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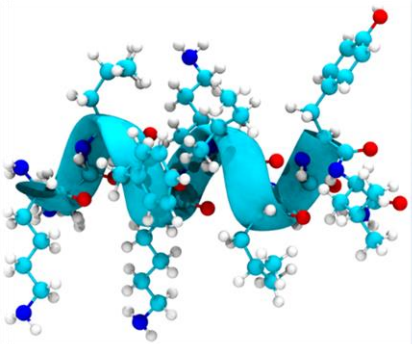
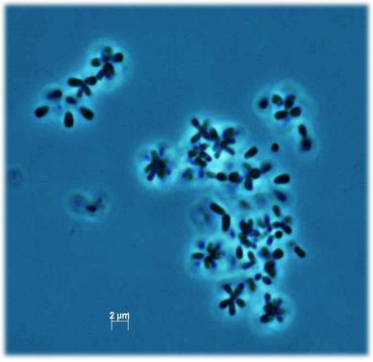

Susceptibility of *Pseudomonas* spp. to new antibacterial compounds and antagonists

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Year 2019/2020

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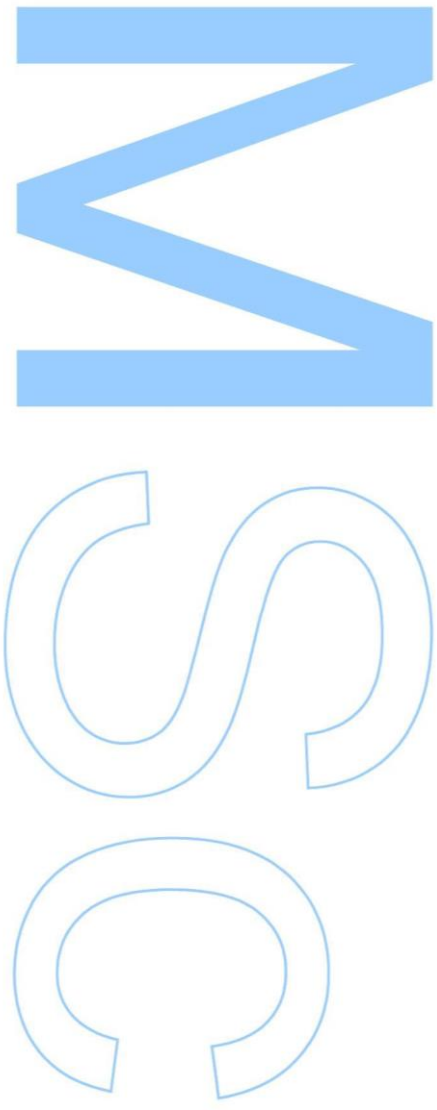

Acknowledgements

First, I would like to thank my advisor, Doctor Prof. Olga Maria Lage, for all the support, availability and guidance of this work.

Special thanks to Doctor Prof. Maria da Conceição Lopes Vieira dos Santos for accepting to co-guide this work and also for the guidance, understanding and opportunity to develop this dissertation thesis.

I thank the entire team of the Microbial Ecophysiology Laboratory of the U.P. (LEMUP) - Eduarda Almeida, Ofélia Godinho, Rita Calisto, Maria da Conceição Marinho for their availability whenever I asked for their help with laboratory tasks, and especially to José Santos and Inês Vitorino for their patience, support, knowledge and help in solving problems and difficulties.

To the group of the Laboratory of Integrative Biology and Biotechnology (IB2) - Cristiana Correia, Laura Regalado, Rafael Mendes, Sara Sario, Marino Santos for their friendship, good times, fun and enormous help and especially, to Nuno Ponte, for the great availability, support and guidance of the work.

Finally, my family who gave me a huge opportunity and allowed me to achieve it.

Abstract

Pseudomonas syringae pv. *actinidiae* (PSA) is the etiological agent of the bacterial canker disease in *Actinidia* spp. that leads to high economic losses. Currently, no effective treatment exists against PSA, with the only existing strategies being essentially preventive measures. These include application of different types of pesticides such as copper based agrochemicals and/or antibiotics. However, the use of antibiotics is forbidden in many countries, such as the included in the European Union, and besides, for both agrochemicals has been found resistance in several bacteria, including PSA. This work aims to characterize the pathogenicity of the four Portuguese PSA isolates (collected from kiwifruit orchards of Entre o Douro e Minho region in 2013) and to evaluate their susceptibility to new potential alternative compounds and current control strategies such as copper-based agrochemicals. Thus, PSA biovar 3 reference strain CFBP 7286, four Portuguese isolates of PSA P84, P85, Am63 and AL13 and, in addition, *P. viridiflava* CFBP 2107, *P. syringae* pv. *syringae* DSM 10604 and *P. cerasi* B65 were selected for this study as kiwifruit pathogens and found in Portugal.

The phenotype characterization demonstrated that all studied strains are oxidase negative, catalase positive, exhibit swimming motility and not able to form biofilm in abiotic surfaces. Furthermore, all strains produce IAA using Trp-dependent pathway and were able to induce hypersensitive response on tobacco leaves. Susceptibility of *Pseudomonas* spp. for AMPs and copper sulphate was assessed through growth curves, determining the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and half inhibitory concentration (IC_{50}). All of the isolates, except for DSM 10604, showed minimum inhibitory concentration of 50 µg/mL and did not demonstrate resistance to copper, with CFBP 7286 (IC_{50} 41.50 µg/mL) and Am63 (IC_{50} 43.02 µg/mL) the most susceptible to this compound. Moreover, at concentrations of 75 µg/ml this compound has been shown to have bactericidal action for CFBP 7286, P84 and P85 isolates, and to have bacteriostatic action for the other five strains.

The activity of the antimicrobial peptides (AMPs) BP100 and 3.1 was assessed through antibiogram assays, growth curves, flow cytometry and hypersensitive response (HR). Both AMPs had strong antimicrobial activity with MIC values ranging from 3.4 to 6.2 µM and from 10 to 25 µM for AMPs BP100 and 3.1, respectively. AMP BP100 (MBC ranged from 3.4 to 25 µM) demonstrated better bactericidal activity than AMP 3.1 (MBC ranged from 25 to 100 µM), but AMP 3.1 showed major membrane permeability activity. The flow cytometry analysis suggested that the activity of both peptides is dependent with peptide-lipid interactions and permeabilization of the bacterial membrane.

Moreover, according to the HR on tobacco leaves, both AMPs showed a reduction in the pathogenicity of the selected strains.

Furthermore, several bacterial isolates from the phylum Actinobacteria and Planctomycetes were tested for the production of bioactive compounds and the antagonistic capacity against phytopathogenic *Pseudomonas*. This study confirmed the bioactive capacity of the crude extracts of actinobacteria and planctomycetes. Extract obtained with liquid-liquid phase (LLPE) extraction protocol exhibited greater antimicrobial activity with percentage of inhibition ranging from 56.48 to 83.08 %, than crude extracts obtained with solid phase extraction (SPE) protocol with activities that presented percentage of inhibition of 50.29 to 75.44 %.

In conclusion, this work discloses the potential of AMPs, BP100 and 3.1, and extracts of actinobacteria and planctomycetes as alternative compounds to control the bacterial canker disease. Further trials should be performed to test the efficiency of these compounds against PSA *in planta* and in field applications.

Keywords: antimicrobial peptides, *Actinobacteria*, copper sulphate resistance, Kiwifruit bacterial canker, *Pseudomonas syringae* pv. *actinidiae*, *Planctomycetes*.

Resumo

Pseudomonas syringae pv. *actinidiae* (PSA) é o agente etiológico do cancro bacteriano em *Actinidia* spp. que é responsável pelas grandes perdas econômicas. Atualmente, não existe nenhum tratamento eficaz contra o PSA, sendo as únicas estratégias existentes são essencialmente medidas preventivas. Estas incluem a aplicação de diferentes tipos de pesticidas, como agroquímicos à base de cobre e / ou antibióticos. Porém, o uso de antibióticos é proibido em muitos países, como os da União Europeia, e, além disso, para ambos os agroquímicos foi encontrada resistência em várias bactérias, incluindo o PSA. Este trabalho tem como objetivo caracterizar a patogenicidade dos quatro isolados portugueses de PSA (recolhidos em pomares de kiwi da região de Entre o Douro e Minho em 2013) e avaliar a sua suscetibilidade a novos potenciais compostos alternativos e meios de controlo atuais como os agroquímicos à base de cobre. Assim, a estirpe de referência PSA biovar 3 (CFBP 7286), quatro isolados portugueses de PSA P84, P85, Am63 e AL13 e, além disso, *P. viridiflava* (CFBP 2107), *P. syringae* pv. *syringae* (DSM 10604) e *P. cerasi* (B65) foram selecionados para este estudo como patogénicos de kiwi e encontrados em Portugal.

A caracterização do fenótipo demonstrou que todas as estirpes estudadas são oxidase negativa e catalase positiva, apresentam motilidade e não são capazes de formar biofilme em superfícies abióticas. Além disso, todas as estirpes produzem IAA utilizando a via dependente de Trp e foram capazes de induzir resposta de hipersensibilidade em folhas de tabaco. Suscetibilidade de *Pseudomonas* spp. para AMPs e sulfato de cobre foi avaliada por meio de curvas de crescimento, determinando a concentração inibitória mínima (MIC), concentração bactericida mínima (MBC) e metade da concentração inibitória (IC_{50}). Todos os estirpes, exceto DSM 10604, apresentaram concentração inibitória mínima de 50 µg/mL e não demonstraram resistência ao cobre, sendo CFBP 7286 (IC_{50} 41.50 µg/mL) e Am63 (IC_{50} 43.02 µg/mL) os mais suscetíveis a este composto. Além disso, em concentrações de 75 µg/ml, este composto demonstrou ter ação bactericida para os isolados de CFBP 7286, P84 e P85, e ação bacteriostática para os outros cinco isolados.

A atividade dos peptídeos antimicrobianos (AMPs) BP100 e 3.1 foi avaliada por meio de ensaios de antibiograma, curvas de crescimento, citometria de fluxo e resposta de hipersensibilidade (HR). Ambos os AMPs tiveram forte atividade antimicrobiana com valores de MIC que variaram entre 3.4 e 6.2 µM e entre 10 e 25 µM, respetivamente para AMPs BP100 e 3.1. O AMP BP100 (MBC variou entre 3.4 e 25 µM) demonstrou

melhor atividade bactericida do que AMP 3.1 (MBC variou entre 25 e 100 µM), mas AMP 3.1 mostrou maior atividade de permeabilidade de membrana. A análise de citometria de fluxo sugeriu que a atividade de ambos os AMPs é dependente das interações péptido-lípido e permeabilização da membrana bacteriana. Além disso, de acordo com a resposta HR em folhas de tabaco, ambos os AMPs apresentaram redução na patogenicidade nas bactérias selecionadas.

Além disso, diversos isolados bacterianos do filo *Actinobacteria* e *Planctomycetes* foram testados quanto à produção de compostos bioativos e capacidade antagónica contra *Pseudomonas* fitopatogénicas. Este estudo confirmou a capacidade bioativa dos extratos brutos de actinobactérias e planctomicetes. O extrato obtido com protocolo de extração em fase líquido-líquido (LLPE) exibiu maior atividade antimicrobiana com percentagens de inibição que variaram entre 56.48 e 83.08 %, do que extratos brutos obtidos com protocolo de extração em fase sólida (SPE) com atividades que apresentaram percentagens de inibição entre 50.29 e 75.44 %.

Em conclusão, este trabalho divulga o potencial dos AMPs, BP100 e 3.1, e de extratos de actinobactérias e planctomicetes como compostos alternativos no controle do cancro bacteriano de kiwi. Outros ensaios devem ser realizados para testar a eficiência desses compostos contra PSA *in planta* e em aplicações de campo.

Palavras-chave: *Actinobacteria*, cancro bacteriano de kiwi, *Planctomycetes*. *Pseudomonas syringae* pv. *actinidiae*, péptidos antimicrobianos, resistência ao sulfato de cobre.

Publications/Presentations

- Poster:

Gimranov, E, Mariz-Ponte, N., Moura, L., Tavares, F., Lage, O, Santos, C., (2020) Phytopathogenic *Pseudomonas* spp. susceptibility against copper treatment and action mechanisms. IJUP 2020.

- Paper (under preparation):

Mariz-Ponte, N., Moura, L., **Gimranov, E.,** Rego, R., Santos, C., Tavares, F., (2020) Genetic and Phenotypic Characterization of *Pseudomonas syringae* pv. *actinidiae* Isolated from Portuguese Kiwifruit Orchards Between 2013 to 2017 Unveil Different Biochemical Profiles Associated to Highly Clonal Population Structure.

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Abbreviations and Symbols

% - Percentage

® - Registered trademark

µmax - The maximum specific growth rate

AMPs - Antimicrobial peptides

CFU - Colony forming units

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

DRAP - Direção Regional de Agricultura e Pescas

e.g. - *Exempli gratia*

EPPO - European and Mediterranean Plant Protection Organization

EU - European Union

G+C - Guanine and Cytosine

IAA - Indol-3-acetic acid

IC_{50} - half inhibitory concentration

INE - National Statistics Institute

LLPE - Liquid-liquid phase extraction

MBC - Minimal bactericidal concentration

MIC - Minimal inhibitory concentration

$OD_{600\ nm}$ - Optical density at 600 nanometres

PBS - Potassium phosphate buffer solution

pH - Potential of hydrogen

PI - Propidium iodide

PKSs/ PKS-I - Polyketide synthases/ Type I Polyketide synthases

PSA - *Pseudomonas syringae* pv. *actinidiae*

r.p.m. - Revolutions per minute

rRNA - Ribosomal ribonucleic acid

SPE - Solid phase extraction

T3SS - Type III secretion system

TMM - Tris minimal medium

™ - Trademark symbol

v/v - Volume/volume

1. Introduction

Plant pathogenic *Pseudomonas* species cause diseases in a wide range of economically important crop species, provoking high economic losses. Most of them are in *Pseudomonas syringae* phylogenetic group (Oueslati *et al*., 2019), wich contains 15 recognized bacterial species and several pathovars (Gomila *et al*., 2017). *P. syringae* is a very studied phytopathogen and were used as a model strain for analysing bacterial pathogenicity and molecular mechanism of plant-bacterial interactions (Xin *et al*., 2018). Among oxidase negative strains, several plant pathogen *Pseudomonas* species were reported. The most economically important strain *P. syringae* with more than 50 pathovars (Xin et al., 2018), like PSA which is the causal agent of bacterial canker of kiwifruit and causes leaf brown necrosis, production of white and red exudates and death of branches (Donati *et al*., 2014). *P. viridiflava* is pathogenic for several citrus plants, including some species of *Actinidia,* causing different plant diseases. Also, this specie was recognized as a member of *P. syringae* species complex (Al-Karablieh *et al*., 2017). Recently isolated specie *P. cerasi* cause shoot blight on pear tree (Choi *et al*., 2020).

The increasing spread of parasites (insects, fungi, bacteria, etc.), mostly due to the trade globalisation (*eg.,* transport of goods and plant material), combined with climate change, favours their adaptation and establishment in new regions, harming not only the agricultural production sector, but also food security. One of the foremost example is the bacterial canker in kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* (PSA). Ever since the first outbreak in China, in the 1990s, this phytopathogenic bacterium has spread rapidly across different kiwifruit-producing regions, causing high economic losses (Flores *et al*., 2018).

The most important mean to control PSA is the use of commercial chemical pesticides (Nawaz *et al*., 2016), which are able to kill or inactivate targeted harmful organisms, with no or little impacts on the plant host. The use of agrochemicals, including pesticides, is an essential practice in combating pests in order to ensure a high productivity world population. On the other hand, their recurrent use, or their use without control (nor adequate technical criteria) can lead to serious environmental and human health problems (Nicolopoulou-Stamati *et al*., 2016). Nevertheless, only in the last 40 years, have measures been taken to minimize the negative impacts caused by these substances. Therefore, the European Union (EU) has adopted risk reduction policies through the implementation of different directives. However, despite the various measures adopted, approximately 500 approved substances remain in the EU, with annual sales of 371 million kilograms (Silva *et al*., 2019).

Phytopathogenic bacterial resistance to agrochemicals is making the treatment of plant disease progressively more difficult and represent a global challenge to crop production (Nicolopoulou-Stamati *et al*., 2016). In recent years, attempts have been made to discover sustainable strategies. Several alternatives to chemical pesticides were proposed like plant resistance inducers compounds, metallic-based nanoparticles, microorganisms, bacteriophages, plant extracts, chitosan (Lamichhane *et al*., 2018), antimicrobial peptides (AMPs) (Sinha & Shukla, 2019), genetic modification of plants and natural substances with pesticidal action produced by the plants or microorganisms themselves (Hossain *et al*., 2017).

1.1. Kiwifruit production in Portugal

The genus *Actinidia* was introduced in Portugal in 1973, with the first kiwifruit orchard being installed in Vila Nova de Gaia by Dr. Ponciano Serrano (Antunes, 2008). Since the 1990s, the kiwifruit production gained increasing importance, due to optimal growth conditions and low production costs (Antunes *et al*., 2018). According to data of the National Statistics Institute (INE), the installation of new orchards allowed high productivity, with increases of production from 17360 tons of kiwifruit in 2005 to 34057 tons in 2018 (Figure 1) (INE, 2018).

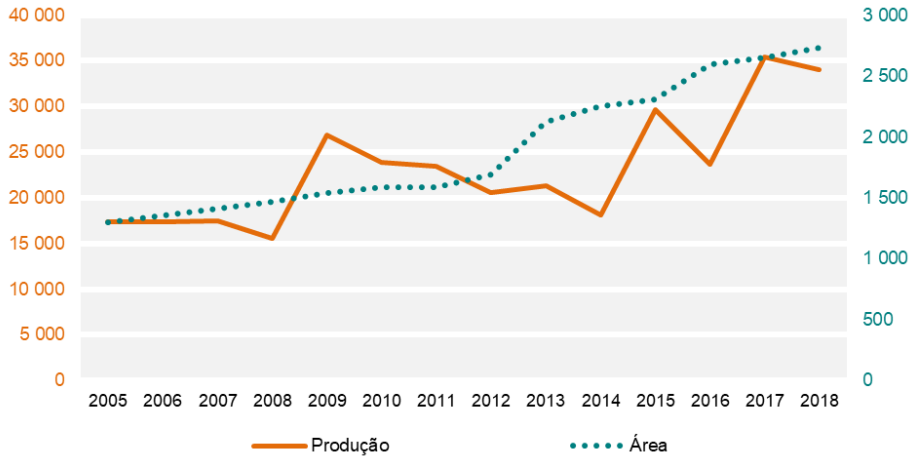

Figure 1 – Kiwifruit production in Portugal between 2005 and 2018. Source: INE I.P., Estatísticas da Produção Vegetal.

Moreover, the main regions of kiwifruit production in Portugal are Entre Douro e Minho and Beira Litoral, because they have the optimal environmental conditions for plant growth, which leads to high levels of productivity (Antunes *et al*., 2018). The increase in kiwifruit production has been remarkable in recent years. In 2017, kiwifruit production reached 35.4 thousand tons (INE, 2018).

1.2. Kiwifruit bacterial canker

Bacterial canker of kiwifruit is caused by PSA. This disease affects various species of the *Actinidia*, including the two most economically important varieties, A. *chinensis* var. *chinensis* and *A. chinensis* var. *deliciosa* (Donati *et al*., 2018). The first PSA isolated was detected in Japan, and few years later, was also identified in China (1989) and in Italy (1992). Since then and after a major disease outbreak of PSA in Italy (2008), this phytopathogenic bacteria has dispersed to the main kiwifruit producing regions in Europe, including Portugal in 2010 (Khandan *et al*., 2013). Therefore, due to the rapid geographical dissemination, high pathogenicity for kiwifruit species and the absence of effective measures of PSA control/prevention, this phytopathogen has been considered as a quarantine bacterium and was included in the A2 list by the European and Mediterranean Plant Protection Organization (EPPO). Nowadays, the bacterial canker of the *Actinidia* is considered the biggest cause of severe losses in world production of kiwifruit. For example, more than 2000 ha of kiwifruit orchards were destroyed by the PSA canker in Italy between 2010 and 2012, causing serious economic losses (Donati *et al*., 2020).

The process of plant colonization and penetration into the host tissues by PSA is not completely clarified. Before infecting the plant, the pathogen grows on the epiphytic surfaces of Actinidia flowers and leaves. After that PSA infects kiwifruit plants by entering through natural openings, like stomata or lenticels, and wounds (Renzi *et al*., 2012). Additionally, in infected kiwifruit plants, the phytopathogen is found in the lenticels, dead phloem tissue (Renzi *et al*., 2012) and in the anthers (Donati *et al*., 2018).

1.3. Symptomatology and disease cycle

Actinidia species have several differences, with the two most economically important varieties being distinct not only in chromosome number, flower and fruit morphology, but also in different susceptibility to PSA. The *Actinidia chinensis* var. c*hinensis* is more susceptible than *Actinidia chinensis* var. *deliciosa* demonstrating a more pronounced symptomatology, faster dieback and enhanced spread (Donati *et al*., 2020).

The symptoms of the disease are linked to its cycle and are easily observed in aerial parts, such as trunks, leaders, canes, leaves, flowers and fruits (EPPO, 2014). While PSA can infect the kiwifuit plants at any time of the year, the critical infectious period occurs around autumn, the end of the winter, and the beginning of spring, since the humidity and temperature conditions are more favourable, and cultural operations occur

during these periods, promoting PSA spread, penetration and colonization of the host plant (Donati *et al*., 2014).

Bacterial canker caused by PSA evolves in two phases: during the first phase (autumn and winter), the bacterial canker affects the overwintering canes and vine structure. In the second phase (spring), the disease affects the new season growth of leaves, canes, and flowers (Figure 2) (Kim *et al*., 2017a).

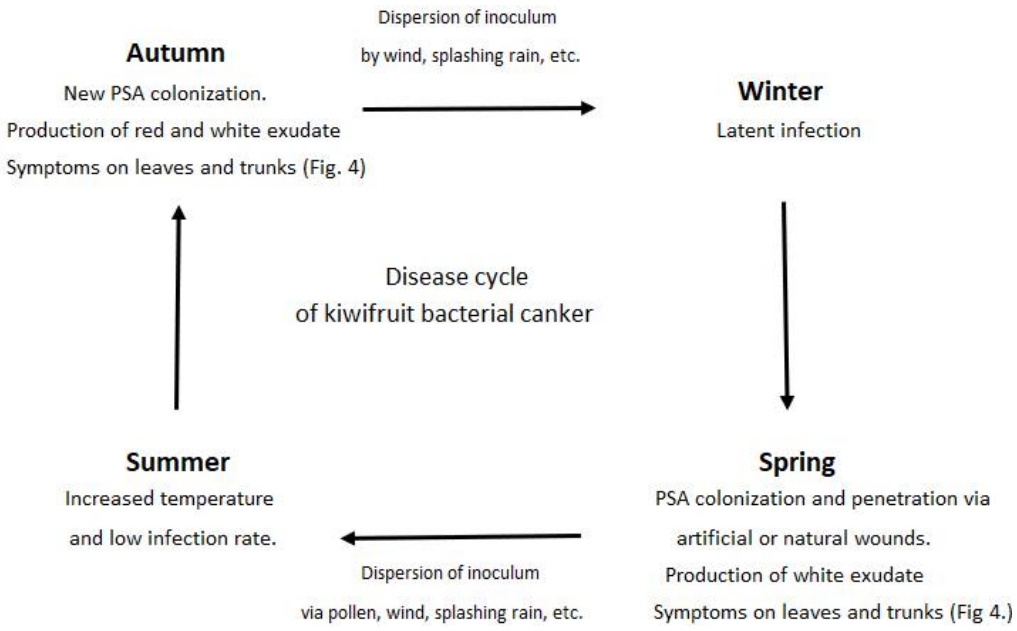

Figure 2 - Disease cycle of bacterial canker on green-fleshed kiwifruit cultivar Hayward (*Actinidia deliciosa*) caused by *P. syringae* pv. *actinidiae* biovar 2 (PSA2) in Korea (Kim *et al*., 2017a).

During the first phase, in the autumn, new infections might occur due to leaf fall and fruit harvesting. However, the symptoms of new infections will appear in the following Spring, but in old infected plants the infection is characterized by the following symptoms: brown necrosis surrounded by yellow halo, leaf with brown necrosis of larger dimensions, wilting and death of branches and canker formation on vines and trunks. During the winter the bacteria survives in latent form in the cortex tissue of infected branches (Figure 2) (Donati *et al*., 2014; Kim *et al*., 2017a).

During the second phase, in spring, bacteria can enter through stomata, broken trichomes, lenticels, flowers, hydathodes, leaf and fruit scars, and artificial or natural wounds. The dissemination of PSA in the plant occurs by xylem vessels. At this stage of the year, infected plants produce bacterial exudates that flow from natural openings or trunks. This is a very effective strategy for bacterial dissemination, which is mainly caused by wind, splashing rain and wind-blown rain (Kim *et al*., 2017a).

PSA may cause leaf brown spots with chlorotic margin, necroses on flower buds, ooze bacterial exudates and plant sap (Figure 3). Similar symptoms on leaves can be caused by others phytopathogenic bacteria that infect kiwifruit plant, for example, *P. syringae* pv. *syringae* (PSS) and *Pseudomonas viridiflava* (EPPO, 2014).

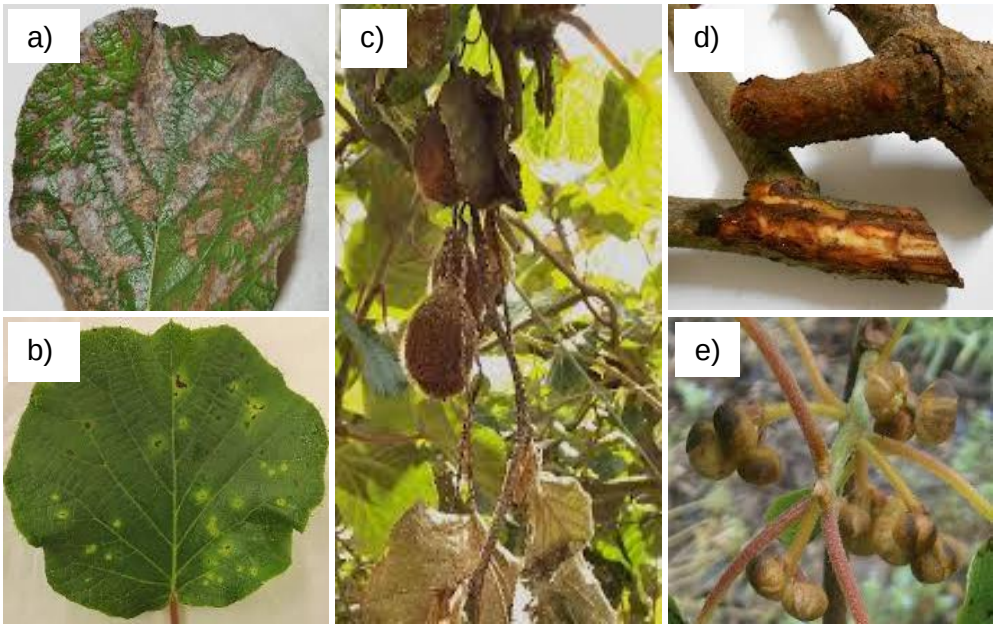

Figure 3 - Characteristic symptoms of PSA in *Actinidia deliciosa*: (a) leaf with larger brown necroses; (b) leaf with surrounded brown necrosis with a yellow halo; (c) wilting and death of branches; (d) areas under the shell with a reddish hue; (e) flower buds with brown necrosis (Moura *et al*., 2015).

In summer, when temperatures often exceed 25 ºC, the degree of infection is drastically decreased. In fact, the optimum temperature for which PSA is most invasive is between 10 ºC and 20 ºC. The bacterium activity increases when the temperatures decrease again in autumn (Donati *et al*., 2014; Kim *et al*., 2017a).

1.4. Geographical distribution of the disease and prevalence in Portugal

The canker of kiwifruit fruit caused by PSA was first reported from Shizuoka Prefecture in Japan (1984). Subsequently, other countries such as South Korea (1988), China (at Yuexi, 1990) and the central parts of Italy (1992) registered the presence of PSA (Khandan *et al*., 2013). However, a highly severe form of the disease was observed in Italy (2010) and quickly dispersed to the others major producing countries of kiwifruit like

New Zealand, South Korea, Iran, France, Portugal, Chile, Spain, Switzerland and Australia, causing grave damage (Wang *et al*., 2018).

Previous molecular work demonstrates that PSA biovar 3 (PSA3) is responsible for a severe form of the disease, which has become a major problem in many countries and has been found that was disseminated from China. The introduction and dispersion of this PSA3 population in the countries of Europe, New Zealand and Chile, was not fully clarified, but three major routes of transmission were observed: a) from China to New Zealand; b) from China to European countries and c) from China to Chile (Sawada & Fujikawa, 2019).

The first report of bacterial canker of kiwifruit in Portugal was in 2010 as mentioned above. This disease was observed in kiwifruit orchards in the Entre Douro and Minho region, more specifically in Santa Maria da Feira and Valença (Balestra *et al*., 2010).

Figure 4 - Results of PSA prospecting in Portugal between 2010 and 2013 (DRAP, 2013).

However, between 2010 and 2013, PSA rapidly spread to other north and centre regions and was detected in different national kiwifruit orchards (Figure 4) (DRAP, 2013). According to molecular studies, it was found that this disease is caused by PSA3, similarly to the data reported for Europe and New Zealand (Renzi *et al*., 2012; Moura *et al*., 2015).

1.5. *Pseudomonas syringae* pv. *actinidiae*

1.5.1. General characterization

Currently, more than 50 pathovars of *P. syringae* are known, in which each is responsible for the infection of a certain plant *species* (Xin *et al*., 2018). Among the different pathovars stands out the *P. syringae* pv*. actinidiae*. This pathogen is the etiological agent

of bacterial canker of kiwifruit as mentioned above. PSA was first described by Takikawa *et al*. (1989) in Japan and belongs to the order Pseudomonadales and family Pseudomonadaceae (Kim *et al*., 2017a). This is a bacillus shaped Gram-negative bacterium with an aerobic metabolism, having one to three flagella, and has the capacity to affect different species of *Actinidia* (EPPO, 2014; Kim *et al*., 2017a).

Based on toxin production, virulence and genetic diversity, PSA can be classified into five groups of biovars. In brief, biovar 1, which includes the first described Japanese strain of PSA that produces phaseolotoxin. Biovar 2 has only been reported in South Korea and can produce coronatine instead of phaseolotoxin. The biovar 3 is currently pandemic and the most virulent isolate and does not have the capacity to produce phaseolotoxin nor coronatine. Biovar 4 is a group of strains with low virulence found in France, New Zealand and Australia, inducing only mild leaf symptoms. This biovar has since been reclassified as a new pathovar called *P. syringae* pv. *actinidifoliorum* (PSAf). Finally, biovar 5 includes Japanese strains isolated in 2012, which is characterized by an inability to produce phytotoxins (Kim *et al*., 2017a).

1.5.2. Virulence factors

P. syringae pathovars developed two main virulence strategies: the suppression of host immunity (Dudnik & Dudler, 2014) and the multiplication into the apoplast (Xin *et al*., 2018). In order to successfully colonize the apoplast, PSA must be able to suppress host defences. Thus, the bacterium has developed several virulence factors, such as, different types of effectors that are secreted via Type III secretion system (T3SS), plant hormones and hormone analogues [*eg.,* indole-3-acetic acid (IAA)], and other compounds like cell wall-degrading enzymes, polysaccharides and low molecular weight toxins (Kunkel & Harper, 2018). Other factors, like mobility and biofilm formation have also been associated to virulence (Ghods *et al*., 2015).

Type III secretion system: One of the unifying characteristics that is present in almost all strains classified as *P. syringae* is the presence of a T3S) involved in suppressing host immunity. This secretion system conducts the different effectors (T3E) within the host cell (Ruinelli *et al*., 2019). T3E present a wide variety of strategies to control and / or inhibit the different mechanisms of the host cells. Therefore, two large families of T3E have been described, HopZ and HopF. Regarding their function, it is known that these can suppress the host immune defence, interfering with signalling cascades and intracellular trafficking, modify the structure of the cytoskeleton and the transcription of genes, and also inhibit callose deposition and production of reactive species of oxygen (Lo *et al*., 2017).

Enzymes and toxins: Small molecules that function as hormone analogues and other compounds like cell wall-degrading enzymes, polysaccharides and low molecular weight toxins (*eg.,* coronatine, syringomycin, syringopeptin, tabtoxin and phaseolotoxin) which improve virulence and help suppressing plant defences (Dudnik & Dudler, 2014; Kunkel & Harper, 2018).

Hormone production: Several phytopathogenic bacteria, including the different pathovars of *P. syringae*, have the capacity to produce auxins (Flores *et al*., 2018). An increase in levels of IAA within the host cell not only promotes epiphytic colonization altering plant signalling and physiology, but also promotes the inhibition of host defences and allows the pathogen growth in plant tissue. As a coronatine, IAA has a similar virulence mechanism via suppression of salicylic acid (SA) - mediated defences (Kunkel & Harper, 2018). Also, Mutka *et al*. (2013) demonstrated that IAA could promote bacteria growth through different mechanisms that are independent of suppression of SA – mediated defence, for example, the production of indole glycosylates that are modulated by auxin signalling (Kunkel & Harper, 2018). The common route described for the production of IAA in PSS and *P. savastanoi* pv. *savastane* (PSAv) is via the indole-3-acetamide (IAM) pathway, in which the enzymes IaaM and IaaH are used. However, in a more recent study, it was shown that PSA and *P. syringae* pv. *tomato* (PST) had the *aldA* and *aldB* genes that were associated with an alternative IAA synthesis pathway (Flores *et al*., 2018).

Currently, six IAA biosynthesis pathways are described using the amino acid tryptophan as a precursor: indole-3-acetamide (IAM), indole-3-acetonitrile (IAN), indole-3-pyruvate (IPyA), tryptamine (TAM) and tripidasphine side chain oxide (TSO). Also, the biosynthesis pathway using indole-3-acetaldehyde dehydrogenase (*aldA*) that has been identified in *P. syringae* (DC3000) (McClerklin *et al*., 2018). In fact, there is evidence that the IAA production pathway may be another distinguishing feature between different PSA biovars, since the common IAA synthesis pathway is only found in PSA4 isolates (Flores *et al*., 2018).

Other virulence factors (motility and biofilm): Leaf and trunk surfaces of the plants is extremely stressful habitat (*eg.,* UV irradiation, shortage of nutrients, environmental parameters) to phytopathogenic microorganisms (Hockett *et al*., 2013). Motility and biofilm production are virulence factors which not only facilitate pathogen-host interactions and promotes colonization, penetration, but also, in the case of production of biofilm, protects bacterial cells against the host's defence mechanisms, UV rays and antimicrobial substances (Ghods *et al*., 2015; Farias et al., 2019). In addition, biofilm matrix contains several biological active molecules (*eg.,* quorum sensing signals,

enzymes, exopolysaccharides, and secondary metabolites), substrates and nutrients which facilitates the survival in the environment (Farias *et al*., 2019).

1.6. Control measures

A variety of available protective strategies have been designed to control plant diseases by killing or by inhibiting the growth of the disease-causing pathogens. Nowadays, kiwifruit industries are affected by PSA and disease management strategies for this pathogen are very important but problematic. There are only preventive measures which includes strict orchard hygiene practices [recommended by EPPO (EPPO, 2014)], and scheduled spraying of bactericidal compound like a copper-based agrochemicals and antibiotics (*eg.,* streptomycin). Interestingly, despite the use of antibiotics for control phytopathogenic agents is illegal in the EU, the resistance of field isolates to copper and/or antibiotics is increasingly being documented worldwide (Cameron & Sarojini, 2014a; Lamichhane *et al*., 2018).

There is a wide spectrum of consequences due to several factors such as the biological action of the active substances, their composition, and the time of exposure (Kim *et al*., 2017b). The presence of pesticides in soils raises several concerns: the leaching of these substances into groundwater and into aquatic ecosystems such as lakes and rivers in surrounding areas (Cruzeiro *et al*., 2017); pest resistance (Hawkins *et al*., 2019); death of benign organisms for soil fertility (Lamichhane *et al*., 2018). Besides, the impact of these agrochemicals on human health has been demonstrated, including neurological, carcinogenic, respiratory, endocrine, dermatological and gastrointestinal effects (Nicolopoulou-Stamati *et al*., 2016).

So, it is extremely important to develop new strategies of control, such as elicitors that activate the plant's immune system and biological control (Cameron & Sarojini, 2014a). This is the case of compounds that can stimulate plant defence, like acibenzolar-S-methyl (ASM) (Reglinski *et al*., 2013) and some vitamins such as riboflavin and thiamine and also other compounds like folic acid, menadione sodium bisulphite and para-aminobenzoic acid that can induce resistance in plants against different phytopathogenic bacteria, including *P. syringae* (Boubakri *et al*., 2016).

1.6.1. Copper-based compounds

Copper is an essential micronutrient for all prokaryotic and eukaryotic organisms and plays important roles in different cellular processes including activity as a cofactor for several enzymes involved in electron transport proteins and respiration (Husak, 2015). At the same time, excessive levels of copper ions induce toxic cellular effects. It has a

broad spectrum of action such as interaction with nucleic acids, interference with the energy transport system, disruption of enzyme active sites and the integrity of cell membranes. Therefore, copper-based compounds are widely used in agriculture to control pathogenic microorganisms (Jeyakumar *at al*., 2014; Lamichhane *et al*., 2018).

The first copper-based compound used in agriculture against phytopathogenic microorganisms was Bordeaux mixture that was discovered in 1885 by Pierre-Marie Alexis Millardet. Following the use of Bordeaux mixture as a fungicide, today a wide range of copper-based fungicides are available to manage crop diseases caused by plant pathogenic microorganism like copper hydroxide, copper chloride, copper oxychloride and copper oxide-based formulations. Likewise, the use of these compounds is considered a very effective practice in protecting against PSA (Lamichhane *et al*., 2018). However, these compounds present several disadvantages such as phytotoxicity (Husak, 2015) and the ability to induce copper resistance of phytopathogenic organisms (Lamichhane *et al*., 2018).

1.6.1.1. Phytotoxicity

The phytotoxicity is one of the severe consequences of the uncontrolled use of copper compounds as mentioned in the previous section. The accumulation of Cu in agricultural soil cause adverse consequences such as plant stress, reduction of soil fertility and the uptake of iron and other nutrients that affects crop quality, fruit number, crop yield and plant height (Husak, 2015; Lamichhane *et al*., 2018). High levels of Cu ions could cause damage to kiwifruit fruit and leaves. Nevertheless, Brun and Max (2012) demonstrated that no significant phytotoxic effect on leaf and fruit development occurred. Many factors determine Cu phytotoxicity, such as, solubility, bioavailability, environmental temperature and humidity levels, the plant sensibility to Cu (Lamichhane *et al*., 2018) and the periodicity and duration of the Cu application (Jeyakumar *et al*., 2014).

Typical toxicity symptoms such as darkening of axial and abaxial surfaces, chlorosis, leaf margin burn and necrotic spots may appear on the leaves and different types of depreciating blemishes on citrus fruit surface (Lamichhane *et al*., 2018). Other symptoms of Cu phytotoxicity are the discolouration and cracking of stalks, the appearance of spots on the leaves and silver-brown leaves that were observed in Japanese kiwifruit orchards (Jeyakumar *et al*., 2014).

1.6.1.2. Development of copper-resistant strains

Nowadays, the scientific literature shows different cases of decreased sensitivity to Cu in *Pseudomonas* spp. isolates. For example, according to the results obtained by Zhang and his collaborators (Zhang *et al*., 2017) several strains of *P. syringae* pv. *phaseolicola* isolated from the bean field in South Florida, showed resistance to Cu. In addition, the

same phenomenon was observed in other phytopathogenic bacteria species such as *Xanthomonas citri* pv. *citri* (Richard *et al*., 2017), *X. campestris* pv. *campestris* (Behlau *et al*., 2017) and *Erwinia amylovora* (Al-Daoude *et al*., 2009).

Studies of the resistance to Cu in seven isolates of PSA allowed the identification of the *czc* / *cusABC* and *copABCD* systems, which were acquired through the incorporation of integrative elements (Colombi *et al*., 2017). However, in an older, Nakajima *et al.* (2002) demonstrated that the copper resistant strains of PST have copper resistance genes similar to those of PSA named *copA*, *copB*, *copC*, and *copD*. The *copA* and *copB* are responsible for homeostasis and the *copC* and *copD* are important to the strength of copper resistance. *CopB* and *copD* encode protein that are located in the inner and outer membranes while *copA* and *copC* encode periplasmic proteins (Jeyakumar *et al*., 2014). Furthermore, Nakajima *et al*. (2002) demonstrated that prolonged use of the Cu-based compounds developed the acquisition of *copR* and *copS* genes that were responsible for maximal Cu resistance in PSA and PST.

1.6.2. Current strategies of biological control

Several species of microorganisms co-inhabit and interact with each other in different habitats. In these, they compete for nutrients producing secondary metabolites that inhibit the growth of their antagonists. In recent years, the study of these interactions has led to the identification of many potential agents for biological control as an alternative to chemical control (Stark *et al*., 2018). Biological pesticides, or biopesticides, represent a wide range of bio-based substances that have different mechanisms of action against a wide variety of pests. As defined by the United States Environmental Protection Agency (EPA), biopesticides are a type of pesticide derived from natural materials such as animals, plants, microorganisms, and certain minerals (Kachhawa, 2017).

Formulations based on microorganisms can control pests through the production of antibiotics, bacteriocins, siderophores, hydrolytic enzymes and other secondary metabolites (Kachhawa, 2017). Recently, the natural pathogen *B. amyloliquefaciens* D747, commercially available as Bacstar ™, was discovered as having a high biocontrol potential on PSA5 when applied as an aerosol (Biondi *et al*., 2012). In addition, there is evidence that the species *B. subtilis*, *Pseudomonas fluorescens* and *Pantoea agglomerans* also efficiently prevent the infection caused by PSA (Donati *et al*., 2014). Furthermore, the lipopeptides produced by *B. subtilis* are involved in stimulating the plant's defence response (Ongena *et al*., 2007). An example of other microorganisms that not only may induce systemic resistance in plants and release antimicrobial compounds but also improve nutrient uptake and increase productivity is *Trichoderma*

spp. (Stark *et al*., 2018). These strains are used in the new product KiwiWax™ which is composed of three *Trichoderma* spp. (*T. atroviride* LU668, *T. atroviride* and *T. virens* LU753) (Agrimm, 2020).

1.6.3. Emerging strategies of biological control

1.6.3.1. The promise of antimicrobial peptides as green compounds

Antimicrobial peptides (AMPs) are short peptide sequences that are evolutionarily conserved and are part of the innate immune system of eukaryotic organisms like plants and animals. These function as the first line of defence against different types of pathogenic organisms, including fungi, bacteria, and parasites. In general, these have a positive charge and are amphipathic in nature (Cameron & Sarojini, 2014a).

Taking into account the composition of the lipid membrane of Gram-negative bacteria [phospholipids and lipopolysaccharides (LPS)] and Gram-positive bacteria (teichoic acid and teichuronic acid) that have a negative charge, allowing the establishment of electrostatic interactions with the cationic AMPs (Cameron & Sarojini, 2014a; Mahlapuu *et al*., 2016; Park *et al*., 2019). AMPs not only alter the physical integrity of the membrane, but also interfere with the synthesis of cell wall components and the transcription, interact with ATP and inhibit the action of certain ATP-dependent enzymes and other cellular processes (Mahlapuu *et al*., 2016; Sinha & Shukla, 2019).

Nowadays, the use of synthetic biology allowed to improve the efficiency of AMPs such as in the antimicrobial activity, the toxicity against non-target organisms and the stability in the environment, which made it possible to create a library of synthetic peptides named CECMEL11 library. Different synthetic peptides are present in this library, for example, the BP100 that is based on a cecropin A-melittin hybrid (Torcato *et al*., 2013) and has strong antibacterial effect against phytopathogenic bacteria and the BP15 and BP12 that show fungicidal activity (Montesinos *et al*., 2017).

The selective action of these peptides against pathogenic microorganisms is based on differences in the composition of the microbial and mammalian membranes (Mahlapuu *et al*., 2016). This selectivity makes them good candidates for replacing chemical pesticides (Sinha & Shukla, 2019). Currently, there are reports on the effectiveness of AMPs against phytopathogenic bacteria. Studies by Cameron *et al*. (2014b) demonstrated the antimicrobial activity of the peptides, structurally modified dodecapeptide amides and two analogues of a hexapeptide amide, against PSA and *E. amylovora*. Additionally, several studies demonstrated strong antibacterial activity of the several AMPs and their analogues like BP100 (Badosa *et al*., 2007; Torcato *et al*., 2013; Park *et al*., 2019) and 3.1 (Kang *et al*., 2009; Gomes *et al*., 2019).

1.6.3.2. The Portuguese coast as source of novel antimicrobial compounds

The increase in the number of multidrug-resistant strains to Cu-based pesticides and antibiotics and their dissemination across the planet are emerging problems that make valueless the control measures currently available.

New antimicrobial molecules are often found in under-explored environments, such as marine habitats. Many marine animals have already proven to be reservoirs for such molecules for example marine sponges, like *Erylus* spp. (Graça *et al*., 2013; Graça *et al*., 2015) and *Theonella swinhoei* (Kuo *et al*., 2019). Yet, many of these molecules are produced by their microbial community (Graça *et al*., 2015).

The marine-sponge associated microorganisms may produce different types of bioactive molecules with antibacterial, antiviral, antifungal, anthelmintic, antiprotozoal, anti-inflammatory, immunosuppressive, antitumor and neurosuppressive properties (Laport *et al*., 2009). Taylor *et al.* (2007) argues that sponges have a diverse microbiological community that includes microalgae, fungi, archaea, and representatives from several bacterial phyla. In recent years, different microorganisms have been isolated in pure culture from marine sponges, and the molecular analysis based on the phylogenetic analysis of the 16S rRNA gene, allowed to identify several bacterial phyla such as *Actinobacteria*, *Planctomycetes*, *Firmicutes*, and *Proteobacteria* (Graça *et al*., 2013; Kuo *et al*., 2019; Santos *et al*., 2020).

Due to the high adaptability and metabolic versatility of the bacteria belonging to the phylum *Planctomycetes* and the phylum *Actinobacteria,* they can be found in a wide range of habitats, including marine environments as mentioned above. As such, they have an interesting diversity of metabolic pathways, large genomes and complex life cycle that make these great candidates for the search of new bioactive molecules (Graça *et al.,* 2016; Santos *et al.*, 2019).

Planctomycetes: *Planctomycetes* are Gram-negative bacteria with distinctive and unique structural, morphological, metabolic, and genomic characteristics. Taxonomically members of this phylum are nearly related to strains of other bacterial phyla, so they belong to the *Planctomycetes*, *Verrucomicrobia*, *Chlamydiae* (PVC) superphylum which include other bacterial phyla such as *Lentisphaerae, Kirimatiellaeota* and *Candidatus* Omni-trophica (Fuerst, 2017; Lage *et al*., 2019). Furthermore, the phylum *Planctomycetes* has two classes: *Planctomycetia* and *Phycisphaerae* (Wiegand *et al*., 2018).

Members of the *Planctomycetes* have relatively large genomes with 50-75 mol % guanine and cytosine ratio in their condensed DNA and a short 5S rRNA (Lage *et al*.,

2019). Furthermore, many genes that are present in the plantomycetal genome have unknown function, for example, two members, *Zavarzinella formosa* and *Fimbriiglobus ruber*, have, respectively, 67.2 % and 68.9 % genes with unknown function. In addition, recent studies have demonstrated the presence of several secondary metabolite-related genes, namely giant genes (> 5 kb) that may encode structural proteins like non-ribosomal peptide synthetase (NRPS) or polyketide synthase (PKS) (Wiegand *et al*., 2018). Recently, Graça *et al*. (2016) proved the presence of both secondary bioactive genes in *Planctomycetes* isolated from macroalgae.

Some *Planctomycetes* divide by binary fission and some of the members may reproduce through polar budding. They are slow growing bacteria (doubling time range from 6 hours to 1 month) (Lage *et al*., 2019). Another interesting feature of this phylum is the dynamic endomembrane system. Further, their cell plan is significantly different from the typical Gram-negative and Gram-positive bacteria (Boedeker *et al*., 2017). Morphologically, *Planctomycetes* form white, orange, pink or red pigmented colonies (Lage *et al*., 2019). Ecologically, they are present in different ecosystems, including terrestrial, aquatic or extreme habitats and can be found in association with other organisms, presenting a wide variety of metabolic pathways and are able to use various organic and inorganic compounds as an energy source (Youssef & Elshahed, 2014). The *Planctomycetes* present novel type of resources, for example, enzymes and bioactive compounds with potential uses in industry and the unique ability to oxidize ammonium anaerobically (Fuerst, 2017).

***Actinobacteria*:** The phylum *Actinobacteria* is one of the largest phyla present in the domain *Bacteria*, containing six classes, 5 subclasses, 6 orders, and 14 suborders (Barka *et al.*, 2016). *Actinobacteria* are Gram-positive bacteria with high guanine and cytosine ratio in their DNA (Lawson, 2018). Additionally, they can reproduce by binary fission or by producing spores or conidia and morphologically, they present enormous range of morphologies (coccoid, rod-coccoid, hyphal and branched forms) (Anandan *et al*., 2016; Lawson, 2018). Another interesting feature of this phylum is its ubiquitous distribution. Different strains could be isolated in habitats, including aquatic and terrestrial environments; they may be found in the human body where they are part of a normal microbiota and also can be plant-associated commensals and nitrogen-fixing symbionts (Lawson, 2018). Furthermore, most *Actinobacteria* are chemoheterotrophic, but they can be chemoautotrophic, using a wide variety of nutritional sources (Barka *et al*., 2016).
Members of this phylum are also able to produce a variety of bioactive metabolites and enzymes with high commercial and pharmacological interest, such as antibiotic compounds, probiotics, biosurfactants, vitamins and pigments (Anandan *et al*., 2016;

Lawson, 2018). Notably *Actinomycetales* and, in particular *Streptomyces* are the major source of antibiotics. However, with the increase of unprecedent levels of antibiotic resistance in pathogens, the necessity to identify novel strategies to combat microbial infections resulted in an increased interest in identifying potential microorganisms that produce new natural bioactive compounds (Lawson, 2018). A wide range of bioactive compounds are synthesized by NRPSs and PKSs as mentioned above. Furthermore, antimicrobial activity was detected in culturable actinomycetes isolated from the tissues of the scleractinian corals *Porites lutea*, *Galaxea fascicularis* and *Acropora milepora*. In addition, it has been demonstrated that, some members of this phylum encode multiple NRPS and PKS genes (Li *et al*., 2014; Santos *et al.*, 2019).

1.7. Objectives

This Master's thesis has two main objectives. The first is the biochemical and morpho-physiological characterization of phytopathogenic *Pseudomonas* strains, including PSA isolates. Their characterization will make it possible to study the differences between the isolates and also to analyse some of the virulence factors like mobility, biofilm formation and hypersensitive reaction (HR). The second objective is to assess the susceptibility of phytopathogenic organisms of the genus *Pseudomonas* to new compounds, namely selected AMPs and bacterial antagonists. This will allow not only to understand the mechanisms of action of these potential means of control, but also to disclose new bioactive compounds against *Pseudomonad* pathogens of kiwifruit.

Besides contributing to better morphologically and biochemically characterise the Portuguese PSA population, achieving these objectives will allow answering three biological questions: 1) Will the bacteria *Pseudomonas* spp. present high susceptibility to Cu-based agrochemicals? 2) What alternative control measures can be good candidates in the control of bacterial canker disease in kiwifruit? 3) How do new antibacterial compounds such as AMPs and other possible antagonists work?

2. Materials and Methods:

2.1. Bacterial strains of *Pseudomonas* spp. and growth condition

Based on previous biochemical and physiological characterization performed by Moura *et al*. (2015), eight phytopathogenic bacteria of the genus *Pseudomonas* were selected for this study (Table 1), one PSA reference strain (CFBP 7286), one *P. viridiflava* strain (CFBP 2107), one *P. cerasi* (B65) and one PSS (DSM 10604). Also, four PSA3 isolates obtained by Moura *et al*. (2015) from symptomatic leaves of *A. deliciosa* cultivar on kiwifruit orchards in Entre Douro e Minho region (north-west Portugal) in 2013, were selected for this study. Furthermore, previous molecular characterization demonstrated that the national isolates belong to PSA3 that was identified in Europe, New Zealand and Chile (Moura *et al*., 2015; Garcia *et al*., 2018).

Table 1 - Selected phytopathogenic bacteria strains used in this study.

Strains	Country/year
P. viridiflava (CFBP 2107)	Switzerland, 1927
P. syringae pv. *syringae* (DSM 10604)	United Kingdom, 1996
P. cerasi (B65)	Portugal, Braga, 2013
P. syringae pv. *actinidiae* (CFBP 7286) [Pt]	Italy, 2009
P. syringae pv. *actinidiae* (P84)	Portugal, Prado, 2013
P. syringae pv. *actinidiae* (P85)	Portugal, Prado, 2013
P. syringae pv. *actinidiae* (AL13)	Portugal, Amares, Lago, 2013
P. syringae pv. *actinidiae* (Am63)	Portugal, Amarante, 2013

[Pt] Pathovar reference strain.

The selected strains used in this study were all grown/maintained in petri dishes containing King's B medium (KB) (Annex I - 1) except when another medium is specified. The bacteria were streaked out from a – 80 °C stock onto KB plates and incubated at 25 ºC in the dark.

2.2. Phenotypic and virulence characterization of phytopathogenic strains of the genus *Pseudomonas*

- Cytochrome oxidase activity: a strip of filter paper is soaked with a freshly made 1 % Kovac's oxidase reagent (N, N, N', N'-Tetramethyl-1,4-phenylenediammonium dichloride, Merc) and a speck of bacterial culture was rubbed onto the filter. The colour change was observed after 10 seconds of incubation. *P. fluorescens* was used as positive control and PSA3 (CFBP 7286) as negative control.

- Catalase assay: catalase activity was evaluated by mixing a small amount of bacterial colony with a drop of H_2O_2 (3 %) in a glass slide. It was considered a positive result when occurred an immediate air bubble formation. PSS (DSM 10604) was used as positive control.

- Indole acetic acid (IAA) production: production of indole acetic acid was quantified using Salkowski's method, according to Flores *et al*. (2018) and based on a previously obtained standard curve with IAA (Annex II). Bacterial strains (Table 1) were grown in Luria-Bertani (LB) (Difco™ LB Agar, Miller) liquid medium supplemented with tryptophan (2 g/L) at 25 ºC for 24 hours in the dark. All liquid cultures were cultivated at 190 rotations per minute (r.p.m). After incubation, the bacterial density was determined ($OD_{600\ nm}$) and the bacterial culture was centrifuged at 10.000 r.p.m. for 10 minutes. The resulting supernatant and pellet were separated. Afterwards, 0.5 mL of Salkowski's reagent (12 g of $FeCl_3$ per litter in 7.9 M H_2SO_4) were added to 4 mL of cell-free supernatants and incubated for 30 minutes in the dark. At the end of the incubation time, the absorbance ($OD_{530\ nm}$) was measured, and the concentration of IAA was calculated using a standard curve of indole acetic acid (0-500 µg/mL). The standard curve obtained presented a R^2 of 0.99 % and the following graph equation y = 0.0009x + 0.0035.

- Biofilm production: the protocol for microtiter plate biofilm production was performed according to Shao *et al*. (2019). Bacterial strains were grown overnight in LB medium. Overnight bacterial culture was adjusted to an optical density of 0.1 ($OD_{600\ nm}$) and 100 µL of each diluted culture were transferred to a well (96-well microtiter plates). The plates were incubated for 7 days at 25 ºC. At the end of the incubation time, violet crystal (0.1 %) was added for biofilm staining and then solubilized with acetic acid solution (30 %). The biofilm production was quantified by measuring the absorbance at 530 nm.

- Bacterial Motility Assay: bacterial motility assay was performed using a semi-solid LB medium (0.3 % of agar) according to Flores *et al*. (2018). Bacterial cultures were grown until stationary phase and adjusted to an optical density of 1.3 ($OD_{600\ nm}$), then 2 µL of each culture were inoculated into the centre of 0.3 % LB agar plates. The plates were incubated for 72 hours at 25 ºC. The zone sizes were record with ChemiDoc Imaging system (Bio-Rad™) and the area (mm^2) was measured using the ImageJ software.

- Hypersensitive reaction (HR): to investigate the capacity of strains to induce disease symptoms and to determine the hypersensitive reaction, tobacco leaves were inoculated using the method described previously by Stefani & Loreti (2014). Tobacco plants (*Nicotiana tabacum* cv. 'Havana') were grown in peat (SIRO GERMINAÇÃO BIO, Portugal) at 25 ºC and 50 % of relative humidity. Bacterial strains were grown in KB

medium at 25 ºC and then a fresh bacterial colony was resuspended in PBS (10 mM, pH 7.0) (see Annex I – 4) and optical density was adjusted to 0.135 ($OD_{600\ nm}$) (10^8 cells). After that, leaves of eight-week old tobacco plants were infiltrated with bacterial suspension (10^8 cells) of selected strains. The development of hypersensitivity response was registered within 24 and 48 hours post infiltration (hpi).

2.3. Copper susceptibility

The Cu susceptibility was determined by minimum inhibitory concentration (MIC), resorting to a 96-well microtiter plate. Bacterial strains were grown overnight in Tris-minimal medium (TMM) (Annex I - 2) and then their optical density was adjusted to 0.1 ($OD_{600\ nm}$). Aliquots (100 µL) of the diluted bacterial cultures were added to each well (96-well microtiter plate) where 100 µL of the TMM supplied with a range of copper sulphate (0 to 125 µg/mL of $CuSO_4$) was previously distributed. The plates were incubated for 24 hours at 25 ºC at 135 (r.p.m.) During the incubation period, the absorbance was hourly measured at $OD_{600\ nm}$ in the Multiskan GO plate reader (Thermo Scientific, USA). Additionally, after the incubation period, 10 µL of the culture in each well were inoculated in copper-free KB medium to determine the minimum bactericidal concentration (MBC). Chlortetracycline (50 µM) was used as positive control and deionized water as negative control. MIC was expressed as the concentration of copper sulphate, at which inhibition of growth was visibly evident after 24 hours of incubation at 25 ºC. The maximum specific growth rate (µmax) and half maximal inhibitory concentration (IC_{50}) were determined from 24 hours growth curves using GraphPad Prism 8 software.

2.4. AMPs susceptibility

- Peptide synthesis: AMPs BP100 and 3.1 (≥ 98 % purity) used in this study were synthesized by solid peptide phase synthesis (SPPS) protocol following Gomes *et al*. (2019) and provided by Doctor Prof. Paula Gomes from Laboratório Associado para a Química Verde da Rede de Química e Tecnologia (LAQV-REQUIMTE). Amino acid sequences and molecular weights are presented in Table 2.

Table 2 - Amino acid sequence and molecular weight of the BP100 and 3.1 peptides.

Peptide	Amino acid sequence	MW(Da)	Purity (%)
BP100	KKLFKKILKYL-NH_2	1419.9	99
3.1	KKLLKWLLKLL-NH_2	1393.9	98

- Antibiogram for antimicrobial peptides (AMPs): preliminary assessment of bacterial susceptibility to AMPs was performed using the antibiogram method. The concentrations of the AMPs used for this assay were 0.4, 1.6, 6.2, 25, 50, 100, 150 and 200 µM. Bacterial strains were grown in Mueller Hinton II Broth (MH) (Liofilchem® s.r.l.) at 25 ºC for 24 hours at 190 (r.p.m.) in the dark. After the incubation period, the bacterial cultures were adjusted to an optical density of 0.135 ($OD_{600\ nm}$) and swabbed uniformly across a petri plate with MH medium. Then, 1 µL droplets of each AMPs were plated. The plates were then incubated at 25 °C for 24 hours and the results were interpreted with (+) for halo formation and (-) for no effect observed.

- Determination of minimal bactericidal concentration and IC_{50}: the susceptibility to AMPs was determined using the same 96-well microtiter plate method that was used to evaluate copper susceptibility. Bacterial strains were grown in double concentrated MH medium (Liofilchem® s.r.l.) at 28 ºC and 135 (r.p.m.) overnight in the dark. Then, bacterial cultures were adjusted to 0.1 $OD_{600\ nm}$ and were incubated for 24 hours with nine concentrations of the AMPs (0.25, 0.75, 1.6, 3.4, 6.2, 10, 25, 50 and 100 µM). Chlortetracycline (50 µM) was used as positive control and deionized water as negative control. After the incubation period, the minimal bactericidal concentration (MBC), IC_{50} and growth curves were obtained, as previously described in 2.3. MIC was expressed as the concentration of AMP, at which inhibition of growth was visibly evident after 24 hours of incubation at 25 ºC.

- Flow cytometry: flow cytometry was used to analyse the capacity of BP100 and 3.1 to permeabilize bacterial membranes of *Pseudomonas* spp. (DSM 10604, CFBP 2107, CFBP 7286 and B65) and to determine the cell viability after exposure to AMPs. Propidium iodide (PI) was used to label the bacterial cells with compromised cell membranes. Strains were grown overnight in KB medium at 25 ºC and 135 (r.p.m.) in the dark. Then, the bacterial cultures were centrifuged at 2500 r.p.m. for 5 minutes and the supernatant was resuspended in PBS (10 mM, pH 7.0) (see Annex I – 4). $OD_{600\ nm}$ was adjusted to 0.1 for each strain, and then they were incubated with 1, 2 and 6.2 µM of each peptide for 1 hour. After the incubation period, 0.5 µL of PI solution (1 mg/mL) were added per 50 µL of bacterial solution. Isopropyl alcohol 23 % (Kennedy *et al*., 2011; Freire *et al*., 2015; Wu *et al*., 2016) was used as positive control and deionized water as negative control. Fluorescence intensities were recorded at t_0, $t_{10\ min}$, $t_{20\ min}$, $t_{60\ min}$ in a DB Acuri™ C6 Plus Flow Cytometer and 20000 events were obtained for each analysis. The wavelength of red fluorescence was 585/40 nm, 670 LP filter for the measurement of PI and were analysed in FL3 detector. The fluorescence intensity of PI above $1x10^3$ was considered as the non-viable cells.

- Hypersensitive reaction (HR): the efficacy of BP100 and 3.1 were analysed in the inhibition of virulence of four *Pseudomonas* spp. (DSM 10604, CFBP 2107, CFBP 7286 and B65) in tobacco plants (*Nicotiana tabacum* cv. 'Havana'). Bacterial strains were grown in KB medium at 25 ºC and 135 r.p.m. Then, bacterial cells were adjusted to 0.1 $OD_{600\ nm}$ and were incubated with 1, 2 and 6.2 µM of each AMP at 25 ºC and 135 r.p.m. for 1 hour according to the flow cytometry assay. After the incubation period, tobacco leaves were infiltrated with the bacterial suspensions. The number of live bacteria cells were calculated and expressed in CFU/mL after and before incubation with AMPs. The development of HR was registered within 24 and 48 hours after infiltration.

2.5. Antimicrobial assays with strain extracts of *Planctomycetes* and *Actinobacteria*

2.5.1. Bacterial strains and selection criteria used

For this study, seven marine bacteria were isolated and characterized by researcher at the Interdisciplinary Centre of Marine and Environmental Research of the University of Porto (CIIMAR) (Table 3). Of the seven isolates, three of these are members of the phylum *Actinobacteria* and were obtained from the marine sponges of the genus *Erylus*, *E. discophorus* (Berg02, from the continental shelf at Berlengas, Portugal) and *E. deficiens* (#91, from the continental shelf at Gorringe, Portugal). Other four bacteria belong to the phylum *Planctomycetes* and were isolated from macroalgal biofilm, *Gracilaria bursa-pastoris* (GR-7, Aveiro, Portugal), *Laminaria* sp. (LF-2, Foz - Porto, Portugal), *Chondrus crispus* (LZ-C2, Praia da Luz, Porto) and also from sea water (LZ-A1, Praia da Luz, Porto) (Table 3).

Table 3 - Selected marine bacterial strains used in this study.

Strain	Affiliation	PKS / NRPS	Previous bioactivity	Reference
Berg02_22.2	*Gordonia* sp.	PKS-I	BS; AF; TC	Graça *et al*., 2013; Santos *et al*., 2019
Berg02_26	*Micrococcus luteus*	ND	AF; CA	Graça *et al*., 2013; Santos *et al*., 2019
#91_17	*Dermacoccus* sp.	NRPS	AF, CA, VA	Graça *et al*., 2015; Santos *et al*., 2019
LZ-C2	*Alienimonas chondri*	PKS-III	NS	Vitorino *et al*., 2020
LF-2	*Rhodopirellula rubra*	PKS-I	N/B	Graça *et al.,* 2016
GR-7	*Rubinisphaera brasiliensis*	PKS-I	CA; BS	Graça *et al.,* 2016
LZ-A1	*Novipirellula caenicola*	NS	NS	--

AF – *Aspergillus fumigatus;* BS - *Bacillus sublilis*; CA - *Candida albicans*; N/B - not bioactive; NS – not studied; ND – not detected; TC - *Trypanosoma cruzi;* VA- *Vibrio anguillarum;*

The selected targets described above were cultivated and maintained in petri dishes with different media. Strains from *Actinobacteria* phylum were grown in Marine Broth (MB) (Annex I – 3) and strains from *Planctomycetes* phylum were cultivated in M607 medium (Annex I – 3) at 25 ºC in darkness.

2.5.2. Contact bioactivity test

Different types of microorganisms compete for space and nutrients through the production of a wide range of bioactive compounds. To determine the potential for the production of bioactive molecules by the selected strains, direct interaction between strains (Table 1) and targets (DSM 10604, CFBP 2107, CFBP 7286 and B65) was executed. *Pseudomonas* spp. were grown overnight in KB medium and strains from the phylum *Actinobacteria* were cultivated in MB medium for 5 days. Liquid cultures of each bacterium were incubated at 190 (r.p.m) at 25 ºC.

After the incubation period, the pure bacterial concentration of culture of *Pseudomonas* spp. was adjusted to 0.1 ($OD_{600\ nm}$) and swabbed uniformly across a petri plate with KB medium. Then, aliquots (10 µL) of active target bacteria (*Actinobacteria*) were added to each plate. Chlortetracycline at 200 µg/mL was used as positive control and deionized water as negative control. Afterwards, the plates were incubated at 25 ºC in darkness and the inhibition zone were record with ChemiDoc Imaging system (Bio-Rad™) and the area (mm) were measured using the ImageJ software after 1, 7, 14 and 21 days of incubation.

2.5.3. Extraction protocol used in this study

Growth medium: for the extraction protocols, different types of culture media were used to ferment the bacterial strains. Actively growing *Planctomycetes* strains were initially pre-cultured in M607 medium for 7 days and *Actinobacteria* in MB medium for 5 days, at 25 ºC and 200 r.p.m. in the dark. A medium-scale culture extraction was performed. *Planctomycetes* were grown in M600 medium. Each planctomycete was cultivated for 7 days, at 25 ºC and 200 r.p.m. in the dark. However, based on previous studies elaborated by Santos *et al*. (2019), only three *Actinobacteria* (#91_17; Berg02_26 and Berg02_22.2) and three types of culture media were selected for these extraction protocols (Table 4).

Table 4 - Culture medium to grow selected strains.

Strain ID	Culture medium
#91_17	CGY[1]
Berg02_26	AB[1]
Berg02_22.2	IN-CRY[1]

[1] Santos, *et al*. 2019.

Extraction protocol using ethyl-acetate (liquid-liquid phase extraction): the extraction protocol using ethyl-acetate was based on the previously described by Santos *et al*. (2019). For extraction with ethyl-acetate four strains from phylum *Planctomycetes* (GR-7, LF-2, LZ-C2 and LZ-A1) were selected. This extraction protocol was done in 200 mL of bacterial culture. Bacterial strains were initially grown in the respective media as mentioned above. After incubation, ethyl-acetate (CARLO ERBA Reagents) was added to each bacterial culture in a 1:1 ratio and the mixture was incubated for one hour at 25 ºC with 200 r.p.m. in the dark. At the end of the incubation period, the organic phase was separated from the aqueous phase in a separatory funnel. Then, three washes of the aqueous phase were performed, and the organic phase was collected. Next, the Rotavapor® R-100 (BUTCHI) was used to remove all ethyl-acetate with a rotatory vacuum evaporator at 25 ºC at 110 mbar. The residue in the balloon was dissolved in a 10 mL of dichloromethane (VWR Chemicals). The vials were stored at 4 ºC for further use.

AmberliteTM XAD16N resin extraction with acetone (solid phase extraction): this protocol was also based on Santos *et al*. (2019). Pure cultures of selected strains (Table 3) were cultivated in 100 mL of their respective media at 25 ºC and 200 r.p.m. in the dark. After the incubation period, 100 mL of acetone (LabChem) were added to each bacterial culture and incubated for one hour at 200 r.p.m. Next, acetone was completely removed from the mixture in a Rotavapor® R-100 (BUTCHI). Then, 10 g of Amberlite™ XAD 16N

(Merk) resin were added and incubated for two hours at 200 r.p.m. At the end of incubation period the resin was collected by filtration and cleaned two times with MilliQ water. Next, 50 mL of acetone were added to the resin and incubated for one hour at 200 r.p.m. After the acetone was collected to a rotary evaporator balloon, it was dried in the rotary evaporator. The resulting extracts were lyophilized and then were stored at 4 ºC before further use.

2.5.4. Well assay using bacterial extracts

The previously obtained extracts were diluted in 20 % dimethyl sulfoxide (DMSO) in a 100 mg/mL concentration. The activity of all extracts was evaluated by a well assay using 96-well plate (Graça *et al.*, 2013). For this assay phytopathogenic strains CFBP 7286, B65, CFBP 2107 and DSM 10604 were selected. Overnight cultures were standardized to 5×10^4 cells and 90 µL aliquots were placed in wells (96-well plates) and completed with 10 µL of each extract. Chlortetracycline (2.5 µg/mL) was used as positive control and deionized water as negative control. The plates were incubated at 25 ºC at 190 r.p.m. in darkness. The absorbances were measured at $OD_{600\ nm}$ in the Multiskan GO plate reader (Thermo Scientific, USA) before and after incubation period (24 hours). Activity of bacterial extract was considered for values equal or above 50 % as determined by the following formulae:

$$OD_E = Abs_{\ time24} - Abs_{\ time0}$$

$$\%\ \text{inhibition for each extract} = 100 - [(OD_E / OD_C) \times 100]$$

OD_E - Optical density of each extract; OD_C - Mean of optical density of negative control.

2.6. Statistical analysis

Statistical analysis of data was performed using GraphPad Prism 8 software. All obtained data were analysed using ordinary one-way ANOVA with $p < 0.05$. All experiments were performed in biological and technical triplicates. Values of $p < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1. Phenotypic and virulence characterization of phytopathogenic strains of the genus *Pseudomonas*

Isolates of the PSA3 were collected in several major kiwifruit production areas. PSA provokes the disease canker of kiwifruit responsible for enormous economical loss in the kiwifruit production sector (Donati *et al*., 2020). Like other phytopathogen of the *Pseudomonas* genus, PSA has developed several mechanisms to overcome multiple barriers of plant defence. For example, virulence factors, like production of IAA, motility, and biofilm production, are extremely important to cause pathogenic effects to the host plant. In this study, the pathogenicity of *Pseudomonas* spp. strains was characterized (Table 1). One of the initial cellular responses against phytopathogens is the production of reactive oxygen species (ROS). In order to survive, different bacterial species produce the enzyme catalase that is a kay enzyme in protecting bacterial cell from oxidative damage. Thus, all selected strains were tested for catalase and cytochrome C oxidase activities. Results showed that, all bacteria were positive for catalase activity and negative for cytochrome activity (Table 5). These characteristics are in conformity with other studies, where similar phenotypes were found in PSA isolates (Garcia *et al*., 2018; Vasebi *et al*., 2019).

Table 5 - Cytochrome oxidase and catalase assay results.

Strains	Catalase	Cytochrome C Oxidase
P. viridiflava (CFBP 2107)	+	-
P. syringae pv. *syringae* (DSM 10604)	+	-
P. cerasi (B65)	+	-
P. syringae pv. actinidiae (CFBP 7286)	+	-
P. syringae pv. actinidiae (P84)	+	-
P. syringae pv. actinidiae (P85)	+	-
P. syringae pv. actinidiae (AL13)	+	-
P. syringae pv. actinidiae (Am63)	+	-
P. fluorescens	NT	+

(+) Presence of the enzyme; (-) Absence of the enzyme, NT – not tested

The motility and biofilm production promote an increased chance to colonize plant tissue and to survive under unfavourable environmental conditions (Ghods *et al*., 2015). Therefore, the ability of *Pseudomonas* spp. to form biofilm, and their motility was evaluated. All the strains used in this study were not able to produce biofilm in 96-well plates. These results are in accordance to Flores *et al*. (2018) who found that Chilean

PSA isolates did not form biofilm in abiotic surfaces. Also, similar results were obtained by Ueda & Saneoka (2015). These authors demonstrated that *P. syringae* does not form biofilm in 96-wll plates with LB medium. However, it was reported that PSA strains can form biofilm on the external and internal surfaces of plants (Renzi *et al*., 2012; Ghods *et al*., 2015). Additionally, the bacterial motility was also analysed in all *Pseudomonas* spp. under study. The results of the motility assay showed that all strains exhibited motility (Figure 5), which agrees with the profile reported for Chilean PSA (Flores *et al*., 2018). The isolates AL13 and Am63 demonstrated a similar level of motility as the reference pathovar strain CFBP 7286 and the strain *P. viridiflava* CFBP 2107. Other two PSA isolates P84 and P85 demonstrated lower motility similar to PSS strain DSM 10604. In contrast, isolate B65 showed higher motility ($p < 0.05$, Figure 5).

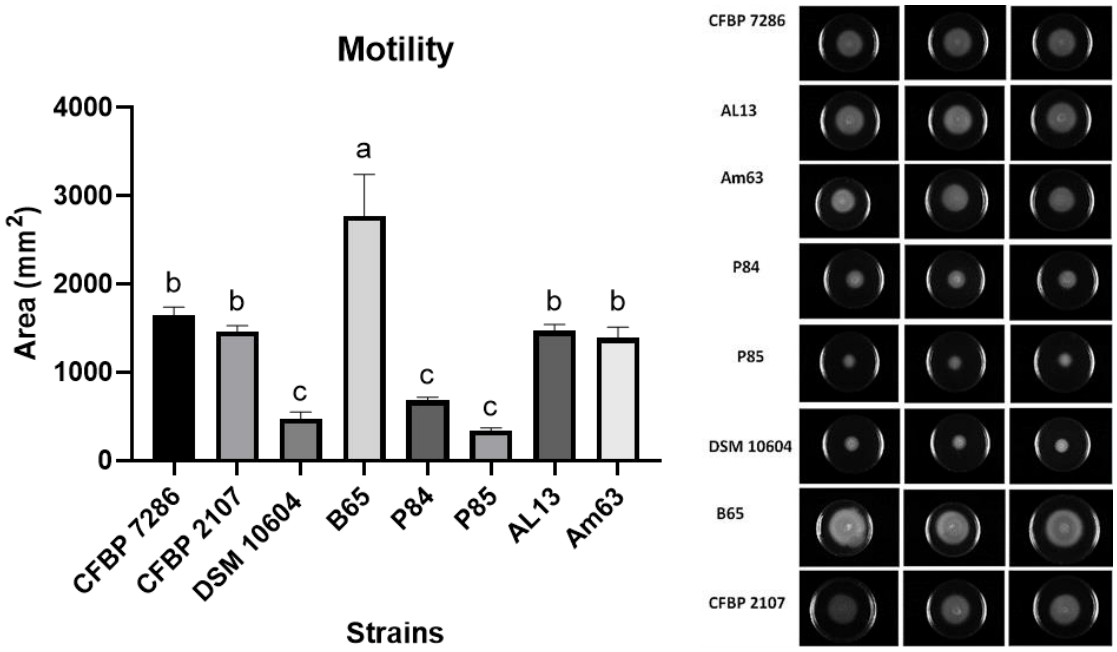

Figure 5 - Quantification of *Pseudomonas* spp. motility used in this study. Different letters mean statistical significance for $p < 0.05$ between strains (one-way Anova).

Phytopathogenic bacteria have several virulent factors and invasive strategies, some of which had been described above. These strategies facilitate their colonization, survival in the intercellular space, growth and division in the host plant. Several plant pathogens are able to produce a wide range of compounds that manipulate physiology of the plant, and suppress its immune system (Kunkel & Harper, 2018). For example, Djami-Tchatchou *et al*. (2020) demonstrated that elevated auxin levels suppress salicylic acid (SA)-mediated host defences during *P. syringae* (PtoDC3000) infection.

As *P. syringae* strains can synthesize plant hormones like auxin (Flores *et al*., 2018), it was decided, in the current study, to evaluate the ability of selected strains to produce IAA. The results show that all isolates produced indole in different concentrations, using tryptophan as a precursor in the IAA synthesis pathways (Figure 6).

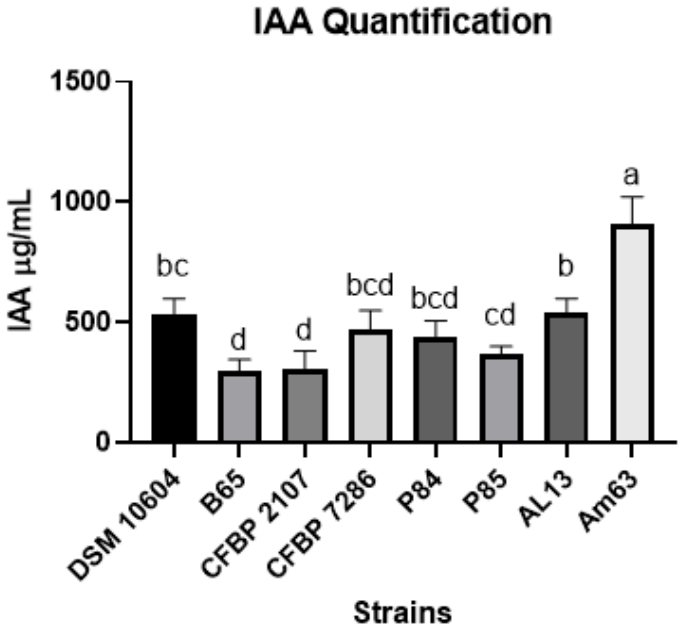

Figure 6 - Quantification of the IAA production of different phytopathogenic *Pseudomonas* spp., *P. viridiflava* CFBP 2107 was used as positive control. Standard curve of IAA (see Annex II) was used for the quantification of IAA (µg/mL). Different letters mean statistical significance for $p < 0.05$ between strains (one-way Anova).

As reported by Glickmann *et al*. (1998), *P. syringae* strains produce IAA particularly when grown in a medium with tryptophan. Isolate Am63 showed higher amount of IAA (909.8 µg/mL), while strains B65 and CFBP 2107 produced lower amounts of IAA compared to isolate Am63 ($p < 0.05$, Figure 6). The other strains showed similar ability to produce IAA with concentration ranging from 297.9 to 535.2 µg/mL, with no significant differences ($p > 0.05$, Figure 6). Interestingly, in the study performed by Flores *et al*. (2018), in which the same quantification method was used, the Chilean PSA isolates produced lower IAA concentrations. These differences in IAA production are probably related to the associated metabolic pathways, bacterial growth, and production conditions. Our results of IAA production are in agreement with some recent studies that have described the capacity to produce IAA by several *P. syringae* pathovars like *P. savastanoi* pv. *phaseolicola*, *P. syringae* pv. *tabaci*, PST (Duca *et al*., 2014) and PSA (Flores *et al*., 2018).

Phytopathogenic bacteria causes disease in host plants by penetrating into the tissues through natural or artificial openings as mentioned above. Tobacco HR assay is an easy and appropriate way to analyse bacterial pathogenicity. Bacterial suspensions were infiltered through stomata. It was noticed that all tested strains induced symptoms in tobacco plants as illustrated in Figure 7.

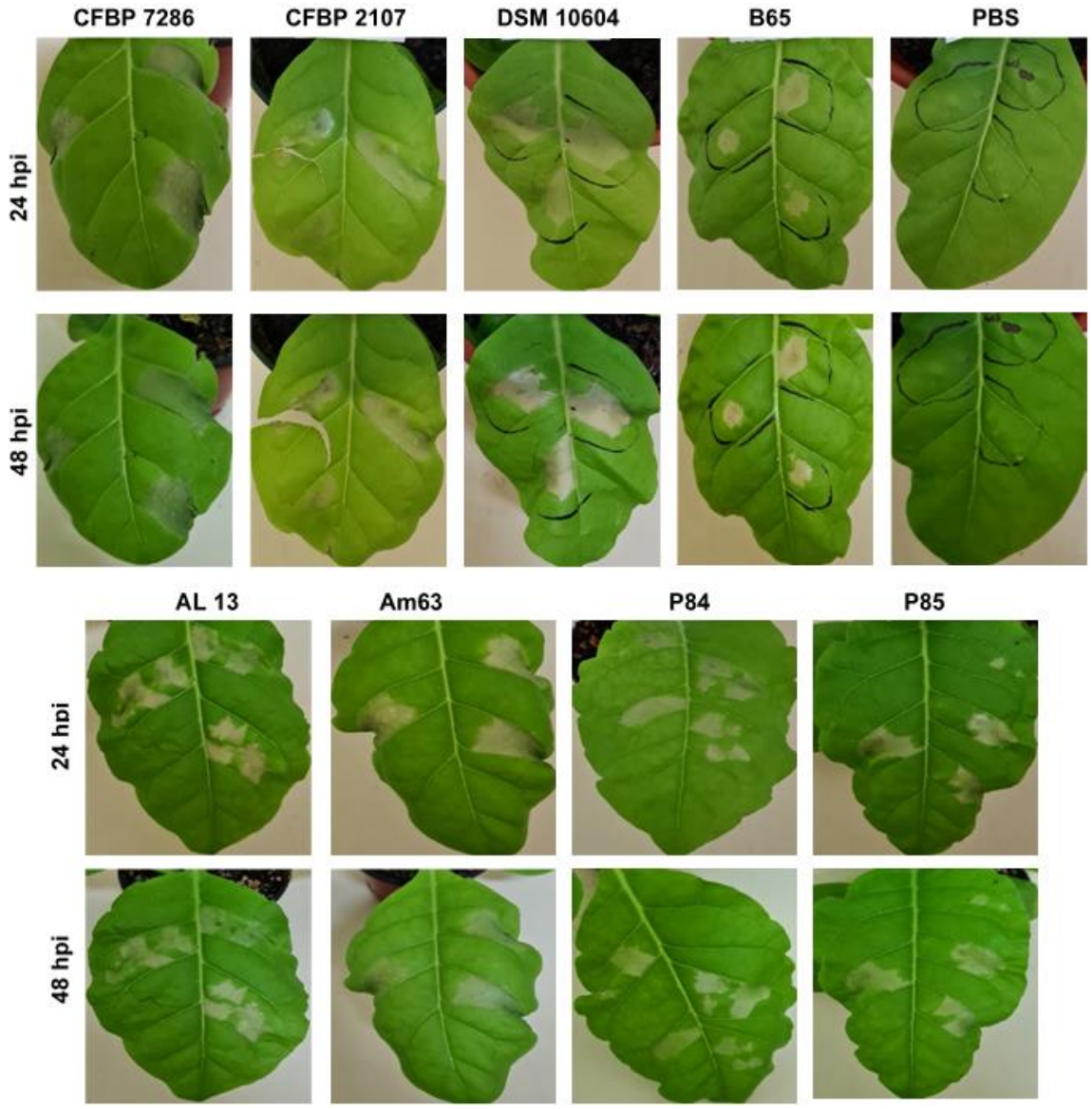

Figure 7 - Hypersensitive response induced by phytopathogenic *Pseudomonas* spp. strains. DSM 10604 and CFBP 7286 were used as positive control and PBS was used as a negative control. For each condition, three different tobacco plants were used as replicates. The development of HR was registered within 24 and 48 hours post-inoculation (hpi).

Visual observations clearly revealed differences between strains. The foliar symptoms caused by the reference strain CFBP 7286 were stronger than by the national PSA isolates AL13, AM63, P84 and P85. Moreover, differences between portuguese isolates were observed. These results are probably related to the virulence factors described

above like production of IAA and motility. For example, the isolate Am63 produces more IAA than other PSA isolates and induces more pronounced symptoms similar to those of the reference PSA strain (Figure 7). The hypersensitive response in tobacco plants was previously described in Chilean PSA isolates by Parada *et al*. (2017). Additionally, similar symptoms on tobacco leaves were caused by other *Pseudomonas* strains such as *P. syringae* pv. *lachrymans* (Shila *et al*., 2013), *P. viridiflava* and PST (Sarris *et al*., 2012).

Phytopathogenic bacteria present several unique virulence strategies to successfully infect host plant. According to our results, all strains were catalase positive, oxidase negative and IAA producers and, also, present motility and ability to induce tobacco leaves. Some studies have already reported the presence of this phenotype in different phytopathogenic bacteria including PSA (Flores *et al*., 2018; Garcia *et al*., 2018; Vasebi *et al*., 2019; Djami-Tchatchou *et al*., 2020). First, as mentioned earlier, the environment outside of the plant is extremely unfavourable for phytopathogenic bacteria (Ghods *et al*., 2015; Farias et al., 2019). So, bacteria developed several mechanisms such as biofilm formation, which will allow them to survive outside the plant and flagella-driven motility, which favours the entry of bacteria into the host plant. through natural or artificial plant openings (Kim *et al*., 2017a). Also, it was demonstrated that flagella-motile-defective and different extracellular matrix components mutants have reduced pathogen virulence (Ichinose *et al*., 2016; Farias *et al.*, 2019). Then, to cause an infection, the bacteria must has to overcome the defence mechanisms of the plant's innate immune system and enter into the apoplast (Pfeilmeier *et al*., 2016). In this case, the ability to produce the enzyme catalase and to produce IAA play a very important role in defend and supress the plant's innate immune system, respectively (Pfeilmeier *et al*., 2016; Djami-Tchatchou *et al*., 2020). Regarding biochemical characterization, B65 isolate revealed high appetency for environmental survivor, once it showed high motility level. Among PSA isolates from Portugal, P84 and P85 demonstrated as less appetency for infection and virulence compared to AL13 and Am63 that showed higher levels of mobility and IAA. The study and analysis of these virulence factors will permit not only to understand the mechanisms of infection and progress of the disease, but also to refine and develop the control strategies against these pests.

3.2. Characterization of copper susceptibility

Nowadays, copper-based agrochemicals are recurrently used as one of the more effective control methods available against PSA. Frequent applications of these compounds have several disadvantages, such as, the increase of copper-resistant PSA

strains in kiwifruit orchards, as was reported by several studies (Lamichhane *et al*., 2018). Despite the emergence of Cu-resistant strains, the use of these agrochemicals persists in several kiwifruit production regions, to control PSA due to the absence of other efficient control measures. As expected, copper resistant PSA strains have been already reported by several studies (Aiello *et al.,* 2015; Colombi *et al*., 2017; Petriccione *et al*., 2017). Therefore, it is extremely important to evaluate the susceptibility of national PSA isolates to copper sulphate that has been used in kiwifruit orchards in Entre Douro e Minho region (north-west Portugal). All strains were grown in TMM medium supplied with a different concentration (0 to 125 µg/mL) of $CuSO_4$.

Therefore, the effect of different concentrations of copper on bacterial growth were analysed namely, the µmax was calculated from 24 hours of bacterial growth curves (see Annex III – 1, 2) and the results are shown in Figure 8. A gradual decrease in the maximum specific growth rate was observed with the increase of copper sulphate concentration revealing a negative effect of copper on bacterial growth. Complete growth inhibition was obtained for $CuSO_4$ concentrations higher than 50 µg/mL with the exception for strain DSM 10604 which showed residual growth in TMM medium supplied with 125 µg/mL $CuSO_4$ (Annex III - 1). Strain DSM 10604 showed medium sensitivity to 50 µg/mL $CuSO_4$ on growth rate reduction (Figure 8).

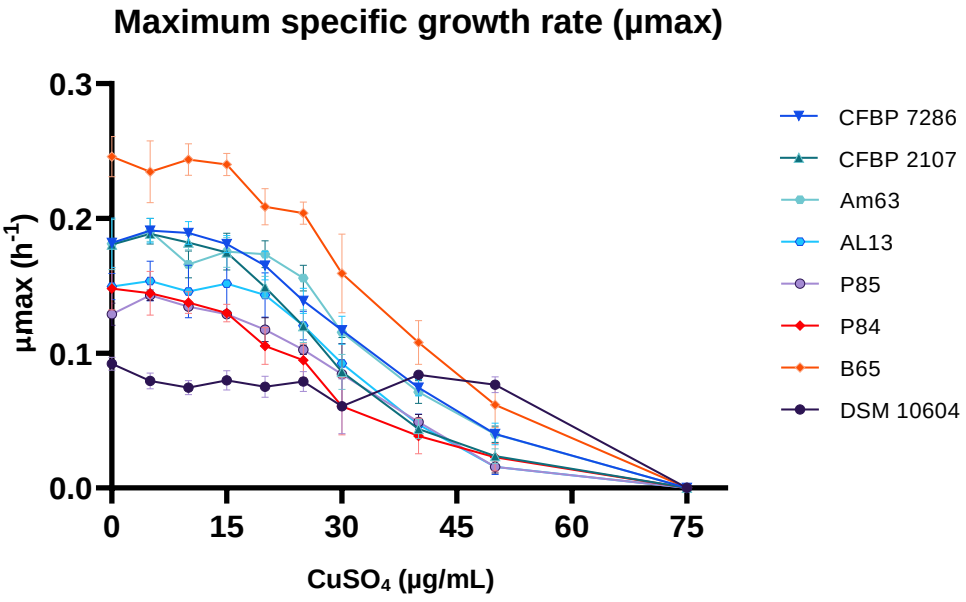

Figure 8 - Maximum specific growth rate (µmax) of eight phytopathogenic bacteria of the genus *Pseudomonas* exposed to increasing concentrations of copper sulphate obtained from 24 hours of bacterial growth curves (see Annex III - 2).

IC_{50} values ranging from 30.83 to 43.02 µg/mL were obtained from eight phytopathogenic bacteria of the genus *Pseudomonas,* as shown in Table 6. According to the results, strains CFBP 7286 and Am63 were less susceptible then other strains, since higher concentrations of the compound were needed to display 50 % inhibition of the microbial population, resulting in a higher IC_{50} value. Strain CFBP 2107 was more susceptible when compared to strains CFBP 7286 and Am63 ($p < 0.05$, Table 1).

Table 6 - Values of half inhibitory concentration (IC_{50}), minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) obtained from 24 hours of bacterial growth curves of *Pseudomonas* spp. strains (see Annex III – 1). Different letters mean statistical significance for $p < 0.05$ between strains (ordinary one-way Anova).

Strain	IC_{50} (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
DSM 10604	36.34 ± 2.810 *ab*	>125	>125
CFBP 2107	30.83 ± 2.585 *b*	50	>125
B65	34.48 ± 3.385 *ab*	50	>125
CFBP 7286	41.50 ± 2.435 *a*	50	75
P84	38.13 ± 3.075 *ab*	50	75
P85	34.03 ± 3.885 *ab*	50	75
AL13	39.18 ± 3.250 *ab*	50	>125
Am63	43.02 ± 2.685 *a*	50	>125

Interestingly, after inoculation into Cu-free medium, only strains CFBP 7286, P84 and P85 did not grow after exposure to 125 µg/mL which shows a bactericide activity of this concentration. These strains had less capacity to tolerate the highest copper concentration tested, presenting MBC value of 75 µg/mL (Table 6). These results could be explained by the differences between strains tested and a wide range of antibacterial mechanisms of copper like the interference with membrane integrity, protein and nucleic acids synthesis (Borkow, 2012; Vincent *et al*., 2018). According to Flores *et al*. (2018), this variation in the sensitivity to copper within the isolates of the same country may be due to several factors such as, the different genetic plasticity of the isolates or the selective pressure of the environment in what concerns the concentration and time of exposure to copper sulphate. The fact that other bacteria are able to recover after exposure to concentration of 125 µg/mL is probably because these strains have acquired one or more of the resistance mechanisms described above (Colombi *et al*., 2017). Some of these mechanisms have already been identified in different *P. syringae* strains (Nakajima *et al.,* 2002; Cameron & Sarojini, 2014a).

MIC values reported for copper resistant strains varies between 1.8 and 3.8 mM (Nakajima *et al*., 2002; Petriccione *et al*., 2017; Flores *et al*., 2018). Accordingly to the MIC values found in other studies, our results show that national PSA isolates were

susceptible to copper compound, showing MIC value of 50 µg/mL (0.3 mM). However, nowadays resistant PSA strains were found in several kiwifruit orchards (Cameron & Sarojini, 2014a), as it is the case of, for example, copper-resistant PSA strains with a MIC value 3.8 mM (Petriccione *et al*., 2017) and 2.6 - 3.2 mM (Aiello *et al.,* 2015). Further investigation of resistant strains shown that this characteristic was conferred by the *copABCDRS* operon (Nakajima *et al*., 2002; Colombi *et al*., 2017). These results are in agreement with some recent studies that have already shown the presence of those genes in PSA resistant strains (Aiello *et al.,* 2015; Petriccione *et al*., 2017). So, to confirm the absence of copper resistance, the next step will be to search for the presence of the copper resistant genes (*copA*, *copB*, *copC*, and *copD)* in PSA isolates.

3.3. Characterization of response to AMPs.

The emergence of copper bacterial resistance among the PSA isolates that has been reported above, and the absence of effective alternative strategies to control PSA infection is a problem of extreme relevance for the cultivation of *Actinidia*. AMPs should be highlighted for their high antimicrobial potential against phytopathogenic bacteria (Cameron *et al*., 2014b; Montesinos *et al*., 2017). In the present study, two synthetic peptides AMPs BP100 and 3.1 were tested for their ability to inhibit the growth through binding and altering the permeability of the cell membrane of the Gram-negative bacteria. These peptides were chosen due to their previously described potential antibacterial activity, their short sequence (11 amino acids) and their physic-chemical characteristics like charge and amphipathicity (Badosa *et al*., 2007; Kang *et al*., 2009).

The selected AMPs were tested against phytopathogenic strains of genus *Pseudomonas* (Table 1). The bioactivity test for the AMPs through antibiogram assay showed that strains DSM 10604, CFBP 7286, CFBP2107 and B65 were resistant up to 25 µM of AMP BP100, but isolates P84, P85, AL13 and Am63 were susceptible to this concentration. Peptide 3.1 was more efficient against strains CFBP 7286, CFBP 2107 and P84 strains, which formed inhibition halos at 6.2 µM (Table 7).

Table 7 - Antibiogram results for different AMPs BP100 and 3.1 concentrations.

		Concentrations (µM)							
Peptide	**Strain**	**0.4**	**1.6**	**6.2**	**25**	**50**	**100**	**150**	**200**
BP100	DSM 10604	-	-	-	-	+	+	+	+
	CFBP 7286	-	-	-	-	+	+	+	+
	CFBP 2107	-	-	-	-	+	+	+	+
	B65	-	-	-	-	+	+	+	+
	P84	-	-	-	+	+	+	+	+
	P85	-	-	-	+	+	+	+	+
	AL13	-	-	-	+	+	+	+	+
	Am63	-	-	-	+	+	+	+	+
3.1	DSM 10604	-	-	-	+	+	+	+	+
	CFBP 7286	-	-	+	+	+	+	+	+
	CFBP 2107	-	-	+	+	+	+	+	+
	B65	-	-	-	+	+	+	+	+
	P84	-	-	+	+	+	+	+	+
	P85	-	-	-	+	+	+	+	+
	AL13	-	-	-	+	+	+	+	+
	Am63	-	-	-	+	+	+	+	+

(+) halo formation and (-) no halo formation.

With these results, MIC, MBC and IC_{50} analysis were carried out to determine the antimicrobial activity of each AMP against the employed bacterial strains. Antimicrobial activity of each peptide is summarized in Table 8. Strains CFBP 7286, B65, P85, AL13 and Am63 were more sensitive to BP100 (MIC 3.4 µM) than the other three strains (MIC 6.2 µM) ($p < 0.05$, Table 8). In case of 3.1 peptide, all bacterial strains exhibited MIC values of 10 µM, except the B65 and P85 that was less susceptible, showing a MIC value of 25 µM. The IC_{50} values were calculated to evaluate the concentration of each peptide that inhibits 50 % of the bacterial population. The observed IC_{50} values showed that BP100 (IC_{50} values ranged from 1.48 to 3.78 µM) exhibit highest antibacterial activity than 3.1 (IC_{50} values ranged from 2.38 to 10.9 µM). B65 showed the higher IC_{50} (10.9 µM) for 3.1, while P84 and P85 were more susceptible compared to B65 ($p < 0.05$, Table 8). Badosa *et al*. (2007) suggested that the variation of susceptibility to the same peptide between bacterial strains could be due to differences in the composition of the membrane (*eg.,* lipid composition and charge). For all strains, the value of MIC was usually x2 IC_{50} value, except for P84 and P85 strains where the MIC value for the peptide 3.1 was ≥ x5 the IC_{50} value.

To assess the bactericidal or bacteriostatic activity of each peptide, the minimal MBC was determined. As shown in Table 8, MBC values of AMP BP100 ranged from 3.4 to 25 µM and for AMP 3.1 ranged from 25 up to 100 µM. Strains DSM 10604 and Am63 were less susceptible to AMP BP100 showing a MBC value of 25 µM. In the case of peptide 3.1, strains B65 and P84 were less susceptible than the other six strains, showing an MBC values of 100 and 50 µM, respectively. However, the same value (3.4

µM) of MIC and MBC was observed for strains CFBP 7286 and B65 for BP100. This observation is consistent with previous investigation but about 3.1 peptide activity which describes the same situation of the MBC value is equal to the MIC value (Gomes *et al*., 2019).

Table 8 – Values of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) and half inhibitory concentration (IC_{50}) obtained from 24 hours of bacterial growth curves of *Pseudomonas* spp. strains (see Annex IV – 1). Different letters mean statistical significance for $p < 0.05$ between strains (ordinary one-way Anova).

Peptide	Strain	MIC (µM)	MBC (µM)	IC_{50} ± sd (µM)
BP100	DSM 10604	6.2	25	3.78 ± 0.514 *a*
	CFBP 7286	3.4	3.4	1.72 ± 0.136 *b*
	CFBP 2107	6.2	6.2	3.71 ± 0.903 *a*
	B65	3.4	3.4	1.86 ± 0.233 *b*
	P84	6.2	10	3.50 ± 0.500 *a*
	P85	3.4	6.2	1.48 ± 0.303 *b*
	AL13	3.4	10	1.84 ± 0.233 *b*
	Am63	3.4	25	1.48 ± 0.131 *b*
3.1	DSM 10604	10	25	6.27 ± 0.911 *B*
	CFBP 7286	10	25	5.81 ± 1.67 *B*
	CFBP 2107	10	25	5.52 ± 0.600 *B*
	B65	25	100	10.9 ± 1.915 *A*
	P84	10	50	2.38 ± 0.271 *D*
	P85	25	25	4.86 ± 1.201 *CB*
	AL13	10	25	6.00 ± 0.835 *B*
	Am63	10	25	5.91 ± 1.35 *B*

Our results confirm previous data on the antimicrobial activity against Gram-negative bacteria of AMPs 3.1 with MIC ranging from 2.2 to 17.9 µM (Kang *et al*., 2009; Gomes *et al*., 2019) and peptide BP100, showing MIC values ranging from 2.5 to 8 µM (Badosa *et al*., 2007; Torcato *et al*., 2013; Park *et al*., 2019).

Several studies showed that antimicrobial activity of the BP100 and 3.1 AMPs is based on membrane integrity alteration mechanism. (Gomes *et al*., 2019; Park *et al*., 2019). Cationic AMPs binds on anionic microbial surface through the electrostatic attractions promoting the formation of pores, which leads to changes in the permeability of the membrane (Kang *et al*., 2009; Torcato *et al*., 2013). In the present study, we evaluate this ability to modify the membrane permeability and the cell viability at the same time, resorting to flow cytometry. We used PI which only binds to DNA, after disruption of the

bacterial membranes occur. The degree of permeability of the membrane was directly proportional to the amount of fluorescence detected by a 670 LP filter and this effect was dependent of the peptide applied, time and concentration as shown in Annex IV – 2, 3. It can be observed that PI fluorescence increased over time for all strains and for all AMPs concentration. Treatment with the positive control isopropyl alcohol 23 % (Kennedy *et al*., 2011; Freire *et al*., 2015; Wu *et al*., 2016) resulted in maximum membrane permeabilization similar to the concentration 6.2 µM of the BP100 and 3.1 AMPs ($p > 0.05$, Annex IV - 4). Interestingly, when bacterial cells were exposure to 2 and 6.2 µM of each peptide, membrane permeability was immediately observed. Additionally, cell viability was measured after 10, 20 and 60 minutes after exposure to AMPs.

The decrease of cell viability was observed over time as shown in Figure 9 – A, C. For CFBP 7286 strains, after 60 minutes of exposure to 1 µM of BP100, 77.8 % of cell were viable. In case of the same concentration for 3.1 AMP, 56.3 % of the cells had permeabilized membranes. After 10 minutes of the 3.1 at 1 and 2 µM exposure, it was observed that the percentage of viable cells does not vary over time and stabilizes, which could be explained by a depletion of the peptide. After 60 minutes of exposure to 6.2 µM, it only was observed 25 % and 14.6 % of viable cells for BP100 and 3.1, respectively (Figure 9 – A, C).

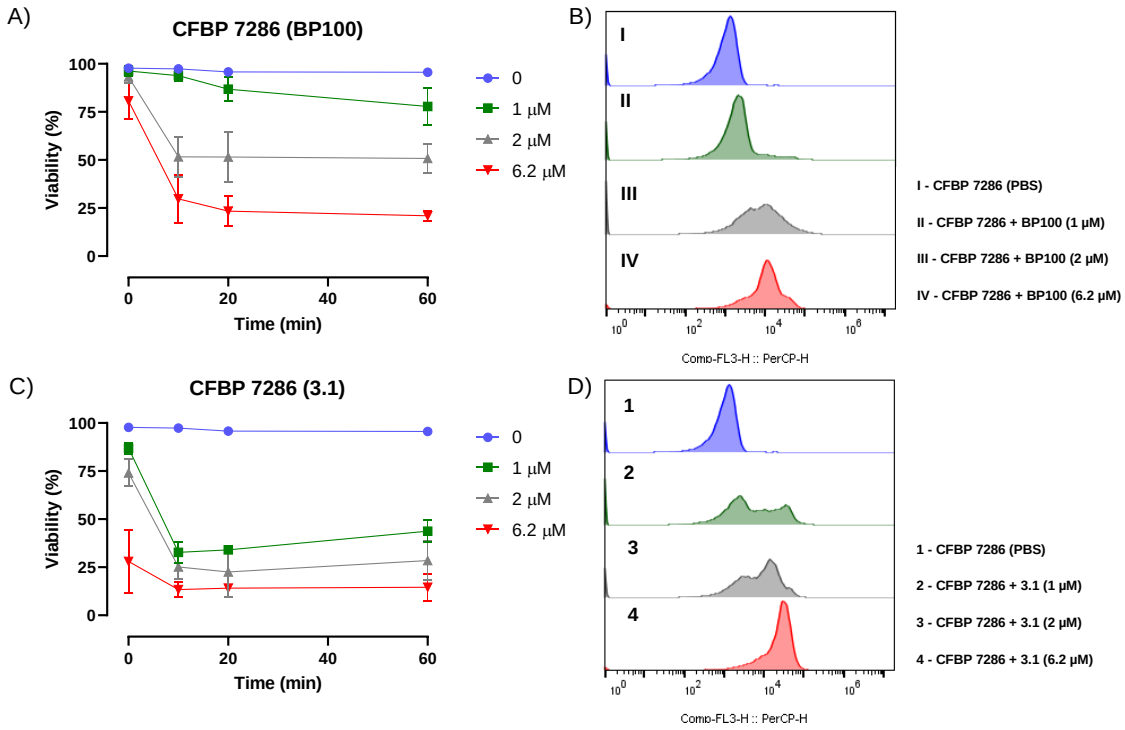

Figure 9 - Evaluation of A) peptide BP100 and C) peptide 3.1 activity against PSA reference pathovar strain CFBP 7286. Cell viability was determined by flow cytometry. Exposure consisted of 10, 20 and 60 minutes for both peptides (1, 2 and 6.2 µM). PI fluorescence was detected at PerCP emission wavelength and the detection was after 60 minutes of exposure to B) BP100 and D) 3.1.

When the effect of those peptides was tested on strain CFBP 2107, the concentration of 1 and 2 µM to AMP BP100 resulted in 71.5 % and 51.2 % of viable cell detected, respectively after 60 minutes of exposure (Figure 10 – A, C). For AMP 3.1 at 1 and 2 µM, the cell viability was, respectively, 56.4 % and 26.5 %. For both peptides, the possible depletion of AMP was observed at 1 and 2 µM (Figure 10 – B, D). For strain CFBP 2107, control (no peptide) showed < 4 % PI-positive bacteria, while for 6 µM of BP100 and 3.1, respectively, 16.6 % and 11.9 % of the bacteria were IP-positive.

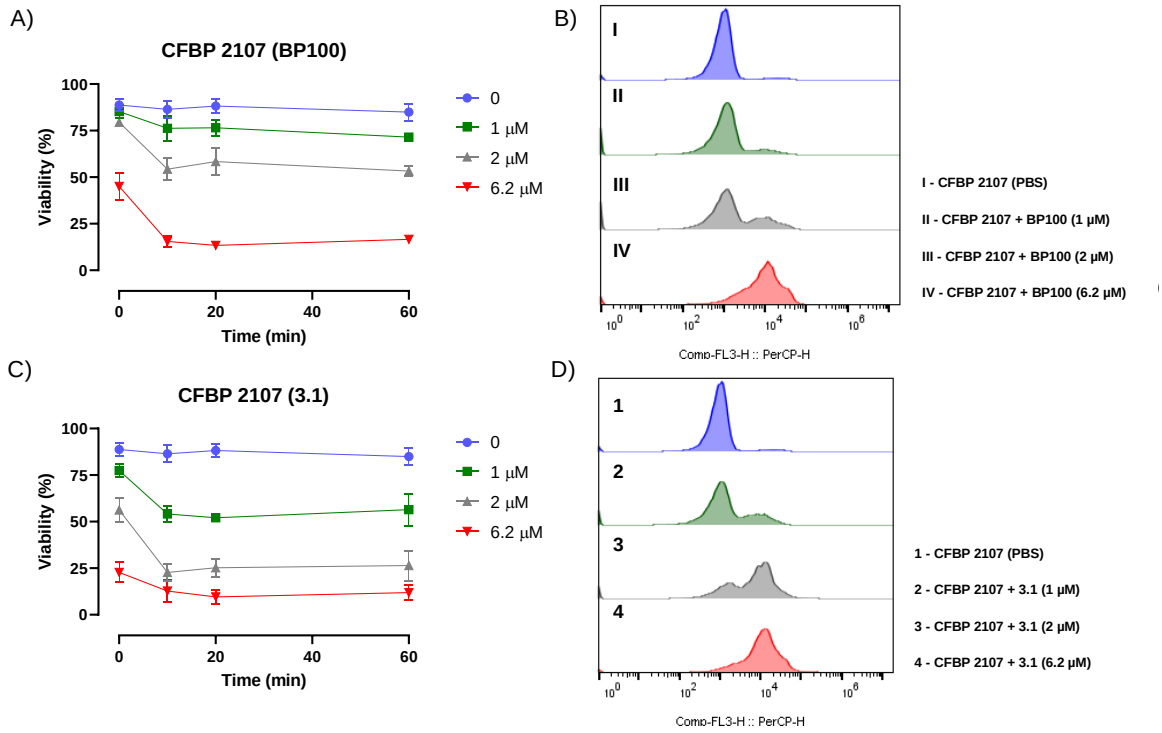

Figure 10 - Evaluation of A) peptide BP100 and C) peptide 3.1 activity against *P. viridiflava* CFBP 2107. Cell viability was determined by flow cytometry. Exposure consisted of 10, 20 and 60 minutes for both peptides (1, 2 and 6.2 µM). PI fluorescence was detected at PerCP emission wavelength and the detection was after 60 minutes of exposure to B) BP100 and D) 3.1.

Both AMPs (BP100 and 3.1) were able to permeabilize cell membranes of the B65 isolate, with effects depending on time, with higher entry of PI after 60 minutes of incubation (Figure 11 – B, D). AMP BP100 (1 µM) permeabilized 13.3 % of B65 cells after 60 minutes of incubation, while 3.1 permeabilized 83.2 % of cells. In this strain, it was observed the possible depletion of the peptide, only for BP100 and 3.1 at 1 and 2 µM. On the other hand, AMP 3.1 demonstrated greater membrane permeabilization efficiency for concentrations of 1 and 2 µM, when compared with BP100. After adding the peptide, the intensity of the PI was analysed, and no significant differences ($p > 0.05$) were observed from the control. The effect of the peptide was only detected after 10 minutes of incubation (Figure 11 – A, C).

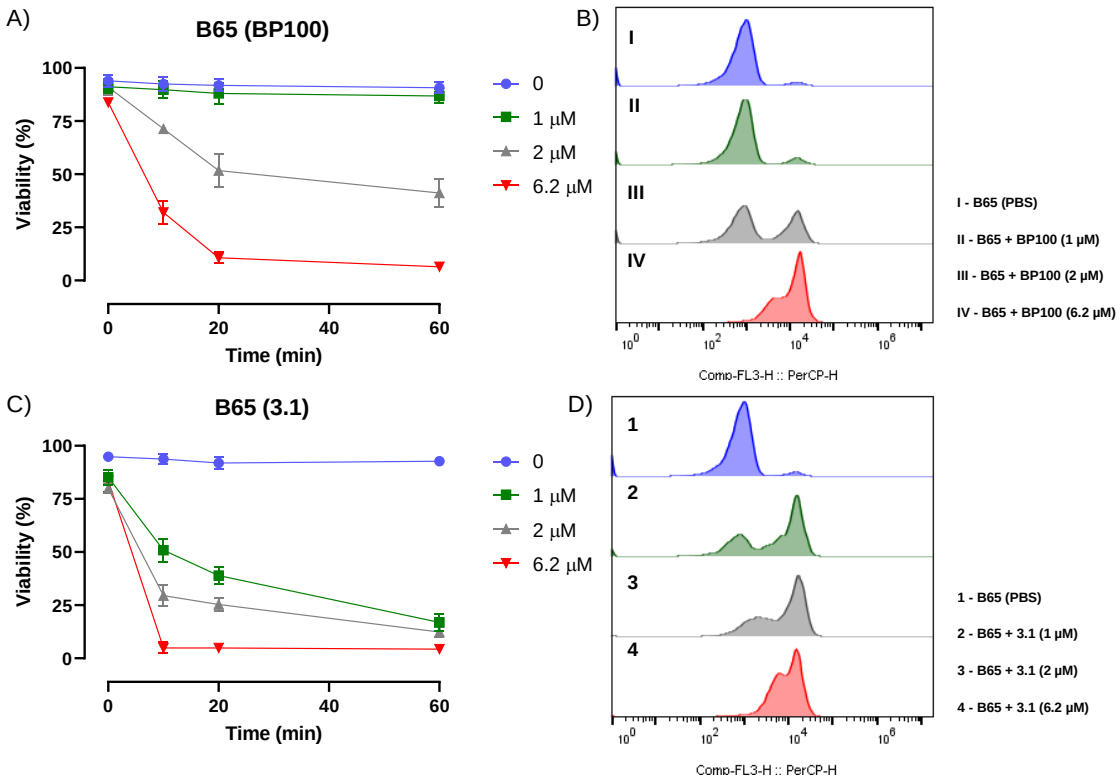

Figure 11 - Evaluation of A) peptide BP100 and C) peptide 3.1 activity against *P. cerasi* B65. Cell viability was determined by flow cytometry. Exposure consisted of 10, 20 and 60 minutes for both peptides (1, 2 and 6.2 µM). PI fluorescence was detected at PerCP emission wavelength and the detection was after 60 minutes of exposure to B) BP100 and D) 3.1.

In the case of the strain DSM 10604, peptide BP100 (6.2 µM) permeabilized the membrane initially in 13.2 % of the cells, a value that increased up to 92.7 % after 60 minutes of incubation, while peptide 3.1 (6.2 µM) showed initially values of 53.4 % which increased to 89.7 % (Figure 12 – A, C). At the beginning, the percentage of viable cells in the control was not significantly different ($p > 0.05$) from concentrations 1, 2 and 6.2 µM of peptide BP100. However, when cells were incubated with peptide 3.1, initially the percentage of viable cells decreased with increasing peptide concentration, namely, 77.8 % for 1 µM, 68.5 % for 2 µM, and 46.6 % for 6.2 µM (Figure 12 – A, C). After 60 minutes of exposure to 6.2 µM, 7.4 % and 10.3 % of viable cells were observed for peptides BP100 and 3.1, respectively. In the case of control (no peptide) showed < 9 % PI-positive bacteria. The possible peptide depletion was also observed for peptide BP100 (1 and 2 µM) as in other strains (Figure 12 – A, C).

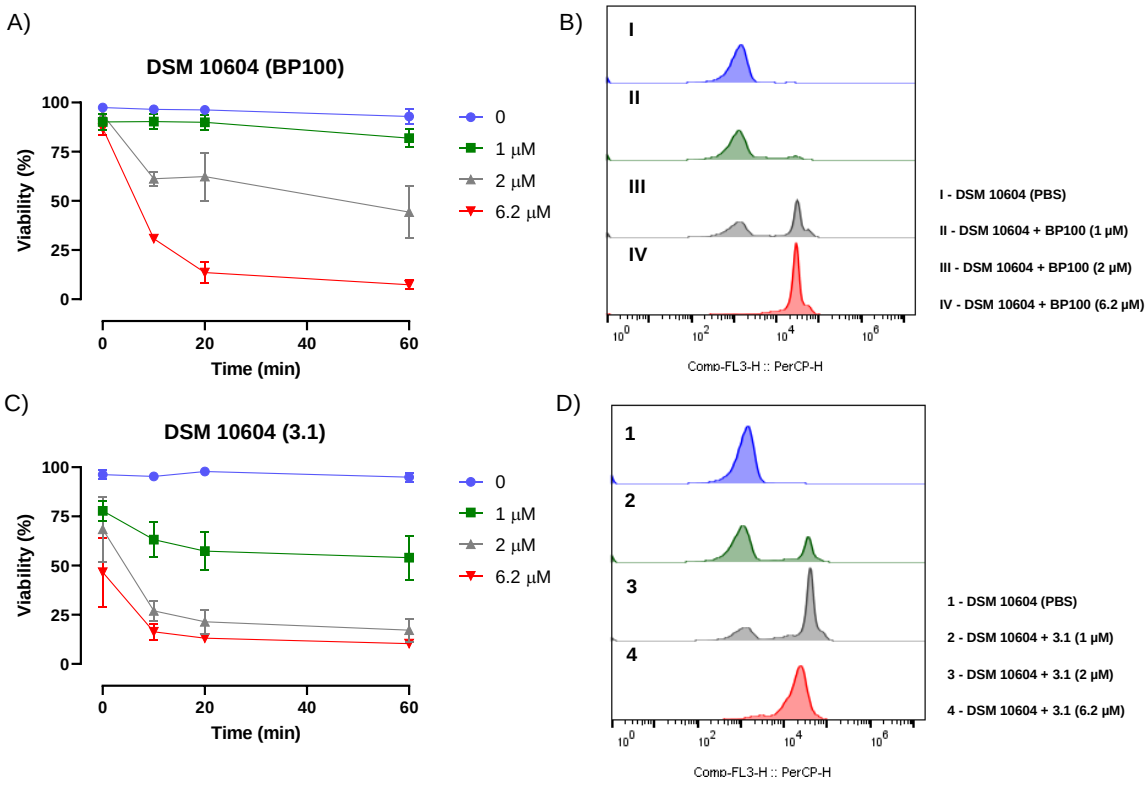

Figure 12 - Evaluation of A) peptide BP100 and C) peptide 3.1 activity against DSM 10604. Cell viability was determined by flow cytometry. Exposure consisted of 10, 20 and 60 minutes for both peptides (1, 2 and 6.2 µM). PI fluorescence was detected at PerCP emission wavelength and the detection was after 60 minutes of exposure to B) BP100 and D) 3.1.

Instantaneous action of the peptides BP100 and 3.1 was observed when, after adding the peptide at 2 and 6 µM to bacterial culture, the increase in PI intensity was observed. Furthermore, the AMP 3.1 demonstrated greater efficiency in membrane permeabilization than peptide BP100. This effect was more evident at lower concentration assayed. For example, after 10 minutes of incubation of strain CFBP 2107 with peptides 3.1 and BP100 at 2 µM, 57.2 % and 20.9 % of the viable cells were detected, respectively (Figure 10 – A, C).

These results suggest that both peptides are able to bind and to depolarize the cell membranes. When the concentration of each peptide increased, the percentage of viable cells decreased. Similar results were obtained by Cabrefiga & Montesinos (2017), who demonstrated the decrease of viable cells after treatment with peptide BP100. Furthermore, they also observed the increase in fluorescence of the SYTOX green in *E. amylovora* cells after 3 hours treatment with peptide BP100 at 15 µM. Similar permeabilization activities was described for other peptides like melamine and Mel4 (Yasir *et al*., 2019).

Cell viability was determined by flow cytometry before and after treatment with peptides. Also, after 60 minutes of incubation, bacterial solution was infiltrated into tobacco leaves to analyse the bacterial ability to cause hypersensitive reaction (HR) on tobacco leaves. The selected concentrations (1 and 6.2 µM) for HR assays were selected due to the results that flow cytometry demonstrated after 60 minutes incubation with 1 µM of peptides BP100 and 3.1, ~ 10 % and ~ 50% of cells were PI-positive, respectively. In case of 6.2 µM, both peptides showed > 80 % PI-positive cells.

Regarding the bacterial suspension of strain CFBP 7286 was treated for 60 minutes with peptides BP100 and 3.1 at 1 and 2 µM. When cells were incubated only in PBS (negative control), PSA was able to induce HR on leaves after 24 and 48 hours (Figure 13 - A). However, after the treatment with peptides 3.1 and BP100 at 6.2 µM, any symptoms of the pathogen infection (only lesions of the infiltration process) were observed. Only treatments with peptides 3.1 and BP100 at 1 µM showed very small necroses after 48 hours inoculation (Figure 13 - A). A decrease was observed in the number of cells of CFBP 7286, with the increase of AMP concentration. Significant differences *($p < 0.05$)* were observed between the treatments with BP100 and 3.1 at 1 µM. The concentration of cells decreased from ~10^8 CFU/mL (control) to ~10^4 CFU/mL after treatment with 1 µM BP100, but no significant differences were observed between the control and the treatment with 3.1 (1 µM). The severity of HR response after treatment of the bacterial suspension with 3.1 (1 µM) was lower than the response observed in the control In the case of peptides BP100 and 3.1 at 6.2 µM, any symptoms on tobacco leaves were observed and no viable cells after the incubation period were detected (Figure 13 - A, B). In the case of 2 µM, significant difference ($p < 0.05$) in value of CFU/mL was obtained between AMPs 3.1 and BP100. AMP BP100 at 2 µM completely inhibited bacterial growth, while AMP 3.1 caused the decrease of bacterial population from ~10^8 CFU/mL to ~10^6 CFU/mL, showing better antimicrobial activity (Figure 13 - B).

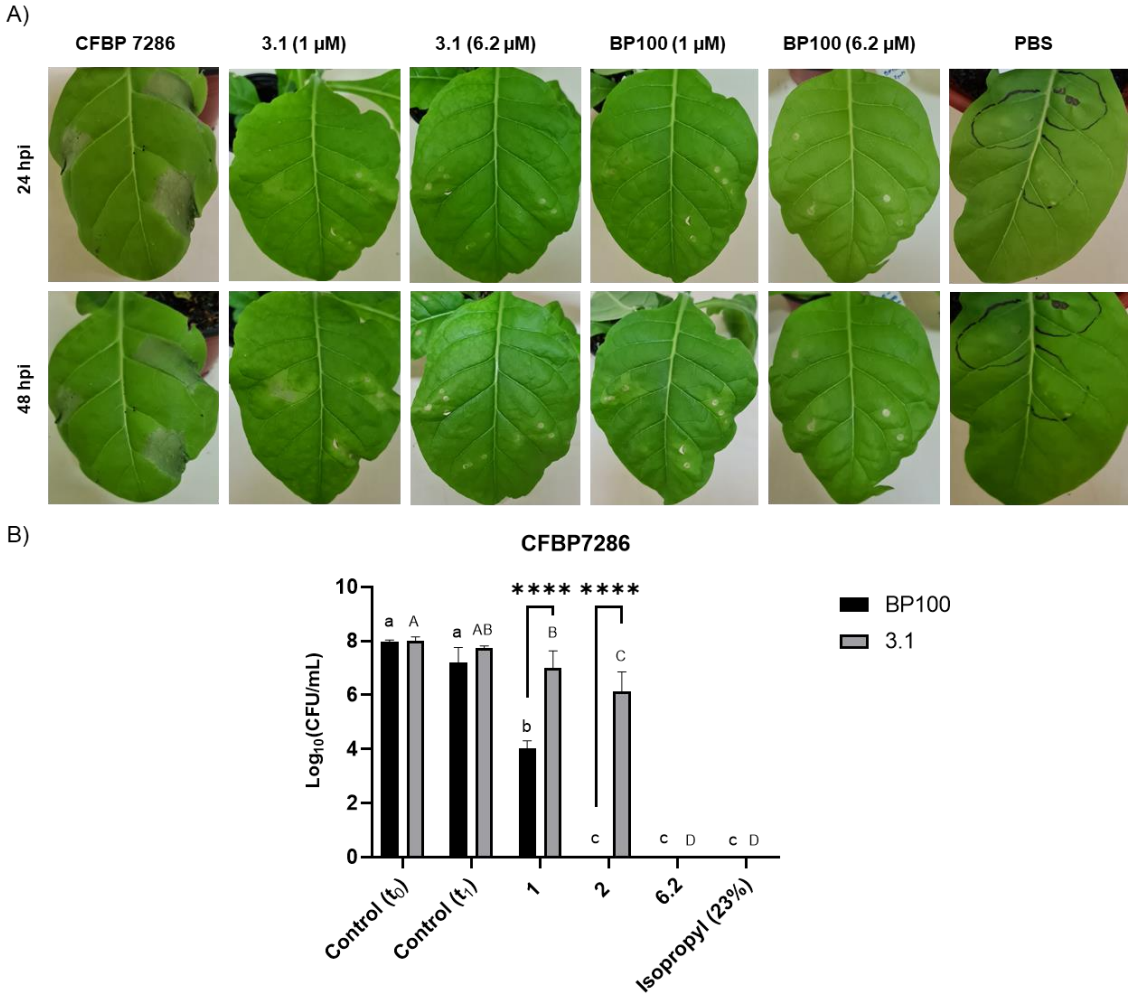

Figure 13 - The effect of peptides BP100 and 3.1 on *P. syringae* pv. *actinidiae* CFBP 7286 cells. A) Hypersensitive response induced by strain CFBP 7286 after 60 minutes of incubation with peptides BP100 and 3.1 at 1 and 6.2 µM. B) Number of active cells. Bacterial suspension (10^8 CFU/mL) incubated for 60 minutes with peptide 3.1 at 1 and 6.2 µM, peptide BP100 at 1 and 6.2 µM or PBS. The development of HR was registered within 24 and 48 hours post-inoculation (hpi). The value of CFU/mL were determined after and before 1 hour treatment with AMPs.

Strain CFBP 2107 (control) induced a HR after 24 and 48 hours after inoculation while treated with peptide BP100 at 1 and 6.2 µM did not show any response. These results are not in order with concentration of cells counted after 60 minutes of exposure to BP100 at 1 µM, concentration of cells was not significantly different *($p > 0.05$)* from the control (~ 10^8 CFU/mL) (Figure 14 - A, B). Only the treatment with peptide 3.1 at 1 µM reduced the severity of symptoms (Figure 14 - A). Peptides BP100 and 3.1 at 6.2 µM completely inhibited bacterial growth after 60 minutes of treatment. Peptide 3.1 was less efficient than peptide BP100, being this effect more evident at 6.2 µM (Figure 14 - B).

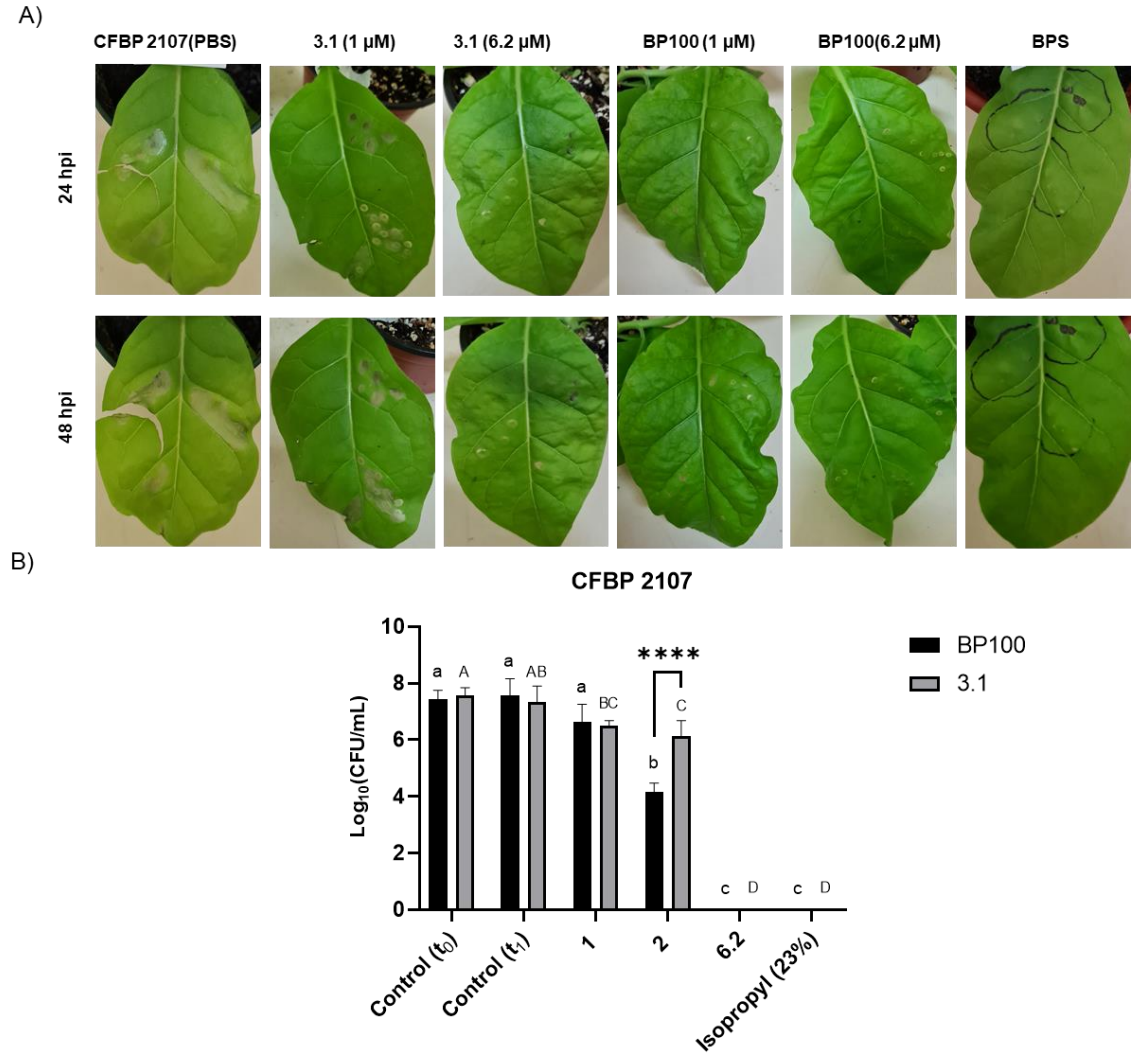

Figure 14 - The effect of peptides BP100 and 3.1 on *P. viridiflava* CFBP 2107 cells. A) Hypersensitive response induced by strain CFBP 2107 after 60 minutes of incubation with peptides BP100 and 3.1 at 1 and 6.2 µM. B) Number of active cells. Bacterial suspension (10^8 CFU/mL) incubated during for 60 minutes with peptide 3.1 at 1 and 6.2 µM, peptide BP100 at 1 and 6.2 µM or PBS. e development of HR was registered within 24 and 48 hours post-inoculation (hpi). The value of CFU/mL were determined after and before 1 hour treatment with AMPs.

Significant difference ($p < 0.05$) in value of CFU/mL was obtained between AMPs 3.1 and BP100. AMP BP100 at 2 µM caused the decrease of bacterial population from ~10^8 CFU/mL to ~10^4 CFU/mL, while AMP 3.1 caused the decrease of bacterial population from ~10^8 CFU/mL to ~10^7 CFU/mL, showing better antimicrobial activity (Figure 14 - B).

Exposure of cells form strain B65 with peptides BP100 and 3.1 at 1 µM did not show any reduction of HR response and the concentration of cells were not significantly different *($p > 0.05$)* from the control (Figure 15 - A). However, increasing the concentration of peptides BP100 and 3.1 to 6.2 µM inhibited bacterial growth and no HR response was

observed (Figure 15 – A, B). As shown in Figure 16 – B, the effect of 2 µM on growth inhibition was significantly different ($p < 0.05$) between peptides BP100 and 3.1.

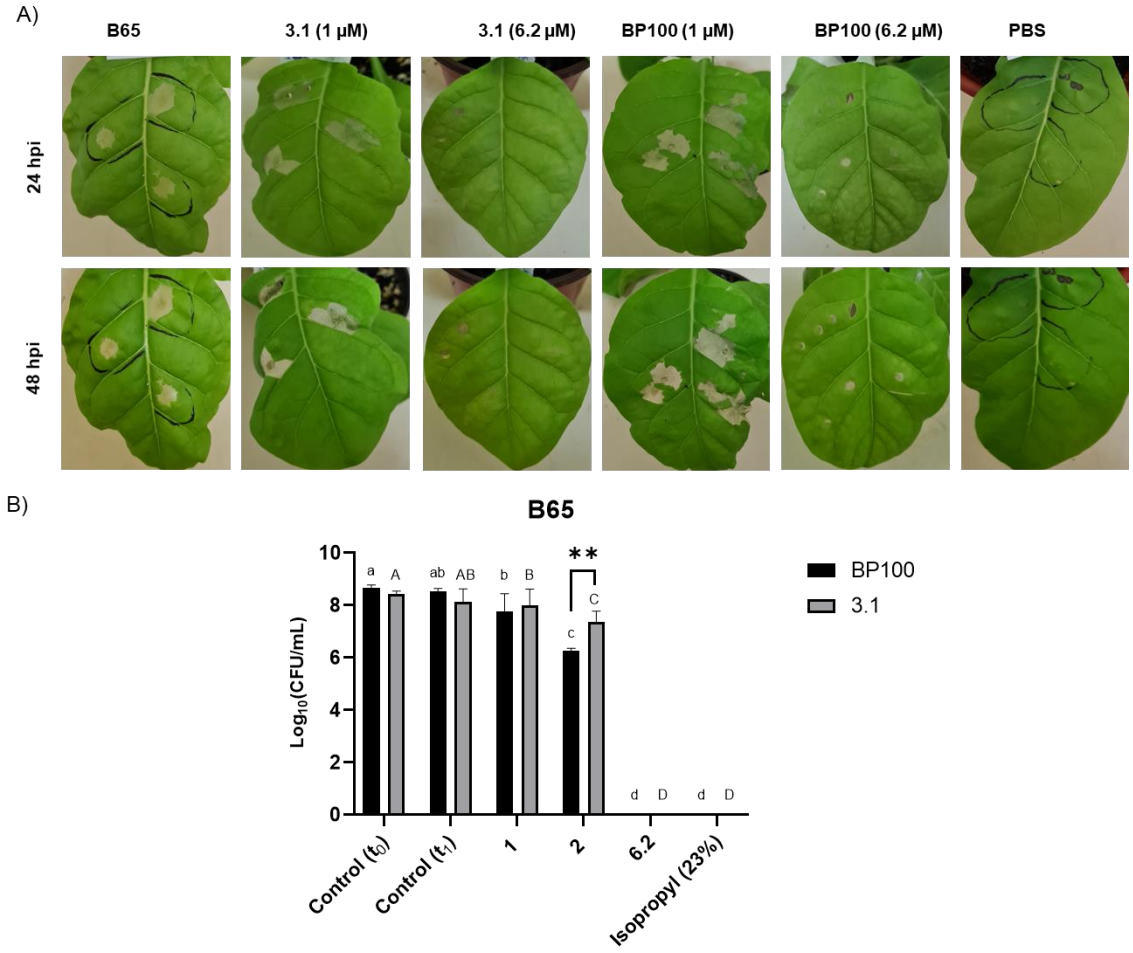

Figure 15 - The effect of peptides BP100 and 3.1 on *P. cerasi* B65 cells. A) Hypersensitive response induced by strain B65 after 60 minutes of incubation with peptides BP100 and 3.1 at 1 and 6.2 µM. B) Number of active cells. Bacterial suspension (10^9 CFU/mL) incubated for 60 minutes with peptide 3.1 at 1 and 6.2 µM, peptide BP100 at 1 and 6.2 µM or PBS. e development of HR was registered within 24 and 48 hours post-inoculation (hpi). The value of CFU/mL were determined after and before 1 hour treatment with AMPs.

AMP BP100 at 2 µM caused the decrease of bacterial population from ~10^9 CFU/mL to ~10^6 CFU/mL, while AMP 3.1 caused the decrease of bacterial population from ~10^8 CFU/mL to ~10^7 CFU/mL, showing better antimicrobial activity (Figure 15 - B).

Similar results were obtained for strain DSM 10604. Treatment with peptides BP100 and 3.1 at 1 µM did not affect the concentration of cells and consequently a HR response was observed, but it was effective at 6.2 µM (Figure 16 – A, B). At this concentration, very weak necroses were observed with peptide 3.1 treatment after 48 hours post-inoculation. These symptoms were not caused by AMP 3.1 solution with at 6.2 µM, when

compared with leaves that were only infiltrated with the peptide, no similar response was observed (see Annex IV - 5). However, no bacterial viable cells were detected. In the case of peptide BP100 (6.2 µM) any symptoms were observed, and bacterial growth was completely inhibited (Figure 16 – A, B). Strain DSM10604 showed low sensitivity to AMPs BP100 and 3.1 at 2 µM, compared with other three strains. Also, the same situation was observed between both AMPs, significant difference ($p < 0.05$) in value of CFU/mL was obtained between AMPs 3.1 and BP100 at 2 µM.

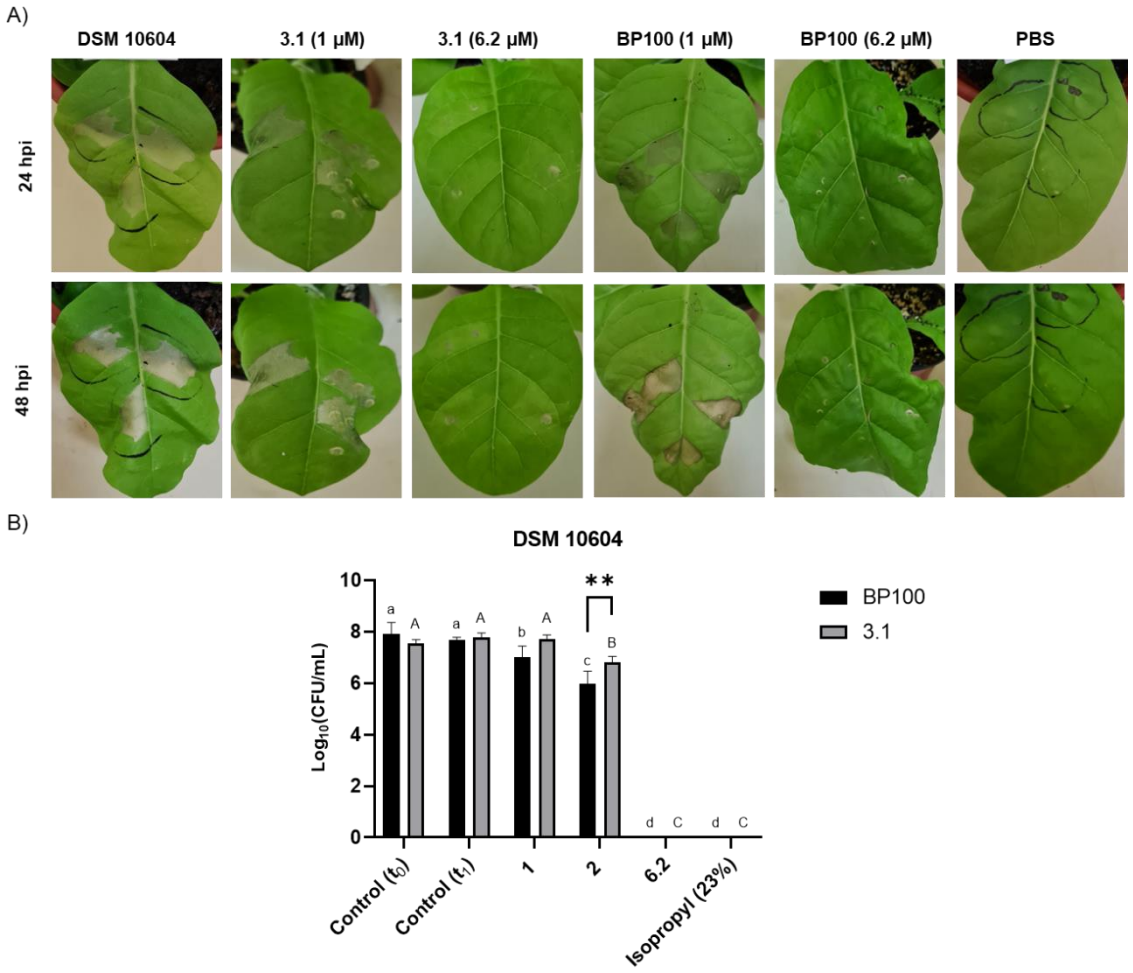

Figure 16 - The effect of peptides BP100 and 3.1 on *P. syringae* pv. *syringae* DSM 10604 cells. A) Hypersensitive response induced by strain DSM 10604 after 60 minutes of incubation with peptides BP100 and 3.1 at 1 and 6.2 µM. B) Number of active cells. Bacterial suspension (10^8 CFU/mL) incubated for 60 minutes with peptide 3.1 at 1 and 6.2 µM, peptide BP100 at 1 and 6.2 µM or PBS. e development of HR was registered within 24 and 48 hours post-inoculation (hpi). The value of CFU/mL were determined after and before 1 hour treatment with AMPs.

Interestingly, although 56.3 % of strain CFBP7286 population was PI positive after treatment with peptide 3.1 (1 µM), the bacterial concentration only decreased from ~10^8 CFU/mL to ~10^7 CFU/mL. While, after treatment with peptide BP100 (1 µM),

concentration of bacterial cells decreased from ~10^8 CFU/mL to ~10^4 CFU/mL and 12.2 % were PI-positive (Figure 9 – C, D). A similar situation was observed for the other three strains. Those were able to recover from peptide 3.1 at 1 µM, but the same effect was not observed for the 6.2 µM concentration. These results suggest that the effect of the peptide is extremely dependent on its concentration, since, in low concentrations, membrane permeabilization occurs, which is confirmed by the increase in PI intensity, but some bacterial cells recover. The damage caused to the membrane is not sufficient to lead to bacterial death.

Peptide BP100 at 2 µM caused death to the entire population of the strain CFBP 7286 (~10^8 CFU/mL) whereas to inhibit the growth of the other isolates it was necessary 6.2 µM (Figure 13 - B). Regarding 3.1, it was necessary the concentration of 6.2 µM to inhibit bacterial growth and cause > 95 % of cells membrane permeabilization. The presence of a bacterial population at ~10^4 CFU/mL and ~10^7 CFU/mL strain CFBP 7286 after treatment with peptides BP100 and 3.1 at 1 µM, respectively, did not cause strong HR response compared to the control (~10^8 CFU/mL) (Figure 13 – A, B). Similar results were obtained with strain CFBP 2107 after exposure to peptide BP100 at 1 µM. The bacterial population only decreased from ~10^8 CFU/mL to ~10^7 CFU/mL and no HR response was observed (Figure 14 – A, B). This result is probably due to the fact that peptides 3.1 and BP100 do not only acts at the membrane, but also interfer at the level of virulence regulation. The mechanism of intracellular regulation of peptides 3.1 and BP100 is not yet studied, however Mahlapuu *et al*. (2016) and Kumar *et al*. (2018) proposed that several AMPs have intracellular targets, which can interfere with the membrane or enter directly into the cell and then accumulate intracellularly, interfering with the DNA/RNA synthesis and protein activity. Furthermore, the absence of HR response after 48 post-inoculation could be due to cell concentration used, which was not sufficient to reach the bacterial *quorum sensing* (QS). In fact, QS is an extremely important factor, not only for the colonization, but also, to the bacterial communication and expression of virulence factors (Javvadi *et al*., 2018).

Globally, we have found that peptide 3.1 exerts membrane permeabilization effects very rapidly when compared to peptide BP100 at the studied timepoints. However, peptide BP100 has shown better bactericidal effects than peptide 3.1. The efficiency in membrane permeabilization could be related with the time it takes to adopt a specific orientation or conformation or the time it takes to interact and to be integrated in the membrane. In this case, the characteristics like charge, amphipathicity, hydrophobicity, structure, target species, growth phase of bacteria, length and the mechanism of action of the peptide, play important roles in activity of the AMP (Le *et al*., 2017; Kumar *et al*.,

2018). Our flow cytometry analyses suggest a membrane permeabilization induced by both AMPs. Were also observed that this effect is dependent on the time and concentration of the peptide (see Annex IV – 2, 3). Moreover, the membrane permeabilization activity of the AMP 3.1 is more efficient than that of peptide BP100 (see Annex IV – 2, 3). The effect of both AMPs was previously characterized as membrane action peptides and the mechanism of action depends on electrostatic interaction with negatively charged membranes molecules altering its permeability (Kang *et al*., 2009; Torcato *et al*., 2013; Manzini *et al*., 2014; Park *et al*., 2019). In the case of the AMP BP100, the mechanism of action and structure have been well studied. Many studies have demonstrated the high affinity to the negatively charged membrane, and in contact with the membrane through electrostatic interactions it adopts a helical conformation and inserts its hydrophobic residues into the lipid bilayer through flip movement (Torcato *et al*., 2013; Park *et al*., 2019). Although the mechanism of action of the peptide 3.1 is not yet well known, it was demonstrated the high amphipathicity of this peptide when it adopts a helical structure (Kang *et al*., 2009) and the capacity of small LK analogues to form ion channels in the membrane (Blondelle & Houghten, 1992). Also, the evidence from the study done by Kang *et al*. (2009) suggests that LK peptides could act via carpet-like or pore-forming mechanism. In this case, carpet-forming peptides acts like detergents when the limit concentration is reached, at this stage specific transmembrane pores can be formed (Kumar *et al*, 2018). Also, several reviews reported that a mechanism of action were extremely correlated with the sequence and physicochemical factors of the peptide (Le *et al*., 2017; Kumar *et al*., 2018).

Furthermore, since both peptides are of the same length, the amino acid composition plays an important role in the activity of the peptides. The presence of tryptophan can be the reason of the difference in the activity between BP100 and 3.1 AMPs. Tryptophan (W) residues in the aminoacidic sequence of a peptide confers an advantage to the peptide in membrane interaction and in helical structure stabilization (Kang *et al*., 2009). Moreover, tryptophan is a hydrophobic residue that plays an important role in the hydrophobicity of the peptide and thus influence its antimicrobial activity (Kumar *et al*, 2018). Torcato *et al*. (2013) observed that changes in BP100 aminoacidic sequence improved peptide activity. They verified that, according to MIC and MBC values, newly designed AMP (RW-BP100) demonstrated better activity than BP100. Similar effect of increased antimicrobial activity was verified by Feng *et al*. (2020) with the derived peptides with (W_4 and W_7) on dCATH, which exhibited better antimicrobial activity than peptides without Trp. Other factors like the number of lysines and leucines that interfere in helical conformation stability were also correlated with antimicrobial potency (Lee *et*

al., 2011). The factors that determine antimicrobial activity and in turn the mechanism of action of the peptides are skewers that are not yet clear.

It is here proposed that the strong membrane permeabilization activity of the 3.1 peptide has additional potential, as it could, for example, be conjugated with the high bactericidal activity of the BP100 to enhance the antibacterial activity of both peptides and further use of this formulation on pest treatment. A similar approach of compounds conjugation was done by Cabrefiga & Montesinos (2017). The authors demonstrated that BP100 conjugated with lysozyme decreased the time required to cause membrane depolarization and subsequent death of the phytopathogen.

3.4. *Actinobacteria* and *Planctomycetes*

Bacteria of the phyla *Actinobacteria* and *Planctomycetes* are great sources of bioactive compounds (Graça *at al*., 2015; Santos *et al.,* 2019). Even though the recent research has been mostly focused on strains of the genus *Streptomyces* that demonstrated a high potential to control phytopathogenic bacteria and fungi (Liotti *et al*., 2019), in this study, were investigated the potential of the less studied marine Actinobacteria and also Planctomycetes that have previously shown the ability to produce bioactive compounds with antibacterial activity (Graça *et al*., 2016; Wiegand *et al*., 2018; Santos *et al*., 2019). These strains were chosen either for the presence of genes like polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) or the previous detected antimicrobial activity as described in Table 3.

Co-cultured bacteria derived from different habitats may simulate the natural competitive environment that could provide the activation of secondary metabolic pathways. Thus, due to physiological and nutritional requirements, namely the presence of salts in the culture medium, only #91_17; Berg02_22.2 and Berg02_26 strains were selected, and the antagonist activity was tested against the phytopathogenic bacterial mentioned above. After 7 days of incubation, no inhibition zones were observed (see Annex V - 1).

Thus, in this work It was decided to change the cultivation parameters using a large-scale liquid culture and then applying two different extraction protocols to detect and analyse the effect of secondary compounds produced during the incubation period. Two extraction protocols were selected: LLPE protocol using ethyl-acetate and a SPE using Amberlite^{TM} XAD16N resin with acetone as solvent. Both protocols are directed predominantly to extract organic compounds. However, ethyl-acetate is frequently used due to its physico-chemical properties such as medium polarity, ability to extract electron donor compounds and low toxicity. SPE is a modern alternative to LLPE in which the

compounds are retained in pores of the resin. The advantage of SPE protocol is the removal of salts present in the culture medium.

Based on a medium-scale (200 mL) bacterial culture, the obtained concentrations of crude extracts are described in Table 9. The mass amounts obtained with the solid phase protocol were, in general, higher than the those obtained with the LLPE protocol. This difference is explained by the fact that SPE protocol uses the resin with high efficiency. However, the resin extracts not only the secondary metabolites but also the constituents of the culture medium.

Table 9 - Mass amount yield with SPE and LLPE extraction protocols. The same volumes were used in both protocols.

	Extraction protocol	
Strain ID	**Solid phase (SPE) (mg)**	**Liquid-liquid phase (LLPE) (mg)**
#91_17	53.6	20.0
Berg02_22.2	61.8	8.3
Berg02_26	70.3	71.6
LZ-A1	20.9	4.1
LF-2	24.2	2.4
GR-7	29.5	8.9
LZ-C2	-	6.0

The activity of each extract is shown in Table 10. One or both extracts of the bacteria tested induced high inhibition levels (> than 50%) in the various *Pseudomonas* tested. Clear differences were observed between extracts obtained from the same bacteria by the two different extraction methods, especially for the extracts derived from strains #91_17, Berg02_26 and LZ_A1. LLPE extracts of strains #91_17 and Berg02_26 induced inhibition percentages below 50 % but higher than 50 % for SPE extracts. Inhibitions lower than 50 % were obtained with SPE extracts of LZ_A1 in strains DSM 10604 and B65, contrary to the inhibitions with the LLPE extracts. These results demonstrated that the extraction method impacts the percentage of inhibition of the extracts.

The inhibitions obtained with extracts from strains #91_17, Berg02_22.2, Berg02_26, LZC-2, LF-2 and GR-7 corroborates data from previous studies (Graça *at al*., 2013; Graça *at al*., 2015; Santos *et al*., 2019; Vitorino *et al*., 2020) which reported antimicrobial activities for these strains and that are here referred in Table 3. Even though the co-culture assays did not show any influence of the Actinobacteria (see Annex V - 1) against *Pseudomonas* spp., their crude extracts demonstrated high antimicrobial activity. Jeske

et al. (2016) observed comparable results because in their work the semi-quantitative agar plate diffusion assay did not show any inhibitory effect of *Rhodopirellula baltica* against *B. subtilis*, but the crude XAD resin extracts exhibited inhibition zones.

Table 10 - Summary of the activities of extracts obtained by solid and liquid-liquid phase extraction protocols against several different PSAs (see Annex V - 2). The positive control used was chlortetracycline (2.5 µg/mL) which induced a 100% of inhibition.

		Bioactivity			
		Extraction protocol			
		Solid phase (SPE)		**Liquid-liquid phase (LLPE)**	
Strain ID	**Target**	**[mg/mL]**	**% of inhibition (±sd)**	**[mg/mL]**	**% of inhibition(±sd)**
#91_17	CFBP 2107	2.5	67.29 ± 5.57	1.0	ND
	B65	2.5	52.97 ± 7.54	1.0	ND
	CFBP 7286	2.5	58.76 ± 6.77	1.0	ND
Berg02_22.2	CFBP 2107	2.5	ND	10.0	66.27 ± 2.70
Berg02_26	CFBP 2107	2.5	65.03 ± 11.20	1.0	ND
	CFBP 7286	2.5	53.75 ± 6.36	1.0	ND
LZ-C2	CFBP 7286	2.5	NT	1.0	69.58 ± 10.30
	CFBP 2107	2.5	NT	1.0	89.99 ± 1.47
	DSM 10604	2.5	NT	1.0	64.59 ± 17.80
LF-2	CFBP 2107	2.5	61.39 ± 11.40	1.0	62.11 ± 12.50
GR-7	CFBP 7286	2.5	50.29 ± 7.95	1.0	64.63 ± 14.70
	CFBP 2107	2.5	61.36 ± 9.71	1.0	83.08 ± 3.67
	DSM 10604	2.5	68.49 ± 7.65	1.0	56.48 ± 19.9
LZ-A1	CFBP 2107	2.5	75.44 ± 3.75	1.0	77.45 ± 3.33
	B65	2.5	ND	1.0	60.08 ± 1.81
	CFBP 7286	2.5	65.49 ± 12.80	1.0	60.60 ± 4.27
	DSM 10604	2.5	ND	1.0	80.96 ± 1.13

ND - not detected (below 50 %); NT – not tested

In this study, only *Alienimonas chondri* LZ-C2 LLPE extract induced activity against pathovar strains CFBP 7286, DSM 10604 and CFBP 2107 with 69.58, 64.59 and 89.99 % of inhibition, respectively (Table 10). This strain encodes a type III PKS (Vitorino *et al*., 2020). In the case of *Novipirellula caenicola* LZ-A1 one or both extracts induced antibacterial activity against four *Pseudomonas* spp.. PKS and NRPS genes were described previously in a *Novipirellula* sp. (Kallscheuer *et al*., 2019). *Rhodopirellula rubra* strain LF-2 induced inhibition against pathovar strain CFBP 2107 with 61.39 % (SPE extract) and 62.11 % (LLPE extract) and in *Rubinisphaera brasiliensis* strain Gr-7 SPE and LLPE extracts were effective against pathovar strains CFBP 7286, CFBP 2107 and DSM 10604 with inhibition values between 50.29 and 83.08 % (Table 10). These strains

have also genomic potential to produce bioactive compounds as both strains possess a type I PKS gene and strain GR-7 have previously demonstrated antimicrobial activity (Graça *et al*., 2015). A NaPDos tool analysis performed by Graça *et al*. (2015) predicted that the type I PKS gene could be responsible for several antibiotic producing pathways like myxalamid in *R. brasiliensis* (GR-7) and myxothiazol and stigmatellin in *Rhodopirellula* spp. (Graça *et al*., 2015). Also, in recent study done by Sandargo *et al*. (2020), new bioactive molecule (stieleriacine) from marine Planctomycete *Stieleria neptunia* sp. nov. were isolated and characterized.

Several bacterial strains of the genus *Gordonia* has a high potential for the production of a wide range of bioactive compounds, due to high percentage of guanine and cytosine content and the presence of PKS-I and NRPS genes (Sowani *et al*., 2018). *Gordonia* sp. Berg02_22.2 extract obtained with LLPE protocol showed 66.27 % of inhibition against CFBP 2107 (Table 10). Moreover, antimicrobial activity of crude extracts obtained from this strain (Graça *et al*., 2013) and from *G. terrae* SPTG 11–1 (Elfalah *et al*., 2013) were previously demonstrated. Members of this genus could produce different types of compounds like canthzanthin, γ-Carotene, circumcin A, kurasoin B, soraphinol C, bendigole (A, B, and C), exopolysachharides, glycolipids (Sowani *et al*., 2018) and also, nocardichelin (A and B), the peptide (Gly-Pro-Phe-Pro-Ile), diketopiperazine cyclo (phenylalanylprolyl) and several compounds with unknown function (Santos *et al*., 2019).

SPE extract of *Micrococcus luteus* Berg02_26 demonstrated 65.3 % and 53.75 % of inhibition against strains CFBP 2107 and CFBP 7286, respectively (Table 10). However, strain Berg02_26, do not possess PKS nor NRPS genes (Graça *at al*., 2013) and, when compared to other species of the phylum *Actinobacteria*, it presents the smallest number of genes associated with secondary metabolism (Young *et al*., 2010). But diketopiperazine, cyclo (prolyltyrosyl) and caerulomycin G were detected after dereplication of its crude extracts (Santos *et al*., 2019). Nisha *et al*. (2020) has detected exopolysaccharide (EPS) and pigments in this genus that have antibacterial activity against *E. coli*, *Klebsiella* sp., *Salmonella typhi*, *Staphylococcus* sp. and *Pseudomonas* sp. In the case of the *Dermacoccus* sp. #91_17 only crude extracts produced with SPE extraction protocol showed 67.29 %, 58.76 % and 52.97 % against pathovar strains CFBP 2107, CFBP 7286 and B65, respectively (Table 10). Graça *et al*. (2015) have demonstrated the presence of NRPS genes associated with secondary metabolism in this bacterium. Several studies have reported a wide range of compounds produced by *Dermococcus* spp. These include phenazines like pigments (dermacozines. A-J and M) (Abdel-Mageed *et al*., 2020), diketopiperazines, the plant hormone (IAA) and several

unidentified molecules (Santos *et al*., 2019). Zhang *et al*. (2016) demonstrated antimicrobial activity of *Dermacoccus* sp. (X4) strain against *S. aureus* and further analysis of the produced metabolites allowed to identify and structurally characterize as 3-(4-hydroxybenzyl) hexahydropyrrolo (1,2-a)pyrazine-1,4-dione and two indole acid glycerides.

4. Conclusions

PSA biovar 3 is a pandemic bacterium, and the most virulent biovar that causes severe consequences in the production of kiwifruit (Kim *et al*., 2017a). In this study different phytopathogenic bacteria of the genus *Pseudomonas*, including the PSA pathovar reference strain (CFBP 7286) and four Portuguese isolates (P84, P85, Am63 and AL13), were characterized regarding their pathogenicity. The results in this study demonstrate that all strains are catalase positive, oxidase negative, were not able to form biofilm in abiotic surfaces, and exhibit a swimming motility. *P. syringae* also produce IAA using Trp-dependent pathway. The strains were able to induce HR response on tobacco leaves.

All studied strains were also evaluated for susceptibility to copper sulphate. This work demonstrated that all strains do not show resistance to this compound, considering the published MICs values for Cu-resistant bacteria (Aiello *et al.,* 2015; Petriccione *et al*., 2017). However, the susceptibility to the copper compound varies between bacterial strains. In case of AMPs, both studied peptides showed strong antimicrobial activity and ability to membrane permeabilization. Additionally, both peptides may decrease the pathogenicity of the bacteria without altering their viability. AMP BP100 demonstrate better bactericidal activity than 3.1, but 3.1 displayed better membrane permeabilization activity. Also, according to the MIC and IC_{50} values, studied strains were more susceptible to BP100 than 3.1 peptide. Due to strong antimicrobial activity, reduced phytotoxicity and low activity against non-target organisms (Kumar *et al*., 2018; Sinha & Shukla, 2019), those peptides could be used for pest treatment. Also, a mixture of both peptides can be applied to enhance the efficiency in pest control. Further studies are necessary to clarify the mechanism of action of both peptides and how they interfere in bacterial pathogenicity.

Finally, strong bioactivity of crude extracts derived from bacterial isolates of the phylum *Actinobacteria* and *Planctomycetes* were observed against PSA strain CFBP 7286, *P. viridiflava* CFBP 2107 and PSS DSM 10604. Extracts obtained with SPE protocol showed better inhibition percentages than extracts obtained with LLPE protocol. Also, the extracts obtained from Actinobacteria only with SPE were bioactive instead the ones

obtained with LLPE, except for the extract from strain Berg02_22.2. Thus, the next step of this study will be to perform the chemical dereplication of the extracts in order to identify and characterize the bioactive compounds with the biotechnological potential.

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6. Supplementary data

Annex I - Culture media and reagents used

1) King's B medium (KB)

	1 L
Peptone	20 g
K_2HPO_4	1.5 g
$MgSO_4$	1.5 g
Glycerol	10 mL
pH	7 – 7.2

2) Tris-minimal medium (TMM)

	1 L
Tris base (1M)	20 mL
Phosphate buffer II[1]	1.0 mL
Solution A[2]	10.0 mL
Hutner's trace elements[1]	1.0 mL
Sodium succinate	2 g
Yeast extract	1 g
pH	7

[3] Hutner *et al*., Proc. Am. Philos. Soc. 94: 152-170 (1950).

[2] Solution A

	500 mL
NH_4Cl	20.0 g
$MgSO_4 \cdot 7H_2O$	5.0 g
$CaCl_2 \cdot 2H_2O$	2.5 g

3) Marine Broth (MB) medium and M607 medium (for 1 L)

	Marine Broth	M607
Peptone	5.0 g	0.25 g
Yeast extract	1.0 g	0.25 g
Tris-HCL	--	50 mL
Deionized water	--	10 mL
Natural sea water [1]	1000 mL	900 mL
Vitamins solution [2]	--	10 mL
Hutner's basal salts solution [3]	--	20 mL
Glucose solution (2.5 %)	--	10 mL
Agar	16.0 g	16.0

[1] Sea water was filtered through a 0.45 µm pore filter

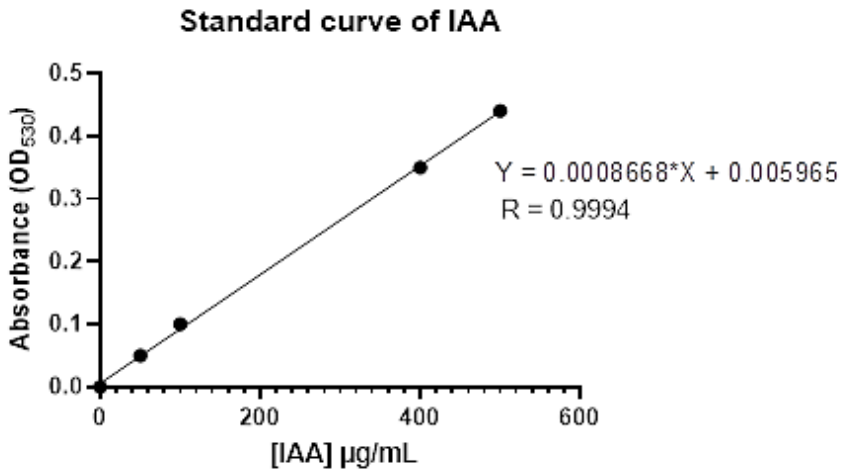
[2] 0.1 µg/mL cobalamin, 2.0 µg/mL biotin, 5.0 µg/mL thiamine-HCl, 5.0 µg/mL Ca-pantothenate, 2.0 µg/mL folic acid, 5.0 µg/mL riboflavin and 5.0 µg/mL nicotinamide.

[3] Cohen-Bazire *et al*., (1957).

4) Potassium phosphate buffer solution (10 mM)

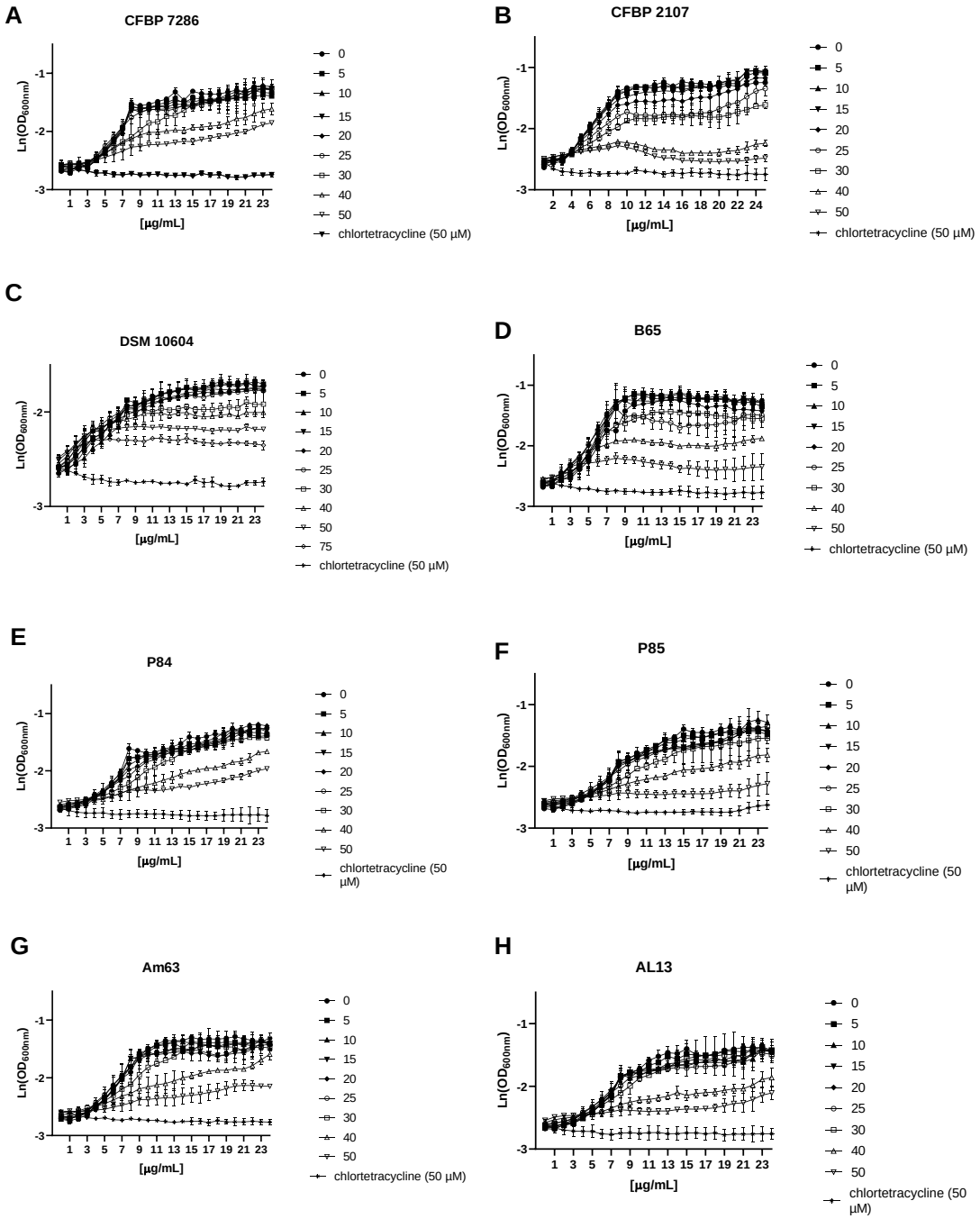
	1 L
NaCl	8.0
KCl	0.2
$Na_2HPO_4 \cdot 12H_2O$	2.9
KH_2PO_4	0.2
pH	7.0 – 7.2

Annex II - Standard curve of IAA



Annex III – Copper susceptibility

1) Bacterial growth curves of A) pathovar reference strain of *P. syringae* pv. *actinidiae* CFB P7286, B) *P. viridiflava* CFBP 2107, C) *P. syringae* pv. *syringae* DSM 10604, D) *P. cerasi* B65, and four national PSA isolates E) P84, F) P85, G) Am63 and H) AL13. Strains were incubated 24 hours in Tris Minimal Medium (TMM) under different $CuSO_4$ concentrations (ranged between 0 to 125 µg/mL). Chlortetracycline (50 µM) was used as positive control.

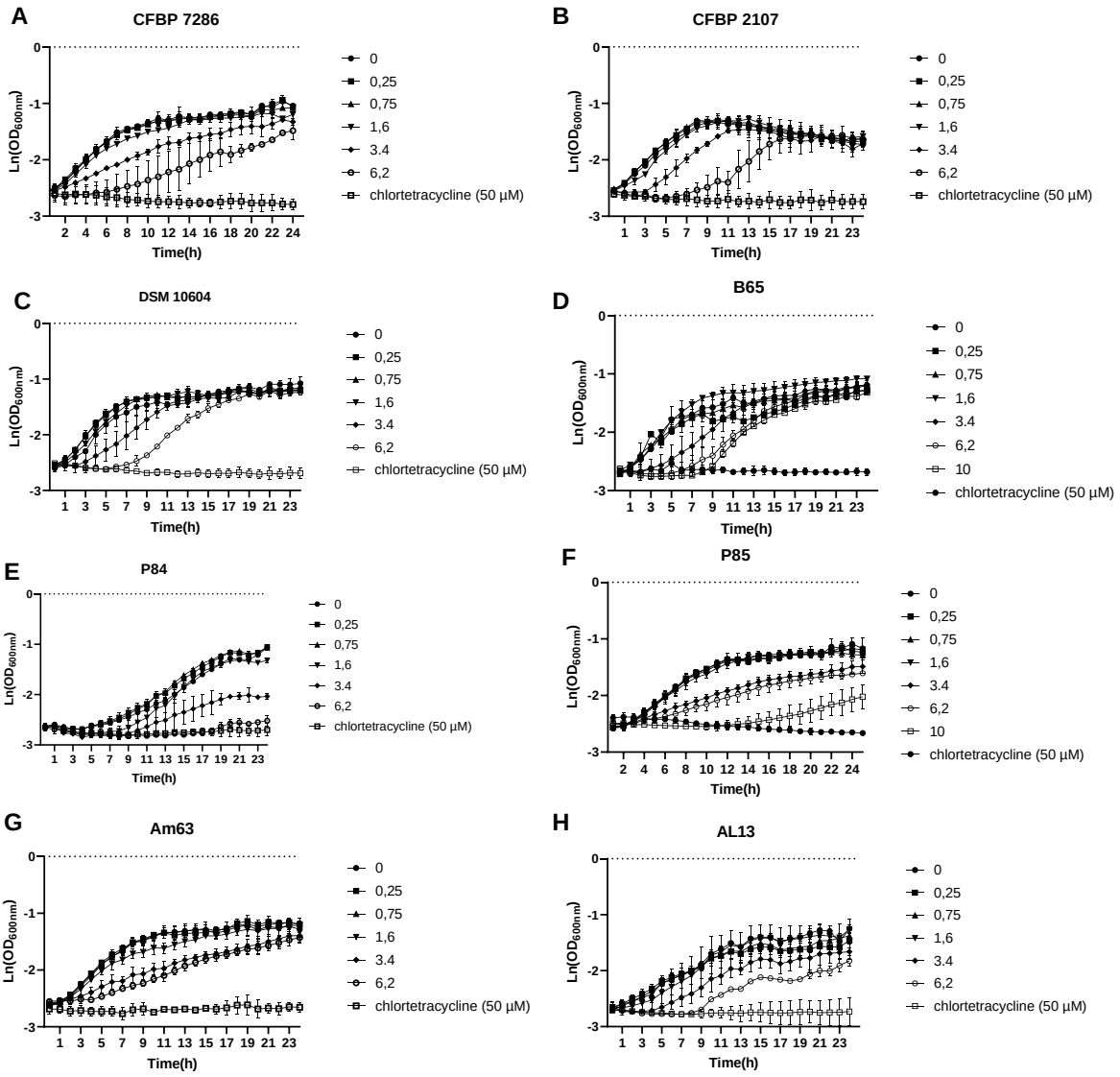


2) Values of maximum specific growth rate obtained from 24 hours bacterial growth curves.

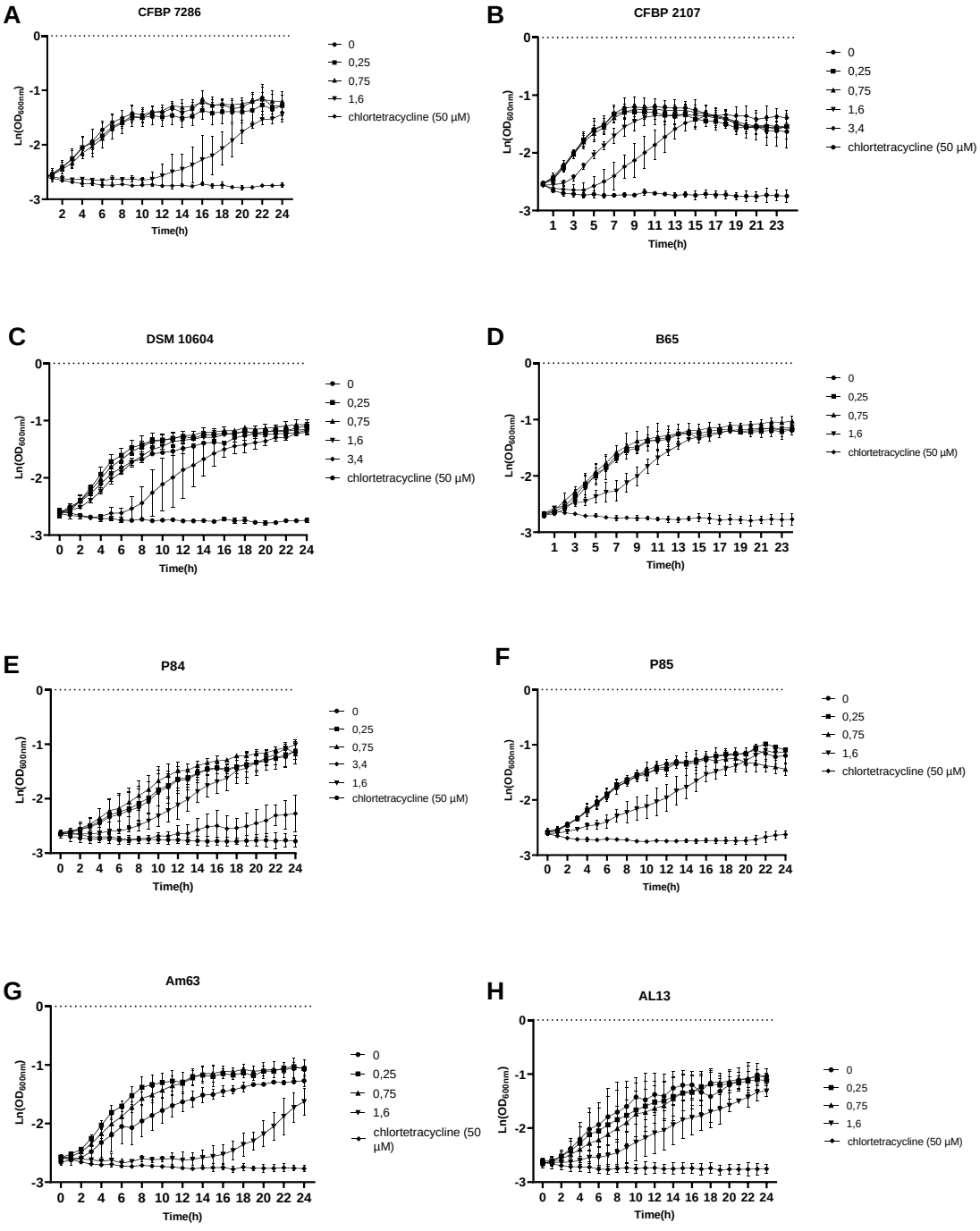
	DSM 10604		**CFBP 7286**		**CFBP 2107**		**B65**	
[$CuSO_4$]	***μmax*** **(h^{-1})**	**sd**	***μmax*** **(h^{-1})**	**sd**	***μmax*** **(h^{-1})**	**sd**	***μmax*** **(h^{-1})**	**sd**
0	0.09	0.008	0.18	0.032	0.18	0.032	0.25	0.026
5	0.08	0.010	0.18	0.016	0.19	0.010	0.23	0.040
10	0.07	0.009	0.18	0.015	0.18	0.009	0.24	0.020
15	0.08	0.012	0.17	0.011	0.17	0.019	0.24	0.014
20	0.08	0.014	0.16	0.018	0.15	0.014	0.21	0.023
25	0.08	0.013	0.13	0.016	0.12	0.021	0.20	0.014
30	0.06	0.029	0.11	0.015	0.09	0.018	0.16	0.041
40	0.08	0.015	0.05	0.011	0.04	0.012	0.11	0.028
50	0.08	0.010	0.02	0.024	0.02	0.010	0.06	0.029
75	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
125	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
	AL13		**Am63**		**P85**		**P84**	
[$CuSO_4$]	***μmax*** **(h^{-1})**	**sd**	***μmax*** **(h^{-1})**	**sd**	***μmax*** **(h^{-1})**	**sd**	***μmax*** **(h^{-1})**	**sd**
0	0.15	0.017	0.18	0.181	0.13	0.014	0.15	0.028
5	0.15	0.026	0.19	0.191	0.14	0.007	0.14	0.028
10	0.15	0.034	0.17	0.166	0.13	0.005	0.14	0.014
15	0.15	0.037	0.18	0.175	0.13	0.004	0.13	0.011
20	0.14	0.028	0.17	0.173	0.12	0.015	0.11	0.023
25	0.12	0.019	0.16	0.156	0.10	0.006	0.09	0.022
30	0.09	0.003	0.12	0.116	0.08	0.032	0.06	0.030
40	0.05	0.014	0.07	0.071	0.05	0.010	0.04	0.023
50	0.02	0.010	0.03	0.033	0.02	0.008	0.02	0.018
75	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000
125	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000

Annex IV – Peptide susceptibility

1) Growth curves of A) pathovar reference strain of *P. syringae* pv. *actinidiae* CFBP 7286, B) *P. viridiflava* CFBP 2107, C) *P. syringae* pv. *syringae* DSM 10604, D) *P. cerasi* B65 and four national PSA isolates E) P84, F) P85, G) Am63 and H) AL13. AMP tested was 3.1 peptide. Strains were incubated 24 hours in MH media under different 3.1 concentrations (ranged between 0 to 100 µM). Chlortetracycline (50 µM) was used as positive control.

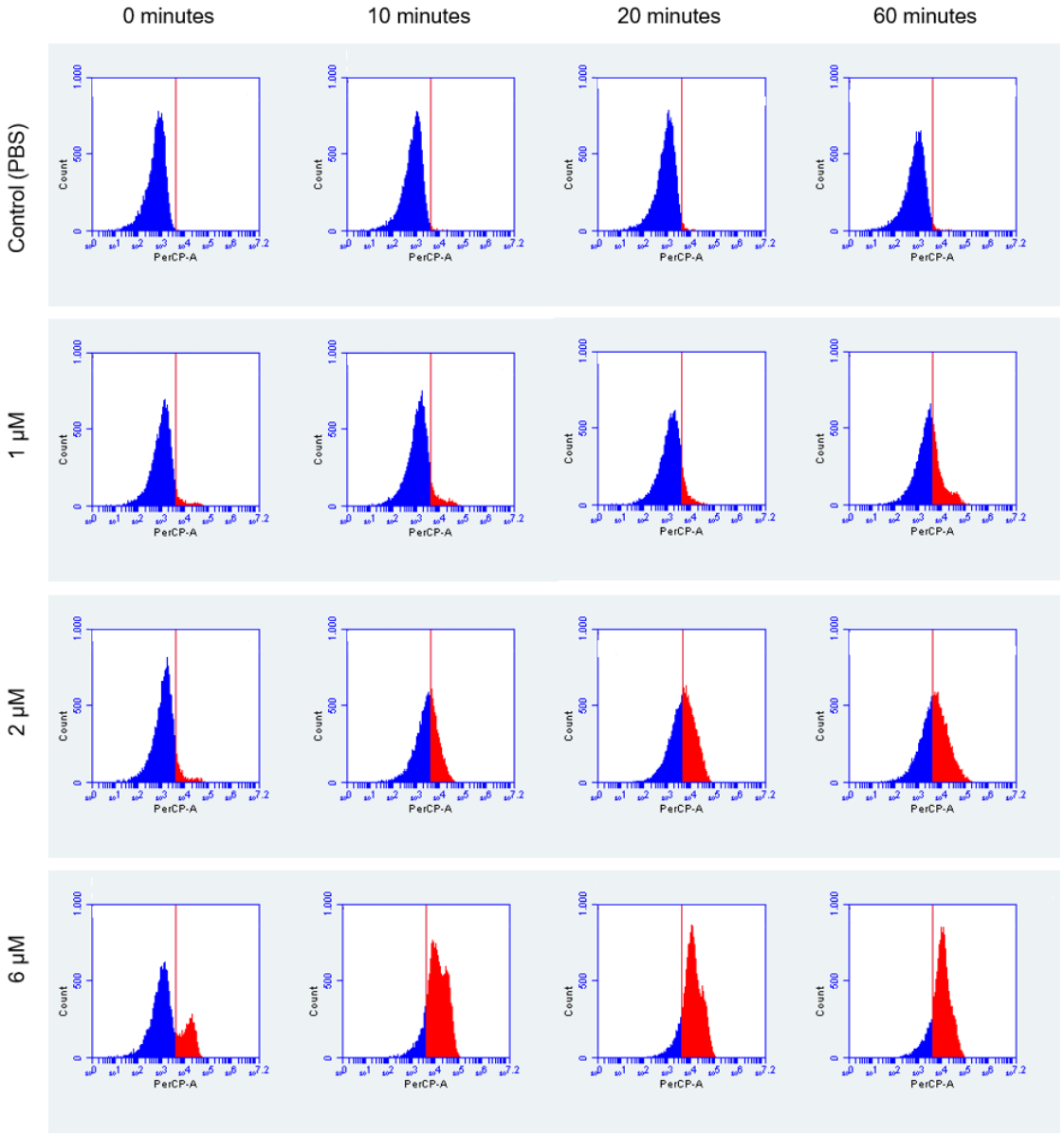


2) Growth curves of A) pathovar reference strain of *P. syringae* pv. *actinidiae* CFB P7286, B) *Pseudomonas viridiflava* CFBP 2107, C) *P. syringae* pv. *syringae* DSM 10604, D) *P. cerasi* B65 and four national PSA isolates E) P84, F) P85, G) Am63 and H) AL13. AMP tested was BP100 peptide. Chlortetracycline (50 µM) was used as positive control. Strains were incubated 24 hours in MH media under different BP100 concentrations (ranged between 0 to 100 µM).

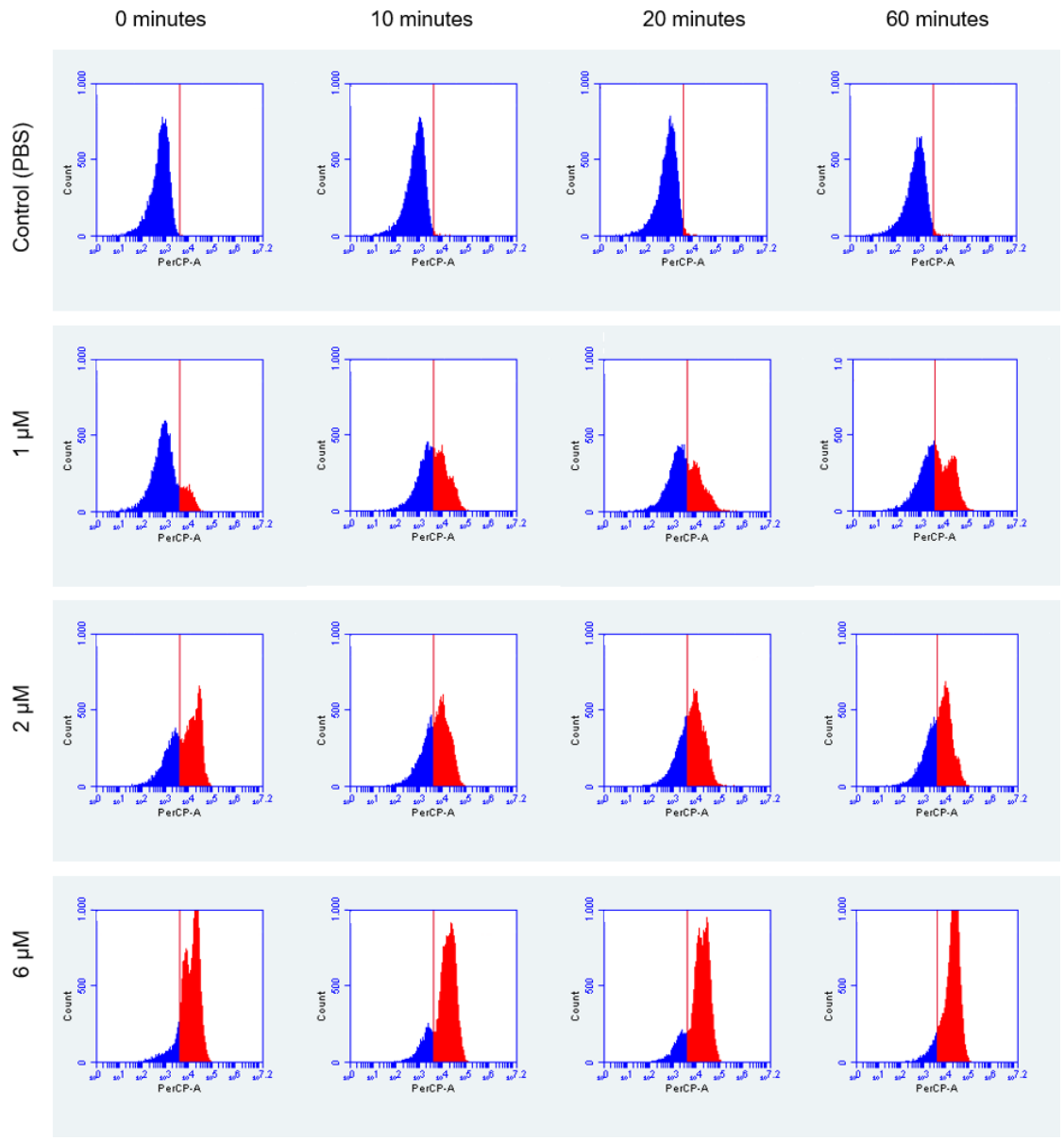


3) Flow cytometry histograms of *Pseudomonas* spp. strains incubated with AMPs BP100 and 3.1 at concentration 1, 2 and 6.2 µM. Membrane permeabilization was determined by flow cytometry with PI. A) CFBP 7286 exposed to BP100. B) CFBP 7286 exposed to 3.1. C) CFBP 2107 exposed to BP100. D) CFBP 2107 exposed 3.1. E) DSM 10604 exposed to BP100. F) DSM 10604 exposed to 3.1. G) B65 exposed to BP100. H) B65 exposed to 3.1. PI fluorescence were detected with PerCP emission wavelength. Fluorescence were analysed after 0, 10, 20 and 60 minutes of incubation. The fluorescence intensity of PI above $1x10^3$ was considered as the non-viable cells.

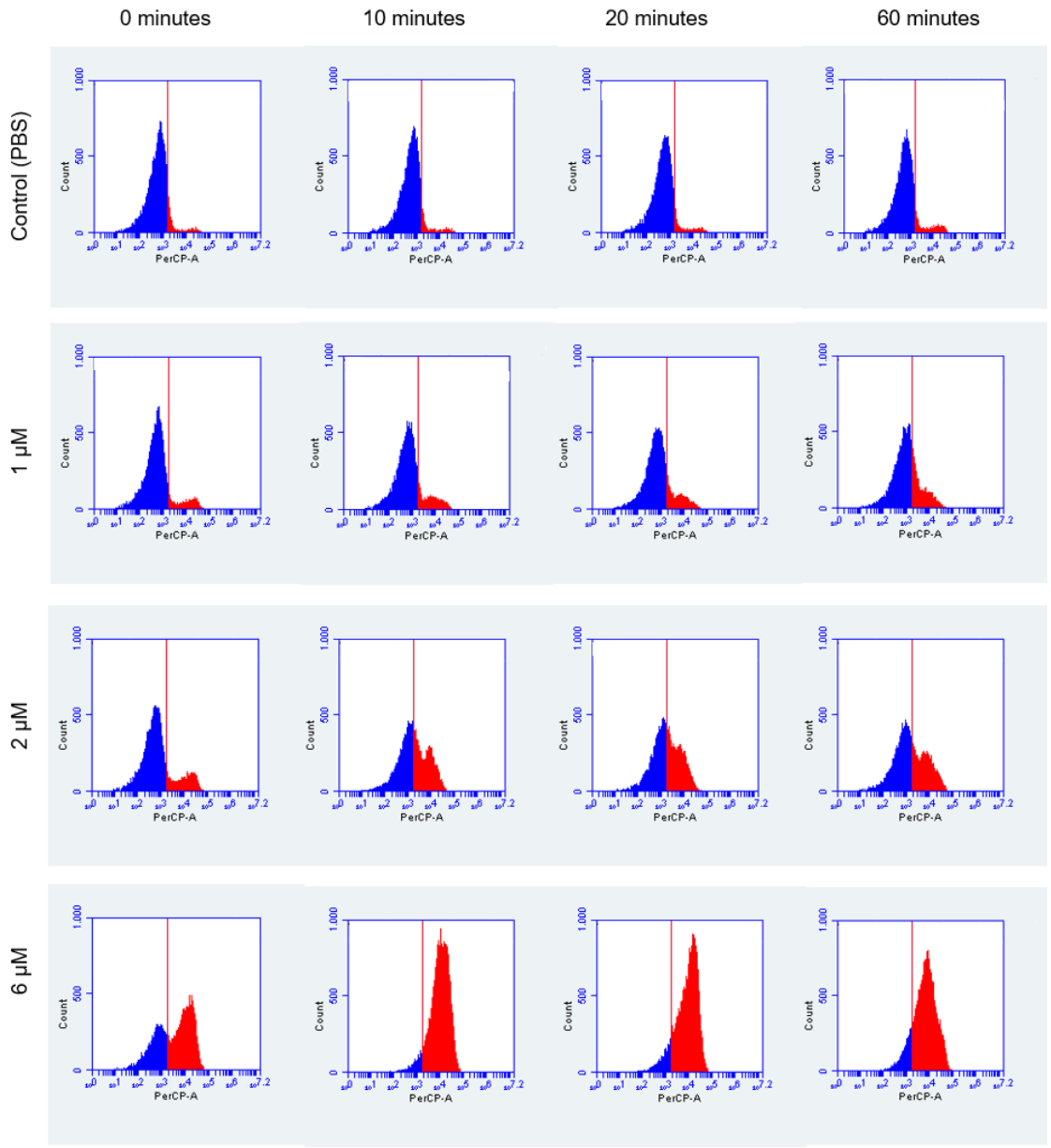
A) CFBP 7286 (BP100)



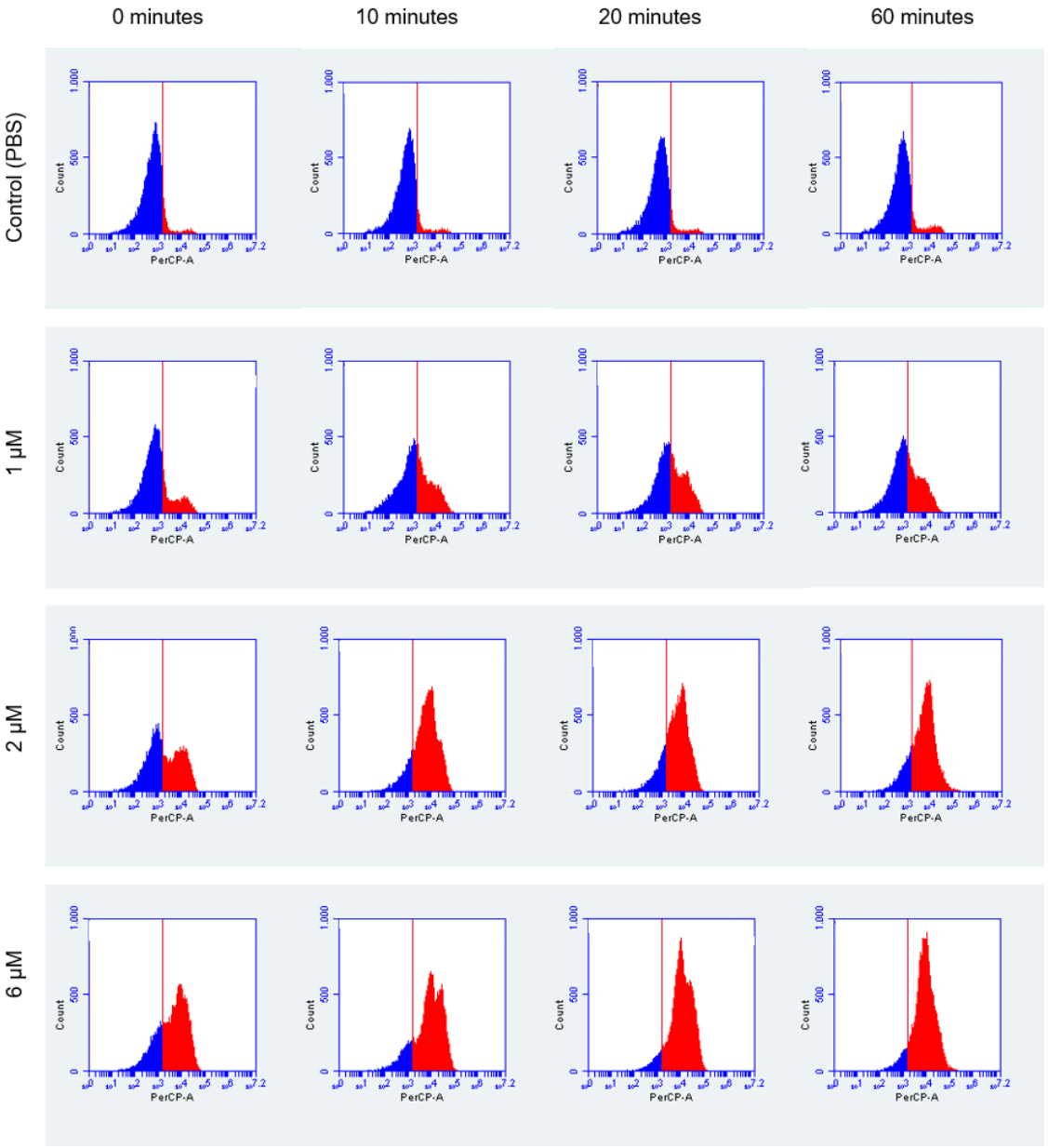
B) CFBP 7286 (3.1)



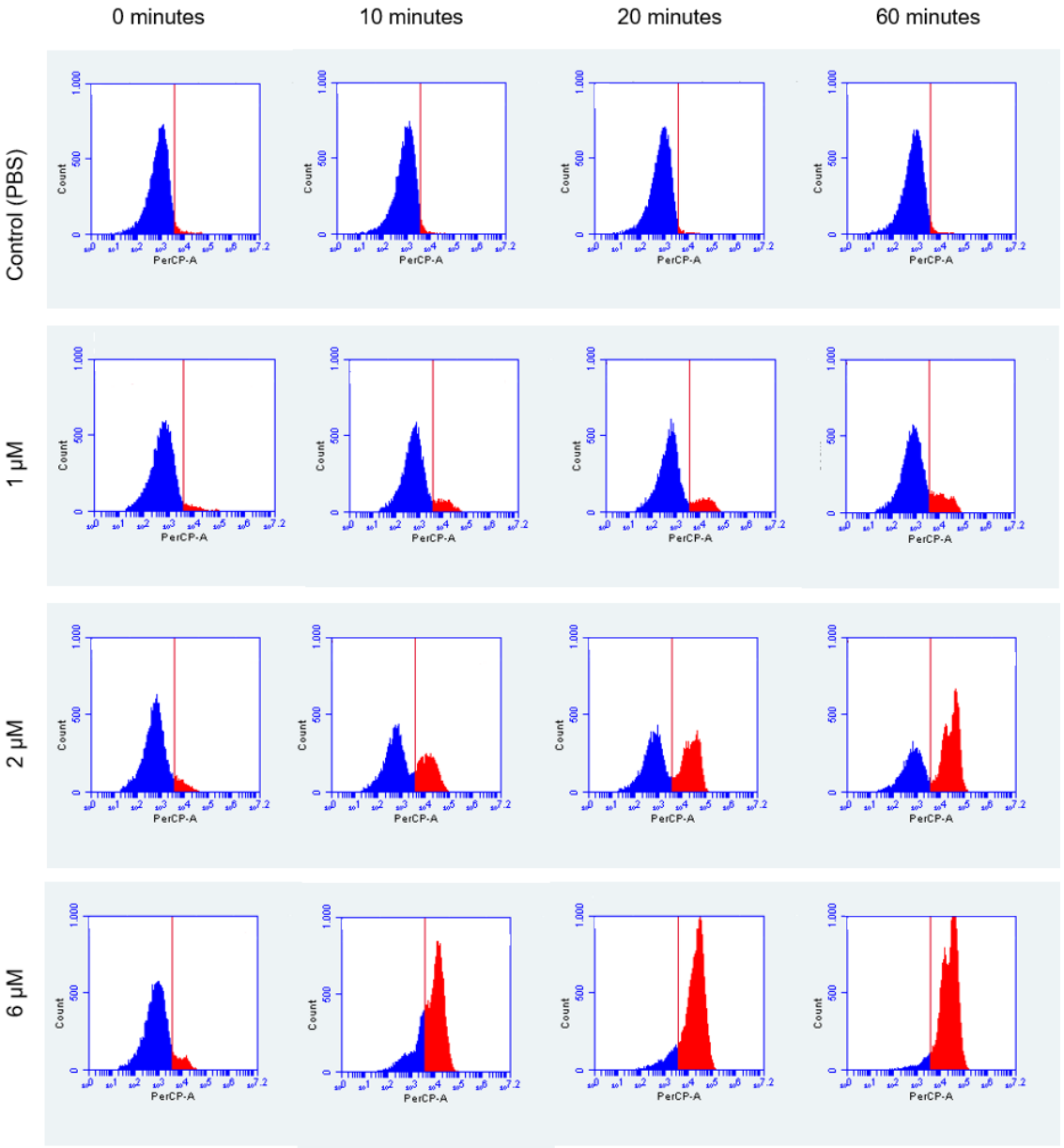
C) CFBP 2107 (BP100)



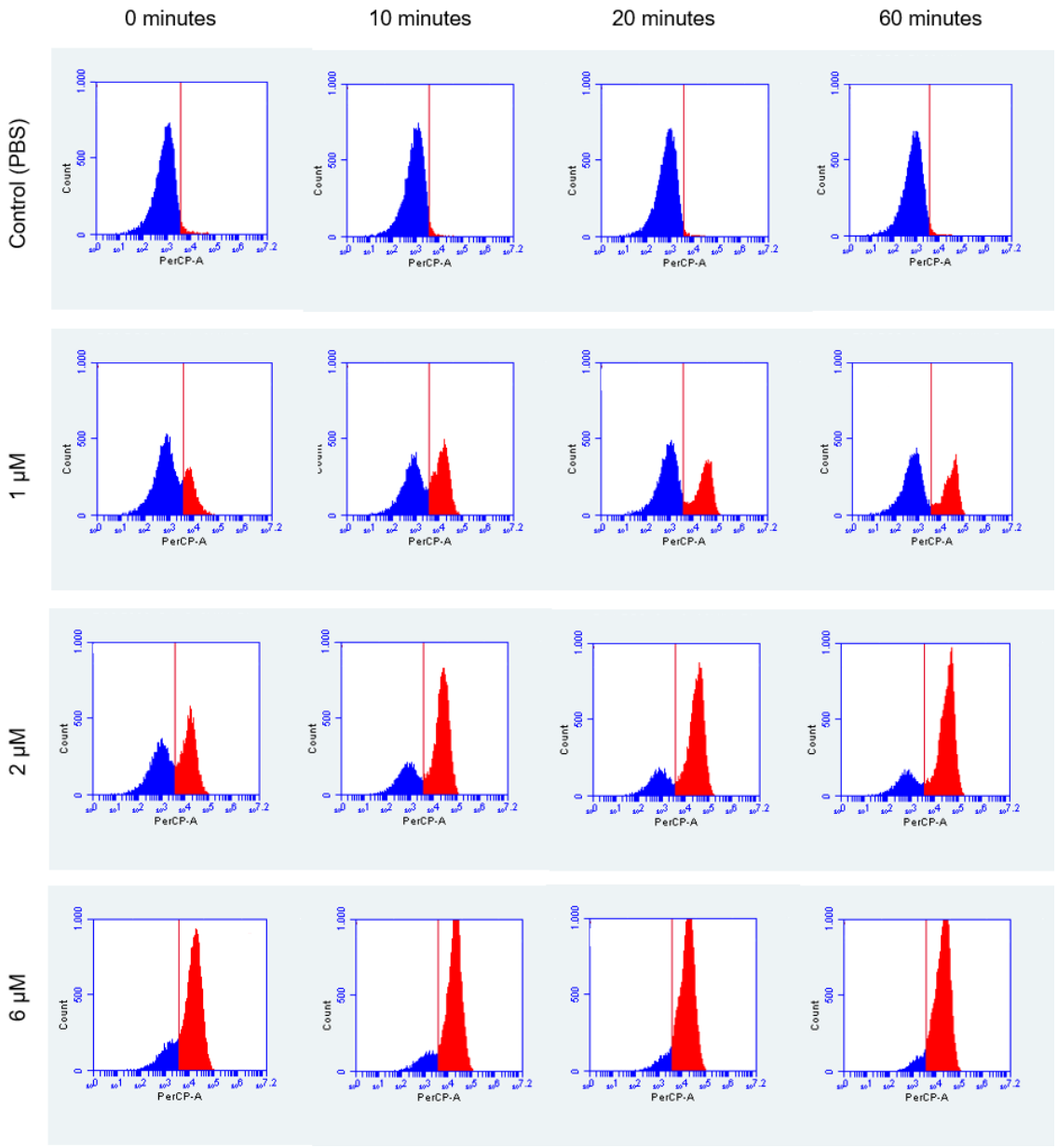
D) CFBP 2107 (3.1)



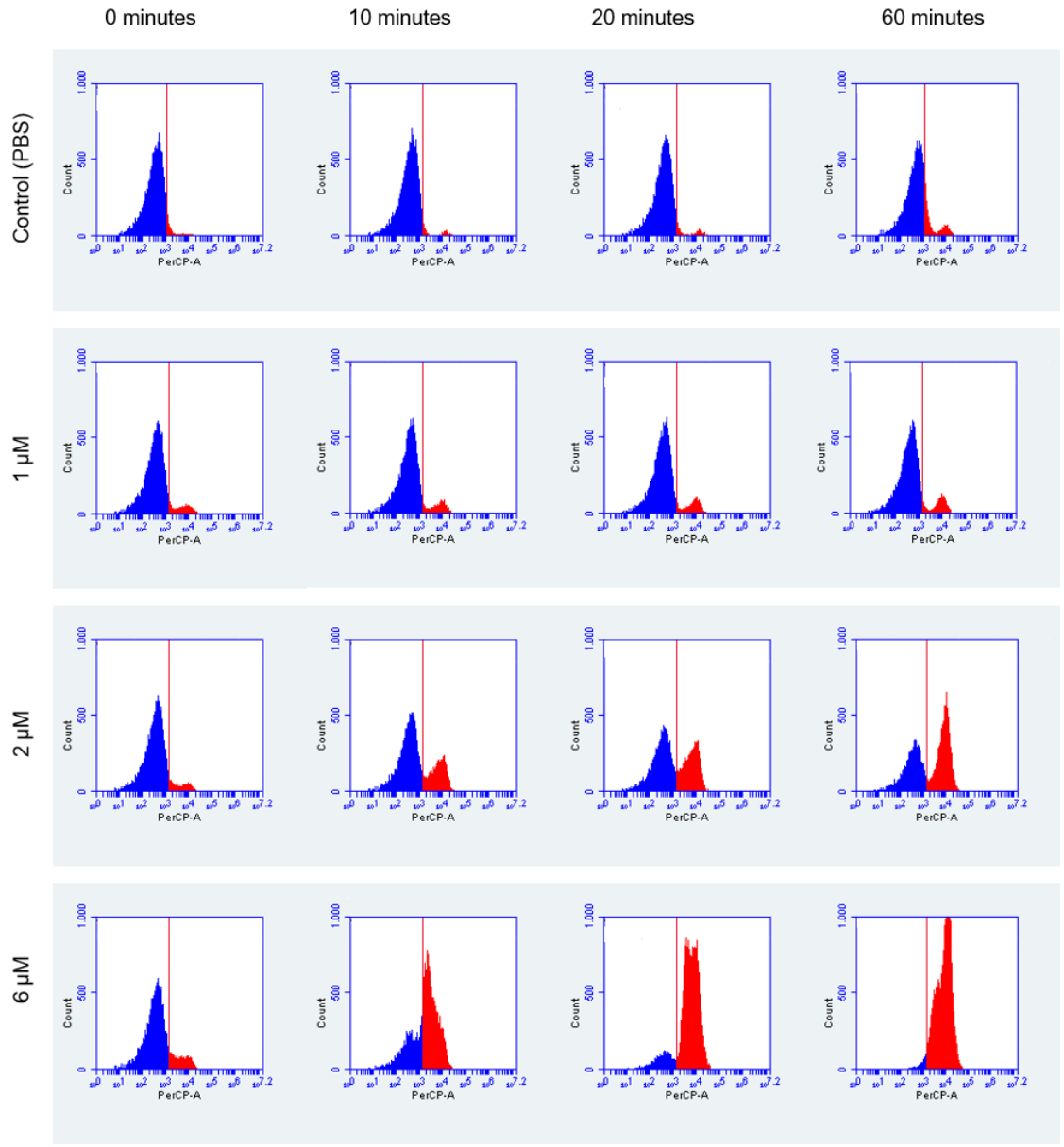
E) DSM 10604 (BP100)



F) DSM 10604 (3.1)



G) B65 (BP100)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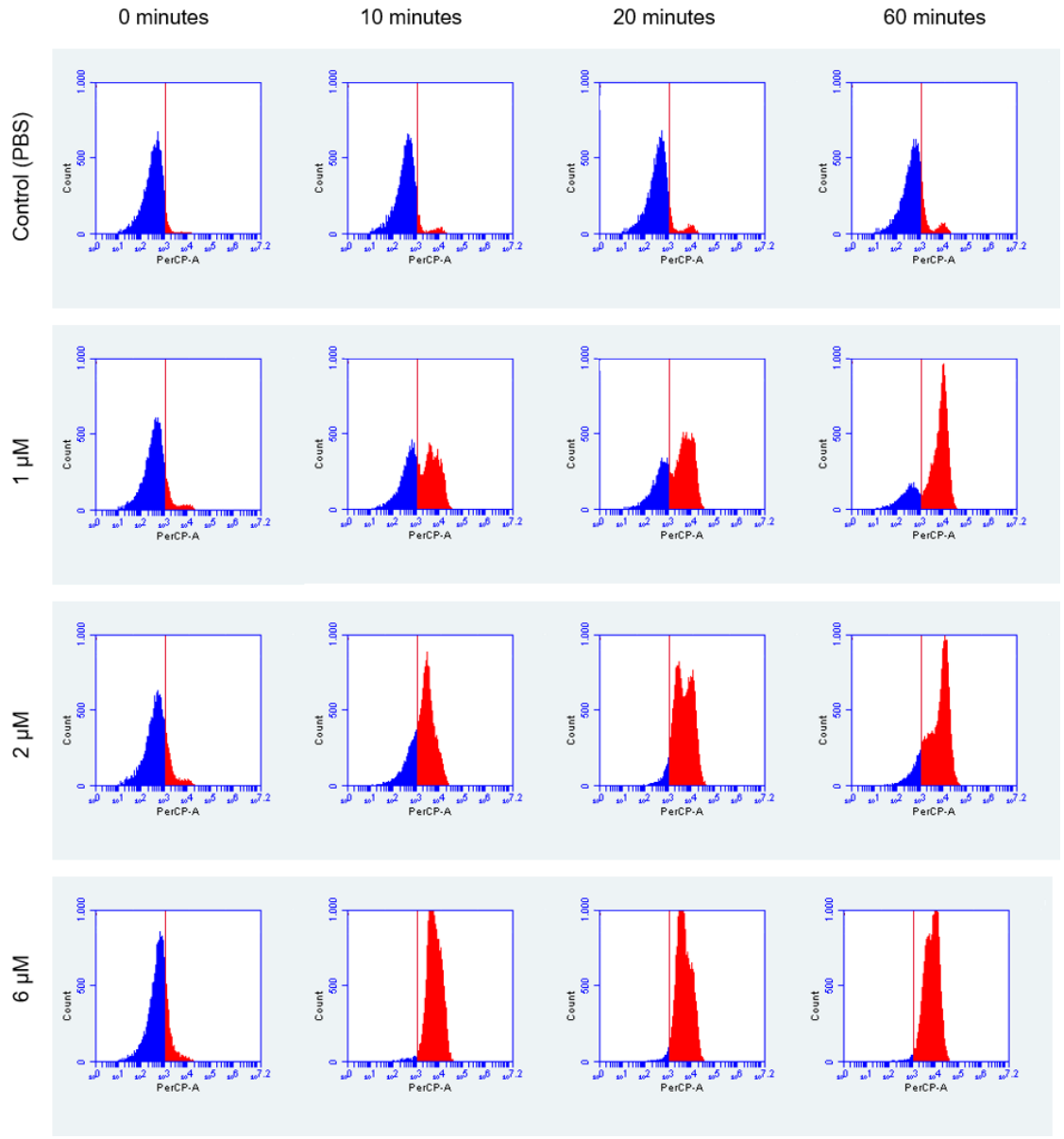
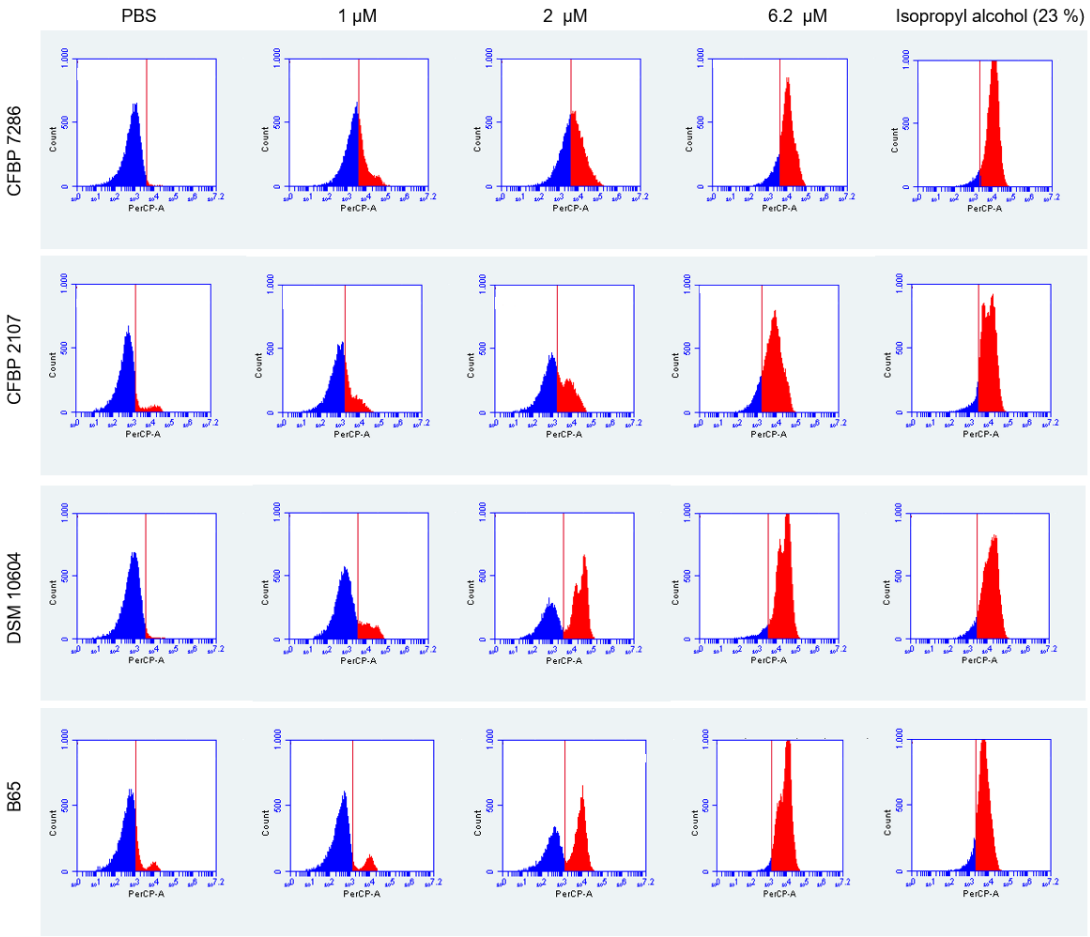
10 minutes
20 minutes
60 minutes
Control (PBS)
1 µM
2 µM
6 µM
Count
PerCP-A

H) B65 (3.1)

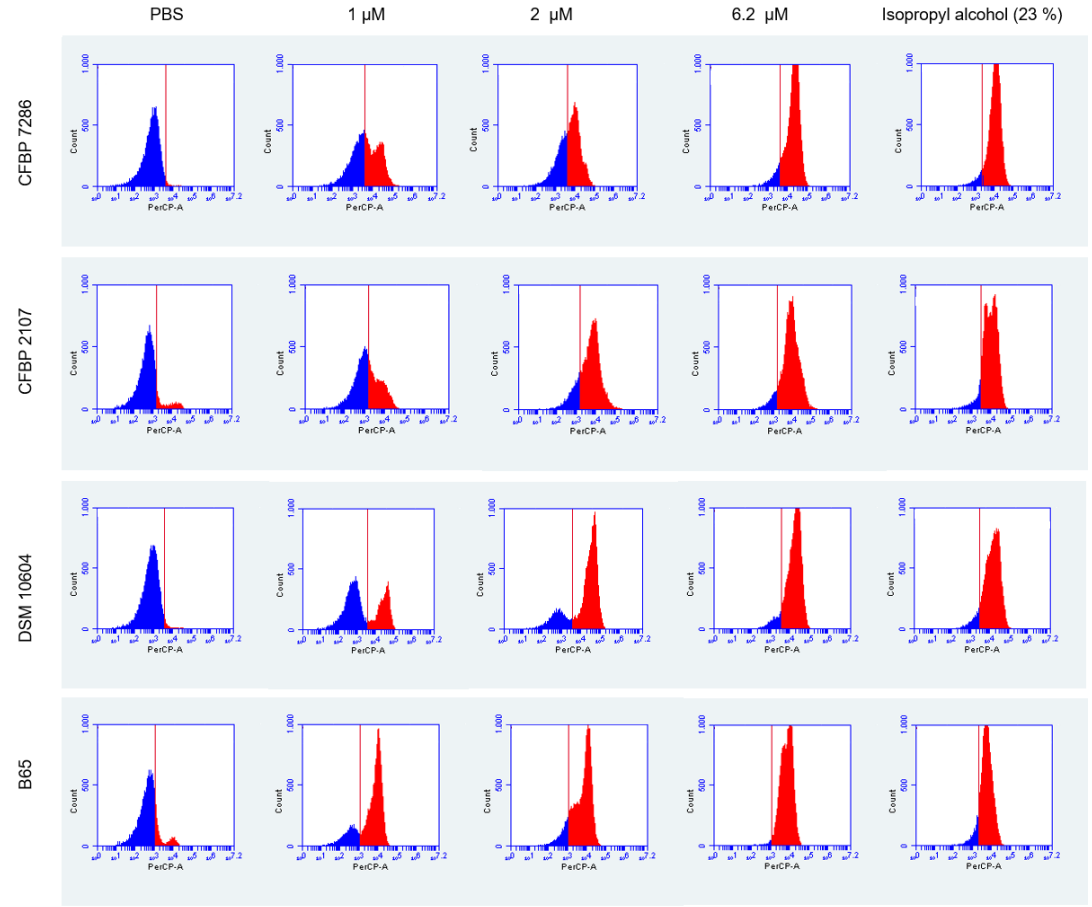

0 minutes
10 minutes
20 minutes
60 minutes
Control (PBS)
1 µM
2 µM
6 µM
Count
PerCP-A

4) Flow cytometry histograms of *Pseudomonas* spp. strains incubated with AMPs BP100 and 3.1 at 1, 2 and 6.2 µM and, isopropyl alcohol (23 %). Membrane permeabilization was determined by flow cytometry with PI. A) BP100 peptide; B) 3.1 peptide. PI fluorescence were detected with PerCP emission wavelength. Fluorescence were analysed after 60 minutes of incubation. Isopropyl alcohol (23 %) was used as positive control. The fluorescence intensity of PI above $1x10^3$ was considered as the non-viable cells.

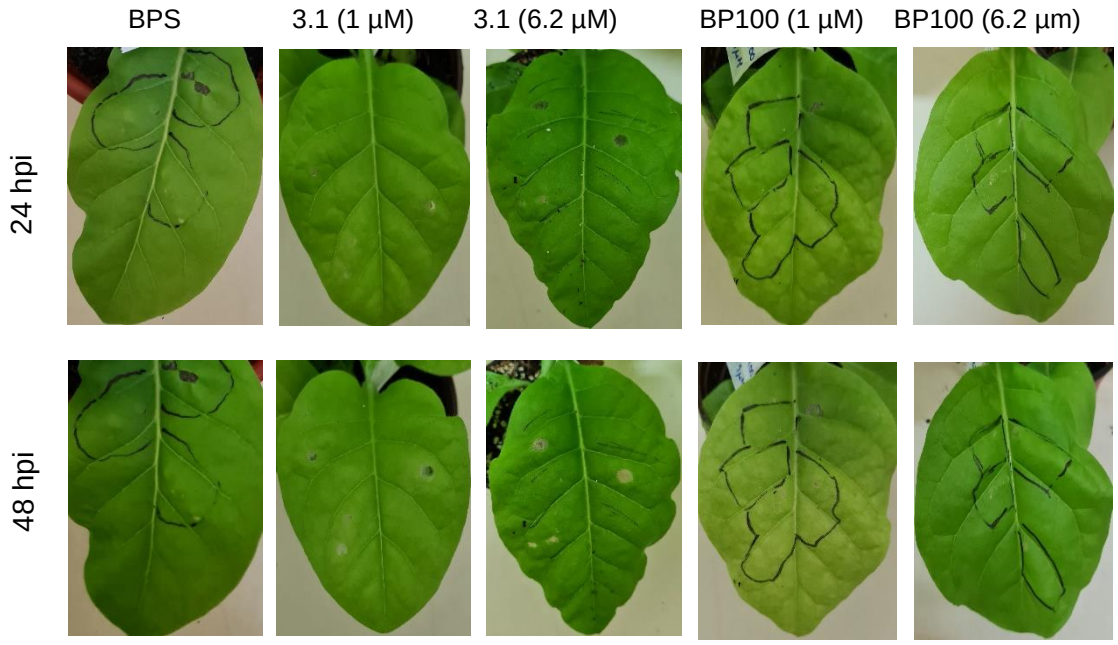
A) BP100 peptide



B) 3.1 peptide

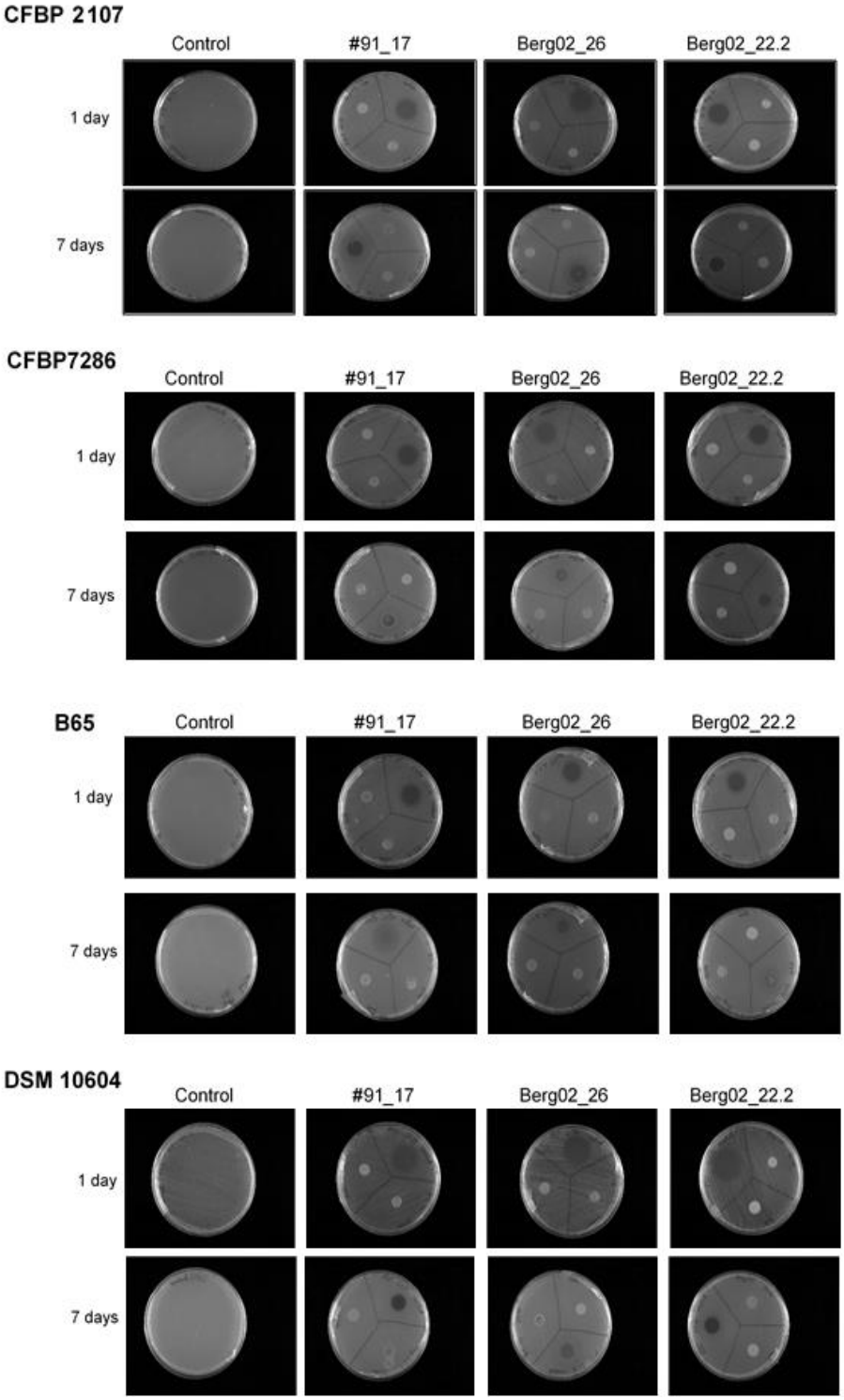


5) Tobacco leaves infiltrated with AMPs BP100 and 3.1 peptides at 1 µM and 6.2 µM. The data were recorded after 24 and 48 post-inoculation.



Annex V – *Actinobacteria* and *Planctomycetes*

1) Results obtained from contact bioactivity assay. Chlortetracycline (200 µg/mL) was used as positive control. Screening of antibacterial activity of strains of the phylum *Actinobacteria* (#91_17; Berg02_22.2 and Berg02_26) on phytopathogenic strains of genus *Pseudomonas* (FCBP 2107; CFBP 7286; B65 and DSM 10604). Results were recorded after 1 and 7 days of incubation period.



2) Percentage of inhibition produced by the 7 bacterial extracts against the phytopathogenic bacterial strains. Chlortetracycline (2.5 µg/mL) was used as positive control and demonstrated 100 % of inhibition. Extracts were obtained with 2 methods:

a) Crude extracts obtained with solid phase extraction protocol (n = 2).

Strain	[mg/mL]	B65		CFBP 2107		CFBP 7286		DSM 10604	
Berg02_ 22.2	2.5	- 34.71	- 15.08	49.96	36.63	37.30	29.50	- 17.47	- 1.43
	1.25	- 57.37	- 8.17	35.75	20.33	14.01	15.38	- 15.60	- 4.59
	0.63	- 37.19	- 8.11	20.24	4.87	3.99	1.34	- 11.58	- 4.44
	0.31	- 36.47	- 6.40	10.85	- 14.86	- 4.06	- 4.14	- 5.73	- 0.47
Berg02_ 26	2.5	- 27.28	- 32.03	57.08	72.96	49.25	58.24	- 16.04	8.50
	1.25	- 42.93	- 7.73	29.42	39.81	29.00	35.29	- 22.50	- 0.89
	0.63	- 41.46	- 7.45	20.90	27.32	18.40	10.90	- 20.93	- 5.70
	0.31	- 32.87	- 6.04	14.10	4.39	3.13	32.32	- 10.56	- 8.72
#91_17	2.5	47.63	58.30	71.23	63.35	53.97	63.54	47.60	24.14
	1.25	13.09	- 2.10	58.42	44.35	44.59	56.02	5.47	1.52
	0.63	- 30.96	- 10.38	41.43	17.36	32.63	40.88	- 16.73	- 8.31
	0.31	- 39.53	- 6.55	31.09	- 2.13	24.17	19.11	- 5.51	- 10.16
LZ-A1	2.5	41.92	41.47	72.78	78.09	56.42	74.54	50.76	14.34
	1.25	26.72	44.39	65.81	74.06	53.71	64.55	26.92	6.36
	0.63	30.67	- 8.93	58.47	55.32	49.13	55.69	6.60	0.38
	0.31	14.82	3.21	53.08	11.43	36.56	43.77	- 7.63	- 5.97
LF-2	2.5	46.01	26.05	69.42	53.36	58.04	35.25	34.06	26.05
	1.25	- 15.46	- 3.34	55.03	15.04	45.41	8.40	- 5.31	7.86
	0.63	4.00	2.69	41.76	- 36.24	32.95	- 22.09	- 7.74	- 0.91
	0.31	10.13	- 12.73	34.74	- 71.34	7.35	- 26.39	10.18	- 0.52
GR-7	2.5	- 22.63	- 6.62	68.22	54.49	55.91	44.66	63.08	73.89
	1.25	- 28.18	- 13.96	54.35	38.73	46.67	25.16	- 13.71	1.48
	0.63	- 26.20	- 8.54	42.11	21.12	23.29	27.05	- 14.13	- 0.66
	0.31	- 49.92	11.13	33.52	1.15	- 8.91	26.86	- 5.05	3.20

a) Crude extracts obtained with liquid-liquid phase extraction protocol (n = 2).

Strain	[mg/mL]	B65		CFBP 2107		CFBP 7286		DSM 10604	
Berg02_22.2	10	22.38	38.08	68.17	64.35	36.28	46.98	15.76	21.35
	5	- 5.70	-5.84	36.13	31.30	- 1.45	28.68	- 14.07	4.59
	2.5	- 7.84	-13.27	13.10	16.26	- 2.93	- 8.19	- 13.66	- 6.05
	1.25	- 8.38	-15.95	- 2.30	3.95	- 3.64	- 8.75	- 5.33	- 11.89
	0.63	- 3.56	- 30.29	- 6.31	0.54	- 12.55	- 17.56	- 4.61	- 3.88
Berg02_26	1	0.60	- 8.46	56.01	35.94	33.41	19.09	- 6.39	8.49
	0.5	- 0.09	-1.65	45.05	18.40	15.26	- 10.54	8.90	7.18
	0.25	1.24	- 23.09	27.07	10.59	26.59	- 11.21	10.21	2.25
	0.13	8.48	- 24.23	7.36	9.51	- 6.87	- 13.78	19.69	- 1.51
	0.06	8.33	- 25.16	- 4.27	1.08	- 15.16	- 14.15	12.71	- 0.38
#91_17	10	- 3.68	- 9.48	43.00	31.56	30.04	26.76	- 1.34	- 6.72
	5	- 8.53	- 17.38	17.47	24.50	22.36	6.48	- 5.73	- 4.82
	2.5	- 8.55	- 22.26	4.60	7.43	14.17	- 6.12	5.56	1.21
	1.25	- 3.94	- 22.36	- 3.59	5.03	20.52	- 1.29	4.81	- 0.60
	0.63	0.75	- 19.78	- 14.71	3.69	- 0.77	- 17.56	- 0.39	- 2.22
LZ-A1	1	58.80	61.35	75.08	79.80	57.57	63.61	80.15	81.75
	0.5	12.57	27.36	64.79	70.03	51.80	48.06	67.34	63.00
	0.25	- 3.34	- 3.54	44.47	49.59	42.76	39.89	50.10	17.99
	0.13	- 8.31	- 40.62	32.89	40.91	24.76	16.83	45.85	5.02
	0.06	- 8.15	- 48.96	18.40	32.58	30.11	11.27	33.79	- 8.52
LF-2	1	- 4.27	- 35.27	53.29	70.92	33.85	39.64	37.98	19.00
	0.5	- 5.76	- 45.13	34.93	55.57	29.54	32.22	26.78	- 2.94
	0.25	- 9.55	- 54.65	15.74	40.32	20.61	9.70	16.05	- 0.52
	0.13	- 3.77	- 47.09	3.31	23.79	23.96	10.42	- 4.52	- 9.98
	0.06	- 5.54	- 31.16	- 16.73	14.97	6.69	- 3.24	- 5.45	- 12.74
LZ-C2	1	- 1.08	8.09	88.95	91.03	76.89	62.26	77.20	51.96
	0.5	- 1.98	- 4.13	76.00	64.17	18.08	58.37	37.08	7.623
	0.25	4.48	- 3.50	62.66	48.50	2.25	49.84	2.88	- 1.63
	0.13	3.50	1.05	54.78	41.31	1.76	6.80	- 24.82	- 3.09
	0.06	5.04	- 1.13	53.77	36.94	- 1.04	9.00	- 35.02	- 10.18
GR-7	1	- 10.48	- 5.37	85.68	80.48	54.24	75.01	70.53	42.43
	0.5	- 26.00	- 20.25	72.01	75.75	19.51	44.43	39.12	13.12
	0.25	- 24.17	0.60	62.58	58.05	11.19	29.87	6.93	- 3.90
	0.13	- 21.39	0.55	55.59	39.36	2.56	6.42	- 18.69	- 10.14
	0.06	10.40	7.89	53.97	35.33	2.32	3.01	- 33.29	- 12.14