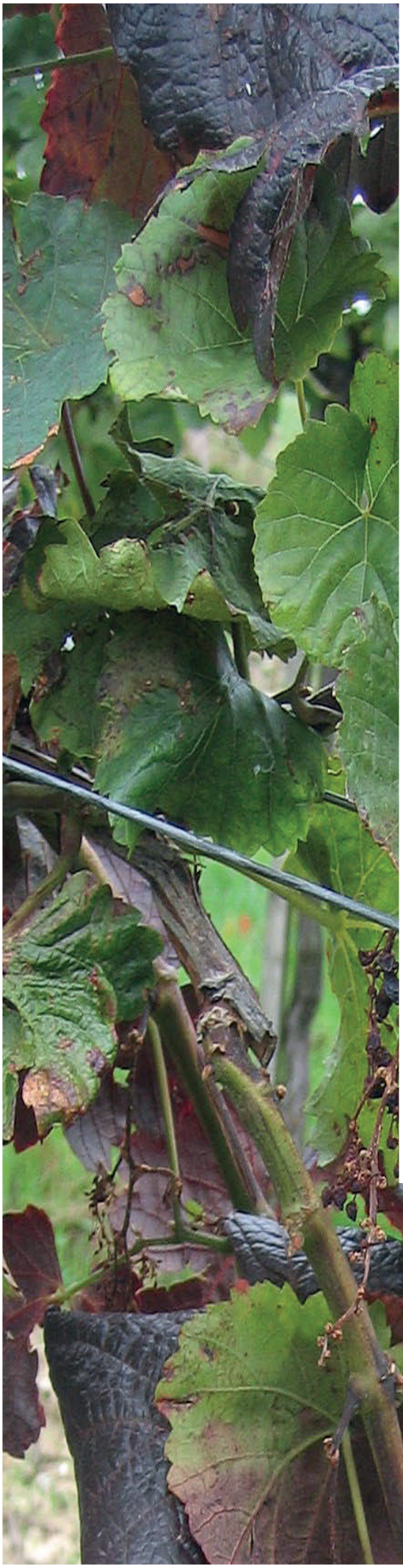

MINISTERIUM
FÜR EIN
LEBENSWERTES
ÖSTERREICH

HBLAuBA KLOSTERNEUBURG
WEIN- UND OBSTBAU

weinobstklosterneuburg.at

MITTEILUNGEN KLOSTERNEUBURG

REBE UND WEIN, OBSTBAU UND FRÜCHTEVERARBEITUNG

VOL. 66/2016 NO. 1 SUPPLEMENT

PAGE 1-92

PROCEEDINGS OF THE 4TH EUROPEAN BOIS NOIR WORKSHOP

9-11 MARCH 2016, KLOSTERNEUBURG, AUSTRIA

TAGUNGSBEITRÄGE ANLÄSSLICH DES 4. EUROPEAN BOIS NOIR WORKSHOP 9.-11. MÄRZ, 2016, KLOSTERNEUBURG ÖSTERREICH

PROCEEDINGS OF THE 4TH EUROPEAN BOIS NOIR WORKSHOP 9-11 MARCH 2016, KLOSTERNEUBURG, AUSTRIA

organized by

Reinhard Eder, Monika Riedle-Bauer, Franz G. Rosner
(Federal College and Research Institute for Viticulture and Pomology, Austria)

Detailed program

Wednesday 9th March 2016

18:00-20:00 Welcome reception, Federal College and Research Institute for Viticulture and Pomology, Koststube

Thursday 10th March 2016

8:30 - 9:00 Registration and poster set up

9:00 - 9:10 **Opening address:** Reinhard Eder (Director of the Federal College and Research Institute for Viticulture and Pomology Klosterneuburg)

9:10-9:30 **Key lecture: Viticulture in Austria**. Ferdinand Regner

9:30 -11:10 **Session 1: Epidemiology of '*Candidatus* Phytoplasma solani'**

Chair: Assunta Bertaccini

Oral presentations:

The role of *Vitex agnus-castus* and associated *Hyalesthes obsoletus* in the epidemiology of *Bois noir* in Mediterranean vineyards. Andrea Kosovac, Sanja Radonjić, Snježana Hrnčić, Oliver Krstić, Ivo Toševski, Jelena Jović

Status of Bois Noir phytoplasma in Turkey. Filiz Ertunc, Didem Canik Orel, Serife Bayram, Gokhan Soylemezoglu

Elucidation of '*Candidatus* Phytoplasma solani' epidemiology through trac(k)ing transmission pathways using field, experimental and molecular data. Andrea Kosovac, Oliver Krstić, Miljana Jakovljević, Tatjana Cvrković, Milana Mitrović, Ivo Toševski, Jelena Jović

Analysis of Bois noir epidemiology in Austrian vineyards by molecular characterization of '*Candidatus* Phytoplasma solani' strains. Monika Riedle-Bauer, Günter Brader, Robert Hack

Epidemiology of '*Candidatus* Phytoplasma solani' associated with potato stolbur disease in Serbia. Milana Mitrović , Miljana Jakovljević , Jelena Jović, Oliver Krstić , Andrea Kosovac, Valeria Trivellone, Ivo Toševski, Tatjana Cvrković

11:10 - 11:30 Coffee break

11:30 - 12:30 **Session 2: Vectors of '*Candidatus* Phytoplasma solani'**

Chair: Jes Johannesen

Oral presentations:

Prevalence of Stolbur phytoplasma in leafhoppers and planthoppers collected in vineyard, corn and potato fields and their surroundings in Switzerland. Valeria Trivellone, Mauro Jermini, Jelena Jović, Tatjana Cvrković, Miljana Jakovljević, Andrea Kosovac, Oliver Krstić, Ivo Toševski, Milana Mitrović

Is *Hyalesthes obsoletus* a species complex undergoing cryptic speciation? More evidence of host-associated genetic differentiation in Southeast

Europe. Andrea Kosovac, Jes Johannesen, Oliver Krstić, Milana Mitrović, Tatjana Cvrković, Ivo Toševski, Jelena Jović

Potential role of *Reptalus panzeri* as a vector of Bois noir in Germany. Friederike Lang, Maria Kölber, Rita Elek, Yvonne Kappel, Dunja Kröhner, Jes Johannesen, Michael Maixner

12:30 - 13:00 Poster Session (short oral presentations)

13:00 - 14:30 Lunch at the Federal College and Research Institute for Viticulture and Pomology

14:30 -16:30 Guided bus tour to Vienna City center

16:30 - 18:00 Free time at Vienna city centre

18:00- 19:30 Technical tour to Schlumberger Kellerwelten (sparkling wine cellar, departure by bus from Vienna city centre)

19:30 - 22:00 Conference dinner at Schlumberger Kellerwelten at the invitation of Federal Minister Andrä Rupprechter

Friday 11th March 2016

9:00-10:40 **Session 3: Characterization of '*Candidatus* Phytoplasma solani'**

Chair: Xavier Foissac

Oral presentations:

***Stamp* gene as the highly discriminative marker for assessment of BN variability in Croatia.** Jelena Plavec, Željko Budinšćak, Ivana Križanac, Dijana Škorić, Martina Šeruga Musić

New '*Candidatus* Phytoplasma solani' strains associated with Bois noir disease in *Vitis vinifera* L. cultivars in Georgia. Fabio Quaglino, David Maghradze, Paola Casati, Nona Chkhaidze, Osvaldo Failla, Piero Attilio Bianco

Multigene characterization of '*Candidatus* phytoplasma solani' strains infecting pepper, celery and maize in Bosnia and Herzegovina. Duška Delić, Nicoletta Contaldo, Biljana Lolić, Đorđe Moravčević, Drago Milošević, Assunta Bertaccini

Multilocus sequence analysis as a powerful tool to monitor molecular epidemiology of '*Candidatus* Phytoplasma solani' at vineyard scale. Sergio Murolo, Gianfranco Romanazzi

Characterization of '*Candidatus* phytoplasma solani' strains from grapevines, *Hyalesthes obsoletus*, reference strains in periwinkle and in colonies. Nicoletta Contaldo, Yuri Zambon, Samantha Paltrinieri, Nicola Mori, Jelena Mitrovic, Bojan Duduk, Assunta Bertaccini

10:40 - 11:00 Coffee break

11:00 - 12:00 **Session 4: Plant pathogen interactions and control strategies**

Chair: Gianfranco Romanazzi

Oral presentations:

Metabolome of grapevine leaf vein-enriched tissue infected with '*Candidatus* Phytoplasma solani'. Nina Prezelj, Lena Fragner, Wolfram Weckwerth, Marina Dermastia

Impact of Bois noir disease on grapevine performance and wine quality of *Vitis vinifera* L. cv. 'Chardonnay' in Hungary. Ibolya Ember, Péter Bodor, Zsolt Zsófi, Xénia Pálfi, Szabolcs Villangó, Zita Pálfi, Márta Ladányi, György Pásti, András Szekeres, Ottó Bencsik, Tamás Deák, Borbála Bálo, László Palkovics, Xavier Foissac, Jacobus Johannes Hunter, György Dénes Bisztray

Strain dependent symptoms and expression of stolbur phytoplasma genes in the experimental host *Catharanthus roseus*. Günter Brader, Amal Aryan, Elisabeth Wischnitzki, Monika Riedle-Bauer

12.00 - 12:40 General discussion: The future of **'*Candidatus* Phytoplasma solani'** research

Moderator: *Michael Maixner*

12:40 –13:00 Conclusions of the meeting

13:00 - 14:30 Lunch at the Federal College and Research Institute for Viticulture and Pomology

14:30-16:00 Guided tour at Klosterneuburg Monastery

Posters

First Report of '*Candidatus* phytoplasma solani' in Sunflower in Bulgaria. Zhelyu Avramov, Jelena Stepanović, Dora Panajotova, Mariana Laginova, Bojan Duduk

Towards a *de novo* genome assembly of *Hyalesthes obsoletus* (Cixiidae) of the stinging nettle host-race. Jes Johannesen, Michael Maixner, Hanno Schmidt

Preliminary results on putative vectors of '*Candidatus* Phytoplasma solani' in Bois noir-affected vineyards in Franciacorta (Lombardy Region, North Italy). Francesco Sanna, Fabio Quaglino, Silvia Filisetti, Paola Casati, Monica Faccincani, Piero Attilio Bianco, Nicola Mori

Microbiomes of the "*Candidatus* Phytoplasma solani" vectors *Hyalesthes obsoletus* Signoret isolated from different host plants. Julien Chuche, Jean-Luc Danet, Sébastien Theil, Xavier Foissac, Denis Thiéry, Nathalie Arricau-Bouvery

Heterologous expression and antigenicity of STAMP Antigenic Membrane Proteins from different '*Candidatus* Phytoplasma solani' genetic clusters. Ibolya Ember, Delphine Desque, Jean-Luc Danet, Marie-Pierre Dubrana, Sybille Duret, Laure Beven, László Palkovics, György Dénes Bisztray, Xavier Foissac

Detection of a Bois noir phytoplasma by a quick-to-use isothermal amplification assay: Preliminary results. Andrea Gentili, Luca Ferretti, Vizzaccaro L., Durante G. , Gianinazzi C., Lopriore S, Chatillon C, Pasquini G

Evaluation of Effective Microorganisms® efficacy on '*Candidatus* Phytoplasma solani'-infected and healthy periwinkle plants. Simon Pierce, Fabio Quaglino, Matteo Montagna, Alberto Spada, Paola Casati, Marcello Iriti

TABLE OF CONTENTS

DETECTION AND CHARACTERIZATION OF '*CANDIDATUS* PHYTOPLASMA SOLANI'

PLANT PATHOGEN INTERACTIONS AND CONTROL STRATEGIES

MITTEILUNGEN KLOSTERNEUBURG
publishes original papers concerning viticulture, enology, pomology and fruit processing as well as abstracts and reviews. Original papers will only be accepted if they have not been published in an identical or similar version or have not been submitted for publication elsewhere (except theses, dissertations, and lectures). Reviews are only to be submitted on request.

Authors of original papers will receive one reprint (max. 10) per page free of charge.

Four issues will be published per year (volume). The annual subscription rate is € 73,- (Austria) and € 79,- (foreign country) respectively; postage is included (surface mail).

NOTICE TO CONTRIBUTORS
Guidelines for authors are available on our homepage http://weinobstklosterneuburg.at

The publications are quoted, abstracted and indexed in the following data-bases:
Thomson Reuter (ISI), Elsevier, CAB International, Vitis Vea und IIR

SUBMISSION OF MANUSCRIPTS
"Mitteilungen Klosterneuburg" publishes original papers, short communications, reviews and surveys on viticulture, enology, pomology and fruit processing as well as enological chemistry, microbiology, hygiene and quality management of wine and fruit.

The official homepage of the Höhere Bundeslehranstalt und Bundesamt für Wein- und Obstbau Klosterneuburg, the editor of "Mitteilungen Klosterneuburg" is: http://weinobstklosterneuburg.at.

Submission of papers should be addressed to the editorial office of the "Mitteilungen Klosterneuburg":
Michael Müller (michael.mueller@weinobst.at)

Höhere Bundeslehranstalt und Bundesamt für Wein- und Obstbau Klosterneuburg
Wiener Straße 74
A- 3400 Klosterneuburg

Online submission of the manuscripts created in a word processing programme is requested. In addition we also ask for submission of the paper or at least the figures in pdf format with a high resolution.
The publication of manuscripts is free of page fees, contributions to printing costs and any other charges.
Authors do not receive fees.

Guidelines for authors can be downloaded from our homepage.
http://weinobstklosterneuburg.at

THE ROLE OF *VITEX AGNUS-CASTUS* AND ASSOCIATED *HYALESTHES OBSOLETUS* IN THE EPIDEMIOLOGY OF *BOIS NOIR* IN MEDITERRANEAN VINEYARDS

Andrea Kosovac[1], Sanja Radonjić[2], Snježana Hrnčić[2], Oliver Krstić[1], Ivo Toševski[1,3] and Jelena Jović[1]

[1] Department of Plant Pests, Institute for Plant Protection and Environment
RS-11080 Zemun, Banatska 33
[2] Montenegro Biotechnical Faculty, University of Montenegro
ME-81000 Podgorica, Mihaila Lalića 1
[3] CABI
CH-2800 Delemont, 1 Rue des Grillons
E-Mail: jovic_biolab@yahoo.com

'Candidatus Phytoplasma solani' (CPs), a 16S rRNA XII-A subgroup belonging phytoplasma (Quaglino et al., 2013), endemic to Europe and the Mediterranean area, causes the most widespread grapevine yellows disease in Europe - Bois noir (BN). Severe damage in grapevine biomass and consequential agroeconomic losses following epidemiological outbreaks (Johannesen et al., 2008) emphasize the importance of clarifying the complex dynamics among inoculum source, pathogen and the vector. *Hyalesthes obsoletus* Signoret, 1865 (Hemiptera: Cixiidae) is the primary vector of CPs to grapevine (Maixner, 1994; Sforza et al., 1998) and as a polyphagous planthopper the species occurs in vineyard ecosystems throughout the Mediterranean basin up to southwestern Germany to the north and Asia Minor in the southeast (Hoch and Remane, 1985). While *Urtica dioica* and *Convolvulus arvensis* are the most frequently recorded hosts of *H. obsoletus* in west and central Europe (Langer and Maixner, 2004; Kessler et al., 2011; Johannesen et al., 2012; Imo et al., 2013), in the Mediterranean several plants characteristic for this bio-geographic area are noted as food source for the adults: *Vitex agnus-castus, Olea europaea, Tamarix sp., Quercus ilex,* etc. (Hoch and Remane, 1985). Among these, *V. agnus-castus* stands out as host plant that provides a niche for larval development (Sharon et al., 2005). Records of *H. obsoletus* occurrence in association with this aromatic woody shrub throughout Greece, Turkey and Israel (Hoch and Remane, 1985; Sharon et al., 2005) prompted the research on its potential role in CPs epidemiology in the Mediterranean littoral, both as a host for the vector populations and as well as pathogen inoculum source.

We investigated in Montenegro, a Mediterranean grape growing country with records of BN (Radonjić et al., 2009), whether *V. agnus-castus* has a role in disease epidemiology and whether it interferes with pathways associated with *U. dioica* and *C. arvensis* (Kosovac et al., 2016). The aim was to employ molecular epidemiology and experimental transmission assays to identify the infection incidence of focal reservoir plants and corresponding vector populations, and to trace transmission pathways of the CPs genotypes from their inoculum source through associated vector populations to symptomatic grapevine (dead-end host).

The *tuf/stamp/vmp1* based multilocus typing revealed 12 genotypes in total, and confirmed a direct, independent pathway of transmission from *U. dioica* by associated *H. obsoletus* populations to grapevine (Langer and Maixner, 2004). Among CPs isolates associated with nettle-sourced cycles some had the typical tuf-a genotype, however some show the intermediate tuf-ab type previously found in Austria (tuf-b2; Aryan et al., 2014). In relation to this, the genotype tuf-b/Rqg50/V17, recently also detected in *H. obsoletus* in BN-diseased vineyards in Austria (CPsM4_At10; Aryan et al., 2014) was found

in grapevine and in two inoculum source plants: *C. arvensis* and *V. agnus-castus* along with their corresponding insect populations, revealing epidemiological routes that overlap and possibly intermix. Based on this finding and assuming that both CPs and *H. obsoletus* are of Mediterranean origin, it is reasonable to suspect that *V. agnus-castus* could be the original host plant of this genotype. Congruently, the same genotype was just recently identified in infected grapevine in Mediterranean vineyards of Bosnia and Herzegovina (Delić et al., 2016). In laboratory controlled transmission assays *Vitex*-affiliated populations of *H. obsoletus* successfully transmitted CPs genotypes to experimental grapevines, thus confirming its vector role. Furthermore, the data unambiguously indicate on natural occurrence of BN in association with *V. agnus-castus* in this region.
Combined results of field collected and experimentally obtained data confirm the role of *V. agnus-castus* in the epidemiology of BN and point out to the importance of this dual host plant as symptomless inoculum source and vector host-plant. This encourages further research in elucidating its occurrence, infection incidence and epidemiological significance, as some of the most important grape growing regions in the world are located in the coastal zone of Spain, France, Italy and Croatia.

ACKNOWLEDGEMENTS

This study was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia (III43001), Ministry of Science of Montenegro (01-550), and partly by the SCOPES programme of the Swiss National Science Foundation (IZ73Z0_152414).

REFERENCES

Aryan, A., Brader, G., Mörtel, J., Pastar, M., and Riedle-Bauer, M. 2014: An abundant '*Candidatus* Phytoplasma solani' tuf b strain is associated with grapevine, stinging nettle and *Hyalesthes obsoletus*. European Journal of Plant Pathology, 140, 213–227.

Delić, D., Balech, B., Radulović, M., Lolić, B., Karačić, A., Vukosavljević, V., Đurić, G., and Jovanović Cvetković, T. 2016: *Vmp*1 and *stamp* genes variability of '*Candidatus* phytoplasma solani'in Bosnian and Herzegovinian grapevine. European Journal of Plant Pathology, DOI 10.1007/s10658-015-0828-z.

Hoch, H., and Remane, R. 1985: Evolution und Speziation der Zikaden-Gattung *Hyalesthes* SIGNORET, 1865 (Homoptera Auchenorrhyncha Fulgoroidea Cixiidae). Marburger Entomologische Publikationen.

Imo, M., Maixner, M., and Johannesen, J. 2013: Sympatric diversification vs. immigration: deciphering host-plant specialization in a polyphagous insect, the stolbur phytoplasma vector *Hyalesthes obsoletus* (Cixiidae). Molecular ecology, 22 (8), 2188-2203.

Johannesen, J., Lux, B., Michel, K., Seitz, A., and Maixner, M. 2008: Invasion biology and host specificity of the grapevine yellows disease vector *Hyalesthes obsoletus* in Europe. Entomologia experimentalis et applicata, 126: 217–227.

Johannesen, J., Foissac, X., Kehrli, P., and Maixner, M. 2012: Impact of vector dispersal and host-plant fidelity on the dissemination of an emerging plant pathogen. PLoS ONE 7, e51809.

KESSLER, S., SCHAERER, S., DELABAYS, N., TURLINGS, T. C. J., TRIVELLONE, V., AND KEHRLI, P. 2011: Host plant preferences of *Hyalesthes obsoletus*, the vector of the grapevine yellows disease '*bois noir'*, in Switzerland. Entomologia Experimentalis et Applicata, 139: 60–67.

KOSOVAC, A., RADONJIĆ, S., HRNČIĆ, S., KRSTIĆ, O., TOŠEVSKI, AND I., JOVIĆ, J. 2016: Molecular tracing of the transmission routes of bois noir in Mediterranean vineyards of Montenegro and experimental evidence for the epidemiological role of *Vitex agnus-castus* (Lamiaceae) and associated *Hyalesthes obsoletus* (Cixiidae). Plant Pathology, 65: 285-298.

LANGER, M., AND MAIXNER, M. 2004: Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur group based on RFLPanalysis of non-ribosomal DNA. Vitis, 43, 191–199.

MAIXNER M. 1994: Transmission of German grapevine yellows (Vergilbungskrankheit) by the planthopper *Hyalesthes obsoletus* (Auchenorrhyncha: Cixiidae). Vitis, 33, 103–104.

QUAGLINO, F., ZHAO, Y., CASATI, P., BULGARI, D., BIANCO, P.A., WEI, W., AND DAVIS, R. E. 2013: '*Candidatus* Phytoplasma solani', a novel taxon associated with stolbur and bois noir-related diseases of plants. International Journal of Systematic and Evolutionary Microbiology, 63, 2879–2894

RADONJIĆ, S., HRNČIĆ, S., JOVIĆ, J., CVRKOVIĆ, T., KRSTIĆ, O., KRNJAJIĆ, AND S., TOŠEVSKI, I. 2009: Occurrence and distribution of grapevine yellows caused by stolbur phytoplasma in Montenegro. Journal of Phytopathology, 157, 682–685.

SHARON, R., SOROKER, V., WESLEY, S., ZAHAVI, T., HARARI, A., AND WEINTRAUB, P. 2005: *Vitex agnus-castus* is a preferred host plant for *Hyalesthes obsoletus.* Journal of Chemical Ecology, 31, 1051–1063.

SFORZA, R., CLAIR, D., DAIRE, X., LARRUE, J., AND BOUDON-PADIEU E. 1998: The role of *Hyalesthes obsoletus* (Hemiptera: Cixiidae) in the occurrence of bois noir of grapevines in France. Journal of Phytopathology, 146, 549–556.

STATUS OF BOIS NOIR PHYTOPLASMA IN TURKEY

Filiz Ertunc[1], Didem Canik Orel[1], Serife Bayram[1] and Gokhan Soylemezoglu[2]

[1] Ankara University, Faculty of Agriculture, Department of Plant Protection
TR-06110, Ankara, Dıskapi
[2] Ankara University, Faculty of Agriculture, Department of Horticulture
TR-06110, Ankara, Dıskapi
E-Mail: fertunc@gmail.com

Grapevine plants bearing symptoms of severe inward curling and redness on leaves were collected from the main viticultural areas in Aegean, Central Anatolia and Eastern Anatolia Regions of Turkey. Nucleic acid extraction was performed to all of the samples, followed by PCR and nested PCR and RFLP analysis for the identification of present phytoplasmas. The majority of the collected infected grapevine samples was infected with Grapevine yellows phytoplasmas, especially '*Candidatus* Phytoplasma solani', the causal agent of Bois Noir in grapevines. This phytoplasma, belonging to 16SrXII-A subgroup, was present in 49 samples. Symptoms of Bois noir were present in all of the regions surveyed.

Turkey is located in the subtropic climatic region in the World, the Northeastern part of Anatolian peninsula and the area between Black Sea and Caspian Sea region is the gene source of some of the important varieties of grapevine (Soylemezoglu et al., 2015). Turkey is one of the nations native to grapevine, therefore our nation has been familiar with grapevine cultivation for more than 6000 years. According to the data of FAO, Turkey has 540.000 ha of grapevine cultivation area and ranks third in terms of cultivation area worldwide. Grape production is 3.923 million tons which means the sixth-largest producer in the world (Soylemezoglu et al., 2015).

The main viticultural region is the Aegean Region with 151.401 ha of vineyard area, comprising 31 % of the total area followed by the Mediterranean, Central Anatolia, Eastern Anatolia and Marmara regions.

Many of the local and international table grape and wine grape varieties are grown, especially in Thrace, Central Anatolia, Meditarrenean, Aegean and Eastern Anatolia regions such as 'Cabarnet Sauvignon', 'Merlot', 'Syrah', 'Malbec', 'Cabarnet Franc', 'Pinot noir', 'Chardonnay', 'Sauvignon blanc' and some famous local varieties such as 'Kalecik Karası', 'Öküzgözü', 'Boğazkere', 'Çal Karasi', 'Bornova Misketi', 'Köhnü', 'Sultani Çekirdeksiz' and 'Yalova Incisi'.

Grapevine is affected by several plant pathogens, including phytoplasma-associated diseases worldwide. Plant pathogenic phytoplasmas are insect-transmitted, wall-less, unculturable phloem-limited bacteria of the class Mollicutes with a small genome size, which ranges from 530 to 1350 kb. In diseased plants, they reside almost exclusively in the phloem sieve tube elements, to which they are introduced by phloem feeding hemipteran insects such as plant- and leafhoppers (Weintraub and Beanland, 2006; Maixner, 2011).

Bois noir (BN) and Flavescense dorée (FD), which are known as Grapevine yellows phytoplasmas (GY), have similar symptoms such as severe redness of the leaves and irregular yellowing of white varieties, backward curling of leaves, lack of lignification and shrivelling of berries, followed by early drying of whole clusters on infected plants (Kuzmanovic et al., 2008; Martini et al., 1999; Martini et al., 2002).

Both cause significant reductions in yields of many European countries and our neighbors Iran, Bulgaria and Syria (Angelini et al., 2001; Avramoz et al., 2008; Karimi et al., 2009; Contaldo et al. 2011). BN is induced by '*Candidatus* (Ca.) Phytoplasma solani', a member of the stolbur group (16SrXII-A group). The planthopper *Hyalestes obsoletus* (Cixiidae), considered as the main vector of BN, is a polyphagous vector that occasionally and erroriously feeds on grapevine (Alma et al., 2002). Recently, severe redness and inward curling of foliage were observed in the wine vineyards, therefore intensive surveys were conducted in the main viticultural areas of Turkey to verify phytoplasma presence and identity.

MATERIALS AND METHODS

PHYTOPLASMA SOURCE

Periodical surveys were conducted in main viticultural areas located in Thrace (Edirne, Kirklareli, Tekirdağ), Aegean region (İzmir, Manisa and Denizli), Central Anatolia (Ankara, Nevşehir) and Eastern Anatolia region (Elazig, Malatya, Diyarbakir and Mardin) of the Anatolian Peninsula from July to September in the years 2009 and 2010 and leaf samples were collected from symptomatic and asymptomatic plants. Survey area is shown in Figure 1.

NUCLEIC ACID EXTRACTION FROM INFECTED LEAVES

Nucleic acids (DNA) were extracted from the midribs and side ribs of the grapevine leaves according to the chloroform/phenol protocol of Prince et al. (1993).

PCR AND NESTED PCR

Direct PCR with the ribosomal P1/P7 universal primer pair (Deng and Hiruki, 1991; Schneider et al., 1995), followed by nested PCR with R16F2n/R2 universal primer pair (Gundersen and Lee, 1996) and R16 (1) F1/R1, R16 (V) F1/R1 (Lee et al., 1994) and also M1/M2 primer pairs were carried out to all of the extracted DNAs from grapevine leaves. PCR mixture for direct PCR was: 2,5 µl 10X PCR buffer, 250 µM dNTP mix,0,3 µM forward primer, 0,3 µM reverse primer, 1 µl target DNA, 0,16U Taq DNA polymerase and 17,875 µl water. Target PCR products were subjected to 1.5 % agarose gel electrophoresis and the gels were stained with ethidium bromide.

PCR cycling conditions for direct and nested PCR were the same and applied as 35 cycles as: 1 min (2 min for the first cycle) denaturation step at 94 °C, 2 min for annealing at 55 °C, 3 min at 72 °C for primer extension. Positive controls of both GY phytoplasmas and pear decline phytoplasma (PD) were supplied by Prof. Assunta Bertaccini (Bologna University, Italy).

PCR-RFLP

Amplified products of nested PCR analysis were further characterized by RFLP analysis with the restriction endonuclease enzyme TruI.

RESULTS

SURVEY RESULTS

During the surveys conducted in main viticultural areas in the years 2009 and 2010 (in 2009 159, in 2010 130) a total of 289 plant samples bearing the symptoms typical to phytoplasma-associated infections was collected. Main symptoms detected on infected vines were severe redness and inward curling of the mature leaves which were also typical for grapevine leaf-roll diseases. Phytoplasma-associated infections were present in all of the surveyed provinces, but the infection was present almost in all of the wine-grape vineyards located in Marmara and Aegean Region of Turkey except Denizli, comparing to the vineyards of South-Eastern Anatolian region. There was no infection in vineyards located in Diyarbakir and Mardin except in the collection vineyard of Dicle University located in Diyarbakır.

Phytoplasma-associated symptoms were detected on some of the local table-grape varieties such as: 'Trakya İlkeren' (Ankara), 'Bogazkere' (Manisa), 'Şirfani' (Elazığ), 'Tahannebi' (Malatya), 'Emir' (Nevşehir) and wine-grape varieties such as: 'Alphonse Lavallee' (İzmir), 'Alicante Bouschet' (İzmir, Manisa, Tekirdağ), 'Chardonnay' (Manisa), 'Shiraz' (Manisa, Çanakkale), 'Cabernet Sauvignon' (Kirklareli, Çanakkale), 'Merlot' (Kırklareli), 'Sauvignon blanc' (Nevşehir) and 'Pinot noir' (Tekirdağ).

PCR – NESTED PCR RESULTS

According to the results of PCR with universal primers and nested PCR with group specific primers,

following enzymatic digestion with TruI enzyme, 53 out of 289 grapevine samples were detected as infected with one of the grapevine phytoplasmas. The detection rate of the phytoplasma infected plant samples was 18.6 %, 49 of the collected samples were detected as infected with Bois Noir phytoplasma (16SrXII-A stolbur group) ('*Candidatus* Phytoplasma solani') (Fig. 2), 2 were detected as group V phytoplasma, one sample was detected as Aster yellows phytoplasma (16SrI group) ('*Candidatus* Phytoplasma asteris') and one sample was infected with Pigeon pea witches broom phytoplasma (16SrIX group) '*Candidatus* Phytoplasma phoenicium' (Canik et al., 2011).

The incidence of grapevine phytoplasma infections in Turkish vineyards was quite low. BN infected samples originated from Ankara (2), İzmir (6), Manisa (6), Çanakkale (2), Tekirdağ (8) Kırklareli (11), Edirne (4), Elazıg (3), Diyarbakır (3), Malatya (1), Nevşehir (3). The phytoplasma was present mostly on wine-grape varieties of imported origin. It was detected from wine-grape varieties such as 'Alphonse Lavallee', 'Chardonnay', 'Alicante Bouschet', 'Shiraz', 'Cabernet Sauvignon', 'Sauvignon blanc', 'Merlot', 'Malbec' and 'Pinot noir' vineyards and it was also detected from local varieties, but in smaller extent: 'Bogazkere' (Manisa), 'Tahannebi' (Malatya), 'Emir' (Nevşehir), 'Papaz karasi' and 'Gamay' (Tekirdağ). None of the detected phytoplasmas was present in Denizli and Mardin provinces. Nineteen of the selected BN amplicons, amplified with R16(I) F1/R1, M1/B6 and M1/M2 primers were sequenced and deposited in genebank NCBI.

DISCUSSION

Epidemics caused by phytoplasma diseases in grapevine have become the major problem in recent years because of their continuous spreading throughout many grape producing regions in different European countries. Phytoplasma diseases are widespread and occur all over the world, besides Europe they

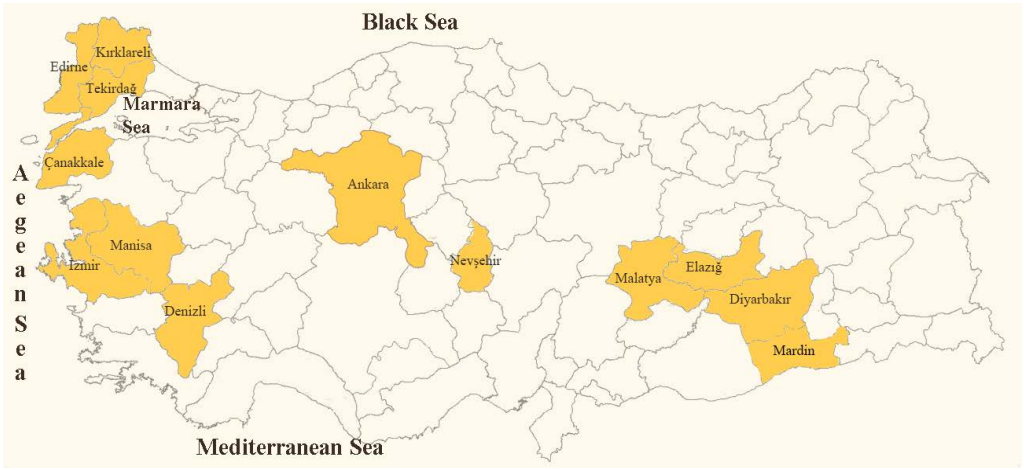

Fig. 1: The survey area

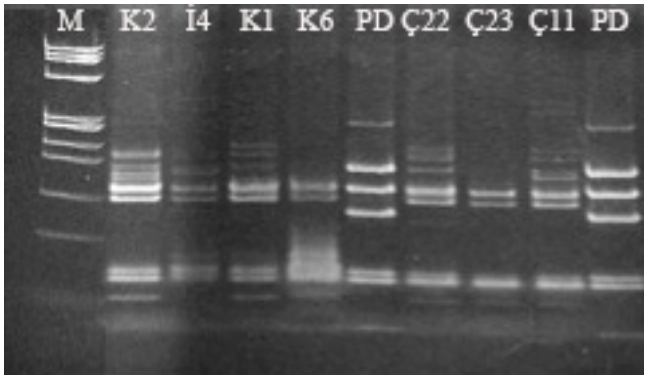

Fig. 2: Restriction profiles of BN infected isolates with *Tru*I enzyme (K 1, 2 and 6: Kirklareli isolates; I4: İzmir isolate; Ç11, 22 and 23: Çanakkale isolates, PD: pear decline phytoplasma used as phytoplasma control

are also present in Ukraine, Iran, Syria, Lebanon, Israel, South Africa and China (Maixner, 2011). Phytoplasmas belonging to the 4 molecularly distinguishable groups (16Sr I, 16Sr V, 16Sr IX and 16Sr XII) were detected on grapevine in Turkey (Ertunc et al., 2015). The other phytoplasmas reported from different locations, belonging to the groups 16Sr II, 16SrIII, 16Sr VII and 16Sr X were not present in the vineyards of Turkey.

It has been clearly stated that transmission of grapevine phytoplasmas occurs through the use of phytoplasma infected multiplication material and vector insects. The main phytoplasma infections present in the vineyards of Turkey were Grapevine yellows phytoplasmas. Widespread phytoplasma infection was determined as BN in Turkey, it has been detected from most of the provinces except Denizli and Mardin. It has been vectored by *Hyalestes obsoletus* which is a polyphagous insect occasionally feeding on grapevine. BN phytoplasma is transmitted to grapevine from several plants which can host both insect and phytoplasma, especially *Urtica dioica* and *Convolvulus arvensis* (Langer and Maixner, 2004). The vector *Hyalestes obsoletus* is present in our country and reported from the potato fields in Erzurum plateau (Guclu and Ozbek, 1988) and rarely from vineyards. Some putative vectors of BN, *Euscelis lineolatus* Brullé and *Dictyophara europaea* (L.) (Lessio and Alma, 2008) were also present in Turkey in low densities. All the confirmed and putative vectors, however, were phytoplasma negative in PCR and nested PCR assays (Bayram et al., 2014).

The incidence of symptomatic vines were higher in the vineyards located in Thrace, Aegean region comparing to Central and Eastern Anatolia regions where local table grape varieties are usually grown and there is also no introduction of new imported grape varieties to this regions.

ACKNOWLEDGEMENTS

The authors are grateful to the Turkish Scientific and Technological Research Council for supporting this research by Project coded COST-109O005 and also COST-FA 0807.

REFERENCES

Angelini, E., Clair, D., Borgo, M., Bertaccini, A. and Boudon-Padieu, E. 2001: Flavescence dorée in France and Italy-occurence of closely related phytoplasma isolates and their near relationships to Palatinate Grapevine Yellows and Alder Phytoplasma. Vitis 40: 79-86

Alma, A., Soldi, G., Tedeschi, R. and Marzachi, C. 2002: Role of *Hyalestes obsoletus* Signoret in transmission of grapevine Bois Noir in Italy. Petria 12: 411-412

Avramoz, Z., Gillet, J. and Laginova, M. 2008: First Detection of Stolbur phytoplasma in Grapevines (*Vitis vinifera* cv Merlot) affected witrh Grapevine Yellows in Bulgaria. J. Phytopathol., 56 (2):112-114 .

Bayram, S., Zeybekoglu, U., Soylemezoglu, G., Canik, D., Karavin, M., Cakir, A. and Ertunc, F. 2014: Putative vectors of Grapevine Yellows Phytoplasmas in Turkey. Phytopathogenic mollicutes 4(1): 22-26

Canik, D., Ertunc, F., Paltrinieri, S., Contaldo, N. and Bertaccini A. 2011: Identification of different phytoplasmas infecting grapevine in Turkey. Bull. Insectology 64: 225-226.

Contaldo, N., Duduk, B., Paltrinieri, S., Dal Molin, F., Mitrovic, J. and Bertaccini A. 2011: Molecular variability of 16 Sr DNA of Bois noir phytoplasmas in grapevine from Italy and Serbia. Petria 21: 136-137.

Deng, S. and Huruki, C. 1991: Amplifications of 16S rRNA genes from culturable and non-culturable mollicutes. Microbiological Methods 14: 53-6

Ertunc, F., Canikorel, D., Bayram, S., Paltrinieri, S., Bertaccini, A., Topkaya, S. and Soylemezoglu, G. 2015: Occurrence and identification of grapevine phytoplasmas in main viticultural regions of Turkey.Phytoparasitica 43:303-310.

Güçlü, S. and Özbek, H. 1988: Some studies on the biology of *Hyalestes obsoletus* Signoret (Homoptera: Cixiidae)in the conditions of Erzurum. Türkiye Entomoloji Dergisi 12:103-111

Gundersen, D. E. and Lee, I-M. 1996: Ultrasensitive detection of phytoplasmas by nested PCR assays using two universal primer pairs. Phytopathologia Mediterranea 35: 144-151

Karimi, M, Contaldo, N., Mahmodi, B., Duduk, B. and Bertaccini, A. 2009: Identification of stolbur related phytoplasma in grapevine showing decline symptoms in Iran. Proceedings of the 16 th meeting of the ICVG, Dijon, August 31– September 4, 2009: 208-209.

Kuzmanovic S., Martini M., Ermacora P., Ferini F., Starovic M. , Tosic M., Carraro L., and Osler R. 2008: Incidence and molecular characterization of Flavescence doree in grapevine cultivars in different viticultural areas in Serbia. Vitis 47: 105-111.

Lee I-M., Gundersen, D. E., Hammond, R. W and Davis, R.E. 1994: Use of Mycoplasma Like Organism (MLO) group-specific oligonucleotide primers for nested PCR assays to detect mixed –MLO infections in a single host plants. Phytopathology 84: 559-566

Lessio, F. and Alma, A. 2008: Host plants and seasonal presence of *Dictyophara europaea* in the vineyard agro-ecosystem. Bull. Insectology 61 (1): 199-200

Maixner, M. 2011: Recent advances in Bois Noir research. Petria 21: 95-108.

Martini, M., Murari, E., Mori, N. and Bertaccini, A. 1999: Identification and epidemic distribution of two Flavescence Doree related phytoplasmas in Veneto (Italy). Plant Disease 83: 925-930

Martini, M., Botti, S., Marcone, C., Marzachi, C., Casati, P., Bianco, P.A., Benedetti, R. and Bertaccini, A .2002: Genetic variability among Flavescence doree phytoplasmas from different origins in Italy and France. Molecular and Cellular Probes 16: 197-208

Prince, J. P., Davis, R. E., Wolf, T. K., Lee, I-M., Mogen, B. D., Dail,Y. E. L., Bertaccini, A., Credi, R. and Barba M. 1993: Molecular detection of diverse Mycoplasmalike Organisms associated with Grapevine Yellows and their classification wİth Aster Yellows, X-Disease and Elm Yellows MLOs. Phytopathology 83: 1130-1137

Schneider, B., Seemüller, E., Smart, C. D. and Kirkpatrick. B. C. 1995: Phyllogenetic Classification of Plant Pathogenic Mycoplasmalike Organisms or Phytoplasmas. In Molecular and Diagnostİc Procedures in Mycoplasmology (Eds. S. Razin and J.G. Tully), Academic Press, New York, pp. 369-380

Soylemezoglu, G., Kunter, B., Akkurt, M., Sağlam, M., Ünal, A., Buzrul, S. and Tahmaz, H. 2015: Bagcılıgın geliştirilmesi yöntemleri ve üretim hedefleri. Türkiye Ziraat Müh. 8.Teknik Kongresi, 606-629.

Weintraub, P. G. and Beanland, L. 2006: Insect vectors of phytoplasmas. Ann. Rev. Entomol. 51: 91-111

ELUCIDATION OF '*CANDIDATUS* PHYTOPLASMA SOLANI' EPIDEMIOLOGY THROUGH TRAC(K)ING TRANSMISSION PATHWAYS USING FIELD, EXPERIMENTAL AND MOLECULAR DATA

Andrea Kosovac[1], Oliver Krstić[1], Miljana Jakovljević[1], Tatjana Cvrković[1], Milana Mitrović[1], Ivo Toševski [1,2]and Jelena Jović[1]

[1] Institute for Plant Protection and Environment, Department of Plant Pests
RS-11080 Zemun, Banatska 33
[2] CABI Europe – Switzerland
CH-2800 Delémont, 1 Rue des Grillons
E-Mail: jovic_biolab@yahoo.com

Bois noir (BN) induced by '*Candidatus* Phytoplasma solani' (CPs), previously referred to as stolbur phytoplasma (Quaglino et al., 2013), is emerging, widespread and economically the most important grapevine yellows disease in Europe. Due to the obligate vector-based transmission of CPs and the case that grapevine is a terminal dead-end host, BN epidemiology is imminently linked to pathogen reservoir plants simultaneously hosting insect vector populations. Additionally, routes of pathogen transmission and dissemination are fundamentally determined by biology, life strategy and feeding preference of vector species. This sets insect vectors and their preferred host plants in the center of epidemiological routes of transmission which could be direct, intermixed, or intermediated through other vectors and their unique or shared transmission pathways (Cvrković et al., 2014; Kosovac et al., 2016).

Cixiid planthoppers (Hemiptera: Cixiidae) are the main vectors of CPs in diverse agroecosystems including vineyards. Hitherto, three cixiid species are proven to be vectors of CPs, namely *Hyalesthes obsoletus, Pentastiridius leporinus* and *Reptalus panzeri* (Maixner, 1994; Sforza et al., 1998; Gatineau et al., 2001; Jović et al., 2007; Bressan et al., 2009), while *Reptalus quinquecostatus* is proven as experimental vector of CPs to artificial feeding medium (Pinzauti et al., 2008) and currently is treated as possible or intermediate vector to grapevine and/or other crops (Cvrković et al., 2014; Mitrović et al., 2016). Among these, *H. obsoletus* and *R. panzeri* are vectors with proven role in BN epidemiology (Maixner, 1994; Sforza et al., 1998; Cvrković et al., 2014), albeit not fully understood due to the complex interactions with environment and diverse host plants acting as pathogen reservoirs.

H. obsoletus, the main and most widespread vector of BN is associated with at least four host plants acting as pathogen inoculum sources in European vineyards: *Convolvulus arvensis, Urtica dioica, Vitex agnus-castus* and *Crepis foetida* (Langer and Maixner, 2004; Kosovac et al., 2013; Kosovac et al., 2016). Host plants of *R. panzeri* and *R. quinquecostatus* are not well-documented especially in regard to their role as pathogen sources. *Sorghum halepense* is evidenced dual host for *R. panzeri* nymphs and CPs in maize redness affected fields in northeastern Serbia (Jović et al., 2009), and tentative inoculum source in vineyards (Cvrković et al., 2014).

How-ever this cixiid is also known as being associated with diverse herbaceous and woody plants such as *Prunus spp., Ulmus spp., Clematis spp., Rosa spp.,* etc. (Nickel, 2003) and situation is similar for *R. quinquecostatus.*

Molecular tracing and tracking CPs routes of transmission in BN affected vineyards helps us to understand complexity of epidemiological cycles or networks, enables prediction of future spread and design of management strategies (Murolo and Romanazzi, 2015; Kosovac et al., 2016). Given that several CPs genotypes, determined based on variable membrane proteins tentatively involved in host plant and/or insect vector recognition and adaptation, are shared by diverse vectors (Aryan et al., 2014; Cvrković et al., 2014; Kosovac et al., 2016) we found that an approach combining field, experimental and molecular data is required to explain transmission pathways and their interconnections. Hence, we attempted to elucidate transmission pathways and epidemiological networks of CPs genotypes associated with so-called *type-b* epidemiology (originally tuf-b; Langer and Maixner, 2004) in Southeastern Europe involving three populations of *H. obsoletus* associated with unique host plants (*C. arvensis, V. agnus-castus* and *C. foetida)* and *R. panzeri associated* with *S. halepense*. The study involved field sampling vectors from their original host plants, genotyping CPs isolates carried by each of the vector population and experimental cross-transmission of CPs between each of the four vector populations and their source/host plant. Results enabled us to conclude that most of the CPs transmission routes are intersected forming complex epidemiological networks comprising several independent epidemiological pathways which can be intermixed and thus solely molecular genotyping cannot give us unambiguous identification of source plant and insect vector.

ACKNOWLEDGEMENTS

This study was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia (III43001) and partly by the SCOPES programme of the Swiss National Science Foundation (IZ73Z0_152414).

REFERENCES

Aryan, A., Brader, G., Mörtel, J., Pastar, M., and Riedle-Bauer, M. 2014: An abundant '*Candidatus* Phytoplasma solani' tuf b strain is associated with grapevine, stinging nettle and *Hyalesthes obsoletus.* European Journal of Plant Pathology, 140: 213–227.

Bressan, A., Holzinger, W.E., Nusillard, B., Semetey, O., Gatineau, F., Simonato, M., and Boudon-Padieu, E. 2009: Identification and biological traits of a planthopper from the genus Pentastiridius (Hemiptera: Cixiidae) adapted to an annual cropping rotation. European Journal of Entomology, 106: 405–413.

Cvrković, T., Jović, J., Mitrović, M., Krstić, O., and Toševski, I. 2014: Experimental and molecular evidence of *Reptalus panzeri* as a natural vector of bois noir. Plant Pathology, 63: 42–53.

Gatineau, F., Larrue, J., Clair, D., Lorton, F., Richard-Molard, M., and Boudon-Padieu, E. 2001: A new natural planthopper vector of stolbur phytoplasma in the genus *Pentastiridius* (Hemiptera: Cixiidae). European Journal of Plant Pathology, 107: 263–271.

Jović, J., Cvrković, T., Mitrović, M., Krnjajić, S., Redinbaugh, M.G., Pratt, R.C., Gingery, R.E., Hogenhout, S.A., and Toševski, I. 2007: Roles of stolbur phytoplasma and *Reptalus panzeri* (Cixiinae, Auchenorrhyncha) in the epidemiology of Maize redness in Serbia. European Journal of Plant Pathology, 118: 85-89.

Jović, J., Cvrković, T., Mitrović, M., Krnjajić, S., Petrović, A., Redinbaugh, M. G., Pratt, R. C., Hogenhout, S. A., and Toševski, I. 2009: Stolbur phytoplasma transmission to maize by *Reptalus panzeri* and the disease cycle of maize redness in Serbia. Phytopathology, 99: 1053–1061.

Kosovac, A., Johannesen, J., Krstić, O., Mitrović, M., Cvrković, T., Maixner, M., Toševski, I., and Jović, J. 2013: Microsatellite and mtDNA evidence of genetic differentiation in *Hyalesthes obsoletus* populations associated with a new major host, stinking hawk's-beard (*Crepis foetida*), in southeast Europe. Proceedings of the 3rd European Bois Noir Workshop, Barcelona, 18-19.

Kosovac, A., Radonjić, S., Hrnčić, S., Krstić, O., Toševski, I., and Jović, J. 2016: Molecular tracing of the transmission routes of bois noir in Mediterranean vineyards of Montenegro and experimental evidence for the epidemiological role of *Vitex agnus-castus* (Lamiaceae) and associated *Hyalesthes obsoletus* (Cixiidae). Plant Pathology, 65: 285-298.

Langer, M. and Maixner, M. 2004: Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur group based on RFLP-analysis of non-ribosomal DNA. Vitis, 43: 191–199.

Maixner, M. 1994. Transmission of German grapevine yellows (Vergilbungskrankheit) by the planthopper *Hyalesthes obsoletus* (Auchenorrhyncha: Cixiidae). Vitis, 33: 103–104.

Mitrović, M., Jakovljević, M., Jović, J., Krstić, O., Kosovac, A., Trivellone, V., Jermini, M., Toševski, I., and Cvrković, T. 2016: *'Candidatus* Phytoplasma solani' genotypes associated with potato stolbur in Serbia and the role of *Hyalesthes obsoletus* and *Reptalus panzeri* (Hemiptera, Cixiidae) as natural vector. European Journal of Plant Pathology, DOI: 10.1007/s10658-015-0800-y.

Murolo, S. and Romanazzi, G. 2015: In-vineyard population structure of '*Candidatus* Phytoplasma solani'using multilocus sequence typing analysis. Infection, Genetics and Evolution, 31: 221-230.

Nickel, H. 2003: The Leafhoppers and Planthoppers of Germany (Hemiptera, Auchenorrhyncha): Patterns and strategies in a highly diverse group of phytophagous insects. Pensoft Publishers.

Pinzauti, F., Trivellone, V., and Bagnoli, B. 2008: Ability of *Reptalus quinquecostatus* (Hemiptera: Cixiidae) to inoculate stolbur phytoplasma to artificial feeding medium. Annals of Applied Biology, 153: 299–305.

Quaglino, F., Zhao, Y., Casati, P., Bulgari, D., Bianco, P.A., Wei, W., and Davis, R.E. 2013: '*Candidatus* Phytoplasma solani', a novel taxon associated with stolbur- and bois noir-related diseases of plants. International Journal of Systematic and Evolutionary Microbiology, 63: 2879–2894.

Sforza, R., Clair, D., Daire, X., Larrue, J., and Boudon-Padieu, E. 1998: The role of *Hyalesthes obsoletus* (Hemiptera: Cixiidae) in the occurrence of bois noir of grapevines in France. Journal of Phytopathology, 146: 549–556.

ANALYSIS OF BOIS NOIR EPIDEMIOLOGY IN AUSTRIAN VINEYARDS BY MOLECULAR CHARACTERIZATION OF '*CANDIDATUS* PHYTOPLASMA SOLANI' STRAINS

Monika Riedle-Bauer[1], Günter Brader[2] and Robert Hack[1]

[1] Höhere Bundeslehranstalt und Bundesamt für Wein- und Obstbau Klosterneuburg
A-3400 Klosterneuburg, Wienerstraße 74
[2] Austrian Institute of Technology, Health and Environment Department, Bioresources Unit
A-3430 Tulln, Konrad-Lorenz-Straße 24

E-Mail: Monika.Riedle-Bauer@weinobst.at
guenter.brader@ait.ac.at

The aim of the present study was to track the spread of Bois noir induced by '*Candidatus* Phytoplasma solani' in Austrian vineyards. *Vitis vinifera, Hyalesthes obsoletus* and *Reptalus cuspidatus* samples were collected at 7 locations in Lower Austria, Styria and Burgenland. The samples were investigated by PCR/RFLP analysis of the marker genes *tuf* and *sec*Y. The results point out that currently the spread of Bois noir happens mostly via nettle associated disease cycles. The vast majority of the detected phytoplasma strains belonged to the newly emerged genotype CPs4M_At1, a nettle associated strain with a tuf b restriction profile. In addition, in Styria "classical" tuf a nettle strains were ascertained in increasing numbers. In contrast, the importance of bindweed associated types seems to decline steadily.

Bois noir (BN) associated with *'Candidatus* Phytoplasma solani' (Stolbur) is regularly found in all Austrian vine growing regions. Disease spread occurs via a cycle including insect vectors and herbaceous hosts, predominantly *Urtica dioica* and *Convolvulus arvensis*, as phytoplasma reservoirs. The pathogen is transmitted by Auchenorrhyncha species. The planthopper *Hyalesthes obsoletus* (Cixiidae) is regarded as main vector in many countries (Maixner et al., 1995; Maixner, 2011). Recently it has been demonstrated that also the Cixiidae *Reptalus panzeri* can transmit BN to grapevine seedlings in South Eastern Europe (Cvrković et al., 2014). Transmission experiments with several Cicadellidae (Hemiptera, Auchenorrhyncha) species have revealed that the leafhopper *Anaceratagallia ribauti* transmits stolbur phytoplasmas to *Vicia faba* (Riedle-Bauer et al., 2008) and *Catharanthus roseus* (Aryan et al., 2014). Up to now, however, the transmission to grapevine has not been proven (Riedle-Bauer, unpublished).

'*Ca*. Phytoplasma solani' strains involved in disease development can be linked to different natural epidemic cycles (nettle and bindweed associated cycles) by analysis of molecular markers. Analysis of the elongation factor *Tu* (*tuf*) gene allows the discrimination into tuf a strains, which are commonly nettle associated and tuf b strains, which are in most cases bindweed associated (Langer and Maixner, 2004; Johannesen et al., 2012). In addition other genes e. g. *sec*Y, *vmp*1 and *stamp* were used for a more fine-tuned characterisation of the phytoplasma strains (Cimerman et al., 2009; Fabre et al., 2011; Pacifico et al., 2009).

BN is a disease characterized by sudden outbreaks and subsequent decreases (Maixner, 2011). This estimation also holds true for the situation in Austria, where dramatic changes in BN epidemiology have been recorded over the past decade. Between 2003 and 2008 almost all BN infections were ascribable to bindweed associated phytoplasma strains. In most parts of the country *H. obsoletus* was rare or not detectable. Only in Southern Styria the insect species was regularly ascertained. The insects were solely collected on *C. arvensis, U. dioica* was never found colonised (Riedle-Bauer et al., 2006; Riedle-Bauer et al., 2008; Tiefenbrunner et al., 2007; Sára and Riedle-Bauer, 2009). In contrast, investigations in 2012 and 2013 revealed a sudden and

unexpected mass occurrence of *H. obsoletus* almost exclusively on stinging nettle. The high population densities of *H. obsoletus* on *Urtica dioica* were accompanied by frequent '*Ca*. Phytoplasma solani' infections of nettles and planthoppers. Phytoplasma sequence analysis of the molecular markers *sec*Y, *stamp*, *tuf* and *vmp*1 revealed a single genotype named CPsM4_At1 in stinging nettles and more than 64 % and 90 % abundance in grapevine and *H. obsoletus*, respectively. Interestingly, this genotype showed tuf b type restriction patterns previously attributed to bindweed associated stolbur strains, but a different sequence assigned as tuf b2 compared to reference tuf b strains and was therefore named Tuf b2. All other marker genes of CPsM4_At1 clustered with nettle derived genotypes. Recently the presence of this nettle associated, but tuf b stolbur strain was also reported from Croatia, Macedonia and Montenegro (Plavec et al., 2015; Atanasova et al., 2015; Kosovac et al., 2016).

Analyses of the Austrian *H. obsoletus* populations by genetic markers revealed two regional populations. The populations in Northeastern and Eastern Austria were related to Pannonian populations, while the population from Southeastern Austria was assigned to the local Eastern Adriatic population. The two vector populations were linked by the exchange of the specific phytoplasma strain mentioned above (Johannesen and Riedle-Bauer, 2014).

The aim of the present study was to track the further spread of the newly emerged genotype CPs4M_At1, to characterise additional '*Ca.* Phytoplasma solani' strains present in and around vineyards and to collect updated data on the epidemiology of BN in Austria.

MATERIAL AND METHODS

All plant and insect samples included in the present study were taken between July and November 2015. Sampling was carried out in7 vineyards and their surroundings in Eastern Austria (in the parts of the country, where grapevines are cultivated) (Table 1). *H. obsoletus* was sampled at all locations, visually infected *Vitis vinifera* at all locations except Kleinschweinbarth, *Reptalus cuspidatus* was sampled in Einöd only. Insects were collected by vacuum sampling directly from *Urtica dioica* and *Convolvulus arvensis* using a modified garden blower-vac (Stihl, Dieburg, Germany). DNA extraction from plants and insects was carried out as published earlier (Maixner et al., 1995; Langer and Maixner, 2004). Plant and insect samples were analyzed by nested PCR procedures with primers P1/P7 (Deng and Hiruki, 1991) and STOLF/STOLR (Maixner et al., 1995), with fTUF1/rTUF1 and fTUFAY/rTUFAY (*tuf*; Schneider et al., 1997) as well as with POSecR1/POSecF1 and POSecF3/POSecR3 (*sec*Y; Fialová et al., 2009). TUFAY fragments were further investigated by RFLP with HpaII (Promega, Mannheim, Germany) as proposed by Langer and Maixner (2004). POSec3 amplicons were characterized by RFLP with *Hinf*I (New England Biolabs, Ipswich, USA) according to the manufacturer's instructions. *Hinf*I RFLP of POSec3 amplicons yields two different restriction profiles. The pattern secY 1 corresponds to classical bindweed associated tuf b1 types, secY 2 corresponds to nettle associated tuf a and tuf b2 types (Aryan et al., 2014).

Table 1: Sampling locations

Location	Federal Province
Falkenstein	Lower Austria
Hagenbrunn	Lower Austria
Kleinschweinbarth	Lower Austria
Klosterneuburg	Lower Austria
Langenzersdorf	Lower Austria
Einöd/Kitzeck	Styria
Rust	Burgenland

RESULTS AND DISCUSSION

Insect sampling was carried out in Einöd/Kitzeck, Rust, Falkenstein and Kleinschweinbarth. In total 197 *H. obsoletus* individuals collected in Einöd/Kitzeck, Rust and Falkenstein were analyzed. In Kleinschweinbarth, however, the *H. obsoletus* population was not detectable anymore. The overall phytoplasma infection rate of the analyzed *H. obsoletus* was 43.2 %. This is considerably higher than in 2012 and in 2013 at the same locations (25.9 and 27 %, respectively). A prominent increase of the infection rate was observed in Falkenstein (from 0 % in 2012 to 12.5 % in 2015). In Rust the infection rate had already been at a high level in 2012 and in 2013 (51 % and 20 %, respectively), in 2015 it reached 60 %. In Einöd, the infection rate stagnated at a high level of 29 % (31.3 % in 2013, 21 % in 2012) (Fig. 1). None of the 5 *Reptalus cuspidatus* collected in Einöd was tested positive.

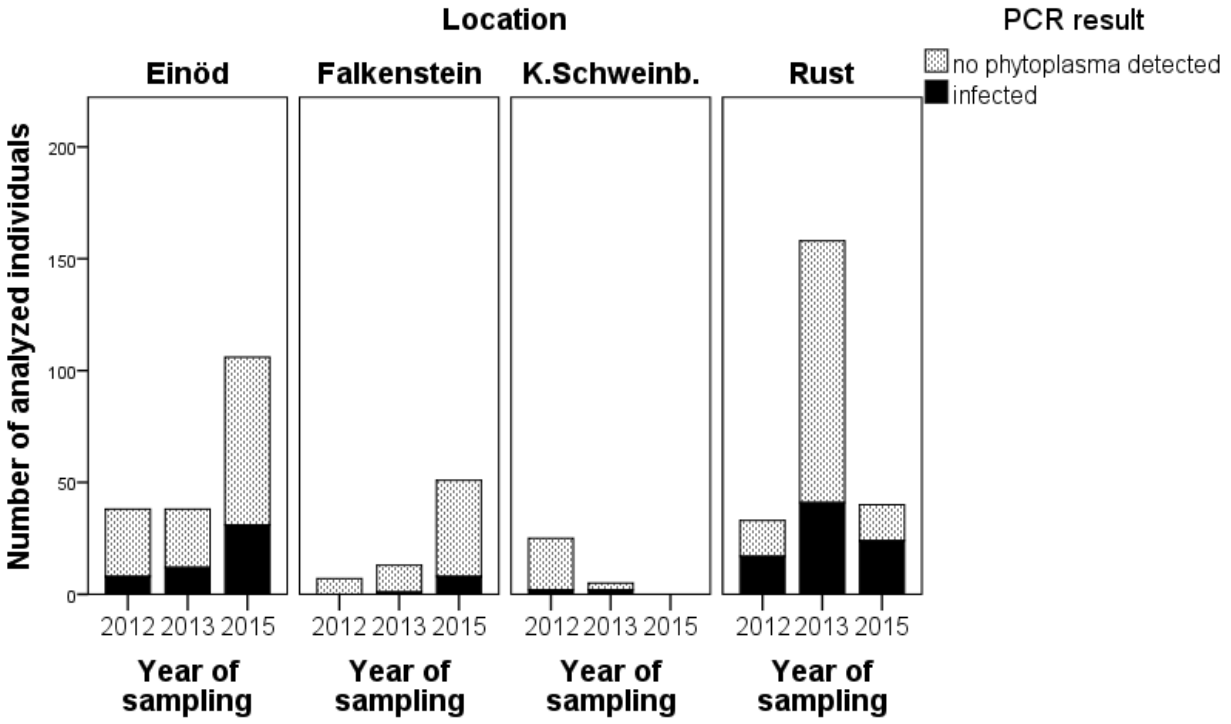

Fig. 1: Infection rates of *H. obsoletus* at selected sampling locations between 2012 and 2015

The majority of stolbur isolates from *V. vinifera* (85 %) and *H. obsoletus* (75 %) in 2015 was infected by a phytoplasma type characterized by a *Hinf*I *sec*Y 2 pattern of the *sec*Y gene and a *tuf* b restriction profile of the *tuf* gene. During our previous analyses (Aryan et al., 2014) the prevalent '*Ca*. Phytoplasma solani' type CPs4M_At1was characterized by an identical combination of restriction profiles. Consequently, we presume that the strains detected this year also belong to the type CPs4M_At1.
In 2015 all infected insects in Falkenstein and in Rust carried CPs4M_At1. In Einöd, in contrast, 52.9 % were infected by a tuf type a phytoplasma strain. Compared to our previous data we can recognise a prominent increase of the classical tuf a associated nettle type in Einöd and a disappearance of bindweed associated types at all locations (Fig. 2).

Figure 3 illustrates the molecular characterization of stolbur types present in *V. vinifera* in 2012 and in 2015. In 2015 the vast majority of symptomatic grapevines was infected with the type CPs4M_At1. This is also the case at locations, where bindweed associated types were common in 2012 (e. g. Falkenstein, Klosterneuburg).

All in all our data prove a considerable spread of the stolbur type CPs4M_At1 in Austria over the last years. In Einöd in addition an increase of tuf a type phytoplasmas in *H. obsoletus* has been noticed. Up to now this type has not been ascertained in grapevines. In the present study no analyses of nettles have been carried out, in 2012 and 2013 no tuf type a phytoplasma strains were found in this host species. In order to track the spread of phytoplasma strains in this area, however, up to date characterisation of the infections of *U. dioica* and of additional marker genes such as *vmp*1 and *stamp* should be performed as soon as possible. In any case the current spread of Bois noir in Austria almost exclusively proceeds via a disease cycle including *H. obsoletus* and nettle. Bindweed associated types in contrast are getting less and less important.

REFERENCES

Atanasova, B., Jakovljević, M., Spasov, D., Jović, J., Mitrović, M., Toševski, I., and Cvrković, T. 2015: The molecular epidemiology of bois noir grapevine yellows caused by 'Candidatus Phytoplasma solani' in the Republic of Macedonia. European Journal of Plant Pathology 142: 759-770

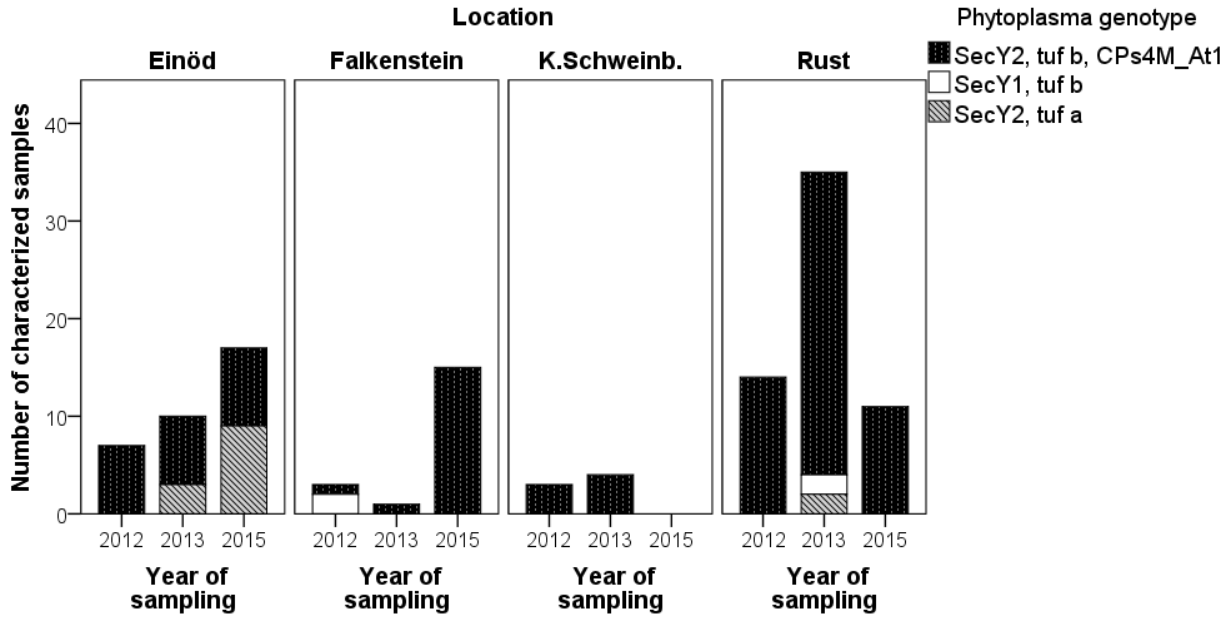

Fig. 2: Characterization of phytoplasma strains in *H. obsoletus*

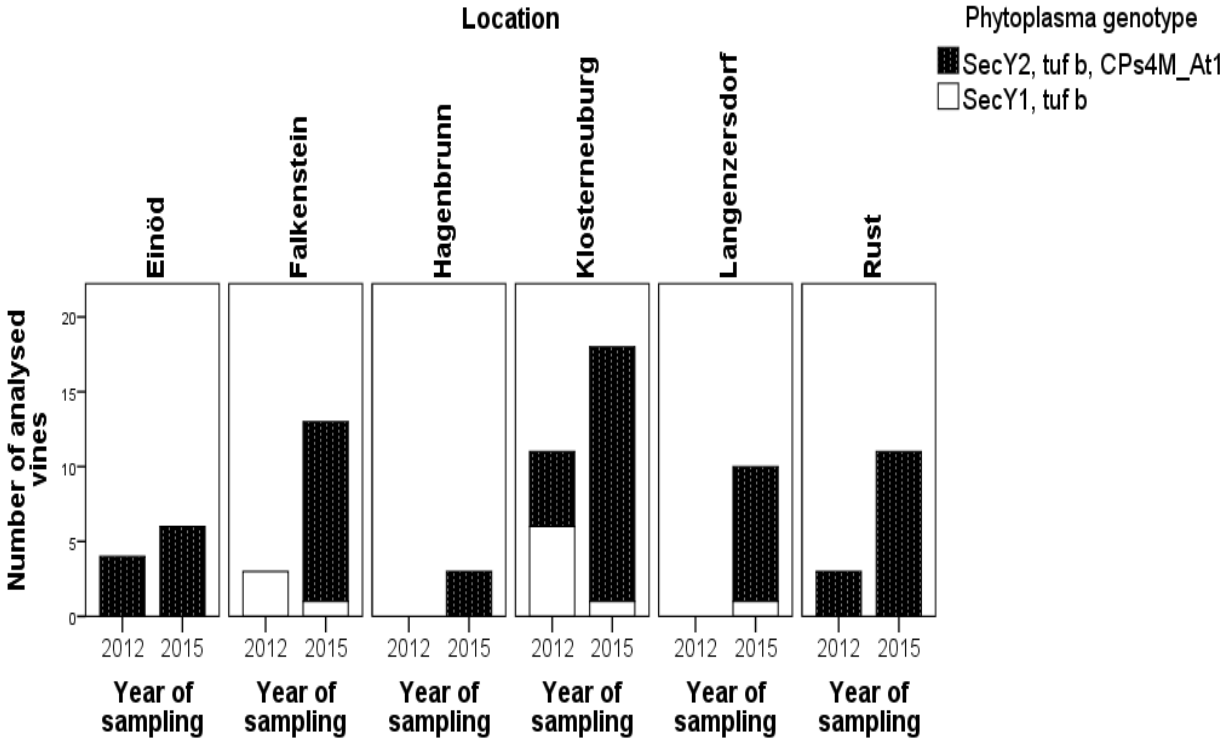

Fig. 3: Characterization of phytoplasma strains in *Vitis vinifera*

ARYAN, A., BRADER, G., MÖRTEL, J., PASTAR, M. AND RIEDLE-BAUER, M. 2014: An abundant '*Candidatus* Phytoplasma solani' Stolbur tuf b phytoplasma strain is associated with grapevine, stinging nettle and Hyalesthes obsoletus. European Journal of Plant Pathology 140: 213-227

CIMERMAN, A., PACIFICO, D., SALAR, P., MARZACHI, C. AND FOISSAC, X. 2009: Striking diversity of *vmp*1, a variable gene encoding a putative membrane protein of the Stolbur phytoplasma. Applied Environmental Microbiology 75: 2951-2957

Cvrković, T., Jović, J., Mitrović, M., Krstić, O. and Toševski, I. 2014: Experimental and molecular evidence of *Reptalus panzeri* as a natural vector of bois noir. Plant Pathology 63: 42-53

Deng, S. and Hiruki, D. 1991: Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. Journal of Microbiological Methods 14:53-61.

Fabre, A., Danet, J.-L. and Foissac, X. 2011: The stolbur phytoplasma antigenic membrane protein gene stamp is submitted to diversifying positive selection. Gene 472: 37-41

Fialová, R., Válová, P., Balakishiyeva, G., Danet, J.-L., Šafárová, D., Foissac, X., and Navrátil, M. 2009: Genetic variability of stolbur phytoplasma in annual crop and wild plant species in south Moravia. Journal of Plant Pathology 91: 411-416

Johannesen, J., Foissac, X., Kehrli, P. and Maixner, M. 2012: Impact of Vector Dispersal and Host-Plant Fidelity on the Dissemination of an Emerging Plant Pathogen. PLoS ONE 7: e51809

Johannesen, J. and Riedle-Bauer, M. 2014: Origin of a sudden mass occurrence of the stolbur phytoplasma vector *Hyalethes obsoletus* (Cixiidae) in Austria. Annals of Applied Biology 165: 488–495

Kosovac, A., Radonjić, S., Hrnčić, S., Krstić, O., Toševski, I. and Jović, J. 2016: Molecular tracing of the transmission routes of bois noir in Mediterranean vineyards of Montenegro and experimental evidence for the epidemiological role of *Vitex agnus-castus* (Lamiaceae) and associated *Hyalesthes obsoletus* (Cixiidae). Plant Pathology 65: 285-298

Langer, M. and Maixner, M. 2004: Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur group based on RFLP analysis of non-ribosomal DNA. Vitis 43: 191-199

Maixner, M., Ahrens, U. and Seemüller, E. 1995: Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. European Journal of Plant Pathology 101: 241-250

Maixner, M. 2011: Recent advances in Bois noir research. Petria 21: 17-32

Pacifico, D., Alma, A., Bagnoli, B., Foissac, X., Pasquini, G., Tessitori, M. and Marzachi, C., 2009: Characterization of Bois Noir Isolates by Restriction Fragment Length Polymorphism of a Stolbur-Specific Putative Membrane Protein Gene. Phytopathology 99: 711-715

Plavec, J., Križanac, Budinšćak, Ž., Škorić, D. and Šeruga Musić, M. 2015: A case study of FD and BN phytoplasma variability in Croatia: multigene sequence analysis approach. European Journal of Plant Pathology 142: 591-601

Riedle-Bauer, M., Tiefenbrunner, W., Otreba, J., Hanak, K., Schildberger, B. and Regner, F. 2006: Epidemiological observations on Bois Noir in Austrian vineyards. Mitteilungen Klosterneuburg 56: 177-181

Riedle-Bauer, M., Sára, A., and Regner, F. 2008: Transmission of a stolbur phytoplasma by the Agalliinae leafhopper *Anaceratagallia ribauti* (Hemiptera, Auchenorrhyncha, Cicadellidae). Journal of Phytopathology 156: 687-690

Sára, A., and Riedle-Bauer, M. 2009: Untersuchungen zur Zikadenfauna (Hemiptera, Auchenorrhyncha) zweier Weingärten nördlich von Wien. Linzer Biologische Beiträge 41: 1767-1792

Schneider, B., Gibb, K.S. and Seemüller, E. 1997: Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas. Microbiology 143: 3381-3389

Tiefenbrunner, W., Leitner, G., Gangl, H., and Riedle-Bauer, M. 2007: Epidemische Ausbreitung der Schwarzholzkrankheit (Stolbur-Phytoplasma) in südostösterreichischen Weingärten. Mitteilungen Klosterneuburg 57: 185-189

EPIDEMIOLOGY OF '*CANDIDATUS* PHYTOPLASMA SOLANI' ASSOCIATED WITH POTATO STOLBUR DISEASE IN SERBIA

Mitrovic Milana[1], Jakovljevic Miljana[1], Jovic Jelena[1], Krstic Oliver[1], Kosovac Andrea[1], Trivellone Valeria[2], Tosevski Ivo[1,3], Cvrkovic Tatjana[1]

[1]Institute for Plant Protection and Environment, Department of Plant Pests, Banatska 33, 11000 Belgrade, Serbia
[2]Agroscope Changins-Wädenswil ACW, A Ramél 18, 6593 Cadenazzo, Switzerland
[3] CABI Europe - Switzerland, 1 Rue des Grillons, 2800 Delémont, Switzerland
E-Mail: milanadesancic@yahoo.co.uk

'Candidatus Phytoplasma solani' is widely distributed worldwide, causing severe diseases in different crops. Over the past decade, it was registered in association with several economically important diseases in Serbia, infecting maize, grapevine, celery, kale, potato and garden bean (JOVIĆ et al., 2007; IVANOVIĆ et al, 2011; TRKULJA et al., 2011; JOVIĆ et al., 2011; CVRKOVIĆ et al, 2014; MITROVIĆ et al. 2015a). A progressive spread with tendency of growing epidemics of potato stolbur disease has been observed in Serbia after its first molecular confirmation (JOVIĆ et al. 2011), imposing the urgency to establish an effective disease management program.

A two years survey of potato fields in Serbia revealed the presence of stolbur phytoplasma in all observed localities, with high incidence of symptomatic plants. The symptoms included rolling and yellowing of the leaves, spongy stems, shortened internodes, purplish discoloration of stems or stem bases and the development of aerial tubers.

Inspection of potato fields in Serbia for potential hemipteran vectors revealed the presence of 19 species with a dominance of *Psammotettix alienus, Hyalesthes obsoletus, Reptalus quinquecostatus, R. panzeri* and *Euscelis incisus* (MITROVIĆ et al. 2015b).

Molecular analyses of Auchenorrhyncha species collected on diseased potato fields determined a presence of stolbur phytoplasma in *Hyalesthes obsoletus, Reptalus panzeri* and *R. quinquecostatus,* all previously reported as natural carriers of '*Ca*. P. solani' (TRIVELLONE et al. 2005; JOVIĆ et al. 2009; CVRKOVIĆ et al. 2014).

Multilocus sequence typing of *tuf*, *stamp* and *vmp*1 genes of phytoplasmas associated with potato plants and insects detected 10 stolbur phytoplasma genotypes without any distinct association to a particular insect vector.

Open field experiments with naturally infected *H. obsoletus* and *R. panzeri* confirmed the ability of both cixiids to successfully transmit stolbur phytoplasma to potato plants, which developed clear symptoms of stolbur phytoplasma infection. Genotypes isolated from the infected plants in the semi-field experiments corresponded to the genotypes identified in field collected plants and insects, confirming the involvement of both cixiids in the propagation of stolbur phytoplasma in potato fields in Serbia.

An additional factor which could contribute to the growing epidemics of potato stolbur disease in terms of alternative pathways of horizontal propagation is the fact that potato fields in Serbia are surrounded by the crops which had previously been found infected with '*Ca. P.* solani', e.g. maize (JOVIĆ et al. 2007), grapevine (CVRKOVIĆ et al. 2014), and garden bean (MITROVIĆ et al. 2015a). Moreover, genotypes detected in potato fields matched the isolates from the vineyards and from maize and also genotypes from insects sampled in these crops in Serbia. Our results strongly suggest an exchange of '*Ca. P.* solani' strains between the crops via polyphagous vectors and their weedy host plants. The findings of the present study confirm the need for urgent actions to be undertaken in terms of prevention of further propagation and yield losses.

ACKNOWLEDGEMENTS
This study was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia (III43001), and by the SCOPES program of the Swiss National Science Foundation (IZ73Z0_152414).

REFERENCES

CVRKOVIĆ, T., JOVIĆ, J., MITROVIĆ, M., KRSTIĆ, O., TOŠEVSKI, I. 2014: Experimental and molecular evidence of *Reptalus panzeri* as a natural vector of bois noir. Plant Pathology 63, 42–53

IVANOVIĆ, Ž., TRKULJA, N., PFAF DOLOVAC, E., DOLOVAC, N., ŽIVKOVIĆ, S., JOVIĆ, J., MITROVIĆ, M. 2011: First report of stolbur phytoplasma infecting celery in Serbia. Bulletin of Insectology, 64, S239-S240

JOVIĆ, J., CVRKOVIĆ, T., MITROVIĆ, M., KRNJAJIĆ, S., REDINBAUGH, M.G., PRATT, R.C., GINGERY, R.E., HOGENHOUT, S.A., TOŠEVSKI, I. 2007: Roles of stolbur phytoplasma and *Reptalus panzeri* (Cixiinae, Auchenorrhyncha) in the epidemiology of Maize redness in Serbia. European Journal of Plant Pathology 118, 85-89

JOVIĆ, J., CVRKOVIĆ, T., MITROVIĆ, M., KRNJAJIĆ, S., PETROVIĆ, A., REDINBAUGH, M. G., PRATT, R. C., HOGENHOUT, S. A., TOŠEVSKI, I. 2009: Stolbur phytoplasma transmission to maize by *Reptalus panzeri* and the disease cycle of maize redness in Serbia. Phytopathology 99, 1053–1061

JOVIĆ, J., EMBER, I., MITROVIĆ, M., CVRKOVIĆ, T., KRSTIĆ, O., KRNJAJIĆ, S., ACS Z., KOLBER M., TOŠEVSKI, I. 2011: Molecular detection of potato stolbur phytoplasma in Serbia. Bulletin of Insectology 64, S83-S84

MITROVIĆ, M., CVRKOVIĆ, T., JOVIĆ, J., KRSTIĆ, O., JAKOVLJEVIĆ, M., KOSOVAC, A., TOŠEVSKI, I. 2015a: First report of '*Candidatus* Phytoplasma solani' infecting garden bean Phaseolus vulgaris L. in Serbia. Plant Disease 99, 551-551

MITROVIĆ, M., TRIVELLONE, V., JOVIĆ, J., CVRKOVIĆ, T., JAKOVLJEVIĆ, M., KOSOVAC, A., KRSTIĆ, O., TOŠEVSKI, I. 2015b: Potential Hemipteran vectors of "stolbur" phytoplasma in potato fields in Serbia. Phytopathogenic Mollicutes 5, S49-S50

TRIVELLONE, V., PINZAUTI, F., BAGNOLI, B. 2005: *Reptalus quinquecostatus* (Dufour) (Auchenorrhyncha Cixiidae) as a possible vector of stolbur phytoplasma in a vineyard in Tuscany. Redia 88, 103-108

TRKULJA, N., IVANOVIĆ, Ž., PFAF DOLOVAC, E., DOLOVAC, N., ŽIVKOVIĆ, S., JOVIĆ, J., MITROVIĆ, M. 2011: Stolbur phytoplasma infection of kale crops (*Brassica oleracea* var. gemmifera L.) in Serbia. Bulletin of Insectology 64, S81-S82

First Report of '*Candidatus* phytoplasma solani' in Sunflower in Bulgaria

Zhelyu Avramov[1], Jelena Stepanović[2], Dora Panajotova[3], Mariana Laginova[3] and Bojan Duduk[2]

[1] University of Forestry, Faculty of Agronomy
BG-1330, Sofia, 10 Kliment Ohridski Blvd.
[2] Institute of Pesticides and Environmental Protection
RS-11080 Belgrade, Banatska 31B
[3] Central Laboratory for Plant Quarantine
BG-1330, Sofia, 120 Nikola Moushanov Blvd.
E-Mail: bojan.duduk@pesting.org.rs

The molecular identification of phytoplasma from infected sunflower in Bulgaria is reported. During a survey in the Boliarsko area in the Yambol region of Bulgaria, phytoplasma-like symptoms were observed on sunflower. The observed symptoms included yellowing, deformations and proliferation of flowers and sterile (empty) seeds. The Polymerase Chain Reaction (PCR) analyses with primers specific for the 16S ribosomal gene of phytoplasmas confirmed that symptomatic samples of sunflower were infected by phytoplasmas. Phytoplasmas were identified using sequence analyses of PCR amplified 16S rDNA. The obtained sequence showed identity with the '*Candidatus* Phytoplasma solani' strain from corn from Bulgaria and other 39 strains deposited in the GenBank. To our knowledge this is the first report of stolbur phytoplasma in sunflower (*Helianthus annuus*) in Bulgaria, adding a new cultivated plant species to the already wide natural host range of stolbur phytoplasma.

'*Candidatus* Phytoplasma solani' (stolbur phytoplasma-STOL) infects a wide range of cultivated and wild plants. Stolbur phytoplasma belongs to the 16SrXII-A ribosomal group and is transmitted by two planthoppers, *Hyalestes obsoletus* and *Reptalus panzeri* (Fos et al., 1992; Maixner, 1994; Jović et al., 2007; Quaglino et al., 2013). Considered as plant pathogen of European and Mediterranean origin, stolbur phytoplasma, also reported in other parts of the world, has been known in Bulgaria in tomato and pepper from 1970 (Kowachevski, 1971). Although known from before, stolbur phytoplasma was molecularly confirmed in Bulgaria in *Prunus avium,* grapevine (Bois noir) and in corn (corn reddening) in 2014 (Garnier, 2000; Duduk et al., 2010; Avramov et al., 2011; Mitrović et al., 2013; Genov et al., 2014). Until now, in sunflower, only phytoplasmas belonging to 16SrIII ribosomal group have been reported in Argentina (Guzmán et al., 2014).
Symptoms resembling those associated with phytoplasma diseases such as yellowing, deformations and proliferation of flowers and sterile (empty) seeds were observed in August 2012 in sunflower in the Boliarsko area, Bulgaria. Molecular analyses were performed to determine whether phytoplasmas are present in the symptomatic plants.

MATERIAL AND METHODS

Leaf samples were collected from three symptomatic and asymptomatic sunflower plants during August 2012, from Boliarsko location in the Yambol region of Bulgaria. Total nucleic acids were extracted from 0.5 g of fresh leaf midrib tissue from each sample, following the CTAB procedure described by Doyle and Doyle (1990), dissolved in TE buffer and stored at -20 °C. Nucleic acids were diluted in sterile distilled water 1:100 before performing PCR assays. For phytoplasma detection in collected samples, direct PCR assays with the universal phytoplasma primer pair P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) and nested PCR assays with primer pair

R16F2n/R2 (Lee et al., 1993; Gundersen and Lee 1996) were carried out. Each 25 µl PCR mix contained 1 µl of DNA template, 1× PCR Master Mix (Fermentas, Vilnius, Lithuania) and 0.4 µM of each primer. Samples lacking DNA were employed as negative controls. As a template for nested PCR, 1 µl of direct PCR amplicon diluted 30× in sterile water was used. Thirty-five PCR cycles were performed, for both amplifications under the following conditions: 1 min (2 min for the first cycle) for denaturation step at 94 °C, 2 min for annealing at 50 °C and 3 min (10 min for the last cycle) for primer extension at 72 °C. Six microliters of PCR products were separated on 1 % agarose gel, stained with ethidium bromide and visualized with a UV transilluminator.
The nested R16F2n/R2-amplified product of the selected symptomatic sample was purified using the mi-PCR purification kit (Metabion International AG, Martinsried, Germany). The product was sequenced by commercial service (Macrogen Inc., Seoul, South Korea) in both directions with the primers used for amplification (R16F2n/R2). The obtained sequences were assembled using Pregap4 from the Staden program package (Staden et al., 2000). The consensus sequence (1169 bp) is deposited in the NCBI, under the accession number KU556855, aligned with similar sequences of stolbur phytoplasmas publicly available in the GenBank using Clustal W (Thompson et al., 1997) from the Molecular Evolutionary Genetics Analysis program-MEGA6 (Tamura et al., 2013) and searched for SNPs in Bioedit program (Hall, 1999).

RESULTS AND DISCUSSION

Amplicons of the expected sizes (1.2 Kbp) were produced with DNA from two out of three symptomatic sunflower samples, while no amplification was observed with the DNA from asymptomatic plants and negative control. The sequence of selected strain obtained from R16F2n/R2 amplicon was 1169 bp in length, containing partial phytoplasma 16S rDNA.
The search for SNPs revealed no differences at any nucleotide position, showing identity (100 %) between the examined sunflower stolbur strain and the strain from corn from Bulgaria (KF907506), as well as with 39 other '*Ca*. Phytoplasma solani' strains. When compared with reference strain of '*Ca*. Phytoplasma solani' (AF248959) the Bulgarian sunflower strain sequence showed four nucleotide differences, making 99,66 % sequence homology.

REFERENCES

Avramov ZH., Contaldo N., Bertaccini A., and Sakalieva D. 2011. First report of stolbur phytoplasmas in *Prunus avium* in Bulgaria. Bulletin of Insectology 64:S71-S72.

Deng, S. and Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. Journal of Microbiological Methods 14: 53-61.

Doyle, J.J. and Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13–15.

Duduk, B., Tian, J., Contaldo, N., Fan, X., Paltrinieri, S., Chen, Q., Zhao, Q., and Bertaccini, A. 2010. Occurrence of Phytoplasmas Related to Stolbur and to '*Candidatus* Phytoplasma japonicum' in Woody Host Plants in China. Journal of Phytopathology 158: 100-104.

Fos, A., Danet, J.L., Zreik, L., Garnier, M., and Bove, J.M. 1992. Use of monoclonal antibody to detect the stolbur mycoplasma like organism in plants and insects and to identify a vector in France. Plant Disease 76: 1092-1096.

Garnier, M. 2000. The stolbur phytoplasma: An ubiquitous agent. Comptes Rendus de l'Academie d'Agriculture France 86: 27-33.

Genov, N., Mitrović, J., Genov, M., AND Duduk, B. 2014. First Report of Corn Reddening caused by '*Candidatus* Phytoplasma solani' in Bulgaria. Plant disease 98(7): 991.

Gundersen, D. E. and Lee, I.-M. 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathologia Mediterranea 35 (3): 144-151.

Guzmán, F., Giolitti, F., Fernández, F., Nome, C., Lenardon, S., and Conci, L. 2014. Identification and molecular characterization of a phytoplasma associated with sunflower in Argentina. European Journal of Plant Pathology 138: 679-683.

Jović, J., Cvrković, T., Mitrović, M., Krnjanjić, S., Petrović, A., Redinbaugh, M. G., Pratt, R.

C., Hogenhout, S. A., and Toševski, I. 2007. Maize redness in Serbia caused by stolbur phytoplasma is transmitted by *Reptalus panzeri*. Bulletin of Insectology 60 (2): 397-398.

Kowachevski I., 1971. Mycoplasmas – a new group pests, causing diseases on plants. Priroda. 20(2): 67 - 68.

Lee, I.-M., Hammond, R. W., Davis, R. E., and Gundersen, D. E. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopathology 83 (8): 834-842.

Lee, I.-M., Bottner-Parker, K. D., Zhao, Y., Davis, R. E. and Harrison, N. A. (2010). Phylogenetic analysis and delineation of phytoplasmas based on *sec*Y gene sequences. International journal of systematic and evolutionary microbiology 60: 2887-2897.

Maixner, M. 1994: Transmission of German grapevine yellows (Vergilbungskrankheit) by the planthopper *Hyalesthes* obsoletus (Auchenorrhyncha: Cixiidae). Vitis 33: 103-104.

Mitrović, J., Contaldo, N., Avramov, Z., Smiljković, M., Bertaccini, A. and Duduk, B. 2013. *GroEL* gene characterization of "bois noir" phytoplasma from Serbia, Bulgaria and Italy. 3rd European Bois Noir workshop, Barcelona, Spain, March 20-21: 63-64.

Schneider, B., Seemuller, E., Smart, C. D., and Kirkpatrick, B. C. 1995. Phylogenetic classification of plant pathogenic mycoplasmalike organisms or phytoplasmas. In: Molecular and Diagnostic Procedures in Mycoplasmology (vol. I). Eds: R. Razin and J. G. Tully. Academic Press, San Diego, pp. 369-380.

Staden R., Beal, K.F., and Bonfield, J.K. 2000. The Staden package. Methods in Molecular Biology 132: 115–130.

Quaglino, F., Zhao, Y., Casati, P., Bulgari, D., Bianco, P. A., Wei, W., and Davis, R.E. 2013. '*Candidatus* Phytoplasma solani', a novel taxon associated with stolbur and bois noir related diseases of plants. International Journal of Systematic and Evolutionary Microbiology 63: 2879-2894

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution 30(12): 2725-2729.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The Clustal X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24: 4876-4882.

PREVALENCE OF STOLBUR PHYTOPLASMA IN LEAFHOPPERS AND PLANTHOPPERS COLLECTED IN VINEYARD, CORN AND POTATO FIELDS AND THEIR SURROUNDINGS IN SWITZERLAND.

Valeria Trivellone[1], Mauro Jermini[1], Jelena Jović[2], Tatjana Cvrković[2], Miljana Jakovljević[2], Andrea Kosovac[2], Oliver Krstić[2], Ivo Toševski[2,3] and Milana Mitrović[2]

[1]Agroscope Changins-Wädenswil ACW, A Ramél 18, 6593 Cadenazzo, Switzerland
[2]Institute for Plant Protection and Environment, Department of Plant Pests, Banatska 33 11080 Zemun, Serbia
[3]CABI Europe - Switzerland, 1 Rue des Grillons, 2800 Delémont, Switzerland

The stolbur phytoplasma ('*Candidatus* Phytoplasma solani', 16SrXII-A subgroup) has a broad host-plant range, including various economically and environmentally important herbaceous and woody plants. A large number of host plants, insect vectors and several independent epidemiological cycles have been reported for stolbur phytoplasma in diverse agroecosystems (Maixner, 2011). In Swiss vineyards, stolbur phytoplasma still can cause grapevine yellows disease (Bois noir, BN) outbreaks. However, the interplay of the phytoplasma, its original host plants, insect vectors and vines is still poorly investigated (Kessler et al., 2011). Potato stolbur was recorded in Switzerland, but it is sporadic, whereas Maize Redness (MR) disease has not yet been reported, however data on insect-vectors and epidemiology of each diseases are still limited or completely unknown.

The main objectives of the present study were to: 1- determine species composition of planthoppers and leafhoppers in three agroecosystems (corn, potato and vineyard); 2- identify potential vectors of stolbur phytoplasma by means of molecular analyses on insects; 3- obtain a geographic map for prevalence of stolbur phytoplasma infected insects in Switzerland.

In the frame of an international joint research project between Switzerland and Serbia, two field samplings were carried out in 2014 and 2015 and planthoppers and leafhoppers specimens were collected in corn, potato and vineyard fields in Switzerland.

The insects were collected from 68 sampling sites in Southern and North-Western Switzerland, and the major crop-growing areas have been selected from 7 out of 26 Cantons: Jura, Neuchatel, Vaud, Geneva, Valais, Bern and Tessin. Overall, 30 vineyards, 28 corn and 10 potato fields were sampled.

In 2014, a preliminary survey was carried out and 10 sites were sampled: 6 vineyards and 4 corn fields. In 2015, an extensive survey was carried out, and 58 sites were inspected: 24 vineyards, 24 corn and 10 potato fields. Leafhoppers and planthoppers were collected from the third week of June to the end of July (one sampling per site) using sweep net and mouth-aspirator directly from crops and the weeds in the surrounding of fields.

All specimens were identified and stored in 96% ethanol. Nomenclature follows Holzinger et al. (2003) and Ribaut (1936, 1952).

For each agroecosystem, Auchenorrhyncha community composition was analysed in terms of number of species and individuals, and the relationship between species mean abundance and species occurrence was examined.

More than 2'000 specimens were collected and they belong to about 90 species encompassed in the families: Cicadellidae (64), Delphacidae (12), Aphrophoridae (4), Cixiidae (3), Issidae (2), Cercopidae

(1), Membracidae (1), Flatidae (1) and Dictyopharidae (1).

The results confirmed the presence of known and potential vectors of stolbur phytoplasma in the investigated areas in Switzerland. Faunistic analysis of Auchenorrhyncha demonstrated that species composition is affected by agroecosystem type and biogeographical region. Knowing the most important species for each agroecosystem, their host plants and where they occur around the crop-growing area is of great importance for their management. With this information, it is possible to design specific strategies and therefore prevent transmission of the phytoplasmas.

Molecular analyses of genomic sequencing of phytoplasma isolates are ongoing.

ACKNOWLEDGEMENTS

We thank the Swiss National Science Foundation for financial support of this study through the SCOPES grant IZ73Z0_152414.

REFERENCES

Holzinger, W. E., Kammerlander, I., & Nickel, H. 2003: The Auchenorrhyncha of Central Europe, Fulgoromorpha, Cicadomorpha Excl. Cicadellidae (p. 673). Leiden: Brill Academic Publishers

Kessler, S., Schaerer, S., Delabays, N., Turlings, T. C. J., Trivellone, V., & Kehrli, P. 2011: Host plant preferences of *Hyalesthes obsoletus,* the vector of the grapevine yellows disease 'bois noir', in Switzerland. Entomologia Experimentalis et Applicata, 139(1), 60-67, doi:10.1111/j.1570-7458.2011.01107.x.

Maixner, M. 2011: Recent advances in bois noir research. Petria 21, 17–32.

Ribaut, H. 1936: Homoptères Auchènorhynches 1. (Typhlocybidae). Faune de France 31, 1-228

Ribaut, H. 1952: Homoptères Auchènorhynches 2. (Jassidae). Faune de France 57, 1-474

Core Team, R. 2012: R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, 3-900051-07-0, doi:http://www.R-project.org/ (09.08.15)

IS *HYALESTHES OBSOLETUS* A SPECIES COMPLEX UNDERGOING CRYPTIC SPECIATION? MORE EVIDENCE OF HOST-ASSOCIATED GENETIC DIFFERENTIATION IN SOUTHEAST EUROPE

Andrea Kosovac[1], Jes Johannesen[2], Oliver Krstić[1], Milana Mitrović[1], Tatjana Cvrković[1], Ivo Toševski [1,3]and Jelena Jović[1]

[1] Institute for Plant Protection and Environment, Department of Plant Pests, Zemun, Serbia
[2] Institute of Zoology, University of Mainz, Mainz, Germany
[3] CABI, Delémont, Switzerland
jovic_biolab@yahoo.com

Research on integrative taxonomy, geographical center of origin and diversity, host-shift patterns and genetic variation in insect pests are of essential importance to perceive their invasive capacity and adaptability to new habitats. These can be clarified by combining population genetic methods with morphological, ecological and biogeography data (Grapputo et al., 2005). In the case of cixid planthoppers (Cixiidae), which establish close relationships with their host plants and affect numerous crops by vectoring phytoplasmas (Nickel, 2003), this combined approach is of particular importance because it can help to trace distinct genetic lineages that potentially have different modes of behaviour and consequently epidemiological cycles.

Hyalesthes obsoletus Signoret, 1865 (Hemiptera: Cixiidae) is the main vector of '*Candidatus* Phytoplasma solani' (CPs) and of the Bois Noir disease of grapevine. It is the type species of the genus and a member of the *Hyalesthes obsoletus* species group (Hoch and Remane, 1985); i.e. a species complex comprising at least six closely related and by outer morphology very similar species. *H. obsoletus sensu stricto* is the most widely distributed of the species within the complex, which has a Mediterranean diversity centre (Hoch and Remane, 1985; Nickel, 2003). It co-occurs in sympatry with all the five other species in the complex: *H. lacotei* in south France, *H. thracicus* in Greece, *H. yozgaticus* and *H. hani* in Turkey and Lebanon, and *H. flavovarius* in Uzbekistan. Although *H. obsoletus* is generally treated as a polyphagous species, so far only few plant species are considered as wild-host plants in central and southeastern Europe: *Convolvulus arvensis*, *Urtica dioica* and *Vitex agnus-castus* (Maixner et al., 1995; Sharon et al., 2005; Kessler et al., 2011). Recent findings of genetic differentiation in *H. obsoletus* populations associated with different host plants revealed segregation of two host races on the north-western edge of the distribution range that were associated with *C. arvensis* and *U. dioca,* respectively (Johannesen et al., 2008; IMO et al., 2013), while preliminary findings from Southeast Europe have revealed the existence of a genetically divergent lineage associated with *Crepis foetida* (Kosovac et al., 2013). In addition, *V. agnus-castus* is now experimentally confirmed as a pathogen source plant in Bois noir epidemiology in the Mediterranean (Kosovac et al., 2016). This new link imposes a new element in the host-plant affiliations of *H. obsoletus* at the population level.

In order to understand ecological specialisation and cryptic speciation potential in *H. obsoletus*, seven nuclear markers (microsatellites) (IMO et al., 2013) were analysed to determine the level of differentiation among 16 populations associated with four host plants in Southeast Europe: *C. arvensis, U. dioca, C. foetida* and *V. agnus-castus*. Multilocus genotypes of 280 specimens were analyzed using Structure

2.3.4 Bayesian-based clustering to determine the most likely number of genetic clusters. The analyzed populations were unambiguously separated into three genetic entities with more than 92% membership assignment per population. The first cluster comprised populations associated with *C. arvensis* and *U. dioca*, while members of the second and third cluster were *H. obsoletus* populations affiliated to *V. agnus-castus* and *C. foetida*, respectively. Molecular variance analysis, preformed with Arlequin 3.5.2, estimated that 82% of the total genetic variance was explained by genetic divergence among the three clusters (host-plant groups) ($p < 0.001$). Phylogenetic analysis based on three mitochondrial gene regions showed separation into three closely related haplotype groups that corresponded to host plant affiliation and clusters obtained from nuclear markers. Sympatric and often syntopic co-occurrence of genetically divergent *H. obsoletus* populations associated with different host plants throughout Southeast Europe implicate ecological specialisation leading to cryptic speciation. It questions the presumed polyphagy of this cixiid and indicates much greater species diversity than is currently recognized within the *Hyalesthes obsoletus* species complex. Considering that the four plant species studied here represent dual host plants for pathogen and vector, i.e. inoculum sources, cryptic species should be of concern in all future epidemiological studies of CPs induced diseases.

ACKNOWLEDGEMENTS

This study was supported by the grant III43001 from the Ministry of Education, Science and Technological Development of the Republic of Serbia, and by grant 15202-386261/861 from Stiftung Rheinland-Pfalz für Innovation to Jes Johannesen.

REFERENCES

Grapputo, A., Boman, S., Lindostroem, L., Lyytinen, A., Mappes, J. 2005: The voyage of an invasive species across continents: genetic diversity of North American and European Colorado potato beetle populations. Molecular ecology, 14 (14), 4207-4219.

Hoch, H., Remane, R. 1985. Evolution und Speziation der Zikaden-Gattung Hyalesthes SIGNORET, 1865 (Homoptera Auchenorrhyncha Fulgoroidea Cixiidae). Marburger Entomologische Publikationen.

Imo, M., Maixner, M., Johannesen, J. 2013: Sympatric diversification vs. immigration: deciphering host-plant specialization in a polyphagous insect, the stolbur phytoplasma vector *Hyalesthes obsoletus* (Cixiidae). Molecular ecology, 22 (8), 2188-2203.

Johannesen, J., Lux, B., Michel, K., Seitz, A., Maixner, M. 2008: Invasion biology and host specificity of the grapevine yellows disease vector *Hyalesthes obsoletus* in Europe. Entomologia experimentalis et applicata, 126: 217–227.

Kessler, S., Schaerer, S., Delabays, N., Turlings, T. C. J., Trivellone, V., Kehrli, P. 2011: Host plant preferences of *Hyalesthes obsoletus*, the vector of the grapevine yellows disease 'bois noir', in Switzerland. Entomologia Experimentalis et Applicata 139: 60–67.

Kosovac, A., Johannesen, J., Krstić, O., Mitrović, M., Cvrković, T., Maixner, M., Toševski, I., Jović, J. 2013: Microsatellite and mtDNA evidence of genetic differentiation in *Hyalesthes obsoletus* populations associated with a new major host, stinking hawk's-beard (*Crepis foetida*), in southeast Europe. Proceedings of the 3rd European Bois Noir Workshop, Barcelona, 18-19.

Kosovac, A., Radonjić, S., Hrnčić, S., Krstić, O., Toševski, I., Jović, J. 2016: Molecular tracing of the transmission routes of bois noir in Mediterranean vineyards of Montenegro and experimental evidence for the epidemiological role of *Vitex agnus-castus* (Lamiaceae) and associated *Hyalesthes obsoletus* (Cixiidae). Plant Pathology, 65: 285-298.

Maixner, M., Ahrens, U., E. Seemüller, E. 1995: Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. European Journal of Plant Pathology, 101: 241-250.

Nickel, H. 2003: The Leafhoppers and Planthoppers of Germany (Hemiptera, Auchenorrhyncha): Patterns and strategies in a highly diverse group of phytophagous insects. Pensoft Publishers.

Sharon, R., Soroker, V., Wesley, S., Zahavi, T., Harari, A., Weintraub, P. 2005: Vitex agnus-castus is a preferred host plant for *Hyalesthes obsoletus*. Journal of Chemical Ecology, 31, 1051–1063.

POTENTIAL ROLE OF *REPTALUS PANZERI* AS A VECTOR OF BOIS NOIR IN GERMANY

Friederike Lang[1], Maria Kölber[2], Rita Elek[2], Yvonne Kappel[1], Dunja Kröhner[1], Jes Johannesen[3] and Michael Maixner[1]

[1]Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for Plant Protecti on in Fruit Crops and Viticulture, Geilweilerhof, 76833 Siebeldingen, Germany, michael.maixner@jki.bund.de

[2]Genlogs Biodiagnostica Ltd, Dioszegi 37, 1113 Budapest, Hungary - maria.kolber@genlogs.com

[3] Institute of Zoology, University of Mainz, Johann-Joachim-Becherweg 13, 55128 Mainz, Germany jesjo@uni-mainz.de

Two planthoppers of the family Cixiidae are confirmed vectors of Bois noir (BN). They transmit *Candidatus* Phytoplasma solani (CPs) to grapevine. *Hyalesthes obsoletus* is the more common vector of the two species, while *Reptalus panzeri* has been reported as a vector in Serbia (CVRKOVIC et al., 2014). *R. panzeri* is a suspected vector in other regions, too, e.g. in Hungary where infected specimens have been identified in corn fields affected by maize redness (ACS et al., 2011). Other Cixiidae and Deltocephalinae have been identified as carriers or as vectors of CPs to other plants, but their ability to transmit CPs to grapevine has not been experimentally proved so far. Nevertheless, reports of an apparent lack of correlation between *H. obsoletus* and BN from various regions prompt further studies on the role of additional species as vectors of BN. Although there is generally a good correlation between *H. obsoletus* and BN in German winegrowing regions, this vector is occasionally rare in some regions with high incidence of BN. On some of these sites, *R. panzeri,* which is otherwise a rather rare species in Germany, has been found. Therefore, we performed a field study to estimate the significance of *R. panzeri* as a vector of BN in Germany with the objectives 1) to collect data on the occurrence, phenology and host range of *R. panzeri* at some of those locations, 2) to estimate the incidence of CPs in grapevine, alternative host plants and *R. panzeri*, and 3) to test the ability of this species to transmit CPs to experimental hosts and grapevine.

MATERIAL AND METHODS

For a first monitoring, vineyards and surrounding uncultivated fields at viticultural sites in four winegrowing regions were checked for the presence of *R. panzeri* from 2009 to 2015 by sweep-netting wild vegetation. One location in the Middle Rhine valley and five locations in the Mosel valley were then chosen as monitoring and collection plots for this planthopper. Sticky traps were setup from May to August to monitor the activity of *R. panzeri.* The insects were also collected by sweep netting in order to gain information about feeding hosts of adults and to obtain specimens for transmission trials. Experimental hosts for transmission experiments were *Catharanthus roseus*, *Vicia faba*, and *Vitis vinifera.* Plants of each species were either exposed to 7 to 25 specimens of *R. panzeri* per cage or caged in groups of three to five plants with 47 to 150 insects (2646 insects in total).

RESULTS AND DISCUSSION

Beside occasional captures of few specimens at different locations in the four winegrowing areas we found *R. panzeri* consistently at one Middle Rhine site (Boppard) and two sites of the Mosel region (Pommern; Platten). Three terraced vineyards at Pommern as well as a vineyard and a fallow field at Platten were sampled regularly between 2012 and 2015. Cixiidae constituted between 11 % and 31 %

of the total number of Auchenorrhyncha captured during this period. The proportion of *R. panzeri* varied between plots from 11 % to 98 % of all Cixiidae specimens. Other common Cixiid species were *H. obsoletus* and *Cixius wagneri*. The density of *R. panzeri* ranged from 0.1 to and 16.5 specimens/trap depending on plot and year. While there was no considerable variation between years at Pommern, the density of the planthopper at Platten oscillated between extremely low and high densities every second year. In general, significantly higher densities were observed in the uncultivated fallow fields compared to the vineyards. *R. panzeri* showed a closer association to grapevine than *H. obsoletus*,since adults were found regularly in the canopy of the vines. In fact, while there was no difference between traps exposed in 0.5 m and 1.25 m in the fallow plot, in the vineyard significantly higher numbers were caught on the upper traps in the height of the canopy compared to the traps on the level of the ground vegetation. Catches of *H. obsoletus*, on the other hand, are usually concentrated on the lower traps.

Adult *R. panzeri* were present on a wide variety of herbaceous plants including *Urtica dioica* and also some woody plants (*Prunus spp., Salix sp., Hedera helix*), but only *Clematis vitalba* was used at all sites. Immature stages were found only on the roots of Ranunculus spp.so far. With the exception of *U. dioica* none of the host plant species tested so far was infected with CPs.

The infestation of *R. panzeri* with CPs was low. Only 1.1 % (n=1192) of the tested specimens collected from 2009 to 2014 at Platten and Pommern were infected by CPs. This is a considerable difference to vineyards in Serbia, where CVRKOVIC et al. (2014) found that 21 % of *R. panzeri* were infected. All specimens (n=6) from Pommern were infected by tuf-b, while insects from Platten carried either tuf-a (n=4) or tuf-b (n=3). *R. panzeri* was regularly found on *U. dioica* on this site. In contrast to the low infestation in *R. panzeri*, the proportions of infected specimens of *H. obsoletus* collected in the same period on *C. arvensis* and *U. dioica* was 33 % and 11 %, respectively.

The results of the transmission trials corresponded to the observation of low infestation of *R. panzeri* by CPs. Only two of 30 *C. roseus* and none of 75 *V. faba* and 18 *V. vinifera* on which the insects were caged became infected. The two periwinkles were inoculated with CPs by *R. panzeri* collected from *U. dioica* at Platten. Both plants were infected by tuf-a.

Although *R. panzeri* is generally rare in Germany, it occurred in high density in some of the investigated vineyard plots where it was the dominant Cixiid species. All of these plots are situated on steep xerothermic slopes, which seem to favor this species. Although adult planthoppers were regularly found feeding on grapevine, the role of *R. panzeri* as a vector of Bois noir in Germany is still unclear. Infestation with CPs is low and the preliminary results of transmission trials hint at a low probability of stolbur transmission by this species. However, the epidemiology of BN is characterized by periodical outbreaks of BN with temporary high infection pressure that lasting a few years only. The outbreaks are followed by longer endemic periods with low infection pressure and decreasing disease incidence (MAIXNER et al., 2006), which is the current situation since 2008. Population density, infestation and transmission efficiency of *R. panzeri* during an epidemic period of BN have to be studied before the significance of this species as a BN vector in Germany can be definitely assessed.

ACKNOWLEDGEMENTS

This study was supported in part by the Federal Ministry of Food and Agriculture through the Federal Office for Agriculture and Food, FKZ 2811HS003. The cooperation between JKI and Genlogs is supported by the Federal Ministry of Education and Science through the International Bureau of the DLR Project Management Agency, FKZ 01DS14026 as well as by the National Research, Development and Innovation Fund from the Hungarian side, TÉT_12_DE-1-2013-0005. JJ was supported by Stiftung Rheinland-Pfalz für Innovation (15202-386261/861)

REFERENCES

ACS, Z., JOVIC, J., EMBER, I., CVRKOVIC, T., NAGY, Z., TALABER, C., GERGELY, L., TOTEVSKI, I., and KOLBER, M. 2011:. First report of maize redness disease in Hungary. Bulletin of Insectology 64: S229-S230.

CVRKOVIC, T., JOVIC, J., MITROVIC, M., KRSTIC, O., and TOSEVSKI, I. 2014: Experimental and molecular evidence of *Reptalus panzeri* as a natural vector of bois noir. Plant Pathology 63: 42-53.

MAIXNER, M., LANGER, M., and GERHARD, Y. (2006). Epidemiological characteristics of Bois noir type I. Extended abstracts 15th Meeting ICVG, Stellenbosch, South Africa,3-7 April 2006, 86-87.

PRELIMINARY RESULTS ON PUTATIVE VECTORS OF '*CANDIDATUS* PHYTOPLASMA SOLANI' IN BOIS NOIR-AFFECTED VINEYARDS IN FRANCIACORTA (LOMBARDY REGION, NORTH ITALY)

Francesco Sanna[1], Fabio Quaglino[2], Silvia Filisetti[3], Paola Casati[2], Monica Faccincani[3], Piero Attilio Bianco[2] and Nicola Mori*

[1]Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente, Università degli Studi di Padova, Legnaro, Italy;
[2]Dipartimento di Scienze Agrarie e Ambientali – Produzione, Territorio e Agroenergia, Università degli Studi di Milano, Milan, Italy; 3Consorzio per la Tutela del Franciacorta, Erbusco (BS), Italy.
*Corresponding author: nicola.mori@unipd.it

Bois noir phytoplasma (BNp) strains are transmitted by the planthopper *Hyalesthes obsoletus* Signoret (Homoptera: Cixiidae), a polyphagous insect living preferentially on weeds. In vine-growing areas where *H. obsoletus* is absent, the presence of BNp implies the existence of alternative vectors. Recently, *Reptalus panzeri* has been reported as a natural vector of BNp in Serbian vineyards. In the present study, field surveys and molecular analyses were carried out in a BN-affected vineyard in Franciacorta (Lombardy region, North Italy) in 2013 to identify putative insect vectors. Insects (1100 specimens) were captured by entomological net and sticky traps from May to October. Phenotypic analysis by stereomicroscope allowed the identification of 42 taxonomic groups at different levels (26 species and 16 genera), grouped in 624 pools for molecular detection. Specific PCR-based amplification of *stamp* gene revealed the presence of BNp in 64 analysed pools (10%) belonging to 20 taxonomic groups (15 species and 5 genera). Further analyses will be carried out to (i) characterize the BNp strains identified in insect specimens by *stamp* gene sequence and compare with strains identified in grapevines and weeds, (ii) determine the transmission capability by trials performed in controlled conditions.

INTRODUCTION

Grapevine yellows (GY) are a phytoplasma-associated disease complex that induces severe crop losses in almost all varieties used for wine production. Among GY, Bois noir (BN), associated with *'Candidatus* Phytoplasma solani', is responsible for serious crop losses in the Euro-Mediterranean area and in other continents (QUAGLINO et al., 2013). BN phytoplasma (BNp) strains are transmitted to grapevine by *Hyalesthes obsoletus* Signoret (Homoptera: Cixiide), a polyphagous vector living preferentially on nettle (*Urtica dioica L.*), bindweed (*Convolvulus arvensis L.*), and chaste tree (*Vitex agnus-castus* L.) inside and/or around vineyards (LANGER und MAIXNER, 2004; KOSOVAC et al., 2015). Furthermore, in vine-growing areas where *H. obsoletus* is absent, the presence of BNp implies the existence of alternative vectors. *Reptalus panzeri* has been reported as a natural vector of BNp in Serbian vineyards (CVRKOVIĆ et al., 2014). Additionally, *Anaceratagallia ribauti* and *Reptalus quinquecostatus* were experimentally confirmed as vectors of 'Ca. P. solani' but not to grapevine (PINZAUTI et al., 2008; RIEDLE-BAUER et al., 2008); therefore, currently, such insects are not considered to be involved in BNp transmission to grapevine. Other studies reported that other Cixiidae and Cicadellidae have been captured within or near BN-diseased vineyards and found to contain BNp. Based on such information, it appears that, even though the role of these nume-

rous hosts in BNp transmission has not been proven, it is probable that other host plants are involved in the epidemiology of BN disease, harboring additional insect species capable of spreading the disease (MORI et al., 2015; OLIVERI et al., 2015). In the present study, field surveys and molecular analyses were carried out in a BN-affected vineyard in Franciacorta (Lombardy region, North Italy) in 2013 to identify putative insect vectors.

MATERIAL AND METHODS

SAMPLING OF INSECT SPECIMENS. A field survey was carried out in a vineyard (variety Chardonnay), located in Gussago (BS), Franciacorta (Lombardy, North Italy), selected for the high incidence of BN disease (>70% affected grapevines). During 2013, insects were monitored and captured every week, from May to September, by yellow sticky traps (placed within, around the borders and in the neighborhood of the vineyard) and entomological nets.

INSECT IDENTIFICATION. All the insect specimens, captured in the examined vineyard, were maintained in ethanol 90% and identified by stereomicroscope based on phenotypic characters. Specimens of the same taxonomic group were pooled (1-3 specimens per pool) for further molecular analyses.

BNP-SPECIFIC DETECTION. Total genomic DNA was extracted from 624 insect pools. Briefly, the ethanol-preserved adults were dried on filter paper and homogenized in CTAB-based buffer plus ascorbic acid 0.5%. After incubation at 60°C for 30min, DNA was extracted with one volume of chloroform:isoamylalcohol 24:1v/v solution and then precipitated with the addition of one volume of isopropanol. The DNA pellet was then washed with 70% ethanol, vacuum dried and resuspended in 100µL TE pH8.0.

Specific detection of BNp ('*Ca*. P. solani', subgroup 16SrXII-A) was carried out by nested-PCR based amplification of *stamp* gene as previously described (FABRE et al., 2011). Total nucleic acids from periwinkle plants infected by phytoplasma strains EY1 ('*Ca*. P. ulmi'), STOL ('*Ca*. P. solani'), and AY1 ('*Ca*. P. asteris') were used as reference controls. Total nucleic acids from healthy periwinkle and PCR mixture devoid of nucleic acids were used as negative controls.

RESULTS AND DISCUSSION

INSECT DIVERSITY IN EXAMINED VINEYARD. Methods employed to capture insects (entomological net and sticky traps) allowed an accurate monitoring of the entomofauna present in the examined vineyard. In detail, during field activities, 1100 insect specimens were collected (717 by net and 383 by sticky traps). Stereomicroscope analysis, based on observation of morphological characters, allowed the identification of 42 distinct taxonomic groups. Based on such evidence, it was possible to classify the majority of captured specimens at species level (28); however, other taxonomic groups were defined only at genus level (16) (Table 1). Further studies will be carried out to describe such groups at species level by integrating the morphological approach with the utilization of the DNA-barcoding techniques.

PHYTOPLASMA IDENTIFICATION. PCR-based amplification of *stamp* gene revealed the presence of BNp in 64 out of 624 analyzed insect pools, belonging to 20 taxonomic groups (15 species and 5 genera) (Table 1).

Interestingly, some of the insects harboring BNp in the present work have been found in previous studies as vectors of other phytoplasmas (i.e. *Fieberiella florii for* '*Ca*. P. mali' and *Scaphoideus titanus* for Flavescence dorée phytoplasma) (WEINTRAUB und BEANLAND, 2006) and/or other bacterial plant pathogens (i.e. *Philaenus spumarius* for *Xylella fastidiosa*) (SAPONARI et al., 2014). Moreover, molecular detection analysis confirmed the presence of BNp in other insects, such as *Euscelis* spp., recently found infected by BNp in vineyards in South Italy (OLIVERI et al., 2015). In the last few years, multiple gene analysis was proposed and employed to describe phytoplasma species distinguished by evident molecular diversity and representing ecologically separated populations. Moreover, this approach was also applied to the investigation of the genetic diversity among phytoplasmas associated with several diseases in order to identify strain-specific molecular markers useful for improving the understanding of complex phytoplasma ecologies (QUAGLINO et al. 2013). To determine the possible vectoring activity of insects found BNp-infected in Franciacorta, further analyses will be carried out to

Table 1. Detection of '*Ca*. P. solani' in insects captured in BN-affected vineyard in Gussago (BS)

Insect (species)	No. of tested pools		Infection %
	collected	BNp-infected	
Allygidius furcatus	24	2	8
Aconurella prolixa	1	-	-
Anoplotettix fuscovenosus	12	1	8
Aphrodes makarovi	21	5	24
Asiraca clavicornis	6	2	33
Caliscelis bonellii	7	-	-
Centrotus corutus	1	-	-
Cercopis vulnerata	120	2	2
Cicadella viridis	35	6	17
Dictyophara europaea	47	-	-
Eupteryx vittata	2	1	50
Evacanthus acuminatus	3	2	67
Fieberiella florii	5	1	20
Goniagnathus brevis	5	-	-
Haematoloma dorsatum	7	-	-
Hephathus nanus	25	-	-
Hishimonus hamatus	4	-	-
Hyalesthes obsoletus	44	13	30
Hyalesthes scotti	6	-	-
Japananus hyalinus	2	-	-
Laodelphax striatella	56	5	9
Metcalfa pruinosa	35	9	26
Mocydia crocea	2	-	-
Neoaliturus fenestratus	2	1	50
Philaneus spumarius	28	2	7
Scaphoideus titanus	1	1	100
Stictocephala bisonia	1	-	-
Toya propinqua	9	-	-

Insect (genus)	No. of tested pools		Infection %
	collected	BNp-infected	
Balcanocerus spp.	5	-	-
Cixus spp.	2	-	-
Dicraneura spp.	1	-	-
Dicranotropis spp.	15	2	13
Empoasca spp.	1	1	100
Euscelis spp.	46	5	11
Kelisia spp.	2	-	-
Macrolestes spp.	4	-	-
Macropsis spp.	6	-	-
Megophthalmus spp.	4	2	50
Mocydiopsis spp.	2	-	-
Psammotettix spp.	12	-	-
Reptalus spp.	2	-	-
Thamnotettix spp.	5	1	20
Typhlocyba spp.	5	-	-
Verdanus spp.	1	-	-

(i) characterize the BNp strains identified in such insects by nucleotide sequence analysis of stamp gene and other genes (*tuf, vmp*1) and comparison with sequences of strains identified in grapevines and weeds, (ii) determine the transmission capability of insects harboring BNp strains genetically identical to those present in grapevine by trials performed in controlled conditions.

ACKNOWLEDGMENTS

This study was supported by the project "Epidemiologia di Legno nero (LN) in Franciacorta: base per lo sviluppo e la messa a punto di metodi di contenimento della malattia", funded by Consorzio per la Tutela del Franciacorta.

REFERENCES

CVRKOVIĆ, T., JOVIĆ, J., MITROVIĆ, M., KRSTIĆ, O., und TOŠEVSKI, I. 2014. Experimental and molecular evidence of *Reptalus panzeri* as a natural vector of bois noir. Plant Pathology 63: 42-53.

FABRE, A., DANET, J.-L., und FOISSAC, X. 2011. The stolbur phytoplasma antigenic membrane protein gene *stamp* is submitted to diversifying positive selection. Gene 472: 37-41.

KOSOVAC, A., RADONJIĆ, S., HRNČIĆ, S., KRSTIĆ, O., TOŠEVSKI, I., und JOVIĆ, J. 2015. Molecular tracing of the transmission routes of bois noir in Mediterranean vineyards of Montenegro and experimental evidence for the epidemiological role of *Vitex agnus-castus* (Lamiaceae) and associated Hyalesthes obsoletus (Cixiidae). Plant Pathology doi:10.1111/ppa.12409.

LANGER, M., und MAIXNER, M. 2004. Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur-group based on RFLP-analysis of non-ribosomal DNA. Vitis 43: 191-200.

MORI, N., QUAGLINO, F., TESSARI, F., POZZEBON, A., BULGARI, D., CASATI, P., und BIANCO, P. A. 2015. Investigation on 'bois noir' epidemiology in north-eastern Italian vineyards through a multidisciplinary approach. Annals of Applied Biology 166: 75-89.

OLIVERI, C., PACIFICO, D., LA ROSA, R., MARZACHÌ, C., und TESSITORI, M. 2015: Bois noir phytoplasma variability in a Mediterranean vineyard system: new plant host and putative vectors. Australasian Plant Pathology 44: 235-244.

PINZAUTI, F., TRIVELLONE, V., und BAGNOLI, B. 2008. Ability of *Reptalus quinquecostatus* (Hemiptera: Cixiidae) to inoculate stolbur phytoplasma to artificial feeding medium. Annals of Applied Biology 153: 299-305.

QUAGLINO, F., ZHAO, Y., CASATI, P., BULGARI, D., BIANCO, P.A., WEI, W., und DAVIS, R.E. 2013. '*Candidatus* Phytoplasma solani', a novel taxon associated with stolbur and bois noir related diseases of plants. International Journal of Systematic and Evolutionary Microbiology 63: 2879-2894.

RIEDLE-BAUER, M., SÁRA, A., und REGNER, F. 2008. Transmission of a stolbur phytoplasma by the Agalliinae leafhopper *Anaceratagallia ribauti* (Hemiptera, Auchenorrhyncha, Cicadellidae). Journal of Phytopathology 156: 687-690.

SAPONARI, M., LOCONSOLE, G., CORNARA, D., YOKOMI, R., DE STRADIS, A., BOSCIA, D., BOSCO, D., MARTELLI, G.P., KRUGNER, R., und PORCELLI, F. 2014: Infectivity and transmission of *Xylella fastidiosa by Philaenus spumarius* (Hemiptera: Aphrophoridae) in Apulia, Italy. Journal of Economic Entomology 107: 1316-1319.

WEINTRAUB, P.G. und BEANLAND, L. 2006: Insect vectors of phytoplasmas. Annual Review of Entomology 51: 91-111.

TOWARDS A *DE NOVO* GENOME ASSEMBLY OF *HYALESTHES OBSOLETUS* (CIXIIDAE) OF THE STINGING NETTLE HOST-RACE

Jes Johannesen[1], Michael Maixner[2], Hanno Schmidt[3]

[1]Institute of Zoology, University of Mainz, Johann-Joachim-Becherweg 13, 55128 Mainz Germany, jesjo@uni-mainz.de
[2]Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, 76833 Siebeldingen, Germany michael.maixner@jki.bund.de
[3]Senckenberg Biodiversity and Climate Research Centre (BiK-F) Georg-Voigt-Straße 14-16 60325 Frankfurt am Main, Germany, hschmidt@senkenberg.de

Next generation sequencing has made *de novo* genome sequencing of non-model organisms possible at comparatively low costs. Genome sequencing of field caught whole specimens represents meta-genomes, which include the genome of the target species as well as the genomes of the associated microorganisms. This poses both a liability and an advantage for research of the target organism. The liability is false inclusion of non-target sequences into the *de novo* assembly of the target species, while the advantage is the additional information obtained on the microorganisms associated therewith. Hemipteran species in particular have a range of endosymbiont bacteria. These include primary, essential symbionts, which typically assist in dietary processes and may determine the diversity of host-plant utilisation, and secondary, facultative symbionts. Facultative symbionts may impact the host negatively, neutrally or positively depending on the associations between them.

The present note reports results of an initial contig assembly based on mate pair reads of the planthopper *Hyalesthes obsoletus*, vector of the Bois noir pathogen *Ca.* Phytoplasma solani (stolbur), and a Blastn search for associated microorganisms. The reference genome will serve to study hereditary (genetic) versus phenotypic (state dependent) components of the ability to acquire and transmit stolbur in *H. obsoletus*. Although it is known that stolbur is acquired by *H. obsoletus* nymphs when feeding on the roots of infected plant hosts, there neither is information about individual variation in the ability to acquire the pathogen nor is there information about interactions between the pathogen and the vector's ability to transmit the pathogen.

MATERIAL AND METHODS

The assembly was performed on DNA obtained from males of the *H. obsoletus* host race associated with *Urtica dioica* (stinging nettle) sampled at Bernkastel-Kues (Moselle), Germany. The *U. dioica* host race is responsible for the majority of Bois noir outbreaks in Germany in recent years. Sequencing was done with Illumina Nextseq 500 technology (sequence length 150bp) and included paired end and mate pair reads (3kb and 8kb inserts). Paired end sequences were obtained from one male. The mate pair reads were obtained from a pool of 10 males. Quality processing was done with the Fastqc and Fastx packages. First, the 5'-ends of raw reads were cut for 8-10bp, adapters clipped, followed by quality trimming and quality filtering. Quality cut off for 3'-trimming was set to 20. Quality filtering was used to discard all reads with less than 80 % bases with quality $\geq$ 20. The presented assembly, based on paired ends only, was done with SOAPdenovo2 (kmer=63) (LOU et al. 2012). The contig assembly was converted to a Blast database for further analysis.

RESULTS AND DISCUSSION

Illumina Nextseq 500 sequencing generated c. 350 Mio. reads. The preliminary de novo assembly based on paired end reads presently has 1.82 Mio contigs of lengths > 500bp and c. 325,000 contigs of lengths of > 1,500bp. The longest contig is 45,000bp. The total number of letters and sequences in the generated database are 3,500 Mio and 22 Mio, respectively. As of writing, an assembly using mate pairs and scaffolding has not been performed. Blastn search for bacterial endosymbionts successfully identified five species known for *H. obsoletus* (BRESSAN et al. 2009; GONELLA et al. 2011) (Table 1). Stolbur was not present in the assembly. This is not surprising as the rate of stolbur infection in German *H. obsoletus* nettle populations typically is 10-15 percent (MAIXNER & JOHANNESEN 2012) and the sequenced individual was assigned for DNA quality rather than stolbur testing prior to sequencing. We blasted 16S of further seven endosymbiotic bacteria and of one spiroplasma known to be associated with Hemiptera. None of these species were found in the assembly (Table 1). It should be noted that

Table 1. Endosymbiont search in contig assembly of *H. obsoletus*. The assembly was based on one male individual. Gene refers to the genes used for search. Insect host is the DNA source of the bacterial endosymbiont used for the search analysis. Sequence length refers to the length of the analysed (published) sequence. Identity value np = not present.

Species	Gene	Insect Host[1]	Present	Sequence length	Identity %	Genbank accession no.
Wolbachia pipientis	wsp	Ho	Yes	501	100	JJ[2]
Cardinium hertigii	16S	Ho	Yes	360	100	JJ[2]
Purcelliella pentastirinorum	16S	Ho	Yes	1415	100	FN428799.1
Vidania fulgoroidae	16S	Ho	Yes	1403	99	FR686932.1
Sulcia muelleri	16S	Ho	Yes	694	100	JJ[2]
Phytoplasma solani	SecY/Stamp	Ho	No	827/489	np	JQ977707.1/ JQ977713.1
Baumannia cicadellinicola	16S	Pm	No	1391	np	AY676896.1
Rickettsia sp.	16S/gltA/Sca1	Mp	No	1422/708/458	np	HE583202.1/ FJ766354.1/ FJ766355.1
Buchnera aphidicola	16S	Ap	No	1460	np	NR074159.1
Arsenophorus sp.	23S	Bm	No	469	np	FJ766372.1
Hamiltonella spp.	GyrB/16S	Bm/Am	No	814/2254	np	FJ766343.1 KF835614.1
Fritschea sp.	16S	Bm	No	1319	np	JQ009299.1
Phlomobacter fragariae	16S	?	No	1462	np	PFU91515
Spiroplasma sp.	16S	Om	No	1439	np	AB775906.1

[1] Ho = *Hyalesthes obsoletus*, Pm = *Paromenia isabellina*. Mp = *Macrolophus pygmaeus*, Ap = *Acyrthosiphon pisum*, Bm = *Bemisia tabaci*, Am = *Aphis mendocina*,? = not reported but *Cixius wagneri* is a known host, Om = *Orius minutus*, [2] JJ = unpublished data from Sanger sequencing of German *H. obsoletus*

environmental 16S sequencing might be preferred to assess the endosymbiont diversity but the initial results presented here were performed to assess data quality for detecting metagenomics sequences.

Although Euhemipteran species are important vectors of a range of plant diseases, there are only eight species with published (NCBI Genbank) genome assemblies, of which only one (*Nilaparvata lugens*) belongs to Archaeorrhyncha. Genome sizes vary considerably, from 500 Mb in *Diaphorina citri* (Asian citrus psyllid) to 2,200 Mb in *Homalodisca vitripennis* (glassy-winged sharpshooter). At present, it is not possible to predict the genome size of *H. obsoletus* from the preliminary contig assembly but it might be around 1 Gb as in the brown planthopper *Nilaparvata lugens* (c. 1,1 Gb). The next steps in this study are inclusion of mate pairs for scaffolding, parameter and re-assembly optimisation followed by filtering of endosymbionts from the assembly.

REFERENCES

BRESSAN, A., ARNEODO; J., SIMONATO, M., HAINES, M. and BOUDON-PADIEU, E. 2009: Characterization and evolution of two bacteriome-inhabiting symbionts in cixiid planthoppers (Hemiptera: Fulgoromorpha: Pentastirini). Environmental Microbiology, 11: 3265-3279

GONELLA, E., NEGRI, I., MARAZORATI, M., MANDRIOLI, M., SACCHI, L., ET AL. 2011: Bacterial Endosymbiont Localization in *Hyalesthes obsoletus*, the Insect Vector of Bois Noir in Vitis vinifera, Applied and Environmental Microbiology, 77: 1423-1435

LUO R., LIU, B., XIE, Y., Li, Z., HUANG, W., ET AL. 2012: SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. GigaScience 1:18, doi:10.1186/2047-217X-1-18

MAIXNER, M. and JOHANNESEN, J. 2012: Spatial and temporal distribution of the infection potential of Stolbur phytoplasma on a fallow vineyard. Proceedings of the 17th Congress of ICVG, Davis, California, USA. Pp. 232-233

MICROBIOMES OF THE "*CANDIDATUS* PHYTOPLASMA SOLANI" VECTORS *HYALESTHES OBSOLETUS* SIGNORET ISOLATED FROM DIFFERENT HOST PLANTS

Julien Chuche[1], Jean-Luc Danet[2], Sébastien Theil[2], Xavier Foissac[2], Denis Thiéry[1] and Nathalie Arricau-Bouvery[2]

[1] INRA, UMR SAVE, ISVV, Bordeaux Sciences Agro
FR-33883 Villenave d'Ornon
[2] INRA, UMR BFP, Université de Bordeaux
FR-33883 Villenave d'Ornon
E-Mail: nbouvery@bordeaux.inra.fr

Because of the potential impact of bacterial symbionts on the adaptation of hemipteran insects to plant hosts, we characterized the microbial community present in various populations of *H. obsoletus* by pyrosequencing. Analysis of the V4V5 variable region of the 16S rDNA showed that insects sampled on *Lavandula* and *Salvia* possessed a bacterial sequence 100 % identical to the 16S rDNA of an endosymbiont of *Irenimus aequalis*. As shown by the two mtDNA markers 16S rRNA-tRNALeu-ND1 and COI-tRNALeu-COII, *Hyalesthes* specimen from *Lavandula* were genetically different from those of *Salvia* as it was the case between specimen from *Urtica* compared to specimen from *Convolvulus*. Insects sampled from *Lavandula* and *Salvia* were also significantly smaller than those sampled from *Urtica* and *Convolvulus.*

Hyalesthes obsoletus is one of the Cixiidae capable of transmitting *'Candidatus* Phytoplasma solani' to cultivated and wild plants, the latter frequently serve as sources of inoculum for crops. In France, *'Ca.* P. solani' is the agent of Bois noir in vineyards, stolbur in solanaceous plants, and lavender decline (Boudon-Padieu, 2003 and 2005). *H. obsoletus* spreads different strains of the *'Ca.* P. solani' belonging to three *tuf* genotypes (Langer and Maixner, 2004). In France, *H. obsoletus* can fully complete its lifecycle on *Convolvulus* arvensis, *Lavandula angustifolia* and *Urtica dioica* (Sforza et al., 1999; Johannesen et al., 2008). All these populations seem to correspond to a single species despite their specialization on different host plants (Maixner, 2007). However, *H. obsoletus* populations from lavender have not been characterized yet, and a novel plant host (*Salvia sclarea*) has been found in the South-East of France (Chuche et al., 2013).

Many symbionts of insects have been described so far and are thought to contribute substantially to the evolutionary and ecological success of insects in broad ecosystems. By their metabolic potential, endosymbionts help insects to feed on imbalanced food resources such as phloem sap (Moya et al., 2008). For the pea aphid, host plant specialization is related both to chromosomal loci of the aphid and facultative endosymbiotic bacteria (Simon et al., 2003; Leonardo and Muiru, 2003; Tsuchida et al., 2004). Symbiotic bacteria can also have a positive effect on pathogen transmission, vector susceptibility to natural enemies and insecticide resistance, or a negative effect such as sex-ratio disturbance. Some endosymbionts have been identified in the Cixiidae (Bressan and Mulligan, 2013), and *Hyalesthes* in particular (Gonella et al., 2011). But while plant specialization has been attributed to chromosomal loci of *H. obsoletus* (Johannesen et al., 2008; Kosovac et al., 2013), the role of their endosymbiotic bacteria on this specialization is not known yet.

Therefore, the goal of this study is to better characterize *H. obsoletus* in relation to the host plant

in the light of its genetic variability and symbiont composition. The genomic variability of insects was analyzed with mitochondrial genetic (mtDNA) markers to test for differentiation among host-plant populations. We used two mtDNA markers previously analysed by Johannesen et al. (2008): 16SrRNA-tRNALeu-ND1 and COI-tRNALeu-COII. The microbiome of sap feeding vectors can be characterized using next generation sequencing approaches (Hail et al., 2012; Jing et al., 2014). Our study also aimed to provide a rDNA-targeted metagenomic study of *Hyalesthes* bacteria isolated from different plant host. Due to its reproducibility and broad range, we chose sequencing the variable region V4-V5 of 16S rRNA gene (Claesson et al., 2010).

MATERIALS AND METHODS

HYALESTHES OBSOLETUS SAMPLING AND OBSERVATION

Insects were collected in France on *Urtica dioica, Salvia sclarea* and *Lavandula angustifolia*, and, in Germany on *Convolvulus arvensis* (M. Maixner).
The size of insect individuals (20 insects per population) were measured under a stereo microscope.

DNA EXTRACTION, AMPLIFICATION AND PYROSEQUENCING

Insect DNA was extracted as described in Maixner et al. (1995). For each plant host, DNA from twenty insects per genus was extracted, quantified using an Epoch spectrophotometer (BioTek Instruments, Inc), pooled and stored at -20 °C until being used for amplification. The V4-V5 variable region of bacterial 16S rDNA was amplified using the primers V4-forward (5'-AYTGGGYDTAAAGNG) and V5-reverse (5'-CCGTCAATTYYTTTRAGTTT) (Claesson et al., 2010), and sequenced using pyrosequencing technology (Genomic and Transcriptomic Facility of Bordeaux).

DATA PROCESSING OF SEQUENCING READS

In total, between 723190 and 1060765 paired end reads were generated for insect samples. Taxonomic identifications were performed according to the pipeline established by USEARCH and Qiime. First, reads were pre-processed, thus sequences were quality trimmed and cleaned from remaining sequencing adapters. Overlapping pairs were assembled to create longer sequences and exactly duplicated sequences and singletons have been filtered out (USEARCH). Remaining sequences have been clustered into OTUs (Operational Taxonomy Unit) with the Bayesian classifier CROP, at a 5 % dissimilarity. Chimeras were excluded by comparison with the 'Gold' database from the Broad Institute. Using Qiime scripts, remaining sequences were then Blast against the GreenGenes 16S database to produce a taxonomic assignation and a global OTU table. The OTU table has been manually curated to remove chimera, duplicated OTU, and low coverage clusters.

MITOCHONDRIAL VARIABILITY STUDIES OF *H. OBSOLETUS*

Genomic DNA of individual insects was analyzed after sequencing the two mtDNA markers CO I-tRNA(Leu)-CO II (CO I) and 16S-tRNA(Leu)-ND1 (16S) as described by Johannesen et al. (2008).

RESULTS

MORPHOLOGICAL DIVERSITY OF *H. OBSOLETUS*

Whatever the plant host, females were systematically taller than males (Fig. 1). Females sampled on *L. angustifolia* and *S. sclarea* were about 20 % smaller than the ones from *C. arvensis* and *U. dioica* and males were about 15 % smaller. This clearly demonstrates that phenotypes of *H. obsoletus* vary according to the host plant.

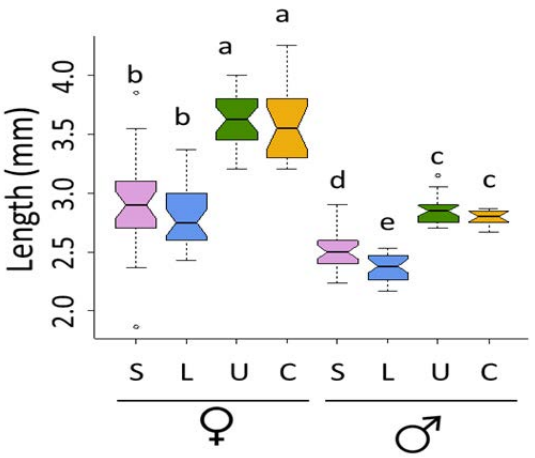

Fig. 1: Length of *H. obsoletus* females and males sampled on *Salvia sclarea* (S), *Lavandula angustifolia* (L), *Urtica dioica* (U) and *Convolvulus arvensis* (C). Statistical differences were measured using the Kruskal-Wallis test ($p < 1$ %).

GENETIC DIFFERENTIATION OF H. OBSOLETUS REVEALED BY MTDNA POLYMORPHISM

To correlate morphological observations with genotypes of *H. obsoletus*, we analysed the genetic diversity of the four populations using mitochondrial markers. The mitochondrial haplotypes are shown in Table 1. All insects sampled from *Salvia* or *Lavandula* showed the same mtDNA haplotype dependent on the host plant. Haplotype diversity was highest for insects sampled from *Convolvulus.*

IDENTIFICATION OF H. OBSOLETUS ASSOCIATED BACTERIA

On average, a total of 431,815 sequences were generated from the different *H. obsoletus* populations in relation with host plants and 0.7 % of the sequences did not find homology in the sequence databanks (Greengene and BLASTn). The overall bacterial diversity was estimated between 8 and 18 genera (Fig. 2).

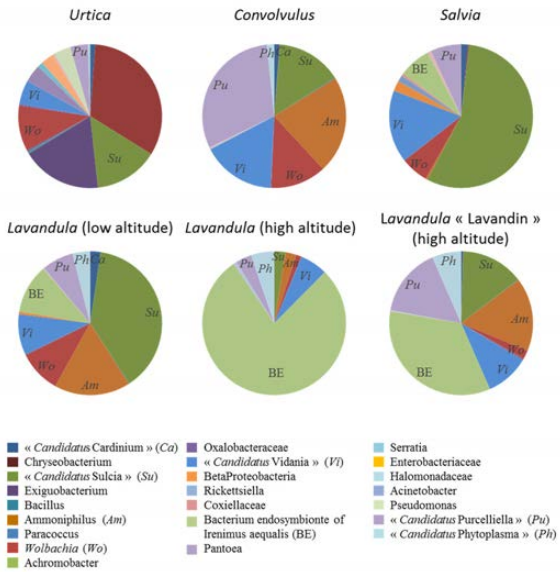

Fig. 2: Relative abundance of OTUs found in *Hyalesthes obsoletus* collected on *Urtica dioica*, *Convolvulus arvensis*, *Salvia sclarea* and *Lavandula angusti*folia (Genera corresponding to more than 0.1 % are shown).

Gamma Proteobacteria contain the higher diversity of bacterial genera among the genera associated to the OTUs. *'Ca*. Purcelliella', *'Ca*. Sulcia', '*Ca*. Vidania' and *Wolbachia* were the most dominant genus associated with *H. obsoletus* and were found in all populations studied. Figure 2 also shows that differences can be observed between host plants. In insects sampled on *Lavandula* cultivated at high altitudes the most abundant OTU was identical to the 16S rDNA of an *Irenimus aequalis* bacterium (GenBank: KJ494864.2). OTUs corresponding to this bacterium were also found in H. *obsoletus* from *Salvia*, but not in insects collected on *Urtica* and *Convolvulus.* The data also show that we were able to detect '*Ca.* Phytoplasma' in all *H. obsoletus* populations except in insects from *Salvia.*

DISCUSSION

The relationship between insect phenotype/haplotype and their host plants is complex. Mitochondrial DNA analysis showed different profiles depending on the host plant and the localization. Our study confirms that the *Urtica Hyalesthes* population differs slightly between years. Indeed, the population of *Urtica H. obsoletus* used in this study was sampled in 2013 from Alsace, a place close to Turckheim from which the population studied by Johannesen was sampled in 2006 (Johannesen et al., 2008). In both cases the mtDNA haplotypes were the same (aa) except for one new haplotype in our case. In the study of Johannesen and collaborators, the population of *Hyalesthes* sampled from *Urtica* in Germany showed the same haplotypes during two consecutive years. By the same time this haplotype was different from those found from *Urtica H. obsoletus* sampled in the South East of France in 2006 (Johannesen et al., 2008). It seems that geographical localization rather than the host plant is responsible for their difference in mitochondrial markers. However, insects sampled in the same area but from *Salvia* or *Lavandula* showed different haplotypes.

To complete the description of *Hyalesthes* and because of the importance of symbionts during insect life, we wanted to characterize the entire microbial community present in *H. obsoletus* sampled from different host plants. The primers used might

not necessarily amplify the 16S rRNA gene of the whole community. For example, it is known that *Chlamydia*-related bacteria have a divergent 16S sequence that could be missed with the consensus primer (Lienard et al., 2011). One goal of this study was to compare the bacteria associated with insects in relation to their host plant. The number of abundant OTUs in these vectors vary between 7 and 11, which is consistent with the composition of the microbiome of phloem feeding insects previously estimated (< 10 bacteria OTUs) (Jing et al., 2014).
The conserved OTUs between all the *Hyalesthes* populations represented the bacterial genera '*Ca*. Sulcia', '*Ca.* Purcelliella'and *'Ca.* Vidania'. These were also found by Gonella et al. using sequencing of 16S rDNA amplified fragments that were separated by denaturing gradient gel electrophoresis (Gonella et al., 2011). These bacteria are supposed to be obligate symbionts. *'Ca.* Sulcia' is besides *'Ca*. Nasuia' (Bennett et al., 2014) one of the two described symbiont genera associated with Auchenorrhyncha (Hemiptera).These two symbionts have the ability to synthetize essential amino acids that are not found in the sap of plants hosts (McCutcheon and Moran, 2010).
Also *Wolbachia* and *'Ca.* Cardinium' were found in all populations of *Hyalesthes* tested here, and were present in half of the tested insects of the study by Gonella et al. (2011). *Wolbachia* and '*Ca.* Cardinium' are interesting symbionts, as it has been shown that they manipulate the reproduction of their insect host (Ma et al., 2014). Further studies are needed to characterize these two symbiont populations more specifically.

ACKNOWLEDGEMENT

We thank Michael Maixner for providing gDNA of *Hyalesthes* sampled from *Convolvulus*. Part of the experiments was performed at the Genomic and Transcriptomic Facility of Bordeaux (grants from the Conseil Regional d'Aquitaine n°20030304002FA and 20040305003FA and from the European Union, FEDER n°2003227 and from Investissements d'avenir, Convention attributive d'aide N°ANR-10-EQPX-16-01). This work was funded by INRA grant from the department of Plant Health and Environment (SPE 2014) and the Structure Fédérative de Recherche 'Biologie Intégrative et Ecologie' (University of Bordeaux).

REFERENCES

Bressan, A., and Mulligan, K.L. (2013) Localization and morphological variation of three bacteriome-inhabiting symbionts within a planthopper of the genus *Oliarus* (Hemiptera: Cixiidae). Environ Microbiol Rep 5: 499–505.

Chuche, J., Yvin, C., Rivoal, J.-B., Danet, J.L., and Thiéry, D. (2013) *Salvia sclarea* (Lamiaceae), new host plant of the stolbur vector *Hyalesthes obsoletus* (Hemiptera: Cixiidae). OILBSROP Work Group "Integrated Prot Prod Vitic Ascona Switz 13-17 October.

Table 1: Genetic polymorphism of *H. obsoletus* using mtDNA markers.

Locality	Country	Host plant	Year	Number of insects	Number of mtDNA haplotypes (CO I - 16S)*			
					aa	ab	bb	new
Haut-Rhin	France	*Urtica*		10	8		1	1 (nd$^{\$}$-p)
Rhineland-Palatinate	Germany	*Convolvulus*		10			5	2 (δ-b) 2 (ε-b) 1 (ζ-b)
Alpes Hautes Provence	France	*Salvia*		14			14	
Vaucluse	France	*Lavandula*		9		9		

* CO I: CO I-tRNA(Leu)-CO II DNA region, ; 16S: ribosomal RNA (16S)-tRNA(Leu)-ND1 DNA region

$^{\$}$ nd: not determined

Claesson, M.J., Wang, Q., O'Sullivan, O., Greene-Diniz, R., Cole, J.R., Ross, R.P., and O'Toole, P.W. (2010) Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. Nucleic Acids Res 38: e200–e200.

Gonella, E., Negri, I., Marzorati, M., Mandrioli, M., Sacchi, L., and Pajoro, M. (2011) Bacterial endosymbiont localization in *Hyalesthes obsoletus*, the insect vector of Bois noir in Vitis vinifera. Appl Environ Microbiol 77: 1423–1435.

Hail, D., Dowd, S.E., and Bextine, B. (2012) Identification And Location Of Symbionts Associated With Potato Psyllid (*Bactericera cockerelli*) Lifestages. Environ Entomol 41: 98–107.

Jing, X., Wong, A.C.-N., Chaston, J.M., Colvin, J., McKenzie, C.L., and Douglas, A.E. (2014) The bacterial communities in plant phloem-sap-feeding insects. Mol Ecol 23: 1433–1444.

Johannesen, J., Lux, B., Michel, K., Seitz, A., and Maixner, M. (2008) Invasion biology and host specificity of the grapevine yellows disease vector *Hyalesthes obsoletus* in Europe. Entomol Exp Appl 126: 217–227.

Kosovac, A., Johannesen, J., Krstić, O., Mitrović, M., Cvrković, T., and Maiden, M.C.J. (2013) Microsatellite and mtDNA evidence for genetic differentiation of *Hyalesthes obsoletus* populations associated with a new major host, stinking hawk's-beard (*Crepis foetida*), in Southeast Europe. 3rd Eur Bois Noir Workshop Barc Spain ICEA 18–19.

Leonardo, T.E., and Muiru, G.T. (2003) Facultative symbionts are associated with host plant specialization in pea aphid populations. Proc Biol Sci 270 Suppl 2: S209–212.

Lienard, J., Croxatto, A., Aeby, S., Jaton, K., Posfay-Barbe, K., Gervaix, A., and Greub, G. (2011) Development of a New Chlamydiales-Specific Real-Time PCR and Its Application to Respiratory Clinical Samples. J Clin Microbiol 49: 2637–2642.

Maixner, M. (2007) Biology of *Hyalesthes obsoletus* and approaches to control this soilborne vector of Bois noir disease. IOCBWPRS Bull 30: 3–9.

Maixner, M., Ahrens, U., and Seemüller, E. (1995) Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. Eur J Plant Pathol 101: 241–250.

Ma, W.-J., Vavre, F., and Beukeboom, L.W. (2014) Manipulation of Arthropod Sex Determination by Endosymbionts: Diversity and Molecular Mechanisms. Sex Dev 8: 59–73.

McCutcheon, J.P., and Moran, N.A. (2010) Functional Convergence in Reduced Genomes of Bacterial Symbionts Spanning 200 My of Evolution. Genome Biol Evol 2: 708–718.

Moya, A., Peretó, J., Gil, R., and Latorre, A. (2008) Learning how to live together: genomic insights into prokaryote-animal symbioses. Nat Rev Genet 9: 218–229.

Sforza, R., Bourgoin, T., Wilson, S., and Boudon-Padieu, E. (1999) Field observations, laboratory rearing and descriptions of immatures of the planthopper (Hemiptera: Cixiidae). EJE 96: 409–418.

Simon, J.-C., Carré, S., Boutin, M., Prunier-Leterme, N., Sabater-Mun, B., Latorre, A., and Bournoville, R. (2003) Host-based divergence in populations of the pea aphid: insights from nuclear markers and the prevalence of facultative symbionts. Proc R Soc B Biol Sci 270: 1703–1712.

Tsuchida, T., Koga, R., and Fukatsu, T. (2004) Host plant specialization governed by facultative symbiont. Science 303: 1989.

HETEROLOGOUS EXPRESSION AND ANTIGENICITY OF STAMP ANTIGENIC MEMBRANE PROTEINS FROM DIFFERENT '*CANDIDATUS* PHYTOPLASMA SOLANI' GENETIC CLUSTERS

Ibolya Ember[1], Delphine Desque[2,3], Jean-Luc Danet[2,3], Marie-Pierre Dubrana, Sybille Duret[2,3], Laure Beven[2,3], László Palkovics[4], György Dénes Bisztray[1] and Xavier Foissac[2,3]

[1]Szent István University, Faculty of Horticultural Science, Institute of Viticulture and Oenology, Department of Viticulture, Villányi str. 29-43, 1118 Budapest, Hungary
[2]INRA, UMR 1332 Biologie du Fruit et Pathologie, 71 avenue Edouard Bourlaux, CS20032 F-33882 Villenave d'Ornon, Cedex, France
[3]Université de Bordeaux, UMR 1332 Biologie du Fruit et Pathologie, 71 avenue Edouard Bourlaux, CS20032, F-33882 Villenave d'Ornon, Cedex, France
[4]Szent István University, Faculty of Horticultural Science, Department of Plant Pathology Villányi str. 29-43, 1118 Budapest, Hungary

Little is known about the mechanisms of insects - phytoplasma interactions which are driving the ecological diversification of phytoplasmas. Phytoplasma surface proteins play an important role in phytoplasma life cycle and polymorphism of this protein might determine the transmission ability of different insect species. Strains of '*Candidatus* Phytoplasma solani' from several European countries, based on variable membrane protein gene (*stamp*), were classified into four distinct phylogenetic clusters which clusters are present in different geographical areas. This may reflect the distribution of the different known insect vectors and the adaptation of the various '*Ca*. P. solani' strain to a specific vector. To detect STAMP protein, monoclonal antibody 2A10 is available. This antibody was produced against StolburC, which is a representative strain of *stamp* clusters I. In this work we performed heterologous expression antigenic membrane proteins STAMP from different '*Ca*. P. solani' genetic clusters and tested their recognition by the 2A10 monoclonal antibody.

Control of phytoplasma diseases is based on prophylaxis. It is crucial to trace the spread of phytoplasma strains and predict their epidemic potential when introduced into an ecological niche. One of the major devastating Grapevine Yellows (GY) in Europe is the Bois noir (BN) disease caused by '*Candidatus* Phytoplasma solani' (QUAGLINO et al., 2013). '*Ca*. P. solani' (Stolbur phytoplasma) is endemic to the Euro-Mediterranean area and is of wild plant origin. It is transmitted from bindweed (*Convolvulus arvensis)* and stinging nettle (*Urtica dioica*) to grapevine (*Vitis vinifera*) and to other crops by different polyphagous planthoppers (*Fulgoromorpha*) of the *Cixiidae* family. In Europe, at least 4 cixiids are vectoring '*Ca.* P. solani' : *Hyalesthes obsoletus, Pentastiridius leporinus, Reptalus panzeri* and *R. quinquecostatus* (FOS et al., 1992; MAIXNER, 1994; SFORZA et al., 1998; GATINEAU et al., 2001; JOVIC et al., 2007; CVRKOVIC et al., 2013; DANET et al., personal comm.). Ecotypes of *H. obsoletus* are vectoring specific '*Ca*. P. solani' genotypes and are related to the plant hosts stinging nettle or bindweed (LANGER and MAIXNER, 2004; ARYAN et al., 2014).

Little is known about the mechanisms of insect-phytoplasma interactions, which are driving the ecological diversification of phytoplasmas. Insect-transmissible pathogens can be transmitted by a particular insect species and not by others. This is due to highly specific interactions between insects and these bacterial pathogens (SUZUKI et al., 2006; GALETTO et al., 2011). Phytoplasma surface proteins play an important role in the phytoplasma life cycle and it has been demonstrated that the antigenic membrane protein (Amp) of '*Candidatus* Phytoplasma asteris' strain OY is in correlation with the phytoplasma-transmission capability of leafhoppers (SUZUKI et al., 2006). The gene encoding for the Stolbur antigenic membrane protein (*stamp*) is the ortholog of *amp.* STAMP is a 16 kDa antigen and likely the most abundant surface protein of 'Ca. P. solani' (FABRE et al., 2011b).

Monoclonal antibodies (2A10 MAb) against a Stolbur phytoplasma (strain StolburC), isolated from field infected tomato plants in France, were produced in mouse spleen hybridoma cells (GARNIER et al., 1990). The specificity of 2A10 MAb was confirmed by immunofluorescence and ELISA (GARNIER et al., 1990, FOS et al., 1992). Additionally, *in situ* immunofluorescence detection demonstrated that the MAb 2A10 recognizes also STAMP of the Stolbur phytoplasma strain PO (FABRE et al., 2011b). Genotyping of the strains StolburC and PO revealed that both belong to STAMP cluster I, one of four clusters identified among strains collected in the Mediterranean Basin (FABRE et al., 2011b).

In European countries BN disease is associated with different strains of '*Ca*. P. solani'. According to the *stamp* genotyping survey we did in Hungary, the most prevalent strains (ST4 and ST9) were clustered into the phylogenetic cluster II. In this work we aimed to perform heterologous expression of STAMP from different '*Ca.* Phytoplasma solani' genetic clusters and test antigenicity of the MAb 2A10.

MATERIALS AND METHODS

To test antigenicity for 2A10 monoclonal antibody STAMP proteins were obtained by two approaches, performing: i) phytoplasma total protein extraction from strains of '*Ca*. P. solani' maintained in *Catharanthus roseus* (periwinkle) of genotypes belonging to ST-I, ST-II, ST-III and ST-IV clusters; ii) heterologous expression of STAMP ST-I, ST-II, ST-III and ST-IV.

i)Proteins were extracted form phytoplasma strains maintained on *C. roseus* (Polka dot hybrid) listed in Table 1. Leaf midribs were excised (0.7 g) and ground in one volume of Laemmli buffer (0.1 % 2-mercaptoethanol, 0.0005 % bromophenol blue, 10 % glycerol, 2 % SDS, 63 mM Tris-HCl, pH 6.8) in a precooled mortar. Extracts were centrifuged for 1 min at 10000 rpm at 4°C. Supernatants were transferred into new Eppendorf tubes and kept on ice until SDS-PAGE.

ii)To amplify the four *stamp* clusters ST4N1 (5'-aatgggtcgggatcccggaagtaaagatttaccat-3'), ST9N1 (5'-aatgggtcgggatcccggaggtaaagatttaccaa-3'), ST4-9-C1 (5'-gtggtggtgctcgagtcaagttgatgtccagaatgaacc-3'), ST1-C1 (5'-gtggtggtgctcgagtcaagttgatgtccataatgaacc-3') primers were used (Table 1). In-Fusion HD Cloning Kit (Clontech) was used for directional cloning of ST1, ST4, ST9, ST6 and ST13 DNA fragments into pET-28b(+) vector carry an N-terminal His6xTag (thrombin/T7) (Novagen, Merck KGaA). Primer design and cloning reactions were performed according to the manufacturer's instruction. A heterologous expression of stamp cluster II, III and IV recombinant proteins was performed in *Escherichia coli*, strain BL21 using standard procedures. His6x-tagged fusion proteins were purified using Ni-affinity chromatography (Sigma-Aldrich).

PD10 desalted 16 kDa fractions were separated on

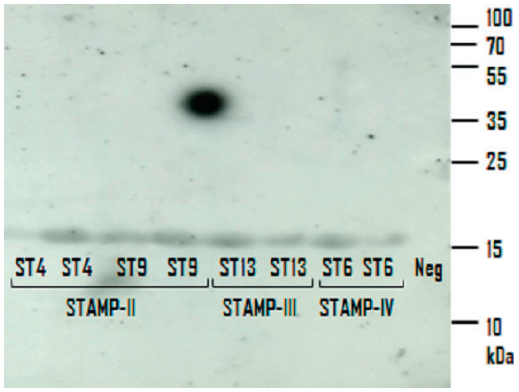

Figure 1. Western blot analysis of recombinant STAMP(s) of cluster II, III, and IV, revealed with anti-His Tag AB (Sigma).

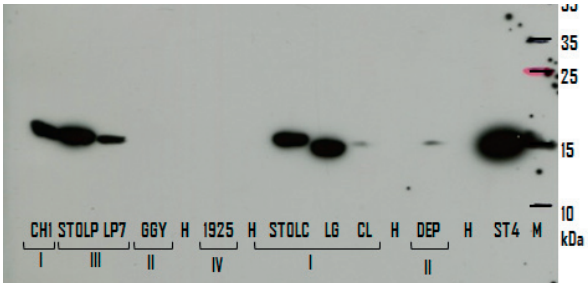

Figure 2. Western blot analysis of proteins extracted from periwinkle of '*Ca.* P. solani' strains, revealed with 2A10 MAb.

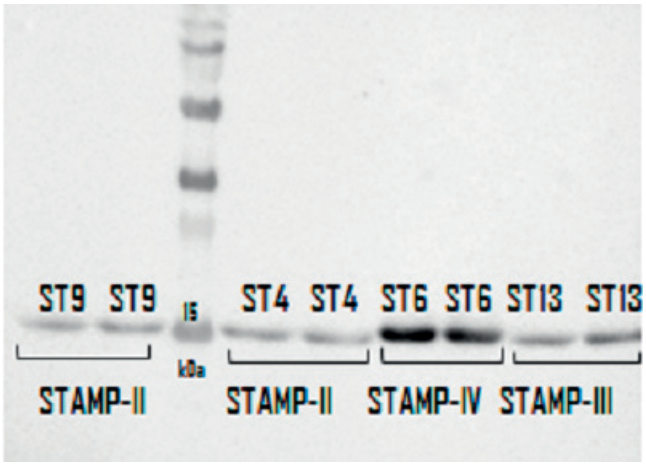

Figure 3. Western blot analysis of recombinant STAMP(s) of cluster II, III, and IV (10µg/lane), revealed with 2A10 MAb.

Figure 1-3: M: page ruler prestained protein ladder (Thermofisher), STAMP(s): 16 kDa, H: healthy periwinkle.

12 % SDS-PAGE, and Western blot (WB) analyses were done according to the protocol described in FABRE et al. (2011b). Used primary antibody was anti-His Tag antibody produced in rabbit (Sigma-Aldrich), secondary antibody was Anti-Rabbit IgG (whole molecule)-peroxidase antibody produced in goat (Sigma-Aldrich). To reveal Super Signal West Pico kit were used (Pierce Biotechnologies).

In order to detect the specificity of 2A10 MAb, total protein of nine '*Ca.* P. solani' strains (Table 1), as well as recombinant STAMPs of each cluster (Table 1) were separated on 12 % SDS-PAGE and WB analyses were performed.

RESULT AND DISCUSSION

Heterologous expression of stolbur antigenic membrane protein of genetic clusters II, III and IV of '*Ca.* P. solani' strains ST4, ST9, ST13, and ST6 were performed. Expression of strain ST1 belonging to cluster I is ongoing. Western blot analyses confirmed a 16 kDa size recombinant protein of each cluster (Figure 1).

Western blot (WB) analysis of STAMP(s) extracted from different phytoplasma strains demonstrated that MAb 2A10 recognizes all genetic clusters (Figure 2). Lack of signal in case of 1925 and GGY strains was due to the loss of these isolates, which was verified by PCR test. WB of recombinant proteins ST4, ST9, ST13, and ST6 confirmed that 2A10 MAb, an antibody produced against a strain belonging to *stamp* cluster I, recognises STAMP of four different stamp genetic clusters (Figure 3).

Study of the genetic diversity of *stamp* gene of '*Ca.* P. solani' strains in Euro-Mediterranean basin showed evidence of four different clusters (FABRE et al., 2011a; FOISSAC et al., 2013). Cluster I, II, III correspond to strains, which propagate on bindweed, and cluster IV corresponds to strains present on stinging nettle (FABRE et al., 2011b; JOHANNESEN et al., 2012). Geographical distribution of *stamp* genotypes seems to correlate to the geographical distribution of different Cixiidae ecotypes (FOISSAC et al. 2013). In our work we demonstrated that different *stamp* clusters are all recognized by the 2A10 MAb, not only cluster I. Further studies are needed to investigate the mechanisms of transmission of '*Ca*. P. solani' and specifically the role of STAMP in interaction between different insect vectors and '*Ca*. P. solani'. For these purposes usage of 2A10 MAb is applicable.

Table 1. List of '*Candidatus* Phytoplasma solani' strains and recombinant proteins

stamp cluster	*stamp* genotype	Strain	Origin	Original plant	Protein size (aa)	Forward primer	Reverse primer
'*Ca.* P. solani' strains maintained on *C. roseus*							
ST-I	ST1	Charente2	France	periwinkle	-	-	-
ST-I	ST2	StolburC	France	tomato	-	-	-
ST-I	ST3	Lot et Garonne	France	tomato	-	-	-
ST-I	ST20	Champlong	France	lavender	-	-	-
ST-II	ST4	GGY	Germany	grapevine	-	-	-
ST-II	ST10	DEP	France	lavender	-	-	-
ST-III	ST13	STOLP	France	red pepper	-	-	-
ST-III	ST14	LP7	Lebanon	periwinkle	-	-	-
ST-IV	ST6	1925	Germany	grapevine	-	-	-
STAMP recombinant proteins							
ST-I	ST1	PO	France	tomato	164	ST4N1	ST1-C1
ST-II	ST4	I6	Hungary	grapevine	164	ST4N1	ST4-9-C1
ST-II	ST9	I22	Hungary	pepper	166	ST9N1	ST4-9-C1
ST-III	ST13	REP2	Hungary	periwinkle	164	ST9N1	ST4-9-C1
ST-IV	ST6	1925	Germany	grapevine	169	ST4N1	ST4-9-C1

REFERENCES

ARYAN, A., BRADER, G., MÖRTER, J., PASTAR, M. and RIEDLE-BAUER, M. 2014: An abundant '*Candidatus* Phytoplasma solani' tuf b strain is associated with grapevine, stinging nettle and *Hyalesthes* obsoletus. European Journal of Plant Pathology 140: 213-227.

CVRKOVIĆ, T., JOVIĆ, J., MITROVIĆ, M., KRSTIĆ, O. and TOŠEVSKI, I. 2013: Experimental and molecular evidence of *Reptalus panzeri* as a natural vector of Bois noir. Plant pathology 63: (1) 42-53.

FABRE, A., BALAKISHIYEVA, G., EMBER, I. OMAR, A., ACS, Z., KOLBER, M., KAUZNER, L., DELLA BARTOLA, M., DANET, J.L. and FOISSAC, X. 2011a: *Stamp* encoding the antigenic membrane protein of Stolbur phytoplasma is useful for molecular epidemiology. Bulletin of Insectology 64: S21-S22.

FABRE, A., DANET, J.L. and FOISSAC, X. 2011b: The stolbur phytoplasma antigenic membrane protein gene STAMP is submitted to diversifying positive selection, Gene 472: 37-41.

FOISSAC, X., CARLE, P., FABRE, A., SALAR, P., DANET, J.L. and THE STOLBUR-EUROMED CONSORTIUM.2013: *'Candidatus* Phytoplasma solani' genome project and genetic diversity in the Euro-Mediterranean basin. 3rd European Bois Noir workshop, Invited lecture, 2013. Barcelona, Spain.

FOS, A., DANET, J.L., ZREIK, L., GARNIER, M. and BOVE, J.M. 1992: Use of monoclonal antibody to detect the stolbur mycoplasma like organism in plants and insects and to identify a vector in France. Plant Disease 76: 1092-1096.

GALETTO, L., BOSCO, D., BALESTRINI, R., GENRE, A., FLETCHER, J. and MARZACHÍ, C. 2011: The major antigenic membrane protein of '*Candidatus* Phytoplasma asteris' selectively interacts with ATP synthase and actin of leafhopper vectors. PLOS ONE 6 (7): e22571.

GARNIER, M., MARTIN-GRAOS, G., ISKRA, M.-L., ZREIK, L., GANDAR, J., FOS, A. and BOVÉ, J.-M. 1990: Monoclonal antibodies against the MLOs associated with tomato stolbur and clover phyllody. Zbl. Suppl. 20. Gustav Fischer Verlag. Suttagart, New York, 1990.

GATINEAU, F., LARRUE, J., CLAIR, D., LORTON, F., RICHARD-MOLARD, M. and BOUDON-PADIEU, E. 2001: A new natural planthopper vector of stolbur phytoplasma in the genus *Pentastiridius* (Hemiptera: Cixiidae). European Journal of Plant Pathology 107 (3): 263-271.

JOHANNESEN, J., FOISSAC, X., KEHRLI, P. and MAIXNER, M. (2012) Impact of vector dispersal and host-plant fidelity on the dissemination of an emerging plant pathogen. PloSONE 7: (12) e51809.

JOVIĆ, J., CVRKOVIĆ, T., MITROVIĆ, M., KRNJAJIĆ, S., REDINBAUGH, M.G., PRATT, R.C., GINGERY, R.E., HOGENHOUT, S.A. and TOŠEVSKI, I. 2007: Roles of stolbur phytoplasma and *Reptalus panzeri* (Cixiidae, Auchenorrhyncha) in the epidemiology of Maize redness in Serbia. European Journal of Plant Pathology 118: 85-89.

KAKIZAWA, S., OSHIMA, K., JUNG, H.Y., SUZUKI, S., NISHIGAWA, H., ARASHIDA, R., MIYATA, S., UGAKI, M., KISHINO, H. and NAMBA, S. 2006a: Positive selection acting on surface membrane protein of the plant-pathogenic phytoplasmas. Journal of Bacteriology 188(9): 324-3428.

LANGER, M. and MAIXNER, M. (2004) Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur-group based on RFLP-analysis of non-ribosomal DNA. Vitis 43: 191-200.

MAIXNER, M. 1994: Transmission of German grapevine yellows (Vergilbungskrankheit) by the planthopper *Hyalesthes obsoletus* (Auchenorrhyncha: Cixiidae). Vitis 33: 103–104.

QUAGLINO, F., ZHAO, Y., CASATI, P., BULGARI, D., BIANCO, P.A., WEI, W. and DAVIS, R.E. 2013: '*Candidatus* Phytoplasma solani', a novel taxon associated with Stolbur- and Bois noir- related diseases of plants. International Journal of Systematic and Evolutionary Microbiology 63: (8) 2879-2894.

SFORZA, R., CLAIR, D., DAIRE, X., LARRUE, J. and BOUDON-PADIEU, E. 1998: The role of *Hyalesthes obsoletus* (*Hemiptera: Cixiidae)* in the occurrence of bois noir of grapevines in France. Journal of Phytopathology 146: 549–556.

SUZUKI, S., OSHIMA, K., KAKIZAWA, S., ARASHIDA, R., JUNG, H.Y., YAMAJI, Y., NISHIGAWA, H., UGAKI, M. and NAMBA, S. 2006: Interactions between a membrane protein of a pathogen and insect microfilament complex determines insect vector specificity. Proceedings of the National Academy of Sciences of the United States of America 103: 4252-4257.

STAMP GENE AS THE HIGHLY DISCRIMINATIVE MARKER FOR ASSESSMENT OF BN VARIABILITY IN CROATIA

Jelena Plavec[1], Željko Budinšćak[1], Ivana Križanac[1], Dijana Škorić[2], Martina Seruga Musić[2]*

[1] Institute for Plant Protection, Croatian Centre for Agriculture, Food and Rural Affairs (CCAFRA) HR-10 000 Zagreb, Rim 98

[2] Department of Biology, Faculty of Science, University of Zagreb HR-10 000 Zagreb, Marulićev trg 9a

* Corresponding author: martina.seruga.music@biol.pmf.hr

Since the specific membrane protein genes have been identified and characterized in '*Candidatus* Phytoplasma solani' genome, their variability has been extensively studied in order to elucidate different BN genotypes as well as the epidemiology of the disease. The aim of this study was to assess a variability of '*Ca*. P. solani' strains from infected grapevine, wild plants and *H. obsoletus* by molecular characterization of *stamp* gene. Genotyping was performed on almost fifty BN strains detected in the scope of a 4-years national survey (2009 to 2012) encompassing 277 samples from all viticultural regions of Croatia. Real-time PCR and conventional 16S rDNA were performed, followed by amplification, sequencing and phylogenetic analyses of *stamp* gene. The comprehensive analyses demonstrated the presence of ten *stamp* genotypes identified so far in Croatia, confirming the significance of this gene as a highly discriminative marker. The most prevalent genotype was identified (38 %) being restricted to the central north-western part of Croatia. Other two highly represented genotypes were also detected with one being present in the central north-western and eastern parts of Croatia (11 %) and the other (9 %) detected exclusively in the coastal west part of Croatia. These results revealed a considerable genetic variability, epidemiological relevance and the geographical distribution of stamp genotypes in Croatia.

'Candidatus phytoplasma solani' (taxonomic group 16SrXII), formerly known as stolbur phytoplasma, which is endemic to Europe and the Mediterranean area affects many herbaceous and woody plants (Quaglino et al., 2013; Schneider et al., 1997; Langer and Maixner, 2004). Different planthopper and leafhopper species are reported to transmit stolbur but cixiid *Hyalesthes obsoletus* Signoret is considered to be the principal vector of the stolbur phytoplasma that causes BN (Maixner et al., 1994). To understand the vector specificity of stolbur phytoplasma to planthoppers of the Cixiidae family, phytoplasma surface proteins and their variability is investigated with special attention (Foissac et al., 2013). Along with *vmp*1 gene (encoding a variable membrane protein specific to stolbur phytoplasma) (Cimerman et al., 2009), *stamp* gene revealed to be a highly variable marker involved in interaction with the insect vector (Fabre et al., 2011). The two variable surface proteins have been shown to be submitted to positive selection and are frequently used to evaluate '*Ca*. P. solani' genetic diversity (Cimerman et al., 2009; Fabre et al., 2011; Foissac et al., 2013). The aim of the present study was to assess a variability of '*Ca.* P. solani' strains from infected grapevine, wild plants and *H. obsoletus* in Croatia by genotyping non-ribosomal gene *stamp* encoding the specific antigenic membrane protein of this phytoplasma.

MATERIAL AND METHODS

Samples with typical Grapevine yellows (GY) symptoms belonging to different grapevine varieties, wild plants and H. *obsoletus* were collected during

the 4-years national survey encompassing all viticultural regions of Croatia (2009 to 2012). Plant and insect total nucleic acids were extracted from 1 g fresh leaf midribs or from single insects following the CTAB extraction protocol (Maixner et al., 1995; Šeruga et al., 2003). Grapevine DNA samples were then analysed by the triplex real-time PCR assay according to Pelletier et al. (2009). Collected weed species together with insect vectors *H. obsoletus* samples were tested by conventional PCR assays with phytoplasma universal primers P1/P7 (Deng and Hiruki, 1991; Smart et al., 1995), followed by R16F2n/R2 (Gundersen and Lee, 1996) in nested PCR to confirm phytoplasma presence. Nested PCR products R16F2n/R2 were subjected to RFLP analysis using enzyme *M*seI. Restriction profiles of positive samples were compared to restriction profiles of reference strains and allowed affiliation of these strains to the group 16SrXII-A. Isolates that had been tested positive for 16SrXII-A (BN) were submitted to nested PCR to amplify *stamp* gene according to Fabre et al. (2011).

Sequencing of the *stamp* gene amplicons was performed by the commercial service Macrogen Inc. (Seoul, Republic of Korea). PCR products were directly sequenced on both strands and subsequent phylogenetic analyses were performed. Raw nucleotide sequences were assembled and edited with the SequencherTM 4.7 software (http://www.genecodes.com/) and then aligned with ClustalX 2.0 (Thompson et al., 1997). Phylogenetic analyses were performed with MEGA 5 software (Tamura et al., 2011) by using the neighbour-joining method with the number of differences model and maximum parsimony with CNI on random trees method. Bootstrap analyses were performed (500 replicates) to estimate the stability of nodes and to support the inferred clades.

RESULTS AND DISCUSSION

Out of 201 grapevine samples analysed by triplex-real time PCR, 41 sample were BN positive belonging to subgroup 16SrXII-A. Phytoplasma of the 16SrXII-A subgroup was also identified in four insect vectors *H. obsoletus* out of 42 and 1 *C. arvensis* sample out of 34 weed samples analysed. In all except one sample *stamp* gene was amplified.

Sequence comparison with the reference strains and phylogenetic analysis using neighbour-joining (NJ) method identified ten *stamp* genotypes designated ST6, ST9, ST13, ST19, ST22, ST23, ST29, ST46, ST48 and ST52. Genotype ST6 was the most prevalent (38 %) and restricted to the central-north western part of Croatia. Other two highly represented genotypes were ST9 (11 %) detected also in the central-north western and eastern part of Croatia and ST46 (9 %) detected only in the coastal-west part of Croatia.

A multiple alignement program ClustalX enabled visualization of *stamp* variations among the detected genotypes. First variation is represented by a 6 bp insertion ATCAAA at position 172 of the gene. This insertion was recorded for all of the *stamp* strains belonging to the genotypes ST46 and ST19, one ST23 genotype and significant part of ST9. Another two insertions of 3 bp (ACC or CCC) and 12 bp (TCAAAAACAACC) occur at positions 258 and 372 in isolates assigned to the genotypes ST6, ST23, ST46 and ST52. These changes allowed discrimination of different *stamp* genotypes.

Stamp gene was characterized by the abundance of mutations. With ten stamp genotypes identified in Croatia, *stamp* revealed to be the highly discriminative marker. As previously reported by Fabre et al. (2011), this tremendous variability of *stamp* gene could be explained by evolutionary necessity for improvement in interaction with microfilaments of a new vector. Adaptations to the new hosts have a significant impact on phytoplasma membrane proteins (changes in the protein sequence) resulting in the number of different genotypes.

The data obtained in the present study reveal considerable genetic variability, epidemiological relevance and the geographical distribution of *stamp* genotypes in Croatia.

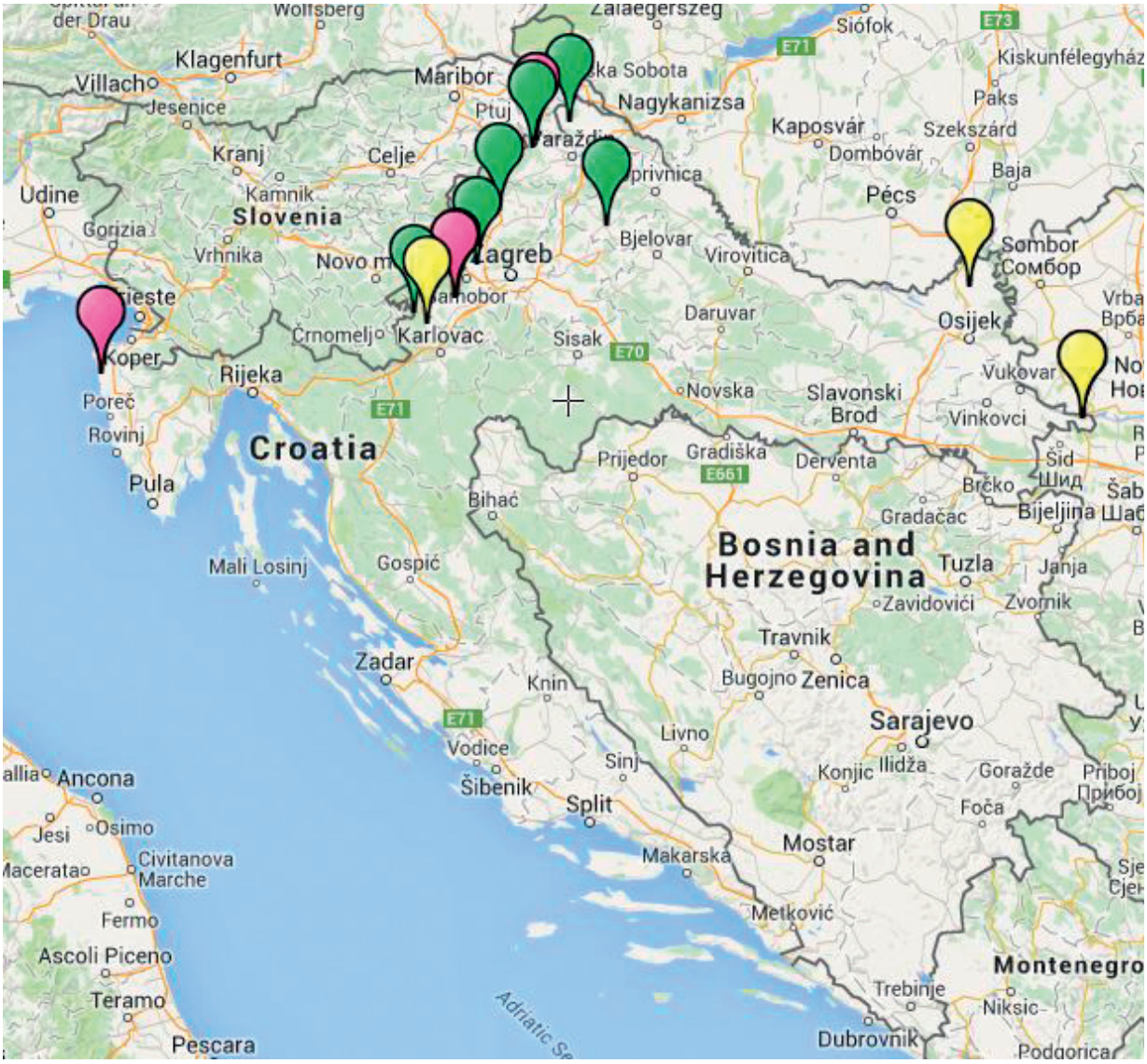

Figure. 1. Map of prevalent stamp genotypes: green pin = ST6 (38 %), yellow pin = ST9 (11 %), pink pin = ST46 (9 %).

ACKNOWLEDGEMENTS

The authors would like to acknowledge Dr. Xavier Foissac for kindly providing reference strains.

REFERENCES

Cimerman, A., Pacifico, D., Salar, P., Marzachi, C. and Foissac, X. 2009: Striking Diversity of vmp1, a Variable Gene Encoding a Putative Membrane Protein of the Stolbur Phytoplasma. Applied and Environmental Microbiology 75: 2951–2957.

Deng, S and Hiruki, C. 1991: Amplification of 16S rRNA genes from culturable and non-culurable Mollicutes. Journal of Microbiological Methods 14: 53-61.

Fabre A., Danet J.L. and Foissac, X. 2011: The stolbur phytoplasma antigenic membrane protein gene *stamp* is submitted to diversifying positive selection. Gene 472: 37–41.

Foissac X., Carle, P., Fabre, A., Salar, P., Danet, J.L and Stolbur-Euromed consortium. 2013b: *'Candidatus* Phytoplasma solani' genome project and genetic diversity in the Euro-Mediterranean basin. Proceedings 3rd European Bois noir Workshop., Barcelona., Spain: 11-13.

Gundersen, D.E. and Lee, I.M. 1996: Ultrasensitive detection of phytoplasmas by nested PCR assays using two universal primer pairs. Phytopathologia Mediterranea 35: 144-151.

Langer M. and Maixner, M. 2004: Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur-group based on RFLP-analysis of non ribosomal DNA. Vitis 43: 191-199.

Maixner, M., Ahrens, U. and Seemüller, E. 1995: Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine., alternative hosts and a vector by a specific PCR procedure. European Journal of Plant Pathology 101: 241–250.

Pelletier, C., Salar, P., Gillet, J., Cloquemin, G., Very, P., Foissac, X. and Malembic Maher, S. 2009: Triplex real-time PCR assay for sensitive and simultaneous detection of grapevine phytoplasmas of the 16SrV and 16SrXII-A groups with an endogenous analytical control. Vitis 48: 87-95.

Quaglino, F., Zhao, Y., Casati, P., Bulgari, D., Bianco, P.A., Wei, W. and Davis, R.E. 2013: *'Candidatus* Phytoplasma solani'., a novel taxon associated with stolbur and bois noir related diseases of plants. International Journal of Systematic and Evolutionary Microbiology 63: 2879–2894.

Schneider, B., Gibb, K.S. and Seemüller, E. 1997: Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas. Microbiology 143: 3381-3389.

Smart, C.D., Schneider, B., Blomquist, C.L., Guerra, L.J., Harrison, N.A., Ahrens, U., Lorenz, K.H., Seemüller, E. and Kirkpatrick, B.C. 1996: Phytoplasma-specific PCR primers based on sequences of 16S rRNA spacer region. Applied and Environmental Microbiology 62: 2988-3033.

Šeruga, M., Škorić, D., Botti, S., Paltrinieri, S., Juretić, N. and Bertaccini,A.F. 2003: Molecular characterization of a phytoplasma from the aster yellows (16SrI) group naturally infecting *Populus nigra* L. 'Italica' trees in Croatia. Forest Pathology 33: 113-125.

Tamura K., Dudley, J., Nei, M. and Kumar, S. 2007: MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596-1599.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. 1997: The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24: 4876-4882.

NEW '*CANDIDATUS* PHYTOPLASMA SOLANI' STRAINS ASSOCIATED WITH BOIS NOIR DISEASE IN *VITIS VINIFERA* L. CULTIVARS IN GEORGIA

Fabio Quaglino[1], David Maghradze[2], Paola Casati[1], Nona Chkhaidze[3], Osvaldo Failla[1] and Piero Attilio Bianco[1]

[1]Department of Agricultural and Environmental Sciences - Production, Landscape, Agroenergy University of Milan, Milano, Italy;
[2]Institute of Horticulture, Viticulture and Oenology, Agricultural University of Georgia, Tbilisi, Georgia;
[3]Laboratory of Plant Anatomy and Physiology, Agricultural University of Georgia, Tbilisi Georgia. *Corresponding author: fabio.quaglino@unimi.it

Preliminary surveys highlighted that bois noir (BN) disease, associated with '*Candidatus* Phytoplasma ('*Ca*. P.') solani', affects grapevine varieties in Georgia (South Caucasus). In this study, further research was carried out to investigate the BN symptom severity in international and Georgian native varieties. Identification and characterization of '*Ca*. P. solani' was performed by analysis of 16S rDNA, *vmp*1 and *stamp* gene nucleotide sequences. During field surveys, moderate/mild and severe symptoms were observed on Georgian grapevine varieties and international cultivars, respectively. Molecular characterization of '*Ca*. P. solani' identified in grapevines revealed the presence of 11 genetically distinct phytoplasma types. Ten of such '*Ca*. P. solani' types were described here for the first time; only one type (VmGe12/StGe7) was identical to a strain previously reported in periwinkle in Lebanon. Phylogenetic analyses of *vmp*1 and *stamp* gene concatenated nucleotide sequences indicated that '*Ca*. P. solani' strains in Georgia are associated mainly with the bindweed-related BN host system. Moreover, the presence of the same '*Ca*. P. solani' strains in grapevine cultivars showing a range of symptom intensity suggested a different susceptibility of Georgian local varieties to BN.

INTRODUCTION

Bois noir (BN) is a phytoplasma-associated disease, belonging to the grapevine yellows (GYs) complex, responsible for serious crop losses in the Euro-Mediterranean area and in other continents. Its etiological agent has been attributed to phytoplasma strains (BNp) belonging to the species '*Candidatus* Phytoplasma solani' ('*Ca*. P. solani'), subgroup 16SrXII-A (QUAGLINO et al., 2013). BNp strains are transmitted to grapevine by *Hyalesthes obsoletus* Signoret (Homoptera: Cixiide), a polyphagous vector living preferentially on nettle, bindweed, and chaste tree (KOSOVAC et al., 2015). Further studies reported the presence of other natural vectors and wild plants playing a role in BN diffusion (MORI et al., 2015). Molecular markers, identified in *tuf, sec*Y, *vmp*1 and *stamp* genes, highlighted the presence of genetically distinct BNp strains, characterized by different distribution and prevalence in the Euro-Mediterranean basin (FOISSAC et al., 2013). Due to this complexity, it is difficult to design efficient control strategies against BN. An ambitious strategy is based on the selection of plant varieties as source of resistance-genes for plant breeding programs. Unfortunately, none of the examined *Vitis* species and *V. vinifera* varieties have been found resistant or tolerant to the GY phytoplasmas (LAIMER et al., 2009). The Georgian native germplasm is composed by more than 500 cultivars constituting a very unique genetic pool (IMAZIO et al., 2013). Recent studies reported that grapevine varieties selected

in domestication centers of V. *vinifera* L., such as Georgia, showed possible tolerance or resistance to plant pathogens, such as *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, associated with downy mildew (BITSADZE et al., 2014). Evidences from a preliminary survey on GYs highlighted that '*Ca*. P. solani' infects international and local varieties in Georgia (QUAGLINO et al., 2014). In the present study, field surveys and multiple gene typing analyses were carried out to study (i) the GY symptom severity in international and Georgian native varieties and (ii) the genetic diversity among '*Ca.* P. solani' strain populations in Georgia.

MATERIAL AND METHODS

SYMPTOM OBSERVATION AND PLANT SAMPLING. In September 2013, surveys on GY symptoms were carried out in vineyards and in field collections of international and native *Vitis vinifera* L. varieties in eastern Georgia. Grapevine varieties were classified in group I (mild symptoms), group II (moderate symptoms), and group III (severe symptoms). Leaf samples were collected from grapevine symptomatic plants of international and native Georgian varieties (Table 1), and from six bindweed plants showing yellowing, reddening, dwarfism and leaf malformation.

PHYTOPLASMA DETECTION. Total DNA was extracted from examined plants as previously described (ANGELINI et al., 2001). Detection of phytoplasmas was carried out by nested-PCR based amplification of 16S rDNA and subsequent RFLP assay as previously described (QUAGLINO et al., 2009). Total nucleic acids from periwinkle plants infected by phytoplasma strains EY1 ('*Ca*. P. ulmi'), STOL ('*Ca*. P. solani'), and AY1 ('*Ca*. P. asteris') were used as reference controls. Total nucleic acids from healthy periwinkle and PCR mixture devoid of nucleic acids were used as negative controls.

MOLECULAR CHARACTERIZATION OF '*CA.* P. SOLANI' STRAINS. *Vmp*1 and *stamp* genes of identified BNp strains were amplified by nested PCR as previously described (FIALOVÁ et al., 2009; FABRE et al., 2011). *Vmp*1 amplicons were further characterized by *Rsa*I-RFLP assays (FIALOVÁ et al., 2009). *Vmp*1 and *stamp* gene fragments amplified from 15 BNp strains, representative of the obtained *Rsa*I-RFLP profiles, were selected for nucleotide sequence analysis. The gene amplicons were sequenced (5x coverage), assembled and deposited in the NCBI GenBank database. *Vmp*1 nucleotide sequences were searched for mutations in *Rsa*I-recognition sites by virtual RFLP analyses using the software pDRAW32. The association between the *vmp*1-RFLP profiles and BN symptom severity was evaluated by $\chi 2$ test using SPSS statistical package for Windows, v. 22.0 (SPSS Inc.). *Vmp*1 and *stamp* gene sequences, obtained in this study and retrieved from GenBank, were aligned and analyzed by the software BioEdit v.7.0.5. Based on sequence identities, BNp strains were grouped in *vmp*1 and *stamp* genetic variants, and in collective *vmp*/*stamp* types. Strains of each variant/type shared 100% sequence identity.

Table 1. Symptom severity and phytoplasmas in Georgian vineyards and field collections

Plant host	Symptom severity	No. of samples	PCR-RFLP						
			16SrXII-A	*vmp1*					
				V1	V14	V15	und1	und2	und3
Chardonnay	+++	20	20	8	5		2	3	
Moscato Bianco	+	3	3		1				
Carignano	+++	1	1					1	
Freisa	+++	1	1					1	
Adznizhi	+	1							
Amlakhu	+	1	1						1
Asuretuli Shavi	+	1							
Buera	++	1	1	1					
Chinuri	+	1							
Chkhaverii	+	1							
Chuberi	+	2							
Goruli Mtsvane	++	4	1		1				
Grdzelmtevana	+	1							
Khikhvi	+	1	1					1	
Khikhvi variation	+	1							
Kikhvi Loladzis	+	1	1			1			
Kisi	+++	3	3	1				1	
Korkaula	+	3	2			1			
Mtredisphekha	+	1							
Mtsvane Kakhuri	+	1							
Mujuretuli	+	1	1						
Rkatsiteli	+	9	4	2		1			
Saperavi	++	13	9	4	1			2	
Saperavi Budeshuri	+	2	1					1	
Saperavi Pachkha	++	1							
Tavkveni Saperaviseburi	+	1	1						
Tavkveri	+	1	1			1			
Tshnoris Tetri	+	1							
Tsitska	+	1	1					1	
Tsolikouri	+	1	1						
Usakhelouri	+	1	1			1			
Convolvulus arvensis	+++	6	6		4		2		
		87	61	16	12	5	4	11	1

PHYLOGENETIC ANALYSIS. *Vmp*1 and *stamp* gene sequences of '*Ca*. P. solani', from this and previous studies, were concatenated and used for minimum evolution analysis (neighbor-joining method, 1000 bootstrap replications) by the software MEGA6.

RESULTS

SYMPTOMS OBSERVED ON GRAPEVINE IN GEORGIA. Severe symptoms were observed in three international varieties (Chardonnay, Carignano, and Freisa) and in one local Georgian variety (Kisi); moderate symptoms were observed in four local Georgian varieties (Buera, Goruli Mtsvane, Saperavi, and Saperavi Pachkha); mild symptoms were observed in 22 local Georgian varieties and in one international variety (Moscato Bianco) (Table 1).

PHYTOPLASMA IDENTIFICATION. PCR-RFLP detection revealed the presence of '*Ca*. P. solani' in 55 out of 81 examined grapevines, and in all six bindweed samples (Table 1). In fact, all the phytoplasma strains had restriction patterns indistinguishable from one another and from the patterns characteristic of the reference strain STOL (data not shown). DNA amplification was obtained from periwinkles infected by strains STOL, AY1 and EY1. No amplification was obtained in the reactions of healthy periwinkle and PCR mixture devoid of DNA.

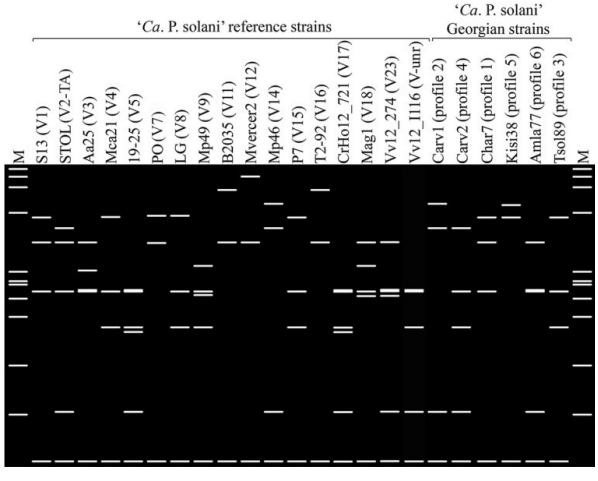

Figure 1. Virtual *Rsa*I-RFLP profiles of vmp1 amplicons obtained from BNp strain populations in Georgia.

'*CA.* P. SOLANI' STRAIN CHARACTERIZATION BY *VMP*1 AND *STAMP* GENE SEQUENCE ANALYSIS. *Vmp*1 gene fragment was amplified from 43 out of 55 infected grapevines, and from all the six infected bindweeds (Table 1). Enzymatic digestions of the 49 *vmp*1 amplicons evidenced the presence of six *R*saI-profiles among BNp strains from Georgia. Virtual RFLP-based comparison of *vmp*1 RFLP profiles evidenced that Georgian BNp strains showed previously described (V1, V14, V15) and new [und1 (undescribed1), und2, und3] restriction patterns (Fig. 1). Strains showing profiles V1, V14 and und2 were prevalent and were identified, with significantly different distribution, in grapevine varieties showing severe, moderate and mild symptoms ($\chi 2 = 16.671$; d.f. = 10; P = 0.029). Nucleotide sequence analysis, performed on 15 representative BNp strains from Georgia, revealed the presence of 12 and 7 genetic variants of *vmp*1 (here designated as VmGe1 to VmGe12) and *stamp* (here designated as StGe1 to StGe7) genes, respectively. Eleven Georgian BNp *vmp*1/*stamp* types were described as the combination of vmp1 and *stamp* genetic variants.

Comparison with *vmp*1 and *stamp* genetic variants from GenBank (63 vmp1 genetic variants, here designated as Vm1 to Vm63, and 35 *stamp* genetic variants, here designated as St1 to St35) evidenced that BNp strains from Georgia showed 11 *vmp*1 (VmGe1 to VmGe7, VmGe9 to VmGe12) and 6 *stamp* (StGe1 to StGe6) novel genetic variants, previously unreported. Only BNp strains Tsol89 and Kiqu94 (VmGe12/StGe7) shared 100% sequence identity with '*Ca*. P. solani' strain P7 (Vm53/St15), identified in periwinkle in Lebanon (CIMERMAN et al., 2009).

PHYLOGENETIC ANALYSIS OF '*CA.* P. SOLANI' STRAINS FROM GEORGIA AND OTHER GEOGRAPHICAL REGIONS. Based on phylogenetic analysis of *vmp*1 and *stamp* concatenated sequences, five *vmp*1/*stamp* clusters were identified (Fig. 2). The cluster *vmp*1/*stamp*-4 included BNp strains associated with nettle, while the other four clusters (*vmp*1/*stamp*-1, -2, -3, -5) included BNp strains associated with bindweed. The majority of Georgian BNp strains (13 out of 14) grouped within bindweed-related clusters *vmp*1/*stamp*-3 and *vmp*/*stamp*-5, while only the strain Amla77 grouped within nettle-related cluster *vmp*1/*stamp*-4. The majority of Georgian BNp strains, grouped within cluster *vmp/stamp*-3, were found to be closely related to strain P7, previously identified in a naturally infected periwinkle plant in Lebanon.

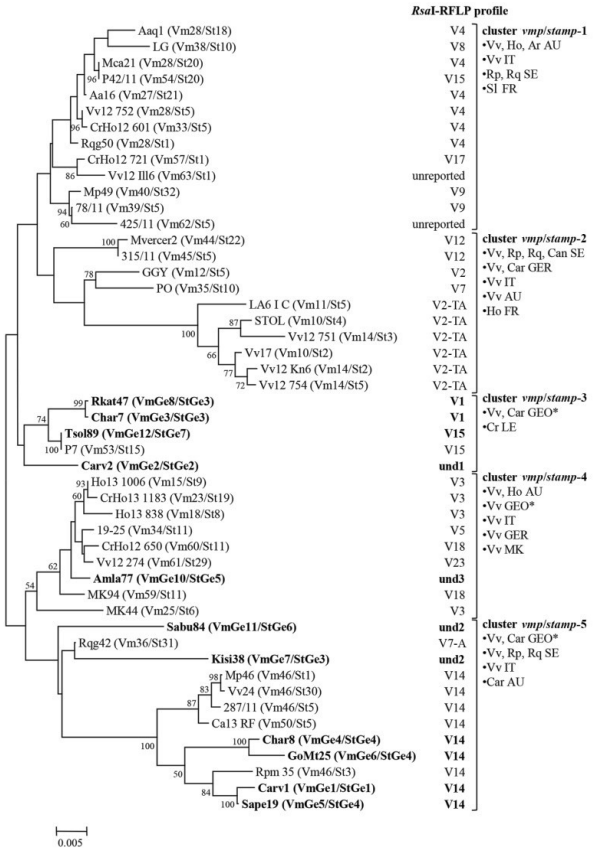

Figure 2. Unrooted phylogenetic tree inferred from '*Ca*. P. solani' strains based on concatenated nucleotide sequences of the genes *vmp*1 and *stamp*. Strains identified in Georgian varieties are showed in bold.

DISCUSSION

The results obtained in this study confirmed the evidences from previous researches indicating the presence BN in Georgia (QUAGLINO et al., 2014). In fact, molecular analyses evidenced the strong association between specific GY disease symptoms and grapevine plant infection by BNp ('*Ca*. P. solani' strains) within the examined vineyards. In order to gain an insight into the genetic diversity among BNp strains in Georgia, nucleotide sequence analysis was performed on two genes (*vmp*1 and *stamp*) coding for membrane proteins putatively involved in the recognition and interaction of BNp with its hosts (CIMERMAN et al., 2009; FABRE et al., 2011). Based on *Rsa*I-RFLP digestions of *vmp*1 gene amplicons, the profiles V1, V14 and und2 were prevalent among the analyzed BNp strains. This data confirmed the specific association of pattern V14 with East Europe (FOISSAC et al., 2013), and highlighted an unexpected diffusion of type V1, reported as the prevalent type in Italy, France and Germany (FOISSAC et al., 2013), in the Caucasian geographic regions. This evidence, along with the prevalence of type V1 in the international cultivar Chardonnay, could suggest the non-indigenous origin of this type, possibly introduced into Georgia through import of planting material. The majority of autochthonous Georgian grapevine cultivars were found mildly symptomatic, maintaining complete berry production. Intriguingly, the presence of BNp strains showing *Rsa*I-RFLP profiles V1, V14 and und2 of the gene *vmp*1 in grapevine cultivars exhibiting severe, moderate and mild symptoms suggested a different susceptibility of the cultivars to these BNp strains.

Molecular characterization by *vmp*1 and *stamp* gene sequence analysis evidenced that BNp populations in Georgia are constituted mainly by previously unreported strains. Only Georgian BNp strains Tsol89 and Kiqu94 shared 100% sequence identity with the sequences of the '*Ca.* P. solani' strain P7 (vmp1/stamp type Vm53/St15), identified in naturally-infected periwinkle in Lebanon in 2001 (CIMERMAN et al., 2009). Phylogenetic analysis revealed that the majority of BNp Georgian strains, identified both in grapevine and bindweed, grouped along with the Lebanese strain P7 within the cluster *vmp*1/*stamp*-3. Interestingly, this cluster is clearly distinct from other *vmp*1/*stamp* clusters including bindweed- and nettle-related BNp strains previously identified in Central and Southern Europe. Only one BNp strain (Amla77) grouped with nettle-related cluster *vmp*1/*stamp*-4. In conclusion, results from the present study evidenced that BNp strain populations in Georgia is constituted mainly by new unreported '*Ca*. P. solani' strains associated with both nettle- and bindweed-related BN host systems. Moreover, the distribution of BNp strains among grapevine cultivars showing a variable range of symptoms intensity suggests a different susceptibility of such local cultivars to BN disease. Further studies are in progress to evaluate this important topic in the perspective of improving breeding programs for the production of novel grapevine cultivars tolerant and/or resistant to phytoplasma diseases.

REFERENCES

ANGELINI, E., CLAIR, D., BORGO, M., BERTACCINI, A., und BOUDON¬-PADIEU, E. 2001: Flavescence dorée in France and Italy: occurrence of closely related phytoplasma isolates and their near relationships to palatinate grapevine yellows and an alder yellows phytoplasma. Vitis 40: 79-86.

BITSADZE, N., CHIPASHVILI, R., TSKHVEDADZE, L., AZNARASHVILI, M., MAGHRADZE, D., VERCESI, A., und FAILLA, O. 2014: Screening of the Georgian grape germplasm to susceptibility of downy mildew: preliminary results. Acta Horticulturae 1032: 191-196.

CIMERMAN, A., PACIFICO, D., SALAR, P., MARZACHÌ, C., und FOISSAC, X. 2009. Striking diversity of *vmp*1, a variable gene encoding a putative membrane protein of the stolbur phytoplasma. Applied and Environmental Microbiology 75: 2951-2957.

FABRE, A., DANET, J.-L., und FOISSAC, X. 2011. The stolbur phytoplasma antigenic membrane protein gene *stamp* is submitted to diversifying positive selection. Gene 472: 37-41.

FIALOVÁ, R., VÁLOVÁ, P., BALAKISHIYEVA, G., DANET, J.-L., ŠAFÁŘOVÁ, D., FOISSAC, X., und NAVRÁTIL, M. 2009. Genetic variability of stolbur phytoplasma in annual crop and wild plant species in South Moravia (Czech Republic). Journal of Plant Pathology 91: 411-416.

FOISSAC, X., CARLE, P., FABRE, A., SALAR, P., DANET, J.-L., und STOLBUR-EUROMED CONSORTIUM. 2013. '*Candidatus* Phytoplasma solani' genome project and genetic diversity in the Euro-Mediterranean basin. Proceedings of the 3rd European Bois Noir Workshop, 2013. Barcelona, Spain. E. Torres, A. Laviña and A. Batlle, eds., 11-13.

KOSOVAC, A., RADONJIĆ, S., HRNČIĆ, S., KRSTIĆ, O., TOŠEVSKI, I., und JOVIĆ, J. 2015. Molecular tracing of the transmission routes of bois noir in Mediterranean vineyards of Montenegro and experimental evidence for the epidemiological role of *Vitex agnus-castus* (Lamiaceae) and associated *Hyalesthes obsoletus* (Cixiidae). Plant Pathology doi:10.1111/ppa.12409.

IMAZIO, S., MAGHRADZE, D., DE LORENZIS, G., BACILIERI, R., LAUCOU, V., THIS, P., SCIENZA, A., und FAILLA, O. 2013. From the cradle of grapevine domestication: molecular overview and description of Georgian grapevine (*Vitis vinifera* L.) germplasm. Tree Genetics and Genomes 9: 641-658.

LAIMER, M., LEMAIRE, O., HERRBACH, E., GOLDSCHMIDT, V., MINAFRA, A., BIANCO, P., und WETZEL, T. 2009. Resistance to viruses, phytoplasmas and their vectors in the grapevine in Europe: a review. Journal of Plant Pathology 91: 7-23.

MORI, N., QUAGLINO, F., TESSARI, F., POZZEBON, A., BULGARI, D., CASATI, P., und BIANCO, P.A. 2015. Investigation on 'bois noir' epidemiology in north-eastern Italian vineyards through a multidisciplinary approach. Annals of Applied Biology 166: 75-89.

QUAGLINO, F., MAGHRADZE, D., CHKHAIDZE, N., FAILLA, O., CASATI, P., und BIANCO, P.A. 2014. First report of '*Candidatus* Phytoplasma solani' and 'Candidatus Phytoplasma convolvuli' associated with grapevine bois noir and bindweed yellows, respectively, in Georgia. Plant Disease 98: 1151.

QUAGLINO, F., ZHAO, Y., CASATI, P., BULGARI, D., BIANCO, P.A., WEI, W., und DAVIS, R.E. 2013. *'Candidatus* Phytoplasma solani', a novel taxon associated with stolbur and bois noir related diseases of plants. International Journal of Systematic and Evolutionary Microbiology 63: 2879-2894.

QUAGLINO, F., ZHAO, Y., BIANCO, P.A., WEI, W., CASATI, P., DURANTE, G., und DAVIS, R.E. 2009. New 16Sr subgroups and distinct single nucleotide polymorphism lineages among grapevine Bois noir phytoplasma populations. Annals of Applied Biology 154: 279-289.

MULTIGENE CHARACTERIZATION OF '*CANDIDATUS* PHYTOPLASMA SOLANI' STRAINS INFECTING PEPPER, CELERY AND MAIZE IN BOSNIA AND HERZEGOVINA

Duška Delić[1], Nicoletta Contaldo[2], Biljana Lolić[1], Đorđe Moravčević[3], Drago Milošević[4] and Assunta Bertaccini[2]

[1]University of Banjaluka, Faculty of Agriculture, Bulevar vojvode Petra Bojovića, 78000-Banjaluka, Bosnia and Herzegovina
[2]Alma Mater Studiorum University of Bologna, DipSA, Plant Pathology, V.le Fanin 42, Bologna, Italy
[3]University of Belgrade, Faculty of Agriculture, Department of Crop and Vegetable Sciences Nemanjina 6, 11080-Beograd, Serbia
[4]University of Kragujevac, Faculty of Agronomy, Cara Dušana 34, Čačak, Serbia

Molecular characterisation was performed on phytoplasma strains collected in Semberia region of Bosnia and Herzegovina from symptomatic maize, pepper and celery and resulted infected with *'Ca.* P. solani', by a multigene RFLP and sequencing study. The RFLP analyses on *tuf, sec*Y, *vmp*1 and *stamp* genes allow to differentiate 8 lineages in the 13 positive samples obtained, indicating the presence of different '*Ca*. P. solani' lineages. Three lineages were detected in the seven samples of maize, each of the four pepper samples was infected by a different lineage, and one of these was also found in one of the two celery samples. The phytoplasmas in the other celery sample represent a further lineage. The identification of a number of lineages in these crops compared to the 4 detected in grapevine in previous surveys, allow to speculate about the presence of insect vectors that could be different in the case of these crops from those reported for grapevine or of possible increasing of phytoplasma biodiversity by their possible seed transmission.

INTRODUCTION

'*Candidatus* phytoplasma solani' is a widely distributed phytoplasma in the Euro-Mediterranean area associated to various diseases in many cultivated plants including grapevine, maize, potato, pepper, celery, and tobacco (Bertaccini et al., 2014). In Bosnia and Herzegovina *'Ca*. P. solani' (16SrXII-A) was detected in association with symptomatic grapevine, maize, pepper and celery (Delić et al., 2011; 2015; Kovačević et al., 2014; Lolić et al., 2014; Delić et al., 2016). Molecular characterisation performed on *tuf* gene on strains from grapevine and maize showed the presence of tuf-type b phytoplasmas, while genotyping of grapevine strains on *vmp*1 and *stamp* genes distinguished five types (Lolić et al., 2014; Delić et al., 2016). Mild continental climate and fertile soil in Semberia region of Bosnia and Herzegovina (B&H) favour traditional crop and vegetable production. '*Ca*. P. solani' was identified for the first time in this area in maize showing reddening disease (Lolić et al., 2014) while, infected pepper and celery were reported recently growing in the vicinity of maize fields (Delić et al., 2016). In the present study a multigene characterisation on '*Ca*. P. solani' strains from maize, pepper and celery was carried

out in order to verify the presence of variability in *tuf*, *sec*Y, *vmp*1 and *stamp* genes and its epidemiologic relevance.

MATERIAL AND METHODS

'Ca. P. solani' strains from symptomatic maize, pepper and celery collected in Semberia region (Lolić et al., 2014; Delić et al., 2016), were employed for further molecular characterisation carried out on *tuf* gene (encoding translation elongation factor Tu); *sec*Y gene (encoding a protein involved in the bacterial secretion mechanism); *vmp*1 gene (encoding a putative membrane protein), and *stamp* gene (encoding an antigenic membrane protein).

The *tuf* gene was amplified in a nested PCR procedure using primer pairs tuf1f/r/TufAYf/r and TufINT1f/TufINT4r (Langer and Maixner, 2004) and amplicons were subjected to the RFLP analyses with *Hpa*II enzyme. The *sec*Y gene was amplified following protocols described by Lee et al. (2010) and the RFLP analyses of StSecYF2/ StSecyR2 nested PCR products were done with *Tru*1I and *Alu*I endonucleases. Nested PCR products of the *vmp*1 gene were obtained with StolH10F1/R1 (Cimerman et al., 2009) and TYPH10F/R (Fialová et al., 2009) primers. The latter amplicons were digested with *RsaI* and AluI restriction enzymes. The nested PCR of the *stamp* gene was done with StampF/R0 followed by StampF1/R1 primers (Fabre at al., 2011). The nested PCR products were subjected also to the restriction analyses with *Tru*1I endonuclease.

Direct sequencing on both strands was done on *sec*Y, *vmp*1 and *stamp* genes after amplification and cleaning of the respective amplicons in Macrogen Europe (Netherlands). The sequences were assembled using the Staden program package (Staden et al., 2000), aligned using Clustal X (Thompson et al., 1997), and deposited **IN GENBANK.**

RESULTS AND DISCUSSION

The overall RFLP profiles and sequences on the four genes were obtained in 7 samples of maize, 4 of pepper and of 2 celery. The RFLP analyses allow to differentiate 8 lineages in the 13 samples studied. Lineages named III, IV and V were only detected in maize, lineages VI and VII and VIII only in pepper; lineage II was detected in one pepper and one celery samples and lineage I was only identified in celery (Table 1).

Table 1. '*Ca*. P. solani' isolates features RFLP profiles associated with infected maize, pepper and celery plants from Semberija region (Bosnia and Herzegovina).

Bosnia and Herzegovina/Semberija		RFLP profiles				
Strain	Plant host	Tuf	Stamp	SecY	Vmp1	LINEAGES
C1	Celery	B	B	B	E/V4	I
C2	Celery	B	B	B	A/V2-TA	II
P10	Pepper	B	B	B	A/ V2-TA	II
M7	Maize	B	A	B	B/V2-TA	III
MB1	Maize	B	A	B	B/ V2-TA	III
MB6	Maize	B	A	B	B/V2-TA	III
MB11	Maize	B	A	B	B/V2-TA	III
MB4	Maize	B	A	A	A/ V2-TA	IV
MB8	Maize	B	A	A	A/V2-TA	IV
MB10	Maize	B	A	B	A/ V2-TA	V
P5	Pepper	B	C	B	C/V4	VI
P6	Pepper	B	B	B	D/V14	VII
P7	Pepper	B	A	B	D/V14	VIII

Amplicons of the *tuf* gene were obtained in all '*Ca*. P. solani' strains and *Hpa*II restriction profiles confirmed that the pepper, celery and maize plants were infected with tuf-type b "stolbur" phytoplasmas (data not shown). On the *stamp* gene three lineages were distinguished, of which two were only detected once in a pepper and in a celery samples (Fig. 1), *sec*Y differentiated only two lineages of which one was only present in two maize samples (Fig. 2). The highest variability was shown by *vmp*1 gene in which two lineages in maize, and two in pepper were differentiated; one lineage in celery was identical to one of the two detected in maize (Fig. 3).

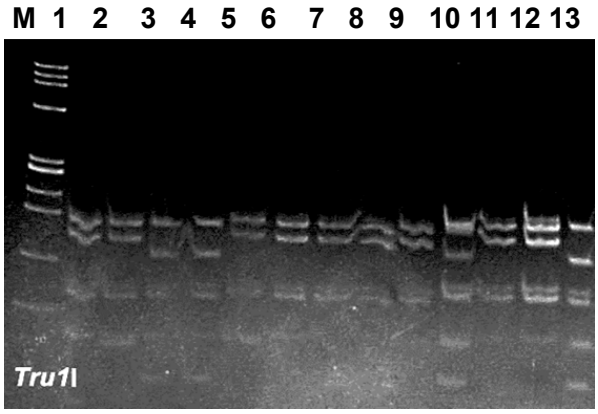

Figure 1. Polyacrylamide (6.7%) gel showing RFLP profiles of *stamp* gene amplicons digested with *Tru1*I. M, Marker DNA phiX174 *Hind*III digest. Samples (acronyms are as in Table 1) 1, M7; 2, P5; 3, P6; 4, C2; 5, P7; 6, MB10; 7, MB11; 8, MB4; 9, MB8; 10, C1; 11, MB6; 12, MB1; 13, STOL (="stolbur" from periwinkle originated from pepper from Serbia).

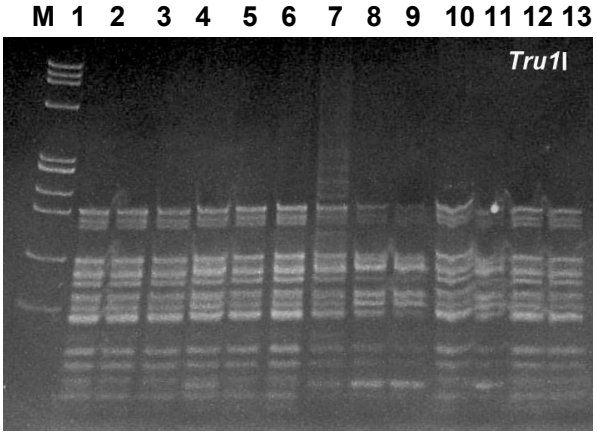

Figure 2. Polyacrylamide 6.7% gel showing RFLP patterns of *sec*Y amplicons digested with *Tru*1I. M, Marker DNA phiX174 *Hind*III digest. Samples (acronyms are as in Table 1) 1, M7; 2, P5; 3, P6; 4, C2; 5, P7; 6, MB10; 7, MB11; 8, MB4; 9, MB8; 10, C1; 11, MB6; 12, MB1; 13, STOL (="stolbur" from periwinkle originated from pepper from Serbia).

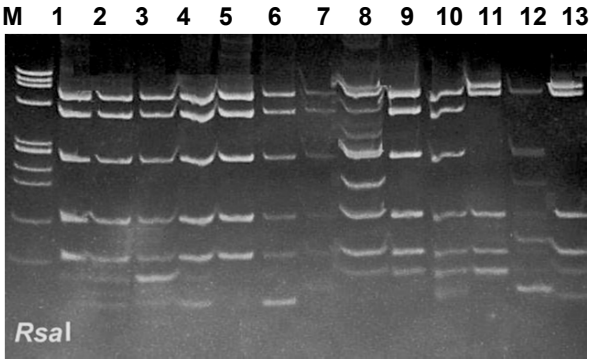

Figure 3. Polyacrylamide (6.7%) gel showing RFLP patterns of *vmp*1 gene nested PCR products digested with *Rsa*I. M, Marker DNA phiX174 *Hind*III digest. Samples (acronyms are as in Table 1) 1, MB10; 2, MB8; 3, MB4; 4, MB6; 5, M7; 6, MB1; 7, MB11; 8, C1; 9, C2; 10, P10; 11, P6; 12, P5; 13, P7.

Homology of sequences of *stamp* gene from maize samples was 100% confirming the RFLP results; the *stamp* sequences of strains from celery showed the presence of SNPs among each other and also with maize strains, two of the pepper strains (P5 and P7) were 100% identical to each other, while the others showed differences confirming the differential RFLP profiles obtained. On *sec*Y gene the maize lineages MB4 and MB8 showed sequences that were different for the presence of one SNP confirming the RFLP differential profiles named A and B (Table 1). The sequences obtained on *vmp*1 gene showed a high degree of heterogeneity that made the comparison among them and to the RFLP profiles not very easy. According to Murolo et al. (2010; 2013) and Cvrković et al. (2014), in the maize samples the V2-TA type was identified while in pepper and celery samples the V2-TA, V4 and V14 profiles were identified. The identification of more lineages in these crops compared to the 4 detected in grapevine in previous surveys (Delić et al., 2015) and to those detected in various "stolbur" infected crops in Ser-

bia (Mitrović et al., 2013; 2015) allow to hypothize the presence of insect vectors such as *Hyalesthes obsoletus* and *Reptalus panzeri* identified in grapevine, corn and potatoes in Serbia (Jović et al., 2009; Cvrković et al., 2014; Mori et al., 2013; Mitrović et al., 2015). Climate and soil structure in Semberija region are closer to those in the region of Serbia where these studies were carried out than to those in Herzegovina region of B&H where the diversity of *'Ca*. P. solani' in grapevine was previously studied. There could be a possible further increase of phytoplasma biodiversity by their possible transmission also by seeds (Calari et al., 2011).

REFERENCES

BERTACCINI, A., DUDUK, B., PALTRINIERI, S. and CONTALDO, N. 2014: Phytoplasmas and Phytoplasma Diseases: A Severe Threat to Agriculture. American Journal of Plant Sciences 5: 1763-1788.

CALARI, A., PALTRINIERI, S., CONTALDO, N., SAKALIEVA, D., MORI, N. and DUDUK, B. and BERTACCINI, A. 2011: Molecular evidence of phytoplasmas in winter oilseed rape, tomato and corn seedlings. Bulletin of Insectology 64(Supplement): S157-S158.

CVRKOVIĆ, T., JOVIĆ, T., MITROVIĆ, M., KRSTIĆ, O., and TOŠEVSKI, I. 2014: Experimental and molecular evidence of *Reptalus panzeri* as a natural vector of bois noir. Plant Pathology 63(1): 42–53.

DELIĆ, D., LOLIĆ, B. and KARAČIĆ, A. 2011: Screening for phytoplasma presence in West Herzegovina vineyards. Phytopathogenic Mollicutes 1(2): 87-90.

DELIĆ, D., BALECH, B., RADULOVIĆ, M., LOLIĆ, B., KARAČIĆ, A., VUKOSAVLJEVIĆ, V., ĐURIĆ, G. and JOVANOVIĆ CVETKOVIĆ, T. 2015: *Vmp*1 and *stamp* genes variability of '*Candidatus* phytoplasma solani' in Bosnian and Herzegovinian grapevine. European Journal of Plant Pathology DOI: 10.1007/s10658-015-0828-z.

DELIĆ, D., CONTALDO, N., LOLIĆ, B., MORAVČEVIĆ, Đ. and BERTACCINI, A. 2016: First report of '*Candidatus* Phytoplasma solani' in pepper and celery in Bosnia and Herzegovina. Journal of Plant Pathology (accepted).

JOVIĆ, J., CVRKOVIĆ, T., MITROVIĆ, M., KRNJAJIĆ, S., PETROVIĆ, A., REDINBAUGH, M.G., PRATT, R.C., HOGENHOUT S.A. and TOSEVSKI, I. 2009: Stolbur phytoplasma transmission to maize by *Reptalus panzeri* and the disease cycle of maize redness in Serbia. Phytopathology 99(9):1053-1061.

LANGER, M. and MAIXNER, M. 2004: Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur-group based on RFLP-analysis of non-ribosomal DNA. Vitis 43: 191–199.

LEE, I-M., BOTTNER-PARKER, K.D., ZHAO, Y., DAVIS, R.E. and HARRISON, N.A. 2010: Phylogenetic analysis and delineation of phytoplasmas based on *sec*Y gene sequences. International Journal of Systematic and Evolutionary Microbiology 60: 1703-1713.

MORI, N., MITROVIĆ, J., SMILJKOVIĆ, M., DUDUK, N., PALTRINIERI, S., BERTACCINI, A. and DUDUK, B. 2013: *Hyalesthes obsoletus* in Serbia and its role in the epidemiology of corn reddening. Bulletin of Insectology 66(2): 245-250.

KOVAČEVIĆ, M., ĐURIĆ, Z., JOVIĆ, J., PERKOVIĆ, G., LOLIĆ, B., HRNČIĆ, S., TOŠEVSKI, I. and DELIĆ, D. 2014: First report of stolbur phytoplasma associated with maize redness disease of maize in Bosnia and Herzegovina. Plant Disease 98(3): 418.

FABRE, A., DANET, JL. and FOISSAC, X. 2011: The stolbur phytoplasma antigenic membrane protein gene *stamp* is submitted to diversifying positive selection. Gene 472: 37-41.

FIALOVA´, R., VA´LOVA´, P., BALAKISHIYEVA, G., DANET, J.-L., SˇAFA´RˇOVA´, D., FOISSAC, X. and NAVRA´TIL M. 2009: Genetic variability of stolbur phytoplasma in annual crop and wild plant species in South Moravia (Czech Republic). Journal of Plant Pathology 91: 411–416.

CIMERMAN, A., PACIFICO, D., SALAR, P., MARZACHI', C. and FOISSAC X. 2009: Striking diversity of *vmp*1, a variable gene encoding a putative membrane protein of the stolbur phytoplasma. Applied and Environmental Microbiology 75: 2951–2957.

CVRKOVIĆ, T., JOVIĆ, J., MITROVIĆ, M., KRSTIĆ, O. and TOŠEVSKI, I. 2014: Experimental and molecular evidence of *Reptalus panzeri* as a natural vector of bois noir. Plant Pathology 63(1): 42-53.

LOLIĆ, B., PERKOVIĆ, G. and DELIĆ, D. 2014: Maize redness disease: current situation in Bosnia and Herzegovina. In: COST FA0807. Phytoplasmas and phytoplasma disease management: how to reduce their economic impact. Ed. A. Bertaccini. IPWG, Bologna Italy, Pp. 143-149.

MITROVIĆ, J., PAVLOVIĆ, S. and DUDUK, B. 2013: Survey and multigene characterization of stolbur phytoplasmas on various plant species in Serbia. Phytopathologia Mediterranea 52(3): 434−441.

MITROVIĆ, M., JAKOVLJEVIĆ, M., JOVIĆ, J., KRSTIĆ, O., KOSOVAC, A., TRIVELLONE V., JERMINI, M., TOŠEVSKI, I. and CVRKOVIĆ, T. 2015: *'Candidatus* phytoplasma solani' genotypes associated with potato stolbur in Serbia and the role of *Hyalesthes obsoletus* and *Reptalus panzeri* (hemiptera, cixiidae) as natural vectors. European Journal of Plant Pathology 107(3): 263-271.

MUROLO, S., MARCONE, C., PROTA, V., GARAU, R., FOISSAC, X. and ROMANAZZI, G. 2013: CORRIGENDUM: Genetic variability of the stolbur phytoplasma *vmp*1 gene in grapevines, bindweeds and vegetables. Journal of Applied Microbiology 115: 631-633.

MUROLO, S., MARCONE, C., PROTA, V., GARAU, R., FOISSAC, X. and ROMANAZZI, G. 2010: Genetic variability of the stolbur phytoplasma *vmp1* gene in grapevines, bindweeds and vegetables. Journal of Applied Microbiology 109: 2049-2059.

STADEN, R., BEAL, K.F. and BONFIELD, J.K. 2000: The Staden package, 1998. Methods in Molecular Biology 132: 115–130.

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. and HIGGINS, D.G. 1997: The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24: 4876–4882.

MULTILOCUS SEQUENCE ANALYSIS AS A POWERFUL TOOL TO MONITOR MOLECULAR EPIDEMIOLOGY OF '*CANDIDATUS* PHYTOPLASMA SOLANI' AT VINEYARD SCALE

Sergio Murolo and Gianfranco Romanazzi

Dipartimento di Scienze Agrarie, Alimentari e Ambientali, Università Politecnica delle Marche, Via Brecce Bianche – 60131 Ancona, Italy. E-mail: g.romanazzi@univpm.it

'*Candidatus* Phytoplasma solani' is a phytoplasma of the stolbur group (16SrXII-A subgroup), associated with grapevine Bois noir (BN), responsible for outbreaks in several European countries, and particularly in the Mediterranean area. It is transmitted by the polyphagous cixiid planthopper *Hyalesthes obsoletus* to a wide range of wild plants (inoculum sources) while grapevine is only occasionally infected. The multiple interactions with wild and cultivated annual and perennial host plants and insect vectors in different ecosystems might be responsible for generating genetic diversity of '*Ca.* P. solani'. Aim of this study was to estimate the '*Ca*. P. solani' molecular genotypes harbored in a single vineyard by multilocus sequence typing analysis for the *vmp*1, *stamp*, and *sec*Y genes. Several haplotypes per gene were detected, showing high genetic diversity even in a restricted area. Further analyses allowed to estimate the pressure of selection within a highly BN infected commercial vineyard by calculating the dN/dS ratio, which resulted particularly high (positive selection) for *stamp* and *vmp*1 genes. The high genetic variability, recorded in particular in genes encoding the membrane proteins, represents an adaptation strategy common to living microorganisms, particularly useful for a generalist pathogen that colonized different environments (host plant and vector tissues).

INTRODUCTION

Bois noir (BN) is a grapevine disease that is associated to '*Candidatus* Phytoplasma solani' ('Ca. P. solani'; 16SrXII-A subgroup) (QUAGLINO et al., 2013), and it is most common and widespread in Euro-Mediterranean regions (MAIXNER et al., 2011), becoming a real limiting factor for the productions. '*Ca*. P. solani' isolates are characterized by different degrees of genetic variability according to the genes involved (FOISSAC et al., 2013; QUAGLINO et al., 2013). The most variable genes are those that code for surface membrane proteins, which are directly exposed to host and vector interactions. In this study, we combined data coming from genotyping by multilocus sequence analysis with estimation of the dN/dS ratio. The latter is the ratio between the non-synonymous (dN) and the synonymous (dS) substitution rates in an alignment of amino-acid-coding sequences (NIELSEN, 2005), in order to estimate the richness of '*Ca*. P. solani' molecular genotypes and the pressure of selection within a highly BN infected commercial vineyard.

MATERIALS AND METHODS

The DNAs, extracted by CTAB protocol, were amplified in nested-PCR with specific primer pairs for *vmp*1, *stamp* and *sec*Y. *vpm*1 amplicons were digested in PCR-RFLP (FIALOVA et al., 2009; FABRE et al., 2011) in order to distinguish the molecular types. On the basis of the RFLP characterisation of *vmp*1 genes, representative samples within the vineyard, amplified with specific primer pairs for the *vmp*1, *stamp* and *sec*Y genes were purified and sequenced. The phylogenetic relationships were reconstructed, using the Mega v. 5.1 software, for the *vmp1, stamp*, and *sec*Y nucleotide sequences of '*Ca*. P. solani' that originated from the study vineyard, with respect to nucleotide sequences from other Italian regions and from Euro-Mediterranean countries that were available in Genebank. Moreover, the ratio between the proportion of non-synonymous and synonymous substitutions (dN/dS ratio), was determined for the nucleotide sequences at the study vineyard level in order to determine the type of selection interfere on *vmp*1 gene. Positive selection happens when dN/dS ratio >1, on the other hand a ratio <1 suggests a purifying selection process (NEI and KUMAR, 2000).

RESULTS AND DISCUSSION

In the study vineyard, the molecular characterization of *vmp*1, allowed to detect eight different *vmp*1 types (V3, V4, V9, V11, V12, V14, V15 and V18), most of them identified in *H. obsoletus* collected in Marche vineyard ecosystems (Landi et al., 2015). The wide genetic diversity of '*Ca*. P. solani' has been reported and generally related to complex interactions between the vector and the wide range of wild host plants (KESSLER et al., 2011). The dominant *vmp*1 genotypes were V14 and V12, while we sporadically detected V3.

The phylogenetic analysis was carried out on nucleotide sequences, which were representative of the RFLP types of the study vineyard, and on those available in GenBank. In the resulting dendrogram, the sequences generally clustered according to the PCR-RFLP patterns. Strains with the same RFLP pattern showed high nucleotide similarity (>99%) of sequences (MUROLO et al., 2010). The selective pressure in the *vmp*1, *stamp* and *sec*Y genes were estimated for the '*Ca*. P. solani' strains according to the abundance of non-synonymous mutations. For the *sec*Y gene, the overall dN/dS ratio was 1.02 (P =0.841), which suggested low neutral selection across this gene. The overall ratio between the non-synonymous to the synonymous mutations (dN/dS) was >1 for *vmp*1 (2.28; P = 0.001) and *stamp* (3.99; P = 0.019). These high values of dN/dS (i.e., >1) indicated detection of a high number of non-silent (dN) mutations. The higher genetic variability in the *vmp*1 and *stamp* genes with respect to the *sec*Y gene arose from the estimation of the rate of non-silent mutation (dN). According to this parameter, which is an indication of selective pressure, FABRE et al. (2011) defined the *sec*Y gene as a housekeeping gene, while the *vmp*1 and *stamp* genes were under positive selection, because they are involved in specific interactions as demonstrated for other phytoplasma (KAKIZAWA et al., 2006). The high genetic variability as well as the dN/dS ratio >1 of '*Ca.* P. solani' in *vmp*1 and *stamp* genes, within a restricted location (i.e. commercial vineyard) provide useful information to trace an inoculum source and the movement of pathogen strains over local and long distances (MUROLO and ROMANAZZI, 2015).

REFERENCES

FABRE, A., DANET, J.L. and FOISSAC, X., 2011. The stolbur phytoplasma antigenic membrane protein gene *stamp* is submitted to diversifying positive selection. Genes 472, 37–41.

FIALOVA, R., VALOVA, P., BALAKISHIYEVA, G. et al., 2009. Genetic variability of stolbur phytoplasma in annual crop and wild plant species in south Moravia (Czech Republic). Journal of Plant Pathology 91, 411–416.

FOISSAC, X., CARLE, P., FABRE, A. Et al., 2013. 'Candidatus Phytoplasma solani' genome project and genetic diversity in the Euro-Mediterranean basin. In: Torres E, Lavina A, Batlle A, eds. Proceedings of 3rd European Bois Noir Workshop 2013, Barcelona (Spain), 20–21, March: International Phytoplasma Working Group, 11–13.

KAKIZAWA, S., OSHIMA, K., NAMBA, S., 2006. Diversity and functional importance of phytoplasma membrane proteins. Trends in Microbiology 14, 254–256.

KESSLER, S., SCHAERER, S., DELABAYS, N., TURLINGS, T.C.J., TRIVELLONE, V., 2011. Host plant preferences of *Hyalesthes obsoletus,* the vector of the grapevine yellows disease Bois noir' in Switzerland. Entomologia Experimentalis et Applicata 139, 60–67.

LANDI, L., RIOLO, P., MUROLO, S., ROMANAZZI, G., NARDI, S., ISIDORO, N., 2015. Genetic variability of stolbur phytoplasma in *Hyalesthes obsoletus* (Hemiptera: Cixiidae) and its main host plants in vineyard agroecosystems. Journal of Economic Entomology 108, 1506-1515.

MAIXNER, M., 2011. Recent advances in Bois noir research. Petria 21, 95–108.

MUROLO, S., MARCONE, C., PROTA, V., GARAU, R., FOISSAC, X., ROMANAZZI, G., 2010. Genetic variability of the stolbur phytoplasma *vmp*1 gene in grapevines, bindweeds and vegetables. Journal of Applied Microbiology 109, 2049–2059.

MUROLO, S., ROMANAZZI, G., 2015. In-vineyard population structure of '*Candidatus* Phytoplasma solani' using multilocus sequence typing analysis. Infection, Genetics and Evolution 31, 221-230.

NEI, M., KUMAR, S., 2000. Molecular evolution and phylogenetics. Oxford University Press

NIELSEN, R., 2005. Molecular signatures of natural selection. Annual Review of Genetics 39,197–218.

QUAGLINO, F., ZHAO, Y., CASATI, P., BULGARI, D., BIANCO, P.A., WEI, W., DAVIS, R.E., 2013. '*Candidatus* Phytoplasma solani', a novel taxon associated with stolbur and Bois noir related diseases of plants. International Journal of Systematic and Evolutionary Microbiology 63, 2879–2894.

CHARACTERIZATION OF '*CANDIDATUS* PHYTOPLASMA SOLANI' STRAINS FROM GRAPEVINES, *HYALESTHES OBSOLETUS* , REFERENCE STRAINS IN PERIWINKLE AND IN COLONIES OF STRAIN STOL

Nicoletta Contaldo[1], Yuri Zambon[1], Samanta Paltrinieri[1], Nicola Mori[2], Jelena Mitrović[3], Bojan Duduk[3] and Assunta Bertaccini[1]

[1] Alma Mater Studiorum, University of Bologna, Plant Pathology
IT-40127, Bologna, Viale Fanin 42
[2] Department of Agronomy, Food, Natural resources, Animals and Environment, University of Padua
IT-35020 Legnaro (Padua), Viale dell'Università 16
[3] Institute of Pesticides and Environmental Protection, Department of Plant Pests
RS-11080 Zemun, Banatska 33

E-Mail: nicoletta.contaldo2@unibo.it

Multilocus typing (MLT) was performed on 72 phytoplasma strains obtained from grapevine samples and *Hyalesthes* obsoletus specimens collected in different Bois noir-infected vineyards, on six phytoplasma strains maintained in the periwinkle reference collections and on a colony obtained from cultivation of a '*Candidatus* Phytoplasma solani' strain derived from the STOL periwinkle reference collection sample. RFLP analyses of *tuf*, *sec*Y, *groel* and *Stamp* genes allowed to differentiate 8 lineages in the 72 field-collected grapevine and *H. obsoletus* samples. In some cases there was a good consistency in the MLT profiles of lineages detected in the grapevine and those identified in *H. obsoletus* indicating the presence of phytoplasma populations shared between the two hosts. On the other hand, the lineages identified in the six "stolbur" strains in collection in periwinkle were all different from those in the vineyard collected samples. The partial molecular characterization of the colonies obtained from the STOL strain after its isolation in a chemically defined medium shows *Stamp* gene congruence with those of the original strain maintained in micropropagation.

Bois noir (BN) phytoplasmas are transmitted by cuttings and predominantly by Cixiidae (Auchenorrhyncha), therefore the study of the epidemiology of the disease is difficult. Disease management of outbreaks and the differentiation of phytoplasma populations in vineyards is of utmost importance. The characterization of BN phytoplasmas was performed in several grapevine growing areas by multilocus sequence analysis on diverse genes. However, very often a consistent differentiation of phytoplasma populations in specific geographic areas was difficult to achieve. This problem could be linked to the molecular markers selected for the differentiation studies, as some of the employed molecular markers are subjected to a high environmental selection pressure and can therefore be problematic for epidemiologic research. Due to the absence of markers directly linked to BN pathogenicity, the use of diverse marker combinations might allow the following of epidemics. Thus, in the present study a multigene characterisation of '*Ca.* P. solani' strains from

different sources was carried out by collective RFLP typing of *tuf*, *sec*Y, *groel* and *Stamp* genes in order to detect their possible usefulness as combined markers in the study of BN epidemiology.

MATERIAL AND METHODS

Seventy-three '*Candidatus* Phytoplasma solani' strains from grapevine and *Hyalesthes obsoletus* samples collected in vineyards located in Northern Italy and Serbia (Table 1) were employed. "Stolbur" reference strains maintained in periwinkle were STOL (from Serbia), ASLO (from Slovenia) STOLC, STOL-PO, STOL-CH, MOL (from France). DNA from colonies grown in chemically defined medium after isolation from STOL periwinkle in micropropagation (Contaldo et al., 2012) was also used. Total nucleic acids were extracted from 1 g of midribs and phloem scrapes of infected samples maintained at -20 °C with a chloroform/phenol method, while *H. obsoletus* samples maintained in -20 °C were extracted by a CTAB procedure (Angelini et al., 2001). Amplicons produced on 16S rDNA (Gundersen and Lee, 1996), *Stamp* (Fabre et al., 2011), and *sec*Y (Lee et al., 2010) genes were subjected to RFLP analyses with *Tru1*I. RFLP on *groEL* gene was performed on nested amplicons obtained with STOLgroesF/STOLstampR followed by AYgroelF/STOLgroelR2 primers (Mitrović et al., 2011, 2012 and 2013) with *Tru1*I. The *tuf* gene was amplified in a nested PCR procedure using primer pairs tuf1f/r/TufAYf/r and TufINT1f/TufINT4r (Langer and Maixner, 2004) and amplicons were subjected to the RFLP analyses with *Hpa*II.

RESULTS AND DISCUSSION

The overall RFLP profiles on four phytoplasma marker genes were obtained from 35 grapevine and 15 *H. obsoletus* samples collected in vineyards as shown in Table 1. Eight lineages were differentiated by RFLP analyses. In 22 field samples not all the four genes could be amplified even if '*Ca.* P. solani'-related phytoplasmas were identified by RFLP analyses on 16S ribosomal gene. The analysis of 5 cixiid and 17 grapevine samples resulted only in amplification of two to three genes, while *Stamp* was amplified in all samples (Fig. 1). Moreover the rate

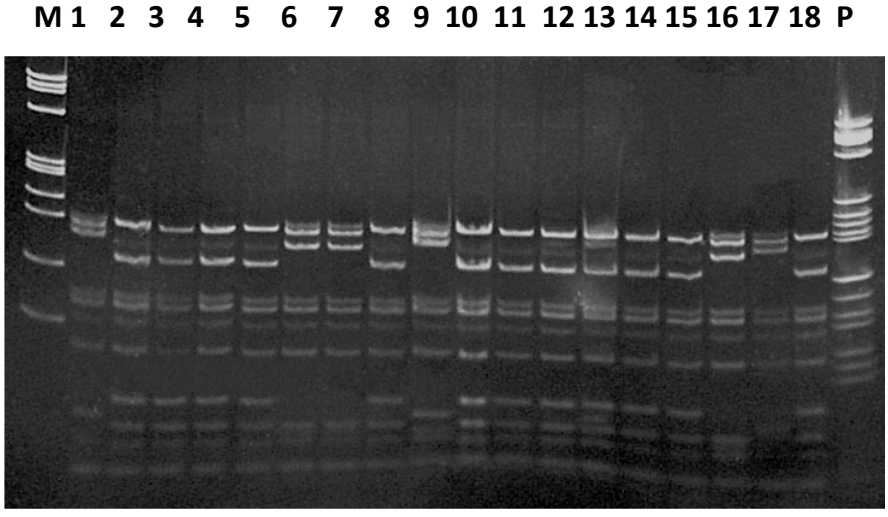

Fig. 1. Polyacrylamide (6.7 %) gel showing RFLP profiles of *Stamp* gene amplicons digested with *Tru*1I. M, Marker DNA phiX174 *Hind*III digest; P, Marker DNA pBR322 *MspI* digested. From 1 to 18 grapevine samples showing 3 of the differential profiles identified on the amplicon from this gene.

of amplification was decreasing from amplification of *tuf* to *sec*Y and *groEL* genes. In the latter, only 9 out of 22 samples were amplified, confirming the reported reduced sensitivity in detection of primers other than those amplifying 16S rDNA and is also in agreement with the expected amplicon length. While in the amplicons of the *Stamp* gene five different restriction profiles were differentiated by *Tru1*I, two profiles were distinguished in the *tuf* gene by *Hpa*II, two in the *groEL* gene by *Tru1*I and three profiles were shown by the same enzyme in *sec*Y gene (Table 1). Overall, 8 lineages could be obtained by the different combination of restriction profiles in the samples from vineyards. Three profiles were only detected in one sample from grapevine or *H. obsoletus* (lineages I, IV and XI); profile X was only detected in 2 grapevine samples from Serbia. The profile III was detected in *H. obsoletus* from Italy as well as in grapevine samples from different localities in Serbia, while the profile VI was present in 7 grapevine samples, two of which are from Serbia and the rest from different provinces of the same Italian region (Emilia-Romagna). On the other hand the profile V was found in both grapevine and *H. obsoletus* in the same Italian region at the time of an epidemic of BN. The results obtained are in agreement with previous results (Contaldo et al., 2009, 2011, 2012 and 2013; Mitrović et al., 2013) and indicate that the MLT of these 4 genes could be useful as combined markers in the study of BN epidemiology under field conditions.

It is important to underline that none of the tested reference strains maintained in periwinkle in micropropagation showed the profiles detected in the field collected samples, but the overall lineage profiles showed to be consistent with the geographic origin of the strains (i.e. France, Slovenia and Serbia). The STOL strain colonies grown on agar media after isolation in broth medium tested positive on both, 16S ribosomal and *Stamp* genes. The RFLP profiles and their sequences obtained after direct sequencing in both directions with primers used for amplification confirmed the presence of STOL DNA, in agreement with the previously reported cultivability of this phytoplasma strain (Contaldo et al., 2012 and 2015).

Table 1: Characteristics of the '*Ca*. P. solani' containing samples, RFLP profiles on the four phytoplasma genes employed and lineages obtained

Acronym	Sample from	ORIGIN					Lineages
			tuf	*Stamp*	*secY*	*groEL*	
CHSM1	grapevine	Veneto	B	B	B	B	I
CHSM2	grapevine	Veneto	B	A	A	A	II
2 E2	*H. obsoletus*	Veneto	B	A	A	A	II
H.o 23	*H. obsoletus*	Emilia Romagna	B	A	A	A	II
H.o. 7	*H. obsoletus*	Emilia Romagna	B	A	A	A	II
H.o. 9	*H. obsoletus*	Emilia Romagna	B	A	A	A	II
H.o. 3	*H. obsoletus*	Emilia Romagna	B	A	A	A	II
H.o. 13	*H. obsoletus*	Emilia Romagna	B	A	A	A	II
H.o. 19	*H. obsoletus*	Emilia Romagna	B	A	A	A	II
H.o. 17	*H. obsoletus*	Emilia Romagna	B	A	A	A	II
2 E1	*H. obsoletus*	Veneto	B	B	B	A	III

Acronym	Sample from	ORIGIN					Lineages
			tuf	*Stamp*	*secY*	*groEL*	
Aleks. 122/10	grapevine	Serbia	B	B	B	A	III
Bela Cr. 132/10	grapevine	Serbia	B	B	B	A	III
Bela Cr. 134/10	grapevine	Serbia	B	B	B	A	III
Radmil. 191/09	grapevine	Serbia	B	B	B	A	III
Radmil. 192/09	grapevine	Serbia	B	B	B	A	III
3 E	*H. obsoletus*	Veneto	A	A	B	A	IV
Ra9818	grapevine	Emilia Romagna	A	A	A	A	V
Ra 9912	grapevine	Emilia Romagna	A	A	A	A	V
Ra9910	grapevine	Emilia Romagna	A	A	A	A	V
Ra 9830	grapevine	Emilia Romagna	A	A	A	A	V
Ra9801	grapevine	Emilia Romagna	A	A	A	A	V
Ra9804	grapevine	Emilia Romagna	A	A	A	A	V
Ra14486	grapevine	Emilia Romagna	A	A	A	A	V
Ra9709	grapevine	Emilia Romagna	A	A	A	A	V
Ra9707	grapevine	Emilia Romagna	A	A	A	A	V
Ra9802	grapevine	Emilia Romagna	A	A	A	A	V
REV8	grapevine	Emilia Romagna	A	A	A	A	V
REV10	grapevine	Emilia Romagna	A	A	A	A	V
REV13	grapevine	Emilia Romagna	A	A	A	A	V
MOV27	grapevine	Emilia Romagna	A	A	A	A	V
FE9805	grapevine	Emilia Romagna	A	A	A	A	V
FC10044	grapevine	Emilia Romagna	A	A	A	A	V
BO14394	grapevine	Emilia Romagna	A	A	A	A	V

Acronym	Sample from	ORIGIN					Lineages
			tuf	*Stamp*	*secY*	*groEL*	
BO9866	grapevine	Emilia Romagna	A	A	A	A	V
H.o. 2	*H. obsoletus*	Emilia Romagna	A	A	A	A	V
H.o. 16	*H. obsoletus*	Emilia Romagna	A	A	A	A	V
H.o. 5	*H. obsoletus*	Emilia Romagna	A	A	A	A	V
H.o. 4	*H. obsoletus*	Emilia Romagna	A	A	A	A	V
H.o. 7b	*H. obsoletus*	Emilia Romagna	A	A	A	A	V
Ra9827	grapevine	Emilia Romagna	B	B	A	A	VI
FE9806	grapevine	Emilia Romagna	B	B	A	A	VI
FE9810	grapevine	Emilia Romagna	B	B	A	A	VI
BO9870	grapevine	Emilia Romagna	B	B	A	A	VI
BO9867	grapevine	Emilia Romagna	B	B	A	A	VI
Aleks. 125/10	grapevine	Serbia	B	B	A	A	VI
Smeder. 66/11	grapevine	Serbia	B	B	A	A	VI
STOL-CH	periwinkle	France	B	C	C	A	VII
MOL	periwinkle	France	B	C	C	A	VII
STOL-C	periwinkle	France	B	C	C	A	VII
STOL-PO	periwinkle	France	B	C	C	A	VII
ASLO	periwinkle	Slovenia	B	D	C	A	VIII
STOL	periwinkle	Serbia	B	A/B	C/B	A	IX
STOL	Colony	Periwinkle-Serbia	-	A	-	-	*
Bela Cr. 144/10	grapevine	Serbia	B	E	B	A	X
Krčedin 93/10	grapevine	Serbia	B	E	B	A	X
Smeder. 69/11	grapevine	Serbia	B	B	A	B	XI
CHCA1	grapevine	Veneto	-	A	A	A	n.d.
5A	*H. obsoletus*	Veneto	-	A	A	A	n.d.
4 A1	*H. obsoletus*	Veneto	-	A	A	A	n.d.

Acronym	Sample from	ORIGIN					Lineages
			tuf	*Stamp*	*secY*	*groEL*	
H.o. 9	*H. obsoletus*	Emilia Romagna	-	B	A	A	n.d.
4 A7	*H. obsoletus*	Veneto	A	A	-	A	n.d.
CH2	grapevine	Veneto	B	A	-	-	n.d.
PiGru1	grapevine	Veneto	B	A	-	A	n.d.
PiGru2	grapevine	Veneto	B	A	-	A	n.d.
4 A12	*H. obsoletus*	Veneto	B	A	-	-	n.d.
Aleks. 123/10	grapevine	Serbia	B	A	-	A	n.d.
Aleks. 127/10	grapevine	Serbia	B	E	-	A	n.d.
Bela Cr. 130/10	grapevine	Serbia	B	A	B	-	n.d.
Bela Cr. 131/10	grapevine	Serbia	B	B	-	-	n.d.
Bela Cr. 140/10	grapevine	Serbia	B	B	-	-	n.d.
Bela Cr. 142/10	grapevine	Serbia	B	B	B	-	n.d.
Bela Cr. 143/10	grapevine	Serbia	B	B	B	-	n.d.
Bela Cr. 145/10	grapevine	Serbia	B	B	-	-	n.d.
Krčedin 95/10	grapevine	Serbia	B	A	B	-	n.d.
Smeder. 65/11	grapevine	Serbia	B	E	-	-	n.d.
Smeder. 67/11	grapevine	Serbia	B	B	B	-	n.d.
Smeder. 68/11	grapevine	Serbia	B	B	-	-	n.d.
Smeder. 70/11	grapevine	Serbia	B	B	-	-	n.d.

REFERENCES

Angelini, E., Clair, D., Borgo, M., Bertaccini, A. and Boudon-Padieu, E. 2001: Flavescence dorée in France and Italy - Occurrence of closely related phytoplasma isolates and their near relationships to Palatinate grapevine yellows and an alder yellows phytoplasma. Vitis 40(2): 79-86

Contaldo, N., Duduk, B., Paltrinieri, S., Kolber, M., Ember, I. and Bertaccini, A. 2009: Towards strain differentiation among grapevine bois noir phytoplasmas. Le Progrès agricole et viticole HS:184-5

Contaldo, N., Duduk, B., Paltrinieri, S., Dal Molin, F., Mitrović, J. and Bertaccini, A. 2011: Molecular variability on 16S rDNA of 'bois noir' phytoplasmas in grapevine from Italy and Serbia. Petria 21:136-137

Contaldo, N., Canel, A., Mitrović, J., Paltrinieri, S., Bertaccini, A. and Duduk, B. 2012: Multilocus analyses on grapevine 'bois noir' phytoplasmas from Italy and Serbia. 17th Meeting of ICVG, Davis California, USA, October 7th-14th: 226-227

Contaldo, N., Bertaccini, A., Paltrinieri, S., Windsor, H.M. and Windsor, D.G. 2012: Axenic culture of plant pathogenic phytoplasmas. Phytopathologia Mediterranea 51(3): 607−617

Contaldo, N., Paltrinieri, S., Mori, N., Canel, A., Zamora, L., Duduk, B. and Bertaccini, A. 2013: Are the "bois noir" - associated phytoplasmas molecularly differentiable from stolbur phytoplasmas infecting other species? 3rd European Bois Noir workshop, Barcelona, Spain March 20-21: 59-60

Contaldo, N., Zambon, Y., Satta, E., Paltrinieri, S. and Bertaccini, A. 2015: Cultivation in chemically defined media of phytoplasmas from field-infected grapevine plants showing yellows symptoms. 18th Congress of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG). September 7-11, Ankara, Turkey: 121-123

Fabre, A., Danet, JL. and Foissac, X. 2011: The stolbur phytoplasma antigenic membrane protein gene *stamp* is submitted to diversifying positive selection. Gene 472: 37-41

Gundersen, D.E. and Lee, I-M. 1996: Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathologia Mediterranea 35:114-151

Langer, M. and Maixner, M. 2004: Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur-group based on RFLP-analysis of non-ribosomal DNA. Vitis 43: 191–199

Lee, I-M., Bottner-Parker, K.D., Zhao, Y., Davis, R.E., Harrison, N.A. 2010: Phylogenetic analysis and delineation of phytoplasmas based on *sec*Y gene sequences. International Journal of Systematic and Evolutionary microbiology 60: 1703-1713

Mitrović, J., Kakizawa, S., Duduk, B., Oshima, K., Namba, S. and Bertaccini A. 2011: The *groEL* gene as an additional marker for finer differentiation of 'Candidatus Phytoplasma asteris' – related strains. Annals of Applied Biology 159: 41-48

Mitrović, J. and Duduk, B. 2012: Variability of groEL gene in stolbur phytoplasmas. Book of Abstracts of the 19th International Congress of the IOM, Toulouse, France: 157-158

Mitrović, J., Contaldo, N., Avramov, Z., Smiljković, M., Bertaccini, A. and Duduk, B. 2013: *GroEL* gene characterization of "bois noir" phytoplasma from Serbia, Bulgaria and Italy. 3rd European Bois Noir workshop, Barcelona, Spain March 20-21: 63-64

DETECTION OF BOIS NOIR PHYTOPLASMA BY A QUICK-TO-USE ISOTHERMAL AMPLIFICATION ASSAY: PRELIMINARY RESULTS

Andrea Gentili[1], Luca Ferretti[1], Lorenzo Vizzaccaro[1], Giuseppe Durante[2], Camilo Gianinazzi[3], Stefano Lopriore[4], Caroline Chatillon[5] and Graziella Pasquini[1]

[1] Council for Agricultural Research and Economics (CREA) - Plant Pathology Research Centre (PAV)
IT-00156 Rome, Via C.G. Bertero 22
[2, 3] International Plant Analysis and Diagnostics s.r.l., Loc. Cascina Codazza
IT-26900 Lodi, Via Einstein
[4] Hyris Ltd.
UK, London NW1 1JD, 41 Chalton St.
[5] Qualiplante SAS, Cap Alpha
FR-34830 Clapiers, Avenue de l'Europe
E-Mail: luca.ferretti@crea.gov.it

Bois noir (BN) is a widespread and economically relevant grapevine disease caused by stolbur phytoplasma (16SrXII-A ribosomal group, '*Candidatus* Phytoplasma solani'). Symptoms induced by this phytoplasma on grapevine are not visually distinguishable from those caused by Flavescence dorée (FD)-related phytoplasmas (16SrV-C/D), a quarantine pathogen regulated by specific control measures at European level. Thus a rapid and specific detection of these two phytoplasmas is crucial. In this work, an assay based on the loop-mediated isothermal amplification (LAMP) technique was developed and has been applied for the detection of '*Candidatus* Phytoplasma solani' in grapevine samples. In order to minimize the risk of contaminations and to further reduce the time for performing the assay a ready-to-use kit will be commercialized by Qualiplante/ Hyris. Here we report the preliminary results obtained from the laboratory trials carried out on naturally BN-infected grapevines, aiming at verifying the ability of the assay to specifically detect the BN phytoplasma.

Bois noir (BN) is a widespread and economically relevant grapevine disease caused by stolbur phytoplasma (16SrXII-A ribosomal group, '*Candidatus* Phytoplasma solani'). Symptoms induced by this phytoplasma on grapevine are not visually distinguishable from those caused by Flavescence dorée (FD)-related phytoplasmas (16SrV-C/D), a quarantine pathogen regulated by specific control measures at European level. A rapid and specific detection of these two phytoplasmas is, thus, crucial. Many molecular diagnostic methods have been developed for the phytoplasmas associated with t FD and BN in the past years including singleplex and multiplex Nested End-Point PCR methods as well as Real-Time PCR methods. More recently, an innovative molecular tool based on isothermal amplification of nucleic acids (Notomi et al., 2000) has been successfully developed and applied for the detection of FD agent phytoplasmas (Kogovšek et al., 2015; Durante et al., 2015). The method, employing a DNA polymerase with a strand displacement activity and a set of six primers that recognized six distinct sequences of

the target DNA, can be performed under isothermal conditions (between 60 °C and 65 °C). It yields results extremely rapidly with an average of amplification time between 8 and 45 minutes.
In this work, an assay based on the loop-mediated isothermal amplification (LAMP) technique was developed and has been applied for the detection of BN agent phytoplasma in grapevine samples. In order to minimize the risk of contaminations and to further reduce the time for performing the assay a ready-to-use kit will be commercialized by Qualiplante/Hyris. Here we report the preliminary results obtained from the laboratory trials carried out on naturally BN-infected grapevines, aiming at verifying the ability of the assay to specifically detect the BN phytoplasma.

MATERIAL AND METHODS

PLANT MATERIAL

DNA extracts belonging to CREA-PAV collection were analysed. Total nucleic acid (TNA) was obtained starting from 0.5 g of leaf midribs powdered in liquid nitrogen using a 2.5 % CTAB buffer as described in Marzachì et al. (1999) with some modifications. Six nested PCR-positive extracts from BN-infected grapevines of different geographical origin were used as target samples. DNA from two grapevines infected by Flavescence dorée phytoplasmas (16SrV-C and -D, respectively) and from one healthy *Vitis* sp. were used as non-target controls. Phytoplasmas belonging to 16SrI group (Aster yellow), often found associated with Grapevine yellows (GY) symptoms, were also included. Specifically, DNA extracted from a periwinkle infected by severe aster yellow (SAY, 16SrI-B) was used. Finally, a sample containing water was used as non-template control (NTC). All the analysed samples and their origin are listed in Table 1.

PRIMER DESIGN AND ISOTHERMAL ASSAY

Different 16S rRNA gene sequences from GenBank database belonging to BN and FD agent phytoplasmas were aligned and used for the design of primers. A primer set was designed using the LAMP Primer Explorer V3 software (http://primerexplorer.jp/elamp3.0.0/index.html) on the 16S rRNA gene sequence EU010007, used as reference.
Isothermal amplification was performed in 96-wells plate in a total final volume of 25 µl using 5 µl of ten-fold diluted DNA extracts as template. The reaction mixture was composed by the Isothermal master mix ISO-001 (Teltec s.r.l., Italy) assembled with a primer mixture containing the following concentration for each primer: 5 pmol for F3 and B3, 20 pmol for F-LOOP and R-LOOP, 40 pmol for FIP and BIP. Isothermal amplification was performed at 65 °C in an ABI Prism 7500 Fast (Applied Biosystems) thermal cycler under the following conditions: 30 cycles with two step, each as 1 s at 65 °C and 55 s at 65 °C with single acquisition of fluorescence. Melting temperature analysis was set as standard melting curve analysis given in the software with continuous acquisition of fluorescence (from 65 °C to 98 °C + 0.5 °C/cycle).

Table 1: Type and origin of the analyzed samples and results of the performed LAMP assay

N° of sample	Host	Origin	Infection status	LAMP assay		
				Signal	*Ct* value	Tm (°C)
1	Grapevine	Latium (central Italy)	Healthy	No	-	-
2	Grapevine	Umbria (central Italy)	BN positive	Yes	10.53	86.35
3	Grapevine	Sicily (south Italy)	BN positive	Yes	10.32	86.10
4	Grapevine	Sicily (south Italy)	BN positive	Yes	10.53	86.60
5	Grapevine	Latium (central Italy)	BN positive	Yes	10.62	86.60
6	Grapevine	Latium (central Italy)	BN positive	Yes	12.34	86.60
7	Grapevine	Latium (central Italy)	BN positive	Yes	11.21	86.85
8	Grapevine	Campania (south Italy)	FD-D positive	No	-	-
9	Grapevine	Veneto (north Italy)	FD-C positive	No	-	-
10	Periwinkle	Latium (central Italy)	16SrI-B positive	Yes	10.71	87.16
11	NTC			No	-	-

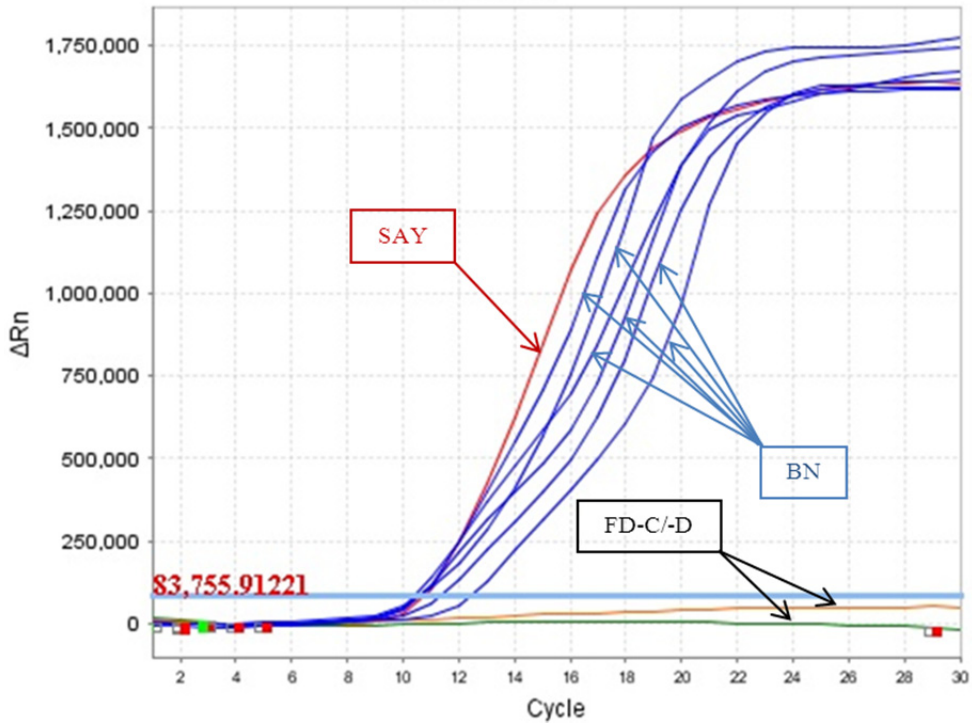

Fig. 1: Amplification plot obtained from the samples infected by tested phytoplasmas

RESULTS AND DISCUSSION

The results obtained from the preliminary trials (Table 1) showed the ability of the developed isothermal assay to detect the BN-phytoplasma in infected grapevines using DNA extracts as template and to discriminate it from the quarantine FD-related phytoplasmas. All samples from BN-infected grapevines showed an amplification signal with Ct values ranging from 10.32 to 12.34 whereas no amplification curve was observed for the healthy grapevines and for FD-C and -D positive samples (Fig. 1). Annealing temperature (Tm) analysis showed that all amplicons from BN-infected samples had a Tm ranging from 86.10 to 86.85 °C. Amplification signal was also obtained from the sample positive to 16SrI-B phytoplasmas but with a Tm slightly higher (87.16 °C). Further experiments with a wider number of samples and strains of this latter phytoplasmas are needed to verify if the different Tm value is consistent with the taxonomic distance of the two phytoplasmas. Finally, no amplification signal was obtained from the water control.

The developed LAMP assay was quick and allowed a rapid detection of BN phytoplasmas. Considering that each amplification cycle had an approximate duration of 1 minute, the Ct values obtained from positive samples indicate that the time required to obtain a positive signal is less than 15 min.

Further experiments on an extended panel of samples will be carried out for a more accurate evaluation of the method especially in terms of analytical sensitivity and specificity and to validate it according to the EPPO standard PM7/98(2) (EPPO, 2014).

REFERENCES

DURANTE, G., GIANINAZZI, C., LOPRIORE, S., AND CHATILLON, C. 2015. Development of an isothermal amplification assay for in-field detection of grapevine flavescence dorée phytoplasma. Proceedings of the 11th International Conference on Plant Diseases 7th, 8th & 9th of December 2015 – Centre de Congrès Vinci - Tours - France. In press.

KOGOVŠEK, P., HODGETTS, J., HALL, J., PREZELJ, N., NIKOLIĆ, P., MEHLE, N., LENARČIČ, R., ROTTER, A., DICKINSON, M., BOONHAM, N., DERMASTIA, M. AND RAVNIKAR, M. 2015. LAMP assay and rapid sample preparation method for on-site detection of flavescence dorée phytoplasma in grapevine. Plant Pathology, 64, 286–296.

MARZACHÌ, C., ALMA, A., D'AQUILIO, M., MINUTO, G., AND BOCCARDO, G. 1999. Detection and identification of phytoplasmas infecting cultivated and wild plants in Liguria (Italian Riviera). J. Plant Pathol. 81, 127-136.

NOTOMI, T., OKAYAMA, H., MASUBUCHI, H., YONEKAWA, T.,WATANABE, K., AMINO, N. AND HASE, T. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research 28 (12), E63.

OEPP/EPPO. 2014. PM7/98 (2) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. EPPO Bulletin, 44 (2), 117-147.

METABOLOME OF GRAPEVINE LEAF VEIN-ENRICHED TISSUE INFECTED WITH '*CANDIDATUS* PHYTOPLASMA SOLANI'

Nina Prezelj[1], Lena Fragner[2], Wolfram Weckwerth[2,3] and Marina Dermastia[1*]

[1]National Institute of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia
[2]Department of Ecogenomics and Systems Biology, Faculty of Life Sciences, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria
[3] Vienna Metabolomics Center (VIME), University of Vienna, Althanstrasse 14, 1090 Vienna Austria
*Corresponding author: marina.dermastia@nib.si

Non-targeted metabolome analysis from leaf vein-enriched tissues of grapevine plants cv. Chardonnay infected with '*Candidatus* Phytoplasma solani' identified 36 annotated or putatively annotated compounds that differed significantly between infected and uninfected samples. The study proved on the metabolome level that infection with phytoplasma significantly affects the carbohydrate metabolism.

INTRODUCTION

In Europe the most widespread phytoplasma associated with grapevine yellows (GY) is '*Candidatus* Phytoplasma solani' (QUAGLINO et al., 2013), which is the agent associated with "bois noir". Several recent studies on molecular mechanisms involved in the interactions between grapevine and '*Ca.* P. solani' suggest that the host plant responses to the infection involved radical changes in the expression patterns of genes (HREN et al., 2009; LANDI and ROMANAZZI; 2011; SANTI et al., 2013). However, although the metabolites are the end products of cellular regulatory processes, and their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes, metabolomic data from phytoplasma infected plants are very rare (CHOI et al., 2004; GAI et al., 2014). The aim of this study was an investigation of the leaf vein-enriched metabolome of grapevine plants cv. Chardonnay infected with '*Ca*. P. solani'.

MATERIAL AND METHODS

The study was carried out in a productive vineyard of grapevine (*Vitis vinifera* L.) cv. Chardonnay in the south-western part of Slovenia in the Brda region (45˚58' N, 13˚32' E). All treatments and sampling were as described (HREN et al., 2007, 2009a). Extraction of the samples and GC-MS analysis was performed according to WECKWERTH et al. (2004). The metabolite identification was done by comparison of retention time indices (RIs) and mass spectra with both an in-house mass spectra library and the Golm Metabolite Database (KOPKA et al., 2005). The metabolites were considered as annotated with a spectral match >800 and a RI deviation <20. Deconvolution was performed with AMDIS (STEIN, 1999) and quantification with LC-Quan 2.6.0 (Thermo Scientific, USA). For each component of a sample, the response factor was determined as the ratio of the peak area of the compound and the internal standard (extracted ion chromatogram of a spe-

cific quantification ion, respectively). The response factor was normalized with the mean response factor of the component from all of the samples. For statistical analysis, Student's t-tests ($p < 0.05$) and PCA (XLSTAT, Addinsoft) were performed.

RESULTS AND DISCUSSION

Metabolome analysis of the leaf vein-enriched tissues allowed the detection of 202 compounds. A total of 84 compounds were annotated or putatively annotated, and 36 of these differed significantly ($p<0.05$) between infected and uninfected samples (Table 1). The majority of the compounds increased in infected samples.

Significant increases in several sugars and sugar derivates are noteworthy. These findings corroborate previous studies showing that phytoplasma infections affect the carbohydrate metabolism of host plants (LEPKA et al., 1999, SANTI et al., 2013). If the increase in fructose-6-phosphate is related with its suggested utilization by phytoplasma (KUBE et al., 2012) is currently not known. In addition, a 25.8-fold increase in salicylic acid-glucopyranoside and several phenolic compounds was also associated with infected samples. The role of flavonoids in response of grapevine to the infection with '*Ca*. P. solani' has been shown before (HREN et al., 2009; LANDI and ROMANZAZI, 2011; RUSJAN et al., 2012).

As the main transport carbohydrate in plant phloem, the major compound in all vein-enriched samples was sucrose. Its amount was in great excess over other compounds and led to detector saturation in GC-MS.

Principal component analysis was performed on the metabolomic dataset. The sanitary status can be resolved by PC1, which contributed 31.45 % of the total variation (Fig. 1).

In conclusion, this study show for the first time at the metabolome level that the products of several genes previously demonstrated to be associated with the grapevine infection with '*Ca*. P. solani' are indeed affected upon the infection.

Table 1. The annotated or putatively annotated compounds in leaf-vein samples of cv. Chardonnay infected with '*Ca.* P. solani'compared to uninfected ones. The infected to uninfected ratios represent the average normalised response factors of each compound between the infected and uninfected samples. $^{\downarrow}$, indicates amounts that were significantly lower in infected samples than in uninfected ones ($p<0.05$); $^{\uparrow}$, similarly, but significantly higher ($p<0.05$).

Compound group	Compound	Infected/Uninfected ratio
Amino acids	β-Alanine	1.061
	Alanine	1.019
	Serine	0.858
	Threonine	2.013
	Valine	1.865
	Leucine	2.900
	Pyroglutamate	2.592$^{\uparrow}$
	Glutamate	0.690
	Proline	7.062
	Aspartate	3.145
	Glycine	1.313
Acids, esters, lactones	Pentahydroxyhexanoic acid peak1	1.031
	Pentahydroxyhexanoic acid peak3	1.103
	Pentahydroxyhexanoic acid peak4	3.836$^{\uparrow}$
	Pentahydroxyhexanoic acid-1,4-lactone	0.975
	Maleate	0.818
	Fumarate	0.617
	Malate	1.462$^{\uparrow}$
	Citramalate	0.688
	2-Oxoglutarate	1.010
	Citrate	1.589$^{\uparrow}$
	Isocitrate	1.213
	Succinate	1.453
	Ascorbate	1.957
	Dehydroascorbate	0.731$^{\downarrow}$
	Pyruvate	0.885
	Lactate	1.310

	Arabinonic acid	0.772
	Quinic acid	1.314
	Shikimate	1.320
	Caffeate	1.343
	Lyxonic acid	1.069
	Threonic acid-1,4-lactone (P)	0.849
	Threonate	0.871
	Butanoic acid-2,4-dihydroxy	0.745↓
	Butanoic acid 4-amino	1.183
	Erythronic acid	0.334↓
	Erythromic acid-1,4-lactone	0.477↓
	Ribonic acid	1.478
	Tartaric acid (putative)	1.755
	Glycerate	0.640↓
Sugars, sugar derivatives	Fructose	1.921↑
	Glucose	2.453↑
	Sucrose	1.037
	Raffinose (putative)	1.727↑
	Galactose/Mannose	3.255↑
	Maltotriose	3.289↑
	Fucose/Rhamnose peak1 (putative)	1.764↑
	Fucose/Rhamnose peak2 (putative)	1.773↑
	Fucose/Rhamnose peak3 (putative)	2.368↑
	Aldopentose peak1	2.887↑
	Aldopentose peak2	3.039↑
	Aldopentose peak3	2.169↑
	Pentose alcohol peak2	0.620
	Pentose alcohol peak3	1.079
	Disaccharide peak1	0.679↓
	Disaccharide peak2	0.531↓
	Disaccharide peak3	0.534↓
	Disaccharide peak4	0.918
	Disacharide peak5	0.874
	Disacharide peak6	1.214
	Disacharide peak7	1.808↑
	Disacharide peak8	1.785↑
	Disacharide peak9	1.173
	Disacharide peak10	1.894
	Ketopentose	0.978
	Glucopyranose	1.235
	Ketohexose	1.127
	Fructose-6-phosphate	2.958↑
	Hexose-6-phosphate	2.765↑
	Hexose-6-phosphate	2.716↑
Phenols, alcohols, ketones	Glycerol	1.375↑
	Glycerate	0.640↓

	Galactinol	1.594↑
	Salicylic acid-glucopyranoside	25.763↑
	Catechin	2.613↑
	Epicatechin	2.885↑
	Epigallocatehin	1.724
	Flavonoid	2.127↑
	Inositol	0.876
	??2-methyl-1,2-propanediol	0.984
	2-methyl-1,3-butandiol	0.704↓
Nitrogen compounds	Ethanolamine	0.774
	Pyridine 2-hydroxy	1.243

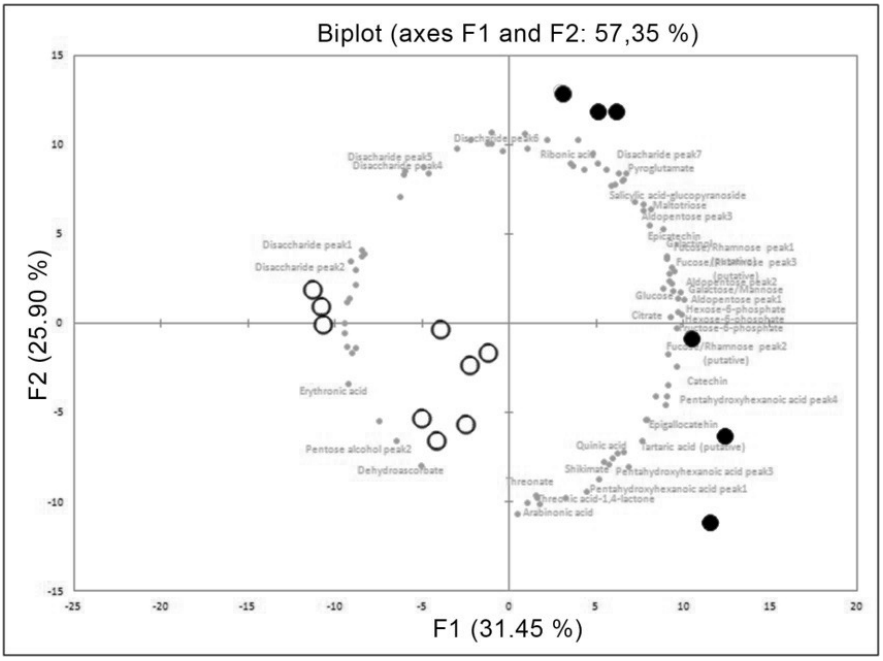

Figure 1: Principal component analysis of the leaf vein-enriched metabolome data from uninfected and grapevine samples infected with '*Ca.* P. solani'. Open circle, uninfected; solid circle, infected.

REFERENCES

CHOI Y. H., TAPIAS E. C., KIM H. K., LEFEBER A.W., ERKELENS C., VERHOEVEN J.T., BRZIN, J, ZEL, J. and VERPOORTE R. 2004: Metabolic discrimination o*f Catharanthus roseus* leaves infected by phytoplasma using 1H-NMR spectroscopy and multivariate data analysis. Plant Physiology 135: 2398-23410

GAI, Y. P., HAN, X. J., LI, Y. Q., YUAN, C.-Z., MO, Y. Y., GUO, F.-Y., QING-XIN, L. and JI, X.-L. 2014: Metabolomic analysis reveals the potential metabolites and pathogenesis involved in mulberry yellow dwarf disease. Plant, Cell and Environment 37: 1474-1490

HREN M., NIKOLIĆ P., ROTTER A., BLEJEC A., TERRIER N., RAVNIKAR M., DERMASTIA, M., GRUDEN K. 2009: "Bois noir" phytoplasma induces significant reprogramming of the leaf transcriptome in the field grown grapevine. BMC Genomics 10: 460

HREN, M., BOBEN, J., ROTTER, A., KRALJ, P., GRUDEN, K., RAVNIKAR, M. 2007: Real-time PCR detection systems for Flavescence dorée and Bois noir phytoplasmas in grapevine: comparison with conventional PCR detection and application in diagnostics. Plant Pathology 56: 785–779

KOPKA, J., SCHAUER, N., KRUEGER, S., BIRKEMEYER, C., USADEL, B., BERGMULLER, E, DÖRMANN, P., WECKWERTH, W., GIBON, Y., STITT, M., WILLMITZER, L., FERNIE, A. R. and STEINHAUSER, D. 2005: GMD@CSB.DB: the Golm Metabolome Database. Bioinformatics 21: 1635-1638

KUBE, M., MITROVIC, J., DUDUK, B., RABUS, R. and SEEMÜLLER, E. 2012: Current view on phytoplasma genomes and encoded metabolism. The Scientific World Journal 185942

LANDI, L. and ROMANAZZI, G. 2011: Seasonal variation of defense-related gene expression in leaves from Bois noir affected and recovered grapevines. Journal of Agricultural and Food Chemistry 59: 6628-6637

LEPKA, P., STITT, M., MOLL, E. and SEEMÜLLER, E. 1999: Effect of phytoplasmal infection on concentration and translocation of carbohydrates and amino acids in periwinkle and tobacco. Physiological and Molecular Plant Pathology 55: 59–68

QUAGLINO, F., ZHAO, Y., CASATI, P., BULGARI, D., BIANCO, P.,A., WEI, W., DAVIS, R.,E. 2013: '*Candidatus* Phytoplasma solani', a novel taxon associated with stolbur-and bois noir-related diseases of plants. International Journal of Systematic and Evolutionary Microbiology 63: 2879–2894

RUSJAN, D., VEBERIČ, R. and MIKULIČ-PETKOVŠEK M. 2012: The response of phenolic compounds in grapes of the variety 'Chardonnay' (*Vitis vinifera* L.) to the infection by phytoplasma Bois noir. European Journal of Plant Pathology 133: 965–974

SANTI, S., DE MARCO, F., POLIZZOTTO, R., GRISAN, S. and MUSETTI, R. 2013: Recovery from stolbur disease in grapevine involves changes in sugar transport and metabolism. Frontiers in Plant Science 4: 171

STEIN, S. E. 1999: An integrated method for spectrum extraction. Journal of The American Society of Mass Spectrometry 10: 770-781

WECKWERTH, W., WENZEL, K., and FIEHN, O. 2004: Process for the integrated extraction, identification and quantification of metabolites, proteins and RNA to reveal their co-regulation in biochemical networks. Proteomics 4: 78-83

IMPACT OF BOIS NOIR DISEASE ON GRAPEVINE PERFORMANCE AND WINE QUALITY OF *VITIS VINIFERA* L. CV. 'CHARDONNAY' IN HUNGARY

Ibolya Ember[1], Péter Bodor[1], Zsolt Zsófi[2], Xénia Pálfi[2], Szabolcs Villangó[2], Zita Pálfi[2], Márta Ladányi[3], György Pásti[4], András Szekeres[5], Ottó Bencsik[5], Tamás Deák[1], Borbála Bálo[1], László Palkovics[6], Xavier Foissac[7,8,] Jacobus Johannes Hunter[9,10] and György Dénes Bisztray[1]

[1]Corvinus University of Budapest, Faculty of Horticultural Science, Institute of Viticulture and Oenology, Department of Viticulture, Villányi str. 29-43, 1118 Budapest, Hungary
[2]Károly Róbert College Research Institute for Viticulture and Enology, Eger, Kőlyuktető, Pf. 83, 3301 Hungary
[3]Corvinus University of Budapest, Faculty of Horticultural Science, Department of Biometrics and Agricultural Informatics, Villányi str. 29-43, 1118 Budapest, Hungary
[4]Corvinus University of Budapest, Faculty of Horticultural Science, Institute of Viticulture and Oenology, Department of Oenology, Ménesi str. 45, 1118 Budapest, Hungary
[5]University of Szeged, Department of Microbiology, Közép fasor 52, 6726, Szeged, Hungary
[6]Corvinus University of Budapest, Faculty of Horticultural Science, Department of Plant Pathology, Villányi str. 29-43, 1118 Budapest, Hungary
[7]INRA, UMR 1332 Biologie du Fruit et Pathologie, 71 avenue Edouard Bourlaux, CS20032 F-33882 Villenave d'Ornon, Cedex, France
[8]Université de Bordeaux, UMR 1332 Biologie du Fruit et Pathologie, 71 avenue Edouard Bourlaux, CS20032, F-33882 Villenave d'Ornon, Cedex, France
[9]Agricultural Research Council of South Africa, ARC Infruitec-Nietvoorbij, Private Bag X5026, 7599 Stellenbosch, South Africa
[10]Stellenbosch University, Department of Viticulture and Oenology, Private Bag X1, 7602 Matieland, Stellenbosch, South Africa

Impact of "bois noir" disease (BN) on performance of 'Chardonnay' grown in Eger (Hungary) is reported from plant (vegetative and reproductive parameters, fruit composition) to wine (wine quality, sensory analysis). BN-associated chlorophyll degradation and leaf rolling resulted in a loss in functioning surface of the canopy. Lack of lignification and non-viable buds evoked unpredictable crop production. Severity and extent of reduction in production varied between years, but the crop loss was always above 53%. In the case of BN-affected grapevines the reduced quantity resulted in a low quality of grape bunch composition, which enhanced unfavourable outcomes in wines. Differences in fruit composition and wine quality between healthy and BN affected grapevines were most pronounced in years with optimal weather conditions and the negative effect was covered up in poor years. In wine regions where mechanical harvesting is applied, harvesting of phytoplasma diseased vineyards can therefore not be selective and qualitative. According to the three years study presented BN induce severe loss, and BN-affected plants compromise the yield and wine quality, and therefore the overall profitability of a vineyard.

INTRODUCTION

Grapevine yellows (GY) diseases have an important economic impact on the world grape and wine production (MAIXNER, 2006). An important GY in Europe is "bois noir" (BN) a disease associated with the presence of '*Candidatus* Phytoplasma solani' ('*Ca.* P. solani') and transmitted from wild reservoirs to grapevines by two phloem-feeding planthoppers (*Hyalesthes obsoletus* Signoret and *Reptalus panzeri* Löw) (MAIXNER, 1994; MAIXNER et al., 1995; QUAGLINO et al., 2013; CVRKOVIC et al., 2013). BN is recognised as less severe or less damaging than other GYs (i.e. "Flavescence dorée"). However, the BN disease cycle is more complex, which is explained by the biology of non-ampelophagous vectors (MAIXNER, 2011). The incidence of BN disease is in correlation with insect vector populations that depends on factors such as temperature, soil, and presence of insect host plants around vineyards (PANASSITI et al., 2015). Although the grapevine cultivar could be an important factor in BN epidemiology, this is also dependent on influence of specific environmental conditions. Higher temperature positively affects not only population dynamics of the insect vectors, but also phytoplasma multiplication rate in the host plant, which may lead to earlier and/or severe symptom expression (SALAR et al., 2013; FOISSAC and WILSON, 2010). Differences in susceptibility of grapevine cultivars to BN direct our interest to the importance of disease management of susceptible cultivars, such as Chardonnay and Riesling (PANASSITI et al., 2015; EFSA Panel on Plant Health, 2014).

As the other agents of GY, BN induces a symptom-complex on grapevine, such as leaf rolling, leaf yellowing or reddening (depending on cultivar), uneven shoot lignification, berry shriveling and bunch drying. In diseased plants phytoplasmas colonise functional phloem elements, the most important effect being the damage of the sieve tube function (MUSETTI et al., 2013). Changes in gene expression involved carbohydrate metabolism and glycolysis impact on the flow of assimilates, i.e. reducing precursor formation in the leaves (sucrose) and translocation via the phloem to the berries, therefore also impacting on grape-based enzyme activity (e.g. acid vacuolar invertase, sucrose synthase) (LEPKA, 1999; JAGOUEIX-EVEILLARD et al., 2001; PRACROS et al., 2006; HREN et al., 2009).

Significant reduction in performance of some grapevine cultivars was investigated in many aspects, such as at physiological as well as yield and fruit quality level (BERTAMINI et al., 2002; GARAU et al., 2007; ENDESHAW et al., 2012; RUSJAN et al., 2012; ZAHAVI et al., 2013; ROMANAZZI et al., 2013). Despite the general decline of plant production in BN infected vineyards, the fluctuations in infection status (incidence and severity) result in an extended range of yield and quality losses, and erratic economic impact. In a 3-year field experiment the effect of "bois noir" disease on vegetative growth, productive performance, fruit and wine quality of *Vitis vinifera* L. cv. Chardonnay in the Eger wine region, Hungary was studied.

MATERIALS AND METHODS

The experimental vineyard belongs to KRC Research Institute of Viticulture and Oenology, situated in the Eger wine region, Eger. Measurements were conducted in a 0.6 ha vineyard of cv. Chardonnay, where three random blocks were selected. In the three blocks phytoplasma infection status of individual plants was recorded before harvest (2011-2014), and 15-15 "bois noir"-affected (BNA) and healthy (H) vines were selected for measurements. Prior to the experiment, '*Ca*. P. solani' infection and health status of the selected plants were also confirmed at molecular level according to PELLETIER et al. (2011).

Table 1. Meteorological data of Eger, Kőlyuktető.

Year	Annual precipitation (mm)	Effective heat sum (°C)	Active heat sum (°C)
2011	945	3727	3727
2012	443	3893	3893
2013	731	3607	3624
2014	768	1655	3760

In all three years of the experiment (2012, 2013 and 2014) and one year prior (2011), meteorological data were recorded (Table 1). The year 2011 was a regular year, followed by an extremely dry one (year 2012). The 2013 season was outstanding in terms of weather conditions that allowed great quality grape production. The year 2014 was an usual year until véraison, during which 50% of annual rainfall was received. This wet condition affected the harvest.

To investigate vegetative growth of BN-affected and healthy plants pruning mass, cane lignification (based on numbers of non-lignified canes), leaf rolling (determination leaf areas of twisted and unfolded flat leaves), leaf fresh and dry mass (weight measurements) and leaf chlorophyll (relative chlorophyll index–SPAD unit) were measured. To assess regenerative performance yield (kg), number of bunches (pc), bunch mass (g) and berry mass (g) were recorded on 15-15 BNA and H vines individually. Fruit composition was determined on these selected BNA and H vines by measuring soluble solids (°Brix), titratable acidity (g/L tartaric acid), and pH. In the case of BN infected plants, all bunches/vine, and in the case of healthy plants, five bunches/vine, were processed.

Small scale wine making was carried out using total yield of BNA and H plants collected separately from the three blocks. Grapes of 2012, 2013 and 2014 were harvested at full maturation at beginning and middle of September, and fermented in the winery of KRC, Research Institute of Viticulture and Oenology, Eger. Yields of BNA and H plants were processed separately and fermented in three oenological replicates/batch in 2012 and 2013. Because of the limited yield in 2014 only one oenological replicate per batch was processed. Dried bunches were not harvested. In each year musts were processed according to the standard white winemaking procedure. Controlled fermentations were conducted in glass jugs at 12°C using a starter yeast culture. Free sulphurous acid concentration of the fermented batches was adjusted to 40 mg/L.

Wine analyses included alcohol (Gibertini distiller) and total extract (densimetry), titratable acidity (titration), organic acids (HPLC), pH, total polyphenols (Folin-Ciocalteu reagent) and colour (spectrophotometry).

Sensory evaluation of experimental wines was done by eleven trained panellists. To characterize wines of BNA and H, appearance, aroma and flavour (acidity, bitterness, body, balance) attributes were used as main descriptors.

For statistical analyses IBM SPSS version 22 (IBM Corp., Armonk, NY, USA) were used. Vegetative and reproductive performance and must quality of the 15 BNA and 15 H grapevines were analysed by two-way ANOVA. Wine analyses of BNA and H plants were done by two-way MANOVA and sensory analysis by Mann-Whitney U test.

RESULTS AND DISCUSSION

Pruning mass and number of canes did not show significant differences between BNA and H grapevines; however significant year effects were detected for both parameters. Lack of lignification of BNA plants occurred in each year. Due to the incomplete maturation, BN symptomatic canes turned to a dark brownish/black colour after winter. An average of 30% of canes of BNA vines showed uneven lignification and the buds of these canes were not viable. Usually, such shoots are less fruitful, thus resulting in unpredictable crop production. A decrease of leaf surface of BNA plants, induced by leaf rolling, occurred (29.5% and 26.4% in 2013 and 2014, respectively). A multi-year average increase of dry mass almost reached 20% and the decrease of relative chlorophyll index exceeded 30% of BNA leaves compared to healthy ones.

Growth, yield and fruit composition of the grapevine eventually depend on the seasonal canopy photosynthetic capacity (HUNTER and RUFFNER, 2001). In each experimental year the yield of BNA vines was significantly lower than that of H vines ($P < 0.001$). Over three years, berry mass, bunch mass and number of bunches/grapevine collectively resulted between 53% and 75% of losses in diseased plants.

Bunches with shriveling berries and dry bunches were frequently produced from diseased grapevines. Results are in agreement with findings of other studies reporting severe yield losses (ENDESAW et al., 2012; GARAU et al., 2007; ZAHAVI et al., 2013; ROMANAZZI et al., 2013). Decline in grape quality caused by different phloem-inhabiting pathogens are well known (BOUDON-PADIEU, 2003; MANNINI et al., 2009). Similar to these findings, the measurements on fruit composition showed significant quality loss. Higher TA (1.6 g/L increase in 3-year average) and lower pH characterized the fruits of BNA grapevines. Soluble solids of must of BNA plants decreased by 6.2% in 3-year average, indicating lower potential alcohol concentration.

Wine analyses confirmed the quality loss in wines of BNA affected vines. In 3-year average alcohol content dropped by 5.3%, and TA and organic acids individually, as well as total phenols increased in each lot of wines from BNA plants. RUSJAN et al. (2012) demonstrated elevated phenolic compound, mainly hydroxycinnamic acids in the berry skin of BN diseased grapevine, which suggested a negative effect on wine quality. In this experiment, results of sensory analyses confirmed these hypotheses. Wines from BNA plants lagged behind wines of healthy plants. Reduced aroma and flavour, flatness, acidity, bitterness and colouring (in some years pinkish discolouration) characterized BNA wines in each experimental year. During wine-making, fermentation of BNA musts was slower than that of H wines. Discoloration of BN-affected wines can be considered a wine default that may decrease the market value of these wines. Thus, importance of sulphur treatments to maintain reductive conditions of wines must be emphasised in cases yields may contain BN-affected bunches.

ACKNOWLEDGEMENTS

We thank our colleagues in the KRC, Institute of Viticulture and Oenology, and in the Corvinus University of Budapest, Department of Viticulture and Department of Oenology for their help with measurements and comments. This project was funded by the National Research, Development and Innovation Fund of the Hungarian Government (KTIA_AIK_12-1-2013-0001).

REFERENCES

BERTAMINI, M., NEDUNCHEZHIAN, N., TOMASI, F. and GRANDO, M.S. 2002: Phytoplasma [Stolbur-subgroup (Bois Noir-BN)] infection inhibits photosynthetic pigments, ribulose-1,5-bisphosphate carboxylase and photosynthetic activities in field grown grapevine (*Vitis vinifera* L. cv. Chardonnay) leaves. Physiological and Molecular Plant Pathology 61: 357-366

BOUDON-PADIEU, E. 2003: The situation of grapevine yellows\and current research directions: distribution, diversity, vectors, diffusion and control. In: Proceedings of the 14th Meeting of ICVG, Locorotondo, Bari (Italy): 47-53

CVRKOVIĆ, T., JOVIĆ, J., MITROVIĆ, M., KRSTIĆ, O. and TOŠEVSKI, I. 2013: Experimental and molecular evidence of *Reptalus panzeri* as a natural vector of Bois noir. Plant Pathology 63: 42-53

EFSA PLH PANEL (EFSA Panel on Plant Health) 2014: Scientific Opinion on the pest categorisation of Candidatus Phytoplasma solani. EFSA Journal 12, (12): 3924-3927

ENDESHAW, S.T., MUROLO, S., ROMANAZZI, G. and NERI, D. 2012: Effects of Bois noir on carbon assimilation, transpiration, stomatal conductance of leaves and yield of grapevine (*Vitis vinifera*) cv. Chardonnay. Physiologia Plantarum 145: 286–295

FOISSAC, X. and WILSON, M.R. 2010: Current and Future Distributions of Phytoplasma. In Weintraub, P.G. and Jones, P. (eds.) Phytoplasmas: genomes, plant hosts and vectors - CAB International, Wallingford, UK

GARAU, R., SECHI, A., PROTA, V.A. and MORO, G. 2007: Productive parameters in Chardonnay and Vermentino grapevines infected with "Bois noir" and recovered in Sardinia. Bulletin of Insectology 60: 233–234

HREN, M., NIKOLIĆ, P., ROTTER, A., BLEJEC, A., TERRIER, N., RAVNIKAR, M., DERMASTIA, M. and GRUDEN, K. 2009: "Bois noir" phytoplasma induces significant reprogramming of the leaf transcriptome in the field grown grapevine. BMC Genomics 10: 460

HUNTER, J.J. and RUFFNER, H.P. 2001: Assimilate transport in grapevines - effect of phloem disruption. Australian Journal of Grape and Wine Research 7: 118-126

JAGOUEIX-EVEILLARD, S, TARENDEAU, F, GUOLTER, K, DANET, J.L., BOVÉ, J.M. and GARNIER, M. 2001: *Catharanthus roseus* genes regulated differentially by mollicute infections. Molecular Plant-Microbe Interactions 14: 225-233

LEPKA, P., STITT, M., MOLL, E. and SEEMÜLLEER, E. 1999: Effect of phytoplasma infection on concentration and translocation of carbohydrates and amino acids in periwinkle and tobacco. Physiological and Molecular Plant Pathology 55: 59-68

MAIXNER, M. 1994: Transmission of German grapevine yellows (Vergilbungskrankheit) by the planthopper *Hyalesthes obsoletus* (Auchenorrhyncha:Cixiidae). Vitis 33: 103–104

MAIXNER, M. 2006: Grapevine yellows–current developments and unsolved questions. In: Extended abstracts 15th International Council for the Study of Virus and Virus-like Diseases of the Grapevine, Stellenbosch, South Africa, 86

MAIXNER, M. 2011: Recent advances in "bois noir" research. Petria 21(2/3): 95-108

MANNINI, F., MOLLO, A., CUOZZO, D. and CREDI, R. 2009: Field performance and wine quality modification in a clone Dolcetto (*Vitis vinifera* L.) after GLRaV-3 elimination. Le Progrès Agricole et Viticole HS: 234-236

MATUS, J.T., VEGA, A., LOYOLA, R., SERRANO, C., CABRERA, S. and ARCE-JOHNSON, P. 2008: Phytoplasma and virus detection in commercial plantings of *Vitis vinifera* cv. Merlot exhibiting premature berry dehydration. Electronic Journal of Biotechnology 11: 1-10

MUSETTI, R., BUXA, S.V, DE MARCO, F., LOSCHI, A., POLIZZOTTO, R., KOGEL, K.H. and VAN BEL, A.J.E. 2013: Phytoplasma-triggered Ca2+ influx is involved in sieve-tube blockage. Molecular Plant Microbe Interactions 26: 379-386

PANASSITI, B., HARTIG, F., BREUER, M., and BIEDERMANN, R. 2015: Bayesian inference of environmental and biotic factors determining the occurrence of the grapevine disease 'bois noir'. Ecosphere 6: 143

PRACROS, P., RENAUDIN, J., EVEILLARD, S., MOURAS, A. and HERNOULD, M. 2006: Tomato flower abnormalities induced by stolbur phytoplasma infection are associated with changes of expression of floral development genes. Molecular Plant-Microbe Interactions 19: 62-68

QUAGLINO, F., ZHAO, Y., CASATI, P., BULGARI, D., BIANCO, P.A., WEI, W. and DAVIS R.E. 2013: *'Candidatus* Phytoplasma solani', a novel taxon associated with stolbur and Bois noir related diseases of plants. International Journal of Systematic and Evolutionary Microbiology 63: 2879-2894

ROMANAZZI, G., MUROLO, S. and FELIZIANI, E. 2013: Effects of an innovative strategy to contain grapevine Bois noir: field treatment with resistance inducers. Phytopathology 103: 785-791

RUSJAN, D., VEBERIČ, R. and MIKULIČ-PETKOVŠEK, M. 2012: The response of phenolic compounds in grapes of the variety "Chardonnay" (*Vitis vinifera* L.) to the infection by phytoplasma Bois noir. European Journal of Plant Pathology 133: 965-974

SALAR, P. CHARENTON, C., FOISSAC, X. and MALEMBIC-MAHER, S. 2013: Multiplication kinetics of Flavescence dorée phytoplasma in broad bean. Effect of phytoplasma strain and temperature. European Journal of Plant Pathology 135: 371-381

ZAHAVI, T., SHARON, R., SAPIR, G., MAWASSI, M., DAFNY-YELIN, M. and NAOR, V. 2013: The long-term effect of Stolbur phytoplasma on grapevines in the Golan Heights. Australian Journal of Grape and Wine Research 19: 277–284

STRAIN DEPENDENT SYMPTOMS AND EXPRESSION OF STOLBUR PHYTOPLASMA GENES IN THE EXPERIMENTAL HOST *CATHARANTHUS ROSEUS*

Günter Brader[1], Amal Aryan[1,2], Elisabeth Wischnitzki[1] and Monika Riedle-Bauer[3]

[1]Austrian Institute of Technology, Health & Environment Department, Bioresources Unit, Konrad-Lorenz-Straße 24, 3430 Tulln, Austria; guenter.brader@ait.ac.at
[2]BOKU –University of Natural Resources and Life Sciences, Department of Crop Sciences Division of Vegetables and Ornamentals, Vienna, Austria
[3]Lehr- und Forschungszentrum für Wein- und Obstbau Klosterneuburg, Wienerstraße 74, 3400 Klosterneuburg, Austria; Monika.Riedle-Bauer@weinobst.at

Bois noir (BN) strains widespread in several winegrowing regions in Austria have been transferred for molecular studies to the lab host *Catharanthus roseus* by wild catches of the Auchenorrhyncha planthopper *Hyalesthes obsoletus*. Interestingly, infection of *C. roseus* with different stolbur genotypes (CPsM4_At1, CPsM4_At4 and CPsM4_At6) resulted in clear differences in symptom development, showing different degrees of virescence and phyllody in *C. roseus* flowers. Here, we report RNA sequencing results to evaluate the expression of stolbur genes of different stolbur genotypes and demonstrate the expression of a large subset of phytoplasma genes including hypothetical proteins in planta.

INTRODUCTION

Bois noir (BN) associated with '*Candidatus* Phytoplasma solani' is widespread in several winegrowing regions in Austria. The transmission of '*Ca.* Phytoplasma solani' to grapevine occurs by Auchenorrhyncha species and the planthopper *Hyalesthes obsoletus* (Cixiidae) is seen as the major vector of the pathogen (MAIXNER 2011). The strains involved in disease development of BN in Europe were linked to cycles involving different wild plants including nettle, bindweed and vitex with the help of molecular markers (LANGER and MAIXNER 2004; JOHANNESEN et al. 2012, KOSOVAC et al., 2016). The marker genes used for typing of the different phytoplasma strains include *sec*Y, *stamp, tuf* and *vmp*1 (CIMERMAN et al. 2009; FABRE et al. 2011; JOHANNESEN et al. 2012; PACIFICO et al. 2009). Previous studies based on these molecular markers have shown dominance of a nettle associated strain named CPsM4_At1 in Austrian vineyard areas (ARYAN et al., 2014).

Symptom developments in plant hosts typically include phyllody, virescence, yellowing, stunting, declines and diebacks (BERTACCINI & DUDUK, 2009; HOGENHOUT et al., 2008). Effector proteins of '*Candidatus* Phytoplasma asteris' have been described to be involved in the development of these phytoplasma symptoms (HOSHI et al., 2009; MACLEAN et al., 2014; SUGIO et al., 2011). The expression of '*Ca*. Phytoplasma asteris' genes is differentiated between insect and plant hosts (OSHIMA et al., 2011), but little is known on the expression of effector like proteins of '*Ca.* Phytoplasma solani' in plants. Here, we used *Catharanthus roseus* infected with different BN strains and showing different symptoms to study the expression of '*Candidatus* Phytoplasma solani' genes in planta.

MATERIAL AND METHODS

Catharanthus roseus (cv. "Sorbas Reinweiß", Austrosaat, Vienna, Austria) were grown in greenhouse on soil "Einheitserde Spezial, Tonsubstrat ED 63"

from (Einheitserdewerke, Sinntal-Altengronau, Germany) at 22-30°C with 14/10 h day/night regime. Wuxal Super (N, P, K fertilizer with trace elements, Kwizda Agro, Wien) was added every second week at the concentration of 0.2%. '*Ca.* Phytoplasma solani' strains were transferred to *C. roseus* plants by aid of infected *H. obsoletus* and *Anaceratagallia ribauti* field collected in Austrian vineyard areas in 2012 and 2013 (ARYAN et al., 2014). The phytoplasma genotypes were characterized by the marker genes *sec*Y, *Stamp*, *tuf* and *vmp*1. *C. roseus* plants infected with the accessions CrHo13_1178, CrHo13_1183 and CrHo12_601 were selected for further analysis. CrHo13_1178 and CrHo13_1183 corresponded to the nettle associated genotypes CPsM4_At1 and CPsM4_At4 respectively, CrHo12_601 corresponded to the bindweed associated genotype CPsM4_At6 (ARYAN et al., 2014).

RNA for RNA sequencing was isolated using a CTAB based protocol (CHANG et al., 1993) from pools of healthy, CPsM4_At1, CPsM4_At4 and CPsM4_At6 infected plants in triplicates. Ribomal RNA was removed using Ribo-Zero rRNA Removal Kit (Epicentre, Madison) for bacteria and for plants according to the instructions of the manufacturer. mRNA sequencing was performed at GATC Biotech (Konstanz) after random primed cDNA synthesis at the HiSeq platform (Illumina) generating 60 million reads per sample. All quality controlled reads (Q30, min seq length 30bp; in house perl scripts) were subjected to a reference based assembly using the available phytoplasma sequences and bowtie2 (LANGMEAD & SALZBERG, 2012). The identified regions were extracted and subjected to further analysis and annotation (information of the triplicate datasets were merged into one representative dataset). All identified regions were annotated using blast (e-value e^{-5}; ALTSCHUL et al., 1990; CAMACHO et al., 2009) and NCBI's NR database (http://ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

With wild catches of *H. obsoletus* and *A. ribauti* we were able to transfer at least six different genotypes to the lab host *Catharanthus roseus*. As in wild populations of *Hyalesthes* obsoletus the majority of the infections belong to genotype CPsM4_At1. Interestingly, infection of *C. roseus* with different stolbur genotypes resulted in clear differences in symptom development, showing different degrees of virescence and phyllody in *C. roseus* flowers (Fig. 1). While CPsM4_At1 infected plants often do not flower at all, CPsM4_At4 infected plants produce small white flowers. CPsM4_At6 infected plants on the other side show strong virescence and phyllody.

Whole genome sequencing of the different genotypes provides the fundament for comparison to understand the molecular basis for these differences. Nevertheless, genome comparison alone might not be sufficient to find and understand the reasons for phenotypic variation. Differential expression of effectors might play an important role in heterogenous symptom development. Little is known on expression of phytoplasma genes in its hosts in general and of the stolbur phytoplasma in particular. Here, we report RNA sequencing results to evaluate the expression of stolbur genes in different stolbur genotypes. Although symptoms were evident and the genotypes were confirmed in all analysed plants, the number of reads assigned to phytoplasmas was clearly different between the strains. The recovery rate and the number of clearly expressed phytoplasma genes in CPsM4_At1 was below hundred, while in CPsM4_At6 several hundred of phytoplasma genes corresponding to >50% of the genome were detected. Interestingly, the expressed genes did not only contain annotated metabolic genes (Fig. 2), but also contained several hypothetical proteins showing very pronounced expression.

Figure 1: Symptoms of BN infections in *C. roseus*. Left the bindweed associated strain CPsM4_At6, in the middle nettle associated CPsM4_At4 and right a healthy control.

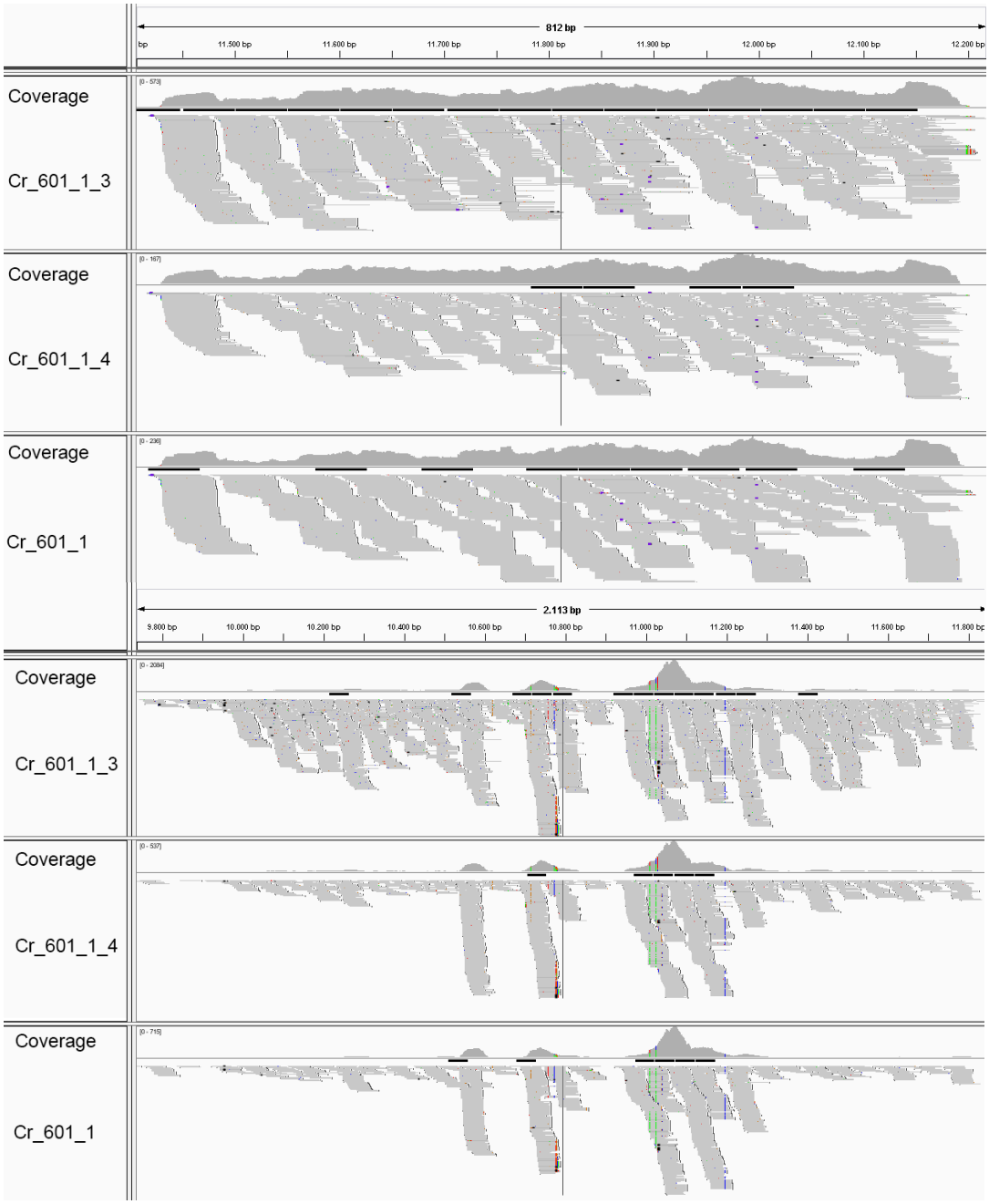

Figure 2: RNAs reads of CPsM4_At6 mapping to gene regions encoding for a diadenosine tetraphosphate hydrolase (top) and a DNA-ligase (below)

Also expression of the genes encoding for the surface proteins Stamp and vmp1 was pronounced in CPsM4_At6 infected *C. roseus*. The clear expression of genes without specific annotation points to a role of the genes in plant colonialization of BN. Knowledge on expression of BN phytoplasma genes in its hosts and its variation will allow a better understanding for the necessary factors for phytoplasma proliferation in its host, but will also pave the way for a better understanding of the virulence factors involved in disease development.

ACKNOWLEDGMENTS

The work was supported by the grant P 24201-B16 of the Austrian Science Fund (FWF).

REFERENCES

ALTSCHUL, S.F., GISH, W., MILLER, W., MYERS, E.W., AND LIPMAN, D.J. 1990: Basic local alignment search tool. Journal of Molecular Biology 215: 403–410.

ARYAN, A., BRADER, G., MÖRTEL, J., PASTAR, M. AND RIEDLE-BAUER, M. 2014: An abundant '*Candidatus* Phytoplasma solani' Stolbur tuf b phytoplasma strain is associated with grapevine, stinging nettle and *Hyalesthes obsoletus.* European Journal of Plant Pathology 140, 213-227

BERTACCINI, A. and DUDUK, B. (2009): Phytoplasma and phytoplasma diseases: a review of recent research. Phytopathologia Mediterranea 48: 355-378.

CAMACHO, C., COULOURIS, G., AVAGYAN, V., MA, N., PAPADOPOULOS, J., BEALER, K., AND MADDEN, T.L. 2009: BLAST+: architecture and applications. BMC Bioinformatics 10:421

CHANG, S., PURYEAR, J. and CAIRNEY, J. 1993: A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11: 113-116.

CIMERMAN, A., PACIFICO, D., SALAR, P., MARZACHI, C. AND FOISSAC, X. 2009: Striking diversity of *vmp*1, a variable gene encoding a putative membrane protein of the Stolbur phytoplasma. Applied Environmental Microbiology 75: 2951-2957.

FABRE, A., DANET, J.-L. AND FOISSAC, X. 2011: The stolbur phytoplasma antigenic membrane protein gene *stamp* is submitted to diversifying positive selection. Gene 472: 37-41

HOGENHOUT, S. A., OSHIMA, K., AMMAR, E.-D., KAKIZAWA, S., KINGDOM, H. N. and NAMBA, S. 2008: Phytoplasmas: bacteria that manipulate plants and insects. Molecular Plant Pathology 9: 403-423.

HOSHI, A., OSHIMA, K., KAKIZAWA, S., ISHII, Y., OZEKI, J., HASHIMOTO, M., KOMATSU, K., KAGIWADA, S., YAMAJI, Y. and NAMBA, S. (2009): A unique virulence factor for proliferation and dwarfism in plants identified from a phytopathogenic bacterium. Proceedings of the National Academy of Sciences of the United States of America 106: 6416-6421.

JOHANNESEN, J., FOISSAC, X., KEHRLI, P. AND MAIXNER, M. 2012: Impact of Vector Dispersal and Host-Plant Fidelity on the Dissemination of an Emerging Plant Pathogen. PLoS ONE 7: e51809

KOSOVAC, A., RADONJIĆ, S., HRNČIĆ, S., KRSTIĆ, O., TOŠEVSKI, I. AND JOVIĆ, J. 2016: Molecular tracing of the transmission routes of bois noir in Mediterranean vineyards of Montenegro and experimental evidence for the epidemiological role of *Vitex agnus-castus* (Lamiaceae) and associated *Hyalesthes obsoletus (*Cixiidae). Plant Pathology 65: 285-298

LANGER, M. AND MAIXNER, M. 2004: Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur group based on RFLP analysis of non-ribosomal DNA. Vitis 43: 191-199

LANGMEAD, B., AND SALZBERG, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nature Methods 9: 357–359.

MAIXNER, M. 2011: Recent advances in Bois noir research. Petria 21: 17-32

MACLEAN, A. M., ORLOVSKIS, Z., KOWITWANICH, K., ZDZIARSKA, A. M., ANGENENT, G. C., IMMINK, R. G. H. and HOGENHOUT, S. A. 2014: Phytoplasma Effector SAP54 Hijacks Plant Reproduction by Degrading MADS-box Proteins and Promotes Insect Colonization in a RAD23-Dependent Manner. PLoS Biology 12: e1001835.

OSHIMA, K., ISHII, Y., KAKIZAWA, S., SUGAWARA, K., NERIYA, Y., HIMENO, M., MINATO, N., MIURA, C., SHIRAISHI, T., YAMAJI, Y. AND NAMBA, S. (2011). Dramatic Transcriptional Changes in an Intracellular Parasite Enable Host Switching between Plant and Insect. PLoS ONE 6: e23242.

PACIFICO, D., ALMA, A., BAGNOLI, B., FOISSAC, X., PASQUINI, G., TESSITORI, M. AND MARZACHI, C., 2009: Characterization of Bois Noir Isolates by Restriction Fragment Length Polymorphism of a Stolbur-Specific Putative Membrane Protein Gene. Phytopathology 99: 711-715

SUGIO, A., KINGDOM, H. N., MACLEAN, A. M., GRIEVE, V. M. and HOGENHOUT, S. A. 2011: Phytoplasma protein effector SAP11 enhances insect vector reproduction by manipulating plant development and defense hormone biosynthesis. Proceedings of the National Academy of Sciences of the United States of America 108, E1254-E1263.

EVALUATION OF EFFECTIVE MICROORGANISMS® EFFICACY ON '*CANDIDATUS* PHYTOPLASMA SOLANI'-INFECTED AND HEALTHY PERIWINKLE PLANTS

Simon Pierce, Fabio Quaglino, Matteo Montagna, Alberto Spada, Paola Casatiand Marcello Iriti

Dipartimento di Scienze Agrarie e Ambientali – Produzione, Territorio e Agroenergia, Università degli Studi di Milano
IT-20122 Milano, Via Festa del Perdono 7
* Corresponding author: marcello.iriti@unimi.it

A sustainable approach to control plant diseases concerns the employment of bacteria promoting induced systemic resistance (ISR) and/or directly antagonizing the pathogens. In the present study, we tested the efficacy of the commercial product EM® (Effective Microorganisms®) on periwinkle plants infected by '*Candidatus* Phytoplasma solani' (CaPsol), the etiological agent of Bois noir disease (BN) of grapevine. Sequence analyses confirmed the affiliation of CaPsol (strain STOL), transmitted to healthy periwinkle plants by grafting, to 16SrXII-A/tuf-B type. Experiments were performed to evaluate both preventive and curative EM® efficacy by treating roots of periwinkles (ungrafted, grafted with healthy scion, grafted with infected scion). Untreated periwinkles were used as controls. Starting from the 20th day, CaPsol-symptoms along with a drastic reduction of the photosynthetic activity, measured as F_v/F_m, were observed in treated and untreated plants infected by grafting, without significant difference. However, higher Fv/Fm values were registered in treated ungrafted plants in comparison with treated plants grafted with healthy scion. Such evidences suggested that (i) EM® treatment stimulated the plant growth; (ii) grafting induced stress to the plants compromising the evaluation of EM® efficacy. Further studies were planned to investigate the effects of EM® on BN-affected grapevine in controlled conditions and in open field.

So far, the management of plant diseases associated with phytoplasmas mainly consists of compulsory insecticide treatments against the vectors. Due to the complexity of the biological cycle of its etiological agent ('*Candidatus* Phytoplasma solani'; CaPsol) (Quaglino et al., 2013), it is difficult to design efficient control strategies against Bois noir (BN) of grapevine (Belli et al., 2010). Since insecticides applied to the grapevine canopy influence neither the disease nor the presence of *Hyalesthes obsoletus*, the insect vector of CaPsol, the management of host plants in the vineyards and surrounding areas is considered crucial for BN control (Mori et al., 2015). Thus, preventive measures such as checking the sanitary status of propagation materials and treating diseased mother plants through thermotherapy are applied to delimit long distance dissemination and in field spread of the disease. Other strategies for reducing BN spread or incidence are based on (i) preventive removal of the grape suckers on which *H. obsoletus* could feed after grass mowing; (ii) trunk cutting above the engagement point of the symptomatic grapevines (Belli et al., 2010). Considering the Horizon 2020 guidelines for the sustainable environmental development, it is necessary to develop new sustainable strategies to contain the spread of plant diseases.

An ambitious strategy for phytoplasma disease control is based on the selection of resistant, tolerant, or not susceptible plant varieties, cultivated or not-cultivated, as source of resistance-genes for plant breeding programs. Previous studies identified the presence of plant species and/or varieties showing low susceptibility to phytoplasma infection (Bianco

et al., 2011). Unfortunately, up to now, none of the examined *Vitis* species and *V. vinifera* varieties have been found immune or resistant to the phytoplasma associated with BN (Laimer et al., 2009). Moreover, a great interest concerns the induced systemic resistance (ISR) promoted by the interaction between bacteria (rhizobacteria and/or endophytes) and plant tissues (mainly the roots) (Choudhary and Johri, 2009). In the case of grapevine yellows (mainly Bois noir and Flavescence dorée), it was suggested that natural plant recovery can be stimulated by grapevine-associated endophytic bacteria capable to induce ISR and/or act directly against the phytoplasmas (Iriti and Faoro, 2007; Bulgari et al., 2011). In the present study, we tested the efficacy of the commercial product EM® (*Effective Microorganisms*®) on periwinkle plants infected by CaPsol reference strain STOL, with the aim to explore innovative sustainable strategies for the control of BN.

MATERIAL AND METHODS

SELECTION OF MODEL PLANTS INFECTED BY 'CA. PHYTOPLASMA SOLANI'

Periwinkle (*Catharanthus roseus* (L.) G. Don) has been selected as universal model plant host of phytoplasmas in order to perform experiments in greenhouse under controlled conditions, independently from the seasonality of natural phytoplasma plant hosts. Symptomatic periwinkles, infected by the phytoplasma strain STOL (reference strain of the species *'Ca.* Phytoplasma solani', genetically identical to CaPsol strains associated with BN of grapevine), were utilized for producing infected scions to be grafted on healthy periwinkles. Before grafting, PCR-based amplification and sequence analyses of 16S rRNA and *tuf* genes were performed using total nucleic acids, extracted from symptomatic periwinkles, as previously described (Langer and Maixner, 2004; Quaglino et al., 2009), to verify the phytoplasma affiliation to the taxonomic group.

TREATMENTS BY EFFECTIVE MICROORGANISMS®: EXPERIMENTAL PLAN

Experiments, designed based on randomized block scheme, were performed to evaluate both preventive and curative EM® efficacy by treating roots of periwinkles (ungrafted, grafted with healthy scion, grafted with infected scion). Untreated periwinkles were used as controls.
Two experimental schemes were adopted for preventive treatments: (i) one treatment pre-grafting, one treatment 24 h post-grafting, four weekly treatments post-grafting (all treatments performed using EM® diluted 1:10); (ii) four treatments pre-grafting (3 treatments performed using EM® diluted 1:10 and 1 treatment using EM® diluted 1:2), one treatment 24 h post-grafting (EM® diluted 1:2), ten weekly treatments post-grafting (EM® diluted 1:2).
One experimental scheme was adopted for curative treatments carried out on symptomatic plants: five weekly treatments (EM® diluted 1:10) and eleven weekly treatments (EM® diluted 1:2).

EVALUATION OF TREATMENT EFFICACY

Visual symptom observation (appearance and progression in plants grafted by infected scions; regression in symptomatic plants) and photosynthetic efficiency analysis (registered as the maximum efficiency of photosystem II ($F_v/F_{m)}$ using a portable fluorometer) were utilized to determine the efficacy of both preventive and curative treatments.

RESULTS AND DISCUSSION

As expected, nucleotide sequence analysis of 16S rRNA and *tuf* genes confirmed the affiliation of the strain STOL, reference strain of the species CaPsol, to 16SrXII-A/tuf-B taxonomic group (Quaglino et al., 2013).
Considering the visual symptom observation, treatments by EM® on periwinkle root system did not

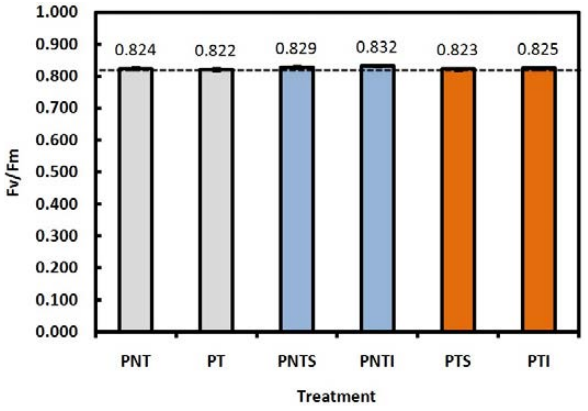

Fig. 1: Maximum efficiency of photosystem II (F_v/F_m) of periwinkle plants in basal conditions (T0). PNT, untreated ungrafted plants; PT, treated ungrafted plants; PNTS, untreated plants grafted with healthy scion; PNTI, untreated plants grafted with infected scion; PTS, treated plants grafted with healthy scion; PTI, treated plants grafted with infected scion

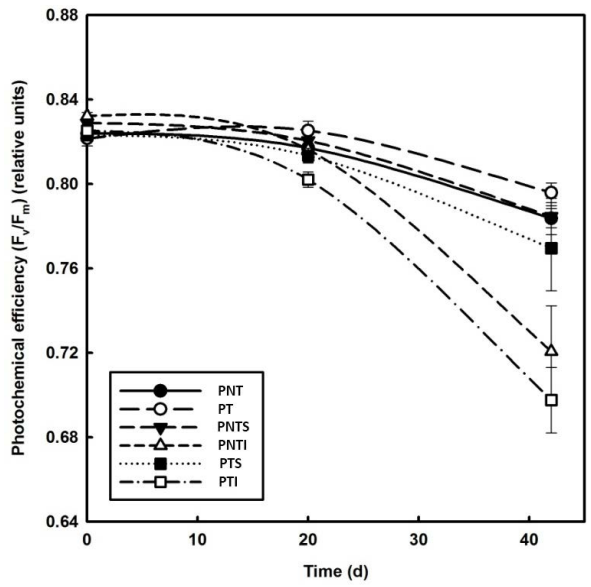

Fig. 2: Effects of phytoplasma infection and EM® treatment on maximum efficiency of photosystem II (Fv/Fm) of periwinkle plants. PNT, untreated ungrafted plants; PT, treated ungrafted plants; PNTS, untreated plants grafted with healthy scion; PNTI, untreated plants grafted with infected scion; PTS, treated plants grafted with healthy scion; PTI, treated plants grafted with infected scion

show any efficacy in all the experiments carried out. In fact, it was not possible to observe symptom regression in curative trials and absence of symptoms in preventive trials on periwinkles grafted by infected scions (data not shown).

Considering the photosynthetic efficiency, basal F_v/F_m values (T0) were identical and underlined the normal physiological conditions of the examined plants (Fig. 1), as previously reported (Pierce et al., 2002).

Starting from the 20th day of the experiments, a drastic reduction of the photosynthetic activity was measured in both treated (PTI) and untreated (PNTI) plants infected by grafting, without significant difference (Fig. 2). The highest F_v/F_m values, even not significant, were measured in treated ungrafted plants (PT) in comparison with treated plants grafted with healthy scion (PTS) (Fig. 2). This evidence suggested that EM® treatment stimulates the plant growth (PT), while grafting induces stress to plants even executed with healthy scions (PTS). Hormonal signals are believed to play an important role in the wound healing and vascular regeneration within the graft union zone. Incomplete and convoluted vascular connections impede the vital upward and downward whole plant transfer routes. Long-distance protein, mRNA and small RNA graft-transmissible signals currently emerge as novel mechanisms which regulate nutritional and developmental root/shoot relations and may play a pivotal role in grafting physiology (Goldsmith, 2014).

Results demonstrated that grafting causes a stress that, independently from infection and treatment, could compromise the EM® efficacy, already proved by promising results obtained on other crops (Iriti, personal comm.). For that reason, further studies will be planned to investigate the effects of EM® on BN-affected grapevine in controlled conditions and in open field.

ACKNOWLEDGMENTS

This study was supported by the project 'Strategie e Tecnologie Innovative nel Controllo e nella Diagnosi delle Fitoplasmosi della Vite' (Piano di Sviluppo UniMI 2014 – Linea L1), funded by Università degli Studi di Milano.

REFERENCES

Belli, G., Bianco, P.A., and Conti, M. 2010: Grapevine yellows: past, present and future. Journal of Plant Pathology 92: 303-326.

Bianco, P.A., Bulgari, D., Casati, P., and Quaglino, F. 2011: Conventional and novel strategies for the phytoplasma diseases containment. Phytopathogenic Mollicutes 1: 77-82.

Bulgari, D., Casati, P., Crepaldi, P., Daffonchio, D., Quaglino, F., Brusetti, L., and Bianco, P.A. 2011: Restructuring of endophytic bacterial communities in grapevine yellows-diseased and recovered *Vitis vinifera* L. plants. Applied and Environmental Microbiology 77: 5018-5022.

Choudhary, D.K., and Johri, B.N. 2009: Interactions of *Bacillus* spp. and plants with special reference to induced systemic resistance (ISR). Microbiological Research 164: 493-513.

Goldschmidt, E.E. 2014: Plant grafting: new mechanisms, evolutionary implications. Frontiers in Plant Science 5: 727.

Iriti, M., and Faoro, F. 2007: Review of innate and specific immunity in plants and animals. Mycopathologia 164: 57-64.

Laimer, M., Lemaire, O., Herrbach, E., Goldschmidt, V., Minafra, A., Bianco, P., and Wetzel, T. 2009. Resistance to viruses, phytoplasmas and their vectors in the grapevine in Europe: a review. Journal of Plant Pathology 91: 7-23.

Langer, M., and Maixner, M. 2004. Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur-group based on RFLP-analysis of non-ribosomal DNA. Vitis 43: 191-200.

Mori, N., Quaglino, F., Tessari, F., Pozzebon, A., Bulgari, D., Casati, P., and Bianco, P. A. 2015. Investigation on 'bois noir' epidemiology in north-eastern Italian vineyards through a multidisciplinary approach. Annals of Applied Biology 166: 75-89.

Pierce, S., Winter, K., and Griffiths, H. 2002: The role of CAM in high rainfall cloud forests: an in situ comparison of photosynthetic pathways in Bromeliaceae. Plant, Cell & Environment 25: 1181-1189.

Quaglino, F., Zhao, Y., Bianco, P.A., Wei, W., Casati, P., Durante, G., and Davis, R.E. 2009. New 16Sr subgroups and distinct single nucleotide polymorphism lineages among grapevine Bois noir phytoplasma populations. Annals of Applied Biology 154: 279-289.

Quaglino, F., Zhao, Y., Casati, P., Bulgari, D., Bianco, P.A., Wei, W., and Davis, R.E. 2013. '*Candidatus* Phytoplasma solani', a novel taxon associated with stolbur and bois noir related diseases of plants. International Journal of Systematic and Evolutionary Microbiology 63: 2879-2894

IMPRESSUM

MITTEILUNGEN KLOSTERNEUBURG ISSN 0007-5922
WWW.WEINOBSTKLOSTERNEUBURG.AT

Medieninhaber und Herausgeber:
HÖHERE BUNDESLEHRANSTALT UND BUNDESAMT FÜR WEIN- UND OBSTBAU
A-3400 Klosterneuburg, Wiener Straße 74
Direktor: Reinhard EDER
Tel.: +43(0)2243/379 10-0 Fax: +43(0)2243/267 05
www.weinobstklosterneuburg.at

Redaktion:
Wissenschaftliche Leiterin: Monika Riedle-Bauer
(monika.riedle-bauer@weinobst.at)
Harald Scheiblhofer, Andrea Kürner, Elisabeth Mantler, Karl Neumann, Michael Müller, Karl Zitta
Alle: Wiener Straße 74, A-3400 Klosterneuburg

Fachbeirat:
Reinhard Eder, Elfriede Fuhrmann, Anton Gangl, Josef Glatt, Manfred Gössinger, Erhard Höbaus, Christian Jaborek, Josef Pleil, Monika Riedle-Bauer, Josef Schuller, Michaela Schwaiger, Robert Steidl, Lothar Wurm

Auskunft und Bestellung:
bibliothek@weinobst.at

Abonnements und Anzeigen:
Martina Schwarz (martina.schwarz@weinobst.at)

Druck:
Fa. Berger A-3580 Horn

Titelblatt:
HBLAuBA für Wein- und Obstbau

MINISTERIUM
FÜR EIN
LEBENSWERTES
ÖSTERREICH

HBLAuBA KLOSTERNEUBURG
WEIN- UND OBSTBAU