COST Action FA0807
Integrated Management of Phytoplasma Epidemics in Different Crop Systems

COST Action FA0701
Arthropod Symbioses: from fundamental studies to pest and diseases management

Phytoplasmas, insect vectors, symbionts and plant endophytes

University of Milan, Faculty of Agriculture, Milan, Italy

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Abstract booklet

Program

09:00 - 09:15 Official welcome
Assunta Bertaccini, Piero Attilio Bianco and Daniele Daffonchio

9:15 - 10:40 Session 1
Phytoplasmas between symbiosis and virulence **Chair A. Bertaccini**

9:15-10:00 Saskia Hogenhout (United Kingdom)
Genomes of phytoplasmas: virulence or symbiosis?

10:00-10:40 Michael Kube (Germany)
Genome analysis: from sequence to protein function

10:40-11:00 Coffee Break

11:00 - 12:50 Session 2
Microbial symbiosis in insects **Chair P. Weintraub**

11:00-11:40 Claudio Bandi (Italy)
Microbial symbiosis in insects: General aspects and the *Rickettsiales* model

11:40-12:10 Daniele Daffonchio (Italy)
Acetic acid bacteria, an emerging group of symbionts of sugar-feeding arthropods

12:10-12:30 Wolfgang Jarausch (Germany)
Symbionts and endophytes in activities of COST FA0807 WG3

12:30-12:50 Barbara Jarausch (Germany)
Symbionts and endophytes in activities of COST FA0807 WG2

12:50-14:00 Lunch

14:00 - 15:45 Session 3
Symbionts in phytoplasma insect vectors and their transmission Chair D. Daffonchio

14:00-14:30 Yupa Hanboonsong (Thailand)
Bacteria symbionts in vectors of sugarcane white leaf disease

14:30-14:55 Elena Gonella & Alberto Alma (Italy)
The insect symbionts of insect vectors of phytoplasmas to grapevine

14:55-15:20 Phyllis Weintraub (Israel)
Symbiotic bacteria associated with vectors of phytoplasma in carrots

15:20-15:45 Noura Raddadi (Italy)
'*Candidatus* Liberibacter europaeus', a novel microorganism associated with *Cacopsylla* spp.: a symbiont, a potential pathogen or an endophyte?

15:45-16:00 Coffee Break

16:00 - 17:40 Session 4
Towards novel disease control strategies Chair P. A. Bianco

16:00-16:40 Stéphane Compant (France)
Endophytic bacteria: niches of colonization and biocontrol properties

16:40-17:10 Daniela Bulgari, Paola Casati, Piero Attilio Bianco (Italy)
Endophytes and recovery from grapevine yellows disease

17:10-17:25 Simone Grisan (Italy)
Fungal endophytic community of grapevine: methodological study and practical approaches

17:20-17:40 Rachele Polizzotto (Italy)
Polyphasic approach for the characterization and the study of fungal endophytes: the case of *Alternaria*

17:40-17:45 Carolin Schneider (Germany)
Presentation of COST Action FA1103: 'Endophytes in biotechnology and agriculture'

17:45-18:30 Discussion and closing remarks

Genomes of phytoplasmas: virulence or symbiosis?

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Arabidopsis thaliana plants infected with the bacterial pathogen Aster Yellows phytoplasma strain Witches' Broom (AY-WB) exhibit witches' broom and leafy flower symptoms and promote reproduction rates of the AY-WB insect vector (the aster leafhopper *Macrosteles quadrilineatus*) by 60% compared to non-infected Arabidopsis plants. We previously sequenced the genome of AY-WB and identified 56 secreted AY-WB proteins (SAPs) that are candidate effector proteins. To investigate which effectors modulate plant development and leafhopper fitness, we generated stable transgenic Arabidopsis lines for these effectors. SAP11 Arabidopsis plants show crinkled leaves and increase in stem numbers resembling the witches' broom phenotype, while SAP54 plants exhibit leafy flowers and SAP05 plants long slender leaves and early flowering. We found that SAP11 binds and destabilizes Arabidopsis *CINCINNATA* (*CIN*)-related TCPs that are conserved plant transcription factors involved in plant development and positively regulate lipoxygenase (*LOX*) genes required for jasmonate (JA) synthesis. *LOX2* expression and JA production are downregulated in the SAP11 plants, and *M. quadrilineatus* produces significantly more progeny on these plants and on *LOX2*-silenced and *jar1* mutant Arabidopsis. Thus, SAP11 suppresses the plant defence response to the AY-WB leafhopper vector by destabilizing TCPs leading to an increase in insect vector progeny. As in nature AY-WB depends on these insects for transmission to other plants, we propose that *SAP11* is a vivid example of a gene that has an extended phenotype beyond the organism in which it resides, a concept put forward in Richard Dawkins' classic book "The extended phenotype – The long reach of the gene".

Genome analysis: from sequence to protein function

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Phytoplasmas are obligate parasites of plant phloem tissue and insects. They have resisted all attempts of cell-free cultivation so far. Genome research offers insights in the genetic endowment of such bacteria. The deduced metabolism of the four completely sequenced '*Candidatus* Phytoplasma' genomes including '*Ca*. P. asteris' strains OY-M and AY-WB, '*Ca*. P. australiense,' and '*Ca*. P. mali' highlights more shared than unique features. These phytoplasmas are characterized by chromosome condensation, which can be interpreted as an evolutionary adaption of the phytoplasmas to their nutrient-rich environments. The loss of genetic modules and increased host dependency are the results of this adaptation process. In consequence, most differences are visible in the content of membrane and secreted proteins indicating the evolutionary development of pathogen-host interactions.
However, major differences in the phytoplamas are present between different phylogenetic subgroups. '*Ca*. P. mali' belonging to a distinct branch separates by deviating chromosome organization, the genetic repertoire for recombination and excision repair of nucleotides and metabolism. Differences, e.g. in the recombination and repair system, may demonstrate the original genetic environment of ancestors of the monophyletic phytoplasmas.
Other features seemed to be shared in general. The sugar metabolism is the key element of the energy metabolism of phytoplasmas and most *Mollicutes*. Transporters for the uptake of sugars but also spermidine/putrescine, manganese/zinc, dipeptides/oligopeptides and methionine are encoded by the phytoplasma genomes. Several encoded incomplete ABC transporters are scattered over the genomes and may increase the uptake of nutrients and metabolites in combination with other subunits. Some of the deduced pathways also appear to be incomplete including the nucleotide and folate synthesis. It remains unclear so far, how many of these fragments are still functional by the uptake of intermediates or so far not understood mechanisms. Several of them may just stand for genetic rudiments and cannot be interpreted due to the few available data from related families. Basic elements such as heterogeneity of organization characterized by chromosome linearity for example make aware of our limited knowledge on the different phytoplasma branches.
However, the genetic environment allow the compensation of many shortfalls and may be also include the lack of a phosphotransferase system by the uptake of phosphorylated hexoses with respect to the location in the host. The connected glycolysis remains to be the central energy pathway for many phytoplasma but the genome of '*Ca.* P. mali' offered us a somewhat different perspective by the loss of the complete energy-yielding part. The energy metabolism of '*Ca*. P. mali' makes clear that a so far uncharacterized alternative pathway fulfilling this function must be present. Genome information supports such a pathway, which may enable all four phytoplasmas to generate pyruvate and ATP.

Microbial symbiosis in insects: general aspects and the Rickettsiales model

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With few exceptions, symbiosis had been a neglected research field for decades, and the fundamental studies effected during the last years of the 19th century and the first half of the 20th century, reviewed for example in the seminal books of B. M. Kozo-Polyansky, U. Pierantoni and P. Buchner, had been overlooked until recently. The interest for symbiosis grew during the decade 1970-1980, and again after 1990, promoted by an unconventional and energetic scientist, Lynn Margulis, and then after the introduction of novel molecular tools, useful to work on unculturable symbionts. Along over a century of symbiosis research, the bacteria of the order Rickettsiales played a central role. For a long period, it seemed that almost all intracellular bacteria observed in arthropods were to be assigned to this order. During the last decade of the 20th century, molecular microbiology tools then revealed that the order was indeed polyphyletic. The order now encompasses a coherent group of intracellular bacteria, associated with a variety of hosts (from ciliate protists to vertebrates), but showing a particular link with insects, and more generally with arthropods. Among the arthropod-associated rickettsiae, a variety of different life styles are encountered, from the vector-borne pathogens that determine diseases like epidemic typhus and the spotted fevers, to the 'parasites of insect reproduction' (e.g. *Wolbachia pipientis*) that cause phenomena like embryonic male killing, parthenogenesis, sperm-mediated female sterility (or cytoplasmic incompatibility), and the feminization of genetic males. In the last years, two main discoveries have further highlighted the unsurpassed biological possibilities of the Rickettsiales. In the agronomic field, there is evidence that some rickettsiae can move horizontally among plant parasitic insects, after a passage into the plant. In the veterinary area, a novel tick-associated Rickettsiales has been discovered, *Midichloria mitochondrii*, characterized by the unique capacity of invading the mitochondria of the host arthropod.

Acetic acid bacteria, an emerging group of symbionts of sugar-feeding arthropods

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Several insects of the orders Diptera, Hymenoptera, Hemiptera and Homoptera have been recently shown to host symbiotic microorganisms of the Acetic Acid Bacteria (AAB) group. All these insects rely on sugar-based diets, such as nectars, fruit sugars or phloem sap. AAB genera *Acetobacter*, *Gluconacetobacter*, *Gluconobacter*, *Asaia*, *Saccharibacter* and the novel genus *Commensalibacter* have been found associated, alternatively or in combination, to the fruit flies *Drosophila melanogaster* and *Bactrocera oleae*, mosquitoes of the genera *Anopheles* and *Aedes*, the honey bee *Apis mellifera*, the leafhopper *Scaphoideus titanus* and the mealybug *Saccharicoccus sacchari*.
AAB are aerobic alpha-Proteobacteria quite well known for their capacity of incomplete oxidation of sugars and alcohols that leads to the accumulation in the growing medium of acetic acid and other organic acids. Such a metabolism makes AAB capable of growing at low pH. AAB establish symbiotic associations with the insect midgut, a niche characterized by the availability of diet-derived carbohydrates and oxygen and by an acidic pH, selective factors that support AAB growth. By using fluorescent *in-situ* hybridization as well as strains that have been genetically tagged with fluorescent proteins like the Gfp or the DsRed, it has been demonstrated that AAB actively colonize different insect tissues and organs, such as the gut, the epithelia of male and female reproductive organs, the Malpighian tubules and the salivary glands. This complex topology of the symbiosis indicates that AAB activate mechanisms for passing through body barriers, allowing them to migrate to different organs of the host. Recently, AAB involvement in the regulation of innate immune system homeostasis of *Drosophila* has been shown, indicating a functional role for host survival. A commensal *Acetobacter pomorum* resulted to be essential for modulating insulin-like growth factor signaling in *Drosophila* to regulate host homeostatic programs that control developmental rate, body size, energy metabolism, and intestinal stem cell activity. Analogously to *Acetobacter pomorum* in *Drosophila*, the removal of *Asaia* sp. from the gut of *Anopheles stephensi* by antibiotic treatment was associated to delays in larval development, even though the mechanism determining such delays remains unknown. All these lines of evidence indicate that AAB may play different roles in insect biology, not being restricted to the feeding habit of the host. The close association of AAB and their insect hosts has been confirmed by the demonstration of multiple modes of transmission between individuals and to the progeny that include vertical and horizontal transmission routes, comprising a venereal one. In particular the sexual transmission from females to the progeny has been demonstrated to occur through egg smearing with the bacterial cells. Taken together, the data indicate that AAB represent novel secondary symbionts of insects that play important roles in the host biology. The importance of AAB opens a novel perspective for using such symbionts as potential tools for affecting the life cycle of the host or blocking the transmission of pathogens by the insect vector hosts.

Symbionts and endophytes in activities of COST ACTION FA0807: WG3

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Working group 3 covers the aspect of „Phytoplasma control in crop systems". As phytoplasmas are phloem-limited plant pathogens which are spread by phloem-feeding insects. In nature their control is difficult and can be carried out either by reducing vectors or eliminating the pathogen from the infected plants. The first requires the knowledge of the vector and its biology, and usually is achieved by repeated insecticide treatments. As European policy aims to reduce the impact of insecticides one major objective of WG3 is to develop and validate innovative environmentally friendly control strategies of phytoplasma vectors. Elimination of phytoplasmas from infected plants is almost impossible and may only be achieved by tetracycline treatments which are usually not allowed in practical application. Therefore, an other objective of WG3 is the identification and use of natural resistance of plants to phytoplasmas. One of the best studied examples of plant resistance to phytoplasmas is apple. Natural resistance to '*Candidatus* Phytoplasma mali' has been found in *Malus sieboldii* and is currently used to develop phytoplasma-resistant apple rootstocks. Studies aiming to elucidate the mechanism of this resistance indicate that general plant defence mechanisms are involved and that some responses are similar to those found in susceptible *Malus* which show a "recovery", a more or less stable remission of symptoms. As natural genetic resistance is rare an objective of WG3 is the identification of alternative control strategies based on biocontrol agents or plant resistance inducers. In this regard, endophytes have gained major interest in WG3, especially for the control of phytoplasma infections in perennial woody crops like grapevine and fruit trees. E.g., an activity of the endophytic fungus *Epicoccum nigrum* against '*Ca.* P. mali' in *Catharanthus roseus* has been reported by Musetti *et al*., 2009. Furthermore, it could be shown that the microbial diversity in healthy, phytoplasma-infected and recovered grapevines is altered. An effect of *Pseudomonas putida* S1Pf1Rif against chrysanthemum yellows phytoplasma infection could be demonstrated by Gamalero *et al*., 2010. The interaction of endophytes with phytoplasmas might be the competition for an ecological niche, the production of allelochemicals or the induction of a systemic resistance. However, before endophytes might be used as biocontrol agent much more knowledge is needed regarding their interaction with phytoplasmas inside the plant. Appropriate model systems are required. Here, two approaches have been reported: the use of herbaceous plants (e.g. daisies for chrysanthemum yellows, *C. roseus*) or the use of micropropagated plants (e.g. *Malus* in the study of the interaction of '*Ca.* P. mali' with latent apple viruses).
An other potential control strategy treated in WG3 is the use of symbionts identified in phytoplasma transmitting insect. This approach will be presented by WG3 members during the workshop. Phytoplasmas live and multiply in two different hosts: in plants and insects. They are highly dependent on host metabolites and there interaction with the environment is complex. A successful, sustainable and environmentally friendly phytoplasma control will have to be also complex: the plant defence to phytoplasmas needs to be strengthened and the spread of the phytoplasma by its insect vector needs to be reduced. A smart combination of different approaches reduces the need for a high efficiency of a single method. In this regard, symbionts and endophytes might have a realistic potential for future phytoplasma control strategies.

Symbionts and endophytes in activities of COST ACTION FA0807: WG2

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Working group 2 is dealing with the epidemiology of phytoplasma diseases and particularly with its dispersal by arthropod vectors. Phytoplasmas are transmitted in a persistent manner by insects belonging to the families Cicadellidae, Cixidae, Psyllidae, Delphacidae, and Derbidae. Micropropagation together with other agricultural practices such as grafting, cutting, stool bed and other systems to propagate plant germplasm avoiding sexual reproduction are other known ways for transmitting phytoplasma diseases, and recently the possibility of transmission through seed has also been under investigation. The objectives of this WG are to establish a vector monitoring system throughout Europe to identify phytoplasma vector species, monitor their spread throughout the COST countries, and to coordinate research into these and other means in which phytoplasmas are spread.
The WG2 tasks are:

1. Establish tools to identify vector species
2. Monitor the presence of phytoplasma diseases and their putative vectors in defined regions throughout Europe
3. Provide data about the infectivity of vector species towards the establishment of a risk assessment system
4. Monitor differences in vector populations to verify correlations between vector populations and efficiencies in disease spread
5. Establish the importance of different means of disease spread, such as seed transmission and transmission by root bridges

Two training schools have been conducted during the past years focused on practical aspects of psyllid and leafhopper vector collection and identification. As a result of these workshop, a wealth of information has been posted on the website including the minutes and lectures from these two schools and an e-key for identification of Middle European psyllid species on *Rosaceae*. A questionnaire concerning presence of phytoplasma diseases and their putative vectors in different European regions has been designed and answers have been compiled in a table. We decided to join forces with Working Group 3 in the preparation of a comprehensive, yet straightforward, questionnaire to address the risk assessment issues. We will be working closely with WG3 to accomplish this task and will request the assistance of all of the COST membership to participate.
Up to date, only few information is available concerning endosymbionts in arthropod vectors of phytoplasmas. But some very interesting studies of WG2 members are presented in this meeting.

Bacteria symbionts in vectors of sugarcane white leaf disease

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Sugarcane white leaf (SCWL) is the most destructive known sugarcane disease in Asia, especially in Thailand which is the world's second largest exporter of sugar. SCWL is associated with plant pathogenic phytoplasmas, and shows symptoms of leaf chlorosis and profuse tillering. The disease is transmitted to the plant by the leafhoppers *Matsumuratettix hiroglyphic*us (Mutsumura) and *Yamatotettix flavovittatus*. So far there is no sugarcane resistant variety or effective method to control SCWL disease. The purpose of this study was to identify and characterize bacterial symbionts in *M. hiroglyphicus* for exploiting in future symbiotic control development. Non-culture and culture techniques were used to identify bacteria associated with the leafhopper. For non culture techniques, the 16S rRNA bacterial gene was amplified from the whole body of *M. hiroglyphicus* and analyzed by cloning and sequencing. Two dominant bacterial types were found; one set of sequences did not closely match any sequences in the database. This sequence is from an unknown Betaproproteobacteria which we called BAMH. '*Candidatus* Sulcia muelleri' was the second most predominant species found in this leafhopper. The distribution of BAMH and '*Ca*. S. muelleri' were comparable in the leafhopper tested. These two bacterial symbionts were found in bacteriome, gut, fat body and ovaries by PCR assays. A detailed localization was obtained by fluorescent *in situ* hybridization and confocal laser scanning microscopy. BAMH and '*Ca*. S. muelleri' were co-localized in the same bacteriome. BAMH was found in all developmental stages from eggs, through nymphs to adults, suggesting it is transmitted from generation to generation. BAMH was localized in the entire gut of leafhopper nymphs. BAMH is proposed to be a new bacterial symbiont co-resident with '*Ca*. S. muelleri'. In addition culturable bacteria were isolated and cultured on common media, analyzed by 16S rDNA bacterial gene sequencing. Two genera were found including Gram-positive *Bacillus* and Gram-negative *Pseudacidovorax*. Determining the part of insect body in which these symbionts are found is in progress.

The insect symbionts of insect vectors of phytoplasmas to grapevine

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Currently, the implementation of sustainable agriculture is achieving a growing relevance at global scale, as in the last century the large use of chemicals for crop production caused severe environmental damages. In the field of crop protection, a promising research topic for the development of sustainability in agriculture is the identification of biocontrol agents. Among the possible control agents, microbial symbionts of insects represent an outstanding source for potential antagonistic activities against insects and insect-borne pathogens. Strategies based on the exploitation of microbial symbionts of insect vectors of phytoplasmas for control of phytoplasmoses represent a very promising approach, considering the severity of these diseases, and the fact that presently there is no direct cure against them.

Among the diseases induced by phytoplasmas, those affecting grapevine are of particular relevance because of their high economic impact. “Flavescence dorée” is the most troubling phytoplasma disease, and it is associated with the 16SrV group phytoplasmas subgroups –C and -D transmitted by the leafhopper *Scaphoideus titanus* Ball. The study of the microbiota of *S. titanus* led to the identification, among the others, of a *Bacteroidetes* of the genus *Cardinium*. The life cycle and the transmission pattern of *Cardinium* in *S. titanus* showed to be complex, as the *Bacteroidetes*, besides being vertically transovarially transmitted, resulted to be released during the insect feeding process, suggesting it is able to undergo a horizontal transmission between co-feeding leafhoppers. Such a complex life cycle opens to the possibility that these symbiotic bacteria could exploit the plant as an additional resource to spread among sap-feeding insects.

“Bois noir” is another important and emerging grapevine phytoplasma disease; it is associated with 16SrXII phytoplasmas transmitted by the polyphagous planthopper *Hyalesthes obsoletus* Signoret. The bacterial community affiliated to *H. obsoletus* was explored as well, underlining the presence of different bacteria stably associated with the planthopper. ‘*Candidatus* Sulcia muelleri’, clustering in the *Bacteroidetes,* ‘*Candidatus* Purcelliella pentastirinorum’ in the *Gammaproteobacteria*, and a newly discovered *Beta-proteobacterium*, named ‘*Candidatus* Vidania fulgoroideae’ were the major symbiotic bacteria, observed with high infection rates, in *H. obsoletus*. All of these bacteria were hosted in the gut, testicles, and oocytes of the cixiid. The knowledge on the potential effects on the host’s biology of these three main symbionts of the planthopper could be useful for the development of control tools against “bois noir” phytoplasmas.

Symbiotic bacteria associated with a phytoplasma vector

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The genus '*Candidatus* Phytoplasma' encloses reduced-genome, phloem-limited bacteria that are responsible for hundreds of plant diseases world-wide. These plant-pathogens are transmitted by a small number of phloem-feeding species in the Hemiptera: leafhoppers (Auchenorrhyncha: Cicadellidae), planthoppers (Auchenorrhyncha: Fulgoroidea) and psyllids (Sternorrhyncha: Psyllidae). Intracellular symbionts of arthropods may influence their host's biology and ecology in various ways. Non-pathogenic symbionts can be divided into two groups: 1) primary symbionts, which are located in specialized cells, supplying essential nutrients and are strictly transmitted from mothers to their offspring; 2) secondary symbionts, which are diverse in terms of their location, function and transmission modes. All Hemiptera tested so far are associated with diverse and dynamic interactions with symbionts. The Auchenorrhyncha have been shown to have an obligate association with the primary symbiont *Sulcia*. To test for a possible correlation between specific symbiotic bacteria and the efficiency of phytoplasma transmission, the diversity of symbionts was assessed. Leafhoppers were collected in carrot fields using vacuum sampling, placed in alcohol and identified as species of *Orosius albicinctus* (Distant), *Batracomorphus glaber* Haupt, *Megopthalmus scabripennis* Edwards, *Anaceratagallia laevis* (Ribaut), *Austroagallia sinuata* (Mulsant & Rey), *Macrosteles quadripunctulatus* (Kirschbaum), *Neoaliturus fenestratus* (Herrich-Schaffer), *Euscelis* sp, and *Psammotettix* sp. An initial screen was conducted using the fingerprinting technique DGGE (Denaturing Gradient Gel Electrophoresis). In addition to the primary symbiont *Sulcia*, which was detected in all of the leafhopper species tested, the analysis showed the presence of *Arsenophonus, Wolbachia* and *Diplorickettsia*. While *Arsenophonus* could be detected in *B. glaber* and *O. albicinctus*, *Wolbachia* was found in *A. sinuate* and *B. glaber* and *Diplorickettsia* was found in *M. scabripennis* and *O. albicinctus*. *Diplorickettsia*, a gamma-proteobacterium which was discovered and described for the first time in 2010 as associated with ixodid ticks and was implied to be a human pathogen. Because this is the first time it was discovered in a phytophagous insect, it was further characterized in the common brown leafhopper *O. albicinctus.* To further determine the phylogenetic affiliation of this newly discovered bacterium, the nearly-full sequence of the 16S rDNA coding gene as well as the rpoB gene, encoding the b-subunit of RNA polymerase, were sequenced. Comparison of these gene sequences to those reported in various databases has confirmed that the symbiont is most closely related to *Diplorickettsia*, found in ixodid ticks, as it showed 99% and 92% similarity of the 16S rDNA and rpoB genes of that bacterium respectively.

A colony of *O. albicinctus* was established from leafhoppers collected in a mint greenhouse, and kept on common bean plants. To test for the infection rate of *Diplorickettsia*, the presence of that symbiont was tested in 100 males and 100 females with species-specific primers. The infection rate of *Arsenophonus* was also tested, and *Sulcia*-specific primers were used a positive control. *Arsenophonus* was detected in 18% of the males and 21% of the females and *Diplorickettsia* in 64 and 65% of males and females, respectively. It was thus established that *Diplorickettsia* is quite common in the *O. albicinctus* populations in Israel. To

determine the localization of the various symbionts within their host, male and female adult leafhoppers were dissected, fixed, hybridized with species-specific fluorescence probes and viewed under a confocal microscope. Symbionts were seen in the midgut and accessory gland of the ovaries, but not in the salivary glands, foregut, hindgut and malpighian tubules, testis, and oocytes.

Orosius albicinctus is a polyphagous leafhopper, found in a number of agricultural crops, flowers, vines, vegetables and herbs, as well as weeds. It is an established vector of phytoplasmas in Europe and the Middle East transmitting sesame phyllody, lucerne witches' broom, purple top and more. *Arsenophonus* is known from a range of hosts including Diptera, Hemiptera, Hymenoptera and Neuroptera, and was previously recorded in the planthoppers *Cixius wagneri* (China) and *Pentastiridius leporinus* L.. It has also been found associated with plant diseases like marginal chlorosis of strawberry and "syndrome basses richesses" in sugar beets. The discovery of *Diplorickettsia* in a plant-feeding insect raises new questions about its host range and phenotype(s) in those hosts. The influence of *Arsenophonus*, *Diplorickettsia* and even *Sulcia* on the ability of various leafhopper species to vector phytoplasma remains to be tested.

'*Candidatus* liberibacter europaeus', a novel microorganism associated with *Cacopsylla* spp.: a symbiont, a potential pathogen or an endophyte?

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Psyllids are serious agricultural pests due to their ability to vector phloem-restricted plant pathogens. In specific, fruit tree phytoplasmas are transmitted by different *Cacopsylla* spp., while other psyllids are acknowledged vectors of liberibacters. '*Candidatus* Liberibacter spp.' were recently associated with severe plant diseases. '*Ca*. L. asiaticus', '*Ca*. L. americanus' and '*Ca*. L. africanus' are associated with citrus greening ("huanglongbing") in Asia, Americas and Africa. '*Ca*. L. solanacearum' causes diseases in solanaceae in America and New Zealand. In this study, a fifth novel species representing the first liberibacter described in Italy and Europe and named '*Ca*. L. europaeus' has been shown to be associated with *Cacopsylla pyri* (L.). It can bloom to high titers in the psyllid host, with more than 10^9 16S rRNA gene copies per individual. Fluorescent in situ hybridization experiments showed that '*Ca*. L. europaeus' is present in the host midgut lumen, salivary glands and Malpighian tubules. '*Ca*. L. europaeus' has a relatively high prevalence (> 51%) in *C. pyri* from different areas in Piedmont and Aosta Valley regions in Italy and it can be transmitted to pear plants in experimental transmission trials. However, even though high titers of the bacterium (more than 10^8 16S rRNA gene copies g-1 of pear plant tissue) could be detected, in the pear tissues no specific disease symptoms could be observed in the infected plants over a 6-months period. This new species is probably a symbiont rather than a pathogen and hence a wide spread of this bacterium could be assumed. Investigations on the distribution of '*Ca*. L. europaeus' in the genus *Cacopsylla* and in the respective host and shelter plants (where psyllids aestivate and overwinter) from north-western Italy, Hungary and Israel as well as its possible co-presence with '*Candidatus* Phytoplasma' spp. were carried out. The screening performed on 421 specimens resulted in the detection of the presence of the bacterium in 17.1% of the individuals (72/421) belonging to 9 out of the 14 species considered. With regard to geographical distribution, '*Ca*. L. europaeus' was found in 67 out of 364 (18.4%) specimens collected in Italy, in 5 out of 28 (17.9%) Hungarian individuals, and in none of the 46 Israeli psyllids. The presence of the bacterium was detected in four species of host plants including apple (the host plant of *C. melanoneura*), blackthorn (the host plant of *C. pruni*), hawthorn (the host plant of both *C. melanoneura* and *C. peregrina*), and pear (the host plant of *C. pyri*, *C. pyricola* and *C. pyrisuga*), whereas none of the shelter plants tested positive for this bacterium. Co-presence of both '*Ca.* L. europaeus' and '*Ca.* Phytoplasma' spp. was observed in most of the 7 host plants, while none of the 4 shelter plants were positive for both bacteria. Altogether, these findings indicate the presence of '*Ca*. L. europaeus' in continental zones, whereas it does not seem to be widespread in the Mediterranean region. Furthermore, lack of specific symptoms in all infected plants confirms an endophytic relationship with this bacterium, while its abundance in insects suggests a beneficial role for the host. Co-infections with phytoplasmas, observed in insects and plants, require further study to evaluate the possible interactions between them.

Endophytic bacteria: niches of colonization and biocontrol properties

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Since works of Galippe, Marcado, Jorissen and others pioneers in the XIXth century, knowledge on endophytic bacteria colonizing various hosts, helping plant growth as well as reducing phytopathogen diseases has increased. Niches of colonization, sources of colonization as well as biocontrol properties by specific strains have been in particular intensively studied. This could however differ from the strains used, as well as if the studies are made under natural conditions. A summary of knowledge related to colonization, establishment inside host plants and biocontrol properties of some strains will be given. This is a pre-requisite to better understand plant/endophyte interaction in a fundamental viewpoint as well as for better use of these microsymbionts for agriculture.

Endophytes and recovery from grapevine yellows disease

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The plant life is naturally associated with different kind of microorganisms. Endophytic bacteria or fungi colonize the host plant systemically, without damaging the host or eliciting symptoms of disease according to widely used definition. Endophytes have beneficial effects on plants and they may confer plant protection against pathogens by induction of plant defense mechanisms (ISR), pathogens-antagonistic substances and competition for root colonization. However, the molecular basis of endophytic interactions is not well understood. Even it is not clear the mechanism, when ISR is activated, it is manifested as a reduction in the rate of disease development, resulting in fewer diseased plants or lesser disease severity. “Flavescence dorée” (FD) and “Bois noir” (BN), the two main phytoplasma associated diseases of grapevine yellows complex, have been seriously affecting the wine production worldwide. In the last decade, in Italian vineyards was also observed the spontaneous remission of symptoms in grapevine plants infected by phytoplasmas (recovery). Although the bases of this phenomenon are still unclear, different hypothesis have been investigated. One of this assumptions is the role of endophytic bacteria in recovery. With this purpose the endophytic bacterial community associated grapevine plants was characterized by cultivation-dependent and –independent methods. Composition and structure of endophytic bacterial community were examined in healthy, phytoplasma-diseased and recovered grapevine plants. Length heterogeneity-polymerase chain reaction (LH-PCR) of total DNA from grapevine leaves was used to generate amplicon profiles that were analyzed with univariate and multivariate statistical methods. Jaccard analyses highlighted that microbial diversity and structure are different in healthy, diseased and recovered grapevine plants. Multivariate analyses confirmed this trend and showed which LH-PCR peaks determined the variation in microbial composition. Furthermore, LH-PCR electrophoretic peaks, assigned to isolated cultivable single bacterial strains, were used to monitor their distribution in total DNAs from analyzed plants. Bacterial community associated with healthy plants was characterized by a greater richness (higher number of LH-PCR peaks) than that present in diseased and recovered plants. Interestingly, some isolated strains showed beneficial traits related to mineral nutrition (phosphate solubilization, siderophore production), development (indole acetic acid production) and health (chitinase). In conclusion, from the above data it can be speculated that the alterations induced by phytoplasmas in the grapevine endophytic bacterial community by selecting those bacterial strains more resistant to ROS and able to eliciting plant defense responses, including ROS as well, may ultimately lead to recovery. This view is supported by previously reported findings showing that recovered grapevine plants have higher level of ROS in respect to diseased and healthy ones. In order to verify this hypothesis, future studies will focus on determining the relative abundance of putative recovery inducers within microbial community living in grapevines. Furthermore, the possibility that endophytic bacteria are involved in the recovery phenomenon opens new perspectives in the control of these detrimental diseases and could be a starting point for developing environmental friendly, biocontrol strategies to be used in open field.

Fungal endophytic community of grapevine: methodological study and practical approaches

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Endophytes were defined by Petrini as "*All organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to the host".* The ecological role of these organisms is still not well determined but most of them exhibit positive effect to host plants by promoting plant growth, improving resistance to multiple stresses and protection from diseases and insects.
As regarding phytoplasma diseases, it has been first reported that endophytic strains of *Aureobasidium pullulans* and *Epicoccum nigrum* are able to change phytoplasma ultrastructure in *Catharanthus roseus* infected plants. In that work, cytological modifications to the plant tissues, connected to defence responses, are also evidenced. The inhibition capability of endophytic *E. nigrum* strains against phytoplasmas has been then confirmed using real-time PCR, so demonstrating that phytoplasma concentration in infected plants treated with *E. nigrum*, was about 2.8 times lower than in untreated.
Starting from these evidences, it is interesting to increase the knowledge on crop fungal endophytic community, above all in relation to the possibility to discover innovative methods for phytoplasma disease management and control.
In this way, we start to develop and set up a method that allow us to describe the fungal endophytic community of grapevine, with the aim to compare plant showing different phytosanitary status. In fact, the described approach represents a strategy that could be applied to different plant–pathogen-endophyte interactions. We combined a culture-dependent and a culture-independent method to estimate the diversity of grapevine fungal endophytic community. In 2009, healthy grapevine leaf, node and internode tissues were randomly collected from fifteen grapevine plants cvs. Tocai and Merlot, in two organic vineyards in Friuli Venezia Giulia (FVG) region, Italy. All samples were surface sterilized according to Mostert *et al.* (2000) and small pieces were placed on PDA medium added with ampicilin and streptomycin. All obtained isolates were maintained in pure culture and grouped as morphospecies. Then, sporulating fungi were identified at genus level by morphological characteristic observations. To identify fungal endophyte at species level, DNA-dependent method was applied to all isolates, performing a DNA extraction followed by amplification of ITS region of fungal rRNA genes by ITS1F-ITS4 primers and by restriction fragment length polymorphism (RFLP) analysis with *Tru*1I and *Hpa*II endonucleases. Identical patterns were grouped into operational taxonomic units (OUT's), and one or more, when possible, representative isolate of each OTU was randomly chosen for sequencing of ITS region and BLAST analysis. This culture dependent approach, allow us to characterize at species level 29 OUT's, these represent a fraction of endophyte community that can grow on artificial media at experimental conditions. Different studies, however, reported that some endophytes are slow or unable to grow on artificial media, so this fraction can be well characterized only by using a culture-independent method.

According to the culture-independent methods, extraction of total genomic DNA from plant shoots, amplification of fungal ITS region and cloning were performed. The culture-independent method allows us to discover other 27 OTUs associated to a most likely non-culturable fraction of fungal endophytic community. From collected data, it resulted that more than 90% of isolates obtained by the culture-dependent method belonged to seven main genera, *Alternaria* sp., *Phoma* sp., *Epicoccum* sp., *Aureobasidium* sp., *Cladosporium* sp., *Pestalotiopsis* sp. and *Pestalotia* sp.

Similarly, the same seven genera represented the 82% of total OTUs obtained from the culture-independent method. The integrated use of both culture-dependent and colture-independent methods is reported as time and labour consuming but, they certainly are a cornerstone for all community analysis studies.

Based on the above reported results, we then developed a new rapid and reliable fingerprinting molecular tool, based on DGGE (Denaturing Gradient Gel Electrophoresis) technique, useful to describe and compare the fungal diversity respectively in healthy, phytoplasma infected or recovered grapevines. Primer pairs ITS1F-GC and ITS2 amplifying ITS1 region, were used to generate amplicons suitable for DGGE analysis from all previously cultivated and non-cultivated endophytes. PCR products were run on 8% polyacrylamide gel in a 30-45% urea/formamide denaturant gradient using the DCode system (Bio-Rad, CA, USA). PCR-based DGGE analysis permitted a very good discrimination among the majority of amplicons obtained from different representative OTUs, derived from both culture-dependent and colture-independent method. In conclusion, the use of integrated approaches, allowed us to increase the knowledge about the diversity of grapevine fungal endophyte community. PCR-based DGGE analyses resulted to be a valuable culture-independent method for the rapid and reliable identification of fungal endophytic species. Further DGGE analyses are in progress to evidence differences among fungal endophytic communities associated with healthy, recovered and phytoplasma infected grapevines, with the final aim to discover fungal endophytes useful as potential biocontrol agents or resistance inducers against phytoplasmas.

Polyphasic approach for the characterization and the study of fungal endophytes: the case of *Alternaria*

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All plants in natural ecosystems appear to be associated to fungal endophytes, microorganisms represented by taxonomically and biologically different species but, all sharing the character of colonizing internal plant tissues without causing apparent harm to their host. Endophytes have profound impacts on plant communities through increasing fitness by conferring abiotic and biotic stress tolerance, increasing biomass and natural defense against insects, mammals or pathogens, or promoting the remediation of contaminated soils and water.
Despite more than 100 yr of research resulting in thousand of journal articles, the ecological significance of fungal endophytes remains poorly characterized.
Genus *Alternaria* Ness is ubiquitous, including species found worldwide in association with a large variety of substrates. Many species are saprophytes, animal/plant pathogens or postharvest pathogens. As well, endophytic *Alternaria* associated to a wide range of plants in different ecosystems, were also reported.
As a genus, *Alternaria* encompasses considerable morphological diversity and there have been a number of attempts to organize *taxa* into subgeneric groupings based on shared morphological characters. Above all, small-spored *Alternaria* species are a taxonomically challenging group of fungi with few morphological or molecular characters that allow unambiguous discrimination among *taxa*.
A precise and correct identification of these species is necessary, not only because of our desire to classify and control, but also because the species name embodies a set of characters (e.g. growth preference, host interaction and metabolite production) that enables us to predict its behavior.
Starting from these statements, goals of this study were to characterize 20 *Alternaria* endophytes, recovered from grapevine in Friuli Venezia Giulia and Tuscany (Italy) by morphological, molecular and chemical analysis, and to investigate the interactions with host plant (grapevine) and their potential in biological control of *Plasmopara viticola*, the most common grapevine pathogen.
Following the morphological analyses, all isolates were grouped according to their three-dimensional sporulation pattern on PCA and the colony characteristics on different substrates. After DNA extraction, all isolates were analyzed by RAPD-PCR. The resulting profiles were subjected to cluster analysis. The metabolites extracted from the 20 *Alternaria* endophytes were analyzed by a HPLC equipped with a diode array detector and the resulting metabolite profiles were subjected to multivariate statistic analyses too.
In comparison with reference small-spored *Alternaria* species, the 20 isolates were differentiated in two morphological groups: one belonging to *A. arborescens* species-group and the second to *A. tenuissima* species-group. None of endophytic isolates recovered from grapevine presented a sporulation pattern similar to *A. alternata* species-group, a relatively rare species.

Analyses of RAPD fragment patterns confirmed the morphological groups. In particular, RAPD analysis revealed that grapevine endophytes belonging to the *A. arborescens* species-group were molecularly distinct from isolates belonging to the *A. alternata* and *A. tenuissima* species-group. In addition, these results clearly placed isolates of the *A. alternata* specie-group in a distinct cluster from isolates of the *A. tenuissima* species-group, whereas in the study of Pryor and Michailides the isolates of *A. alternata* and *A. tenuissima* species-group did not separated through cluster analysis.

To complete the endophyte fungal characterization, chemotaxonomy based on secondary metabolite profiles gave clear-cut classification. In this study, the grapevine endophytic isolates belong to *A. arborescens* and *A. tenuissima* species-groups produced known metabolites typical of these species-groups, in particular tenuazonic acid that differentiated them from the *A. alternata* group; these data in agreement with those reported by Andersen *et al.* The 20 grapevine endophytes resulted to produce also a number of unknown metabolites, whose characterization could be useful not only for a more precise segregation of the two species but also by a practical point of view. In fact, according to antagonistic trials, performed against grapevine downy mildew, all the endophytic *Alternaria* strains demonstrated efficiency in the control of *P. viticola* in greenhouse-maintained grapevines, mainly when used as pre-infection treatment.

In conclusion, our data demonstrate that grapevine is associated with a different *Alternaria* endophyte community and that this genus constitute the dominant fungal consortium in the tissues of this plant. Complementary morphological, molecular and chemical data can clarify relationships among endophyte species-groups of low morphological divergence, improving the knowledge about the identity, diversity and phylogeny of these microorganisms.