LIFE PROJECT
AFTER CU

LIFE12 ENV/IT/000336

"Anti-infective environmental friendly molecules
against plant pathogenic bacteria for reducing Cu"

ANNEX 11

DELIVERABLE ACTION C4

Monitoring of the absence of a direct selection operated by the anti-virulence peptides towards the emergence of bacteria resistant to the anti-virulence peptides themselves at laboratory level

TABLE OF CONTENTS

1. INTRODUCTION

This action was focused to monitor at laboratory level the absence of the emergence of bacteria resistant to the anti-virulence peptides following repeated treatments with these molecules. This action has the objective to evaluate a phenomenon usually occurring on bacterial populations when treated with anti-bacterial molecules used, where selective toxicity means that the chemical being used should inhibit or kill the pathogen of interest without damaging the host. Among the most important antimicrobial chemotherapeutic agents there are antibiotics, which are metabolic products of one microorganism inhibiting or killing other microorganisms. But bacteria can become resistant to antibiotics by several mechanisms coded by genes that are present in the mobile and easily transmissible parts of the bacterial genome, called plasmids. Copper treatment induced resistance on bacterial population as well, and generally this leds to the simultaneous emergence of resistance to antibiotics, because of a cross-resistance mechanism based on the presence on the same plasmids of both the gene for copper- and antibiotic-resistance. The AFTER CU anti-virulence peptides specifically target T3SS, a system essential for bacterial pathogenicity but not for life. This means that the selective pressure generated by anti-virulence peptides application would be very low and thus a null or neglegible risk of resistance can be hypothesized. The activities carried out until now, by using a standard dilution micro-method and several reporter genes, demonstrated that repeated anti-virulence peptides' treatments on the *Pseudomonas* species here used as a model did not decrease their inhibition activity on T3SS, thus indirectly confirming the absence of selection for resistance to anti-virulence peptides themselves.

2. DELIVERABLE ACTION C4

2.1 Anti-virulence peptides bacterial treatment

Bacterial strains *Psn23 (Pseudomonas savastanoi* pv. *nerii)*, *Psa* (*Pseudomonas syringae* pv. *actinidiae*) and *Pss* (*Pseudomonas syringae* pv. *syringae*) were subjected to an anti-virulence peptides stress, a bacterial growth on minimal medium (MM), resembling plant apoplast conditions, performed during 48 hours in orbital shaking (100 rpm) at 26°C. Peptides AP17 and Li27 were added at 100 µM concentration, in a flask of 100 ml, with a starting inoculum of 0.5 OD (600 nm) from an overnight culture. Every 12 hours, a sample of 1 ml was collected from each bacterial preparation in order to evaluate its capability to reduce HrpA promoter activation and T3 gene expression cluster, and to induce HR response on Tobacco leaves. The same strain, not treated, was used for each bacterial species as a positive control.

The anti-virulence peptides treatment produced 20 (20^{th}), 40 (40^{th}), 60 (60^{th}) and 80 (80^{th}) bacterial generation at 12, 24, 36 and 48 hours of incubation respectively (Gullberg *et al*., 2011). All these strains were used in subsequent experimental tests, to identify differences between generations treated and positive control (wild type, untreated).

2.2 *In vitro* growth on rich and minimal medium

Anti-virulence peptide treated bacteria were compared in their capability to growth *in vitro* in minimal medium (MM) and in rich medium (KB). The growth curves were built analyzing absorbance peak at 600 nm during 70 hours, through a real-time evaluation of each bacterial strain in UV spectrophotometer.

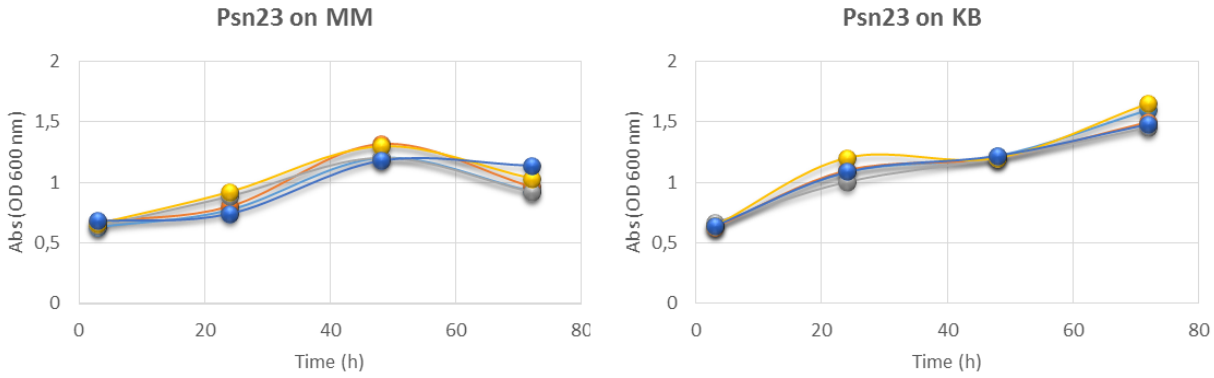

Fig. 1 Bacterial growth of Psn23 in MM and KB medium. Wild type (light blue), 20^{th} generation (orange), 40^{th} generation (grey), 60^{th} generation (yellow), 80^{th} generation (blue).

As reported in Figure 1, we did not observe significant changes in bacterial growth during 72 h of analysis for *Psn23*, demonstrating the inability of anti-virulence peptides to induce selection. The same results were obtained with *Pss* and *Psn23* (Fig. 2).

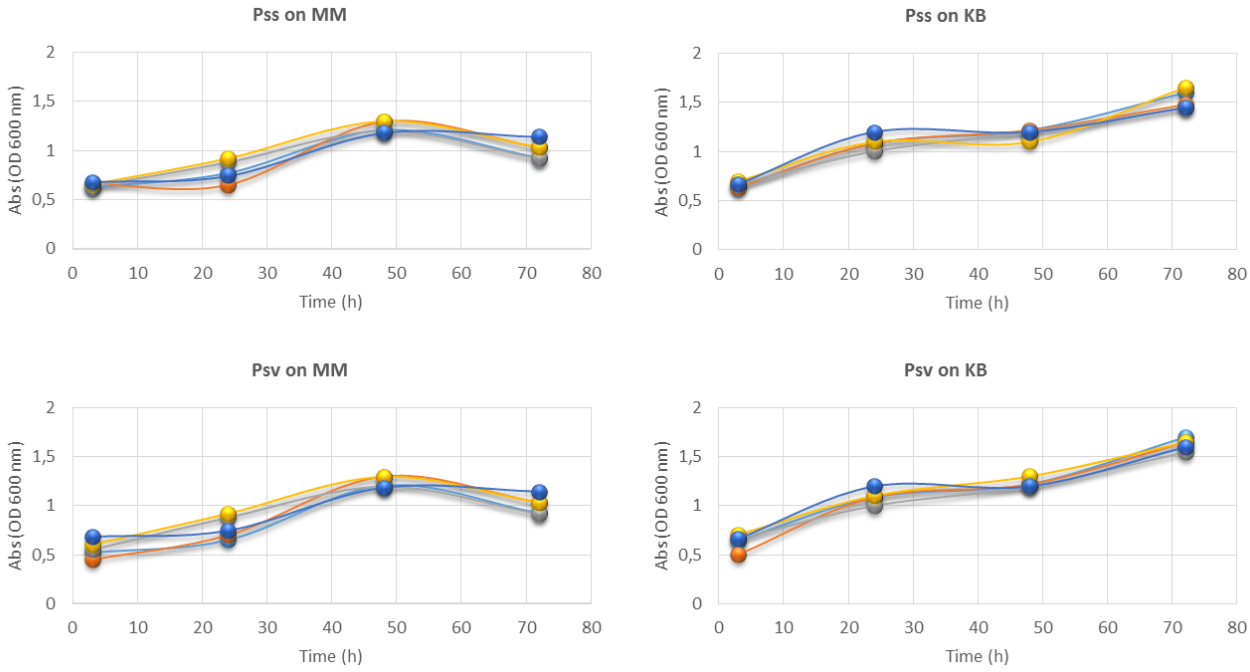

Fig. 2 Bacterial growth of *Pss* and *Psv* strain *Psn23* in MM and KB medium. Wild type (light blue), 20th generation (orange), 40th generation (grey), 60th generation (yellow), 80th generation (blue).

2.3 *In vitro* T3SS promoter reduction

The inhibition of T3SS on the phytopathogenic bacteria, here used as a model (the other two species, *Pss* and *Psn23*, will not be analysed in all subsequent experiments), was monitored and demonstrated also following a different and complementary technical approach, which consists in the use of reporter genes (e.g. *gfp* and *luc*) to evaluate activation of the promoter driving the expression of the *hrpA* gene, hereafter indicated as pT3. The *Psn23* bacteria were transformed with the plasmid pLPVM-pT3-GFP, and then grown in MM, with or without adding the anti-virulence peptides. Bacterial cells were grown overnight in KB from 0.1 OD (Abs 600 nm), washed twice with sterile physiological solution (SPS, 0.85% NaCl in distilled water), and transferred into MM alone or supplemented with 30 µM of AP17 and Li27 solution. Data were obtained evaluating the fluorescence activity, given by the activation of pT3 promoter, after 16 h at 26°C in culture plates, shacked at 100 rpm. The results obtained are reported in Figure 3, where it is possible to observe that peptides AP17 and Li27, in wild type strain, caused a strong reduction in the fluorescence emitted, as a consequence of a decrease in pT3

activation. This phenomenon is related to HrpA working on its promoter with a positive feedback: when HrpA is sequestered by AP17 or Li27, no more HrpA protein is available to trigger pT3 activation. In treated bacteria with anti-virulence peptides, we did not observe any differences in comparison with untreated.

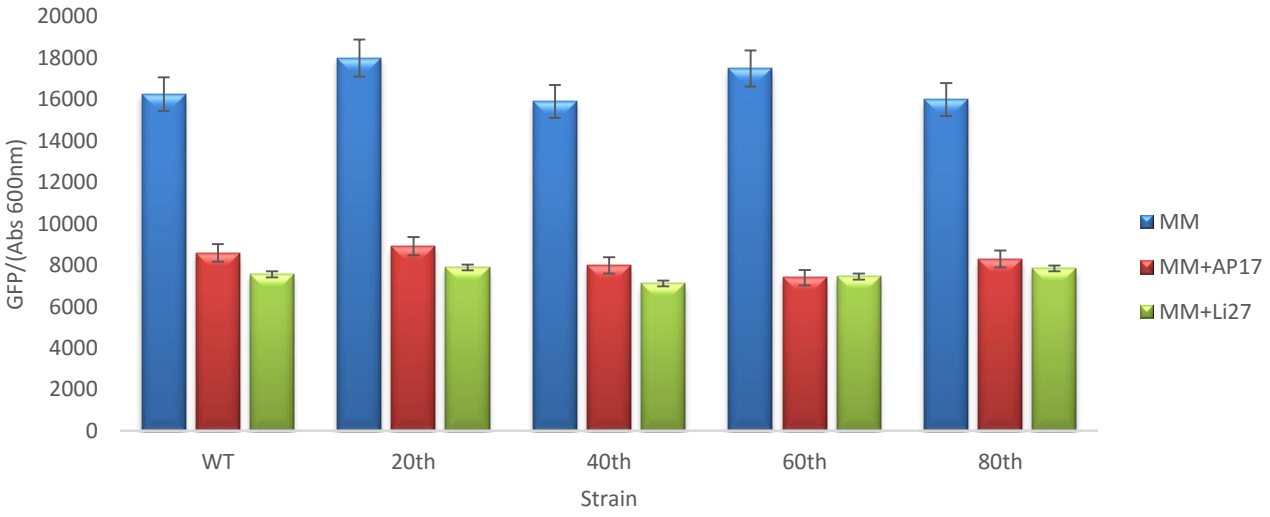

Fig. 3 Fluorescence emitted by *Psv* wild type *Psn23* transformed with pLPVM-pT3-GFP recombinant plasmid, when grown in minimal medium, resembling plant apoplast. Fluorescence was normalised with bacterial growth, expressed in abs at 600nm, after 16h of analysis.

2.4 *In vitro* gene expression downregulation

The activity of anti-virulence peptides against T3 promoter activation was demonstrated in previous section (2.3), therefore gene expression of *hrp* genes (involved in T3 pilus assembly and protein transport) should be modulated by the presence of these peptides. Unfortunately, this was the first time we had evaluated the relationship between *hrpA* promoter activity (used as representative of all T3 promoters) and gene transcription, in particular concerning *hrpA* (the main component of the T3 pilus), *hrpL* (a sigma factor involved in T3SS upregulation) and *hopAB1* (a protein translocated in plant cell through T3SS, correlated to host defence suppress). Bacterial cells of *Psn23* wild type were grown overnight in KB from 0.1 OD (Abs 600 nm), washed twice with sterile physiological solution (SPS, 0.85% NaCl in distilled water), and transferred into MM alone or supplemented with 30 µM of AP17 and Li27 solution. Cells were collected after 24 hours of incubation at 26°C under shaking (100 rpm) and used for RNA extraction performed with NucleoSpin® RNA Plus (Macherey-Nagel GmbH and Co. KG, Düren, Germany) and genomic DNA was eliminated by treatment with NucleoSpin® gDNA Removal Column (Macherey-Nagel GmbH and Co. KG, Düren, Germany). RNA Reverse transcription was performed using iScript™ Advanced cDNA Synthesis kit (Bio-Rad Laboratories

Inc., Hercules, CA, USA) with about 2 µg of total RNA. Diluted cDNA was analyzed with SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) using the CFX96 cycler - RealTime PCR Detection System and CFX-manager software v1.6 (Bio-Rad, Laboratories Inc., Hercules, CA, USA). To normalize the expression of each gene, the 16S rDNA expression level was used as housekeeping. The quantification results were based on triplicate samples. It is important to notice that there was a direct correlation in *Psn23* between the promoter inhibition and the gene downregulation, as reported in Figure 4.

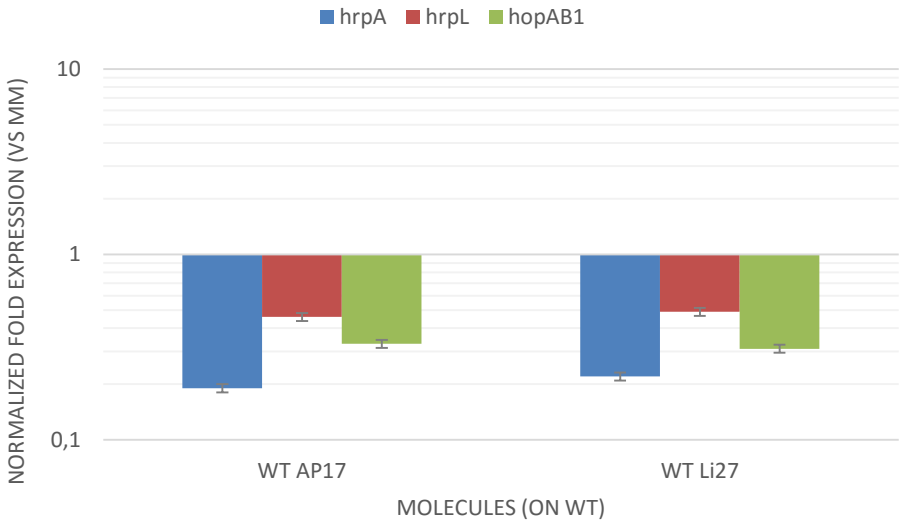

Fig. 4 Gene expression analysis of *Psn23* grown *in vitro* on MM supplemented with AP17 (30 µM) and Li27 (30 µM), evaluated in comparison with MM.

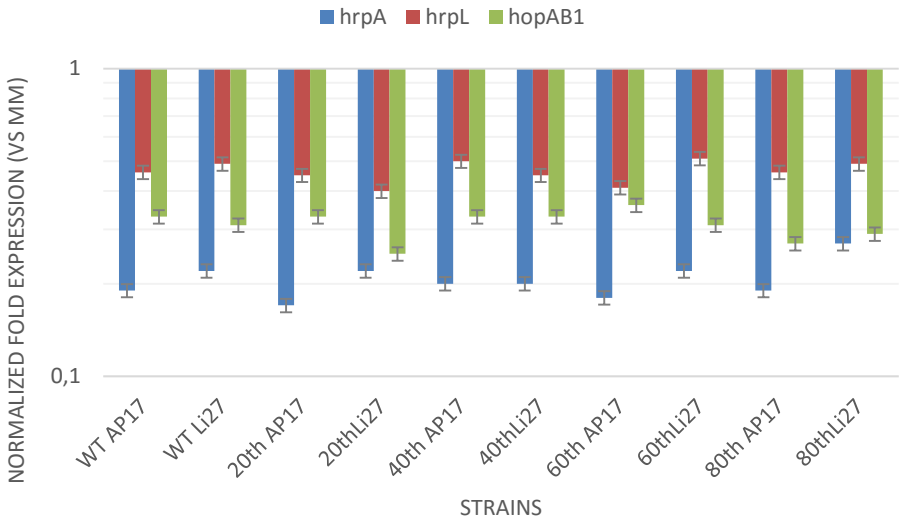

Fig. 5 Gene expression analysis of *Psn23* grown *in vitro* on MM supplemented with AP17 (30 µM) and Li27 (30 µM), evaluated in comparison with MM. Wild type (WT), 20th generation (20th), 40th generation (40th), 60th generation (60th), 80th generation (80th).

Results obtained for wild type strain *Psn23* should be comparable with the same bacteria treated until 80th generation with anti-virulence peptide (AP17 and Li27). As reported in figure 5, this phenomenon has been proven true and we can observe the same trend for all three genes both in wild type and in treated bacteria.

2.5 Hypersensitive response assays on Tobacco leaves

Nicotiana tabacum var. Burley White was grown at 24°C, at 75% relative humidity and a photoperiod of 16/8 h of light/dark. Bacterial cultures were grown overnight in KB medium at 26°C, resuspended in SPS to an 0.5 OD (Abs 600 nm), and bacterial cell concentration was confirmed by a serial dilution plating method. Using a 2 ml blunt-end syringe, approximately 100 µl of bacterial suspension was injected into abaxial mesophyll of fully expanded leaves of three tobacco plants (Baker *et al*., 1978), with six replicates for each strain tested, and for each of the three independent experiments carried out. The development of HR was assessed according to the macroscopic tissue collapse at 24 h post-inoculation, taking photographic record of the results obtained.

Psn23 wild type and anti-virulence treated strains were inoculated into non-host plant. All the bacteria were demonstrated able to triggered the hypersensitive response (HR) on this test with non-host plant (Figure 6). These data further confirmed that there are no considerable differences between wild type strains and treated bacteria.

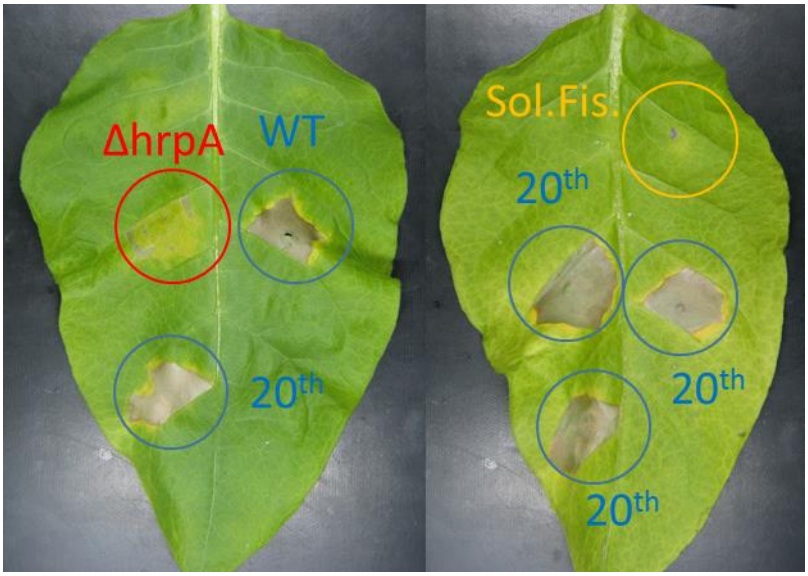

Fig. 6 Inoculation trials on Tobacco plants challenged with wild type *Psn23*, and its anti-virulence treated strains. Pictures taken 48 h after inoculation. *Psn23* wild type (WT), 20th generation (20th), 40th generation (40th), 60th generation (60th), 80th generation (80th), ΔhrpA *Psn23* mutant (ΔhrpA) and negative control, SPS (Sol.Fis.).

2.6 Pathogenicity trials on *in vitro* micropropagated Oleander plants

In vitro micropropagated Oleander (*Nerium oleander* L.) (Vitroplant Italia s.r.l., Cesena, Italy), with red double flowers, were grown for 3 weeks at 26°C on MS (Murashige and Skoog, 1962) without addition of phytohormones, with a photoperiod of 16 h/light-8 h/dark. Then, plants were wounded on the stem at the second internode, using a 1 ml syringe needle, and immediately inoculated with 1 µl of a bacterial suspension in sterile physiological solution (SPS, NaCl 0.85% in distilled water), with a 0.5 OD (Abs 600 nm) (about 0.5×108 CFU/ml). Negative control plants were inoculated with SPS alone. Plants were then incubated at 26°C, and a 16 h/light-8 h/dark photoperiod, periodically monitored for symptoms appearance and photographic record was made at 21 days. Three independent experiments were performed, and nine plants for each *P. savastanoi* strain were used in each run.

Psn 23 wild type and anti-virulence treated strains (20^{th}, 40^{th}, 60^{th} and 80^{th}), inoculated into *in vitro* micropropagated Oleander plants, have demonstrated that their ability to typical hyperplastic induce symptoms on host plant was not impaired by peptide treatments (Figure 7). Results obtained further confirmed previous data.

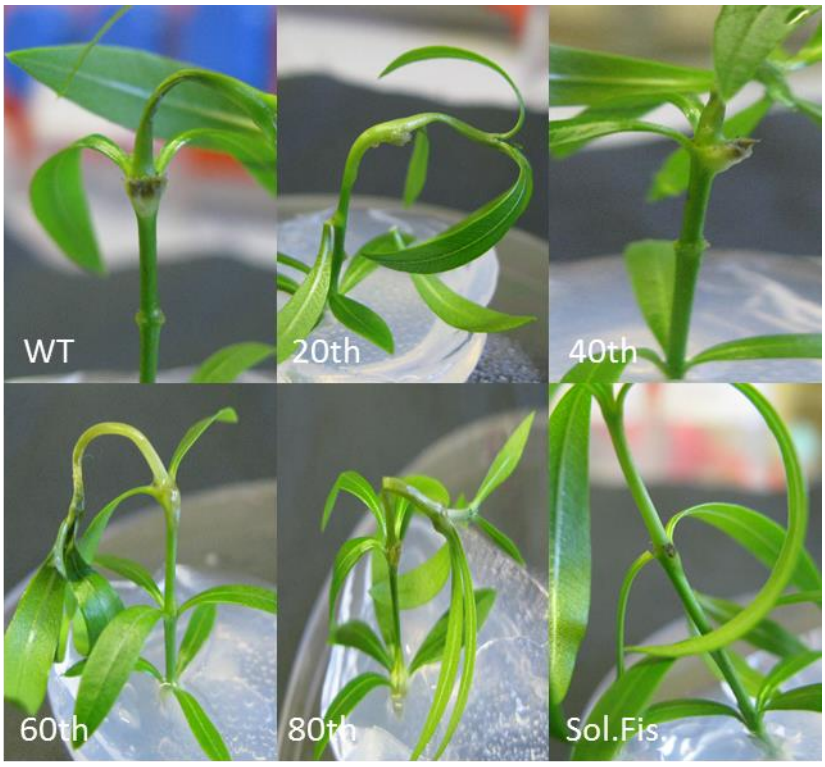

Fig. 7 Inoculation trials on *in vitro* Oleander plants challenged with wild type *Psn23*, and its anti-virulence treated strains. Pictures taken 21 days after inoculation. *Psn23* wild type (WT), 20^{th} generation (20^{th}), 40^{th} generation (40^{th}), 60^{th} generation (60^{th}), 80^{th} generation (80^{th}) and negative control, SPS (Sol.Fis.).

3. CONCLUSIONS

Activities performed in this action had the objective to demonstrated that repeated anti-virulence peptides' treatments on the *Pseudomonas* species, in particular *Psn23 (Pseudomonas savastanoi* pv. *nerii)*, *Psa* (*Pseudomonas syringae* pv. *actinidiae*) and *Pss* (*Pseudomonas syringae* pv. *syringae*), did not decrease their inhibition activity on T3SS, thus indirectly confirming the absence of selection for resistance to anti-virulence peptides themselves. This phenomenon usually occur on bacterial populations when treated with antibiotics, where selective toxicity means that the chemical being used should inhibit or kill the pathogen without damaging the host. In fact bacteria can become resistant to these molecules by several mechanisms coded by specific genes, that are present in their plasmid genome. Anti-virulence peptides used in this project to treat *Pseudomonas* species, until 80 generations, did not induce any type of resistance. Bacteria treated with the anti-virulence peptides have been shown to have the same behaviors of type strains in all the tests performed.

Based on these results we can affirm that the anti-virulence peptides designed, synthesized and used in this demonstration study not induce resistance phenomena in phytopathogens and thus they will extremely useful for plant diseases control.

4. REFERENCES

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