LIFE PROJECT
AFTER CU

LIFE12 ENV/IT/000336

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

ANNEX 5

DELIVERABLE ACTION B5

Demonstration of the null toxicity profile of the anti-virulence peptides on model organisms and microorganisms

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1. INTRODUCTION

The use of copper in agriculture has been sometimes indiscriminate and led to an urgent search for new plant protection compounds/molecules to be used against phytopathogenic bacteria and fungi. Besides their biological activity against phytopathogens, the candidates selected have to be tested also for their toxicity.

The toxicity of a molecule is usually defined in terms of the biological response obtained by a particular organism/microorganism following the treatment with the molecule to be tested. In other terms, the toxicity so evaluated reflects the harmful effects on the test organism/microorganism upon exposure to well defined concentrations of the chemical to be assayed and for a given period of time. Therefore, in a toxicity test organism/microorganism are used to identify the minimum concentration of the molecule to be tested that results in a defined disturbance, thus determining the level at which exposure becomes harmful. Usually the toxicity tests can fall in two main categories, which are evaluating acute and chronic toxicity, respectively. The models for measuring acute toxicity evaluate the harmful effects on the test organism/microorganism usually as its mortality occurring within a brief period of exposure to the molecule to be tested. The experimental models for chronic tests are more difficult to be performed, and generally evaluate those harmful effects occurring on the test organism/microorganism and related to several of its significant biological functions, within a period of exposure on the timescale of the full life cycle (Beelen, 2003).

It should be underlined that no one organism/microorganism can be defined and considered to be the ideal indicator for a toxicity test. For instance, algae are more sensitive to toxic metal ions than are vascular plants. Conversely, vascular plants are more sensitive to herbicides than are algae. Still concerning herbicides, it was found that *Rhizobium* spp. is much more sensitive to the toxicity of herbicides than are rats or birds, but was not as sensitive as *Daphnia magna* (Guimaraes *et al.*, 2012). Therefore, the choice of a specific organism/microorganism as indicator does strictly depend on the nature of the chemical which has to be tested. Current *in vivo* tests on laboratory animals are performed according to outdated guidelines and are going to be replaced entirely by sophisticated *in vitro* procedures carried out on several microorganisms and organisms used as models. In general terms, toxicity assays using bacteria as test organisms are generally included in the short-term toxicity tests. Undoubtfully, they have several advantages mainly given by the biochemical cycles of bacteria that are not as complex as the cycles of other organisms, and by the short life cycle displaying a rapid response to changes in environmental conditions. Moreover, it is possible to perform these bioassays in

small sample volumes, with many replicates, high reproducibility and lower costs than those of other toxicity tests (Parvez *et al.*, 2006).

2. DELIVERABLE ACTION B5

2.1 Experimental design and results

In the present Action B5, the absence of any toxicity of the AFTER CU anti-virulence peptides has to be demonstrated by applying several *in vitro* tests, already validated and accepted by international regulatory authorities. According to the AFTER Cu project plan, the *in vitro* toxicity tests of these anti-virulence peptides was performed using prokaryotic and eukaryotic organisms.

The anti-virulence peptide AP17, Li27 and Psa21were at the end the most studied, because in the meantime they were demonstrated the most biologically active in the other Actions.

By using several model bacteria, such as *E. coli*, *Bacillus cereus* and *Staphylococcus aureus*, and fungi and yeasts (*Candida albicans* and *Saccaromyces cerevisiae*), the antibiotic activity of the AFTER Cu peptides was confirmed to be above 300-1,000 µM. Experiments were carried out by traditional plating tests on solid media, amended with several concentrations of the the AFTER Cu peptides (from nM to mM). As a negative control, no amended plates were used. As a positive control, copper sulphate at different concentrations ranging from 10 µM to 6 mM were used. On each plate bacterial or fungal spots (5 µL each) were separately plated, having a concentration around 10^6 CFU/ml. After 24-48 h of incubation at the optimal temperature for each organism used (*e.g.* 37°C for *E. coli*), the colonies growing were estimated (Figure 1).

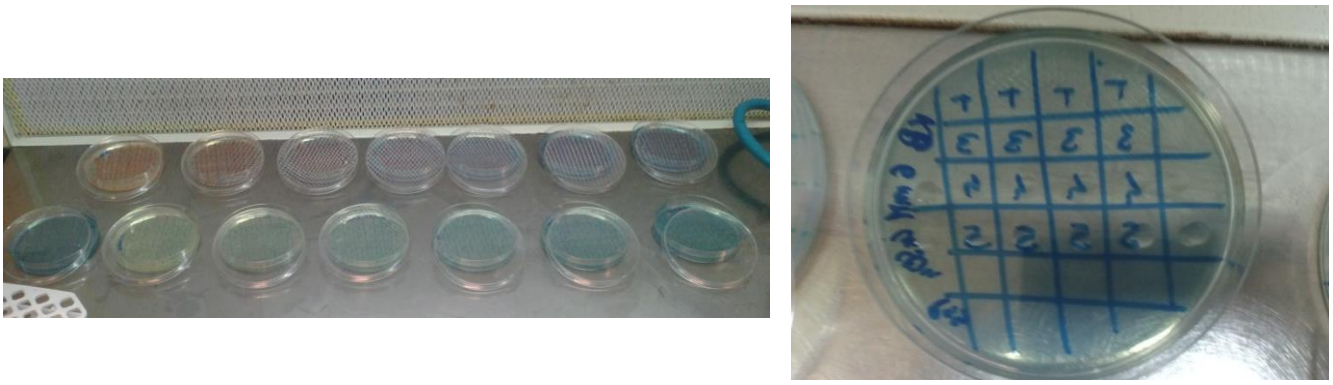

Fig. 1 Toxicity test on *E. coli* performed on solid LB an KB media, amended with the AFTER CU anti-virulence peptides AP17, Li27 and Psa21 (here showed from 10 µM to 2 mM), and using as positive control copper sulphate (10 µM to 6 mM).

On the basis of these data, then the experiments were carried out in liquid media, using microliter 96 well plates (Figure 2). The bacterial growth was evaluated as optical density at 600 nm (OD_{600}). The results obtained so far are reported in Table 1 and Table 2, and they showed the absence of any toxicity for the AFTER CU anti-virulence peptides, unless very high concentration were used such as 1,000 and 2,000 µM.

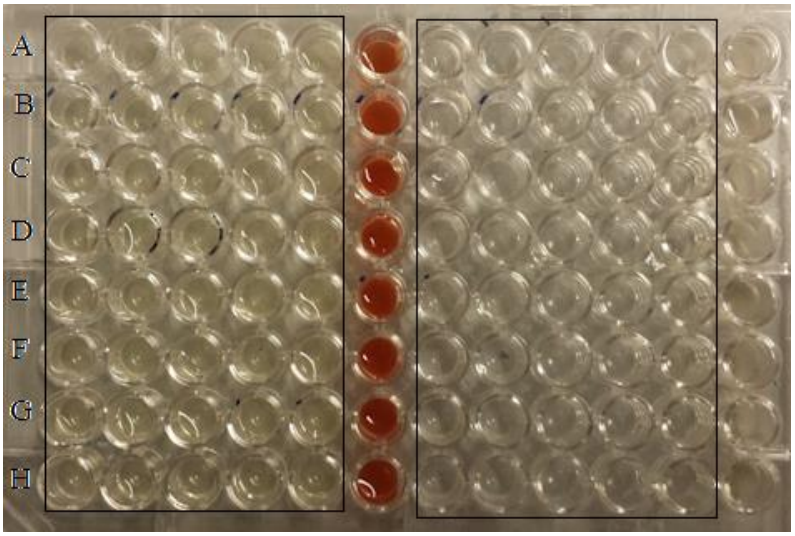

Fig. 2 Microliter 96 well plates used to perform toxicity test on *E. coli* on liquid LB an KB media, amended with the AFTER CU anti-virulence peptides AP17 or Li27 (10 µM to 2 mM).

It is worth to underline that the AFTER CU anti-virulence peptides were demonstrated to be active as plant disease protection products against bacteria belonging to the group *Pseudomonas syringae sensu lato* at concentrations of 30 µM, as fully described in the Annexes for Deliverables B3 and C2.

A	B	C	D	E	F	G	H
0	10 µM	30 µM	50 µM	100 µM	500 µM	1,000 µM	2,000 µM
100%	100%	100%	100%	100%	93%	89%	77%

Table 1. Growth inhibition of *E. coli* grown in LB solid medium amended with several concentrations of the anti-virulence peptide AP17, evaluated as percentage of the growth on the untreated medium.

A	B	C	D	E	F	G	H
0	10 µM	30 µM	50 µM	100 µM	500 µM	1,000 µM	2,000 µM
100%	100%	100%	100%	100%	91%	85%	72%

Table 2. Growth inhibition of *E. coli* grown in LB solid medium amended with several concentrations of the anti-virulence peptide Li27, evaluated as percentage of the growth on the untreated medium.

Concerning the eukaryotic organisms *Daphnia magna* and *Artemia salina*, no toxicity was detected below 300 or 1,000 µM concentration (Fig. 3).

Daphnia magna *Artemia salina*

Fig. 3 *Daphnia magna* and *Artemia salina* used for toxicity tests on the AFTER Cu peptides.

Moreover, a toxicity test was also carried out using luminescent bacteria (Microtox®), in which the inhibition of the luminescence of *Vibrio fischeri* was measured using a luminometer (Kapanen and Itävaara, 2001) after adding extracts of the samples. This assay uses a suspension of the luminescent bacteria *Vibrio fischeri* as bioassay organism for measuring acute toxicity in aqueous extracts. *V. fischeri* are non-pathogenic, marine, bacteria that luminesce as a natural part of their metabolism. When exposed to a toxic substance, the respiratory process of the bacteria is disrupted, reducing light output. *V. fischeri* has been demonstrated to be high sensitive across a wide variety of toxic substances, and a strong response to toxicity is observed as a change in luminescence, which is a by-product of cellular respiration. This change can be used to calculate a percent inhibition of *V. fischeri* natural luminescence, that directly correlates to toxicity of the compound tested.

Lyophilized bacteria were used after rehydration in the commercial solution of the test. All assays were carried out at 15 ºC, with 15 min and 30 min contact periods between 0.5 ml of bacterial suspension and compost suspension. Compost suspension was prepared by mixing 1g sample with 10ml of 2 % NaCl (w/w) solution. According to the official guidelines, a substance is to be considered toxic when giving EC50 (15 minutes at 15ºC) under 3000 mg/L by using Microtox®. As shown in Figure 4, the AFTER CU anti-virulence peptides AP17, Li27 and Psa21 have much higher concentrations before to be toxic under Microtox® test (from about 4 to 160 fold). Therefore, our results indicate that no toxicity was produced by AP17, Li27 and Psa21 peptides.

Reference	Ecotoxicity (mg/l)
Li 27	479.105.000
Ap 17	16.678,000
PSA 21	13.475.000

Fig. 4 Toxicity of AP17, Li27 and Psa21 peptides evaluated by Microtox® test.

In view of the application in the field of the AFTER Cu peptides for plant protection against Gram negative bacteria, we thought essential to determine if these peptides had some phytotoxic activity, that could make them inadvisable for agronomic use. To this aim, germination tests using seeds of rye grass (*Lollium perenne*) and *Lepidium sativum* seeds were carried out. This study was carried out in laboratory growth chambers, with control of temperature, humidity, and light conditions.

The potential phytotoxicity was established by seed germination test on filter paper. In Petri dishes with filter paper and 15 seeds of rye-grass (*L. perenne*), 2ml of different peptides dilution in distilled water was added and dishes were put in a germination chamber at 28 ºC, 75% relative humidity and darkness. After germination, the number of germinated seeds was recorded and the length of the seedling roots and shoots was measured. All treatments were carried out by quadruplicate, and Petri dishes with 2 ml of distilled water instead of peptides was used as control (Figure 5).

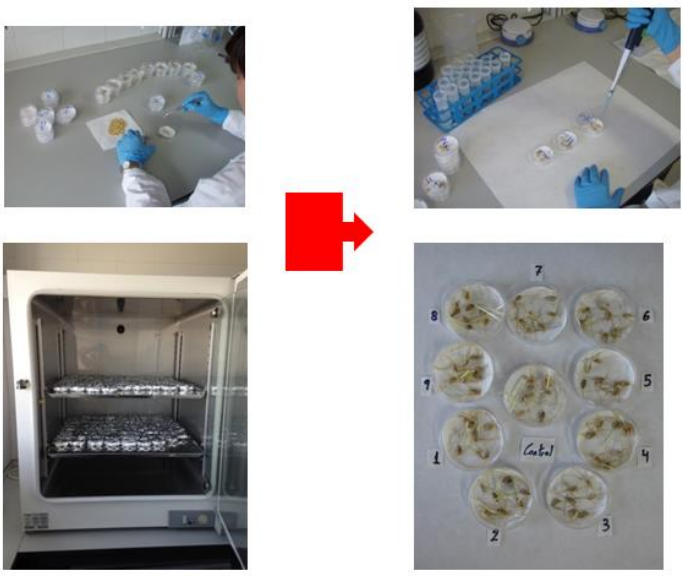

Fig. 5 Procedures for phytotoxicity tests on AP17, Li27 and Psa21 peptides.

Germination index (GI) was calculated according to the following equation (Zucconi and De Bertoldi 1987): $GI = \% GS (L_R/L_{RC})$; where GI = Germination index in percentage; %GS = Percentage of germinated seed with respect to the control; L_R = Average root length in the treated seedling; and L_{RC} = Average root length in the control seedling (Figure 6).

Germination index is a widely accepted indicator of potential phytotoxicity, for instance for organic amendments in agriculture. It combines the effect of the studied material on both, seed germination capacity and root elongation. This index was established by Zucconi and De Bertoldi (1987) using *Lepidium sativum* for evaluating compost maturity. They reported that GI values lower than 50% indicated phytotoxicity, composts being immatured. However, scientific community has generalized its use for any kind of organic material and with different kind of seeds. GI values lower than 50% indicate phytotoxicity; IG values between 80 and 50% indicate moderate phytotoxicity; GI higher than 80% indicate absence of phytotoxicity and values higher than 100% indicate a bio-stimulant effect.

Fig. 6 Germinated seeds under treatment with AP17, Li27 and Psa21 peptides.

The results obtained are shown in Table 3 and Figure 7. The peptides assayed increased the Germination Index value regarding the control. For root length, the highest value of GI was for Li27, and the lowest for Ap17.

Reference	Germination Index (%)	$\bar{x}$ (%)	Root Length (cm)	$\bar{x}$ (cm)
Control. 1	100,0	100,0	0,5	1,6
Control. 2	100,0		2,5	
Control. 3	100,0		0,6	
Control. 4	100,0		1,5	
Control. 5	100,0		2,9	
Li 27. 1	250,0	204,7	2,0	2,3
Li 27. 2	257,8		2,4	
Li 27. 3	179,7		2,3	
Li 27. 4	164,1		2,6	

Li 27. 5	171,9		2,8	
Ap 17. 1	203,1		1,9	
Ap 17. 2	156,3		5,0	
Ap 17. 3	195,3	192,2	3,1	3,1
Ap 17. 4	156,3		2,5	
Ap 17. 5	250,0		3,2	
PSA 21. 1	156,3		2,5	
PSA 21. 2	179,7		1,6	
PSA 21. 3	210,9	198,4	3,4	2,7
PSA 21. 4	195,3		3,1	
PSA 21. 5	250,0		2,7	

Table 3. Germination index and root length data under peptides treatment

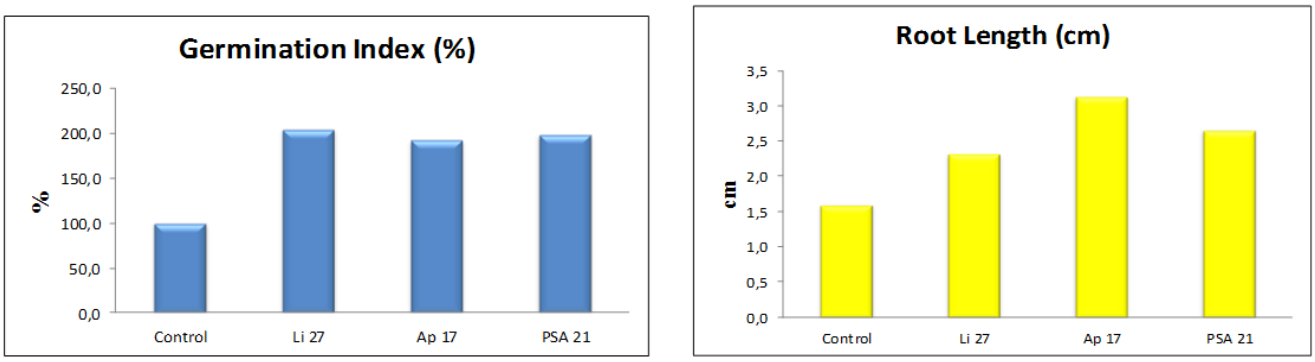

Fig. 7 Germination index and root lenght following treatments with AP17, Li27 and Psa21 peptides.

3. CONCLUSIONS

According to the results obtained for Action B5, it is possible to affirm that no any toxicity was found for the AFTER CU anti-virulence peptides AP17, Li27 and Psa21, using as model organisms *E. coli*, *B. cereus* and *S. aureus*, *C. albicans* and *S. cerevisiae*. No effect of these peptides were demonstrated on their growth and survival at concentrations demonstrated to be effective for plant disease control against *Pseudomonas syringae sensu lato* (*e.g.* 30 µM). Similarly, no toxicity was detected with the eukaryotic organisms *D. magna* and *A. salina*, below 300 or 1,000 µM concentration. Moreover, no toxicity was found for the AFTER CU anti-virulence peptides AP17, Li27 and Psa21 when tested by Microtox®, which is officially accepted for several environmental and industrial applications, such as for testing the toxicity of pesticides and other inorganic and organic chemicals.

At last, in view of their potential application for plant protection against Gram negative bacteria, the AFTER Cu peptides were tested for their phytotoxic activity. The results were really encouraging, because theyt were demonstrated to be no phyotoxic. Conversely, they showed a sort of biostimulant effect.

4. REFERENCES

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