Current status and perspectives of phytoplasma disease research and management

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Integrated Management of Phytoplasma Epidemics in Different Crop Systems

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Integrated Management of Phytoplasma Epidemics
in Different Crop Systems

Current status and perspectives of phytoplasma disease research and management

Abstract book of the combined meeting of Work Groups 1-4

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Preface

The abstracts contained in this book are the presentations of participants of the first COST Action FA0807 meeting held on the 1 and 2 February 2010 in Sitges, Spain.

The main objective of this Action is the enhancement and exchange of scientific knowledge and technologies related to phytoplasma diseases, through the establishment of a multidisciplinary scientific European Network, aimed at developing strategies to detect and prevent biological invasion, and the spread of phytoplasma diseases of plants.

Phytoplasmas are insect-transmitted plant pathogenic prokaryotes causing serious diseases in important crops such as grapevine, vegetables, corn, sugar beet, oil-seed crops and fruit trees. Recent advances in phytoplasma genomics have generated an impetus for research into control and management of these diseases. New approaches for disease management based on understanding the phytoplasma-plant interaction at a molecular level are one of the main research aims. This will result in improved diagnostic methods; reduction of disease spread; improvement of insect-vector monitoring and a reduction in the pesticides used for control.

Information on the activities of the COST Action FA0807 is available on the WebPages:

http://www.costphytoplasma.eu/index.htm

http://www.cost.esf.org/domains_actions/fa/Actions/integrated_management_of_phytoplasma_epidemics

http://www.cost.esf.org/index.php?id=181&action_number=FA0807

We would like to dedicate this book to our colleague Dr. Luigi Carraro who very recently passed away while still in the blooming of his research work on epidemiology and management of stone fruit phytoplasma diseases.

The editors

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WG2

Working Group 1: Early detection and diagnostics

Early and sensitive detection and diagnosis of phytoplasmas is of paramount importance for effective prevention strategies, particularly because phytoplasmas may have a very long latency period. The main objectives of this WG are to compare diagnostic procedures already available for most phytoplasma pathogens and/or develop novel methods and integrate these into sensitive and simple early detection protocols, suitable for monitoring propagation material and for screening in plant-inspection services. To accomplish the goals in this task marker genes that show sufficient polymorphism will be selected as DNA bar-coding regions, and a database of available collections of phytoplasma strains and/or DNA will be established.

Coordinators

Dr. Bojan Duduk - Republic of Serbia

Dr. Jana Franova - Czech Republic

Tasks

1. Develop a database of available collections of phytoplasma strains and DNA collections in the EU

2. Identify suitable marker genes for differentiation at species and sub-species level forming the basis of new diagnostic protocols

3. Apply the above mentioned collections and methods to study pathogen diversity throughout EU and neighbouring countries

4. Develop new detection protocols for selected phytoplasmas and optimize and validate these and existing protocols

5. Standardise protocols based on results from the above tasks evaluated and validated in ring tests among laboratories

Tuf-type characterization of Hungarian stolbur strains from different host species

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The stolbur disease was described in Hungary more that fifty years ago on potato, pepper, tomato, tobacco and thorn apple (*Datura stramonium*). In the years of the '50s, '70s as well as in 2003-2005 stolbur disease caused mainly outbreaks in potatoes. The first molecular identification of stolbur phytoplasma was done by Viczian *et al*., (Növényvédelem, 34, 11. 1998) on the following species: pepper, tomato, parsley, rape, bladder campion (*Silene vulgaris*), and thorn apple. Later further plants and insect species joint to the list as carrot, celery, bindweed, nettle, common dandelion as well as grapevine, and *Reptalus panzeri*, indicating the wide spread presence of this phytoplasma in Hungary on crops and on wild plants as well. Molecular characterization of Hungarian stolbur strains has high importance to study epidemiology and to devise management of the disease in the field.

In this study the Tuf-type characterization of different Hungarian strains from grapevine, potato and tomato was performed. Stolbur positive samples identified on 16S ribosomal gene R16F2/R2 amplicons followed by *Tru*I restriction digestion were amplified with fTuf1/rTuf1 and fTufAy/rTufAy primers in nested PCR, and digested with *Hpa*II restriction enzyme (Langer and Maixner, Vitis, 43, 191-199. 2004) to identify the tuf-type. RFLP profiles obtained for the three species were referable to the stolbur tuf-type b. These results suggest possibility that stolbur phytoplasmas associated with major diseases of grapevine and potatoes are maintained in *Convolvulus arvensis* as wild host plant considering that this species is widespread in Hungary. This hypothesis is also supported by the high density of bindweed in all cultivated areas of Hungary. Further characterization of stolbur strains from the same species as well as from other species and insect vector or potential vector of stolbur phytoplasmas is in progress.

This study was (partly) supported by the 'National Office for Research and Development' as well as financed by the 'Research and Technology Innovation Fund in Hungary'.

Association of '*Candidatus* Phytoplasma asteris' with witches' broom and little leaf disease of *Zinnia elegans* in India

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Different gardens and nurseries were surveyed in different region of Eastern Uttar Pradesh, India during 2008-2009 for the incidence of phytoplasma disease. A typical little leaf, chlorosis, witches' broom, yellowing and phyllody symptoms were recorded on *Zinnia elegans* plants growing in different gardens Sugarcane Research Station, Kunraghat , Gorakhpur of district Uttar Pradesh, India. The symptomatic plants were further processed for phytoplasma detection and characterization. Universal primer pair of P1/P7 amplified the 1.8 kb DNA fragment of phytoplasma 16S-23S rDNA from nucleic acid extracted from ten symptomatic *Z. elegans* plants showing witches broom and little leaf disease symptoms. In a nested PCR assay, the amplification of 16SrDNA from 1.8 kb PCR products produced a DNA fragment of 530 bp (P1/P7 and P4/P7). Neither by direct (one-round) nor by nested PCR assays was DNA amplified from template DNA isolated from any of the non-symptomatic plants. Sequence analysis through Mega 4.0 tool revealed 99% sequence similarity of the *Zinnia* phytoplasma isolate in the present study with the 16SrRNA gene of mulberry yellow dwarf phytoplasma (GQ249410); aster yellows phytoplasma (FJ824597) and Italian *Empoasca* phytoplasma (AM990990) respectively belonging to the '*Candidatus* Phytoplasma asteris' (16SrI) group. Therefore, the strain of *Zinnia* phytoplasma in the present study has been identified as related to '*Ca*. P. asteris'. This is the first report of '*Ca.* P. asteris' associated with yellowing, witches' broom and little leaf disease of *Zinnia elegans* in India.

Identification of phytoplasmas infecting pine trees

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Symptoms of abnormal shoot branching and formation of dense, ball-like structure were observed on *Pinus sylvestris*, *P. sylvestris* var. *lapponica*, *P. mugo*, *P. mugo* subsp. *uncinata*, *P. banksiana* and *P. tabulaeformis* trees grown in natural habitat as well as propagated in commercial nurseries in Poland and Czech Republic. The presence of the phytoplasmas in those pine trees was demonstrated using nested PCR with P1/P7 followed by R16F2n/R16R2 primer pairs, as described earlier (Kamińska *et al*., Journal of Phytopathology, 151, 469-476. 2003).

Identification of detected phytoplasmas was done using restriction fragment length polymorphism analysis (RFLP) of 16S rRNA gene fragment with *Alu*I, *Hha*I, *Mse*I and *Rsa*I endonucleases. After enzymatic digestion, all tested samples showed restriction pattern similar to that of '*Candidatus* Phytoplasma pini', computer-calculated on the basis of the sequence of isolate Pin127S (GenBank acc. no. AJ632155) found in *P. halepensis* tree (Schneider *et al*., International Journal of Systematic and Evolutionary Microbiology, 55, 303-307. 2005).

Nested PCR-amplified rDNA fragments, obtained with primers R16F2n/R16R2, were sequenced. GenBank accession numbers of obtained sequences were: EF128037, FJ409230, FJ409231, FJ409232, FJ409233, FJ409234, GQ290115 and GQ290143. Comparison of the tested 16S rDNA fragments revealed high nucleotide sequence identity between analyzed phytoplasma isolates (99.8%-100%). They were also nearly identical (99.8-99.9%) with the sequences of three other phytoplasma isolates found in pine trees previously (AJ310849, AJ632155, AJ632156). Based on the results of RFLP and sequence analyses, tested phytoplasma isolates were classified as '*Ca.* P. pini'-related.

Phytoplasma detection in corn with reddening in Italy

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During the second half of August 2009 in corn fields located in Northern Italy scattered plants showing reddening symptoms were observed, mainly located at the edge of the fields. Symptoms were clearly visible on the main leaf midribs, and/or on the stalks, and eventually affect the whole plant. Symptomatic plants had smaller size than healthy ones, and corn cobs were sometime malformed and of very little size. In some of the symptomatic plants the cobs produced were of regular size and contains poor shrivelled grains as reported for reddening disease of corn in Serbia (Duduk & Bertaccini, Plant Disease, 90, 1313-1319. 2006). Ten samples of symptomatic, and 4 of asymptomatic corn plants were collected in two different locations and nested PCR assays were carried out on total nucleic acids from 1 g of main leaf midrib and phloem stalk tissues chloroform/phenol extracted. Direct PCR assays with phytoplasma universal primer pair P1/P7 followed by nested PCR with 16S758F/16S1242R (Gibb *et al*., Phytopathology, 85, 169-174. 1995) primers allowed amplification of 500 bp amplicons from all samples from symptomatic plants, no bands were obtained from asymptomatic samples. Identification of detected phytoplasmas done using RFLP analyses with *Tru*I, *Tsp509*I and *Mbo*II restriction enzymes allow preliminary identification of phytoplasmas belonging to 16SrI (aster yellows), 16SrIII (X disease) and 16SrXII (stolbur) groups, in same cases in mixed infection. Further molecular characterization of these phytoplasmas is in progress together with epidemiological studies to verify the presence of phytoplasma sources, and of possible insect vectors in the two environments. Presence of stolbur phytoplasmas in corn samples with reddening symptoms is confirming the finding in Serbia (Duduk & Bertaccini, above), however this is the first report in Europe of 16SrI group phytoplasmas, and the first report of 16SrIII in corn. The diverse phytoplasmas are associated with indistinguishable symptoms in plants as already worldwide reported in this and in other plant species for phytoplasma infection.

New SNP genetic lineages among '*Candidatus* Phytoplasma mali' populations in northern Italy

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'*Candidatus* Phytoplasma mali' ('*Ca.* P. mali') is the aetiological agent of apple proliferation (AP), a quarantine disease widespread in the most important apple-growing regions in Europe (http://www.eppo.org). The aim of the present study was to evaluate the genetic diversity among strains of '*Ca.* P. mali' in orchards of north-western Italy, where AP induces severe economical losses.

'*Ca*. P. mali' has been detected through PCR/RFLP analysis of phytoplasma 16S rDNA, in 89% (101/114) of the examined samples (apple trees and insect vectors). Collective RFLP patterns, obtained by analyses of genomic fragments including 16S-23S rDNA, PR-1, PR-2 and PR-3 non-ribosomal region, ribosomal protein genes *rplV-rpsC*, and gene *secY*, revealed the presence of 12 distinct genetic lineages among 60 selected representative '*Ca.* P. mali' isolates. These findings underscored an unexpected high degree of genetic heterogeneity among '*Ca.* P. mali' populations in north-western Italy. Prevalence of distinct '*Ca.* P. mali' genetic lineages in diverse geographic regions opens new interesting perspectives for studying the epidemiology of AP disease. Molecular markers determining diverse '*Ca.* P. mali' genetic lineages, identified in the present work, could be useful for investigate the biological life cycle of AP phytoplasma, with the perspective of developing new strategies for the control of AP epidemics.

Association of sugar beet yellow wilt disease with phytoplasmas belonging to 16SrIII group and their detection in the insect vectors

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Sugar beet yellow wilt disease is the most important disease of sugar beet in the Central valley of Chile. The symptoms include yellowing, typical leaf roll called "capucha de monge" (monk hood), proliferating shoots, necrosis of roots and plant dead. The hypothesis of phytoplasmas associated to this disease was reported many years ago, as the possible insect vector *Paratanus exitiosus* (Beamer) (Urbina-Vidal and Hirumi*,* Journal of American Society Sugar Beet Technology, 18, 142-162. 1974).

A survey in many fields of sugar beet of IANSA (Industry Sugar National Anonymous Society) in the Central valley of Chile was made to recollect sugar beet plants with symptoms of the disease and cicadellidae insects that were distinguished in *Paratanus* and non *Paratanus*. The disease etiology was investigated using polymerase chain reaction with phytoplasma-specific primers, sequencing, and phylogenetic analysis. No amplification products from symptomless plants, whereas all the analyses were positive from samples collected in plants with symptoms. Phylogenetic analysis indicated that this phytoplasma clustered in the 16SrIII group, reference strain of which is X-disease phytoplasma. To analyse the presence of phytoplasmas in *P. exitiosus*, and in other cicadellidae, PCR were made in batches of 1, 5, 10 *Paratanus* and non *Paratanus* insects. PCR products were observed in the three batches of *Paratanus* and in the 5 and 10 batches of non *Paratanus* insects. The PCR products were cloned and sequenced and showed 100% homology to phytoplasmas associated to the sugar beet yellow disease. These results will be discussed with the control measures that the farmers use for this disease.

[+] Past away in 2008.

Occurrence of phytoplasmas infecting stone fruit trees in Poland

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In Poland, pear decline is the only fruit tree phytoplasma known to be widespread, although studies showed the occurrence of phytoplasmas in strawberry, *Rubus* sp., blueberry, apple, sweet cherry, sour cherry, peach, apricot, nectarine, plum, and hazelnut. Epidemiological study for stone fruits phytoplasmas was carried out in commercial and experimental orchards located in several regions of Poland. Characteristic symptoms such as chlorotic leaf roll, leaves deformation, die-back and decline of the infected trees were observed on some tested trees of sweet cherry, sour cherry, peach, apricot, nectarine and plum. Nucleic acids extracted from phloem tissue were subjected to a nested PCR with phytoplasma universal primers P1/P7 and R16F2n/R16R2 as well as primers specific for 16SrI, 16SrIII, 16SrV, and 16SrX groups. RFLP analysis of R16F2n/R16R2 products was performed using *Rsa*I, *Mse*I, *Alu*I, *Ssp*I enzymes. Nested PCR products from two sweet cherry ('Trzebnica' and 'Kordia I/8'), two apricot (EO, I/5), one nectarine, one plum (II/3), one peach (III/5) and one sour cherry ('Sokowka') trees were purified and sequenced. The results of nested-PCR with R16F2n/R16R2 universal primers and with R16(X)F1/R1 primers specific for apple proliferation (AP) group (16SrX) and RFLP analyses indicated that eleven peaches, six sweet cherry plants, three apricots, three plums, three sour cherry plants, and one nectarine out of 412 tested trees were infected by phytoplasmas from AP group. RFLP profiles with *Ssp*I and *Rsa*I enzymes indicated that peach III/5 was infected by phytoplasma related to '*Candidatus* Phytoplasma mali' and sweet cherry 'Kordia I/8' - to '*Candidatus* Phytoplasma pyri'. The profiles for phytoplasmas infected the other stone fruit trees were indistinguishable from restriction patterns for the '*Candidatus* Phytoplasma prunorum'. PCR/RFLP results were confirmed by sequence analysis results. The study on comparison of diagnostic methods based on molecular tools, diversity of phytoplasma infecting fruit crops will be carried out in the frame of the project.

Lavender decline is caused by several genetic variants of the stolbur phytoplasma in south eastern France

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Lavender decline has affected *Lavandula* sp. in south eastern France since the 1970s. The disease can be transmitted by *Hyalesthes obsoletus*, the vector of stolbur phytoplasma, but can be confused with damage due to heavy frost and drought. In order to ascertain the aetiological role of stolbur phytoplasma in the disease a large epidemiological survey was undertaken. The origin of the phytoplasma inoculum was obtained by genotyping the phytoplasma strains detected. Twenty lavender fields both planted with *Lavandula angustifolia* and *Lavandula* hybrids were surveyed and sampled during spring and early fall 2008. Disease incidence ranged from 1% to 68% at spring and increased from 17% to 99% during fall. In the same time the mean severity was significantly increased. DNA was extracted from 15 diseased lavender plants per field and tested by a Taqman realtime PCR assay with an internal analytical control to detect false negative (Pelletier *et al.,* Vitis 48**,** 87-95. 2009). Results indicate that 37% of the diseased lavenders were positive for stolbur infection at spring whereas the proportion of positives reached 46% at fall 2008. The phytoplasma strains detected in lavender were submitted to *sec*Y genotyping (Fialova *et al.,* J. Pl. Pathol., 91, 411-416. 2009). Over 45 strains analyzed 17 *sec*Y different genotypes were evidenced. Only three genotypes accounting for 16 samples corresponded to genotypes commonly found in France in wild plant reservoirs and in the vineyards, where only these three genotypes are detected. The 14 remaining genotypes were specific to lavender. This study confirms the role of stolbur phytoplasma in the etiology of lavender decline and demonstrates that the epidemics mainly propagate from lavender to lavender. In addition an important genetic diversity characterizes the phytoplasma populations associated with the disease.

"Bois noir" phytoplasma infecting grapevine in Srpska - Bosnia and Herzegovina

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The presence of "bois noir" (BN) phytoplasmas in Bosnia and Herzegovina (B&H) was reported for the first time in 2005, in a survey of grapevine growing region, *i.e.* Mostar, Trebinje and Banjaluka areas (Delić *et al*., Journal of Plant Pathology, 88: 225. 2006). Moreover, "flavescence dorée" phytoplasma vector, *Scaphoideus titanus* Ball was identified in Trebinje region (Delić *et al*., Bulletin of Insectology, 60, 369-370. 2007). The aim of this work was to check the presence and geographical distribution of BN phytoplasmas in the grapevine growing region. During summer 2008, grapevine (*Vitis vinifera*) and weeds (*Setaria* spp., *Convolvulus arvensis* and *Clematis vitalba*) were visually inspected and samples were collected. In general, 85 samples were collected for the laboratory analyses. All the samples were analyzed by means of molecular methods. DNA was extracted from midribs tissue using DNeasy Plant Mini kit protocol with slight modification. Nested PCR assays were carried out with phytoplasma universal and specific primer pairs, targeting ribosomal and non-ribosomal gene fragments. The following primer pairs were used: P1/P7 (direct) (Deng and Hiruki, Journal of Microbiological Methods, 14, 53-61. 1991; Schneider *et al*., Molecular and Diagnostic Procedures in Mycoplasmology, 369-380. 1995); R16F2nR16R2 (nested) (Gundersen & Lee, Phytopathologia Mediterranea, 35, 144-150. 1996); fstol/rstol (nested) (Maixner *et al*., Vitis, 39, 83-84. 1995); FD9R/FDF (direct) (Daire *et al*., European Journal of Plant Pathology 103, 507–514. 1997) and FD9R2/FD9F3b (nested) (Clair *et al*., Vitis, 42, 151-157. 2003). From 85 tested, 35 samples were phytoplasma positive in nested PCR with phytoplasma universal primer pairs (P1/P7 and R16F2n/R16R2). Specific nested PCR with fstol/rstol primer pair confirmed that 35 positive samples are infected with BN phytoplasma. The presence of BN phytoplasma was detected only in grapevine samples but not in weeds. Further studies will be dedicated to the characterization of the phytoplasma strains and to the identification of the BN vectors involved in transmission of the phytoplasmas in the area.

Development of a one-hour DNA extraction and loop-mediated isothermal amplification assay for rapid detection of phytoplasmas

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A rapid DNA extraction and loop-mediated isothermal amplification (LAMP) procedure has been developed and evaluated for the detection of two specific groups of phytoplasmas from infected plant material. Sets of primers based on 16S-23S rDNA sequences were developed and tested for the 16SrI aster yellows types, and a second set of primers was developed for the 16SrXXII Cape St Paul wilt disease of coconuts. DNA could be extracted from leaf material (16SrI phytoplasmas) or coconut trunk borings (lethal yellowing, 16SrXXII phytoplasmas, Wei *et al*., International Journal of Systematic and Evolutionary Microbiology, 57, 1855-1867. 2007) onto the membranes of lateral flow devices, and small sections of these membranes were then added directly into the LAMP reaction mixture and incubated for 45 mins at 65°C. Positive reactions were detected through the hydroxyl napthol blue colorimetric assay within 1 hour of the start of DNA extraction, and were confirmed by subsequent agarose gel electrophoresis of the LAMP products. The level of detection was comparable to that obtained by nested PCR using conventional 16S rDNA phytoplasma-specific primers. Furthermore, the assays were specific for the phytoplasmas they were designed to detect – the 16SrI assay only detected 16SrI phytoplasmas and not those from any other phylogenetic groups, whilst the 16SrXXII assay only detected 16SrXXII phytoplasmas. The DNA extractions and LAMP assay are easy to perform, requiring minimal equipment, and may therefore form the basis of a rapid and reliable field-detection system for phytoplasmas.

Multigene analysis of an aster yellows phytoplasma strain showing interoperon heterogeneity

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Identification of phytoplasmas associated with carrot yellows in Serbia allow to identify 16SrI-A and 16SrI-B subgroups (Duduk *et al*., Annals of Applied Biology, 154, 219-229. 2009). However, PCR amplification of 16SrDNA followed by RFLP analysis, cloning and sequencing showed clearly presence of interoperon heterogeneity in one of the samples: two different sequences were obtained associated with two different RFLP profiles. Homology comparison among the sequences clustered in 16SrI-B clade showed that cloned sequences of the strain are closer to some other 16SrI-B strain sequences than to each other. Moreover phylogenetic analyses of the two operons showed that, while one operon is clustering in the 16SrI-B clade, the other operon is clustering out of it. The two operons of the same phytoplasma can be affiliated to different 16SrI subgroups according to RFLP analyses and this is supported also by phylogenetic analyses. Therefore, additional genes such as the *l22* and *s3* ribosomal protein genes, the *tuf* gene coding the elongation factor Ef-Tu, the putative aa kinase gene and ribosomal recycling factor gene, and a phytoplasma DNA helicase gene were studied to molecularly characterize this aster yellows strain from carrot. The RFLP and sequence analyses of PCR amplified ribosomal protein genes clearly showed that the strain is different from those affiliated with rpI-B and from all other strains in rpI subgroup tested. This strain was also differentiable from all other strains by RFLP analyses of putative aa kinase gene and ribosomal recycling factor gene, while analyses of *tuf* gene and of DNA helicase gene did not supported the difference and did not show any polymorphism, respectively. The presence of 16S rRNA interoperon sequence heterogeneity is not uncommon in phytoplasmas, and although the difference in homology between two operons is relatively small, when differences occur in restriction sites, misidentification or assignment of the same phytoplasma to two different 16S rRNA subgroups is possible. However, the use of other genes present as single copy in the phytoplasma genome can be helpful in discriminating when different phytoplasma populations are present in mixed infection from the presence of interoperon sequence heterogeneity.

Study of the molecular variability of phytoplasmas by *tuf* gene analysis

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The identification of molecular markers for the study of the genetic diversity is one of the main topics in phytoplasma research and the *tuf* gene was proved to be useful towards this aim (Schneider *et al.*, Microbiology, 143, 3381-3389. 1997). Starting from this evidence a molecular analysis of the *tuf* gene was carried out to investigate on the genomic variability of phytoplasmas associated to widespread and economically important diseases such as tomato stolbur and bois noir (BN), caused by stolbur 16SrXII-A phytoplasma and European stone fruit yellows (ESFY), induced by '*Candidatus* Phytoplasma prunorum' (16SrX-B). Genomic variability was investigated by specific PCR/RFLP based methods and nucleotide sequence analysis. Molecular characterization of Stolbur phytoplasma from infected samples collected from different Italian grapevine growing areas confirmed the presence of two distinct isolate types, referable to the tuf-type A and tuf-type B previously described (Langer & Maixner, Vitis, 43, 191-200. 2004). The two types showed a well defined geographical distribution and were identified also in alternative host plants and insect vectors (Pasquini *et al.,* Bulletin of Insectology, 60, 355-356. 2007). This molecular variability was not observed in stolbur phytoplasma isolates from infected tomato, since only the tuf-type B, identical and not distinguishable from the stolbur referable strain from pepper, was found in all analyzed samples (Mazzoni *et al.*, Petria, 18, 333-334. 2008). Two groups of isolates with a well defined geographical distribution were also identified in '*Ca.* P. prunorum'. According with the ESFY epidemiological cycle this molecular variability was recognized in phytoplasma isolates from cultivated plants, *Cacopsylla pruni* insect vector and wild *Prunus* species (Ferretti *et al*., 21st ICVF, 82. 2009). The genetic heterogeneity of phytoplasma *tuf* gene resulted to be useful to improve the knowledge on the possible correlation between the molecular and biological phytoplasma properties. Moreover the selected molecular markers could be considered an effective tool also for epidemiological investigations.

Identification of "flavescence dorée" - related phytoplasma in plants of *Ailanthus altissima* in Italy

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Ailanthus altissima (tree of heaven) is an invasive tree species, originally introduced from China, which arrived in Italy two centuries ago (Celesti-Grapow *et al*., Plant invasion in Italy, 22. 2009). Nowadays it is widely found in urban and rural areas of Italy, due to its efficient spread.

Leaf samples were collected several areas in North of Italy (Friuli Venezia Giulia, Veneto, Piedmont and Lombardy regions). Molecular analyses showed that 7 samples out of 26 were infected with a "flavescence dorée" (FD-C)-related phytoplasma. No clear association between yellowing symptoms and presence of phytoplasma was observed.

PCR and RFLP experiments were carried out on three genomic regions: the 16S-23S rRNA, *secY* and *rpl22-rps3* genes. All strains showed the same RFLP pattern in the 16S ribosomal gene, identical to the reference strains belonging to the 16SrV-C subgroup. Different patterns were obtained in the *secY* and *rpl22-rps3* genes, which allowed three clusters to be distinguished. A correlation between FD-C molecular variants on these genes and geographic area was found. Interestingly, FD-C phytoplasma isolates from *A. altissima* collected in Friuli Venezia Giulia showed the same RFLP patterns as *Clematis vitalba* of the same areas. Moreover, the FD-C isolate found in *A. altissima* samples from Piedmont was identical to the FD-C isolate found in *C. vitalba* collected in the same region, which showed 100% nucleotide sequence identity with a French FD strains found in grapevine in Savoie (Arnaud *et al*., Applied Environmental Microbiology, 73, 4001-4010. 2007; Filippin *et al.,* Plant Pathology, 58, 826-837. 2009).

This finding suggests that FD-C phytoplasma exchange can occur among different plant species. The tree of heaven could therefore play a role in the FD evolutionary history in Europe, along with grapevine, clematis and alder.

Investigation of phytoplasma diseases at the Department of Plant Virology BC ASCR v.v.i. IPMB – past, present and future

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A research program on occurrence and identification of phytoplasmas began at our department in 1992. During the screening of strawberry viruses, symptoms resembling strawberry green petal disease were observed. Thanks to cooperation with the lab of Prof. A. Bertaccini, strawberry green petal phytoplasma was found by electron microscopy in infected plants and characterized by molecular methods. There is valuable partnership between our department and other institutes in the Czech Republic (Palacký University in Olomouc, University of South Bohemia, RBIP Holovousy), breeders of different crops and many crop producers.

For phytoplasma characterization, various methods are used, ranging from symptoms observation, biological transmission, cultivation of infected and healthy plants *in vitro* cultures, electron microscopy examination, PCR assay, RFLP analysis, cloning and sequencing. Phytoplasmas have been detected cca in 25 plant species and identify as a members of 16SrI-B, 16SrI-C, 16SrIII-B, 16SrV-A, 16SrVI-A, 16SrX-A, 16SrX-B, 16SrX-C and 16SrXII-A ribosomal subgroups in single and mixed infection, sometimes in co-infection with viruses.

The objectivities of the current and future activities are: - to clarify the etiology of diseases of important grown crops as well as wild plants associated with symptoms typical for phytoplasmas (especially quarantine organisms: apple proliferation (differentiation of AP, AT1, AT2 subtypes), pear decline, European stone fruit yellows); molecular hybridization approach for universal detection on microarray platform should be developed based on the ribosomal internal transcribed spacer and/or other genes; and study on the presence and variability of plasmids in phytoplasmas inducing different level of disease symptoms.

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Seasonal variations of '*Candidatus* Phytoplasma pyri' in pear trees under field conditions in Turkey

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Pear decline disease (PD) caused by '*Candidatus* Phytoplasma pyri' was monitored in pear trees cv Deveci in two orchards in Bursa, Turkey. PD infections on twenty pear trees in each orchard were previously determined by nested PCR. Fluctuations of PD were determined throughout a year monthly. The tests were performed by using roots, shoots, leaf midribs, fruit columellas and flowers of the trees depending on the season. Samples were analyzed with PCR using P1/P7 and fU5/rU3 universal primer pairs. Nested PCR products were digested with *Rsa*I restriction enzyme. All digested products revealed the same profile as PD positive control. RFLP results were supported by sequencing of three selected PD isolates. The results revealed that the infection rate of PD had different averages according to the sampling tissue and the period. The flower tissues were sampled only in March and the infection rate was 75% whereas the fruit tissues which were only sampled in September, was 100%. Root, shoot and leaf samples were collected longer period of the year, but the infection rate of PD was comparatively less comparing to flower and fruits. The infection rate in shoots, roots and leaves was found as 19, 18 and 10%, respectively. The present result has revealed that the best period to detect PD infection was in September by using fruits followed by flowers in March. Shoot samples were found a good inoculum source due to the possibility to detect phytoplasmas during the whole year except July and August. Root samples can be used from November to March and best time for leaf midribs was found as April, October, November and December.

Development of real-time PCR assays for improved universal and group specific detection of phytoplasmas

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In the UK detection of all phytoplasma isolates is key to try and prevent ingress of pathogens into the country, whist in many other European countries specific isolate identification is the key priority. To this end there are many specific real-time assays primarily for fruit tree and grapevine phytoplasmas, yet there is only a single assay aimed at universal phytoplasma detection (Christensen *et al*., Molecular Plant Microbe Interactions, 17, 1175-1184. 2004).

To this end we designed primers and TaqMan probes based on the 23S rRNA gene to design a range of real-time PCR assays for routine universal phytoplasma diagnostics. A universal assay to detect all phytoplasmas has been developed, along with a multiplex assay to discriminate 16SrI group phytoplasmas from members of all of the other 16Sr groups. Individual assays for 16SrII, 16SrIV, and 16SrXII have also been developed to confirm that the 23S rRNA gene can be used to design group-specific assays (Hodgetts *et al*., Applied Environmental Microbiology, 75, 2945-2950. 2009).

These assays have been authenticated as phytoplasma specific and shown to be at least as sensitive as nested PCR. These assays are now used routinely in the Fera diagnostic laboratories.

Genetic variability among '*Candidatus* Phytoplasma ulmi' strains infecting elms in Serbia and survey of potential vectors

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Presence of elm yellows phytoplasmas (EY) belonging to 16Sr DNA group 16SrV-A infecting *Ulmus minor* and *U. laevis* in Serbia was reported in 2008 (Jović *et al*., Plant Pathology, 57, 1174). Molecular characterization of these strains and additionally collected samples of different geographical origin in Serbia was performed. RFLP and nucleotide sequence analyzes of four conserved genes: 16S rDNA, *rpl22-rps3*, *SecY* and *map* were carried out. Comparison of obtained sequences with representative phytoplasma strains in the EY phytoplasma group (Lee *et al*., Int. J. Syst. Bacteriol., 54, 337-347. 2004; Arnaud *et al.*, Appl. Environ. Microbiol., 73, 4001-4010. 2007) revealed presence of five different strains. Four strains exhibited nucleotide changes located inside a range of unique regions of 16S, *rp* and *SecY* genes determined by Lee *et al*. (2004), while the fifth strain had sequences most similar to strain EY626. Based on sequence analyses of FD9 genetic loci and virtual digestion of FD9f3/r2 amplicons with *Mse*I endonuclease, a routine typing method was determined for all five strains. Topology of phylogenetic trees constructed for the *rp*, *SecY* and *map* genes was the same with EY phytoplasma strains from Serbia forming a separate cluster inside the '*Candidatus* Phytoplasma ulmi' branch. Survey of potential hemipteran vectors on two localities in East Serbia where symptomatic, phytoplasma infected elm trees were present resulted in identifying 14 species of planthoppers and leafhoppers which were analyzed for EY phytoplasma presence. Most abundant species were *Reptalus quinquecostatus* and *Hyalesthes luteipes*. On both sites only *H. luteipes* individuals regularly present on elms, proved to be infected (6% and 10%). RFLP analyses of FD9 amplicons with *Mse*I endonuclease showed that all phytoplasma strains from *H. luteipes* had a profile similar to the fifth strain described above and therefore related to EY strain EY626.

Diseases of forest trees associated with phytoplasma infection

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Diseases of forest trees of uncertain aetiology as shoot proliferation and fasciation symptoms have been widely distributed throughout the world. These aberrations are potentially attractive and they are used for propagation to get new selections of dwarf types of evergreens. The grafting of witches' brooms tissues has been done since 1874 and is origin of such dwarf pines as *Pinus sylvestris* 'Beauvronensis' and *P. nigra* 'Hornibrookiana'. However, up to date little is known on the nature of the virus-like symptoms of coniferous plants in Europe.

In the last decade an economic importance of some plant diseases associated with phytoplasma infection has increased considerably in many countries. Using molecular techniques for detection and identification, several phytoplasmas were found to be associated with diseases in about a thousand plant species, almost exclusively angiosperms. There are only three reports on the electron microscope detection of phytoplasmas in conifer plants with leaf yellowing, shoot proliferation and stunting. More recently it was reported that in Italy *Cypress* species were naturally infected with phytoplasma related to the X disease phytoplasma and the new taxon '*Candidatus* Phytoplasma pini' was associated with witches' broom formations in *Pinus sylvestris* and *P. halpensis* trees in Germany and Spain (Schneider *et al*., Int. J. Syst. Evol. Microbiol. 55, 303-307. 2005).

Conspicuous symptoms of stunted growth, abnormal shoot branching with dwarfed needles, or shoot fasciation were observed in coniferous trees of several species in a forest ecosystem in Poland. The symptomatic plants were phytoplasma affected (Śliwa *et al*., Journal of Phytopathology 156, 88-92. 2007) and they were vegetatively propagated to get new dwarf forms.

The objective of this study is to report on the occurrence of growth abnormalities in coniferous trees in Poland, evaluation of their association with phytoplasma infection using molecular methods, and development of diagnostic protocols for phytoplasmas to use for producing phytoplasma–free plant material.

Phytoplasma detection in declining pistachio orchards in Iran

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Pistachio (*Pistacia vera* L.) is one of the important economic crops in Iran and the reach of high quality with low production cost is an important task for the Iranian pistachio industry. Orchards are mainly obtained by seedlings while also grafting is employed and mainly the Ohadi variety on Badami variety as rootstock is used. Northeastern part of Iran (Khorassan province) is one of the important regions for growing pistachio; this species is known to be tolerant to salts, however Iranian pistachio plantations are on sodic soils and irrigated with low quality, saline water resulting over the recent years in reduction of yields. Decline symptoms such as yellowing of the leaves and reduced vigour in some of the branches were observed in orchard where poor production yield is also reported. Nucleic acid samples from 10 pistachio plants grafted on the Badami rootstok and showing decline symptoms were collected in two different locations Feizabab and Bardaskan (South of Khorassan province) in Iran during spring 2009. Molecular analyses were carried out to verify phytoplasma presence and preliminary identification was achieved by PCR/RFLP analyses on R16F2/R2 amplicons obtained after nested PCR on P1/P7 amplicons. Nine out of the 10 samples were positive in nested-PCR and phytoplasma detected could be affiliated to ribosomal groups 16SrI ('*Candidatus* Phytoplasma asteris' – related), 16SrII ('*Ca.* P. aurantifoliae' - related), 16SrIX ('*Ca*. P. foenicium' - related) and 16SrXII (stolbur-related). Preliminary surveys allow to verify presence of potential phytoplasma vectors such as psyllids and leafhoppers. In particular pistachio psylla (*Agonoscena pistaciae*) and pistachio leafhopper (*Idiocerus stali* Fieb) were abundantly present in affected fields. Researches are in progress to clarify epidemiological aspect of the phytoplasma presence and spreading in these Iranian pistachio cultivations.

Could carrot leaf discolouration symptoms be associated to phytoplasma infection in Finland?

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In previous research carrot seedlings, exposed to carrot psyllid (*Trioza apicalis)* feeding in a greenhouse experiment, showed yellow, orange and purple discolouration approximately one month after the psyllids were removed (Nissinen *et al.*, Entomological Experimental Applications, 125, 277-283. 2007). Therefore, a study about whether discolouration of foliage was caused by psyllid transmitted pathogens was carried out under greenhouse conditions. Carrots were exposed to carrot psyllid feeding at 1- or 2-leaf stage for a 3 day period and at harvest, 30 samples of carrot petioles and midribs were collected for phytoplasma analysis. Other 20 samples showing carrot psyllid damage and discolouration were collected from commercial carrot fields. Total DNA was extracted from 2 g of leaf petioles and midribs of healthy and symptomatic plants using a slight modification of CTAB extraction procedure. Samples were analysed using nested PCR reaction (P1/16S-Sr and R16F2n/R2). The positive samples were confirmed with selected restriction enzymes for RFLP analysis and sequencing. No amplicons of expected size were obtained from 16S ribosomal DNA from any carrots in the greenhouse experiment. A phytoplasma fragment of the expected length (1.2 kb) of 16S rDNA was amplified in nested PCR from 2 out of 20 field samples of carrots showing leaf reddening and proliferation of hairy roots. Direct comparison to reference strains verified that the detected phytoplasmas belong to the aster yellows subgroup 16SrI-A. Further study on the reason for the phytoplasma-like symptoms in the carrot psyllid-damaged carrots under greenhouse conditions and on the vector of aster yellows phytoplasma in the field collected carrot are necessary.

Use of vmpA gene for fine typing of 16SrV group phytoplasmas

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From the sequencing of the flavescence dorée phytoplasma genome, a coding sequence sharing some similarity with a variable surface protein of animal mycoplasmas and with the Vmp1 putative membrane protein of stolbur phytoplasma was identified (Cimerman *et al.,* Appl. Envir. Microb., 75, 2951-2957. 2009). The *vmpA* gene of FD92 strain encodes a protein of 381 amino acids predicted to possess a putative signal peptide and a C-terminal transmembrane domain. It is supposed to be anchored in the phytoplasma membrane with a large N-terminal hydrophilic part exposed to the phytoplasma cell surface. Primers were designed for the amplification by nested-PCR and the sequencing of v*mpA* on a set of 37 European phytoplasma isolates representative of the 16SrV group (EY, RS, PGY, AldY and FD phytoplasmas). *VmpA* is variable in size and also in nucleotide sequence, much more than other studied markers (13% max. against 3% for *map* and 5% for *secY* genes). Virtual RFLP analyses by single digestion with *Bfm*I allowed the distinction of 9 different profiles. The topology of the phylogenetic tree realised with *vmpA* sequences is different from the trees derived from house-keeping genes. 16SrV strains from grapevine and alder (PGY, FD and AldY) are clearly divided in three clusters. Cluster I comprises AldY and PGY from Germany, France, Hungary and Serbia and no FD phytoplasmas, cluster II comprises all the FD strains from the 16SrV-D subgroup and some AldY strains from France and Serbia. Cluster III encloses all the FD strains from the 16SrV-C subgroup and some AldY strains from France and Italy. RFLP with *Bfm*I should allow distinguish epidemic from non-epidemic strains infecting grapevine.

Specific detection of '*Candidatus* Phytoplasma mali' by a new real-time PCR method based on ribosomal protein gene

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'*Candidatus* Phytoplasma mali' is the causal agent of apple proliferation, a quarantine phytoplasma disease present mainly in central and southern Europe. It belongs to the apple proliferation (AP) phytoplasma group (16SrX), together with '*Ca.* P. prunorum' and '*Ca.* P. pyri'. Conventional detection of '*Ca.* P. mali' is mainly based on nested-PCR using 16S rDNA universal or group specific primers; a multi-step procedure that increases the risk of cross-contamination. Real-time PCR represents one of the most recent innovations in the detection of phytoplasmas; previously published real-time PCR methods '*Ca.* P. mali' detection were based on 16S rRNA or nitroreductase gene sequences. In the present work a real-time PCR assay conjugated with the fluorescent SYBR® Green I dye has been developed for rapid, sensitive and quantitative detection of '*Ca.* P. mali' in its natural hosts, apple plants and the insect vectors *Cacopsylla picta* and *C. melanoneura*. Ribosomal protein (rp) gene *rplV* (*rpl22*, EF193366) was chosen as target for specific amplification of '*Ca.* P. mali'. Total DNAs were extracted from phytoplasma infected apple, apricot and pear trees, from batches of 5-6 phytoplasma infected *C. picta* and *C. melanoneura*, *C. pruni*, and from periwinkle-maintained phytoplasma reference strains AP15, AT, PD and LNp using CTAB extraction methods. In real-time PCR experiments, the selected primers rpAP15f-mod/rpAP15r3 amplified specifically a fragment 238 bp long from the *rplV* (*rpl22*) gene of '*Ca.* P. mali' and not from closely related '*Ca.* P. prunorum' infecting apricot trees and *C. pruni*, and '*Ca.* P. pyri' infecting pear trees. A unique melting peak at about 78.0°C was observed after real-time PCR with DNA from infected apples and *C. picta* and *C. melanoneura*, and reference strains AP15 and AT. Sensitivity of the method was also evaluated. The standard curve established with serial dilutions of the plasmid containing '*Ca.* P. mali' *rpl22* target gene in 20ng/µl of total DNA from healthy apple, presented a high R^2 = 99.8 and a slope value which indicated a PCR efficiency close to 100% demonstrating that the method is a useful tool for quantitative detection of '*Ca.* P. mali'. [+]Past away in 2009.

Molecular diversity of "flavescence dorée" – associated phytoplasmas in Slovenian grapevine, *Clematis vitalba* and other potential vector

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In Slovenia, a systematic official survey for the presence of grapevine phytoplasmas has been conducted since 2002. At the beginning, the detection methods were based on using PCR, nested PCR and RFLP, from 2007, a new real-time PCR detection system was developed (Hren *et al.,* Plant Pathology, 56, 785-796. 2007), which allows sensitive detection of different phytoplasmas groups and distinguishes between "bois noir" (BN) and "flavescence dorée" (FD) phytoplasmas in grapevine, insect vectors and other hosts. Together with improved extraction method with magnetic beads, the new method enables pulling of five plants in one sample.

The first detection of phytoplasma associated with "flavescence dorée" (FD) in grapevine plants in Slovenia was in 2005. Since then FD was found on different locations in Slovenia and appears to be a serious threat to the vineyards. In 2008 FD phytoplasma was also detected in *Clematis vitalba*, which has been reported as a putative host for this phytoplasma.

The aim of reported work is to compare FD isolates from *C. vitalba* with those present in grapevine in the vicinity by means of RFLP and nucleotide sequencing. The preliminary results showed the presence of different FD strains related to different potential hosts.

First report of '*Candidatus* Phytoplasma asteris' associated with several cultivars of oilseed rape in Italy

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In April and May 2009, phytoplasma symptoms were observed in several cultivars of oilseed rape (*Brassica napus* L. var. oleifera) growing in three trials located in the Experimental Farm of Padova University. The fields were established in October 2008 to test agronomic performance of different oilseed rape cultivars. Affected plants presented symptoms such as stunting, leaf reddening, green petals and extensive malformations of floral parts. When an inflorescence was affected only a small proportion of flowers set seeds at the end of the cycle. Among the 51 varieties cultivated in the fields 27 showed the described symptoms with a disease presence varying from 0.03 – 1.89% of the crop.

Symptomatic samples from 6 plots of 5 varieties were collected and phytoplasma detection and identification was obtained with PCR assay using R16F2/R16R2 universal primers specific to the phytoplasma 16S rRNA gene (Lee *et al*., Phytopathology, 85, 728-735. 1995). PCR products of expected size (~1.2 kb) were obtained from all samples with symptoms but not from the 2 symptomless samples, collected as negative controls. RFLP analyses using *Tru*I, *Bfa*I, and *Hha*I restriction enzymes allowed to verify that amplicons from all positive samples showed identical profiles, undistinguishable from those of the European aster yellows phytoplasma (16SrI-B) reference strain. The results indicate that a '*Ca.* P. asteris' related phytoplasma was associated with the disease. The same phytoplasma was already detected in winter oilseed rape in Czech Republic and, recently, in Greece (Bertaccini *et al*., Plant Pathology, 47, 317-324. 1998; Maliogka *et al*., Plant Pathology, 88, 792. 2009); therefore this new finding indicates some epidemic spreading of the disease. This is the first report of the association between '*Ca*. P. asteris' and oilseed rape in Italy and studies to verify epidemiological behaviours of the disease in insect and weeds are in progress.

Genetic variability of the coconut lethal decline phytoplasma in Tanzania

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In Tanzania, the lethal decline (LDT), lethal yellowing-type disease, is the most destructive disease of the coconut palm. It is due to one phytoplasma (Mpunami *et al.*, Plant Patholology, 48, 109-114. 1999) belonging to the 16SrIV group. Although the disease spreads the full coastal belt of Tanzania, its incidence varies between the north, where it is low, and the south of the country where the incidence is very high.

To evaluate the LDT phytoplasma diversity, the 16S rRNA gene and the 16S-23S rRNA spacer region have been amplified using the P1/P7 primer pair (Smart *et al.*, Applied Environmental Microbiology, 62, 2988-2993. 1996), and the PCR product sequenced for 15 samples of diseased coconut palms collected in the different coastal region of Tanzania.

Comparison of the sequences revealed 5 different genotypes named TZ-I to TZ-V and distributed from the north up to the south of the country. The genotype TZ-I and TZ-II are observed in the north of Tanzania only, whereas the genotype TZ-III and TZ-IV are present in the central regions and the genotype TZ-V in the south exclusively.

The distribution of the different genotypes could explain the differences in the incidence of the disease, and could be associated with the history of the coconut introduction in Tanzania also.

QBOL – Development of a new diagnostic tool using DNA barcoding to identify quarantine organisms in support of plant health

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Recently DNA barcoding has arisen as a robust and standardised approach to species identification (Hebert *et al*., PNAS 270, S96-S99. 2003)**.** QBOL, a new project that has been funded by EU FP7, wants to make DNA barcoding available for plant health diagnostics and to focus on strengthening the link between traditional and molecular taxonomy as a sustainable diagnostic resource. Each group of relevant quarantine organisms (fungi, nematodes, arthropods, bacteria, viruses, and phytoplasmas) are covered in specific workpackages in the project.

Phytoplasma 'barcoding' has been performed for many years, particularly using the 16S rDNA, but also in other genes such as secY, secA, tuf and ribosomal proteins, however most of these regions span more than 1 kb and/or primers are not generic, which make them impractical for routine barcoding.

In this project we will develop robust markers of a size that can easily be sequenced (4-600 bp) and that can be obtained from most if not all phytoplasma ribosomal groups and/or '*Candidatus* Phytoplasma' species using generic primers. The markers are not intended for phylogenetic analysis but only for diagnostic purposes. First target phytoplasmas will be those associated with diseases enclosed in European quarantine lists of pest and pathogens (EPPO and EU Council directive). In this presentation, preliminary results on the selection of suitable marker regions will be described.

A new real-time PCR detection system for AP, ESFY and PD phytoplasmas in fruit trees

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A new real-time PCR detection system was developed for apple proliferation (AP), European stone fruit yellows (ESFY) and pear decline (PD) phytoplasmas using TaqMan minor groove binder probes. All three amplicons were designed to amplify species-specific spacer region between 16S and 23S ribosomal DNA region. Efficiency of PCR amplification, limit of detection, range of linearity and dynamic range were assessed for all three amplicons. Specificity of detection systems was tested on several other isolates of phytoplasmas, bacteria that are normally present in fruit trees and on healthy field fruit trees. No cross reactivity with other phytoplasma strains, bacteria or plant DNA was detected. The assays were compared with conventional PCR on 241 field samples; 105 samples of apple trees, 44 samples of pear trees, 29 samples of plum trees, 46 samples of peach trees, 14 samples of apricot trees, 2 samples of nectarine trees and 1 sample of cherry tree. In comparison with conventional PCR, real-time PCR showed higher sensitivity as phytoplasmas were detected in several samples, which were previosly identified with conventional PCR as negative. The developed procedures for phytoplasma detection in fruit trees also included amplification of plant DNA co-extracted with phytoplasmic DNA, providing additional quality control for the DNA extraction and PCR amplification for each sample and it also included amplification of universal phytoplasma amplicon that amplify the 16S ribosomal DNA region (Hren *et al*., Plant Pathology, 56, 785-796. 2007). This amplicon served as an additional specificity control, which provides more reliable results. All real-time PCR-positive samples were positive with universal amplicon as well as with specific one. The newly developed assays are reliable, specific and sensitive methods easy applicable to high-throughput diagnosis of AP, ESFY and PD phytoplasmas.

Molecular characterization of the phytoplasmas associated with toon (*Toona ciliata*) trees and periwinkles in India

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Samples from toon trees (*Toona ciliata*) showing little leaf and witches' broom symptoms were collected from the Palampur region. Tissues of periwinkle plants exhibiting yellowing, virescence and witches' broom symptoms were collected from the Palampur and Chandigarh regions in November of 2008. DNA from symptomatic toon trees and periwinkles originating from India as well as from Egyptian periwinkle was extracted. All symptomatic toon and periwinkle samples tested gave positive results in PCR amplification of the 16S rDNA, rplB-rpsC, secA and SecY genes using primers P1/P7 (Deng and Hiruki, Proceedings of Japanese Academy, 81: 1475–1479. 1991), rpL2F (Martini *et al*., International Journal of Systematic and Evolutionary Microbiology, 57: 2037–2051. 2007) /rp(I)R1A (Lee *et al*., Phytopathology, 93: 1368-1377. 2003), AYsecYF1/AYsecYR1 (Lee *et al*., Mol. Cell. Probes, 20: 87–91. 2006) and SecAfor1/SecArev3 (Hodgetts *et al*., International Journal of Systematic and Evolutionary Microbiology, 58: 1826–1837. 2008), respectively. The four phytoplasma isolates were identified as belonging to ribosomal subgroup 16SrI-B by sequencing and subsequent phylogenetic analysis of 16S rDNA using neighbor-Joining methods and of rp, secA and SecY genes using Maximum Parsimony method. Phylogenetic tree of the 16S rDNA and *rp1*B-*rps*C sequences showed that the four isolates clustered together. However, phylogenetic analyses of a fragment of the *sec*A gene demonstrated that three of the isolates; Chandigarh, Himachal and Egyptian periwinkle phytoplasmas are most closely related and clustered together with OY-M ('*Candidatus* Phytoplasma asteris') whereas the toon witches' broom phytoplasma stood alone outside of the cluster. The SecY phylogenetic tree showed that the Chandigarh and Himachal periwinkle phytoplasmas appeared identical and more related to the Egyptian strain, while the toon witches' broom phytoplasma was more related to the Y3-chinaberry witches' broom phytoplasma.

LNA probe-based Real-Time PCR for the detection of phytoplasmas in *Solanum tuberosum*

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Phytoplasmas are unculturable, wall-less prokaryotes that cause disease in many plant species world-wide. A number of different phytoplasmas have been associated with disease in potato, including 2 quarantine diseases: potato purple top wilt and stolbur. Following classification based on RFLP analysis of the 16S rRNA gene sequence, potato-associated phytoplasma were found to belong to 16SrI, 16SrII, 16SrVI and 16SrXII groups. More recently a 16SrIII group phytoplasma has been reported in Montana (Lee *et. al,* Plant Disease, 93, 970-970. 2009), and a new species, '*Candidatus* Phytoplasma americanum' proposed (Lee *et al*., International Journal of Systematic and Evolutionary Microbiology, 56, 1593. 2006). Due to the wide diversity found in phytoplasmas affecting this host a detection method which is specific, yet sensitive and reliable is required. Phytoplasma detection using the available universal primers designed from the 16S rRNA gene, produced many false positives resulting from the presence of other bacteria naturally present in the potato samples analyzed. Once sequenced these bacteria were found to be close relatives of phytoplasmas, on the basis of their 16S rRNA gene. A similar approach based on nested-PCR improved the specificity of this diagnostic test but with inconsistent results using different primer combinations. As a consequence, an alternative approach based on the use of locked nucleic acid (LNA) probes and real-time PCR was investigated. The chemistry of LNA probes offers advantages of improved specificity and sensitivity over conventional DNA probes (Costa *et al.,* Clinical Biochemistry, 37, 930-932. 2004; Josefsen *et al*., Molecular and Cellular Probes, 23, 201-203. 2009). The detection assay developed using this approach has been trialled with 100 potato microplant samples and improvements in specificity, repeatability, and sensitivity were all evident when compared against results obtained using conventional PCR. This is the first report of use of LNA probe in Real Time PCR as diagnostic tool for phytoplasmas.

Differentiation among '*Candidatus* Phytoplasma mali' strains by multiple genes analyses

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Samples from apple plants infected by apple proliferation (AP) phytoplasmas of different varieties and from diverse areas were examined by different molecular marker to verify strains differentiation. In the 16S plus spacer region two profiles were distinguished with RFLP on F1/B6 amplicons. P-I profile was detected in reference strains, in samples from Serbia, and in the majority of samples from Trentino (Italy); the P-II profile was prevalent in samples from Veneto (Italy); both profiles were identified in samples from Hungary, in some cases both together in the same sample. The analyses of rpl22-s3 genes allow to identify in all the samples showing a P-I profile presence of phytoplasmas belonging to rpX-A subgroup, while in the samples showing a P-II profile it was possible to distinguish the other three reported rpX subgroups. In samples from Italy phytoplasmas belonging to rpX-D, rpX-B and rpX-C subgroups were identified with further local differences. RFLP analyses on AP13/AP10 amplicons differentiate among strains belonging to the rpX-A subgroup: the samples from Serbia show AP profiles, while those from Italy show AT-2 profiles. In the samples from Hungary the presence of AT1, AT2, and AP profiles was identified. The combined use of these markers allows differentiating '*Ca.* P. mali' strains according with geographical and, in some cases, also with epidemic distribution. In several orchards of Veneto vector monitoring by yellow sticky traps was carried out and *Cacopsylla melanoneura* was consistently detected, while *Fieberiella florii* was erratically found, and only one specimen of *Cacopsylla picta* was captured. Work is in progress to further verify epidemiological application of these molecular markers for AP strain characterization in insect vector and in alternative host plants.

Identification of phytoplasma of 16SrXII-A group infecting two *Echinacea* species in Serbia

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Species belonging to the genus *Echinacea* have been introduced from North America and cultivated fields of *Echinacea purpurea* and *E. angustifolia* were introduced in Serbia in last seven years. Since 2003, phytoplasma-like symptoms have been observed at plantations located in Pančevo and Indjija. The symptoms on *Echinacea purpurea* are yellowing in the early stages of disease development; reddening, plant stunting and proliferation of axillary shoots appear as the disease progresses, and infected plants showed bunchy or witches' broom appearance. Symptoms on *Echinacea angustifolia* were stunt, with shoteren internodia and purplish-reddening leaves and stalks. Flowers on such plants are smaller and do not produced seeds. Phytoplasma presence was found using electron microscopy, molecular identification of pathogen on *Echinacea angustifolia* confirmed infection by 16SrXII-A group phytoplasmas. Large numbers of *E. angustifolia* and presence of pathogen on *E. purpurea* was therefore carried out. Samples from both plant species were collected for phytoplasma tissue culture preparations and molecular identification of pathogen. Total DNA was employed for PCR assays with the universal primer pair P1/P7 for the amplification of phytoplasma 16S rRNA gene, and R16F2n/R16R2 primer pair for nested PCR. The PCR products showed expected lenghts of about 1,800 and 1,200 bp, respectively. No PCR product was obtained from healthy plants. RFLP patterns were obtained by restriction endonucleases *Tru*I, *Hha*I and *Alu*I of R16F2n/R16R2 amplified products. The results showed the presence of 16SrXII-A group (stolbur) phytoplasmas in both investigated species. This is the first report of 16SrXII-A group phytoplasma identification in *E. purpurea* in Serbia.

To the problem of early and reliable detection of European stone fruit yellows phytoplasma in peach trees

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A collection of sixteen peach trees eight years old growing in orchard where symptoms resembling those of European stone fruit yellows phytoplasma (ESFY) appeared was evaluated during 2007-2009. The presence of ESFY was evaluated by indexing on the peach indicator GF-305, by PCR detection, and by inspection of symptoms. Eleven trees showed ESFY symptoms, interveinal yellowing and partial rolling of leaves. Five trees remained without ESFY symptoms in 2009. Seven trees with ESFY symptoms died, from that four trees in 2008 and three trees in 2009. Three different procedures were used for PCR detection. First, primers ECA1/ECA2 according to Jarausch *et al.* (European Journal of Plant Pathology, 104, 17-27. 1998) were used for PCR assays. For the second procedure, primers fAT/rPRUS according to Smart *et al.* (Applied and Environmental Microbiology, 86, 2988-2993. 1996) were applied. Third procedure was nested PCR with primers R16F1/R16R0 in a first step and primers R16F2/R16R2 in a second step (Lee *et al.*, Phytopathology, 85, 728-735. 1995). All three mentioned procedures gave identical results, the same plants from tested set were positive. The presence of ESFY was proved in eight trees with symptoms in leaves. Results of PCR were negative in five symptomless trees in 2007 and 2008. Results of PCR were positive in one tree without ESFY symptoms in 2009. The presence of ESFY was proved in six symptomatic trees by indexing on the peach GF-305. The evaluation of symptoms and molecular diagnostic based on PCR are a reliable criterion for detection of ESFY in peach trees. Further research of PCR detection systems is necessary for early detection of ESFY in peach trees.

Biological complexity among populations of '*Candidatus* Phytoplasma solani'*-related strains in Italy is plausibly associated with molecular markers in genes *tuf* and *hlyC*

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The biological complexity of bois noir (BN), a grapevine disease associated with infections by '*Candidatus* Phytoplasma solani'* (stolbur), has stimulated research on molecular markers of grapevine-affecting stolbur phytoplasma genetic diversity. Three BN phytoplasma *tuf* gene lineages (*tuf*-I, *tuf*-II and *tuf*-III) were found consistently associated with different herbaceous hosts (Langer & Maixner, Vitis, 43, 191-200. 2004). In the present study, PCR-RFLP analyses of genes *tuf* and *hlyC*, amplified from more than 90 BN-infected grapevines from vineyards of north-eastern and central Italy, revealed the presence of two genetic lineages *tuf*-I/*hlyC*-I and *tuf*-II/*hlyC*-II. Sequence analyses underscored the presence of four single nucleotide polymorphisms (SNPs) in *tuf* gene and 14 SNPs in *hlyC* gene, distinguishing the BN phytoplasma lineages here identified and encoding differences at amino acid composition of Elongation Factor-Tu (EF-Tu) and Hemolysin III. Conceivably, critical amino acid substitutions encoded in diverse phytoplasma *tuf* and *hlyC* genes could alter protein interactions. Other studies have reported that EF-Tu and Hemolysin III can play, directly or indirectly, a key role in virulence processes of pathogenic bacteria in plants and animals (Goebel *et al.*, A. Van Leeuw. J. Microb., 54, 453-463. 1998; Archambaud *et al.*, Mol. Microbiol. 56, 383-396. 2005; Kunert *et al.*, J. Immunol., 179, 2979-2988. 2007). These findings supported the hypothesis that EF-Tu and Hemolysin III participate in interactions of '*Ca*. P. solani'-related strains with host plants and/or insect vectors, driving the adaptation of diverse phytoplasma genetic lineages to varied vineyard ecosystems. (*) '*Candidatus*' species proposed but not yet formally described, and reported here as incidental citations which do not constitute prior citations, according to rule 28b of the bacteriological code (Lapage *et al*., 1992).

Survey of phytoplasma diversity in heavily grapevine yellows affected areas of Croatia

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Surveys of grapevine yellows (GY) in Croatia are being conducted since 1997 with only bois noir (BN) and aster yellows phytoplasmas confirmed so far as the associated with the disease. On the bases of visual surveys conducted from 2006 to 2009, Eastern Slavonia, Međimurje and Istria were assessed to be the most heavily affected by GY. Symptomatology, geographical occurrence of the principal flavescence dorée (FD) vector *Scaphoideus titanus* and distribution of FD in the neighboring countries lead us to believe that these regions are potentially under the most infective pressure from FD phytoplasmas. In the last 4 years, molecular analyses confirmed the presence of BN phytoplasmas in 47 out of 87 tested vines, with equal distribution of infected plants among the three regions. The identity of phytoplasmas was confirmed by sensitive triplex real-time PCR procedure simultaneously detecting the presence of BN and FD phytoplasma *map* genes (Pelletier *et al.*, Vitis, 48, 87-95. 2009), as well as the routine PCR-RFLP analyses. Only 1 out of 3 *S. titanus* from Istria in 2006 was found to carry phytoplasmas but of the AY type (16SrI-B). In the following years, 33 *S. titanus* samples from all regions tested negative for phytoplasma presence. Four out of ten *Clematis vitalba* samples were found positive for the presence of 16SrV group phytoplasma both in the PCR-RFLP and real-time PCR assays. Three of the positive *C. vitalba* plants were sampled in Međimurje and the other in Istria. Since the high content of phenolic compounds in the plant tissues influence the detection sensitivity of PCR-based methods, we are currently testing the impact of the tissue type and the extraction method on the sensitivity of the real-time PCR phytoplasma detection. Aside from the classical CTAB-extraction method, various commercial kits for nucleic acid extraction are used. Preliminary results of the detection protocol comparisons will be presented as well as the new findings on GY phytoplasma diversity in grapevines, insects and weeds from 2009.

The complex "flavescence dorée" phytoplasma/*Scaphoideus titanus* Ball in the Northeast and Central regions of Portugal

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The "flavescence dorée" (FD) disease agent in grapevine is a quarantine organism included in the A2 EPPO List (Nº 2000/297CE Directive) well known in several European countries since 1950's but only detected in Portugal in 2002 (Sousa *et al*., 14th ICVG Conference, 78. 2003) after the first identification of its specific vector, the leafhopper *Scaphoideus titanus* Ball in 1998 (Quartau *et al*., Reunião grupo trabalho de Protecção Integrada da Vinha OILB/SROP. 2001). "Flavescence dorée" phytoplasmas belong to the 16SrV group (elm yellows group), subgroups 16SrV-D or 16SrV-C, and is identified mainly by molecular techniques because reliable antisera are not yet commercially available.

In 2007, in Portugal, FD was identified in grapevine tissues (leaves and bark) by nested-PCR, RFLP or sequencing of PCR product. Until now, only the subgroup 16SrV-D was found (Sousa *et al*., 7º Simpósio de Vitivinicultura do Alentejo, 86-93. 2007; Sousa *et al*., 16th ICVG Conference, 178. 2009).

An eradication program was established by the Portuguese Agricultural Plant Protection Services and since then surveys for the vector and the disease have been carried out in the grapevines of the Northeast part of Portugal, region for D.O.C. wines well know as "Vinho Verde" and "Vinho do Porto", as well as in the Central region of Portugal, where the presence of the vector was also reported. The objectives of this work were to present the life cycle of *S. titanus* and its monitoring in the Northeast Portuguese vineyards since 2001 and the molecular identification of the disease agent in the Northeast and Central regions of Portugal.

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Detection of phytoplasmas: evaluation of sampling seasons and plant material and development of new methods

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Seasonal detection of pear decline phytoplasma was studied in pear and grapevine, during all year and in different plant parts. The best season for detection ranged from October until the end of winter, and the best tissues were stems (Garcia-Chapa *et al*., Plant Pathology, 52, 513-520. 2003; Batlle *et al*., Phytopathology, 95, 153. 2005).

Different works were developed in order to improve detection methods. Primers designed against ribosomal and non-ribosomal sequences were evaluated for PCR applications (Garcia-Chapa *et al*., Journal of Microbiological Methods 56, 231-242. 2004) and also dot-blot probes have been designed (Garcia- Chapa *et al*., Acta Horticulturae, 657, 431-436. 2004). A Real Time PCR (Torres *et al*., Molecular and Cellular Probes, 19, 334-40. 2005) and a co-operational PCR coupled with dot blot hybridization (Bertolini *et al*., Plant Pathology, 56, 677-682. 2007) were developed for the detection of the AP-group phytoplasmas.

A project is being developed at IRTA with the collaboration of the Plant Health Laboratory and supported financially by the grant RTA2009-00079 (M.A.P.A., Spain). The aim of the project will be to evaluate the possibilities of the Real time PCR for the routine detection of phytoplasmas with different types of primers, universal and specifics, ribosomics as well as not ribosomic.

Other methodologies for phytoplasma detection as the use of primers designed for sequences repeated and preserved of the genome (RCS) and the evaluation of the PCR-dot blot, already proved for some phytoplasmas with specific probes will be evaluated. We are also interested in the design of specific primers for '*Candidatus* Phytoplasma pini'.

A possible threat to the timber industry: '*Candidatus* Phytoplasma pini' in Scots pine (*Pinus sylvestris* L.*)* in Lithuania

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The timber industry generates about 2% of industrial production and engages 13% of the workforce in Lithuania. Timber, including wood from pine trees, is a very important Lithuanian export commodity. In 2008, in Southern Lithuania, we noticed several diseased pine trees with unusual symptoms similar to those caused by phytoplasmas. Although phytoplasmas mainly affect angiosperms, recently they have been detected in coniferous plants in Europe (Schneider *et al*., International Journal of Systematic and Evolutionary Microbiology, 55, 303–307. 2005; Śliwa *et al*., Journal of Phytopathology, 156, 88–92. 2008). The observed pine trees (*Pinus sylvestris* L.) in Lithuania exhibited excessive branching, dwarfed needles and dry shoots. DNA from dwarfed needles was extracted using Genomic DNA Purification Kit (Fermentas, Lithuania) according to manufacturer's instructions. Nested PCR assays using extracted DNA, primer pairs P1/R16-SR (Deng & Hiruki, Journal of Microbiological Methods 14, 53–61. 1991; Lee *et al*., Int. J. Syst. Evol. Microbiol., 54, 337-347. 2004) and R16F2n/R16R2 (Lee *et al*., International Journal of Systematic Bacteriology, 48, 1153–1169. 1998), and AmpliTaq Gold polymerase (Applied Biosystems, USA) were carried out, and the amplified products were analysed as previously described (Lee *et al*., International Journal of Systematic Bacteriology, 48, 1153–1169. 1998). Products from nested PCR primed by R16F2n/R16R2 were analysed by single enzyme digestion. The restriction fragment length polymorphism (RFLP) profiles of digested DNA were similar to RFLP profiles of rDNAs of '*Candidatus* Phytoplasma pini'. The PCR product primed by R16F2n/R16R2 was cloned in *E. coli* using the TOPO-TA cloning kit (Invitrogen, USA) and sequenced. Sequence analysis confirmed that the tested symptomatic Scots pine trees are infected by '*Ca*. P. pini' in Lithuania. The phytoplasma disease of pine trees possibly can cause tangible losses in the wood industry. This is the first report of '*Ca*. P. pini' in Lithuania.

Genetic diversity of phytoplasmas identified in peach (*Prunus persica*) accessions at the Canadian Clonal Genebank

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Peach [*Prunus persica* (L.) Bastch] is mainly cultivated in Canada in Ontario province (82% of national production). A variety of symptoms typical of a phytoplasma infection were observed in some of the 83 *P. persica* accessions during June-August 2009 at the Canadian Clonal Genebank. PRU0430 ('HW274', from Canada), PRU0380 ('GF305' from France), PRU0334 ('RedSkin' from USA), PRU0155 ('Harblaze', from Canada) and PRU0375 ('Babygold #5', from USA) showed decline, leaf reddening, yellowing, shortening of internodes, witches' broom and reduced fruit size; while 'PRU0382' (peach-almond 'Kando' from the Czech Republic) and PRU0445 (peach 'HW271' from Canada) exhibited peach rosette-like symptoms. Leaf samples from symptomatic and symptomless plants were collected and total DNA was extracted. Phytoplasma universal 16S rRNA primers R16mF2/mR1 were used for direct amplification followed by R16F2n/R2 and fU5/rU3 in nested PCR assays. R16F2n/R2 nested PCR products were obtained for 'PRU0430', 'PRU0380', 'PRU0155', 'PRU0334' and 'PRU0375'; while fU5/rU3 PCR products were obtained for 'PRU0382' and 'PRU0445'. Plants lacking symptoms yielded no PCR products. Amplicons were purified, cloned, and sequenced. BLAST analysis showed that R16F2n/R2 sequences (GU223903) were 99% identical to 16S rRNA phytoplasma sequences for group 16SrVII '*Candidatus* Phytoplasma fraxini', while those from fU5/rU3 amplicons (GU223904) shared 99% identity with those of group 16SrI '*Candidatus* Phytoplasma asteris'. *Alu*I, *Rsa*I and *Mse*I RFLP analysis yielded profiles consistent with those of phytoplasma in subgroups 16SrVII-A and 16SrI-B, respectively. To our knowledge, this is the first report of a 16SrI-related phytoplasma in peach in Canada, and the first world report of a 16SrVII-related phytoplasma strain identified in peach after a case in southern Italy.

Detection and identification of '*Candidatus* Phytoplasma asteris' isolate associated with atypical symptoms on grapevine plants in Poland

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Grapevine plants exhibiting atypical disease symptoms were collected from Wielkopolska region of Poland. Symptoms included curling and dwarfing of leaves on single shoots. Infected parts of the plants were also brighter than nonsymptomatic parts of plants. To determine a disease causal agent the tested grapevine plants were checked for the presence of bacteria, fungi and viruses using universal media for axenic growth, optical microscopy, electron microscopy and biological tests. The presence of phytoplasma in tested samples was investigated using nucleic acid extraction and PCR detection. The total genomic DNA was extracted from plant material using the modified cetyltrimethylammoniumbromide (CTAB) buffer method. The direct PCR was done with universal phytoplasma primers P1/P7 for amplification of ribosomal 16S rDNA fragment. Diluted PCR product of direct PCR was used as a template for nested PCR with primers R16F2n/R16R2. The total DNA extracted from *Catharanthus sp.* infected with phytoplasma of 16SrI-C subgroup and from a healthy grapevine plant were used as positive and negative assay controls, respectively. The amplified fragments of phytoplasma rRNA gene obtained from infected grapevine plants were cloned in *Escherichia coli* cells and its nucleotide sequence was obtained using universal sequencing primers. Restriction fragment length polymorphism (RFLP) analysis of nested PCR product was also performed using enzymes *Mse*I, *Alu*I, *Hha*I, *Hpa*II, *Rsa*I and *Taq*I on R16F2n/R16R2 amplicons. Restriction fragment length polymorphism (RFLP) analysis of the PCR products showed that the RFLP profile of tested phytoplasma isolate and comparative phylogenetic analysis of the obtained 16S rRNA gene sequence of the phytoplasma isolate against other phytoplasma sequences available in the GenBank database indicated that the Polish grapevine phytoplasma isolate are closely related to the '*Candidatus* Phytoplasma asteris'.

Working Group 2: Epidemiology and vector ecology

Epidemiology will study the dispersal of phytoplasma diseases. Phytoplasmas are transmitted in a persistent manner by insects belonging to the families Cicadellidae, Cixidae, Psyllidae, Delphacidae, and Derbidae.
Micropropagation together with other agricultural practices such as grafting, cutting, stool bed and other systems to propagate plant germplasm avoiding sexual reproduction are other known ways for transmitting phytoplasma diseases, and recently the possibility of transmission through seed has also been under investigation.
The objectives of this WG are to establish a vector monitoring system throughout Europe to identify phytoplasma vector species, monitor their spread throughout the COST countries, and to coordinate research into these and other means in which phytoplasmas are spread.

Coordinators

Dr. Phyllis Weintraub - Israel

Dr. Barbara Jarausch - Germany

Tasks

1. Establish tools to identify vector species

2. Monitor the presence of phytoplasma diseases and their putative vectors in defined regions throughout Europe

3. Provide data about the infectivity of vector species towards the establishment of a risk assessment system

4. Monitor differences in vector populations to verify correlations between vector populations and efficiencies in disease spread

5. Establish the importance of different means of disease spread, such as seed transmission and transmission by root bridges

Distribution of *Scaphoideus titanus* eggs on grapevine

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In the context of the EUPHRESCO-PROPSCAPH project, a survey of *Scaphoideus titanus* egg distribution on grapevine has been under way since January 2009 through laboratory and field trials. The tested material was represented by wood samples of different varieties collected in untreated or abandoned vineyards in Latium region (central Italy) and in Veneto region (northern Italy). To set up laboratory trials the wood material was cut in pieces of different length and put into rearing cages that were kept in climatic chambers set at 24°C, 70-75% RH and 16: 8 photoperiod to obtain the first instar *S. titanus* larvae. Regarding field experiments, in May-June, in two Latium vineyards 18 plants were repeatedly sampled with devices suitable for catching *S. titanus* larvae hatching from eggs laid in the bark of different parts of the plant. Laboratory tests confirmed that *S. titanus* females prefer to oviposit in the bark of wood two or more years old; very few specimens were obtained from the one-year old wood. Moreover, tests carried out on the different portions of wood showed that the node area was the most affected by the presence of eggs. The field experiments with the capture devices showed that even the old and abundant bark of the trunk serves as a preferential site for *S. titanus* egg-laying (Bagnoli & Gargani, IOBC/wprs Meeting, WG-IPPV, Staufen im Breisgau, Germany, 45. 2009). Our results concerning the one-year old wood, agree with those of Linder & Schaub (IOBC/wprs Meeting, WG-IPPV, Staufen im Breisgau, Germany, 5. 2009) and highlight the fact that adoption of all the appropriate phytosanitary measures are important to avoid the risk of the spread of *S. titanus* through the grapevine propagation materials.

Bio-ethological observations on *Reptalus quinquecostatus* and its relationship with stolbur phytoplasma in Tuscany vineyards

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In recent years studies were carried out in a Tuscan vineyard (central Italy), in order to define the planthoppers and leafhoppers associated to the vineyard agro-ecosystem and to increase knowledge on the grapevine phytoplasma vectors. Insect samples were mostly conducted with yellow sticky traps and sweep nets on three different habitats: grapevine (Sangiovese and Syrah varieties), elm, border and inter-row weeds from May to October.

Among the over 40 Auchenorrhyncha species collected, besides *Scaphoideus titanus* Ball (Cicadellidae) and *Hyalesthes obsoletus* Signoret (Cixiidae), *Reptalus quinquecostatus* (Dufour) (Cixiidae) assumed a considerable interest. In fact, about 50% of the *R. quinquecostatus* adults, collected from the three habitats, tested positive for Stolbur phytoplasma (represented mainly by the isolate tuf type a but also by tuf type b) (Trivellone *et al.*, Redia, 88, 103-108. 2005; Bagnoli *et al.*, Petria, 18, 225-228. 2008).

In the monitored vineyard as well as in many other Tuscan vine growing areas, *R. quinquecostatus* was the more abundant cixiid. Adults, usually detected from the third week of June to the first of August, showed a fairly good mobility among the different habitats and a clear tendency to visit the vine where they were seen to insert their stylets into the leaf midribs and green shoots.

From laboratory tests performed using a membrane feeding method it was demonstrated that male and female *R. quinquecostatus*, naturally infected by stolbur, are able to inoculate the phytoplasma in the artificial medium with an efficiency of 40% despite the brief survival (Pinzauti *et al.*, Annals of Applied Biology, 153, 299-305. 2008).

These bio-ecological characteristics allow to define *R. quinquecostatus* as a competent species to transmit the stolbur phytoplasma and strengthen the hypothesis that the cixiid is a vector of BN phytoplasma to grapevine. Transmission tests on grapevine to verify this hypothesis are in progress.

Molecular identification of *Hyalesthes* and *Reptalus* species (Hemiptera: Cixiidae) allows monitoring of vector species throughout the year

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There is a growing interest in *Hyalesthes* and *Reptalus* species (Hemiptera: Cixiidae) because of their role as vectors of stolbur phytoplasmas (16SrXII-A group). *Hyalesthes obsoletus* (Signoret) is the vector of grapevine bois noir (BN), *Reptalus panzeri* (Löw) transmits stolbur to maize and it is suspected, along with *Reptalus quinquecostatus* (Dufour), to be an alternative vector of BN.

To date, cixiid vectors recognition is based on morphological characters and it is restricted to few specialist entomologists. Furthermore, the main taxonomic characters concern male genitalia, thus hampering the identification of nymphs and adult females. DNA-based approaches can offer valuable support to the traditional taxonomic methods.

PCR and PCR-RFLP assays carried out on the mitochondrial cytochrome oxidase I gene (COI) and on a ribosomal internal transcribed spacer region (ITS2) provided species-specific profiles for four common *Reptalus* species: *R. quinquecostatus*, *R. cuspidatus* (Fieber), *R. panzeri* and *R. melanochaetus* (Fieber). Similarly ITS2 amplicon length and RFLP assays performed after COI amplification allowed the unambiguous identification of three *Hyalesthes* species: *H. obsoletus*, *H. scotti* (Ferrari) and *H. luteipes* (Fieber). Our molecular identification assays have been tested on a large number of samples collected on different host-plants in several European regions.

Both COI and ITS2 sequences proved to be fast and reliable tools for the correct cixiid identification and, making feasible the species identification of females and nymphs, greatly extend the vector monitoring period.

Phytoplasma diseases in Lebanon

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During the last decade, the evaluation of the sanitary status of main agricultural crops in Lebanon, field visits and inspections was carried out in commercial fruit trees orchards in both traditional and new cropping areas of the country, in vineyards and in main solanaceous crops. Here are briefly described the main phytoplasma diseases presently known in Lebanon (Table 1).

Table 1. Primary crops and phytoplasma diseases in Lebanon

Location	Crop	Phytoplasma
Bekaa Valley	Almond, peach	*'Candidatus* Phytoplasma phoenicium'
Bekaa Valley	Pear	Pear decline
Bekaa Valley	Wine grapes	Bois noir disease
Bekaa Valley	Tomato, pepper	*'Candidatus* Phytoplasma trifolii'
Bekaa, Sghbine	Cactus	16SrII group phytoplasmas

The 16SrDNA from phytoplasmas present in symptomatic leaves and proliferating shoots of almond and peach samples was sequenced and found to be in the pigeon pea witches' broom cluster (PPWB). '*Candidatus* Phytoplasma phoenicium' was identified from a specific PCR test. Symptoms of pear decline have been observed in different commercial orchards of pear and a nested PCR assay confirmed the presence of pear decline. *Vitis vinifera* cultivars showing yellows were collected and analyzed and results infected by bois noir-associated phytoplasma. A survey for phytoplasma diseases in fields where tomato and pepper plants were symptomatic revealed that '*Candidatus* Phytoplasma trifolii' was associated with the diseased plants. Symptomatic cactus samples (*Opuntia monocantha*) were found to be infected with a 16SrII group phytoplasma. Although a preliminary inventory of insects was conducted, further studies should be done on the insect vector(s) and reservoir plant(s) in order to manage the diseases and reduce their incidence.

Diversity of Auchenorrhyncha species and potential "bois noir" vectors in Serbian vineyards

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"Bois noir" (BN) represents an important grapevine disease caused by stolbur phytoplasma belonging to 16SrXII-A ribosomal subgroup (Lee *et al*., International Journal of Systematic Bacteriology, 48, 1153-1169. 1998). It has a wide distribution in all European countries where grapevine is growing. The diversity of Auchenorrhyncha species was studied in three vineyards, in central, northern and eastern Serbia, where there was a high percentage of BN infected plants. Hemipteran vectors were collected using sweep nets and mouth-aspirators from grapevine and weeds present in vineyard inter-rows and borders. DNA was isolated from individual insects and amplified using a modification of the stolbur phytoplasma-specific nested PCR protocol (Clair *et al*., Vitis, 42, 151–157. 2003). A total of 4,971 specimens belonging to 8 families and 49 species were collected. The most numerous was Cicadellidae with 30 species, followed by Cixiidae 7 species, Delphacidae 4 species, Aphrophoridae 3 species, and Dictyopharidae, Issidae, Cercopidae and Membracidae with only one species recorded. The most abundant species from all inspected sites were *Psammotettix alienus* (29.4%), *Dictyophara europaea* (10%), *Hyalesthes obsoletus* (9.2%), *Euscelis incisus* (6.4%) and *Reptalus quinquecostatus* (5.8%), *Neoaliturus fenestratus* and *Errastunus ocellaris* (about 4.2%), while presence of *Philaenus spumarius, Laodelphax striatella, Doratura impudica* and *Zyginidia pullula* was between 2.5 and 3.3%. PCR analyses for stolbur phytoplasma presence indicated that 4 out of 49 collected species harbored the BN phytoplasma: 38% of *H. obsoletus* (91/240), 15% of *R. quinquecostatus* (44/289), 8% of *R. panzeri* (4/49) and 12% of *D. europaea* (41/341) specimens. This is the first record of stolbur phytoplasma presence in *D. europaea*, but further studies are required to determine if it is a vector.

Nymphocixia caribbea (Fennah) (Homoptera: Cixiidae) potential candidate as coconut lethal yellowing vector in the Carribean

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Phytoplasmas are associated with coconut lethal yellowing (CLY), a devastating disease of palms in the Caribbean, from Florida to Honduras (actual southwest limit) and Nevis (eastern limit). Only in Florida an insect vector, *Myndus (Haplaxius) crudus* (Homoptera: Cixiidae) was claimed to be the vector of CLY (Howard *et al*., Tropical Agriculture, 60, 168-171. 1983). However, to date, all experimental trials to transmit CLY by *M. crudus* in Jamaica and Mexico failed. In Cuba, in the province of Granma, on the narrow coast line overlooked by the hills of the Sierra Maestra, coconut plantations have been severely affected by CLY since the beginning of the 2000s. In 2005 we detected for the first time in Cuba, the cixiid *Nymphocixia caribbea* (Fennah) in an affected plantation, in the locality of Pilon. In 2005, the same species was found in Jamaica, in regions severely affected with CLY. Between 2006 and 2009 several *N. caribbea* were screened by direct PCR using Caribbean CLY non-ribosomal primers (Harrison *et al.,* Plant Pathology, 43, 998-1008. 1994), and/or direct PCR using P1/P7 and if required, nested-PCR. Cloning and sequencing showed that sequences obtained from the insects matched the sequences of some phytoplasmas isolated from LY affected palms from Cuba. The phytoplasmas detected in the insects could have originated in the alimentary bolus of insects feeding on diseased coconuts. However, the lack of success of experimental transmissions with *M. crudus* in several regions of the Caribbean outside Florida, the large quantity of *N. caribbea* found in focuses of CLY in Pilon area, the important number of insects harbouring the LY phytoplasma, are in favour of their involvement in the transmission of the disease, at least in the Granma state of Cuba. Preliminary data (not published) showed there are different sub-groups of CLY phytoplasmas in Cuba, which are different from Florida phytoplasmas. It is therefore possible to speculate that there could be different vectors, especially in such ecosysytems like the narrow cost line in Pilon region.

Transmission of aster yellows to grapevine by *Mgenia fuscovaria* (Stål) (Hemiptera, Cicadellidae)

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Aster yellows phytoplasma has recently been discovered in grapevine in the Western Cape Province of South Africa. It is considered a quarantine disease in South Africa and appears to have spread in the Vredendal and Slanghoek areas of the Western Cape. The disease has a broad host range and has been reported to infect both fruit and vegetable crops and weeds elsewhere (Hogenhout *et al.*, Molecular Plant Pathology, 9, 403-423. 2008). However, very little is known about the disease epidemiology of aster yellows in grapevine in South Africa, and the identification of insect vectors is of paramount importance if the disease is to be managed. The objective of this study was rapid identification of the insect vector(s) of aster yellows in grapevine with field collected leafhoppers. The rationale is that insects collected from highly infected vineyards are likely to be infected with phytoplasma and when transferred to phytoplasma-free plants for the transmission of the pathogen, the insect species that are vectors would infect the plants. *Vitis vinifera* L. (Vitaceae; cv. Chardonnay) plants were used as indicator plants for the transmission experiments. Plants were tested prior to trials to confirm their disease-free status. Field collected *Mgenia fuscovaria* (Stål) (Hemiptera, Cicadellidae) were transferred in groups of 20 to 10 indicator plants each, for an inoculation access period (IAP) until they died. *Mgenia fuscovaria* was used, as individuals of it tested positive for aster yellows in four independent molecular laboratories in South Africa. So far six plants were tested five weeks after transmission trials for aster yellows presence using a 3% CTAB DNA extraction method and real-time PCR protocol (Angelini *et al.*, Journal of Microbiological Methods, 68, 613-622. 2007). Two of the five plants tested positive for aster yellows, suggesting that *Mgenia fuscovaria* could be a vector of aster yellows in South Africa.

Identifying potential phytoplasma vectors in infected carrot fields in Serbia

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Aster yellows (AY) phytoplasmas were reported in carrot fields in Serbia associated with yellows symptoms (Duduk *et al*., Bulletin of Insectology, 60, 341-342. 2007) at low epidemic percentages (about 3%). To prevent dangerous spreading of the disease, identification of the insect species vectoring phytoplasmas and detection of insect-carried phytoplasmas were carried out. During 2007 and 2008 leafhoppers were trapped from the beginning of April till the end of October, in two sites of South Bačka, where phytoplasma-associated diseases were reported. Adult leafhoppers were sampled every two-week, in carrot fields and weeds nearby, by using double-sided, yellow sticky traps (25x10 cm) and by sweep netting. Leafhoppers were stored into 96% ethanol and identified before nucleic acid extraction. Phytoplasma detection was carried out by nested PCR-RFLP assays on 16S ribosomal gene. Four known vectors of AY phytoplasmas were recorded: *Anaceratagallia laevis* (Ribaut), *Macrosteles laevis* (Ribaut), *M. quadripunctulatus* (Kirschbaum) and *M. sexnotatus* (Fallen). The following six leafhopper species, still unknown as AY vectors but reported as vectors of other phytoplasmas and/or viruses according to the literature: *Anaceratagallia ribauti* (Ossiannilsson), *A. venosa* (Fourcroy), *Empoasca* spp., *Scaphoideus titanus* (Ball), *Psammotettix alienus* (Dahlbom), *P. striatus* (Linnaeus) were identified. The other five identified species, *Eupteryx mellissae* (Curtis), *Ophiola decumana* (Kontkanen), *Psammotettix confinis* (Dahlbom), *P. notatus* (Melichar) and *Errastanus ocellaris* (Fallén), are not known described as vectors. AY phytoplasmas were detected in the following vector and non-vector leafhopper species: *A. laevis, A. ribauti, M. quadripunctulatus, M. sexnotatus*, *O. decumana and P. confinis.* Transmission trials with mentioned unreported vector species are in progress to provide evidence of their transmission capabilities.

Study of stolbur phytoplasma tuber transmission in potato

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The transmission of potato stolbur (16SrXII-A) phytoplasma, through potato tubers has not been clarified so far. In the international literature there are a few and contradictory results published about it. Work was carried out to clarify stolbur phytoplasma tuber transmission, in order to determine its role in the epidemics.

Tubers of variety Lady Rosetta originating from stolbur infected potato field of Romania were planted in April 2009 and kept in an isolated plant growing room, under controlled climatic conditions. Plants were cultivated until the end of the season, in average of 4 months or until the vegetation was dry. Visual inspections for symptoms were performed regularly. Samplings for molecular tests was done four times at the following stages of plants: on tubers before planting; on plant leaves three times during the growing season. Universal phytoplasma primers, amplifying the 16Sr region, were applied in PCR (P1/P7, R16F2n/R16R2), and followed by RFLP for identification of phytoplasma.

Symptomatology: 27% of the tubers had spongy appearance and most of them showed hairy sprouting. After germination the plants grew weakly and stunted, but after one month, in most of the cases the shape and vigour of the plants returned to normal. The first phytoplasma symptoms appeared at the middle of the season. Two plants showed symptoms such as purple top, yellowing and leaf rolling. Molecular tests: all 118 tubers were tested for phytoplasma infection immediately before planting; 73% of tubers proved to be positive for stolbur. During the season, 3 samples were taken for molecular tests, but the total number of plants decreased due to wilting. In 1 of the 58 plants remained alive stolbur phytoplasma was identified at the 2nd and 3rd sampling dates.

Based on this result the tuber transmission of stolbur can be assumed in case of Lady Rosetta variety however in a very low percentage. Further experiments have to be performed to confirm these data, to identify the pathogen(s) causing the wilting as well as to study their possible influence on the percentage of phytoplasma transmission.

Transmission dynamics of European stone fruit yellows on thirteen *Prunus* species in controlled conditions

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The aim of this study was to improve knowledge about the susceptibility and sensitivity of several *Prunus* species to European stone fruit yellows (ESFY) infection by way of the vector *Cacopsylla pruni* under semi-controlled conditions. At the same time, data on the host-feeding preference of *C. pruni* and ESFY spread were recorded.

In March–April 2007 and 2008, naturally infected reimmigrant *C. pruni* were collected in productive orchards of the Friuli Venezia Giulia region (northern Italy) and released in a large screenhouse containing a total of 130 test plants belonging to 13 different *Prunus* species. The presence of *C. pruni* on the test plants was recorded one, two and three weeks after release, and the new generation of insects was also monitored for its presence and distribution on the different species. Individual *C. pruni* were also tested for '*Candidatus* Phytoplasma prunorum' infection by nested-PCR analyses. All test plants were visually inspected for symptoms and analysed for '*Ca.* P. prunorum' infection in September 2007, 2008 and 2009.

The highest ESFY incidence rate was recorded on *P. salicina*; the species that were most susceptible to ESFY in the trial conditions were *P. salicina*, *P. armeniaca*, *P. persica* and *P. tomentosa*. These species were characterized by a high sensitivity to ESFY, showing the typical symptoms of the disease; on the other hand the high tolerance of *P. domestica*, *P. cerasifera,* and *P. spinosa* to ESFY was confirmed, as previously reported from field observations.

The *C. pruni* reimmigrant adults showed a clear preference for *P. salicina*, and in decreasing order of importance *P. domestica*, *P. armeniaca*, *P. persica* and other *Prunus* species.

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Detection and characterisation of grapevine phytoplasmas and vectors by molecular techniques in Turkey and the reactions of common cultivars to those infections

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The northeastern part of Anatolia peninsula, located between Black sea and Caspian sea region, is the gene source and culture area of the most important varieties of grapevine, *Vitis vinifera* L.. Grape is widely produced in Turkey and suffers yield reduction because of diseases and pests. Flavescence dorée (FD) and bois noir (BN) phytoplasmas cause serious loses in vineyards in Europe. FD phytoplasma is a member of 16SrV phytoplasma group and transmitted by *Scaphoideus titanus* (Ball) with strains in 16SrV-C and 16SrV-D subgroup. Bois noir belong to group 16SrXII and it is transmitted by *Hyalestes obselatus* (Signoret). There is no report on phytoplasma infections in vineyards in Turkey, so a project was started to survey wine and table grape production areas in Marmara, Aegean, Central Anotolia and eastern Anatolia region of Turkey with periodical surveys to collect symptomatic leaves, plants and vector samples. The initial surveys were conducted in mainly grape cultivated areas in July and September 2009 and a total of 167 samples were collected. Main symptoms were chlorosis of veins, or dark colorisation and redness of the leaves. These symptoms were prevalent on wine cultivars as compared to table grape varieties. DNA isolation was made according to Leford *et al*. (Silvae Genet., 47, 5-6. 1998) and Ahrens and Seemüller (Phytopathology, 82, 828-832. 1992) and all of the DNA was subjected to PCR by P1/P7 universal primers. They all were subjected to nested-PCR by group specific primers for the presence of FD and BN phytoplasmas.

In total 1,306 insects were collected during the surveys; belonging to 16 species in 7 families of Hemiptera. *Arboridia* sp. was prevalent in the survey area, as were *Dictyophara europaea and Laodelphax striatellus* which were all reported as potential vectors of grapevine phytoplasma in different countries of the world.

Imaginal phenology of *Cacopsylla picta* and *C. melanoneura* in Belgian apple orchards and hawthorn hedges

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In order to verify the presence and to determine the relative abundance of '*Candidatus* phytoplasma mali' psyllid vectors in Belgian apple orchards, weekly beatings of branches were carried out from 2007 to 2009, throughout the year, both in commercial apple orchards and in their immediate environment (where hawthorn hedges were present), and also in an untreated repository apple orchard. During this inventory, numerous specimens of psyllids were caught, counted and identified to species level. The two species *Cacopsylla picta* and *C. melanoneura*, known as apple proliferation (AP) vectors and already detected in Belgian orchards in previous inventories (Haleng, dissertation, 1991; Baugnée *et al.*, Bulletin I.R.S.N.B., Biologie, 72-Suppl., 125-127. 2002), were recorded.

The periodic sampling of adult insects through the year in the orchards and hedges showed 1) the absence of these two species during all the winter, 2) the beginning of the imaginal activity just at the end of this season, namely in February and March, and 3) the occurrence of the population peaks between April and June. For the two species, the phenology was variable from one year to another, with a possible shift of several weeks between the peaks, but *C. melanoneura* was always the earliest species.

The two species where represented in nearly all kinds of apple orchards but always with a low number of individuals. *C. melanoneura* was yet very abundant only in the hawthorn hedge adjacent to the orchard, while *C. picta* was always rare in the orchard as well in the hedges.

Scaphoideus titanus egg hatching rates from grapevine propagation material

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Commercial grapevine propagation material (approximately 5000 scions, rootstocks and grafted plants) from 9 nurseries in Italy, France, Slovenia and Switzerland were analysed for the presence of *Scaphoideus titanus* eggs. Wood was placed in a growth chamber at 25°C and 70% humidity for 4 months and emerging nymphs were counted. Positive controls were scions and rootstocks from *S. titanus* infested vineyards.

No *S. titanus* individuals were born from any woody material coming from the nurseries, irrespective of the country of origin or the kind of material. Therefore, in spite of limited sampling, the results of this trial showed that commercial grapevine propagation material used nowadays in trade exchanges in Europe is probably reliable as far as the absence of *S. titanus* eggs.

In contrast, several hundred *S. titanus* individuals were born from 2 year old canes collected from an infested vineyard, as expected. Surprisingly, several individuals were also born from 1 year old rootstock canes collected from an abandoned wild rootstock plot. This finding demonstrated that 1 year old wood from rootstocks can be suitable for the oviposition of *S. titanus* females, although the egg hatching rates were approximately 20-30 times lower than those from 2 year old wood. These results are in agreement with preliminary data recently obtained from other Italian and Switzerland research groups (Bagnoli *et al.,* Abstract Book, IOBC/WPRS – OILB/SROP Meeting, 45. 2009; Linder *et al.,* Abstract Book, IOBC/WPRS – OILB/SROP Meeting, 5. 2009).

Transmission of '*Candidatus* Phytoplasma prunorum' by formation of root bridges in Turkish apricot cultivars

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Research has been carried out under greenhouse conditions to determine whether '*Candidatus* Phytoplasma prunorum' could be transmitted by formation of root bridges. Two Turkish local apricot cultivars (cv. Sakıt and cv. Hacıhaliloğlu) grafted on wild apricot seedlings were used for this experiment. Two plants from same cultivars were planted together in a 30 liter plastic containers and one of them was inoculated with '*Ca*. P. prunorum' by two chip budding in March, 2009. The study was replicated 15 times for each cultivar. Three months after the inoculation, the all inoculated plants were tested by PCR using P1/P7 and F01/R01 primer pairs. If one of the inoculated plants was found infected with the pathogen, its pair was also tested by PCR. Leaves, shoots and roots of inoculated plants and their pairs were used for total nucleic acid extractions. The first infections were recorded in July, one plant for cv Sakıt, two plants for Hacıhaliloğlu, but their pairs were negative. Infection rate in inoculated plants increased in August and September for cv. Sakıt and cv. Hacıhaliloğlu, respectively. The first infections of pairs were detected for cv. Sakıt in root samples in August. Although no infection was detected in leaves and shoots of inoculated plants and in their pairs in September, the pathogen was present in root samples of both cultivars. In November, infection rate of inoculated plants was 14.28% and 7.14% for shoot and root samples of cv. Sakıt whereas it was 87.5% and 25.0% for shoot and root samples of cv. Hacıhaliloğlu, respectively. In the same month un-inoculated pairs of cv Sakıt were 85.71% for shoot and 7.14% for root samples whereas no phytoplasma was detected for shoots of cv. Hacıhaliloğlu but infection rate of roots was 18.75%. This research is in progress and the data will be collected at least for two years.

Microsatellite markers for the study of host races and dispersal biology of the "bois noir" vector *Hyalesthes obsoletus*

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Epidemiological cycles of vector-transmitted diseases are highly influenced by the dispersal biology and host specificity of both vector and pathogen. The polyphagous planthopper *Hyalesthes obsoletus* is the main vector of stolbur 16SrXII-A phytoplasma to grapevine. The epidemiology of the associated yellows disease "bois noir" is primarily determined by vector populations infesting field bindweed (*Convolvulus arvensis*) and stinging nettle (*Urtica dioica*). The existence of phenological differences between the two vector populations and the presence of plant-specific stolbur strains indicate separate host races of both pathogen and vector, hence two independent epidemiological disease cycles. However, the ability to test for genetic host-races of the vector populations, as well as their dispersal biology, has been hampered by extremely low genetic polymorphism of the markers so far analysed (mtDNA, RAPD-DNA, allozymes). The lack of polymorphism is most likely due to genetic drift resulting from recent range expansion to large parts of the current European distribution range. To obtain information about the dispersal biology and to gain insights into the evolution of plant-specific host races of *H. obsoletus*, we developed microsatellite genetic markers for *H. obsoletus*. We have successfully tested eight polymorphic loci in 95 specimens caught on both host plants over two flight periods (2005 and 2006) at a syntopic site in Germany. Gene diversity and alleles per locus and population varied between 0.50-0.90 and 5-15, respectively. Genetic differentiation, F_{ST}, between *H. obsoletus* populations sampled in 2005 and 2006 on bindweed (F_{ST} = 0.004) and on stinging nettle (F_{ST} = 0.030) was lower than between host plant related populations (F_{ST} = 0.066-0.108). The results indicate host-plant associated genetic differentiation at this site. The applicability of the microsatellite markers is being tested on further European populations.

Development of specific primers for the molecular identification of *Cacopsylla picta*, the main vector of apple proliferation disease

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Cacopsylla picta has been shown to be the main vector of apple proliferation (AP) disease. However, identification of this psyllid by morphological means is difficult for people lacking experienced. On the other hand, molecular screening for phytoplasma infection in insects has become increasingly importance to identify the vectors of AP and to analyse the disease spread in different apple growing regions. Therefore, molecular markers for the identification of *C. picta* were developed. At the beginning of this study, DNA nucleotide sequence data of psyllids were only available for one genetic locus, the wingless (*wg*) gene. Based on these sequence data primers were selected which amplified a wide range of *Cacopsylla* species. So far, sequence data of a fragment of the *wg* gene were produced for 23 different psyllid species, including the known phytoplasma vector species: *C. picta*, *C. melanoneura*, *C. pruni*, *C. pyri* and *C. pyricola*. The sequence comparison enabled the development of specific primers for *C. picta*. The specificity of the primers was tested for a range of more than 40 psyllid species, predominantly those which are known to occur in apple and stone fruit orchards in central Europe. The universality of the primers was tested for *C. picta* samples originating from 33 different locations in Germany, France, Italy, Czech Republic and Switzerland. Furthermore, the available sequence data were used to establish a first phylogenetic tree of psyllid species based on the *wg* locus.

Hyalesthes obsoletus, vector of "bois noir": distribution and host plant preferences in Switzerland

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In Europe, the polyphagous planthopper *Hyalesthes obsoletus* Signoret (Hemiptera, Cixiidae) is assumed to be the most important vector of the grapevine yellows disease "bois noir" that is caused by phytoplasmas of the stolbur, 16SrXII-A group. For a better understanding of the epidemiology of bois noir in Switzerland, distribution and host plant preferences of *H. obsoletus* were studied in the field as well as in the laboratory. A national survey revealed that *H. obsoletus* is present in vineyards of southern, western and northern Switzerland; however, no specimens were caught in the east of Switzerland, where evidence for the disease is lacking. Even though field bindweed (*Convolvulus arvensis* L.) is much more abundant in Swiss vineyards than stinging nettle (*Urtica dioica* L.), *H. obsoletus* was captured almost exclusively on the latter. Molecular analyses revealed that specimens captured were infected with stolbur strains tuf-type a and tuf-type b, associated with *U. dioica* and *Convolvulus arvensis* or *Calystegia sepium* L., respectively. Two-choice experiments in the laboratory showed that *H. obsoletus* adults preferred to feed and to oviposite on stinging nettle compared to field bindweed. Similar two-choice experiments also indicated that nymphs do not inherit a host plant preference even though they developed significantly better on stinging nettle compared to field bindweed. Likewise, adults survived significantly longer on stinging nettle compared to bindweed or any other plant species tested. In conclusion, there is good evidence that *H. obsoletus* is the most important insect vector of bois noir in Switzerland and that the insect prefers to feed, to oviposite and to develop on *U. dioica*. Stinging nettle therefore plays a central role in the epidemiology of "bois noir" in Swiss vineyards.

Studies on phytoplasmas in Norway – apple proliferation and poinsettia-branch inducing phytoplasma

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Apple proliferation phytoplasma in Norway

Suspicious symptoms were reported at first in 1996. A few trees of apple 'Summerred' in Gvarv, Telmark County, had symptoms looking like apple proliferation. This case initiated a survey. The first samples were analyzed in England. Later we did the PCR-diagnosis in our own lab.

Apple proliferation phytoplasma was detected in the surveys carried out in 1996-1998 in 14 orchards: 1 experimental orchard in Telemark County, 4 orchards in Vestfold County, 4 orchards in Hordaland County and 5 orchards in the County Sogn og Fjordane.

Apple proliferation disease was later also found at other locations. From this we can conclude that this quarantine organism is present in almost all important fruit districts. It is important to follow the situation to stop the further spread of this disease. All infected trees have been eradicated.

Branch-inducing phytoplasma in poinsettia

Free-branching poinsettias (*Euphorbia pulcherrima*) were first introduced during the sixties through the Norwegian 'Annette Hegg' cultivars. The identity of the "branching agent" was proven to be a phytoplasma, termed poinsettia branch-inducing phytoplasma (PoiBI) (Lee *et al.*, International Journal of Systematic Bacteriology, 48, 1153-1169. 1998). Without phytoplasma, poinsettias grow tall, and produce very few branches.

We have studied the relative amount and distribution of PoiBI in poinsettia and how this relates to branching in different cultivars grown under different light levels and temperatures using a quantitative PCR assay (TaqMan). Results from this work will be presented.

Psyllid vectors of phytoplasmas in pome and stone fruit trees in Austria

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European stone fruit yellows and pear decline are quarantine diseases caused by phytoplasmas ('*Candidatus* Phytoplasma prunorum' and '*Candidatus* Phytoplasma pyri' respectively), both already distributed in Austrian orchards widely. For both quarantine diseases spreading is due to infected planting material or insect vectors, especially psyllids (Psyllidae, Homoptera).

Considering that these phytoplasma diseases were already reported in Austria a survey about the occurrence of psyllids as potential vectors was carried out. Samples were collected by using the beat tray method in the orchards, on apricot trees in Lower Austria and Burgenland in 2005 and 2006 as well as on pear trees in Lower Austria in 2009. The collected psyllids were analysed by PCR and RFLP assays for the presence of phytoplasmas.

Cacopsylla pruni was captured in all surveyed apricot orchards. This is the first report of *C. pruni* in apricot trees in Austria. Interesting was the frequent occurrence of *Cacopsylla melanoneura* in apricot trees in the investigation sites. *Cacopsylla pyricola*, *Cacopsylla pyri*, *Cacopsylla pyrisuga* and *Cacopsylla melanoneura* were the main species on pear trees. PCR and RFLP analyses showed few infections of *C. pruni* with '*Ca.* P. prunorum' and few individuals of *C. pyricola*, *C. pyri* and *C. pyrisuga* were carrier of '*Ca.* P. pyri'.

Occurrence of '*Candidatus* Phytoplasma pyri' in pear growing areas of Srpska - Bosnia and Herzegovina

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Pear (*Pyrus communis* L.) is one of the most important fruit crops in the Republic of Srpska (Bosnia and Herzegovina, B&H) basically used for local consumption or fruit industry. The presence of '*Candidatus* Phytoplasma pyri' as well of the vector *Cacopsylla pyri* was detected in several pear growing regions in B&H (Gradiska, Banjaluka, Maglaj, Sarajevo) (Duduk *et al*., Journal of Plant Pathology, 87, 75. 2005; Delić *et al*., OEPP/EPPO Bulletin, 37, 444–448. 2007). Symptoms of poor shoot and spur growth, dieback of shoots, upper rolling and reddening of leaves, reduced leaf and fruit size, and premature leaf drop were observed in the last 3 years. All these symptoms frequently appeared in pear orchards. During July 2008 leaf samples were collected from the symptomatic trees. Total DNA was extracted from midribs tissue according Angelini *et al.* (Vitis*,* 40, 79-86. 2001). Nested PCR assays were carried out with universal and specific in phytoplasma primers: P1/P7 (direct) (Deng & Hiruki, Journal of Microbiological Methods, 14, 53-61. 1991; Schneider *et al*., Molecular and Diagnostic Procedures in Mycoplasmology, 369-380. 1995); R16F2/R16R2 (nested) (Gundersen & Lee, Phytopathologia mediterranea, 35, 144-150. 1996); f01/r01 (nested) (Lorenz *et al*., Phytopathology, 85, 771-776. 1995). All positive f01/r01 PCR products were then submitted to the RFLP using the restriction enzymes *Ssp*I end *Bsa*AI. '*Ca.* P pyri' was identified in six tested samples. With reference to these results as well as results of the surveys conducted during the period of 2004-2007, we can conclude that '*Ca.* P. pyri' is widely distributed and threatening pear production in the region. *Cacopsylla pyri* is a well known pest of the pear in the region with a worsening situation. Therefore, further studies will be the design of appropriate control strategies as well as controlling the vector.

Determination of the parameters for a day-degree method to predict the flight of host populations of *Hyalesthes obsoletus*

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Hyalesthes obsoletus the vector of bois noir (BN) is a univoltine planthopper. Like many Cixiidae species the immature stages of this insect live in the soil where they feed on the roots of herbaceous plant hosts. Nymphs hibernate in deeper soil levels. When they move back to the surface in spring they come under the influence of air temperature. Since growth and development of insects are significantly influenced by temperature, the time of emergence of adult vectors depends on accumulated heat units. A preliminary phenology model for *H. obsoletus* populations on *Convolvulus arvensis* (bindweed) based on degree days has been developed by Maixner & Langer (IOBC/wprs Bullettin, 29, 161-166. 2006). Since the flight of adult *H. obsoletus* from populations on *Urtica dioica* (stinging nettle) is delayed compared to bindweed populations, the model required an adaptation for nettle populations. An inverse modelling approach was used to determine the most appropriate parameters (starting date, threshold temperature, required degree-days) for a degree-day calculation of the start of flight activity of both populations, based on weather data and field observations of adult flight from different years and locations. The estimated parameter sets allow already a rather exact prediction of the flight activity of adult *H. obsoletus*, although additional field data will help to improve the accuracy. The precise information on the flight activity is useful for both, an optimal monitoring of the presence, infestation and activity of this vector, and the proper timing of weed control activities in order to avoid an increased flight of infective vectors from their natural host plants to grapevine or other susceptible crops.

Entomofauna of Hemiptera Auchenorrhyncha in chayote (*Sechium edule*) fields with chayote witches' broom (ChWB) disease

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Chayote (*Sechium edule*) is a commercially important vegetable crop which is affected by chayote witches' broom (ChWB) disease, associated with 16SrIII-J phytoplasma reported also in *Momordica charantia* (Cucurbitaceae) growing as weeds in fields of chayote in Brazil (Montano *et al*., Plant Disease, 84, 429-436. 2000). *M. charantia* is likely the main reservoir of chayote witches' broom phytoplasma, and it is important to investigate the presence of putative insect vectors. At the location of Mendanha (State of Rio de Janeiro), a survey was conducted to examine Auchenorrhyncha fauna, in chayote fields with ChWB. Individuals observed were collect from Malaise and yellow adhesive traps. Sweeping net method was also utilized to collect leafhoppers. Specimens examined could be identified to the family/subfamily levels, distributed among Achilidae, Agallinae, Cicadellidae, Cicadellinae, Cixiidae, Delphacidae, Delthocephalinae, Gyponinae, Membracidae, Nogodinidae and Thyphlocibinae. Species identified were *Acrogonia* sp., *Balclutha hebe, Bucephalogonia xanthopis*, *Copididonus hyalipennis*, *Curtara concava, Curtara curtara, Fonseicaiulus* sp., *Hortensia similis*, *Ileopeltans aberrans, Macugonalia cavifrons*, *Oncometopia facialis, Oragua triplehorni*, *Plesiommata comiculata, Scaphytopius (convelinos) marginelineatos, Scopogonalia altinani*, *Tettisama quinquemaculata, Xerophloea* sp. and *Xerophloea veridis.* In fields next to chayote plantings, 16SrIII-J phytoplasma was found associated with diseased pumpkin (*Cucurbita moschata*) plants, and the disease was named pumpkin yellows (Montano *et. al.,* J. Pl. Pathol., 88, 226. 2006). This finding suggests the involvement of insect vectors in the dissemination of 16SrIII-J phytoplasma among species of the family Cucurbitaceae. It is paramount to search for potential insect vectors and to gain understanding of the spread of ChWBIII phytoplasma to chayote and other plant species.

Epidemiological investigation on bois noir disease in Central and Southern Italy

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Bois noir (BN) is one of the main grapevine yellows diseases. It is wide-spread in several grapevine growing areas and it is induced by stolbur phytoplasma, belonging to 16SrXII-A subgroup. In order to improve on the knowledge of the disease, one nursery and several infected vineyards were surveyed in the central and southern Italy regions as models for epidemiological investigations. The infection rate and the distribution of grapevine symptomatic plants were evaluated. Moreover monitoring and sampling of Auchenorrhyncha fauna and wild plant species were performed for several years and the stolbur isolates from the different hosts were molecularly characterized (Langer & Maixner, Vitis, 43, 191-200. 2004). In the selected vineyards results showed that several insect and weed species were infected by the same Stolbur type identified in symptomatic grapevines, suggesting their possible involvement in the disease epidemiology. *Reptalus quinquecostatus* (Dufour), *R. panzeri* (Low), *Exitianus capicola* (Stål), *Toya propinqua* (Fieber), *Hyalesthes luteipes* Fieber, *Thamnotettix zelleri* (Kirschbaum) and *Anoplotettix putoni* Ribaut (Pasquini *et al.,* Bull. Insectol., 60, 355-356. 2007) could be considered possible Stolbur vectors together with *Hyalesthes obsoletus* Signoret. *Urtica dioica* L. and *Convolvulus arvensis* L. are certainly involved in stolbur cycle (Maixner, 15th ICGV, 103-104. 2006), but also *Cirsium arvense* L. Scopoli in southern regions and some annual species as *Solanum nigrum* L. and *Amaranthus* spp. could be involved as source of inoculum (Pasquini *et al*., Petria, 18, 218-221. 2008). In the investigated nursery a high population density of *H. obsoletus* was found on nettle plants growing along side the border and 12% of the collected specimens resulted in the presence of stolbur-infection. Although no symptomatic grapevine plantlets were observed, the presence of infected insect vectors could play an important role in spreading the disease.

Phytoplasma research in The Netherlands – Preparation for upcoming diseases

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As result of a marine climate with cold winters, phytoplasma infections in The Netherlands did not previously result in high economic losses. Phytoplasma infections were generally limited to small scale 16SrX phytoplasma infections in pear and apple and small-scale 16SrI infections in bulbous crops like hyacinth and gladiolus. However, most likely due to global warming, recent Dutch winters were milder and average summer temperatures have increased. Interestingly in the last few years phytoplasma infections in hyacinth and gladiolus have occurred more frequently, and small-scale 16SrI phytoplasma infections were also detected in several ornamental crops. It is likely that the increase in vector survival rates during Dutch winters will result in increased vector population during the Dutch summer. As a consequence, economic losses may increase if the Dutch agricultural sector is not prepared, informed or educated about phytoplasma infections.

The Netherlands participates in the EU-COST initiative to access reliable diagnostics tools and to obtain knowledge in order to formulate efficient crop protection measures. In addition, our research institute runs several epidemiological studies on phytoplasma-vector-crop interactions and operates Cicadellidae monitoring for 16SrI phytoplasmas. The main objectives of these applied agronomical studies are firstly to introduce tools in The Netherlands for phytoplasma risk assessment and secondly to provide measures to protect crops of high economic value. Results of these studies will be shared within the EU-COST initiative and will contribute to a description of phytoplasma epidemiology, vector ecology and phytoplasma control in Northern European crop systems.

Search for possible vectors of “bois noir” in Austrian vineyards

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During the past years, inspections revealed a significant increase of “bois noir” (BN) disease in Austrian vineyards. Known natural BN vectors belong to the Cixiidae family (Hemiptera, Auchenorrhyncha). In some parts of Austria, however, Cixiidae species have not or are rarely found. The aim of our work was to study other Auchenorrhyncha species for their ability to transmit stolbur phytoplasma.

The Auchenorrhyncha fauna was analyzed in two severely infected vineyards in Lower Austria between 2006 and 2009. Surveys were carried out by yellow sticky traps and by vacuum sampling. Yellow sticky traps were mounted at three different levels (10-20 cm above herb layer, 120-150 cm above ground level in the canopy and 320-400 cm above ground level).

Transmission trials with several Auchenorrhyncha species (field trapped and laboratory reared insects) were carried out. *Vicia faba*, *Convolvulus arvensis* and *Vitis vinifera* were selected as host plants. Infected *C. arvensis* plants were used as phytoplasma source.

Altogether 155 Auchenorrhyncha species were recorded. Among them were 20 Fulgoromorpha from the families Cixiidae (5 species), Delphacidae (11 species), Dictyopharidae (1 species), Issidae (1 species) and Tettigometridae (2 species) and 136 Cicadomorpha from the families Aphrophoridae (4 species), Cercopidae (1 species), Membracidae (2 species) and Cicadellidae (subfamily Agallinae 4 species, Aphrodinae 2 species, Cicadellinae 3 species, Dorycephalinae 1 species, Iassinae 1 species, Idiocerinae 4 species, Macropsinae 3 species, Megophthalminae 1 species, Penthimiinae 1 species, Ulopinae 1 species, Typhlocybinae 51 species, Deltocephalinae 54 species).

We observed a transmission of stolbur phytoplasma to *Vicia faba* by *Anacertagallia ribauti*, but up to now we have not succeeded to find a species transmitting BN of grapevine.

Vectors identification of phytoplasmas belonging to apple proliferation and stolbur groups in Spain

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Vectors and host plants of '*Candidatus* Phytoplasma pyri' and '*Ca.* P. prunorum' were studied in Spain. For '*Ca.* P. pyri' its vector, *Cacopsylla pyri* was identified, the vector population dynamics was determined as well as the percentage of infective individuals, and the transmission efficiency throughout the year (Garcia-Chapa *et al*., European Journal of Plant Pathology, 152, 432-437. 2005). In the case of '*Ca*. P. prunorum' its vector *C. pruni* was identified (Sabaté *et al.* Bulletin of Insectology, 60, 193-194. 2007). The cycle of *C. pruni* was studied during four years in wild *Prunus* (*P. mahaleb* and *P. spinosa*) and in commercial orchards of *P. salicina.* The populations reached two maximums, at the end of March (re-immigrant with the higher percentage of phytoplasma carriers) and in June, with inter-annual fluctuations *(*Sabaté *et al.,* XXI ICVF, 47. 2009*).*

The stolbur phytoplasma was identified in Spain in several woody and vegetable crops. In grapevine areas a positive correlation between the disease incidence and the importance of *Hyalesthes obsoletus* populations was found, although these are always low (Sabaté *et al*., Bulletin of Insectology, 60, 367-368. 2007). The percentage of stolbur-bearing *H. obsoletus* individuals ranged from 20 to 100%. The study of stolbur isolates revealed the presence in Spain of two Tuf and three stol 1-H10 strains (Batlle *et al*., XVI ICGV, 190-192. 2009). In other areas with stolbur affected crops, *H. obsolethus* was not identified and other leaf and planthoppers were identified as potential vectors. Transmission was obtained to different plant species with *Macrosteles quadripunctulatus* (Batlle *et al*., Annals of Applied Biology, 152, 235-242. 2008). Transmission assays to insect feeding medium and to *in vitro* plants showed transmission with several leafhoppers (Laviña *et al*., XV ICGV, 218-220. 2006).

The objectives for the next years are to continue the studies on transmission of different stolbur isolates by *H. obsoletus* and by other plant and leafhopper vectors. Epidemiological studies on '*Ca*. P. mali' with identification of the vectors and host plants will be started.

European stone fruit yellows: identifying factors affecting the dissemination of the disease

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The psyllid *Cacopsylla pruni* is the vector of '*Candidatus* Phytoplasma prunorum', the causal agent of European stone fruit yellows (ESFY). Using an approach combining population genetics and molecular epidemiology, we searched to determine the possible distances involved in ESFY dissemination by its vector. We first aimed at clarifying the taxonomic status of *C. pruni.* A preliminary population genetics study on this psyllid in Europe (mainly in France) has shown the existence of two strongly differentiated genetic groups (A and B), co-occurring in several zones of sympatry (Sauvion *et al.*, Bulletin of Insectology, 60, 185-186. 2007). To better understand the origin and the genetic structure of both groups, we have initiated phylogenetic analyses based on mitochondrial and nuclear markers. Sequences within the internal transcribed spacer (ITS) indicate an ancient divergence of the 2 groups, and suggest that they are in fact two distinct species. Based on this deep divergence, we have developed diagnostic markers to assign thousands of individuals to either species by PCR. Based on the abundance of each species at sampling points 20-100 km apart, we established distribution maps for the two species across France. Using microsatellite markers (Sauvion *et al.*, Molecular and Ecological Research, 9, 1196-1199. 2009), we are also studying the genetic structure of *C. pruni* populations within each group in order to infer dispersion patterns. The first results show that geographic barriers, distance and host plants may play an essential role. Concurrently, we are investigating what the genetic structure of '*Ca*. P. prunorum' tells us about pathogen flows between wild and cultivated plants. A first screening of thousands of insects shows that ESFY prevalence in *C. pruni* is rather even throughout France. Trees from orchards and wild host plants are being screened for ESFY with the available molecular markers. Comparing the frequency of ESFY genotypes in orchards, wild plants, and psyllids will help to assess the role of each ecological compartment in ESFY epidemiology.

Role of wild plants in epidemiology of fruit tree phytoplasmas and in ecology of the insect vectors. The case of hawthorn plants

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Fruit tree phytoplasmas are transmitted mainly by psyllids, most of which spend part of their life cycle on wild host plants. For example, *Cacopsylla melanoneura* the vector of apple proliferation (AP), requires hawthorn plants (*Crataegus monogyna*) as reproduction and oviposition hosts, an alternative to apple (Lauterer, Acta Museica Morava, 84, 71-151. 1999).

The role of hawthorn and its psyllid population was investigated to better understand its role in the epidemiology of AP; eventually, it proved to be important for other fruit tree phytoplasmas as well. *C. melanoneura* was the dominant psyllid species, followed by *C. peregrina*, *C. affinis* and *C. crataegi*. PCR and RFLP analyses revealed the presence of '*Ca.* P. mali' and '*Ca.* P. pyri' in hawthorn plants. The same analyses detected '*Ca.* P. mali' in specimens of *C. melanoneura* and *C. peregrina*, '*Ca.* P. prunorum' in *C. peregrina* and *C. affinis* and "*Ca.* P. pyri' in *C. melanoneura*, *C. peregrina*, *C. affinis*, *C. crataegi*. For more reliable data, a molecular tool was developed to discriminate between *C. affinis* and *C. melanoneura*. Hawthorn can now be considered as a new source of inoculum for '*Ca*. P. mali' and '*Ca.* P. pyri'. Until now the transmission ability of hawthorn psyllids has been proven only with *C. melanoneura* and '*Ca.* P. mali' (Tedeschi & Alma, Journal of Economical Entomology, 97, 8-13. 2004), but further studies are now required. First, transmission trials are required to prove the ability of the four psyllid species to transmit the three phytoplasmas to healthy plants. Moreover the ability of *C. peregrina*, *C. affinis* and *C. crataegi* to feed on plants different from *Cr. monogyna*, even occasionally, should also be investigated, to define the possible risk of phytoplasmas spreading.

Disseminating information on leafhopper, planthopper and psyllid vectors of phytoplasma disease

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Phytoplasma disease vectors are found within the leafhoppers and planthoppers (Hemiptera: Auchenorrhyncha) and among the psyllids (jumping plant lice) (Hemiptera: Psylloidea). Around 100 vectors of phytoplasma are already known on a world basis but many more are likely to be recognized. Few comprehensive identification keys are available and details of pest species are mostly widely scattered in the specialist literature. They are not easily accessible with the exception of the account by Nielson (U.S.D.A. Technical Bulletin, 1382. 1968) “Leafhopper vectors of phytopathogenic viruses”. This volume remains a specialist reference work – there are no whole insect figures or photographs, only some morphological drawings to assist in identification. However, in the past 40 years there have been many taxonomic changes in the status of the insect names. Also Nielson’s volume only covered leafhoppers (Cicadellidae) and no work has been produced for the (less numerous) planthopper vectors. As well as changes in insect taxonomy, perhaps a more confusing problem in using Nielson’s 1968 work is that phytoplasmas were then thought to be viruses, but his work does not differentiate between the two. Weintraub & Beanland summarized information on phytoplasma vectors (Annual Revue of Entomology, 51, 91-111. 2006). The identification of known insects needs to be facilitated as well as a means to be able to assist in characterising potential vector species. A challenge in Europe and elsewhere is to detect new vector species and also monitor movements due to climate change. Our approach (funded by The Leverhulme Trust) will provide a comprehensive and accessible guide to the leafhopper, planthopper and psyllid vectors of phytoplasma, bacteria and virus diseases. Datasheets to known vectors will include high quality digital images of adult insects (and nymphs when available), taxonomic drawings of morphological features, and text on the identification, biology, pest status and distribution of each species. Introductory keys will assist in identification of different vector groups.

Working Group 3: Phytoplasma control in crop systems

Control of epidemic outbreak can be carried out either by controlling the vector or by eliminating the pathogen from the infected plants by tetracycline, or by other chemicals. However, these protection measures have proved to be quite ineffective under field conditions therefore the only effective way to control phytoplasma infection has been to prevent the outbreaks.
Identification of alternative control strategies against these diseases, such as the possibility to use biocontrol organisms or phytoplasma mild strains could also provide innovative and promising tools for limiting phytoplasma spread in an environmentally sustainable approach.
This WG will coordinate the results from epidemiological and molecular studies to formulate new and improved strategies for the control and management of phytoplasma diseases.

Coordinators

Dr. Wolfgang Jarausch - Germany

Dr. Ester Torres - Spain

Tasks

1. Identification and breeding of crop plant varieties that are resistant (or less susceptible) to the phytoplasma

2. Effects of biotic and abiotic factors on disease and symptom development

3. Vector control with low-impact insecticides and treatment schedules and development of environmentally sustainable vector control strategies

4. Devising the best practices in phytoplasma disease control

5. Interaction of endophytes with host plant and phytoplasma

6 Effect of mild phytoplasma strains for protection from infection by virulent strains

Survey of almond witches'-broom phytoplasma ('*Candidatus* Phytoplasma phoenicium') and of leafhopper species in infected orchards

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Almond witches' broom phytoplasma ('*Candidatus* Phytoplasma phoenicium') was reported as a very devastating disease that has killed over a hundred thousand almond (*Prunus dulcis*) trees in Lebanon within a few years. This phytoplasma belongs to the pigeon pea witches' broom group (16SrIX). Since it was first reported in 2001, no action was taken to stop or delay its spread. A preliminary survey, in 2008/2009, showed that the disease is spreading rapidly in North Lebanon. Several *foci* of infection on almond, peach and nectarine were also detected in several regions in South Lebanon and West Bekaa plain. DNA sequencing of samples collected from all infected regions in Lebanon showed over 99% sequence identity suggesting a same origin. A survey of leafhoppers present in two infected almond orchards, in South and North Lebanon, revealed that the most abundant species was *Asymetrasca decedens*. Potential phytoplasma vectors in members of the subfamilies Aphrodinae, Deltocephalinae and Megophthalminae were present in very low numbers. PCR analysis showed that at least seven species carried phytoplasmas. A survey is planned to cover all stone fruit production regions in Lebanon and more detailed studies are planned to identify the almond witches' broom (AlmWB) vector(s). The heavy losses incurred in Lebanon coupled with the reported spread of AlmWB in Iran call for a rapid action to consider this disease as a quarantine pest.

Field and molecular studies on grapevine tolerance to phytoplasma infection

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The pathology group of the Centre for Research in Viticulture is focused on the study of all grapevine pathogens, including phytoplasmas. Since the first discovery of grapevine yellows in Italy *(*'flavescence dorée': FD, and 'bois noir': BN), field observation on the susceptibility of different varieties of *Vitis vinifera*, hybrids and rootstocks to phytoplasma has been carried out in the ampelographic collection and clonal comparison vineyards of the Centre. It was observed that a few *V. vinifera* varieties, although growing in heavily FD-infected sites, were almost never infected with phytoplasmas, suggesting the possibility that they possess an unknown tolerance mechanism. Moreover, different susceptibilities in diverse clones of the same variety were observed. The occurrence of diseased plants varied between 0 and 40% (Borgo *et al*., Proceedings XXIX Congrés Mondial de l'OIV., CD 1-11. 2006). Different susceptibilities were observed also in the rootstock varieties which are usually thought to be resistant to phytoplasmas; indeed, in some rootstock varieties several plants showed symptoms and were infected by FD phytoplasma as revealed by molecular analysis (Borgo *et al.,* 16th ICVG Meeting, Dijion-France, 162-163. 2009).

A molecular approach to the analysis of tolerance and susceptibility was started. As epigenetic regulation plays a potential role in abiotic and biotic stress adaptation by plants, the first step is to study one class of the most important epigenetic regulator: the microRNAs (miRNAs). The miRNAs are small conserved RNA molecules which play an important role in plant post-transcriptional gene regulation by means of modulating the expression of transcriptional factors. They were demonstrated to be involved also in the plant response to pathogen invasion (Zhang *et al*., Dev. Biol., 289, 3-16. 2006). The study of the quantitative and qualitative miRNA expression in healthy and infected plants of varieties displaying susceptibility or tolerance to phytoplasma infection is in progress. Indeed, the differences in grapevine miRNA expression are potentially related to the differences in susceptibility to phytoplasmas.

Optimal control strategies of phytoplasma vectors: an overview of efficacy results of low-impact insecticides and alternative products against pear psyllids

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Pear decline (PD) is an important disease of *Pyrus communis* fruiting cultivars in Europe, caused by the phloem-limited phytoplasma *'Candidatus* Phytoplasma pyri'. Pear psyllids (*Cacopsylla* sp.) are considered to be mainly responsible for the transmission and spread of this disease in pear orchards. Although it is impossible to eliminate all vectors from the environment, well-managed vector control strategies significantly reduce the chance of an epidemic outbreak. Efficient control relies on a perfect tuning of treatment schedules, the efficacies of (preferably) low-impact insecticides, the side-(repellent)-effects of alternative products (e.g. kaolin, mineral oils and fungicides), the optimal positioning of these crop protection agents, and the best possible presence of beneficial predators. The department of Zoology of the pcfruit vzw research institute (Belgium) has a long tradition of executing insecticide field trails according to EPPO guidelines. We present an overview of the results of currently recognized products (diflubenzuron, abamectine, bifenthrin, thiacloprid, spinosad, deltamethrin, spirodiclofen, kaolin, mineral oils), tested in efficacy trials against pear psylla (winterform/summerform, different life stages) during the last decade. The application of any product – alternative or insecticide - has of course an effect on the development of beneficial organisms throughout the season, and hence, known data concerning effects on natural enemies of pear suckers will be included.

Epidemiological relevance of "flavescence dorée" phytoplasma strains

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"Flavescence dorée" (FD) is a quarantine disease associated with phytoplasmas showing great ability to differentiate new strains in short periods of time; two strains were distinguished more than ten years ago on 16S ribosomal gene (Bertaccini *et al*., 12th ICVG Meeting, Lisbon, 28 Sept./2 Oct., 57-58. 1997), and more strains were identified later on other genes. The identification of FD strains is of major relevance towards a correct disease management in the different affected areas, considering that some strains were only sporadically detected without epidemic diffusion. FD-C and FD-D strains from major FD-affected viticultural areas in Serbia, Italy and France were identified by RFLP analyses on rpS3 (ribosomal protein) and SecY (translocase) genes. Strains belonging to the FD-C ribosomal group showed the presence of diverse polymorphisms after RFLP analyses with *Tru*I and *Taq*I on the SecY gene and the resulting strains were partially related to their geographic distribution; however the analyses on the rpS3 gene did not allow the amplification of all examined samples and did not show polymorphisms. RFLP analyses on the SecY gene from 16SrV-D strains collected in Veneto region showed identical profiles with reference strain FD-88 from France; these profiles were clearly differentiable from the one identified in the majority of samples from Emilia (Lambrusco varieties) indicating for the first time a strain differentiation in FD-D phytoplasmas according to both geographic distribution and variety. The FD-D strains from France showed profiles identical to each other on SecY and on rpS3 genes. Their collective profiles on the SecY gene were however distinguishable from that of the reference strain FD-88 from France and from those of both strains from Veneto and from Emilia regions in Italy. These data confirm that FD-D type phytoplasmas are starting to differentiate strains mainly according to the geographic/varietal distribution of the disease.

Establishment of an *in vitro* system to study the interaction between '*Candidatus* Phytoplasma mali' and susceptible and resistant *Malus* genotypes

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Previous work showed that all cultivated apple varieties and rootstocks are susceptible to apple proliferation disease whereas the wild species *M. sieboldii* showed high level of resistance (Seemüller *et al.,* Acta Horticult., 309, 245-251. 1992; Bisognin *et al*., Phytopath., 98, 153-158. 2008). Field trials demonstrated that the use of *M. sieboldii* as rootstock can prevent the disease, reduce its impact and is sufficient to confer resistance to the whole tree as overwintering of '*Ca.* P. mali' occurs only in the roots. In order to obtain AP resistant rootstocks of agronomic value a breeding program was started by crossing *M. sieboldii* and some of its selections with standard dwarfing stocks (Bisognin *et al*., Plant Breeding, 128, 507-513. 2009). In the present study an *in vitro* system for testing AP resistance has been developed as alternative to time-consuming and labour-intensive screening in the field. Hybrids obtained by different cross combinations were inoculated *in vitro* by grafting with '*Ca.* P. mali' - strain PM6 maintained in micropropagated apple. Specific symptoms of the disease, height, basal proliferation and phytoplasma concentration were recorded for each plant shoot and used to define an *in vitro* disease index which finally ranged from 0 (resistant) to 10 (highly susceptible). Seven hybrid genotypes out of the seventy-two tested showed a low *in vitro* disease index and were selected for further agronomic evaluation. When tested with a different strain (PM11) five of these hybrids showed again a low disease index while the other two genotypes were more affected by the disease. Furthermore, micropropagation offers the possibility of producing homogenous plant material *ex vitro* for each promising single progeny genotype and resistance could also be confirmed in these *ex vitro* plants. Thus, *in vitro* and *ex vitro* results are in agreement with *in vivo* observations. They support application of the *in vitro* system for simultaneous evaluation of resistance to different strains and further investigation of the plant-pathogen interaction.

Detection of phytoplasmas and bacterial endophytes in the plant model *Catharanthus roseus* by fluorescence *in situ* hybridization

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Endophytic bacteria can be termed all the bacteria colonizing the interior of plants without inducing diseases, including those that become pathogenic under certain conditions. Though their relationship with the host is not well understood, they may reduce the disease severity by activating systemic resistance, antibiosis, competition of nutrients and niches (Lugtenberg *et al*., Annual Revue of Microbiology, 63, 541-556. 2009). For these abilities bacterial endophytes are candidates for biological control of plant diseases. A bacterium to be suitable for biocontrol, should not only synthesize secondary metabolites, but would also compete successfully with other organisms and maintains its interaction with the host. A possible way to monitor its presence in host tissues and its interaction with host is the use of Fluorescence in situ Hybridisation (FISH).

This study aimed to experiment FISH in localizing phytoplasmas and bacterial endophytes in the plant model *Catharanthus roseus* L.. Shoots of periwinkle plants, inoculated by grafting with genetically different phytoplasma strains, were cut in 50-70 µm slides with a vibratome. Slides were permeated as described by Webb *et al*. (Phytopathology, 89, 894-901. 1999), then hybridized with 16SrV phytoplasmal probe labelled with FAM and bacterial probes labelled with fluorophores emitting in the far-red (i.e. CY5). Some slides were stained with DAPI as control. The detection of targeted bacterium was performed with a confocal microscope. Phytoplasma probe, labelled with FAM, was able to detect and discriminate genetically different phytoplasmas, in stem phloem tissues. Endophytic bacteria were instead detected in the phloem, xylem and leaf parenchyma. These results, though preliminary, show the great potentiality of FISH in analyzing the interaction between phytoplasmas and endophytic bacteria, opening new perspectives in the study of microbial antagonism to control phytoplasma diseases.

Characterization of endophytic bacterial community associated with healthy and grapevine yellows-diseased *Vitis vinifera* L. plants

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Endophytic bacterial community associated with grapevine leaf tissues was characterized by cultivation-independent 16S rRNA gene library analysis and cultivation methods. In order to identify endophytes directly from metagenome, a protocol for bacteria enrichment and DNA extraction was optimized. Library analysis of 16S rDNA identified five diverse Operational Taxonomic Units (OTUs), showing best sequence matches with γ-Proteobacteria, family *Enterobacteriaceae*, with a dominance of the genus *Pantoea*. More than 85% of the cloned sequences yielded best matches with the species *Pantoea agglomerans.* Bacteria isolation through cultivation revealed the presence of six OTUs, showing best sequence matches with *Curtobacterium*, *Bacillus* and *Enterococcus*. Specific electrophoretic peaks, associated with bacterial species identified in this study, were inserted in a reference LH-PCR database. The creation of this database was the first essential step for an extended large survey on bacterial diversity in vineyards in Italy. Preliminary data registered several additional peaks in healthy and phytoplasma-infected grapevine plants from Lombardy vineyards. Although bacteria identified in the present study probably do not represent the whole microbial diversity in grapevine plants, the cultivation-independent approach could be used in order to compare endophytic bacterial communities in different ecological niches.

Biochemical and epigenetic changes in phytoplasma-recovered periwinkle after indole-3-butyric acid treatment

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Phytoplasmas cause deregulation of developmentally important genes in infected hosts and disturb the normal transport through the phloem causing numerous physiological and biochemical changes including changes in plant growth regulators balance. This fact was the basis for the idea to try to eliminate phytoplasmas by treating infected shoots with auxins. *In vitro* grown *Catharanthus roseus* shoots infected with different '*Candidatus* Phytoplasma' strains were treated with two auxins. Tested plant growth regulators, indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), induced remission of symptoms (recovery) in all phytoplasma-infected shoots (Perica *et al*., FEMS Microbiology Letters, 268, 171-177. 2007; Perica, J. Applied Microbiology, 105, 1826-1834. 2008; Ćurković-Perica, Chemical Croatica Acta, 81, 641-646. 2008). The time period and concentration of the auxin needed to induce recovery was dependent on the phytoplasma strain and the type of auxin. IBA-treatment eliminated '*Ca.* P. asteris' (strain HYDB) from the shoots, while two other strains, '*Ca.* P. ulmi' (strain EY-C) and stolbur (strain SA-I) persisted in the host tissue despite the recovery of infected shoots. To elucidate the possible mechanism of host recovery and '*Ca.* P. asteris' elimination from *C. roseus* shoots H_2O_2 and related enzymes, endogenous auxin levels and general methylation levels were measured and compared for infected, non-infected and recovered periwinkles. Differences in methylation of the plant host genome after the treatment with IBA revealed that epigenetic changes might be responsible for the elimination of '*Ca.* P. asteris' from the infected periwinkle. A similar experiment is in progress on *Vitis vinifera*.

Resistance elicitors to control phytoplasma diseases

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Systemic acquired resistance (SAR) is an inducible resistance mechanism in plants that provides resistance against a broad spectrum of plant pathogens. Arbuscular mycorrhizal fungi form mutualistic associations with most plant species, increase plant growth by improving mineral nutrition, and enhance plant tolerance towards biotic and abiotic stresses. Bacteria living on the roots may promote plant growth by a number of mechanisms including hormone synthesis, phosphate solubilization, nitrogen fixation and root architecture modifications. Among the synthetic inducers of plant disease resistance, acibenzolar-S-methyl (BTH) is known to induce SAR in many monocots, solanaceous and leguminous plants and fruit trees. The role of different SAR inducers as possible tools for the integrated pest management of phytoplasma diseases has been studied on '*Candidatus* Phytoplasma asteris' infecting chrysanthemum plants (chrysanthemum yellows, CY). SAR was induced before leafhopper transmission of the disease by application of *Pseudomonas fluorescens* strain S1Pf1 or *Glomus mosseae* BEG12 under the appropriate conditions, or by foliar spraying of different BTH concentrations. The application of any of these elicitors always resulted in a delay of symptom expression, although only little protection from the disease was achieved. Rhizosphere bacteria and arbuscular fungi induced enhanced fitness in treated plants. This was evident also after CY infection, since *P. fluorescens* and *G. mosseae* were able to rescue some of the damages associated with CY. Application of BTH had no effect on plant fitness, but resulted in a decrease of phytoplasma concentration in the treated plants. None of the microorganisms showed any significant effect on phytoplasma multiplication or viability. The eliciting effect induced by BTH was temporary, while the long-lasting persistence of microorganisms in the plants was not affected by the presence of the phytoplasma. Our results should now be tentatively transferred onto more important crop systems before designing new integrated management strategies to develop more environmentally-friendly phytoplasma diseases control methods.

Asaia, the acetic acid bacterial symbiont of *Scaphoideus titanus*, is a potential symbiotic control agent against "flavescence dorée"

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Symbiotic microorganisms of microbial pathogen vectors may be used in symbiotic control (SC) approaches with the aim of interfering with the disease transmission or to impair the biological cycle of the insect. Secondary symbionts, facultative and recently acquired by the hosts, have ecological traits for the efficacy of SC like the ability to grow outside the insect or to colonize different hosts. The acetic acid bacterium *Asaia*, previously found in the malaria mosquito vector *Anopheles stephensi* Liston, is also an important secondary symbiont of the leafhopper *Scaphoideus titanus* Ball, the vector of the "flavescence dorée" phytoplasma (Crotti *et al.*, Environmental Microbiology, 11, 3252-3264. 2009). To explore the association between *Asaia* and the insect, different analyses were conducted, e.g. the evaluation of symbiont abundance and its localization within the host's body. By using specific primers for *Asaia* it has been estimated that its 16S rRNA gene copies accounts in average for 4.9% of the 16S rRNA gene copies of total bacteria in the insect. By means of specific in situ hybridization, the acetic acid bacterium was found in *S. titanus* malpighian tubes, spermatic boundless, testes of males and all around ovarian eggs of females. Colonizing experiments with a GFP-labelled *Asaia* strain isolated from *A. stephensi* showed that, after acquisition with the diet, *Asaia* reaches the gut of *S. titanus* and is able to colonize different body parts. The efficiency of *Asaia* in colonizing its hosts, together with the ability to be widespread and abundant in insect populations, and the easy cultivability and transformability, makes it an interesting candidate for SC of "flavescence dorée".

Use of infochemicals for trapping phytoplasma vectoring psyllids

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We work on the chemical ecology of jumping plant lice (Hemiptera: Psyllidae) and their interactions with vectored phytoplasmas and their different host plants. We investigate the olfactory reactions of these insects to host plants used for reproduction or overwintering, and elucidate chemically mediated interactions between all players in such multitrophic systems. By identification of chemical compounds responsible for the migration of these insects between their different host plants, we make a valuable source accessible for the development of innovative strategies using attractive and repellent infochemicals for control of these insect vectors.

Here an example for attempt to develop a biotechnical control method for psyllid species vectoring fruit tree phytoplasmas by sticky traps lured with newly detected infochemicals will be presented. The apple psyllid *Cacopsylla picta* is the main vector of '*Candidatus* Phytoplasma mali', the causal agent of the apple proliferation disease. Complex interactions between *Malus domestica,* the psyllid *C. picta*, and the phytoplasma were investigated in the laboratory and in the field. Results from Y-tube shaped olfactometer trials showed that immature adults of *C. picta* are able to distinguish the odours of healthy and infected apple trees and preferred the odours of infected trees. Thus, the phytoplasma directly manipulates both the plant physiology by producing an attractive compound and the psyllid behaviour, resulting in a better spread within its host plant population. The compound responsible for the attraction of the vector was collected from headspace of infected apple plants and identified by gaschromatography coupled with mass spectrometry. This sesquiterpen attracts both genders of *C. picta* and is now used for the development of traps for monitoring or mass trapping of this vector.

Monitoring of cross protection activity of a '*Candidatus* Phytoplasma mali' strain in periwinkle

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Cross protection is a phenomenon whereby prior infection with a mild or avirulent strain of a pathogen prevents or interferes with superinfection by another, usually related pathogen. Here we studied for the system apple proliferation the cross protection effect of the mild '*Ca*. P. mali' strain 1/93 on infection by the virulent '*Ca.* P. mali' strain AT and the virulent but only distantly related American aster yellows phytoplasma (AAY). Our aim was to clarify, how the avirulent strain inhibits development and virulence of the highly virulent strains in periwinkle. In the experiments conducted the plants were either pre-inoculated with the mild strain and after 2 months graft-inoculated with the severe strain or, both types of strains were grafted simultaneously. The infection was monitored by examining a *hfl*B gene fragment using polymerase chain reaction (PCR), single strand conformation polymorphism (SSCP) analysis and real-time (RT-PCR). In the RT-PCR experiments the two '*Ca*. P. mali' strains were distinguished by specific TaqMan probes. In the pre-inoculation trials the mild strain quickly colonized the entire plants whereas the severe strain was not detectable anymore after about six months post inoculation, even in the inoculation scions. When both strains were inoculated simultaneously the mild became by far predominant. However, the severe strain was still present in the inoculation scions one year post inoculation. The AP strain 1/93 had no inhibitory effect on symptom development of the non-related AAY strain. These results show that cross protection in periwinkle plants is only successful against the related severe AT strain. These results might help understanding the basic phenomenon of phytoplasma cross protection.

Susceptibility of new and old plum varieties to '*Candidatus* Phytoplasma prunorum'

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The production losses associated with European stone fruit yellows (ESFY, 16SrX-B phytoplasmas) in Italian plum orchards reach up to 40% in Japanese plum. During six years a trial was carried out in Vignola area (Northern Italy) to assess the susceptibility of several plum varieties to the infection by '*Ca.* P. prunorum'. In the surrounding of the experimental orchard ESFY presence was identified in declining cherry; *Cacopsylla pruni* was negative to phytoplasma presence, while *Fieberiella florii* specimens resulted infected by 16SrX-B phytoplasmas (Landi *et al*., Bulletin of Insectology, 60, 163-164. 2007), providing evidence for the pathogen presence in the environment. Varieties, cultivars and new selections of European and Japanese plum employed were grafted on Myrabolan 29C, and derived from commercial nurseries and from breeding programs. Plants were evaluated in 2-4 plots of four plants each. Yearly monitoring by visual inspection and PCR/RFLP identification of phytoplasmas allowed verifying the phytoplasma presence in the orchard since the first year of plantation. After a scattered phytoplasma presence detected in the year of plantation mainly in asymptomatic plants, an increasing ESFY presence in both symptomatic and asymptomatic plums was observed in subsequent years. After six years monitoring, eight out of the 30 Japanese plums selections showed ESFY symptoms or pathogen presence in 50% of the plants and nine selections showed 20% of infection. Only nine cultivars and selections - Bragialla, Brarossa, Fortune, Ruby Crunch, n. 89.030.020, n. 89.030.031, n. 89.036.131, n. IFF/260, and n. IFF271- showed absence of both symptoms and pathogen. Although the majority of the 35 European types of plum was not symptomatic, some of the genotypes - Rheingold, Valcean, Valerie, n. 3018 – showed one to three symptomatic plants each, and one asymptomatic selection, n. 1474, was tested phytoplasma-positive in one plant.

Control strategies of phytoplasma diseases affecting fruit trees and grapevine in Spain

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Susceptibility or resistance of different cultivars and rootstocks of *Pyrus* and *Prunus* to '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', respectively, was studied. For this, symptom expression and phytoplasma concentration, as determined by quantitative real-time PCR, were evaluated. Higher phytoplasma concentrations were determined in more susceptible pear cultivars. In inoculated *Prunus* rootstocks, a higher concentration was determined in crossings *P. persicae* x *P. amygdalus* whereas in *P. persicae* x *P. davidiana* a lower concentration was found .(Torres *et al.*, XXI International Conference on Virus and other Graft Transmissible Diseases of Fruit Crops, 81. 2009).

The influence of mycorrhiza on symptom development was evaluated in rootstock *P. communis* OHF 333. The results showed that pear decline (PD)-inoculated plants were more affected by the disease than mycorrhizal PD-infected plants (Garcia-Chapa *et al.,* Acta Horticulturae, 657, 437-441. 2004). These studies will be continued. Recovery of trees with pear decline was observed in plots with an effective psyllids control.

Containment measures such as elimination of affected grapevines and insecticide treatments against *Scaphoideus titanus* were applied to control flavescence dorée (FD) in Catalonia. The aerial treatments with pyrethroid insecticides plus two more treatments done by the farmers were effective in limiting the FD spread (Torres *et al.*, XVI International Council for the Study of Virus and Virus-like Diseases of the Grapevine, 176-177. 2009). The remission of bois noir symptoms was also observed in grapevines despite the increase of the disease incidence (Batlle *et al.,* XII International Council for the Study of Virus and Virus-like Diseases of the Grapevine, 69-70. 1997).

Effectiveness of hot water treatments against the eggs of *Scaphoideus titanus*

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The project PROPSCAPH united four European research institutes in order to improve risk management schemes against the propagation of the vector of flavescence dorée. Amongst others, the impact of hot water treatments on the development of eggs of *Scaphoideus titanus* was studied. In autumn 2008, one-year-old and two-years-old cuttings were collected in *S. titanus*-infested vineyards in France, Italy and Switzerland and sent to our institute. After storage in a cooling chamber until March 2009, half of the cuttings were exposed for 45 minutes to a hot water treatment at 50°C. Thereafter, treated and untreated cuttings were placed in cages and the hatching of *S. titanus* nymphs was assessed. About 100-times more nymphs hatched from two-years-old cuttings than from the one-year-old ones. Moreover, hot water treatments significantly reduced the number of hatched nymphs, e.g. the exposure to hot water killed about 80% of deposited eggs. In conclusion, hot water treatments do not only have a major impact on flavescence dorée phytoplasma, they also kill most of the eggs of its vector. Thus, the hot water treatment of stocks and scions before grafting as well as of grafts before commercialisation is strongly recommended in order to reduce the risk of the propagation of *S. titanus*.

Field trials to study the efficiency of weed control in reducing the density of adult *Hyalesthes obsoletus*

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The principal vector of bois noir of grapevine (BN) and other diseases caused by stolbur phytoplasmas is *Hyalesthes obsoletus*, a polyphagous planthopper living on wild herbaceous plants. Two major hosts of both, the vector and the pathogen are *Convolvulus arvensis* (bindweed) and *Urtica dioica* (nettle). *H. obsoletus* is not affected by insecticide sprays on vines, because it lacks a close association with grapevine but occurs on its wild plant hosts inside and outside of vineyards as well. Control strategies should focus on larval instars that are rather immobile and constrained to their host plants. While bindweed grows in a dispersed pattern throughout the vineyards and in abandoned fields, nettle is more common along the vineyard borders. Within the plots it grows along terraces or in small stands. An obstacle to all field trials with *H. obsoletus* is its extremely uneven distribution. This study aimed at the assessment of methods for estimating the numbers of *H. obsoletus* and at the evaluation of the efficiency of herbicide treatments against host plants as a measure to decrease *H. obsoletus* density by depriving the immature vectors of their food source.

Individual stands of nettle were treated selectively with herbicides within an experimental plot, while at the vineyard's border herbicides and an insecticide were applied with two replications each on continuous plots. All treatments were carried out in April approximately two month before the emergence of adults. Inside the vineyard the planthoppers were caught by sticky traps exposed directly above the host plants, while emergence traps were used at the border. Although the numbers of caught vectors varied strongly between traps, a significant decrease of emerging vectors could be achieved with all methods. The data show that weed control is an efficient measure to significantly decrease *H. obsoletus* population density and thereby to reduce the infection pressure on grapevine.

Defense response induced by fungal endophytes in phytoplasma-infected plants

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Spontaneous 'recovery' from phytoplasma disease symptoms has been reported in grapevine and fruit crops. Cytological and molecular modifications, related to resistance, have been observed in 'recovered' plant tissues (Musetti *et al*., Functional Plant Biology, 34, 750-758. 2007; Musetti *et al*., Phytopathology, in press), however the physiological bases of 'recovery' remain not completely understood. It has been hypothesized that endophytic microorganisms may take part in this resistance phenomenon, and fungal endophyte strains were isolated from grapevines and apple plants grown in areas where 'recovery' phenomenon was recurrent. Some of these endophytes, such as *Epicoccum nigrum* Link and *Aureobasidium pullulans* (de Bary) Arnaud, are very interesting because reported as biocontrol agents or resistance inducers. A study on phenological and ultrastructural interactions between phytoplasmas and an endophytic strain of *E. nigrum* in the experimental host *Catharanthus roseus* has been recently reported (Musetti *et al*., Petria, 19, 43-46. 2009). Moreover an efficient DNA extraction method and a sensitive PCR protocol were developed for the detection of endophytes in the host plants (Martini *et al*., Plant Disease, 93, 993-998. 2009). Investigations on plant/endophyte/phytoplasma relationships at molecular level will be performed. We intend to optimize procedures and protocols for isolation and characterization of nucleic acids from the phloem, both from experimental (*C. roseus*) and natural hosts (grapevine, apple). In particular, we will set up methods for expression analyses of defense-related genes by quantitative-real time PCR. Expression of SAR genes as well as of SA-independent transcriptional and phloem structural modification markers will be investigated. In the same time, biochemical experiments on the role of secondary metabolites, produced by endophytic *E. nigrum* and *A. pullulans* against phytoplasmas, will be carried out.

'Recovery' from apple proliferation disease: an integrated approach

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Recovery is the spontaneous remission, sometimes permanent, from disease symptoms. Phytoplasmas surviving in the roots are not able to recolonise the plant crown. The causes that induce recovery remain still unknown and its physiological bases are poorly understood. In this research the modifications in the phloem tissue related to recovery-induced resistance in apple have been investigated through ultrastructural, chemical, cytochemical and gene expression analyses of leaf tissues from recovered, healthy and apple proliferation-diseased plants. Ultrastructural observations detected abnormal callose and P-protein accumulations in the phloem of recovered apple plants. Callose synthesis and P-protein plugging, which are Ca^{2+}-dependent, would form physical barriers preventing the *in planta* movement. The cytochemical localization by potassium pyroantimonate detected the presence of Ca^{2+} ions in the phloem in all the three groups of plants; however the Ca^{2+} concentration was remarkably higher in the cytosol of the recovered apple plants. This observation would support the hypothesis that resistance mechanisms would be related to an increased Ca^{2+}-dependent signaling activities. Apple genes coding for callose synthases and phloem proteins were identified by an *in silico* approach. The expression patterns of five genes encoding callose synthases (MDCALS1/5) and of four genes encoding phloem proteins (MDPP2-1/3 and MDERG1) were analysed by quantitative real time RT-PCR. Four of the nine analysed genes were up-regulated in recovered plants in comparison to healthy and diseased ones, supporting the hypothesis that recovered apple plants were able to develop resistance mechanisms dependent from Ca^{2+} signal activities.

‘Recovery’ and phytoplasma presence in Chardonnay affected by bois noir disease

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Over a number of years, studies have been carried out in a Chardonnay vineyard in north Sardinia (Italy) in which 893 grapevine plants, affected by “bois noir” (BN) disease, were surveyed. The aim of these studies was to evaluate recovery occurrence and its significance in terms of etiological latency of BN phytoplasma. Monitoring conducted from 2004 to 2009 revealed infection rates between 4% (2008 and 2009) and 17% (2004) and variable annual levels of symptom remission. Plants that had been recovered for one to five years were recorded in proportions varying between 73% (recovery for one year in 2008) and 16% (recovery for four years in 2009). Plants recovered for five years reached a total of 32% in 2009. In the same year, molecular analyses were carried out on 13 plants which showed symptoms for at least 4 out of 6 years and on 25 plants which recovered for at least 1 year. Samples, taken in August 2009 were subjected to nested PCR analysis using universal primers P1 (Deng and Hiruki, Journal of Microbiological Methods, 14, 53-61. 1991) and P7 (Schneider *et al*., Molecular and Diagnostic Procedures in Mycoplasmology, 369-380. 1995) and specific primers R16(I)F1/R1 (Lee *et al.,* Phytopathology, 84, 559-566. 1994). In total, 77% of samples from symptomatic plants tested positive for stolbur phytoplasmas (16SrXII-A), as did 28% of samples from asymptomatic plants. This result clearly indicates the inconsistency of the symptomatic evidence with etiological positivity in *V. vinifera* affected by BN and, at the same time, a condition of recovery very often as synonymous with latency. These results, which are also interesting on an epidemiological basis, are alarming in the area of selection and production of plants free from phytoplasmas. As the production parameters of recovered plants are near to the standard of healthy plants (Garau *et al*., Bulletin of Insectology, 60, 233-234. 2007), they could not be a discriminating factor in the selection of phytoplasma free plants either.

Ten years of apple proliferation epidemics in the apple orchards of Pelion Mountain in Greece

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Apple proliferation disease symptoms in Pelion region were reported in 1999, when a number of mature apple trees were observed bearing unusually small fruits. During the following four years, the appearance of the disease was limited and sporadic, affecting mainly orchards in higher altitudes. In 2004 investigation of nutritional and physiological disorders or environmental factors as potential causes of small fruit symptoms did not provide any reasonable explanation for the phenomenon and the use of methods to alter tree physiology did not succeed to increase fruit size. Therefore, the studies were focused on the detection of '*Candidatus* Phytoplasma mali'. The presence of '*Ca.* P. mali' was proven in 2005 by: a) increased percentage of recovery in symptomatic trees treated with oxy-tetracycline, b) PCR-detection of the pathogen in cv. 'Firiki' samples grafted on symptomatic cv. 'Starking Delicious' trees and c) PCR-detection of the pathogen in a considerable number of trees. The majority of the infected trees were symptomatic, grafted on seedling rootstock and aged (35-40 years) from cvs. 'Starking Delicious' and, to a lower extent, 'Starkrimson', 'Imperial', and 'Firiki'. 'Golden Delicious' apple trees showed no symptom development. The pathogen was also detected in young (2-3 years) non-symptomatic trees used to replace the removed infected trees. Later, the pathogen was found to be widely spread in many orchards all around the Pelion. Recovery phenomena and reappearance of symptoms were observed over the years. Today, ten years later, because no coordinated measure for the control of the epidemic was taken, we face a dramatic situation, where ~ 90% of the apple trees are infected, the losses range from 20-40% in the lower elevations (≤ 400 m), 40-70% in the main production zone (400-600 m), and in the higher elevations, where apple orchards neighbour beech forest, the losses reach 70-100%.

Activity of bioactive compounds on apple proliferation on potted apple trees

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The apple proliferation (AP) disease, caused by the phytoplasma '*Candidatus* Phytoplasma mali', can lead to serious economic losses due to its negative impact on fruit growth and quality. Up to now, management of the disease has been based on the vector control and on the suppression of the inoculum by the eradication of symptomatic plants; no direct control strategy is available.

In the present research different bioactive compounds (SAR inducers and growth regulators) were applied on potted 1-2 year-old '*Ca.* P. mali'-infected apple trees ('Golden Delicious' on M9) to evaluate their activity on symptom suppression or ability to induce a stable asymptomatic infection status (recovery-phenomena). In February 2008, 600 M9 rootstocks were grafted with infected scions and 400 rootstocks with healthy scions as control plants. The phytoplasma strain AT2 was chosen as it is recently the most common in the province. Sixty infected and 30 healthy plants were gathered in blocks in an insect-proof field-tunnel. Each block was treated curatively with a different bioactive compound in both growing seasons 2008 and 2009, except for the untreated control block. We tested Bion® (50% Acibenzolar-S-Methyl), Messenger® (3% Harpin protein), Aliette® (80% Al-Phosethyl) and Regalis® (10% Prohexadion-Ca) through foliar applications, whereas Dormex® (49% Cyanamid) was applied through watering the pot. In both years at the end of the period of treatments in July, Dormex showed a significant difference in symptom expression compared to the untreated control. This effect was not long-lasting, indicating a non-bactericidal activity.

In order to determine the effect of the compound on healthy and infected plants, pathogen levels, peroxidase activity and hydrogen peroxide, as well as reducing sugars and starch content will be measured in the next year from different plant tissue throughout the growing season.

Screening for resistant rootstocks to control pear decline in pomiculture

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Pear decline is a phytoplasma disease caused by '*Candidatus* Phytoplasma pyri'. The disease is present in most pear growing regions in the northern hemisphere. The pathogen is vectored by psyllids which are difficult to control. Pear trees in extensive and abandoned orchards are infected to a high degree and represent a permanent inoculum source for commercial orchards and nurseries. '*Ca*. P. pyri' is a quarantine pathogen in the EU and pear plant material is subjected to rules and regulations prior shipment. The commercial rootstocks based on *Pyrus communis* are not resistant and the low-vigour quince based rootstocks are demanding in terms of soil and winter temperature.

The current pear rootstock screening which started in May 2009 is based on the results of an 18-year old field trial with offspring from 39 open pollinated *Pyrus* genotypes (Seemüller & Schneider, European Journal of Plant Pathology, 123, 217-223. 2009) Twenty *Pyrus* species, some represented by accessions of different provenience, which performed well after experimental inoculation with infected scions were selected for re-evaluation and assessment of agronomic characteristics. Shoots emerging from the rootstock were cut in February, tested for phytoplasma infection by PCR before grafting on seedlings of 'Kirchensaller Mostbirne' for maintenance. *In vitro* cultures were established from buds of the grafted *Pyrus* genotypes. *Ex vitro* material was produced from all twenty *Pyrus* species, although, a number of genotypes could not be propagated *in vitro*. In total about 1300 *ex vitro* plants were grafted in October 2009 with four accessions of pear decline infected material.

The genetic variability of '*Ca*. P. pyri' accessions was tested by SSCP- and sequence analysis of the *hfl*B- and *imp* gene, respectively. Several SSCP patterns of the different strains could be discerned, although the heterogeneity within this species was less compared to '*Ca*. P. mali'.

Physiological changes in grapevine leaves infected with bois noir

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The concentrations of seven mineral elements (Ca, K, N, Mg, Mn, Fe and P) were analyzed in leaves of healthy grapevines and of grapevines infected with bois noir (BN) in the field. The calcium levels were lowered significantly in BN-infected leaves of all five cultivars tested (Chardonnay, Müller-Thurgau, Pinot Noir, Lagrein and Zweigelt). The other six elements also showed a trend towards decreasing concentrations; however, the effects were less consistent. The cultivars reacted differently to BN infection both by visual symptoms and mineral contents, with Chardonnay and Zweigelt being the most susceptible cultivars. The dynamics of BN outbreak also depends on the cultivar: whereas the first leaf symptoms on Chardonnay can be found around mid June, normally they do not appear on Pinot Noir leaves before early August. First studies on the dynamics of mineral elements in the leaves over a five-month period from May until September also showed that differences in calcium levels between healthy and BN-infected leaves could be detected in Chardonnay as early as June/July and increased until the end of September, whereas leaves of Pinot Noir showed the decrease of calcium levels only later in the season and at a lower rate (Schweigkofler *et al*., Mitt. Klosterneuburg, 4, 117-122. 2008). Monthly foliar treatment of grapevines showing symptoms of BN during the summer of 2008 using commercial fertilizers had no significant effect on the recovery rate compared to the control plants. Our results indicate that BN infected grape leaves suffer from severe malnutrition of several mineral elements, especially calcium. For the next field season we plan to study the effect of a bioactive ingredient, which showed promising effects in other experiments, in the field on the mineral contents of the leaves. Analysis of the biochemical pathways leading from phytoplasma infection to these leaf symptoms might help to gain a better understanding of this complex pathosystem and eventually to establish strategies for symptom reduction.

Resistance to fruit tree phytoplasmosis – solution or illusion?

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Phytoplasma diseases are difficult to control, mainly due to the lifestyle of the pathogens. The most promising approach seems to be the use of resistant plants. Although intra- and interspecific variation in susceptibility to phytoplasma has been reported for several decades, resistance-based control is rare. Chances to design systems for producing resistant plants appear particularly good in pomaceous fruit trees because of the annual fluctuation of the phytoplasma colonization in the trees. As the pathogens overwinter in the roots, apple proliferation and pear decline can be controlled by the use of resistant rootstocks. Extensive screening of many *Pyrus* taxa revealed great differences in resistance. However, in each taxon resistant and susceptible offspring occurred in variable numbers so that careful selection of resistant genotypes is required. Great differences in resistance were also observed in apple rootstocks. Screening of trees on many established and more recent stocks has shown that there is little resistance in this group. Satisfactory resistance was observed in a few experimental rootstock selections consisting of interspecific hybrids of apomictic *M. sieboldii* and genotypes of *M.* x *domestica* and *M* x *purpurea*. However, trees on these stocks are more vigorous and less productive than trees on standard stock M9. A breeding program has been initiated to reduce vigour and improve yield. From 2001 to 2009 35 major crosses were made in which *M. sieboldii* and *M. sieboldii* F1 to F3 hybrids were crossed mainly to M9. The seedlings obtained were examined by simple sequence repeat (SSR) analysis to identify sexually derived offspring for selection. In the most successful crosses about half of the progenies showed good resistance traits. The strategy to control stone fruit phytoplasma diseases by the use of resistant plants differs from that of pome fruits in that the pathogen persists throughout the year in the canopy. Due to this condition disease control is not possible with resistant rootstocks alone but also requires resistant scion cultivars.

Endophytic bacteria in phytoplasma infected bindweeds (*Convolvolus arvensis*)

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Endophytic bacteria are defined as bacteria that are detected after surface sterilization of a plant part and analysed by cultural or molecular methods. Relationships of phytoplasmas and endophytic bacteria are practically unknown. The first step to understand how these microorganisms influence each other is the evaluation of the endophytic diversity of bacteria in phytoplasma infected and healthy plants. In the present study molecular and cultural methods were used for identifying endophytic bacteria isolated from bindweeds (*C. arvensis*) infected with stolbur phytoplasmas. A surface-sterilized 2 cm long part of a stem from the soil line was homogenized and dilution plated onto nutrient agar plates containing cycloheximide against fungal growth. Colonies from the highest dilutions were picked on the basis of differences in morphology. The resulting isolates were tested for their ability to suppress the growth of *Curtobacterium flaccumfaciens* (a Gram+ bacterium). The identity of isolates was determined by PCR amplification of the 16S rRNA gene using primers 27F/1492R (Lane D.L., In: Nucleic Acid Techniques in Bacterial Systematics, 115-175. 1991) followed by DNA sequencing of the PCR amplicons. Sequence similarities were searched for in Genbank databases (http://www.ncbi.nlm.nih.gov). Forty-two isolates were ranged in nine different groups of duplicated bacteria and eighteen were identified as unique. Sequencing of 16S ribosomal gene revealed that *Pantoea agglomerans*, *Curtobacterium flaccumfaciens, Bacillus megaterium* and *Microbacterium sp.* were the most frequenty species. The culturable endophytic bacterial communities detected in bindweed stem bases were in most cases in the order of 10^3 to 10^5 CFU g^{-1} of fresh plant tissue. Dilution plating revealed that a range of bacterial types dominated these populations. Many bacterial isolates gave amplicons in PCR with primers P1/P7 and in nested PCR with R16F2n/R16R2 in similar sizes as phytoplasmas. However RFLP analysis of the amplicons clearly differentiated phytoplasmas from bacteria.

Working Group 4: Phytoplasma/host interactions

European research teams have been involved in a number of phytoplasma full genome sequencing projects and some of this sequence information is available in public access databases. These projects have resulted in major advances in understanding phytoplasma genomics. The genomes encode between 496 and 839 genes and whilst the main housekeeping genes appear to be conserved among phytoplasmas, there are also other genes that are unique to specific strains. Compared to other organisms, phytoplasmas lack genes encoding components of the pentose phosphate pathway, lack most genes for nucleotide synthesis, and also lack genes for the F0F1-type ATP synthase, which was previously thought to be a component of the minimal gene set required for all living organisms. Studies are currently identifying the various biosynthetic pathways that exist in phytoplasmas and the changes in host gene expression that occur in infected plants to devise possible practical use of these relevant information.

Coordinators

Dr. Saskia Hogenhout - United Kingdom

Dr. Xavier Foissac – France

Tasks

1. Integration all genomics and bioinformatics information into a single website

2. Identification of model systems in which to co-ordinate effort and research to enhance understanding of phytoplasma/host interactions

3. Eplore the phytoplasma genomes available for differences and test individual candidate genes for their relevance in pathogenicity or host pathogen interaction

4. Clarify aspects of population genetics of vector species and phytoplasma diversity by molecular typing of phytoplasmas and vectors with respect to their host plant affiliation

Modification of secondary metabolites production in medicinal herbs infected by phytoplasmas

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Recent Italian reports indicate the presence of different phytoplasmas in medicinal herbs showing stunting, witches' broom and yellowing: stolbur in *Hyssopus officinalis* (hyssop) and in *Parietaria* sp., aster yellows in *Digitalis lanata* (woolly foxglove) and *Grindelia robusta* (gumweed), and ash yellows in *Hypericum perforatum* (St. John's worth). Comparative biochemical analyses by GC-MS, reversed-phase HPLC, and other methodologies on healthy and infected plants provide indication about metabolic modifications related to the presence of phytoplasmas. Hyssop oil samples showed content of isopinocamphone and pinocamphone of 44.7% and 29.1%, and of 6.2% and 3.92% respectively from healthy and infected plants, with a reduction of antimicrobial action for the oil from infected plants. In the same oil higher levels of bicyclogermacrene, germacrene D and caryophyllene oxide were measured. Parietaria flavonoid quality and quantity reduction was observed: quercetin, canferol and isorhamnetin were detected only in healthy plants, while from infected ones only rutin (a quercetin glycoside) was identified but 83% less concentrated. Qualitative and quantitative analysis of cardiac glycosides from woolly foxglove demonstrated that the secondary metabolite mainly affected was lanatoside C with a 50% of reduction in infected plants (Pellati *et al*., J. Chromatogr. A., 1216, 3260-3269. 2009). Among 42 components analysed in oil from infected gumweed, a higher percentage of selected monoterpenes was reported, concentration of limonene and borneol acetate was almost 50%, and that of borneol was about 30% higher. Infected St. John's worth plants showed a decreased amount of rutin (1,96 vs. 4,96 mg/g), hyperoside (2.38 vs 3.04 mg/g), isoquercitrin (1.47 vs. 3.50 mg/g), amentoflavone (0.12 vs. 0.39 mg/g), pseudohypericin (1.41 vs. 2.29 mg/g), whereas chlorogenic acid content was doubled (1.56 vs. 0.77 mg/g); essential oil revealed an increased abundance of sesquiterpenes (β-caryophyllene, δ-elemene and germacrene D in particular) and a matching decrease in monoterpene hydrocarbons and aliphatics (Bruni *et al*., J. Agric. Food Chem., 53, 964-968. 2005).

A study of floral symptoms in phytoplasma infected *Arabidopsis thaliana*

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Despite the significant progresses in the genomic and molecular biology of the phytoplasmas, it is still largely unknown how these pathogens manipulate plant host physiology to induce a complex, diverse, yet unique array of symptoms. In order to provide a reliable plant-phytoplasma interaction model for the study of floral symptoms, we developed an efficient infection protocol for *Arabidopsis thaliana*, the plant for which floral development is best understood at the molecular level. Infection of different phytoplasma strains (X disease phytoplasma, strain Italian Clover Phyllody [ICPh] phytoplasma and '*Ca.* P. asteris' strain Chrysanthemum yellows [CY] phytoplasma), on different *A. thaliana* ecotypes (Col-0, Ms-0, Loh-0, Bu-6, *Ler*) in different growing stages and conditions, resulted in different symptom patterns. By inoculating Col-0 at 30 days after germination with ICPh phytoplasma in short day (9 hours light/ 15 dark), flowers were produced with increasingly severe floral symptoms for two months. Gene expression analysis showed strong down regulation of some, but not all homeotic genes involved in flowering and genes involved in the gibberellin metabolism that allowed elaboration of a preliminary hypothesis on the molecular mechanism underlying floral symptoms. Common morphological features were detected in phytoplasma infected wild type plants and not infected mutants impaired in the gene functions that were found to be down regulated.

Characterization of the *imp* gene in "flavescence dorée" and related phytoplasmas

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The *imp* gene of phytoplasmas encodes for an immunodominant membrane protein which is thought to be involved in host-phytoplasma interactions (Kakizawa *et al.,* Trends in Microbiology, 14, 254-256. 2006). Thus far the *imp* gene was characterized in a few phytoplasmas, belonging to 16SrI, 16SrII, 16SrIII, 16SrX, 16SrXI and 16SrXII groups (Kakizawa *et al.*, FEMS Microbiology Letters, 293, 92-101. 2009).

Several forward and reverse primers for the PCR amplification of the *dna*D-*imp*-*pyr*G genomic fragment in isolates belonging to the 16SrV phylogenetic group were designed and tested. The best results were obtained with two pairs of primers which amplified about 800-1,000 nt, including the whole *imp* gene.

The amplicons of 20 different "flavescence dorée" and related phytoplasma strains detected in grapevine, clematis and alder, mostly from Italy, were amplified and sequenced. Reference strains from the 16SrV-A, -C and -D subgroup were included.

Data analyses showed that the *imp* gene in the 16SrV group of phytoplasmas is characterized by a genetic variability much higher than the one described in other previously studied genes of this group. For example, the genetic diversity between FD-D and FD-C reference strains is approximately 25%. The lengths of the putative encoded proteins varied between 153 and 158 aminoacids in the strains studied so far. In particular, insertions and deletions (from 3 to 9 bp) were detected in several strains.

The antigenic membrane protein of chrysanthemum yellows phytoplasma specifically interacts with vector ATP synthase

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Phytoplasmas are transmitted by insect vectors in a persistent propagative way that implies recognition specificity. Membrane proteins of these wall-less prokaryotes are in direct contact with host cells and probably are involved in recognition. To study these interactions, the antigenic membrane protein "Amp" and an arginine transporter "ArtI", two membrane proteins of '*Candidatus* Phytoplasma asteris' chrysanthemum yellows strain (CY), were expressed as fusion antigens (Galetto *et al.*, Canadian Journal of Microbiology, 54, 341-351. 2008). The interactions between these phytoplasma membrane proteins and total as well as membrane insect vector proteins were analysed with different serological methods. Total and membrane proteins from several CY insect vector and non vector species were included in the study. Dot blot Far Western experiments showed the interaction between Amp protein and total protein extracts from several vector species. CY Amp-packed affinity chromatography assay showed that only few insect vector proteins interacted with Amp, while no interaction was present when proteins from non insect vector species were analysed. MS/MS spectrometry identified actin and ATP synthase α and β subunits as three of the vector proteins interacting with Amp. Western blots with specific antibodies, Far Western and affinity chromatography experiments, confirmed the MS/MS spectrometry identification. Insect vector actin was reported as involved in interaction with phytoplasma Amp (Suzuki *et al.*, PNAS, 103, 4252-4257. 2006). The role of α and β subunits of ATP synthase in the phytoplasma – vector interaction is a new finding and requires further confirmation in other phytoplasma – insect vector combinations, although the ability of ATP synthase to act as receptor for various ligands together with its localization on the cell membrane surface have been reported in other biological systems (Chi & Pizzo, Ann. Med., 38, 429-438. 2006).

Analysis of the acquisition and multiplication efficiency of different strains of '*Candidatus* Phytoplasma mali' by the vector *Cacopsylla picta*

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Based on previous observations during long-term acquisition and transmission trials, studies were carried out under standardized conditions in order to analyse the acquisition and multiplication efficiencies of different strains of '*Ca*. P. mali' by different developmental stages of *C. picta*. The acquisition of '*Ca*. P. mali' from micropropagated plants infected with different strains was tested for nymphs, larval stages and new adults of *C. picta*. When born on infected plants a nearly 100% acquisition was achieved for all strains of '*Ca*. P. mali' by *C. picta*. Differences in acquisition efficiency were observed for new generation adults which acquired the phytoplasma as imagines. The multiplication efficiency of the different '*Ca*. P. mali' strains inside the insects was analysed by quantitative real-time PCR. Despite high acquisition rates only few subsequent transmission events to healthy test plants could be recorded.

The extended phenotype, a phytoplasma effector protein that improves vector fitness

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Phytoplasmas induce morphological changes in their plant hosts and affect plant-insect interactions. We studied transmission of Aster Yellows phytoplasma strain Witches' Broom (AY-WB) by the aster leafhopper *Macrosteles quadrilineatus*. We found that infection of AY-WB within the insect, plant or both hosts increases the fecundity of *M. quadrilineatus* by 60 to 70%. In addition, the maize specialist leafhopper *Dalbulus maidis* survives and produces nymphs on AY-WB-infected *Arabidopsis thaliana*, but die on healthy *Arabidopsis* plants. In order to identify proteins responsible for these changes in these interactions with insects, the fully sequenced AY-WB genome was mined for genes encoding secreted proteins. These proteins are candidate virulence factors (effectors) that may manipulate the plant or insect hosts. Fifty-six secreted AY-WB proteins (SAPs) were identified (Bai *et al.*, Molecular Plant Microbe Interactions, 22, 18-30. 2009). One candidate effector is SAP11, which carries a nuclear localization signal and accumulates in the plant cell nuclei. In this study, we found that stable over-expression of *SAP11* in Arabidopsis increased the fecundity of *M. quadrilineatus* by on average 25%. Moreover, compared to wild type Arabidopsis, the SAP11 lines have severely crinkled leaves and higher number of stems. More recent data suggests that SAP11 may interact with plant proteins that regulate plant defense responses to pathogens and pests. This may affect *M. quadrilineatus* nymph production. Thus, the SAP11 effector function extends beyond its direct interaction with the plant host; it stimulates the generation of more insect vectors that subsequently can disseminate the phytoplasmas to other plant hosts.

Comparative genome analysis of '*Candidatus* Phytoplasma mali' strain AT and strain 1/93

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'*Ca.* P. mali' is causing apple proliferation (AP) disease severely impairing fruit quality and productivity of the trees. The complete genome sequence of the virulent strain AT was determined and analysed (Kube *et al*., BMC Genomics, 9, 306. 2008). Results separate '*Ca*. P. mali' from the other completed phytoplasma genomes by its linear chromosome organisation (core and terminal inverted repeat structure), and its reduced genome size, at least. However, genome analysis provided no promising candidates for virulence factors. It also remains unclear if the genome organisation is instable due to putative phage integration events, and subsequent re-arrangements. Additional information on that is expected from the genome sequence of the nearly avirulent '*Ca*. P. mali' strain 1/93. A draft sequence was generated by 454 sequencing reaching a >30-fold sequencing coverage. Problems in read length and quality correspond to homopolymer sequences and the low GC content of 22% as it is known for pyrosequencing. Nevertheless, up to 84% percent of all reads could be mapped on the strain AT chromosome. Unmapped reads were assigned to other bacteria, to genome of tobacco host and to a few genes absent in '*Ca*. P. mali' strain AT, but present in other phytoplasmas. It was unexpected that ~87 kb of the strain AT chromosome could not be covered by strain 1/93 reads by mapping approaches or reference guided assembly. These preliminary results indicate a genome size below the 602 kb of strain AT. Absent regions could be predicted as phage associated gene clusters. These results demonstrate that the amount of integrated regions was underestimated in previous study. Apart from these modulations highlighting a rapid evolution, it is clear that the general chromosome organisation of '*Ca*. P. mali' is stable. Furthermore, results also highlight that virulence-related genes may be located within the integrated regions present in strain AT, but absent in strain 1/93 showing no symptoms on infected plants. The sequence range to be searched for candidate genes is limited for ongoing studies in consequence.

Model systems to study phytoplasma-host interactions

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Phytoplasma infection depends on the interactions of at least three partners: the plant, the pathogen and the vector. The complexity of such interactions makes most of phytoplasma-host associations hard to investigate. In the last ten years we have developed a model patho-system represented by '*Candidatus* Phytoplasma asteris', strain chrysanthemum yellows (CY), its host plant *Chrysanthemum carinatum* and its leafhopper vectors *Macrosteles quadripunctulatus* and *Euscelidius variegatus*. CY infects a variety of dicotyledonous plants and is transmitted with different efficiencies by several species of leafhoppers that can be easily reared under controlled conditions (Bosco *et al*., Journal of Economic Entomology, 100, 1504-1511. 2007). Due to its short incubation in the plant and latency in the vector, rapid and obvious symptom development and extremely high transmission efficiency, CY represents an optimal model system. Moreover, '*Ca*. P. asteris' genome is fully sequenced (Oshima *et al*., Nature Genetics, 36, 27-29. 2004). We studied pattern of multiplication and movement of the phytoplasma in both plant and vector, pathogenic effects on insect vectors, competition of different phytoplasmas inside the same vector, possible intraspecific variation in transmission capability, interactions between phytoplasma and vector proteins possibly regulating transmission specificity, activity of resistance elicitors on phytoplasma infection. Studies on the effect of changing temperature and environmental factors on phytoplasma epidemiology are currently undergoing using this model system.

To which extent can results obtained on one model system be applied to other phytoplasma-host associations? The development of other model systems, based on different host plants - i.e. woody vs herbaceous, fully sequenced - , phytoplasma (genetically unrelated) and vector species (i.e. other than leafhoppers) would be useful to increase knowledge on phytoplasma diseases.

Evaluation of infection process in European stone fruit yellows strains from various apricot cultivars grafted on fifteen *Prunus* rootstocks

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In years 2004-2009, symptoms of phytoplasma infection were observed in trees of 14 different apricot varieties and in the peach cultivar Jantze. The presence of European stone fruit yellows (ESFY) phytoplasmas was detected by nested PCR with universal primers P1/P7 (Schneider *et al*., Molecular and diagnostic procedures in mycoplasmology. 1995) and specific primers f01/r01 (Lorenz *et al*., Phytopathology, 85, 771-776. 1995). Fifteen *Prunus* rootstocks, grown in pots, were then infected by grafting with buds from 12 infected trees. Primary symptoms in apricot trees were: cv. Murfatlar/chlorotic leaf roll, cv. Poljus Južnij/weak chlorotic leaf roll, cv. Hargrand 4/no symptoms, cv. Hargrand 2/weak chlorotic leaf roll, cv. Hargrand 1/leaf roll, cv. Poyer/no symptoms, cv. Saldcot/leaf roll, reduction of growth, weak yellowing of leaves and delayed beginning of flowering, cv. Churmai/leaf yellowing and premature leaf drop, cv. Vestar/leaf yellowing and premature leaf drop, cv. Olimp/no symptoms, cv. Veselka/premature fruit drop, cv. Jantze/chlorotic leaf roll. To study the infection process were used the following rootstock: MRS 2/5, AP-1, Myrobalan 29C, MY-KL-A, Strážovický myrobalan, Lesiberian, GF-8-1, GF 677, GF 31, GF 305, VVA-1, Shirofugen, St. Julien A, Torinel and M-LE-1. Each combination was replicated 15 times and statistical correlation with symptoms observed was calculated by using Kruskal-Wallis one-factor analysis of variance for *t*-division (*t*=95%). The chlorotic leaf roll symptom was observed in 98% of combinations using the peach cv. Jantze, in 58% for the apricot cv. Poljus Južnyj and in 34% for Hargrand 2. All combinations using the rootstock GF-8-1 had leaf yellowing, all those with Torinel had leaf roll and reddening, and all with GF305 had early leaf drop. Symptoms were also observed after using buds taken from trees ESFY positive but showing no symptoms. In the case of buds from the cv. Poyer, 55% of shoots showed no symptoms and 36% leaf roll. In peach rootstocks Lesiberian and GF305 pronounced leaf reddening premature drop occurred together with leaf roll in 80% of the cases.

Characterization of phytoplasmal extrachromosomal DNAs

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Several phytoplasmas bear extrachromosomal DNAs (EC-DNAs) of various sizes. We identify by Southern blot analysis the smallest EcDNA in our strain collection (designed EcDNA-NJAY, carried by the New Jersey Aster Yellows strain of '*Candidatus* Phytoplasma asteris'), that was cloned and sequenced. This 2,443 bp EC-DNA, had a nucleotide content of about 28% G+C and contained only two open reading frames that share high similarity with genes coding geminivirus replication-associated protein (*RepA*) and single-strand DNA-binding protein (*SSB*), respectively. In addition, the EcDNA-NJAY included a non coding region of about 1,200 bp in length containing remnants of genes potentially implicated in vector transmission, possibly in the process of being lost as this strain has been propagated by grafting for the last ten years. RepA showed a high identity (25% to 94%) with the homologous gene products in EcDNAs detected in different species such as '*Ca.* P. asteris', '*Ca.* P. trifolii', '*Ca.* P. pruni'*, '*Ca.* P. australiense', but shares low identity (<15%) with the replication associated protein of true plasmids such as pOYW of '*Ca.* P. asteris'; conversely SSB is highly similar in plasmid DNAs and in RepA-containing EcDNAs. Although associated in this and other EcDNAs, *RepA* and *SSB* are not phylogenetically related with each other: *SSB* genealogies indicated an origin from the *Bacillus/Clostridium* clade, and are congruent with the genealogies of phytoplasma plasmids such as pOYW and the phytoplasma 16S rDNA, while a similar ancestor cannot be found for *RepA*, that conversely is similar to viral genes for replication associated proteins. This suggest a recombinant origin of EcDNA-NJAY, as chimeric molecule containing a SSB from plasmid such as pOYW and a virus replication associated protein. The interspecies spread of recombinant EcDNAs, that is wider than that of the true plasmids, is an intriguing evidence that may be related to the expansion of vector range.

(*) '*Candidatus*' species proposed but not yet formally described, and reported here as incidental citations which do not constitute prior citations, according to rule 28b of the bacteriological code (Lapage *et al*., 1992).

PMUs are true mobile genetic elements that can influence phytoplasma genome evolution and adaptation to plant and insect hosts

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Phytoplasmas replicate intracellularly in plants and insects and are dependent on both hosts for dissemination in nature. The four completely sequenced phytoplasma genomes are reduced in size. They also contain ca. 20-kb repeats, named potential mobile units (PMUs), which are characterized by the presence of ca. 21 genes encoding full-length transposases (*tra5*), transcription factors (*sigF*), DNA recombination and replication proteins (*ssb*, *himA*, *dnaB, dnaG*) and several predicted membrane-targeted proteins of unknown function. Here we show that PMU1 of Aster Yellows phytoplasma strain Witches' Broom (AY-WB) exists in both linear (L-PMU1) and covalently closed circular (C-PMU1) forms. L-PMU1 is flanked by inverted 237/238-bp repeats upstream of the first gene, *sigF*, and downstream of the last gene, *tra5*. C-PMU1 contains one 238-bp repeat between *sigF* and *tra5*. This repeat is oriented in the opposite direction in C-PMU1 compared to L-PMU1. We also found that C-PMU1 copy number increases during AY-WB infection of insects compared to that of plants and this coincides with higher PMU1 gene transcript levels in insects. Thus, PMUs can exist as extrachromosomal DNAs and are therefore true mobile genomic units. These findings provide novel insights into the impact of PMUs on phytoplasma host adaptation and genome evolution.

cost
EUROPEAN COOPERATION
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COST Action FA0806

Plant Virus Control Employing RNA-Based Vaccines: A Novel Non-Transgenic Strategy

PlantiVax

Time frame: 2009-2013

For more information:
www.aua.gr/COSTFA0806

1. Background Information

- The currently applied virus control methods are limited in number, efficacy and environmental suitability. Thus new methods are urgently called forth.
- A very promising approach is the exploitation of a natural, endogenous mechanism in plants providing virus resistance known as RNA silencing. Since current EU decisions restrict transgenic plant usage, non-transgenic approaches exploiting the silencing mechanism for plant virus control are sought.
- This COST Action networks several European labs, working on the subject, in order to develop suitable, efficient and cost-effective methods to induce anti-viral silencing in crops by the transient application of dsRNA, siRNAs and/or artificial small RNAs (collectively designated as "**RNA-based vaccines**").

2. Main Objectives

- Co-ordinate frontier European research on plant virus control through gene silencing approaches.
- Develop novel non-transgenic control strategies for managing plant viral diseases in Europe and world-wide
- Optimize protocols for high-throughput production and delivery of suitable resistance inducer molecules.
- Transfer innovations to end-user groups (*i.e.* SMEs, plant protection organizations, growers' groups).
- Disseminate research accomplishments via publications and establish long-term networking.

3. Stucture of the Action

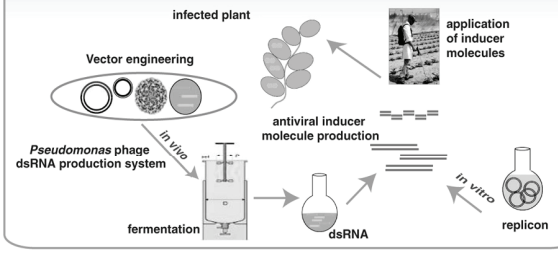
There are three Working Groups (WGs)

WG1	WG2	WG3
Development of novel non-transgenic strategies for plant virus control	***Application of novel non-transgenic strategies for plant virus control***	***Socio-economic evaluation of the impact of the novel application methods***
• Development of novel non-transgenic strategies • Selection of the best inducer molecule • Establish high throughput production methods	• Standardize protocols for inducer molecule delivery • Establish high throughput application methods • Monitor of the efficacy of the developped methods in non lab conditions	• Evaluate the economics of the established methods • Dissemination of the obtained results • Establish a web-site, publish an information pamphlet

4. Training and targeted research opportunitites

In the frame of COST Action FA0806, **Training Schools** and **Short Term Scientific Missions** (STSMs) will be funded to disseminate the outcomes of research activities that are part of the Action, provide intensive training on a new emerging subject, support research work related to the objectives of the Action. They are open to early-stage and senior researchers alike.

5. Strategic plan



6. Methods for production of resistance inducing molecules

(a) *In vitro approach:*

A suitable DNA template produced by PCR amplification is used together with commercially available reagents ('kits') for transcription and production of double stranded RNA molecules (resistance inducing molecules).

(b) *In vivo approach:*

Suitable bacterial cells are engineered to produce double stranded RNA molecules (resistance inducing molecules) in large quantities.

7. Technology for application of inducer molecules

in the lab *in the nursery* *in the field*

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