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Study of the bacterial community affiliated to *Hyalesthes obsoletus*, the insect vector of "bois noir" phytoplasma of grape

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Abstract

Grape yellows caused by phytoplasmas afflict several important wine-producing areas of Europe. A grape yellows with increasing incidence in European vineyards is "bois noir" (BN), caused by '*Candidatus* Phytoplasma solani'. Its vector is the planthopper *Hyalesthes obsoletus* Signoret (Hemiptera Cixiidae), occasionally feeding on grapevine. An innovative strategy for reducing the diffusion of the disease could be symbiotic control, exploiting the action of symbiotic microorganisms of the insect host. To investigate the occurrence of possible microbial candidates for symbiotic control we performed a molecular characterization of the bacteria associated to *H. obsoletus*. Length heterogeneity PCR was applied for a preliminary population screening. Taxonomic affiliations of the bacterial species were analyzed by denaturing gradient gel electrophoresis, showing, within the microbial diversity, the intracellular reproductive parasite *Wolbachia pipientis* and a Bacteroidetes symbiont with 92% nt identity with '*Candidatus* Sulcia muelleri'. PCR essays specific for these bacteria showed they co-localize in several organs of *H. obsoletus*. Fluorescent *in situ* hybridization was performed to assess the distribution of these microorganisms within the insect body, showing interesting localization patterns, particularly in insect gonads and salivary glands. These results could be a starting point for a deeper investigation of functions and relationships between microbial species.

Key words: American grapevine, "bois noir", vector, polymerase chain reaction.

Introduction

Grape yellows are a group of severe diseases of grapevine caused by phytoplasmas in several important wine-producing areas across Europe, generating significant losses in production. Phytoplasmas are cell wall-less bacteria spread throughout the vineyards by leafhoppers and planthoppers, which can inoculate the phytopathogenic organisms while feeding on lymph of healthy grapevine. A grape yellow with increasing incidence in European vineyards is "bois noir" (BN), caused by '*Candidatus* Phytoplasma solani', a phytoplasma belonging to the Stolbur group (16Sr XII). (The IRPCM Phytoplasma/Spiroplasma Working Team, 2004). The insect vector of '*Ca.* Phytoplasma solani' is the planthopper *Hyalesthes obsoletus* Signoret (Hemiptera Cixiidae), which lives on dicotyledonous weeds, but can be occasionally found on grapevine (Alma *et al.,* 2002). Direct control of phytoplasmas is not yet possible. Therefore application of control strategies against *H. obsoletus* is necessary, mainly by means of a good management of weeds in the vineyard. Furthermore, infected plants must be destroyed, in order to avoid development of new reservoirs for the disease.

Use of biocontrol agents is recently acquiring increasing interest in pest management. Biocontrol microorganisms can impair the insect life cycle in many cases by producing toxic factors for the target insect species. Another innovative strategy in this field, which does not require spraying the biocontrol agent at each growing season, is the 'symbiotic control' approach, in which microorganisms, by establishing a strict symbiosis with the insect host, exploit mechanisms for reducing vector competence (Beard *et al*., 1998) or the manipulation of undesirable host traits (Rio *et al*., 2004).

In the field of insect-borne grapevine pathogens, studies on the symbiotic microbiota obtained promising results toward control strategies in the case of Pierce's disease of grape, caused by the xylematic bacterium *Xylella fastidiosa* Wells et al., spread by the sharpshooter *Homalodisca coagulata* (Say) (Hemiptera Cicadellidae) (Bextine *et al*., 2004).

Despite the economic relevance of BN, no studies have been carried out about the microflora associated with its vector. We performed a characterization of the bacteria stably associated to the body of *H. obsoletus*, to investigate the occurrence of possible microbial candidates to be used as biocontrol agents.

Materials and methods

A cultivation-independent analysis of the microflora of *H. obsoletus* was performed by molecular tools on individuals sampled from uncultivated fields close to vineyards in Piedmont region in north Italy. 16S rRNA gene-length heterogeneity PCR (LH-PCR) (Ritchie *et al*., 2000) was applied with universal primers targeting bacteria, in order to identify the presence of microbes associated to the majority of specimens. Taxonomic affiliations of the bacterial population observed by LH-PCR were analyzed by 16S rRNA gene PCR and dena-

turing gradient gel electrophoresis (DGGE) followed by band excision and sequencing (Sass *et al*., 2001). To investigate the abundance of bacteria in the host, we executed specific PCR assays for interesting organisms: '*Ca*. Phytoplasma solani', the pathogen causing BN (Langer and Maixner, 2004), *Wolbachia pipientis* Hertig, an important reproductive parasite associated to several insect species (Werren *et al*., 1995), and a bacterium related to '*Candidatus* Sulcia muelleri' (Moran *et al*., 2005). The latter is interesting because of the low homology with the closest relative. We also obtained the almost complete 16S rRNA gene sequence of this bacterium by using a combination of specific and universal primers. Fluorescent *in situ* hybridization (FISH) on 16S rRNA gene (Amann *et al*., 1990) was performed to assess the distribution of these microorganisms within the insect body, by using order- and species-specific probes.

Results and discussion

Preliminary population screening executed by LH-PCR essays showed some peaks (representing different organisms) associated with most of the individuals examined, both in females and males. This gave evidence of a rich microbial community inside the body of *H. obsoletus*. DGGE analysis of taxonomic affiliations of bacteria found in the insect body confirmed the diversity detected by LH-PCR. Among the bacteria associated to BN insect vector, in addition to '*Ca*. Phytoplasma solani', the intracellular reproductive parasite *W. pipientis*, a Bacteroidetes endosymbiont with 92% nt identity with '*Ca*. Sulcia muelleri', and other Bacteroidetes, Beta- and Gamma-Proteobacteria, were found. Bacteria considered particularly interesting were tested by specific PCR essays. A co-localization of '*Ca*. Phytoplasma solani', *W. pipientis* and the bacterium related to '*Ca*. Sulcia muelleri' was observed in several organs of *H. obsoletus*. The distribution of these three microorganisms was then investigated by FISH. Confocal microscopy images obtained by FISH showed interesting localization patterns of bacteria, particularly in insect gonads and salivary glands. These results could be a starting point for a deeper investigation of functions and relationships among microbial species.

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