LIFE PROJECT
AFTER CU

LIFE12 ENV/IT/000336

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

ANNEX 13

DELIVERABLE ACTION C6

Monitoring of the absence of an indirect selection operated by the anti-virulence peptides on copper compounds and antibiotic resistant bacteria at pilot scale level in field screening

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1. INTRODUCTION

Copper containing fungicides have been used to protect crops from bacterial diseases since about 200 years. Whilst farmer's objective is to apply fungicides to the agricultural crop/plant, inevitably a proportion of the chemical spray will miss its target. Much of the lost chemical will enter the soil surface where it will persist for a period of time and potentially migrate off-site due to leaching and/or runoff (Wightwick *et al.*, 2010).

Organic fungicide compounds persist for varying lengths of time in the environment, which is often expressed in terms of their expected half-life (Kookana *et al.*, 1998; Arias-Estevez *et al.*, 2008; Katayama *et al.*, 2010). The repeated use of copper-based bactericides and fungicides to control plant diseases has led to long-term accumulation of copper in the surface of some agricultural soils throughout the world (Mackie *et al.*, 2012).

Copper is an enzymatic cofactor in several metabolic processes and an essential trace element for crop growth at low concentrations (Dewey *et al.*, 2012). However, it is also a common soil contaminant, so, copper is known to accumulate within topsoil following sprays (Pietrzak & McPhail, 2004; Rusjan *et al.*, 2007) and can never be degraded (McBride *et al.*, 1981), its potential to have adverse eco-toxicological effects on the environment is large. Currently, EC regulation 473/2002 (European Commission, 2002) restricts the annual dose of applied Cu to 6 $kgCuHa^{-1}$, which corresponds to an annual accumulation of about 5 $mgCuKg^{-1}$ soil in the top 10 cm assuming no losses (Ruyters *et al.*, 2013).

It has been demonstrated that copper heavily affects the composition of microbial communities, including bacteria. Several studies highlighted a correspondence between copper contamination and the frequency of copper-resistant bacterial isolates. Changes in bacterial community structure to adapt to the stress given by the presence of copper have been demonstrated, and it mainly occurs by horizontal transfer of copper-resistance genes. More importantly, copper-contaminated soils also contain a higher percentage of antibiotic resistant bacteria than non-contaminated soils. It was demonstrated that the widespread accumulation of copper in agricultural soils contribute worldwide to increase antibiotic resistance through an indirect and environmental selection, with huge risks for the humans and animals health.

This action aimed to monitor the absence of an indirect selection operated by the anti-virulence peptides on copper- and antibiotic-resistant bacteria, to assess the further benefits deriving from the use of these innovative plant disease control molecules in comparison to the conventional copper

compounds. This action had the objective to demonstrate this essential feature of the anti-virulence peptides at field scale.

2. DELIVERABLE ACTION C6

2.1 Experimental design and methods

In this project, one of the most important objectives was to demonstrate the environmentally impact of anti-virulence peptides on soil and plant epiphytic microbiological communities, by examining how these affects soil and leaf surface microbiological activities and composition. Similar experiments were carried out in action C1, where demonstration activities started with investigations concerning how copper pollution could influence the physical-chemical features of soil. Instead, in this action we would demonstrate the null activity of anti-virulence peptides on changing of soil and plant surface microflora, to better highlight the potential of these compounds in bacterial control diseases.

All these analysis were carried out at field scale, using controlled land portion, to test the ability of anti-virulence peptide to maintain unchanged bacterial microflora in soil and plant surfaces. As reported in Figure 1, we used six plastic tunnel at ASTRA S.r.l., which contained over 300 plants each (about 2000 plants).

Fig. 1 Plastic tunnels at ASTRA (Faenza, Italy).

In the last few years, there has been an increase in molecular-based methods to evaluate soil microflora, such as as Denaturing Gradient Gel Electrophoresis (DGGE), and more recently also High Resolution Melting Analysis (HRMA), which is a very informative and cutting edge PCR-based method (as reported in Figure 2). In HRMA, when present saturating concentrations of DNA binding dyes (*i.e.* Syber Green), the specific sequence of the amplicon determines the melting behavior as the temperature of the solution is increased. Fluorescence intensity decreases as the double stranded DNA becomes single stranded and the dye is released. The melting temperature (Tm) at which 50% of the DNA is in the double stranded state can be approximated by taking the derivative of the melting curve.

The distinctive melting curve can used to detect DNA sequence variations in the amplicon without the need for any post-PCR processing.

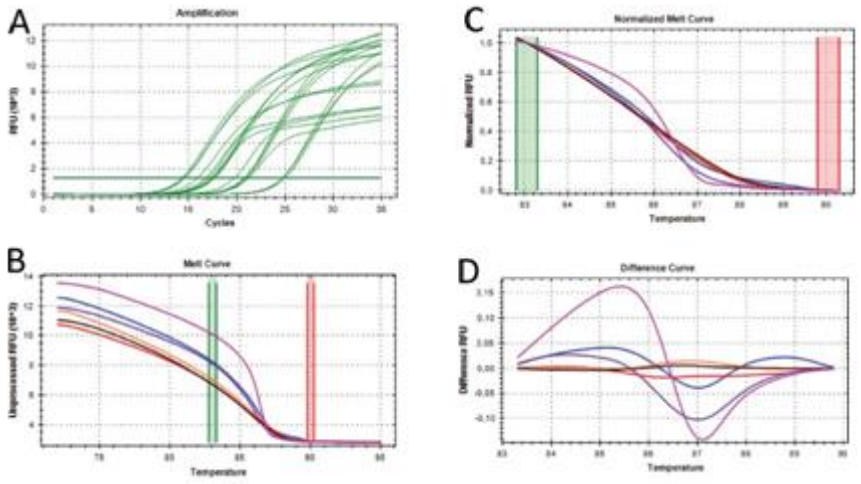

Fig. 2 Overview of the steps in 16S rRNA gene HRM analysis. Amplification and quantification of the 16S rRNA gene (A), melting of the PCR product in 0.1°C increments (B), normalization of the melt curves (C), conversion into difference curves in relation to control sample (black curve) (D).

The method is easy to use, highly sensitive, specific, low cost and yields rapid sample turn-around, in the identification of the different composition of soil bacterial communities (De Leeneer *et al.*, 2008; Kramer *et al.*, 2009; Whitehall *et al.*, 2009). Furthermore, HRMA is a non-destructive method.
Therefore, subsequent analysis of the sample by other techniques, such as gel-electrophoresis or DNA sequencing, can still be performed after HRMA analysis. These characteristics make HRMA ideal for use in routine settings. Due to its numerous advantages, HRMA has been widely applied in diagnostic laboratories for screening for bacterial biodiversity both at soil level that epiphytic microflora.

2.2 Absence of copper- and antibiotic-resistant bacteria due to anti-virulence peptides treatments

Pathogenicity trials and anti-virulence treatments were carried out on Olive and Kiwifruit plants in six plastic tunnels (about 400 square meters each) at ASTRA. Citrus was not interesting for Emilia-Romagna region and the climate conditions aren't good for its cultivation. ASTRA bought 850 Olive plants (5 years old) and 1050 Kiwifruit plants (3 years old).
Plants were treated once a week for three weeks by ASTRA with a single rate of AP17 or PSA21 (100µM solution in water). Control plants were not treated and used for comparison. For the evaluation of the anti-virulence peptide treatment, the plants have been periodically irrigated, fertilized and cleaned from weeds, and carefully observed. No visible differences were observed between treated

and untreated plants. After 28 days from anti-virulence peptide treatment, DISPAA researchers have collected soil and leaves in each plastic tunnel by following the sampling plan reported in Figure 3.

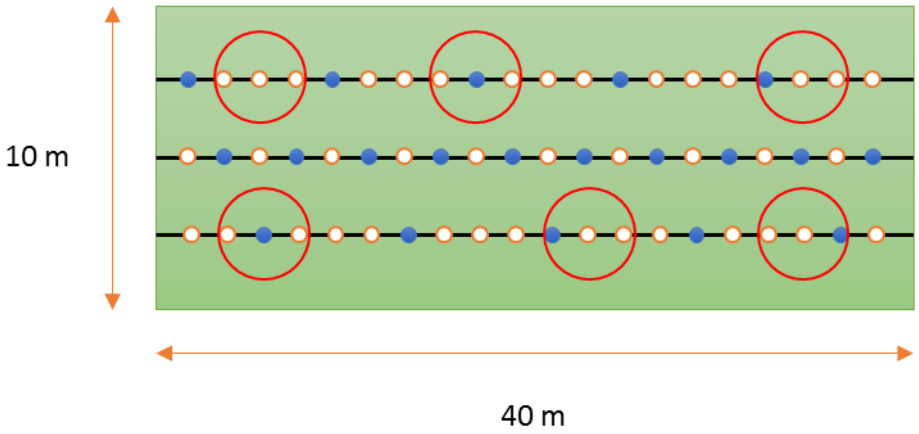

Fig. 3 Sampling plan for each plastic tunnel. Four hundreds square meters were parted in three lines and used to place about three hundreds Olive or Kiwifruit plants. In black were reported the seeding lines and each spot represent five plants in the ground. Blue spots indicate the plant group from which a single leaf was collected. Red circles identify specific areas from which 100 grams of soil were sampled.

2.2.1 Epiphytic microflora after AP17 and Psa21 treatment

Collected leaves obtained from plastic tunnels were weighed and put on flasks with sterilised water (500 ml), in order to resuspend bacterial microflora present in epiphytic surface. After 30 minutes of shacking (100 rpm) at room temperature, an aliquot of 50 ml were collected and centrifuged at 5.000 rpm for 10 minutes.

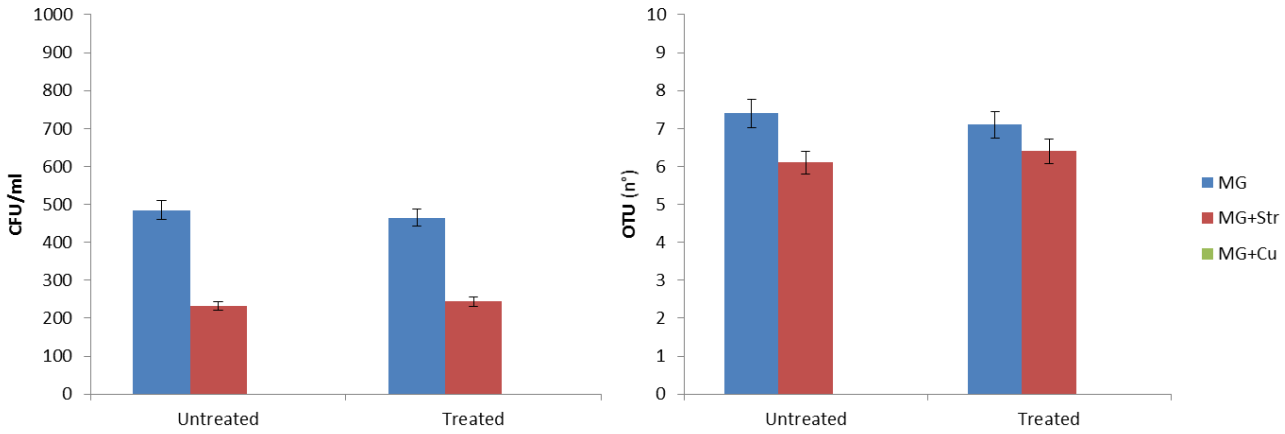

Fig. 4 Epiphytic microflora on Olive plants after 28 days of AP17 treatment. (On the left) Number of colonies counted in different plates and reported as CFU (colony-forming unit) per ml of leaf washing water. (On the right) High-resolution melting analysis to identify the number of species present in epiphytic microflora and reported as number of OTU (operational taxonomic unit).

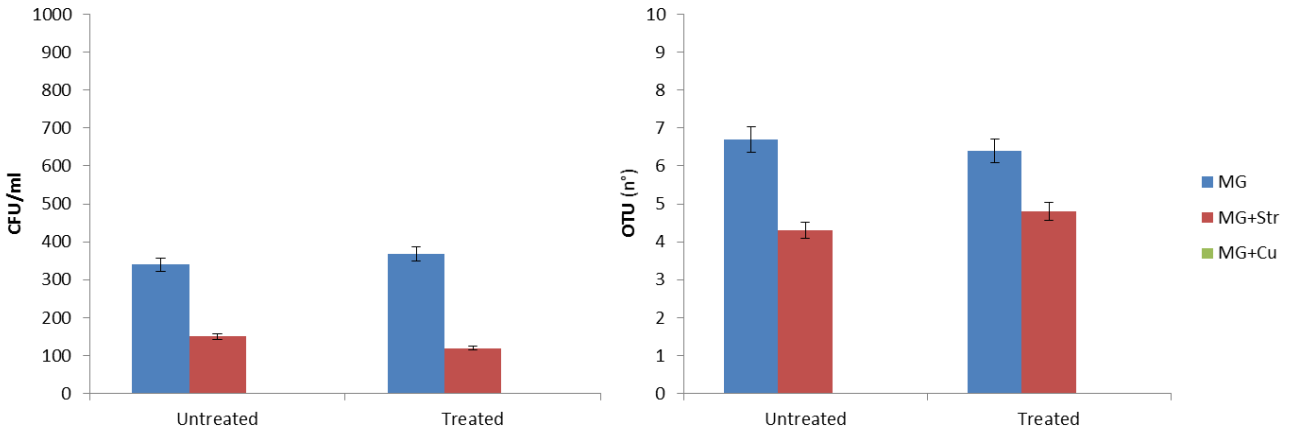

Fig. 5 Epiphytic microflora on Kiwifruit plants after 28 days of Psa21 treatment. (On the left) Number of colonies counted in different plates and reported as CFU (colony-forming unit) per ml of leaf washing water. (On the right) High-resolution melting analysis to identify the number of species present in epiphytic microflora and reported as number of OTU (operational taxonomic unit).

Supernatant were removed and bacterial pellet were resuspended in 5 ml of sterilised water. Each suspension was inoculated on Mannitol Glutamate (or MG) (Ghoddusi, 2008) agar plates supplemented with 10 µl Topas® 10EC (Syngenta Crop Protection Spa, Milan, Italy), a systemic triazole fungicide. Each sample was spread on three different plates: MG only (negative control), MG with Streptomycin (10 µg/ml), and MG with Copper (0.8 mM). After two days of incubation at 26°C, bacterial colonies were counted and selected by colour and morphology (clustering). One colony for each cluster was analysed by HRMA. Data obtained from Olive and Kiwifruit plants were reported in Figure 4 and Figure 5 respectively. No differences were identified between treated and untreated conditions, and this situation has confirmed the absence of copper- and antibiotic-resistant bacteria due to anti-virulence usage.

2.2.2 Soil microflora after AP17 and Psa21 treatment

Collected soil obtained from plastic tunnels were weighed and put on flasks with sterilised water (2 litres), in order to resuspend bacterial microflora present in the ground. After 30 minutes of shacking (100 rpm) at room temperature, an aliquot of 50 ml were collected and centrifuged at 5.000 rpm for 10 minutes. Supernatant were removed and bacterial pellet were resuspended in 5 ml of sterilised water. Each suspension was inoculated on Mannitol Glutamate (or MG) (Ghoddusi, 2008) agar plates supplemented with 10 µl Topas® 10EC (Syngenta Crop Protection Spa, Milan, Italy), a systemic triazole fungicide. Each sample was spread on three different plates: MG only (negative control), MG with Streptomycin (10 µg/ml), and MG with Copper (0.8 mM). After two days of incubation at 26°C, bacterial colonies were counted and selected by colour and morphology (clustering). One colony for

each cluster was analysed by HRMA. Data obtained from Olive and Kiwifruit plants were reported in Figure 6 and Figure 7 respectively.

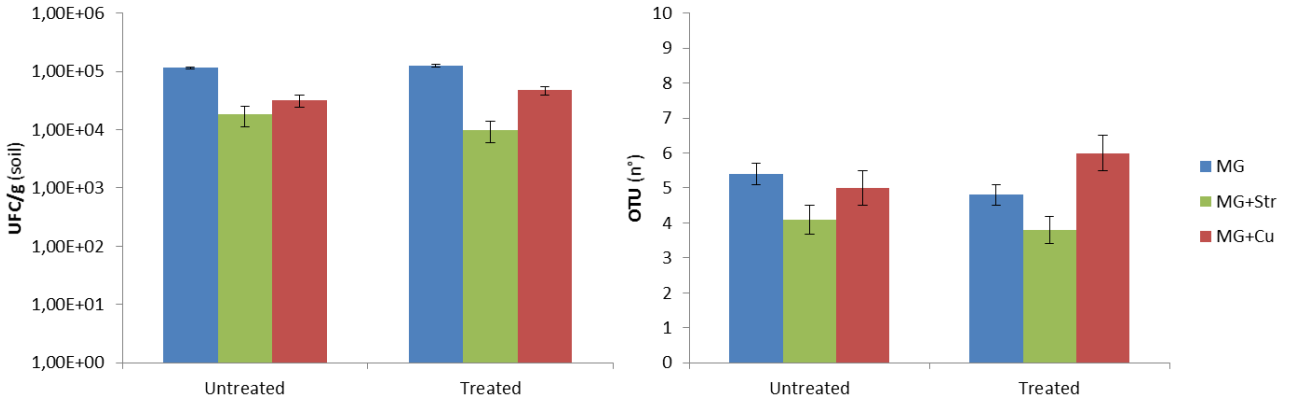

Fig. 6 Soil microflora on Olive plants after 28 days of AP17 treatment. (On the left) Number of colonies counted in different plates and reported as CFU (colony-forming unit) per gram of soil. (On the right) High-resolution melting analysis to identify the number of species present in epiphytic microflora and reported as number of OTU (operational taxonomic unit).

No differences were identified between treated and untreated conditions, and this situation has confirmed the absence of copper- and antibiotic-resistant bacteria due to anti-virulence usage.

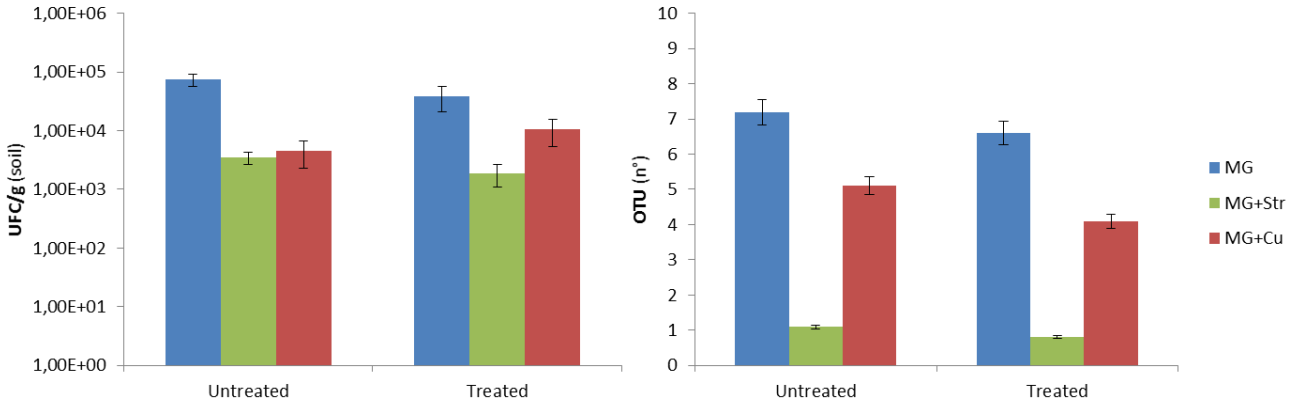

Fig. 7 Soil microflora on Kiwifruit plants after 28 days of Psa21 treatment. (On the left) Number of colonies counted in different plates and reported as CFU (colony-forming unit) per gram of soil. (On the right) High-resolution melting analysis to identify the number of species present in epiphytic microflora and reported as number of OTU (operational taxonomic unit).

3. CONCLUSIONS

In order to study the influence of anti-virulence treatment on biological properties of soil and plant surfaces, we have carried out an experiment at field level on 2000 plants, Olive and Kiwifruit plants, using peptide AP17 and Psa21. We decide to apply only AP17 treatment, and not also Li27, for some principal reasons: i) AP17 was the best performer monitored during laboratory and pilot scale analysis; ii) AP17 is a portion of Li27; iii) two different treatments at field scale would require an excessive number of plants.

It has been demonstrated that copper, as other chemical compounds used in agriculture, affects the composition of microbial communities, both at ground level that foliar surfaces. It is noteworthy that copper-contaminated soils also contain a higher percentage of antibiotic resistant bacteria than non-contaminated soils. It was demonstrated that the widespread accumulation of copper in agricultural soils contribute worldwide to increase antibiotic resistance through an indirect and environmental selection, with huge risks for the humans and animals health.

In conclusion, we demonstrated the absence of an indirect selection operated by the anti-virulence peptides, specifically AP17 and Psa21, on copper- and antibiotic-resistant bacteria, to assess the further benefits deriving from the use of these innovative plant disease control molecules, in comparison to the conventional copper compounds. It was possible to prove this feature at field scale for AP17 and Psa21, on Olive and Kiwifruit plant, respectively.

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