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ABSTRACT

Xylella fastidiosa and *Candidatus* Liberibacter are two highly widespread phytopathogens throughout the world that cause multiple diseases having a huge economic impact on agriculture. Although multiple studies aiming to controll these pathogens, no therapeutic solutions have been found for its reduction or suppression in infected plants. Antimicrobial peptides were identified as possible candidates of treating and preventing diseases caused by these pathogens because of their low cytotoxicity and wide range of antibacterial activity against a variety of phytopathogens. Antibacterial, bactericidal and viability assessment allow to identify AMP with high antibacterial activity against *Liberibacter crescens*. BP5, BP3, BP24 and BP31 are among AMPs that exhibited strong antibacterial activity against *Liberibacter crescens*, with MIC values in the range of 6.25-50 µM. BP5 has the overall highest antibacterial activity and has been selected to be tested *in planta* for the control of *Candidatus* Liberibacter in citrus plants. In parallel, a combined peptide treatment strategy (preventive and curative) confirmed that BP2 controls the growth of *Xylella fastidiosa subsp. pauca* in *Nicotiana benthamiana* plants, 24 days after pathogen inoculation.

Keywords: *Xylella fastidiosa, Candidatus* Liberibacter, phytopathogen, *Liberibacter crescens,* Antimicrobial peptides.

LIST OF ABBREVIATIONS

ACES	*N*-(2-Acetamido)-2-aminoethanesulfonic acid
AFT	Buffered physiological water
AMP	Antimicrobial peptide
ANOVA	Variation analytics
BCYE agar	Bufferd charcoal yeast extract agar
BP	Bactericidial Peptide
CFU	Colonial forming units
CT	Cycle treshold
DEPC water	Diethyl pyrocarbonate treated water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
FBS	Fetal Bovine serum
GC content	Procentual degree of guanine and cytosine againts total of bases
HCl	Hydrochloric acid
HLB	Huanglongbing disease
KOH	Potassium hydroxide
LB	Luria Bertani media
MATAB	Mixed alkyl trimethyl ammonium bromide
MIC	Minimum inhibitory concentration
min	minutes
mg	Miligrams
ml	Mililiters
MKC	Minimal killing concentration
NaOH	Sodium hydroxide
NTC	Non treated control
OD	Optical density
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
rpm	Revolutions per minute
SYBR Green	$C_{32}H_{37}N_4S^+$
TAMRA	Tetramethylrhodamine
Taq polymerase	Thermus aquaticus polymerase
TBS	Tryptone soya broth
T_m	Melting temperature
TNM-FH	Grace insect media
V	Volt
µl	Microliters
µM	Micromolars

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INTRODUCTION

Crops around the world are subject to infection and damage from bacteria, viruses and fungi. There is a need to control plant pathogens to ensure the quality and quantity of all agricultural production [1]. Conventional plant disease control has been routinely achieved by applying high amounts of synthetic chemical pesticides which are subject to strong regulations and restrictions to reduce their toxicity and environmental impact. In addition, plants often develop resistance to fungicides or bactericides that may compromise disease control [2]. The active components of these pesticides, insecticides and fungicides are getting more and more reduced because of regulations. There is a strong need to develop novel compounds with antimicrobial activity that can fulfill the strict regulations about phytotoxicity, environmental impact and biodegradability included in national regulations mainly in USA and Europe [3].

Antimicrobial peptides to control bacterial and fungal diseases

Antimicrobial peptides (AMPs) are an alternative for plant disease management and comply with regulations and restrictions that the other disease controls have trouble with [4]. These regulations are all set by the European Commission [5] who work together with the European Food Safety Authority or EFSA [6]. All the control measures that do not live up to the strict regulations of the European Commission are no longer viable options to use against plant pathogen. However, due to its physicochemical characteristics, AMPs can fulfill these strict regulations. AMPs are small peptide sequences of generally 12-50 amino acids. These AMPs include positively charged residues provided by arginine, lysine or, in acidic environments, histidine, and a large proportion (generally >50%) of hydrophobic residues conferring a net positive charge (cationic peptides). AMPs have the ability to adopt an amphipathic conformation that gives them stability in both aqueous and hydrophobic environments. These AMPs are present in all living-organisms, from the smallest prokaryotes to mammals including humans [7]. AMPs being considered to be the first response of the innate immune system. Apart from their higher biodegradability compared to most pesticides, they are stable at high temperatures and of course have high antimicrobial activity (Minimal inhibitory concentration (MIC) at the same order than conventional antibiotics) which is all good criteria for a sustainable disease control methods[8].

A widely accepted mode of action of AMPs include a direct electrostatic interaction with the cell membrane that could reform its structure to make holes in the membrane and induce apoptosis or necrosis trough: membrane depolarization, micellization and diffusion of peptide to intracellular targets (**¡Error! No se encuentra el origen de la referencia.¡Error! No se encuentra el origen de la referencia.**).

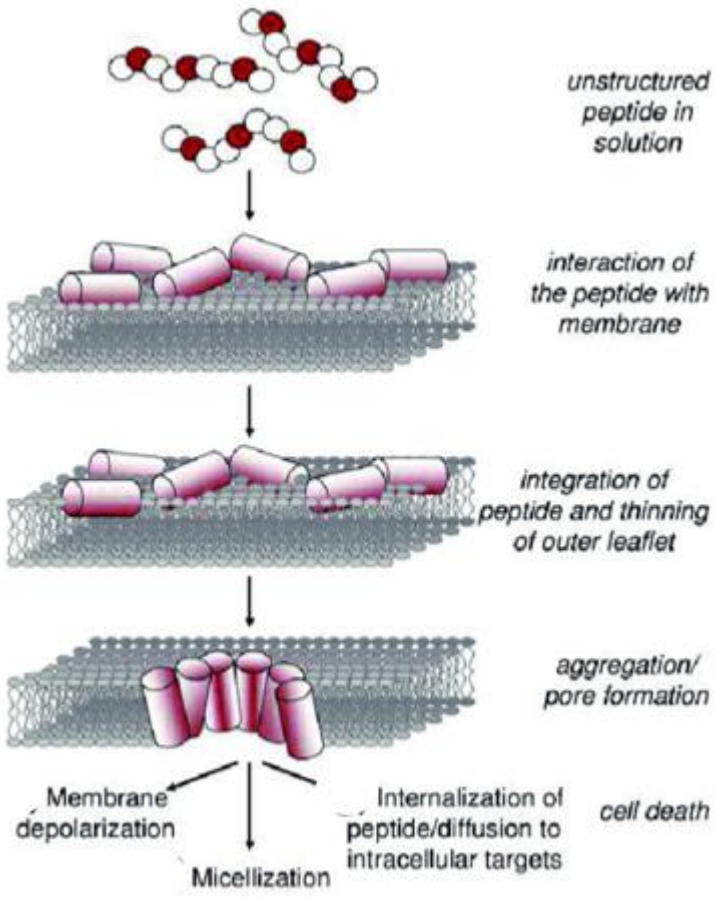

Figure 1: Cationic antimicrobial peptide mechanism of action [9]

AMPs have a lower probability of developing bacterial or fungal resistance unlike antibiotics. This is because the antibiotics attack the microorganisms directly and always via the same way, this creates resistance to the antibiotics. As AMPs display multiple modes of action, the emergence of resistant mechanisms in target pathogens would require significant alteration of the membrane composition. Because of this, bacteria and fungus have a harder time developing resistance to AMPs [10].

The innate immune system can produce AMPs trough ribosomal synthesis or non-ribosomal synthesis. Despite their diversity and advantages they still have limitations to plant disease control as not all natural AMPs shows efficacy in controlling plant diseases [4]. It is also difficult to obtain these natural AMPs because it is only produced by organisms in low concentrations. Along with this, modifications were made to the natural AMPs to produce more efficient synthetic AMPs that have better selectivity, less cytotoxicity and require lower concentrations for higher antimicrobial activity. To make these modifications the amino acid sequence natural AMPs were used as a model template to synthesize AMPs with improved physicochemical characteristics. It is important to have in consideration that any changes in the amino acid sequence of AMPs can alter their mechanism of action, and consequently their antimicrobial activity. The synthetic AMPs still needed to be able to make direct electrostatic interactions from there positive charges to the anionic surface of the target cell [11]. In this study, newly designed and synthesized AMPs (CIDSAV and LIPPSO research groups at the University of

Girona) were used to evaluate their antimicrobial activity against *Liberibacter crescens* and *Xylella fastidiosa*. The codes and sequences of the AMPs are not named in this paper due to confidentiality, they are referred as BP1,2,3,4,5 – 37-

The aim of this study was to identify synthetic antimicrobial pepides exhibiting high antimicrobial activity against *Liberibacter crescens* and *Xylella fastidiosa.* Both bacteria have a big impact or are related to bacteria that have a big impact on the agriculture around the globe.

The *Candidatus* Liberibacter phytopathogen is the causal agent of the Huanglongbing disease (HLB) or more commonly known as the citrus greening disease/yellow-shoot-disease [12]. HLB is a quarantine pathogen that causes blockages in the vessels of the plant reducing sap flow avoiding the necessary nutrient intake to leaves and fruits/flowers and plants can die prematurely. The symptoms of HLB are: yellowing of the veins and adjacent tissue of the leaves followed by mottling of the entire leaf(Figure 2), premature defoliation, decay of feeder rootlets and lateral roots, decline in vigor and followed by death of the plant [13].

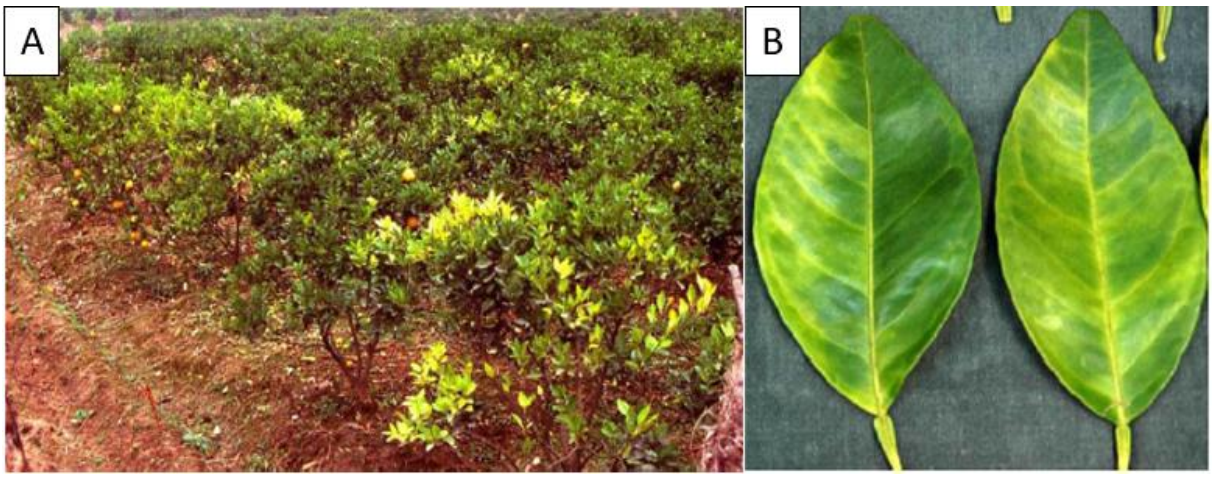

Figure 2: A) Symptoms of HLB in trees, B) yellowing of the veins and adjacent tissue of the leaves [13]

HLB is considered to be the most destructive bacterial disease in the citrus industry. It is carried over via psyllids or jumping plant lice [13]. HLB is present in Asia, Africa, Oceania and America but not in Europa. To prevent an epidemic of this pathogen in Europa, the present study was carried out with *Liberibacter crescens* (*Lc*), a non-pathogenic bacterium closely related to members of the genus *Candidatus* Liberibacter. *Lc* is the only member of the genus that can be grown in a media outside of plants [14]. For this reason, *Lc* has become a model organism for studying the plant pathogenic *Candidatus* Liberibacter. *Lc* has only been isolated once from an environment in Puerto Rico in a mountain papaya [15]. The pathogens are located in the vessels of the plant namely the phloem (Figure 3). Because the pathogen is located in the vascular vessels of the plant, conventional treatments like copper that are usually applied by spraying (and are not systemic) shows no or less efficacy in controlling this type of bacteria in comparison to other epiphytic bacteria as *Erwinia amylovora* or *Pseudomonas* sp. or *Xanthomonas* sp. among others. See the working of copper treatment in figure below (Figure 4).

Currently the common technique used for preventing spread of HLB between the plants is to control the psyllids population with insecticides and pesticides and destroying all the plants and trees that show symptoms [16] but there are a lot of new treatments in development like: gene modification of plants, gene modification of the psyllids or the use of AMPs among others.

In this study, the antimicrobial activity of synthetic AMPs against *Liberibacter crescens* was evaluated with the aim to identify AMPs that can be used in controlling HLB in infected plants [17].

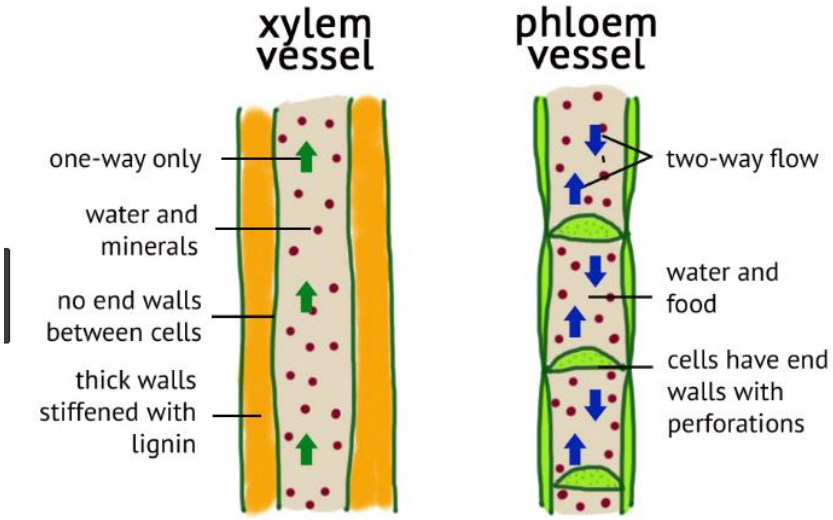

Figure 3: Difference between xylem and phloem vessels [18]

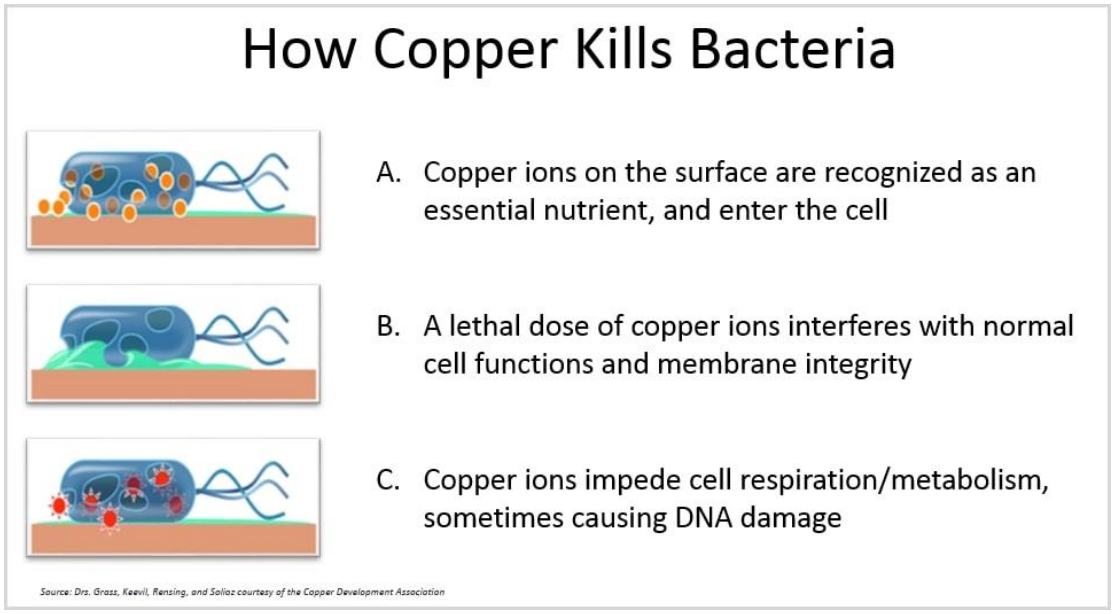

Figure 4: Copper bacteria killing mechanism [19]

Xylella fastidiosa or *Xf* is a plant pathogen responsible for multiple diseases in different host plants as Pierce's disease of grapevines, phony peach disease, plum leaf scald, citrus variegated chlorosis disease and olive scorch disease [20]. Its symptoms are: in Pierce's disease shirveled fruit, leaf scorching and premature abscission of leaves (Figure 5); in citrus variegated chlorosis disease the symptoms are: small and hard fruits, spotty chlorosis (Figure 6)(chlorosis is a condition of the plant when it doesn't make enough chlorophyll to stay green and turns yellow/white) of the citrus leaves [21].

Figure 5: A) Leaf scorching on almond leaves, B) shriveled grapes by Pierce disease [21]

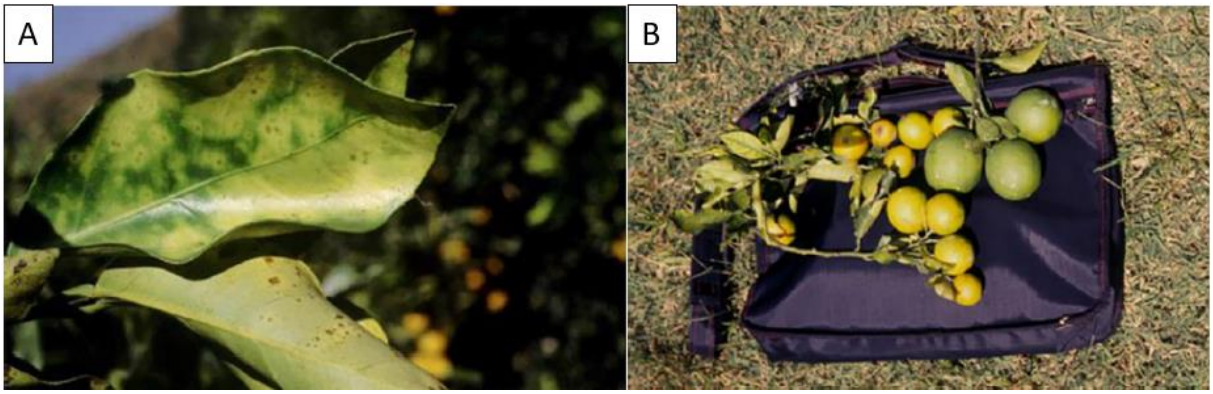

Figure 6: A) Spotty chlorosis on orange leaves, B) On the left side small and hard fruit and on right side healthy fruit (Citrus variegated chlorosis) [21]

The other diseases have the same symptoms but in other plants like the coffee plant or peach trees. *Xf* works almost in the same way as *Lc* does when infecting plants. It travels through the xylem vessels (Figure 3) of the plant and creates biofilm layers with the xylem cells to create blockages in the vessels that restrict sap flow and cut off important nutrients from the plant [20]. *Xf* is carried over by a range of xylem-feeding insects. This pathogen is present in all the continents around the globe so with this pathogen could be worked with in the quarantine greenhouse. Like *Lc*, conventional treatments like antibiotics and copper are difficult to use on this pathogen because of its location inside the vascular vessels [22]. Its only treatment plan for preventing spread of *Xf* is by eradication of the infected plants and carrier insects [23]. AMPs have been identified in earlier studies to be a potential candidate in the disease control [7]. In this study, the efficacy of AMPs in controlling *Xf* infections in *Nicotiana benthamiana* plants is evaluated. As *Xf* resides in the xylem tissue, for both AMP application and *Xf* inoculation, a high precision microinjector (NanoJet, Chemyx, Stafford, USA) was used to access the vascular vessels(Figure 7).

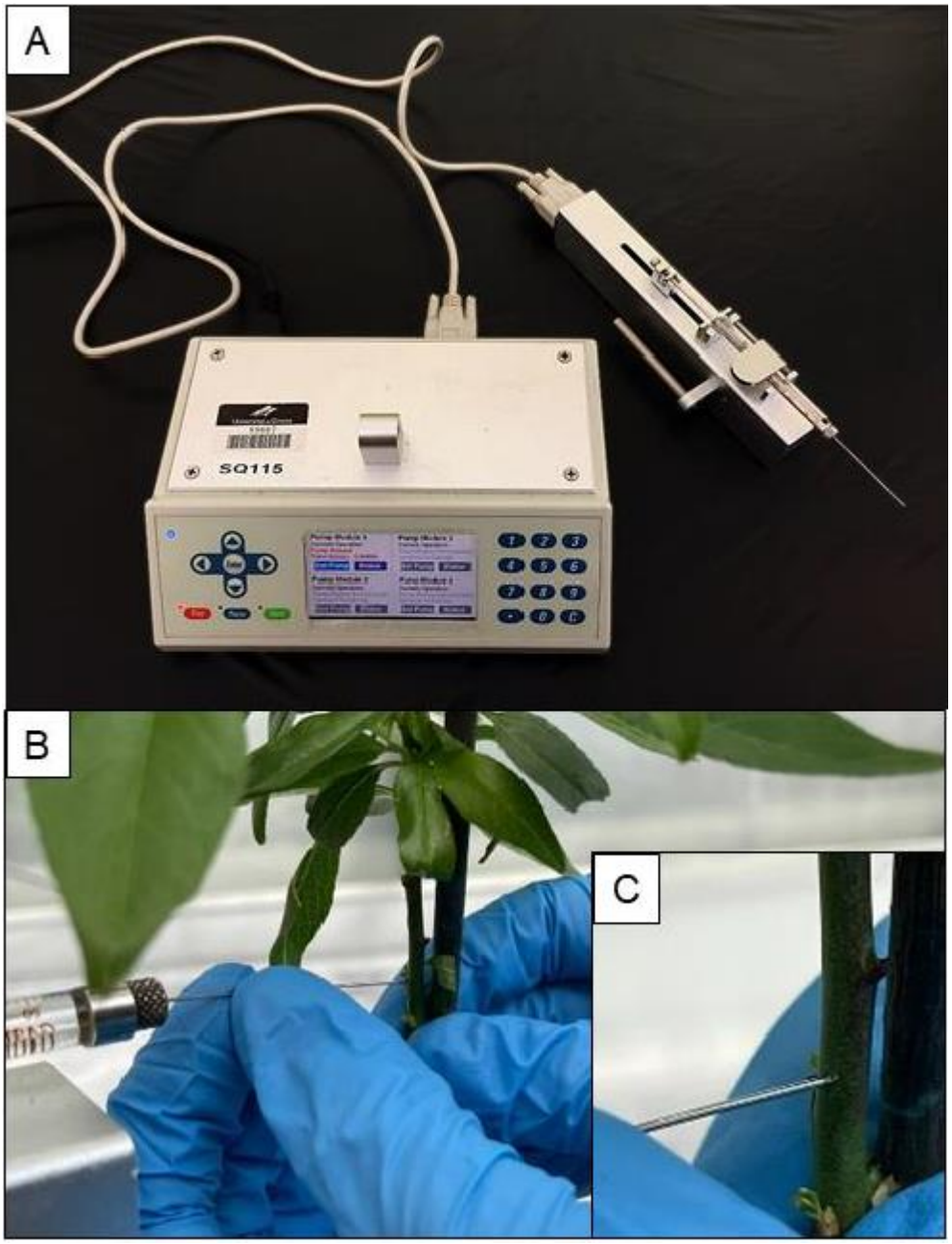

Figure 7: A) High precision microinjector (NanoJet, Chemyx, Stafford, USA) used together with a Hamilton 250 µL syringe with needle and bevel tip, B) Inoculation of Xf in one-year old almond plants, C) Zoom of inoculation process [24]

Alternative treatments for *Xf* and *Candidatus* Liberibacter infections

Candidatus Liberibacter

Other studies have identified antimicrobial compounds with antimicrobial activity against *Candidatus* Liberibacter. The University of California identified a novel class of stable antimicrobial peptides from *Microcitrus australiasica* that rapidly kill *Liberibacter crescens* [25]. In China, at the Guanxi University, there were 8 antimicrobial compounds identified that are effective against *Candidatus* Liberibacter asiaticus namely: aluminum hydroxide, D,L-buthionine sulfoximine, nicotine, surfactin from *Bacillus subtilis*, SilverDYNE, colloidal silver, EBI-601, and EBI-602 [26]. In another study, the efficacy of Nano-Zinc Oxide and their combinations demonstrated against *C*Las viz. 2S albumin (ICAR-Central Citrus Research Institute together with Indian Institute of Technology Roorkee and the University of Central

Florida)[27]. Also, a study in China by the Yunnan Agricultural University and National and Local Joint Engineering Research Center for Screening and Application of Microbial Strains that have researched the effectivity of endophyte *Bacillus subtilis* L1-21 in the management of diseases caused by *Candidatus* Liberibacter asiaticus [28].

Xylella fastidiosa

The following studies have identified possible antimicrobial compounds that work against *Xylella fastidiosa.* A study from the University of Florida, New Mexico Consortium Biolab, Boyce Thompson Institute in Ithaca have identified Menadione- and Benzethonium Choride as potential treatment methods that work for Pierce disease caused by *Xylella fastidiosa* [29]. The EFSA panel on plant health has also conducted a study to asses the effect of zinc, copper, and citric acid biocomplex, of N-acetylcysteine, and of 'diffusible signal factor'. These control measures were sometimes able to reduce the symptoms caused by *Xf* [30]. A antagonistic bacteria like *Bacillus* strains were used in a study from The Institute for Sustainable Plant Protection and the Institute of Sciences of Food Production in Italy to use as biocontrol agents against *Xf* in olive trees [31]. As a last example of alternate antimicrobial compounds to use against *Xf* was a study from the Centro de Citricultura Sylvio Moreira, Universida de Estadual de Campinas, , Universida de Federal de São Carlos, Brazil that has investigated the inhibitory effect of N-Acetylcysteine, a cysteine analogue used mainly to treat human diseases, on *Xf* in different experimental conditions [32].

CONTENT

DETAILS PLACEMENT

ABSTRACT

INTRODUCTION

1 OBJECTIVE AND SCOPE

The main objective of this internship was to identify antimicrobial peptides with high antibacterial activity against the quarantine plant pathogens *Xylella fastidiosa* and *Candidatus* Liberibacter. Specific objectives were:

- To evaluate the *in vitro* antimicrobial activity of synthetic antimicrobial peptides against *Liberibacter crescens*.

- To study the mode of action of synthetic antimicrobial peptides in *Liberibacter crescens* cells.

- To evaluate the *in planta* efficacy of synthetic antimicrobial peptides in controlling infections caused by *Xylella fastidiosa* in *Nicotiana benthamina* plants.

To the impossibility to artificially inoculate citrus plants with Candidatus *Liberibacter* suspension (quarantine pathogen not present in the EU), the patosystem *Xylella fastidiosa*-*Nicotiana benthaminana* was used to study the efficacy of the AMP in controlling infections developed after artificial inoculation.

2 MATERIALS AND METHODS

2.1 MICROBIOLOGICAL TECHNIQUES AND MATERIALS

The materials used in the experiments on microorganisms were subjected to an autoclave cycle of 20 minutes on 121 °C for non-quarantine pathogens. For the quarantined pathogens an autoclave cycle of 25 minutes on 125 °C was used. In addition, all the experiments were carried out in a laminar flow cabinet to ensure a sterile environment after applying UV radiation for 15 minutes before use.

As *Liberibacter crescens* (*Lc*) is a strain closely related to the quarantine pathogen *Candidatus* Liberibacter (List A1 of the EPPO), and considered a quarantine pathogen in other EU countries. The handling of *Lc*, *Xylella fastidiosa* (*Xf)* and the antimicrobial products were carried out in a laboratory of SL2 level biosafety (EPPO criteria), following strict quarantine measures, and in a biosafety greenhouse. The biosafety laboratory and greenhouse are authorized by the General Directorate of Agriculture and Livestock, of the Ministry of Agriculture of the Generalitat de Catalunya, for research with quarantine pathogens in the European Union (level A2), and specifically for *Xf*.

2.1.1 Bacterial strains used in the experiments

The bacterial strains used in the experiments were *Lc* strain BT1 (kindly provided by Ester Marco, IVIA, Valencia) and *Xf* subsp. pauca strain DeDonno (Maria Saponiari, CNR-IPSP, bari, Italy).

2.1.2 Culture conditions

Liberibacter crescens

For growing *Lc* the bacterial strain was spread out over a BM7-agar plate and incubated on 28 °C for 1 week. The BM7-agar plate contains: Fetal bovine serum, Grace's insect medium (TNM-FH), α-ketoglutaric acid, ACES-buffer (N-(2-acetamido)-2-aminoethanesulfonic acid) and potassium hydroxide (concentration are detailed in section 2.4.2) at pH 6.8 (BM7 needed to be made at a pH of 6,8 because changes in the pH can kill the *Lc* during the stationary phase). During incubation the plates were wrapped with parafilm to prevent moisture loss.

Xylella fastidiosa

The efficacy of the antimicrobial peptides was evaluated against *Xf* subsp. *pauca* De Donno. The strain was deposited in the CIDSAV quarantine pathogens collection. The inoculum was obtained from an actively growing pure culture after seeding on BCYE agar (concentration is detailed in section 2.4.5) and incubation at 28 °C for 7-10 days. The bacterial suspension was prepared at a final concentration of 1x108 CFU/ml. The pathogen was inoculated by microinjection applied to the trunk of the plant, making a total of 3 applications of 10 µl each (three inoculation points in a section of 4-5 cm, 3x106 CFU / plant). Precision injection equipment was used namely NanoJet, Chemyx, Stafford, USA.

2.1.3 Conservation of the bacterial strain

All the strains were stored at -80 °C. Briefly, *Lc* was resuspended in liquid BM7 medium containing 20 % of glycerol to protect the cells. This suspension was then transferred to sterile cryotubes and left at room temperature for 2 hours. The same methodology was followed for

the *Xf* strain, resuspending in liquid PD2 media, instead of BM7. Both bacterial strains were stored at at -80 °C.

2.1.4 Preparation of the bacterial inoculum

Liberibacter crescens

The bacterial suspension was made from living *Lc* growing on a BM7-agar plate for 1 week at 28 °C. The bacterial biomass was collected via an inoculation needle and resuspended in sterile AFT buffer stored at 28 °C. The suspension was homogenized by pipetting and not by vortex because *Lc* is sensitive to vortexing. The bacterial suspension was adjusted to 1.0 O.D. (optical density) what correlates to a bacterial concentration of ~ 10^9. This optical density was measured by a spectrophotometer at a wavelength of 600 nm.

When necessary a bank of serial dilutions was made from the bacterial suspension. 100 µL of initial suspension (DO of 1) was diluted in 900 µL of sterilized AFT buffer to a final volume of 1 ml. The process was repeated as many times as dilutions were needed (each dilution decreased a log the bacterial concentration).

Xylella fastidiosa

The bacterial suspension was prepared from living *Xf growing* on BCYE agar plate for 1 week at 28 °C. The bacterial biomass was collected via an inoculation needle and resuspended in DEPC water stored at 28 °C. The suspension was homogenized by pipetting and not by vortex (*Xf* is sensitive to vortexing). The bacterial suspension was adjusted to 0,3 O.D. what correlates to a bacterial concentration of ~ 10^8. This optical density was measured by a spectrophotometer at a wavelength of 600 nm.

When necessary a bank of serial dilutions was made from the bacterial suspension. 100 µL of initial suspension (DO of 0,1) was diluted in 900 µL of DEPC water to a final volume of 1 mL. The process was repeated as many times as dilutions were needed (each dilution decreased a log the bacterial concentration).

2.1.5 Preparation of peptides dilution

Antimicrobial peptides were prepared at different dilutions depending on the assay. The stock concentration of each peptide was 10 mM and the needed concentration of peptides varies between 2 mM to 62,5 µM. In most cases 1 ml of each peptide dilution was prepared. As example, to prepare peptides at 1 mM, 800 µl of DEPC water mix with 200 µl of 10 mM of the corresponding peptide.

2.1.6 *In vitro* antibacterial activity assessment of synthetic antimicrobial peptides [33]

Peptide antibacterial activity was tested according to the following protocol. Lyophilized peptides were solubilized in double distilled H_2O to a final concentration of 1 mM and filter sterilized through a 0.2 µm pore filter (Whatman®). For minimum inhibitory concentration (MIC) assessment, dilutions of the synthetic reference peptides were made to obtain a final concentration of 200, 100, 50, 25, 12.5, 6.25 µM. Twenty µL of each dilution were mixed in a microtiter plate with 20 µL of the corresponding suspension of *Lc* and added to 160 µL of Tryptone soya Broth (TBS) (Biòmereux, France) to obtain a total volume of 200 µL. Three replicates for each strain, peptide and concentration were used (Figure 8). Also, positive controls containing water instead of peptide and negative controls containing peptide without bacterial suspension were included. Bacterial cell concentration in suspension was

automatically determined by optical density measurement at 600 nm (Bioscreen C, Labsystem, Helsinki, Finland). Microplates were incubated at 28 °C with 20 s shaking before hourly absorbance measurement for 48-168 h. The MIC value was taken as the lowest peptide concentration with no growth at the end of the experiment.

PLACA 1

	directe				1/10					
	1	11	21	31	41	51	61	71	81	91
A	BP1_200	BP1_25	BP2_200	BP2_12,5	BP1_200	BP1_25	BP2_200	BP2_12,5		
B	BP1_200	BP1_25	BP2_100	BP2_12,5	BP1_200	BP1_25	BP2_100	BP2_12,5		
C	BP1_200	BP1_12,5	BP2_100	BP2_12,5	BP1_200	BP1_12,5	BP2_100	BP2_12,5		
D	BP1_100	BP1_12,5	BP2_100	BP2_6,25	BP1_100	BP1_12,5	BP2_100	BP2_6,25		
E	BP1_100	BP1_12,5	BP2_50	BP2_6,25	BP1_100	BP1_12,5	BP2_50	BP2_6,25		
F	BP1_100	BP1_6,25	BP2_50	BP2_6,25	BP1_100	BP1_6,25	BP2_50	BP2_6,25		
G	BP1_50	BP1_6,25	BP2_50	C+	BP1_50	BP1_6,25	BP2_50	C+		
H	BP1_50	BP1_6,25	BP2_25	C+	BP1_50	BP1_6,25	BP2_25	C+		
I	BP1_50	BP2_200	BP2_25	C+	BP1_50	BP2_200	BP2_25	C+		
J	BP1_25	BP2_200	BP2_25	C- medi	BP1_25	BP2_200	BP2_25	C- medi		

PLACA 2

	directe				1/10					
	1	11	21	31	41	51	61	71	81	91
A	BP4_200	BP4_25	BP3_200	BP3_12,5	BP4_200	BP4_25	BP3_200	BP3_12,5		
B	BP4_200	BP4_25	BP3_100	BP3_12,5	BP4_200	BP4_25	BP3_100	BP3_12,5		
C	BP4_200	BP4_12,5	BP3_100	BP3_12,5	BP4_200	BP4_12,5	BP3_100	BP3_12,5		
D	BP4_100	BP4_12,5	BP3_100	BP3_6,25	BP4_100	BP4_12,5	BP3_100	BP3_6,25		
E	BP4_100	BP4_12,5	BP3_50	BP3_6,25	BP4_100	BP4_12,5	BP3_50	BP3_6,25		
F	BP4_100	BP4_6,25	BP3_50	BP3_6,25	BP4_100	BP4_6,25	BP3_50	BP3_6,25		
G	BP4_50	BP4_6,25	BP3_50	C+	BP4_50	BP4_6,25	BP3_50	C+		
H	BP4_50	BP4_6,25	BP3_25	C+	BP4_50	BP4_6,25	BP3_25	C+		
I	BP4_50	BP3_200	BP3_25	C+	BP4_50	BP3_200	BP3_25	C+		
J	BP4_25	BP3_200	BP3_25	C- medi	BP4_25	BP3_200	BP3_25	C- medi		

Figure 8: Example of templates for antibacterial activity tests

2.1.7 Viability assessment of *Liberibacter crescens* cells after peptide treatment.

For the determination of the viability of the *Lc* cells after the peptides treatments, an assay was performed with the compound resazurin. Resazurin is a compound that was used as an indicator REDOX (oxidation-reduction) based on detection of cell metabolic activity. Resazurin assays are based on the reduction by living cells of the oxidized blue dye to a pink fluorescent resorufin product. This fluorescens was measured by Varioskan (Thermo Fischer Scientific). After the antimicrobial test was completed (section 2.1.6), it was determined whether the cells of each treatment are alive by resazurin test.

To carry out the test 90 µl were taken from each well of the antibacterial activity test and transferred to a new well in a Varioskan plate (96 wells), 10 ul of the resazurin product (5µM) was added to each well and incubated for 3 hours at 28 ° C with a shaking of 150 rpm. After 3 hours samples were exposed to an excitation wavelength of 560 nm and tracking emission wavelength of 590 nm, by multimode plate reader Varioskan for determine the quantity of fluorescence for each treatment.

2.1.8 Screening of bactericidal activity of antimicrobial peptides

Bactericidal activity of chemically synthesized peptides was assessed by a contact time exposure test of the pathogenic bacteria to a peptide solution in water. This method is a killing assay which differs from continuous exposure (bacteriostatic assay) used to screening of antibacterial activity (Growth inhibition test, section 2.1.6).

Lyophilized peptides were solubilized in double distilled H_2O to a final concentration of 10 mM and filter sterilized through a 0.2 µm pore filter (Whatman®). For minimum killing concentration (MKC) assessment, dilutions of the synthetic reference peptides were made to obtain a final concentration of 200, 100, 50, 25, 12.5, 6.25 µM. 50 µL of each dilution were mixed in a microtiter plate with 450 µL of the corresponding suspension of *Lc* to obtain a total volume of 500 µL. Three replicates of each peptide and concentration were used as well as positive controls containing AFT buffer instead of peptide and negative controls containing peptide without bacterial suspension.
Tubes were incubated at 28 °C with shaking and the MKC was evaluated by viable plate counting method (three times: 2h, 24h, 48h) value was taken as the lowest peptide concentration with no growth at the end of the experiment (Figure 9).

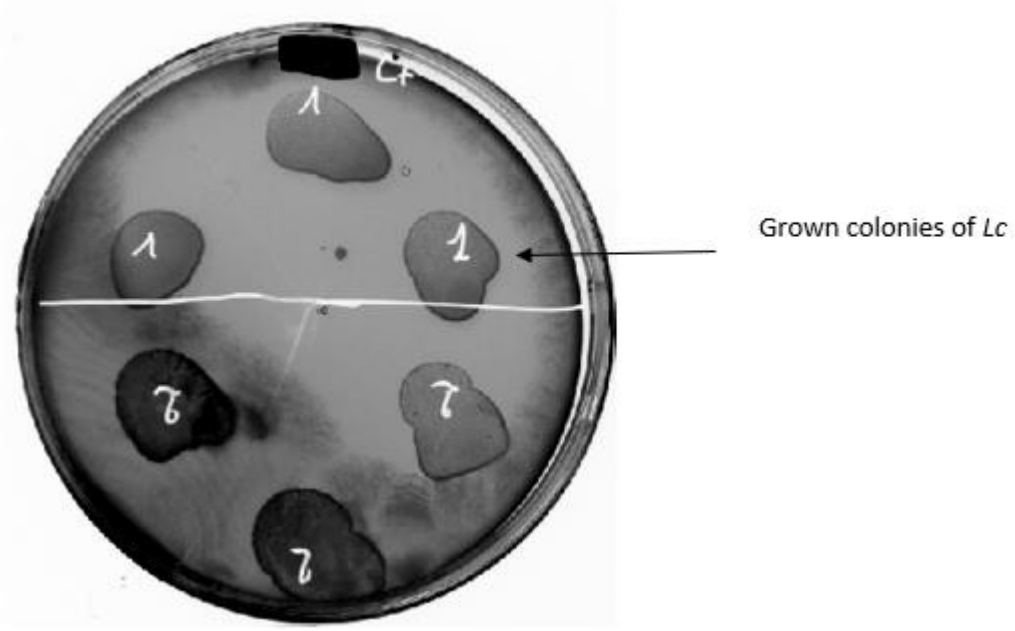

Figure 9: Growth of Lc in BM7 agar plates.

2.2 PLANT MATERIAL

2.2.1 Preparation and cultivation of the *Nicotiana benthamiana* plants

The efficacy of antimicrobial peptides in controlling diseases caused by *Xf* in potted *Nicotiana benthaminana* plants was evaluated. Seeds of *N. benthaminana* plants were sown in a seedbed with substrate, germinated and grown under controlled greenhouse conditions. Three-week old seedlings were then transplanted into 250 ml pot containers filled with substrate. After three weeks, the corresponding peptide treatments were applied to *N. benthaminana* plants.

2.2.2 Nutrients and grow conditions

The substrate and growth conditions are detailed below:
Substrate composition: Fertilized substrate (Substrat 1, Klasmann Deilmann), supplemented with number 3 vermiculite (Europerlita Española) and Osmocote (NPK 11:11:13 +2MgO, Scotts). The soil substrate surface was kept well-watered at all times by underwatering.
Controlled environment conditions: 25±2 ºC, 16 h light/ 15±2 ºC, 8 h dark, and 60% RH in the CA greenhouse.

During the assay, standard treatments with insecticides and acaricides were carried out. The use of fungicides and bactericides was avoided to exclude interferences in the development of the study.

2.2.3 Controlled environmental conditions

During the tests, the plants were kept in the biological safety greenhouse with controlled environmental conditions of temperature and relative humidity. Specifically, a photoperiod of 16 hours of light, 8 hours of darkness and 60% relative humidity were maintained at 28 ºC during the day, 25 ºC during the night.

2.2.4 Efficacy of antimicrobial peptide *in planta*

To determine the effect of the peptides on the population levels of *Xf* subsp. *pauca*, peptide 1 and peptide 2 were applied by microinjection using a combined application strategy (preventive and curative). Specifically, the first peptide application was performed 24 h before pathogen inoculation (preventive strategy) and 3 days after pathogen inoculation (curative strategy). An untreated control was also included in the test, where the product was replaced by sterile distilled water. The injector was needed so the peptides could enter the phloem and xylem vessels to interact with the *Xf*. The injector worked by filling the syringe with the bacterium inoculum or the peptide suspension and programming the injector to infuse 10 µl every cycle, this way the amount of peptide and pathogen in the plants were for every plant the same. After the plants were infected and treated they were processed for DNA extraction and viable qPCR to discover the effect of the synthetic AMPs on the *Xf* and the plants.
The experimental design consisted of 4 replications of 3 plants per treatment (peptide 1, peptide 2 and NTC; 12 plants per treament).

36 plants were used for this experiment: 12 plants that were inoculated with peptide 1 and *Xf*, 12 plants that were inoculated with peptide 2 and *Xf* and 12 plants that were the control group and only get inoculated with *Xf*. These inoculations happened like seen on the picture (Figure 10).

The following figure (Figure 11) shows the *Nicotiana benthamiana* plants used in this assay, and an example of the application of AMP by microinjection in a combined strategy (preventive and curative).

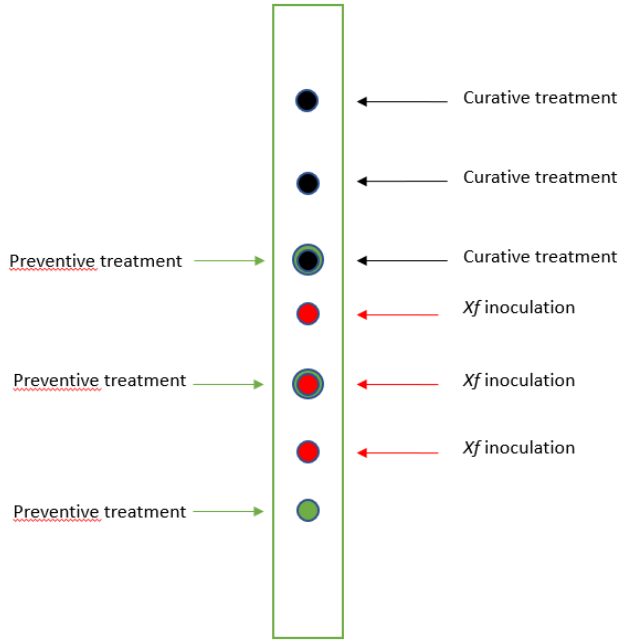

Figure 10: Treatments and Inoculation points for Nicotiana benthamiana

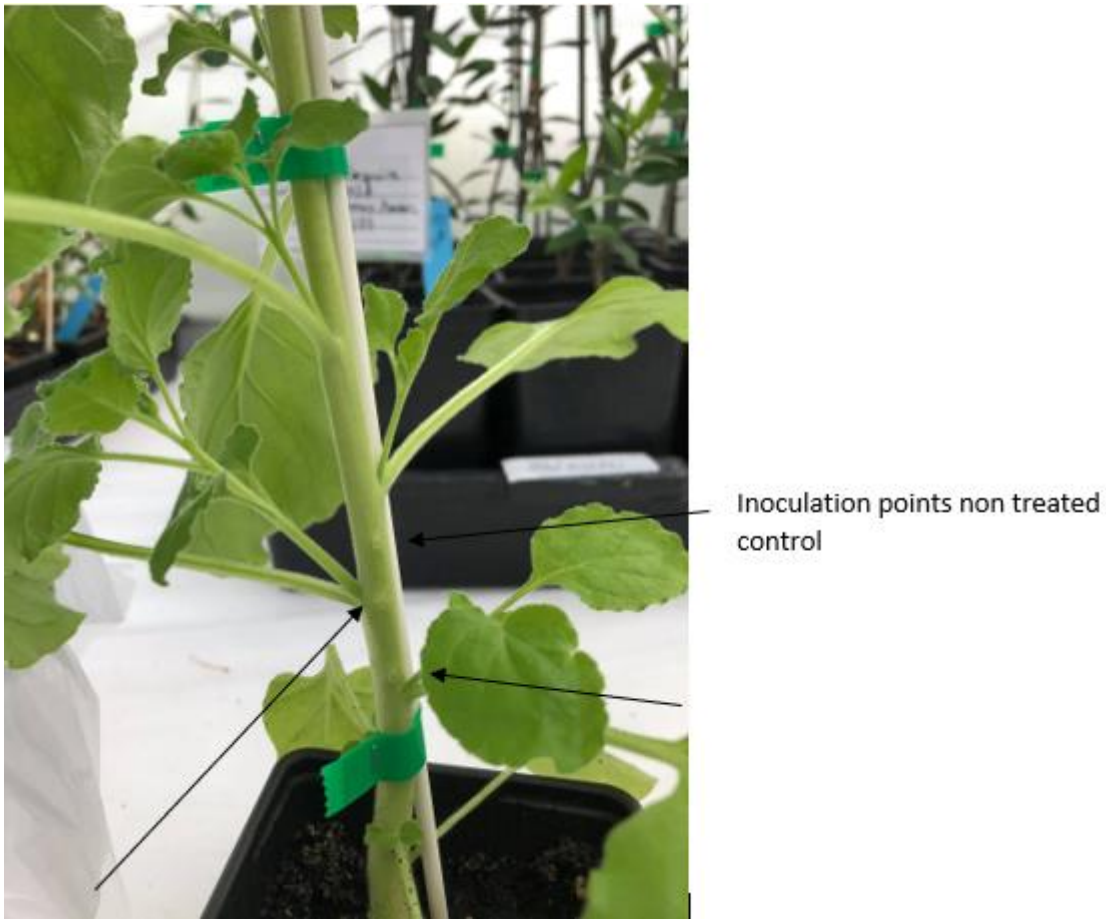

Figure 11: *Xf* Inoculated plant from NTC. Arrows, indicate the three Xf inoculation points

2.2.5 Evaluation of the severity of the disease caused by *Xylella fastidiosa* subsp. *pauca* in *N. benthaminana* plants after treatment with antimicrobial peptides

The evaluation of the *in planta* efficacy of the treatments was carried out according to the severity of the disease caused by *Xylella fastidiosa*.

Disease severity was evaluated following a severity scale adapted from the one already described for grapevines [34]: 0 = no symptoms, 1 = from one to five leaves just beginning to show decay, 2 = from five to ten leaves showing significant decay, 3 = one-half or more of the leaves showing decay and few showing necrosis, 4 = all of the leaves showing decay and one-half or more showing necrosis, 5 = dead plant.

Once the severity in each treatment was determined, the significant differences between treatments were determined by ANOVA and the separation of means was performed with the Tukey test using the SPSS statistical package (IBM SPSS Statistics v25).

2.3 MOLEQULAR TECHNIQUES

In this study, DNA extraction was performed if purified DNA was needed for qPCR in order to detect or quantify bacterial cells. Also viable qPCR was performed when the quantity of viable cells in a sample was needed to be determined.

2.3.1 Genomic DNA extraction

2.3.1.1 DNA extraction via MATAB

This protocol was based on the original from Murray and Thompson [35] with some modifications. This method allows the extraction and purification of DNA, both from plant eukaryotic and prokaryotic cells, of high molecular weight (≥50,000 bp). In the procedure, genomic DNA was purified by using organic solvents (chloroform: isoamyl alcohol) that allowed the DNA to be isolated from the proteins and suspended in the aqueous phase, to later precipitate it with isopropanol.

Briefly, 30-50 mg of frozen sample was crushed and homogenized in the Tissue Lyser II with liquid nitrogen for 30 seconds. Next 750 µl of MATAB solution (Mixed AlkyTrimethyl Ammonium Bromide) was added and mixed via vortex or pipetting. The tubes were then incubated in an thermoblock for 30-60 minutes at 74 °C. After the incubation, 900 µl of chloroform/isopropyl alcohol (24/1) solution was added and mixed by inverting the tubes 3-5 times. The tubes were centrifuged at 13 000 rpm for 5 minutes at room temperature. After centrifugation the supernatant (the aqueous phase) was transferred to a new set of tubes. Next 2 µl of RNase T1 was added and incubate the tubes at 37 °C for 30 minutes. After this 900 µl of chloroform/isopropyl alcohol (24V/1V) solution was added again and mixed by inverting the tubes 3-5 times. Then another centrifuge followed at 13 000 rpm for 5 minutes and the supernatant was again transferred to a new set of tubes. In de following step de DNA was precipitated by adding 750 µl of isopropanol and inverting the tubes. To obtain the pelleted DNA, the tubes were centrifuged again at 13 000 rpm for 5 minutes, and supernatant was removed. Next, pelleted DNA was washed with 100 µl of 70% ethanol (repeated 3 times). After ethanol removing, pelleted DNA was air dried and resuspended in 50 µl of DEPC water. Tubes were stored at -80°C until used [36].

2.3.1.2 DNA extraction via DNA extraction kit

The GeneJet Genomic DNA purification kit for Gram negative bacteria (Thermo Fisher Scientific, Walthman, EEUU) was used when highly purified DNA was required. Bacterial suspension was centrifuged at 5000 rpm for 10 min to obtain pelleted bacterial cells, the supernatant needs to be removed. In the next step 180 µl of digestion solution was added to the pellet to break the pellet down into various components. After digestion, 20 µl of Proteinase K solution was added and mixed by vortex or pipetting (to break down the proteins in the suspension). To activate the Digestion buffer and Proteinase K the tubes were incubated in a thermoblock for 30 minutes at 56 °C (it's important to vortex the tubes by hand every 10 minutes to make sure all the proteins were affected by the enzyme). After incubation there was still RNA in the suspension, this is why 20 µl of RNase A solution was added to the suspension and incubated for 10 minutes at room temperature. To clean up the remaining impurities in the suspension, 200 µl of Lysis solution was added and mixed by pipetting. Next, 400 µl of 50 % ethanol was added to precipitate the DNA. To clean the DNA further, the sample lysate was loaded onto a GeneJet Genomic DNA column assembled with a collection tube and centrifuged at 8000 rpm for 1 min, and the collection tube was discarded. To wash the DNA, the column was transferred to a new collection tube and 500 µl of Wash buffer I was added. The column was centrifugated at 10 000 rpm for 1 minute were after the contents of the collection tube were discarded and the column was put back in the same collection tube. For the last wash step, 500 µl of Wash buffer II was added to the column and centrifugated for 3 minutes at 13 000 rpm. After centrifugation the column was put back in the collection tube and centrifugated for 1 minute at 13 000 rpm to make sure all the Wash buffer was removed from the column. Now all the purified DNA was on the membrane in the column (Figure 12). Finally, genomic DNA was eluted by adding 30 µl DEPC water in the column and leaving the column in a sterile 1,5 ml Eppendorf for 15 minutes, and centrifugated on 10 000 rpm for 1 minute. Genomic DNA was stored at -20°C until used.

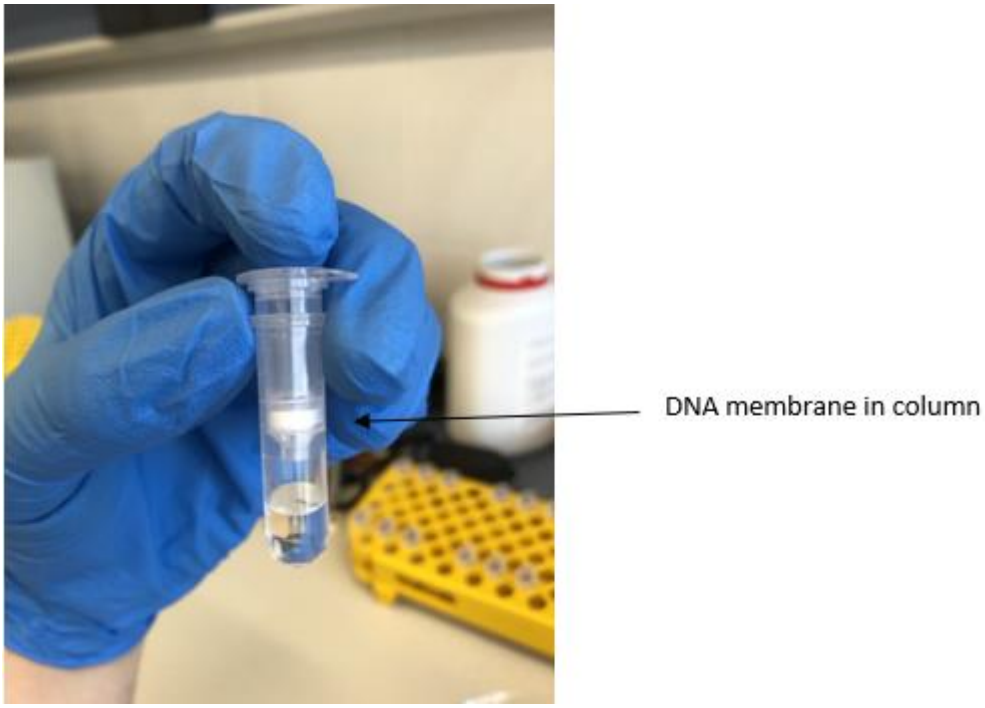

Figure 12: DNA on purifying spin column using the GeneJet Genomic DNA purification kit

2.3.2 qPCR amplification

Principle of qPCR [37]

qPCR or Real Time quantitative polymerase chain reactive is a method for measuring DNA by amplification and fluorescence. The reaction is followed in the Thermocycler (QuantStudio 5 Real-Time PCR system (Figure 13). The Thermocycler used for performing qPCR are included with a fluorometer for detecting fluorescence (due to the hydrolysis of the probe, described below). When the thermocycler is running fluorescence can be detected during the amplification process of PCR by the fluorometer. The real-time values that are detected by the fluorometer can be translated in a graph into information about relative and absolute amounts of DNA.

Figure 13: QuantStudio 5 Real-Time PCR system [38]

Amplification steps [37]

Each PCR cycle goes over the following steps: Denaturation, Annealing and extension.
During the denaturation high temperature incubation was used to break the bonds between the double- stranded DNA into single strands and weaken secondary structure in single-stranded DNA so it can be copied later on. The highest temperature used is 95°C because it is also the highest temperature the DNA polymerase can withstand. The denaturation time can be increased if the guanine-cytosine content is high enough.

During annealing, complementary sequences will hybridize to the single strands together with the primer and probe. The used temperature with this step is determined on the melting temperature of the primers (5°C below the T_m of the primer).

The last step is the DNA polymerase which happens at 70-72°C, at this moment the primer extension also occurs at rates up to 100 bases per second.

Detection [37]

The two most common markers used in qPCR are: Taqman probe and SYBR Green (Figure 14). In this paper only Taqman probe was used. This was because the Taqman probe has a better specificity than SYBR Green because the Taqman is a labeled oligonucleotide while SYBR Green is a non-specific dsDNA-binding dye [39].

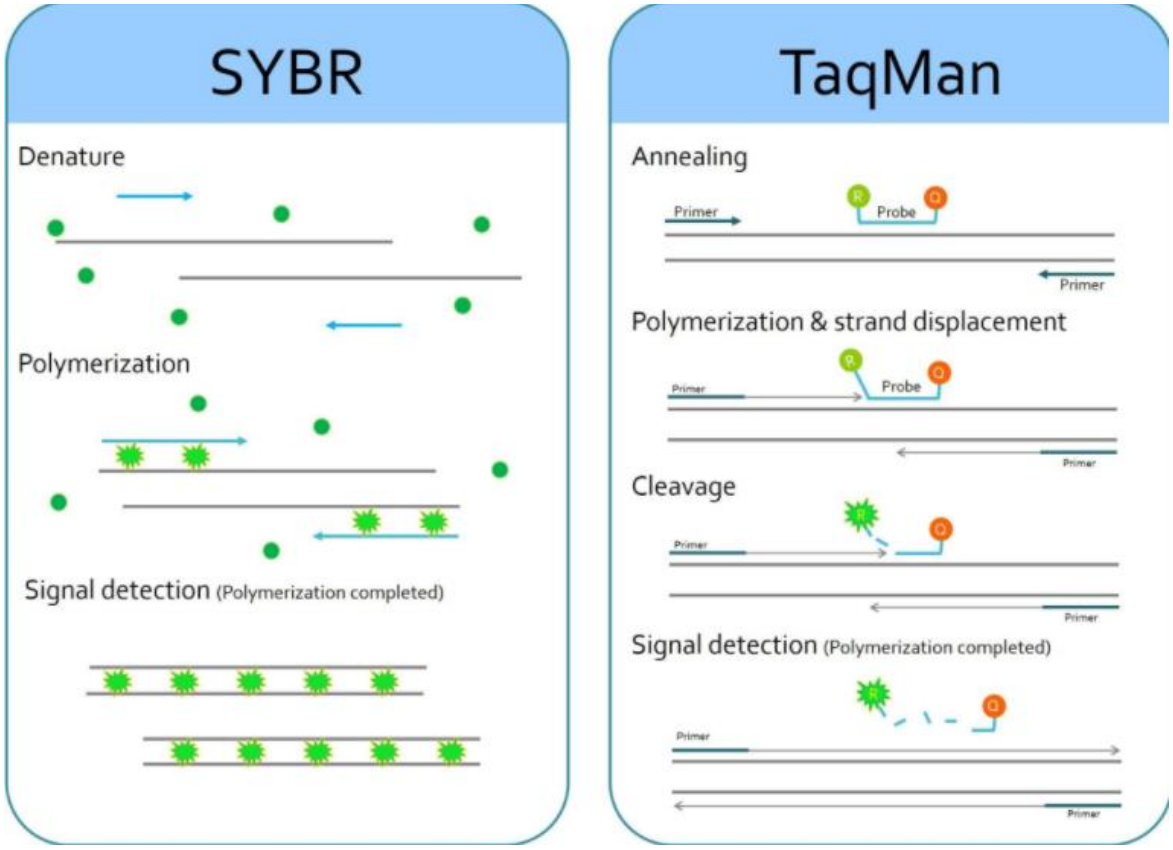

Figure 14: Difference between SYBR Green and TaqMan probe mechanism during qPCR [37]

A: SYBR Green [37]

This is a dye that emits fluorescent signal when it binds at the minor groove of DNA, these binds happen nonspecifically. SYBR GREEN has a high signal intensity and this is why it is most of the times preferred over other fluorescent dyes like Ethidium Bromide or Acridine Orange. However, when it comes to specificity the Taqman Probe takes the lead over fluorescent dyes like SYBR Green [39].

B: Taqman Probe [37]

The Taqman Probe is a hydrolysis probe which holds a reporter dye, often fluorescein (FAM) at its 5' end and a quencher tetramethylrhodamine (TAMRA), attached to the 3' end of the oligonucleotide. Under normal conditions, the probe is coiled which inhibits fluorescent signal of the dye because it is too close to the quencher. The oligonucleotide of the Taqman probe has a homologous region with the target gene so when it comes close to the target sequence it will bind with the DNA. As the taq polymerase start to synthesize new DNA strand in the extension stage, it causes degradation of the probe by 5' end nuclease activity and the fluorescein will be further apart from the quencher and for this reason the fluorescence signal will be released. Each cycle this release of fluorescein will increase, causing an increase in fluorescence what can be translated to the increase of DNA [39].

In this study, qPCR the mix consisted of DEPC water, a Forward and Reverse primer (10 µM), Taqman universal PCR mix (2x) and a Taqman probe (100 µM)(Table 1). This probe is sensitive

to light so when working with this probe the lights were off. The following program was run (Figure 15).

Table 1: qPCR Primer and probe information

Bacteria	qPCR assay	Primer/probe	Sequence
Xf	XF16S-1	Rev-1	CCGATGTATTCCTCACCCGTC
	XF16S-2	Rev-2	CTAATCGGACATCGGCTCAT
	XF16S-3	Rev-3	GTAGGAGTCTGGACCGTGTCTC
		For	CGGCAGCACGTTGGTAGTAA
		probe	FAM/–CATGGGTGGCGAGTGGC–/TAMRA
Lc	HA14068125	for	CCGTCACCAATTTCCACACC
	HA14068126	rev	TGATCGCTCTCGATGGGATT
	HA14068127	probe	(FAM)AGGAGCTCCTAGTCCTGCAGGAACA(TAM)

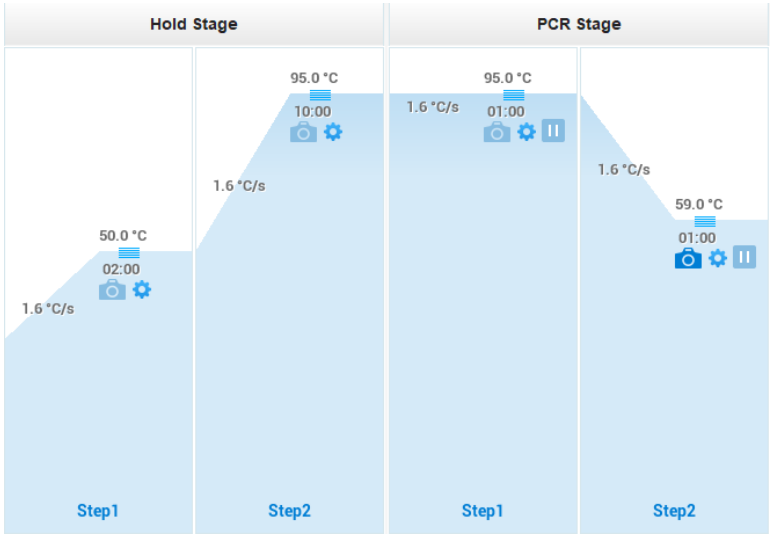

Figure 15: Thermocycler conditions for qPCR used to detect Lc and Xf qPCR program

2.3.3 Viable qPCR using PMAxx reagent

2.3.3.1 PMAxx principle [40]

In this study, PMAxx reagent was used for viability qPCR, allowing to distinguish between dead and viable cells.

PMAxx is a high-affinity photoreactive DNA binding dye for viability qPCR (v-qPCR) of bacteria and other organisms. In v-qPCR the target cell cultures are treated with a viability dye such as PMAxx or PMA that will allow visualization of all the live and dead cells after qPCR.
PMAxx will bind to the dsDNA inside the dead cells and will modify the DNA permanently in these cells after photolysis. (Figure 16). PMAxx is cell membrane-impermeant, and can be used to selectively modify only the DNA in dead cells while leaving the DNA in viable cells intact. Because of the PMAxx modifications the DNA cannot be amplified by the DNA polymerase. This causes the selective detection of viable cells by quantitative real-time PCR.

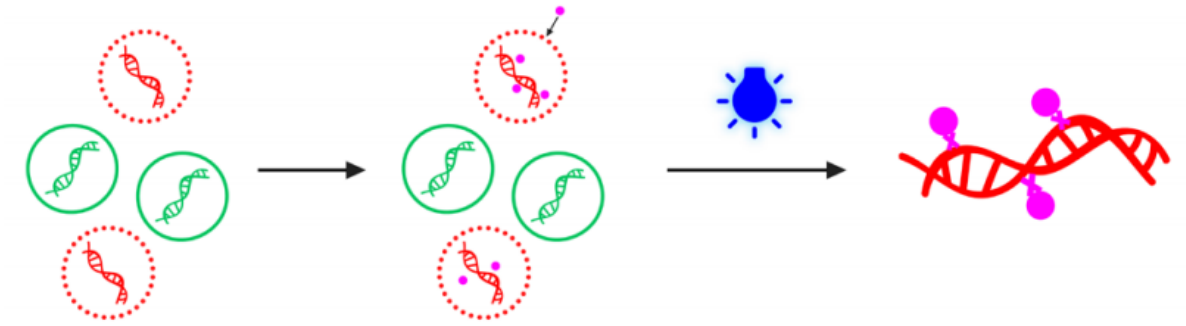

Figure 16: PMAxx simplified working mechanism [40]

2.3.3.2 Use of PMAxx in *in vivo* plant experiments

In the plant experiments, 3 plants were used every harvest and subjected to a DNA extraction and viable qPCR analysis. For each sampling time, 3 plants of each treatment were stripped from the bark and cut into 3 equal parts: The basal part (D: this was all from the bottom of the plant until the inoculation points), the middle part (U1: this was all from the start of inoculation points to end of inoculation points) and lastly the top part (U2: this was all from the end of A1 to the top of the plant)(Figure 17)[24].

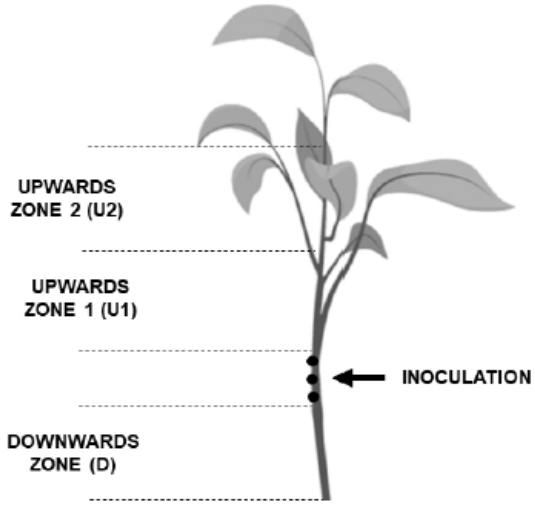

Figure 17: Sample zones of in vivo plant experiments with Nicotiana Benthamiana and Xf [24]

From these parts the sap and the branches needed to be separated. Branches were put into an Eppendorf tube that was previously punctured. This tube was deposited into 5 ml Eppendorf to collect the sap. These tubes were centrifuged for 25 min at 13000 rpm. After centrifugation the branches were frozen and the sap was processed.

Sap samples: 200 µl of the sap was treated with PMAxx and 200 µl was treated without PMAxx. The tubes with the PMAxx were put in aluminum paper and left at room temperature for 7 min. After this the tubes were put under a blue LED light to start the photolysis for 15 min. Next, all the tubes from the PMAxx treated and non-PMAxx treated sap was centrifuged for 10 min at 13000 rpm. From the non-PMAxx tubes 150 µl of supernatant was removed and the pelleted cells was used in DNA extraction like explained in section 2.3.1.2. From the PMAxx tubes the supernatant was also removed and 450 µl of PBS was added to wash the PMAxx (PMAxx is toxic to the cells). The samples were centrifuged for 10 min at 13000 rpm and 400

µL of the supernatant was removed. From the pelleted cells, DNA extraction was performed like explained in section 2.3.1.2.
Branch samples: To process the branches 3 ml of PBS was added to the branch in a homogenizer bag (BioReba, Reinach, Switzerland). The branches were crushed with a hammer in the bag and then homogenized in the homogenizer. The bags were put in the fridge for 15 min. After this 200 µl of each branch sample was put in a tube and centrifuged for 10 min at 13000 rpm. 150 µl of the supernatant was removed after centrifugation and the pelleted cells was used in DNA extraction like explained in section 2.3.1.2.

After all the DNA was purified the samples were subjected to a v-qPCR. This way after qPCR the DNA values of the PMAxx samples was all the DNA from only the viable cells and the DNA values of the samples that were not treated with PMAxx was all the DNA from viable and non-viable cells. If there was no difference in ct value between the 2 treatments then all the cells were viable, but if the ct value of the PMAxx treatment was higher than the non-PMAxx treatment then there were non-viable cells in the sap. From these results was then concluded if the peptide treatment in the plants worked or not.

2.4 PREPARATION OF CULTURE MEDIA, BUFFERS AND REAGENTS

2.4.1 Production of PD2 media 1 l

PD2 media is a media that was used to grow *Xf*.
To start the preparing of the media a beaker was filled with 900 ml of distilled water and a magnetic flea was added for stirring. When the beaker was placed on the magnetic stirrer the ingredients could be added: 2g of Soy Peptone, 4 g of Bacto tryptone, 1 g of Disodium succinate, 1 g of Trisodium citrate, 1.5 g of K_2HPO_4, 1 g of KH_2PO_4, 10 ml of hemin chloride solution, 1 g of $MgSO_4 * 7H_2O$. Before the agar was added the pH of the media had to be adjusted to 6,9. This was done with a pH meter and HCl (0.2 M) and NaOH (0.2M). When the pH was 6,9 the 15 g of antimicrobial agar was added together with the last 100 ml of distilled water. Now the media needed to be sterilized in an autoclave for 20 minutes at 121°C. To do this the media was transferred in a glass flask. After the sterilization the media had to cool down to 50°C before plating in petri dishes, if the media wasn't going to be plated after sterilization the flask was put in an oven on 56°C until plating. Before the media was plated a last ingredient had to be added to the media, 10 ml of BSA fraction 20%. This ingredient was only added at the end because it was sensitive to the heat. Because the BSA fraction wasn't sterilized the BSA fraction first goes through a Whattman filter. The media was poured in plates in a laminar flow cabinet. When the media was poured in the plates and hardened, the plates were closed and stored in a dry bag.

2.4.2 Production of BM7 media 500 ml

2.4.2.1 Solid BM7 media

BM7 media is the media used to grow the *Lc*.
To start the preparing of the media a beaker was filled with 900 ml of distilled water and a magnetic flea was added for stirring. When the beaker was placed on the magnetic stirrer the ingredients could be added: 1 g of α-ketoglutaric acid, 5 g of ACES buffer, 1.875 g of KOH. Before the agar was added the pH of the media had to be adjusted to 6,8. This was done with a pH meter and HCl (0.2 M) and NaOH (0.2M). When the pH was 6,8 the 7.5 g of agar was added to the media. Now the media needed to be sterilized in an autoclave for 20 minutes at 121°C. To do this the media was transferred in a glass flask. After the sterilization the media

had to cool down to 50°C before plating in petri dishes, if the media wasn't going to be plated after sterilization the flask was put in an oven on 56°C until plating. Before the media was plated 2 last ingredients have to be added to the media, 150 ml of TNM-FH insect medium and 75 ml of Fetal bovine serum. These ingredients were only added at the end because they are sensitive to the heat. Because the FBS and TNM-FH weren't sterilized they first went through a Whattman filter. The media was poured in plates in a laminar flow cabinet. When the media was poured in the plates and hardened, the plates were closed and stored in a dry bag (Figure 184).

2.4.2.2 Liquid BM7 media

To start the preparing of the media a beaker was filled with 900 ml of distilled water and a magnetic flea was added for stirring. When the beaker was placed on the magnetic stirrer the ingredients could be added: 1 g of α-ketoglutaric acid, 5 g of ACES buffer, 1.875 g of KOH. Before the methyl-β-cyclodextrin was added the pH of the media had to be adjusted to 6,8. This was done with a pH meter and HCl (0.2 M) and NaOH (0.2M). When the pH was 6,8 the 0.1875 g of methyl-β-cyclodextrin could be added to the media. . Now the media needed to be sterilized in an autoclave for 20 minutes at 121°C. To do this the media was transferred in a glass flask. After the sterilization the media had to cool down to 50°C before plating in petri dishes, if the media wasn't going to be plated after sterilization the flask was put in an oven on 56°C until plating. Before the media was plated 2 last ingredients had to be added to the media, 150 ml of TNM-FH insect medium and 75 ml of Fetal bovine serum. These ingredients were only added at the end because they are sensitive to the heat. Because the FBS and TNM-FH weren't sterilized they first went through a Whattman filter. The media was poured in plates in a laminar flow cabinet. The template for production of these medias is located below.(Figure 18)

BM7 agar

Fridge	Molecular grade water	550	**275**	137,5	
	α-ketoglutaric acid	2	**1**	0,5	
	ACES buffer	10	**5**	2,5	
	Potassium hydroxide	3,75	**1,875**	0,9375	Adjust pH to 6,8
	TNM-FH insect medium	300	**150**	75	
	Fetal bovine serum	150	**75**	37,5	Add after autoclavation
	Agar	15	**7,5**	3,75	
	Total	1000	**500**	250	

BM7 liquid

Fridge	Molecular grade water	550	275	**137,5**	27,5	
	α-ketoglutaric acid	2	1	**0,5**	0,1	
	ACES buffer	10	5	**2,5**	0,5	
	Potassium hydroxide	3,75	1,875	**0,9375**	0,1875	Adjust pH to 6,8
	TNM-FH insect medium	300	150	**75**	15	
	Fetal bovine serum	150	75	**37,5**	7,5	Add after autoclavation
	Methyl-β-cyclodextrin	0,75	0,375	**0,1875**	0,0375	
	Total	1000	500	**250**	50	

Figure 18: BM7 production template

2.4.3 Production of LB media

LB media is a generic media used to grow a variety of bacteria.
To start the preparing of the media a beaker was filled with 1000 ml of distilled water and a magnetic flea was added for stirring. When the beaker was placed on the magnetic stirrer the ingredients could be added: 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl and 15 g of agar. Now the media needed to be sterilized in an autoclave for 20 minutes at 121°C. To do this the media was transferred in a glass flask. After the sterilization the media had to cool down to 50°C before plating in petri dishes, if the media wasn't going to be plated after sterilization the flask was put in an oven on 56°C until plating. The media was poured in plates in a laminar flow cabinet. When the media was poured in the plates and hardened, the plates were closed and stored in a dry bag.

2.4.4 Production of AFT buffer media

AFT buffer is a media used in most experiments for making bacterium inoculum.
To start the preparing of the media a beaker was filled with 1000 ml of distilled water and a magnetic flea was added for stirring. When the beaker was placed on the magnetic stirrer the ingredients could be added: 8 g of NaCl, 0.4 g of NaH_2PO_4 . $2H_2O$, 2.7 g of Na_2HPO_4 . $12H_2O$. Now the media needed to be sterilized in an autoclave for 20 minutes at 121°C. To do this the media was transferred in a glass flask. After sterilization the buffer media was ready to use.

2.4.5 Production of BYCE media

BCYE is a media used to grow *Xf*. To start the preparing of the media a beaker was filled with 1000 ml of distilled water and a magnetic flea was added for stirring. When the beaker was placed on the magnetic stirrer the ingredients could be added: 1.7 g of charcoal, 0.37 g of yeast extract, 2 g of glycerol and 15 g of agar. Now the media needed to be sterilized in an autoclave for 20 minutes at 121°C. To do this the media was transferred in a glass flask. After the sterilization the media had to cool down to 50°C before plating in petri dishes, if the media wasn't going to be plated after sterilization the flask was put in an oven on 56°C until plating. The media was poured in plates in a laminar flow cabinet. When the media was poured in the plates and hardened, the plates were closed and stored in a dry bag.

3 RESULTS AND DISCUSSION

3.1 EVALUATION OF ANTIBACTERIAL, BACTERICIDAL AND VIABILITY ASSESSMENTS THAT ALLOW IDENTIFICATION OF ANTIMICROBIAL PEPTIDES WITH HIGH ANTIBACTERIAL ACTIVITY AGAINST *LIBERIBACTER CRESCENS*

3.1.1 Antibacterial activity of synthetic peptides

We tested the antimicrobial and bactericidal activity of BP1 trough BP36 synthetic peptides by using *in vitro* growth inhibition assays and contact exposure tests, respectively. For these studies we used the non-pathogenic bacteria *Liberibacter crescens*. The MIC values of different peptides were assessed by using increasing concentrations of the corresponding peptides. Specifically, 6 antibacterial activity tests were carried out to determine the MIC of the different peptides against *Lc*. The MIC value was determined as the lowest peptide concentration with no bacterial growth at the end of experiment. With this methodology cannot be determined if the antibacterial activity is due a bactericidal or bacteriostatic effect of the AMPs.

In this experiment were 3 possible outcomes: (1) the MIC was determined between the tested concentrations of the peptide; (2) the MIC was not determined because the tested concentrations were too low or (3) the MIC was not determined because the tested concentrations were too high.

In Figure 19, there is an example of *Lc* growth in the presence of increasing concentrations of BP5 peptide. The determined MIC of BP5 was between 6.25 µM and 12.5 µM. This was visible on the graph because no bacterial growth (absorbance did not increase overtime) was observed at 12.5 µM peptide and 25 µM peptide, compared to C+ (AFT instead of peptide). At a peptide concentration of 6.25 µM to the absorbance increased overtime which indicates that BP5 at 6.25 µM allowed bacterial growth, although this growth was 50% less than that observed in C+

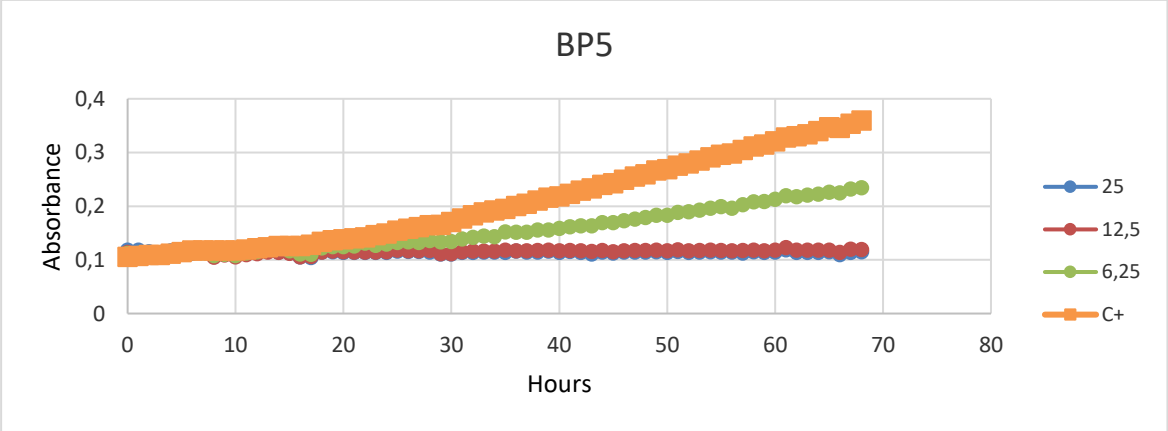

Figure 19: Lc growth in presence of increasing BP5 peptide dilutions.

In some cases, the MIC was determined but there was no precise value obtained. This is because the used concentrations were too high and then the graph will look something like this (Figure 20). On the graph is seen that the two used peptide concentrations of 50 µM and 100 µM have a constant absorbance and going nowhere near the positive control what means

there was no bacterial activity and full inhibition of the *Lc*. So if this is the case then the test has to be repeated with lower concentrations to find a more precise value of the MIC.

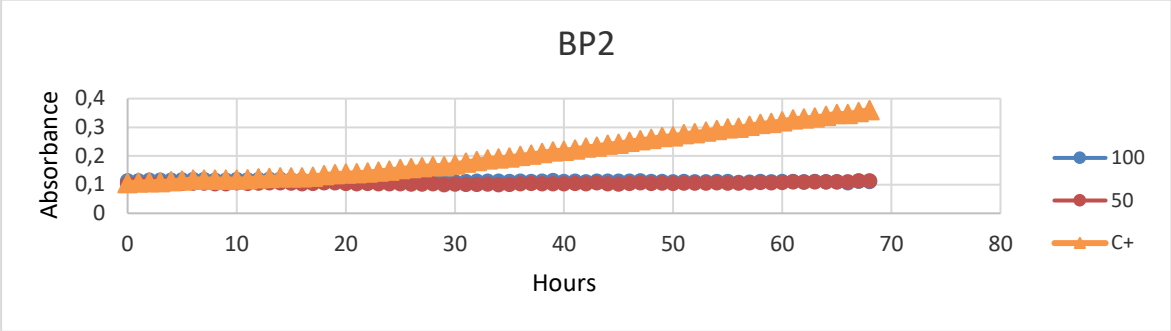

Figure 20: Lc growth in presence of increasing BP2 peptide dilutions.

For some AMPs, the MIC was determined but there was no precise value obtained. This is because the highest did not inhibit the bacterial growth of *Lc* (Figure 21Figure 21). All the used peptide concentrations of 25 µM, 50 µM and 100 µM have a similar absorbance as the C+. The peptide concentration of 50µM and 100 µM are elevated just below the C+ low or no inhibition of the *Lc* at the studied peptide dilutions. The concentration of 25 µM had the same absorbance as the C+ which indicates no inhibition of the *Lc* and full bacterial activity. If this is the case the test should to be repeated with higher concentrations of peptide.

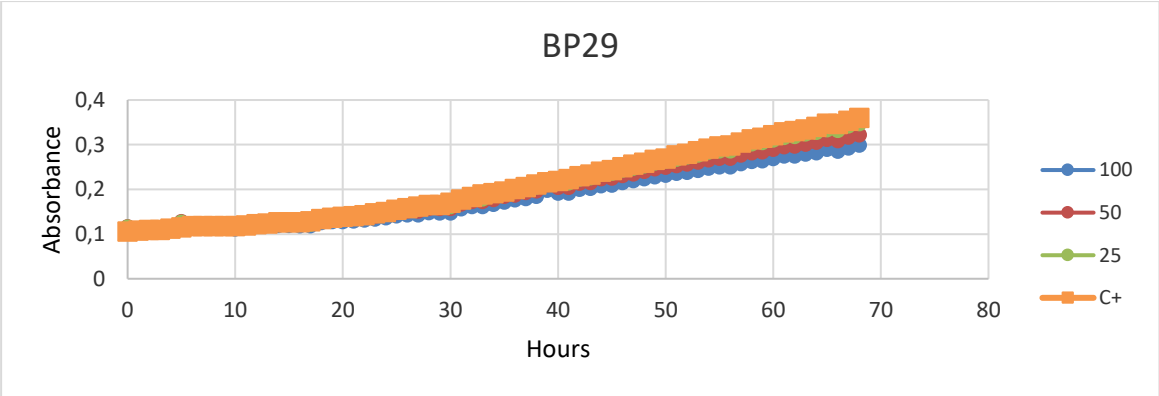

Figure 21: Lc growth in presence of increasing BP29 peptide dilutions.

The value of MIC obtained for AMP are detailed in the Table 2

Table 2: MIC results BP1 – BP35 against Lc

	MIC (µM)
BP1	<100
BP2	<50
BP3	<25
BP4	<100
BP5	6.25-12.5
BP6	25-50
BP7	>200
BP9	100-200
BP11	<25
BP12	50-100

BP13	<50
BP16	>100
BP17	>100
BP18	N/D
BP24	<25
BP25	<50
BP26	<50
BP27	50-100
BP28	50-100
BP29	>100
BP31	<25
BP33	>50
BP34	>50
BP35	>50

As shown in the growth inhibition graphs (Figure 22)(Figure 23)(Figure 24)(Figure 25) and table above (Table 2) MIC from the variety of peptides differ among them. This is because not all peptides have the same effect on the tested pathogen, some of them will need very low concentrations for full inhibition and some of them will need very high concentrations for full inhibition. This is why multiple peptides were tested against the *Lc* to determine which peptide can be used for practical applications. We have confirmed that peptides BP3,5,11,24,32 were the most active peptides against *Lc* because their MIC was <25 μM. Peptides BP1,2,4,6,12,13,25,26,27,28,33,34,35 all presented full inhibition between 100 μM and 50 μM, these peptides were less active than the previous ones because they need a higher concentration to inhibit growth of the *Lc*. Lastly, BP7,9,16,17,29 presented a MIC interval in the range of 100 μM – 200 μM or more, being not very active against *Lc*.

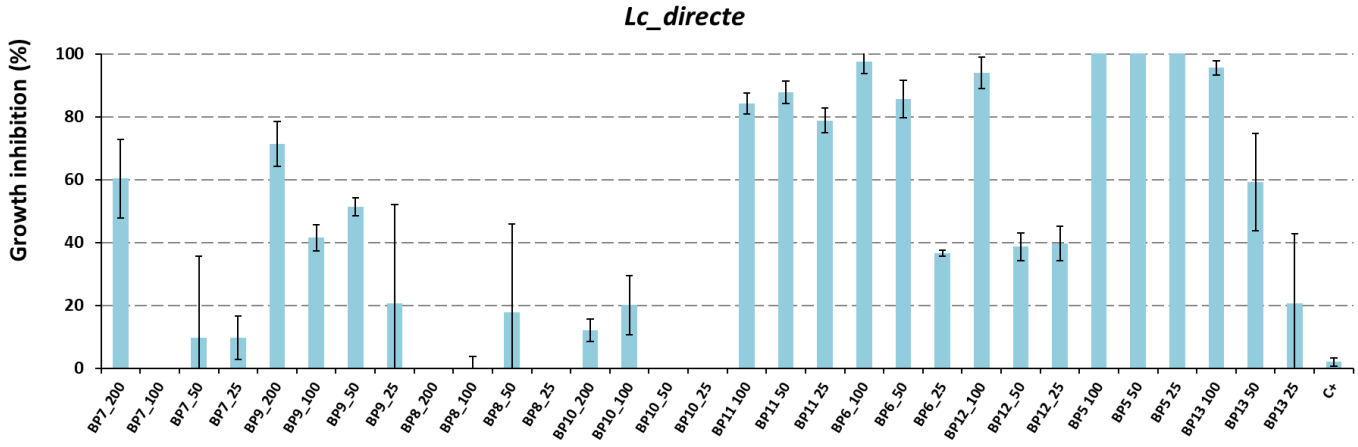

Figure 22: Growth inhibition graph of Lc against BP5,6,7,8,9,10,11,12,13

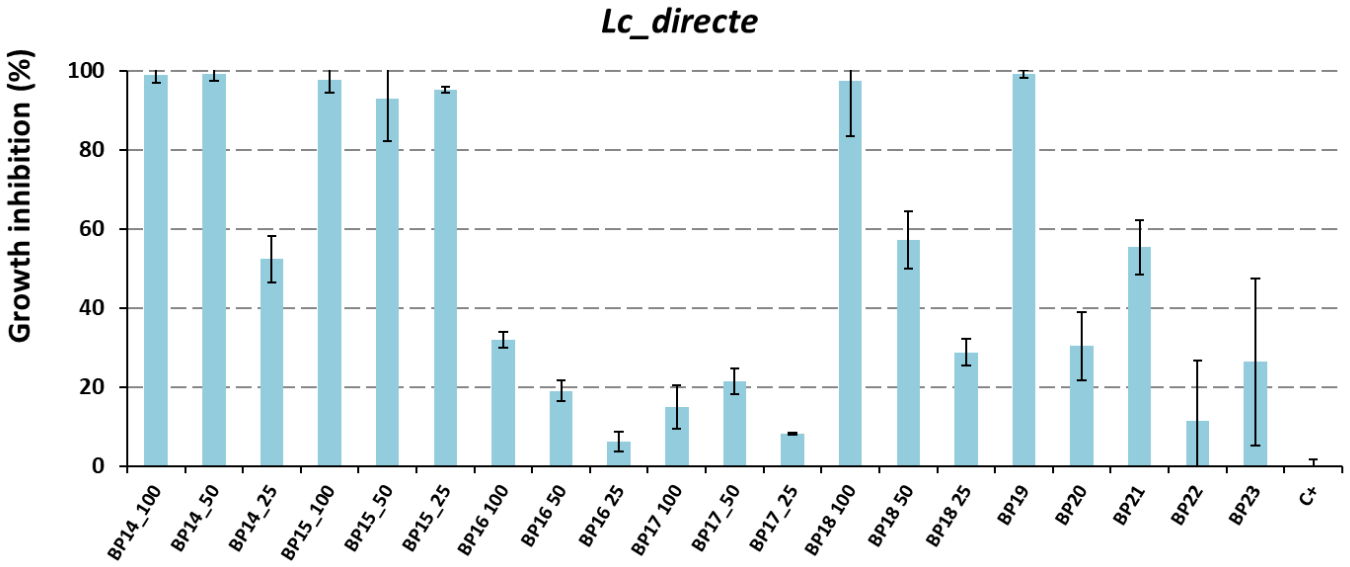

Figure 23: Growth inhibition graph of Lc against BP14,15,16,17,18,19,20,21,22,23

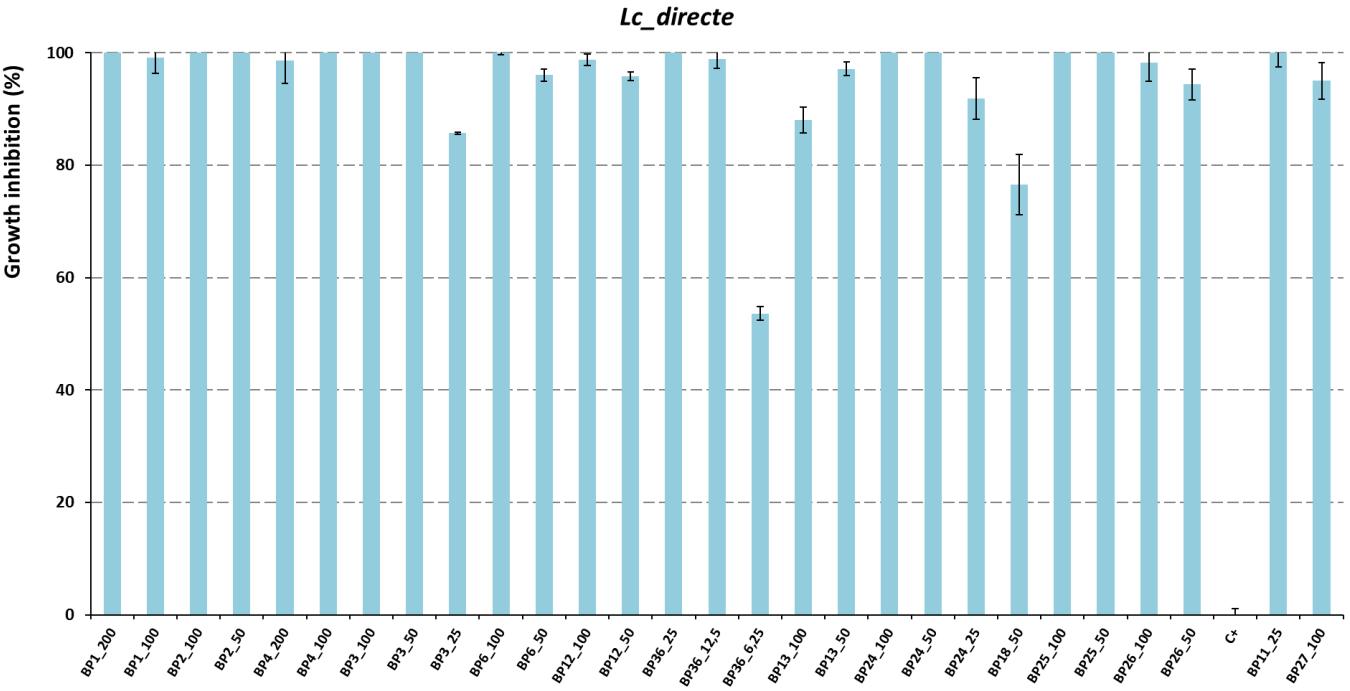

Figure 24: Growth inhibition graph of Lc against BP1,2,3,4,6,11,12,13,18,24,25,26,27,36

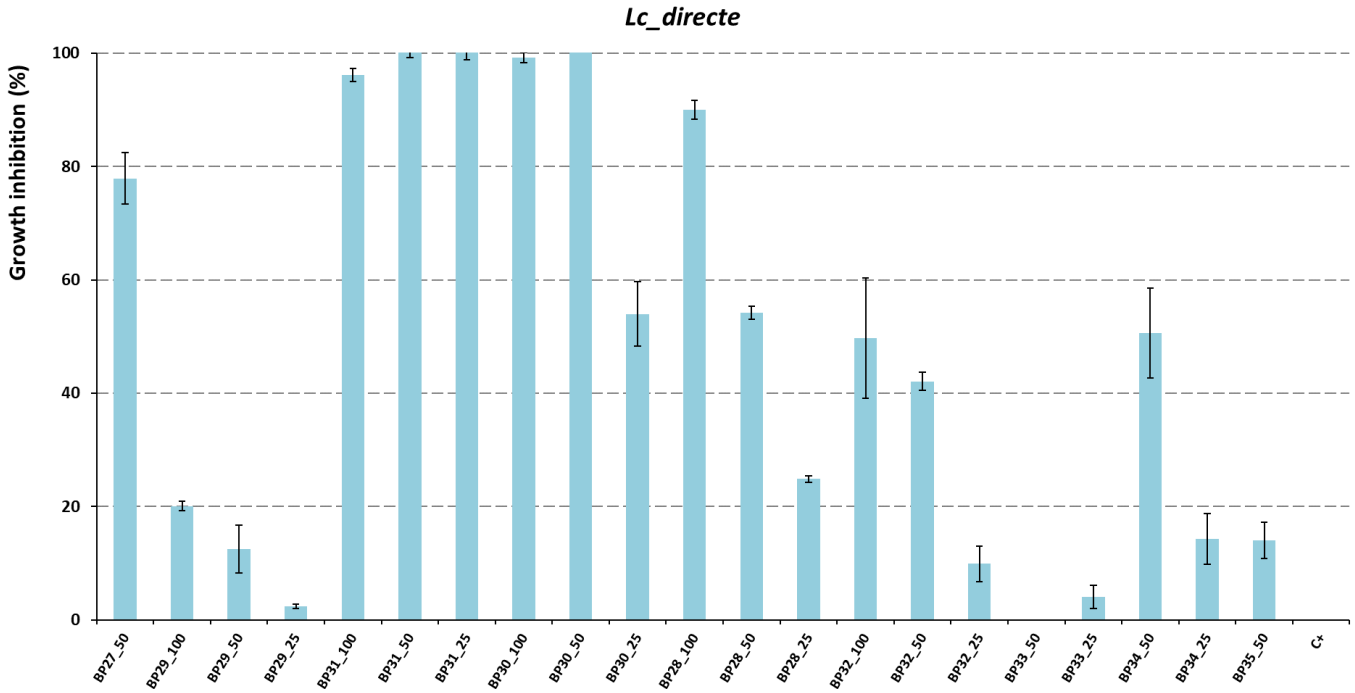

Figure 25: Growth inhibition graph of Lc against BP27,28,29,30,31,32,33,34,35

3.1.2 Viability assessment of *Liberibacter crescens* cells after peptide treatment.

Once antibacterial activity assays confirmed that some peptides caused growth inhibition of *Lc* cells, a test to confirm the viability of this cells were performed. Viability assessment allow to detect viable but not culturable cells (VBNC) as well as viable cells. On the contrary, a killing assay followed by a counting plate method (bactericidal assay, section 3.1.3) only allow quantification of viable and culturable cells.

For the determination of the viability of the *Lc* cells after the peptides treatments, an assay was performed with the compound resazurin. This particular graph (Figure 26) shows an example of how the viability of *Lc* cells can be assessed with fluorescence. It is seen on the graph that there was a high fluorescence spike with a concentration of 25 µM from BP3 and 6.25 µM of BP5. This high fluorescence value means that the *Lc* cells were viable at that concentration of peptide. So from this example was concluded that BP5 had a higher antimicrobial activity than BP3 because the concentration from where the cells were viable was lower than the concentration from BP3.
The other peptides that were tested by viability assessment are found in the graph below (Figure 27). From the fluorescence graph viability could be determined and are visible in the table below (Relative fluorescence units (RFU) after BP3 or BP5 treatment.

Table 3).

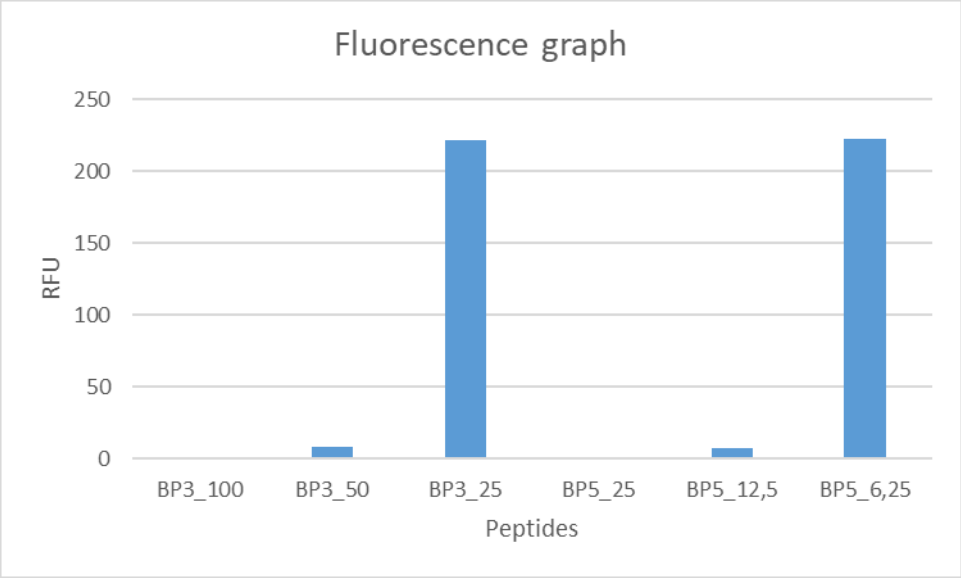

Figure 26: Relative fluorescence units (RFU) after BP3 or BP5 treatment.

Table 3: MKC determined by viability assessment

	MKC (µM)
BP1	200-100
BP2	200-100
BP3	50-25
BP4	<100
BP5	6.25-12.5
BP6	>100
BP11	>25
BP12	50-100
BP13	<100
BP18	>50
BP24	<25
BP25	>50
BP26	50-100
BP27	>100
BP29	>100
BP31	25-50
BP34	>50
BP37	>100

The determined results show that the peptides BP3,5,11,24,31 have high bactericidal activity because the *Lc* were only viable at low concentrations of 50 µM and lower. Peptides BP4,12,13,18,25,26,34 have moderate bactericidal activity against the *Lc* because there were only viable cells and viable non culturable cells at concentrations between 50 µM and 100 µM. Lastly the peptides that have low antimicrobial activity against *Lc* were BP1,2,6,27,29,37. These peptides only show non-viable cells at high concentrations of 100 µM – 200 µM.

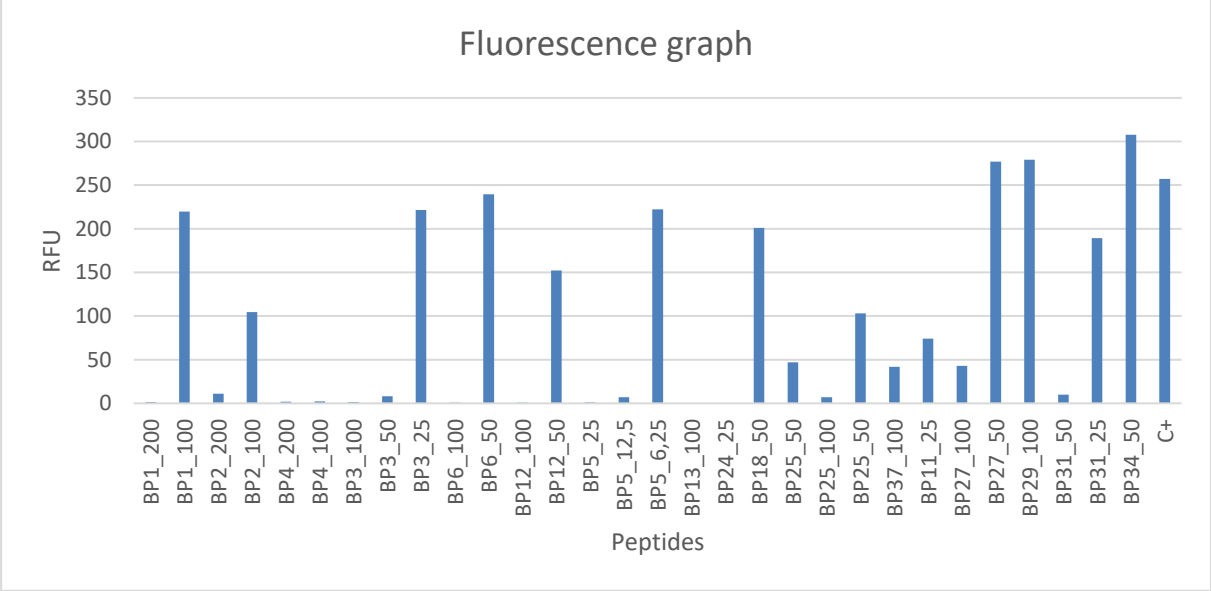

Figure 27: Relative fluorescence units (RFU) after antimicrobial peptide treatment.

Figure 28: Resazurin viability test visual results

3.1.3 Bactericidal activity of synthetic peptides to *Liberibacter crescens*

3.1.3.1 Determination of Minimal killing concentration

Bactericidal activity of synthetic peptides was assessed by a contact exposure test of *Lc* to a peptide solution. This method was a killing assay which differs from the continuous exposure (growth inhibition assay, bacteriostatic assay, 2.1.6) used for screening of antibacterial activity. Four contact tests were carried out to determine the MKC values of the different peptides against *Lc* (Figure 29). The MKC value was determined as the lowest peptide concentration with no bacterial survival at the end of the experiment, and are shown in the table below (Table 4) For example if there was no growth at 50 µM peptide but there was growth at 25 µM peptide then the MKC from that peptide against *Lc* is between 25 µM and 50 µM [33].

Table 4: Bactericidal activity (MKC, µM) of BP1,2,3,5 against Lc. Survival of Liberibacter crescens after the contact test with the different concentrations of the AMPs after 24 or 48 h. The viable count was performed after 15 days of incubation at 28 ºC in BM

	MKC (µM)
BP1	25-50
BP2	100-200
BP3	<12.5
BP5	12.5-25

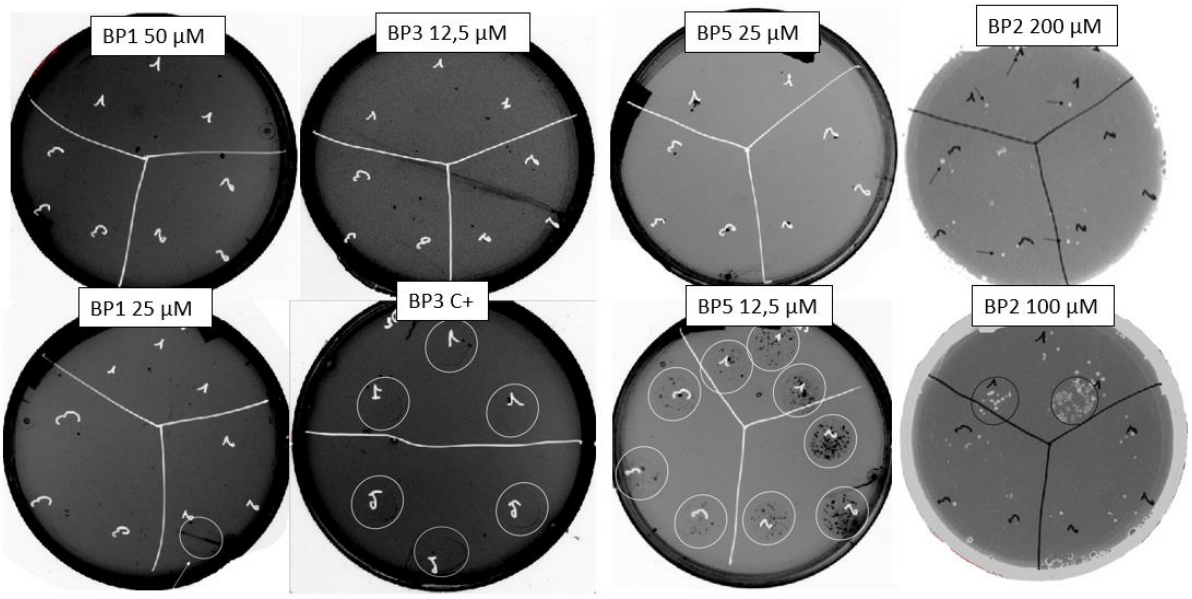

Figure 29: Visual results of contact test with Lc and BP1,2,3,5

In this experiment BP3 showed a MKC lower than 12.5 µM and BP5 showed a MKC between 12.5 µM and 25 µM. Both of these peptides only need a low concentration to kill *Lc* this means they have high bactericidal activity against the *Lc* strain. BP1 has a determined MKC between 25 µM and 50 µM. This is not as low as BP3 and BP5 but it still a low enough concentrations with a moderate bactericidal activity to be a viable option against *Lc*. BP1 has lower antimicrobial activity then BP3 and BP5 against *Lc*. The last peptide that was researched was BP2 which has a high MKC between 100 µM and 200 µM. This peptide has lower bactericidal activity then the other tested peptides BP1,3,5. The results for BP1,3,5 are not the same as for the MKC determined by the viability assessment.

From all the results obtained from the different antimicrobial peptide activity analysis, we can confirm that BP5 and BP3 inhibited *Lc* growth and showed batericidal activity against *Lc,* at concentrations ranging from 6.25 µM to 25 µM and <12.5 µM to 50 µM, respectively. BP24 presented in both the viability assessment and the antibacterial assessment a high bactericidal activity under the concentration of 25 µM. From the same assessments as BP24 there was determined that BP31 presented high bactericidal activity between the concentration lower than 25 µM to 50 µM. Several peptides similar to those used in this study have been tested in other studies against other plant pathogens including *Xanthomonas sp., Pseudomonas* sp., *Erwinia amylovora*, *Xylella fastidiosa*, presenting potent antimicrobial activity [22][20][41][8]. Our results agree with these previous studies a we can confirm that the BP3,5,24,31 presented a high antimicrobial effect against *Lc*. However, in our study, the MIC or MKC values against *Lc* were higher than that observed for the other phytopathogens described in other studies. In this context, although in other studies BP2 was identified as a highly active peptide against other plant pathogens, the majority of our results point to a moderate antibacterial effect of BP2 against *Lc*. Probably, as AMPs acts directly at the microbial membrane, it could be that *Lc* has a different membrane structure or composition. On the other hand, the possibility that *Lc* has the ability to release some type of protease to the extracellular medium that degrades the peptide should be considered.

3.1.4 Standard curves of *Lc* and qPCR detection

Standard curves were performed using *Lc* suspension in both BM7 liquid media and AFT to correlated CFU/ml with ct value. Also the efficiency of the qPCR primers was evaluated. After the dilution banks were made, the DNA was extracted via GeneJet kit. This purified DNA was then used in these qPCR test to make a standard curve to see what media was best to grow *Lc* (Figure 30)(Figure 31). Standard curves showed a good linearity between 10^9 to 10^3, with R^2 values of above 0.99, in both cases. As seen on the two graphs there was minimal difference between the two but the standard curve of AFT has a lower ct max what should mean that AFT is better to use for the experiments then BM7 liquid media because the lower the ct value is the lower the cycles needed for detection therefor higher DNA content as is explained in paper [42]. But the composition of AFT and BM7 differs from each other which can cause the slight ct difference.

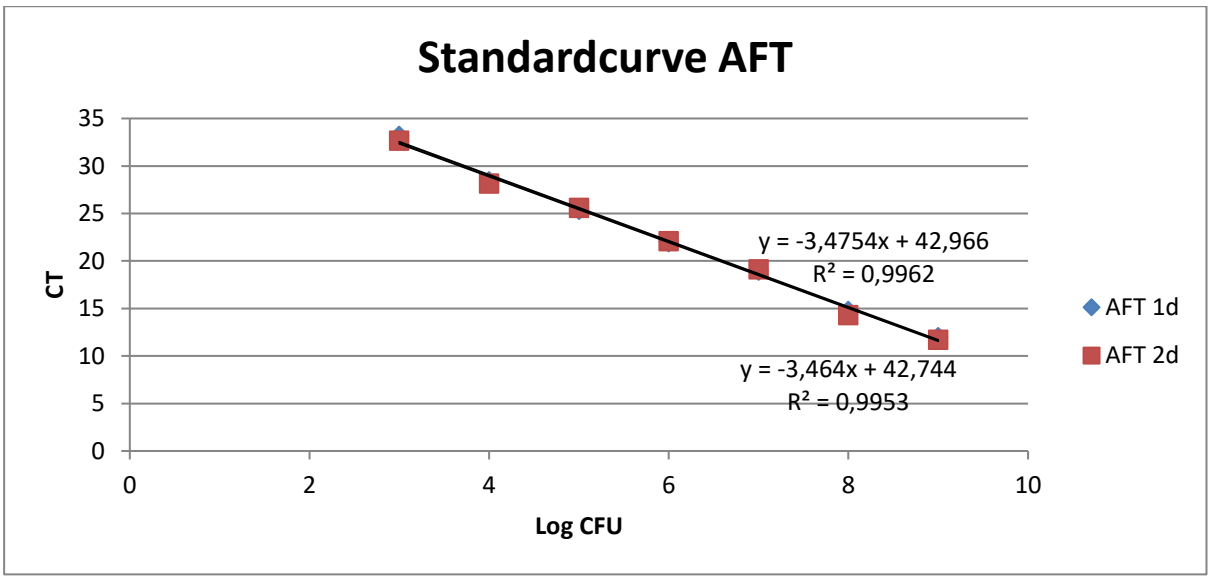

Figure 30: Graph of qPCR standard curve AFT

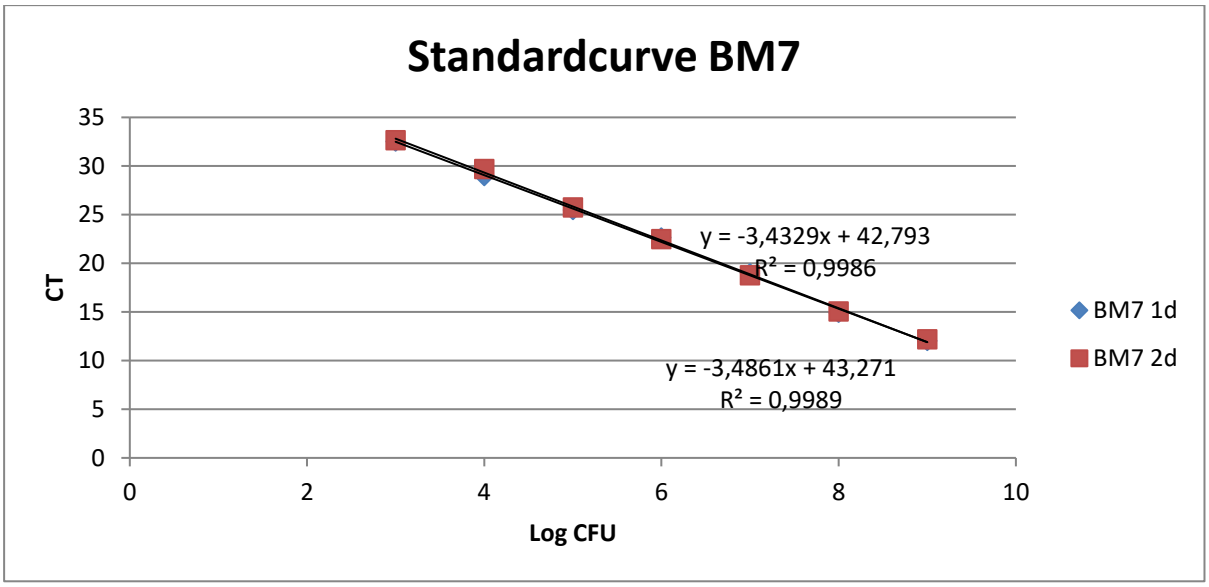

Figure 31: Graph of qPCR standard curve BM7

3.2 Efficacy of BP1 and BP2 in controlling *Xf* in *Nicotiana benthamiana* plants.

Throughout the experiment, the severity of infections caused by *Xf* in *N. benthamiana* plants treated with BP1 and BP2 was evaluated. Up to 24 days after *Xf* inoculation (the severity of infections will also be quantified at 40 and 50 days after inoculation) no clear symptoms attributed to the typical symptoms in leaves associated with *Xf* diseases were observed in *N. benthaminana*. Only one leaf from BP1 and BP2 began to show marginal necrosis(Figure 32). The intensity of symptoms did not increase overtime (13 dpi and 24 dpi). In this context [24] confirm that almond plants inoculated with *Xf* begins to show symptoms at 60 dpi, with a maximum of 120 dpi.

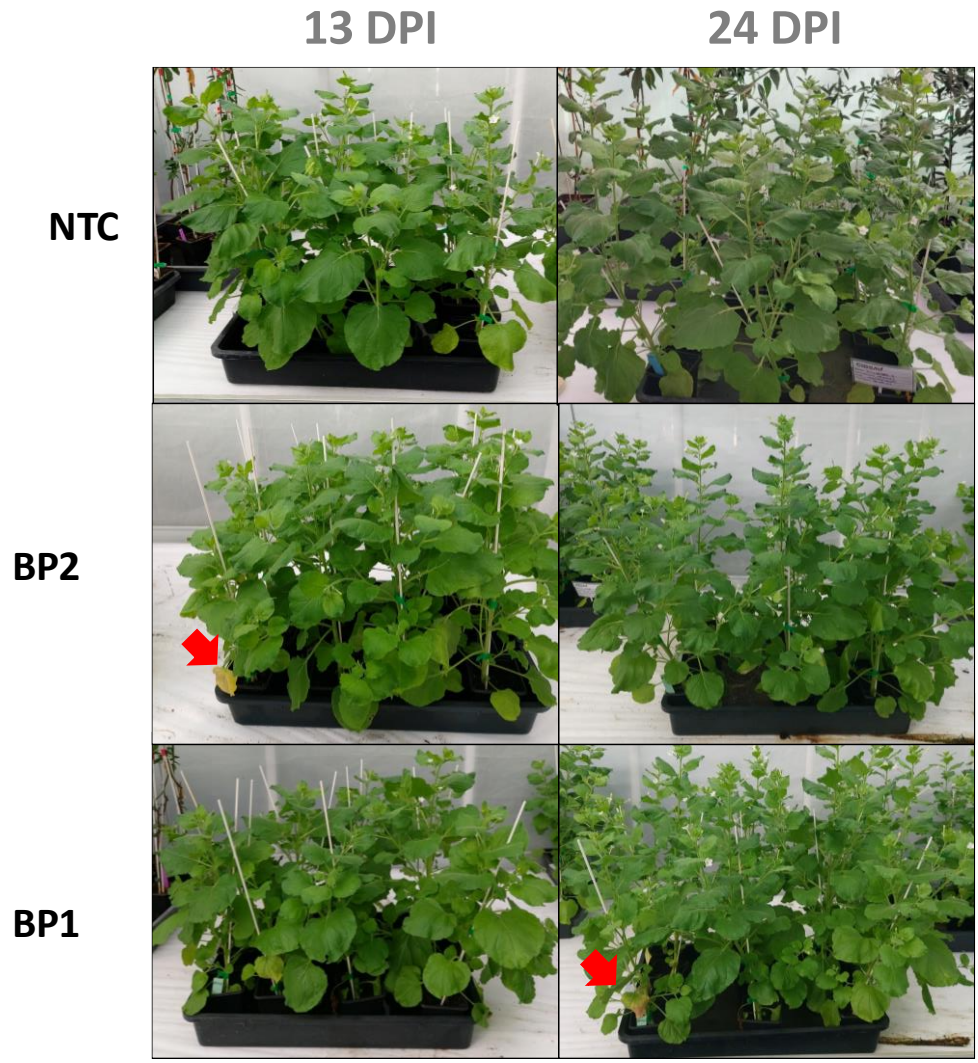

Figure 32: Images of the N. benthamiana plants in the efficacy test of BP1 and BP2 peptides in the control of Xf, at 13 and 24 days after the inoculation of the pathogen (dpi). The red arrow indicates typical symptoms on leaves associated with diseases caused by Xf in plants

3.1.5 Spread of *Xylella fastidiosa* from inoculation point to basal and apical zones of the plant

Quantification of total and viable cells in sap and branch of plants treated with BP1 and BP2 was performed by qPCR. The tables (Table 5)(Table 6) shows the ct values in relation to the treatment and the sampling zone at 13 and 24 dpi, respectively.

Table 5: Quantification of viable and total Xf cells in sap and branch 13 dpi (Ct values)

			SAP		BRANCH
	PLANT	ZONE	Viable cells	Total cells	Total cells
NTC	1	U1	38,3	39,4	38,0
		U2	ND	ND	39,1
		D	ND	ND	ND
	2	U1	39,8	ND	35,9
		U2	41,4	ND	ND
		D	ND	ND	38,4
	3	U1	37,4	39,7	30,8
		U2	ND	ND	ND
		D	37,0	40,3	35,7
BP1	1	U1	36,2	33,2	36,3
		U2	36,4	34,6	39,5
		D	40,2	38,8	37,9
	2	U1	37,6	32,9	35,8
		U2	38,3	36,9	38,5
		D	40,6	38,0	40,5
	3	U1	39,1	35,6	33,5
		U2	38,5	35,0	39,1
		D	40,9	40,4	ND
BP2	1	U1	39,6	34,2	33,6
		U2	43,7	31,4	38,9
		D	37,7	39,4	35,4
	2	U1	36,6	33,6	33,7
		U2	37,6	35,9	39,4
		D	38,7	37,3	39,8
	3	U1	35,1	30,7	31,5
		U2	35,2	32,2	37,0
		D	ND	ND	ND

Upwards zone 1 (U1), the upwards zone 2 (U2), and downwards zone (D); ND, not detected. In red, ct values <35, positive detection of DNA of *Xf*.

Table 6: Quantification of viable and total Xf cells in sap and branch 24 dpi (Ct values)

			SAP		BRANCH
	PLANT	ZONE	Viable cells	Total cells	Total cells
NTC	1	U1	38,8	39,1	33,5
		U2	39,0	37,7	38,2
		D	ND	40,7	36,6
	2	U1	30,1	30,2	30,4
		U2	38,1	38,5	38,2
		D	32,9	33,4	29,9
	3	U1	36,3	34,9	30,2
		U2	39,0	38,6	37,1
		D	37,8	41,2	33,0
BP1	1	U1	38,5	34,2	32,1
		U2	ND	36,8	36,5
		D	38,1	36,7	37,6
	2	U1	39,5	35,4	31,3
		U2	35,7	33,0	35,5
		D	39,4	36,2	32,5
	3	U1	30,1	28,1	31,6
		U2	38,9	35,3	35,9
		D	37,3	35,2	34,9
BP2	1	U1	36,2	37,7	28,9
		U2	ND	38,6	35,4
		D	37,7	37,1	30,4
	2	U1	35,9	33,3	30,2
		U2	39,6	38,6	35,0
		D	40,4	37,8	32,9
	3	U1	38,9	35,7	29,5
		U2	38,1	35,6	36,1
		D	39,2	38,1	37,5

Upwards zone 1 (U1), the upwards zone 2 (U2), and downwards zone (D); ND, not detected
In red, ct values <35, positive detection of DNA of *Xf*.

At 13 dpi, as expected, *Xf* levels were still low in all three treatments analyzed (values close to 35, which is the limit of detection, EPPO standards, PM7/024(4) *Xylella fastidiosa*). Specifically, in NTC plants *Xf* was detected in the U1 zone (which include the inoculation point) of the branch of one of the three sampled plants, but not in any sap sample. By contrast, in plants treated with BP1, no *Xf* was detected in any of the branch analyzed. In sap tissue, DNA of *Xf* was detected in two of the three plants analyzed (Ct values <35) although no viable cells were observed (PMAxx ct values are >35). Concerning BP2, *Xf* was detected in branch of 100 % of analyzed plants. Similarly, DNA of *Xf* was detected in all sap samples, but in none of the plants *Xf* is alive (PMAxx >35 ct). Clearly, BP1 and BP2 exhibit bactericidal activity against *Xf*, after preventive and curative treatment at 13 dpi. These results are corroborated by the results of a paper from last year [22].
At 24 dpi the levels of *Xf* increase along the different zones of the plant. *Xf* was detected in all U1 zones of the 100 % of plants analyzed, and for all treatments. *Xf* also was detected in most of D zones of these plants. In one of the three plants of the NTC and BP1 treatment, viable *Xf*

can already be detected in sap (ct values of PMAxx and NoPMAxx samples <35). By contrast, viable *Xf* cells were not detected in in sap of any BP2 plants analyzed. Therefore, the application of BP2 seems to continue controlling *Xf* growth in *N. benthaminana* plants at 24 dpi.

It is important to note that, during plant sampling, in some of the plants treated with BP1 or BP2, phytotoxicity was observed along the vascular vessels (without compromising the nutrients or water intake, as observed in figure 33). Probably, this phytotoxicity can be related with AMP application [41].

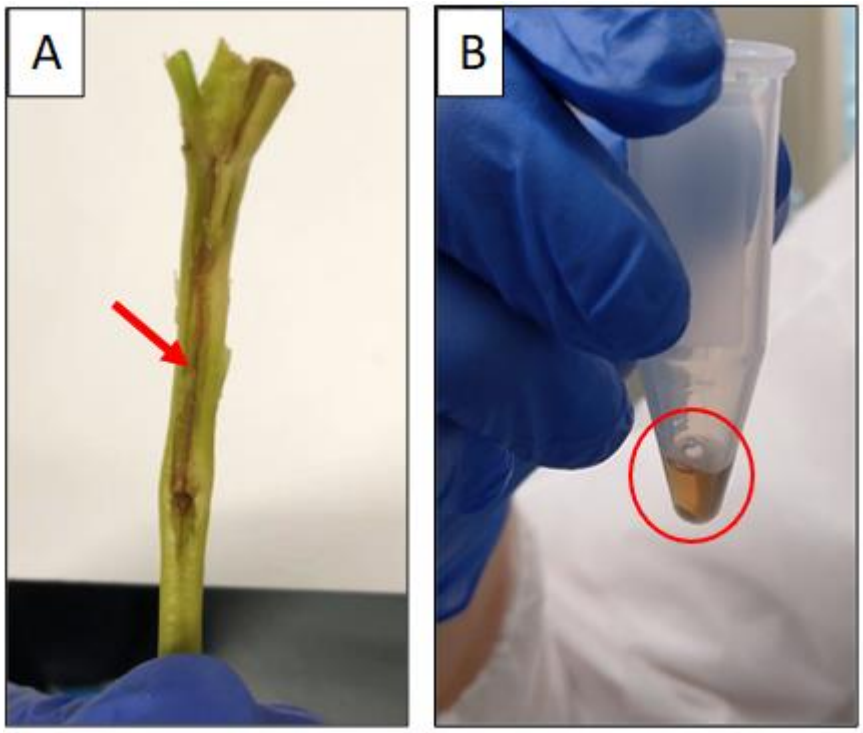

Figure 33: Phytotoxicity observed in vascular tissue of N. benthaminana plants treated with the AMP BP2. Necrotic zone is indicated with a red arrow. (B) Brown sap obtained from branch where phytotoxicity is observed.

The next sampling (at 40 and 50 dpi) will allow us to confirm the efficacy of the BP1 and BP2 treatment in controlling bacterial diseases caused by *Xylella fastidiosa* in *Nicotiana benthamiana* plants. Analyzing the different zones of the plants we will study the progression of the *Xf* through the vascular tissue.

4 GENERAL CONCLUSION

1) Antibacterial, bactercidal and viability assessment allow to identify antimicrobail peptides with high antibacterial activity against *Liberibacter crescens*.

2) BP5, BP3, BP24 and BP31 are among antimicrobial peptides that showed strong antibacterial activity against *Liberibacter crescens*, with MIC values in the range of 6.25 µM and 50 µM. BP5 has the overal highest antibacterial actitivity and has been selected to be tested *in planta* for the control of *Candidatus* liberibacter in citrus plants.

3) A combined peptide treatment strategy (preventive and curative) confirmed that BP2 controls the growth of *Xylella fastidiosa subsp. pauca* in *Nicotiana benthamiana* plants during the first stages of infection.

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