EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

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: Optimization of controlled in vitro growth of *Xylella fastidiosa* strains (STSM reference number 39497)
STSM start and end date: 26/11/2017 - 15/12/2017
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PURPOSE OF THE STSM

In vitro cultivation of *Xylella fastidiosa* is a challenge and poses limitations to the work with this bacterium in different areas of research and diagnosis. With the commonly used cultivation methods, in vitro growth of *X. fastidiosa* is inconveniently slow and, depending on the strain, may also be unpredictable. Its fastidious nature and challenging cultivation means that *X. fastidiosa* is not routinely isolated in pure culture leading to culture collections that do not fully represent the diversity of the species. In addition, even strains that have been isolated are often lost in collections and laboratories. Outside xylem, planktonic growth of *X. fastidiosa* is difficult to control and cells tend to aggregate and form biofilms. Preliminary results at ILVO show that high concentrations of a homogeneous bacterial suspension are impossible or difficult to produce. This severely hampers repeatable preparation of well-defined *X. fastidiosa* suspensions, leading to variable results in different tests and experiments.

At ILVO, a promising alternative method has been developed in which *X. fastidiosa* cells grow in vitro to high densities in an imbedded environment from which they are eluted into a liquid medium. In addition, different liquid media are compared and preliminary results suggest that *X. fastidiosa* cells can be preconditioned to the xylem habitat, which increases the success of infection upon inoculation. The purpose of the STSM is to exchange current knowledge on the in vitro growth of *X. fastidiosa* and to fine-tune the growing protocol.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSM

It has previously been observed that different *X. fastidiosa* subspecies and strains have different but yet limited capacity to grow in vitro to high concentrations and to produce planktonic, quantifiable suspensions. On plates, the cells are stressed and growth is often limited to spots where high cell densities were deposited (which also gives uncertainty about the purity of the culture in these spots compared to single colonies). Also in liquid medium, cells only tend to grow further when already surrounded by high concentrations and growth initiated from low density suspensions stops without reaching the concentrations needed. Timely transfers to new medium can reduce a premature lag phase and scale up cell density, but are a highly risky practice exposing the inocula to contamination. For its growth in the plant *X. fastidiosa* cells rely on the nutrients present in the xylem fluid which is known to be poor. However, in this environment *X. fastidiosa* survives and can even rise to high numbers in case of disease expression.

During this STSM a growing protocol adapted to *X. fastidiosa* requirements previously developed at ILVO was applied to different *X. fastidiosa* subspecies and genotypes relevant for Europe i.e. subspecies of *fastidiosa*, *multiplex* and *pauca*. The current protocol was further tested using different variations of the media with and without addition of the plant xylem fluid and under different growing conditions. During the

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experiments, several methods were implemented and used with the aim to improve the assessment of the concentrations, morphological forms and metabolic activity of *X. fastidiosa*. These methods included measurements of optical density, RNA concentration, immunofluorescence, real-time PCR, and digital PCR. Biofilm formation was determined at the conclusion of the experiments. In addition, contamination checks were done at each sampling point.

DESCRIPTION OF THE MAIN RESULTS OBTAINED

For our purpose, two aspects of *X. fastidiosa* growth were essential to consider: on the one hand, the in vitro growth of separate *X. fastidiosa* cells for reliable *X. fastidiosa* quantification, and on the other hand the infectivity of the in vitro grown *X. fastidiosa* cells upon introduction in the host xylem. An alternative method of growing *X. fastidiosa*, entails growing *X. fastidiosa* cells in vitro to high densities in an imbedded environment from which they are eluted into a liquid medium.

Using several different strains of *X. fastidiosa* of subspecies *fastidiosa*, *multiplex*, and *pauca*, we have confirmed previous observations that the growth of *X. fastidiosa* is often unpredictable and unreliable. Even lines of the same strain can lead to variable results in terms of both qualitative and quantitative results as assessed by molecular methods. However, at the start of the experiments it was not clear whether the results of bacterial quantification are accurate and how they relate to bot, concentration of bacteria and their DNA released into the media during growth. Therefore, to improve the accuracy of assessing the *X. fastidiosa* growth and condition in our experiments we have combined several methods, and addressed their critical points. Mainly, that was the sample preparation step i.e. the use of adequate dilutions. The use of adequate dilutions was found to be necessary to prevent distorted or inaccurate results because of high cell concentrations and their increased inhibitory effects on methods over time. Suitable dilutions were defined for each method and each time point of the experiment; they represent a significant improvement in the standardization of *X. fastidiosa* growth experiments.

The following methods were found particularly useful to follow the growth of *X. fastidiosa*:

- digital PCR. This method was implemented at ILVO during this STSM. It enables us to quantify bacterial DNA with higher accuracy over time than in real-time PCR. It was found to be less influenced by the differences among strains and media used, and is independent of the validation data (previous validation data is used as a reference for quantification in real-time PCR however, it is based on a different type of samples). Using dPCR we were able to assess optimal conditions (sample dilutions) also for real-time PCR.

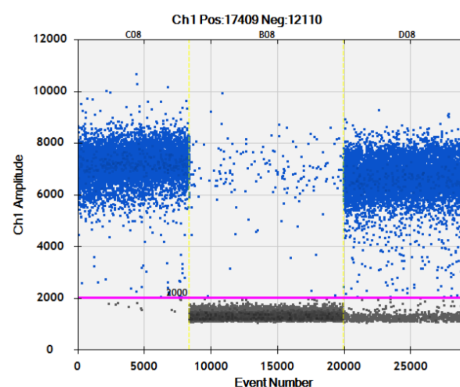

Figure 1: Digital PCR results of inocula quantification of three Xylella fastidiosa *subspecies. From left to right: subsp.* fastidiosa, multiplex *and* pauca. *Similar fluorescence intensity of positive droplets was observed for all three subspecies.*

- immunofluorescence. In contrast to molecular methods, which detect DNA, and therefore both living and dead cells, as well as free DNA, this method allows us to see bacterial cells. This has allowed us to estimate both the number of cells (however, living and recently dead cells can still not be differentiated) and, more importantly, their variations in shape, size and general morphology.

The use of combination of methods has allowed us (i) to identify optimal combinations of *X. fastidiosa* strains and their growing conditions, (ii) differentiate between inert increase of *X. fastidiosa* concentrations because of diffusion and growth / amplification, and (iii) optimize the experimental set-up for future experiments.

FUTURE COLLABORATIONS (if applicable)

Because of the time scale of the experiments, some were continuing beyond the end of the STSM stay; a more detailed analysis of the final results is therefore ongoing. Future collaboration is envisioned to exploit fully the results of this STSM, the new experimental set-up and to test its repeatability and robustness. Together with the results of this STSM, the results are planned to be presented in a publication (draft under preparation).