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BOOK OF ABSTRACTS

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PHYTOPLASMA IN FRUIT TREES: MULTIDISCIPLINARY APPROACHES TOWARD

Invited lecture

GLOBAL GENE EXPRESSION ANALYSIS OF PHYTOPLASMA IN THE HOST SWITCHING BETWEEN PLANT AND INSECT

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Phytoplasmas are biologically unique since they can parasitise a diverse range of hosts, including plants and insects. Phytoplasmas reside endocellularly within the plant phloem and feeding insects (mainly leafhoppers), and are spread among plants also by insects. It is of interest how phytoplasmas can adapt to two diverse intracellular environments (i.e., plant and insect cells). We have previously demonstrated that a complex between Amp, a surface membrane protein of phytoplasma, and insect microfilament may play a major role in the insect transmissibility of phytoplasma. However, the mechanisms enabling the switch between plant and insect hosts are poorly understood. To investigate the molecular mechanism underlying the "host switching" between plant and insect, we performed the global gene expression analysis of '*Candidatus* Phytoplasma asteris' OY-M strain. As a result, the phytoplasma alters many gene expressions in response to the plant and insect host, such as genes for transporters, secreted proteins, and metabolic enzymes. These results suggest that the phytoplasma may use transporters, secreted proteins, and metabolic enzymes in a host-specific manner. As phytoplasmas reside within the host cell, the proteins secreted from phytoplasmas are thought to play crucial roles in the interplay between phytoplasmas and host cells. Our microarray analysis revealed that the expression of the gene encoding the secreted protein PAM486 was highly upregulated in the plant host, which is also observed by immunohistochemical analysis, suggesting that this protein functions mainly when the phytoplasma grows in the plant host

DETECTION AND IDENTIFICATION OF A PHYTOPLASMA AFFECTING BLACKCURRANT AND REDCURRANT SHRUBS

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During October-November/2011, blackcurrant (*Ribes nigrum* L.) and redcurrant (*Ribes rubrum* L.) shrubs exhibiting symptoms of leaf redness and downward curling in Ontario, Canada were tested for phytoplasma presence. Total DNA was extracted (Fast DNA Spin kit, MP Biomedicals, USA) and used in a nested PCR assay with universal primers that target the phytoplasma 16S rRNA gene, P1/P7 followed by either R16F2n/R2 or fU5/rU3 for the nested reaction. R16F2n/R2 PCR amplicons were obtained for all the symptomatic plants collected, purified (Cycle Pure kit, Omega, USA) and sequenced bi-directionally (University of Health Network, Toronto, Canada). Sequences were compared to those of reference phytoplasmas in GeneBank. BLAST analysis showed a 100% of 16S rDNA sequence identity between the two phytoplasmas detected in both blackcurrant and redcurrant plants, and a 99% of sequence identity to those of members of group 16SrX, subgroup 16SrX-A. Virtual and actual RFLP of the R16F2n/R2 and fU5/rU3 amplicons with *Alu*I, *Rsa*I, *Ssp*I and *Mse*I restriction endonucleases yielded RFLP patterns similar to those of phytoplasma members of the group 16SrX 'apple proliferation', subgroup 16SrX-A. Phylogenetic analysis (MEGA version 4.1, USA) based on the 16S rDNA sequences confirmed RFLP and BLAST results. Phytoplasmas of group 16SrX have been previously reported in Ontario, affecting *Prunus* and *Pyrus* species. However, results represent the first record of a 16SrX-A infecting blackcurrant and redcurrant, and provide a valuable tool for further epidemiological studies of a 16SrX phytoplasma in these two plant species.

MOLECULAR POLYMORPHISM IN PHYTOPLASMAS INFECTING PEACH TREES IN SERBIA

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During a survey carried out in 2008 to verify phytoplasma presence in fruit trees in Serbia, samples were collected from two single peach plants (30-Niš and 103-Radmilovac) showing yellows disease. Phytoplasma detection was carried out using PCR assays with primer pair P1/P7 in direct reaction, followed by nested PCR with F1/B6, R16F2n/R2, and R16(I)F1/R1. RFLP analyses carried out on R16F2n/R2 amplicons with *Tru1*I, *Taq*I, *Tsp509*I, *Alu*I, and *Bfa*I restriction enzymes showed that in peach 30, a mixed phytoplasma infection was present. Identification of 16SrII and 16SrXII phytoplasmas was confirmed by RFLP analyses carried out on amplicons obtained with primers R16(I)F1/R1 with *Tru1*I and *Tsp509*I restriction enzymes. RFLP analyses on F1/B6 and R16F2n/R2 amplicons with *Tru1*I showed that 16SrII group phytoplasmas were present also in peach 103. F1/B6 and R16F2n/R2 amplified products (about 1,700 bp and 1,200 bp respectively) of both peach samples were purified using Qiagen PCR Purification Kit, cloned in DH5 alfa and a number of clones was screened by PCR with M13 primers followed in nested PCR by F1/B6 and R6F2n/R2 primers (according with amplicon). RFLP analyses with *Tru1*I on R16F2n/R2 amplicons of seven M13 clones obtained from peach 30 show the presence of 6 profiles referable to 16SrXII and two referable to 16SrII phytoplasmas. *Alu*I restriction enzyme did not show polymorphisms but distinguished between groups 16SrII and 16SrXII while *Tsp509*I showed different profiles between the two 16SrII group phytoplasmas. Similar results were obtained with *Tru1*I and *Alu*I restriction enzymes on 3 clones amplified with R16(I)F1/R1. Peach 103 clones were all identical after RFLP analyses. This is the first report of 16SrII phytoplasmas in peach and the 16Sr DNA variability detected in both 16SrXII and 16SrII phytoplasmas is an indication of phytoplasma population presence in the infected plant.

SEROLOGICAL PROOFS FOR RELATEDNESS OF THE MYCOPLASMALIKE ORGANISMS (PHYTOPLASMAS) FROM APPLE PROLIFERATION GROUP

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Two polyclonal antibodies were produced in rabbits for apricot chlorotic leaf roll (ACLR) MLO purified from apricot, and for European Aster yellows (EAY) cultivated in artificial media. Both antisera were used as primary antibody to detect apple proliferation (AP15), pear decline (PD) and ACLR, in a comparative study with EAY MLO purified from periwinkle. AP15 (from Udine, Italy) was kindly provided by E. Seemüller, Germany, and in our laboratory, it was identical by symptoms (virescence and proliferation) in periwinkle with EAY isolated in Romania and with American severe western yellows. An indirect dot blot ELISA method was performed on nitrocellulose membrane stripes using secondary antibodies Goat anti-rabbit IgG conjugated with Alkaline Phosphatase (Sigma product No. A3937) and Goat anti-rabbit IgG conjugated with colloidal gold (Sigma product No. G3779). Positive reactions were identified for alkaline phosphatase using SIGMAFAST-BCIP/NTB (Sigma product No. B0274 substrate). When EAY and AP15 MLOs purified from periwinkle were tested with antiserum for ACLR, these gave positive reaction. Pear decline MLO extracted from pepper infected by Cacopsylla pyri and from pear trees was also detected with anti ACLR MLO serum. MLO purified from C. pyri insects used in experimental transmissions was also detected. These results support the hypothesis that EAY, AP15, PD and ACLR MLOs are identical serologic. The EAY antiserum recognized AP15 and EAY MLOs (phytoplasmas) purified from periwinkle when both molecular markers (AP and Colloidal gold) were used. The MLOs from AP15 and AP15 group (PD, ACLR) were also identical with EAY which infect more than 500 species of cultivated and spontaneous plants. Recent investigation in Germany, based on PCR analyses of 16S ribosomal DNA revealed that AP, PD and ACLR are identical pathogens.

MULTILOCUS GENE ANALYSES OF '*CANDIDATUS* PHYTOPLASMA MALI' CONFIRMS THE GENETIC DIVERSITY OF PHYTOPLASMA POPULATION IN THE CZECH REPUBLIC

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Apple trees are affected by the apple proliferation (AP) disease in several European countries. The AP is usually associated with the presence of '*Candidatus* Phytoplasma mali' ('*Ca.* P. mali'), a quarantine pathogen in EU. Samples from several apple trees showing proliferation disease symptoms were collected during 2010 at 28 different locations all over the Czech Republic. Phytoplasma strains were detected through polymerase chain reaction and restriction fragment length polymorphism analyses of 16S rDNA-spacer region-23rDNA, ribosomal protein gene sequences *rpl22* and *rps3* and non ribosomal DNA fragment (nitroreductase-like gene). All 74 apple trees examined were positive for phytoplasmas, predominantly for '*Ca.* P. mali' (60 trees). '*Ca.* P. asteris' alone and in also mixed infection with '*Ca.* P. mali' was detected sporadically. In the 16S plus spacer region of '*Ca.* P. mali' two profiles P-I (39 trees) and P-II (5 trees) singly or together (8 trees) were identified. The presence of two genetic lineages designated in literature as pattern '1' (7 trees) and '2' (31 trees) singly and also mixed (7 apple trees) was detected while the only pattern named 'a' was identified in 45 apple trees. '*Ca.* P. mali' strains belonging to ribosomal protein rpX-A subgroup were identified in the majority of apple samples (51 out of 55 plants positive in direct PCR amplification with rpAP15f/rpAP15r primers), while phytoplasmas belonging to rpX-B subgroup were detected sporadically (four trees). Nearly equal distribution of apple proliferation subtypes AP-15 and AT-2 (in 21 and 22 trees, respectively) was determined, while apple proliferation subtype AT-1 was detected in 7 trees. Nucleotide sequence analyses of the 16S-23S ribosomal operon, ribosomal proteins L22, S3 and nitroreductase-like protein gene of five selected apple proliferation phytoplasma strains confirmed the PCR/RFLP analyses results. This is the first study of molecular diversity among '*Ca.* P. mali' strains in the Czech Republic.

EARLY AND RELIABLE DETECTION OF EUROPEAN STONE FRUIT YELLOWS PHYTOPLASMA IN PEACH TREES

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The presence of European stone fruit yellows (ESFY), syn. ´*Candidatus* Phytoplasma pruni´ detection in peach trees grown in South Moravia, Czech Republic was studied during five years. ESFY symptoms were evaluated visually and biological indexing was carried out on the peach indicator GF-305. Three different procedures of molecular detection of ESFY were used. The first was PCR with primers ECA1/ECA2 according to Jarausch et al. (1998), the second one with primers fAT/rPRUS according to Smart et al. (1996), and third procedure was nested PCR with primers R16R0/R16F1 in a first step and primers R16F2/R16R2 in a second step according to Lee et al. (1995). Detection of ESFY in peach trees by biological indexing was found to be less suitable, the presence of pathogen was proved in less than 60% of symptomatic trees. Results of the all three applied PCR procedures were the same, but reactions of nested PCR were more strong. The presence of ESFY was detected in ca 75% of symptomatic trees. Results of PCR were negative in symptomless peach trees. The presence of ESFY symptoms is still most reliable criterion for detection of ESFY disease in peach trees grown in the Czech Republic. Further improving of PCR procedures is necessary for early and reliable detection of ESFY in peach trees.

DETECTABILITY OF PHYTOPLASMAS IN NATURALLY INFECTED *PICEA* AND *PINUS* SPP. TREES BY PCR

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Coniferous plants in Europe are natural hosts of three phytoplasmas - *'Candidatus* Phytoplasma asteris', phytoplasma belonging to X-disease phytoplasma group and '*Ca*. P. pini'. Very recently '*Ca*. P. pini' was identified in trees and shrubs of eight species. Unfortunately, the previous studies yielded unsatisfactory results concerning phytoplasma detection in coniferous plants. This work was undertaken to study round-year detectability of phytoplasmas in the needles of naturally infected coniferous plant species and to examine detectability of '*Ca*. P. pini' in plant material stored at different conditions (fresh needles, needles dried at room temperature and stored at 20°C or -18°C, lyophilized material stored at 4°C and frozen needles stored at -70°C). Experiments with phytoplasma infected pine and spruce trees indicated that phytoplasma detection measured by PCR assay was different depending on the host plant as well as time of testing. In *Picea tabuliformis* tree, '*Ca*. P. pini' was detected in samples of needles collected every month. In contrast, in genotypes such as *Picea pungens* and *P. abies,* phtytoplasma detectability was lower. In two *P. abies* trees infected with phytoplasma X-disease, the pathogen was detected in 70% of the tested samples mainly in nested PCR using P1/P7 followed by R16F2n/R16R2 primer pairs. In two *P. pungens* trees affected with '*Ca*. P. pini' phytoplasma was detected only in spring and summer months. In affected *Pinus banksiana* and *P. sylvestris* plants *'Ca*. P. pini' could be detected in direct PCR in fresh plant material as well in needles stored for 12 months at -70^{o}C or stored at 4^{o}C after lyophilization. Detectability of phytoplasma in needles dried at room temperature and stored at -18^{o}C or at 20^{o}C for 12 months was relatively lower; in these samples phytoplasma could be detected only by nested PCR.

FIRST OCCURRENCE OF PEAR DECLINE DISEASE IN PORTUGAL

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All over the world over than a thousand phytoplasma-associated diseases is known, causing high economic losses. They affect all kind of plant species, such as vegetables, fruit trees and ornamental plants. There is no treatment to this kind of organisms, being most of them included in EPPO quarantine list. Throughout the years, biomolecular techniques were developed to detect and identify phytoplasmas, where the most important is nested-PCR for detection complemented with RFLP for identification and recently the real-time PCR. Fruit trees' phytoplasmas are widespread, mainly in Europe, and cause severe fruit production losses. This study concerned the apple proliferation group phytoplasmas that gathers three major phytoplasmas: '*Candidatus* Phytoplasma mali', '*Ca.* P. pyri' and '*Ca.* P. prunorum', associated with apple proliferation (AP) and pear decline (PD) diseases in pome fruit trees and European stone fruit yellows (ESFY) in stone fruit trees. These phytoplasmas are vectored by psillids *Cacopsylla picta*, *C. pyri* and *C. pruni*, respectively. Until now, there is no evidence of any of these diseases in Portugal, as well as of their vectors, except for *C. pyri*, the only one reported until now in Portuguese pear orchards. Portuguese pear cv. Rocha is widely cultivated in Portugal, being a very important icon to national agriculture. This cultivar is exported worldwide, representing a major income in Portuguese economy. Due to its importance, it is fundamental to control all diseases infecting pear trees. As *C. pyri* is very abundant in Portuguese pear orchards and cause severe damages by itself, it is important to know if the insect or the trees are infected with PD. As there are no previous records of the disease in Portugal, the main goal of this study was to assess the presence of disease in order to prevent and control future spread. As the other two vectors were never found in Portugal, one of the goals of this study was to look for them in apple orchards (where *C. picta* feeds) and prunus (peach, apricot, and cherry) orchards (where *C. pruni* feeds). The search was not successful so testing for AP and ESFY phytoplasmas was limited to vegetal samples, all negative for Portuguese orchards. Some of the captured insects revealed to be positive for the PD phytoplasma, as well as some pear tree samples. The positive results, obtained by nested-PCR were confirmed by RFLP, nucleotide sequencing and PD specific real-time PCR assays. Work on this disease is now in progress.

SURVEY FOR APPLE PROLIFERATION IN ORCHARDS CLOSE TO NURSERIES IN NORWAY IN 2011

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Apple proliferation (AP) is one of the most serious plant diseases in the main apple production areas all over Europe. The losses are estimated to be very high. Apple production is important in specific areas in both the western and the eastern parts of Norway. AP is listed as a quarantine disease in Norway and until 1996 it was never reported in Norway. A survey in the years 1996 and 1997 revealed 14 diseased trees in orchards throughout the country. It turned out that several of these cases were relatively old trees and it was suspected that some infected material entered the country in the 1970-ties and has been spread to some extent by vegetative propagation. No conclusive evidence for natural spread by vectors was found. The infected trees at these locations were eradicated. From 2000 until 2008 only two or three more infected trees were detected and eradicated. In 2010, however, some new serious cases of AP disease were found in the western parts of Norway. This led to the initiation of a survey program for orchards close to nurseries in the most important fruit districts. During autumn 2011 orchards close to nurseries were visited for visual inspection and sampled by the Norwegian food safety authorities. Small branches from sampled trees were sent to Bioforsk for testing by PCR analysis. In the orchards where no AP-like symptoms were found a random sampling was carried out. DNA was extracted using the DNeasy® Plant Mini Kit (Qiagen), according to the manufacturer's instructions. PCR was performed according to the TaqMan real-time PCR protocol as described by Nicolić et al. 2009. In 2011, altogether 250 samples from 126 orchards close to nurseries were analyzed for AP. Altogether 139 samples representing 43 orchards were infected. AP was found in both symptomatic and asymptomatic trees. There was not found any infected trees in the nursery production in any of the nurseries in Norway. This is due to a strict use of healthy propagation material and good control of potential vector populations.

EVIDENCE FOR *'CA*. P. PYRI' AND *'CA*. P. PRUNORUM' INTER-SPECIES RECOMBINATION

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The genetic diversity of three temperate fruit tree phytoplasmas; '*Candidatus* Phytoplasma prunorum', '*Ca.* P. mali' and '*Ca*. P. pyri' has been established by multilocus sequence analysis. Genotyping of some Spanish and Azerbaijanese '*Ca*. P. pyri' isolates revealed that they possess both '*Ca.* P. pyri' and '*Ca*. P. prunorum' genetic markers, supporting for the first time the existence of inter-species recombination between these two species. Particularly, isolate Spa3 had aceF and imp genes of '*Ca.* P. prunorum' while its pnp and secY genes corresponded to '*Ca.* P. pyri' genotypes. Isolate Spa2 also had two genes corresponding to '*Ca*. P. prunorum' genotypes and two genes clustering within the '*Ca.* P. pyri' phylogenetic branch, whereas AZ-POI45 had only a '*Ca.* P. prunorum' pnp gene and three other gene markers corresponding to '*Ca.* P. pyri'. Altogether, these results indicated that these isolates of '*Ca.* P. pyri' are inter-species recombinants. Surprisingly, for the Spa2 isolate, two different imp sequences could be detected, one corresponding to the one of '*Ca.* P. prunorum' and the other clustering with 'Ca. P. pyri'. This could not be associated with a mixed infection as only '*Ca.* P. pyri' 16S rDNA sequence could be detected. A simple recombination event between '*Ca.* P. pyri' and '*Ca.* P. prunorum' can lead to the exchange of many gene markers at the same time. However, to allow recombination between two phytoplasma species, they have to share a common host. Peach has been described as a common host for these two phytoplasmas. Recombination might also have occurred in a common insect vector, such as Cacopsylla pyri, which is more polyphagous in autumn and might acquire both phytoplasmas from their respective host plant. Because none of the three '*Ca*. P. pyri' recombinants had the same MLST genotype, it is likely that the recombination event is quite frequent. Indeed, it represented three cases from two distinct geographical areas over 19 different '*Ca*. P. pyri' isolates tested. One could certainly consider that if recombination occurs between species it could also occur within species. We cannot confirm such a phenomenon because, despite incongruence between the phylogenetic trees of the different markers, the bootstrap validity levels were not high enough to consider it evident.

ENRICHMENT OF PHYTOPLASMA DNA BY SELECTED OLIGONUCLEOTIDES AND PHI29 POLYMERASE AMPLIFICATION

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Phytoplasma have resisted all attempts of cell-free cultivation so far. This problem hampers genome research. Elaborate and material intensive approaches are used to enrich the phytoplasma DNA. We present an amplification-based approach to obtain phytoplasma DNA from a few grams of plant tissue for downstream applications such as genomic draft sequencing. Total DNA was extracted by CTAB extraction from tobacco and parsley infected by stolbur strains 284/09 and 231/09, respectively. For enrichment, genomic DNA was amplified using oligonucleotides deduced from the four published complete phytoplasma genomes, random hexamers and Phi29 polymerase. Twenty-eight oligonucleotides were selected by frequency and distribution in the complete genomes. In additional experiments, the application of phytoplasma-specific primers P1/P7 was evaluated. *De novo* assemblies of short-reads with a length of 36 bases were generated. BLASTX against NCBI's NRPROT database using contigs with a minlength of at least 300 bp was analysed with MEGAN and taxonomical assignment of the contigs performed. Enrichment was measured by illumina's sequencing by synthesis approach. Up to a fifteen-fold increase of obtained phytoplasma draft sequence resulted from the usage of the determined oligonucleotides. Individual assemblies of short single-reads resulted in an average contig length of 1.3 kb for strain 231/09 and 2.5 kb for strain 284/09 and a total contig length of >474 kb and >498 kb, respectively. Preliminary, results indicate only weak phytoplasma enrichment in the amplification experiments supplemented with P1 and P7 oligonucleotides. Combining the reads of the individual experiments resulted in a draft sequence of >516kb for strain 231/09 and >557 kb for strain 284/09. Sequencing and annotation results highlight the potential of this strategy for uncharacterized phytoplasma genomes in particular. It is shown that cost-saving short-read sequencing can be used to generate efficient draft sequences from these templates.

GENETIC DIVERSITY, MEMBRANE TOPOLOGY, AND RELATIONSHIP TO VIRULENCE OF THE AAA+ ATPASES AND HFLB PROTEASES OF '*CANDIDATUS* PHYTOPLASMA MALI'

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One of the economically most important phytoplasma pathogen in Europe is '*Candidatus* Phytoplasma mali', the agent associated with apple proliferation disease. As other phytoplasmas, '*Ca*. P. mali' resides in plants in the sieve elements of the conducting phloem tissue where it may induce severe histopathological aberrations including the deposition of pathological callose and sieve tube necrosis. These symptoms lead to the accumulation of starch in the aerial parts of the trees and depletion of starch in the roots and may severely affect the performance of the trees. In more recent studies, accumulation of hydrogen peroxide and phloem protein and the upregulation of genes involved in synthesis of these compounds were also identified. In addition to this data on possible pathogenicity factors, phylogenetic comparison of sequences of one of the *hflB* genes (AP464) annotated in the '*Ca*. P. mali' chromosome revealed that mild and severe strains cluster separately, according to their virulence. *HflB* (synonym *ftsH*) genes encode membrane-associated ATP- and Zn^{2+}-depending proteases that are conserved among bacteria and degrade misassembled and short-lived proteins and are thus contributing to quality maintenance of proteins in the membrane and the cytoplasma. The HflBs are composed of ATPase domains and a protease module. The high copy numbers of *hflB* genes in '*Ca*. P. mali' and other phytoplasmas is unusual in prokaryotes and seems to indicate their importance for these plant pathogens. To further investigate the possible role of HflB proteins in the virulence of '*Ca*. P. mali', we PCR-amplified and sequenced all genes annotated as *hflB* in the chromosome of strain AT from a representative number of mild and severe strains. Analysis of deduced amino acid sequences revealed that seven of the annotated HflBs lack the protease module and have thus to be classified as AAA+ ATPases (ATPases Associated with various cellular Activities). Furthermore, several predictors indicated that the enzymatically relevant C-termini of most of the AAA+ ATPases and about half of the HflB proteases are facing the extracellular space and may thus be involved in the impairment of sieve element function. Other findings are that full-length genes from the only cytoplasmatic ATPase (AP11) can only be amplified from mild and moderately virulent strains whereas severe strains yielded truncated genes only. Degeneration was also observed in one of the cytoplasmatic proteases (AP454) from which only a fragment could be amplified from all strains. In contrast, amplification of all AAA+ proteins facing the extracellular space resulted in full-length genes. In addition, phylogenetic analysis showed that the amino acid sequences of mild and severe strains of most of the proteins examined cluster distantly, mostly associated with the presence of virulence-related amino acid markers. In conclusion, the data reported here support previous indications that the AAA+ ATPases and HflB proteases of '*Ca*. P. mali' appear to be involved in pathogenicity.

TRANSCRIPTOME ANALYSIS OF *VITIS VINIFERA* cv. CHARDONNAY INFECTED WITH GLRAV-3 AND ASTER YELLOWS PHYTOPLASMA IN A SOUTH AFRICAN VINEYARD

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Grapevine is one of the most important fruit crops grown in South Africa. Fruit productivity and quality can be greatly affected by diseases caused by various pathogens. Grapevine leafroll disease is prevalent in the grapevine production areas of South African. More recently, vineyards with aster yellows (AY) phytoplasma infection were reported in parts of the Western Cape winelands of South Africa and AY is becoming a threat to the grapevine industry. Using next-generation sequencing, we investigated the effects of *Grapevine leafroll associated virus-3* (GLRaV-3), the predominant virus associated with leafroll disease in South Africa and aster yellows phytoplasma on the transcriptome of Vitis vinifera cv. Chardonnay plants grown in the field. Young shoots were collected from a 6 year-old vineyard from the Western Cape region infected with GLRaV-3 and AY. The samples were tested for the presence of common grapevine viruses in South Africa, GLRaV-3 and aster yellows phytoplasma. Twelve samples were selected for RNA sequencing: three samples negative for common viruses, GLRaV-3 and AY; three positive for GLRaV-3; three for aster yellows and the remaining three were positive for both GLRaV-3 and AY. Total RNA was extracted from these samples and sequenced using the Illumina Hiseq 2000 platform. The infected samples were compared to non-infected samples. Preliminary data showed that more genes are differentially expressed in the mixed infected plants (GLRaV-3 and aster yellow) compared to singly infected plants. The significance of the data will be discussed. To our knowledge, this is the first report investigating the effects of mixed infection between a grapevine virus and phytoplasma from field samples.

SUMMARY OF QUESTIONNAIRE DATA FOR THE PRESENCE OF PHYTOPLASMA DISEASES AND THEIR PUTATIVE VECTORS THROUGH EUROPE AND MIDDLE EAST

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Phytoplasmas occur in hundreds of commercial and wild host plants and are associated with many different diseases. In nature they are mainly spread by insects of the families Cicadellidae (leafhoppers), Cixiidae, Delphacidae and Derbidae (planthoppers), and Psyllidae (psyllids). With the development of diagnostic techniques the number of new phytoplasma diseases described worldwide markedly increased. However, there are still no effective control measures able to reduce the spread and the damage of phytoplasma diseases in many crops, mainly due to the lack of information on their epidemiology. Therefore, one of the main tasks of the WG2, which concerns phytoplasma epidemiology and vector ecology, is the establishment of a phytoplasma and vector monitoring system throughout Europe. For this purpose, a questionnaire about phytoplasma diseases and their putative vectors throughout European regions have been drafted and distributed to all members of the COST action FA0807. The obtained data have been used to create a database on the presence of phytoplasma diseases and their putative insect vectors in different European and Middle East regions. Information on the collection methods as well as on the phytoplasma detection tools is provided. Moreover a series of maps summarizing the five major phytoplasma-associated diseases (AP, ESFY, PD, BN and FD) and their known and putative vectors have been drawn up.

PSYLLID VECTORS OF THE AP GROUP (16SrX) PHYTOPLASMAS IN TURKEY

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A survey was conducted in 2010 and 2011 in order to determine the presence of known vectors of apple proliferation group phytoplasmas (16SrX), namely '*Candidatus* Phytoplasma prunorum', '*Ca*. P. mali' and 'Ca. P. pyri', in different plants hosts (wild plums, conifers, and/or orchard trees). *C. pruni* individuals were collected from *Prunus spinosa*, some other *Prunus* spp. and *Abies* spp. from different provinces of Turkey. Molecular typing based on COI genes and an ITS region indicated that all individuals were from the species B. '*Ca*. P. prunorum' infection rate in overwintered adults of *C. pruni* was estimated as 4.70%. Phytoplasma infection rate in new generation adults of *C. pruni* collected only from Mersin province was estimated as 4.64%. Psyllids, collected from wild and cultured forms of pome fruit trees, were identified as *C. picta, C. melanoneura, C. affinis, C. crataegi, C. pyrisuga, C. pyri* and *C. pyricola* according to their morphological characters. The most common psyllid among the collected ones, *C. picta,* was infected with '*Ca.* P. mali' with the ratio of 4.36%, but one sample was infected by '*Ca*. P. prunorum'. *C. crataegi* individuals collected from hawthorn were also infected by '*Ca.* P. mali' (2.90%). *C. melanoneura* and *C. affinis* were infected by '*Ca*. P. pyri' with respectively a ratio of 3.63% and 2.27%, and never by '*Ca*. P. mali'. The psyllid *C. pyri* were found positive for '*Ca*. P. pyri' (2.75%). The individuals of the species *C. pyrisuga* and *C. pyricola* were negative for phytoplasma presence.These preliminary data indicated that, *C. pruni*, the main vector of '*Ca*. P. prunorum' is prevalent in the country. The main vector of '*Ca.* P. pyri' was *C. pyri,* and *C. picta* might be the main vector of '*Ca*. P. mali' Experimental transmission assays by using these vectors are in progress.

DEGREE OF SPECIFICITY AMONG THE PSYLLID-PHYTOPLASMA INTERACTIONS AND CONSEQUENCES IN EPIDEMIOLOGY

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The psyllid vectors often show close association with the pathogens they transmit. In particular, the eight known vectors of the genus Cacopsylla transmit only one phytoplasma species, and on the contrary a single phytoplasma species may be transmitted by only one or no more than two psyllid species. When two species are described they are generally very closely related from a taxonomic point of view, such as for the vectors of ‘Candidatus Phytoplasma pyri’. Recent results confirmed this degree of specificity of the vector-psyllid interaction (C. pruni species complex) or suggested that this could be even more specific (different populations of the same species would be able to have a distinct transmission efficiency towards ‘Ca. P. mali’). On the other hand, other reports are suggesting that several species living on the same host plant may transmit the same phytoplasma (‘Ca. P. mali’ and the psyllids on hawthorn). We discuss why these new results have important consequences for the investigation and the risk assessment of the role of a specific psyllid species in the spread of a phytoplasma disease.

PHYTOPLASMAS ASSOCIATED WITH APRICOT CHLOROTIC LEAFROLL DISEASE

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Apricot trees are affected by apricot chlorotic leafroll disease in all major cultivation areas in Europe: the disease is killing the plants few years and it widely associated with European stone fruit yellows (ESFY) '*Candidatus* Phytoplasma prunorum' belonging to 16SrX-B. Samples from apricot trees showing typical chlorotic leafroll symptoms were collected in June/July 2010 in Serbia and Italy in orchards where the disease was present in scattered distribution in 7 and 8 year old orchards. Ten symptomatic and two asymptomatic plants were tested in total. Phytoplasma detection was achieved by polymerase chain reaction and restriction fragment length polymorphism analyses using nested-PCR assays. Primers employed were P1/P7 followed by F1/B6 or R16F2n/R2. RFLP analyses on F1/B6 amplicons indicated the presence of 16SrXII in one of the samples from Serbia and of 16SrX-B in one of the samples from Italy. RFLP analyses with *Tru1*I, *Rsa*I, *Hinf*I, *Hha*I, and *Hpa*II on R16F2n/R2 amplicons showed the presence of 16SrX-B phytoplasmas in 4 samples, two from the Italian orchard and two from the Serbian one. In the remaining three Serbian apricot samples a mixed infection of 16SrX-B and 16SrXII and a mixed infection of 16SrX-B, 16SrXII and 16SrI phytoplasmas were identified; one of the samples was negative. In two of the remaining samples from the Italian orchard a 16SrX-B and 16SrXII mixed infection was detected, while in the last sample 16SrX-B and 16SrIX-C phytoplasmas were identified. The use of nested PCR with PA2 primers on P1/P7 allow to detect 16rXII phytoplasma in the last sample from Serbia. In all the experiments carried out the two asymptomatic plants were negative. This is the first report of presence in symptomatic apricot of phytoplasmas different from ESFY; work to verify the epidemic relevance of these phytoplasmas in the apricot chlorotic leafroll disease is in progress.

ALMOND WITCHES' BROOM PHYTOPLASMA, OFFICIALLY DECLARED AS A REGULATED PEST IN LEBANON

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Surveys conducted during 2009/10 showed that almond witches' broom phytoplasma (AlmWB) disease, incited by 'Candidatus Phytoplasma phoenicium', was widespread in Lebanon and was detected in 16 out of 26 districts. Almond, peach and nectarine were severely affected. The North of Lebanon was considered as the epidemic center from which the disease had spread to the other regions. Due to the devastating losses already incurred and in order to reduce further future losses, HE the Minister of agriculture held a press conference on January 17, 2011 during which he officially declared AlmWB as a regulated pest in Lebanon and that a National plan will be implemented for the integrated management of the disease. Farmers will be encouraged to eliminate infected trees in all areas and will be compensated by providing free seedlings of replacement crops. The major stone fruit cropping regions in Bekaa (West Bekaa and Rachayya) will be given priority and the extension service will actively help in the eradication process. The field activities started on March 2012 with financial support from the Italian government. Updates on the actions taken will be presented during the meeting.

MONITORING DISTRIBUTION OF FRUIT TREE PHYTOPLASMAS IN BULGARIA FROM 2007 UNTIL 2011

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A survey was conducted from August to November during the years 2007 until 2011 in selected fruit tree orchards in the territory of all 28 districts of Bulgaria, as part of the official fruit trees monitoring program of National Service on Plant Protection (now Bulgarian Food Safety Agency). The plant material was collected from symptomatic and non-symptomatic trees and was sampled in late autumn when the concentration of the phytoplasma in the tree is highest. The priority was to collect samples from plant material originating from fruit tree nurseries and mother stock samples. All samples were analyzed by PCR in the Central Laboratory for Plant Quarantine. During the described period phytoplasma-infected plants were found in 10 of the 28 investigated regions. '*Candidatus* Phytoplasma mali' the agent of apple proliferation (AP) and '*Candidatus* Phytoplasma prunorum', the agent of European stone fruit yellows (ESFY) were found in samples from five different regions. Pear samples from 7 different regions in Bulgaria were found to be infected with '*Candidatus* Phytoplasma pyri', the agent of pear decline (PD). The incidence of apple proliferation in 2009 and 2010 was reduced compared to 2007 and 2008 because of the applied phytosanitary measures. As a result, in 2011 no cases of AP infection were found. ESFY infection was found in 2 samples in 2009, in 5 samples in 2010, and as well in 5 samples in 2011. PD infection was found in 2 samples in 2009, in 6 samples in 2010, and in 4 samples in 2011. The infected plants have been destroyed through burning and the affected areas have been taken under quarantine. Phytosanitary prevention measures should be taken for ESFY and especially for PD to minimize infection spreading.

INDUCTION OF DEFENSE GENES IN APPLE LEAF TISSUES BY AN ENDOPHYTIC STRAIN OF *EPICOCCUM NIGRUM* AND ITS POSSIBLE USE IN THE CONTROL OF THE APPLE PROLIFERATION DISEASE

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Endophytes are micro-organisms living inside host plants without causing disease symptoms or visible injury. The ecological role of these organisms is still not well determined but most of them exhibit positive effect for the host plants by promoting growth, improving resistance to multiple stresses and protecting them against pathogens and insects. Apple proliferation (AP), associated to '*Candidatus* Phytoplasma mali' ('*Ca*. P. mali') is one of the most economically important disease affecting apple in Europe. The possibility to use endophytes as biocontrol agents or resistance-inducers against phytoplasmas has been recently reported. Musetti et al., reported that an endophytic strain of *Epicoccum nigrum*, inoculated in *Catharanthus roseus*, experimentally infected with '*Ca*. P. mali', reduced symptoms severity and phytoplasma titre inside the plant tissues, inducing ultrastructural modifications both to the phytoplasma and to the host. Starting from these promising results and aiming to assess the possibility of using *E. nigrum* in the AP control an experiment has been carried out using the natural host of '*Ca.* P. mali', *Malus domestica*. Four groups of young apple trees (10 individuals each group) were set up: only *E. nigrum* inoculated, E^{+} AP^{-}; *E. nigrum* inoculated then infected with '*Ca.* P. mali', E^{+} AP^{+}; only '*Ca.* P. mali' infected, E^{-} AP^{+}; control plants E^{-} AP^{-}. AP symptom expression, plant and phytoplasma ultrastructural modifications as well as phytoplasma titre in the different groups of plant are evaluated, together with the expression of some defence-related genes. In this work, we investigated the expression of apple genes coding for three pathogenesis-related (PR) proteins, PR 1, PR 2, PR 5 and for three jasmonate (JA)-pathway marker enzymes, Allene Oxide Synthase 2 (AOS 2); 12-Oxyphytodienoate reductase 3 (12-OPR 3); JA-inducible Proteinase Inhibitor II (PI II), comparing their relative expression levels in apple plants inoculated or not by *E. nigrum* ($E^{+}AP^{-}$ and $E^{-}AP^{-}$ plant groups). Expression profiling of the selected genes revealed a different expression pattern according the two different plant groups. In particular, PR1 and PR5 resulted significantly induced (respectively 8 and 4 folds) in $E^{+}AP^{-}$ plants 3 day after endophyte inoculation (dai) compared to the uninoculated controls ($E^{-}AP^{-}$). The expression level of the induced genes tends to return comparable to the control two weeks and two months after endophyte inoculation. On the other hand variations in the expression level of the genes encoding the three JA-pathway marker enzymes at 3 dai seem to be not considerable in $E^{+}AP^{-}$ leaf tissues compared to the control. From these preliminary results, we can hypothesize that *E. nigrum* inoculation can induce in apple defense responses related to salicylic acid pathway and PR protein gene expression, inhibiting, on the other hand, JA-related defense pathway. Further analyses will be carried out aimed to understand the mechanism(s) by which *E. nigrum* interacts with apple plants and to assess the real possibility of its usefulness in AP disease control.