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: Viability quantitative PCR for the specific detection and quantification of Xanthomonas citri pv. citri, the causal agent of citrus bacterial canker disease
STSM start and end date: 10/01/2019 to 01/03/2019
Grantee name: Pilar Sabuquillo Castrillo

PURPOSE OF THE STSM:

To determinate the metabolic status and infective capacity of the bacterial cells is required the use of reliable detection and identification methods. International plant protection organizations recommend techniques (serology, PCR) that would allow better risk management, especially in the case of quarantine pathogens, and establish recommendations for health management for species of economic importance for agriculture. But these techniques do not allow assessing the viability of target organisms. The development, implementation, comparison and standardization of detection methods using molecular techniques should has a clear potential to be applied in research programs and also for further applications in other Xanthomonadaceae.
Xanthomonas citri pv. *citri*(Xcc) causes citrus bacterial canker and is listed as a quarantine pathogen in the European Union (Directive 2000/29/EC). Xcc infects all aerial organs of citrus and nearby species in the Rutaceae family. The purpose of the STSM was to evaluate the host status of non-citrus rutaceae plant species by assessing disease development and the population dynamics of three Xcc strains on leaves using several technologies (plating on a semi-selective medium and qPCR). Experiments have been focused on the unclear host status of *Atalantia*, which has been used for example as an ornamental tree for landscaping in Southern USA.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSMS

(max.500 words)
The objective of the project was focused on the optimization of Xcc detection from plant tissues, a research program started by the group of CIRAD. The viability assay consists in using the nucleic acid intercalating dyes propidium monoazide (PMA) prior to PCR amplification (Fittipaldi et al., 2012, doi: 10.1016/j.mimet.2012.08.007; Cangelosi and Meschke, 2014, doi:10.1128/AEM.01763-14). The dye penetrates only dead cells with compromised membranes and intercalates covalently into the DNA after photoactivation, inhibiting DNA amplification. No scientific papers on this technology have been issued on Xanthomonadaceae family. CIRAD recently optimized a real-time quantitative PCR assay specifically targeting viable cells using PMA. This methodology has been validated *in vitro* and *in planta* on several genetically distinct strains and host species (Mexican lime, sweet orange and kumquat) and proved reliable for estimating Xcc population sizes *in planta* as compared to plating data on a semi-selective medium.
The assay was carried out in a non-citrus rutaceae plant and three Xcc strains, one type A* and the two one type A, as follow:
- *Atalantia* leaves were poked in two places with a sterilized needle and then 5µl with a bacterial suspension at 10^8cfu/ml was injected in each wound (60 inoculations per strain). Control host was inoculated with

0.01MTris buffer (60 inoculations). Plants were incubated in a chamber at 26ºC with a 95-80%HR during the assay.

- 4 leaves, with two wounds each, were sampled at 1,4,6,14,21,28 and 60 days post inoculation (dpi). 8 lesions on leaves, per strain, were observed at visible microscopy to control the evolution of symptom. 1cm diameter wounded leaf disks were cut out and introduced into an eppendorf. Four ceramics beads and 1ml of 2%PVP (Polyvinylpyrrolidone) Tris buffer pH 7.2 were added. The samples were lysed 30s at $300s^{-1}$ in a Lyser II.

- All the grind was deposited into a falcon contained 1.9ml of 2%PVP Tris buffer. 50µl were plated on YPGA medium for cfu counting and the rest was divided in 6 parts of 400µl each. The samples were centrifuged at 500g for 3min.

- 3 of the supernatant obtained were deposited into a new eppendorf contained 2µl of 25mM PMA and the other 3 in an eppendorf contained 2µl of water. All the supernatant were incubated in darkness for 10min and photoactivated 15min in a Lite™ LED photolysis Device. Supernatant were centrifuged 10min at 20000g. For each time, 156 samples were processed.

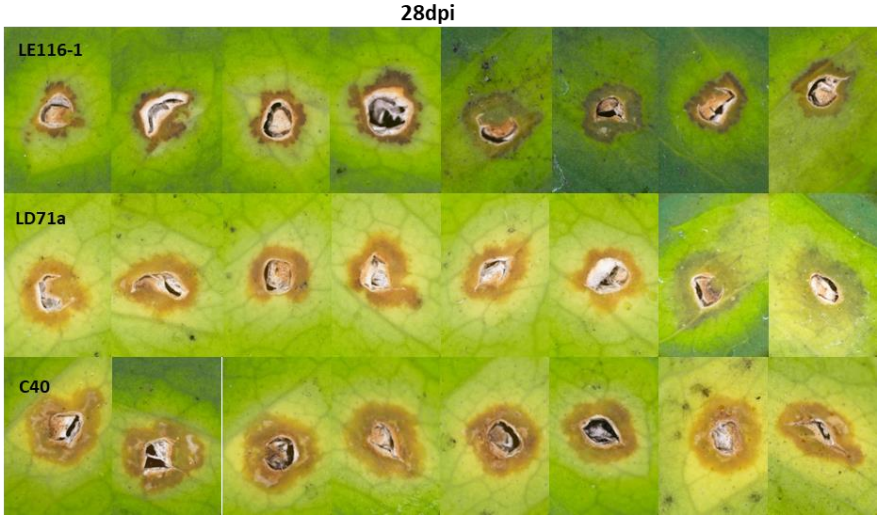
- DNA, from the supernatant, were extracted with the Quiagen kit Dnasy 96Plant Mini Kit (Ref. 69181). Number of DNA extracted 936.

- qPCR were performed to assess the viability of bacteria in plant. 2µl of extracted DNA was added into an eppendorf contained the Maxter Mix solution (7.5µl of Fast SybrGreen(2x), 10µl of forward and reverse primers (10µm) and 4.54µl of Milli Q water). qPCR conditions: 95ºC 20s, 95ºC 3s and 62ºC 30s 40times, 95ºC 15s, 60ºC 60s, 95ºC 15s.
The standard consisted in ten fold dilutions (10^3-10^8 CFU/ml) of pure strains suspended in vegetal grinded tissue as is described above. Total qPCR:28.

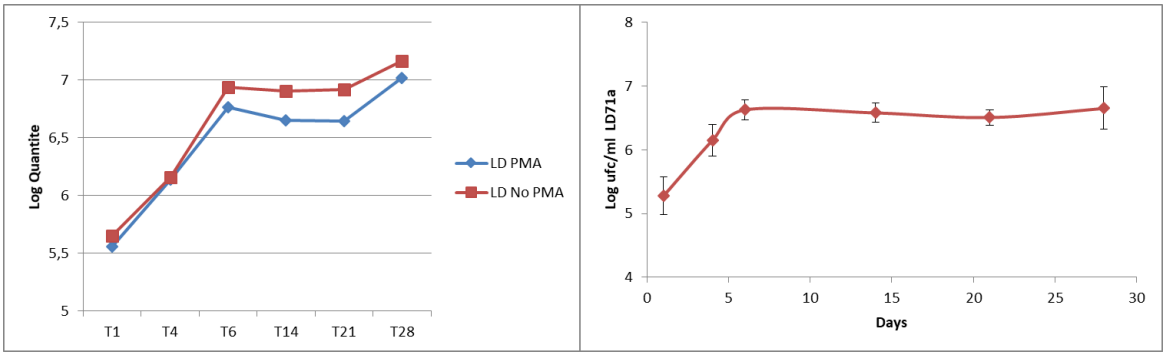
DESCRIPTION OF THE MAIN RESULTS OBTAINED

The aim of the STSM was the development and application of molecular viability assays for reliable detection and quantification of Xcc from Rutacea tissue. The main results obtained are:

1. The host status of non-citrus rutacea plant specie was evaluated by assessing disease development and the population dynamics of three Xcc strains on leaves using several technologies (plating on a semi-selective medium, qPCR and PMA-qPCR). The experiments has been focused on the unclear host status of *Atalantia*, an ornamental tree for landscaping in Southern USA (Campbell, 1979, Proc. Fla. State Hort. Soc. 92:215).
After inoculation of *Atalantia* leaves, symptoms appeared 6dpi for all the three Xcc strains assayed. They consisted in a typical brown spot with a yellowish halo but in any case there were observed canker.



The bacteria population showed an exponential growth till 6dpi and after all they remain constant. As by cfus as by qPCR, no differences was observed between the population treated with or without PMA because no decrease in population was observed.



These results showed the lack of typical canker symptoms in *Atalantia* when infected by Xcc. Nevertheless, the three different pathotypes were able to multiply and survive in paremchyma tissue.

2. I have received training from the team at CIRAD, Réunion (France) that developed this methodology.

3. We have exchanged experiences in the area for pathogen diagnosis complying with the objectives with this Action. The mutual interest for this research topic involved training the researcher, the exchange of infrastructures data and experience.

4. We have discussed further areas of possible collaborative research involving these new molecular viability assessment and quantification techniques. This STMS will reinforce the coordination and coherence in the management of research currently ongoing on citrus canker in the institutions and will open the possibilities of new common projects.

FUTURE COLLABORATIONS (if applicable)

In future collaborations, INIA-CIRAD, PMA-qPCR can used for studies of biofilm evolution, an important virulent factor in pathogenic bacteria. The INIA group has a project related with the study of biofilm formation in different species belonging to Xanthomonadaceae family. This is a clear option to apply for a project in common.