# COST STSM- Final Report by *Rumbou Artemis*

COST Action FA0807 for

'Integrated Management of Phytoplasma Epidemics in Different Crop Systems'

COST STSM Reference Number: COST-STSM-FA0807-5583

STSM Topic:
**"Isolation and genetic characterisation of *Candidatus* Phytoplasma mali strains from the Pelion region (Thessaly, Greece) and genetic comparison with other European strains"**

Period: 10 February - 19 March 2010

STSM Applicant: Dr Rumbou Artemis: National Agricultural Research Foundation of Greece, Plant Protection Institute, Volos, Greece / amirumbou@yahoo.gr

Hosts:
- **Dr Schneider Bernd**: Julius Kühn Institute / Institute for Plant Protection in Fruit Crops and Viticulture, Dossenheim, Germany / b.schneider@bba.de
- **Dr Kube Michael**: Max Planck Institute / Institute for Molecular Genetics, Berlin, Germany / kube@molgen.mpg.de

COST Office Science Officer: Mr Albino Maggio / amaggio@cost.esf.org

COST STSM Manager: Matthew Dickinson / Matthew.Dickinson@nottingham.ac.uk

Purpose of the visit

The aim of this scientific mission was to improve the understanding of the apple proliferation disease occurring in the Pelion orchards the last decade. Leaves and roots from apple trees exhibiting symptoms of the apple proliferation disease were used for isolation of phytoplasma DNA and detection of the pathogen. Molecular characterization of local greek isolates was intended by cloning and sequencing PCR-amplified DNA fragments. Methods routinely used for phytoplasma detection and genetic analysis in the Dossenheim and Berlin laboratories were to be performed.

Methods performed

**A. At the Julius Kühn Institute, Dossenheim**

1. CTAB DNA extraction and separation of phytoplasma DNA from plant DNA by CsCl buoyant density gradient centrifugation
2. DNA isolation from leaf and root samples
3. Phytoplasma detection by PCR techniques (direct, nested, real-time)
4. Cloning and sequencing of phytoplasma-derived PCR products

5. Purification of phytoplasma DNA for pulsed-field gel electrophoresis (PFGE) by an enrichment procedure
6. SSCP analysis of *Ca.* Phytoplasma mali strains by polyacrylamide gel electrophoresis and silver staining
7. Detection of phytoplasmas by fluorescence microscopy after DAPI staining

**B. At the Max Planck Institute for Molecular Genetics, Berlin**

1. Methods for phytoplasma DNA amplification based on PCR and Restriction Fragment Length Polymorphism analysis of PCR products (RFLP-PCR)
2. Sequencing and sequence assembly
3. Shot-gun cloning for DNA library construction

Results

- Screening of symptomatic apple material from the Pelion region. Total DNA was extracted from 24 leaf and 24 root samples from diseased apple trees from different orchards in the Pelion Mountain. PCR screening with common primer combinations was performed. Positive amplicons were obtained only from root samples as they exhibited better durability during transportation. The PCR products were cloned and sequenced. From the 24 root samples, nine samples originating from 8 different orchards were assigned as *Ca.* P. mali, while three samples originating from three different orchards were assigned as *Ca.* P. pyri. Two samples coming from the same orchard (samples 3 and 4), were found to be infected by different phytoplasma species, namely from *Ca.* P. pyri and *Ca.* P. mali, respectively.

- Isolation of Lime Witches' Broom phytoplasma from infected periwinkle. Following the CTAB DNA extraction and separation of phytoplasma DNA by CsCl buoyant density gradient centrifugation, approx. 2.5 mg of phytoplasma DNA were isolated in total from the symptomatic- and the non-symptomatic material. This material was obtained in the Julius Kűhn Institute in Dossenheim and then was carried to the Max Planck Institute in Berlin.

- Subcloning and sequencing of the isolated lime witches' broom DNA. The construction of a whole-genome shot-gun library of lime witches' broom phytoplasma was initiated in the laboratory of the Max Planck Institute. Randomly chosen clones from the small insert shotgun library were end-sequenced. However, only 10% of the shotgun sequences could be assigned to phytoplasmas. In contrast, 80% could be assigned to a plant origin by BLASTX. Consequently, the isolated lime witches' broom DNA is not suitable for the Sanger sequencing due to the high plant background.

Discussion

The presence of *Candidatus* Phytoplasma mali in tissues from apple trees exhibiting symptoms of apple proliferation was confirmed for one more time using the PCR-RFLP diagnostic method. As it was previously experienced (Rumbou *et al*., 2007), this method has very low detection rate when applied to material from the Pelion region. Although the fidelity of the reactions was high, both direct and nested amplification was tried and many modifications on PCR conditions were applied, difficulties were faced in obtaining PCR amplicons with the primer pairs used (P1/P7, fO1/rO1, fu5/ru3, fHflB3-1/rHflB3). This fact

suggests the need for using other primer pairs or even designing new ones to be able to study isolates from this region. Despite the difficulties, PCR amplicons were obtained from 11 root samples and fragments of the 16S rDNA from those samples were sequenced for first time. Interestingly, some fragments (samples 3, 18 and 21) were assigned as *Candidatus* Phytoplasma pyri, the causal agent of Pear decline.

Due to difficulties in amplifying the phytoplasma DNA from the Pelion samples, it was not possible to apply the SSCP analysis and proceed with the molecular differentiation of the local strains. However, for practicing the technique, the method was performed with strains from the institute of Dossenheim.

Subcloning of the isolated lime witches' broom DNA showed the typical preference to small inserts. This aspect has a heavy impact for phytoplasma DNA, which is more difficult to clone due to its low GC content. Due to the high plant background estimated in the isolation performed, DNA isolation of lime witches' broom phytoplasma will be repeated within this year. Unfortunately, repetition of phytoplasma DNA isolations at the beginning of a genome project is more a rule than an exception.

Concluding, this Short Term Scientific Mission was highly interesting, first from the aspect of obtaining phytoplasma sequences from a newly and very vaguely studied population, second from the aspect of knowledge and techniques transfer and exchange and finally from the aspect of new collaboration established which hopefully will be continued in the future.

Rumbou A., Carraro L., Nanos G., Boutla I., Rumbos I.C., 2007. First report of *Candidatus* Phytoplasma mali in Greece and correlation with small apple fruit disorder occurring in the orchards of the Pelion Mountain. *Acta Horticultura* (ISHS) 781:505-510.