COST Action FA0807 Integrated Management of Phytoplasma Epidemics in Different Crop Systems

Short-term Scientific Mission (STSM) Report

COST STSM Reference codes: COST-STSM-FA0807-7486
Grant letter: COST-STSM-ECOST-FA0807-090511-005067

STSM dates: from 09/05/2011 to 27/05/2011

STSM Aplicant: Constantina Chireceanu, cchireceanu@yahoo.com, Laboratory of Harmful Organisms, Research Development Institute for Plant Protection Bucharest Romania

STSM Host: Dr. Malembic-Maher Sylvie, smalembi@bordeaux.inra.fr, UMR 1090 Génomique Diversité et Pouvoir Pathogène, Institut National de la Recherche Agronomique, Centre de Bordeaux, France

STSM Topic: Improved methods for identifying of grapevine phytoplasmas and detection of phytoplasma from Romania

Purpose of the visit
The purpose of this work was to learn the techniques used for detect the presence of the grapevine phytoplasmas but also to detect and identify the phytoplasmas infecting vineyards in Romania. I was interested in acquiring knowledge on the molecular characterization of phytoplasma strains and to learn using bioinformatics tools for sequencing and phylogenetic analysis.

Description of the work
Samples of DNA were extracted from vines (40), insects (3) and alder (6) collected in Romania in 2010 and were transported and investigated in the laboratories of INRA Bordeaux between 09 – 27.05.2011.
At first, the grapevine and insect DNA samples were tested for the presence of phytoplasma infection of 16SrV and 16SrXII-A groups using the Triplex assay and by Quantitative real-time polymerase chain reaction assay. It was performed PCR then nested PCR with specific the primers: first PCR - FD9F1/FD9R1 (16SrV group) and stol11F2/stol11R1 (16SrXII-A group) and nested PCR - FD9F3b/FD9R2 (16SrV group) and stol11F3/stol11R2 (16SrXII-A group). It was used 2 µl of diluted DNA for first PCR and 5 µl of diluted PCR product for nested PCR. The final volume was 22 µl for the first PCR and 25 µl for the nested reaction. As positive reference isolates for the both phytoplasma groups there were used the internal samples kindly provided by the LABSA. Nine samples were found to be infected with the phytoplasma belonging to 16SrXII-A group and no sample with 16SrV group.

The DNA samples from vine and insects were further analysed by molecular typing in order to characterize of the phytoplasma isolates according to the non-ribosomal genes

protocols: SecY, Stamp and vmp1. For each non-ribosomal gene, the amplification was carried out by first PCR then nested PCR with specific the primers:

*sec*Y non ribosomal typing (Fialova *et al.* 2009)
- First PCR:PosecF1/ PosecR1;
- Nested PCR: PosecN2 / PosecR3;

Stamp non ribosomal typing (Fabre et al., 2011)
- First PCR StampF / StampR0;
- Nested PCR StampF1/ StampR1;

Vmp1 (former name Stol1H10) non ribosomal typing (Fialova *et al.* 2009; (Murolo *et al.* 2010, with different primers: Cimerman et al. 2009, pacifico et al., 2009)
- First PCR STOLH10F1 / STOLH10R1;
- Nested PCR TYPH10F / TYPH10R;

Composition of PCR mixtures contained: 1 µM of each primer, 2 units of Taq Polymerase (Biolabs), 200 µM of dNTPs and 1X buffer containing 2 mM $MgCl_2$. It was used 1ul of diluted DNA for first PCR and 1µl of PCR product for nested PCR. All PCR reactions were performing in a final total volume of 25 µl for the first PCR (24µl PCR mixture and 1µl of DNA) and 50 µl (49µl PCR mixture and 1µl of first PCR product) for the nested reaction. Migration of PCR products was observed in 1% agarose gel stained with ethidium bromide. Capillary electrophoresis (Qiaxcel apparatus) has also been performed.

Positive Vmp1 PCR products were submitted to R*saI* digestion in 40µl volume, containing 1X *Rsa*I buffer, 1unit of *Rsa*I enzyme, 15 µl of PCR (TYPH10F/R) products and water. Results were analyzed on 2,5% agarose gel stained with ethidium bromide and on polyacrylamide 8% and visualized on a UV transilluminator after electrophoresis.

SecY: Three DNA samples of grapevines were shown to be positive by *secY* gene testing. They were selected for registering and preparing to be sequenced. Sequence analysis showed that the isolates 3T and 14CT belong respectively to the genotypes S4 and S5, which are common genotypes propagated from bindweed.

Stamp: Using the Stamp gene test, six samples were amplified. The all of 6 samples were subjected for sequencing. Stamp sequences revealed that isolate 6CT is of genotype ST1 (*tuf*B cluster I), isolates 3T and 19CT are of genotype ST9, 14CT is of genotype ST11 (*tuf*B cluster II) and isolate 10CT is of a new genotype named ST33. This ST33 genotype grouped in *tuf*B cluster III which corresponds to isolates of South East Europe and East of the Mediterranean basin. So the genetic diversity of stolbur phytoplasma isolates in Romania seems to be important.

The samples from alder were submitted to Map-PCR, first PCR FD9F5 / MAPR1; Nested PCR FD9F6 / MAPR2. 0.5µl of diluted DNA for first PCR and 0.5µl PCR product

for nested PCR were used; PCR reactions were performing in a final total volume of 25 µl for the first PCR and 50 µl for the nested reaction. The PCR products were analyzed on 1% agarose gel stained with ethidium bromide and visualized on a UV trans-illuminator after electrophoresis. None of the alders resulted infected with a group 16SrV phytoplasma.

Vmp1 : the reference strain 3T could be characterized by PCR-RFLP with the enzyme *Rsa*I. It belongs to the genotype V4. The genotype V4 is quite common in Europe.

Conclusion: The results obtained in framework of this scientific mission allowed us to better characterize our phytoplasma strains, especially the stolbur phytoplasma strain we transmitted to periwinkle and to be trained for the molecular characterization of grapevine phytoplasmas.