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: "Studies on *Xanthomonas citri* subsp. *citri* (*Xcc*) survival on *Citrus* rutaceous relatives"
STSM start and end date: 01/02/2018 to 07/03/2018
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PURPOSE OF THE STSM

Xanthomonas citri subsp. *citri* (*Xcc*) is the causal agent of asiatic citrus bacterial canker (CBC), a serious disease affecting mostly *Citrus* species plants (Graham et al., 2004; Schaad et al., 2005). Biofilms are described as bacterial structures used for plant infection and a resistance strategy by several plant pathogenic bacteria and among them *Xcc* (Cubero et al., 2011; Rigano et al., 2007). Aggregation and biofilm formation have been described important in host plant colonization by *Xcc* (Cubero et al., 2011; Rigano et al., 2007) and play a role in bacterial survival on plant surface, although this bacterium is considered mainly an endophytic microorganism.
The aim of the work performed during this short stay was to evaluate the effect of plant extracts (apoplast fluids) from several *Citrus* and *Citrus*-relatives species, on aggregation and biofilms formation of *Xcc* compared to another phytopathogen, such *Xanthomonas arboricola* pv. *pruni*, in the apoplast from ruteaceae plants. *X. citri* pv. *citri* (strain C40, Xcc C40) and *X. arboricola* pv. *prunus* (strain 33; *Xap* 33) were chosen for the assays, and two different growth media –LB and XMV2– were also alternatively used in order to show putative differences between rich and basic nutritive condition, respectively. On the other hand, to investigate all the genes modulated during biofilm production, RNA-sequencing of *Xcc* C40 in mature biofilms compared to planktonic state and testing both growth media, was also planned.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSM

Apoplast fluid -AF- from *Murraya paniculata*, *Murraya ovatifoliolata*, *Murraya koenigii*, *Severinia distica*, *Severinia buxifolia*, *Microcitrus australasica*, *Citrus sinensis* var. *tarocco*, *Citrus paradisii* var. *campaniformis*, have been previously obtained at the CREA-OFA of Acireale (Italy), according to the protocol described by O'Leary et al., 2014. AF from two *Prunus* species (*P. prunus* and *P. cerasus*), obtained by the host group, were also included in the analysis as control of Xap 33 activity, being them target hosts. To observe effects of the AF onto *Xcc* C40 and *Xap* 33 aggregation and biofilm formation, interaction assays were carried out in 96-multiwell plates, as reported by O'Tool, 1999.
We decided to compare *Xcc* C40 to *Xap* 33, as species affecting different hosts and as bacterial models with different behavior regarding endophytic and epiphytic stages. Moreover high and low nutrient conditions were evaluated using LB or XVM2 media, alternatively.
Methods
Bacterial cells of both strains were retrieved from glycerol stocks and seeded on LB plates at 27 °C. After 48 h, a single colony was distributed into 5 mL of LB broth and incubated overnight (27 °C, with shaking). 60 mL of the bacterial inoculum were finally diluted in 30 mL of LB and leaved for further 24 h in the same

previous conditions. A final bacterial concentration of 10^8 cfu (colony forming units) mL^{-1} (corresponding to 0.1 OD_{600}) was used for the assays. AF was firstly filtered with the support of a syringe and a 0.22-μm filter, then diluted in the specific growth medium in order to reach four different dilution points (from 10^{-1} to 10^{-4}). Three technical replicates were performed for each dilution point. After 72 h of growth in the presence of the specific AF dilution, medium was removed and the bacterial aggregates with the possibly formed biofilms were left for further 72 h in dry conditions. Staining with crystal violet and spectrophotometric absorbance read (570 nm) allowed to quantify the biofilm deposited on the bottom of the wells (Davey and O'Tool, 2000). Absorbance values for each strain and AF dilution were the mean of three different measures. Statistical analysis were also carried out to confirm the significance of results.

To compare whole transcriptomic profile of *Xcc* C40 within biofilm matrix *vs* the planktonic state, RNA-sequencing was organized.
Four different sets of samples were treated: bacteria grown in static *vs* in shaking conditions (biofilm and planktonic culture, respectively) and both were tested in LB and XMV2 media.
Methods *Nine flasks for each experimental condition were prepared, with* Xcc *C40 inoculum at 0.1 OD_{600} seeded in 25 mL of the specific medium and growth along 6 days at 27 °C.*
Bacterial inoculum was obtained as described above.
After 72 h, media exclusively from the flasks in static condition were removed, taking care to not interfere with possible biofilms on the bottom of the flasks. Then, the flasks were incubated for further 72 h in dry conditions. Control samples were also prepared, incubating medium without bacteria.
RNA extraction will be carried out using TRIZOL® Reagent according to manufacturer's instructions, and concentration evaluated by nanodrop, in order to get ready for further quality analysis and RNAseq at STABVIDA company.

DESCRIPTION OF THE MAIN RESULTS OBTAINED

Analysis of the data obtained during this month is currently ongoing but several preliminary interesting results were retrieved.
Comparative analysis are being done to elucidate differences among the different species tested, mainly to determine variances between *Rutaceae* plants and *Prunus* species and also to evaluate possible differences within *Rutaceous* between citrus and non citrus.
When XVM2 medium was used, the addition of apoplast from the two *Prunus* species, *M. paniculata*, *C. sinensis*, *M. Koenigi* and *S. buxifolia* resulted in a significant positive effect on bacterial aggregation for the C40 strain. Aggregation was also induced by *C. paradisi*, *C. sinensis*, *M. australasica*, *M. koenigi* and *S. buxifolia* in presence of high nutrient content when LB medium was used.
Different results were obtained when the strain *Xap* 33 of *X. arboricola* pv. *pruni* was used. Almost no effect on aggregation was shown in high nutrient content condition with any of the apoplasts tested, while positive induction was shown with *M. australasica*, *M. koenigi* and *S. buxifolia* in addition to the *Prunus* species evaluated, when XVM2 was used, and therefore, low nutrient was available.
In this study differences among apoplast concentrations were shown, giving a clue for developing future assays, not only concerning aggregation but also for expression analysis.
Finally, although all these results are preliminary and must be confirmed by new assays, it looks like clear that the effect of apoplast in bacterial aggregation depends on the host. For instance, while a positive effect in aggregation of C40 was recorded for both lime (*C. aurantifolia*, by the host group) and *Prunus* apoplasts, on the other hand these two hosts appear significantly different. Moreover, no or inhibition effect in aggregation was observed for *Xap* 33 in many cases, when *Rutaceous* apoplast fluids were added.
New assays are planned to confirm these results and to define differences in gene expression of the strains under exposure to different apoplast fluids.

FUTURE COLLABORATIONS (if applicable)

As written above, different collaborative studies are currently being done between the Spanish and the Italian group and some of the results obtained will be presented at the next Spanish Phytopatology Society meeting in October 2018.