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BOOK OF ABSTRACTS

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AIPP

INVITED LECTURES

The Good, the Bad and the Ugly: Which processes shape phytoplasma-insect-plant relationship?

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The association between insect herbivores and vascular plants represents one of the greatest success stories in terrestrial evolution. Microbes play important roles in mediating plant-insect trophic interactions, for example by supplementing nutritionally unbalanced diets, endosymbionts help facilitate adaptation of herbivores to their host plants. Parasitic bacteria (such as phytoplasmas) that manipulate host behavior and phenotype may have even more dramatic effects on evolutionary trajectories in insect-plant associations. Phytoplasmas, a diverse group of bacterial plant pathogens, alter the phenotypes of infected plants and hemipteran insect vectors, thereby affecting functional traits (such as diet breadth) and mediating host shifts (in ecological time) and diversification (in evolutionary time). Previous research focusing on bipartite patterns of hemipteran insect-phytoplasma associations has shown that strict co-speciation appears to be quite rare. In contrast, our preliminary cophylogenetic analyses reveal that host shifts among distantly related phytoplasmas occurred frequently in many different hemipteran lineages. We provide a first insight toward understanding coevolutionary and cospeciation processes of phytoplasmas and their hemipteran hosts. We reveal that subnetworks of phytoplasma linageages (e.g., 16SrX group) are confined to particular host groups (i.e., Psyllidae) including taxa that are strongly restricted to specific biogeographic region.Future research may benefit from exploring the patterns of host-phytoplasma phylogenetic congruence to predict possible host switches of phytoplasmas among host plants or vectors.

'*Candidatus* Phytoplasma phoenicium' associated with devastating diseases of stone fruits

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'*Candidatus* Phytoplasma phoenicium', taxonomic subgroup 16SrIX-B, was found in association with a lethal devastating almond disease (almond witches'-broom, AlmWB) in Lebanon in the early 1990s. It was later reported in Iran starting in 1995. During the last two decades, the outbreak of AlmWB has led to a rapid decline of almond trees in northern regions and in the Bekaa Valley in Lebanon and in Fars province and in other southern provinces in Iran. In 2009, '*Ca*. P. phoenicium' was also identified in association with a severe disease of peach and nectarine in southern Lebanon. From 2012 to 2017, '*Ca*. P. phoenicium' was found associated with apricot yellows and peach witches'-broom in Iran. The most characteristic symptoms in almond trees are shoot proliferation on the main trunk with the appearance of a witches' broom, perpendicular development of many axillary buds with small and yellowish leaves, and general tree decline with final dieback. The presence of witches'-broom is more common in almond trees than in peach/nectarine, while phyllody was observed only in peach. A total loss of production happens 1–2 years after the initial appearance of the symptoms. In Lebanon, AlmWB epidemiological cycle involves *Asymmetrasca decedens* (prevalent in almond), possibly responsible for the transmission of '*Ca*. P. phoenicium' from almond to almond, and cixiids of the genus *Tachycixius* (prevalent in *Smilax aspera* and *Anthemis* sp.), possibly responsible for the transmission from weeds to almond. In Iran, *Prunus scoparia*, a wild almond species harboring '*Ca*. P. phoenicium', could play a role in the phytoplasma transmission pathways to fruit trees. Based on detection of '*Ca*. P. phoenicium' in insect body and saliva and the presence of consistent populations, the leafhopper *Frutioidea bisignata* can be considered as potential vector of this phytoplasma in Iran. Alignment of 16S rDNA nucleotide sequences of '*Ca*. P. phoenicium' strains from Lebanon and Iran allowed the identification of 21 SNPs mutually exclusive in the phytoplasma strain populations identified in the two countries. The combination of such SNPs allowed the recognition of nine SNP lineages in Lebanon and eight in Iran. Multiple gene typing analyses of '*Ca*. P. phoenicium' strains infecting almond, peach, and nectarine in Lebanon allowed the identification of distinct AlmWB-associated phytoplasma strains from diverse host plants based on *inmp* (integral membrane protein) gene sequence analysis. This evidence suggests that AlmWB could be associated with phytoplasma strains derived from the adaptation of an original strain to diverse hosts. Healthy plant material and vector control are the main measures applied for AlmWB containment. The first report of a '*Ca*. P. phoenicium' strain, identical to the species reference strain, on almond in Italy in 2019 opened a worrying scenario on its impact on production of stone fruits and other hosts.

Insights into the emergence of the grapevine "flavescence dorée" epidemics. From European scale to local case studies

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"Flavescence dorée" (FD) is a European quarantine grapevine disease transmitted by the Deltocephalinae leafhopper *Scaphoideus titanus*. Whereas, this vector had been introduced from North America, the origin of the etiological agent, *i.e.* phytoplasmas of taxonomic subgroups 16SrV-C and -D, remained unclear. A survey of genetic diversity of FD-related phytoplasmas detected in grapevines, *S. titanus*, alders, alder leafhoppers and clematis were conducted in five European countries (Hungary, Serbia, Germany, Italy and France). Out of 132 genotypes identified based on *map* gene, only 11 were associated to FD outbreaks, including 3 also detected in clematis, whereas 128 were detected in alder trees, alder leafhoppers or in grapevines not related to FD outbreaks. Most of the alder trees were found infected, including 8 % with FD genotypes M6, M38 and M50, also present in alders neighboring FD-free vineyards and vineyard-free areas. The Macropsinae *Oncopsis alni* could transmit genotypes unable to achieve transmission by *S. titanus*, while the Deltocephalinae *Allygus* spp. and *Orientus ishidae* transmitted M38 and M50 that proved to be compatible with *S. titanus*. Variability of *vmp*A and *vmp*B adhesin-like genes of FD-related phytoplasma clearly discriminated 3 genetic clusters. Cluster Vmp-I grouped genotypes only transmitted by *O. alni*, while clusters Vmp-II and -III grouped genotypes transmitted by Deltocephalinae leafhoppers. Interestingly, adhesin repeated domains evolved independently in cluster Vmp-I, whereas in clusters Vmp-II and –III showed recent duplications. Our data demonstrate that most FD phytoplasmas are endemic to European alders. Their emergence as grapevine epidemic pathogens appeared restricted to some genetic variants pre-existing in alders, whose compatibility to *S. titanus* possibly resulted from the pre-adaptation of Vmp adhesins to other Deltocephalinae leafhoppers living on European alders. In diverse vineyards ecosystems in Bordelais and Burgundy, we investigated the risk of FD phytoplasma transfer from infected wild alders or clematis to the surrounding cultivated vine plots. By monitoring phytoplasma infection in plants and insects and by characterizing phytoplasma genetic diversity on local case studies, we confirmed that phytoplasma transfer could occur, but at a low frequency.

Multitrophic interactions in plant-insect-phytoplasma disease systems and advances in control of psyllid vectors

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Phytoplasmas causing the three most important diseases on fruit crops in Europe, '*Candidatus* Phytoplasma mali' (apple proliferation), '*Ca*. P. pyri' (pear decline), and '*Ca*. P. prunorum' (European stone fruit yellows (ESFY), have been investigated for the chemistry of their interactions in a multitrophic context. The chemically mediated ecological interactions of the phytoplasmas with vector insects (psyllids), their (alternate) host plants, and antagonists (entomopathogenic fungi) were studied. Attractive and repellent compounds were identified and tested in laboratory studies and field surveys. Additionally, a new entomopathogenic fungus was isolated from psyllid host. The identified new chemical compounds, blends and antagonists are used for the development of biotechnical control methods using the complete spectrum of available methods and materials for application. Traps and dispensers, microencapsulated volatiles, and also nanofibers are used for the development of appropriate formulations for field applications of semiochemicals for phytoplasma vector control. Entomopathogenic fungi from the order Entomophthorales are known for their comparatively high host specificity and rapid speed-to-kill and are therefore considered a promising alternative to conventional synthetic insecticides. We isolated a new species of the genus Pandora from *Cacopsylla pyri*. In a collaborative project we aimed to make this new Pandora species usable for biological psyllid control in Central European orchards and practically applicable through a suitable formulation. In infection trials under laboratory conditions, the target insects *Cacopsylla picta* and *C. pyri* were successfully infected and killed by the encapsulated fungus. In addition, attractive semiochemicals were encapsulated to attract the respective leaf-sucking insects and kill them by infections with the fungus.

I SESSIONE

DIAGNOSI, CARATTERIZZAZIONE E NUOVE SEGNALAZIONI

Etiology of phytoplasma-associated diseases of almond, pomegranate, and grapevine in Jordan

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In this study, a national survey on phytoplasma-associated diseases was conducted in Jordan from 2019 to 2021 targeting almond, pomegranate, and grapevine, three of the main crops cultivated in all country as commercials and family farming. The activities included: (i) monitoring and sampling symptomatic and symptomless plants from early summer to autumn; (ii) total nucleic acids extraction and phytoplama detection by 16S rDNA amplification in nested PCRs using the primer pairs P1/P7 followed by F1/R0; (iii) sequencing and bioinformatic analyses (BlastN, iPhyClassifier) of F1/R0 amplicons. During field surveys, almond yellows and witches'-broom (incidence around 40%), pomegranate exhibiting leaf chromatic alteration and rolling, little leaf and witches'-broom (incidence around 70%), and grapevine yellows (incidence around 10%) were observed. Molecular detection and 16S rDNA nucleotide sequence analyses revealed the presence of different '*Candidatus* Phytoplasma' species within samples from symptomatic plants, while no amplification was obtained from symptomless plant samples. '*Ca*. P. solani'-related strains was identified in all almond trees showing yellowing, while seven phytoplasma species were found in almond witches'-broom affected plants, including '*Ca*. P. ulmi'-related strains (identified in 28% of analyzed trees), '*Ca*. P. omanense'-related strains (24%), '*Ca*. P. asteris'-related strains (12%), '*Ca*. P. aurantifolia'-related strains (12%), '*Ca*. P. pyri'-related strains (12%), '*Ca*. P. trifolii'-related strains (6%), and '*Ca*. P. phoenicium'-related strains (6%). In pomegranate symptomatic plants, four different phytoplasma species were identified: '*Ca*. P. solani'-related strains (identified in 52% of analyzed trees), '*Ca*. P. aurantifolia'-related strains (21%), '*Ca*. P. ulmi'-related strains (16%), and '*Ca*. P. asteris'-related strains (11%). Interestingly, '*Ca*. P. ulmi', '*Ca*. P. pyri', and '*Ca*. P. omanense' in association with almond, and '*Ca*. P. ulmi' in association with pomegranate are reported for the first time in this study. The other phytoplasma species identified in almond and pomegranate were previously reported in the Middle East. In grapevine yellows affected plants, three phytoplasma species were found: '*Ca*. P. solani'-related strains (identified in 70% of analyzed plants), '*Ca*. P. omanense'-related strains (25%), and '*Ca*. P. aurantifolia'-related strains (5%). Such phytoplasmas were largely reported in previous works in association with grapevine yellows in the Middle East. Data obtained in this study revealed a great genetic diversity of phytoplasmas infecting important crops in Jordan. Further studies concerning the epidemiology of these phytoplasma-associated diseases, including the identification of putative insect vectors and reservoir plants, are in progress. Overall results will allow developing integrated strategies for the management of such diseases.

Genome assembly and annotation of eight '*Candidatus* Phytoplasma mali' strains

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'*Candidatus* Phytoplasma mali', the causal agent of apple proliferation (AP) disease, represents an important threat to the apple culture in Central-northern Europe being responsible for significant damages to the quality and quantity of apple production. Still little is known about the molecular basis of the '*Ca*. P mali' infection and host-interaction. Infected apple trees were shown to contain several '*Ca*. P. mali' strains characterized by a relatively high genetic variability associated with genes thought to be involved in strain virulence. Differences at the molecular level between '*Ca*. P mali' strains could explain the variation in symptoms severity, a scenario that could characterize all phytoplasma diseases. In addition, recent studies have demonstrated the secretion of phytoplasma-specific proteins, namely effector proteins. These are able to enter the host plant cells and are identified during the infection, influencing the disease development. Homologs of SAP11 (effector protein of '*Candidatus* Phytoplasma asteris'), can be also found in '*Ca*. P. mali', therefore suggesting paramount importance of these proteins in the disease progression. Here we present the comparative analysis on eight '*Ca*. P. mali' strain genomes, sequenced using the Oxford Nanopore Technologies (ONT). Long read sequencing allowed us to perform downstream genome assembly and annotation analysis with unprecedented resolution at genomic structure level. Our experimental approach did not require laborious enrichment of the phytoplasma DNA and, despite the sequenced material contains both host (~95%) and phytoplasma (~5%) genome, we developed an analysis pipeline to resolve the mixed dataset facilitating genome assembly, gene prediction and gene annotation. Whole comparative genome analysis has been performed to detect genetic differences between the eight '*Ca*. P. mali' strains and to assess the variability associated with already known effector proteins. Moreover, these results provide novel insights about the genomic differences between phytoplasma strains in terms of pathogenicity and invasiveness. This will also allow the identification of possible novel effector proteins that will be experimentally validated.

Molecular characterization of different '*Candidatus* P. solani' strains transmitted to tomato cv. Micro-Tom by *Hyalesthes obsoletus*

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'*Candidatus* Phytoplasma solani' is a species that includes strains classified in the 16SrXII group and in most cases associated in Europe and the Mediterranean basin with "bois noir" (BN) disease of grapevine and with stolbur (STOL) diseases of wild and cultivated herbaceous plants. '*Ca*. P. solani' can be transmitted both by vegetative propagation of infected hosts and by insect vectors of the family Cixiidae. Its most common vector is the polyphagous planthopper *Hyalesthes obsoletus*. The aim of our study was to investigate the molecular phytoplasma-host interactions exploiting the tomato model plant cv. Micro-Tom and several strains of '*Ca*. P. solani'. Tomato is a natural plant host of '*Ca*. P. solani', besides being an economically important crop; moreover cv. Micro-Tom is particularly suited for molecular investigations due to its small size, rapid growth and its completely sequenced genome. We transmitted several strains of '*Ca*. P. solani' to tomato cv. Micro-Tom plants by means of *H. obsoletus.* Adult insects were captured in vineyards with high incidence of BN in Friuli-Venezia-Giulia. They were collected with sweep nets from bindweed (in June) and stinging nettle (in July) and confined in cages with tomato plants. Another strain (named Sol), originally found in tomato in Puglia, was already available in greenhouse collection. All the strains are currently maintained by grafting. During their maintenance the strains showed remarkable differences in the symptoms induced on Micro-Tom plants: strains from bindweed as long as the strain Sol developed symptoms of leaf chlorosis and miniaturization, phyllody, big bud and cauliflower-like inflorescence, while strains from stinging nettle showed symptoms of leaf yellowing and miniaturization, shoot thickness and necrosis that from the apex and lateral shoots progressed towards the main stem, eventually resulting in plant death. With the aim to molecularly differentiate our strains, we performed multilocus sequence typing (MLST) on *tuf*, *vmp1*, *secY* and *stamp* genes by means of Sanger sequencing. Phylogenetic analyses on these genes delineated three major clades: one including the stinging nettle-associated strains and two comprising the bindweed-associated strains. To further investigate the molecular variability resulted from MLST, we carried out a whole genome sequencing of several strains using MinION (Oxford Nanopore Technologies). Tomato genome reads were filtered out, genome assemblies were generated using CANU optimized for repetitive genomes and annotated with RAST and MG-RAST. Once completed, the genomes were aligned with Mauve to search for structural variations. Interestingly, the resulting chromosomes of '*Ca*. P. solani' strains from the three major clades differed for several structural features.

Identification of epidemic "flavescence dorée" molecular variants in Emilia-Romagna and Veneto regions

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The study of the genetic variability of phytoplasmas is a fundamental tool to clarify their epidemiology and to implement an effective monitoring and management of their associated diseases. "Flavescence dorée" (FD), a threatening disease of grapevine associated to phytoplasmas belonging to 16SrV group and their insect vector(s), is distributed within the most important European wine-producing areas and has severe effects on both vineyard productivity and landscape management. FD is a quarantine disease in Europe, mainly transmitted by the ampelophagous leafhopper *Scaphoideus titanus* and, despite the efforts to contain the pathogen dissemination, the disease is still epidemic in several viticultural areas of Northern Italy. Based on sequence and restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene two FD ribosomal groups were described as present in Italy during the last century: 16SrV-C and 16SrV-D. However, the sequencing of non-ribosomal *loci*, such as *secY*, *map* and *rpsC*, allowed the identification over the years of several variants within the FD phytoplasma populations. A multilocus analysis approach was carried out on symptomatic FD-infected samples collected in Emilia-Romagna and Veneto regions, in different areas where the disease is spreading in the last 2-3 years. The geographic distribution of the two strains was confirmed to be different, with areas (Treviso province) with the prevalence of FD-C and others (Verona and Modena provinces) with only FD-D strains presence. Interestingly, FD-D phytoplasmas were identified for the first time also in a vineyard located in Modigliana (Forlì-Cesena province) and surrounded by forests. The molecular analyses allowed the identification of genetic variants among FD-D populations in both regions, that is quite a novel finding for these phytoplasmas that showed, since their first molecular identification in 1996, very little variability. In particular, the sequencing of *secY* amplicons highlighted the presence of the same SNP in samples of cultivars Teroldego and Trebbiano collected in Verona province and in grapevines cultivar Sangiovese located in Modigliana (Forlì-Cesena province). On the other hand, among the FD-C phytoplasma strains, the highest variability was shown on *rpsC* gene, that highlighted 5 restriction profiles after RFLP analyses on samples from cultivar Glera collected in Treviso and Belluno provinces. The epidemiology of the disease is therefore still to be monitored, especially since it is now involving different vectors/plant hosts species that are very likely responsible for the emergence of these FD variants. Therefore, a continuous and capillary monitoring of the presence and emergence of FD strains associated with the disease in the areas where it is present is necessary for the application of the most appropriate and stringent control measures aimed to avoid their epidemic spreading.

First report of '*Candidatus* Phytoplasma pyri' associated to pear decline (PD) in Sicily (Italy)

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Pear decline is an important disease of pear associated with '*Candidatus* Phytoplasma pyri', a phytoplasma belonging to the apple proliferation group (16SX). It is included in the EPPO A2 List and transmitted by two vectors, *Cacopsylla pyricola* and *Cacopsylla pyri*. '*Ca.* P. pyri' is present in almost all European pear tree producing countries but has never been reported in Sicily. In the autumn of 2019, premature leaf reddening and curling were observed in two young commercial pear orchards (2 years old) of Coscia (Bronte, CT) and Abate Fetel (Castronovo di Sicilia, PA) cvs. Five samples of Coscia pear and five of Abate Fetel pear were collected (4 showing symptoms and 1 asymptomatic). Total nucleic acids extraction was obtained from a mixture of midribs and petioles of leaves according to CTAB-based protocol followed by nested PCR using P1/P7 and R16F2n/R16R2 primers for 16S rRNA gene and using SecYMalF1/secYMalR1 and SecYMalF2/secYMalR2 for SecY gene of group 16SrX. Amplicons of the expected size (about 1200 and 664 bp respectively) were obtained from symptomatic plants. No amplification was obtained from the asymptomatic controls. Two representative 16Sr amplicons (one per site) were purified, cloned and sequenced in both directions, whereas five SecY amplicons (3 from Bronte and 2 from Castronovo sites) were purified and directly sequenced in both directions. The 16S rRNA gene sequences showed a similarity of 99.8% (LV72-Castronovo) and 100% (DSM-Bronte) with reference strain AJ542543. Virtual RFLP of the partial ribosomal amplicons evidenced matching (similarity coefficient 1.00) with 16Sr group X, subgroup C for both isolates. The phylogenetic analysis of the partial SecY genes clustered all sequences with the reference strain. Representative sequences of both genes were deposited in GenBank. This is the first report of '*Ca* Phytoplasma pyri' associated with pear decline (PD) on pear trees in Sicily. Further investigation might clarify epidemiological aspects related to insect vectors, and disease distribution, and will allow to hypothesize pathways of pathogen introduction in the region.

Phytoplasma strains maintained in micropropagation since 30 years and the EPPO-Qbank collection

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The *Alma Mater Studiorum*, University of Bologna, DISTAL is hosting a collection of more than 140 phytoplasma strains maintained mainly in micropropagated *Catharanthus roseus* shoots. The collection was initiated in 1989 and maintained over the years with strains provided from greenhouse collections located in Udine (Italy), Dossenheim (Germany), Bordeaux (France) and other Institutions in Serbia, Chile, and USA. The different strains were insects or dodder transmitted from original host plants by researchers from around the world after the discovery of these pathogens in 1967 in Japan. The shoots were kept in a growth chamber and the presence and identity of phytoplasmas was verified before micropropagation by PCR followed by RFLP and/or sequencing of the 16S rRNA gene. For the micropropagation 1-3 cm long symptomatic shoots are subjected to sterilization in 10% sodium hypochlorite and rinsed in sterile water before their insertion in a MS based solid medium added with low amount of BAP. The shoots are kept under artificial lights (16 h photoperiod) at the constant temperature of 24°C $\pm$ 2°C and multiplied by microcuttings every 3 to 6 months. When necessary, strains still maintained *in vivo* under greenhouse conditions are micropropagated again after phytoplasma molecular identity further verification. The strains were all sequenced on 16Sr and *tuf* genes to produce specific barcodes for the quarantine strains and their look-alike strains, under the EU founded project QBol, ended in 2013. The collection was made officially available for general consultation and strain identification through barcode firstly as Q-Bank (Q-Collect EU founded project) and, since May 1 2019, as EPPO-QBank (ttps://qbank.eppo.int/phytoplasmas/). Shoots of other phytoplasma-infected plant species such as cactus pear, tobacco and paulownia are presently maintained in the same collection only for research purposes. Over the time also other phytoplasma-infected species such as hydrangea, jujube, bindweed, *Prunus* spp., apple and grapevine infected by phytoplasmas were micropropagated, however their survival was, in many cases, limited in time and their micropropagation performance very poor, probably because of the strong effect of the pathogens and the reduced suitability of the medium (same for all the strains) used for the micropropagation. Molecular testing was performed on micropropagated shoots to verify presence and genetic identity of phytoplasma strains over the years on several genes. Among the studied genes the 16S rRNA, leucyl-tRNA synthase (*leuS*), *secA* and *tuf* resulted the most reliable in providing results, while *secY* and *rp* only resulted amplifiable in some of the tested strains. The comparison of RFLP profiles obtained from these with the same amplicons from the original strains before the micropropagation showed the presence of different restriction profiles in some of the strains. After about 25 years in micropropagation phytoplasma strains enclosed in groups 16SrII, -III, and -IX exhibited different profiles in amplicons of *rp*, *leuS* and *secA* genes. Moreover, the sequencing of *secA* gene of strain SOYP (16SrII-C) showed the presence of a differential SNP confirming the presence of some selective pressure related to the micropropagation maintenance. Periodic verification is performed by PCR/RFLP analyses on 16S rRNA gene to confirm phytoplasma presence and identity. The strains present in the collection (http://www.ipwgnet.org/collection) are available for sale for diagnostic and research purposes.

Detection of apple proliferation disease in *Malus* × *domestica* by near infrared reflectance analysis of leaves

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'*Candidatus* Phytoplasma mali' is the causative agent of apple proliferation, one of the most threatening diseases in commercial apple growing regions. In a two-year study, leaves were sampled from three apple orchards, at different sampling events throughout the vegetation period. Dried and ground leaves of *Malus* × *domestica* were analysed by near infrared reflectance and multivariate data analysis was performed on the spectra to distinguish '*Candidatus* Phytoplasma mali' infected from non-infected apple trees. A principal component analysis and classification models were developed for this purpose. The model performance for the differentiation of apple proliferation diseased from non-infected trees increased throughout the vegetation period and gained best results in autumn. Even with asymptomatic leaves from infected trees a correct classification was achieved indicating that the spectral-based method provides reliable results even if samples without visible symptoms are analyzed. The wavelength regions that contributed to the differentiation of infected and non-infected trees could be mainly assigned to a reduction of carbohydrates and N-containing organic compounds. Wet chemical analyses confirmed that N-containing compounds were reduced in leaves from infected trees. The results of our study provide a valuable first indication that spectral analysis is a promising technique for apple proliferation detection in future smart farming approaches.

Multigene differentiation of '*Candidatus* Phytoplasma solani' strains from different geographic origins and diverse host species

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The knowledge of genetic variability in phytoplasmas is a fundamental resource for the study of their epidemiology and a valuable support to improve the monitoring and the management of their associated diseases. Multigene analyses have been used widely to characterize phytoplasmas strains providing relevant information about their epidemiology. '*Candidatus* Phytoplasma solani' is associated with "bois noir" (BN) in grapevines and yellowing in many other cultivated and wild plant species such as tomato, pepper, bindweed, causing serious damages in many parts of the world. The epidemiology of the diseases associated with this phytoplasma is very complex considering its wide host range distribution, the interactions with both host plant and insect vectors, and the diffusion of the bacterium through infected plant propagation material. The genetic variability of '*Ca*. P. solani' strains, in different host species and in several geographic areas was widely studied by genotyping selected non-ribosomal genes. The polymerase chain reaction coupled with restriction fragment length polymorphism analyses and sequencing was applied to samples collected during more than 20 years in Italy, Portugal, Hungary and Serbia in a multigene analysis on *vmp1*, *stamp* and *tuf* genes. A total of 116 '*Ca*. P. solani' strains was examined and allowed the differentiation of 26 genetic variants confirming the differential variability of the studied genes. In particular, the *vmp1* and *stamp* genes showed the presence of 14 and 5 RFLP profiles, respectively, while the *tuf* gene grouped all tested strains into two profiles. In the *vmp1* gene sequences it was registered the largest variability; the V3-*Rsa*I RFLP profile was the most present in the Italian grapevine samples tested. A large part of the samples tested were collected in Italy and Serbia; in the comparison between the two geographic areas only one genetic variant was found in common, confirming the local distribution of the strains. Considering the time frame of the sample collection and the geographical distribution of the variants identified, it appears that populations of this phytoplasma are generally less variable on a local scale leading in some cases to the emergence of epidemic strains. In particular, the grapevine samples collected during 2020 in Tuscany revealed the predominance of *tuf* type-a strain and showed the same variant in all the samples, indicating the possible emergence of an epidemic BN strain in that region. In addition, the identification of two *stamp* variants (St5 and St10) that have shown specific epidemiological characteristics linked to different virulence in the field indicates the need to continue to study and to monitor the disease with specific molecular analyses considering that the strains with different virulence can lead to epidemic infections with significant economic damages. The presence of mixed infections with different phytoplasmas indicate another important element to consider for the '*Ca*. P. solani'-associated diseases epidemiology. These results confirm that except for *tuf* gene there is no specificity of '*Ca*. P. solani' variants linked to different host species and different epidemiological cycles. Their differentiation can be explained by geographic distribution of host and insect vectors, year of infection and epidemic outbreaks registered during the time. Strains diversity combined with epidemiological data are useful also to identify sources of inoculum, new host species and to monitor the spreading of the phytoplasma both locally and on a larger environmental scale for focused management purposes.

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New genetic diversity of '*Candidatus* Phytoplasma solani' in Iranian vineyards

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Recent outbreaks of the grapevine yellows "bois noir" that is associated with phytoplasma strains related to '*Candidatus* Phytoplasma solani' (16SrXII group) were recorded in several Iranian regions. Surveys were carried out from 2015 up to 2017, followed by collecting of leaf samples from symptomatic grapevines and weeds, and study of molecular epidemiology of '*Ca*. P. solani' in Iranian vineyards. In order to investigate the genetic diversity, multiple gene analyses were carried out according to molecular characterization of the *tuf* and *vmp*1 genes. From the molecular characterization, all grapevine and weed samples were infected with *tuf* b type. The abundance and infection of *C. arvensis* in Iranian vineyards supports the hypothesis that '*Ca*. P. solani' *tuf* b type predominantly spreads via a cycle that involves bindweed. According to the molecular characterization, three molecular types - *tuf* b1, *tuf* b5 and *tuf* b6 - were found, with *tuf* b1 type being the most prevalent. These data provide further knowledge of *tuf* gene diversity and question the ecological role of such "minor" *tuf* types in Iranian vineyards, which have been detected only in grapevines. Detailed molecular characterization of the *vmp*1 gene (i.e., PCR-RFLP, sequence analysis) defined five molecular types: V1, V4, V10, V15, and V20. V1 and V10 were detected both in grapevines and in weeds, while V4 and V20 were detected only in grapevines samples, and V15 only in *C. arvensis*. The data obtained on '*Ca*. P. solani' molecular types in Iranian vineyards is basilar to apply proper management strategies based on the eradication of weedy reservoir plants and the monitoring and control of the insect vectors.

Recording of *Spartium junceum* whitches' broom in Riviera del Conero (Marche, Ancona, Italy)

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Spanish broom (*Spartium junceum*), is a shrub known for its outstanding pioneer qualities, for its ability to adapt to difficult soil conditions and rather prolonged periods of dryness. For these reasons, *S. junceum* is widely distributed in Italian landascape in a variety of soil conditions, altitudes and temperature ranges. On the Marche Apennines up to the Conero Riviera, it forms patches on escarpments and lowland areas, playing an important role in mitigating the erosion of steep slopes and in the recovery of degraded areas (quarries and landfills). Particularly evident and considerable beauty during flowering is Spanish broom, so much as to inspire in the past poems by the famous Marche poet Giacomo Leopardi. During the spring of 2019 and 2020, a monitoring was carried out in the Marche region, to assess the presence of *S. junceum* bushes showing symptoms associated with phytoplasma infections. The main symptoms were the witches' broom and branch fasciation. Witches' broom gives to the plant a compacted appearance and are accompanied by a light green color tending to yellowing, which can turn into drying of even extended portions of the plant. The other less common symptom, but still present on some monitored plants, is the branch fasciation. Symptomatic plants, sporadic in 2019, were recorded more frequently in 2020, mainly in location near the coast. From 52 symptomatic and 12 symptomless plants, we collected few branches, which were subjected to DNA extraction and to amplification with universal primers P1/P7, followed in nested PCR with R16F2n/R2. All the symptomatic plants resulted positive to phytoplasma infection, while for asymptomatic plants and water control we did not yield any amplicon. Representative amplicons were purified and sequenced. From blast analysis, the samples showed a high homology (100%) with the reference nucleotide sequence MT629815, detected in *Livilla spectabilis*, reported to be the potential vector of SpaWB-associated phytoplasma, and MT629806, detected in the isolate Sj2MS of *Spartium junceum*. Spanish broom was identified as a reservoir and potential inoculum source of phytoplasma, that can cause severe disease in other crops. The recording of several infected plants of *S. junceum* can induce to hypothesize the active presence of vectors and a potential effect on the regional landscape.

Detection of phytoplasma infection in seed-bearing cabbage (*Brassica oleracea* var. *capitata*) in Marche region, Italy

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Brassica oleracea is one of the most important vegetables and includes many common crops with a long consumption tradition in Europe, such as the cabbage (*B. oleracea* var. *capitata*). Marche Region, Central Eastern Italy, has an ideal geographical exposure for growing seed-bearing crops, since there are several valleys orthogonal to the sea, then highly ventilated, and it allows to get high quality seed with reduced risk of contamination from pathogens. In 2019, the regional surface devoted to the multiplication of seed-bearing vegetables, largely destinated to the export, exceeded 3,000 ha, ranking second in Italy after Emilia-Romagna, mainly used for the multiplication of *Brassicaceae* (34%). The productivity and quality of *B. oleracea* crops are seriously affected by many diseases, which result in substantial economic losses for agricultural producers every year. In the framework of the project "CleanSeed", two seed production fields of cabbage were investigated for the presence of disease symptoms. During survey in 2021, phyllody was observed in one cabbage field in Montefiore dell'Aso (Ascoli Piceno Province, Italy), with incidence ranging from 1 to 3% of plants. The main symptoms of the disease were malformed flowers and little leaves, inducing later a complete loss of the production. A total of 15 cabbage plants with typical symptoms were collected and DNA was extracted, grinding and homogenizing 0.5 g greenish, malformed floral organs using CTAB protocol. The detection of phytoplasma was carried out through polymerase chain reaction (PCR) with universal primer pairs P1/P7 and in nested PCR with R16F2n/R2. The amplifications were checked in the agarose gel. All 11 symptomatic samples showed specific amplified products of expected size (1.8 and 1.2 kb, respectively). In four asymptomatic plants, no DNA bands were amplified. Further investigations are in progress to identify the phytoplasma and to investigate routes for dissemination, to set up sustainable management strategies.

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II SESSIONE
EPIDEMIOLOGIA, VETTORI E GESTIONE

Finding of parasitized *Scaphoideus titanus* in a vineyard in Veneto region

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The North American leafhopper *Scaphoideus titanus* was accidentally introduced in Europe during the fifties. Its presence was first reported in 1958 in France, and shortly after in northwestern Italy and southern Switzerland. *S. titanus* is monophagous on *Vitis vinifera* and is the main vector of the quarantine pest "flavescence dorée" (FD). This epidemic disease caused by phytoplasmas (FDp) belonging to 16SrV-C and -D subgroups threatens European viticulture. The systematic mandatory control applied in FD-infected areas mainly consists in uprooting of infected plants, certification of the material for planting and insecticide applications against the vector. However, in the last years recurrences of the disease have been observed, mainly justified by the decrease in allowed insecticide molecules. In addition, the remaining active ingredients have a more complex use, which often results in a loss of efficacy. Given this background, alternative strategies could become a valid support in insect control. One of these consists in parasitoids as possible biocontrol agents. This work reports the observations and the subsequent studies made in a vineyard in Corbanese (Treviso, Veneto Region) during the 2020 growing season. In this study area some specimens of *S. titanus* with parasitoid's sac were observed. In order to determine the percentage of parasitized insects, monitoring and sampling were carried out. Sampling consisted in the collection of 1000 *S. titanus* from grapevine canopy with an entomological aspirator. The collected insects were transferred on vine seedlings inside a wire mesh cylinder covered by a dense knit of pantyhose. Two types of parasitoids larvae emerged, Drynidae (*Gonatopus lunatus* and *G. clavipes*) and Pipunculidae. The parasitization rate of collected *S. titanus* was 2.9% (29 out of 1000 specimens). The last instar larvae of the wasp, after having split the sac, moved away by means of peristaltic contractions, and eventually spun a pupal cocoon on the surface of the vine leaf. The eclosion occurred in about 10 days and the adults (all apterous females) were collected and placed in Petri plates. Five adults of *S. titanus* were daily presented to each of the Drynidae females. Every *S. titanus* specimen, during the attack, was immobilized and, after being released, remained paralyzed for the next few minutes. All *S. titanus* which were fed on died within two days as a result of host-feeding. Attempts of oviposition have also been observed, but the host specimens used died anyway. On the other side, pipunculids larvae (3 out 29) emerged by rupturing an intersegmental membrane of *S. titanus* and then pupated in the soil. Only one of the three dipteran cocoons opened, but the adults died within a few days. This preliminary study highlights the importance of continuing to deepen the knowledge about parasitoids, which could in some environments constitute a support to traditional methods of control. Since *S. titanus* is a vector, there is no acceptable threshold value for its presence in the vineyard, and a natural limiter would not be able to guarantee a satisfactory reduction of the population. Nevertheless, it must be considered that uncultivated areas with wild grapevines, abandoned vineyards, hedgerows or wood edging are not covered by insecticides and the presence of parasitoids can be a resource. Further studies will be needed to investigate how to implement the role of these natural antagonists in order to increase the number and the tools for *S. titanus* control.

TROPICSAFE project: identification and management of phytoplasma-associated diseases in coconut palms in Ghana, Mexico, Jamaica and Cuba and in grapevine in South Africa, Chile and Italy

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The TROPICSAFE project covers the diseases associated with phloem limited bacteria associated diseases in coconut palms, grapevine, and citrus species in diverse countries (http://www.tropicsafe.eu/). A part for the citrus "huanglongbing" disease associated with the presence of '*Candidatus* Liberibacter' species mainly studied in Cuba, France (Guadeloupe), South Africa and Spain where for the moment only one of its insect vectors, *Trioza erytreae* is present, the project studies the epidemiology and management of coconut palms and grapevine associated phytoplasma diseases. Coconut palm lethal yellowing (LY) is studied in Ghana, Cuba, Jamaica and Mexico and grapevine yellows (GY) are studied in Italy, South Africa, and Chile. The presence and the identity of the pathogen strains present in the different countries was verified through their molecular characterization and in some cases also cultivation in artificial media to acquire the data necessary to detect and manage in sustainable manner the studied diseases. The pathogen identification in alternative host plants and insect vectors and potential vectors was also performed together with the improvement of specific diagnostic techniques. The confirmation of the presence of 16SrXXII-B and 16SrIV phytoplasmas in Ghana and in the Caribbean (Jamaica, Cuba, and Mexico), respectively, allowed to study alternative plant species and potential insect vectors to define the epidemiological cycles of this disease towards its focused management. In Jamaica where the LY disease has been managed since some time with sustainable results a newly discovered species *Oecleus mackaspringii* was associated with coconut palm in plots with still active cases of the disease acting as possible insect vector. In Cuba also symptomatic palms positive for phytoplasmas belonging to the ribosomal groups 16SrI, 16SrVII and 16SrXII were identified and citrus with "huanglongbing" symptoms the 16SrIV phytoplasma was found in mixed infection with '*Ca*. L. asiaticus' or other phytoplasmas. In Mexico transmission trials with *Haplaxius crudus* confirmed its vector role in this Country. Dwarf coconut varieties showed promising results in relation to LY resistance in Ghana. GY disease are associated in South Africa with '*Candidatus* Phytoplasma asteris'-related strains transmitted by *Mgenia fuscovaria,* while Chile and in Italy phytoplasmas belonging to diverse ribosomal subgroups, several alternative host plants, and some insect species have been described as phytoplasma vectors or potential vectors in grapevine or in vineyard environments. In Chile the 16SrIII-J phytoplasma is transmitted by *Paratanus exitiosus* and *Bergallia valdiviana*. Surveys in selected north Italy vineyards detected '*Ca*. P. solani', '*Ca*. P. fraxini', '*Ca*. P. asteris' and "flavescence dorée" phytoplasmas and new potential insect vectors. It is therefore clear that only a constant monitoring will allow for the prompt detection of phytoplasmas or new phytoplasmas that may infect the studied crops. Appropriate management is linked to the diverse geographical location and agro-ecosystem conditions but with the appropriated epidemiologic knowledge can be applied as sustainable tool to reduce economic losses and the environmental pollution.

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Transmission trials using *in vitro* plants: a new protocol to confirm phytoplasma insect vector species

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In Chile the phytoplasmas associated to grapevine yellows belong to the ribosomal subgroups 16SrI-B and 16SrI-C ('*Candidatus* Phytoplasma asteris'-related), 16SrIII-J ('*Ca*. P. pruni'-related), 16SrV-A ('*Ca*. P. ulmi'), 16SrVII-A ('*Ca*. P. fraxini'), and 16SrXII-A ('*Ca*. P. solani' or "stolbur"). The prevalent are phytoplasmas 16SrIII-J that are widely distributed mainly in South America. The "flavescence dorée" phytoplasmas and its vector *Scaphoideus titanus*, have not been found in Chile. Previous epidemiological studies indicate that weed, shrub, and non-grapevine woody plants from different botanical families present in the vineyards and in their surroundings (*Convolvulus arvensis*, *Galega officinalis*, *Polygonum aviculare*, *Malva* sp., *Brassica rapa*, *Rubus ulmifolius*, and *Rosa* sp.) are reservoirs of 16SrIII-J phytoplasma, and the leafhoppers *Paratanus exitiosus* and *Bergallia valdiviana* are vectors of the same pathogen. Other leafhopper species, *Bergallia* sp., *Amplicephalus ornatus*, *Amplicephalus curtulus*, *Amplicephalus pallidus*, and *Exitianus obscurinervis*, captured in a vineyard planted with Pinot noir, were identified as new potential vectors of the 16SrIII-J phytoplasma. To carry out the transmission trials (TT), adult individuals were captured monthly from October 2017 to June 2021, using an entomological sweeping net. The insects were identified at the species level based on morphological characteristics. Periwinkle and grapevine cultivar Cabernet Sauvignon micropropagated phytoplasmas-free plantlets, were used in the TT. Between 4 and 6 insects of the same species were introduced to each *in vitro* plant tube. For each TT, the insects have been allowed to feed for a maximum of 7 days and were collected as they dye or after 7 days and stored in 70% ethanol. At the end of the TT, the plants were treated with fungicides (Captan and Tebuconazole), transferred in a solid sterilized substrate composed of peat and perlite in a 2:1 ratio, and kept in a conditioned incubator at 25ºC under 16 h/day light. To date, 235 *in vitro* TT have been carried out, using 123 and 112 grapevine and periwinkle plants, respectively. Eight plants without contact with insects have been used as control. Plants and insects used in the TT have been analyzed by nested-PCR with P1/P7 followed by R16F2n/R2 primers. The amplification products (1,250 bp) were sequenced, and the identification of phytoplasmas was carried out by in *silico* RFLP using the enzymes *Hha*I, *Bst*UI and *Rsa*I. All the plants were analyzed 3, 10 and 18 months after the start of TT. All the insect species were able to transmit the 16SrIII-J phytoplasma to grapevine and/or periwinkle plants: *A. ornatus* (1 grapevine); *A. pallidus* (4 grapevine, 1 periwinkle); *Bergallia* sp. (3 grapevine, 2 periwinkle); *E. obscurinervis* (1 grapevine, 3 periwinkle); *A. curtulus* (5 grapevine, 2 periwinkle). The plants used as control were negative for phytoplasma presence. The plants positive for phytoplasmas, start to show symptoms two months after the beginning of TT. Plants that were kept in contact with insects and that were negative for phytoplasmas, are still under observation and will be tested again in the next months. The use of *in vitro* plants for TT has been successful in identifying new insect vector species. This is due very likely to the reduced stress the insects suffer during the experiments of transmission.

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Identification and characterization of '*Candidatus* Phytoplasma solani' associated with selected Auchenorrhyncha species

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Phytoplasma are phytopathogenic bacteria that inhabit the phloem of plants as well as tissues of their insect vectors. "bois noir" (BN) is the most widespread grapevine yellows in Europe and Mediterranean area, which can cause severe production losses. '*Candidatus* Phytoplasma solani', associated with BN, is transmitted from host plants to grapevine by two cixiid species, *Hyalesthes obsoletus* and *Reptalus panzeri*. However, other polyphagous planthoppers and/or leafhoppers are probably involved in the epidemiological cycle of '*Ca*. P. solani' when these two vectors are absent, or their population density are not correlated with BN incidence in some viticultural areas. According the phytoplasma gene, which encodes the translation elongation factor Tu (*tuf*), the '*Ca*. P. solani' consists of two genetically divergent strain types, *tuf*-a and *tuf*-b, which are involved in two diverse epidemiological cycles: the stolbur *tuf*-b type is generally associated with field bindweed, while stinging nettle acts as *tuf*-a type reservoir. The aims of this study were: i) to investigate the natural infection of '*Ca*. P. solani' in selected phloem-feeding Auchenorrhyncha species; ii) molecular characterization using RFLP method of *tuf* gene in stolbur isolates from insects to better understanding the epidemiological cycle. The survey on Auchenorrhyncha populations was carried out in a vineyard, located in Montalto delle Marche (AP) (central-eastern Italy), where a high incidence of BN has been recorded. Insect sampling was performed using yellow sticky traps and a D-vac aspirator. Molecular investigations were carried out on *Anaceratagallia ribautii* (No. 48), *Euscelis lineolatus* (No. 212), *Exitianus capicola* (No. 53), *Hyalesthes obsoletus* (No. 38), *Laodelphax striatellus* (No. 64), *Neoaliturus fenestratus* (No. 482) and *Psammotettix alienus* (No. 235). Overall, according to the sampled insects, 32% of *H. obsoletus*, 10% of *N. fenestratus*, 5% of *E. lineolatus*, 50% of *A. ribauti*, 9% of *L. striatellus*, 9% of *E. capicola* and 16% of *P. alienus* were positive to stolbur. The *tuf*-type investigation revealed that all the insect specimens were infected by the *tuf*-b, except one *H. obsoletus* specimen, that harbored *tuf*-a type. Investigation on vectors and putative vectors in vineyard agroecosystem can be useful to gain information on disease cycles of '*Ca*. P. solani' that can be applied in BN management strategies.

Widespread occurrence of elm yellows and alder yellows diseases in southern Italy

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Elm yellows (EY) and alder yellows (ALY) are yellows and decline disease that affects several *Ulmus* (elm) and *Alnus* (alder) species, respectively. These diseases are known to occur in Europe and North America and are caused by closely related phytoplasmas, the EY agent '*Candidatus* Phytoplasma ulmi' and the ALY agent, which are members of the EY phytoplasma group or 16SrV group, subgroups 16SrV-A and -C, respectively. EY symptoms vary among the species. Foliar yellowing, extensive phloem necrosis and death are predominant in North American species. In contrast, European and Asian species are primarily characterized by witches'-brooms, do not show phloem necrosis and are less prone to severe decline. In all ALY-affected species the symptoms are similar. These include yellowing, sparse foliage, premature autumn coloration, reduced terminal growth, die-back and decline. However, latent infections are also common. Visual symptom assessment and PCR amplification using primers directed to rDNA sequences were used to survey the occurrence of EY and ALY diseases in several areas of Basilicata and Campania regions. The elm trees examined consisted of *U. minor* (syn.: *U. carpinifolia*), *U. glabra*, *U. laevis* and hybrids whereas the alder trees examined consisted mostly of *A. glutinosa* and to a lesser extent of *A. cordata*. The survey revealed that a high percentage of elm and alder trees were infected reaching more than 80% in some areas, *e.g.*, Agri valley of Basilicata region. The symptoms recorded in diseased elm trees were leaf epinasty, yellowing, stunting, small leaves, premature leaf shedding and pronounced witches'-brooms present at the tips of twigs and branches and at the root level. In ALY-affected alder trees, in addition to the above-mentioned symptoms, pronounced shoot proliferation at the base of trunk of several-year-old trees were very often observed. More than half of alder trees examined proved to be latently infected. On the basis of primer specificity and RFLP analysis of PCR-amplified rDNA sequences employing suitable restriction endonucleases the phytoplasmas detected in elms and alders were assigned to '*Ca.* Phytoplasma ulmi' and ALY agent, respectively. Although the diseases examined were known in southern Italy, results of our study indicate that EY and ALY diseases are more widespread than previously thought and are of considerable ecological and epidemiological significance.

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Acibenzolar-S-methyl: effects on the "flavescence dorée" pathosystem

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A recent derogation from the 2021 Integrated Production Regulations has authorized the exceptional use of the Bion 50 WG formulation to control grapevine "flavescence dorée" (FD) in Italy. This commercial preparation of Acibenzolar-S-methyl (ASM) product by Syngenta Crop Protection, is a derivate of benzo-1,2,3-thiadiazole which is able to mimic the role of salicylic acid in the activation of resistance genes in plants, eliciting systemic resistance to a broad range of plant-pathogens, including fungi and bacteria. Preliminary experiments on various plants exposed to insect or graft inoculation with different '*Candidatus* Phytoplasma species' suggested that the resistance elicitor may delay symptom development and phytoplasma multiplication in treated plants and reduce infection rates in some cases. As for grapevine yellows, BTH applications seem to elicit plant resistance as repeated field applications reduced vector transmission of FD to the highly susceptible Barbera cultivar and increased recovery of "bois noir" infected vines. On the contrary, no effect on recovery of FD-infected Barbera vines was recorded. This work describes the induction of pathogenesis-related protein gene expression (PR-1) and the incidence reduction of new FD infections on ASM treated grapevines in productive vineyards. Moreover, in order to verify the possible effects of ASM on FD-vector, recording trials of the nutritional behavior of *Scaphoideus titanus* on treated and untreated grapevines are underway by electropenetrography (EPG).

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Evaluation of biostimulant effectiveness in grapevine "bois noir" management

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This work, carried out from 2017 to 2020, aimed to evaluate the effectiveness of four commercial products (Delfan plus, Phylgreen, Phylgreen Kuma, Vegenergy), which use as biostimulants is allowed in organic farming, in the management of "bois noir" (BN), a grapevine disease associated with '*Candidatus* Phytoplasma solani'. The field trials were carried out in a Chardonnay BN-affected organic vineyard located in Gussago (Franciacorta, North Italy), monitored since 2012. In September 2017, the vineyard was monitored for grapevine yellows symptoms and a map reporting the phytosanitary state of each plant was created. Based on this map, four treated blocks (each one treated with one biostimulant), and one untreated (control) block were arranged. The activities, conducted from 2018 to 2020, included: (i) applications with the biostimulants from mid-April (10 cm long grapevine shoots) to the beginning of August (every two weeks (7 treatments/year); (ii) mapping and sampling of symptomatic and asymptomatic vines in September; (iii) extraction of total nucleic acids and molecular identification of '*Ca.* P. solani' by nested PCR-based amplification of *stamp* gene; (iv) molecular characterization of phytoplasma strains by means of *stamp* gene nucleotide sequence analysis; (v) statistical analysis to evaluate any differences in the curative and preventive effect on BN-symptoms. The statistical analysis, conducted on the increase of symptomatic grapevines from 2017 (pre-treatment) to 2020 (cumulative effect of the treatments conducted from 2018 to 20), showed a significant decrease in symptomatic vines in the Delfan plus block (-4.5 percentage points) in comparison to the other blocks. The curative effect (percentage of recovered grapevines) was calculated considering the health status of symptomatic plants in 2017 over the following three years. Percentage of recovered plants in the Delfan plus block was higher than in all the other blocks, although the difference observed towards the block treated with Vegenergy and the untreated one was not statistically significant. The preventive effect was calculated considering the percentage of new symptomatic plants (grapevines showing symptoms for the first time) in the three-year period 2018-20. Statistical analyses did not reveal significant differences on the preventive effect observed in the different blocks. However, the percentage of new symptomatic grapevines in the block treated with Delfan plus was lower than in the other blocks. Molecular and bioinformatics analyses showed the presence of eight distinct '*Ca.* P. solani' strains carrying different *stamp* gene variants (St1, St5, St8, St10, St16, St18, St19, St30). The statistical analysis showed a uniform distribution of such phytoplasma strains within the vineyard, reinforcing the evidence that the effect on BN incidence in the blocks is due to the action of the biostimulants and not to possible differences in the '*Ca.* P. solani' strain virulence. Based on the obtained results, it emerged that a reduction in the percentage of symptomatic vines was observed exclusively in the block treated with Delfan plus. Further open field trials carry out in different grape growing areas and on different cultivars are necessary to confirm the effectiveness of biostimulants BN control.

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Role in "bois noir" epidemiology of inter-row vineyard groundcover vegetation used for green manure

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'*Candidatus* Phytoplasma solani' (CaPsol), associated with grapevine "bois noir" (BN), has a broad range of host plants and can be transmitted to grapevine by several insect vectors. *Hyalesthes obsoletus*, the main CaPsol vector in European and Mediterranean countries, feeds preferentially and completes its biological cycle on *Convolvulus arvensis* and *Urtica dioica*, occasionally transmitting the phytoplasma to grapevine, a dead-end host for the pathogen. Recent studies evidenced that other weeds, naturally present within and around the vineyards, can play a role in BN spreading. This study investigated the role of eight groundcover plant species (*Eruca sativa*, *Sinapis arvensis*, *Phacelia tanacetifolia*., *Vicia sativa*, *Vicia faba* var. minor, *Trifolium incarnatum*, *Trifolium alexandrinum*, *Polygonum fagopyrum*), commonly utilized for inter-row vineyard green manure in Franciacorta (North Italy), in BN epidemiology. The activities, conducted in the years 2019 and 2020 in two BN-affected vineyards in Gussago and Provaglio d'Iseo (Franciacorta), included: (i) monitoring and sampling groundcover plant species and *C. arvensis* (known host plant) in July, and symptomatic grapevines in September; (ii) total nucleic acids extraction followed by CaPsol-specific identification by nested PCR amplification of *stamp* gene; (iii) sequencing and bioinformatic analyses (comparison with *stamp* sequence variants dataset available in literature) of *stamp* amplicons obtained from symptomatic grapevines and groundcover plants. Molecular analyses were carried out on 341, 55, and 108 samples collected during the field surveys from groundcover plants, *C. arvensis* and symptomatic grapevines, respectively. Nested PCR allowed identifying CaPsol in 7.6% of groundcover plants, 25.5% of *C. arvensis* plants, and 76.9% of grapevines. Within groundcover plants, only *E. sativa*, *V. sativa*, and *P. fagopyrum* were found CaPsol infected. Based on nucleotide sequence analysis of *stamp* amplicons, CaPsol strains harboring three *stamp* sequence variants were identified in grapevines: St5, St19, and St30. The variant St19 was found exclusively in the analyzed grapevines. The variants St5 and St30 were found also in CaPsol strains infecting *E. sativa* (St5), *P. fagopyrum* (St5), *V. sativa* (St5, St30), and *C. arvensis* (St5), suggesting their possible involvement in CaPsol transmission routes to grapevine. Interestingly, previous study demonstrated that *H. obsoletus* survival on *V. sativa* and *C. arvensis* are comparable, reinforcing its association to BN epidemiology. Results from this and further studies can indicate how to select the groundcover plants for green manure mixture, excluding the species putatively involved in BN diffusion.

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Observation about relationship between *Scaphoideus titanus* and two differently susceptible cultivars to "flavescence dorée"

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"Flavescence dorée" (FD) is one of the most important diseases in Europe, due to the rapidity of the spreading and the importance of the damage. It is caused by a quarantine pathogen, a phytoplasma belonging to the 16SrV phylogenetic group, and transmitted from vine to vine by *Scaphoideus titanus*. FD symptoms are indistinguishable from those associated with the grapevine yellows' disease. They include leaf colour alterations, absence of inflorescences and irregular growth of the bud. *S. titanus* is a North America leafhopper, introduced in Italy in the '50s. It is closely ampelophagous, it only feeds on grapevine. During the years it has been observed that there are grapevine varieties more or less susceptible to "flavescence dorée". Chardonnay and Pinot grigio are much more susceptible to "flavescence dorée" compared to other varieties such as Tocai friulano and Moscato bianco. In fact, T. friulano and M. bianco show few symptoms or the damages are limited to some shoots, even if they are under the same disease pressure. In this work are reported two types of observations on two varieties differently susceptible to FD (Tocai friulano and Chardonnay): i) vector survival on vine seedlings, and ii) vector distribution on vineyard. In 2019 a test was made on vine seedlings, comparing four plants of T. friulano with as much of Chardonnay, in order to study the survival of three different ages of the vector: L2-3, L4-5 and adult. In every plant from 10 (on adult test) to 25 (on L2-3 test) *S. titanus* have been placed, enclosed in a wire mesh cylinder covered by a dense knit of pantyhose. The specimens have been counted every 2-3 days, for a maximum of 40 days. Regarding the field test, in 2019 ten vineyards were chosen in Friuli Venezia Giulia region, grown with Tocai friulano and Chardonnay with these characteristics: the two different varieties needed to be close to each other, they had to share the same landscape and had to have the same cultivation practices. In every vineyard from 2 to 10 yellow sticky traps for monitoring of *S. titanus* were placed. They have been positioned symmetrically to each other within the same parcel and they were changed every 15 days from July to August. Among these vineyards, in 2020 only those with a considerable number of vectors were monitored again. For each vineyard 10 yellow sticky traps were posed and were changed every 15 days from July to September. On vine seedling test, statistically significant differences were observed only in the first test, in L2-3 ages, where approximately double mortality was found in Tocai compared to Chardonnay. In the other two tests no significant differences were highlighted. In the first year of field monitoring, 4 plots have not captured specimens of *S.titanus*. Among the other six, three of them had a significant difference in the number of caught specimens, in both monitoring periods. In all these cases the number of vector specimens was larger on the traps positioned in the Chardonnay vineyards than those on the T. friulano. In 2020, however, this trend was confirmed only in one plot and only for the first monitoring period. This preliminary evidence indicates that a close relationship may exist between *S.titanus.* and varietal susceptibility. This relationship could be inherent to the active choice by the vector and/or the production of volatile and metabolite compounds by the plant. Further studies are certainly needed.

High infection rate of "flavescence dorée" phytoplasmas in *Orientus ishidae* and *Alnus glutinosa* in Valtellina

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This work, carried out in the summer of 2020, focused on investigating the "flavescence dorée" (FD) phytoplasma diffusion in *Scaphoideus titanus* (epidemic vector), *Orientus ishidae* (secondary vector), and *Alnus glutinosa* (plant host) in viticultural (Fracia vineyard, Valgella sub-area) and forest (Castello dell'Acqua and Chiuro) areas of Media Valtellina (Sondrio, North Italy). The conducted activities were: (i) starting from June, surveys on grapevine yellows (GY) symptoms and monitoring of *S. titanus* and *O. ishidae* by chromatic traps in the Fracia vineyard, Valgella sub-area; (ii) starting from mid-July, monitoring of *S. titanus* and *O. ishidae* adults by chromatic traps, and collection of 39 leaf samples from 13 *A. glutinosa* plants in forest areas (municipalities of Castello dell'Acqua and Chiuro); (iii) total nucleic acids extraction from collected plant samples and insects; (iv) 16SrV phytoplasma identification by nested PCR-based amplification of *map* gene; (v) phytoplasma typing and phylogeny by sequencing and bioinformatics analyses of *map* gene amplicons. In Fracia vineyard, GY symptoms were observed only in two grapevines, one of which was found infected by FD phytoplasma (based on analysis by Lombardy Region Phytosanitary Service). *S. titanus* was captured only within the vineyard (7 larvae, 6 adults), while *O. ishidae* adults were found both in the vineyard (28) and, prevalently, in the forest areas (45 adults). PCRs identified FD phytoplasma in 31.1% (14/45) of *O. ishidae* adults from forest areas and 100% of the *A. glutinosa* samples, while all the *S. titanus* and *O. ishidae* specimens, captured in the vineyard, tested negative. Amplicons of the *map* gene, obtained from 14 *O. ishidae* specimes and 33 alder samples (from 13 plants), were sequenced. The nucleotide sequences were compared by alignment with the database (https://doi.org/10.1371/journal.ppat.1007967). FD phytoplasmas, clusters FD1 and FD2, were found in 8 *A. glutinosa* plants (strains M50, M58 and M121) and in 13 *O. ishidae* specimens (strains M38 and M50). Phytoplasmas belonging to the Alder yellows (AldY) (strains M51, M78, M106, M117) and Palatinate grapevine yellows (PGY) (strain M48) clusters were found in the remaining samples. This investigation indicated that *A. glutinosa* is an important source of 16SrV phytoplasmas but has low relative abundance in the territory and is distributed at a significant distance from vineyards. The results also indicated that *S. titanus* has a sporadic presence in the examined area and given the absence of positive specimens, suggested that its role in the epidemiology of FD is negligible. On the contrary, *O. ishidae* is widespread in the territory (including vineyards) and frequently tested positive for FD phytoplasmas. However, the distance of the alders from the wine-growing areas made the probability of transmission of FD phytoplasma to grapevines extremely low. In conclusion, this study evidenced that important sources of 16SrV phytoplasmas (alders) as well as secondary non-epidemic vectors (*O. ishidae*) are present in Media Valtellina. In the monitored vineyard, the incidence of FD is very low, and this may be due to the lack of pathogen plant hosts in the vicinity of the crops. In wine-growing areas located near woods with the presence of alders, the *A. glutinosa* - *O. ishidae* system can play an important role in maintaining a reduced but constant presence of new FD phytoplasma infections in the vineyards.

First report of "flavescence dorée" phytoplasma identification and characterization in the leafhopper species *Graphocephala fennahi, Japananus hyalinus, Hishimonus hamatus*

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During a survey conducted in Canton Ticino (Southern Switzerland) and focused on edges and woods surrounding vineyards infected with "flavescence dorée" (FD) 5,137 leafhoppers and planthoppers were captured, by means of yellow sticky traps exposed from June 22nd to October 21st, 2017. In particular, ten traps were placed in a plot in Stabio, twelve in a plot in Bedano and eight in a vineyard in Rovio and changed every week. Among the captured insects, 53 were *Graphocephala fennahi*, 38 *Japananus hyalinus*, and 17 *Hishimonus hamatus*. *G. fennahi* specimens were caught during all the vegetative season, while the captures of *J. hyalinus* were limited from the end of June until the end of August, and those of *H. hamatus* between the second week of July and late October. The specimens of the three species were detached from the traps for DNA extraction and subsequent molecular analyses for the identification of 16SrV group phytoplasmas. After a first screening conducted with a group specific real time PCR, the positive samples were amplified by nested PCR both on the ribosomal gene region and on the *secY-map* genetic *locus*. RFLP analysis was performed on the ribosomal gene amplicons, whereas double-stranded sequencing was conducted for the purified *secY-map* PCR products. The molecular analyses evidenced the presence of phytoplasmas in three samples: one specimen of *H. hamatus* out of eight analysed samples; one pooled sample of *J. hyalinus*, grouping five insects, out of ten; one pooled sample of *G. fennahi*, grouping three insects, out of 14. The RFLP analyses showed that all the phytoplasmas present in the three samples belonged to the 16SrV-C ribosomal subgroup. The *secY-map* sequencing revealed that *H. hamatus* was infected with the M12 genotype and *J. hyalinus* with the M50 one. On the opposite, at least two different phytoplasma genotypes were present in the positive pooled sample of *G. fennahi*, as various double peaks could be observed in its chromatograms. Phytoplasmas with both M12 and M50 *secY-map* genotypes can be transmitted by the vector of FD *Scaphoideus titanus* and they are probably involved in FD outbreaks in some European regions. So far M12 had been found in grapevines from North-Western Italy; M50 had been detected in grapevines and in alders among plants, and in *S. titanus, Orientus ishidae* and *Oncopsis alni* among insects, in Italy, France, Hungary and Germany. In this study, the entomofauna present on the vine canopy was not addressed, however *H. hamatus* was already sporadically identified in Switzerland on the vineyard floor vegetation and on the vine canopy. Moreover, specimens of all the species caught were observed on plants facing towards the vineyards. In conclusion, the finding and characterization of FD phytoplasma genotypes in the three insect species examined suggest that these leafhoppers could play a role in the epidemiological cycle of FD, if, in the future, their ability to acquire and transmit the phytoplasma to other plants and in particular to grapevine is demonstrated. The low density of captures and positivity suggests that this role could be marginal.

Epidemiological investigations and molecular characterization of '*Candidatus* Phytoplasma solani' in grapevines, weeds, vectors and putative vectors in western Sicily

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"Bois noir", caused by '*Candidatus* Phytoplasma solani', is one of the most widespread diseases in the Euro-Mediterranean basin. Particularly complex result the interactions between phytoplasma, grapevines, weeds, and vectors. To solve this intrigue ecological relationships, molecular epidemiology was applied. In 2014 and 2015, we tracked incidence and spatial distribution of "bois noir" in a vineyard with three grapevine varieties in Sicily, Southern Italy, and we identified molecular types of the *tuf* and *vmp*1 genes in these naturally infected grapevines, according to the potential reservoir plants and vectors. Disease incidence in 2015 was significantly higher in 'Chardonnay' (up to 35%) than for 'Nero d'Avola' and 'Pinot noir' (<5%). All grapevine, weed, and insect samples were infected by '*Ca*. P. solani' *tuf*-type b. Most of the collected insects were related to *Vitis* spp. and belonged to Cicadellidae *Neoaliturus fenestratus*, *Empoasca* spp., and *Zygina rhamni*. The characterization of the *vmp*1 gene revealed six different *vmp* types in grapevines (V1, V4, V9, V11, V12, V24), three in weeds (V4, V9, V11), and four in insects (V4, V9, V11, V24). V4 and V9 were found both in hosts and vectors, with V9 that was predominant. Virtual restriction fragment length polymorphism (RFLP) analysis based on the nucleotide sequences of *vmp*1 gene supported the data of the conventional RFLP. Connections between the molecular data recorded in the vineyard ecosystems, further molecular investigations based on geographical related genes in phytoplasma and their vectors, and the application of innovative tools based on the geostatistical analysis will contribute to further clarification of the specific ecological and epidemiological aspects of '*Ca*. P. solani' in Sicilian vineyards.

This work is dedicated to the memory of Dr Gaetano Conigliaro, that recently passed away

Possible implication of *Orientus ishidae* in apple proliferation epidemiology. Preliminary study in Trentino (Italy)

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Apple proliferation (AP) is one of the most dangerous phytoplasma diseases in apple orchards and can cause severe economic losses due to the production of small fruits with low organoleptic qualities in the infected plants. The causal agent is '*Candidatus* Phytoplasma mali', belonging to the 16SrX phytoplasma group, and nowadays it is distributed within the most important European apple-producing areas. The phytoplasma is transmitted by insect vectors, mainly by two psyllids *Cacopsylla picta* and *C. melanoneura*, only occasionally by the leafhopper *Fieberiella florii*, but the research for new insect vectors is still ongoing. *Orientus ishidae*, or mosaic leafhopper (MLH) is an East Palearctic species introduced first into USA with the import of ornamental species that is spreading also in Europe. MLH has a wide range of host plants, including some cultivated species like grapevine and apple, it is able to transmit 16SrV phytoplasmas (*i.e.*, grapevine "flavescence dorée") from infected broad bean to grapevine and '*Ca.* P. pruni' (peach-X disease) from symptomatic chokecherry to peach. In 2019, high MLH populations were found, for the first time, in apple orchards in Trentino province. Given its ability to transmit phytoplasmas, an investigation was started in the summer of 2020 to establish its biology on apple and whether MLH could play a role in the AP epidemiology. The results indicate that MLH can conduct the full life cycle on apple trees: nymphs emerged during June from eggs laid under the bark of apple wood and adults are presents from early July to late October. Moreover, in the MLH adults collected at the end of July and individually tested by PCR techniques (primer fAT-rAS) the AP phytoplasma was detected in the 25% of investigated specimens. These preliminary results revealed that an important percentage of MLH population can acquire and carry '*Ca.* P. mali'. Studies on the ability of MLH to transmit AP to apples are currently ongoing to better understand its role in the AP epidemiology and the possible consequence in the AP management.

Frequency of "flavescence dorée" detected in adults of *Scaphoideus titanus* collected in vineyards with different epidemic pressure of the disease

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As per the routine control of the spread of quarantine diseases and harmful insects (PEST project) in the vineyards of the province of Trento, carried out by the staff of the Technological Transfer Center of the E. Mach Foundation, since 2016 molecular analysis' have been carried out (PCR) to verify the presence of "flavescence dorée" phytoplasma (FDp) in adults of the leafhopper *Scaphoideus titanus*. The insects are collecting during late summer in vineyards where their population density is known and the presence of diseased vines is monitored. The aim is to evaluate the potential role of leafhopper in the adult stage in the spread of FDp in the Trentino viticultural environment. The survey took into consideration, in the various vintages, the period of late August-early September, a potentially critical phase for the epidemiology of FD as the following factors may coexist: 1) the populations of *S. titanus* are potentially abundant in vineyards as the peak of flight occurs, usually, in August, 2) the insect vector is in adult stage with the ability to move 3) the presence in the field of most of the plants that have already manifested symptoms for that season 4) maximum concentration of phytoplasma in the tissues of the infected vines and high efficiency of acquisition of phytoplasma by the leafhopper. To avoid possible degradation (and therefore alteration of the results) and to have a photograph of the situation at a precise moment of the season, the insects to be analyzed were captured alive through the *frappage* technique performed on the canopy of the vines. For each vineyard monitored, the *frappage* operations lasted 60 minutes and involved a variable number of plants chosen randomly within the site. Molecular investigations were performed at the phytopathological diagnosis laboratories in S. Michele all'Adige (TN); the adults of *S. titanus* were analyzed individually using a qPCR with a probe TaqMan® specific for the pathogen *FD-related* phytoplasma. In total 49 vineyards were monitored for 5 years and 2087 individuals were collected and analyzed: those that tested positive for FD were 181 (8.7%) and come from 26 different sites (53% of the total). The number of plantations with infected individuals and the insect positivity rate varies according to the epidemic situation of FD in the vineyard. The sites where the insects were collected were divided into three "disease pressure" classes based on the results of visual inspections in the field: 1) low: no presence of symptomatic plants and low presence of cases of FD in that area, 2) medium: sporadic or limited presence of diseased vines in the vineyard (<1%) and in the surrounding area, 3) high: plants with many symptomatic plants in outbreak areas. In areas with "low disease pressure", insects were collected from 17 vineyards for a total of 842 individuals. Only one sample was found positive to FD and, specifically, only to one individual among the 179 captured at that site (0.6%). In the 16 "medium disease pressure" vineyards, infected specimens were found in 63% of the samples with a generally low positivity rate: in 8 vineyards it was between 1 and 5.9%, but in 2 sites it reached values relatively high, 12.5% and 25%. In the "high disease pressure" situations, in 15 out of 16 samples of *S. titanus* adults were found positive for FD and with an average rate of 26.2%. The vineyards in which the highest levels of infected individuals were found (from 38.2% to 58.6%) were all characterized by an incidence of symptomatic vines greater than 20%. This work confirms therefore the role of primary importance of *S.titanus* in Trentino in spreading the disease vineyards during late summer.

Three-year monitoring of potential insect vectors of "flavescence dorée" in Trentino vineyards through use of chromotropic traps.

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Since the appearance of "flavescence dorée" (FD) in the Trentino vineyards in 2001, the Technological Transfer Center of the E. Mach Foundation, on behalf of the Phytosanitary Office of the Autonomous Province of Trento, has carried out annual monitoring activities to evaluate the spread in the viticulture areas of the main insect vector of the disease: the leafhopper *Scaphoideus titanus*. From 2015 the presence of other leafhoppers/ planthoppers of interest for viticulture has also been evaluated by the use of adhesive chromotropic traps (model "Glutor yello" by Biogard®, 10x25 cm, positioned vertically in the center of the vineyard, exposed from the beginning of July until the beginning of November and replaced every 14 days). In this first year of the survey other potential vectors of FD were found: *Orientus ishidae*, *Dictyophara europaea* and *Phlogotettix cyclops* and other species more or less relevant for viticulture were identified, including: *Fieberiella florii*, *Hishimonus* cfr *hamatus, Hyalesthes obsoletus, Neoaliturus fenestratus* and *Philaenus spumarius*. However, the discovery of *Erasmoneura vulnerata* in the traps only began since 2017. The monitoring network is composed of 101 stations distributed in all the main provincial vineyard areas across 39 municipalities. The vineyards, located between 82 and 669 meters above sea level, belong to private farms, in the 86% of the cases the training system is "pergola" and the variety most represented are Chardonnay (39% of sites) and Pinot Gris (28%). This work summarizes the results of the last three years of monitoring, from 2018 to 2020, a period during which the insecticide defense was carried out in all the vineyards according to the indications given annually by the mandatory control for FD and its vector: in 72% of cases with active substances that can be used in IPM strategies and the remainder with pyrethrum-based insecticides; in 2018 and in 2019 generally with a single insecticide treatment in the post-flowering period of the vine, while in 2020 with two insecticide treatments, both in organic and integrated viticulture. The survey shows that the main insect vector of FD, *S. titanus*, is present in almost all the monitored sites, and the population densities are generally significant: the portion of the sites that recorded more than 10 catches per season was, in three years, on average 77%, while those more than 100 were 23%. The flight of the insect had a similar trend in the three years of observation, with a peak detected in the period between 5th and 18th August, the month in which 50% of total annual catches are recorded. The observations showed that in the Trentino environment, the presence of adults is very prolonged and in some cases it can last up to the phase immediately preceding the fall of the leaves of the vines, with a duration of the flight, therefore, of about 18 weeks. As for the other potential FD vector species, in general, low density populations were found and a fairly limited spread in the vineyards. More relevant levels of catches were recorded for *O. ishidae* (23% of the sites); the insect is confirmed to have a biological cycle very similar to *S. titanus*, although earlier in the season. For *D. europaea* and *P. cyclops* most of the findings consist of single and sporadic catches which took place during the season in a limited number of locations (10%) scattered throughout the various wine-growing districts of the province.

Ecology-based analysis of association between *Spartium junceum* and 16SrV phytoplasmas

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Spartium witches' broom (SpaWB) disease has spread in Italy and Spain and has been associated with single or mixed infection of phytoplasma members of the 16SrX-D and 16SrV groups. A SpaWB outbreak started in Sicily in 2010 prompted us to identify and characterize associated phytoplasmas. Over 80 samples of Spanish broom (*Spartium junceum*) and around 270 individuals of the potential vector *Livilla spectabilis* were collected in 17 sites all over the island and analyzed. Single and mixed infections of 16SrV and '*Candidatus* Phytoplasma spartii' were detected in Spanish broom samples and for the first time in *L. spectabilis*. The 16SrV isolates were further characterized by multilocus sequence typing (MLST) to determine their phylogenetic relationship with "flavescence dorée" phytoplasma (FDp) and evaluate the risk of host-jumping to the grapevine. Phylogenetic analysis of most of the analyzed genes grouped *S. junceum* 16SrV-C isolates with FDp isolates infecting grapevine and *Scaphoideus titanus*. Notably, phylogenetic analysis of the *vmp*A gene clustered the *S. junceum* isolates with FDp genotypes transmitted by *S. titanus*. This study extends the knowledge of SpaWB epidemiology, focusing on the possible risk of a 16SrV host jump from Spanish broom to the grapevine. Spanish broom was identified as a reservoir and potential inoculum source of phytoplasmas that cause severe disease in cultivated crops. Furthermore, the *L. spectabilis* psyllid may be involved in the epidemiology of this 16SrV-C phytoplasma, although in the absence of *in vivo* transmission trials. The study further confirms the strong ability of phytoplasmas to adapt to new hosts and vectors, thus leading to potential phytosanitary emergencies.

Culture dependent analysis of the microbial community associated to phytoplasma recovered apple trees

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Beneficial endophytes are microorganisms that, thanks to their symbiotic relationship with plants, represent an ideal mean to counteract the deleterious effects caused by plant pathogens. Little information is available about the role of endophytes as potential control agents against apple proliferation (AP), a disease caused by '*Candidatus* Phytoplasma mali'. This study aims to isolate and identify rhizospheric and endophytic microorganisms from trees that recovered from AP phytoplasma infection. The goal is to discover endophytes that can be used for treating or preventing AP. Roots, shoots, leaves and material from the rhizosphere were collected from symptomatic, recovered, and infected trees that never displayed AP symptoms. Bacteria and fungi were isolated from the collected samples using selective media. Roots, shoots, and leaves were surface sterilized to isolate only endophytic microorganisms. For the isolation of the rhizospheric microorganisms, a lavage of the roots was prepared and plated. Bacteria and fungi were differentiated based on morphological colony characteristics and identified by sequencing the bacterial 16S rRNA gene and the fungal ITS region. A total of 558 bacterial and 76 fungal isolates representing 77 and 36 genera were recovered from the plant tissues and the rhizosphere. To assess the potential of these microorganisms as biological control against AP phytoplasma an *in vitro* surrogate assay will be performed. In this assay the most promising endophytic and rhizospheric isolates will be tested regarding their antimicrobial activity against the easily cultivable surrogate organisms *Spiroplasma melliferum* and *Spiroplasma citri*. Furthermore, selected isolates will be evaluated regarding their plant growth promoting activities. The analysis of the endophytic and rhizospheric microbial community associated to symptomatic, asymptomatic and recovered plants is sought to help to gain information about the mechanisms involved in the recovery process.

III SESSIONE

INTERAZIONE OSPITE-PATOGENO-VETTORE

New insight into seasonal vector competence of *Cacopsylla melanoneura* in Northwest Italy

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The transmission of phytoplasmas is the result of an intricate interplay involving pathogens, insect vectors and host plants. The knowledge of the vector competence during its lifespan allows to define more sustainable well-timed control strategies targeted towards the most worrisome life stages and, therefore, to the management of the spread of the diseases. Control strategies should consider not only the vector abundance, but also its competence and efficiency during the growing season in relation to its phytoplasma load. The quantity of '*Candidatus* Phytoplasma mali' load in *Cacopsylla melanoneura* was analysed in order to assess the relative acquisition efficiency and the vector infective risk in Northwest Italy. All the insects used in the experiments were originated from *C. melanoneura* overwintered adults collected from asymptomatic plants in commercial apple orchards located in the Aosta Valley region. '*Ca.* P. mali' was quantified in overwintering adults, nymphs and newly emerged adults after different acquisition access periods. Moreover, immediately after emergence, newly emerged adults were periodically transferred to shelter plants (conifers) in order to follow the multiplication of the pathogen during the aestivation and the overwintering period at different time points. '*Ca.* P. mali' was detected in all stages, highlighting a higher acquisition efficiency in 3^{rd}-5^{th} instar nymphs and newly emerged adults due to the longer acquisition access period. The phytoplasma load significantly increased during the period spent on conifers for aestivation and overwintering, demonstrating a long latency period. Overwintered adults retained high quantities of phytoplasmas, representing the most worrisome stage. However, in several nymphs and newly emerged adults it was possible to detect a sufficient quantity of phytoplasmas to consider these stages infective. Therefore, in the investigated area, particular attention should be paid to the management of overwintered *C. melanoneura* as soon as they return to the orchards, but also to newly emerged adults, particularly in orchards with a high infection rate and when the migration to conifers is delayed due to weather conditions.

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Population genomics of factors influencing '*Candidatus* Phytoplasma mali' transmission

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Apple proliferation (AP) is a disease caused by '*Candidatus* Phytoplasma mali', causing proliferation of auxiliary shoots and a decrease in fruit size and quality. '*Ca*. P. mali' is mainly transmitted between apple trees by two psyllids, *Cacopsylla picta* and *Cacopsylla melanoneura*, with regional differences. *C. melanoneura* is considered the main vector of AP phytoplasma in Northwestern Italy, but it is a poor transmitter in Northeastern Italy and other parts of Europe. In contrast, *C. picta* is the primary vector in most European populations with variable transmission rates among populations. Other occurring *Cacopsylla* species do not transmit phytoplasmas. Knowledge about factors influencing the transmission efficiency of '*Ca*. P. mali' is currently scarce. To compare the acquisition efficiency of different populations of *C. melanoneura*, we collected several individuals in orchards in South Tyrol, Trentino and Aosta Valley. We reared them on plants infected by '*Ca.* P. mali' strains from Aosta Valley and subtypes AT1, AT2 from South Tyrol. After an inoculation period of several weeks, F1 individuals were collected and tested for the presence of phytoplasma. Preliminary results show that all families from Aosta Valley became infected when reared on the trees containing the phytoplasma strains from Aosta Valley. On the other hand, only one third of Aosta Valley families were infected with phytoplasma subtype AT2 and none with AT1. We observed differences in the acquisition efficiency of the insects collected in various locations of Trentino and South Tyrol. For instance, families from Eisacktal were able to acquire only phytoplasma subtypes AT1 and AT2, whereas families from Dorf Tirol could acquire phytoplasma subtype AT1 as well as the one from Aosta Valley. Overall, 66% of families from Trentino – South Tyrol could acquire the phytoplasma strains from Aosta Valley, 33% the subtype AT1 and 50% the subtype AT2. The concentration of phytoplasma in the analyzed insects varied among members of the same family, with $3.88x10^7$ being the highest value. Moreover, not all families reared on different branches of the same infected tree were able to acquire phytoplasma. For comparison, a similar acquisition experiment with *C. picta* individuals collected in Trentino is on-going.Based on these results, we will select some candidates for insect metagenome sequencing to determine key factors affecting phytoplasma acquisition in different *Cacopsylla* species. These data will provide novel insights into the complex biology of the apple proliferation caused by '*Ca*. P. mali' and will be therefore an important milestone in combatting this disease.

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Transcriptional profile of "flavescence dorée" phytoplasma during different infection stages of host plants and insect vectors

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The expression of 11 genes of "flavescence dorée" phytoplasma (FDp) was studied at different times after infection of natural hosts (plant *Vitis vinifera* and vector *Scaphoideus titanus*) and experimental laboratory ones (plant *Vicia faba* and vector *Euscelidius variegatus*), to highlight different transcriptional patterns of the pathogen during the infection of host plants and insect vectors. In particular, the infection in grapevine was studied under natural field conditions, analysing the same plants in three moments of the vegetative season (late June, late July and late August). On the other hand, the analysis of broad bean samples was carried out at 20, 30 and 40 days after the experimental inoculation with infectious *E. variegatus*, while the specimens of both vector species were tested at 21, 28 and 35 days after the starting of phytoplasma acquisition from infected broad bean plants. The collected samples were subjected to simultaneous extraction of DNA, intended for FDp diagnosis and quantification of the number of phytoplasma cells, and RNA, intended for gene expression analysis of FDp transcripts. The genes selected for the latter analysis encode the immunodominant membrane protein (imp), a group II catalytic intron, two hypothetical proteins with transmembrane domains, two transporters (ftsY and CoABC) and other proteins involved in the cell cycle (spoVG), in the protein (tldD) and sugar (ysdC) metabolisms, in the oxidative stress response (osmC) and in the transcription promotion (rpoD). All the genes analysed were expressed in the four hosts and those encoding the intron and the immunodominant membrane protein imp were the most transcribed, suggesting key roles during the infection cycle for these pathogen proteins.

Transmission of "flavescence dorée" phytoplasma requires a reduced latency period in *Euscelidius variegatus* when acquired by adults

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Vector-mediated transmission of phytoplasmas requires an acquisition access period (AAP), followed by a latency period (LP) and finally an inoculation access period (IAP). In the vectors, the nymphal stages are generally thought to be the most efficient for acquisition, because the long LP that is necessary for allowing successful transmission may exceed the adult life span. However, it has been previously shown that if the acquisition takes place at the adult stage, a shorter LP may be requested than in nymphs. On the other hand, the LP temporal dynamics in adult vectors is still mostly unknown. This study aims at determining the minimum time required for phytoplasmas to colonize the gut system and salivary glands of the vector, finally allowing successful inoculation. Therefore, the model leafhopper *Euscelidius variegatus* was employed for characterizing the phases of phytoplasma transmission process; "flavescence dorée" phytoplasma (FDp) was used for controlled transmission experiments. Healthy *E. variegatus* adults were exposed to FDp-infected broad bean plants for an AAP during seven days, subsequently they were individually transferred to a healthy broad bean to undergo IAP. Seven distinct IAPs (IAP 1-7) were applied, each one during 24 hours, from day 8 to day 14; every IAP followed a LP with different duration, from 0 to 7 days. FDp infection was investigated in vectors (in the midgut and salivary glands separately) and in inoculated plants, by means of molecular diagnosis as well as fluorescence *in situ* hybridization. The phytoplasma was detected in midgut samples already in IAP1, showing an infection rate of 50%, whereas it was found in salivary glands starting from IAP2, reaching a 30% infection rate. Additionally, FDp was successfully inoculated to broad beans from IAP4 (10% infection). Our results indicated that *E. variegatus*, after acquisition as an adult, may become infective as soon as nine days from the beginning of phytoplasma acquisition. Consequently, these findings significantly contribute to the knowledge on phytoplasma transmission, as they point out the need for a reconsideration of the role of adults in influencing the epidemiology of "flavescence doreé", in the light of their enhanced potential capability to complete the transmission process.

Chloroplasts under attack!

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Symptoms of phytoplasma diseases vary between the different affected species, however leaf yellowing or chlorosis are seen in most infected plants. Those symptoms amongst others are a hint that chloroplasts and their components are affected by phytoplasma infections. Indeed, perturbations of the photosynthetic apparatus and carbohydrate metabolic pathways of the host plant are the most prominent alterations seen in diverse phytoplasma pathosystems. The chloroplast is the photosynthetic organelle of the plant cell and besides its role in energy metabolism and CO_2 fixation, it is also a key player in the plant's response against pathogens. Different studies have shown that the chloroplast plays an important role in plant immunity and might thus be an interesting target for effector-mediated plant manipulation by pathogens. The chloroplast is an important signaling hub during the immune defense response because it is the production site of many phytohormones involved in plant defense, the major site of reactive oxygen species (ROS) production and a site for nitric oxide synthesis, both acting as signaling molecules involved in hypersensitive response (HR). This makes the chloroplast an interesting target for plant pathogens. While necrotrophic pathogens use effectors to induce ROS production and HR, some biotrophic pathogens positively influence photosynthesis by effectors. HopBB1 and HopR1, two effectors of the bacterial pathogen *Pseudomonas syringae*, modulate jasmonate synthesis and bind to the transcription factor TCP13, that is involved in the regulation of chloroplastic gene expression. The phytoplasmal effector protein SAP11 binds also to TCP13 and jasmonate levels are altered upon '*Candidatus* Phytoplasma mali' infection. Phytoplasma infection causes massive perturbations in chloroplasts, but detailed studies about the interaction of the pathogen and the chloroplast are missing. We developed three possible hypotheses regarding the role of chloroplasts in phytoplasma infection:
Hypothesis 1: The phytoplasma actively attacks the chloroplast for its own benefit: '*Candidatus* Phytoplasma mali' suppresses H_2O_2 production to better colonize the canopy and applies 'remote control' strategies to manipulate the chloroplast.
Hypothesis 2: Phytoplasma infection induces a nonspecific immune response, which leads to general perturbations at the chloroplast level: A general pathogen recognition takes place upon phytoplasma infection and also induces changes in the chloroplast. The observed aberrations might thus be a general defense or stress response.
Hypothesis 3: Chloroplast aberrations are a collateral damage of infection that is neither beneficial for the phytoplasma nor for the plant: In some phytoplasma-pathosystems a fast decline of the plant host occurs, while in others a co-existence between the pathogen and its host can be observed. Until now, experimental corroboration is missing for any of the three hypotheses. Thus, further research is necessary to unravel missing links between phytoplasma infection and the chloroplast involvement.

Discovering insect viral resources and modelling their potential for biocontrol of phytoplasma vectors

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Virus-based biocontrol technologies in crop protection have received much attention in recent years due to their narrowed target and their environmentally-safe properties, which make them harmless both to human and non-target organisms. The H2020-funded VIROPLANT project aims at identifying potential biocontrol agents from the virosphere of phytoplasma vectors. In particular, this study focuses on "flavescence dorée" phytoplasma (FDp) of grapevine, its principal vector *Scaphoideus titanus* (Hemiptera: Cicadellidae), and *Euscelidius variegatus* (Hemiptera: Cicadellidae), used as an efficient vector of FDp in laboratory condition. Through transcriptomic analyses, four novel +ssRNA viruses putatively belonging to the family Iflaviridae have been found to be naturally present in the above mentioned leafhopper species. Scaphoideus titanus iflavirus 1 (STiV-1) and Scaphoideus titanus iflavirus 2 (StiV-2) were identified during an NGS analysis of *S.titanus* wild populations sampled in Europe and in the US, respectively. Finally, other two iflaviruses, named Euscelidius variegatus virus 1 (EVV-1) and Euscelidus variegatus virus 2 (EVV-2), were serendipitously found in our in-house rearing of *E. variegatus* during an RNA-seq analysis. In particular, EVV-1 was found to be present with a 100% prevalence. The discovery of a virus-free lab population coming from France allowed us to characterize the transmission routes of this iflavirus. The ability to transmit FDp was also tested for both EVV-1 infected and non-infected individuals. Preliminary results showed that the virus was constantly detected both in phytoplasma-exposed and non-exposed vectors, with no differences in viral load. Further experiments aiming at setting up an efficient inoculative strategy of EVV-1 in *E. variegatus* virus-free individuals are ongoing. Indeed, an infectious clone derived from EVV-1 was able to infect and replicate in virus-free insects. Even though the infection rates still need to be improved, an EVV-1-derived VIGS might provide the unprecedented opportunity to manipulate the expression of endogenous insect genes by promoting virus-induced gene silencing by the iflavirus and thus unfold the host-pathogen mechanisms involved in phytoplasmas transmission in both leafhopper targets.

Fitness of *Scaphoideus titanus* varies on grapevine cultivars with different susceptibility to "flavescence dorée"

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Scaphoideus titanus is a Nearctic leafhopper, naturalized in Europe were it became an efficient vector of the "flavescence dorée" (FD) phytoplasma to grapevine. We investigated the existence of differences in the leafhopper fitness on different grapevine genotypes. Four fitness parameters were considered: developmental time of nymphs, survival rate of nymphs and adults, female prolificacy. *S. titanus* fitness parameters were described on three grapevine cultivars, known to have different susceptibilities to FD: Barbera as FD-highly susceptible, Brachetto and Moscato as FD-poorly susceptible. Results showed significant differences in nymph developmental time, with shorter time required from 1st instar nymph to adult stage on Barbera cultivar. Consistently, both nymph and adult of *S. titanus* showed better survival on this genotype. Finally, females reared on Barbera developed more mature eggs in their ovaries, compared to the other two cultivars, especially considering the first sampling time (14 days post emergence, dpe). Accordingly, Barbera-reared females expressed high levels of vitellogenin mRNA already at 14 dpe, while those reared on Brachetto and Moscato showed a delayed expression. These results suggest a preference of *S. titanus* for Barbera, and pair with the high susceptibility of this cultivar to the disease. On the other hand, fitness parameters of *S. titanus* on Moscato, in particular low survival, slow development and low female prolificacy, may suggest the presence of antibiotic compounds that actively interfere with *S. titanus* fitness. Our work suggests that plant resistance to FD may be partly mediated by resistance towards the vector. The implications of these results are discussed with special focus on their application for conventional or NGS-based exploitation of plant genetics to improve grapevine resistance to FD.

Immune activation in the vector *Euscelidius variegatus* in response to phytoplasmas and symbiotic bacteria

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In insects, the immune response is a crucial phase defining the interaction with microorganisms, including pathogens, commensals, and mutualists. Hemipteran vectors of phytopalsmas support high phytopathogen load; moreover they are stably colonized by a diversity of bacterial symbionts. These bacteria have evolved over time to be retained by the host, implementing strategies such as immune adaptation or modulation. Several studies have been addressed to characterize insect response to entomopatogens, whereas little work has been conducted on the role of immunity in regulating gut microbiota homeostasis and phytopathogen transmission. The objective of this research is to investigate the expression of immune-related genes in the leafhopper vector *Euscelidius variegatus* in response to infection by the symbiotic acetic acid bacterium *Asaia* ap. and by "flavescence dorée" phytoplasma (FDp). *Asaia* has been previously shown to be capable to limit FDp acquisition by the vector. The expression of four genes was measured to explore different elements of *E. variegatus* immunity. Specifically, selected genes are involved in the expression of: i) the antimicrobial peptide defensin; ii) the phenoloxidase enzyme; iii) the kazal type 1 serine protease inhibitor, involved in melanization and oxidative stress induction; iv) the Ras/Raf pathway, contributing to hemocyte proliferation (the Raf gene was selected as a marker of this pathway). The response to *Asaia* infection was separately analyzed in whole insects, in midgut samples and in hemocyte primary cultures, and compared between FDp-free leafhoppers and insects undergoing different stages of phytoplasma colonization (early and late infection). The results indicated the specific activation of Raf gene in the midgut samples as a consequence of double infection by *Asaia* and FDp. *Asaia*-induced overexpression of Raf was observed already in the early phytoplasma infection stage. The specific gut localization and the early activation of this gene are consistent with the capability of *Asaia* to limit FDp acquisition in *E. variegatus*, suggesting the involvement of the related pathway in determining the antagonistic role exerted by the symbiont against the phytoplasma. The demonstrated immune modulation performed by *Asaia* in *E. variegatus* contributes to clarify the molecular machinery regulating the interference with phytoplasma acquisition. The involvement of Raf gene suggests that the reduced genetic array available in Hemiptera may have led to the reorganization of immune genes to play additional functions, normally supported by those genes that are missing in this order.

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The effector protein SAP11$_{CaPm}$ interacts with MdTCP18

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Phytoplasma as well as other pathogens release effector proteins to manipulate their hosts. The so far best characterized phytoplasmal effector is SAP11 from aster yellow witches broom phytoplasma and its homologs from other phytoplasma species. All SAP11-like effectors target transcription factors that belong to the protein family of TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR (TCP) 1 and 2. For SAP11$_{CaPm}$ from '*Candidatus* Phytoplasma mali' it was demonstrated that it interacts and destabilizes MdTCP4 (a.k.a. MdTCP25) and MdTCP13 (a.k.a. MdTCP24), two class II CINCINNATA (CIN)-like TCP transcription factors in apple. The destabilization of MdTCP4 leads to the downregulation of jasmonic acid (JA) biosynthesis and results in a reduced JA-response to pathogen attack. The degradation of MdTCP13 affects the phosphate metabolism, anthocyanin accumulation and the root architecture. Interestingly, leaf reddening and altered root growth are characteristic symptoms of a '*Candidatus* Pyhtoplasma mali' infection in apple. In addition, SAP11-like effectors from different phytoplasma species destabilize the class II CYC/TB1-like TCP transcription factor BRANCHED1, also known as TCP18, BRC1 or TB1. TCP18 is a regulator of branching in plants and suppresses the growth of axillary buds, represses the floral transition, and plays a role in plant immunity. Thus, the degradation of TCP18 by SAP11-like effector proteins is hypothesized to induce increased shoot branching. It has been shown that SWP1, the SAP11-like effector from wheat blue dwarf phytoplasma interacts with AtTCP18 and induces witches' broom symptoms when transiently overexpressed in *Nicotiana benthamiana* plants. Even though several SAP11-like effectors target TCP18-like TFs, an interaction between SAP11$_{CaPm}$ and MdTCP18 (a.k.a. MdTCP16) has never been proven and assays with AtTCP18 failed to show an interaction with SAP11$_{CaPm}$. Therefore, a Y2H screen with SAP11$_{CaPm}$ against Malus cDNA library was performed and resulted in the identification of a putative interaction between SAP11$_{CaPm}$ and a MdTCP18 fragment. It was, however, not possible to confirm the interaction with that fragment. Thus, the full length of the coding MdTCP18 sequence was amplified from apple, subcloned into a Y2H vector and used as a prey in a targeted Y2H approach against a SAP11$_{CaPm}$ carrying bait vector. The targeted Y2H approach revealed for the first time the interaction of SAP11$_{CaPm}$ with MdTCP18. The interaction was further confirmed *in planta* by bimolecular fluorescence complementation (BiFC) in *N. benthamiana* protoplasts. These results led to the assumption that SAP11$_{CaPm}$ might not only be responsible for decreased JA levels and leaf reddening, but also for the typical growth aberrations that cause the so-called witches' brooms in apple and give the disease its name, apple proliferation.

Investigating the role of *MdTCP4* of *Malus* × *domestica* and its role during phytoplasma infection

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Phytoplasmas secrete so called effector proteins, that manipulate the host physiology for the bacteria's benefit. The best characterized phytoplasmal effector is SAP11 from aster yellow witches broom phytoplasma. $SAP11_{CaPM}$ from apple proliferation (AP)-associated '*Candidatus* Phytoplasma mali' ('*Ca*. P. mali') is known to target and deactivate *Malus* × *domestica* transcription factors of the *TCP* family, amongst these MdTCP4 (a.k.a. MdTCP25). The present work aims to shed light on the role of the interaction between MdTCP4 and $SAP11_{CaPM}$ in the context of the phenomenon of tolerance against AP observed in certain Malus accessions. Independent *Malus* × *domestica* plants expressing *MdTCP4* under the control of the strong 35S CaMV promoter were generated *via* Agrobacterium-mediated transformation. Transgenic lines kept *in vitro* showed a ~30%-fold increase in the expression and no differences in phenotype compared to non-transformed plants. Following the soil acclimatization, the transgenic plants demonstrated a substantial increase in the overexpression level and displayed some peculiar phenotype characteristics, such as smaller and crinkled leaves, loss of apical dominance, and generation of more shoots than non-transformed control plants. *In vitro* transgenic plants were infected with '*Ca*. P. mali' using the micrografting technique and subsequentially screened for the presence of phytoplasma by qPCR. Results show that two of three transgenic lines displayed significantly lower phytoplasma concentrations than non-transformed plants. Low phytoplasma concentration and reduced or no symptom expression is a commonly observed characteristic of tolerant Malus accessions. Furthermore, the transcriptomes of three soil-acclimatized lines have been analyzed *via* RNAseq, and preliminary analyses show an up-regulation of several genes associated with plant development, abiotic stress responses and down-regulation of many chloroplast-related genes. To shed light on the potential role of MdTCP4 during tolerance against AP, the gene sequences in susceptible and tolerant genotypes have been compared. Sequence comparison and *in silico* analysis led to the prediction of two rare amino acid substitutions in the *MdTCP4* encoding genes of tolerant genotypes. One of these substitutions is in the predicted $SAP11_{CaPM}$ binding site and has never been reported to date. The interaction between the bacterial effector and the MdTCP4 variant from tolerant genotypes was analyzed in a heterologous yeast expression system (Y2H) and *in planta* (BiFC). In both assays the MdTCP4 variant from the tolerant Malus accessions interacted with $SAP11_{CaPM}$. Currently, the MdTCP4 protein variants from susceptible and tolerant accessions are analyzed to define their effector-binding characteristics.

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