Summary of P/TF

Theoretical background of AMP's and used bacteria

Antimicrobial peptides (AMPs) are small peptide sequences of generally 12-50 amino acids and are an alternative for plant disease management. These AMPs are present in all living-organisms and are 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. 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. As AMPs display multiple modes of action, the emergence of resistant mechanisms in target pathogens would require significant alteration of the membrane composition. Despite their diversity and advantages they still have limitations to plant disease control as not all natural AMPs shows efficacy in controlling plant diseases. 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. 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. HLB is a quarantine pathogen that causes blockages in the vessels of the plant reducing sap flow. Because *Candidatus* Liberibacter is a quarantined pathogen a non-pathogenic bacterium closely related to members of the genus *Candidatus* Liberibacter namely *Liberibacter crescens* was used. *Lc* is the only member of the genus that can be grown in a media outside of plants. 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.

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. *Xf* works almost in the same way as *Lc* does when infecting plants. It travels through the xylem vessels 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. In this study, the efficacy of AMPs in controlling *Xf* infections in *Nicotiana benthamiana* plants is evaluated.

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).

Screening of peptides against *Liberibacter crescens*

Peptide antibacterial activity was tested trough measuring the optical density of samples with *Lc* suspension in the presence of different antimicrobial peptides and a variety of different

concentrations. These optical density values were then put into a graph to visualise the growth inhibition of the different peptides at their respective concentrations. With this test the MIC or the Minimum inhibitory concentration of the peptides could be determined.

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). To carry out this assay, the samples of the antibacterial activity test was needed. These samples were then transferred to new wells and accompanied by resazurin. During the test, fluorescence was measured and the values were put in a graph to see the viability of *Lc* against the different peptides and their concentrations.

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 for the screening of antibacterial activity. In this test, *Lc* inoculum was made and brought into contact with the different tested peptides and their concentrations. This mixture of *Lc* inoculum and peptides was incubated for 2 h and then plated on BM7-agar plates. The mixture was then further incubated and plated over a course of 2 days. This way could be seen if the peptides and their respective concentrations could fully inhibit the culturable *Lc* cells for a period of time. From the results of these experiments a MKC value or Minimum Killing Concentration was determined. It was evaluated by viable plate counting method, value was taken as the lowest peptide concentration with no growth at the end of the experiment.

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. 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.

Screening of peptides against *Xylella fastidiosa*

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.

To determine the effect of the peptides on the population levels of *Xf* subsp. *pauca*, peptides 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*. 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. To perform v-qPCR of bacteria and other organisms a high-affinity photoreactive DNA binding dye named PMAxx was used. 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. 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.

The spread of *Xf* trough out the *Niotiana benthamiana* plants was evaluated at 13 days after inoculation and 24 days after inoculation. Quantification of total and viable cells in sap and branch of plants treated with BP1 and BP2 was performed by qPCR.

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.

DNA purification and detection

When there was a need for purified DNA in any of the above mentioned experiments, DNA extraction was carried out. This purified DNA was then used in qPCR or v-qPCR to detect or quantify the bacterial cells after the experiments. For the DNA extraction the GeneJet Genomic DNA purification kit for Gram negative bacteria (Thermo Fisher Scientific, Walthman, EEUU) was used. The protocol that was described on the website of Thermo Fisher Scientific was followed for all the extractions. The Thermocycler from Quantstudio used for performing qPCR was included with a fluorometer for detecting fluorescence that was due to the hydrolysis of the Taqman probe. When the thermocycler was running fluorescence could be detected during the amplification process of PCR by the fluorometer. The real-time values that were detected by the fluorometer were translated in a graph into information about relative and absolute amounts of DNA.

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.