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: Phenotypic characterization and genetic diversity of *Xanthomonas hortorum* isolates from ornamentals
STSM start and end date: 25/02/2019 to 22/03/2019
Grantee name: Nay Dia

PURPOSE OF THE STSM:

Xanthomonas hortorum causes diseases such as bacterial leaf spot and bacterial blight on ornamental and economically important plants. Based on host specificity, the species *X. hortorum* has been divided into six pathovars: *X. hortorum* pv. hederae, *X. hortorum* pv. pelargonii, *X. hortorum* pv. vitians, *X. hortorum* pv. taraxaci, *X. hortorum* pv. carotae, and *X. hortorum* pv. nigromaculans.
The aim of the work performed during this STSM was to examine the phylogeny and pathogenicity of strains recently isolated from disease symptoms and found to be closely related to *X. hortorum*.
In order to evaluate their virulence and host range, pathogenicity tests were done on their host of isolation as well as on hosts described for the known pathovars of *X. hortorum*. Different inoculation techniques were used on whole plants (spraying, pricking, infiltration) and on detached artichoke bracts (pricking, incision, injection).
To evaluate their phylogenetic relationship, the studied strains together with the pathotype reference strains of *X. hortorum* pathovars and a selection of known *X. hortorum* strains were included for MLSA of seven housekeeping genes. In order to complete the MLSA, another aim of this STSM was to optimize the PCR amplification of those genes that did not yield an amplicon in previous PCR amplification efforts.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSM:

I) Pathogenicity tests on different host plants for the recently isolates together with pathotypesof the described *X. hortorum*

Pathogenicity tests were scheduled over four days. Fresh, pure bacterial cultures grown for 48h at 28°C on PAF-Sucrose media were used to prepare the inoculum in a mixture of 10 mM Phosphate-Buffer Saline (PBS)-Tween 0.02%. The optical density of the inoculum was measured and the inoculum was diluted to 10^6 colony forming units (cfu)/ml for all inoculation methods, except for the infiltration of the leaf mesophyll (10^5 cfu/ml). All the plants, except the artichoke bracts, were placed in a greenhouse. The artichoke bracts were kept in sterile glass jars with Petri dish lids. Plants were checked for symptoms daily and irrigated when to needed. Seven inoculation methods were tested (Table 1); Table 2 provides details of the bacterial strains, host plants and inoculation methods.

First pathogencity tests: these tests consisted of inoculating positive controls, i.e.: inoculating the type (T) or pathotype (PT) strains on their known hosts, using stem infilatrion (for *Pelargonium* and *Geranium* plants), entomological needle pricking (for the artichoke bracts) and pressurized spraying for the other plants (Table 2).

Second pathogencity tests: lettuce, ivy, pelargonium leaves and stem, dandelion, lavender were inoculated with ornamental isolates using either pressurized spraying or stem infiltration (Table 2).

Third pathogencity tests: artichoke bracts were inoculated with strain CFBP4188^{T}, using toothpick pricking, hypodermic needle injection or scalpel incision (Table 2). Violet, young inner bracts and older bracts were inoculated using each method.
The inoculated bracts were put in sterile glass jars with filter paper at the bottom, permeated with fungicide cycloheximide (100ppm), and covered with Petri dish lids.

Fourth pathogencity tests: lettuce, ivy and dandelion plants were reinoculated using two methods: leaf mesophyll infiltration (description in Table 1) and pressurized spraying (Table 2). The abaxial side of the leaves of hydrangea was sprayed with recently isolated strains GBBC2123 and GBBC2128, while carrot leaves were sprayed with CFBP7900^{PT} and CFBP7807.

Bacteria were reisolated from symptomats following classical protocols of diagnostics at ILVO (Merelbeke, Belgium). Their indentity will be confirmed using MALDI-TOF MS and *gyrB* sequencing at the home institution ZHAW(Wädenswil, Switzerland).

II) MLSA of the recently isolated strains in relation to reference strains of *X. hortorum*

The quality of six housekeeping genes sequences (*gyrB*, *atpD*, *rpoD*, *dnaK*, *efp* and *lepA*) was checked with BioNumerics version 7.6.2. (Applied Maths, Sint-Martens-Latem, Belgium). Based on the quality check and the gaps to be filled, DNA was extracted using the CTAB protocol. DNA was quantifed using a NanoDrop ND-1000 device (Thermo Fischer Scientific, Waltham, Massachusetts, United States). PCRs were then performed for seven housekeeping genes (genes mentioned above plus *glnA*) and the quality of the PCR products was checked using QIAxcel (Qiagen, Hilden, Germany). The PCR products were cleaned using the SMARTPURE Protocol (Eurogentec, Liège, Belgium). PCR products with amplicon size higher than 600 bp were sent for sequencing to Genewiz (Leipzig, Germany), while the others were sent to Macrogen (Amsterdam, Netherlands). The obtained sequences were checked for quality and aligned with BioNumerics.

DESCRIPTION OF THE MAIN RESULTS OBTAINED

This STSM contributed to the development of my technical skills and to the broadening of my theoretical knowledge, especially pertaining to the methodology used for investigating the pathogenicity of plant bacteria. I am now familiar with the different inoculation techniques used for inoculating *X. hortorum* pathovars, how to recognize and describe the symptoms caused by the different pathovars of *X. hortorum* on various hosts and how to isolate bacteria from symptomatic leaf tissue.

I) Results of the pathogenicity tests
Symptomatic leaves were photographed; an example is presented in Figure 1. *Geranium*, *Pelargonium* and lettuce showed symptoms 14 days post inoculation (dpi), while ivy and dandelion showed symptoms at 19 dpi.
Geranium leaves spray-inoculated with CFBP2533 ^{PT} showed small brown colored spots with yellow halo on both their abaxial and adaxial parts, while the plants inoculated by stem infiltration exhibited stem rot and brown spots on leaves.
The leaves of *Pelargonium* plants inoculated with the same bacterium showed leaf spots with dark centers, and exhibited typical V-shaped yellowing patternson both sides of the leaves. The leaves of the plants inoculated by stem infiltration were yellowing and the stems rotting.

Ivy leaves showed lesions at 19 dpi for type train CFBP2528^{T} and isolate GBBC2128. Young leaves were more affected than older ones. Aphids at various life stages (nymphs, winged adults and adults) present on ivy plants were collected for further barcoding analysis. Lettuce plants inoculated with ornamental isolates GBBC3001 and SB4092 showed lesions. Dandelion leaves inoculated with strain CFBP410^{PT} showed angular leaf spots.
Artichoke bracts inoculated during the first inoculation tests were checked at 4 dpi and no symptoms of *X. cynarae* infection were observed. The bracts were then checked again at 11 dpi. The bracts were almost fully black. Rotting was ruled out as the bracts were still rigid and not “mushy”. A lack of light is suspected to have contributed to this observation.

The artichoke bracts inoculated during the third inoculation day were checked 3 dpi and 5 dpi. There were no distinctive symptoms at 3 dpi or at 5 dpi.

II) MLSA results

The sequences were analyzed using BioNumerics. Nineteen sequences were judged inadequate and will thus have to be resequenced. A phylogenetic tree based on the seven housekeeping genes will be constructed once the remainder gaps will be filled.

FUTURE COLLABORATIONS (if applicable)

Further collaboration is envisioned and the results of this STSM will be part of a joint publication describing the up-to-date status of the *X. hortorum* complex. The obtained pathogenicity results have to be repeated and confirmed as the work during this STSM has to be considered as first experiments.

Table 1. Description of the inoculation methods used for the pathogenicity tests during the STSM

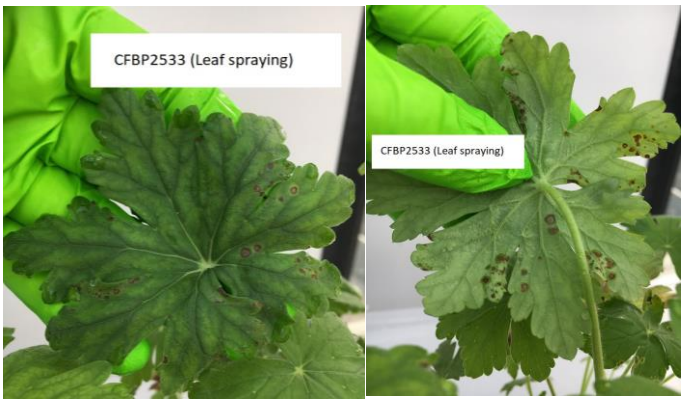
Inoculation method	Description
Pressurized spraying	Inoculum sprayed on the dorsal and abaxial sides of the leaves until runoff
Pricking with a entomological needle	10 µl of inoculum deposited on the bract then pricked in with a needle
Pricking with a sterile toothpick	10 µl of inoculum deposited on the bract then pricked with a toothpick
Injection with syringe + hypodermic needle 23G	100 µl of inoculum injected into the bract
Scalpel incision	Scalpel sterilized using isopropanol and flame; dipped in inoculum and used to incise the artichoke bracts in a grid manner
Stem infiltration	Deposit 10 µl of inoculum above a support leaf, push need through the inoculum drop and onto the stem, then pull it out in a upward slit fashion.
Leaf mesophyll infiltration	Small puncture made; infiltrate the inoculumn with a syringe into the puncture. The inoculum can be seen spreading through leaf veins.

Table 2. Pathogenicity tests carried out during this STSM.

Inoculation day 1 (27/02/2019)	CFBP4188	Artichoke leaves	Artichoke bracts	Lettuce		
	GBBC3001					
	SB4092					
	CFBP5858	Atlantic ivy				
	CFBP2533	Pelargonium leaves	Pelargonium stem			
	CFBP410	Common dandelion				
	CFBP3994	Lettuce				
Inoculation day 2 (06/03/2019)	GBBC2123		Lettuce	Ivy	Pelargonium leaves	Common dandelion
	GBBC2128					
	GBBC1934	Lavender				
	SB3727	Pelargonium stem				
	Xc407					
	GBBC950					
Inoculation day 3 (15/03/2019)	CFBP4188	Artichoke bracts	Artichoke bracts	Artichoke bracts		
Inoculation day 4 (20/03/2019)	CFBP3994	Lettuce	Lettuce			
	CFBP3996					
	CFBP5858	Ivy	Ivy			
	CFBP410		Dandelion	Dandelion		
	CFBP7807	Carrots				
	GBBC2123	Hydrangea				
	GBBC2128					
	CFBP7900	Carrots				

Pressurized spraying
Pricking w/entomological needle
Pricking w/toothpick
Syringe w/hypodermic needle 23G
Scalpel incision
Stem infiltration
Leaf mesophyl infiltration

Figure 1. Symptoms on abaxial and adaxial Geranium plants inoculated by sprayingwith an inoculum solution of strain CFBP2533^{PT}.



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