. pH experiments

When different pH values (6.5, 7 and 7.5) were tested, it was observed that the phage concentration slightly decreased with the decrease of pH; however, the differences among the three values of pH were not statistically significant (Figure 7b, ANOVA, $p > 0.05$). In the three pH values studied, phage ϕ6 persisted viable for at least 67 days at 25 °C (Figure 2.7b). The abundance of phage ϕ6 decreased about two orders of magnitude during 67 days (Figure 2.7b, ANOVA, $p < 0.05$).

2.4.9.3. UV-B irradiation experiments

When phage ϕ6 was exposed to UV-B irradiation, it was observed that the phage concentration decreased (Figure 2.7c, ANOVA, $p < 0.05$) during 12 h of incubation when compared with the phage control (UV-B C). The abundance of phage exposed to UV-B irradiation decreased about 0.8 log PFU/mL after 2 h of incubation, but after 6 h the decrease was of 3.5 log PFU/mL. A maximum decrease of 7.3 log PFU/mL was observed after 8 h when compared with the phage control (UV-B C) (Figure 2.7c, ANOVA, $p < 0.05$) (Figure 2.7c). The concentration of the phage ϕ6 not exposed to UV-B irradiation (UV-B C) remained constant (Figure 2.7c, ANOVA, $p > 0.05$) during 12 h of incubation.

2.4.9.4. Solar radiation experiments

When phage ϕ6 was exposed to solar radiation, the abundance of phage ϕ6 decreased 2.1 log PFU/mL (Figure 2.7d, ANOVA, $p < 0.05$), when compared with the phage control (SR C). The decrease in phage abundance was 0.6 log PFU/mL (Figure 2.7d).

2.5. Discussion

Phage ϕ6, one of the best-studied phages and a commercially available one, to the best of our knowledge, was only used to control infections by the plant-pathogenic *P. syringae* pv. *phaseolicola* (Vidaver et al., 1973). According to our results, this phage can be used also against other *Pseudomonas syringae* pathovars such as *P. syringae* pv. *syringae*, which are important phytopathogens for the agriculture sector because they can easily infect several horticultural plants (He, R.; Liu, P.; Jia, B.; Xue, S.; Wang, X.; Hu, J.; Al Shoffe, Y.; Gallipoli, L.; Mazzaglia, A.; Balestra, G. M.; Zhu, 2019; McGrane and Beattie, 2017; Moore, 1988; Pscheidt and Ocamb, 2018; Sistrom et al., 2015; Vasebi et al., 2019; Ward, 2014), causing severe economic losses worldwide.

Although the major advantage of phage treatment is phage specificity, since the non-target bacterial populations should remain undisturbed, phages should be capable to lyse the majority of strains of a given bacterial species (Rios et al., 2016; Pereira et al., 2011; Hawkins et al., 2010; Almeida et al., 2009; Park and Nakai, 2003). Phage ϕ6 besides the *P. syringae* pv. *syringae* infects also *P. syringae* pv. *actinidiae* CRA-FRU 12.54 and CRA-FRU 14.10. These results suggest that phage ϕ6 can be potentially used not only to control bacterial canker and blast in *Citrus* species (such as orange, mandarin) and *Prunus* species (including sweet cherry, apricot, tart cherry and almond) (Ivanović et al., 2017; Kennelly et al., 2007), but also to control *P. syringae* pv. *actinidiae*, which is the causal agent of the kiwifruit bacterial canker worldwide (Fujikawa and Sawada, 2016; McCann et al., 2017). The effectiveness of phage ϕ6 was also tested in this study against other species of *Pseudomonas* and of other bacterial genera, but none of these bacteria were infected by the phage. As the host range of phage ϕ6 is quite narrow, natural non-pathogenic bacteria of infected plants will not be affected by the treatment with this phage. However, phage ϕ6 has been shown to alter its host range, mostly through mutations in its receptor binding proteins (Ferris et al., 2007). The high mutation rate associated with its RNA-based genome allow it to exploit new niches by infecting closely related *Pseudomonas* species (Simpson, 2016; Sistrom et al., 2015). Nevertheless, in the future, new phages need to be isolated and

tested together with phage ϕ6 in order to produce a cocktail with a broader spectrum of activity to control several pathovars of *P. syringae*.

As phage ϕ6 is already available commercially and has been the subject of extensive genomic characterization, with its genome sequence available in the GenBank Genomes database (Yang et al., 2016), it is possible to assure at the outset that this phage is a safe biological control agent since it does not code for integrase genes nor genes coding for virulence factors and antibiotic resistance (Li and Dennehy, 2011; Wei et al., 2009; Wickner, 1993; Yang et al., 2016).

Before the application of phages to inactivate pathogenic bacteria, it is important to characterize *in vitro* the dynamics of phage-host replication. The first step of phage infection is the attachment of the phage virion onto a susceptible bacterial host cell (Ceyssens, 2009; Hyman and Abedon, 2009; Moldovan et al., 2007). This adsorption process is usually described by mass-action kinetics (Storms and Sauvageau, 2015), which implicitly assumes an equal influence of host density and phage adsorption rate (Ceyssens, 2009; Shao and Wang, 2008). Therefore, an environment with high bacterial host density can be considered as equivalent to a phage endowed with a high adsorption rate and vice-versa. The result obtained in this study for the phage ϕ6 adsorption rate is in close agreement with results published by Shao and Wang (2008), Lindberg et al. (2014) and Santos et al. (2014) for *Pseudomonas* phages isolated from environmental sources. The growth characteristics of phage ϕ6 showed a relatively high burst size (60 ± 1 PFU/ host cell), indicating that phage ϕ6 replicates efficiently in *P. syringae* pv. *syringae* but need a long latency period (100 min). Consequently, phage ϕ6 caused a high reduction in *P. syringae* pv. *syringae* growth, but its effect occurs only after 10 h of treatment.

P. syringae pv. *syringae* was effectively inactivated by phage ϕ6, reaching the maximum of inactivation of ≈4 log CFU/mL after 12 h treatment at a MOI of 1. After that, although some bacteria were not inactivated by the phage ϕ6, around 3 log CFU/mL, most of the inactivated bacteria did not regrow after treatment. In fact, between 12 h and 24 h of phage treatment, bacterial concentration was significantly lower than that observed for the non-treated cultures. Vidaver et al. (1973) characterized phage ϕ6 using the plant-pathogenic *P. syringae* pv. *phaseolicola* HB10Y, but no inactivation studies were performed

by those authors. To our best knowledge, this phage has not yet been tested to inactivate *P. syringae* pv. *syringae*.

The kinetic theory of phage therapy predicts that the MOI could be critical to the efficiency of bacterial inactivation. It has been shown, both *in vitro* and *in vivo*, that the reduction of pathogenic bacteria increases in parallel with MOI or that bacterial reduction occurs sooner at higher MOI values (ChiHsin et al., 2000; Pasharawipas et al., 2011; Prasad et al., 2011). However, other studies (Lopes et al., 2018; Nakai, 2010) show that precise initial doses of phage may not be essential due to the self-perpetuating nature of phages, revealed by an increasing of phage titers along with bacteria. In the present study, the increase in MOI from 1 to 100 did not promote an increase in the efficiency of phage ϕ6. The number of phage particles during 24 h of treatment in the presence of the host at a MOI of 1 increased more (by 4.1 log PFU/mL) than at a MOI of 100 (by 1.9 log PFU/mL). This confirms the hypothesis that, due to the self-perpetuating nature of phages, precise initial doses of phage may not be essential.

A major concern of bacterial inactivation by phages is the emergence of phage-resistant bacteria (Lima et al., 2019; Lopes et al., 2018; Pereira et al., 2016a; Rio et al., 2016; Mateus et al., 2014; Filippov et al., 2011; Gill and Hyman, 2010; Levin and Bull, 2004). Phage ϕ6 did not prevent the bacterial regrowth during treatment. After 24 h of incubation, 33.3% of *P. syringae* pv. *syringae* cells showed to be insensitive to phage ϕ6. However, some of these insensitive bacterial cells may be the result of not having had contact with the phage during the incubation period, not all being phage-resistant. In fact, the development of resistant mutants was limited (1.20×10^{-3}). Some authors have suggested that exposure to phage could cost bacteria their fitness, which can lead to their removal from the environment at a faster rate than their wild-type counterparts (Bohannan et al., 1999; Brockhurst et al., 2005). Recently, (Sistrom et al., 2015) showed that *P. syringae* pv. *phaseolicola* can evolve resistance to phage ϕ6 by eliminating type-IV pili, but the phage-mutants presented reduced virulence. In our study, the experimental results of the fitness of phage showed that the concentration of sensitive bacteria and resistant mutants, when grown in the absence of phage ϕ6, are similar. Nevertheless, these experiments were carried out in nutrient-rich (culture) medium and in the absence of competition, from

which, according to some authors, the cost of resistance can vary across environmental factors and degree of competition for resources (Lennon et al., 2007; Quance and Travisano, 2009). Meaden et al. (2015) obtained similar results *for P. syringae* pv. *tomato* under standard laboratory conditions (*in vitro*). However, when the experiments were carried out in tomato plants (*Solanum lycopersicum*), the phage-resistant bacterial mutants exhibited reduced densities relative to the sensitive bacterial population. In the future, further studies will be needed to evaluate the cost of bacterial resistance to phage ϕ6 in plants. Moreover, according to several authors, the resistance drawback can be overcome by the use of phage cocktails (Costa et al., 2019; Lopes et al. 2018; Pereira et al., 2017, 2017b, 2016a,b; Mateus et al., 2014; Hooton et al., 2011; Gill and Hyman, 2010; Scott et al., 2007; Sandeep, 2006).

For the design and implementation of an effective phage therapy protocol to control plant diseases, the study of the stability of phages to environmental factors such as temperature, soil pH, solar and ultraviolet radiation is crucial. One important factor that influences phage stability is the pH of the environment (Jończyk et al., 2011), influencing attachment, infectivity, intracellular replication and amplification of phages (Leverentz et al., 2001, 2004; Pirisi, 2000). Unfavorable pH values can interfere with the lysozyme enzyme and/or with other phage capsid proteins, thus preventing phage attachment to receptor sites on the host cell (Leverentz et al., 2001, 2004). In this study, pH values ranging from 6.5 to 7.5 were tested, which are included within the optimum neutral range of pH values for plant cultivation, and survival of phage ϕ6 was not significantly affected. Generally, 10 < pH values < 5 have shown to be less efficient in studies on the lytic activity of phages, with the optimum conditions being around a neutral pH of 6 - 8 (Endersen et al., 2017; Pirisi, 2000; Nakai et al., 1999). Since there is a positive correlation between soil pH and pH of fresh leaves (Cornelissen et al., 2011; Masoero and Cugnetto, 2018), the range of pH values studied lie within the optimum pH range for the soil in orchards and, consequently, for the surface pH of plant leaves. This is clearly important in the context of a putative phage therapy application in the field.

Temperature is a crucial factor for phage viability in the environment (Nasser and Oman, 1999; Olson et al., 2004), playing a fundamental role in the attachment, penetration

and amplification of phage particles in their host cells (Jończyk et al., 2011). At low temperatures, only a few phages genetic material enters into bacterial host cells and hence fewer phage particles can be involved in the multiplication phase. On the other hand, high temperatures can promote an extended phage latency period (Tey et al., 2009). In this study, phage ϕ6 was completely inactivated at 37 °C after 6 days of incubation (maximum decrease of 8.5 log PFU/mL). However, when the temperature was decreased to 15 °C and 25 °C, the rate of maximum reduction in phage viability decreased to 2.0 log PFU/mL after 67 days of incubation. This means that in summer, when temperatures occasionally rise to 37 °C, bacteria may not be inactivated by the phage. However, as the most critical period for plants is autumn/winter and early spring and the infection ability of *P. syringae* pathovars at temperatures above 25 °C is reduced (CABI, 2015; A Fox, 2011; Kiwifruit Vine Health Inc., 2017), temperature would not be a problem for the implementation of phage therapy.

Solar radiation or, more specifically, UV irradiation, has been recognized as the most important factor for the loss of phage infectivity in the environment (Duarte et al., 2018; Lytle and Sagripanti, 2005; Mojica and Brussaard, 2014; Yolanda J Silva et al., 2014; Wommack et al., 1996). Solar radiation can directly affect free viruses by degrading proteins, altering structure and decreasing infectivity (Mojica and Brussaard, 2014). Shorter wavelengths (UV-B radiation, ranging from 290 nm - 320 nm) impart irreversible damages to the genomic material and can result in the modification of viral proteins and formation of (lethal) photoproducts (Hotze et al., 2009; Wigginton et al., 2010; Mojica and Brussaard, 2014). In fact, the abundance of phage ϕ6 particles decreased when it was exposed to solar radiation (decrease of 2.1 log PFU/mL after 6 h of incubation, with a solar radiation of 83.3 kWh/m^2/day (data obtained from IPMA - Portuguese Institute of the Sea and the Atmosphere). Notwithstanding the fact that phage particles are sensitive to UV radiation, their sensitivity to UV wavelengths from solar radiation can be overcome by applying the phages at high titers and at the end of the day or at night, period during which radiation is limited (Duarte et al., 2018). The phages can be applied in free form (as a spray of concentrated phage particles or cocktail of phage particles) or trapped within micro- or nanocarriers (Balcao et al., 2014; Rios et al., 2016).

2.6. Conclusions

The results of this study clearly show that the commercially available phage ϕ6 can be an effective alternative to control *P. syringae* pv. *syringae* infections. However, phage ϕ6 should be applied at the end of the day or during night periods, either in free form as a spray of concentrated phage particles or trapped within micro- or nanocarriers, in order to produce a higher bacterial control. Nonetheless, further studies are needed, namely in the field, in order to fully understand the true potential of this phage to control infections caused by *P. syringae* pathovars.

Chapter 3. Biocontrol of *Pseudomonas syringae* pv. *actinidiae* infection of kiwifruit plants using phage ϕ6: *in vitro* and *ex vivo* experiments

3.1 Abstract

In the last decade, the worldwide production of kiwi fruit has been highly affected by P*seudomonas syringae* pv. *actinidiae*, a phytopathogenic bacterium responsible by bacterial canker of kiwifruit. The available treatments for this disease are still scarce, with the most common involving frequently spraying the orchards with copper derivatives and/or antibiotics. Moreover, these treatments should be avoided due to their high toxicity to the environment and promotion of bacterial resistance. Phage therapy may be an alternative approach to inactivate Psa. The present study investigated the potential application of the already commercially available bacteriophage (or phage) ϕ6 to control Psa infections. The inactivation of Psa was assessed *in vitro*, using liquid culture medium, and *ex vivo*, using artificially contaminated kiwifruit leaves with two biovar 3 (a highly aggressive pathogen) strains (Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10). In *In vitro* experiments, the phage ϕ6 was effective against both strains (maximum reduction of 2.2 and 1.9 CFU/mL for Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10, respectively). In the *ex vivo* tests, the decrease was lower (maximum reduction 1.1 log and 1.8 CFU/mL for Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10, respectively). The results of this study suggest that the commercially available phage ϕ6 can be an effective alternative to control Psa infections in kiwifruit orchards.

Keywords: Phage treatment, phage ϕ6, Psa*,* kiwifruit canker

3.2 Introduction

The production of kiwifruit is an important industry worldwide, particularly in China, Italy, New Zealand, and Chile (Guroo et al., 2017). In the last decade, bacterial canker in kiwifruit orchards, caused by Psa, has become quite problematic worldwide (Yu et al., 2016; Wilstermann et al., 2017; Poulter et al., 2018). Psa damages both *Actinidia deliciosa* (green kiwifruit) and *Actinidia chinensis* (gold kiwifruit), resulting in severe worldwide economic losses. Psa infection of kiwifruit plants causes browning and spotting with yellow haloes in leaves, necrosis and brown discolouration of both buds and flowers (Bender et al., 1999;

Hwang et al., 2005; Jesus et al., 2018; Martins et al., 2018; Pscheidt and Ocamb, 2018). Psa infection not only significantly reduces the fruit yield but also leads to thicker pericarp, higher acidity, inconsistent shape and undesirable colour in kiwifruits (Liu, Y.; Zhu, T.; Fan, F.; Shao, 2013).

Psa was first isolated in Japan in 1984 as the causative agent of this disease in *A. deliciosa* (Takikawa et al., 1989) and was subsequently recorded in Korea (Bastas and Karakaya, 2011; Koh et al., 1996), China (Baolin et al., 2016), Italy (Scortichini, 1994), Portugal (Balestra et al., 2010), Spain (Balestra et al., 2011), France (Vanneste et al., 2012), Turkey (Vanneste et al., 2012), South America (Everett et al., 2011) and New Zealand (Everett et al., 2011). As kiwifruit plants is clonally propagated, other outbreaks could happen due to the emergence of new strains that may be rapidly spread throughout growing areas worldwide (McCann et al., 2017, 2013). Psa can be classified into six biovars based on their biochemical, pathogenicity and molecular characteristics (Fujikawa and Sawada, 2016). The biovar 3, also referred to as Psa 3 or PsaV, is a highly aggressive pathogen and is responsible for the global pandemic of kiwifruit, which was first reported in Italy in 2008 (McCann et al., 2013).

The available treatments for this disease are still scarce, with the most common involving frequent spraying the orchards with copper derivatives, in particular cuprous oxide (Cu_2O), and/or antibiotics (such as streptomycin) (Cameron and Sarojini, 2014; Marcelletti et al., 2011). However, these strategies are not completely effective and their massive use can promote the development of resistance in the pathogen and changes in the structure of bacterial communities (Altimira et al., 2012). In addition, the streptomycin is not a viable control option in many countries because of antibiotic residues in the fruits (Frampton et al., 2014). Therefore, efficient approaches are required to control Psa-driven kiwifruit canker disease. One of the most promising strategies is the application of lytic phages to prevent and/or to treat the infection (Yu et al., 2016; Park et al., 2018).

As specific pathogen-killers, phages are effective agents for controlling bacterial infections, without affecting the normal microbiota (Almeida et al., 2009; Pereira et al., 2011; Rios et al., 2016; Silva et al., 2016; Yolanda J. Silva et al., 2014). Currently, phages have been characterized and studied as agents for phage therapy of plant diseases caused

by pathogenic bacteria (Bae et al., 2012; Czajkowski et al., 2014; Frampton et al., 2012). There are already on the market some phage-based products approved for the agriculture area [(EcoShield™ from Intralytix Inc. (Baltimore MD, U.S.A.), Agriphage™ from Omnilyticus (Salt Lake City UT, USA), Phagelux Inc. (Sanghai, China)] (Abuladze et al., 2008; Balogh et al., 2010, 2003; Moye et al., 2018; Soffer et al., 2017). However, there are still no approved phages for the biocontrol of Psa-induced kiwifruit canker. Although some Psa phages were already isolated and characterized, only two *in vitro* studies were performed to evaluate phage treatment to inactivate Psa (Park et al., 2018; Yu et al., 2016) and no *ex-vivo* or *in vivo*/field studies were done. Phage ϕ6, one of the best-studied phages, and already commercially available, was used in a first approach with plant-pathogenic *P. syringae* pv. *phaseolicola* (Vidaver et al., 1973), but no attempt was still done to evaluate its effectiveness to control Psa infections. The results of our previous study (Pinheiro *et al.*, 2019) showed that phage ϕ6 besides infecting its host *Pseudomonas syringae* pv. *syringae* (DSM 21482), infect also two biovar 3 Psa strains, CRA-FRU 12.54 and CRA-FRU 14.10. So, in the present study, we extended our research to evaluate the efficiency of phage ϕ6 to control Psa infections. The assays were performed *in vitro* and *ex vivo* using kiwi leaves artificially contaminated with Psa CRA-FRU 12.54 and CRA-FRU 14.10 strains.

3.3 Materials and methods

3.3.1 Bacterial strains and growth conditions

The bacterial strain *P. syringae* pv. *syringae* (DSM 21482, natural host of phage ϕ6) was used to produce the phage ϕ6 suspensions. Psa strains CRA-FRU 14.10 and CRA-FRU 12.54 were isolated in Lazio, Italy, in 2008, and were obtained from the Culture Collection of Centro di Ricerca per la Frutticoltura (Rome, Italy). All bacteria were grown in Tryptic Soy Broth (TSB, Liofilchem, Roseto degli Abruzzi (Te), Italy). The bacterial strains were stored at -80 °C in 10% (v/v) glycerol. Before each assay, stock cultures of each bacterium were aseptically inoculated in 30 mL of Tryptic Soy Broth (TSB, Liofilchem, Italy) and grown during 18 h at 25 °C and 120 rpm. Then, an aliquot (300 µL) of each bacterial culture was

transferred to 30 mL of fresh TSB and grown during 18 h at 25 °C under orbital shaking (120 rpm). The viable cell density was approximately 10^9 colony-forming units (CFU) /mL.

3.3.2. Phage preparation

The phage ϕ6 (DSM 21518) was purchased from Leibniz-Institute DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmmH (Braunschweig, Germany). Phage ϕ6 is a double-stranded RNA phage and belongs to the family *Cystoviridae* (Callanan et al., 2018; Mäntynen et al., 2018). Lysogeny genes (viz. integrase genes) genes codifying for virulence factors and antibiotic resistant, were not found in the phage ϕ6 genome (Wei et al., 2009; Wickner, 1993; Yang et al., 2016), make it safe for phage treatments.

Phage ϕ6 suspensions, produced using *Pseudomonas syringae* pv. *syringae* (DSM 21482)**,** were prepared in SM buffer [0.1 M NaCl (Sigma, Molsheim MO, USA), 8 mM $MgSO_4$ (Sigma), 20 mM Tris-HCl (Sigma), 2% (w/v) gelatin, pH 7.5]. Three hundred microliters of the phage stock were added to thirty millilitres of SM buffer and one millilitre of *P. syringae* pv. *syringae* in the exponential growth phase. The suspension was grown overnight and incubated at 25 °C under orbital shaking set at 50 rpm. The lysates were incubated with chloroform (final volume of 0.5% (v/v)) during 1 h at 120 rpm. After incubation, the lysate was centrifuged at 13000 rpm for 10 min at 4 °C and the supernatant was filtered through a polyethersulphate membrane with 0.22 µm pore size, to remove intact bacteria or bacterial debris. The phage suspension was stored at 4 °C and the titre was determined by the double-layer agar method (Adams, 1959).

Successive dilutions of the phage suspension were performed in phosphate buffered saline [PBS; 137 $mmol^{-1}$ NaCl (Sigma), 2.7 $mmol^{-1}$ KCl (Sigma), 8.1 $mmol^{-1}$ $Na_2HPO_4 \cdot 2H_2O$, 1.76 $mmol^{-1}$ KH_2PO_4 (Sigma), pH 7.4] and 500 µL of each dilution were added to 200 µL of both fresh Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10 cultures, mixed with 5 mL of TSB 0.6% top agar layer [30 g/L TSB (Liofilchem), 6 g/L agar (Liofilchem), 0.05 g/L $CaCl_2$ (Sigma), 0.12 g/L $MgSO_4$ (Sigma), pH 7.4] and poured over a Petri plate containing solid TSA agar. The plates were incubated at 25 °C for 18 h. The results were expressed as plaque forming units per milliliter (PFU/mL).

3.3.3. One-step growth analysis

Phage ϕ6 suspension (final concentration of 10^5 PFU/mL) was added to 10 mL of each of the bacterial cultures of Psa CRA-FRU 14.10 and Psa CRA-FRU 12.54 (cell density of 10^9 CFU/mL) in order to have a multiplicity of infection (MOI) of 0.001 and the resulting suspensions incubated without shaking during 5 min at 25 °C (Mateus et al., 2014). The mixtures were centrifuged at 10000g for 5 min, the pellets were re-suspended in 10 mL of TSB (Liofilchem) and incubated at 25 °C. Samples (1 mL) were collected at time 0 and at time intervals of 10 min up to 150 min of incubation and immediately tittered by the double-layer agar method (Adams, 1959). The plates were incubated at 25 °C and examined for the presence of plaques after 18 h. Three independent assays were performed for each Psa strain. The results were subsequently plotted to determine the phage eclipse period, latent period, intracellular accumulation period and burst size in each Psa strain. The one-step growth curve data produced was better adjusted via nonlinear fitting the data to a typical sigmoidal curve (or 4-parameter logistic regression model) (Equation (1)):

$$Log\ (average\ PFU's/mL)\ =\ m_1 + \frac{m_2 - m_1}{1+\left(\frac{t}{m_3}\right)^{m_4}} \quad (1)$$

where m_1 is the response at t = 0, m_2 is the response at t = ∞, m_3 is the curve inflection point, m_4 is the slope that defines the steepness of the curve, and t is the time (min). Nonlinear fitting of the phage growth data to the model in Equation (1) was performed using the software KaleidaGraph v. 4.5.2 for MacOS X.

3.3.4. Adsorption curves

Phage ϕ6 suspension (final concentration of 10^5 PFU/mL) was added to 10 mL of each of the bacterial cultures (final concentration10^8 CFU/mL) to obtain a multiplicity of infection (MOI) of 0.001 (Stuer-Lauridsen et al., 2003) and the resulting suspensions were

incubated at 25 °C. The mixtures were centrifuged at 10000g for 5 min and supernatants were immediately filtered through 0.20 µm pore-size filters (Millipore Bedford, MA, USA). The filtrates containing unadsorbed or reversibly adsorbed phage particles were diluted and titrated. The plates were incubated at 25 °C and examined for plaques after 18 h. Adsorption was expressed as the percentage decrease of phage titre in the supernatant, as compared to the time zero. Suspensions of phage without any bacterial cells were used as no-adsorption standard for calculations (Stuer-Lauridsen et al., 2003). Three independent assays were performed for each Psa strain. The adsorption rates were estimated via nonlinear fitting the experimental data to the model depicted in Equation (2) (García et al., 2019; Santos et al., 2014; Shao and Wang, 2008), viz:

$$Ln\left(\frac{P_t}{P_0}\right) = -\delta\left(\frac{X_0}{\mu(t)}\right)\left(e^{\mu(t)\cdot t} - 1\right) \quad (2)$$

where P_t and P_0 are phage concentrations at times t and 0, respectively, δ is the adsorption rate to be estimated, X_0 is the concentration of (susceptible, uninfected) bacterial cells at time 0, $\mu(t)$ is the bacteria multiplication rate and t is the infection time. Nonlinear fitting of the phage adsorption data to the model in Equation (2) was performed using the software KaleidaGraph v. 4.5.2 for MacOS X.

3.3.5. *In vitro* phage assays

Bacterial inactivation by phage ϕ6 was determined for Psa CRA-FRU 14.10 and Psa CRA-FRU 12.54, at a MOI of 1. Exponential bacterial cultures of Psa CRA-FRU 14.10 and Psa CRA-FRU 12.54 were adjusted to a 0.7 O.D. at 600 nm (corresponding to a cell density of 10^8 CFU/mL). In order to obtain a MOI of 1, the exponential cultures of bacteria (final concentration of 10^5 CFU/mL) and phage suspension (final concentration of 10^5 PFU/mL) were inoculated in sterilized glass Erlenmeyer flasks with 30 mL of TSB medium and incubated at 25 °C without agitation (BP). For each assay, two control samples were included: the bacterial control (BC) and the phage control (PC). The bacterial controls were inoculated with either Psa CRA-FRU 12.54 or Psa CRA-FRU 14.10 but not with phage ϕ6 and

the phage controls were inoculated with phage ϕ6 but not with any bacterial cells. Controls and test samples were incubated exactly under the same conditions. Aliquots of test samples (BP, bacteria and phage) and of the bacterial and phage controls were collected at time 0 and after 2, 4, 6, 8, 10, 12, 14, 18 and 24 h of incubation. In all assays, the phage titre was determined in triplicate by the double-layer agar method after an incubation period of 18 h at 25 °C. Bacterial concentration was determined in triplicate in solid TSA medium by the drop-plate method after an incubation period of 48 h at 25 °C. Three independent experiments were performed for each condition.

3.3.6. *Ex vivo* phage treatment experiments in artificially contaminated kiwifruit leaves

Leaf samples from kiwifruit trees (without any apparent signs of Psa contamination) were collected from a kiwifruit orchard in the region of Aveiro (Portugal) and kept at 4 °C. Before assays, all leaves were labelled, cut into square samples (3 cm x 3 cm) and duly sterilized via immersion in H_2O_2 at 3% (v/v) during 15 min followed by whashing in PBS for 10 min and UV irradiation on both sides for an extra 15 min. In these experiments, 12 groups of leaves (4 groups for each incubation time) were used. Each group included 3 replicates of leaves (for a total of 12 groups x 3 replicates = 36 square leaves). Each replicate was stocked in an independent Petri plate. Psa CRA-FRU 12. 58 was added to 6 of the 12 groups to obtain a final concentration of 10^8 CFU/mL (1mL of fresh overnight bacterial suspension at 10^9 CFU/mL diluted in 9 mL of PBS). To the other 6 groups was added the same volume of the TSB. All groups were incubated during 1 h at 25 °C. From the 6 groups of leaves infected with Psa CRA-FRU 12.58, 3 of them were inoculated with phage ϕ6 at a MOI of 1 (bacteria + phage - BP) and the remaining 3 groups were not inoculated with phage (bacteria control - BC). Concerning the 6 groups of leaves not infected with Psa CRA-FRU 12.58, 3 of them was inoculated with phage ϕ6 at a MOI of 1 (phage control - PC) and the remaining 3 groups were not inoculated with phages (leaf control - LC). To maintain a moist surface on the leaf samples, the Petri plates (60 mm) with the leaf samples were placed inside larger Petri plates (90 mm) with 10 mL of sterile PBS. All 12 groups were incubated exactly under the same conditions. Leaves of test samples and controls were

sampled at time 0 and after 12 and 24 h of incubation. Leaves were placed in 10 mL of sterile PBS and incubated during 30 min at 25°C with stirring (130 rpm). The phage titer was determined in duplicate for all assays through the double agar layer method after an incubation period of 18 h at 25 °C. Bacterial concentration was determined by the drop-plate method spread method in triplicate in TSA medium after an incubation period of 48 h at 25°C. The same procedure was used to determine the effect of phage to control of Psa CRA-FRU 14.10 in leaves artificially contaminated. This assay was repeated three times in different periods to ensure independent replicates. A simplified scheme of the procedure followed is depicted in Figure 3.1.

3.3.7 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 Levene's test. Significance was accepted at $p < 0.05$. For a pairwise comparison of the means one used the Tukey's multiple comparison test. The significance of bacterial and viral concentrations between treatments, and along the experiments, was tested using two-way ANOVA and the Bonferroni *post-hoc* tests (Sections 3.4.4 and 3.4.5). For different treatments, the significance of differences was evaluated by comparing the results obtained in the test samples with the results obtained for the correspondent control samples, for the different times. A value of $p < 0.05$ was considered to be statistically significant.

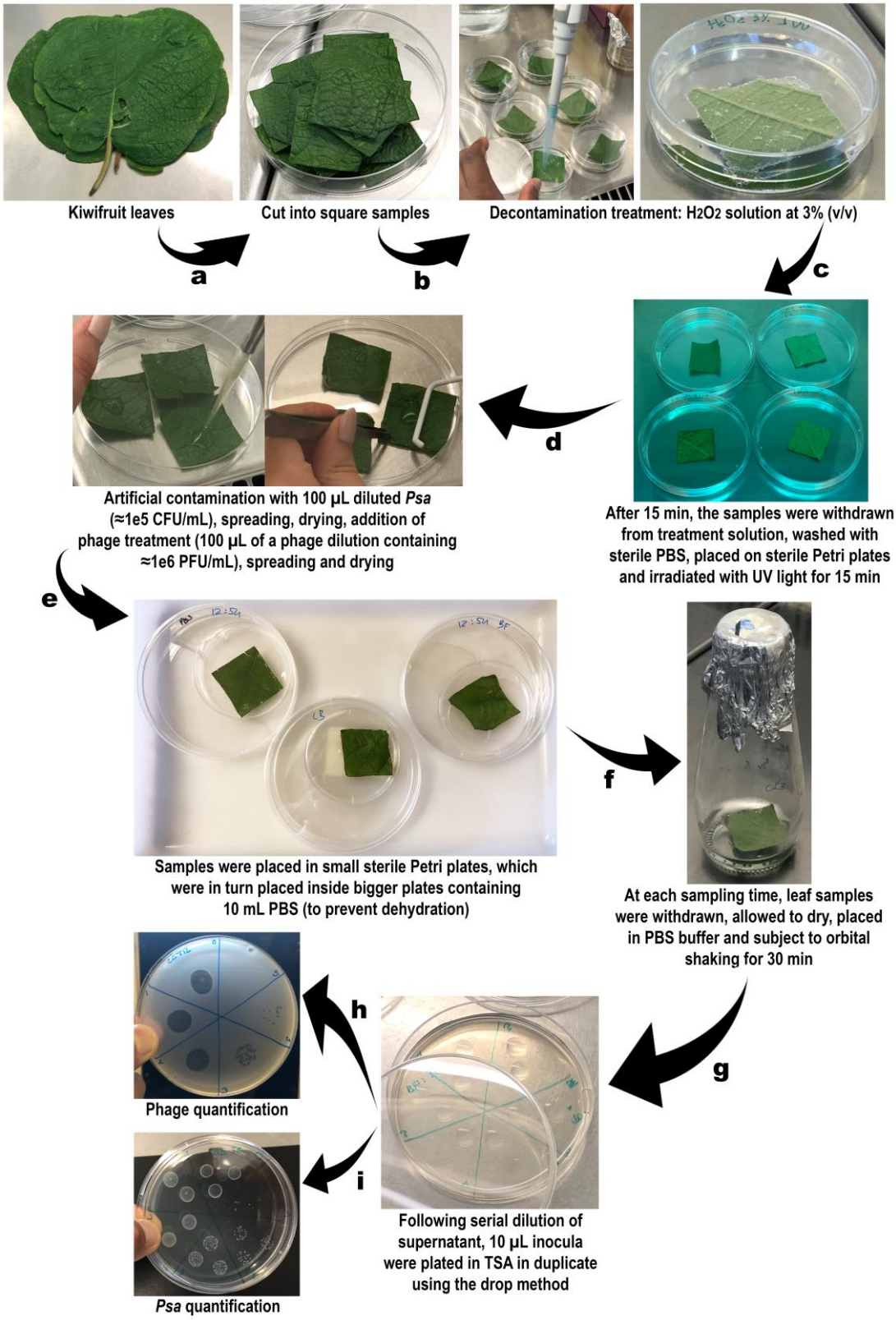

Figure 3.1. Simplified scheme of the *ex vivo* phage treatment procedure using phage ϕ6 on artificially contaminated kiwifruit leaves.

3.4. Results

3.4.1. Phage ϕ6 plaque morphologies on infected Psa strains

Phage ϕ6 formed clear plaques on both Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10 and with diameters of 1-2 mm (Figures 2a and 2b, respectively). Phage plaques exhibited a thin secondary halo in the frontier of the lysis plaques of phage ϕ6 (Figure 2), indicative of the production of phage endolysins. The phage titre in both Psa strains varied from 10^8 to10^9 PFU/mL.

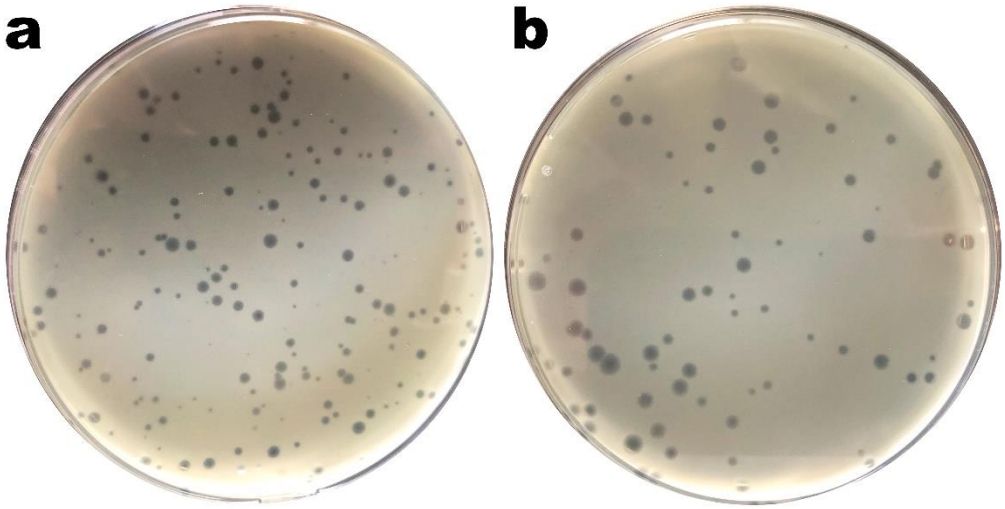

Figure 3.2. Morphology of the phage ϕ6 lysis plaques on Psa CRA-FRU 12.54 (a) and on Psa CRA-FRU 14.10 (b).

3.4.2. One-step growth curve analyses

Non-linear fitting the one-step growth data of phage ϕ6 to the 4-parameter logistic model depicted as Equation (1) resulted in good correlation coefficients (viz. 0.99793 for Psa CRA-FRU 12.54 and 0.99959 for Psa CRA-FRU 14.10) and showed that the eclipse period, latent period and intracellular accumulation period lasts 60 min, 100 min and 40 min, respectively (Figure 3.3), for both Psa strains. The burst size of phage ϕ6 was 148 ± 1 PFU for Psa CRA-FRU 12.54 and 172 ± 0 PFU for Psa CRA-FRU 14.10 and (Figure 3.3).

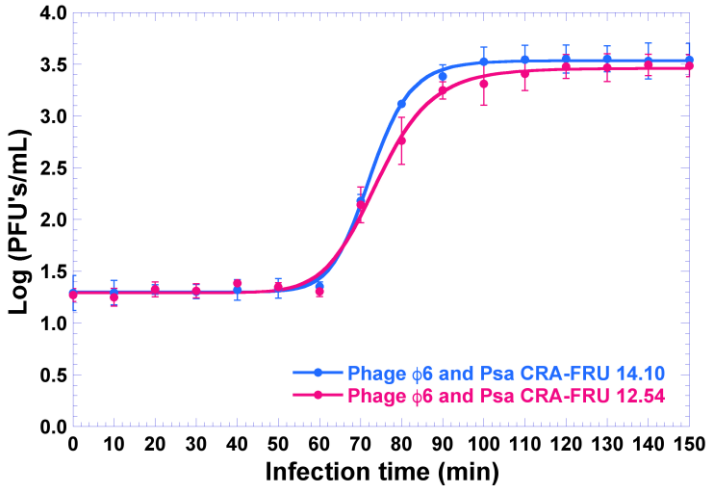

Figure 3.3. One-step growth curves of phage ϕ6 in the presence of Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10 as hosts. Values represent the mean of three experiments; error bars represent the standard deviation.

3.4.3. Adsorption curves

Phage ϕ6 adsorption assays showed that approximately 10% of the phage particles adsorb to Psa CRA-FRU 12.54 after 30 min, 38% adsorbed after 60 min and 70% adsorbed after 120 min (Figure 3.4), and that approximately 18% of the phage particles adsorb to Psa CRA-FRU 14.10 after 30 min, 54% adsorbed after 60 min and 67% adsorbed after 120 min (Figure 3.4). Nonlinear fitting the experimental data to the model depicted in Equation (2) resulted in good correlation coefficients (viz. 0.96980 for Psa CRA-FRU 12.54 and 0.977 for Psa CRA-FRU 14.10) and allowed the determination of the adsorption rate of phage ϕ6 onto Psa CRA-FRU 12.54 cells (concentration of susceptible, uninfected bacterial cells at t_0 of 8.85×10^7 CFU/mL) as $\delta_{PsaCRA\text{-}FRU12.54} = (7.971 \pm 1.401) \times 10^{-11}$ PFU^{-1} CFU^{-1} mL^{-1} hr^{-1} and a Psa CRA-FRU 12.54 multiplication rate $\mu(t)_{PsaCRA\text{-}FRU12.54} = (6.093 \pm 3.091) \times 10^{-3}$ hr^{-1} an adsorption rate of phage ϕ6 onto Psa CRA-FRU 14.10 cells (concentration of susceptible, uninfected bacterial cells at t_0 of 6.55×10^7 CFU/mL) as $\delta_{PsaCRA\text{-}FRU14.10} = (1.832 \pm 0.230) \times 10^{-10}$ PFU^{-1} CFU^{-1} mL^{-1} hr^{-1}, a Psa CRA-FRU 14.10 multiplication rate $\mu(t)_{PsaCRA\text{-}FRU14.10} = (3.918 \pm 2.714) \times 10^{-3}$ hr^{-1}.

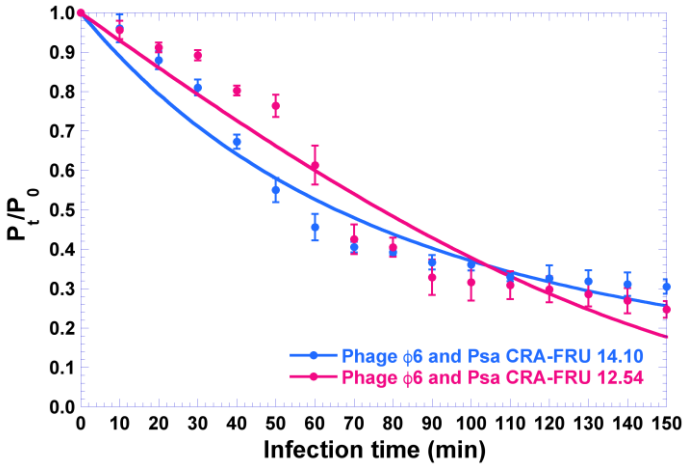

Figure 3.4. Adsorption curves of phage ϕ6 particles onto Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10 host cells, allowing to calculate the phage particles adsorption rate on either strain following non-linear fitting of a logarithmic function to the experimental data.

3.4.4. *In vitro* phage assays

Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10 density in the BC increased by 3.7 and 3.5 log CFU/mL, respectively (Figure 5a, ANOVA, $p < 0.05$) during the 24 h of incubation (Figure 3.5a). The maximum of Psa CRA-FRU 12.54 inactivation with phage ϕ6 was 2.2 log CFU/mL (Figure 3.5a, ANOVA, $p < 0.05$), achieved after 24 h of incubation, when compared with those of the bacterial control (BC). However, after 12 h of incubation, the rate of inactivation of CRA-FRU 12.54 (1.0 CFU/mL, respectively) was significantly higher (ANOVA, $p < 0.05$). After 18 h of incubation, the bacterial inactivation was 1.4 log CFU/mL (Psa CRA-FRU 12.54, BP), which was statistically different (Figure 3.5a, ANOVA, $p < 0.05$) from the values obtained in the bacterial control (Psa CRA-FRU 12.54, BC).

The maximum of Psa CRA-FRU 14.10 inactivation with phage ϕ6 was 1.9 log CFU/mL (Figure 5a, ANOVA, $p < 0.05$), achieved after 24 h of incubation, when compared with those of the bacterial control (BC). The Psa CRA-FRU 14.10 inactivation by phage ϕ6 started after 12 h and the rate of inactivation was 0.9 CFU/mL. After 18 h of incubation, the bacterial inactivation was 1.6 log CFU/mL (Psa CRA-FRU 14.10, BP), respectively, which was statistically different (Figure 3.5a, ANOVA, $p < 0.05$) from the value obtained in the bacterial control (Psa CRA-FRU 14.10, BC).

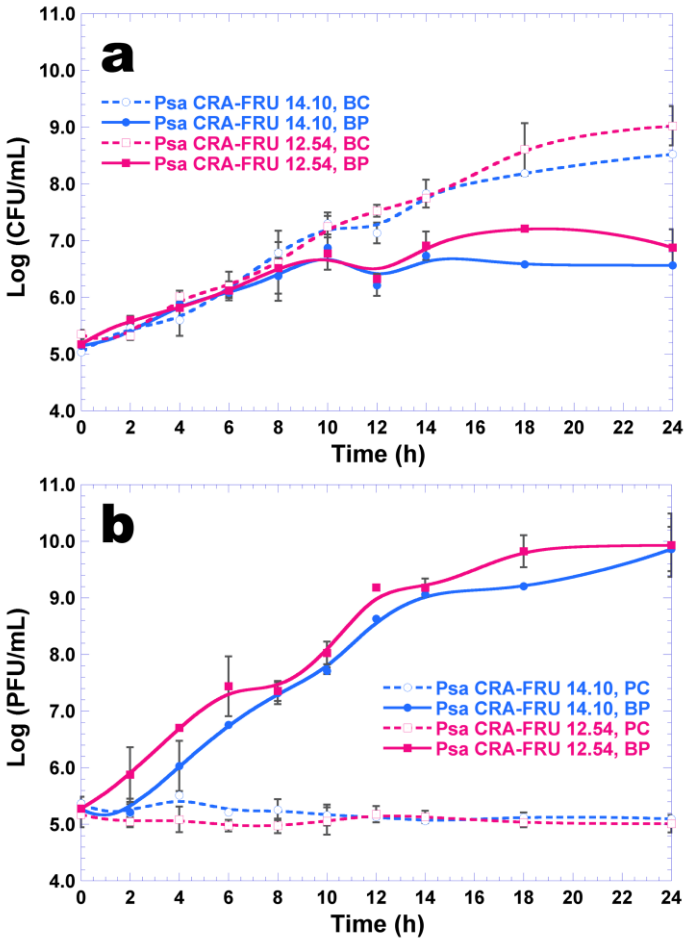

Figure 3.5. *In vitro* inactivation of Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10 by phage ϕ6 at a MOI of 1, during 24 h. a: Bacterial concentration (BC, bacteria control; BP, bacteria plus phage); b: Phage concentration (PC, phage control; BP, bacteria plus phage). Values represent the mean of three independent assays; error bars represent the standard deviation.

The phage controls (PC) remained constant throughout the experiment (ANOVA, $p > 0.05$), in both assays. When phage ϕ6 was incubated in the presence of of Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10, a significant increase in phage particle concentration (4.7 and 4.6 log PFU/mL, respectively, ANOVA, $p < 0.05$) was observed (Figure 3.5b).

3.4.5. *Ex vivo* phage treatment experiments in kiwifruit leaves

Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10 density in the BC increased by 2.2 and 2.9 log CFU/mL, respectively (Figure 3.6 a, ANOVA, $p < 0.05$) during the 24 h of incubation. Phage ϕ6 was effective against Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10 in artificially contaminated leaves. The maximum of Psa CRA-FRU 12.54 and 14.10 inactivation was 1.1 and 1.8 CFU/mL, respectively (Figure 3.6a, ANOVA, $p < 0.05$), after 24 h of incubation. After 12 h of incubation, the Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10 counts (reduction of 0.4 and 0.6 CFU/mL, respectively) were statistically similar (ANOVA, $p < 0.05$) from the values obtained in the bacterial control.

The phage controls (PC) remained constant throughout the experiment (ANOVA, $p > 0.05$), in both assays. When phage ϕ6 was incubated in the presence of Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10, a significant increase in phage particle concentration (1.4 and 2.8 log PFU/mL, respectively, ANOVA, $p < 0.05$) was observed (Figure 3.6b).

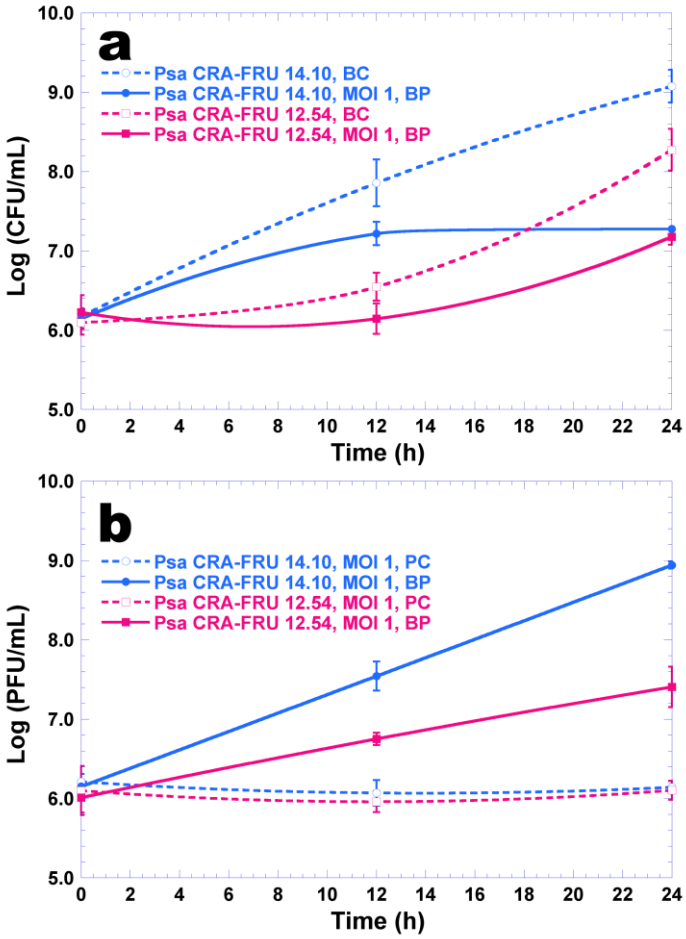

Figure 3.6. *Ex vivo* inactivation of Psa CRA-FRU 14.10 and Psa CRA-FRU 12.54 in artificially contaminated kiwifruit leaves by phage ϕ6 at a MOI of 1, during 24 h. a: Bacterial concentration (BC, bacteria control; BP, bacteria plus phage); b: Phage concentration (PC, phage control; BP, bacteria plus phage). Values represent the mean of three independent assays; error bars represent the standard deviation.

3.5. Discussion

The elimination of Psa in kiwifruit orchards is of utmost importance for the agriculture sector, but the treatments for this disease are still scarce and are not fully efficient and environmentally safe. While several studies have demonstrated that phages can be used to successfully control bacterial diseases in plants (Eman and Afaf, 2014; Flaherty et al., 2000; Fujiwara et al., 2011; Kalpage and Costa, 2014; Kim et al., 2016; Monk et al., 2010; Nguyen et al., 2012), little effort has been done to evaluate the effectiveness of phages to

control the Psa infection. There are only two *in vitro* studies of Psa inactivation by new isolated phages and no study using phages already characterized and even commercially available, such as phage ϕ6.

Our previous study showed that the commercially available phage ϕ6 besides infecting its original host, *P. syringae* pv *syringae*, infects also the two Psa biovar 3 phytopatogen strains CRA-FRU 12.54 and Psa CRA-FRU 14.10 (Pinheiro *et al.,* 2019). These results prompted us to evaluate the efficiency of phage ϕ6 to control Psa in order to improve/complement the non-environmentally friend conventional treatments already approved and in use. The evaluation was done first in *in vitro* conditions and after on kiwifruit leaves (*ex vivo*) artificially contaminated with Psa.

The efficacy of phage ϕ6 to inactivate Psa *in vitro* was evaluated by determining the dynamics of phage-host replication in liquid medium for both Psa strains. The adsorption rate of phage ϕ6 to Psa CRA-FRU 14.10 was higher than that of Psa CRA-FRU 12.54 ($\delta_{PsaCRA-FRU14.10}$ = (1.832±0.230) x 10^{-10} PFU^{-1} CFU^{-1} mL^{-1} hr^{-1} and $\delta_{PsaCRA-FRU12.54}$ = (7.971±1.401) x 10^{-11} PFU^{-1} CFU^{-1} mL^{-1} hr^{-1}, respectively). These results are in agreement with our previous results obtained for this phage with its original host *P. syringe* pv. *syringae* ($\delta_{P.\ syringae\ pv.\ syringae}$ = (9.495 ± 0.660) x 10^{-12} PFU^{-1} CFU^{-1} mL^{-1} hr^{-1} (Pinheiro *et al*., 2019) and with the results obtained by other authors (1.96x10^{-9} to 5.49x10^{-8} PFU^{-1} CFU^{-1} mL^{-1} hr^{-1}, (H. M. Lindberg et al., 2014; Santos et al., 2014; Shao and Wang, 2008) for other *Pseudomonas* phages isolated from environmental sources. When compared with its natural host, *P. syringae* pv. *syringae* ((60 ± 1 PFU/cell), phage ϕ6 showed a high burst size for both Psa strains (172 ± 0 PFU for Psa CRA-FRU 14.10 and 148 ± 1 PFU for Psa CRA-FRU 12.54, respectively)indicating that phage ϕ6 replicates efficiently in these Psa strains, but at the expense of long latency periods (100 min). Consequently, phage ϕ6 caused a significant reduction in Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10 concentration when compared with the non-treated bacteria, but its effect occurs only after 24 h of incubation. Although the phage adsorption rate and burst size have been higher for the strain Psa CRA-FRU 14.10 than for Psa CRA-FRU 12.54, the *in vitro* phage inactivation was similar for both bacteria (maximum inactivation of 2.0 log CFU/mL for Psa CRA-FRU 14.10 and 2.1 log CFU/ml for Psa CRA-FRU 12.54), corresponding to a reduction of 38.0% for Psa CRA-FRU 14.10 and

41.5% for Psa CRA-FRU 12.54 in bacterial concentration after 24 h of incubation, when compared to the non-treated cultures. Other phages (KHUϕ34, KHUϕ38 and KHUϕ44) already tested against Psa strain KBE9 (biovar 2) reduced the $OD_{600\ nm}$/mL of the bacteria by 61.1% to 81.5%, over a period of 80 h, but two other phages (KHUϕ59 and KHU□74) were innefective against the same bacteria (Yu et al., 2016). In another study, Park et al. (2018) tested the phage PPPL-1 against the Psa strain KBE9 (biovar 2) and observed a reduction of 50% in $OD_{600\ nm}$ after 12 h of treatment. Although in our study the *in vitro* phage inactivation of both Psa strains (biovar 3) was in general lower than the values reported in the literature for other Psa strains (biovar 2), one must keep in mind that Psa strains of the biovar 3 (as the ones tested in this study) are more significant in the context of kiwifruit canker. The results obtained with phage ϕ6 in these Psa strains are promising, however, more *in vitro* studies are needed, extending for instance the treatment time.

One of the current challenges faced when performing phage biocontrol studies is to demonstrate its feasibility in real matrices (Vieira *et al.*, 2012; Silva *et al.*, 2014; Pereira *et al.*, 2016, 2017; Fong *et al.*, 2017), and hence *ex vivo* phage experiments were carried out using artificially contaminated kiwifruit leaves. The results of the *ex vivo* assays showed that the phage inactivated both Psa strains, but the efficacy was lower, than that observed *in vitro*, namely for the strain CRA-FRU 12.54 (1.1 and 1.8 log CFU/mL, respectively, for Psa CRA-FRU 14.10 and Psa CRA-FRU 12.54), corresponding to a reduction of 29.3% for Psa CRA-FRU 14.10 and 17.6% log CFU/ml for Psa CRA-FRU 12.54 in bacterial concentration after 24 h of incubation, when compared to the non-treated cultures. These differences to the *in vitro* experiments might be explained by the different nanoenvironmental conditions prevailing around each phage particle and/or Psa bacterium cell. In the artificially contaminated kiwifruit leaves, the Brownian motions of both bacterial cells and phage ϕ6 particles are slower than those prevailing in the *in vitro* assays in liquid culture medium (Joiner *et al.*, 2019).

The reduction of Psa concentration by phage ϕ6 in both *in vitro* and *ex vivo* phage experiments is an important step forward in the development of an efficient and ecofriendly alternative to the conventional copper and antibiotic-based treatments. The field application of phages can be similar to that already used for copper, that is, by

spraying the kiwifruit plants with the phages in free form, or trapped within micro- or nanocarriers. As phage ϕ6 is affected by solar radiation and ultraviolet radiation (Pinheiro *et al.*, 2019), the phage should be applied during the night period. However, to translate the application of this strategy to the field, more studies are needed using whole kiwifruit plants, first in a laboratory scale and, afterwards, scaling up to kiwifruit plantations, essentially in naturally contaminated orchards.

Chapter 4. Conclusions and future perspectives

Conclusions

In this work a series of experiments were performed to evaluate the sustainability of phage therapy to biocontrol of Psa in kiwifruit plants.

The main conclusions of this work are summarized in the following topics.

Phage ϕ6 tested in this study is a potential candidate to be used on the biocontrol of *P. syringae* in plants. The characterization of phage replication in the host bacterial strain is an important aspect to select the most adequate phages.

- Phages with high burst sizes and short lytic cycles increase the efficiency of phage therapy.

  - Phage ϕ6 presents a high burst size (60 ± 1 PFU/ host cell), indicating that phage replicates efficiently in *P. syringae* pv*. syringae,* but the latency period in long (100 min).

- The efficiency of plating against Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10 strains, suggest that phage ϕ6 can be used in the control of bacterial canker of kiwifruit

  - Spot tests and Efficiency of plating results indicated that phage ϕ6, besides its host, formed phage lysis plaques on 2 of the 25 strains tested.

  - Phage ϕ6 infected Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10, with an efficiency of 101.3% and 96.8%, respectively.

- The viability of phage ϕ6 was affected by ultraviolet-B (UV-B) radiation, solar radiation and high temperatures, but this can be overcome by the application of phage at the end of the day or at night.

  - The viability of phage ϕ6 was mostly affected by UV-B radiation exposure (decrease of 7.3 log PFU/mL after 8 h), solar radiation exposure (maximum reduction of 2.1 PFU/mL after 6 h) and high temperatures (decrease of 8.5 PFU/mL after 6 days at 37 °C).

- The viability of the phage was not significantly affected by conditions as lower temperatures (decrease 2.0 log PFU/mL after 67 days at 15 °C and 25 °C) and pH (decrease 2.3 log PFU/mL at pH 7 and 7.5 and 2.7 log PFU/mL at pH 6.5).

- The emergence of phage-resistant mutants should not be a major problem to the application of phages to control bacterial pathogens in plants.
  - The frequency of phage-resistant mutation was low (10^{-3} CFU/mL).

***Ex vivo* studies are needed to transpose phage therapy for kiwifruit plants. The results obtained in the *ex vivo* experiments were different from those obtained *in vitro*.**

- The rate of Psa CRA-FRU 12.54 and Psa CRA-FRU 14.10 inactivation, *in vitro* assays, using phage ϕ6 was significantly higher than the results recorded *ex vivo*.
- In the *in vitro* assays, a reduction of approximately 2.0 log CFU/mL for both Psa strains was observed after 24 h of incubation.
- In the *ex vivo* assays, the decrease of Psa CRA-FRU 12.54 and Psa CRA.FRU 14.10 was 1.1 log CFU/mL and 1.8 log CFU/mL, respectively, after 24 h of incubation.

Future perspectives

While successfully used *in vitro* and *ex vivo* assays, the feasibility of phage therapy *in vivo* must be validated. The isolation of new phages and their evaluation to inactivate other strains of Psa will be also done in a near future. Further studies are needed in order to evaluate the potential development of phage-resistant strains in *ex-vivo* and *in vivo* assays. As it has been stated that phage resistance development can be overcome by the combined use of two or more phages (phage cocktails), so further studies using phage cocktails, including the phage ϕ6 and the new isolated phages, are needed in order to optimize the application of phages in kiwifruit plants to treat Psa.

Chapter 5. References

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