COST action FA 0807

Integrated Management of Phytoplasma Epidemics in Different Crop Systems

Scientific Report of Short-Term Scientific Missions (STSM)

STSM Topic: Diagnostics of grapevine phytoplasma diseases: from symptomatology to PCR detection

STSM grantee: Nikolay Genov, n_genov@mail.bg

Institute of Viticulture and Enology (Agricultural Academy),

1, "Kala tepe" str., Pleven, BULGARIA

Host: Dr. Elisa Angelini, elisa.angelini@entecra.it,

CRA-VIT Centre for Research in Viticulture

Period: from 24/09/2012 to 21/10/2012

Place: CRA - VIT CONEGLIANO (Italy)

Reference code: COST-STSM-ECOST-STSM-FA0807-240912-018471

COST STSM Reference Number: COST-STSM-FA0807-10731

State of the art

Grapevine yellows (GY) are serious diseases caused by phytoplasmas and spread worldwide in vine growing countries. GY present in Europe are essentially two:

• *Flavescence dorée* (FD), a quarantine disease in the European Community, associated to FD phytoplasma (phylogenetically belonging to 16SrV ribosomal group). It is specifically transmitted by *Scaphoideus titanus* Ball. The disease shows an epidemical behaviour.

• *Bois noir* (BN), associated to Stolbur (STOL) phytoplasma (phylogenetically belonging to 16SrXII ribosomal group). It is specifically transmitted by *Hyalesthes obsoletus* Signoret. Usually the disease shows an endemic behaviour.

In Italy both diseases occur. FD and its vector have been spread to North and Central Italy in the last 20 years, while BN is common in all Italian regions (Borgo *et al.,* 2005; Borgo and Angelini, 2007; Bertaccini *et al.,* 2008; Belli *et al.,* 2010). Arrival of FD in Italy caused huge damages to Italian viticulture, especially due to the lack of knowledge on the correct disease control strategies. Preventive control strategies include early diagnosis of the disease, carried out by field observation and molecular diagnosis.

In Bulgaria the presence of BN and its vector *H. obsoletus* has been reported (EPPO, 2006; Sakalieva *et al.*, 2007; Avramov *et al*., 2008). FD have not been yet detected, but its vector *S. titanus* was recently found in the country (Avramov *et al.*, 2011) Moreover, FD and its insect vector have been present at least since 2003 in the nearby Serbia (Duduk *et al.*, 2003; Magud and Toševski, 2003; Duduk *et al.*, 2004), where they caused very serious economical losses to wine growers. Therefore, there is the serious and real risk that FD spreads out in Bulgaria soon,

causing epidemics in vineyards. Thus, a strict surveillance and a joint effort by all Bulgarian teams working on grapevine are necessary.

Purpose of the visit

The main purpose of this stage was the transfer of knowledge on GY diagnostic from the Centre for Research in Viticulture (CRA-VIT) to the Institute of Viticulture and Enology (IVE). It is very precious in order to face the problem of GY in the grapevine germplasm collection at IVE and, more generally, in Bulgaria, and to allow the survey of the country for the possible entry of FD.

To be achieved the purpose, activities including field observation of GY symptoms and molecular diagnosis of phytoplasmas were implemented. This comprised recognizing symptoms of grapevine phytoplasma diseases; collecting and maintenance of samples; isolation of DNA from samples; detection of grapevine phytoplasmas with nested and real-time PCR; basic knowledge of GY epidemiology.

Description of the work

Field work: Identification of GY symptoms on different varieties in vineyards were demonstrated by the specialists from CRA-VIT. Distinction of grapevine yellows symptoms from different symptoms associated to other biotic (such as viruses and leafhopper damages) or abiotic pathologies and way of sampling and maintenance of the grapevine samples, insects and weeds were also presented.

Laboratory work: Molecular analyses were carried out for detecting presence of phytoplasmas in different kinds of samples (grapevine, insect and weed tissue). Four Bulgarian grapevine samples were also included in the analyses. DNA extractions from plant tissues and insects were performed by using the CTAB procedures as described in Angelini *et al*. (2001).

Phytoplasma detection and characterization were carried out by means of DNA amplification with nested PCR, followed by restriction fragments length polymorphism (RFLP) analysis of amplicons. Ribosomal DNA was amplified in nested-PCR procedure with universal and specific primer pairs for phytoplasmas. The first direct PCR was performed with universal primer pair P1/P7 (Deng and Hiruki, 1991; Smart *et al*., 1996). The obtained amplimers, after dilution 1:50 in water, were used as target DNA in three different nested-PCR amplification: with 16r758f/M23Sr primers (Gibb *et al*., 1995; Padovan *et al*., 1995) for universal detection of phytoplasmas; with R16(V)F1/R1 and R16(I)F1/R1 primer pairs (Lee *et al*., 1994), which are specific for phytoplasmas belonging to 16SrV and 16SrI/16SrXII groups, respectively.

Primers targeting the nonribosomal *tuf* gene of phytoplasmas belonging to 16SrI/16SrXII groups were also used in direct and nested PCR using primer pairs f Tuf1/rTuf1 and fTufAy/rTufAy respectively (Schneider *et al*., 1997; Langer and Maixner, 2004).

PCR products were analyzed by electrophoresis in 1% agarose gel, stained with GelRed^{TM} Nucleic Acid Gel Stain (Biotium) and visualized in UV transilluminator.

Results showed that three of the four Bulgarian samples were collected from BN diseased grapevines (Fig. 1. and Fig. 2.). This is the first report of detecting Stolbur phytoplasma in grapevine tissue in Pleven region.

The phytoplasma group in the positive DNA samples was determined by means of RFLP analyses after enzymatic digestions with the restriction endonucleases *Taq* I for 16r758f/M23Sr amplicons and *Hpa* II for fTufAy/rTufAy amplicons (Angelini *et al.*, 2001; Langer and Maixner, 2004; Botti and Bertaccini, 2007). The products of

digestions were processed on 13% polyacrylamide gel electrophoresis (PAGE), stained with Gel Red™ Stain and visualized in UV transilluminator (Fig. 3. and Fig. 4.). The Bulgarian isolates were determined to belong to tuf – b type, which is common in Eastern Europe countries (Maixner, 2011).

The results from detection with conventional PCR/RLFP assays were confirmed by *TaqMan* real-time PCR (Angelini *et al.*, 2007). All DNA samples were diluted 1:50 and 1:500 prior to amplification. Reactions were performed in 96-well plates using Bio-Rad thermal cycler in 25µl total volume, including 5µl of DNA and 2X Platinum qPCR Supermix-UDG (Invitrogen). The concentration of primers was 0.15µM and of the probe 0.2µM. The program of thermal cycler included a decontamination step of 3 min at 50°C for optimal UDG enzymatic activity, followed by 3 min at 95°C and 50 cycles of two-step protocol including 15 s of denaturation at 95°C and 1 min of annealing/extension at 60°C (Angelini *et al.*, 2007).

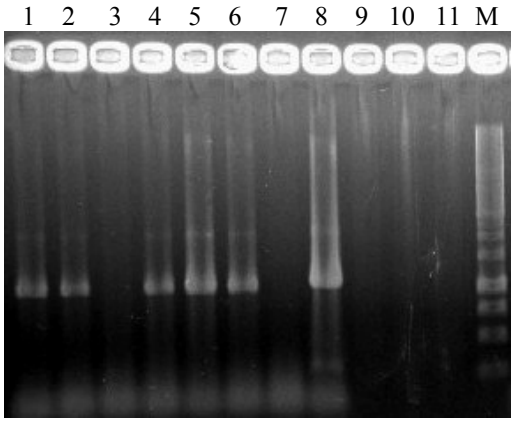

Fig. 1. Agarose gel showing the fragments from nested PCR with primers 16r758f/M23Sr. Line - **1** - sample № 39; **2** - № 40; **3 - 6** - №№ 59, 60, 61, 62; **7** - B (negative control); **8** - positive controle; **9-11** - empty wells; **M** - 1 Kb ladder.

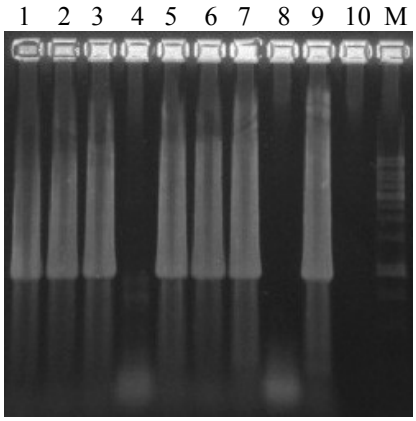

Fig. 2. Agarose gel showing the fragments from nested PCR with primers fTufAy/rTufAy. Line - - **1 -3** - samples №№ 38, 39, 40; **4 - 7** - №№ 59, 60, 61, 62; **8 -** B (negative control); **9** - positive controle; **10** - empty well; **M** - 1 Kb ladder.

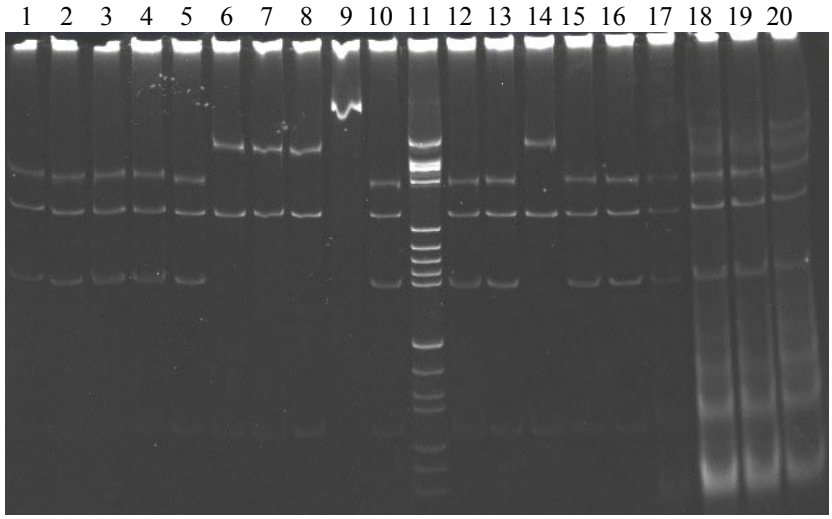

Fig.3. RFLP profiles of nested PCR amplicons (fTufAy/rTufAy), digested with *Hpa*II and divided in 13% PAGE. Lines – **1 - 5** samples №№ 29, 30, 31, 32, 33; **6 - 8** - №№ 60, 61, 62; **9** - phytoplasma reference isolate subgroup 16SrI-C; **10** - № 34; **11** - DNA ladder (pBR322 Hae III digest); **12 -15** - №№ 35, 36, 37, 38; **16** - № 40; **17** - № 41; **18** - № 43; **19** - № 44; **20** - № 46.

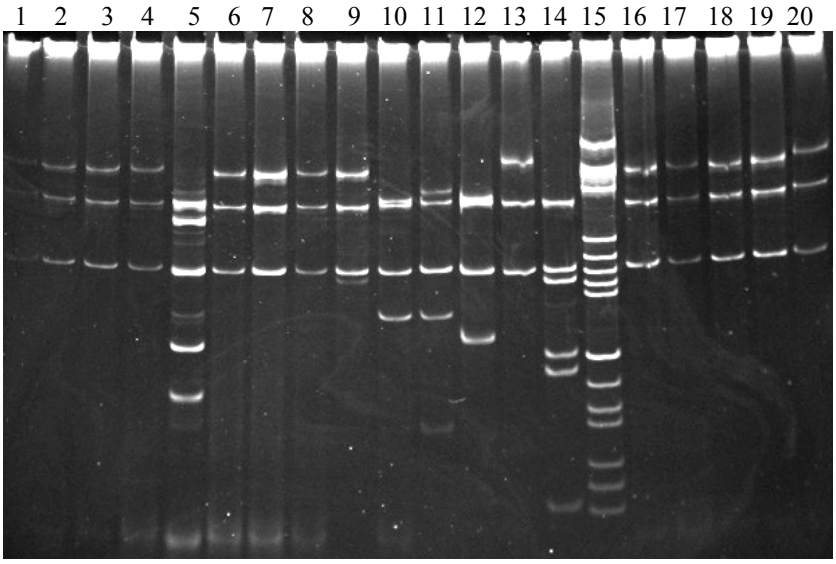

Fig.4. RFLP profiles of nested PCR amplicons (16r758f/M23Sr), digested with *Taq*I and divided in 13% PAGE. **Lines - 1 - 4** - samples №№ 29, 30, 31, 32; **5** - № 52; 6 - 8 - №№ 60, 61, 62; **9 - 14** - phytoplasma reference isolates, subgroups 16SrXII-A; I-B; I-C; II-C; V-A; X; **15** – DNA ladder (pBR322 Hae III digest); **16 - 20** - №№ 33, 34, 35, 36, 37.

* №№ 59, 60, 61 and 62 – Bulgarian grapevine samples.

Achievements for the applicant

This STSM allowed the applicant to learn to: recognize symptoms of grapevine phytoplasma diseases; collect and maintain samples; isolate DNA from samples; perform detection of grapevine phytoplasmas with nested PCR; perform detection of grapevine phytoplasmas with real-time PCR; analyse and document the obtained results. As a result, it was complemented the possibilities of the IVE plant protection group to study and protect sanitary state of the grapevine germplasm in Bulgaria.

Future collaboration with host institution

This collaboration will lead to a better control and protection against GY epidemics in Bulgaria and in particular in the IVE grapevine germplasm collection. Moreover, it will allow a scientific and technical collaboration among the two Institutes, both working on grapevine. This collaboration could be very useful in the future, also in other topics concerning grapevine and viticulture.

References

Angelini E., Clair D., Borgo M., Bertaccini A., Boudon-Padieu E., 2001. Flavescence dorée in France and Italy - Occurrence of closely related phytoplasma isolates and their near relationships to Palatinate grapevine yellows and an alder phytoplasma. Vitis 40: 79-86.

Angelini E., Bianchi GL., Filippin L., Morassutti C., Borgo M., 2007. A new TaqMan method for the identification of phytoplasmas associated with grapevine yellows by real-time PCR assay. J. Microbiol. Methods 68, 613-622.

Avramov, Z., Gillet J., Laginova M., 2008. First Detection of stolbur phytoplasma in grapevines (*Vitis vinifera* cv. Merlot) affected with grapevine yellows in Bulgaria. Journal of Phytopathology, 156: 112–114.

Avramov Z., Ivanova I., Laginova M., 2011. Screening for phytoplasma presence in leafhoppers and planthoppers collected in Bulgarian vineyards. Bulletin of Insectology, 64: 115-116

Bertaccini A., Angelini E., Bianco P.A., Botti S., Casati P., Durante G., Filippin L., Marzachì C., Pacifico D., Paltrinieri S., Quaglino F., 2008. Caratterizzazione dei ceppi di flavescenza dorata individuati nel territorio italiano nel periodo 2004-2008. Petria 18 (2): 268-271.

Belli G., Bianco P.A., Conti M., 2010. Grapevine yellows in italy: past, present and future. Journal of Plant Pathology, 92: 303-326.

Borgo M., Angelini E., Filippin L., Botti S., Marzachì C., Casati P., Quaglino F., Zorloni A., Albanese G., La Rosa R., Tessitori M., Pasquini G., Bertaccini A., 2005. Monitoring of grapevine yellows and molecular characterization of associated phytoplasmas in "GIA.VI" project during 2004. Petria, 15, 161-164.

Borgo M., Angelini E., 2007. Giallumi: mai abbassare la guardia. VQ Vite vino & qualità, 2: 48-56.

Botti S, Bertaccini A, 2007. Grapevine yellows in Northern Italy: molecular identification of Flavescence dorée phytoplasma strains and of Bois noir phytoplasmas. J Appl Microbiol 103:2325-2330.

Duduk B., Ivanovic M., Dudik N., Botti S., Bertaccini A., 2003. First report of an elm yellows subgroup 16SrV-C phytoplasma infecting grapevine in Serbia. Plant Disease 87: 599.

Duduk B., Botti S., Ivanovic M., Krstic B., Dukic N., Bertaccini A., 2004. Identification of phytoplasmas associated with grapevine yellows in Serbia. J. Phytopathol. 152: 575-579.

EPPO Reporting Service, 2006. First report of stolbur phytoplasma causing bois noir on grapevine in Bulgaria, 8: 2006/167.

Langer M., Maixner M., 2004. Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur-group based on RFLP-analysis of non ribosomal DNA. Vitis, 43, 191-199.

Magud B., Toševski I., 2003. Scaphoideus titanus Ball. (Homoptera, Cicadellidae) Nova tetoina na teritoriji Srbije. VI savetovanje o zatiti bilja, Zbornik reszimea, Zlatibor, 96.

Maixner M., 2011. Recent advances in Bois noir research. Petria 21 (2/3), 85-190.

Sakalieva, D., Paltrinieri S., Calari A., Bertaccini A., 2007. Molecular identification of "Bois Noir" phytoplasmas in grapevine in Bulgaria. Bulletin of Insectology, 60: 153-154.

Schneider, B.; Gibb K. S.; Seemüller E.; 1997 b: Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas. Microbiol. 143, 3381-3389.