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INDEX

DETAILED PROGRAM

Sunday 27th February 2011

18,00-20,00 Registration of participants, reception, poster set up

Monday 28th February 2011

8,00-8,30 Registration and poster set up

8,30-9,00 Opening addresses

9,00-9,30 Invited review, M. Maixner: Recent advances in Bois noir research

9,30-11,00 Session 1 "Epidemiology of the disease"
Chairpersons: Marina Barba, Xavier Foissac
Oral presentations:

M. Navrátil, P. Válová, D. Šavářová, P. Lauterer, M. Starý, Z. Korbášová: Occurrence and molecular characterization of stolbur phytoplasma infecting grapevine in South Moravia (Czech Republic)

S. Mitrev, I. Karov, E. Kostadinovska: Grapevine yellows in the Republic of Macedonia: molecular identification of stolbur phytoplasma strains in grapevine and weeds

G. Marchi, P. Braccini, S. Paltrinieri, D. Rizzo, N. Contaldo, T. Cinelli, A. Bertaccini: Spread of Bois noir in organic vineyards in Tuscany: spatial pattern analysis and identification of the phytoplasma in weeds

P. Ermacora, N. Loi, F. Ferrini, M. Martini, R. Osler: A five-year study on the dynamics of Bois noir spreading in a Chardonnay vineyard in Friuli Venezia Giulia region (N.E. Italy)

D. Canik, S. Topkaya, S. Bayram, G. Soylemezoglu, F. Ertunc: Occurrence and distribution of Bois noir phytoplasma in Turkey

Short oral presentation of posters by D. Skoric

11,15-11,45 Coffee break

11,45-13,00 Poster session

13,00-14,00 Lunch

14,00-15,30 Session 2 "Detection and characterization of the stolbur phytoplasma in grapevine and other natural hosts"

Chairpersons: Assunta Bertaccini, Dijana Skoric

Oral presentations:

N. Prezelj, P. Nikolić, M. Ravnikar, M. Dermastia: RNA-based phytoplasma detection

H. Bouyahia, M. Della Bartola, D. Rizzo, S. Paltrinieri, P. Braccini, C. Milano, A. Bertaccini, A. Materazzi: Detection and tuf-type characterization of Bois noir phytoplasma in Tuscany by an improved real-time assay or nested PCR

N. Contaldo, B. Duduk, S. Paltrinieri, F. Dal Molin, J. Mitrovic, A. Bertaccini: Molecular variability on 16S rDNA of Bois noir phytoplasmas in grapevine from Italy and Serbia

J. Johannesen, X. Foissac, M. Maixner: Genetic structure and dissemination of tuf-a type stolbur phytoplasma associated with stinging nettle (*Urtica dioica* L.)

Short oral presentation of posters by A. Bertaccini

15,30-19,00 Technical guided tour to a winery and wine tasting

20,30 Social dinner

Tuesday 1st March 2011

8,30-10,00 Session 3 "Plant-pathogen interactions and control strategies"

Chairpersons: Piero Attilio Bianco, Gianfranco Romanazzi

Oral presentations:

A. Fabre, G. Balakishiyeva, J.L. Danet, X. Foissac: Heterologous expression and genetic diversity of StAMP, the antigenic membrane protein of stolbur phytoplasma

G. Brader, A. Sessitsch: Tomatoes and 16SrXII phytoplasma as tools to evaluate the role of plant signalling in phytoplasma-plant interactions

N. Reggiani, N. Mori, L. Maistrello: Control of *Hyalesthes obsoletus* Signoret, vector of Bois noir, using entomopathogenic agents: preliminary results

P. Kehrli, N. Delabaysl: Herbicide control of stinging nettle: does the date of application affect the emergence of *Hyalesthes obsoletus* Signoret?

Short oral presentation of posters by G. Romanazzi

10,00-10,30 Coffee break

10,30-12,00 Session 4 "Vectors of the stolbur phytoplasma"

Chairpersons: Alberto Alma, Jes Johannesen

Oral presentations:

U. Wyss, H. Hoch, M. Breuer: Film on the behaviour and development of the planthopper *Hyalesthes obsoletus* Signoret

F. Pavan, E. Cargnus, N. Mori: New acquisitions on *Hyalesthes obsoletus* Signoret nymphs

M. Imo, M. Maixner, J. Johannesen: Host races of the Bois noir vector *Hyalesthes obsoletus* Signoret in Germany

M. Riedle-Bauer, K. Hanak, A. Sára, H. Bauer: Influence of cover crops on the Auchenorrynca fauna in Bois noir infected vineyards

Short oral presentation of posters by J. Johannesen

12,00-12,30 Conclusions

12,30-13,30 Lunch

LIST OF CONTRIBUTIONS

A. Schartl, A. Baumann, P. Schwappach	Epidemiology and detection of Bois noir in Franconia, Germany	1	P	49
E. Angelini, I. Bazzo, D. Bellotto, N. Bertazzon, M. Borgo, L. Dalla Cia, L. Filippin, V. Forte, E. Patriarca, G. Perini, A. Rainato, L. Stringher	Occurrence of Bois noir in nurseries and young vineyards	1	P	51
N. Mori, F. Quaglino, A. Zorloni, P. Casati, F. Tessari, G. Zanini, P.A. Bianco	Epidemiologic surveys of Bois noir in nursery industry of Veneto region	1	P	53
M. Baránek, J. Čechová, J. Raddová, V. Holleinová	Zigzag growth of grapevine nodes and the role of phytoplasma diseases in this syndrome	1	P	55
N. Prezelj, P. Nikolić, M. Ravnikar, M. Dermastia	RNA-based phytoplasma detection	2	O	57
H. Bouyahia, M. Della Bartola, D. Rizzo, S. Paltrinieri, P. Braccini, C. Milano, A. Bertaccini, A. Materazzi	Detection and tuf-type characterization of Bois noir phytoplasma in Tuscany by an improved real-time assay or nested PCR	2	O	59
N. Contaldo, B. Duduk, S. Paltrinieri, F. Dal Molin, J. Mitrovic, A. Bertaccini	Molecular variability on 16S rDNA of Bois noir phytoplasmas in grapevine from Italy and Serbia	2	O	61
J. Johannesen, X. Foissac, M. Maixner	Genetic structure and dissemination of tuf-a type stolbur phytoplasma associated with stinging nettle (*Urtica dioica* L.)	2	O	63

F. Terlizzi, R. Credi	Molecular characterization of stolbur phytoplasma isolates from vineyards of Emilia-Romagna (Northern Italy)	2	P	65
D. Delić, N. Contaldo, S. Paltrinieri, A. Bertaccini	Molecular characterization of Bois noir phytoplasma from Bosnia and Herzegovina	2	P	67
A. Gentili, F. Punelli, L. Ferretti, G. Pasquini	Innovative diagnostic methods in stolbur detection: preliminary results	2	P	69
Z. Acs, N. Contaldo, I. Ember, S. Paltrinieri, M. Kolber, B. Duduk, A. Bertaccini	Bois noir in Hungary: tuf-b type strain variability on 16S ribosomal gene	2	P	71
M. Martini, P. Ermacora, N. Tosone, A. Loschi, F. Pavan, N. Loi	Genetic diversity of stolbur phytoplasma strains from different host plants in Friuli Venezia Giulia	2	P	73
J. Sabaté, A. Laviña, A. Batlle	Genetic variability of the stolbur phytoplasma in plants and insects from different viticulture areas of Spain	2	P	75
S. Murolo, C. Marcone, V. Prota, R. Garau, X. Foissac, G. Romanazzi	Molecular characterization of grapevine Bois noir phytoplasma in Central-Southern Italian regions	2	P	77
A. Fabre, G. Balakishiyeva, J.L. Danet, X. Foissac	Heterologous expression and genetic diversity of StAMP, the antigenic membrane protein of stolbur phytoplasma	3	O	79
G. Brader, A. Sessitsch	Tomatoes and 16SrXII phytoplasma as tools to evaluate the role of plant signalling in phytoplasma-plant interactions	3	O	81

U. Wyss, H. Hoch, M. Breuer	Film on the behaviour and development of the planthopper *Hyalesthes obsoletus* Signoret	4	O	99
F. Pavan, E. Cargnus, N. Mori	New acquisitions on *Hyalesthes obsoletus* Signoret nymphs	4	O	101
M. Imo, M. Maixner, J. Johannesen	Host races of the Bois noir vector *Hyalesthes obsoletus* Signoret in Germany	4	O	103
M. Riedle-Bauer, K. Hanak, A. Sára, H. Bauer	Influence of cover crops on the Auchenorrynca fauna in Bois noir infected vineyards	4	O	105
B. Panassiti, M. Breuer, R. Biedermann	Analysis of major enviromental factors determining the distribution of *Hyalesthes obsoletus* Signoret in Baden (Germany)	4	P	107
D. Škorić, I. Ember, Z. Ács, M. Kölber, Ž. Budinšćak, J. Plavec, M. Šeruga-Musić, I. Križanac	Insects in the Boir noir pathosystems of neighbouring viticulture regions along Croatian-Hungarian state border	4	P	109
C. Olivier, B. Galka, R. Johnson, M. Green, C. Vincent, J. Saguez, L. Stobbs, N. Greig	Potential Bois noir vectors in Canadian vineyards and their feeding behaviour on grapevines	4	P	111
W. Schweigkofler, C. Roschatt, G. Kunz	Planthoppers (Auchenorrynca) in vineyards infected by the Bois noir phytoplasma in South Tyrol (Northern Italy)	4	P	113
G. Torrini, E. Gargani, S. Caradonna, B. Bagnoli	Preliminary results of a survey on the Auchenorrynca in some vineyards of the Chianti district (Tuscany)	4	P	115

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 *vmp1* RFLP profiles are associated with different tuf-types. 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 H_2O_2 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 (Cvrkovic *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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ABSTRACTS

Occurrence and molecular characterization of stolbur phytoplasma infecting grapevine in South Moravia (Czech Republic)

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The symptoms of leaf reddening or yellowing as well as incomplete lignification of canes and shrivelling of berries in grapevine were observed in vineyards in South Moravia region for several years. Because of this, symptomatic plants (105) were sampled from seven locations to study occurrence and genetic variability of phytoplasma. Furthermore, survey for stolbur phytoplasma vectors was done at the locality Perná.

Using nested-PCR with ribosomal primers P1/P7 followed by R16F2/R2 and by RFLP analysis (*Rsa*I, *Mse*I) stolbur phytoplasma was identified in six locations and in *Hyalesthes obsoletus* Signoret vector. Moreover non-ribosomal genes manifesting a higher variability than ribosomal genes were used (namely *vmp1, tuf* genes) to further characterize stolbur phytoplasma. The RFLP analyses of nested PCR products of *vmp1* gene (Fialová *et al.*, 2009) revealed restriction fragments variability; six different *Rsa*I profiles were discriminated. In case of *tuf* gene, PCR/RFLP analysis of all samples revealed the same *Hpa*II restriction profile, corresponding to the tuf-b type described by Langer and Maixner (2004).

In addition, a preliminary study showed approximately 6 % of stolbur symptomatic shrubs (cv. Frankovka modrá) at locality Perná. The occurrence of stolbur phytoplasma was confirmed in all PCR tested symptomatic shrubs. During the year 2010, 1657 individuals of bugs, leafhoppers, planthoppers and psyllids were collected in this locality and determined. The most important vector of stolbur phytoplasma, *H. obsoletus*, was sampled especially on nettle plants at locality Perná from 8th July to 12th August (peak of occurrence 8th July). Other potential stolbur vectors such as *Aphrodes bicinctus* Schrank, *Dictyophara europaea* Linneaus, *Euscelidius variegatus* Kirschbaum, *Euscelis incisus* Kirschbaum, *Lygus rugulipennis* Poppius, *Psammotettix alienus* Dahlbom, *Psammotettix confinis* Dahlbom, and *Reptalus panzeri* Löw were caught too. A molecular typing of stolbur phytoplasma isolates from grapevine, wild plant sources (nettle, bindweed) and vectors is in progress.

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Key words: Bois noir, Hyalesthes obsoletus*, genetic variability.*

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Grapevine yellows in the Republic of Macedonia: molecular identification of stolbur phytoplasma strains in grapevine and weeds

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During the period from 2006 to 2010, a survey for presence of Bois noir (BN) phytoplasmas on *Vitis vinifera* L. and wild spontaneous vegetation (*Clematis vitalba* L.*, Solanum nigrum* L.*, Amaranthus retroflexus* L. and *Convolvulus arvensis* L.) was conducted. The aims of this study were: i) to check the presence of BN phytoplasmas on grapevines and wild vegetation in investigated vineyards in the Eastern part of Macedonia, and ii) to molecularly characterize and compare the isolates from grapevine with those from weeds.

A total of 485 grapevine samples were collected from all Macedonian regions where grapevine is cultivated and tested by PCR. In the same vineyards, some weeds with suspected symptomatology were collected. The samples were analyzed by conventional PCR with the following primers that amplify phytoplasma rDNA: P1/P7 in direct PCR, 16r758f/M23Sr and R16(I)F1R1 in nested-PCR (Angelini *et al*., 2001). Stolbur specific primer pairs, STOL4f/r and STOL11f2/r1, that specifically amplified a 1,7 kb and a 0,9 kb DNA fragment, respectively, were used (Rott *et al*., 2007). Positive samples were characterized by restriction fragment length polymorphic (RFLP) analysis, using a combination of three primer-enzyme combinations (*Taq*I, *Tru*9I, *Hpa*II) (Duduk *et al*., 2004).

In all regions, most frequently affected cultivars were "Vranec" and "Chardonnay" (Seruga *et al*., 2003). Within the period of five investigative years, 254 grapevine samples (52 %) were PCR positive to stolbur phytoplasma, confirmed with specific primers. RFLP analyses on non-ribosomal amplicons from 50 selected grapevine samples confirmed that stol4 type-B was present.

Concerning weeds, 10 bindweed samples out of 21 were detected as primary host plants of stolbur phytoplasma. RFLP analyses on non-ribosomal amplicons with *Taq*I and *Hpa*II enzymes showed that stol4 type-B strain was present also in bindweed. On the contrary, solanum (five plants) and amaranthus (seven plants) were always negative to PCR tests.

Key words: Vitis vinifera*, Bois noir, solanum, amaranthus, convolvulus.*

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Spread of Bois noir in organic vineyards in Tuscany: spatial pattern analysis and identification of the phytoplasma in weeds

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In this study the spread of Bois noir (BN) was monitored from 2005 to 2010 in three organic vineyards of cv. Sangiovese established in 2001 and 2002 in the province of Florence, Tuscany (Central Italy). Maps with locations of all symptomatic and non-symptomatic vines, a complete census of binary data, were made for each plot and year of assessment. Three indexes (annual incidence, infection and recovery rates) were calculated from the original data sets to describe changes in the epidemic with time. Moreover, with the purpose of determining the spatial pattern of the disease (random vs. non random or clustered), for each year of assessment, field data on annual incidence were analyzed at three spatial levels (hierarchies).

The values of annual incidence, infection and recovery rates in each year were different at each studied site but with a similar trend over time. In general symptomatic plants showed the phenomenon of recovery in a relatively short time, meanwhile the infection rate decreased rapidly after the second year of assessments. Results of spatial pattern analysis were consistent at all considered levels and suggested that the arrangement of diseased vines was random across all disease assessment dates in two out of three studied sites. On the other hand, in the third vineyard the distribution of the disease was always non random, although an intensification of clustering over time never became evident.

Since the epidemics were different in different environments, a survey on the herbaceous vineyard weeds was carried out in 2010 to study how organic vineyard management practices may influence disease spread. Symptoms of yellowing or reddening were recorded on some species, meanwhile others showed symptoms typical for those associated with phytoplasma infections like dwarfing, virescence and phyllody. Total DNA was isolated from root and/or

leaf tissues of about 200 samples and nested PCR was carried out using P1/P7 followed by R16F2/R2 primers. RFLP analyses carried out with *Tru*1I restriction enzyme allowed to identify the following species infected by stolbur phytoplasmas: *Picris hieracioides* L., *Daucus carota* L., *Lactuca saligna* L., *Lactuca* sp., *Bupleurum tenuissimum* L., *Cichorium intybus* L., *Convolvolus* spp., *Cuscuta* spp., *Mercurialis annua* L., *Linaria vulgaris* Miller, and *Medicago lupulina* L.. In some of these species further polymorphisms in R16F2/R2 amplicons were detected after *Mbo*II restriction, and in one case also with *Hpy188*I; the same samples digested with *Alu*I, did not show any polymorphism. The different profiles observed could be referred to some of those identified in grapevine from diverse geographical areas including Tuscany (Contaldo *et al.*, 2011). Further studies to verify the relevance of these polymorphisms in the BN epidemiology are in progress.

Key words: epidemiology, disease, molecular detection, weeds.

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A five-year study on the dynamics of Bois noir spreading in a Chardonnay vineyard in Friuli Venezia Giulia region (N.E. Italy)

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Grapevine yellows caused by phytoplasmas have been reported in Friuli Venezia Giulia region since late eighties (Osler *et al.*, 1993); recently, stolbur phytoplasma epidemics were reported in this region and Bois noir (BN), unlike Flavescence dorée, have become one of the most relevant problems for the vineyard management (Carraro *et al.*, 2009).

Aims of this study were to evaluate spreading of BN in a vineyard located in Friuli Venezia Giulia region, with a particular attention to the recovery phenomenon and the evaluation of the effect of different rootstocks on the epidemiology of the disease. Dynamics of the disease were monitored by symptom expression; results were confirmed applying randomly nested-PCR assays with phytoplasma universal primers (P1/16S-SR followed by R16F2n/R16R2 primers) and RFLP analysis with *Tru*1I (Lee *et al.*, 1998; Lee *et al.*, 2004).

The inspected vineyard is located near Gorizia (N.E. Italy: +45°55', +13°34') in an area where BN is the only grapevine yellows disease present. The considered vineyard is constituted by a total of 2,400 grapevines cv. Chardonnay (ENTAV-INRA® Clone 548) grafted on three different rootstocks: 420A, SO4 and 1103 Paulsen; it was established in 2000 and monitored for symptom expression since 2006 when a serious outbreak of BN occurred.

Highest values of prevalence (percentage of symptomatic plants) of BN were observed in the years 2006-2008 on vines grafted on 420A and SO4. In particular, the major prevalence of the disease was respectively 18.7 % in 2006 on grapevines grafted on 420A and 16,7 % in 2007 on SO4 grafted plants. In the same period prevalence on vines grafted on 1103 Paulsen was considerably lower. In the following years of observation the percentage of symptomatic plants decreased significantly. At the same time, also the incidence (new cases of disease) decreased during the period. In the case studied, the recovery (disappearance of symptoms) has been quite a common phenomenon; its annual rate has been very variable year by year, and does not seem to be strictly

related to the different rootstocks considered. Highest percentages of recovery were recorded in 2009, ranging from 65,4 % of 420A grafted plants to 60,0 % of SO4 grafted ones. Stability of the recovery phenomenon is still under investigation; actually, among the 44 grapevines symptomatic in 2006 and recovered in 2007, 32 (72,7 %) of them did not re-present symptoms until 2010.

Key words: recovery, prevalence, incidence.

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Occurrence and distribution of Bois noir phytoplasma in Turkey

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Turkey is one of the intensive grape producers and is the 6th for grape production in the world according to FAO. Although mostly wine varieties are imported from Europe, there are no investigations on phytoplasma infections in quarantine offices in Turkey. This research was conducted in order to determine the phytoplasma infections on local and imported table grape and wine grape varieties.

Extensive surveys were carried out in the vineyards of Aegean, Thrace, Central Anatolian and Eastern Anatolian regions in years 2009 and 2010. In total 289 plant samples were collected. Major symptoms were chlorosis of veins, inward curling of leaf blades, reddening of the leaves. There was no symptomatic plant belonging to the local varieties; however, the symptoms were prevalent on wine grape varieties that were originated from the vine multiplication material imported from different European countries. For determination of potential insect vectors, 1306 individuals in the year 2009 and 968 individuals in the year 2010 were collected from vineyards. Individuals collected were from 16 species belonging to 7 families of Hemiptera order. The most prevalent species in the surveyed area were *Arboridia adanae* Dlabola and *Eupteryx filicum* (Newman), in years 2009 and 2010, respectively.

Plant DNA was extracted from midribs according to Prince *et al.* (1993). Direct PCR assays were performed with ribosomal P1/P7 universal primer pair, followed by nested PCR with R16F2n/R2 and R16(I)F1/R1 primer pairs (Lee *et al.*, 1995; 1994). These primers amplified a single fragment of approximately 1100 bp from infected samples. The PCR products were digested by the restriction enzyme *Tru*1I for the identification of subgroups. Among all tested plants (including year 2009 and 2010 samples), 17 samples from wine grape varieties were detected as infected by Bois noir (BN) phytoplasma. Positive samples showed the same restriction pattern of a reference stolbur strain provided by A. Bertaccini (University of Bologna, Italy). There was no infection on table grape varieties. On the other hand, all the samples of insect individuals, collected in the year 2009, were also tested

and they were found to be free of BN phytoplasma. This is the first report on the presence of BN phytoplasma in Turkey.

Key words: wine grape, Turkey, Bois noir, phytoplasma.

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Bois noir in Trentino: field observation on the recovery phenomenon

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In the province of Trento, the 2010 grape harvest, recorded over approximately 10,000 hectares, was equivalent to a production of 125,012 tonnes. In this most recent vintage, the ratio between white and red varieties was 70 % to 30 %. The major part of grape production (more than 55 %) in Trentino consists of Chardonnay and Pinot grigio varieties. The widespread presence of these two varieties makes the Trentino vineyards particularly susceptible to grapevine yellows.

In Trentino the first affected plants were observed in the mid-eighties; the analyses carried out in the following years identified the presence of Bois noir (BN) phytoplasma (Dal Ri *et al.*, 1991). At the moment grapevine yellows are not a limiting factor in the viticulture production anywhere in Trentino. The BN disease occurs in all vine-growing zones of the province, but the incidence is generally low and the cases of vineyards with high rates of symptomatic plants are very rare. BN infected grapevines were found in almost all the main cultivated varieties, but mostly in Chardonnay and Pinot grigio (Gelmetti *et al.*, 2009).

The manifestation of BN symptoms in the vineyards, the study of the disease evolution and the recovery phenomenon were carried out by means of vineyard mapping system. In 2010 the maps were produced in Chardonnay vineyards in two different areas: Vallagarina and Valsugana. In Vallagarina the disease evolution was followed in the period 2003-2010 in a vineyard planted on 1990. In Valsugana (Vindimian *et al.*, 1997) the study comprised five Chardonnay vineyards planted between the years 1989 and 1991, where the presence of BN infected vines has been recorded between 1990 and 2010. Comparing the maps of the last 20 years (Valsugana) and the last 8 years (Vallagarina), it has been possible to quantify a very important aspect of the epidemiology of BN: the recovery phenomenon. Field observations have made it possible to determine the number of plants which have shown symptoms for a single year, for various consecutive years and for non-consecutive years and to determine the relationship between symptom intensity and the recovery phenomenon.

Key words: Bois noir, mapping vineyard, symptom intensity

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Monitoring of grapevine yellows by molecular tools in South Tyrol (Northern Italy)

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Two grapevine yellows diseases, Flavescence dorée and Bois noir, have been causing considerable damages in European viticultural areas. Both phytoplasmas associated with these diseases are present in the Northern part of Italy and in some regions they occur simultaneously.

In order to understand which pathogen is linked with the occurrence of grapevine yellows symptoms in the Autonomous Province of Bozen/Bolzano (South Tyrol), a monitoring program involving molecular tools has been carried out since 2002. Leaf samples of more than 600 symptomatic grapevines of different cultivars were tested by a PCR procedure to determine the phytoplasma involved (Baric and Dalla Via, 2007).

So far, primarily BN phytoplasma (16SrXII or stolbur group) has been identified in South Tyrol. The samples tested positive were further analysed using a molecular typing approach based on a single nucleotide polymorphism in the gene encoding elongation factor Tu (Langer and Maixner, 2004; Berger *et al.*, 2009a). While in the first two years of the monitoring period exclusively tuf-a type was found, tuf-b type first appeared in 2004 and since then spread throughout the territory, reaching an average frequency of 19 %. The extension of the analyses to the insect vector *Hyalesthes obsoletus* Signoret and its herbaceous host plants from BN affected vineyards helped to gain a clearer picture of the epidemiology of this grapevine yellows disease in South Tyrol (Berger *et al.*, 2009b).

Key words: Convolvulus arvensis, Urtica dioica, *Vergilbungskrankheit*, Vitis vinifera.

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Epidemiology and detection of Bois noir in Franconia, Germany

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Since its first detection in one vineyard in 1999 (Gilge *et al.*, 2003), Bois noir (BN) has been under scrutiny in the Franconian wine-growing region located along the Main River in Central Germany. Besides visual inspections for symptom bearing vines in the complete region, vector surveillance with yellow sticky traps and by vacuuming host plants was performed at some selected locations. For detection of BN phytoplasmas, hundreds of DNA samples from plants and insect vectors were analyzed by PCR with the primer pair fstol/rstol (Schneider *et al.*, 1995) or tufAY (Schneider *et al.*, 1997), followed by *HpaII* restriction for further characterization.

In 2010 BN infections proved to be present at seven locations spread over the Franconian wine-growing region. In addition visual symptoms such as typical leaf discoloration, downward rolling of leaves, shrunken berries, black pustules on the shoots and irregular lignifications were reported from five additional vineyards. Different white and red varieties were affected, including Scheurebe, Riesling, Mueller-Thurgau, Domina, Pinot noir, and Blaufraenkisch. In a few cases even young vines not older than three years displayed symptoms and tested positive in molecular analyses.

Yellow sticky traps for *Hyalesthes obsoletus* Signoret revealed that the vector was present at various sites, mainly on bindweed (*Convolvulus arvensis* L). At two locations *Reptalus panzer* (Löw), a putative vector, was also identified.

Molecular analysis of vine samples as well as samples of *H. obsoletus* showed that mainly tuf-b type is present in Franconia. At three sites a few samples tested positive for the tuf-a type. From a vector population living on nettle and detected in 2006, the first *H. obsoletus* individuals tested positive for stolbur phytoplasma in 2009.

In a model vineyard for BN phytoplasma tuf-b type, which had been monitored since 2005, single vine assessment of typical symptomatology showed that strict weed management led to a decrease of symptoms. It appears that also water balance is a factor influencing the abundance of BN symptoms. Often affected vines did not show symptoms the following year and vice versa.

In spring 2010 about 100 *H. obsoletus* nymphs reared on nettle in the greenhouse were used for an herbicide experiment. After treatment of the host

plant with 1 % Roundup in May, larvae were still present in the soil at the end of June. They all had reached L5 but were smaller than the larvae living on a control nettle host. One adult *H. obsoletus* hatched in July, two weeks after the control population. New offspring in the greenhouse will be used for testing different herbicides and different times of application.

Key words: Hyalesthes obsoletus, Reptalus panzeri*, bindweed, nettle, laboratory rearing.*

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Occurrence of Bois noir in nurseries and young vineyards

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Bois noir disease (BN), caused by stolbur phytoplasma, occurs in grapevine in all Italian regions. Although prevention strategies and strong insecticide treatments are applied in nurseries, often the grape growers complain about the occurrence of the disease in young grapevine plants and wonder if the nurseries are involved. The work investigated the presence of Bois noir in grapevine in nurseries, one- and two-year-old vineyards of Veneto, Friuli Venezia Giulia and Piedmont, from 2006 to 2010.

Approximately 19 nurseries and 250 young vineyards (200 one-year-old and 50 two-year-old vineyards) were inspected. On the whole, more than 8,000,000 plants were visually observed and about 300 leaf samples exhibiting suspected symptoms were collected. Molecular diagnosis and characterization were carried out according to Angelini *et al.* (2001; 2007).

Results showed the presence of the phytoplasmas associated to BN and Flavescence dorée (FD) in nurseries, in one- and two-year-old vineyards. In particular, approximately 0.001% of the rooted grafts observed were infected by stolbur phytoplasma. The occurrence of BN-infected plants increased in one- and two-year-old vineyards, being 0.005% and 0.037%, respectively. FD phytoplasma infection was found at the same extent as stolbur in all the areas investigated, both in nurseries and in one- and two-year-old vineyards.

Stolbur phytoplasma host plants and vectors were then surveyed in the young vineyards and nurseries where BN had been found. Indeed, the vector and the host plants were spread in those areas. In conclusion, the results suggested that most of the infected grapevines could have acquired the stolbur phytoplasma in the field.

Key words: host plant, Hyalesthes obsoletus, *rooted grafts.*

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Epidemiologic surveys of Bois noir in nursery industry of Veneto region

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Grapevine Bois noir (BN) was firstly observed in Veneto region in 1983 (Egger and Borgo, 1983) and is expanding in vineyards of all North-Eastern Italy (Milanesi *et al.*, 2005; Barba *et al.*, 2006; Sancassani *et al.*, 2007), including also mother plant vineyards.

A three-year research project, supported by Regione Veneto, "Prevention and control of grapevine Bois noir in Veneto Region", promotes the study of (i) the presence of *Hyalesthes obsoletus* Signoret in nursery industry (rootstock mother plants – RMP, mother plants – MP, and rootling plantlets, RP), (ii) the role of rootstocks in transmission of BN phytoplasmas to the scions.

Considering the effectiveness of insecticide treatments against *H. obsoletus*, BN control is difficult and preventive measures are crucial. Even if the role of nursery activities is marginal, marketing of a few diseased plants in areas where BN is absent is critical (Credi *et al.,* 2007).

In the present work, *H. obsoletus* was identified in RMP, MP and RP and its frequency was strictly associated with the presence of *Urtica dioica* L. Insects (90 %) were prevalently captured by using sweep net, more frequently at the vineyard borders (95 % of captured individuals). Molecular analyses identified BN phytoplasma in 9.2 and in 8.1 % of insects captured in 2008 and 2009, respectively. These results confirmed that specific elimination of nettle and bindweed is the sole efficient BN control strategy.

In order to clarify the role of rootstocks Kober 5BB, SO4 and 420A in BN transmission, a double grafted cutting, called "trionte", constituted of a BN infected Chardonnay cane as phytoplasma source at the bottom, and an healthy Chardonnay bud grafted on an healthy rootstock at the top, was created. Molecular analyses were performed on symptomatic and symptomless grapevines and in rootstocks for phytoplasma detection.

Double grafts have been executed with a bench omega-type grafting machine; as a whole 1175 triontes have been grafted: 385 in 2009, 790 in 2010. Grafted cuttings have been forced, potted and maintained in a screen house. Sprouting percentage was very low: 59 out of 385 (15.3 %) rooted cuttings sprouted on the first year, 125 out of 790 (15.8 %) on the second year.

Leaf yellowing and rolling were observed, in September 2009, on the originally healthy Chardonnay sprout of one SO4 grafted vine, suggesting the translocation of phytoplasmas from the infected cane to the healthy bud, through the rootstock. Analyses on triontes obtained in 2010 are still in progress.

Key words: Bois noir, Hyalesthes obsoletus*, grapevine nursery.*

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Zigzag growth of grapevine nodes and the role of phytoplasma diseases in this syndrome

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Grapevine plants showing shortened internodes and a zigzag pattern of growth are sometimes noticeable in South Moravian vineyards (Czech Republic). These symptoms were observed on a wider range of cultivars, but most frequently affected cultivars are Neuburg and its relative Aurelius (crossing of Neuburg and Riesling). Shortened internodes and a zigzag pattern of growth are in the literature explained as consequences of different factors. They are very often connected with infection by biological pathogens Grapevine fanleaf virus (GFLV) and Arabic mosaic virus (ArMV). Zigzag pattern of growth was observed in the case of plants infected by Pierce's disease of grapevine too. Another explanation is molybdenum or zinc deficiency, which may play a role in growth disorders including mere zigzag or distorted growth habit of shoots (Walton *et al.*, 2009). Last but not least, discussed symptoms were observed in the case of plants infected by phytoplasma diseases (Canadian Food Inspection Agency, 2006).

For better understanding the causes of these symptoms, samples showing shortened internodes and zigzag pattern of growth were collected from four different locations within vineyards of South Moravia. Finally 28 plants (17 plants of Neuburg cultivar, 11 plants of Aurelius cultivar) were tested for the presence of phytoplasmas. DNA isolation was performed according to Ahrens and Seemuller (1992), with minor modifications. Testing was performed via nested PCR using two universal primer pairs for grapevine phytoplasma diseases: P1/P7 and R16F2n/R16R2 (Kuzmanovic *et al.*, 2008). Due to the occurrence of stolbur phytoplasma in Czech Republic, an additional nested PCR test, specific for stolbur phytoplasma, was applied using STOL11F2/R1 and STOL11F3/R2 primer pairs (Clair *et al.*, 2003).

Stolbur phytoplasma was detected in only one plant (cultivar Aurelius) out of the 28 tested plants. Thus, our results ruled out that phytoplasma diseases are the main reason for observed shortened internodes and a zigzag pattern of growth. Moreover, in another study we tested the same group of plants for six viruses, including GFLV and ArMV. There was no clear correlation between symptoms and virus infection again. Thus, the reasons for occurrence of above described symptoms are still ambiguous and need further studies. Our next

observation will be focused on other known factors as nutrient deficiency or rust and bud mite infestation (Walton *et al.*, 2009).

Keywords: phytoplasma, zigzag growth, shortened internodes.

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RNA-based phytoplasma detection

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Grapevine yellows diseases (GYs) are associated with different phytoplasmas infecting grapevine plants in the majority of grapevine growing countries worldwide, causing serious economical damages. The main phytoplasmas associated with GYs are those causing Bois noir (BN) and Flavescence dorée (FD) diseases. To control the dissemination of GYs, a good diagnostic is crucial. Detection of pathogens depends on the available methods and high specificity and sensitivity are the most relevant characteristics together with reliability. Phytoplasma detection is usually based on DNA molecular methods, such as conventional polymerase chain reaction (PCR) or real-time PCR. It has been suggested that mRNA could be a better target for pathogen detection, since more copies of RNA than of DNA are present (Firrao *et al.*, 2007). A simple phytoplasma extraction protocol based on RNA detection using PCR or real-time PCR was already successfully applied to pathogens infecting grapevine (Osman and Rowhani, 2006; Margaria *et al.*, 2009).

Using real-time PCR we compared our current DNA-based detection of BN and FD phytoplasmas (Hren *et al.*, 2007; Boben *et al.*, 2007) to the RNA-based detection. We isolated RNA (RNeasy Plant Mini Kit, Qiagen) and DNA (magnetic beads, Bionobile) from midribs of BN or FD infected grapevine plants. Real-time PCR for BN was based on amplification of Stol11 genomic fragment for BN phytoplasmas and on *secY* gene for FD phytoplasmas.

Preliminary results indicated that DNA and RNA detection reliability is approximately the same for both phytoplasmas and for this experimental setup. There was also no difference in BN detection from RNA samples processed immediately in the field or one day later, indicating the stability of phytoplasma RNA. Comparison of FD detection from older and younger leaves of the same plant showed that also from older, more senescent, leaves RNA can be used for phytoplasma detection.

We showed that RNA-based method is working with the same reliability as specific Stol11 and *secY* DNA-based real-time PCR. However this method allowed to verify not only phytoplasma presence but also its activity. Improvement of detection sensitivity is still possible, using different RNA extractions or choosing a suitable phytoplasma gene for real-time PCR that is present in many copies.

Key words: Bois noir, Flavescence dorée, real-time PCR, detection.

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Detection and tuf-type characterization of Bois noir phytoplasma in Tuscany by an improved real-time assay or nested PCR

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In agreement with a regional strategy aimed to monitor grapevine yellows (GY) disease, an extensive survey was carried out covering the ten provinces of the Tuscany region. Symptomatic leaf samples were collected from commercial vineyards and nurseries during 2009 and 2010. Laboratory analyses performed by nested PCR (Botti *et al.*, 2005) and by real-time PCR (Angelini *et al.*, 2007), both on 16S ribosomal gene, confirmed that Bois noir (BN) is largely the most important GY in Tuscany with ubiquitous distribution. Molecular characterization of BN phytoplasmas relied on genetic diversity studies through multiple step amplification process of one or more genes, followed by RFLP analysis. In recent times the latter technique was flanked by sequencing of amplicons and/or cloned amplicons, followed by *in silico* RFLP analyses. This procedure enabled to find in a short time a wider BN genetic diversity through the identification of several single nucleotide polymorphism (SNP) lineages. Nevertheless, little is known about the ecology of these newly defined subgroups and their putative role in the epidemiology of BN. Consequently, the model proposed by Langer and Maixner (2004), permitting the distinction of three biologically differentiable sequence variants (tuf-a, tuf-b and tuf-c types), is still widely used to study BN epidemiology. Recently, a new TaqMan allelic discrimination assay was developed and proposed for the distinction of tuf-a and tuf-b types (Berger *et al.*, 2009).

A real-time PCR assay for the rapid and specific detection of tuf-a and tuf-b types was developed and applied in alternative to nested PCR assays carried out on the same gene. For this purpose, two newly designed BHQplus probes (Biosearch Tech, CA, USA) were used in an allelic discrimination test by

multiplex real-time PCR, in order to distinguish between tuf-a and tuf-b types among selected BN positive samples identified in grapevine in Tuscany. Real-time PCR analysis was performed in duplicates in 12 µl reaction assays, containing 6 µl IQ Powermix (Bio-Rad Laboratories, USA), 500 nM of each primer, 100 nM of FAM-tuf-a probe, 100 nM of CalFluor orange 560-tuf-b probe, and 1 µl of target DNA. Assays were carried out on Rotor-Gene Q (Qiagen, Germany). Standard curves obtained from serial dilutions of synthetic oligonucleotides permitted to calculate the reaction efficiency. The slopes of the linear fits reached -3.329 for the green channel, indicating an efficiency of 99.70 % (R^2= 0.992) for the tuf-a type assay. Similar results were obtained with the tuf-b type probe (efficiency = 98.64 %, R^2= 0.996). When applied to grapevine samples, results were automatically generated through the allelic discrimination option of the Rotor-Gene Q software (version 2.0.2, Qiagen).

According to preliminary results, the newly designed assay for the simultaneous detection of tuf-a and tuf-b types resulted to be very efficient. A number of BN positive samples are currently under characterization with both methods, real-time and nested-PCR, to establish the presence of tuf-a and tuf-b types and to trace a distribution map in Tuscany.

Key words: grapevine, stolbur, tuf-a, tuf-b, allelic discrimination.

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Molecular variability of 16S rDNA of Bois noir phytoplasmas in grapevine from Italy and Serbia

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Surveys to identify Bois noir (BN) phytoplasmas in grapevine yellows outbreaks in vineyards located in Serbia and in several Italian regions were carried out from 2008 to 2010. The presence of BN phytoplasmas was preliminarily demonstrated by RFLP analyses with *Tru*I restriction enzyme on R16F2/R2 amplicons. About 60 samples were selected for further molecular characterization. Reference strains maintained in periwinkle were STOL (from Serbia), STOLC and STOL-PO (from France). RFLP analyses on R16F2/R2 amplicons with *Hpy188*I, *Hpy8*I, *Mbo*I, *Mbo*II, *Tru*I, *Rsa*I, *BstU*I, *Alu*I, and *Tsp509*I restriction enzymes were carried out. *Tsp509*I was only differentiating one sample from Veneto region in Italy, while different profiles were observed in several of the examined samples when *Mbo*II, *Hpy188*I, and *Alu*I were used. In particular, three different profiles were observed with *Alu*I in samples from Veneto and Tuscany (Italy), and from Serbia. Using *Mbo*II four different profiles (*a-b-c-d*) were observed in samples from Veneto, Tuscany and Emilia, while only one of them (profile *a*) was observed in the samples from Serbia. RFLP profiles of reference strains were *a* for STOL, *b* for STOL-PO and *c* for STOLC. Considering that the profile *c* is referring to an amplicon longer than the expected one, the presence of inter operon heterogeneity and/or of mixed BN strains infection could be hypothesized for this profile. Some of the samples having profile *c* when digested with *Hpy188*I showed further polymorphism.

The reference strains plus seven field-collected BN strains chosen among those showing the above described polymorphism were sequenced on the full 16S gene. The sequences were assembled using DNA STAR software, and compared with selected sequences of phytoplasmas in GenBank database using Blast program. Obtained aligned sequences ranged from 1,300 to 1,500 bp, all showing 99 % homology among themselves and with several of the 16SrXII-related strains deposited in Genbank. Virtual RFLP analyses on R16F2/R2

uncloned amplicons were carried out, using pDRAW32 program (AcaClone Software) and it was possible to confirm the variability detected in real RFLP analyses in the majority of BN sequenced strains (Contaldo *et al*., 2009). The comparison between real and virtual RFLP analyses showed in some cases different profiles when digested with *Mbo*II. Strain STOLC showed in virtual analyses a *b* profile, confirming that profile *c* obtained in real RFLP is formed by mixed infection or inter operon heterogeneity. Moreover the presence of a further *d* virtual profile (different from all those above described) was detected in one of the sequenced strains from Serbia showing an *a* profile in the real RFLP analyses. BN strains differentiation on 16S rDNA obtained from some field collected samples indicates that the detected variability (this work; Acs *et al*., 2011; Delic *et al*., 2011; Marchi *et al*., 2011) could be related with the presence of non yet epidemically spreading BN strains.

Key words: phytoplasmas, molecular variability, strains, classification, Bois noir.

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Genetic structure and dissemination of tuf-a type stolbur phytoplasma associated with stinging nettle (*Urtica dioica* L.)

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Stolbur 16SrXII-A phytoplasma associated with bindweed (*Convolvulus arvensis* L.) and stinging nettle (*Urtica dioica* L.) are associated with tuf-b type and tuf-a type RFLP patterns, respectively. Sequence analyses of other stolbur genes have detected further polymorphism, thus confirming a phylogenetic split between stolbur tuf-a and tuf-b types. Whereas the bindweed-associated tuf-b type is found in many other herbaceous plants, the tuf-a type is associated, as far as we know, only with stinging nettle. This species is reported to be the main host plant of the vector of stolbur in Italy and being a new host plant in Germany, where it bears the main responsibility for the increase in Bois noir disease.

We report sequence diversity of *vmp1* gene (Cimerman *et al.*, 2009*)* from 41 stolbur isolates (1300bp) of the tuf-a type with the aim of evaluating the geographic origin and dissemination of tuf-a type in Europe. By contrast to tuf-b type stolbur (i.e. the bindweed type), where *vmp1* is phylogenetically difficult to interpret, *vmp1* of tuf-a type stolbur (i.e. the stinging nettle type) is phylogenetically informative due to being more conserved. *Vmp1* of the tuf-a type strain was detected for a penta-peptide sequence "Asp-Val-Ala-Asn-Asn", thus confirming the uniqueness of the tuf-a type strain. We found 14 genotypes with maximum 2 % sequence divergence. Diversity in Germany and Switzerland was extremely low. Nineteen sequences out of 20 were identical (average nucleotide diversity over loci = 0.000230). Ten isolates analysed from Northern and Central Italy (including one from South-Eastern France) all showed different genotypes and were very diverse (average nucleotide diversity = 0.010729). We observed three genotypes among 11 Slovenian and Croatian isolates (average nucleotide diversity = 0.000780). The most common genotype was found in seven isolates; this genotype was also found once in Italy. As high genetic diversity is often associated with centres of origins, Italy may constitute the geographic origin of tuf-a type stolbur phytoplasmas. Genetic diversity decreased away from Italy, indicating a longer evolution in Italy on stinging nettle, and bottleneck effects in more recently colonised areas. Phylogenetic

analysis of the nettle-stolbur genotypes suggests that nettle-stolbur arrived in Germany via Italy or France and not via Slovenia, as indicated for the vector *Hyalesthes obsoletus* Signoret (Johannesen *et al.*, 2008). The lack of monophyly of Slovenian/Croatian and German genotypes coupled with the observation of genetic bottlenecks in both areas but for different genotypes, suggests that Slovenia/Croatia was not the source area of German nettle-stolbur. If our hypothesis is correct, it implies that stolbur tuf-a type and the vector *H. obsoletus* have experienced independent dispersal dynamics in Europe. This hypothesis is presently being tested.

Keywords: VMP1*, host-plant strains, dispersal.*

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Molecular characterization of stolbur phytoplasma isolates from vineyards of Emilia-Romagna (Northern Italy)

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Stolbur (16SrXII-A), the etiological agent of grapevine Bois noir (BN), occurs in most Italian viticulture regions. As known, this phytoplasma is vectored from some weeds to grapevines by the planthopper *Hyalesthes obsoletus* Signoret. BN epidemiology, however, appears very complex and still not well elucidated. To improve knowledge on disease epidemics occurring in Emilia-Romagna, a study was initiated and some preliminary results are reported here.

Vineyard surveys were made in different provinces, leaf samples were collected from symptomatic grapevines and crude extracts were tested by a TaqMan-based real-time RT-PCR technique (Margaria *et al.*, 2007; Terlizzi *et al.*, 2009). With this detection assay, 106 BN positive samples were identified and subsequently characterized. The *tuf* non-ribosomal gene was amplified in nested-PCR using primer pairs Tuf1f/r and TufAYf/r; PCR products were then analyzed by RFLP (Schneider *et al.*, 1997; Langer and Maixner, 2004). A newly developed multiplex real-time PCR protocol, with a few modifications, was also adopted for this purpose (Berger *et al.*, 2009).

TufAY/*Hpa*II restriction profiles showed a polymorphism corresponding to the stolbur types reported by Langer and Maixner (2004): tuf-a type, associated with *Urtica dioica* L., and tuf-b type, associated with *Convolvulus arvensis* L. or *Calystegia sepium* (L.) R. Br. These types were also distinguished using the multiplex real-time PCR procedure, confirming previous nested-PCR/RFLP results. In total, infection of stolbur tuf-a type strain was demonstrated in 78 BN-affected grapevines, while strain tuf-b type was only found in 28 symptomatic plants.

Nettle and bindweeds play an important epidemiological role, being the main hosts and sources of phytoplasma inoculum for the insect vector (Langer and Maixner, 2004). Elimination of these wild plants could be an important part of an integrated control strategy for BN disease in vineyards.

Key words: grapevine, stolbur phytoplasma, PCR-RFLP, tuf-a type, tuf-b type.

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Molecular characterization of Bois noir phytoplasmas from Bosnia and Herzegovina

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Bois noir (BN) phytoplasmas have been associated with grapevine yellows disease in Bosnia and Herzegovina (Delic and Lolic, 2010). The disease is widely to moderately distributed in vineyards with both domestic and imported grapevine cultivars. Molecular characterization of selected strains of BN phytoplasma detected in heavily BN-infected vineyards was done to verify the presence of genetic variability. Thirty-five DNA samples of grapevines that were shown to be positive by PCR assays on the 16S rDNA were selected for the study. Polymorphism was studied in the 16S rDNA, *tuf* and ribosomal protein genes of the selected samples. Tuf1f/r, TufAYf/r, and TufINT1f/TufINT4r (Langer and Maixner, 2004) primer pairs were used in nested PCR for amplification of *tuf* genes. RFLP analyses with *Hpa*II showed the presence of the tuf-b type in all the samples tested.

The BN-infected samples were further amplified with rpS3 primers (Martini *et al*., 2007) and PCR products were digested with *Tai*I and *Ssp*I restriction enzymes. Finally 16S rDNA genes were amplified with P1/P7 primers in direct PCR and R16F2n/R16R2 primers in nested PCR assays; both types of the obtained amplicons were restricted with *Mbo*II and *Rsa*I, and *Mbo*II and *Hpy*188I enzymes, respectively.

Restriction profiles obtained after RFLP analyses on 16S rDNA and ribosomal protein amplicons were not uniform. Some samples showed profiles comparable to some of those recently published (Contaldo *et al*., 2009), while some of the profiles of the other samples were totally different from any of those reported in literature. Therefore, polymorphism of these regions should be further studied to better understand BN epidemiology in Bosnia and Herzegovina.

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Key words: Bois noir, strains, Bosnia and Herzegovina, PCR, RFLP.

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Innovative diagnostic methods in stolbur detection: preliminary results

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Bois noir is an important grapevine disease associated with phytoplasmas belonging to ribosomal subgroup 16SrXII (stolbur), commonly spread in a wide range of wild and cultivated host plants. Phytoplasmas are wall-less bacteria generally present in very low concentration in phloem plant tissues. Detection is predominantly based on phytoplasma DNA amplification using two consecutive PCR procedures. This approach is time consuming and increases the risk of contamination.

Two different innovative methods, a LAMP PCR (Loop mediated Isothermal Amplification) and a "nested" SYBR® Green /TaqMan RT-PCR, were developed in order to improve stolbur phytoplasma detection.

LAMP PCR is a nucleic acid amplification method, performed under isothermal condition, based on a set of four primers specific for six different target genomic regions and using a DNA polymerase (*Bst* DNA polymerase) with strand displacement activity. This technology offers advantages in terms of specificity, sensitivity and rapidity and requires less expensive equipment than classic PCR (Notomi *et al.*, 2000). Particularly, a RT-LAMP PCR protocol was developed starting from total RNA (TRNA) extracted using a commercial kit (Qiagen RNeasy Plant mini kit), in order to improve the amplification performance (Minguzzi *et al.,* 2010). Different phytoplasma groups, commonly retrieved in grapevine (16SrI, 16SrV, 16SrXII) were used as reference controls. Stolbur phytoplasma was obtained from infected grapevine and tomato plants; Flavescence dorée phytoplasma was obtained from infected grapevine plants, and aster yellows phytoplasma was extracted from infected periwinkle plantlets. Two different targets (16S rDNA and *tuf* genes) were used to design universal and specific primer sets. A primer set designed on 16S rDNA gene gave positive results with TRNA from 16SrI and 16Sr XII-A infected samples, whereas group specificity was obtained using a primer set designed on *tuf* gene. No reactions were observed with 16SrV-infected and healthy samples with both primer sets.

Alternative "nested" SYBR® Green I/TaqMan RT-PCR method was used to increase the analytical sensitivity of grapevine phytoplasma detection

methods. A fragment of 150 bp in the 16S rDNA gene was firstly amplified with a universal pair of primers, followed by amplification with a 16SrI-16SrXII specific TaqMan probe-primers set. Two different thermal protocols were tested: 1) starting at 68 °C with a gradually constant decrease along to 40 steps; 2) 68 °C for 15 steps, followed by 25 steps at 60 °C. Preliminary results showed a specific amplification with 16SrI and 16SrXII phytoplasma reference strains.

Further analyses are in progress for the validation of these methods.

Key words: Bois noir, diagnosis, LAMP PCR, SYBR® Green/TaqMan RT-PCR.

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Bois noir in Hungary: tuf-b type strain variability on 16S ribosomal gene

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Bois noir (BN) - associated phytoplasmas were detected in Hungary since long time (Kolber *et al.,* 1997); recently the presence of polymorphism in some of the genes used as markers for strain differentiation was also reported (Contaldo *et al.,* 2009).

To further verify the consistence of molecular variability, BN-infected samples were collected in 2008-2010 from symptomatic grapevine plants from vineyards of variety Zweigelt located in Sopron area (near to the Austrian border), varieties Riesling-Sylvaner and Chardonnay from Etyek (near Budapest), unknown variety from South-East Hungary, and from variety Chardonnay from Eger. PCR/RFLP characterization was carried out on *tuf* gene (Langer and Maixner, 2004) using nested-PCR procedures, and restriction with *Hpa*II enzyme showed that all the strains belonged to tuf-b type. Further molecular characterization on 16S rDNA gene, spacer region and beginning of 23S gene was carried out on 12 selected samples by RFLP analyses with *Trn*I, *Bst*UI, *Hpy188*I, and *Mbo*II restriction enzymes on length diverse amplicons. RFLP analyses on R16F2/R2 with *Bst*UI and *Hpy188*I did not show differences among BN samples. The same amplicons digested with *Mbo*II showed variability among BN strains: in particular three different profiles (*a, b,* and *d*) were observed. While the *a* profile was present in four samples, profile *d* was observed in two samples, and profile *c* was observed in all the others. This last profile is very likely derived from of a mixed infection by two BN strains recently differentiated on reference strains STOL and STOL-C (Contaldo *et al.*, 2009; 2011).

Selected grapevine samples were sequenced on 16S ribosomal region (about 1,500 bp), and virtual RFLP analyses carried out on R16F2/R2 amplicons showed in some cases differences between real and virtual RFLP profiles, confirming the presence of mixed infection in samples with profile *c*. After GenBank search, similar analyses on deposited BN strains showed

identical *Hpy188*I profiles among a sample from Hungary and BN samples from Canada and Spain (EU086529 and AJ964960). These results clearly indicate that among BN strains belonging to tuf-b type strain differentiation on 16S gene is achievable using selected restriction enzymes. Further studies will clarify the epidemiological relevance of these strains in BN epidemics.

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Key words: RFLP analyses, strain differentiation, epidemiology.

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Genetic diversity of stolbur phytoplasma strains from different host plants in Friuli Venezia Giulia

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Stolbur phytoplasma, belonging to 16SrXII group, is associated with several diseases of herbaceous and woody plants, such as grapevine Bois noir (BN). *Hyalesthes obsoletus* Signoret of the family *Cixiidae* is its most important known vector. In Friuli Venezia Giulia (FVG) region (North-East Italy) stolbur phytoplasma has been reported since 1986 in Chardonnay and other grapevine cultivars, then it has been described in wild and cultivated plants. During the last years severe outbreaks occurred also in some important crops such as celery and tomato (Carraro *et al.*, 2009). Non ribosomal genes (*tuf*, *vmp*1, *sec*Y) together with 16S rRNA gene are currently proposed as genetic markers for a finer differentiation of the stolbur phytoplasmas (Langer and Maixner, 2004; Cimerman *et al.*, 2009; Fialova *et al.*, 2009; Quaglino *et al.*, 2009). In the present study, the genetic variability of stolbur phytoplasma strains in the FVG region was investigated through PCR-RFLP analysis of *tuf* and *vmp*1 genes.

Weed, crop and grapevine samples were collected in summer and autumn of 2007 and 2010 in several localities. Five stolbur phytoplasma strains obtained by dodder and insect transmission and maintained in periwinkle at the Department of Scienze Agrarie ed Ambientali (University of Udine) were used as reference strains. The presence of phytoplasmas in symptomatic samples was determined either by a nested-PCR procedure using universal primer pairs having 16S rDNA as a target or by a stolbur-specific SYBR® Green I real-time PCR having *rplV* gene as a target. Among wild plants the presence of stolbur phytoplasma was ascertained in two plants of *Phytolacca americana* L. showing symptoms of virescence and phyllody. To our knowledge this is the first report of a phytoplasma infection in *Phytolacca americana*.

More than 70 stolbur isolates from grapevines, crops and weeds were characterized by RFLP analysis with *Hpa*II of direct-PCR products TufAYf/TufAYr (Langer and Maixner, 2004) and with *Rsa*I of nested-PCR products TYPH10F/TYPH10R (Fialová *et al.*, 2009). While both tuf-a type and tuf-b type were identified in grapevine samples, in all herbaceous plants, wild or cultivated, only tuf-b type was retrieved. Several *vmp1* RFLP-types were obtained after RFLP analyses of nested-PCR products with *Rsa*I, proving the

presence of genetic variability of the pathogen in this gene also in North-Eastern Italy, especially among tuf-b type strains.

Key words: PCR-RFLP, tuf *gene,* vmp1 *gene,* Phytolacca americana

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Genetic variability of the stolbur phytoplasma in plants and insects from different viticulture areas of Spain

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Bois noir disease was first identified in Spain in 1994 (Laviña *et al.,* 1995), and in the last decade an increase of the incidence in different wine regions has been reported (Batlle *et al.,* 2009). The identification and characterization of isolates is an important tool to identify which are the vectors and the host plants implicated in the disease dissemination. The amplification of sequences of the gene encoding the elongation factor Tu (*tuf* gene) have allowed to distinguish three different stolbur isolates in grapevine and in the vector, *Hyalesthes obsoletus* Signoret. The tuf-a, -b and -c types, showed correlations with the host plants, *Urtica dioica* L.*, Convolvolus arvensis* L. and *Calystegia sepium* (L.) R. Br.*,* respectively (Langer and Maixner, 2004). Also studies on the *vmp*1 gene, encoding a putative membrane protein, confirmed the genetic diversity of stolbur isolates (Pacifico *et al.,* 2009; Murolo *et al.,* 2010).

The main infected varieties in the viticulture regions of Spain are Chardonnay in Catalonia and Aragón and Grenache and Tempranillo in La Rioja, Álava and Navarra. Samplings conducted in the last years showed that in plots of the variety Grenache in La Rioja the percentage of plants with symptoms was as high as 70 %. The incidence in Chardonnay ranged from 5 % in some plots of Catalonia to 70 % in plots of Aragón (Somontano).

The aim of this study was the evaluation of the genetic variability of stolbur phytoplasma infecting grapevines, host plants and individuals of the vector *H. obsoletus*, collected in different viticulture areas of Spain.

The presence of different stolbur genotypes in the different regions was evaluated using PCR-RFLP analyses of *tuf* and *vmp*1 genes. Tuf-types were analyzed by nested-PCR with Tuf1f/r primers in the first step and TufAYf/r primers in the second step (Langer and Maixner, 2004). Variability in the *vmp*1 gene was analyzed by nested-PCR with StolH10F1/R1 primers in the first step and TYPH10F/R primers in the second step (Murolo *et al.,* 2010). RFLPs were carried out with *Hpa*II and R*sa*I enzymes, respectively (Langer and Maixner, 2004; Pacifico *et al.,* 2009).

According to the results obtained in this study, the tuf-b type was actually the most prevalent in most of the studied areas of Spain, with the exception of La Rioja. Most of the grapevine samples from Catalonia and Aragón, 100 %

and 85 %, respectively, showed the profile tuf-b, whereas in La Rioja only 5.5 % of positive samples showed the latter profile. The rest of grapevine samples from La Rioja showed the tuf-a type. Samplings from all the geographical areas showed that in all *H. obsoletus* specimens, as well as in all *C. arvensis* sampled, the stolbur tuf-b type was identified.

Concerning diversity of the *vmp1* gene, only three V types were identified in the 51 grapevine samples analyzed: V1 (49 %), V3 (43 %) and V15 (8%). Also in 19 individuals of *H. obsoletus* analyzed three profiles were identified: V1 (74 %), V15 (21 %) and V4 (5 %). No *H. obsoletus* carrier of V3 type has been identified at the moment.

The V3 profile was correlated with isolates characterized as tuf-a type, whereas V1, V4 and V15 profiles were correlated with isolates characterized as tuf-b type.

The prevalence of different genetic isolates in insects, host plants and vineyards explains the dissemination patterns in the studied areas.

Key words: Bois noir, stolbur, tuf*,* vmp*, grapevine.*

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Molecular characterization of grapevine Bois noir phytoplasma in Central-Southern Italian regions

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Bois noir (BN) is one of the most important grapevine yellows in Europe. It is associated with the stolbur phytoplasma, which belongs to the 16SrXII-A subgroup. BN has a heavy impact on European viticulture and herbaceous crops (Maixner, 2006). The complex interactions of the stolbur phytoplasma with wild and cultivated annual and perennial host plants and insect vectors in different ecosystems might be responsible for generating genetic and phenotypic diversity. Indeed, wide genetic diversity of stolbur has been shown in the analysis of the *vmp1* gene by PCR/RFLP (Cimerman *et al.*, 2009; Fialová *et al.*, 2009; Pacifico *et al.*, 2009; Murolo *et al.*, 2010).

The aim of the present study was to carry out molecular characterization of stolbur phytoplasma isolates from infected grapevines, vegetables, weeds and insects in different Central and Southern Italian regions, with reference to *vmp1* as a genetic marker, together with the *tuf* gene.

The total DNA extracted from 143 samples of grapevine varieties representative of five Italian regions and collected from 2004 to 2008 was amplified by TYPH10F/R primer pair. The PCR products were digested with *Rsa*I at 37 °C. The PCR/RFLP analyses revealed high genetic diversity of *vmp1* in the grapevine samples, and relevant correlation with molecular characterization based on the *tuf* gene. The nine different *vmp1* types defined were differently distributed in the five regions analysed, and sporadically mixed infections of BN strains were detected. The genetic data, correlated with the biological properties and the grapevine varietal susceptibility, could contribute to the definition of control strategies in the management of grapevine BN.

Keywords: PCR-RFLP, vmp1, Vitis vinifera.

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Heterologous expression and genetic diversity of StAMP, the antigenic membrane protein of stolbur phytoplasma

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Surface proteins play an important role in phytoplasma life cycle. The antigenic membrane protein (AMP) of '*Candidatus* Phytoplasma asteris' has been shown to interact with the insect microfilaments (Suzuki *et al.,* 2006).

Due to the synteny of the *groEL-amp-nadE* genetic locus between phytoplasma genomes, the gene *stamp* that encodes the antigenic membrane protein of stolbur phytoplasma has been cloned and characterized. It encodes a 157 aminoacid-long protein with a predicted signal peptide and a C-terminal hydrophobic alpha helix. StAMP was 26-40 % identical to AMP of '*Ca.* P. asteris' strains and 40 % identical to AMP of '*Ca.* P. japonicum'. The expression of StAMP in *Escherichia coli* produced a 16 kDa peptide recognized by an anti-stolbur monoclonal antibody.

Stamp was more variable than the house-keeping gene *secY* and the ratio between non synonymous over synonymous mutations (dN/dS) was 2.78 for *stamp* as compared to 0.64 for *secY*. This indicates that *stamp* is submitted to a positive diversifying selection pressure (Fabre *et al.,* 2010).

The genetic diversity of *stamp* was evaluated among a collection of isolates representative of the genetic diversity of stolbur phytoplasma in the Euro-Mediterranean basin. Regarding to the geographic origin of the isolates and the *stamp* sequences, it must be noticed that most of the French isolates clustered on the same branch supported by high bootstrap values. One branch of the phylogenetic tree corresponded to isolates of Central and Eastern Europe, while another branch grouped isolates of the East of the Mediterranean basin, namely P7 from Lebanon, GR328 from Greece, STOL from Serbia and six isolates from Azerbaijan. However, a higher number of isolates should be sequenced in order to ascertain correlation between sequence genotype and geographic origin.

Key words: Bois noir epidemiology, genotyping, variable surface protein, positive selection.

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Tomatoes and 16SrXII phytoplasma as tools to evaluate the role of plant signalling in the phytoplasma-plant interaction

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Bois noir (BN) disease associated with 16SrXII-A phytoplasmas occurs frequently in vineyards in Burgenland, Eastern Austria. In 2010, in a vineyard with cv. "Zweigelt" in the Seewinkel East of Lake Neusiedl, BN symptoms were sporadically visible. 16S and *tuf* sequence analysis confirmed 16SrXII-A phytoplasma infection of the tuf-a type, frequently found in *Convolvulus arvensis* L. (Langer *et al.*, 2004). The 16S sequences matched GenBank entries of phytoplasmas in grapevine and Solanaceae hosts such as tomato, green pepper and potato.

As 16SrXII-A is associated with Solanaceae stolbur, tomatoes provide potentially valuable tools to understand and dissect plant disease and plant defence responses to phytoplasma. Apart from fast growth, fairly easy cutting and grafting, tomato research has also the advantage that several plant signalling and plant defence mutants are available for the research community. Since phytoplasmas have not been cultivated so far and controlled infection with phytoplasmas is not easily achievable, little is known on the role of hormonal and defence signalling in phytoplasma infection.

Typical witches' broom symptoms and recent research advances in studies of the Aster Yellows group (16SrI) phytoplasmas (Hoshi *et al.*, 2009; Leljak-Levanic *et al.*, 2010) point to a specific role of auxin signalling in plant-phytoplasma interactions. We will discuss the use of tomato signalling mutants and hormonal treatments to evaluate the contribution of specific signalling pathways to the plant-stolbur interaction and the potential of hormonal treatment or treatment with endophytes influencing the plant hormonal balance for containment of BN.

Key words: tomato, stolbur, plant defence signalling.

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Control of *Hyalesthes obsoletus* Signoret, vector of Bois noir, using entomopathogenic agents: preliminary results

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The planthopper *Hyalesthes obsoletus* Signoret (Homoptera, Cixidae) is the vector of the grapevine yellows Bois noir (BN), one of the most important phytoplasma disease in Europe. Reduction of incidence of phytoplasma-associated diseases relies on the control of their insect vectors and on the eradication of affected host plants. Management of BN is especially difficult because 1) both the vector and the phytoplasma can develop also in plants other than grapevines and 2) the overwintering younger instars of the insect live underground feeding on the roots of wild plants nearby vineyards (such as nettle and bindweed) and chemical treatments are ineffective and unsustainable (Mori *et al.*, 2008). The aim of this work was to evaluate the possibility to control the younger forms of the planthopper by means of entomopathogenic agents applied to nettle roots (AAVV, 1997; Vanesa Toledo *et al.*, 2007; Reggiani and Maistrello, 2010).

A first semi-field test was performed on young planthoppers obtained from hundreds of wild *H. obsoletus* adults that had been captured and allowed to breed in a greenhouse on potted nettles during the previous summer. In late spring, pots containing groups of these juveniles were treated by sprinkling the ground with selected strains of entomopathogenic fungi (*Beauveria bassiana* (Bals.-Criv.) Vuill. and *Paecilomyces lilacinus* (Thom) Samson) and nematodes (*Heterorhabditis bacteriophora* Poinar and *Steinernema feltiae* Filipjev). The mortality of *H. obsoletus* was recorded on day 7, 14, and 21 after treatment. A second trial was made in field conditions selecting plots (3 m^2 in surface) in the proximity of vineyards with a high quantity of nettle. These plots were sprayed on the middle of July at the ground level with the same entomopathogenic agents strains as used in the first test. The plots were isolated with proper cages and the number of emerged adults was monitored during the whole flight period using yellow sticky traps.

The obtained results show a good activity of the tested biopesticides, in particular for *B. bassiana* in the semi-field trial, and for nematodes in the field trials. The experiments suggest that microbiological control of the planthopper

vector could represent a promising option for the integrated management of Bois noir.

Key words: Bois noir, vectors, microbiological control, biopesticides.

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Herbicide control of stinging nettle: does the date of application affect the emergence of *Hyalesthes obsoletus* Signoret?

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Hyalesthes obsoletus Signoret (Hemiptera, Cixiidae) is the principal vector of Bois noir (BN) in Switzerland (Kehrli *et al.*, 2010). Captures on different viticultural weeds indicated that the nymphs as well as the adults of this polyphagous planthopper mainly use stinging nettle (*Urtica dioica* L.) as a host plant (Kessler *et al.*, 2010). Stinging nettle therefore plays a central role in the epidemiology of BN in Swiss vineyards. Since BN disease can hardly be cured and direct control measures against *H. obsoletus* are ineffective, viticultural control practices should target stinging nettle, the actual reservoir and source of the phytoplasma associated to BN and of the vector. Even though experimental evidences are, to our knowledge, lacking, it is currently recommended to apply herbicides against stinging nettle in the end of the season in order to kill all developing nymphs of *H. obsoletus*. In Switzerland, this is the only herbicide treatment authorised in autumn.

In order to verify the proper date of herbicide application, stinging nettle patches were treated with glyphosate in the autumn, in the spring or not at all (=untreated control). Significantly more stinging nettle grew in the untreated control plots over the coming summer compared to the plots treated with glyphosate in autumn or spring. Herbicide applications at both dates showed a very high efficacy and there was no statistical difference between them.

To study the direct impact of herbicide on the development of *H. obsoletus* nymphs, emergence traps (=photo-eclectors) were placed directly in the centre of treated and untreated plots. Emerging adults were captured in all three treatments and there was no significant difference in the number of emerged adults among them. Thus, the aerial application of glyphosate does not impede the development of nymphs on the roots of stinging nettle in the soil, neither if it is applied in autumn nor in spring. Government agencies, therefore, have to reconsider if the exceptional authorisation of herbicide applications against stinging nettle in autumn is really justified or if stinging nettle could also be controlled in early spring, alike other viticultural weeds.

Key words: viticulture, Vitis vinifera*, weed control, insect development.*

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Gene expression pattern in grapevine infected with Bois noir phytoplasma

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Phytoplasmas are insect-transmitted, plant-pathogenic bacteria, without cell wall and are responsible for significant crop yield losses. Due to inability to culture them without the presence of their hosts and consequently greater inaccessibility to our experimental systems the knowledge about their biology and their interactions with hosts is scarse.

The gene expression patterns were followed in leaf midribs of grapevine cv. Chardonnay naturally infected with stolbur phytoplasma, which is associated with the grapevine yellows disease called Bois noir (BN).

A five-season long experiment in a production vineyard has been conducted and has been a continuation of our previous study (Hren *et al.*, 2009). For the duration of the experiment the same set of plants has been maintained where we monitored changes in plant health (presence/absence of phytoplasmas) and their correlation with specific gene expression pattern. From global gene expression profiling analysis with microarrays (Hren *et al.*, 2009) a set of 17 or 22 candidate genes from differentially expressed metabolic pathways have been selected and analysed more precisely with quantitative real-time PCR.

The selected genes are involved in primary and secondary metabolic pathways. The obtained results showed significant changes in levels of gene expression that were compared between phytoplasma-infected and phytoplasma-free grapevine samples. The most prominent gene up-regulation was observed in genes involved in defence against biotic stress in infected plants in comparison to healthy ones. Similar response was detected in carbohydrate metabolism and secondary metabolism. On the contrary, some ROS genes were significantly down-regulated, where the others were up-regulated.

Furthermore, our PCA (principal component analysis) results showed a clear separation between healthy and infected samples in all five seasons. Additionally, these results were supported with another statistical analysis - support vector machines algorithm (SVM) - where only 2-5 % of the samples regarding to the season were categorised into wrong group.

Lastly, screening of disease symptoms in different sampling seasons revealed changes in symptoms intensity, which is also reflected in different gene expression pattern.

Our experiment revealed complex interactions among grapevine and the BN phytoplasma. The results indicate that phytoplasma infection induced both the reprogramming of primary metabolic pathways and the activation of secondary ones, especially those related to the defense mechanisms.

Key words: gene expression profile, stolbur, grapevine.

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Real-time PCR validation of microarray expression profiles obtained in comparing healthy, recovered and stolbur-affected grapevine plants

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Diseases caused by phytoplasmas reduce quality and quantity of grapevine production with important consequent management costs. In this view, physiological studies of mechanisms involved in plant diseases become relevant to understand plant responses and to develop preventive control measures.

In order to obtain detailed expression profile, allowing to increase knowledge of differentially expressed genes in investigated plant and relative involved pathways, the microarray Roche NimbleGen® technology was used with chips representing the whole *Vitis* genome. Specifically, a study was performed to understand gene expression level in phytoplasma-plant interactions, comparing biological replicates of different grapevine varieties affected by Bois noir (BN, Stolbur phytoplasma, 16SrXII-A) with recovered and healthy samples.

Obtained results showed a differential expression of hundreds genes in plants infected by phytoplasma, involving different Gene Ontology (GO) classes and metabolic pathways, as previously reported (Punelli *et al.*, 2010a; 2010b).

In this paper the validation of expression profiles obtained using the microarrays system is reported.

A quantitative real-time PCR (q rt PCR) was performed to examine the differential transcription of five not annotated gene sequences in *Vitis*, corresponding to probes that showed up- or down-regulation in microarrays analysis.

Quantitative rt PCR analysis was performed using SYBR® Green chemistry and normalizing the expression level of each sample with the housekeeping gene level (actine, AF369524). The quantitative analysis confirmed the differential expression observed in microarray assay.

The validation and confirmation of microarray results obtained comparing differentially expressed genes in healthy, phytoplasma-infected and recovered plants open new prospective in the comprehension of the mechanisms involved in the phytoplasma-grapevine interaction.

Key words: host-plant interaction, real-time PCR, gene expression, phytoplasma, Vitis.

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Phloem cytological and molecular modifications induced by Bois noir infection in grapevine plants

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Phytoplasmas colonize the sieve tubes of the phloem in most organs of the plant and manipulate the host to ensure for themselves an efficient spread inside. Recent developments have improved our knowledge of the molecular and biochemical effects of phytoplasma infection, mainly on herbaceous host plants (Musetti, 2010), but the literature available is still scarce regarding the physiology of phytoplasma infection in grapevine (Bertamini *et al.*, 2002; Musetti *et al.*, 2007).

To gain insights into the phytoplasma-grapevine interaction, defence reactions such as callose accumulations and P-protein aggregations in the phloem have been evaluated at the ultra structural level by means of transmission electron microscopy, comparing healthy, Bois noir-symptomatic and recovered grapevines. In parallel, the expression of some plant defence-related genes, as well as those involved in sugar metabolism, or in the generation and transduction of mobile molecular signals leading to the establishment of the recovery syndrome (Hren *et al.*, 2009), have been analysed in leaves. A single cell approach aimed to focus the expression analysis to the phloemic complexes has been optimised.

TEM investigations showed ultrastructural differences in the phloem of the grapevine plants, according to their different status (healthy, symptomatic or recovered). The leaf tissues from healthy plants were well preserved and without ultrastructural modifications. Phytoplasmas were not detected in the sieve tubes of these plants. Callose was not present in sieve plate pores and the P-protein was uniformly dispersed into the lumen of most sieve elements. Phytoplasmas were instead observed in the lumen of the sieve elements in diseased leaves, although the pathogen was scarcely detected because many sieve tubes were collapsed, necrotized or filled with starch, often showing, moreover, plasmolysis and cytoplasm condensation. In the leaves of recovered plants, on the contrary, phytoplasmas were not detected but callose depositions were observed in the sieve tubes, particularly occluding sieve pores. Moreover, in the mature phloem elements of recovered plants, P-protein aggregates were observed, forming P-protein plugs and filling the cell lumen.

Real-time RT-PCR analysis of symptomatic leaves confirmed an up-regulation of genes involved in pathogenesis (*VvOLP*), in callose formation (*VvCASY*), and in sugar metabolism (*VvSUSY, VvINV2*). Genes for sucrose transport were also examined (*VvSUC11, VvSUC12* and *VvSUC27*) as putative markers of the phloem. Phloemic complexes from symptomatic leaves and shoots were isolated by Laser Microdissection (LM). Leaf and shoot were fixed by acetone overnight at 4 °C, then dehydrated and infiltrated with paraffin. Cells were cut from 13 mm-thin cross-sections, and RNA isolated with a common filter absorption-based micro-kit. Two sucrose transporters (*VvSUC11* and *VvSUC27)* and a vacuolar acid invertase (*VvINV2*) were analysed in preliminary experiments of RT-PCR; *VvSUC11* was found expressed in phloemic complexes of both leaf and shoot, while *VvINV2* and *VvSUC27* specifically in leaf and shoot, respectively. Experiments are carried out to extend the analysis to different genes and plants with different healthy status.

Key words: laser microdissection, phytoplasma-plant interaction, recovery.

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Recovery experiences on adult vine plants affected by grapevine yellows in North-West of Italy

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Increasing occurrence of grapevine yellows disease has been observed in North West of Italy in the last 15 years. Grapevine yellows showed different spread and recovery phenomena according to the year, growing area, vine variety, viticulture techniques (Osler *et al.*, 2006; Bellomo *et al.*, 2007; Zorloni *et al.*, 2008). Grapevine yellows, as vascular disorders, are affecting plant longevity and wine economy. Previous experiences showed yellows to be closely related to plant stress like over cropping, bad soil management and deleterious wounds on the trunk (Corino et *al.*, 2004; Corino *et al.*, 2010). This paper is a report of a series of trials started in 2001 with the aim to verify pruning as a tool to recover vines affected by yellows.

The experiences were carried out on 7 adult vineyards planted with the varieties Barbera, Grignolino and Nebbiolo. By a convenient pruning, based on shoots from the bottom of the trunk and far from wounds, a consistent recovery of affected plants was observed. Best results were achieved on Grignolino (89 %) and Nebbiolo (87 %); Barbera was less promising (73 %). The recovery was greater on Bois noir (BN) infected plants (91 %) whilst it was not as good where Flavescence dorée (FD) was present (33 %).

Concerning the two phytoplasmas, BN was definitely more common and the symptoms were wide spread mostly at veraison period; FD was detected early in the season and affected all the flowers, causing no fruit-setting. Often, one part of the same plant was found completely asymptomatic and the other with symptoms; sometimes this behaviour was observed on the same shoot as well, during the growing season.

These results highlight the vascular disorder on plant tissues during development of yellows infected grapevines. Functional tissues help for sap regular flow; therefore, by means of correct pruning, specifically avoiding deleterious wounds and searching for promising buds on the trunk, it is possible to recover, with different success, the affected plants.

Key words: phytoplasma, pruning, wounds, recovery.

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Effects of treatment with resistance inducers on incidence of Bois noir symptomatic plants and on qualitative and quantitative parameters of production

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Bois noir (BN) is the main phytoplasma disease of grapevine in Italy. It is caused by the stolbur phytoplasma, and it is widespread in all Italian regions, with the consequent severe losses in production. At present, no effective measures of disease containment are known and no chemicals are available to eradicate the pathogen from infected plants in field.

A possibility that is under investigation in different fields consists of increasing the plant defences (Romanazzi *et al.*, 2009a). Recently, treatments with resistance inducers have proven to be effective in reducing the number of symptomatic plants and in the induction of recovery, an asymptomatic status of the plant, that can be accompanied with the disappearance of the phytoplasma from the plant canopy. Weekly treatments with the compounds Bion, Olivis and Kendal have proven to be the most effective for the induction of recovery in grapevines of cv Chardonnay, the most sensitive grapevine variety to BN (Romanazzi *et al.*, 2009b).

The present study was aimed to assess the changes that occur in grapevines of cv Chardonnay with and without BN symptoms after treatment with these resistance inducers. The weekly application of resistance inducers from the beginning of May to the end of July confirmed the reduction in the number of symptomatic plants. Moreover, there were also changes in the quantity and quality of grapevine production. Although it has been reported that application of resistance inducers can have negative effects on production (e.g., Vallad and Goodman, 2004), this behaviour was not observed in our trials on grapevine plants treated with the resistance inducers.

Keywords: phytoplasma, induced resistance, Vitis vinifera.

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Biochemical pathways and seasonal variations in Bois noir affected and recovered grapevines

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Bois noir (BN) is a severe disease of grapevines (*Vitis vinifera*) that is associated with the stolbur (16SrXII-A subgroup) phytoplasma. Typical BN symptoms appear on the grapevine leaves, shoots and clusters, with different extents seen according to cultivar. Plants infected by this phytoplasma can show recovery, which is seen as the disappearance of disease symptoms. This phenomenon can be spontaneous or induced (Romanazzi *et al*., 2009). Recent studies at a genomic scale in infected BN plants have underlined alterations in the expression of many genes that are involved in different pathways, which are again cultivar specific (Albertazzi *et al.,* 2009; Hren *et al.,* 2009). However, the physiological changes in BN-infected symptomatic and recovered plants according to phenologic plant development and the presence or absence of symptoms are unknown.

The goal of this study was to investigate the changes in gene expression in leaves of the Sangiovese and Chardonnay cultivars, which are moderately and highly susceptible to BN, respectively. The genes selected related to defence mechanisms (superoxide dismutase, catalase, class III peroxidase, class III chitinases, and β-1, 3-glucanase), secondary metabolism (phenylalanine ammonia-lyase, chalcone synthase, and flavanone 3-hydroxylase) and electron transport (NADPH). The study was performed in healthy (control), symptomatic and recovered plants, with samples collected in September, when BN symptoms are clearly visible on the canopy, and in June, when BN symptoms are not expressed. The recovered plants were obtained after application of abiotic stress (Romanazzi and Murolo, 2008). Moreover, in these plants the enzyme activities of β-1,3-glucanase, chitinase, phenylalanine ammonia-lyase and superoxide dismutase were determined. The gene expression study was performed by reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR) with the SYBR-green dye system, with spectro-photometric assays performed for the measurement of enzymatic activities.

In symptomatic leaves of both cultivars, gene expression of β-1,3-glucanase, class III chitinase, phenylalanine ammonia-lyase, chalcone synthase

and flavanone 3-hydroxylase was up-regulated, while that of NADPH dehydrogenase was down-regulated. In Sangiovese, the gene expression pattern showed a similar trend for all of the genes analyzed, independent of the presence of BN leaf symptoms; however, in Chardonnay, there was increased gene expression associated with the seasonal phenology and the appearance of symptoms on leaves. In recovered plants of both cultivars, gene expression of class III chitinase, phenylalanine ammonia-lyase and chalcone synthase increased, which was not affected by plant phenology. The enzyme activities analyzed generally correlated with gene expression.

Our data show that in BN-infected grapevines the expression of defence-related genes depends on the BN sensitivity of the cultivar, the plant phenology, and the presence of disease symptoms. This study contributes to investigations into the optimal timing for application of BN control measures.

Keywords: plant-phytoplasma interaction, plant phenology, recovery, stolbur.

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Film on the behaviour and development of the planthopper *Hyalesthes obsoletus* Signoret

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During the last decade, the grapevine yellows disease termed Bois noir (BN) has caused severe problems to viticulture across Central Europe and the Mediterranean. The disease is induced by phytoplasmas, which are vectored by *Hyalesthes obsoletus* Signoret, a Cixiid planthopper (Mainer *et al.,* 1995). Due to an ongoing geographical range expansion of the thermophilous vector and in support to defined control strategies, stakeholders, researchers, vine growers and their advisors should have sound knowledge of the behaviour and the biological life cycle of *H. obsoletus*. The documentary film is aimed to address this need.

The documentary shows close-up images of the vector and its anatomic aspects. Further images provide insight in the different life cycle stages, e.g. how the juvenile stages feed and develop on the roots of their herbaceous host plants. It is interesting to see how the adults even suck on lignified stalks. The probing behaviour and the action of the stylet are well documented. Amazing is the acoustic communication of the adults. Habitat characteristics, such as preferred host plants, but also how to recognize symptoms on the vine plant caused by BN, are explained.

With an emphasis on the biological life cycle, the habitat preferences and the disease symptoms, this film might contribute to better understand measures that effectively reduce the further increase and spread of the grapevine BN disease.

Key words: Hyalesthes obsoletus*, Bois noir, grapevine, life cycle, behaviour.*

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New acquisitions on *Hyalesthes obsoletus* Signoret nymphs

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Hyalesthes obsoletus Signoret (Homoptera: Cixiidae) is the only known vector of stolbur phytoplasma associated with Bois noir (BN). Grapevine is an occasional host for this planthopper. It is monovoltine and overwinters as nymphs on the roots of several wide, mainly herbaceous, plants. Five nymphal instars were reported in literature (Musil, 1956; Alma *et al.*, 1988; Güclü and Ozbek, 1988). A dichotomous key for the identification of the instars was proposed (Sforza *et al.*, 1999).

Since nymphs of *H. obsoletus* collected on *Urtica dioica* L. in North Italy showed characteristics not always in agreement with those reported in Sforza *et al.* (1999), morphological and morphometric studies were carried out on nymphs associated with nettle.

Both nymphs either collected on nettle grown along a vineyard border (Cormons, North-Eastern Italy) or obtained in the laboratory from *H. obsoletus* adults sampled in the same locality were slide mounted. Count of morphological parts (e.g. sensory pits and metatarsomeres), morphometric parameters (e.g. head width, head-thoracic length) and ratio between parameters (e.g. metatarsomere and wingpad lengths) were considered. Measurements were grouped in frequency classes and plotted to verify the existence of a series of discrete size classes.

Adults collected in the field or emerged in the laboratory all belonged to *H. obsoletus* according to Holzinger *et al.* (2003). Frequency distributions of morphometric parameters were in agreement with five nymphal instars. The most discriminant characters were metatarsomere and pit numbers, head-thoracic length and wingpad length ratio. The nymphs differed from those studied by Sforza *et al.* (1999) for both morphological and morphometric characteristics.

Key words: Cixiidae, Bois noir, morphology, morphometry.

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Host races of the Bois noir vector *Hyalesthes obsoletus* Signoret in Germany

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The epidemiology of the grapevine yellows disease Bois noir (BN) is determined by host-plant populations of the vector *Hyalesthes obsoletus* Signoret (Cixiidae) associated with bindweed or stinging nettle. Bindweed used to be the predominant host plant in Germany, but over the last 15 years the abundance of *H. obsoletus* on nettle has been increasing and nettle is now considered to be the main host plant (Langer and Maixner, 2004). This increase in abundance of *H. obsoletus* on nettle coincides with an increase of BN caused by the stolbur tuf-a type. The shift in host-plant use, as well as observed phenological differences between *H. obsoletus* on the two host plants (Maixner *et al.*, 2006), suggest two host races associated with bindweed and nettle, respectively, in Germany. If two host races exist, did they evolve locally in Germany or arise via immigration of nettle-adapted individuals from Southern Europe, maybe Italy, where nettle is the main host plant? Microsatellite genetic analyses of *H. obsoletus* populations caught on different host plants from Germany, Switzerland, France, Italy, Slovenia, Romania, Russia, and Israel were analysed to answer these questions.

Populations across Europe were significantly geographically differentiated. Significant differentiation between *H. obsoletus* populations associated with the two host plants was found in Germany but not elsewhere in the European distribution range. The German *H. obsoletus* populations associated with bindweed and nettle were closer related to each other than to populations from the same host plants in other countries. Combined, the results provide evidence for local host-race evolution in Germany and the lack of host races elsewhere. The host-shift in Germany might be based on a single founder event involving a shift from bindweed to nettle. The results from Germany obey three of the four criteria for defining host races 1) host association and fidelity, 2) sympatry, and 3) genetic differentiation (Dres and Mallet, 2002). The fourth criterium, appreciable gene flow between host races, is likely, but has not been proven yet.

Key words: Hyalesthes obsoletus*, host races, vector.*

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Influence of cover crops on the Auchenorrhyncha fauna in Bois noir infected vineyards

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Various monocotyledonous and dicotyledonous plant species were cultivated as green covers in fallows and vineyard rows in or next to three Bois noir (BN) tuf-b type infected vineyards. The presence of Auchenorrhyncha as real or possible vectors of the disease was analyzed. Insect counts in the cover crops, in naturally established green covers and on bare soil were gathered by vacuum sampling, in the vine canopies by yellow sticky traps.

Raphanus sativus L., *Fagopyrum esculentum* Moench*, Phacelia tanacetifolia* Bentham and *Medicago sativa* L. covers were only rarely exploited by Auchenorrhyncha. Low numbers of insects belonging to few species were present in the ground layer of these cover crops. Presence of insects in the canopy, however, was not very much influenced by low numbers of insects in the ground cover. Analyses of yellow sticky traps showed no significant difference between *R. sativus, F. esculentum* and *P. tanacetifolia* plots and naturally green covers. The highest numbers of Auchenorrhyncha (species and individuals) in the canopy were observed on bare soil.

Our results showed that a large part of the Auchenorrhyncha moved into the vineyards from outside. It seems likely that bare soil or "unfavourable" cover crops induced immigrating insects to colonize the vines. In general Cixiidae were rare in the investigated area. Single individuals were observed on fallows with naturally established green covers but they were never found in the ground cover of the vineyards. On sticky traps in the canopy, however, a few specimens were ascertained, mainly on plots with open soil or cover crops. Although their number was too low to draw definite conclusions, it seems likely that Cixiidae, as many other species, migrated into the vineyards and switched to the vines due to lack of food in the ground cover.

We can conclude that fallows or ruderal areas letting weeds take over or leaving some soil bare carry the risk to harbour both infected field bindweeds and high vector populations. Dramatic increases of BN might be the consequences. Cover crops are far less colonized by real or possible vectors and might help to suppress field bindweed. Vegetation management of fallows by cover cropping is therefore advisable. Cover cropping in the vineyard itself needs to be seen in a nuanced light. On one hand cover crops can suppress

both field bindweed and presence and multiplication of vectors within the vineyard. On the other hand they are not food plants for incoming insects and might provoke a switch of these insects to the vines.

Key words: vector, stolbur phytoplasma, fallow, green cover, bare soil.

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Analysis of major environmental factors determining the distribution of *Hyalesthes obsoletus* Signoret in Baden (Germany)

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In recent years, there has been an alarming spread of the yellows disease Bois noir (BN) in Germany (Breuer and Maixner, 2007). Phytoplasmas causing BN are accidentally transmitted to grapevine by *Hyalesthes obsoletus* Signoret. Hence, sound knowledge about the habitat preferences of this cixiid plant hopper is important to formulate guidelines for action to advisers of vine growers. However, the causal, driving factors determining the vector distribution remain unknown (Strauss and Biedermann, 2006). Given the high sensitivity to ecological disturbances, planthoppers are a predestined group for habitat suitability modelling.

The objective of our study was to develop a spatially explicit species distribution model (SDM) in order to examine whether range expansions of *H. obsoletus* are related to different environmental conditions. Therefore, we recorded the occurrence of the planthopper at 111 randomly selected locations across Baden and collected an extensive range of local habitat characteristics. Further explanatory variables on landscape scale were derived from GIS maps. Statistical diagnostic models expanded with ecological expertise were used to derive habitat-response relationships. The results show that occurrence characteristics of *H. obsoletus* are associated with a combination of environmental factors at different scales. Response differences between the endpoints are discussed.

Key words: Hyalesthes obsoletus*, Bois noir, grapevine, species distribution models, analysis*

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Insects in the Bois noir pathosystems of neighbouring viticulture regions along Croatian-Hungarian state border

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In the frame of the bilateral Croatian-Hungarian project, viticulture regions with widespread Bois noir (BN) and potential vector species were identified. Several areas adjoining state borders potentially having similar BN pathosystems were targeted for molecular characterization of pathogens in grapevines, weeds and insects. A total of 91 insect samples collected in 2010 was tested for phytoplasma presence. Most samples (71) consisted of individual *Hyalesthes obsoletus* Signoret adults, out of which 25 were caught in the vineyard of Vukanovec (Međimurje), with *Urtica dioica* L. as a dominant weed of known importance for BN epidemiology. The rest of the insects were collected from other vineyards in Međimurje, Zagreb and Baranja, with *Convolvulus arvensis* L. as a dominant weed. Insect populations screened in Međimurje included also *Reptalus cuspidatus* (Fieber), whilst Baranja vineyards harboured more diverse insect populations including *Reptalus quinquecostatus* (Dufour), *Anaceratagallia ribauti* (Ossiannilsson), *Lygus rugulipennis* Poppius, *Cixius* sp., *Philaenus spumarius* L., and *Laodelphax striatella* (Fallen), besides the above mentioned species.

Total DNA was isolated by using MagNA Pure LC Instrument and MagNA Pure LC DNA Tissue Isolation Kit II (Roche) mostly from individual insects. Templates obtained from this magnetic-bead technology procedure were used for conventional direct and nested PCR employing P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) and R16F2n/R2 (Gundersen and Lee, 1996) generic phytoplasma primers, respectively. Identification of stolbur phytoplasma DNA was performed by RFLP after digestion with the enzyme *Tru*1I (Fermentas). BN-positive insect samples were subjected to tuf-typing (Langer and Maixner, 2004). In parallel, the DNA was tested by real-time PCR using a protocol modified from Pelletier *et al.* (2009).

BN phytoplasma DNA was amplified from 20 individual insects in conventional PCR-RFLP and real-time PCR experiments. The individual

insects harboring BN DNA were *H. obsoletus* from Vukanovec (2), Jastrebarsko (9) and Baranja (8), as well as a pooled sample of *L. striatella* from Baranja. Tuf-types were obtained for 15 *H. obsoletus*. Only one *H. obsoletus* from Vukanovec, a pathosystem dominated by *U. dioica*, was found to harbour tuf-a type. The rest was infected by tuf-b type, including the other positive *H. obsoletus* from Vukanovec, indicating that tuf-b type is more common and geographically more widespread along the Croatian-Hungarian border. Finding both tuf-types in insects caught in close proximity (Vukanovec) indicate that this ecological niche may involve both *Urtica* and *Convolvulus* type BN pathosystems.

This research was performed in the frame of the Hungarian – Croatian project (No. HR- 12/2008) "The role of wild plant species in the epidemiology of grapevine phytoplasmoses", supported by the National Office for Research and Technology from the Fund for Research and Technology Innovation in Hungary, and the Croatian Ministry of Science, Education and Sports.

Key words: 16S rDNA, MagNA Pure LC, PCR-RFLP, real-time PCR, tuf-typing.

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Potential Bois noir vectors in Canadian vineyards and their feeding behaviour on grapevines

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Canada is importing between 1.5 to 2 million grapevines every year, from France and Germany, where Bois noir (BN) is still spreading. BN was recently detected in grapevines imported from Europe and grown in Canadian vineyards in British Columbia and Ontario (Rott *et al.*, 2007). BN is a quarantine disease in Canada (Canadian Food Inspection Agency 1995) and all the infected or suspect grapevines were destroyed, followed by three years of surveys to confirm eradication. In Europe, the main European vector of BN is *Hyalesthes obsoletus* Signoret. Although this pest has not been found in Canada, other suspected secondary vectors are present in Canada (Maw *et al.*, 2000). Given the possibility of BN being re-introduced to Canada, research was conducted to determine the abundance of secondary BN vectors and their potential vector ability in Canadian vineyards.

Leafhopper population surveys were conducted in several vineyards from British Columbia, Ontario and Québec since 2006 and established the presence and incidence of three potential BN vectors in vineyards: *Aphrodes bicinctus* (Schranck), *Euscelis obsoleta* Kirschbaum and *Fieberiella florii* Stål (Laviña *et al.*, 2006). *Macrosteles quadrilineatus* (Forbes), the aster leafhopper, was also found in very high numbers in and around Canadian vineyards. BN phytoplasma was not detected in leafhopper specimens during this study. Feeding behavior of the potential BN vectors, *F. florii* and *A. bicinctus,* on *in vitro*-grown grapevines is being conducted and will be discussed. *M. quadrilineatus* is not known to be a BN vector but was included in the feeding behaviour study because of its high number.

Key words: Bois noir, grapevine, Canada, leafhopper vector

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Planthoppers (Auchenorrhyncha) in vineyards infected by Bois noir phytoplasma in South Tyrol (Northern Italy)

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The presence and density of planthoppers and leafhoppers was investigated in eleven vineyards infected with stolbur phytoplasma, the causal agent of Bois noir (BN), in South Tyrol (Northern Italy) using insect nets for sampling the understory vegetation.

The confirmed vector *Hyalesthes obsoletus* Signoret was sampled from early June to mid August in 2006; its abundance was positively correlated to the presence of BN symptoms on grapevines. The understory vegetation of the sampled vineyards was composed by 12 to 24 herbaceous plant species on average, included the known host plants *Urtica dioica* L. and *Convolvulus arvensis* L. Both subtypes of the stolbur phytoplasma (VK type I and VK type II) were detected in *H. obsoletus* and plant samples.

An additional 56 Auchenorrhyncha species were sampled; the most numerous being *Psammotettix confinis* (Dahlbom), *Laodelphax striatella* (Fallén), *Dicranotropis hamata* (Boheman), *Psammotettix alienus* (Dahlbom), *Falcotoya minuscula* (Horváth), *Macrosteles cristatus* (Ribaut), *Dictyophara europaea* (L.), *Philaenus spumarius* (L.), *Anaceratagallia ribauti* (Ossiannilson) and *Neoaliturus fenestratus* (Herrich-Schäffer). Several invasive species, such as *Stictocephala bisonia* Kopp & Yonke and *Metcalfa pruinosa* (Say) were sampled in the investigated vineyards, whereas *Scaphoideus titanus* Ball, the vector of the Grapevine yellow Flavescence dorée, was not found. *Recilia horvathi* (Then) (Cicadellidae) was found for the first time in South Tyrol.

Key words: Vitis vinifera*, planthoppers, Bois noir, biodiversity, invasive species, Auchenorrhyncha.*

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Preliminary results of a survey on the Auchenorrhyncha in some vineyards of the Chianti district (Tuscany)

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Due to the recent increase of Bois noir in Central Italy, during the last four years a survey was carried out in some areas of the Chianti district (Tuscany), in order to define the planthoppers and leafhoppers associated to the vineyard agro-ecosystem and to improve knowledge on the grapevine phytoplasma vectors.

In forty five vineyards belonging to seven different macro-areas, one to three monitoring stations were set up with yellow sticky traps (40x20 cm) exposed from June to September in four rounds of three weeks. Overall, the stations amounted to 69 in 2007 and 2008, 65 in 2009 and 64 in 2010. Each monitoring station was geo-referenced and characterized by agronomic and cultural parameters.

During the four years no vine plant affected by Flavescence dorée was detected and no Bois noir epidemic phenomena were observed. The survey allowed to detect, in addition to *Scaphoideus titanus* Ball and *Hyalesthes obsoletus* Signoret, many other species of planthoppers and leafhoppers associated with grapevine yellow phytoplasmas, such as *Dictyophara europaea* (L.), *H. luteipes* Fieber, *H. scotti* Ferrari, *Reptalus panzeri* (Löw), *R. quinquecostatus* (Dufour), *Neoaliturus fenestratus* (Herrich-Schäffer), *Anoplotettix fuscovenosus* (Ferrari), *Thamnotettix zelleri* (Kirschbaum) (Bosco *et al.*, 1997; Orenstein *et al.*, 2003; Alma *et al.*, 2008; Alma *et al.*, 2010; Maixner, 2010).

Auchenorrhyncha population (Typhlocibinae excluded) showed a significant increase in the last three-year period. In fact, taxa and specimens collected during the survey, were respectively 47 and 2928 in 2007, 30 and 1373 in 2008, 36 and 9432 in 2009, 53 and 14402 in 2010. *N. fenestratus*, of which only males were captured, was the most abundant species and represented in most cases over 50 % of all specimens counted. As regards *S. titanus*, the percentage of affected stations and the average number of specimens per station increased progressively in the four-year period. The percentage of stations with captures of *H. obsoletus* increased in the last three years as well, but the average number of specimens per station remained rather low. The corresponding values of these variables were: 57 % and 1.46 ± 2.57 in 2007; 55 % and 1.46 ± 1.88 in 2008; 74 % and 1.74 ± 1.99 in 2009; 83 %

and 2.28 ± 2.38 in 2010. A similar trend was observed for the other *Hyalesthes* species. Concerning *Reptalus* spp., the percentage of affected stations and the average number of specimens per station did not exceed respectively the 58 % and 1.58 ± 2.23 even in 2010. While the distribution of *S. titanus* seemed influenced by anthropic and microclimatic factors, a certain association between the captures of the cixiids and the flora growing in and around the surveyed vineyards was observed. However, a better evaluation of the influence of the environmental factors on the distribution of the intercepted species needs further investigations.

Key words: Hyalesthes obsoletus*, cixiids, phytoplasma vectors, sticky traps*

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AUTHORS' ANALYTICAL INDEX

S

T

U

V

W

Z

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