Analysis of the acquisition and multiplication efficiency of different strains of Ca. 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 realtime PCR. Despite high acquisition rates only few subsequent transmission events to healthy test plants could be recorded.

Oral Session IX

Strain differentiation of *Candidatus* Phytoplasma Mali by SSCP- and sequence analyses of the HFLB gene

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Apple proliferation caused by *Candidatus* Phytoplasma mali is an important disease in Central and Southern Europe and reports from Greece and Turkey reflect the spread of the disease. The pathogen itself is known for almost forty years but due to a lacking in vitro culture little biological information is available on the causal agent. Field trials, however, have demonstrated that differences, expressed in virulence, are present. Although, the complete genome sequence of one Ca. P. mali strain is known, the search for genes related to pathogenicity is ongoing. A number of putative candidate genes have been identified, amongst others the *hflB* gene. This gene is present in multiple copies which might reflect its importance for the organism. To explore the *hflB* gene of pathogen strains and isolates in more detail a 530 bp PCR fragment from 42 German, Italian and French accessions were examined. The amplimers were analyzed by singlestrand conformation polymorphism (SSCP) analysis and revealed an unexpected variability. More than 20 different profiles could be discerned. Sequencing of the PCR fragments showed that the nucleic acid homology of the strains ranged from 94.2% to full identity. Although the differences in gene sequence could not yet be related to pathogenicity traits the fragment is predestined as molecular marker for tracking strains in epidemiological studies as well as defining and monitoring inoculum strains in resistance breeding projects.

Molecular characterization of *Candidatus* Phytoplasma mali strains in outbreaks of apple proliferation in north eastern Italy, Hungary, and Serbia

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During 2005-2008 apple plants showing proliferation symptoms were observed in diverse cvs in different areas of north eastern Italy (Veneto and Trentino), Hungary and Serbia. Leaves and young shoots were collected from June to October in orchards with epidemic presence of apple proliferation, and in others where the symptomatic plants were present in a scattered way. PCR/RFLP amplification carried out on R16F2/R2 amplicons showed that all the samples were infected with 'Candidatus Phytoplasma mali'. Further strain characterization was carried out using RFLP analyses with HpaII and FauI on almost full ribosomal DNA plus spacer region, *Alu*I on *rpl22-s3* genes, and RcaI and HincII on AP13/AP10

amplicons from representative samples collected in these geographic areas. Analyses of 16S plus spacer region distinguished two phytoplasma profiles (P-I and P-II). P-I was detected in reference strains AP, AT1, AT2, in samples from Serbia, and in the majority of samples from Trentino. P-II profile was prevalent in samples from Veneto, and both profiles were identified in samples from Hungary, in the majority of the cases together. The analyses of *rpl22-s3* genes allow to identify in all the samples showing P-I profile the presence of phytoplasmas belonging to rpX-A subgroup, while in samples showing P-II profile it was possible to distinguish the four described rp subgroups. In the majority of samples from Veneto region phytoplasmas belonging to rpX-D subgroup were identified, while rpX-B and X-C subgroups were identified in a few samples from Trentino and Veneto regions respectively. Further RFLP characterization on AP13/AP10 amplicons differentiates among strains belonging to rpX-A subgroup: the Serbian samples show AP profiles, while those from Trentino show AT-2 profiles. In the samples from Hungary the presence of AT1, AT2, and AP profiles was identified. The combined use of these molecular markers allows differentiating 'Ca. P. mali' strains.

Breeding of rootstocks resistant to apple proliferation disease

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Apple proliferation (AP) is caused by the wall-less bacterium *Candidatus* Phytoplasma mali and is spread by psyllids. Previous work indicated that, due to the colonization behavior of the causal agent, the disease could be controlled by the use of resistant rootstocks. Unfortunately, extensive screening revealed no satisfactory resistance in established rootstocks. In contrast, substantial levels of resistance were identified in experimental rootstocks derived from crosses of the apomictic species *M. sieboldii* and genotypes of $^{M.\ x\ domestica}$ and *M.* x *purpurea*. However, these hybrids were more vigorous and less productive than standard stock M 9. For this reasons, a program was initiated to reduce vigor and improve yield by crossing and backcrossing M. sieboldii and its apomictic hybrids with M 9 and other dwarfing stocks. From 2001 through 2006 a total of 36 crosses were made. However, only 23 progenies consisted of a substantial number of seedlings while the other crosses largely failed due to pollen incompatibility. The 3.500 seedlings obtained were DNA-typed using codominant SSR markers to distinguish apomicts and recombinants in the progenies. A total of 1.800 seedlings consisting of all recombinants and a representative number of apomicts were screened for AP resistance by graft inoculation followed by observation in the nursery and under commercial growing conditions. Several progenies showed a good inheritance of resistance. In two of them (4608 x M 9 and D2212 x M 9) more than 50% of the individuals never developed symptoms.

Influence of apple stem grooving virus on *Malus sieboldii*-derived apple proliferation resistant rootstocks

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Apple stem grooving virus (ASGV, Capillovirus) is widely spread in apple growing regions. As it causes no symptoms on most cultivated apple varieties and rootstocks it is considered latent in *Malus* x *domestica*. In Asia, however, ASGV has been found associated with topworking disease of apple rootstocks originating from *Malus sieboldii*. Recently, *M. sieboldii* and its hybrids have gained new interest in Europe as they confer resistance to apple proliferation (AP) disease. A new breeding program aiming to develop AP-resistant rootstocks of agronomic value for modern apple culture, reported unexpected tree decline and found it to be associated with ASGV. As little information is available on the variability of ASGV isolates in Germany, the complete genome of a German isolate of ASGV associated with tree decline was cloned and sequenced. Sequence comparisons with available ASGV isolates revealed two regions of high variability in the genome. The genetic variability of additional isolates from Germany and other countries were collected and the variable areas characterised. In addition ASGV was successfully maintained in micropropagated apple trees and could be transmitted by

in vitro grafting to various genotypes, making it possible to study the effect of the virus and virus/phytoplama combination on *M. sieboldii*-derived genotypes.

Identification of host plants for *Candidatus* Phytoplasma prunorum and of his vector *Cacopsylla pruni* in SPAIN

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Candidatus phytoplasma prunorum is the causal agent of the "European stone fruit yellows" disease. This phytoplasma infects different wild and cultivated Prunus species that are tolerant to the disease, favouring a natural cycle: vector- Prunus-pathogen, making the disease unnoticed. The introduction of a more sensitive species like the Japanese plum-tree (*Prunus salicina*) in an agroecosystem with many several inoculum sources, like the Baix Llobregat area (Barcelona), and the presence of a very efficient vector in the acquisition and the transmission, has caused a quick dissemination of the disease.

Cacopsylla pruni was identified for the first time in Spain in the Baix Llobregat (Catalonia) in 2003 and later in other Spanish regions. The cycle of *C. pruni* has been studied during four years in this area. The insect population was evaluated in two geographical areas of wild Prunus (*P. mahaleb*) and in two commercial orchards of *P. salicina* with a high incidence of the disease. Adults appeared in the middle of February, and the populations reached two maximums, at the end of March and at the beginning of June. In the commercial plantations the species followed a similar evolution with interannual fluctuations. *C. pruni* was captured also in specific samplings on *P.spinosa* in different areas. The percentage of individuals of *C.pruni* carrying the phytoplasma was about 15 %, the high number of positives were identified in the re-immigrant adults (January - March) whereas in the new generations (April - June) the percentage of positives is lower. The phytoplasma was detected both in wild and cultivated Prunus. The fact that the vector might accomplish his life cycle on wild infected hosts, and emigrate to the commercial plots, despite the direction of the plantations, points out that wild Prunus has an important role in the epidemiology of the ESFY and therefore in his potential control.

Infection rates of natural psyllid populations with *Candidatus* 'Phytoplasma mali' in South Tyrol (northern Italy)

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Apple proliferation is a severe disease of apple trees spreading in many European apple growing areas. It is caused by *Candidatus* 'Phytoplasma mali' that was shown to be transmitted through infected grafting material, via natural root grafts and by sap-sucking insects. Two psyllid species, *Cacopsylla picta* and *C. melanoneura*, that are recognised as the vectors of the disease occur in orchards of South Tyrol (northern Italy). The aim of the study was to assess the infection rates of natural populations of these insect species with *Ca.* 'Phytoplasma mali'. Two additional psyllid species (*C.* mal*i* and *Trioza urticae*), which are frequent in apple orchards of South Tyrol, were also investigated. A total of 800 specimens from 18 orchards were analysed using a real-time PCR procedure. While no specimen of T. urticae was found to be infected with *Ca.* 'Phytoplasma mali', the mean infection rate of *C. melanoneura* and *C.* mali was below 1%. The highest infection rate was found for *C. picta*, with a mean value of 11%. Based on these results, it can be concluded that *C. picta* plays the major role as the vector of apple proliferation in South Tyrol.

Comparison of European stone fruit yellows phytoplasma strains differing in virulence by multi-gene sequence analyses

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Twenty strains of the ESFY phytoplasma, which on the basis of graft-inoculation experiments greatly differ in aggressiveness, were examined by sequence analyses of several PCR-amplified non-ribosomal genes in order to identify molecular markers linked to virulence. These strains, which were maintained in various stone fruit genotypes, were indistinguishable with techniques for routine phytoplasma differentiation and characterization such as sequence and RFLP analyses of PCR-amplified rDNA. Also, the virulent ESFY strains maintained in periwinkle, namely GSFY1, GSFY2 and ESFY1 as well as an avirulent strain of the same phytoplasma, maintained in apricot, which was identified in recovered apricot trees, in France, and used there as a cross protecting agent, were included in the work for comparison. For PCR amplification, primers were designed from a number of genes distributed over the chromosome of strain AT of the apple proliferation phytoplasma. Visible PCR products were only obtained with primer pairs derived from the tuf gene which encodes the elongation factor Tu (EF-Tu), *rps*C (*rps*3) gene encoding the ribosomal protein S3, tlyC gene which encodes hemolysins, a membrane-damaging agents that serve as important virulence factors for many bacteria, the imp and fol genes encoding an immunodominant membrane protein and an enzyme involved in the folate biosynthesis, respectively. Nucleotide sequence comparisons revealed that the highest genomic variability occurred within the imp gene sequence with dissimilarity values ranging from 0.2 to 4.6%. For the remaining genes, the strains examined proved to be identical o nearly identical. Within the tuf gene, the presence of an additional TaqI restriction site, which had already known to occur in the strain GSFY1, was not identified for the other strains. The genetic differences observed among the strains examined are neither suitable markers for strain differentiation nor linked to pathological traits.

Hypo and hyper-virulence in apricot trees infected by European stone fruit yellows

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An apricot orchard, located in an area of northeast Italy under high European stone fruit yellows (ESFY) infection pressure, has been monitored starting from the year of planting (1990). By time, most of the plants showed symptoms or resulted infected by PCR analyses. Particularly interesting resulted two groups of apricots: some asymptomatically infected and others recovered from the symptoms but not from the pathogen. With the aim to isolate strains of the phytoplasma characterised by different virulence, each group was used as mother plants and propagated. The new plants were used to constitute experimental orchards, where the plants were observed for the presence of symptoms and in part tested by PCR starting from 2003. The obtained results confirmed the presence of strains of the pathogen characterised by different virulence. The strains originally present in infected apricots recovered from the symptoms of ESFY resulted hypo-virulent; all the propagated infected plants never showed symptoms of the disease. Surprisingly, the strains present in asymptomatic mother plants of apricot resulted hyper-virulent and the propagated plants always showed severe symptoms. In the propagated plants, the transmission of the pathogen resulted higher for the hyper-virulent strains in comparison with the hypo-virulent ones. A graft transmission trial carried out in the greenhouse using some of the identified hypo and hyper-virulent strains, confirmed the results obtained in open field. Real time PCR analyses showed that in the plants infected by hypo-virulent strains the colonisation of the pathogen was lower than in the plants infected by the hyper-virulent ones. It is possible to affirm that the hypo-virulent strains were present in the originally recovered mother plants of apricot. The research will continue with the aim to verify the possibility of cross protection among the identified hypo and hyper-virulent strains.

Poster Presentations

First survey on blueberry viruses and phytoplasma in the Czech Republic

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First survey on the occurrence of viruses and phytoplasma in blueberry Vaccinium corymbosum (highbush blueberry) and naturally occurring Vaccinium species is currently conducted in the Czech Republic. Plantations, germplasma collections, propagation materials and wild plants are monitored for