SZENT ISTVÁN UNIVERSITY
FACULTY OF HORTICULTURAL SCIENCE, BUDAPEST

SZENT ISTVÁN UNIVERSITY
FACULTY OF HORTICULTURAL SCIENCE

Epidemiology of Bois noir disease and effect of disease on grapevine performance and wine quality in Hungary

Theses of doctoral (PhD) dissertation

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1. INTRODUCTION AND OBJECTIVES

Grapevine Yellows (GY) diseases caused by phytoplasmas occur in vineyards, inducing chronic damages worldwide. One of the major devastating GY in Europe is the Bois noir (BN) disease caused by '*Candidatus* Phytoplasma solani', which is difficult to manage, and control is based on prophylaxis.

BN disease is common in European and Hungarian vineyards, as the causal agent is endemic to the Euro-Mediterranean area and is of wild plant origin. '*Ca*. P. solani' is transmitted from bindweed and stinging nettle to grapevine and to other crops by different planthoppers of the *Cixiidae* family (Maixner 2011). In Europe, at least four planthoppers are vectoring '*Ca*. P. solani'. Among them, only *H. obsoletus* and *R. panzeri* are proven vectors to grapevine, however vectoring ability of others species cannot be excluded (Cvrkovic *et al*. 2013, Maixner and Mori 2013).

Genetically different '*Ca*. P. solani' strains are shown to be associated with specific insect vector ecotypes living on different wild plant reservoirs (i.e. stinging nettle and bindweed). 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 cycles, and polymorphism of this protein might determine the transmission ability of different insect species (Suzuki *et al*. 2006, Fabre *et al*. 2011). Tracing the route of phytoplasma strains propagation and predicting their epidemic potential when introduced in a given ecological niche is of high interest. Multi locus sequence typing (MLST) tool is used to investigate epidemiological properties and population genetics of bacteria, which is based on sequence analyses of different genetic loci. Robust and precise characterization of bacterial strains requires mainly housekeeping genes under neutrality. However, to increase the predictive value regarding epidemic properties, functional markers, which are linked to interaction properties with insect vectors, can also be sequenced.

Differences in sensitivity of vine cultivars to BN direct our interest to the importance of disease management of susceptible cultivars, such as 'Chardonnay'. Phytoplasma symptomatic grapevines can undergo remission which corresponds to a temporary disappearing of symptoms, and in some cases leading to recovery which remains permanent (Caudwell 1961). The recovery can be spontaneous or induced, as has been observed in the case of Bois noir and Flavescence dorée affected plants (Osler *et al*. 1993, Romanazzi *et al*. 2009). This mechanism can be assisted by exposing grapevines to abiotic stresses or induced by certain molecules. Recently, an innovative strategy has been assessed to control BN by applying resistance inducers and steady recoveries were induced (Romanazzi *et al*. 2013).

BN is widespread in Hungarian wine regions (Kölber *et al*. 2003), however information is not available on biodiversity of Hungarian '*Ca*. P. solani' isolates. Thus the epidemiological properties of the pathogen (i.e. source of the pathogen and the vectors) is not known in our Hungary. The aim of viticulture is to produce a good quality final product. Although chronic damages of GY infected plants are noticeable worldwide, the decline in growth of '*Ca*. P. solani' infected grapevines and its effect on wine quality have not been investigated. Apart from hot-water treatment of planting material, there is no effective and satisfactory treatment against BN. As '*Ca*. P. solani' vector insects are not feeding permanently on grapevine the insecticide treatments against them are not efficient, thus rendering it hard to control the spreading of BN disease.

<u>Therefore we aimed to investigate:</u>

1. The epidemiology of Bois noir disease in Hungary:
   - to picture genetic diversity of Hungarian '*Ca.* P. solani' strains,
   - to survey vectors in different wine producing regions and perform insect transmission trials,
   - new generation sequencing of Hungarian '*Ca*. P. solani' strains,
   - to investigate insect-pathogen protein interactions.

2. In a multi-year field experiment we aimed to comprehensively describe the impact of BN on *Vitis vinifera* L., cv. 'Chardonnay' grown in the Eger wine region:
   - vegetative and reproductive performance,
   - yield and fruit quality,
   - wine analyses and sensory evaluations were carried out.

3. To attempt an applicable control strategy against BN disease, field treatments applying resistance inducers benzothiadiazole and glutathione-oligosaccharine active ingredients of two commercial products were set up to investigate their curative effect on BN-affected cv. 'Chardonnay' in the Eger wine region.

2. MATERIALS AND METHODS

2.1. Epidemiology of Bois noir disease in Hungary

Genetic diversity of '*Ca.* P. solani' strains in Hungarian wine regions. For phytoplasma test 136 plant samples (mainly grapevine and wild reservoir and potential reservoir plats, as well as lavender and Solanaceous crops) were collected in August or September of the years 2011, 2012, 2013 and 2014 in five wine regions of Hungary: Egri, Tokaji, Kunsági, Villányi, Soproni and Etyek-Budai, from 16 locations in seven counties. To obtain phytoplasma-reach phloem, main leaf veins were cut and DNA were extracted using the CTAB method (Clair *et al*. 2003). To amplify 16S rDNA universal primers P1/P7 (Deng and Hiruki 1991, Smart *et al*. 1996), followed by R16F2n/R16R2 (Lee *et al*. 1995) were used. Positive samples were subjected to restriction fragment length polymorphism to identify phytoplasma 16Sr subgroups. Among the samples testing positive for '*Ca.* P. solani', 46 isolates were selected for molecular characterisation (Multi Locus Sequence Typing - MLST) which was carried out using five genetic markers i.e. housekeeping genes: *secY, tuf* and *yidC* and functional genes: *vmp1* and *stamp*. New markers were developed based on the investigation of the genome of '*Ca*. P. solani' PO strain: *yidC*, *ligA*, *priA*, *alaS* and *pheT* and their variability were tested. Direct sequencing of the selected loci were performed (Macrogen, Amsterdam, The Netherlands, or Base-Clear, Leiden, The Netherlands). Staden Package Version 3.3 was used for assembling and sequence editing. Nucleotide sequences were aligned with CLUSTAL W. A neighbour joining (NJ) method with Tamura-Nei model was applied to construct phylogenetic trees using MEGA 6 software (Tamura *et al*. 2011). Reference sequences for each gene were used for phylogenetic analyses to which access was obtained (provided by Dr. X. Foissac and the Stolbur-Euromed Consortium).

Insect transmission of Hungarian '*Ca.* P. solani' strains. In July 2013, planthoppers of *Cixiidae* family were collected by sweep netting in eight locations in: Sopron, Fertőd, Etyek, Monorierdő, Eger, Andornaktálya and Tolcsva. Insects from all locations were placed on healthy *Catharanthus roseus* (periwinkle, Polka dot XP hybrid) plants. After 8-10 days planthoppers were gathered from the plants and kept for morphological and/or molecular determination at species level, as well as for protein interaction experiments. Molecular determination was applied for *Reptalus* species based on PCR-RFLP assays on the mitochondrial cytochrome oxidase I gene (Bertin *et al*. 2010). Symptom development was monitored regularly.

New generation sequencing of Hungarian '*Ca.* P. solani' strains. In order to gain new genome data, two of the insect transmitted '*Ca*. P. solani' strains (HO11 and REP2) were subjects for new generation sequencing (NGS). These two Hungarian strains were chosen based on the results of

stamp genotypes which belong to different *stamp* clusters II and III, respectively. The DNA was extracted (Claire *et al*. 2003) from HO11 and REP2 isolates maintained on periwinkles, and NGS of phytoplasmas were enriched isopycnic cesium chloride density gradient in the presence of bisbenzymide (Kollar *et al*. 1990). Illumina Solexa new generation sequencing were performed.

Insect-pathogen protein interaction. As *stamp*-ST4 and *stamp*-ST9 were the most prevalent genotypes in Hungary, we selected strains ST4 and ST9 (*stamp* cluster II) for heterologous protein expression. To amplify the central part of *stamp* gene that correspond to the hydrophilic domain ST4-N1, ST9-N1, ST4-9-C1 and ST1-C1 primers were designed. In-Fusion HD Cloning Kit (Clontech) was used for directional cloning of ST4 and ST9 DNA fragments into pET-28b(+) vector infused with an N-terminal His6xTag (thrombin/T7) (Novagen, Merck). Heterologous expressions of recombinant proteins were performed in *Escherichia coli* strain BL21* (Invitrogen Corporations) using standard procedures. His6x-tagged fusion proteins were purified using Ni-affinity chromatography according to the HIS-Select Nickel Affinity manual (Sigma-Aldrich). Protein was eluted using a gradient of imidazole.

To test the 2A10 monoclonal antibody (MAb) capability to recognise all four *stamp* clusters, total proteins of periwinkle infected with representative strains of clusters I, II, III and IV of '*Ca*. P. solani' maintained in periwinkle was extracted. Extracted '*Ca*. P. solani' proteins and STAMP fusion proteins were tested by western blot analyses using monoclonal anti-polyhistidine antibody produced in mouse (Sigma-Aldrich) as the primary antibody, and horseradish peroxidase conjugated goat anti-mouse IgG (Sigma-Aldrich) as the secondary antibody (Fabre *et al*. 2011a). To reveal signals a Super Signal West Pico kit was used (Thermo Scientific Pierce Protein Biology).

Interaction capabilities of insects' proteins with fusion proteins fp_ST4 and fp_ST9 were investigated in dot-western-blot analyses. Total protein was extracted from vector: *H. obsoletus* (different ecotypes: bindweed, stinging nettle and lavender), *R. panzeri*; potential vector: *R. quinquecostatus*, *R. cuspidatus*; and non-vector*: Euscelidius variegatus*, *Circulifer haematoceps* species (Galetto *et al*. 2011). In dot-western-blot hybridisation, to detect STAMP-insect protein interaction primary antibody (2A10 MAb) and secondary antibody (horseradish conjugated anti-mouse peroxidase MAb; Sigma-Aldrich) were applied. Results were developed the same as described above.

2.2. Effects of Bois noir disease on performance of cv. 'Chardonnay' in Eger wine region

A three-year field experiment was performed from 2012 to 2014 in the experimental vineyard situated in the Eger wine region of Hungary, belonging to Károly Róbert College, Research Institute of Viticulture and Enology. Measurements were performed in a 0.6 ha vineyard of *V. vinifera* L. cv. 'Chardonnay'. Vines were spaced 1.2 m within rows and 3.0 m between rows. They were cordon trained with 4-bud spurs (18–20 buds/vine) and shoots were vertically positioned. Three random blocks contained 50 plants per block in which the phytoplasma infection status of the individual plants was visually evaluated before harvest in each year of the experiment (2012–2014).

Vegetative and reproductive performance measurements. In each block, in total 15-15 healthy (H) and BN-affected (BNA) grapevines were assigned for viticultural measurements, with each replicate consisting of a single vine. Vegetative performance measurements were pruning mass, cane lignification, leaf fresh and dry mass, leaf rolling and leaf chlorophyll content. Reproductive performance measurements were fruit set (determined based on the proportion of flower conversion into berries), bunch mass, rachis mass, and number of normal and abnormal berries. In terms of yield bunch mass, number of bunches/vine, and 100 berries mass, as well as numbers of asymptomatic, symptomatic (i.e. shrivelled) and dried bunches per vine were also recorded. Fruit composition was characterized by measuring soluble solids (°Brix), titratable acidity (TA) (g/L tartaric acid) and pH.

Small-scale wine production. Grapes of three vintages (2012, 2013 and 2014) were hand-harvested at full maturity in the middle of September 2014. Musts were fermented in the winery of Károly Róbert College, Research Institute of Viticulture and Enology. Grapes (60 kg/batch) from healthy (total yield) and BNA vines (total yield) were processed separately. H and BNA grapes were sectioned out in three parts and fermented in three oenological replicates of each batch in 2012 and 2013. Fruit of each batch was processed and fermented using the normal white wine process.

Wine analyses. To characterise experimental wines of each replicate alcohol (Gibertini distiller), total extract (densimetry using hydrostatic balance), residual sugar (Luff-Schoorl method), TA (after titration), pH, tartaric acid (spectrophotometry), malic and lactic acids (Boehringer Mannheim enzyme test), total polyphenols (Folin-Ciocalteu reagent calibrated for gallic acid), colour (spectrophotometrically, 420 nm) and mineral substance and ion content (ICP-AES, ICAP-9000 spectrophotometer, Thermo-Jarell-Ash, USA) were measured in the laboratory of Department of Oenology (SZIU, Faculty of Horticultural Science).

Flavonoid, organic acid, ethanol and sugar compounds of wine samples were analysed with HPLC and the results were kindly provided by Dr. A. Szekeres and Dr. O. Bencsik (University of Szeged).

Sensory analyses. Wines were subjected to sensory evaluation by 11 trained panellists. To characterize wines prepared from H, BNA, and BNS grapes, appearance (colour and clarity), aroma (quality, intensity, fruitiness, and varietal character), and flavour (acidity, bitterness, body, and balance) attributes were considered as the main descriptors. Aroma or taste defaults, overall quality, and preferences were also recorded. For the profile analysis, wine attributes were evaluated on a line scale from 0 (poor) to 100 (prominent).

Statistical analyses. For statistical, including neural networks and discriminant analyses, IBM SPSS version 22 (IBM Corp., Armonk, NY, USA) was used. Vegetative and reproductive performance and must quality of healthy and BN-affected grapevines were analysed by two-way ANOVA; analyses of wines was done by two-way MANOVA; and sensory analysis of wines by Mann-Whitney's U test. To determine most significant parameters characterising BN disease neural network model and discriminant analyses was applied only for vegetative and reproductive parameters.

2.3. Curative field treatments of BN-affected grapevines applying resistance inducers

The experimental site was the same as described in 2.2. Three treatments, in three replicates each, were set up with two commercial products and an untreated control. For three years (2012-2014) spraying was performed applying glutathione-oligosaccharine (3 l/ha) and benzothiadiazole (0.2 kg/ha) active compounds according to Romanazzi *et al*. (2009). These elicitors were sprayed from the beginning of shoot development (stage EL-12 according to Coombe 1995) to the beginning of bunch closure (stage EL-32 according to Coombe 1995) with a 7-10 day frequency. Disease severity and incidence, as well as the disappearance of the symptoms were recorded in each experimental year in September from 2011 to 2015. Effectiveness of the treatments was determined by calculations of the relative frequencies of recovery, duration/carry of symptomless status, and relapses. Those of described comparisons were tested using MANOVA, as well as Marascuillo's procedure (Marascuillo and McSweeney 1977).

3. RESULTS

3.1. Epidemiology of Bois noir disease in Hungary

Genetic diversity of '*Ca.* P. solani' strains in Hungarian wine regions. In order to determine the extensive source of the pathogen as well as the level of phytoplasma exchange between grapevine and their wild reservoirs, the ecosystems of BN disease in five geographically representative wine regions of Hungary were surveyed. In Hungary, infection of '*Ca.* P. solani' on grapevine, celery and bindweed were confirmed. First time in Hungary this pathogen were detected on lavender, blackberry, red dead nettle and field elm.

For '*Ca*. P. solani genotyping, *tuf* and *secY* housekeeping and *vmp1* and *stamp* functional markers are used routinely. In order to improve genotyping tools for describing genetic diversity of '*Ca*. P. solani' at the Euro-Mediterranean scale five we developed new housekeeping markers. Variability of the markers was tested on representative strains of '*Ca*. P. solani', among them *yidC* (gene encoding protein involved in protein integration into the cell membrane) appeared to be the most variable.

Based on MLST of five genetic loci, namely *tuf, secY, yidC, vmp1* and *stamp* biodiversity of '*Ca.* P. solani' isolates of viticultural areas of Hungary were investigated. In our experiment *tuf*-b1 (bindweed related) and *tuf*-b2 (stinging nettle related) genotypes were found. Despite the attempt phytoplasma infection on stinging nettle has not been detected; however nettle type *tuf*-b2 genotype was found on grapevine. Investigation of *vmp1* gene revealed the presence of V2, V9, V13 and V18 genotypes in Hungary. In Europe the V18 genotype was present both in grapevine and stinging nettle, confirming stinging nettle as the most likely infection source of V18 genotype to grapevine. The *secY* S1, S4 and S6 genotypes are dominant in Hungary, similarly as in other central European countries. In general S1 and S4 are present on bindweed and on grapevine, and S6 is mostly reported on stinging nettle and on grapevine, all of which correspond to our results. High diversity of *stamp* was found in Hungary. On grapevine *stamp* ST6 (cluster IV) was dominant, followed by a lower incidence of ST4 and ST9 (cluster II). Only ST4 and ST9 were detected on bindweed, revealing that bindweed is the main infection source of these genotypes.

In summary the high variability of '*Ca*. P. solani' strains on grapevine implicates the role of polyphagous vectors and different host plants (as inoculum source) in the infection.

Insect transmission of Hungarian '*Ca.* P. solani' strains. In Hungary, experimental transmissions were done in 2013. Planthoppers of the *Cixiidae* family: *H. obsoletus*, *R. panzeri*, *R. quinquecostatus*, *R. cuspidatus* were collected from four locations and experimental host Madagascar periwinkle (*C. roseus*) were exposed to them for feeding. Strain ST4 (*stamp* clusters II) and ST13 (*stamp* cluster III) were successfully transmitted to Madagascar periwinkle by *H.*

obsoletus and *R. quinquecostatus* respectively. These species were the most abundant in the examined regions. In accordance with Elekes *et al*. (2006), populations of *H. obsoletus* were mainly found on stinging nettle and only very few individuals on bindweed.

New generation sequencing of Hungarian '*Ca*. P. solani' strains. In this study successful transmission of '*Ca*. P. solani' ST4 and ST13 genotypes with *H. obsoletus* (HO11, collected from bindweed) and *R. quinquecostatus* (REP2, collected from wild vegetation in the vicinity of grapevine) was obtained. Prior to NGS phytoplasma enrichment using isopycnic cesium chloride density gradient in presence of bisbenzymide was successfully performed. As a result of this process, phytoplasma genome were separated from genome of *C. roseus*, and high quantity and good quality DNA of HO11 and REP2 were obtained. New generation genome sequencing of the strains was achieved. The *de novo* assembling and annotation is in progress.

Insect-pathogen protein interaction. In order to better understand the vector specificity, an interaction study was initiated. To perform this experiment, we demonstrated that 2A10 MAb - raised against STAMP of cluster I- is able to recognise *stamp* clusters II, III and IV, and is therefore a very useful tool for investigation interaction between vector protein - '*Ca*. P. solani' protein.
Based on the *stamp* genotyping the Hungarian isolates dominantly clustered into the *stamp* II phylogenetic group, with the most prevalent strains being ST4 and ST9. BN vectors, potential vectors and non-vectors and the different ecotypes of *H. obsoletus* (population from bindweed, stinging nettle and lavender) were compared. It was demonstrated that STAMP cluster II (both fp_ST4 and fp_ST9) is capable of interacting with proteins of *H. obsoletus* bindweed and lavender ecotypes, and *R. quinquecostatus* with high intensity. Interaction with *H. obsoletus* stinging nettle ecotype and *R. panzeri* showed lower intensity. These results suggest that *H. obsoletus* bindweed ecotype might be a more competent vector of *stamp* genotypes of cluster II than the others tested. The '*Ca*. P. solani' vectoring ability of *R. quinquecostatus* to grapevine has not been demonstrated so far (Cvrkovic *et al*. 2013). However, based on our results (i.e. presence of ST13 on grapevine, transmission of ST13 by *R. quinquecostatus* and the results of interaction experiment) it can be hypothesized that *R. quinquecostatus* could be a competent vector of '*Ca*. P. solani' ST13 *stamp* genotype to grapevine and/or other herbaceous hosts. *In vitro* interaction between '*Ca*. P. solani' STAMP and insect proteins was demonstrated for the first time in this study.

3.2. Effect of Bois noir disease on performance of cv. 'Chardonnay' in Eger wine region

Vegetative and reproductive performance measurements. Significant increases/decreases in vegetative and reproductive parameters of BN-affected grapevines were found in the three-year average, compared to healthy vines: leaf rolling caused a decrease in leaf surface (-28.0%), leaf fresh and dry mass were increased (+11.2 and +19.5%), relative chlorophyll index was lower (-30.4 %), yield/vine decreased (-68.4%), berry mass decreased (-32.5%), bunch number/vine was lower (-56.7%), symptomatic bunch/vine (+97.2%), dry bunches/vine increased (+90.4%), titratable acidity increased (+16.4%), pH decreased (-2.7%), and soluble solids decreased (-6.2%). BN resulted in less photosynthetically active canopy, lack of lignification, and non-viable buds. Crop losses always exceeded 53%.

Wine analyses. It was demonstrated for the first time that '*Ca*. P. solani'-caused BN disease negatively affects the wine quality of cv. 'Chardonnay' grown in the Eger wine region (Hungary). Significant increases/decreases of parameters compared to those of healthy plants were demonstrated: lower alcohol (-5.3%), deeper colour (in some years pink) (+22.2%), higher titratable acidity (+7.9%), elevated malic- and citric acids (+4.5% and +6.0%), higher calcium and magnesium (+8.4% and 4.4%), lower iron (-5.4%), elevated hydroxycinnamic acid (caftaric +3.6% and caffeic +36.1%), and lower flavonoids (catechin -8.9% and epicatechin -14.4%). The extent and proportion of yield loss, bunch mass and berry mass varied in years and there was a significant year × infection interaction detected. A strong dependence of BN-affection on environmental factors in different years was demonstrated. Negative effects were slightly masked in the years with unfavourable weather. Differences between vintages were observed in this study, e.g. between the 2012 vintage, marked by warm and extremely dry weather, and the 2013 vintage, when balanced conditions favoured great wine quantity and quality. In 2014, a poor vintage was produced due to high precipitation at ripening stage.

Sensory analyses. There were noticeable differences in analytical parameters among wines produced from healthy and BN-affected grapes which was confirmed by sensory evaluations, and were most pronounced in 2013. Elevated organic acid and phenolic compound contents were responsible for the acidity and likely the bitterness of the wines produced from BN-affected and shrivelled grapes. These wines, due to the lack of sufficient sugar accumulation in berries, resulted in lower alcohol contents. The pink discolouration of BN-affected wines was considered a wine fault and certainly decreases the market value of these wines. The BN-affected grapevines may decrease economic sustainability of vineyards with vegetative and reproductive growth, as well as wine quality being detrimentally affected.

3.3. Curative field treatments of BN-affected grapevines applying resistance inducers

In the three-year experiment (2012-2014) commercial products with an active agent of glutathione-oligosaccharine and benzothiadiazole, were applied to study their curative effect on BN-affected grapevines. The most intensive curative effect, in short term remission, was observed in the case of glutathione-oligosaccharine treatment followed by untreated control, then benzothiadiazole. The extents of remissions were moderate in all cases, and differences in long term (2, 3 or 4 year symptomless status after remission) have not yet been observed. However, long term positive effects on symptom remission of the performed treatments may be expected. Side effects from the treatments were not observed.

4. NEW SCIENTIFIC RESULTS

1. Significant negative effects of '*Cadidatus* Phytoplasma solani'-caused Bois noir disease on vegetative and regenerative performance, and on fruit composition of BN-affected grapevines cv. 'Chardonnay' in the Eger wine region (Hungary) were demonstrated.

2. First demonstration that '*Ca*. P. solani'-caused Bois noir disease negatively effects the wine quality of cv. 'Chardonnay' grown in the Eger wine region (Hungary).

3. The extent and proportion of the loss of yield, bunch mass and berry mass varied in years and there was a significant year × infection interaction detected.

4. Two new housekeeping markers were developed to improve multi locus sequence typing tools of '*Ca*. P. solani': *yidC*, the gene encoding protein involved in protein integration into the cell membrane, and *ligA*, encoding NAD(+)-dependent DNA ligase.

5. Hungarian '*Ca*. P. solani' isolates were genotyped based on *tuf*, *secY*, *yidC*, *vmp1* and *stamp* genes. It was demonstrated that the following genotypes are dominantly present in Hungarian wine regions: *Tuf*: *tuf*-b1 and *tuf*-b2; *secY*: S1, S4 and S6; *vmp1*: V2, V9, V13 and V18; *stamp*: ST6 (cluster IV), ST4 and ST9, ST9D (cluster II), and sporadically ST13 and ST22 (cluster III). The most prevalent genotypes on grapevine are S6/V18/ST6, S1/V2/ST4 and S1/V2/ST9.

6. '*Ca.* P. solani' infection on lavender (*Lavandula angustifolia*) is reported for the first time in Hungary. Infection of '*Ca*. P. solani' on red deadnettle (*Lamium purpureum*) and field elm (*Ulmus minor*) was also detected for the first time.

7. Experimental transmissions were performed with planthoppers and it was shown for the first time that '*Ca*. P. solani' ST4 (clusters II) and ST13 (clusters III) of *stamp* genotypes were transmitted to Madagascar periwinkle by *Hyalesthes obsoletus* and *Reptalus quinquecostatus* respectively.

8. It was demonstrated for the first time that 2A10 monoclonal antibody, produced against '*Ca*. P. solani' strain of *stamp* cluster I, recognises strains of *stamp* cluster II, III and IV.

9. *In vitro* interaction between '*Ca*. P. solani' STAMP and insect protein was demonstrated for the first time. It was shown that STAMP cluster II (both fp_ST4 and fp_ST9) is capable of interacting with proteins of *H. obsoletus* bindweed and lavender ecotypes, and with *R. quinquecostatus*. Interaction with *H. obsoletus* singing nettle ecotype and *R. panzeri* showed less intensity.

5. CONCLUSIONS AND PERSPECTIVES

To increase robustness and precise characterization of '*Ca*. P. solani' strains we developed five new housekeeping markers. Due to its high variability *yidC* appeared to be an appropriate genotyping marker, but *ligA* could also be suitable for '*Ca*. P. solani' MLST. Applicability of these genetic loci has to be evaluated on a larger number of isolates.

Genotyping of Hungarian '*Ca*. P. solani' isolates revealed useful epidemiological information that facilitates the tracing of pathogens and predicts the risk of BN disease in wine regions. In Hungary the most prevalent genotypes on grapevine were S6/V18/ST6 (genotypes related to stinging nettle reservoir in Europe), S1/V2/ST4 and S1/V2/ST9 (genotypes related to bindweed reservoir in Europe). The role of bindweed in spreading of S1/V2/ST4 and S1/V2/ST9 genotypes to grapevine in Hungarian vineyards was confirmed by our experiment. Based on our results, the importance of stinging nettle and/or red deadnettle as the main dissemination source of S6/V18/ST6 to grapevine in Hungarian vineyards can be suggested. However, the roles of these plants in BN ecosystems in Hungary have to be confirmed. The presence of *stamp* ST13 genotype on grapevine -the genotype transmitted by *Reptalus quinquecostatus* to periwinkle- suggests that this planthopper could be a competent vector of '*Ca*. P. solani' to grapevine.

High variability of '*Ca*. P. solani' strains on grapevine suggests that the pathogen might not have been introduced with propagation material. When a pathogen is introduced by propagation material a lower genetic variability is expected. High genetic diversity implies the crucial role of polyphagous vectors and different host plants as inoculum sources of the infections. According to the prevalence of stinging nettle related genotypes on grapevine in Hungary, the primary infection sources are most likely the stinging nettle, and assuredly the bindweed with somewhat/slightly less relevance. However, information is very limited on which '*Ca*. P. solani' genotypes are present on Hungarian vector populations (i.e. *H. obsoletus*), as well as wild plants acting as infection sources (mainly stinging nettle). Therefore additional studies are needed in other wine growing areas/locations/sites to evaluate the risk of the disease and clarify the role of wild hosts and vectors in the spreading of the Bois noir disease.

In vitro interaction between '*Ca*. P. solani' STAMP (cluster II) and insect vectors' proteins was demonstrated. To complete this study testing of the interacting activity of each representative STAMP of four *stamp* genetic clusters is required. For this purpose, recombinant protein fp_ST4, fp_ST9, fp_ST6 (cluster III) and fp_ST13 (cluster IV) were successfully expressed.

Factors like lifetime of the cultivar, planting density and the proportion of symptomatic plants affect the productivity of a diseased vineyard, and influence the decision to replace the BN-affected vines. Pavan *et al*. (2012) stated that in the case of 'Chardonnay', the maintenance of BN-diseased

plants is more profitable than their elimination, even though BN is a chronic disease. On the other hand, because of significant yield and quality losses, the economic sustainability of BN affected vineyards is compromised enough to support replanting (Garau *et al*. 2007, Endeshaw *et al*. 2012, Rusjan *et al*. 2012). In this study, heavy yield and quality losses were found for 'Chardonnay' in Hungarian pedo-climatic conditions. This would certainly have a negative effect on economic sustainability. Regarding the vectors themselves, climate changes can alter, in both the short and long term, the distribution and behaviour of several insect species (Boudon-Padieu and Maixner 2007). At higher temperatures, the geographical distribution of insect species and the colonisation of plants by phytoplasma are more efficient and lead to earlier onset and/or higher severity of the disease (Foissac and Wilson 2010, Salar *et al*. 2013). Altogether, these factors together influence the economic damage caused by '*Ca*. P. solani'. More pronounced negative effects on fruit composition and wine quality occurred in a year with optimal weather conditions for the grapevine. These negative effects were slightly masked in the years with unfavourable weather, suggesting that BN effects are more pronounced in vineyards which are less exposed to extreme weather conditions. However further studies are needed to clarify this. In many wine producing regions, especially in areas producing grapes for industrially manufactured wines, the grapes are mechanically harvested. This practice prevents wine producers from selecting only disease-free grapes. As the most important factor in viticulture is the maintenance of grape quality, in balance with quantity, the elimination of BN-affected grapevines is advisable. As different '*Ca.* P. solani' strains might affect a given cultivar differently, it is important to determine which '*Ca.* P. solani' genotype/s is/are prevalent in the Eger and other wine regions of Hungary.

Also important could be the masking effect of BN over other GY disease management. Indeed, Bois noir and Flavescence dorée (the only GY classified as quarantine pathogens in Europe), induce identical symptoms, and BN cases lead to a hiding of early FD outbreaks. Therefore further studies need to be undertaken in Hungary to survey BN, as well as investigate its effect on other cultivars in order to better evaluate the economic impact of the disease. Moreover continuation of '*Ca*. P. solani' genotyping, the STAMP-insect protein interaction studies, as well as the treatment using resistance inducers would be crucial, in order to develop new effective pest management strategies against Bois noir disease.

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