SHORT TERM SCIENTIFIC MISSION (STSM) SCIENTIFIC REPORT

This report is submitted for approval by the STSM applicant to the STSM coordinator.

Action number: CA16107
STSM title: Microbiome of *Prunus dulcis* and *Prunus avium* by Next Generation Sequencing (NGS) and bioinformatics data analysis
STSM start and end date: 11/09/2018 to 15/12/2018
Grantee name: Aitana Ares Yebra

PURPOSE OF THE STSM

Metagenomics is an effective and valuable methodology to study the impact of casual agents of plant diseases and its interaction with other naturally occurring microorganism, as part of innovative approaches to mitigate or control the disease. During this short-term scientific mission, was learned the methodology using NGS techniques (Illumina's MiSeq platform) to characterize the structural diversity of bacterial community's naturally present in *Prunus dulcis* and in *Olea europaea* by sequencing hypervariable regions from 16S rRNA gene. For other hand, for try to understand the plant pathogen interactions was characterize the structural diversity of bacterial community's in healthy and diseased plants of *Prunus dulcis,*negative and positive for *Xylella fastidiosa,* using this NGS techniques.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSM

Samples analyzed:

From samples obtained in Córdoba province the microbiota composition was determined in healthy trees. The trees were choosen within the same experimental farm in order to avoid the influence of abiotic conditions or plant genotype into the microbiota structure.

For study the plant pathogen-interaction were analyzed samples of DNA from branches of *Prunus dulcis* sampled from field plots located in the province of Alicante, where the demarcated area of the *X. fastidiosa* outbreak. We selected samples that tested positive, negative and doubtful for *X. fastidiosa* by using Harper an Francis qPCR EPPO standard protocols.

The work done during this short-term scientific missionincluded:

- Sampling strategies: the plant material was obtained from two plantations of *Prunus dulcis* and *Olea europaea*, located in the Experimental farm where the IAS-CSIC is located in the province of Córdoba. To carry out the study, three asymptomatic trees were selected from each plantation. The samples were collected in the month of September and from each tree were picked up four branches located at the top of it and of different orientation. The branches were sprayed with water and kept in a closed bag at 4ºC until analysis in the laboratory.
- Testing different protocols to compare their efficiency forextracting the xylem plant microbiome:
    - Extraction of the xylem sap with a Scholander pressure chamber instrument accoplated with an external arm to introduce tree branches.

- Extraction of the microorganisms present inside the branches washing this with water and with the help of a syringe.
- Extraction of the xylem tissue by peeling the bark and making wood chips with the help of a blade.

- Extraction of DNA of the microbiome contained in the xylem sap extracted by the three protocols referd above:
  - The bleeding sap and the branches washingfirst were filteredwith membrane filters of 0.22 µm pore size and then DNA was extracted from the membrane with the Power Plant Pro Kit (Qiagen).
  - Bark slice branches were macerated them in PBS and afterward the same kit for DNA extraction was used.
- Testing of different PCR primers targeting 16S rRNA:
  - We attempt to lower the total DNA amplification of plant material (chloroplasts and mitochondria), because a high number of sequences classified as chloroplasts can mask microbiome results, obtaining a low number of bacterial sequences in the analysis of the samples. PCR-primers analyzed were 799F-1193R, 967F-1391R, 799F-1115R and 799-1062R.
  - A total of 30 samples extracted at IAS-CSIC were processed including 15 from olive and 15 from almond.
  - Furthermore a total of 48 samples from almond from Alicante 12 tested as positive or negative for *X. fastidiosa* were used and DNA extraction was performed from xylem bark samples.
- Library preparation for NGS amalysis:
  - Incorporation of Acces array Barcodes for the Illumina Sequencing Systems by PCR.
  - Clean up the final library before quantification using magnetics beads.
  - Library Quantification using picogreen, Normalization and Pooling.
- Bioinformatic analyses with QIIME™ 2, microbiome analysis package. The competences for this software were acquired with samples provided by Institute for sustainable agriculture (IAS). Basically, I learned these different steps:
  - Import raw sequence (FASTQ) data into QIIME 2
  - Demultiplex data
  - Remove non-biological parts of the sequences (i.e. primers)
  - Performe quality control
    denoise sequences with DADA2 or deblur, and/or
    quality filtering, length trimming, and clustering with VSEARCH or db OTU
  - OUT assignation
  - Diversity analysis: alpha---beta
  - Generate a tree for phylogenetic diversity analyses
  - Alpha rarefaction plotting
  - Taxonomic analysis

DESCRIPTION OF THE MAIN RESULTS OBTAINED

It was observed that was easier to extract bleeding sap with the pressure chamber instrument from branches of *Olea europea* than from *Prunus dulcis.* In olive-trees the branches were more flexible and this facilitate the extraction because high pressures from the pressure chamber instrument can break the plant tissues and the branch can get out from the external arm of the pressure chamber. This happened sometimes for the branches of almond-trees.

DNA extraction generated better yields from the branches than from bleeding sap and from the branches washing. In bleeding sap and in the branches washings were obtained low DNA concentrations but there were differences between *Prunus dulcils* and *Olea europea*. In samples of almond-trees there were more PCR-inhibitors than in olive-trees. Bovine serum albumin (BSA) was used in PCR reactions to solve these problems.

With DNA extracted from wood chips were obtained double amplicon with the primers 799F-1193R and 967F-1391R. Some times this double amplicon were obtained with DNA extracted from bleeding sap and from the branches washing. In general, this problem was more common in the almond tree than in the olive tree. When this happened, before to put the Barcodes for the Illumina Sequencing Systems the bands of interest were extracted with a Cut & Spin Gel Extraction Column.

In total 96 samples were sent to analyze to the sequencing service of the Parque Científico de Madrid, we are waiting for these results. When we receive the sequences, they will be analyzed with the microbiome analysis package QIIME™ 2. This methodology will include quality analysis and filtering of sequences (filtering / preprocessing of sequences), selection of optimal parameters for filtering sequences and assigning OTUs. Different exploratory analyzes of multiple variables will be carried out to test the effect of the *Xylella fastidiosa*, environmental factors, host genotype or ecological niche on the microbiome composition.

FUTURE COLLABORATIONS (if applicable)

The results and the competences acquired in this short-term scientific mission will be applied in research projects and writing a manuscript