COST FA 0807 WG1 School

Methodologies to Improve Phytoplasma DNA Extraction from plants and insects

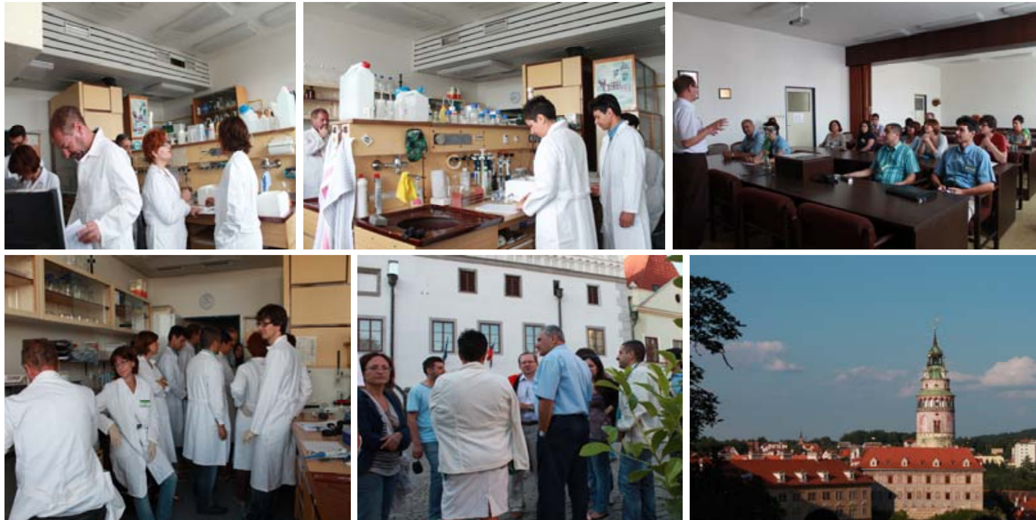
15 -18 July, 2013 Biology Centre AS CR, v.v.i. IPMB Czech Republic

The aim of the course was to teach phytoplasma extraction methods from fruit trees as host plants and insects. Below the final schedule.

July 15, 2013	**8:30-16:30** **Plant sample DNA extraction** **Lunch (between 11AM -01PM)** **Plant sample DNA extraction** **17:30 Field trip**
July 16, 2013	**8:00-12:00** **End of plant sample DNA extraction - Insect extraction** **Lunch (between 11AM -01PM)** **13:00-17:00** **DNA concentration reading and PCR setting** **19:30 České Budějovice city visiting and social dinner**
July 17, 2013	**8:00-12:00** **Nested-PCR - Electron microscopy demonstration - Discussion** **Lunch (between 11AM -01PM)** **13:15- 17:00** **PCR gels, second nested PCR setting and RFLP digestion - Lectures** **18:00 Field trip**
July 18, 2013	**8:30-12:00** **RFLP and second nested PCR gels; discussion about overall procedures** **Lunch and departure**

Monday – July 15, 2013

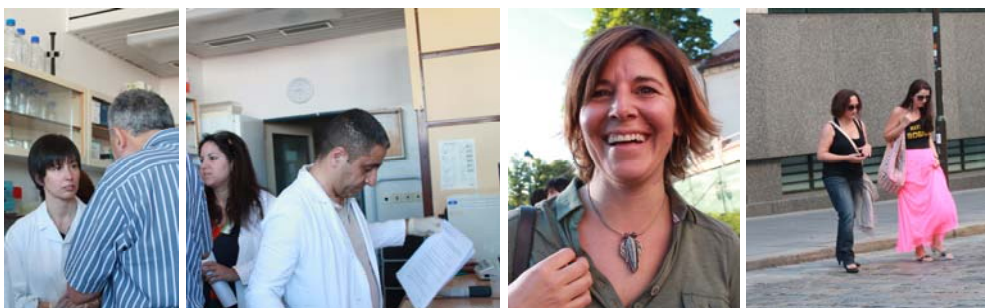
Prof. Assunta Bertaccini, dr. Jana Fránová, dr. Nicoletta Contaldo and dr. Bojan Duduk prepared final course schedule and materials needed. Leaf midribs and phloem tissue from diseased and asymptomatic apple and apricot trees were prepared before the beginning of the course and frozen (-20°C); insects *Euscelidius variegatus* and *Cacopsylla* sp. were caught before the course and maintained in 99% ethanol by dr. Elena Gonella.
The course started immediately in the lab. Frozen plant tissues were distributed to the participants according to the list identifying the names of the participants and the different infected and healthy host samples. Drs. N. Contaldo and J. Fránová trained the DNA extraction. Trainees grinded samples in liquid nitrogen with sterile mortar and pestle. Plant total nucleic acid (DNA) was extracted according to modified protocol of Lee *et al*., 1991 and Prince *et al*., 1993. The protocol was applied up to the stage of 4°C overnight.
In the time of sample incubation, prof. Josef Špak and prof. A. Bertaccini made a welcome speech and gave brief information about the course. Each participant introduced himself to the audience and gave brief report on research topics and reasons for joining the training school.



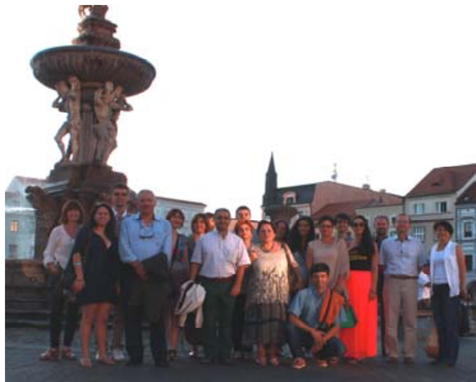
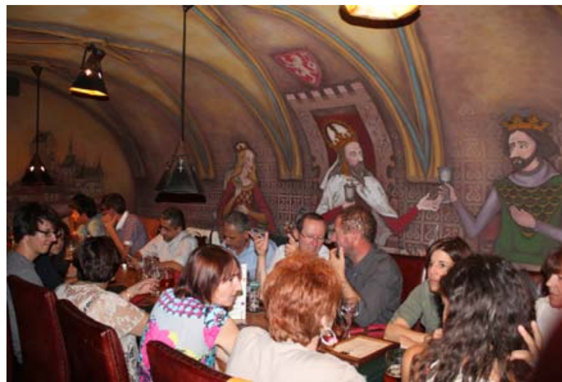
After the end of the laboratory day schedule, participants observed symptoms of apple proliferation phytoplasma disease (especially growth of young shoots from old branches and branch proliferation) in private garden and a tour to historical part of Český Krumlov (UNESCO) was also carried out.

Tuesday – July 16, 2013

Samples were taken from 4°C and plant total nucleic acid extraction protocol was completed. Trainees started DNA extraction from insect tissues (supplied and taught by Dr. Elena Gonella) according to the insect DNA extraction protocol of Marzachi *et al*., 1994. *Euscelidius variegatus* and *Cacopsylla* sp. were used and insect total nucleic acid extraction protocol was completed on the same day. Concentration of plants and insect vector DNAs were measured by nanodrop reading. DNAs were prepared for direct PCR that was started using universal phytoplasma specific primers P1/P7.



In the evening there was a tour guided by prof. J. Špak through the historical part of České Budějovice that finished by social dinner held in a typical Czech restaurant.

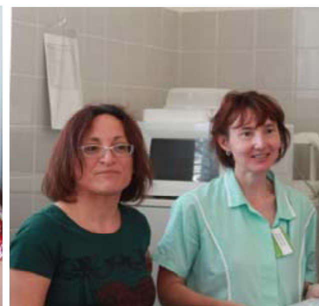
Wednesday –July 17, 2013

In the morning, participants diluted direct PCR products for nested PCR. PCR master mix was prepared and nested PCR was performed with the universal, phytoplasma specific primer pair R16F2n/R16R2. Prof. Bertaccini additionally also taught every participant how to cut the main leaf veins which includes phloem tissues from fruit trees leaves and J. Fránová taught how to separate phloem tissues from branches using scalpel.

After a visit to the electron microscopy laboratory (scanning electron microscopy: Jeol JSM-6300, Jeol JSM-740 1F; transmission electron microscopy: Jeol JEM-1010, Delong LVEM-5, Jeol JEM-2100F and equipment for sample preparation) in which the observation of phytoplasma bodies on ultrathin sections was performed by each trainee, a general lecture about phytoplasmas was presented by prof. A. Bertaccini.

In early afternoon, agarose gels (1%) were prepared and amplified PCR products were loaded to the gels, the gels were run and stained by GelRed and visualised under image analysis system of DOC-PRINT VX2 (Vilber). Second nested PCR with the group specific primers R16(I)F1/R1 and R16(X) F1/R1 was performed. Mixture for endonuclease digestion (enzymes: *Mse*I, *Rsa*I, *Ssp*I) of samples which gave positive reaction was prepared according to manufacturer´s instructions and incubated at 37°C overnight.

In the evening, participants visited private garden with apple trees showing especially enlarged stipules as symptom of apple proliferation phytoplasma disease, strawberry plant with leaf malformation, yellowing/reddening and dwarf resembling phytoplasma infection and also apple tree with typical symptoms after herbicide treatment. Castle with park in Hluboká nad Vltavou was visited subsequently.



Thursday – July 18, 2013

Dr. Bojan Duduk and J. Fránova trained acrylamide gel (8%) preparation including loading of samples after endonuclease digestion. Gels were stained by GelRed and visualised under image analysis system.

Phytoplasma DNA was successfully amplified from both plant and insect samples. Negative controls (DNA prepared from asymptomatic apple trees and also water controls) gave no results in PCR and RFLP digestion confirmed the presence of phytoplasmas in the examined samples. Discussion about RFLP results and overall procedures was also carried out.

PCR products from samples which gave positive reaction in second nested PCR with group specific primers were digested with *Mse*I and *Rsa*I endonucleases. Additionally, samples were loaded to acrylamide gels and copy of results was sent to all participants by e-mail.

Participants left the Department of Plant Virology, Academy of Sciences of the Czech Republic, v. v. i. in České Budějovice in the afternoon or on Friday (July 19th) for the departure to their countries.