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Recent advances in Bois noir research

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Bois noir (synonyms: Vergilbungskrankheit, Legno nero) and other grapevine yellows diseases (GY) are caused by phytoplasmas, plant pathogenic bacteria of the class Mollicutes. Phytoplasmas of the 16SrXII-A or 'stolbur' group are associated with Bois noir (BN) and other diseases of various woody and herbaceous plants. They are endemic to Europe and the Mediterranean area. Different planthopper and leafhopper species have been reported to transmit stolbur, but only the Cixiid planthopper Hyalesthes obsoletus Signoret is known so far to transmit this pathogen to grapevine.

Symptoms of BN are not distinguishable from symptoms of other GY, but they differ considerably between cultivars. Symptom expression is further influenced by environmental conditions and agricultural practice. Typical symptoms comprise discoloration of leaves including the veins, often associated with downcurling of the leave blade, lack of or incomplete lignification of shoots that later turn black, abortion of clusters or shrivelling of the ripening fruit. In most cultivars the symptoms of BN remain restricted to parts of the infected vines for several years. BN does usually not kill the infected vines usually although their vigour can be significantly reduced. Remission of symptoms and even a complete recovery of infected vines are common phenomena.

BN is a still emerging disease in Europe and it can cause severe economic loss by reducing yields, fruit quality and vitality of the plants. It does not spread as fast as Flavescence dorée (FD), the other important GY in Europe, but it is more widespread and more difficult to control. New outbreaks of BN have been reported from various vine growing areas during the last decade. Changes between epidemic and endemic periods are typical for the disease. BN is not only an economically important disease but also of great scientific interest, because it is a phytoplasma disease with an intricate epidemiology that includes wild herbaceous host plants, different vector populations and grapevine as an important cultivated plant. Since plant host adapted vector populations and phytoplasma strains lead to different epidemiological cycles, BN can also serve as a model system for studies of host adaptation and the co-evolution of pathogens and vectors. The first "Bois noir Workshop" was held at Weinsberg, Germany in November 2008. The progress made in BN research since then will be summarized in this paper.

BN occurs from Spain to Ukraine and from Germany and Northern France to Lebanon and Israel. Symptoms of GY and the detection of phytoplasmas related to stolbur have recently been reported from Iran (Karimi et al., 2009) and China (Duduk et al., 2010). Stolbur is also widespread in Hungary in wild plants, grapevine and solanaceous crops (Acs et al., 2010). BN was the only GY detected during a survey in Croatia (Seruga Music et al., 2009), while Delic and Lolic (2010) reported it from Bosnia and Herzegovina, and Radonjic et al. (2009) from Montenegro. The disease is still spreading in some regions of Northern Italy (Mori et al., 2010), where it is considered the predominant GY (Berger et al., 2009b; Cainelli et al., 2010; Forte et al., 2010; Quaglino et al., 2009a).

Phytoplasmas of group 16SrXII-A have an extremely wide range of herbaceous and woody plant hosts. It is necessary to distinguish between occasionally infected plant species and principal host plants that can serve as natural reservoirs of the pathogen. Since the juvenile stages of the vector H. obsoletus acquire the phytoplasma from the roots of herbaceous plants after hibernation, only perennial plants are of epidemiological significance. Plants like Solanum nigrum L. or Amaranthus retroflexus L. seem to be preferred feeding hosts for the adult vectors because they are frequently found infected (Pasquini et al., 2010); however, they cannot play a role in stolbur epidemiology because they are annual plants. Convolvulus arvensis L. and Urtica dioica L., on the other hand, are principal hosts of stolbur phytoplasmas all over Europe, though with varying regional importance (Acs et al., 2010; Berger et al., 2009b; Cainelli et al., 2010; Fialova et al., 2009; Kessler, 2009; Langer and Maixner, 2004; Navratil et al., 2009; Pasquini et al., 2007). Regular or occasional infection was recently reported for Taraxacum sp., Polygonum aviculare L., Rumex acetosa L., Cirsium arvense (L.) Scop., (Berger et al., 2009b; Cainelli et al., 2010; Fialova et al., 2009; Kessler, 2009; Navratil et al., 2009; Pasquini et al., 2010), Tussilago farfara L. (Berger et al., 2009a; Romanazzi et al., 2009b), Conyza bonariensis (L.) Cronq. (Tessitori et al., 2009) and some other herbaceous hosts.

Phytoplasmas as graft transmissible disease agents can be disseminated with propagation material collected from infected rootstock or scion motherplants. The assessment of the risk of dissemination has to consider the probability of propagating infected wood, the chance of producing contaminated rootlings from this material (Mannini et al., 2009), and the infection pressure in nurseries. Another important factor is the significance of infected vines for the further spread of the disease in the field, which depends on the epidemiology of a particular GY. The presence of infected plant hosts of stolbur and of H. obsoletus in and around nurseries and mother-plots (Pasquini et al., 2010; Quaglino et al., 2009a; Tessari et al., 2010) increases the risk of an unintentional dissemination of infected wood or young plants.

Infected grapevine is considered a dead end host of the stolbur phytoplasma with negligible significance for the epidemiology of the disease due to the host preferences of the BN vector that feeds on grapevine only erroneously. A phytosanitary risk is nevertheless associated with the dissemination of infected plants in disease free areas, since potential vectors might be able to acquire the phytoplasma from infected vines and transmit it to other hosts. An extensive study of nurseries and young vineyards in Northern Italy revealed a very low incidence of FD and BN infestation of planting material, but an increasing proportion of infected vines in the young vineyards due to infection in the field (Borgo et al., 2010). Although symptomatic vines found in the year of planting are most likely a consequence of the contamination of planting material, field inoculation experiments proved that early infection and favorable weather conditions can cause symptom development in young vines in the same season (Maixner, unpublished). Hot water treatment (Boudon-Padieu and Grenan, 2002) can be applied as a phytosanitary method to propagation wood or rootlings to reduce the risk of unintentional dissemination of material infected by GY. The efficiency of this method against BN was confirmed (Bianco et al., 2010; Mannini et al., 2009).

The effective exclusion of infected grapevine wood from propagation but also disease monitoring and epidemiological studies depend on reliable diagnostic procedures to detect phytoplasmas in infected plants and insects. A principal problem for the detection of GY associated phytoplasmas is the uneven distribution of the pathogens in infected vines and the seasonal variation in pathogen titres (Marcone, 2010). The routine detection of BN and other GY depends on DNA based techniques. New protocols are still developed for the detection and characterization of GY associated phytoplasmas or of specific strains in order to enhance the sensitivity, the practicability for routine applications, or to reduce labour. Multiplex real-time PCR protocols for the simultaneous detection of BN and FD together with a grapevine specific internal control were developed based on ribosomal (Terlizzi et al., 2009) and non-ribosomal (Pelletier et al., 2009) markers. A reverse transcription real-time PCR protocol was developed by Margaria et al. (2009) to detect BN and FD associated phytoplasmas as well as important grapevine viruses simultaneously and with high sensitivity. However, while FD was readily detected, a nested protocol was required for the sensitive detection to BN. A TaqMan allelic discrimination assay for a one tube detection and differentiation of the two major tuf-types (Berger et al., 2009a) is a useful tool for studies of the different epidemiological systems of BN and for field surveys.

BN is a disease with a complex epidemiology. One reason is the existence of stolbur strains with biological as well as genomic differences (Pacifico et al.,

2009), most likely the result of the wide geographic and host range of stolbur. The genetic polymorphism of stolbur strains is considered both the cause of their host and vector specificity (Cimerman et al., 2009; Quaglino et al., 2009c) and the consequence of distinct host associations (Johannesen et al., 2009). The genetic polymorphism of BN related stolbur strains is studied intensively in order to develop more specific diagnostic tools and to gain information about the association of strains with different ecological niches, their geographic variation and the ways of spread and dissemination.

New subgroups of the 16SrXII group have been proposed after a virtual RFLP analysis of stolbur strains from Italy (Quaglino et al., 2009b). Results achieved with this method need to be confirmed by real RFLP analysis, since differing results were occasionally achieved with both techniques (Contaldo et al., 2009). The correlation of the observed intra-group diversity with biological and epidemiological traits of the BN associated phytoplasmas requires further investigations (Quaglino et al., 2009b).

The tuf gene encoding for a translational elongation factor (Schneider et al., 1997) is of particular use for epidemiological studies because it is diagnostic for the plant host association of stolbur strains that depend on distinct epidemiological systems in the field (Langer and Maixner, 2004). Unfortunately, the nomenclature for tuf types is not consistently used. Three tuf types were originally described as 'tuf-a', 'tuf-b', and 'tuf-c' by Langer and Maixner (2004), while the analysis of the same isolates with the marker Stol4 led to four different groups ('A', 'B', 'C', and 'no amplification'). Only those isolates that were found in diseased vines were classified as 'VK1', 'VK2', and 'VK3' (VK='Vergilbungskrankheit'), according to the combination of the results of tuf- and Stol4-typing. Tuf-types and VK-types are therefore not just synonyms. If only the tuf-marker is used for analyses the strains should be classified as 'tuf-a', 'tuf-b', 'tuf-c' etc. only.

Recent studies confirmed the specific association of tuf-a type to *U. dioica* and the significance of *C. arvensis* as the principal plant host of tuf-b type, although this type occurs in other herbaceous hosts, too. They also show the geographic variation in the prevalence of the different tuf-types. Tuf-b type seems to be the more widespread and the predominant type in Eastern and Southern winegrowing regions. It was the only type found in Hungary, Serbia and the South Moravia region of the Czech Republic (Acs et al., 2010; Contaldo et al., 2009; Fialova et al., 2009). Both tuf-types are present in Italy but with different regional prevalence (Berger et al., 2009b; Filippin et al., 2009; Murolo et al., 2010a; Pacifico et al., 2009; Pasquini et al., 2007; Quaglino et al., 2009a; Quaglino et al., 2009c; Tessitori et al., 2009). Only tuf-b type was found in different viticultural areas of Spain except of the Rioja region where both types are present (Batlle et al., 2009).

Other housekeeping genes like ribosomal protein genes (Contaldo et al., 2009) and secY are used beside tuf for the further characterization of stolbur isolates. The phylogenetic analysis of stolbur strains from Italy and France revealed two main strain clusters of the variable secY marker that corresponded to the two major tuf-types (Filippin et al., 2009). The secY marker shows not only diagnostic differences in strains corresponding to tuf-types, but also allows the differentiation of regional strains (Johannesen et al., 2009). The comparison of secY sequences of stolbur isolates from grapevine and lavender revealed a much higher genetic diversity of strains from the latter host (Danet et al., 2010).

Membrane proteins are considered to play a central role in the molecular mechanisms of phytoplasma host interactions and vector specificity (Cimerman et al., 2009). They are therefore subject to a positive diversifying selection that leads to a high variability (Fabre et al., 2011). Analyses of the variability of the vmp1 gene (Cimerman et al., 2009) in the Czech Republic (Fialova et al., 2009), Germany (Maixner et al., 2009), France (Cimerman et al., 2009; Pacifico et al., 2009), Italy (Murolo et al., 2010a; Pacifico et al., 2009) and Spain (Batlle et al., 2009) revealed regional differences with respect to the presence and predominance of vmp1 profiles. Unfortunately, RFLP-typing of this marker turned out to be not diagnostic for the host association of the strains because identical ump1 RFLP profiles are associated with different tuftypes. Sequence analyses, on the other hand, revealed diagnostic differences in this gene that correspond to tuf-a and tuf-b types (Johannesen et al., 2009). Repeated motifs in the vmp1 gene (Cimerman et al., 2009) make co-evolution analysis in the tuf-b group of strains difficult, but the nettle isolates (tuf-a) show geographic-genetic concordances (Johannesen et al., 2009). The observation of plant host specific mutations in multiple markers supports the hypothesis, that the tuf-a and tuf-b types are independently evolving lineages (Johannesen et al., 2009). Another highly variable marker is the stamp gene that encodes the antigenic membrane protein of stolbur phytoplasma, which is presumably involved in phytoplasma-vector interactions (Fabre et al., 2011). This gene might be an additional marker for further studies of host plant and vector specificity of the stolbur phytoplasma strains.

The physiological and molecular aspects of phytoplasma-host interactions and of plant defence mechanisms with respect to pathogenesis and the recovery phenomenon gained more interest recently. A better understanding of these mechanisms could help to develop more specific control and management strategies for BN and other GY. Comparisons of physiological parameters between asymptomatic, symptomatic and recovered vines demonstrated the negative influence of BN infection on photosynthesis and transpiration (Endeshaw et al., 2010; Murolo et al., 2009) and the mineral

contents in symptomatic leaves (Schweigkofler et al., 2010). Yields of symptomatic but also of lately recovered vines were reduced (Endeshaw et al., 2010; Zahavi et al., 2009).

Microarray techniques were applied to analyze changes in gene expression profiles between healthy, infected and recovered vines (Albertazzi et al., 2009; Dermastia et al., 2009; Hren et al., 2009a; Punelli et al., 2010). Effects of the phytoplasma infection on primary and secondary metabolic pathways were observed, including enzymes of the photosynthetic chain, calvin cycle and lipid metabolism, the induction of defence genes (Dermastia et al., 2009; Hren et al., 2009a) and the suppression of cell wall degradation (Albertazzi et al., 2009). Hren et al. (2009a) identified a set of genes whose expression patterns allowed the grouping of vines according to their infection status. The classification of grapevines into disease status groups based on the levels of expression of selected genes was highly accurate (Hren et al., 2009b). Recovered vines could be distinguished from healthy plants by "metabolic scars" (Punelli et al., 2010). However, significant differences in the expression of genes involved in disease response were not only observed between vines of different disease status but also between cultivars (Landi and Romanazzi, 2009). Phytoplasmas are not only sinks of carbohydrates in the infected plants but influence the balance of growth regulators, too. Consequently, Curkovic-Perica et al. (2010) achieved a remission of symptoms in infected Catharanthus roseus L. plants after application of auxins. While some phytoplasmas were apparently eradicated by the treatment, the stolbur phytoplasmas persisted in the symptom-free shoots.

Cyclic variations in disease incidence are typical for BN. The visible incidence is a result of new and retained infection, remission and re-occurrence of symptoms and complete recovery of previously infected vines. Remission and recovery are often used as synonymous terms. However, it is reasonable to distinguish between the two phenomena. Remission could be defined as a temporal vanishing of symptoms or a period of latent infection, while the permanent disappearance of phytoplasmas from previously infected plants should be considered as true recovery. Field observations (Maixner, 2006) as well as phytoplasmas diagnosis (Osler et al., 2003; Zahavi et al., 2009) suggest that grapevines can be considered recovered if they remain symptomless for at least three years. The rate of symptom remission and recovery of GY infected vines depends on the type of phytoplasma associated with the disease, varieties and rootstocks, environmental conditions and agronomic practices (Prota and Garau, 2010; Romanazzi et al., 2009c). Increased levels of H₂O₂ in the phloem of recovered plants are thought to counteract the pathogen virulence (Musetti et al., 2007). Further physiological and molecular effects are involved in the recovery phenomenon as described above, but the physiological base of recovery is still not completely clear. On the other hand, means to stimulate

recovery are investigated in order to reduce the detrimental effects of BN to the vineyards and to minimize economic damage. Specific pruning techniques aim at the elimination of infected tissues. They have to take account of the differing susceptibility of cultivars to BN (Murolo et al., 2010b). Pollarding seems to be the appropriate technique for highly susceptible cultivars and young vines (Ipach et al., 2009; Riedle-Bauer et al., 2010), while cane pruning is sufficient for less susceptible varieties (Ipach et al., 2009). Other attempts to increase recovery are based on the application of abiotic stress or the repeated application of resistance inducers to stimulate the defence response of infected vines (Romanazzi et al., 2009a). A new field of interest are the interactions between endophytic organisms in grapevine with BN. Studies of the composition of bacterial communities in vines of different health status using a length heterogeneity PCR assay revealed significant differences between healthy vines on the one hand and infected as well as recovered vines on the other hand (Bulgari et al., 2009a; Bulgari et al., 2009b). A reduced diversity of bacterial communities was observed in the latter group. The role of fungal endophytes for the induction of grapevine defence response is currently also investigated (Musetti et al., 2010).

The Cixiid planthopper H. obsoletus is the principal vector of BN although other species of the families Cixiidae and Cicadellidae are known or suspected to transmit stolbur phytoplasma, too. The juvenile stages of H. obsoletus live in the soil, feeding on the roots of host plants from which they also acquire the stolbur phytoplasma. The proportion of infected nymphs is significantly increasing from the third to the fifth and last larval instar (Kaul et al., 2009). Preferred herbaceous hosts of H. obsoletus are field bindweed (C. arvensis), hedge bindweed (Calystegia sepium (L.) R. BR.) and stinging nettle (U. dioica) (Alma and Tedeschi, 2010). Dead nettle (Lamium orvala L.) was identified as a new host plant in Northern Italy (Forte et al., 2010). Lavender (Lavandula sp.) and monk's pepper (Vitex agnus-castus L.) are natural woody host plants in France and Israel, respectively (Sforza et al., 1999; Sharon et al., 2005), while grapevine is only an erratic feeding host for the adult planthoppers.

H. obsoletus is often not very abundant in vineyard agro-ecosystems, and the time for surveys for the presence of this species is limited to about two month in summer, when the adult planthoppers are present (Berger et al., 2009b; Forte et al., 2010; Kehrli et al., 2010b; Maixner et al., 2009). Therefore, a lot of effort is sometimes taken to identify alternative vectors instead of searching for H. obsoletus appropriately, particularly in areas of new outbreaks of BN. In some areas, however, there seems to be no close correlation between the occurrence of H. obsoletus and BN (Belli et al., 2010). The role of H. obsoletus as the principal vector of BN to grapevine was confirmed in Switzerland (Kehrli et al., 2010c), Serbia (Cvrkovic et al., 2010), and Spain (Sabate et al.,

2010), where H. obsoletus is considered responsible for the stolbur transmission to grapevine while Macrosteles quadripunctulatus Kirschbaum transmits the same phytoplasma to other crops. Other Cixiid and Cicadellid species were found carrying stolbur phytoplasmas in vineyard environments, but their ability to infect grapevine was not yet confirmed. Reptalus panzeri Löw transmits stolbur phytoplasmas to corn (Jovic et al., 2009), but up to 15 % infected specimens were found in vineyards and other crops in Hungary (Acs et al., 2010), Italy (Mori et al., 2010; Pasquini et al., 2010), and Serbia (Cvrkovic et al., 2010), too. Infected Reptalus quinquecostatus (Dufour) are present in Italian (Pasquini et al., 2010) and Serbian vineyards (Cyrkovic et al., 2010). It is the most abundant Cixiid species in Tuscany vineyards, where up to 50 % of the tested specimens were infected by stolbur (Bagnoli and Gargani, 2010). Since R. quinquecostatus is able to transmit stolbur to artificial feeding medium (Pinzauti et al., 2008), its ability to inoculate grapevine is currently checked. Another planthopper, Dictyophara europaea (L.), was found infected in Serbia (Cvrkovic et al., 2010) and Northern Italy (Filippin et al., 2009). Stolbur infection was also detected in leafhoppers, e.g. Euscelis lineolatus Brullé and Exitianus capicola Stål in Northern (Landi et al., 2009) and Southern Italy (Pacifico et al., 2009; Pasquini et al., 2010), respectively. E. lineolatus transmitted stolbur to artificial feeding medium (Landi et al., 2009), while Anaceratagallia ribauti (Ossiannilsson) was able to infect experimental hosts (Riedle-Bauer and Sara, 2009). The ability of this leafhopper to infect grapevine needs still to be confirmed. The correct identification of Auchenorrhyncha species is a prerequisite for epidemiological studies. However, the identification of Cixiids in particular is restricted to specialist, while the juvenile stages of closely related species are almost indistinguishable. Protocols for the molecular identification and differentiation of Cixiids of the genera Hyalesthes and Reptalus (Bertin et al., 2010a; Bertin et al., 2010b) are therefore valuable tools for stolbur epidemiological research.

Not only the different genotypes of stolbur are associated with either nettle (tuf-a) or bindweed (tuf-b), but also populations of the vector *H. obsoletus* are specifically affiliated to these plant hosts. This specific plant-host association results in distinct epidemic systems of stolbur that both branch to grapevine as a dead end host (Maixner, 2010b). The background of the host adaptation of *H. obsoletus* populations is not yet clear. While mtDNA based genetic population analysis demonstrated a recent expansion of this species to the Northern winegrowing areas, it also implicated an intrinsic ability of the planthopper to use both plant species as hosts (Johannesen *et al.*, 2008). On the other hand, host populations show biological differences with respect to flight phenology (Forte *et al.*, 2010; Maixner, 2010a), wing size (Johannesen *et al.*, 2009), vibrational signals (Grube, unpublished), feeding and oviposition preferences (Kehrli *et al.*, 2010a), and survival (Albert, unpublished). First

results of studies using microsatellite markers indicate indeed a genetic differentiation of host populations of *H. obsoletus* in Germany (Imo *et al.*, 2010).

Vectors like H. obsoletus which are not closely associated with the diseased crop are difficult to control directly by insecticide applications. The management of their herbaceous host plants is the most practicable alternative strategy to reduce vector populations and to decrease infection pressure on grapevine thereby. Chemical weeding of nettle stands inside and along the borders of vineyards reduced vector numbers significantly (Maixner et al., 2010; Mori et al., 2009). All weed control activities, either chemical or mechanical, must be ceased during the period of flight activity of the adult vectors to prevent their migration into vineyards (Mori et al., 2009). Innovative and specific control strategies require detailed information about the biology and behaviour of H. obsoletus. Romani et al. (2009) studied the sensory structures of the antennae and identified different types of sensillae that are suspected to be involved in the perception of mechanical stimuli, temperature, and other environmental parameters. Studies of the olfactory response of adult H. obsoletus revealed the preference of males and females for different host plants (Riolo et al., 2010) and led to the isolation of plant volatiles that could elicit responses of the antennae. Mazzoni et al. (2010) analyzed the mating behaviour of H. obsoletus and identified different vibrational signals that are used in partner recognition, pair formation and courtship. The detailed understanding of such behavioural elements could lead to new control approaches in the future (Mazzoni et al., 2009). Endosymbiontic microorganisms are likely involved in the host specificity of sap-sucking insects and might be functionalized in future for innovative control strategies (Alma et al., 2010). A molecular characterization of symbiotic bacteria in Cixiids identified four species that either colonized different tissues of planthoppers of the genus Pentastiridius or inhabited bacteriomes (Bressan et al., 2009). A characterization of the endosymbiont community of H. obsoletus indicated that the planthopper is colonized by several bacterial symbionts, including a previously unknown betaproteobacterium that appeared closely associated with this vector (Gonella et al., 2011).

The great importance of Bois noir in European and Mediterranean viticulture is represented by the amount of recent research activities and publications. Although the basic features of the aetiology and epidemiology of this yellows disease are known, new as well as still unsolved questions stimulate further research. The genetic variability of the pathogens and vectors and the possible identification of additional vectors in some regions add even more complexity to the already complicated epidemiology of BN and require further analysis. It is still difficult to predict the temporal dynamics of the disease and

to assess the future risks for viticulture. Our ability to interfere specifically with the natural epidemiological systems of stolbur phytoplasmas is not sufficient to provide an efficient control of the spread of BN to vineyards. Additional studies on the physiology of phytoplasma-grapevine interactions and the recovery phenomenon could help to adjust cultural practices in order to prevent permanent infection and damage to vines. Further investigations of the vector biology, communication, population structure, and interaction with host plants and endosymbiontic bacteria are required to gather the necessary information to develop more specific strategies for the control of *H. obsoletus* or to prevent the transmission of stolbur phytoplasmas to grapevine.

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The following acronyms are used in the references for the proceedings of meetings:

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