COST Action FA0807

Integrated Management of Phytoplasma Epidemics in Different Crop Systems

Short-term Scientific Mission (STSM) Report

STSM Grantee: Gulnara Balakishiyeva, gbalakishiyeva@yahoo.com, Institute of Botany, Azerbaijan National Academy of Sciences, Patamdar shosse 40, AZ-1073, Baku, Azerbaijan.
STSM Host: Xavier Foissac, foissac@bordeaux.inra.fr, UMR Fruit Biology and Pathology, INRA and University of Bordeaux, 71 avenue Edouard Bourlaux, BP81, F-33883 Villenave d'Ornon cedex, France
STSM Topic: Genetic diversity of Stolbur phytoplasma in Azerbaijan
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Purpose of the visit

In Azerbaijan, Stolbur phytoplasma has recently been detected in annual crops such as eggplant, pepper and tomatoes, but also in declining cherry and common medlar trees [1]. We are now focusing on the determination of the insect vectors and plant reservoirs of stolbur phytoplasma in Azerbaijan. The aim of the proposed STSM visit are detection and genotyping of stolbur isolates in insect vectors and plants collected in Baku, Pirshagi, Mehdiabad and Guba regions.

Description of the work

In the first week of July 2012 we have realized with Dr. Xavier Foissac (Research director in UMR-1332 Biologie BFP, INRA - Centre de Bordeaux) the surveys in Baku, in Absheron peninsula (Pirshagi) and in Guba region, and during these surveys were captured planthoppers (Cixiidae) and leafhoppers (Cicadellidae) insects in the fields and the vicinity of solanaceous crop fields. Transmission assays to periwinkles and tomato plants were set-up. In September I realized a new crop survey to evaluate the incidence of stolbur phytoplasma diseases in Azerbaijan. Stunted cherry trees exhibiting yellowing were collected. A cherry-plum tree exhibiting early flowering was also sampled during this survey. For solanaceous crop, yellowing and stunted peppers and eggplant with virescence and phyllody symptoms were collected. Symptomless plants were also sampled as negative control. I extracted nucleic acids from plants and insects as previously described by Maixner et al. (1995) at the laboratory in Baku and brought them at INRA laboratory (see list of samples in the following table 1 (plant samples collected in Baku, Mehdiabad and Guba regions) and table 2 (insects and plant samples issued from transmission)). The DNA concentrations were measured by a nanospectrophotometer and results were recorded. First I tested the DNA extracts by 16S-rDNA nested PCR with the universal primers for phytoplasmas R16mF2 / R16mR1 and R16F2n / R16R2 [4]. For every plant species I tested the DNA extract of diseased plants and the DNA extract of healthy plants as negative control. For each PCR reaction one positive control (reference phytoplasma isolate maintained in periwinkle experimental host at INRA greenhouse), and healthy periwinkle from INRA greenhouse and water as negative controls were used to check the reliability of the reaction. PCR results were analysed on 1% agarose gels and visualized by staining with ethidium bromide under UV transillumination.

Table 1. Plant samples collected in Baku, Mehdiabad and Guba regions

N°	Sample ID	Latin name (H)- healthy control	Place	DNA concen - tration ng/µl	PCR 16S R16R1/ F2	PCR 16S R16R2 /F2n
1.	Ce1.AZ.2012	*Prunus avium*	Mehdiabad	195	-	+
2.	Ce2.AZ.2012	*Prunus avium*	Mehdiabad	173	-	-
3.	Ce3.AZ.2012	*Prunus avium*	Mehdiabad	152	-	+
4.	Ce4.AZ.2012	*Prunus avium*	Mehdiabad	116	-	-
5.	Ce5.AZ.2012	*Prunus avium*	Baku	225	-	-
6.	Ce6.AZ.2012	*Prunus avium*	Baku	332	-	-
7.	Ce7.AZ.2012	*Prunus avium*	Baku	140	-	-
8.	Ce8.AZ.2012	*Prunus avium*	Bakou	145	-	+
9.	Ce9.AZ.2012	*Prunus avium*	Guba	315	-	-
10	Ce10.AZ.2012	*Prunus avium*	Guba	70	-	-
11	Ce11AZ.2012	*Prunus avium*	Guba	264	-	+
12	Ce12.AZ.2012	*Prunus avium*	Guba	120	-	-
13	Ce13.AZ.2012	*Prunus avium*	Guba	29	-	-
14	Ce14.AZ.2012	*Prunus avium*	Guba	156	-	-
15	Ce15.AZ.2012	*Prunus avium*	Guba	58	-	-
16	Ce16.AZ.2012	*Prunus avium*	Guba	225	-	-
17	Ce17.AZ.2012	*Prunus avium*	Guba	68	-	-
18	Ce18.AZ.2012	*Prunus avium*	Guba	235	-	-
19	Ce19.AZ.2012	*Prunus avium*	Guba	195	-	-
20	Ce20.AZ.2012	*Prunus avium*	Guba	107	-	-
21	Ce21.AZ.2012	*Prunus avium*	Guba	115	-	-
22	Ce22.AZ.2012	*Prunus avium*	Guba	144	-	-
23	Ce23.AZ.2012	*Prunus avium*	Guba	118	-	-
24	Ce24.AZ.2012	*Prunus avium*	Guba	566	-	-
25	Ce25.AZ.2012	*Prunus avium*	Guba	206	-	+
26	Ce26.AZ.2012	*Prunus avium* **(H)**	Guba	166	-	-
27	Prm13.AZ.2012	*Prunus cerasifera*	Mehdiabad	366	-	+
28	Peach14.AZ.12	*Prunus persica*	Mehdiabad	1014	-	+
29	Peach15.AZ.12	*Prunus persica*	Guba	135	-	-
30	Peach19.AZ.12	*Prunus persica*	Guba	161	-	+
31	Pv1.AZ.2012	*Capsicum annuum*	Mehdiabad	191	+	+
32	Pv2.AZ.2012	*Capsicum annuum*	Mehdiabad	374	-	+
33	Pv3.AZ.2012	*Capsicum annuum*	Mehdiabad	410	-	+
34	Pv4.AZ.2012	*Capsicum annuum* **(H)**	Mehdiabad	493	-	-
35	Au5.AZ.2012	*Solanum melongena* **(H)**	Mehdiabad	345	-	-
36	Au6.AZ.2012	*Solanum melongena*	Mehdiabad	380	+	+

Table2 List of insects and plant samples issued from transmission *

N°	Sample ID	Latin name	Place	DNA concen-tration ng/µl	PCR result
1	EC5-I	Cixiids	Pirshagi	137	-
2	EC10-I	Cixiids	Pirshagi	39	-
3	EC11-I	Cixiids	Pirshagi	134,5	-
4	EC12-I	Cixiids	Pirshagi	12	-
5	EC13-I	Cixiids	Pirshagi	118,5	-
6	EC14-I	*Hyalesthes obsoletus*	Guba	31	-
7	PHO1-I	*Hyalesthes obsoletus*	Guba	376	+
8	PHO1*-I	*Hyalesthes obsoletus*	Guba	613	-
9	PHO2-I	*Hyalesthes obsoletus*	Guba	57,5	-
10	THO1-I	*Hyalesthes obsoletus*	Guba	552	-
11	THO1*-I	*Hyalesthes obsoletus*	Guba	148	-
12	THO2*-I	*Hyalesthes obsoletus*	Guba	1790	-
13	THO3-I	*Hyalesthes obsoletus*	Guba	89,5	-
14	THO4-I	*Solanum lycopersicum* (transmission by *H. obselutus*)	Greenhouse	44,4	-
15	THO1-P	*S. lycopersicum* (transmission by *H. obselutus*)	Greenhouse	987	-
16	THO2-P	*S. lycopersicum* (transmission by *H. obselutus*)	Greenhouse	481	-
17	THO3-P	*Catharanthus roseus* (transmission by Cixiids)	Greenhouse	1447	-
18	EC1-P	*C. roseus* (transmission by Cixiids)	Greenhouse	699	-
19	EC3-P	*C. roseus* (transmission by Cixiids)	Greenhouse	570	-
20	EC4-P	*C. roseus* (transmission by Cixiids)	Greenhouse	499	-
21	EC7-P	*C. roseus* (transmission by Cixiids)	Greenhouse	325	-
22	EC9-P	*C. roseus* (transmission by Cixiids)	Greenhouse	308,5	-
23	EC10-P	*C. roseus* (transmission by Cixiids)	Greenhouse	491,5	-
24	EC12-P	*C. roseus* (transmission by Cixiids)	Greenhouse	2150	-
25	EC14-P	*C. roseus* (transmission by Cixiids)	Greenhouse	888	-
26	PHO4-P	*C. roseus* (transmission by *H. obselutus*)	Greenhouse	159,5	-

* See STSM report Xavier Foissac, 2012

PCR results of DNAs of insects and plants issued from transmission exhibited that only one *Hyalesthes obsoletus* was positive for phytoplasma infections. Due to the high DNA concentrations of some of the isolate (potential inhibition of PCR), these isolate will be tested again after sample dilution in TE 1X buffer.

We continued our analysis to identify the detected phytoplasmas. For that I used RFLP analysis of the PCR products. 20 µl the enzymatic digestion mixture contained 0,5 µl of each enzyme (5 U/µl), 2 µl of 10x buffer, 13,5 µl sterilized H_2O and 4 µl of PCR product. Digested PCR products were then loaded on 2% agarose gel, stained with ethidium bromide and visualized on a transilluminator after electrophoresis. Results acquired from RFLP analyses

with enzymes *Taq* I, *Alu*I and *Rsa*I showed that the 16S-rDNA from the infected and cherry trees, peppers and eggplant (Au6.AZ.2012) gave the same profile that the Stolbur phytoplasma reference strain (Molière) maintained at INRA Bordeaux in periwinkle *Catharanthus roseus*.

All stolbur phytoplasma isolates obtained were subjected to genotyping on non-ribosomal gene *stamp*. To amplify *stamp* gene used primer pair Stamp-F/Stamp-R0 and StampF1/StampR1 as previously reported [2]. Expected 637 bp amplicons were submitted to sequences in BECKMAN-COULTER GENOMICS Company in Grenoble, France on MegaBACE capillary sequencing instruments. The raw sequence chromatograms were assembled and edited using two different sequence-editing and assembling programs (Chromas and GAP4 Staden package). Multiple sequences alignments were performed using the Clustal W program [6]. Phylogenetic analyses were conducted with MEGA version 5 [7] using maximum parsimony with randomized bootstrapping evaluation of branching validity (Fig.1).

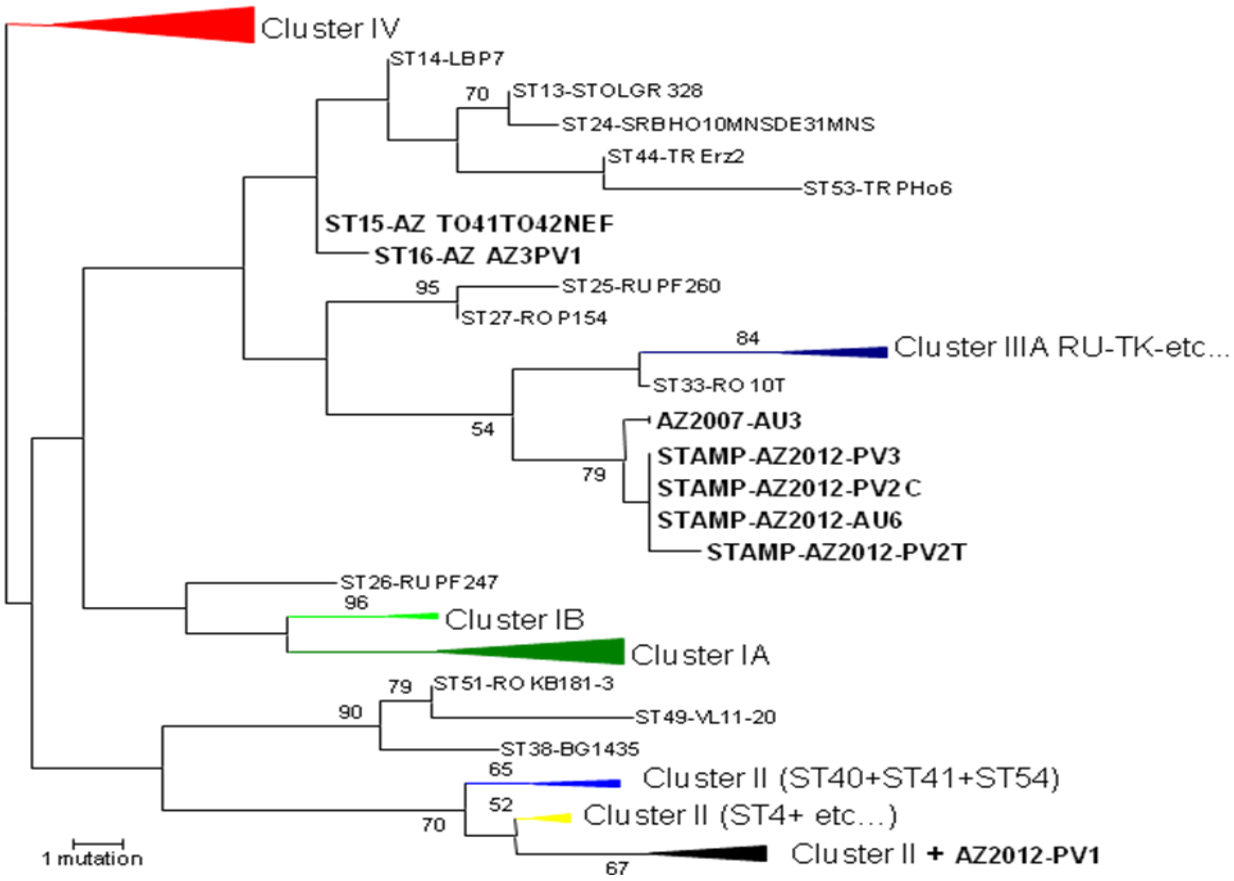

Figure 1. Maximum Parsimony analysis of STAMP sequences. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 52 most parsimonious trees (length = 140) is shown. The consistency index is (0.452830), the retention index is (0.871965), and the composite index is 0.510722 (0.394852) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (200 replicates) are shown next to the branches [3]. The MP tree was obtained using the Close-Neighbor-Interchange algorithm (pg. 128 in ref. [5]) with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The analysis involved 60 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 613 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [7].

Genotyping by sequencing the *stamp* gene that encodes the antigenic membrane protein of Stolbur showed that stolbur isolates detected in Azerbaijan have eight genotypes; seven genotypes grouped in the branch of *tuf-type* b *stamp* cluster III. This branch groups Stolbur isolates of the east of the Mediterranean basin (Azerbaijan, Serbia and Lebanon). The isolate AZ2012-PV1 have a Stamp genotype different from others and took place in Stamp cluster II. The isolate AZ2012-PV2 is a mixed infection of 2 different stolbur phytoplasma strains (AZ2012-PV2C and AZ2012-PV2T). PHO1 stamp amplicon turned out to be not stamp sequence but an alien DNA.

Conclusion and future collaboration

I am very grateful to all the collaborators and especially to the Dr. Xavier Foissac's group for the assistance in organization of my investigations. We will continue our investigations on genotyping of stolbur isolates by MLSA based on non-ribosomal genetic loci. INRA Bordeaux have agreed to collaborate with us in the future. They will help us to perform transmission assays and also in the identification of vector insects and the molecular characterization of stolbur phytoplasmas detected in insects and plants.

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