10.14751/SZIE.2016.070

DOCTORAL (PhD) THESIS

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Gödöllő

2016

10.14751/SZIE.2016.070

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

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2016

10.14751/SZIE.2016.070

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CONTENT

1. LIST OF ABBREVIATIONS

AM-complex	AMP-actin microphilament complex
AMP	Antigenic membrane protein
ATP	Adenosine triphosphate
AY	Aster yellows, '*Candidatu*s Phytoplasma asteris'
AY-M	Aster yellows, '*Candidatu*s Phytoplasma asteris' Mild strain
AY-OY	Aster yellows, Onion yellows strain, '*Candidatu*s Phytoplasma asteris'
AY-WB	Aster yellows Witches' broom strain, '*Candidatu*s Phytoplasma asteris'
'*Ca*. P. asteris'	'*Candidatu*s Phytoplasma asteris'
'*Ca*. P. solani'	'*Candidatu*s Phytoplasma solani'
'*Ca*. P. vitis'	'*Candidatu*s Phytoplasma vitis'
BBZ-CsCl	Isopycnic caesium chloride density gradient in presence of Bisbenzymide
BN	Bois noir
Chl	Chlorophyll
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide solution mix
dpi	Day post inoculation
ER	Endoplasmic reticulum
FD	Flavescence dorée
fp_ST1	fusion protein STAMP 1 genotype of *stamp* cluster I
fp_ST13	fusion protein STAMP 13 genotype of *stamp* cluster III
fp_ST4	fusion protein STAMP 4 genotype of *stamp* cluster II
fp_ST6	fusion protein STAMP 6 genotype of *stamp* cluster IV
fp_ST9	fusion protein STAMP 9 genotype of *stamp* cluster II
GY	Grapevine Yellows
IDPs	Immunodominant membrane proteins
IMP	Immunodominant membrane protein
IMP A	Immunodominant membrane protein A
IP	Insect total protein
IPTG	Lactose isopropyl-beta-D-thiogalactopyranoside
JA	Jasmonic acid phytohormone
MAb	Monoclonal antibody
MLOs	Mycoplasma-like organisms
MLST	Multi locus sequence typing
NGS	New generation sequenceing
NJ	Neighbor Joining
PAb	Polyclonal antibody
PCR	Polymerase chain reaction
poly-His MAb	Anti-polyhistidine monoclonal antibody
PTS	Phosphoenolpyruvate-dependent sugar phosphotransferase system
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SAP	Secreted AY-WB protein
SE	Sieve element
SNP	Single-nucleotide polymorphism
stamp	Stolbur antigenic membrane protein gene; lowercase, italics refers to gene encoding a protein
STAMP	Stolbur antigenic membrane protein, upercase refers to translated protein
SVM	Sequence variable mosaics
WB	Western blot

2. LIST OF FIGURES AND TABLES

10.14751/SZIE.2016.070

3. INTRODUCTION

Phytoplasmas cause Grapevine Yellow (GY) diseases which have economic impact. In Europe, the Flavescence dorée caused by '*Candidatus* Phytoplasma vitis' (taxonomic groups 16SrV-C and D) transmitted by the grapevine leafhopper *Scaphoideus titanus* and the agent of Bois noir '*Candidatus* Phytoplasma solani' (taxonomic group 16SrXII-A) transmitted by polyfagous planthoppers of the family *Cixiidae*, induce GYs with high impact in viticulture. The quarantine Flavescence dorée (FD) affects many European vineyards from Portugal to Serbia, while Bois noir (BN) is much more widespread. The epidemiology of FD and BN greatly differ because of distinct pathogens, host range and vectors. As FD and BN symptoms are identical and as phytoplasma cannot be cultivated they can only be distinguished by DNA-based molecular diagnosis. Phytoplasma disease cannot be cured and disease control is based on the implementation of prophylactic measures such as usage of pathogen-free propagation material, hot water treatments of propagation material, destruction of infected grapevines and wild reservoir plants and chemical control of the insect vector. Unfortunately, none of these approaches are fully satisfactory and plants cannot be protected against new infections.

As it cannot propagate from grapevine to grapevine, Bois noir disease is recognised as less dangerous than other GYs (i.e. FD). However, BN management is difficult as the BN disease cycle is more complex due to the biology of non-ampelophagous vectors living on weeds such as bindweed and stinging nettles (Maixner 2011). The number of BN cases has increased in recent years, and correlate with higher population of insect vectors, which depend on factors such as temperature, soil, and presence of insect host plants around vineyards (Maixner 2011, Panassiti *et al*. 2015). Differences in sensitivity of vine cultivars to BN direct our interest to the importance of disease management of susceptible cultivars, such as 'Chardonnay' (Panassiti *et al*. 2015, EFSA Panel on Plant Health 2014). The GY diseases are classified as 'auxonic disease' indicating a possible interaction with the hormonal balance of the host causing severe symptoms on leaves, shoots and fruits. Symptom appearance is anticipated by cellular modifications, such as callose deposition in phloem sieve elements, and eventually phloem necrosis (Lepka *et al*. 1999, Musetti *et al*. 2007). Significant reduction in performance of certain grapevine cultivars was investigated in many aspects, such as at physiological as well as yield and fruit quality level, and remarkable losses had been reported (Bertamini *et al*. 2002b, Garau *et al*. 2007, Endeshaw *et al*. 2012, Rusjan *et al*. 2012, Romanazzi *et al*. 2013, Zahavi *et al*. 2013). In BN diseased vineyards, fluctuation in infection status results in an extended range of yield and quality loss, and erratic economic impact. In the last few years, the number of Bois noir cases increased in European vineyards (Maixner 2011). Some genetically different '*Ca*. P. solani' strains shown to be associated with specific insect vector ecotypes living on different wild plant reservoirs, suggests the adaptation of phytoplasma

strains to different ecological niches. Knowledge on mechanisms of insects-phytoplasma interactions, which are driving the ecological diversification of phytoplasmas, is limited. We know that insect-transmissible pathogens can be transmitted by a certain insect species and not by others. This is due to the highly specific interaction between vectoring insects and the bacterial pathogen (Suzuki *et al*. 2006, Galetto *et al*. 2011). Phytoplasma surface proteins such as antigenic membrane protein - AMP, play an important role in the phytoplasma life cycle, and are in correlation with the phytoplasma-transmission capability of leafhoppers (Suzuki *et al*. 2006, Galetto *et al*. 2011). In the genome of '*Ca*. P. solani' the ortholog of *amp* named *stamp* (stolbur antigenic membrane protein -STAMP) has been identified might have major importance in phytoplasma pathogenicity (Fabre *et al*. 2011a). *Stamp* gene showed high variability in Europe, and genotypes were grouped in different clusters (Fabre *et al.* 2011b).

Phytoplasma symptomatic grapevines can undergo remission which corresponds to a temporary disappearing of symptoms sometime leading to recovery which remains permanent (Caudwell 1961). The recovery can be spontaneous or induced, as has been observed in the case of BN and FD affected plants (Osler *et al*. 1993, Romanazzi *et al*. 2009). This mechanism can be assisted by exposing grapevines to abiotic stresses and agronomical practices. Recently, an innovative strategy has been assessed to control BN by applying resistance inducers and steady recoveries were induced (Romanazzi *et al*. 2013).

Bois noir disease is widespread in Hungarian wine regions. 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. Its presence raises the need for detailed information on Bois noir disease i.e. '*Ca.* P. solani'- caused damage on grapevines and its epidemiological spread. Such knowledge may help the development of environmental-safe pest management programmes for more profitable and safer grape production.

4. OBJECTIVES

4.1. To study the epidemiology of Bois noir disease the genetic diversity of Hungarian '*Ca.* P. solani' strains, as well as occurrence of planthopper vectors in different wine producing regions were investigated. Additionally, insect-pathogen interaction experiments were performed.

- Multi Locus Sequence Typing (MLST) of Hungarian '*Ca.* P. solani' strains were conducted based on conserved (housekeeping genes: *tuf, secY*) and variable (surface protein genes: *vmp1* and *stamp*) genetic markers.
- Transmission trials to experimental hosts with cixiid planthoppers collected in Hungary were performed to identify vector species of '*Ca.* P. solani'.
- To characterise '*Ca.* P. solani' strains at a genomic level, new generation sequencing (NSG, Illumina Solexa) of two Hungarian '*Ca*. P. solani' strains was initiated.
- To evaluate the ability of various STAMP proteins to interact with proteins of insect vectors, the heterologous expression of recombinant STAMPs of '*Ca*. P. solani' strains belonging to *stamp* cluster I, II, III and IV were carried out.
- To investigate STAMP-insect proteins interactions, a serological tool to detect STAMP of all clusters is needed. The polyvalence of an anti-STAMP monoclonal antibody (2A10 MAb) -initially raised against the strain StolburC of *stamp* cluster I- against *stamp* of all genetic clusters was evaluated.
- Preliminary results of *in vitro* interaction assays between recombinant STAMP and planthoppers proteins have been produced.

4.2. In a three-year experiment, the impact of Bois noir disease in terms of yield and wine quality loss on *Vitis vinifera* L., cv. 'Chardonnay' in the Eger wine region was defined.

- To picture BN disease comprehensively, vegetative and reproductive performance, morphological measurements, physiological analyses, small-scale winemaking (microvinification), berry must and wine analyses, and sensory evaluations were carried out.

4.3. 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.

10.14751/SZIE.2016.070

5. BACKGROUND

5.1. Phytoplasmoses

Phytoplasmas are plant-pathogenic bacteria causing diseases on several crops worldwide (Lee *et al*. 2000, Bertaccini 2007). These endogenous bacteria are strictly limited to the phloem sieve tube elements, and due to their vascular habitat have a systemic distribution in the plant (Bové and Garnier 2002). Phytoplasmas are transmitted from plant to plant by phloem-feeding insect species such as leafhoppers, planthoppers and psyllids in a persistent manner (Weintraub and Beanland 2006), as well as by graft inoculation. Plant parasite *Cuscuta* spp. (dodder) are also able to transmit experimentally phytoplasmas by attaching to vessel tissue of dicotyledonous plants through haustoria. Because they were able, like viruses, to pass filters of 0.45 μm porosity, phytoplasma-caused diseases were for long considered as viruses. Phytoplasmas were first observed by electron microscopy in Japan in 1967, when Doi and colleagues described them as mycoplasma-like organisms (MLOs) because they resembled human and animal pathogens mycoplasmas, wall-less bacteria of the bacterial class *Mollicutes* (Doi *et al*. 1967). MLOs were classified into class *Mollicutes*, and in 1992, the name phytoplasma was proposed by the Subcommittee on the Taxonomy of Mollicutes. In the following years, based on ribosomal protein sequences and 16S rRNA gene sequences it was demonstrated that MLOs represent a distinct, monophyletic clade within the class *Mollicutes* and were renamed phytoplasma (Lim and Sears 1989, 1992a, Kuske and Kirkpatrick 1992, Namba *et al*. 1993, Gundersen *et al*. 1994, Seemüller *et al*. 1994, Lee *et al*. 2000). In 2004, the '*Candidatus* Phytoplasma' genus name was introduced, with Candidatus status used for bacteria that cannot be cultured (Murry and Schleifer 1994, IRPCM 2004). Up till now 37 '*Candidatus* Phytoplasma' species have been described so far and a further 12 suggested for approval (Harrison *et al*. 2014).

Phytoplasmas inhabit the functional sieve elements of the phloem or the insects' body, where phloem sap or insect cytoplasm provides them a nutrient-rich environment for growth. Phytoplasmas are pleomorphic particles with the size 0.1-0.8 μm. They have no cell wall and are bounded by a lipidic bilayered membrane. The cell membrane contains membrane proteins some of which can be immunogenic when phytoplasmas are injected to rabbit or mouse and are named immunodominant membrane proteins (Kakizawa *et al*. 2006b). Phytoplasmas have limited genome, lacks genes encoding certain functions, making them entirely dependent on their host (Hogenhout *et al*. 2008).

In the plant, phytoplasmas are restricted exclusively to the phloem sieve tubes. In the phloem, phytoplasmas spread systemically throughout the plants by passing through phloem sieve plate pores. Sieve tubes are enucleated living cells of the phloem, surrounded by companion cells.

Companion cells are important in loading sieve tubes with photosynthates in the source area (i.e. mature leaves) and unloading in the sink area (i.e. young leaves, fruits and roots). They also serve essential proteins and ATP for ribosome and mitochondry-poor sieve elements. Both sieve tubes and companion cells are metabolically highly active (reviewed in Cayla *et al*. 2015). Adjacent sieve elements are joined by altered plasmodesmata, forming sieve pores that enable translocation of assimilates. Due to the lack of cell-wall phytoplasmas are able to elongate or modify their shape to pass through the sieve pores. It was also hypothesized that interaction with plant actin might be involved in this process (Musetti *et al*. 2016). It had been shown that the Immunodominant Membrane Protein of '*Ca.* P. mali' is able to interact with *Malus* actin (Boonrod *et al*. 2012).

5.2. Genetic background and taxonomy

Phytoplasmas are wall-less non-helical *Mollicutes* that have this far been unable to grow *in vitro*. Recent report on establishing axenic cultures of phytoplasmas (Contaldo *et al*. 2012) has not yet been introduced into practice. Therefore, methods used for prokaryotes classification are not applicable for these pathogens.Within *Mollicutes* which represents a single branch that evolved from Gram-positive bacteria, phytoplasmas constitute a single clade that diverged from *Acholeplasma* spp. (Figure 1) (Lee *et al*. 2000). However it was demonstrated that phytoplasmas have membrane properties similar to that of *Acholeplasma* because they resist to the sterol chelator 'digitonin' and are more sensitive to lysis in hypotonic salt solutions than does mycoplasma membrane (Lim and Sears 1992b). The fact that phytoplasmas use UGA as a stop codon, not as a tryptophan, also supports the evolutionary relationship with *Acholeplasmas* which, on the contrary of *Mycoplasmas* and *Spiroplasmas*, also use UGA as stop codon (Lim and Sears 1992a). Therefore the genus '*Candidatus* Phytoplasma' belongs to Class *Mollicutes*, and is currently placed under *Insertae sedis* within Order *Acholeplasmatales*, Family *Acholeplasmataceae* (Table 1) (Bergey's Manual of Systematic Bacteriology, Krieg *et al*. 2010). However, based on biochemical and physiological characteristics, as well as genome structure and phylogenetic association; taxonomic placement and establishment of a new provisional order and family were recently suggested to accommodate the Genus '*Candidatus* Phytoplasma' (Zhao *et al*. 2015). At present a new '*Candidatus* (*Ca*.) Phytoplasma' species can be described if its 16S rRNA gene sequence has < 97.5 % similarity to that of any previously described '*Ca*. Phytoplasma' species (IRPCM 2004). Phenotypic characteristics such as habitat specificity, life cycle, and genomic data have also to be considered to use to separate Orders in the Class *Mollicutes* (Zhao *et al*. 2015).

5.3. Reductive evolution of plant-pathogenic phytoplasma

Mollicutes have the smallest genome among the bacteria and most likely diverged from a Gram-positive ancestor (*Clostridium* or *Lactobacillus* spp.) (Weisburg *et al*. 1989). During genome reduction, *Mollicutes* lost their outer cell wall, possessing only a single cell membrane (Sirand-Pugnet *et al*. 2007). The size of phytoplasma genomes range from 530 to 1350 kbp as estimated on pulse-field gel electrophoresis (Marcone *et al*. 1999) and contains less than 30 % GC (Sears *et al*. 1989). Evidence of gene adaptations to insect hosts environment were reported (Ishii *et al*. 2009, Chuche *et al*. 2013). Habitat specificity is an important feature of the Genus '*Ca.* Phytoplasma' as these intracellular parasites can live and multiply only in a highly specialized niche. As the environment determine the lifecycle of a given pathogene, the nutritional demand and other physiological properties of mollicute are largely determined by its habitat, such attributes can be inferred from genomics data (Zhao *et al*. 2015). Complete sequence data of five phytoplasmas revealed that these pathogens have a minimal gene set for life, and a lack of certain metabolic functions (Oshima *et al*. 2004, Bai *et al*. 2006, Kube *et al*. 2008, Tran-Nguyen *et al*. 2008, Andersen *et al*. 2013). The massive gene loss is most likely the result of the nutrient-rich environment they live in (Oshima *et al*. 2004, Hogenhout *et al*. 2008). However, while genes coding basic cellular functions i.e. DNA replication, transcription, translation and protein translocation are present in the phytoplasma genome, it lacks certain others such as those coding for amino acid and fatty acid biosynthesis, the tricarboxylic acid cycle, oxidative phosphorylation, the pentose phosphate cycle and F_1F_0-type ATP synthase subunits. The pentose phosphate cycle is essential for synthesizing NADPH to maintain redox homeostasis and also supplies the ribose 5-phosphate to synthesize nucleotides. Due to the lack of the pentose phosphate cycle, phytoplasmas are likely not able to synthesize nucleotides, thus they withdraw them from their environment (Razin *et al*. 1998, Oshima *et al*. 2004). Transport and metabolism of sugar molecules is different in phytoplasmas compared to other bacteria. Phytoplasmas lack the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) and hexokinase to phosphorylate hexoses, whereas this system is present in other *Mollicutes* and is considered essential to the minimal genome of a free-living bacterium (Glass *et al*. 2006). It also suggests that other forms of carbohydrate have to be utilized by phytoplasmas. Possessing genes coding malate/citrate symporters and malic enzyme that convert malate to pyruvate, phytoplasmas also possess pyruvate dehydrogenase that cans oxydatively decarboxylate pyruvate to acetyl-coA (Kube *et al*. 2012).The malic enzyme of '*Ca.* P. asteris' phosphotransacetylase that convert acetyl-coA to acetyl-phosphate have been expressed in *E. coli* and their enzymatic activity was confirmed (Saigo *et al*. 2014). Presence of such enzymes implies that malate could be the main carbon source for phytoplasmas.

Table 1. Taxonomy of class Mollicutes*
(Adapted from Zhao *et al*. 2015 with modifications: **)

Classification	Habitat	Sterol requirement	Distinctive properties
Order I:*Mycoplasmatales*	Parasites of humans and animals	+	Surface parasites
Family I: *Mycoplasmataceae*			
Genus I: *Mycoplasma*			Urease negative
Genus II: *Ureaplasma*			Urease positive
Order II: *Entomoplasmatales*	Inhabitants of arthropod gut, and few species infect plants	+	
Family I: *Entomoplasmataceae*			
Genus I: *Entomoplasma*			Non-motile, non-helical, do not infect plants
Genus II: *Mesoplasma*			
Family II: *Spiroplasmataceae*			Motile, helical filaments, three species infect both plants and insects
Genus I: *Spiroplasma*			
Order III: *Acholeplasmatales*	Saprophytic, free-living	-	
Family I: *Acholeplasmataceae*			No parasitic life stage
Genus I: *Acholeplasma*			
Genus II: '*Candidatus* Phytoplasma'**	Obligate parasites of plants and phloem-feeding insects		Lack PTS and redox self-regulating capability. Possess SVM genome architecture. Transkingdom parasites.
Order IV: *Anaeroplasmatales*	Parasites in bovine and ovine rumen	±	Obligately anaerobic
Family I: *Anaeroplasmatacea*			
Genus I: Anaeroplasma			Sterol required in media
Genus II: Asteroleplasma			Sterol non required
Order V: *'Candidatus* Phytoplasmatales'	Obligate parasites of plants and phloem-feeding insects	-	Lack PTS and redox self-regulating capability. Possess SVM genome architecture. Transkingdom parasites.
Family I: '*Candidatus* Phytoplasmataceae'			
Genus I: '*Candidatus* Phytoplasma'			

Legend: *: based on Razin (1992) and Bergey's Manual of Systematic Bacteriology (Krieg *et al*. 2010); **in green: current taxonomic classification of the genus '*Candidatus* Phytoplasma'; red colour: suggested new Order and Family for the genus '*Candidatus* Phytoplasma' by Zhao *et al*. 2015.

The Phytoplasma genome has gone through a reductive evolution losing numerous genes considered essential for autonomous cell replication. The presence of 27 genes coding the transport system in multiple copies implies that phytoplasmas withdraw certain metabolites from the host cell (Oshima *et al*. 2004). Despite their small genome, they contain certain mobile genetic elements which have significantly influenced their evolution (Bai *et al*. 2006). Phytoplasma genome reduction due to gene loss and horizontal gene transfer was counterbalanced by the integration of repeated sequences and gene acquisitions (Jomantiene *et al*. 2007, Wei *et al*. 2008). "While the loss of genes encoding diverse metabolic pathways led to increased host dependence", phytoplasma-host interaction competencies evolved in parallel (Zhao *et al*. 2015).

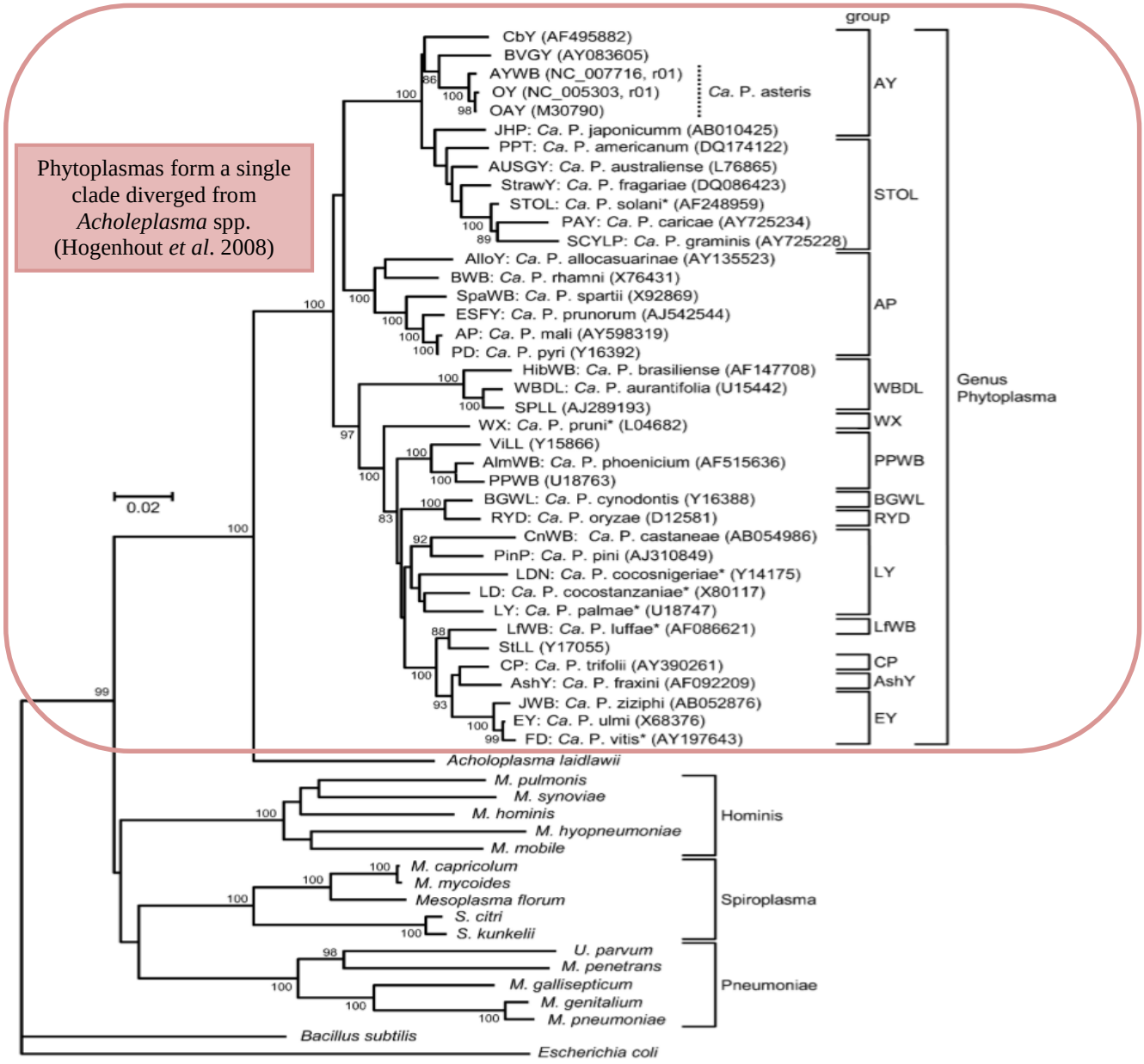

Figure 1. Phylogenetic tree of phytoplasmas, acholeplasmas, mycoplasmas, spiroplasmas based on 16S rRNA gene (Adapted from Hogenhout *et al*. 2008)

5.4. Structural membrane proteins

During infection, biotrophic plant pathogens form a relationship with the living cells of the host. To overcome plant host defences, pathogenic organisms produce or secrete effector molecules, which induce various physiological changes in the host to provide a competitive advantage for themselves and their vector(s) (Staskawitz 2001, Hogenhout *et al*. 2008, Sugio *et al*. 2011a, MacLean *et al*. 2014). A high proportion of bacterial proteins (30 %) are destined to function at the cell envelope or outside the cell, such as for nutrient uptake, metabolism, communication, virulence and defence. Secretory (Sec) pathway is the primary protein export system of bacteria which is essential for cell viability (Economuo 1999, Driessen *et al*. 2008). In *Echerichia coli* the Sec system involves a set of conserved proteins (Figure 2). Among them, three proteins -namely

SecA, SecY and SecE- are the most important, and were sufficient to remodel protein translocation *in vitro*, demonstrating their fundamental role in protein secretion and likely in pathogenicity. YidC is also a relevant secretory pathway and, has a role in the integration of newly synthesized membrane proteins. Previously, YidC was thought to be part of the Sec pathway, as it serves together with the Sec system. However, recently it was demonstrated that YidC is independent from the Sec system (reviewed in Hogenhout *et al*. 2008). Both systems, Sec for protein integration and secretion, and YidC for protein integration of membrane proteins, are present in phytoplasmas (Kakizawa *et al*. 2004, 2004, Oshima *et al*. 2004, Bai *et al*. 2006).

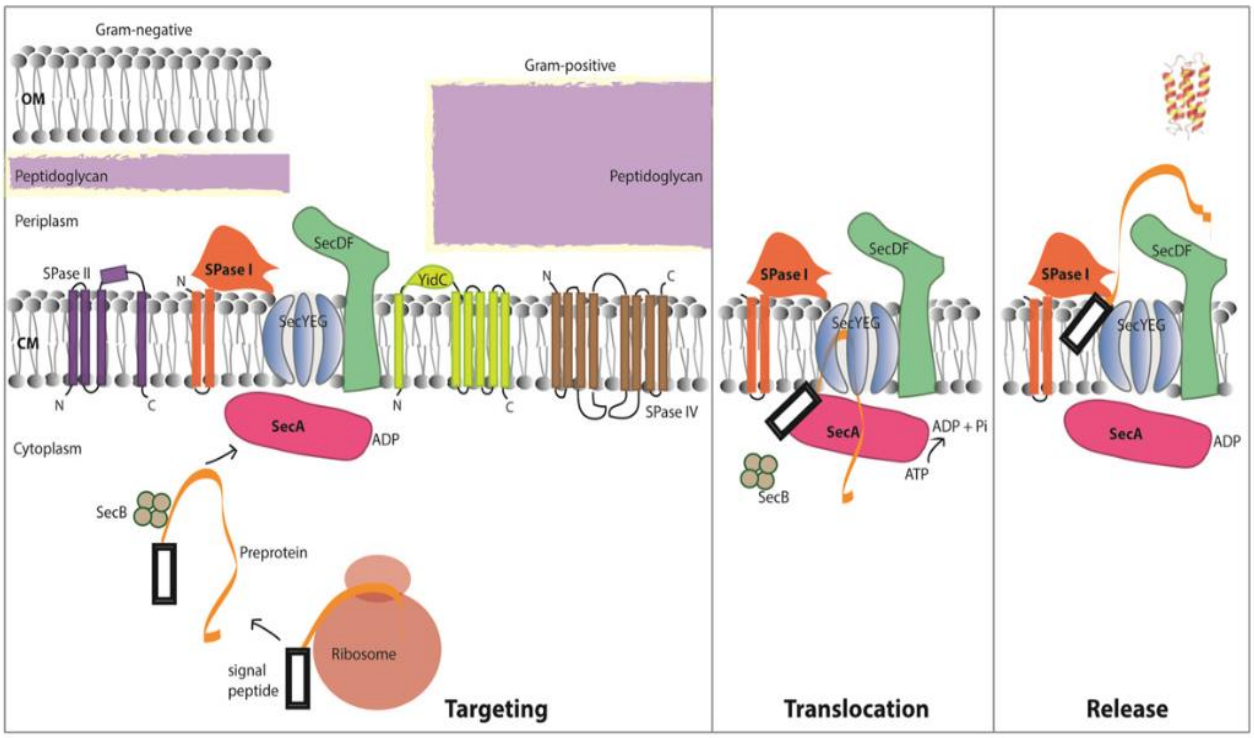

Figure 2. The Sec-pathway in *E.coli*
(Adapted from Dalbey *et al*. 2012 and Rao *et al*. 2014)

Legend:
"Preproteins synthesized at the ribosomes are targeted to the membrane translocase, assisted by the cytosolic chaperone, SecB. The Sec-translocase consists of the SecYEG protein conducting channel, accessory proteins SecDF and the peripherally associated motor protein, SecA. SecA translocates the preprotein through the channel utilizing the energy from ATP hydrolysis. SPaseI, a serine protease, cleaves non-lipoprotein substrates at the extracytoplasmic side, releasing the mature protein. SPaseII is an aspartic acid protease which cleaves lipoprotein substrates beneath the extracytoplasmic membrane surface. SPaseIV, also an aspartic acid protease, cleaves prepilins and pseudopilins at the cytoplasmic side of the membrane. YidC, a membrane protein insertase, together with the SecYEG channel, inserts membrane proteins via the SRP pathway (not shown). The locations of theN- and C-termini of the membrane enzymes are indicated. The transmembrane helices are depicted as barrels."

Proteins containing a signal peptide (a specific hydrophobic sequence at the N-terminal), and /or a transmembrane domain are membrane-targeted proteins or destined to interact with host cells (effectors) and therefore are important virulence factors. The most abundant proteins of the

phytoplasma cell membrane are the immunodominant membrane proteins (IDPs), which may have important roles in the pathogenicity of a given phytoplasma (Barbara *et al*. 2002, Kakizawa *et al*. 2006a). Genes encoding IDPs are present in several phytoplasmas and are classified in three different types: immunodominant membrane protein (IMP), immunodominant membrane protein A (IDP A), and antigenic membrane protein (AMP) according to their type of insertion in the phytoplasma membrane (Barbara *et al*. 2002, Morton *et al*. 2003, Kakizawa *et al*. 2004). However, types of IDPs are non-homologous (i.e. have no amino acid similarity, as well as have different predicted structure and position in the genome); phylogenetically related phytoplasmas possess the same type of IDP, which most likely have the same role (Figure 3) (Kakizawa *et al*. 2006b).

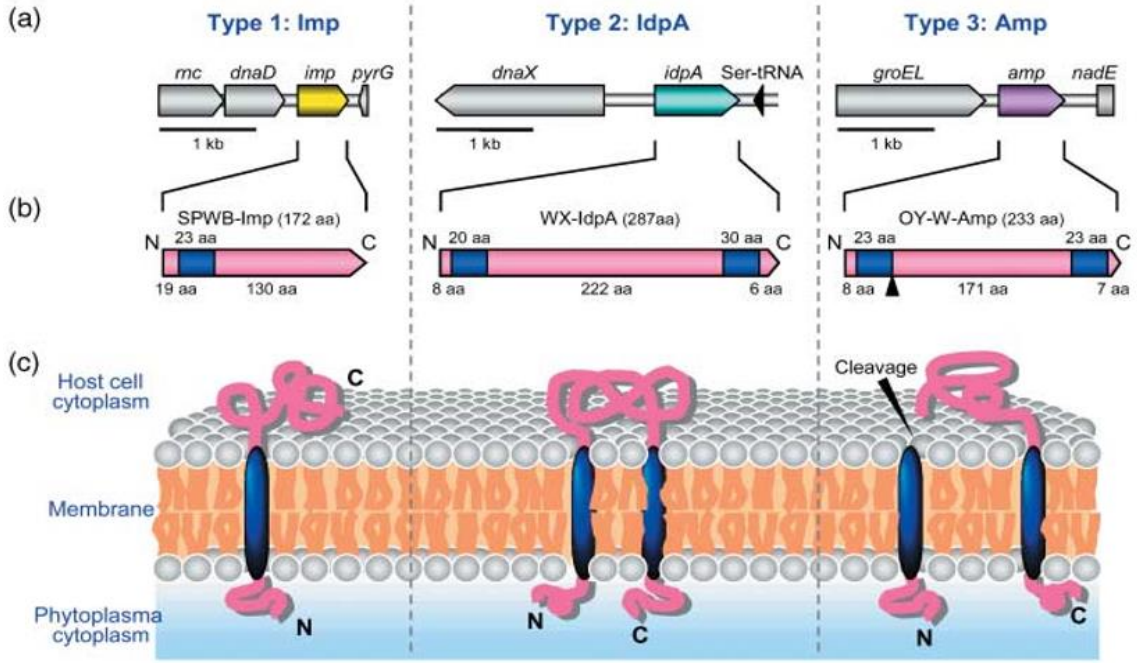

Figure 3. Immunodominant membrane proteins (IDPs) of phytoplasmas (Adapted from Hogenhout *et al*. 2008)

Legend:
"(a) gene organizations around the genes encoding three IDPs (SPWB (U15224), WX (AF533231) and OY-W (AB124806).
(b) schematic representation of the putative translocation products of three IDPs.
(c) schematic graphic of the hypothetical transmembrane structures of three types of IDP.
Blue: transmembrane regions, pink: non-transmembrane regions, black triangle: cleavage site, the N-terminal transmembrane region of AMP (type 3) is cleaved during protein localization (Kakizawa *et al*. 2004), C: C terminus, N: N terminus, aa: amino acids."

Gene encoding Stolbur antigenic membrane protein (*stamp*) has been identified recently, which is an ortholog of *amp* of '*Ca*. P. asteris' (Suzuki *et al*. 2006, Fabre *et al*. 2011a). The STAMP is a 16 kDa antigen and likely an abundant antigenic surface protein of '*Ca*. P. solani' (Fabre *et al*. 2011a). The *idp* genes showed higher variability than 16S rDNA, suggesting that many *idp*(s) have been subjected to strong selection pressure. It was anticipated that their function in the adaptation to changing environment such as new insect vectors played a role in their diversification (Kakizawa

et al. 2006a, Fabre *et al*. 2011a). To overcome the immune system of the vector and to adhere to the host protein IDPs are decisive elements in establishing bacterial infection, which requires co-evolution with the given host or positive changes to adapt to a new host (reviewed in Kakizawa *et al*. 2006a).

5.5. Phytoplasma interaction with plant and insect hosts

Phytoplasma reside in plant. Phytoplasmas indeed establish interaction with plant cells. It was demonstrated that in '*Ca*. P. solani' infected tomato plants, phytoplasmas attach to plasma membrane of sieve elements which result in an adjacent association between phytoplasma cells and sieve-element reticulum (SER) and sieve elements (SE) cytoskeleton (Buxa *et al*. 2015). Musetti *et al*. (2016) speculated the phytoplasma accommodation in the phloem SE based on observations of TEM micrographs of the vein section of phytoplasma infected tomato: after the phytoplasmas get into the plant phloem (via feeding by the vector), they are floating in the SE lumen. The sieve-element containing free-floating phytoplasma were exhibiting unipolar plant actin, which most likely plays a role in phytoplasma adhering and anchoring to the plasma membrane of sieve elements. In this process phytoplasma transmembrane proteins are also involved (Kube *et al*. 2012, Oshima *et al*. 2004, Neriya *et al*. 2014). Many intracellular pathogens evolved a nearly identical way for the intracellular movements inside the host, utilizing host-cell actin cytoskeleton. The binding to host actin is dependent on unipolar polymerization of the actin. *In situ* demonstration of direct contact between phytoplasma and actin revealed that actin is localizing at on one side of phytoplasma cells. After phytoplasmas are anchored to the parietal position of the endoplasmic reticulum (ER) by minute clamps, they establish a relationship with SER, which makes it possible to withdraw proteins and metabolites from the host. Interestingly, actin labelling could not be observed on the surface of phytoplasmas when it was adhered to the SER (Buxa *et al*. 2015, Musetti *et al*. 2016).

Effectors. As phytoplasmas are endocellular parasites, they secrete proteins directly into the host cells. The secreted proteins and also transmembrane proteins are in direct contact with the plant or insect host cells (Kakizawa *et al*. 2006a). A study of the '*Ca*. P. asteris' AY-WB strain revealed 76 proteins with a signal peptide (SP), of which 20 have a transmembrane domain (reviewed in Hogenhout *et al*. 2008). From those 76 proteins, 56 had only a signal peptide, implying that these are destined for an extracellular environment: moving first to phloem, then to the companion cells and in some case to the nucleus of the mesophyll cells (Sugio *et al*. 2011b). The size of the effector proteins (most of them are < 40 kDa) and the size exclusion of plasmodesmata (10-67 kDa) depending on the plant tissue, are not contradictory (reviewed Sugio and Hogenhout 2012). Among those effectors certain proteins were identified, such as SBPs (solute-binding proteins) and

SAPs (secreted AY-WB proteins) which are involved in bacterial virulence (reviewed Sugio and Hogenhout 2012). '*Ca.* P. asteris' effectors such as SAP11, SAP54 and TENGU manipulate plant cells to facilitate their multiplication in the host (Sugio and Hogenhout 2012). TENGU was the first phytoplasma secreted effector to be discovered as causing leaf-branching and dwarfism when expressed in transgenic *Nicotiana benthamiana* and *Arabidopsis thaliana* plants (Hoshi *et al*. 2009). Although the localization of phytoplasma was restricted to the phloem, TENGU protein was detected in apical buds by immunohistochemical analysis, suggesting that TENGU was transported from the phloem to other cells. Microarray analyses showed that auxin-responsive genes were significantly down-regulated in the tengu-transgenic plants. Also it was shown that TENGU was not addressed to the nucleus when expressed in fusion to GFP in onion epidermal cells. However it is now known that TENGU is processed in planta into a 13 amino acid peptide that could have a different subcellular localization (Hoshi *et al*. 2009, Sugawara *et al*. 2013). TENGU also causes plant sterility by downregulating of the jasmonic acid and auxin pathways (Minato *et al*. 2014). SAP11 that contains nuclear localisation domain targets the nuclei of the plant cell. It causes leaf crinkle and stem proliferation symptoms, has an impact on flower development and decreases jasmonic acid (JA) synthesis (Sugio *et al*. 2011). JA is a phytohormone regulating cell maturation and senescence and plays a role in plant defence against herbivores including leafhoppers and planthoppers. Downregulation of JA in the plant led to the increased colonisation ability of *Macrosteles quadrilineatus*. This leafhopper, vectoring '*Ca*. P. asteris', had 60 % higher progeny on AY-WB-affected *Arabidopsis thaliana* than on healthy ones (Sugio and Hogenhout 2012). SAP54 and its ortholog PHYLLOGEN are targeting MADS box homeotic transcription factor family in the infected plant, trigger its degradation resulting in impaired flower development such as green pigmentation of flowers (virescence) and the abnormal development of floral parts into leaf structures (phyllody) (MacLean *et al*. 2011, Maejima *et al*. 2014, Maejima *et al*. 2015). In summary, phytoplasma effectors induce: (i) proliferation in the plant to generate more stems and consequently more vascular tissue including phloem, that results in habitat expanding for phytoplasmas to replicate; (ii) virescence and phyllody to increase young vegetative tissue that increases the attractiveness of the infected plant to vectors; virescence also delays flowering and senescence of annual plants, therefore extending the lifespan of infected plants and rendering better vector colonisation; (iii) decreased JA synthesis provides advances for the insect vector, i.e. increased fecundity. All these modifications in plant or insect host caused by effectors increase phytoplasma fitness and their dispersal in nature (Sugio *et al*. 2011a).

Competent insect vector. Phytoplasmas are transmitted by phloem sap-feeding insects of families *Cixiidae* (planthoppers), *Cicadellidae* (leafhoppers) or *Psyllidae* (psyllids) in a persistent propagative manner (Weintraub and Beanland 2006). Phytoplasmas are able to live and multiply

in the body of phloem-feeding insect vectors. Little is known about the mechanisms of insects-phytoplasma interactions which are driving their ecological diversification. Phytoplasmas can be transmitted by a particular insect species and not by others. This is due to the highly specific interaction between the phytoplasma and its vector (Suzuki *et al*. 2006, Galetto *et al*. 2011). Once phytoplasmas have been acquired from the phloem via feeding, the sap enters the intestinal lumen of the insect. In order to be transmitted to another plant, phytoplasmas have to multiply in the vector and reach the salivary glands (Maillet and Gouranton 1971). This requires the ability to overcome barriers such as intestine and salivary glands which are anatomically very different. Only high titers in the salivary glands make successful transmission possible. After acquisition feeding, colonisation takes 7 to 20 days, or even more, depending on the phytoplasma strain, insect species and further environmental factors such as temperature. As soon as the insect has been colonised by the phytoplasma, it becomes a competent vector and will be able to infect new plants by inoculation feeding. Once colonised by phytoplasma, the insect remains infectious for its whole life. After inoculation feeding, phytoplasma systemically colonize the plant and symptoms appear on it. Phytoplasma has various effects on fitness, survival, fecundity or feeding preference of the insect vector, which can be positive, negative or neutral (reviewed in Hogenhout *et al*. 2008). For example, the Flavescence dorée phytoplasma reduce fecundity and longevity of its leafhopper vector *Scaphoideus titanus* (Bressan *et al*. 2005). Positive interaction between phytoplasma and a competent vector can form with longer evolutionary time; longer co-existence renders more benefit for the insect vector (Nault 1990).

Importance of phytoplasma membrane proteins. Certain studies reported interaction between actin of host (plant and insect) and phytoplasma. Phytoplasma surface membrane proteins play an important role in this interaction. It was demonstrated that plant actin is capable to interact *in vivo* and *in vitro* with immunodominant membrane protein (IMP) (Boonrod *et al*. 2012). Furthermore the capability of AMP of '*Ca.* P. asteris' strain OY to bind with leafhopper actin is in correlation with the phytoplasma-transmission capability of leafhoppers (Suzuki *et al*. 2006). In this study AMP co-localized in the intestinal muscle cells (containing actin and myosin light chain) of '*Ca*. P. asteris' vector. Whereas AMP co-localisation with actin was detected in vector species, it was not the case in non-vectors, this is supported by the finding of Galetto *et al*. 2011. An ortholog of *amp* named Stolbur antigenic membrane protein (*stamp*) has been identified recently. The *stamp* gene -similarly to *amp*- is submitted to positive diversifying selection in '*Ca*. P. solani' (Kakizawa *et al*. 2006a, Fabre *et al*. 2011a). Genetic diversity of the *stamp* gene in the Euro-Mediterranean basin is high (Fabre *et al*. 2011b, Foissac *et al*. 2013). Some genetically different '*Ca*. P. solani' strains shown to be associated with specific insect vector ecotypes living on different wild plant reservoirs, suggests the specialization of phytoplasma strains to different epidemiological cycles

(Johannesen *et al*. 2012). Whereas no physical interaction was demonstrated for VMP1, the variable membrane protein 1 of '*Ca.* P. solani' which is also submitted to diversifying selection pressure (Cimerman *et al*. 2006).

5.6. Epidemiology of Grapevine Yellow diseases

The grapevine is an important cultivated entity with 7554 mha total world area (4060 mha in Europe) under vines (OIV 2015), thus vitiviniculture represents a great value in the global economy. Grapevine production faces certain challenges such as Grapevine Yellow (GY) diseases. These diseases are caused by different '*Candidatus* Phytoplasma' species (Table 2). Among them are two phytoplasmoses which have significance in Europe: the quarantine Flavescence dorée (FD) and the endemic Bois noir (BN) (Figure 4) (Foissac and Maixner 2013). Although, FD and BN cause identical symptoms on grapevines, the causal agents, the insect vectors, and the biological cycle of the vectors and pathogens differ greatly (Figure 5, 6). FD and BN can only be differentiated by means of molecular methods. Disease control of FD and BN is also different.

Table 2. Grapevine Yellow diseases worldwide

Disease	*Pathogen*	*16Sr group*	*Distribution*	*Proved insect vector*
Aster Yellows	'*Ca*. Phytoplasma asteris'	16SrI-B	North America South Africa, Europe (rare)	*Mgenia fuscovaria* *Macrosteles ssp.*
North American Grapevine Yellows	'*Ca.* Phytoplasma asteris' '*Ca.* Phytoplasma pruni'	16SrI 16SrIII	North America Canada	*Agallia consticta* *Macrosteles spp.* *Scaphoideus titanus*
Stolbur/Bois Noir	'*Ca*. Phytoplasma solani'	16SrXII-A	Europe Asia	*Hyalesthes obsoletus* *Reptalus panzeri*
Australian Grapevine Yellows	'*Ca*. Phytoplasma australiense'	16SrXII-B	Australia New Zealand	*Oliarius atkinsoni*
Flavescence dorée	('*Ca*. Phytoplasma vitis')	16SrV-C,D	Europe	*Scaphoideus titanus*
Palatinate Grapevine Yellows	('*Ca*. Phytoplasma vitis')	16SrV-C	Europe (Germany)	*Oncopsis alni*

Legend: bracket indicates that species name has not been officially approved.

Management of FD is based on the control of ampelophagous vectors and elimination of infection sources from the vineyard, whereas BN control is more difficult, due to the non-ampelophagous vector and more abundant infection sources of the disease. Pathogen-free propagation material is essential in order to avoid the spreading of GYs over long distances.

One of the major GY in Europe is Bois noir caused by '*Candidatus* Phytoplasma solani' ('*Ca*. P. solani') (Figure 4) (Foissac and Maixner 2013, Quaglino *et al*. 2013). This disease has also been reported in Canada, Chile, and Turkey, and is a potential threat to table grape and wine-producing regions of other countries (Rott *et al*. 2007, Gajardo *et al*. 2009, Ertunc *et al*. 2015). '*Ca.* P. solani' is endemic to Europe, Asia Minor and the Mediterranea, where it infects several crops, including grapevine, vegetables and natural vegetation (Lee *et al*. 2000). It was previously known as stolbur phytoplasma; the causal agent of sterility of *Solanaceous* plants and its main plant hosts are weeds

such as the bindweed (*Convolvulus arvensis)* and the stinging nettle (*Urtica dioica)* (Maixner 2011). The economic importance of a pathogen (e.g. '*Ca*. P. solani') causing monocyclic epidemics is strongly correlated with vector dispersal and infectivity of a population, as well as distribution of host plants (Foissac and Wilson 2010). Therefore, BN control is based on prophylactic measures reservoir weed control, and the use of pathogen-free propagation material (Maixner and Mori 2013, Mori *et al*. 2014). Although BN is considered less damaging than the epidemic Flavescence dorée (FD), the only GY classified as quarantine pathogens in the world, its disease cycle is more complex because of the biology of its polyphagous vectors *Hyalesthes obsoletus* Signoret and *Reptalus panzeri* Löw (Mori *et al*. 2008, Foissac and Maixner 2013).

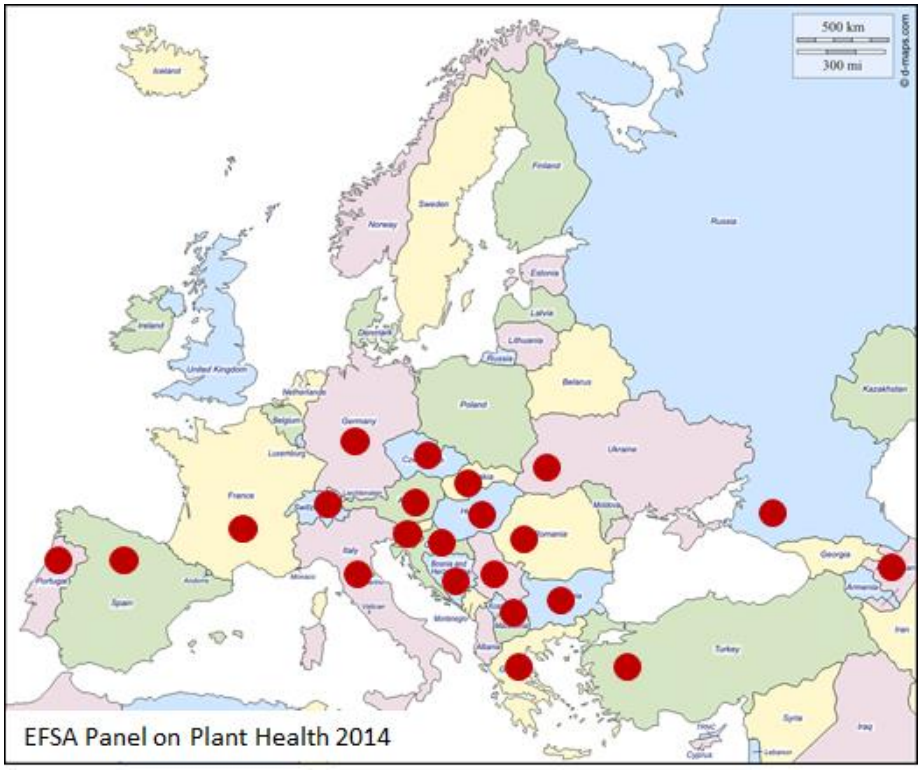

Figure 4. Bois noir disease in Europe

The masking effect of BN over FD deserves attention as it has implication in FD disease management. Indeed, BN and FD, as any other GY, induce identical symptoms, and BN cases result in masking early FD outbreaks. In southeast France, after the year 2003, which corresponded to a peak in BN incidence in southern France, Alsace and the neighbouring German states, the uprooting of BN affected grapevine plants was made compulsory on the French side and removal of nettles and bindweed is strongly recommended. Since then, BN incidence has consistently decreased in France (Foissac personal communication, Maixner 2011, Kuntzmann *et al*. 2014). Recent detection of FD in Hungarian vineyards (Kriston *et al*. 2013), widespread presence and disregards of BN cases in Hungarian vineyards may create a similar situation in Hungary. Despite difficulties in BN management, certain knowledge available on the biology of the main BN vector,

which has enabled the development of alternative control strategies i.e. indirect control of *H. obsoletus* and habitat management (Maixner 2007, Forte *et al*. 2010, Mori *et al*. 2014).

There are ranges of wild and cultivated plants, which are reservoirs of '*Ca*. P. solnai' (stolbur phytoplasma). These are *Solanaceous* crops, *Solanum nigrum, Datura stramonium, Asteraceae* (carrot, celery, parsley, wild chicory and chervil), grapevine, strawberry, lavender, maize, sugar beet and *Prunus* spp. (peach, plum, cherry and almond) (reviewed in EFSA Panel on Plant Health 2014). These cultivated plants, except lavender, are known as dead-end host for this pathogen, as its planthopper vectors do not develop on them. Epidemiological status of certain weed hosts (such as *Ranunculus, Taraxacum*, or *Cirsium* spp.) is very similair to those of corp hosts. Bindweeds (*C. arvensis* and *C. sepium*) and stinging nettle (*U. dioica*) are important weeds in phytoplasma lifecycle, as plant hosts of both '*Ca*. P. solani' and its vector (Maixner 1994, Sforza *et al*. 1998, Langer and Maixner 2004, Bressan *et al*. 2007). It was recently demonstrated that further wild plants i.e. *Salvia sclarea, Crepis foetida* and *Vitex agnus-castus* are also act as host plant of *H. obsoletus* and is a pathogen source of '*Ca*. P. solani' (Kosovac *et al*. 2013, 2016; Chuche *et al*. 2013). Although the main hosts of *H. obsoletus* are bindweed and stinging nettle, both of which are present inside and outside of vineyards. Insecticide treatment of these weeds (using systemic insecticide which accumulates in the plant and is acquired by the insect during feeding) may lead to a decrease of vector density and decrease infection pressure on grapevines (Maixner 2007, Mori *et al*. 2014). Eradication of bindweed, which commonly grows underneath the grapevine, is very difficult and may raises problem. Mechanical or chemical clearing can significantly increase the '*Ca*. P. solani' infections of grapevine, as planthoppers from bindweeds can shift to the vine. Thus, timing of weed control and the insecticide treatments are important, it should be executed at least four weeks before the flight period of cixiids (Maixner 2007). Green cover between rows has the effect to displace bindweed. Use of ground covering rosette plants (e.g. *Hieracium pilosella*) is advisable (Maixner 2007). The *H. obsoletus* prefers to migrate to open soils, green cover is less attractive for them. Additionally, green cover reduces the density of the vector's host plants (Maixner 2007). Control measures are important to decrease infection pressure and reduce economic damage in a BN-affected vineyard. However, economic damage also depends on the biological properties of the given phytoplasma strain.

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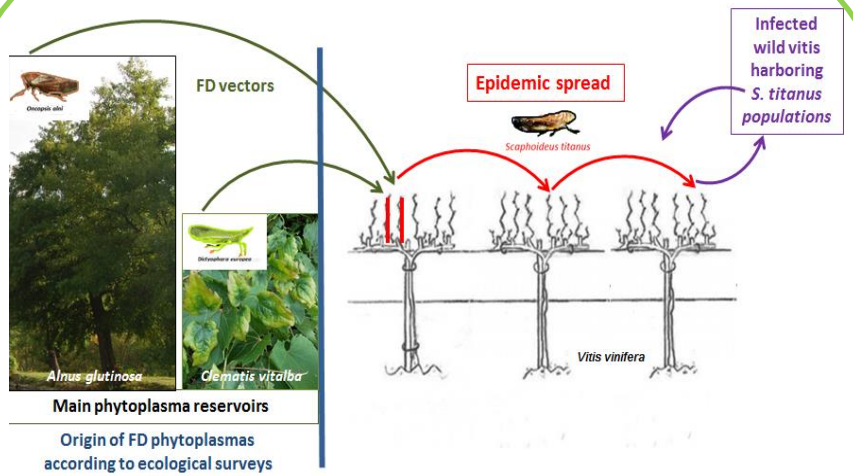

Figure 5. Epidemiology of Flavescence dorée disease

Originally FD phytoplasmas (taxonomic groups 16SrV-C and D) have been transmitted from wild reservoirs (*Alnus glutinosa* and *Clematis vitalba*) to grapevine by different leafhoppers (from alder: *Oncopsis alni* or *Allygus* species and from clematis: *Dictyophara europea*).

In the vineyard the American origin *Scaphoideus titanus* is transmitting the disease. However, wild reservoirs can have importance on the long-term, an FD outbreak can effectively emerge only in the presence of *S. titanus*. Since *S. titanus* is ampelophagous it completes its biological cycle on grapevine: lays eggs on vine canes, where the eggs overwinter; after hatching larvae appear on lower leaves of the vine in May, and L2 are able to acquire phytoplasma. First adults are visible in July. Effective pesticide control can be performed in May and June against larvae.

Source: Maixner *et al*. 1999,; Filippin *et al*. 2009, Schvester *et al*. 1961.

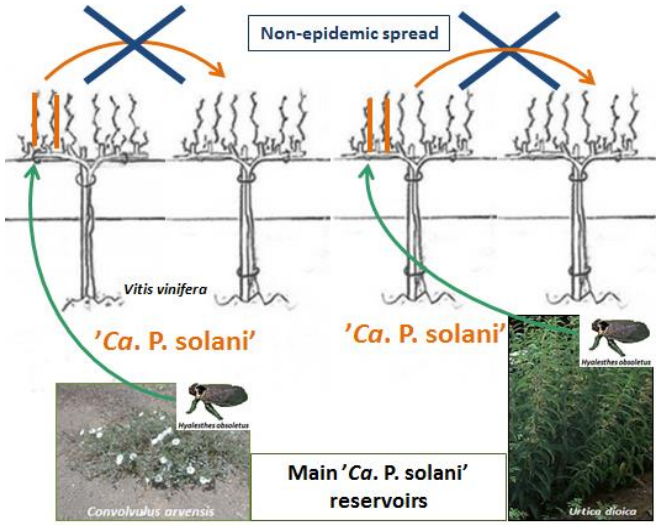

Figure 6. Epidemiology of Bois noir disease

During non-epidemic spread (i.e. crops like *Solanaceous* plant and grapevine which are a dead-end host for the pathogen), '*Ca.* P. solani' is transmitted mainly from bindweed (*C. arvensis*) and stinging nettle (*U. dioica*) to grapevine by different phloem-feeding planthoppers cixiids. However, further weeds are reservoir plants of '*Ca.* P. solani' (i.e. *Ranunculus*, *Cirsium* and *Taraxacum* spp. etc.), the bindweed and stinging nettle are the main hosts of the '*Ca.* P. solani' vector *Hyalesthes obsoletus*. *H. obsoletus* lay eggs on roots of bindweed and stinging nettle. However further wild plants i.e. *S. sclarea*, *C. foetida* and *V. agnus-castus* are also the host of this planthopper, thus may play an important role in '*Ca*. P. solani' biology. After hatching, larvae feed, as well as overwinter on roots. Adults appear in June. As the proven '*Ca.* P. solani' vectors (*H. obsoletus* and *Reptalus panzeri*) are non-ampelophagous, they associate and feed on grapevine just randomly. Therefore spraying of the vine canopy, which is effective against FD vector, is not effective to control '*Ca.* P. solani' vectors.

Source: Maixner 1994, Sforza *et al*. 1998, Cvrkovic *et al*. 2013, Kosovac *et al*. 2013, 2016; Chuche *et al*. 2013.

5.7. Bois noir disease in Hungary

Stolbur, the disease of *Solanaceous* crops caused by '*Ca*. P. solani' and the vector *Hyalesthes obsoletus,* were reported in Hungary in the middle of the twentieth century (Szirmai 1956, Sáringer 1961). Certain scientist was working on Stolbur disease on different hosts, as well as on the biology and population dymanics of the vector *Hyalesthes obsoletus* (Petróczy 1958, 1962, 1965; Kuroli 1969, 1970; Gáborjányi és Lönhárd 1967; Horváth 1970). Stolbur infections on certain crops (tomato, parsley, celery, carrot, celery and sugarbeet), and weeds (*Datura stramonium, Silene otites, Taraxacum officinale*) were detetected in late '90 (Viczián *et al*. 1998a, 1998b).

Symptoms of Grapevine Yellow diseases were found on 'Aligoté' and 'Rhine Riesling' cultivars in the Tokaj wine region by János Lehoczky in 1970. This case was the first report of GY in Hungary; however the identification of the pathogen was not possible at that time. The few symptomatic vines were eradicated and phytoplasma infection was not reported from the Tokaj wine region for a long time. In 1994, GY symptoms on certain cultivars were discovered in Tolna and Heves counties (Szendrey *et al*. 1997). Molecular identification confirmed the presence of '*Ca*. P. solani' (at that time 16SrXII-A, stolbur phytoplasma) (Kölber *et al*. 1997, 1998). Between 1997 and 2002, a nation-wide survey of GYs, coordinated by the Hungarian Plant Protection Service, revealed the presence of Bois noir disease on 21 cultivars in 11 counties of Hungary. Molecular analysis confirmed '*Ca.* P. solani' (16SrXII-A) infection in white cultivars: 'Aligoté', 'Chardonnay', 'Chasselas', 'Ezerfürtű', 'Kerner', 'Muscat lunel', 'Olaszrizling', 'Pinot blanc', 'Pintes', 'Rhine Riesling', 'Semillon', 'Pinot Gris' and 'Zöld veltelini'; as well as in red cultivars: 'Alicante bouchet', 'Blauburger', 'Cabernet franc', 'Kékfrankos', 'Merlot', 'Pinot noir', 'Vranac' and 'Zweigelt' (Kölber *et al*. 2003). Cultivars 'Chardonnay', 'Pinot gris' and 'Zweigelt' cultivars exhibited the most severe symptoms. From 1998, a multi-year survey of vector and potential vector species of GYs was conducted, which resulted in a report of 70 species, including *H. obsoletus* and *Reptalus panzer*i, belonging to the *Auchenorrhyncha* suborder (Orosz *et al*. 1996, Elekes *et al*. 2006). The '*Ca.* P. solani' infection rate of *H. obsoletus* and *R. panzeri* species was determined which were 18 % and 9.2 %, respectively (Palermo *et al*. 2004).

In parallel with these studies on BN, a survey of FD disease and monitoring of its insect vector was carried out by the National Plant Protection Service (Figure 7). As a result, in 2006, the FD vector *Scaphoideus titanus* was found in Bács-Kiskun, Somogy and Zala counties (Dér *et al*. 2007). The highest populations were recorded in abandoned vineyards near the Serbian border. Since then, continuous monitoring has revealed an almost country-wide distribution of *S. titanus* (Kriston *et al*. 2013).

In order to evaluate the risk represented by the wild reservoir as a source of Flavescence dorée outbreaks in Hungary, diverse wild perennial plants growing in vineyard areas were tested for the presence of 16SrV-C and D subgroup phytoplasmas. The 16SrV phytoplasmas were detected in alders (86 % infected) and in clematis (71 % infected). Further characterisation by sequencing of the *map* gene revealed that isolates of both plants had the same *map* sequence as Flavescence dorée epidemic isolates (Ember *et al*. 2011b). Until 2013, pathogens of FD on grapevine had not been found. The first report of quarantine Flavescence dorée disease on grapevine in Hungary was released in 2013. Phytoplasma belonging to 16SrV-C subgroup were found in south Hungary, close to the Croatian and Serbian borders (Kriston *et al*. 2013). Since then, the FD pathogen was found sporadically in other parts of the country (Szőnyegi *et al*. 2015). Publication about the molecular characterisation of Hungarian '*Ca*. P solani and '*Ca*. P. vitis' isolates is currently being written.

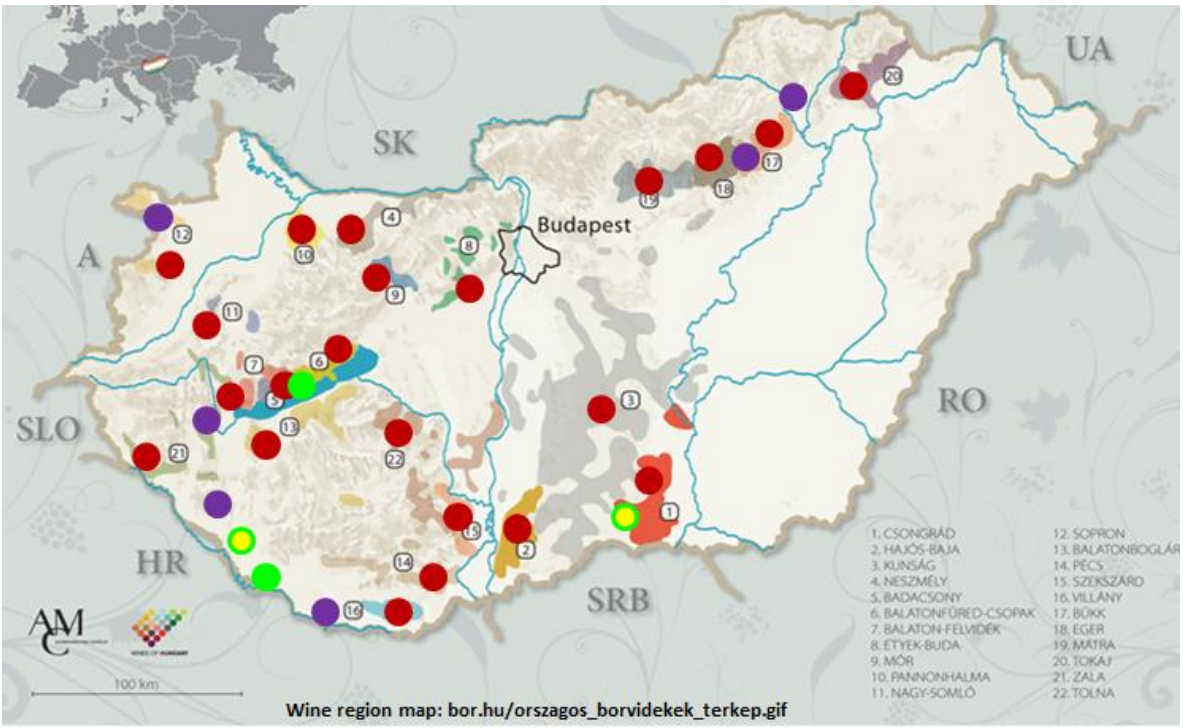

Figure 7. History of GYs in Hungary

5.8. Diversity of '*Candidatus* Phytoplasma solani' strains

Phytoplasma disease control is based on prophylaxis, thus it is crucial to trace the spread of phytoplasma strains and predict their epidemic potential when introduced in a given ecological niche. '*Candidatus* Phytoplasma solani' is endemic to the Euro-Mediterranean area and is of wild plant origin. It is transmitted from bindweed and stinging nettle to grapevine and to other crops by

different planthoppers (*Fulguromorpha)* of the *Cixiidae* family (Figure 6). In Europe, at least 4 cixids are vectoring '*Ca*. P. solani', namely *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, Chuche *et al*. 2016a). Among them, only *H. obsoletus* and *R. panzeri* are proven vectors of '*Ca*. P. solani' to grapevine. Ecotypes of *H. obsoletus* are vectoring specific '*Ca*. P. solani' genotypes and are related to plant hosts bindweed or stinging nettle (Langer and Maixner 2004, Aryan *et al*. 2014).

Multi locus sequence typing (MLST) is used to investigate epidemiological properties and population genetics of prokaryotes (Maiden *et al*. 1998, Urwin and Maiden 2003). It is a useful tool to investigate phytoplasma genetic variability and to trace the route of introduction of phytoplasma strains into a vineyard. Use of various genetic markers facilitates deciphering epidemiological properties such as vector specificity and infection source. MLST is based on sequence analyses of different genetic loci. Robust and precise characterization of bacterial strains requires mainly housekeeping genes under neutrality. In order to increase the predictive value regarding epidemic properties, markers linked to interaction with insect vectors can also be sequenced. For standard MLST analyses 5-10 loci are used (Scally *et al*. 2005, Yuan *et al*. 2010). Different markers can be used based on their variability or sensitivity to biological traits. Neutral markers like housekeeping genes (e.g. 16S RNA, *secY*) are not decently selective thus provide only partial insight into genetic diversity or local adaptation of a given phytoplasma strain. Highly variable functional markers are sensitive to biological traits and encode proteins involved in adaption to a vector, i.e. *vmp1* encoding phytoplasma variable membrane protein and *stamp* encoding a surface antigenic membrane protein.

16S rRNA gene. This gene is a conserved, structural gene present in all bacteria, used for taxonomic classification of prokaryotes including phytoplasmas. Based on PCR-amplification followed by restriction fragment length polymorphism (RFLP) of the 16S rRNA gene and the 16S/23S rRNA region phytoplasmas were grouped into 19 groups and 50 subgroups (Lee *et al*. 1998). *In silico* RFLP of the ribosomal DNA sequence revealed 29 groups and 89 subgroups (Wei *et al*. 2007). Several studies revealed that phytoplasmas belonging to the same group/subgroup or '*Candidatus* Phytoplasma species' (e.g. '*Ca*. P. solani') are harbouring different biological and epidemiological properties i.e. plant host and insect vector. Due to this intraspecific diversity of '*Ca*. P. solani' it is important to know which strain is present in a given ecological niche. The 16S rRNA is widely used and accurate, however the high degree of similarity between closely related species/strains limits its usefulness for further differentiation. Therefore molecular markers with higher variability were required to better characterise phytoplasma strains (Lee *et al*. 2010).

***Tuf* gene**. *Tuf* encodes EF-Tu elongation factor, an essential gene in the bacterial genome and involved in peptide chain formation. This gene is often used for differentiation, as well as classification of phytoplasmas (Schneider *et al*. 1997). In the case of phytoplasma species, the resolution efficacy of *tuf* is nearly the same as 16S rRNA. However, in some cases *tuf* proved to be very useful in strain differentiation (Langer and Maixner 2004). Two distinct epidemiological cycles of BN, i.e. *tuf*-a associated with stinging nettle, and *tuf*-b associated with bindweed as the primary host plant and pathogen reservoir, were established based on PCR-RFLP of *tuf* gene in Germany (Figure 8) (Langer and Maixner 2004). A *tuf*-a genotype is predominantly present in Western Europe (France, Germany, Italy, Switzerland and Austria), while *tuf*-b is commonly found in Eastern and Central Europe (Croatia, Serbia, Czech Republic and Hungary) (Maixner 2011).

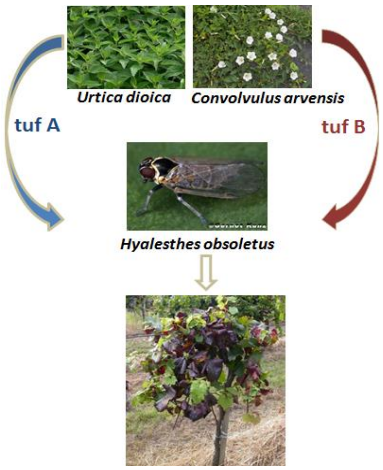

Figure 8. Different epidemiological cycles of '*Ca*. P. solani' *tuf* genotypes in Germany

H. obsoletus ecotypes vectoring specific '*Ca*. P. solani' genotypes: *tuf*-a and *tuf*-b constitute different epidemic cycles (Langer and Maixner 2004).

Differentiation of *tuf*-a and *tuf*-b genotypes was based on PCR/RFLP (Langer and Maixner 2004), which provides limited sequence information. Recent information obtained based on sequence analysis of *tuf* fragments revealed further genotypes which were renamed (Table 3). The nettle-related genotypes are *tuf*-a, *tuf*-b2 and *tuf*-b3; the bindweed related is *tuf*-b1 (Foissac personal communication, accordance of 4th Bois noir Workshop, Klosterneuburg, Austria).

Table 3. Nomenclature of tuf genotypes based on SNPs of FtufAY/RtufStol fragment

Name based on sequence**	Name based on RFLP*	Plant origin/type	SNPs			
			1st	2nd	3rd	4th
tuf-a	*tuf*-a	*U. dioica*	A	C	G	T
tuf-b1	*tuf*-b	*C. arvensis*	G	T	A	T
tuf-b2	*tuf*-b	*U. dioica*	A	T	A	T
tuf-b3	*tuf*-b	*U. dioica*	A	T	G	T
tuf-c	*tuf*-c	*C. sepium*	A	T	G	C

Legend: * Langer and Maixner 2004; ** 4th Europen Bois noir Workshop, 2016.

***SecY* gene**. This housekeeping gene is an essential element of the Sec protein secretory pathway of phytoplasmas encoding the protein translocase subunit. *SecY* is widely used for phytoplasma genotyping (Fialová *et al*. 2009, Cvrkovic *et al*. 2013, Aryan *et al*. 2014, Kosovac *et al*. 2015). Diversity of *secY* in Europe is shown in Figure 9.

***Vmp1* gene**. This gene encodes variable membrane proteins which present in several phytoplasma genomes and shows high variability (Cimerman *et al*. 2009, Pacifico *et al*. 2009, Murolo *et al*. 2010). A PCR amplification of *vmp1* of several '*Ca*. P. solani' strains' revealed different fragment sizes (S-small, M-medium and L-large), which was due to the deletion or insertion of 240-bp direct repeats (repeated domains) (Cimerman *et al*. 2009, Pacifico *et al*. 2009).

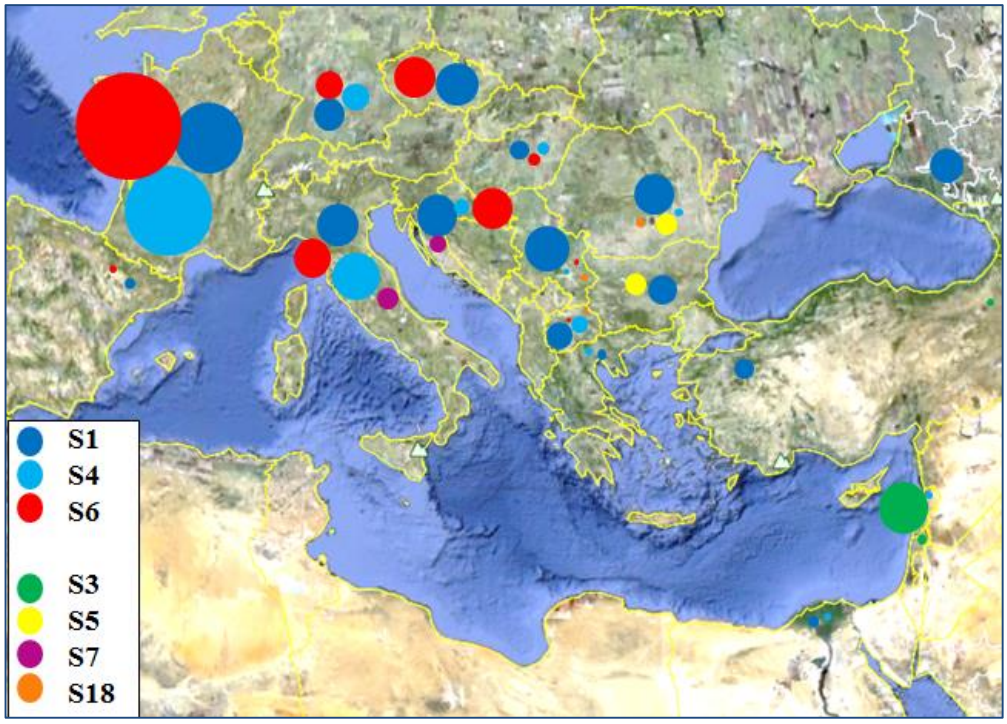

Figure 9. Diversity of *secY* gene in Europe (Adapted from Foissac *et al*. 2013)

*Legend: p*lants and insects were surveyed in the frame of the Stolbur-Euromed Consortium (2004-2012); S1-S18: *secY* genotypes.

***Stamp* gene**. The stolbur antigenic membrane protein (*stamp*) gene has been identified recently. This gene is submitted to positive selection which explains its high variability (Fabre *et al*. 2011a). Investigation of the *stamp* gene of '*Ca*. P. solani' strains in the Euro-Mediterranean basin showed evidence of four different clusters (Fabre *et al*. 2011b, Foissac *et al*. 2013). *Stamp* cluster I, II and III correspond to strains which propagate on bindweed as a wild reservoir, and cluster IV corresponds to strains present on stinging nettle (Figure 10) (Johannesen *et al*. 2012). Geographical distribution of *stamp* genotypes seems to correlate to the geographical distribution of different *Cixiidae* ecotypes (Figure 11) (Foissac *et al*. 2013).

A monoclonal antibody (2A10 MAb) against '*Ca.* P. solani' strain Tomato Stolbur (StolburC), isolated from field infected tomato plants in France, was produced in mouse spleen hybridoma

cells (Garnier *et al*. 1990). 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 2A10 recognizes STAMP of '*Ca*. P. solani' PO strain (Fabre *et al*. 2011a). Genotyping of strains StolburC and PO also revealed that they belong to *stamp* cluster I, one of the four clusters identified among strains collected in the Mediterranean Basin (Fabre *et al*. 2011a).

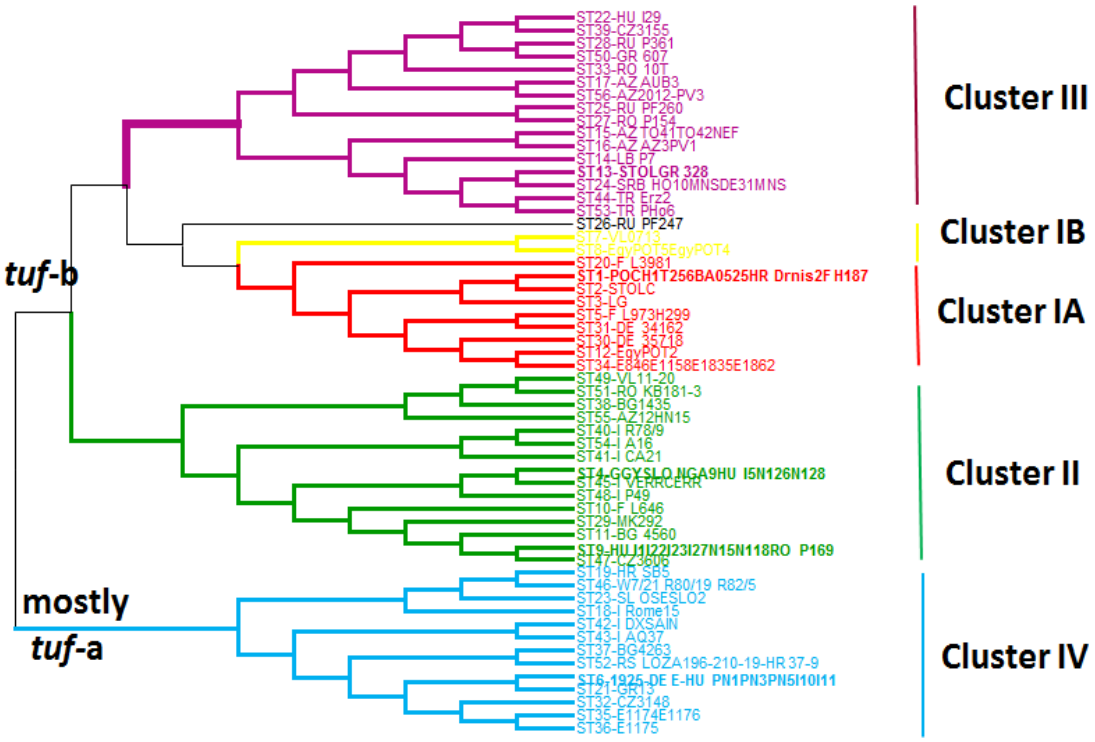

Figure 10. Diversity of *stamp*: four genetic clusters present in Europe (Adapted from Fabre *et al*. 2011a and Foissac *et al*. 2013)

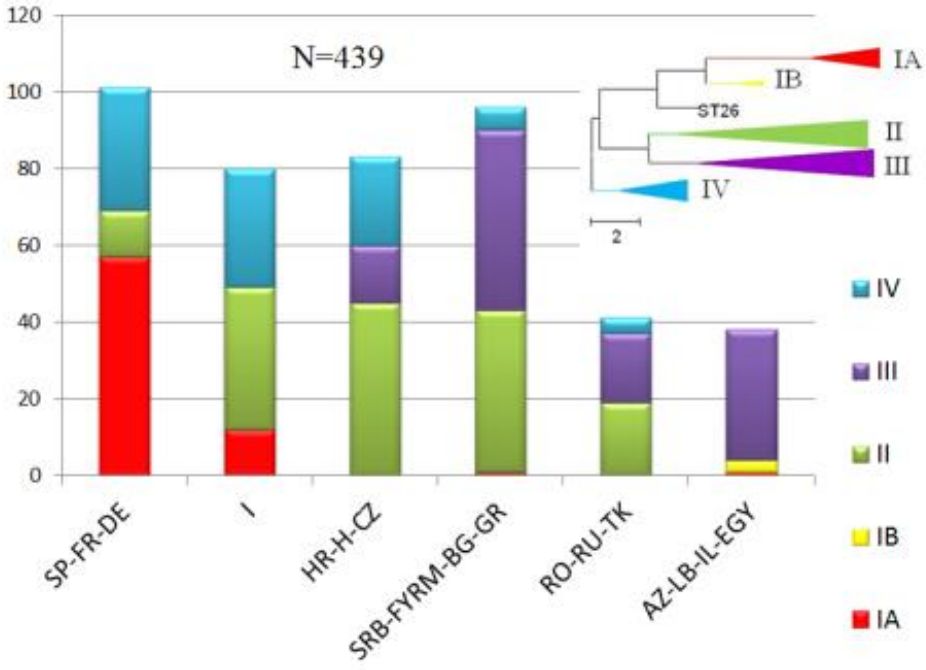

Figure 11. Geographical distribution of s*tamp* clusters in Europe (Adapted from Fabre *et al*. 2011a and Foissac *et al*. 2013)

5.9. Phytoplasma-caused changes in the plant/grapevine

The GY diseases cause severe symptoms on leaf, shoots and fruits: leaf rolling, leaf yellowing/reddening and necrosis, incomplete shoot lignification, immature and/or dried fruit. As phytoplasmas live and multiply in functional sieve elements, one of the main consequences of the infection is the disturbance of the phloem function (Lepka *et al*. 1999, Musetti *et al*. 2007, Santi *et al*. 2013). The energy demands of phytoplasmas regarding growth induce changes in the infected plants (Hren *et al*. 2009, Landi and Romanazzi 2011, Margaria *et al*. 2013). Photosynthesis and hormone metabolism are heavily affected in diseased model plants and grapevines. Changes in expression of genes involved in carbohydrate metabolism and glycolysis certainly impact the flow of assimilates to the grapes (Jagoueix-Eveillard *et al*. 2001, Pracros *et al*. 2006). In BN-affected grapevines, damaged phloem sieve elements have also been associated with callose aggregation (Figure 12). These effects, combined with other impaired physiological functions, reduce growth and affect plant development (Bertamini 2002a, Musetti *et al*. 2011, Santi *et al*. 2013). It had been shown that photosynthetic activity is impaired in BN-affected plants (Guthrie *et al*. 2001, Bertamini *et al*. 2002b). In plants, phytoplasma infection induces the production of defence proteins and phenolic compounds, as well as signal molecules, i.e. Ca^{2+}, H_2O_2 and salicylic acid (Musetti and Favali 2003, Musetti *et al*. 2005).

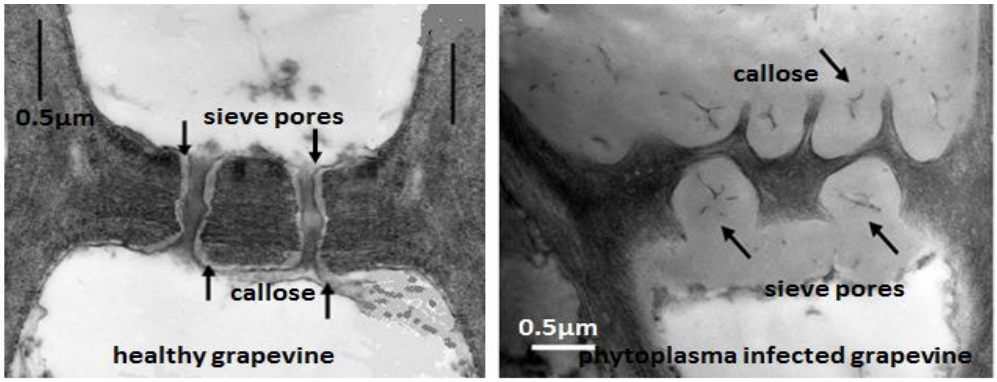

Figure 12. Callose deposition in the phloem of grapevine
(Adapted from Santi *et al*. 2013)

BN-diseased ‘Chardonnay’ and ‘Primitivo’ cultivars showed decreased transpiration (Endeshaw *et al*. 2012). Yield and fruit composition of grapevines depend on the seasonal photosynthetic capacity of the canopy (Hunter and Visser 1988). Bois noir affection leads to a decrease in bunch mass, berry mass, and number of bunches per vine resulting in a 70 % yield loss (Endeshaw *et al*. 2012, Ember *et al*. 2014, 2016). According to Panassiti *et al*. (2015) BN incidence is affected by environmental conditions and grapevine cultivars. Phytoplasmas require a living host to survive and virulence depends on temperature, plant age and genetic backgrounds of phytoplasma and plant host (Foissac and Wilson 2010, Sugio *et al*. 2011b, Jarausch *et al*. 2013). Phloem-limited

pathogens are known to affect grapevine performance and fruit quality (Boudon-Padieu 2003). Decreases in yield and fruit soluble solids caused by Grapevine leaf roll associated viruses are well described (Mannini *et al*. 2009, 2012b; Endeshaw *et al*. 2014). Interestingly, Fleck virus positively changed grape composition (i.e. colour intensity) of cv. 'Nebbiolo', but the yield drop was a remarkable 40 % (Mannini *et al*. 2012a). Premature berry dehydration that occurred in 'Merlot' cultivars was associated with phytoplasma infection, suggesting that the phytoplasma-caused partitioning between the nutrient source and berries resulted in inhibited sugar transport, poor synthesis of anthocyanins and restricted organic acid respiration (Matus *et al*. 2008).

5.10. Plant defence mechanism: recovery and remission phenomenon

The phytoplasma symptomatic grapevines can undergo a spontaneous remission (disappearing of phytoplasma symptoms), which may involve the elimination of the causal agent from the plant (Caudwell 1961, 1966). The recovery can be spontaneous or induced, as has been observed in the case of BN and FD-affected plants (Osler *et al*. 1993, Romanazzi *et al*. 2009). This mechanism can be promoted by exposing grapevines to abiotic stresses, such as transplanting (Osler *et al*. 1993) or partial uprooting and agronomical practices such as pruning or topping (Borgo and Angelini 2002). In the recovered vines, elevated reactive H_2O_2, jasmonic acid content and increased NAD(P)H peroxidase activity were measured, accompanied by a decreasing amount of salicylic acid, indicating systemic acquired resistance (SAR). Disappearance of symptoms can be temporary (i.e. remission) or permanent (i.e. recovery). Vines are considered to be recovered after a minimum of three consecutive years without symptoms (Maixner 2006). Induced resistance is non-specific for the disease. The resistance in plants can act against a wide range of pathogens and can be activated by several non-specific inducers, also known as elicitors (Romanazzi *et al*. 2009). However, it is important to mention that recovery appears to be induced by different factors (mentioned above), among them differences can be observed in cultivar-rootstock combinations and climatic conditions (Bellomo *et al*. 2007, Garau *et al*. 2007, Romanazzi *et al*. 2009). Mechanisms involved in recovery from phytoplasma disease were studied in different crops (apple, apricot and grapevine) (Musetti *et al*. 2005, 2007). Studies showed that the reaction in these plants appears to be induced by systemic acquired resistance (SAR). This suggests that different metabolic pathways might be involved in recovery and are identical to those involved in the mechanisms of defence responses in compatible and incompatible pathogen interactions (Landi and Romanazzi 2011).

Recently, an innovative treatment has been investigated to control Grapevine Yellows disease, applying resistance inducer by spraying the grapevine canopy during the growth season (Romanazzi *et al*. 2013). In the three-year experiment different commercial resistance inducers

were applied (Product/Active ingredient: Aliette/phsetyl-Al, Kendal/glutathione and oligosaccharines, Chito Plant/chitosan, Bion/benzothiadiazole, Olivis/glutathione and oligosaccharines). All treatments increased the number of recovered plants, wherein phytoplasmas were not detectable (Romanazzi *et al*. 2009, 2013). As part of this study, gene expressions related to the plant defence mechanisms and enzyme activities were examined in a highly sensitive ('Chardonnay') and moderately sensitive ('Sangiovese') cultivars (Landi and Romanazzi 2011). They analysed the relative expression level of several marker genes that covered a large set of defence proteins involved in grapevine-pathogen interactions (*pathogenesis related*: β-1,3-glucanase and class III chitinase; *secondary metabolism related*: phenylalanine ammonia-lyase, chalcone synthase and flavanone 3-hydroxylase; *oxidative stress related*: superoxide dismutase, catalase, class III peroxidase; *electron transport*: NADPH). In recovered plants of both cultivars, class III chitinase and almost always phenylalanine ammonia-lyase and chalcone synthase expression were increased in each collection period. In symptomatic leaves of both cultivars, the expressions of the same genes were up-regulated: those of β-1,3-glucanase and flavanone 3-hydroxylase. For the moderately susceptible 'Sangiovese' the defence genes were generally up-regulated in both symptomatic and asymptomatic leaves. This behaviour was not observed in the highly susceptible 'Chardonnay' (Landi and Romanazzi 2011). In summary, in the three-year experiment, the greatest degree of curative effect was found with use of the benzothiadiazole and the two glutathione-oligosaccharine formulations, curative level of 25 % was reached, and no phytotoxic effects of the products were observed (Romanazzi *et al*. 2013).

5.11. Symptoms of Grapevine Yellows and disease susceptibility of *V. vinifera* cultivars

As all GY, BN corresponds to a complex of symptoms which include leaf rolling, leaf yellowing or reddening (depending on the cultivar), uneven shoot lignification, berry shrivelling and bunch drying. Symptoms usually appear in summer (Figure 13).

The incidence and severity of the symptoms vary among cultivars, and no resistant cultivars have been identified so far. 'Chardonnay' is considered to be one of the most sensitive cultivars (reviewed in EFSA Panel on Plant Health 2014, Panassiti *et al*. 2015) (Table 4). Sensitivity of a given cultivar is also in correlation with the feeding behaviour i.e. preference and activity of the insect vector (Martelli and Boudon-Padieu 2006). Susceptibility of rootstocks was also investigated and it was found that *Vitis riparia*, American rootstocks and hybrids do not exhibit typical symptoms or show no symptoms of GY (Martelli and Boudon-Padieu 2006). However, according to Moutous (1977) phytoplasma symptoms could be observed in species *V. riparia*, and *V. rupestris*, and rootstock cvs. K5BB, 420A, R99, 3309C and SO4, after experimental transmission by *S. titanus*.

Table 4. Phytoplasma sensitivity of grapevine cultivars and rootstocks (EFSA Panel on Plant Health 2014, Panassiti *et al*. 2015, Borgo *et al*. 2009)

Species	Low susceptibility	Medium susceptibility	High susceptibility
V. vinifera cultivars	'Merlot'	'Sangiovese'	'Chardonnay', 'Riesling', 'Cabernet Sauvignon', 'Barbera', 'Sauvignon blanc', 'Pinot blanc', 'Pinot gris', 'Pinot noir', 'Sémillon'
American rootstocks	*V. rupestris*, *V. berlandieri* x *V. riparia* 420A		125-2 Millardet, *V. rupestris* Constatia

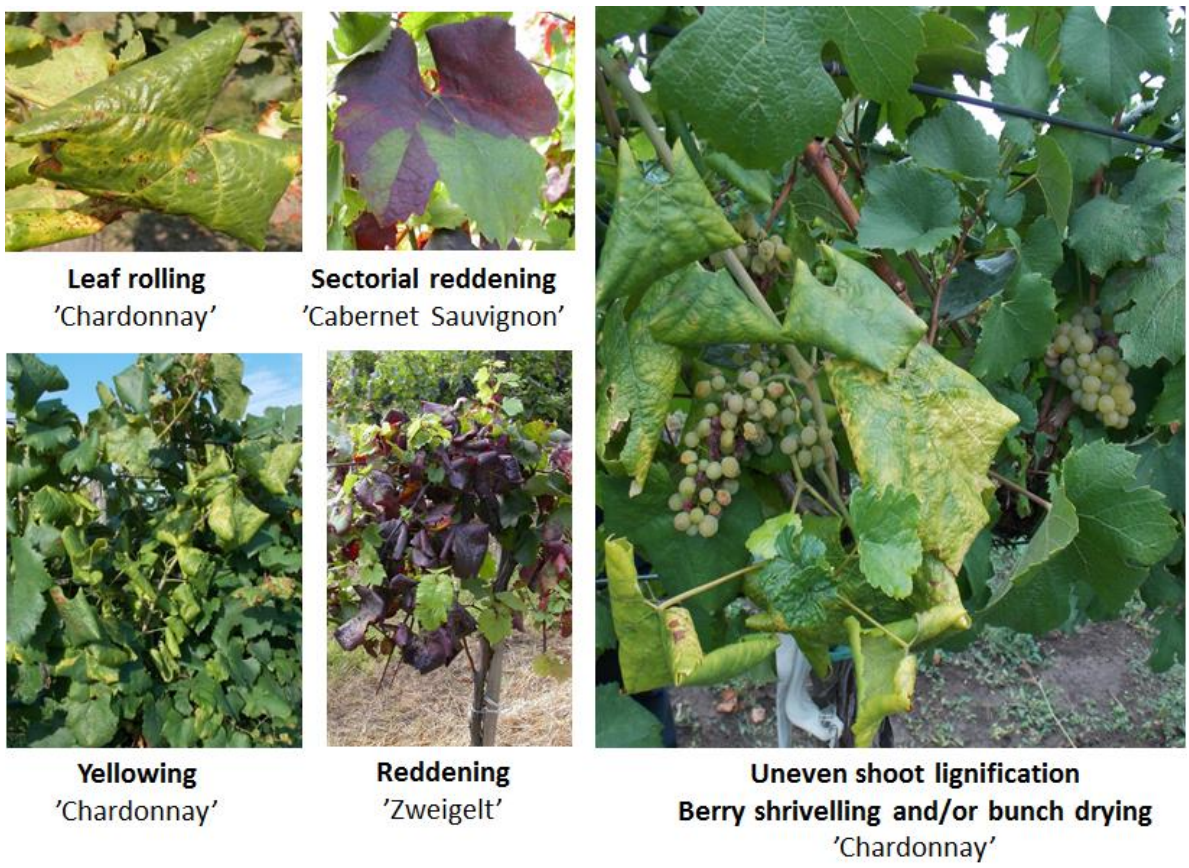

Figure 13. Symptoms of phytoplasma-caused Grapevine Yellow disease

The aim of viticulture is to produce a good quality final product. The BN effect was already studied from some aspects, however multi-year field experiment using comprehensive approaches has not been performed. As well as, the impact of BN disease on wine attributes has not been deciphered so far. Furthermore information are not available on biodiversity of Hungarian '*Ca*. P. solani' isolates, thus the epidemiological cycle of the pathogen is unravelled.

6. MATERIALS AND METHODS

6.1. Epidemiology of Bois noir disease in Hungary

6.1.1. Genetic diversity of '*Ca*. P. solani' strains in Hungarian wine regions

6.1.1.1. Plant material

Plant samples (136) were collected in August or September of years 2011, 2012, 2013 and 2014 in five wine regions of Hungary (Egri, Tokaji, Kunsági, Villányi, Soproni and Etyek-Budai) from 16 locations of 7 counties. Most of the samples were exhibiting phytoplasma symptoms such as yellowing/reddening, leaf rolling or proliferation. In a few cases asymptomatic individuals were collected. All samples were tested for phytoplasma infection (Table 5).

Table 5. List of plants collected in Hungary

County	Location	Sampling site	Plant species	Cultivar	Plant organ	Number of collected sample
Bács-Kiskun	Csikéria	abandoned field	*Vitis vinifera*	white variety	midrib	5
Bács-Kiskun	Kecskemét	vineyard	*Vitis vinifera*	-	midrib	4
Bács-Kiskun	Kecskemét	vicinity of vineyard	*Urtica dioica*	-	midrib	7
Baranya	Barcs	private vineyard	*Vitis vinifera*	-	midrib	3
Baranya	Siklós-Göntér	vineyard	*Vitis vinifera*	Cabernet franc	midrib	3
BAZ	Mád	Kakasok	*Rubus fruticosus*	-	midrib	1
BAZ	Mád	Kakasok	*Vitis vinifera*	Hárslevelű	midrib	3
BAZ	Tolcsva	Gyopáros (low)	*Vitis vinifera*	Furmint	midrib	3
BAZ	Tolcsva	Gyopáros (low)	*Vitis vinifera*	Pinot noir	midrib	3
BAZ	Tolcsva	Gyopáros (up)	*Convolvulus arvensis*	-	midrib	3
BAZ	Tolcsva	Gyopáros (up)	*Vitis vinifera*	Furmint	midrib	3
BAZ	Tolcsva	Kútpadka	*Vitis vinifera*	Furmint	midrib	3
BAZ	Tolcsva	Mánd	*Convolvulus arvensis*	-	midrib	3
BAZ	Tolcsva	Petrács (bak)	*Vitis vinifera*	Furmint	midrib	3
BAZ	Tolcsva	vicinity of vineyard	*Urtica dioica*	-	midrib	7
GYMS	Fertőszentmiklos	vineyard	*Vitis vinifera*	-	midrib	3
GYMS	Fertőszentmiklos	vicinity of vineyard	*Urtica dioica*	-	midrib	7
GYMS	Röjtökmuzsaly	plot	*Solanum tuberosum*	Demon	midrib	2
GYMS	Sopron	vineyard	*Vitis vinifera*	Zweigelt	midrib	4
Heves	Andornaktálya	abandoned field	*Vitis vinifera*	Chardonnay	midrib	4
Heves	Andornaktálya	vineyard	*Vitis vinifera*	Merlot	midrib	4
Heves	Eger	Kőlyuktető	*Convolvulus arvensis*	-	midrib	2
Heves	Eger	Kőlyuktető	*Vitis vinifera*	Chardonnay	midrib	3
Heves	Eger	vineyard	*Vitis vinifera*	Pinot noir	midrib	2
Heves	Eger	vicinity of vineyard	*Urtica dioica*	-	midrib	7
Heves	Vécs	vineyard	*Vitis vinifera*	Chardonnay	midrib	3
Pest	Budapest	Kopaszi barrier	*Lavandula angustifolia*	-	midrib	5
Pest	Etyek	Orban statue	*Vitis vinifera*	Chardonnay	midrib	3
Pest	Etyek	vineyard	*Convolvulus arvensis*	-	midrib	3
Pest	Etyek	vineyard	*Vitis vinifera*	red variety	midrib	3
Pest	Monorierdő	plot	*Apium graveolens*	-	midrib	3
Pest	Monorierdő	plot	*Capsicum annuum*	-	midrib	3
Pest	Monorierdő	plot	*Solanum lycopersicum*	-	midrib	3
Pest	Monorierdő	plot	*Solanum tuberosum*	-	midrib	3
Pest	Monorierdő	vicinity	*Convolvulus arvensis*	-	midrib	2
Pest	Monorierdő	vicinity	*Urtica dioica*	-	midrib	7
Pest	Monorierdő	vicinity	*Lamium purpureum*	-	midrib	2
Pest	Monorierdő	vicinity	*Ulmus minor*	-	midrib	1
Somogy	Homokszentgyörgy	vineyard	*Vitis vinifera*	Zweigelt	midrib	3

6.1.1.2. DNA extraction and phytoplasma detection

DNA extraction. To obtain phytoplasma-reach phloem, main leaf veins were cut, 1 g of tissue was frozen and kept at -20°C until nucleic acid isolation. The DNA extraction was carried out using the CTAB method described in Clair *et al*. (2003). DNA was quantified and concentrations were adjusted to 20-60 ng/µl.

Nested PCR and gel electrophoresis. Samples were tested for phytoplasma infection in nested PCR-RFLP system. 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. The PCR reactions were performed based on protocol described in Ember *et al*. (2011a, 2011b).The 1.2 kb nested PCR products were separated in 1.2 % agarose gel and visualised under UV.

RFLP analysis. Positive samples were subjected to restriction fragment length polymorphism. The 1.2 kb R16F2n/R16R2 amplicons were digested with *Tru1*I (Fermentas, Vilnius, Lithuania) restriction enzyme according to the manufacturer's instructions. Digested products were separated on 2.5 % agarose gel and restriction profiles were compared with those of reference strains from 16Sr groups I, V and XII. Conrols EAY 16Sr-B, FD-D 16SrV-C, and MOL 16SrXII-A were provided by A. Bertaccini (DiSTA, Bologna, Italy); AY27 16SrI-A, CPh 16SrI-C, PaWB 16SrI-D, CP and PWB 16SrVI-A were obtained from I.-M. Lee (USDA-ARS, Beltsville, USA). The DNA extraction and tests for phytoplasma infection of insects, sampled for the transmission trial (see 6.1.1.2.), were the same as described above.

6.1.1.3. Multi locus sequence typing of Hungarian '*Ca.* P. solani' isolates

Among the samples testing positive for '*Ca.* P. solani', 46 isolates were selected for molecular characterisation which was carried out using five genetic markers.

Markers for MLST. Multi Locus Sequence Typing (MLST) was done based on conserved and variable genetic markers. Housekeeping genes were *secY, tuf* and *yidC,* and variable genes encoding surface protein were *vmp1* and *stamp* (Table 6). New markers were developed based on the investigation of the genome of '*Ca.* P. solani' PO strain (iANT 2.0 platform, http://iant.toulouse.inra.fr/bacteria/annotation/cgi/phytoplasma.cgi). In PO genome further constitutive markers were selected and primers were designed for genes: *yidC, ligA, priA, alaS* and *pheT* (Table 6). Variability of the markers was tested on reference strains of '*Ca.* P. solani': PO, T292, LG, P7, CL, TOTK10, AZ-AU06, I5, I1, REP5, HO11, GGY, I29, 1925, DEP, AZ-12HN and KB181/3. The PCR reaction mixture and amplicon visualisation were the same as described in 6.1.1.2., with the exception that a 2.5 mM final concentration of $MgCl_2$ was applied in the case of newly designed markers.

RFLP. The majority of the isolates *vmp1* and *tuf* genotypes were determined by RFLP analyses and only a minor part of the isolates were sequenced. The fTufAy/rTufAy products were digested with *Hpa*II restriction enzyme (Fermentas, Vilnius, Lithuania) for genotype identification according to Langer and Maixner (2004). The RFLP profiles were compared with reference strains 1925 (*tuf*-a type) and GGY (*tuf*-b type), obtained from Dr. M. Maixner (Julius Kühn-Institut, Bernkastel-Kues, Germany).

In the case of *vmp1* gene TYPH10F/TYPH10R amplicons were evaluated firstly based on fragment size. To date, three different amplicon sizes (small, medium and large) were recorded, which range between 1189 to 1438 bp, according to the strain. Medium size is the most frequent (Pacifico *et al*. 2009). The *vmp1* products were digested with *Rsa*I and/or *Alu*I restriction enzymes (Fermentas, Vilnius, Lithuania). Digestions were performed according to the manufacturer's instructions. Fragment length profiles were analysed on 2.5 % agarose gel. Reference strains for *vmp1* and RFLP profiles were provided by Dr. X. Foissac (INRA, Bordeaux, France).

Direct sequencing. Prior to direct sequencing purification of PCR products was carried out to eliminate primers. One volume of 20 % polyethylene glycol (6.000) and 2.5 M NaCl was added to PCR fragments. After 20 min incubation at room temperature, the mixture was centrifuged at 20.000 rpm for 20 min at 4 °C. Pellets were washed twice in 70 % ethanol, and after evaporation of ethanol, pellets were resuspended in 25 µl sterile water. The DNA quantity of the purified samples were checked on 1.2 % agarose gel, and 15 ng per 100 bp of product/sample were sent for sequencing (both strands to achieve a 2x coverage) (Macrogen, Amsterdam, The Netherlands, or Base-Clear, Leiden, The Netherlands).

Sequence analysis. 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). To test the reliability of the inferred tree, bootstrap analyses (500 replicates) were applied. I got access to a large set of reference sequences for each gene which were used for phylogenetic analyses (provided by Dr. X. Foissac and the Stolbur-Euromed Consortium).

Table 6. Primers and PCR conditions used for MLST of Hungarian '*Ca*. P. solani' isolates

	Marker	Forward and reverse primer sequence 3'-5'	Size bp	PCR conditions	Reference
Housekeeping genes	***tuf*: TU EF elongation factor**				
	StoltufF0 StoltufR0	gcacgttgatcacggcaaaac ctgtttttccaccttcacgg	1185	94°C 4 min, 35 cycles: 94°C 30 sec, 52°C 30 sec, 72°C 1 min	Foissac *unpublished*
	FtufAY RtufStol	gctaaaagtagagcttatga cgttgtcacctggcataacc	969	94°C 4 min, 35 cycles: 94°C 30 sec, 55°C 30 sec, 72°C 1 min	Schneider *et al*. 1997, Langer and Maixner 2004
	***secY*: protein translocation system**				
	POsecF1 POsecR1	tctgctttgcctttgccttt attagtaaactagttcctcc	1052	94°C 4 min, 35 cycles: 94°C 30 sec, 54°C 30 sec, 72°C 1 min	modified from Fialová *et al*. 2009
	POsecN2 POsecR3	ccatcaaaacttttttggtttaggc gcccctataacggtgattttga	887	94°C 4 min, 35 cycles: 94°C 30 sec, 52°C 30 sec, 72°C 1 min	Fialová *et al*. 2009
	***yidC*: protein translocation system**				
	YidCF0 YidCR0	ggctggaagaaaaacgcactga gtgtttttttaggctagtgccaac	1304	94°C 4 min, 35 cycles: 94°C 45 sec, 53°C 45 sec, 72°C 1 min 30 sec	Ember and Foissac unpublished
	YidcF1 YidCR1	ctgacagttccagctggcac aagcgcattgtttttttaatgcaag	1056	94°C 4 min, 35 cycles: 94°C 30 sec, 58°C 30 sec, 72°C 1 min	
	***ligA*: NAD(+)-dependent DNA ligase**				
	LigAF1 LigAR1	cccagaaatgccgcttccgg ttagaaccagcattagtgcc	1341	94°C 4 min, 35 cycles: 94°C 30 sec, 56°C 45 sec, 72°C 1 min	Ember and Foissac unpublished
	LigAF2 LigAR2	catgccttaattggggcgac cccacgtgtttaattcctaa	674	94°C 4 min, 35 cycles: 94°C 30 sec, 56°C 30 sec, 72°C 1 min	
	***priA*: primosomal protein**				
	PriaF1 PriaR1	agagtcgttgttccttttgg ctggcacaacgaccagcggc	1856	94°C 4 min, 35 cycles: 94°C 45 sec, 58°C 45 sec, 72°C 1 min 30 sec	Ember and Foissac unpublished
	PriaF2 PriaR2	ggcaaaacaggttccggcaa cgacataaaacaaaaggcgaaaatcc	653	94°C 4 min, 35 cycles: 94°C 30 sec, 58°C 30 sec, 72°C 1 min	
	***alaS*: alanyl-tRNA synthetase**				
	AlasF1 AlasR1	ggcccagggccttcaggacc ccgccgccagaacctagagc	2033	94°C 4 min, 35 cycles: 94°C 45 sec, 63°C 45 sec, 72°C 1 min	Ember and Foissac unpublished
	AlasF2 AlasR2	gcaatcaacgacggagctac actgcttcaatacgataaatccc	169	94°C 4 min, 35 cycles: 94°C 30 sec, 56°C 30 sec, 72°C 1 min	
	***pheT*: phenylalanine tRNA synthetase, beta subunit**				
	PheTF1 PheTR1	gtaaaaattgtctgtggagc gtcaatccctctttcaaaacg	934	94°C 4 min, 35 cycles: 94°C 30 sec, 53°C 45 sec, 72°C 1 min	Ember and Foissac unpublished
	PheTF2 PheTR2	caaggttcatttttggcagg ctaaaagacccacaatcccagc	742	94°C 4 min, 35 cycles: 94°C 30 sec, 55°C 1 min, 72°C 1 min	
Variable genes	***vmp1*: variable membrane protein (former name Stol1H10)**				
	STOLH10F1 STOLH10R1	aggttgtaaaatcttttatgt gcggatggcttttcattatttgac	1189 to 1438	94°C 4 min, 35 cycles: 94°C 30 sec, 52°C 30 sec, 72°C 2 min	Fialová *et al*. 2009
	TYPH10F* TYPH10R*	aacgttcatcaacaatcagtc cacttctttcaggcaacttc		94°C 4 min, 35 cycles: 94°C 30 sec, 55°C 30 sec, 72°C 1 min 30 sec	
	***stamp*: stolbur antigenic membrane protein**				
	StampF StampR0	gtaggttttggatgttttaag aaataaaagaacaagtatagacga	637	94°C 4 min, 35 cycles: 94°C 30 sec, 56°C 30 sec, 72°C 1 min 30 sec	Fabre *et al.* 2011a
	StampF1 StampR1	ttctttaaacacaccaagac aagccagaatttaatctagc	578	94°C 4 min, 35 cycles: 94°C 30 sec, 52°C 30 sec, 72°C 1 min 30 sec	

6.1.2. Insect transmission of Hungarian '*Ca.* P. solani' strains

In July 2013, planthoppers of *Cixiidae* family were collected by sweep netting in eight locations in Hungary where '*Ca.* P. solani' was detected previously: Sopron, Fertőd, Etyek, Monorierdő, Eger, Andornaktálya and Tolcsva (Table 7).

Table 7. List of planthoppers collected in Hungary for transmission trial

Location	Sampling site	Collection date	Insect species	Host	No. of collected insects	Trans-mission code
Eger	vineyard	2/7/2013	*Hyalesthes obsoletus*	bindweed	4	HO1
Sopron	meadow	3/7/2013	*Hyalesthes obsoletus*	stinging nettle	20	HO2-9
Monorierdő	potato field and grassy road	3/7/2013	*Hyalesthes obsoletus*	potato, bindweed	4	HO10
			Hyalesthes obsoletus		5	HO11
Etyek	vineyard	3/7/2014	*Hyalesthes obsoletus*	bindweed	2	HO12
Lovászi	vicinity of vine, nettle patch	18/7/2013	*Hyalesthes obsoletus*	stinging nettle	33	HO13-14
Fertőd	potato field	3/7/2013	*Reptalus quinquecostatus*	Potato	3	REP1
Andornak-tálya	dirty road with *Polygonum aviculare*, vicinity of vineyard and cereal field	2/7/2013	*Reptalus quinquecostatus*	myrobalan plum, common knotgrass	6	REP2
			Reptalus quinquecostatus		9	REP4
			Reptalus quinquecostatus		6	REP4
			Reptalus quinquecostatus		8	REP5
Eger	Chardonnay plot	2/7/2013	*Reptalus quinquecostatus*	weeds	1	REP3
Etyek	vicinity and grassy in-between rows of vineyard	4/7/2013	*Reptalus cuspidatus*	common hawthorn, grass	20	REP6
			Reptalus cuspidatus		20	REP7-12

Insects from all locations were kept alive and placed on healthy *Catharanthus roseus* (periwinkle, Polka dot XP hybrid) plants at 8 to 10 leaf stages for feeding (Figure 14). After 8-10 days planthoppers were gathered from the plants and kept in 96 % ethanol until 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 (COI) (Bertin *et al*. 2010).

Figure 14. Feeding planthoppers on periwinkle

Plants were grown in a plant growth chamber at 25 °C, 70 % RH, with a 16/8 h light/dark photoperiod. Symptom appearance was observed regularly. In 2013 September periwinkles with symptoms (four plants) were brought to INRA (Bordeaux, France) for genetic characterization and

to be maintained in the phytoplasma collection of INRA. Each of the four phytoplasma symptomatic plants were grafted on healthy periwinkle in two replicates to multiply the phytoplasma strains. Symptom development was monitored regularly.

6.1.3. New generation sequencing of Hungarian '*Ca*. P. solani' strains

In order to gain new genome data, two of the transmitted and maintained strains (on periwinkle in Bordeaux): HO11 and REP2 were subjects for Illumina Solexa new generation sequencing (NGS). These two strains were chosen based on the results of *stamp* genotypes belonging to different *stamp* clusters II and III, respectively.

Isopycnic cesium chloride density gradient in presence of bisbenzymide (BBZ-CsCl) (Kollar *et al*. 1990). Cesium chloride (CsCl) solution under intense gravitational forces form a continuous density gradient. During ultracentrifugation DNA with different densities is placed in a zone of the gradient where the CsCl density is identical to their own density. The bisbenzymide fluorescent agent inserts between bases A and T. An AT rich DNA is swelling when incorporating more bisbenzimide and therefore reduces its density. This permits separation of phytoplasmal, mitochondrial and chloroplastic DNAs, which have high AT content by comparison to the nuclear DNA of most plant species. Under UV light a good visualization allows to compare the 2 gradients "healthy and infected" plant in order to take the upper supplementary band that corresponds to phytoplasmal, mitochondrial and chloroplastic DNAs. Two successive gradients procedures are needed to perform for a good purification. In this way, the GC poor phytoplasma genome (20-28 %) can be enriched by eliminating most of the periwinkle nuclear DNA which has a higher GC content. The obtained phytoplasma rich fraction was used for genome sequencing.

Phytoplasma enrichment and NGS. For NGS of phytoplasmas were enriched. This experiment was performed at INRA, Mollicutes team, Phytoplasma group (Bordeaux, France). The DNA was extracted from main veins of HO11 and REP2 isolates maintained on periwinkles, as well as from healthy periwinkles (as reference control) by CTAB method (same as described at 6.1.1.2.). The DNA concentration was determined by spectrophotometry. Separation of different fractions based on their density was performed in two rounds using isopycnic CsCl density gradient in the presence of bisbenzymide-Hoescht reagent. After 72 hours isopycnic centrifugation at 38000 rpm at 20 °C the upper phytoplasma band was eliminated under UV. In order to obtain higher phytoplasma quantity, two subsequent gradient procedures were performed (Figure 15). At the end of the procedure bisbenzymide were eliminated by isopropanol extraction, which was followed by CsCl elimination by 48 h repeated dialysis against 2 liter 1x TE (pH 8) at 4 °C. The DNA was concentrated by embedding the dialysis tube with solid polyethylene glycol, then quantified by UV spectrometry, and 50 ng of each strain were sent for Illumina Solexa NGS, which was done

by UDGGenomed and University of Debrecen (Hungary). This work was funded by the National Research, Development and Innovation Fund of the Hungarian Government (KTIA_AIK_12-1-2013-0001).

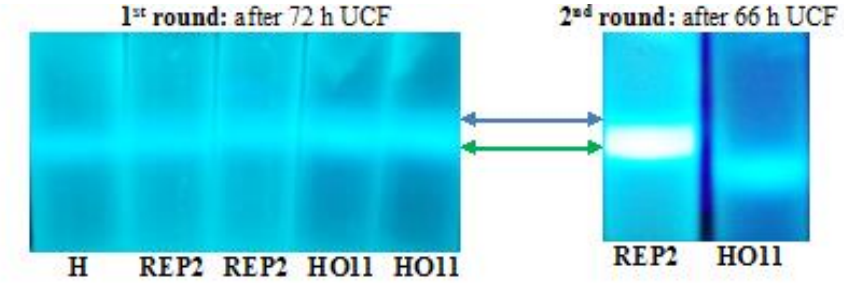

Figure 15. Separation of AT reach phytoplasmal, mitochondrial and chloroplastic DNAs from periwinkle DNA after gradient ultracentrifugation

Legend: bule arrow: mitochondrial and chloroplastic DNAs, green arrow: periwinkle DNA

6.1.4. Insect-pathogen protein interaction

6.1.4.1. Heterologous expression of recombinant STAMPs of '*Ca.* P. solani' strains

Selection of '*Ca.* P. solani' strains. Based on our preliminary result (2013) on *stamp* genotyping, *stamp*-ST4 and *stamp*-ST9 were the most prevalent genotypes in Hungary. Therefore we selected strains ST4 and ST9 belonging to cluster II, as well as strains belonging to *stamp* clusters I: ST1, cluster III: ST13 and cluster IV: ST6 for heterologous protein expression (Table 8).

Table 8. '*Ca*. P. solani' strains for total protein extraction (*list a*) and recombinant protein expression (*list b*)

stamp cluster	*stamp* genotype	Strain	Origin	Original plant	Protein size (aa)	Forward primer	Reverse primer
List a **'*Ca.* P. solani' plant strains**							
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	P7	Lebanon	periwinkle	-	-	-
ST-IV	ST6	1925	Germany	grapevine	-	-	-
List b **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

Amplification *stamp* gene and cloning. To amplify the central part of *stamp* gene that correspond to the hydrophilic domain of the protein of four *stamp* clusters ST4-N1 (5'-aatgggtcgggatcccggaagtaaagatttaccat-3'), ST9-N1 (5'-aatgggtcgggatcccggaggtaaagatttaccaa-3'), ST4-9-C1 (5'-gtggtggtgctcgagtcaagttgatgtccagaatgaacc-3') and ST1-C1 (5'-gtggtggtgctcgagtcaa gttgatgtccataatgaacc-3') primers were designed (Table 8). Amplification was carried out using proofreading Q5 High-Fidelity DNA Polymerase (New England Biolabs) using the following PCR cycles 98 °C 43 sec, 35 cycles: 98 °C 10 sec, 59 °C 10 sec, 72 °C 2 min. In-Fusion HD Cloning Kit (Clontech, Mountain View, USA) was used for directional cloning of ST1, ST4, ST9, ST6 and ST13 DNA fragments into pET-28b(+) vector infusioned with a N-terminal His6xTag (thrombin/T7) (Novagen, Merck) (Figure 16).

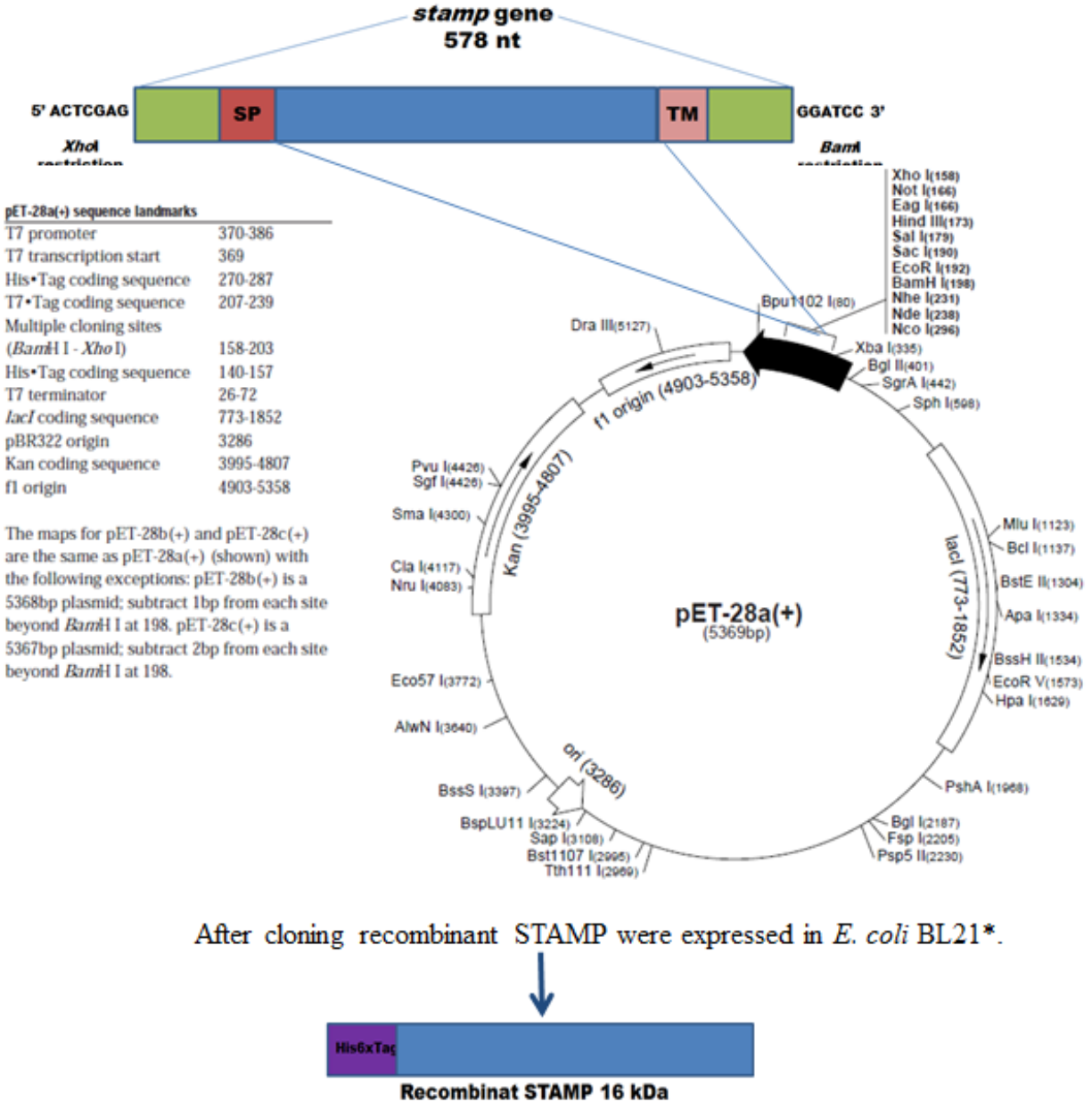

Figure 16. Cloning STAMP into pET-28b(+) vector
Legend: pET-28a-c(+) map, SP: signal peptide, TM: transmembrane domain.

Primer design and cloning reactions were performed according to the manufacturer's instructions. Recombinant pET28b(+) vectors (5 ng each): Rec_pET28_ST1, Rec_pET28_ST4, Rec_pET28_ST9, Rec_pET28_ST13 and Rec_pET28_ST6 (later the fusion proteins were named: fp_ST1, fp_ST4, fp_ST9, fp_ST13 and fp_ST6) were transformed into Stellar chemical competent cells (Clontech) by heat shock for 45 sec at 42 °C. After cell regeneration in SOC medium (1 h at 37 °C) 50 µl, 100 µl and 250 µl cell suspensions were plated on LB plates (K50: contain 50 µg/mL Kanamicin). Plates were incubated overnight at 37 °C and colonies were counted the next morning. Twelve selected colonies were pre-cultured in 3 ml LB (K50) overnight at 37 °C in a shaker incubator (200 rpm). The following day, plasmids of the pre-cultured clones were extracted using Wizard Plus SV Minipreps DNA Purification System (Promega Corporation). To check the presence of the insert, plasmids were sequentially digested with *Xho*I and *Bam*HI restriction enzymes according to manufacturer's instructions (Takara Bio Europe), and visualised on 1.2 % agarose gel. Inserts were observed in all 12 clones in the case of each *stamp* cluster. Two of each circular recombinant pET28 clones: Rec_pET28_ST1, Rec_pET28_ST4, Rec_pET28_ST9, Rec_pET28_ST13 and Rec_pET28_ST6) were sent for sequencing using primers of T7P-promoter and T7 terminator (Figure 16, 17).

STAMP	Position	Protein sequence
>PO_ST1	1-54	GSKDLPSGTETKEVA I STDD
>fp_ST9	1-54	MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRDPGGKDLPTGTDTKEVA I STDD
>fp_ST4	1-54	MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRDPG SKDLP SGTETKEVA I STDD
>fp_ST13	1-54	MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRDPGGKDLPTGTDTKEVA I SADD
>fp_ST6	1-54	MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRDPG SKDLP SGTETKEVA I S I DD
>PO_ST1	55-111	VTNQSELVKALKKIDALKDVKENDFDPSLS - - DKKEITLKSKDGGQFKTGEIKVKRR
>fp_ST9	55-111	VTNQSELVKALKKIEALKDVTEKDFDAVLST - DKREITLKSKDGGQFKAGEIKVKRR
>fp_ST4	55-111	VTNQSELVKALKKIEALKDVTEKDFDAVLST - DKREITLKSKDGGQFKAGEIKVKRR
>fp_ST13	55-111	VTNQSELVKALKKIEALKDVKENDFDPS LSP - DKKEITLKSKDGGQFKSGEIKVKRR
>fp_ST6	55-111	VTNQSELVKALKKIDALKDVKENDFDASLNTTDKKEITLKSKDGGQFKPGEIKVKRR
>PO_ST1	112-136	DLNDTEKAEKEA - - - - EKGSLWTST
>fp_ST9	112-136	DLNDTEKAEKEA - - - - EKGSFWTST
>fp_ST4	112-136	DLNDTEKAEKEA - - - - EKGSFWTST
>fp_ST13	112-136	DLTDTEKAEKEA - - - - EKGSFWTST
>fp_ST6	112-136	DLTDTEKAEKEAQKEAEKGSFWTST

Figure 17. Protein sequences of clones of recombinant STAMPs of cluster II, II and IV

Legend: amino acids (aa) differing between strins are highlighted in red, green and blue; aa identical has the same colour; >fp_ST9: strain I22 isolated from pepper, Monorierdő; >fp_ST4: strain I6 isolated form grapevine, Sopron; >fp_ST13: strain REP2 isolated from *R. quinquecostatus* and transmitted to periwinkle, Andornaktálya; >fp_ST6: 1925 isolated from grapevine, Germany, maintained on periwinkle at INRA.

Recombinant STAMP expression. Heterologous expressions of *stamp* cluster II, III and IV recombinant proteins was performed in *Escherichia coli*, strain BL21* (Invitrogen Corporations) using standard procedures. This high-level expression strain (1 x 10^8 cfu/µg transformation efficiency) carries the gene for T7 RNA polymerase which is under control of the lacUV5 promoter. To induce expression of the T7 RNA polymerase IPTG (isopropyl-beta-D-thiogalactopyranoside), a highly stable synthetic analogue of lactose were used. To transform clones of *stamp* clusters into BL21* - strain recommended to express heterologous genes of low-copy number-, chemical competent cells were prepared using standard protocol (Hanahan *et al*. 1991). Transformation and plating were the same as described before. One discrete colony of each *stamp* cluster was cultured until OD reached 0.6. For induction, 500 mM IPTG (final concentration) was applied and incubated for 3 h at 30 °C. Bacteria lysis was done in 10 ml lysis buffer (0.5 M Tris-HCl, 5 M NaCl) with DNase (100 U/mL), lysozime (0.2 g/mL) and PMSF (1 mM final concentration) and incubated for 20 min on ice. Soluble proteins were sonicated 10 x 1 min (duty 40%, microtip limit 3) (Vibra-Cell Ultrasonic Processor, Sonon & Materials Inc.). Separation of lysate was done on 8000 rpm for 10 min at 4 °C. Supernatant (as well as the resuspended pellet) was kept at -20 °C until Ni-affinity chromatography.

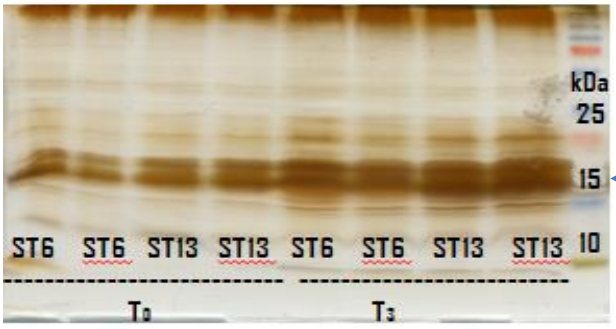

Figure 18. Verification induction on silver stained SDS-PAGE

Legend: T0: *E. coli* BL21* cells with recombinant ST6 and ST13 plasmids before induction, T3: 3 hours after IPTG induction, arrow: shows the induction in T3. M: protein PageRuler™ Prestained Protein Ladder (ThermoFicher Scientific): 10, 15, 25, 35, 55, 70, 100, 130 kDa.

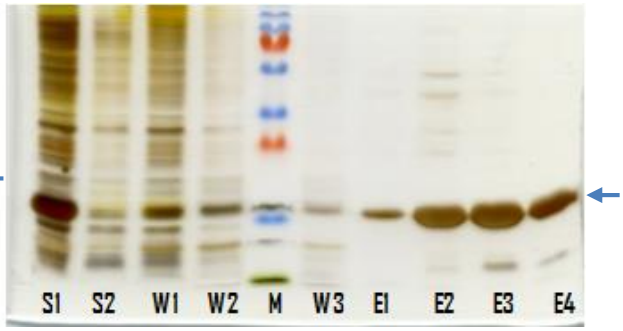

Figure 19. Verification protein purification (fp_ST9) on silver stained SDS-PAGE

Legend: S1: protein before column, S2: protein after column, W1-W2-W3: wash buffer between elutions steps, E1-E2-E3-E4: fractions of eluted fusion protein, arrow: 16 kDa fusion protein, M: protein PageRuler™ Prestained Protein Ladder (ThermoFicher Scientific).

Inductions were checked on silver stained (Sigma-Aldrich, Saint Louis, USA) SDS-PAGE (polyacrylamide 40 %, Tris-HCl 1.5 M pH 8, SDS 20 %, APS 10 %, TEMED) (Figure 18). His6x-tagged fusion proteins were purified using Ni-affinity chromatography according to the HIS-Select Nickel Affinity manual (Sigma-Aldrich, Saint Louis, USA). Protein was eluted using a gradient of imidazole 0.1 M, 0.25 M, 0.5 M and 1 M (coded from E1 to E4), and each fraction E1-E2-E3-E4 was collected separately. To check purification steps, 50 µL of each elution step and protein

before column was kept at 4 °C and subjected to SDS-PAGE (Figure 19). Fractions of 16 kDa protein of each recombinant STAMP were desalted using PD10 column (GE HealthCare, Freiburg, Germany).

Extraction of total proteins from phytoplasma-infected periwinkles. To test the 2A10 MAb capability to recognise all four *stamp* clusters, total proteins of periwinkle infected with different strains of '*Ca*. P. solani' maintained in periwinkle was extracted. Representative strains of clusters I, II, III and IV: Charente2, StolburC, Lot et Garonne, Champlong, GGY, DEP, STOLP, P7, 1925 were used (Table 8). 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 precooled mortar. Extracts were centrifuged for 1 min on 10000 rpm at 4 °C. Supernatants were transferred into Eppendorf tubes and kept on ice until SDS-PAGE.

Western blot (WB) analyses. To confirm that the produced proteins are STAMP fusion proteins western blot analyses were performed using monoclonal anti-polyhistidine antibody produced in mouse (poly-His MAb) (Sigma-Aldrich, Saint Louis, USA) as the primary antibody, and horseradish peroxidase conjugated goat anti-mouse IgG (Sigma-Aldrich, Saint Louis, USA) as the secondary antibody (Fabre *et al*. 2011a). Antibodies were diluted in PBS + 2 % low fat milk (1:6750 and 1:6250, respectively) and incubated for 2 h on a 3D shaker at room temperature. Between steps membrane was washed 3 times with PBS + 0.1 % Tween 20 for 20 min. To reveal signals a Super Signal West Pico kit was used (Thermo Scientific Pierce Protein Biology). Results were developed after 20 sec, 1 min, 3 min and 10 min exposure time, and evaluated according to the signal intensity.

6.1.4.2. STAMP-insect protein interaction

Total protein (IP) was extracted from vector: *H. obsoletus* (different ecotypes: population from bindweed, stinging nettle and lavender), *R. panzeri*; potential vector: *R. quinquecostatus*, *R. cuspidatus*; and non-vector*: Euscelidius variegatus*, *Circulifer haematoceps* species. Interaction capabilities of these insect proteins with fp_ST4, fp_ST9 (*stamp* cluster II) were investigated in dot-blot analyses (Table 9).

Insects' total protein extraction from planthoppers. Ten to 30 insect individuals, depending on their size (Table 9), were homogenized in 1.5 ml Eppendorf in 300 µl Rx buffer (Galetto *et al*. 2011) and incubated 10 min at 37 °C. Extract were centrifuged at 5000 rpm for 1 min. The supernatants were pipetted into Eppendorf tubes and sonicated for 3 seconds. Proteins were concentrated in sped vac (room temperature for 30 minutes) to obtain ~100 µl protein extract/sample.

Dot-blot hybridisation. An aliquot of 35 µl of insect proteins was dropped on nitrocellulose membrane (Amersham Hybond ECL; GE HealthCare, Freiburg, Germany) and fixed using VAC apparatus (under vacuum for 10 min). The membrane was blocked in saline buffer (PBS) with 5 % low fat milk for 2 hours. Membranes were incubated with the interacting protein fp_ST4 (32 µg), or fp_ST9 (85 µg), or negative control (without interacting protein) overnight on a 3D shaker. To detect if STAMP is interacting with insect protein primary antibody (2A10 MAb) and secondary antibody (horseradish conjugated anti-mouse peroxidase MAb; Sigma-Aldrich, Saint Louis, USA) were applied. Antibodies were diluted in PBS + 2 % low fat milk (1:6750 and 1:6250, respectively) and incubated 2 h on 3D shaker at room temperature. Between steps membranes were washed 3 times with PBS + 0.1% Tween 20 for 20 min. Membranes were revealed with the use of the Super Signal West Pico kit (Thermo Scientific Pierce Protein Biology). Results were developed after 20 sec, 1 min, 3 min and 10 min exposure time, and evaluated based on the signal intensity of the dots.

Table 9. List of insect used in dot-blot hybridisation with STAMP pf_ST4 and pf_ST9

Sample	Origin	Host plant	No. of used insects	Stored at
Circulifer haematoceps	INRA, insectarium	*Triticum aestivum*	30	10 min at -20°C
Euscelidius variegatus	insectarium	*T. aestivum*	10	Dry -20°C
Hyalesthes obsoletus ecotype Ca (HoCa)	FR, Charante	*Convolvulus arvensis*	14	Ethanol -20°C
Hyalesthes obsoletus ecotype La (HoLa)	FR, Champlong	*Lavandula officinalis*	20	Ethanol -20°C
Hyalesthes obsoletus ecotype Ud (HoUd)	FR, Elsas	*Urtica dioica*	20	Ethanol -20°C
Hyalesthes obsoletus ecotype Ud (HoUd)	DE, JKI,BKS, Kesten	*U. dioica*	20	Ethanol -20°C
Reptalus quinquecostatus	SR, Plant Prot. Inst. Belgrade	*Wild plants*	10	Ethanol -20°C
Reptalus panzeri	FR, Triazol	*Zea mays*	26	Triazol ext., -20°C
Replaus cuspdatus	HU, Etyek	*Crategus monogyna*	10	Ethanol -20°C
Control fp_ST4	purified protein			
Control fp_ST9	purified protein			

6.2. Effects of Bois noir disease on performance of *V. vinifera* L. cv. Chardonnay in Eger wine region

6.2.1. Experimental site and plant material

The experimental vineyard is situated in the Eger wine region of Hungary (47°86′N, 20°38′E, 185 m), belonging to Károly Róbert College, Research Institute of Viticulture and Enology. The climatic conditions of the region can be described as humid continental with a mean annual temperature of 10.5 °C and average rainfall of 600 mm/year. Measurements were performed in a 0.6 ha vineyard of *V. vinifera* L. cv. 'Chardonnay', planted in 1993, and grafted onto Teleki 5C rootstock. 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. The experimental vineyard was managed, with regards to fertilisation, canopy management and disease control, according to the normal practices applied to a commercial vineyard.

Three random blocks contained 50 plants per block (Figure 20) in which the phytoplasma infection status of the individual plants was visually evaluated before harvest in each year of the experiment (2011–2014).

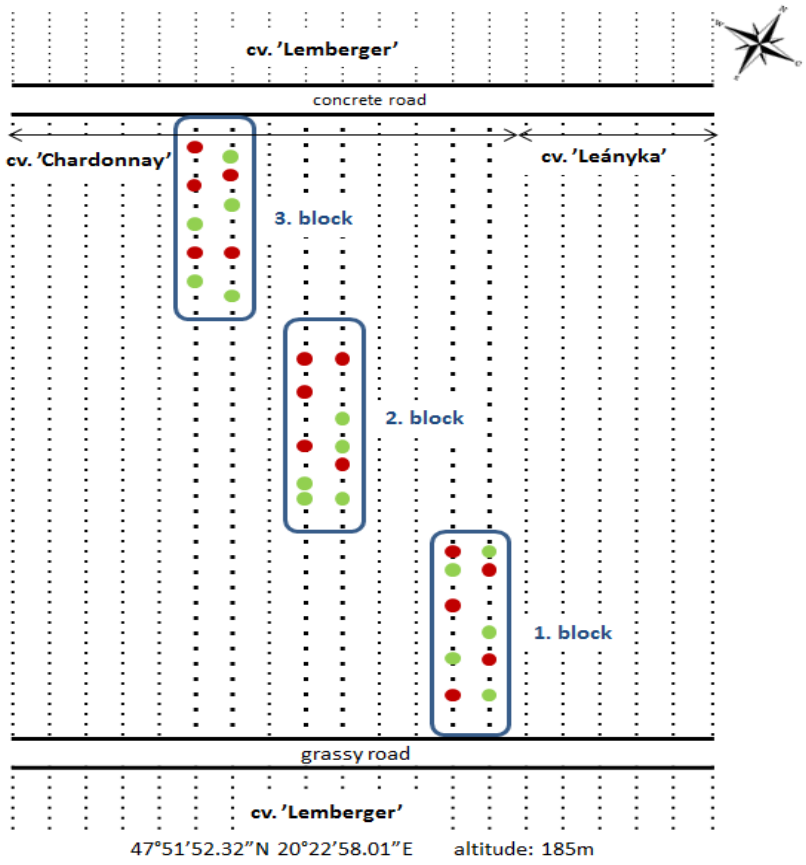

Figure 20. Experimental lay out of the plot cv. 'Chardonnay', Eger, Kőlyuktető

Legend: dotted lines: rows (80 vine/row), in bold: experimental rows, blue boxes: blocks contain 50 vines each, 15-15 red and green dots: BN-affected and healthy plants assigned for measurements (five each/block).

Severity and incidence of phytoplasma disease were recorded individually and scored from + (mild) to +++(+) (extreme) according to the observed symptoms (leaf roll, yellowing, necrosis, uneven shoot lignification, and berry shrivelling or drying) (Table 10). 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. To reduce the standard deviation due to the heterogeneity of the infected plants, grapevines that received a score of ++ (i.e. moderately infected, symptoms on 15–25 % of the shoots) were analysed. Prior to the experiment the '*Ca*. P. solani' infection as well as the health status of the 15-15 selected plants was confirmed at molecular level (data not shown) according to the protocol described in 6.1.1.2. Grape yields from the remaining H and BN-affected (mild to severe) plants of the experimental blocks were used for microvinification. Meteorological data for every year of the experiment (2012, 2013 and 2014) and the preceding year was recorded.

Table 10. Severity and incidence of phytoplasma disease grapevines

Infection status (score)	**Severity** (occurring symptoms)	**Incidence** (infected shoots/vine)
+ (mild)	leaf roll, mild yellowing	below 15 %
++ (moderate)	leaf roll, yellowing, necrosis, uneven shoot lignification, berry shrivelling and/or drying	15-25 %
+++ (severe)	leaf roll, yellowing, necrosis, uneven shoot lignification, berry shrivelling and drying	25-35 %
+++(+) (extreme)	leaf roll, yellowing, necrosis, uneven shoot lignification, berry shrivelling and/or drying	40-100 %

6.2.2. Vegetative performance measurements

Measurements were recorded on 15-15 healthy (H) and BN-affected (BNA) grapevines (5-5 H and BNA vines from each block of each replicates) in 2012, 2013 and 2014. Deviations in year and/or sample number are indicated where applicable.

Pruning mass and cane lignification. Vines were pruned in winter (February) and cane mass was measured individually. The number of non-lignified canes was recorded on 15-15 H and BNA grapevines and bud viability was observed by means of bud dissection. Additionally, in 2014, 15 shoots each of H and BNA, were collected to measure the diameter and determine the pith:wood (xylem + phloem) tissue ratio in the first, second and fourth internode sections (Dardeniz *et al*. 2008).

Leaf rolling. Decreases in leaf area caused by leaf rolling were recorded on 10-10 H and BNA symptomatic leaves in 2013 and 2014. Twisted and unfolded flat leaves were photographed with

scale. Individual leaf areas for BNA (twisted and unfolded) and H leaves (not twisted) were determined using ImageTool (UTHSCSA, 3.0; San Antonio, Texas, USA) and expressed as cm^2/leaf (Figure 21).

Figure 21. BN-affected outstretched (left) and rolled leaf (right)
Legend: contour shows the rolling-caused decrease

Figure 22. Healthy (left) and BN-affected (right) leaves used for fresh and dry mass measurments

Leaf fresh and dry mass. Leaves from 20-20 H and BNA vines were collected in the middle of August in each experimental year at nodes 8–10. Four leaf discs (1 cm diameter) were punched out next to the central, main vein of each leaf (Figure 22). Fresh and dry (5 h at 55 °C in an oven) mass of the discs, as well as leaf water content (fresh mass minus dry mass) were determined and expressed as mg/cm^2.

Leaf chlorophyll content. At the end of August in 2012 and 2014, 20-20 H and BNA symptomatic leaves were collected to measure the relative chlorophyll (Chl) index in soil plant analysis development (SPAD) units (Steele *et al*. 2008). A Minolta SPAD-502 leaf Chl meter (Konica-Minolta, Osaka, Japan) was used to measure leaf transmittance in four positions per leaf, collected in the middle of the shoot. In 2012, additional measurements were recorded for leaves from the basal and apical shoot positions. To assess the Chl content corresponding to each SPAD unit, 12 leaf discs ranging in colour from yellow (lowest SPAD reading) to dark green (highest SPAD reading) were subjected to Chl extraction and spectrophotometric determination (Coste *et al*. 2010, Steele *et al*. 2008). Chlorophyll calculations were completed using Arnon's equation (1949).

6.2.3. Reproductive performance measurements

Fruit set. In 2013 and 2014, net bags were placed on inflorescences of H and BNA plants (20 each) before flowering at stage EL-17 according to Coombe (1995) (Figure 23). The fruit-set rate was determined based on the proportion of flower conversion into berries (Figure 24). Bunch mass, rachis mass, and the number of normal and abnormal berries (i.e. stenospermocarpic, parthenocarpic and dried) were also recorded.

Figure 23. Net bags from inflorescences (left) were eliminated after flowering (right)

Figure 24. To determine fruit-set rate flowers (left) and berries (right) were counted

Yield. Yield (by weight) and the number of bunches were recorded on 15-15 H and BNA vines. Bunch mass was recorded based on yield/number of bunches. Berry mass was calculated based on the weight of 100 berries, where 20 and 10 berries were collected from five H and 10 BNA bunches, respectively. The numbers of asymptomatic, symptomatic (i.e. shrivelled berries) and dried bunches per vine were also recorded.

Fruit composition. Fruit composition was characterized on 15-15 H and BNA vines by measuring soluble solids (°Brix) with a digital refractometer (Atago PAL-1, Japan), titratable acidity (TA) (g/L tartaric acid, after titration), and pH (Thermo, Orion TriStar, USA). All bunches from BNA plants and five randomly selected bunches from H plants were analysed.

6.2.4. Small-scale wine production

Grapes of three vintages (2012, 2013 and 2014) were hand-harvested at full maturity on 31st August 2012, 17th September 2013, and 18th September 2014, which was determined based on °Brix and titratable acidity values. Musts were fermented in the winery of Károly Róbert College, Research Institute of Viticulture and Enology. In all three experimental blocks, total yield was gathered separately for H and BNA vines. Grapes (60 kg/batch) from healthy (total yield) and BNA vines (total yield) were processed separately. H and BNA grapes were sectioned out three parts and fermented in three oenological replicates of each batch in 2012 and 2013. In 2014, one replicate of each batch was processed because of a limited quantity of grape yield. Additional wine was made (BNS) in 2013 and 2014, when only affected bunches (BN-shrivelled) were selectively gathered from infected shoots. Due to the limited number of symptomatic bunches, one oenological replicate of BNS wine (40 kg /batch) was made in each year. It is important to note that dried bunches were avoided during harvesting. Quick crushing and destemming were followed by the addition of sulphite (20 mL/hL Sterisol) and treatment with pectolitic enzymes. After pressing (balloon press, 1.5 Bar), the musts were settled for 24 h at 5 °C. Controlled fermentations were conducted in 20-L glass jugs at 12 °C using a starter yeast culture (Uvaferm; Lallemand S.A.S, Saint Simon, France). Complex fertilizer (Uvavital Komplex; Danstar Ferment AG,

Switzerland) was added three times. The free sulphur concentration of the fermented batches was adjusted to 30 mg/L. Wines were fined with calcium/sodium bentonite, and the final concentration of free sulphur was adjusted to 40 mg/L in each batch. Wine was bottled in February in all years.

6.2.5. Wine analyses

The following parameters were measured for 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 (Al, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Sr, Ti, V, Zn, Li, and Si) (ICP-AES, ICAP-9000 spectrophotometer, Thermo-Jarell-Ash, USA) 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 András Szekeres and Ottó Bencsik (University of Szeged, Department of Microbiology). The detailed mesurment protocol is being published (Ember *et al*. under submission).

6.2.6. 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 an unmarked line scale from 0 (poor) to 100 (prominent).

6.2.7. Statistical analyses

Two-way ANOVA models with factors ‘disease’ (BNA vs. H) and years (2012, 2013 and 2014) were used to detect the differences in the following variables: pruning mass; number of the respective lignified and non-lignified canes per vine; leaf Chl content (2012 and 2014); and leaf water content. The significance of the decreases in leaf area due to leaf rolling was determined using a one-sample *t*-test (with test parameter 0%). Results for 2013 and 2014 were compared by a two-sample *t*-test for independent samples. Leaf fresh and dry mass were highly correlated ($R = 0.6$; $P < 0.001$). Therefore, a two-way MANOVA model with factors ‘disease’ (BNA vs. H) and years (2012, 2013 and 2014) was used to assess the significance of the differences between

factor levels. Yield, number of bunches, bunch mass, and 100-berry mass were also dependent variables which indicated the application of two-way MANOVA model for statistical evaluation with factors ‘disease’ (BNA vs. H) and years (2012, 2013 and 2014). The same method was used to analyse the dependent fruit composition parameters, soluble solids (°Brix), TA, and pH. The significance of the numbers of diseased or dry bunches per vine was tested using a one-sample *t*-test (with test parameter 0). Results for all three experimental years were compared by one-way ANOVA. Bunch and rachis fresh mass, and the number of berries were evaluated as dependent variables using the two-way MANOVA model with factors ‘disease’ (BNA and H) and years (2013 and 2014). The same method was used for the comparisons of the numbers of normal, abnormal, and dry berries per bunch. The number of flowers was analysed using a two-way ANOVA model with the same two factors. Results of wine analyses were evaluated by a two-way MANOVA model with disease factors (BNA and H) and years (2012 and 2013) for basic parameters, organic acids and elements, and simple phenols. The pith:wood ratios of H and BNA vines were compared using a one-way MANOVA model.

When normality of the residuals was required, the absolute values of skewness and kurtosis of the error terms were proved to be below 1. Homogeneity of variances was cheked by Levene’s test ($P > 0.05$). If the MANOVA test indicated significance (with a significant Wilk’s lambda; $P < 0.05$), a follow-up one-way ANOVA was run to detect the factor effects regarding each variable. The sensory analysis evaluations of the 11 panellists were analysed using Mann–Whitney’s U test. The most significant factors for BN disease were defined by neural networks and discriminant analysis (Haykin 1999, Tabachnic and Fidell 2007).

For statistical, including neural networks and discriminant analyses, IBM SPSS version 22 (IBM Corp., Armonk, NY, USA) was used.

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

6.3.1. Experimental site

The experimental site was the same as described in 6.2.1. Three random blocks per treatment (containing 50 plants per block) were assigned (Figure 25), in which the phytoplasma infection status (severity and incidence) of each individual plant was visually evaluated before harvest during each year of the experiment (2011–2014) in the same way as described in 6.2.1. (Table 10). At the beginning of the experiment, in 2011, the infection rate of the blocks ranged from 50-70 % (data not shown).

6.3.2. Resistance inducer treatments

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 Kendal (Valagro, Italy) with glutathione-oligosaccharine (3 l/ha) and Bion (Syngenta, Switzerland) with benzothiadiazole (0.2 kg/ha) active compounds according to Romanazzi *et al*. (2009). The commercial products were kindly provided for experimental use by the Hungarian Representative Agencies of Syngenta and Valagro Companies. These elicitors were dissolved in tap water and sprayed using a knapsack sprayer 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. The volume equivalent of the first treatment was 300 l/ha and later 600 L/ha. Disease severity and incidence (based on Table 10), as well as the disappearance of the symptoms were recorded in each experimental year in September from 2011 to 2015.

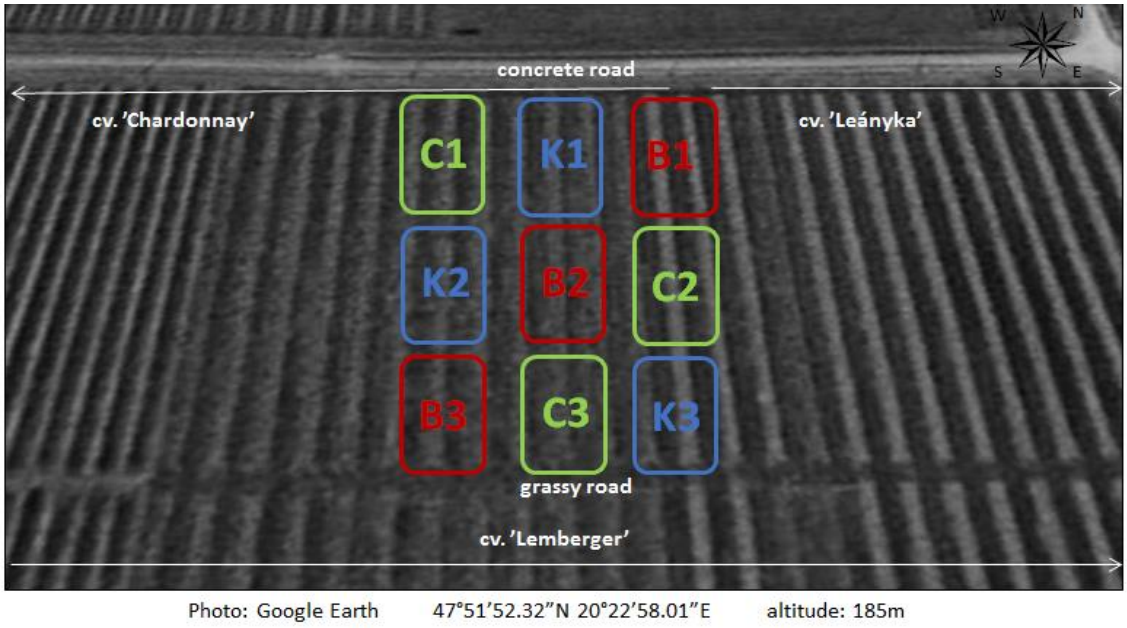

Figure 25. Arial photo of the experimental plot of resistance inducer treatments

Legend: 3 blocks of treatments marked in different colours, green: C1-C2-C3 untreated control, blue K1-K2-K3 Kendal treatment, red: B1-B2-B3 Bion treatment.

Effectiveness of the treatments was determined by calculations of the relative frequencies of recovery (N), duration/carry of symptomless status, and relapses (Table 11, 12).

The R values i.e. R0, R1, R2, R3 and R4 values were calculated for all blocks of each treatments/groups i.e. Control (C), Kendal (K) and Bion (B).

i) In each year the R values of the treatments/groups C, K, B were separately/severally compared. Thus, the numbers of comparisons were four for R0 and R1, three for R2, two for R3 and one for R4.

ii) In each group/treatment years were compared. The coparisons between R values were the followings: the R0 and R1 in four years (2012-2015), R2 in three years (2013-2015) and R3 in two years (2014-2015). In the case of R4 (2015) the result was compared to zerus values.

In each case of R0, R1, R2 and R3 three coparisons were carried out. For R4 there was an only nonzero results which was tested against zero.

Those of described comparions were tested using MANOVA, as well as Marascuillo's procedure (Marascuillo and McSweeney 1977).

Table 11. Evaluation of the effectiveness of the treatments

Status (n-1)	S	GY symptomatic vines in year n-1
	A	Asymptomatic vines in year n-1
Frequencies	II	Stayed symptomatic in year n compared to year n-1
	I	Become symptomatic in year n compared to year n-1
	N0	Stayed asymptomatic in year n compared to year n-1
	N1	Remissoned in year n compared to year n-1
	N2	Remissoned and stayed asymptomatic in year n compared to year n-2
	N3	Remissoned and stayed asymptomatic in year n compared to year n-3
	N4	Remissoned and stayed asymptomatic in year n compared to year n-4
Relative frequencies	R0	Stayed asymptomatic in year n compared to year n-1
	R1	Remissoned in year n compared to year n-1
	R2	Remissoned and stayed asymptomatic in year n compared to year n-2
	R3	Remissoned and stayed asymptomatic in year n compared to year n-3
	R4	Remissoned and stayed asymptomatic in year n compared to year n-4

Table 12. Calculation of relative frequencies of remission, duration of symptomless status and relapses

Meaning of R value		**Equation for 2012**	**Equation for 2013**	**Equation for 2014**	**Equation for 2015**
R0	Stayed asymptomatic in year n compared to year n-1	N0 (2012)/A (2012)	N0 (2013)/A (2013)	N0 (2014)/A (2014)	N0 (2015)/A (2015)
R1	Recovered in year n compared to year n-1	N1 (2012)/S (2012)	N1 (2013)/S (2013)	N1 (2014)/S (2014)	N1 (2015)/S (2015)
R2	Recovered and stayed asymptomatic in year n compared to year n-2		N2 (2013)/S (2012)	N2 (2014)/S (2013)	N2 (2015)/S (2014)
R3	Recovered and stayed asymptomatic in year n compared to year n-3			N3 (2014)/S (2012)	N3 (2015)/S (2013)
R4	Recovered and stayed asymptomatic in year n compared to year n-4				N4 (2015)/S (2012)

7. RESULTS

7.1. Epidemiology of Bois noir disease in Hungary

7.1.1. Genetic diversity of '*Ca.* P. solani' strains in Hungarian wine regions

7.1.1.1. Phytoplasma detection using conserved marker (16S rRNA)

The ecosystem of BN disease was surveyed in five wine regions of Hungary: Egri, Tokaji, Kunsági, Villányi, Soproni and Etyek-Budai. The results revealed that '*Ca*. P. solani', the causal agent of Bois noir disease, is present in all 16 locations of the wine regions, on both grapevine and wild reservoir plants. The pathogen was also detected on other crops such as tomato, potato, pepper celery and lavender (Table 14). Using nested PCR/RFLP the presence of phytoplasma belonging to 16SrXII-A subgroup ('*Ca*. P. solani') was identified in 78 samples out of 136, none of the other phytoplasmas were detected. Phytoplasma-infected stinging nettles were not found (data not shown). Among the '*Ca*. P. solani' positive samples 46 were selected for MLST (Table 14).

7.1.1.2. MLST of '*Ca.* P. solani' strains using variable markers

Development of new markers. To expand the list of genotyping markers primers for *yidC, ligA, priA, alaS* and *pheT* housekeeping genes were developed (Table 6). Nested PCR for *yidC, ligA* and *priA* was successfully optimized, obtaining a discrete band necesarry for direct sequencing. In the cases of *alaS* and *pheT* non-specific backgrounds were obtained and these genes were therefore excluded from sequence analyses. Variability of the markers *yidC, ligA* and *priA* were tested on reference strains of '*Ca*. P. solani'. Amplicons of each gene obtained from these strains were sequenced. In the case of *yidC* gene sequences were grouped into nine genotypes, and we were able to differentiate GGY and 1925 strains based on six mutations (Table 13). Genetic markers *ligA* and *priA* showed less variability, as strains grouped in three and two genotypes, respectively (Table 13). Based on these results, the *yidC* and *ligA* markers appeared to be a useful marker for '*Ca*. P. solani' MLST.

Table 13. Results of *yidC, ligA* and *priaA* genotyping

Gene	Genotypes					
yidC	**Y1** PO	**Y2** T292	**Y3** P7, CL, TOTK10, AZ-AU06, I5, I1, REP5, HO11	**Y4** GGY	**Y5** I29	**Y6** 1925
				Y7 DEP	**Y8** 12HN15	**Y9** KB181/3
ligA	**L1** PO, GGY, 1925, AZ-12HN15, CL, LG DEP, P7, TOTK10, I5	**L2** REP5, HO11, I1	**L3** POT4			
priA	**P1** PO, GGY, 1925, I29 I5, CL, HO11, I1, TOTK10, AZ_12HN15	**P2** DEP				

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Table 14. Result of phytoplasma detection and molecular characterisation of Hungarian '*Ca*. P. solani' isolates

Code	Plant species	Cultivar	Origin	Sampling site	Observed symptoms	16Sr DNA	*tuf*	*vmp1*	*secY*	*stamp*	*yidC*	Code
B85	*Vitis vinifera*	white variety	Csikéria	abandoned field	yellowing, leaf rolling	16SrXII-A	tuf-b	-	S6	ST6	Y6	**B85**
N15	*Vitis vinifera*	-	Kecskemét	vineyard	reddening, leaf rolling	16SrXII-A	tuf-b1	V14	S7	ST9	nt	**N15**
H20	*Vitis vinifera*	-	Barcs	private vineyard	yellowing, leaf rolling	16SrXII-A	tuf-b	V5	S7	-	nt	**H20**
ZA14	*Vitis vinifera*	Cabernet Franc	Siklós-Göntér	vineyard	sectorial reddening	16SrXII-A	tuf-b	-	S1	ST22	Y5	**ZA14**
ZA34	*Vitis vinifera*	Zweigelt	Homokszentgyörgy	vineyard	reddening, leaf rolling	16SrXII-A	tuf-b	V5	S6	ST6	Y6	**ZA34**
B201	*Rubus fruticosus*	-	Mád	Kakasok	reddening	16SrXII-A	tuf-b	-	S1	-	nt	**B201**
B200	*Vitis vinifera*	Hárslevelű	Mád	Kakasok	asymptomatic	16SrXII-A	tuf-b1	-	-	-	Y3	**B200**
B204	*Convolvulus arvensis*	-	Tolcsva	Mand	proliferation, herbicide suspect	16SrXII-A	tuf-b	V4	S1	ST9	Y3	**B204**
B319	*Convolvulus arvensis*	-	Tolcsva	Gyopáros	proliferation	16SrXII-A	tuf-b	-	S4	-	Y3	**B319**
B207	*Vitis vinifera*	Furmint	Tolcsva	Gyopáros	leaf rolling, yellowing, necrosis	16SrXII-A	tuf-b1	-	S1	ST9	Y3	**B207**
B208	*Vitis vinifera*	Furmint	Tolcsva	Kútpadka	leaf rolling, yellowing, bunch drying	16SrXII-A	tuf-b2	-	S6	ST6	-	**B208**
B209	*Vitis vinifera*	Furmint	Tolcsva	Gyopáros	leaf rolling, yellowing, bunch drying	16SrXII-A	tuf-b2	-	S6	ST6	nt	**B209**
B313	*Vitis vinifera*	Furmint	Tolcsva	Gyopáros (up)	yellowing, leaf rolling	16SrXII-A	tuf-b	-	S1	ST13	nt	**B313**
B320	*Vitis vinifera*	Pinot noir	Tolcsva	Gyopáros (low)	reddening total , leaf rolling	16SrXII-A	tuf-b1	-	S4	-	Y3	**B320**
B322	*Vitis vinifera*	Furmint	Tolcsva	Petrács	leaf rolling, yellowing, bunch drying	16SrXII-A	tuf-b	-	S1	ST9	nt	**B322**
B48	*Vitis vinifera*	-	Fertőszentmiklós	vineyard	leaf rolling	16SrXII-A	tuf-b	-	S6	ST6	Y6	**B48**
N128	*Solanum tuberosum*	Desiree	Röjtökmuzsaly	plot	reddening, leaf rolling	16SrXII-A	tuf-b1	V2	S1	ST4	nt	**N128**
N126	*Solanum tuberosum*	Demon	Röjtökmuzsaly	plot	reddening, lea rolling	16SrXII-A	tuf-b1	V14	S1	ST4	nt	**N126**
I11	*Vitis vinifera*	Zweigelt	Sopron	vineyard	sectorial reddening	16SrXII-A	-	V18	S6	ST6	Y6	**I11**
I1	*Vitis vinifera*	Zweigelt	Sopron	vineyard	sectorial reddening	16SrXII-A	tuf-b1	V9	S1	ST9D	Y1	**I1**
I5	*Vitis vinifera*	Zweigelt	Sopron	vineyard	sectorial reddening	16SrXII-A	tuf-b1	V2	S1	ST4	Y5	**I5**
I6	*Vitis vinifera*	Zweigelt	Sopron	vineyard	sectorial reddening	16SrXII-A	tuf-b1	V2	S1	ST4	nt	**I6**
B12	*Vitis vinifera*	Chardonnay	Andornaktálya	abandoned field	yellowing, leaf rolling	16SrXII-A	tuf-b	V13	S1	ST9	nt	**B12**
B17	*Vitis vinifera*	Merlot	Andornaktálya	vineyard	leaf rolling, reddening,	16SrXII-A	tuf-b	V13	S1	ST9	Y3	**B17**
B138	*Convolvulus arvensis*	-	Eger	Kőlyuktető	proliferation, small leaves	16SrXII-A	tuf-b	V13	S1	ST4	Y1	**B138**
B133	*Vitis vinifera*	Chardonnay	Eger	Kőlyuktető	leaf rolling, yellowing	16SrXII-A	tuf-b	-	S1	-	-	**B133**
B134	*Vitis vinifera*	Chardonnay	Eger	Kőlyuktető	leaf rolling, yellowing	16SrXII-A	tuf-b	V13	S1	-	nt	**B134**
B135	*Vitis vinifera*	Chardonnay	Eger	Kőlyuktető	leaf rolling, yellowing	16SrXII-A	tuf-b	V13	S1	-	nt	**B135**
PN1	*Vitis vinifera*	Pinot noir	Eger	vineyard	reddening, leaf rolling	16SrXII-A	tuf-b1	V18	S6	ST6	Y6	**PN1**
PN3	*Vitis vinifera*	Pinot noir	Eger	vineyard	reddening, leaf rolling	16SrXII-A	tuf-b1	V18	S6	ST6	Y6	**PN3**
ZA54	*Vitis vinifera*	Chardonnay	Vécs	vineyard	yellowing, leaf rolling, necrosis	16SrXII-A	tuf-b	-	S1	-	nt	**ZA54**

Table 14. continue

Code	Plant species	Cultivar	Origin	Sampling site	Observed symptoms	16Sr DNA	*tuf*	*vmp1*	*secY*	*stamp*	*yidC*	Code
B77	*Lavandula angustifolia*	-	Budapest	Kopaszi barrier	partial dry out	16SrXII-A	tuf-b	-	S6	ST52	Y6	**B77**
B91	*Convolvulus arvensis*	-	Etyek	vineyard	bushy shape, stunt	16SrXII-A	tuf-b	-	-	-	nt	**B91**
B88	*Vitis vinifera*	red variety	Etyek	vineyard	reddening, leaf rolling	16SrXII-A	tuf-b	-	S1	ST9D	Y1	**B88**
B126	*Vitis vinifera*	Chardonnay	Etyek	Orbán statue	yellowing, leaf rolling, dried branches	16SrXII-A	tuf-b	-	-	-	nt	**B126**
I31	*Apium graveolens*	-	Monorierdő	plot	yellowing	16SrXII-A	tuf-b	V18	S4	ST9*	nt	**I31**
I22	*Capsicum annuum*	-	Monorierdő	plot	yellowing	16SrXII-A	tuf-b1	V2	S4	ST9	Y1	**I22**
I23	*Capsicum annuum*	-	Monorierdő	plot	yellowing	16SrXII-A	tuf-b1	V2	S4	ST9	nt	**I23**
B129	*Convolvulus arvensis*	-	Monorierdő	Vicinity	proliferation, yellowing	16SrXII-A	tuf-b	-	S1	ST9D	-	**B129**
B130	*Lamium purpureum*	-	Monorierdő	Vicinity	reddening	16SrXII-A	tuf-b	-	S6	ST6	-	**B130**
B131	*Lamium purpureum*	-	Monorierdő	Vicinity	asymptomatic	16SrXII-A	tuf-b	-	S1	ST9D	nt	**B131**
I27	*Solanum lycopersicum*	-	Monorierdő	plot	lace-like shoots, elongated leaves	16SrXII-A	tuf-b	-	S4	ST9	nt	**I27**
I29	*Solanum lycopersicum*	-	Monorierdő	plot	lace-like shoots, elongated leaves	16SrXII-A	tuf-b	V2	S1	ST22	Y5	**I29**
P179	*Solanum tuberosum*	-	Monorierdő	plot	reddening, leaf rolling	16SrXII-A	tuf-b1	V9	S1	ST9	nt	**P179**
P184	*Solanum tuberosum*	-	Monorierdő	plot	reddening, leaf rolling	16SrXII-A	tuf-b1	V9	S1	ST9	nt	**P184**
B132	*Ulmus minor*	-	Monorierdő	Vicinity	proliferation, yellowing	16SrXII-A	tuf-b	-	S1	ST9	nt	**B132**
Total:	***10 plant species***	***7 counties***	***16 locations***	***24 site***		***46 'Ca. P. solani' positive samples***	2	7	3	7	4	

Legend: -: no results obtained, nt: not tested, blue: *tuf* genotype results based on nested PCR-RFLP, red: *tuf* genotype results based on sequencing, ST9 and ST9D are different in 6 nt deletion (AAATCA), dotted line separate locations of the same county.

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

MLST of Hungarian '*Ca.* P. solani' isolates. Genetic markers*: tuf, secY, yidC, vmp1* and *stamp* were used to characterise '*Ca*. P. solani' positive samples/isolates. Altogether 46 isolates of which: 27 grapevine (*Vitis vinifera*), 2 tomato (*Solanum lycopersicum*), 4 potato (*Solanum tuberosum*), 2 pepper (*Capsicum annuum*), 1 celery (*Apium graveolens*), 1 lavender (*Lavandula angustifolia*), 5 bindweed (*Convolvulus arvensis*), 1 blackberry (*Rubus fruticosus*), 2 red deadnettle (*Lamium purpureum*) and 1 field elm (*Ulmus minor*) were genotyped (Table 14). Multilocus genotyping was not fully completed for some isolates certainly due to lower sensitivity of nested-PCR protocols when compared to the higly sensitive 16S rDNA nested-PCR. Additionally, in a few cases the received sequences were poor quality. Concerning the *yidC* gene, only part of the isolates was genotyped, because the results of this gene gave nearly identical results to the *secY* result.

Tuf. Based on PCR/RFLP of the *tuf* gene all the characterised isolates proved to be *tuf*-b 'bindweed' genotype (Figure 27). Sequence analyses confirmed *tuf*-b1 which is a variant of *tuf*-b and *tuf*-b2 which is a variant of *tuf*-a 'stinging nettle' type (Figure 26; Table 3, 14).

Vmp1. Amplification of the *vmp1* fragment of Hungarian isolates revealed a medium size (1450 bp) amplicon (Figure 28). The RFLP and sequence analyses confirmed the dominant presence of *vmp1* genotypes: V2, V9, V13 and V18 (Figure 29, 30, 31; Table 14).

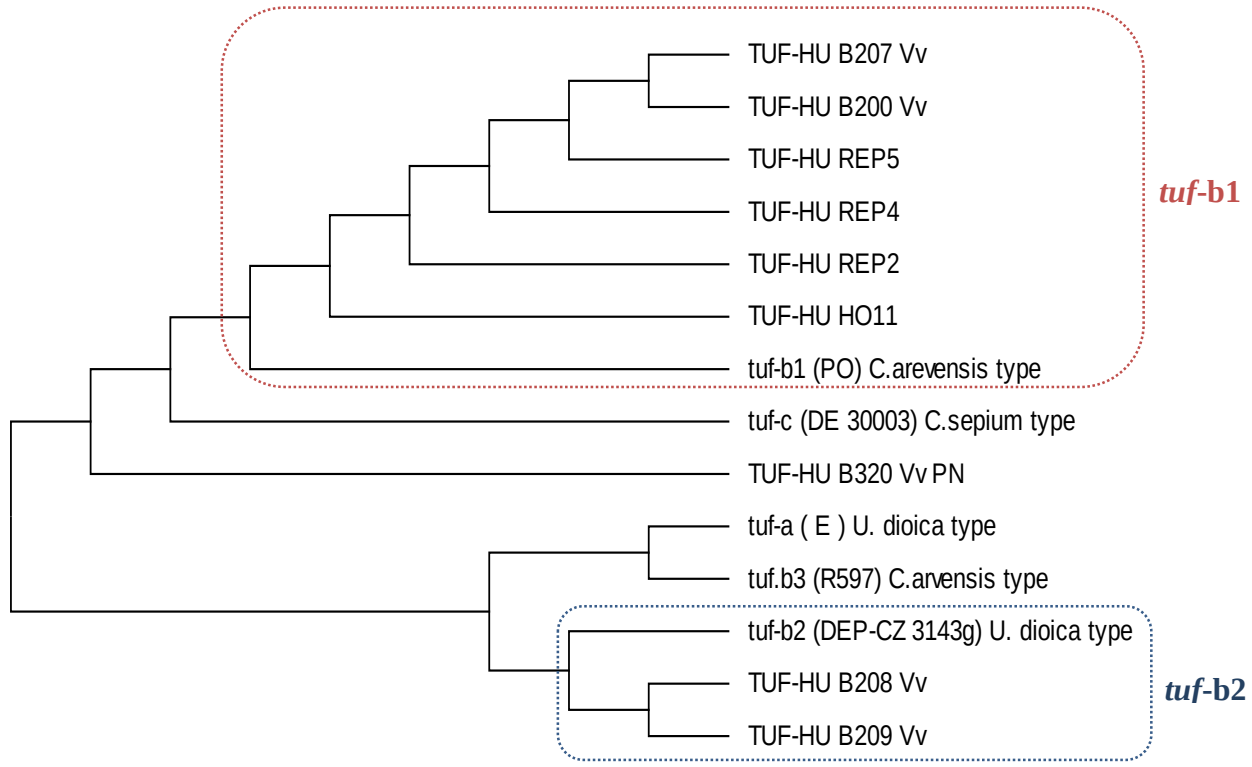

Figure 26. Phylogenetic tree (NJ) of *tuf* genetic locus

Legend: to consctruct Neighnor-Joining tree *tuf* reference secquences *tuf*-a, *tuf*-b1, *tuf*-b2, *tuf*-b3 were provided by Dr. X. Foissac and the Stolbur-Euromed Consortium (2004-2012).

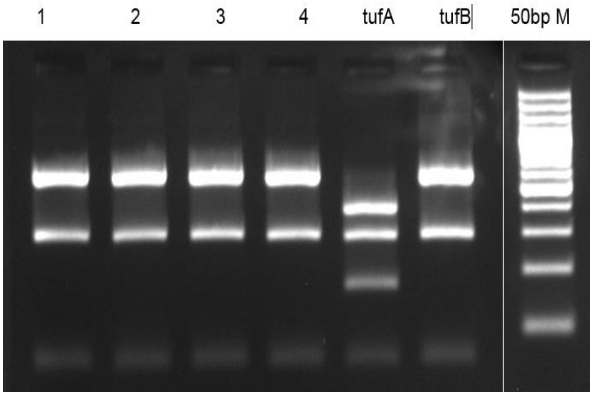

Figure 27. RFLP profile of *Hpa*II digested *tuf* FtufAY/RtufStol amplicon

Legend: 1-4 grapevine samples, tufA and tufB references, M: 50 bp Marker.

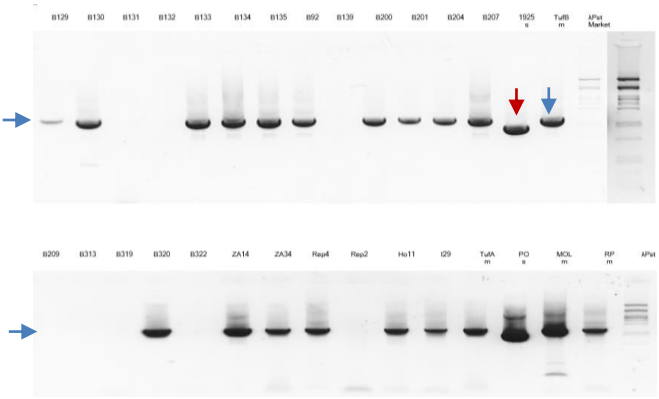

Figure 28. PCR of *vmp1* with TYPH10F/TYPH10R primers

Legend Figure 28-30: B numbers: samples (Table 14), ref.: 1925, TufB, TufA, PO, MOL, RP, blue arrow: medium size amplicon, red arrow: small size amplicon, M: λPst marker.

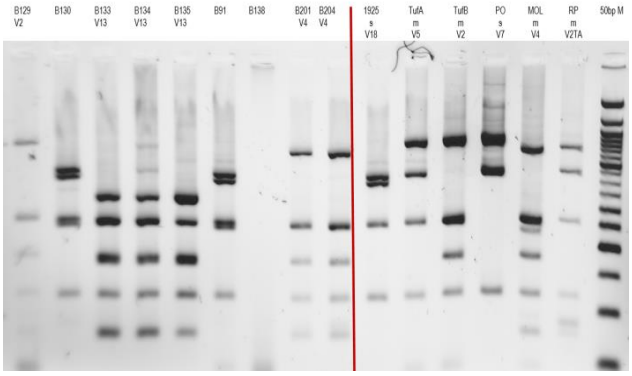

Figure 29. RFLP profile of *Rsa*I digested *vmp1* TYPH10F/TYPH10R amplicon

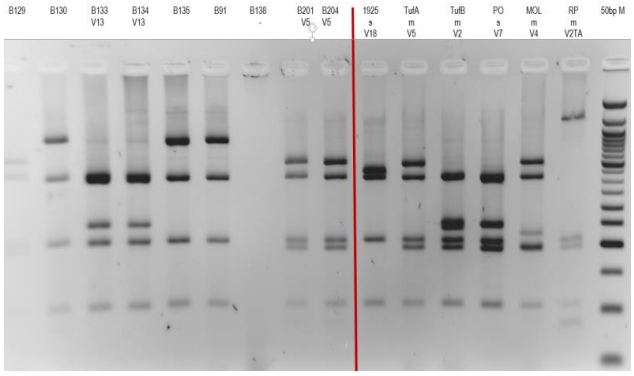

Figure 30. RFLP profile of *Alu*I digested *vmp1* TYPH10F/TYPH10R amplicon

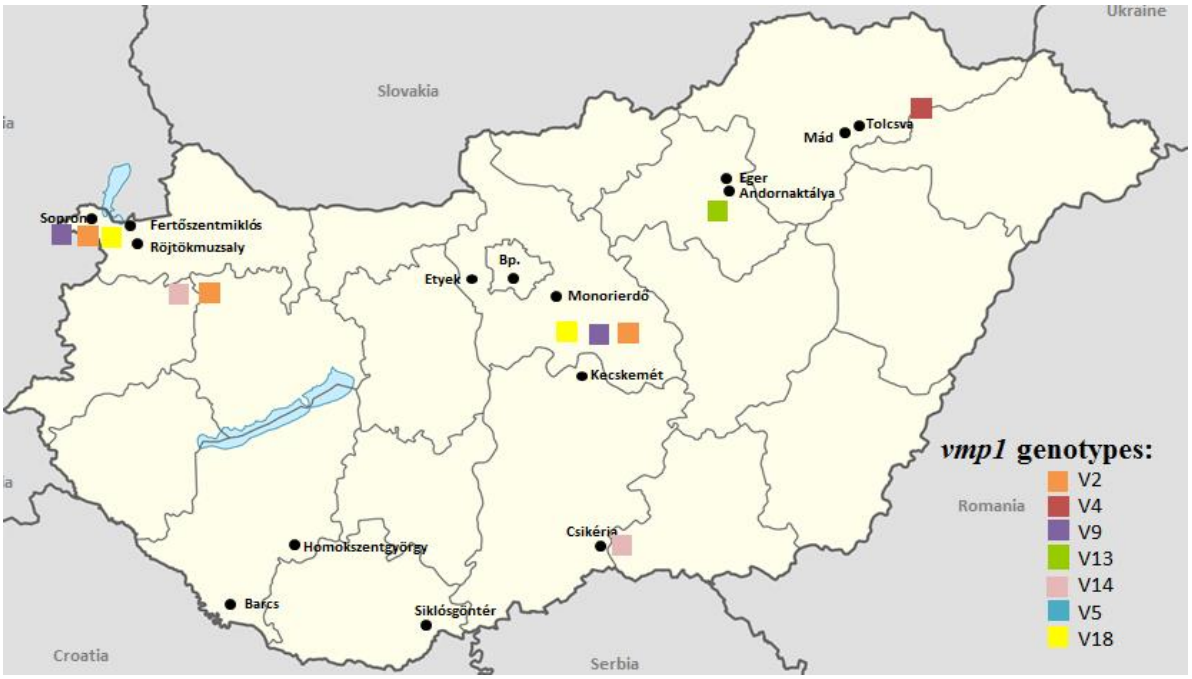

Figure 31. Geographical distribution of *vmp1* genotypes in Hungary

SecY. Four genotypes were detected with this conserved marker: S1, S4, S6 and S7 (Table 14; Figure 32, 33). The most prevalent were S1 (59 %) and S6 (24 %) (Table 14, Figure 32 A and C). On grapevine the most frequent genotype was S1 which was found on bindweed with very high abundance (Figure 32 D). Although in lower percentage, S4 genotypes were present on both plants (Figure 32 B). Sporadically S7 genotype was also present on grapevine. The *secY* S1 and S4 genotypes occurred on bindweed, red deadnettle (asymptomatic), field elm, and the S6 on lavender and on one of the red deadnettle (showing a reddening symptom) (Figure 32 B). Similar to the grapevine, S1 and S4 genotypes were detected on the other '*Ca*. P. solani' dead-end hosts i.e. *Solanaceous* plants and celery (Figure 32 D). Country-wide occurrence of *secY* genotypes showed consistent distribution of S1 and S6, while S7 was present only in the southern part of the country (Figure 34).

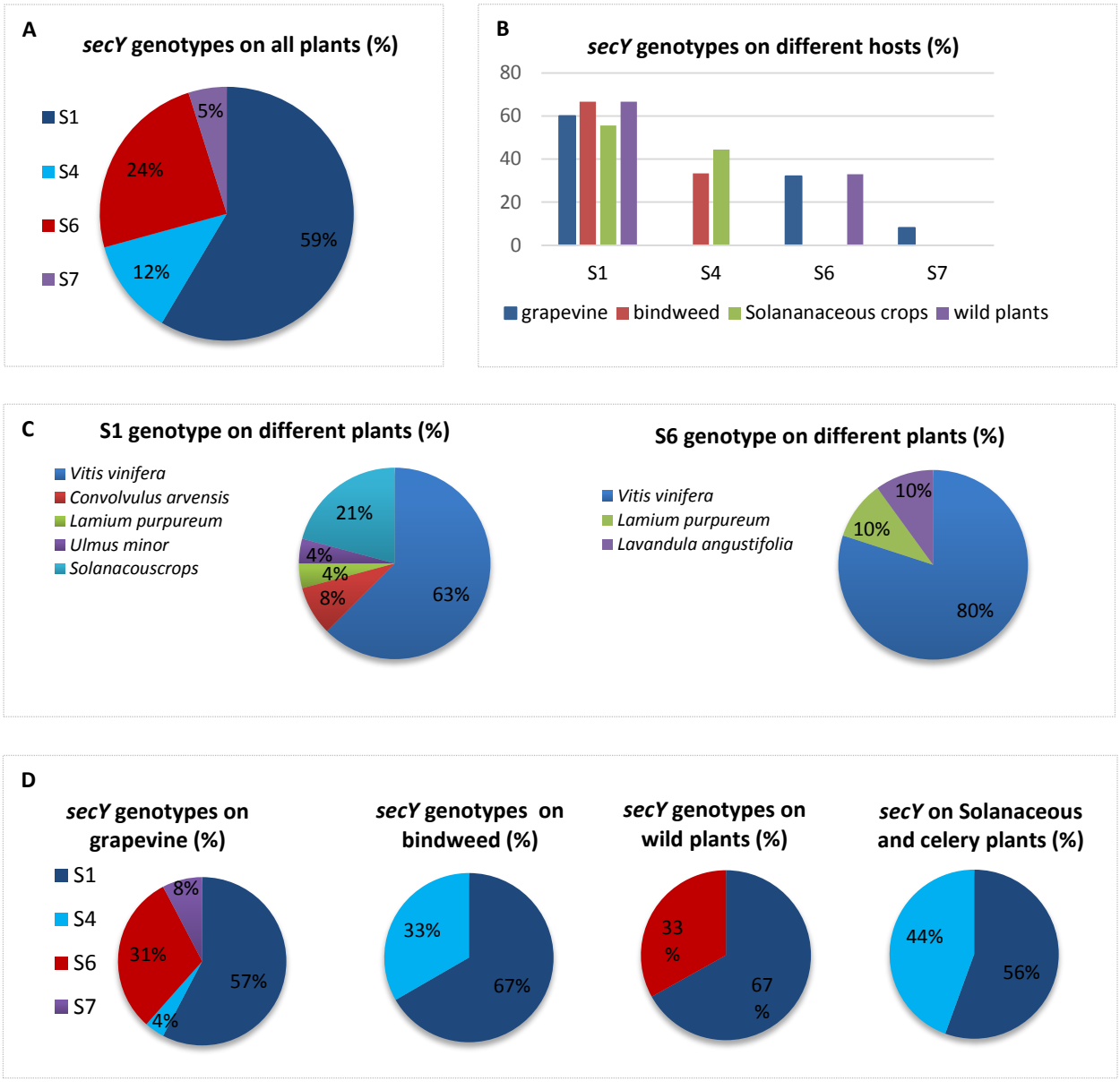

Figure 32. A-D. Distribution of *secY* genotypes in BN ecosystem

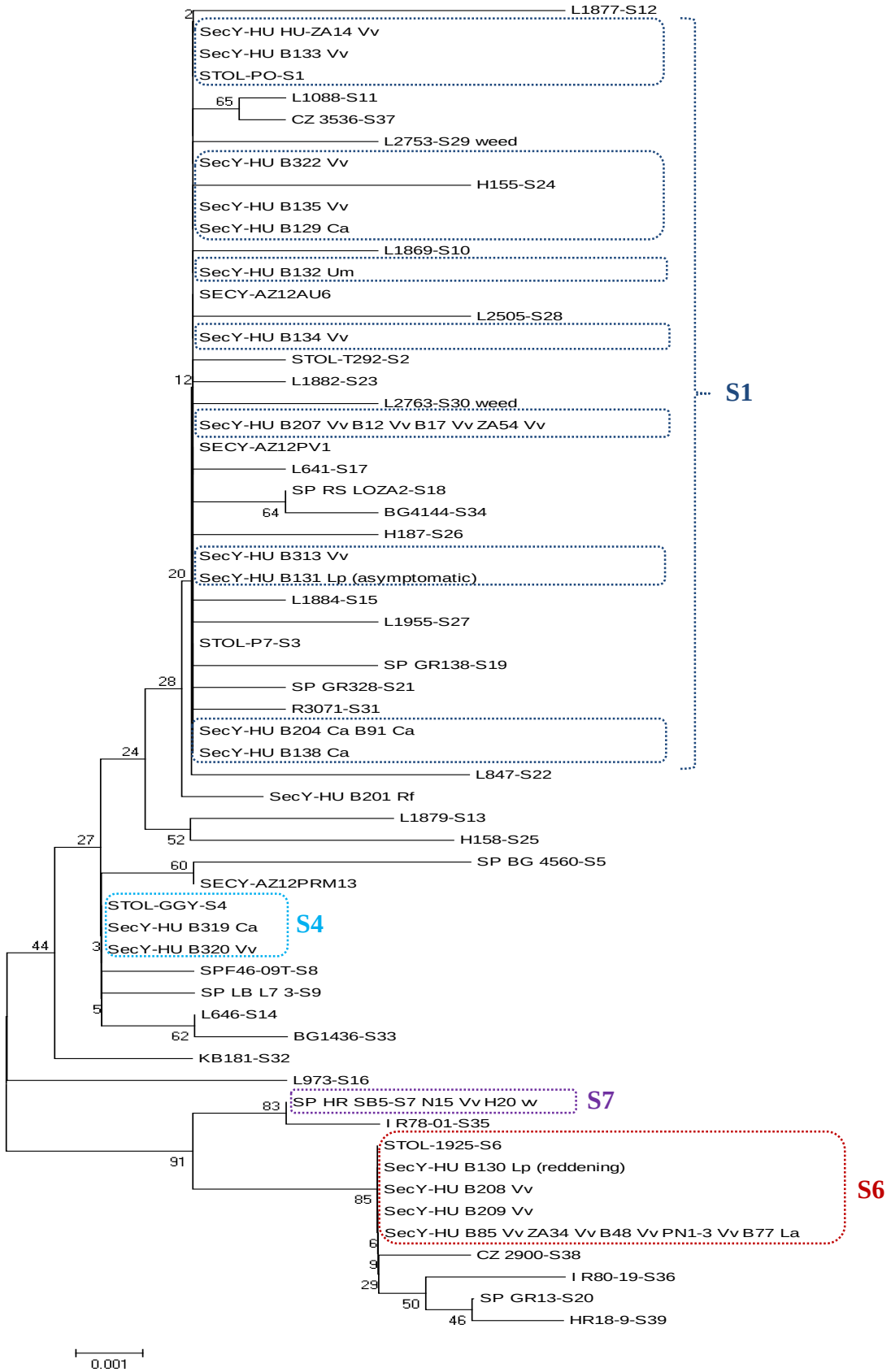

Figure 33. Phylogenetic tree of *secY* genetic locus (NJ)

Legend: to consctruct Neighnor-Joining tree (Tamura-Nei modell) *secY* reference secquences S1-S39 were provided by Dr. X. Foissac and the Stolbur-Euromed Consortium (2004-2012).

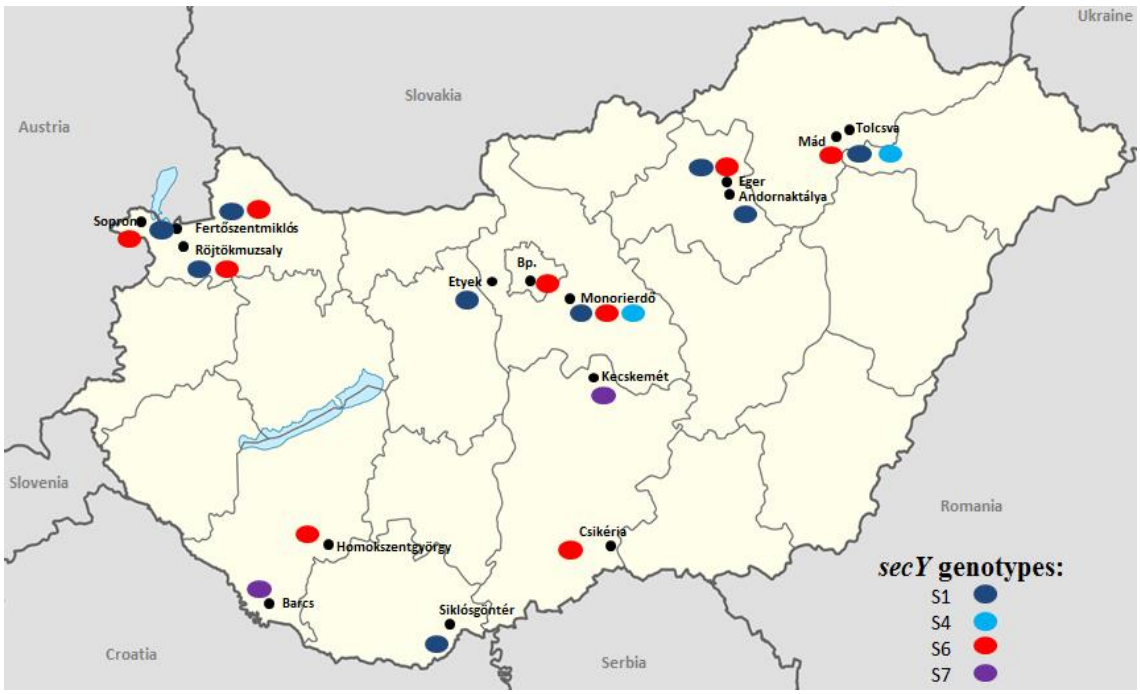

Figure 34. Geographical distribution of *secY* genotypes in Hungary

YidC. This housekeeping marker encoding protein plays an important role in protein translocation, resulting in three known genotypes (Table 14, Figure 35). These results were almost identical to *secY* results, which is not surprising as *secY* is also an essential protein for the protein secretory system of phytoplasmas (Figure 2).

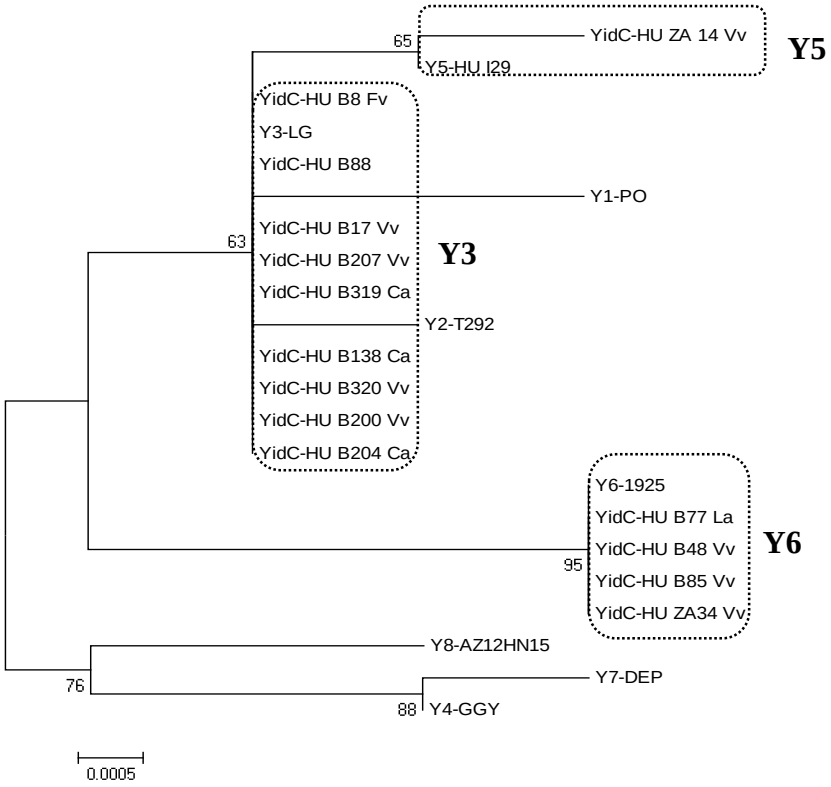

Figure 35. Phylogenetic tree of *yidC* genetic locus (NJ)

Legend: to consctruct Neighnor-Joining tree (Tamura-Nei modell) *yidC* reference secquences Y1-Y8 were provided by Dr. X. Foissac and the Stolbur-Euromed Consortium (2004-2012).

Stamp. This gene showed the highest variability among the examined loci (Table 14; Figure 36, 37, 38). Seven different known genotypes were found: ST4, ST9, ST9D (different from ST9 with a deletion), ST13, ST22, ST52 and ST6 (Figure 36 A, B). The most prevalent were the ST9 and ST9D, but ST6 and ST4 were also frequent (Table 14, Figure 36 A, C). On grapevine the most frequent genotypes were the ST6, ST4 and ST9, also ST13 was present (Figure 36 B, C, D). On bindweed ST9 and ST9D, and on *Solanaceous* plants both ST9, ST9D and sporadically ST22 were detected (Figure 36 B, D). Geographic distribution of the *stamp* locus could also be observed (Figure 38).

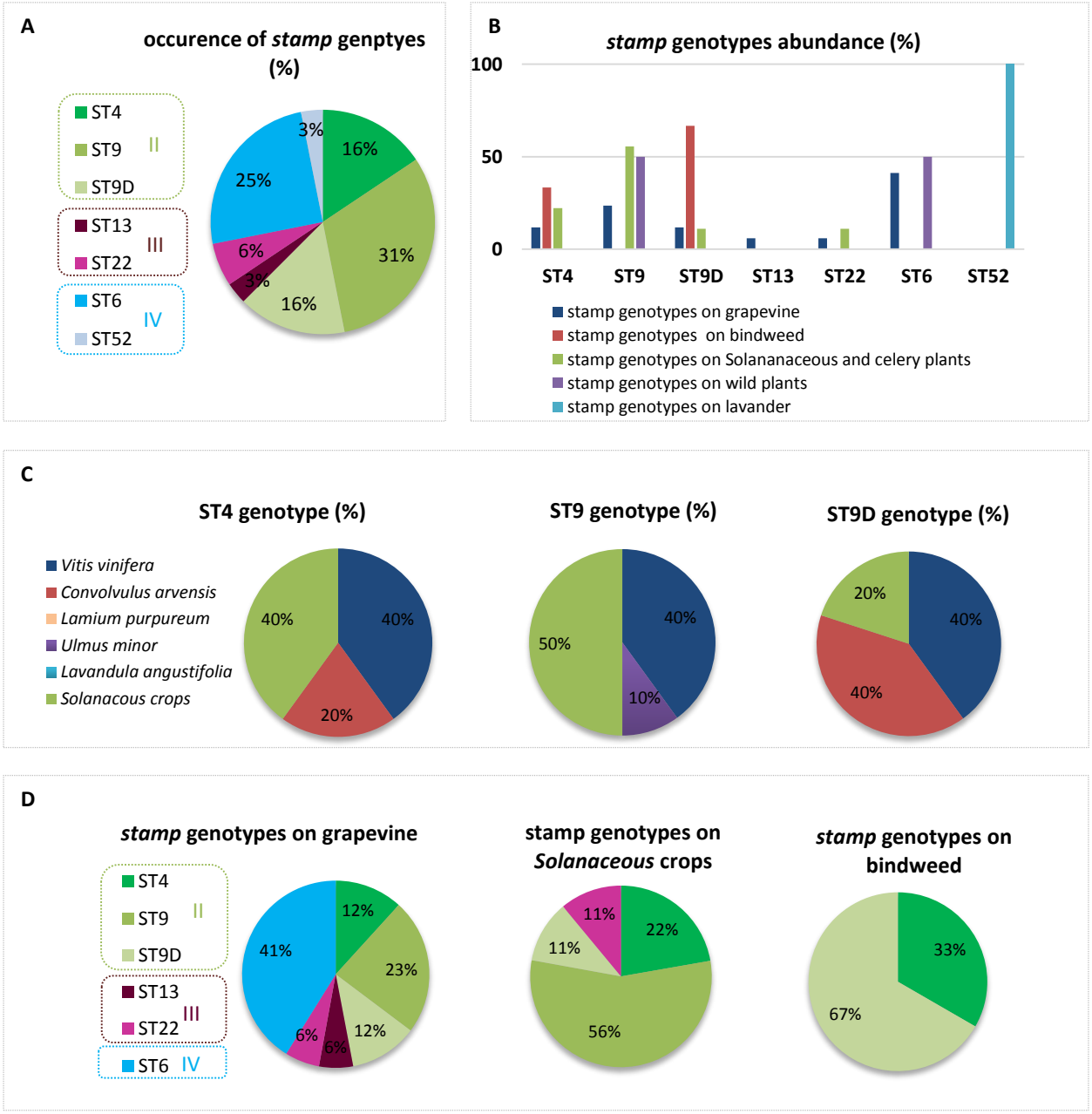

Figure 36. A-D. Distribution of *stamp* genotypes in BN ecosystem

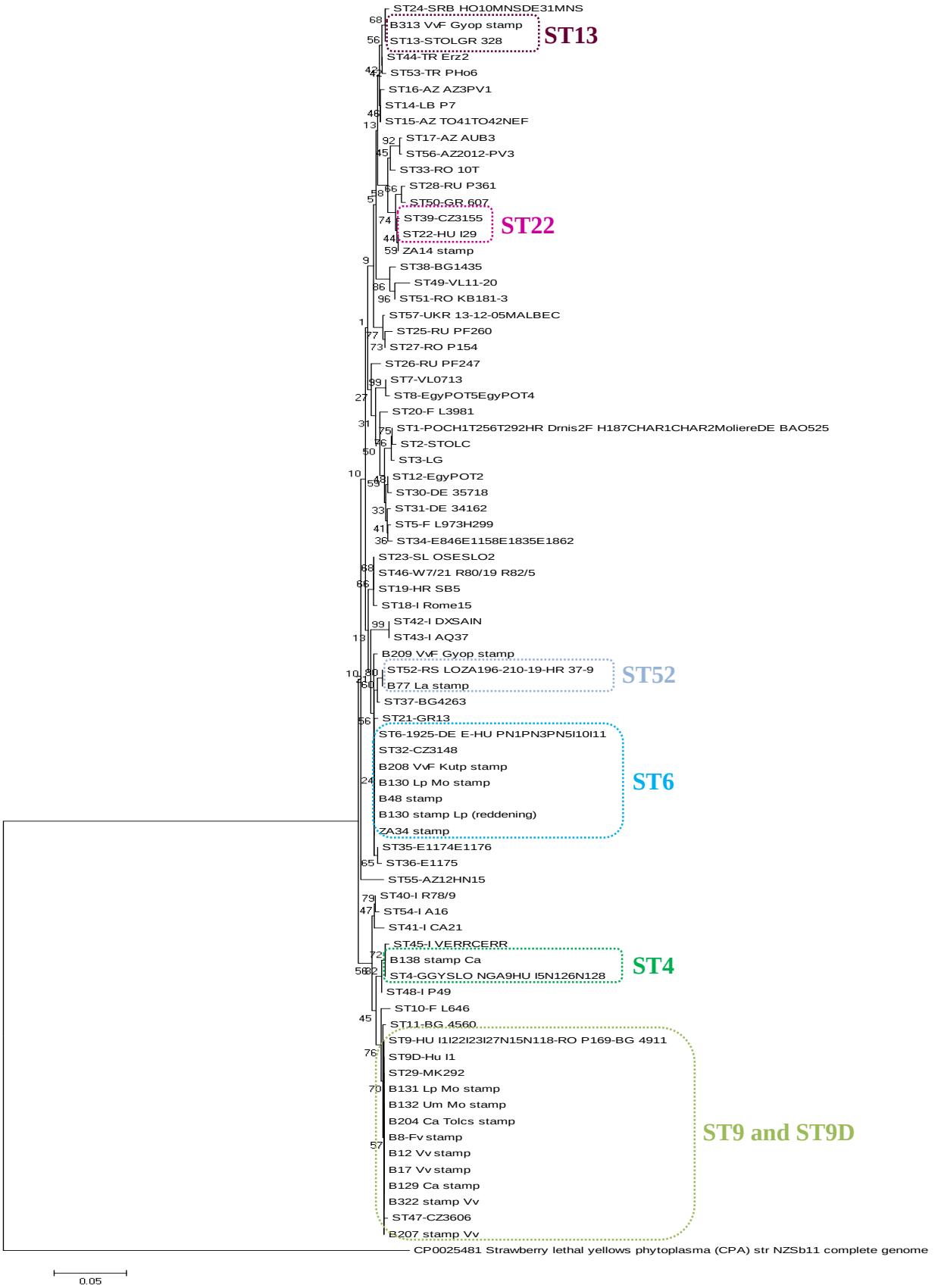

Figure 37. Phylogenetic tree of *stamp* genetic locus (NJ)

Legend: to consctruct Neighnor-Joining tree *stamp* reference secquences ST1-S57 were provided by Dr. X. Foissac and the Stolbur-Euromed Consortium (2004-2012), out group was Strawberry lethal yellows phytoplasma (CP0025481). In the case of strains of ST9 and ST9D strains the 6 nt deletion (AAATCA) was confirmed in each case (Table 14).

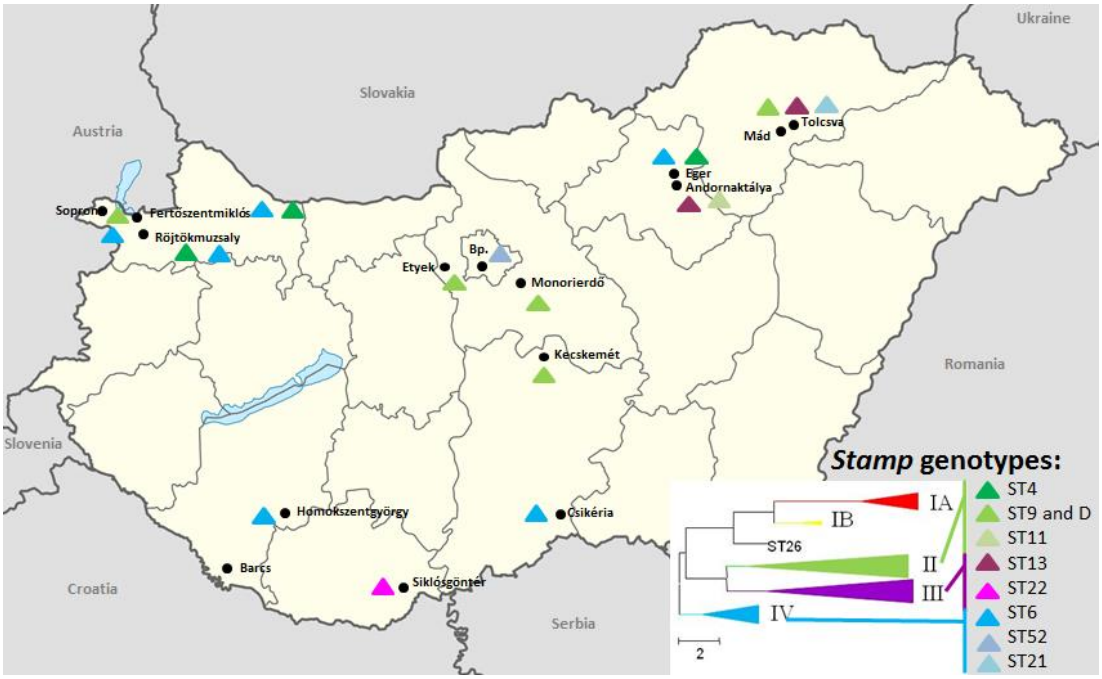

Figure 38. Geographical distribution of *stamp* genotypes in Hungary (cladogram Foissac *et al*. 2013)

In Hungary the most prevalent genotypes on grapevine were S6/V18/ST6, S1/V2/ST4 and S1/V2/ST9. The role of bindweed in spreading of S1/V2/ST4 and S1/V2/ST9 genotypes to grapevine was confirmed. Based on European and also on our results, the importance of stinging nettle and red deadnettle as main dissemination source of S6/V18/ST6 to grapevine can be suggested. However, the role of these plants in BN ecosystem in Hungary has to be confirmed. The presence of *stamp* ST13 genotype on grapevine -the genotype transmitted by *Reptalus quinquecostatus* to periwinkle- suggesting that this planthopper could be a competent vector of '*Ca*. P. solani' to grapevine (Figure 39).

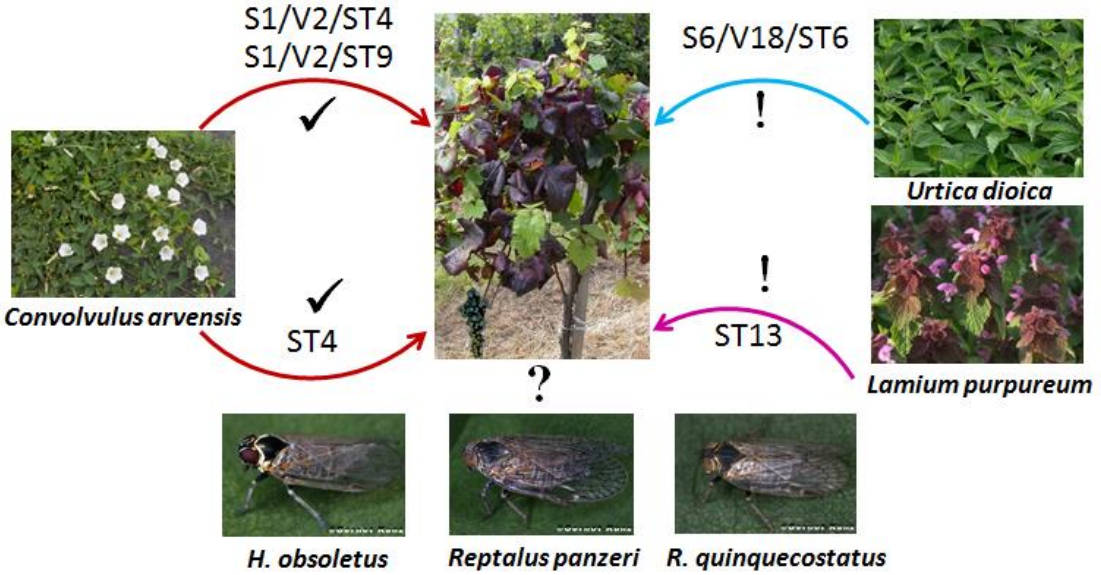

Figure 39. Role of '*Ca*. P. solani' genotypes in BN disease in Hungary

Legend: ✓: confirmed, !: confirmation needed, ?: no information available in Hungary, insect's photos: Gernot Kunz (gallery.kunzweb.net).

7.1.2. Insect transmission of Hungarian '*Ca.* P. solani' strains

In 2013, a transmission experiments were performed with planthoppers of the *Cixiidae* family collected in five wine regions of Hungary. Planthopper species were identified based on genitalia (done with the help and supervision of Jean-Luc Danet, entomologist, INRA, Bordeaux, France). As well as molecular testing of the COI gene was carried out. It was confirmed that *H. obsoletus*, *Reptalus panzeri*, *R. quinquecostatus*, *R. cuspidatus* and *R. melanochaetus* are present in Hungarian vineyards and/or in their vicinity on wild plants (Table 15). In the nine locations 152 specimens were collected and 14 transmission experiments were performed with *H. obsoletus* and 12 transmissions with *R. panzeri* and *R. quinquecostatus* species. The experimental host, Madagascar periwinkles (*C. roseus*) was exposed (5 insects/plant) to the insects for feeding. In four plants (HO11, REP2, REP4 and REP5) symptoms such as mild yellowing (arrow shape) and virescence appeared after 28 days (counted from the first day of feeding: day post inoculation=dpi) and severe yellowing was observed after 45-50 days (Figure 40).

Figure 40. Phytoplasma symptom appearance on periwinkles after transmission trials

These plants were re-grafted and are maintained in the phytoplasma collections of INRA (Bordeaux, France). The grafted plants showed mild symptoms of yellowing, virescence and phyllody after four weeks and severe symptoms after 8 weeks (Figure 41). Genotyping of HO11, REP2, REP4 and REP5 revealed that the isolates were harbouring *tuf*-b1, and *secY* S1 genotype. The result of *stamp* typing was the following: isolate HO11 ('*Ca.* P. solani' transmitted by *H. obsoletus)* was ST4 belonging to *stamp* clusters II; and REP2, REP4 and REP5 isolates ('*Ca.* P.

solani' transmitted by *R. quinquecostatus)* were ST13 of cluster III. In this insect transmission experiment it was demonstrated that *H. obsoletus* is able to transmit ST4 *stamp* genotype, and *R. quinquecostatus* is able to transmit ST13 *stamp* genotype.

HO11
top and bottom: leaf yellowing, without virescence

REP2
top: virescence and phyllody, bottom: leaf yellowing

REP5
top: virescence and phyllody, bottom: leaf yellowing

Figure 41. Phytoplasma symptom appearance on periwinkle plants after grafting

7.1.3. New generation sequencing of Hungarian '*Ca.* P. solani' strains

In the frame of a collaboration between INRA, UMR 1332 Biologie du Fruit et Pathologie, Mollicute Team, Phytoplasma group (Bordeaux, France) and Corvinus University of Budapest (from 1st January 2016 Szent István University), Department of Viticulture two strains of '*Ca.* P. solani', transmitted by *H. obsoletus* (HO11) and *R. quinquecostatus* (REP2) were subjected to genome sequencing. Prior to NGS phytoplasma enrichment using isopycnic cesium chloride density gradient in presence of bisbenzymide was successfully performed in France, and 5.7 mg DNA of (38 ng/µl) REP2 and 11.16 mg (186 ng/µl) DNA of HO11 strains were obtained. 2*100 pair-end libraries have been prepared by UDGenomed and the University of Debrecen Hungary. Sequencing has been carried out on an Illumina HiSeq platform. Read counts for three raw libraries were considered as pair-end reads, and base count has been calculated as read count * 200. Isolate read count base was 179.268.496 35 Gb for REP2 and 137.266.492 27 Gb for HO11. Adapter trimming and quality filtering has been carried out in one step by trimmomatic. Quality statistics of the filtered libraries have been created with fastQC. *De novo* assembly is in progress using SOAPdenovo and Velvet by Dr. Tamás Deák.

Table 15. Results of insect transmission trial, grafting, and the MLST of transmitted isolates

Location	Sampling site	Insect species	Host	No. of insects	Id. of male genit.*	Transm. code 2-4/7/2013	Symptom appearance		MLST plant			Insects	Symptoms /date of grafting	
									stamp	*secY*	*tuf*	*COI*		
Eger	Kőlyuktető	*Hyalesthes obsoletus*	*C. arvensis*	4	-	HO1	no	-	-	-	-	-	-	
Sopron	over the fences	*Hyalesthes obsoletus*	*U. dioica*	20	-	HO2-9	no	-	-	-	-	-	-	
Monorierdő	Quality control Inst., potato field	*Hyalesthes obsoletus*	*S. tuberosum*	4	-	HO10	no	-	-	-	-	-	-	
		Hyalesthes obsoletus	*C. arvensis*	5	-	HO11	V, sY	29/7/2013	ST4	S1	TufB1	-	Y	25/9/2013
Etyek	Vineyard close to St. Orban statue	*Hyalesthes obsoletus*	C. arvensis	2	-	HO12	no	-	-	-	-	-	-	-
Lovászi	next to vineyard, nettle patch	*Hyalesthes obsoletus*	*U. dioica*	33	-	HO1-14	no	-	-	-	-	-	-	-
Eger	Chardonnay/1st field	*Reptalus quinquecostatus*	Weeds	1	✓	REP1	no	-	-	-	-	R.q.	-	-
Andornak-tálya	Dirty road with *Polygonum aviculare, Prunus cerasifera,* next to vineyard and cereal field	*Reptalus quinquecostatus*	*P. cerasifera, Polygonum aviculare*	4	✓	REP2	V, sY	29/7/2013	-	-	-	R.q.	Strong Phy, Y	-
		Reptalus quinquecostatus		6	✓	REP2	V, sY	29/7/2014	ST13	S1	TufB1	R.q.		25/9/2014
		Reptalus panzeri		1	✓	REP4	Y	4/8/2013	ST13	S1	TufB1	R.p.	Phy, Vir, Y	-
		Reptalus quinquecostatus		9	✓	REP4	Y	4/8/2013	-	-	-	-		25/9/2016
		Reptalus quinquecostatus		6	✓	REP5	V, Y	29/7/2013	ST13	S1	TufB1	-	sPhy , Y	25/9/2018
		Reptalus quinquecostatus		8	✓	REP3	-	-	-	-	-	-	-	-
Fertőd	Potato field	*Reptalus quinquecostatus*	*S. tuberosum*	3	✓	REP6	-	-	-	-	-	R.q.	-	-
Etyek	Vineyard close to St. Orbán statue, middle of rows of vine	*Reptalus cuspidatus*	*Crategus monogyna,* weeds	20	✓	REP7-12	-	-	-	-	-	R.c.	-	-
		Reptalus cuspidatus		20	✓	REP7-12	-	-	-	-	-	R.c.	-	-
Tolcsva	Upper plot	*Reptalus melanochaetus*	*Prunus spinosa*	6	✓	NO	-	-	-	-	-	R.m.	-	-

Legend: Phy: phyllody, V: virescence, Y: yellowing, s: severe, -: no result/not tested, *: identification based on male genitalia, ✓: identification was performed, R.p.: *R. panzeri*, R.q.: *R. quinquecostatus*, R.c.: *R. cuspidatus*, R.m.: *R. mealnochaetus*.

7.1.4. Insect-pathogen protein interaction

To investigate interaction ability between STAMP and insect protein (IP), recombinant STAMPs were first produced and then dot-blot analyses were performed using purified 16 kDa STAMP(s) fp_ST4, fp_ST9 with protein of different planthopper species.

7.1.4.1. Heterologous expression of recombinant STAMPs of '*Ca.* P. solani' strains

Heterologous expression of *stamp* genetic clusters II, III and IV of the central hydrophilic part of stolbur antigenic membrane protein (STAMP) of '*Ca*. P. solani' strains ST4, ST9, ST13, and ST6 was performed. Using poly-His MAb in western blot analyses a 16 kDa size His6X-tagged fusion protein of each cluster was confirmed (Figure 42).

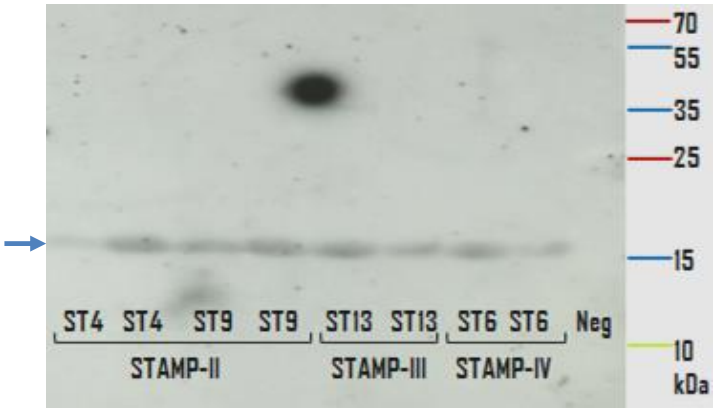

Figure 42. Western blot of recombinant STAMP(s) of cluster II, III, and IV, revealed with poly-His MAb

Legend: ST4: fp_ST4, ST9: fp_ST9, ST6: fp_ST6, ST13: fp_ST13, arrow: 16 kDa STAMP(s), Neg: BSA control, M: protein PageRuler™ Prestained Protein Ladder (ThermoFicher Scientific): 10, 15, 25, 35, 55, 70, 100, 130 kDa.

In western blot analysis of the extracted phytoplasma protein (from different phytoplasma strains maintained on periwinkle), *stamp* cluster II and III strain recognition by MAb 2A10 was first demonstrated (Figure 43 A, B). Lack of signal in 1925 and GGY strains was due to the loss of these isolates (Figure 43 A), which was verified by PCR test (data not shown).

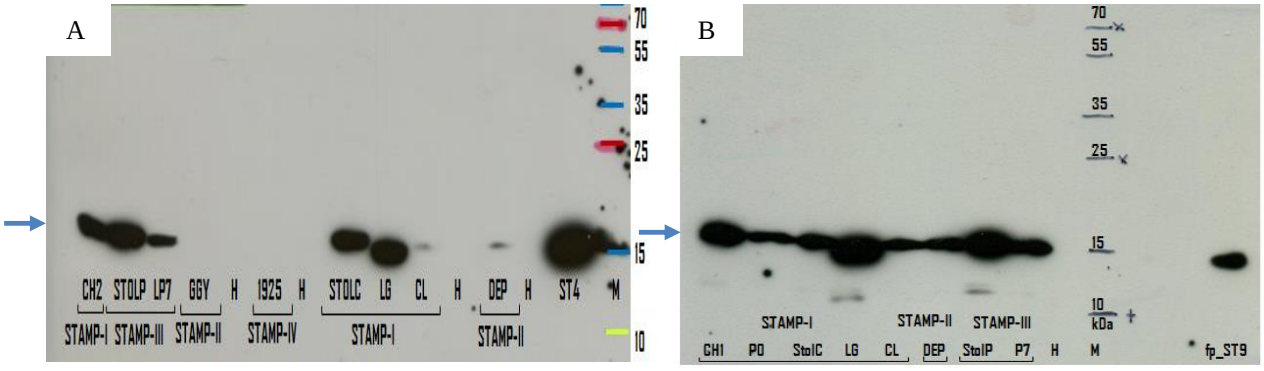

Figure 43. A-B. Western blot analysis of extracted phytoplasma protein from different phytoplasma strains demonstrated that MAb 2A10 recognizes genetic cluster II and III

Legend: arrow: 16 kDa STAMP(s), Neg: BSA control, M: protein PageRuler™ Prestained Protein Ladder (ThermoFicher Scientific): 10, 15, 25, 35, 55, 70, 100, 130 kDa.

Furthermore, it was confirmed that 2A10 MAb -antibody produced against strain belonging to *stamp* cluster I- recognises all four *stamp* genetic clusters (Figure 44). In this experiment slight difference in sensitivity of MAb 2A10 was observed. In the case of clusters II and III the signal was weaker suggesting that 2A10 MAb is more specific to cluster I and cluster IV than to cluster II (Figure 44). However, further experiments are needed to confirm this.

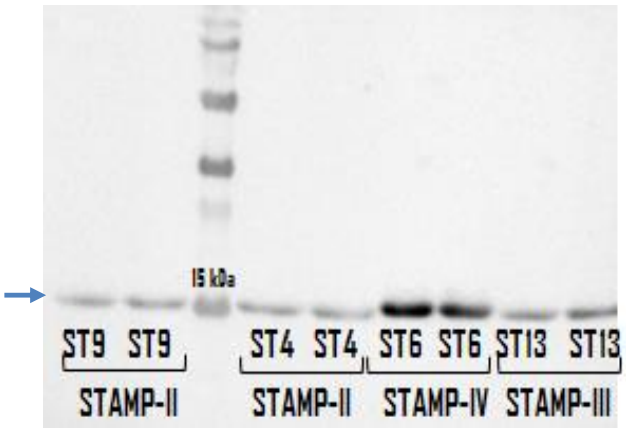

Figure 44. 2A10 MAb produced against strain *stamp* cluster I- recognises all four *stamp* genetic clusters

Legend: ST4: fp_ST4, ST9: fp_ST9, ST6: fp_ST6, ST13: fp_ST13, 10µg/lane fusion protein, SDS-PAGE revealed with 2A10 MAb, M: M: protein PageRuler™ Prestained Protein Ladder (Thermo Ficher Scientific): 10, 15, 25, 35, 55, 70, 100, 130 kDa.

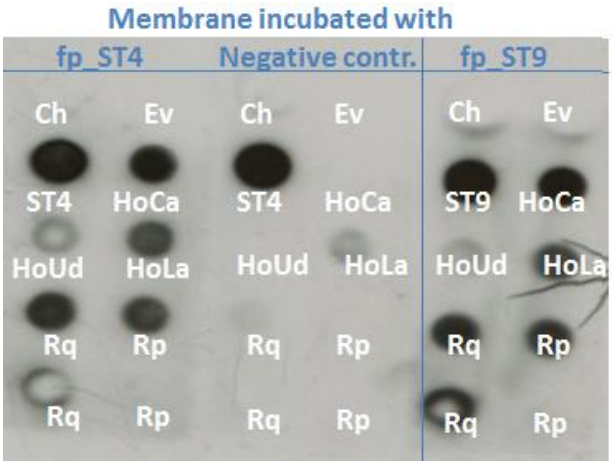

Figure 45. Dot-WB-blot between fp_ST4/fp_ST9/negative control and different insect total protein revealed with 2A10 MAb

Legend: HoUd: *H. obsoletus* ecotype stinging nettle, HoCa: *H. obsoletus* ecotype bindweed, HoLa: *H. obsoletus* ecotype lavender, Rq: *R. quinquecostatus*, Rp: *R. panzeri*, Ch: *Circulifer haematoceps*, Ev: *Euscelidius variegatus*
● very strong signal, ● strong signal, ● medium signal, ● mild signal, ○ no signal (non specific reaction).

7.1.4.2. STAMP-insect protein interaction

Assessment of interaction ability of the16 kDa recombinant STAMP(s) fp_ST4 and fp_ST9 with various insects' protein extracted from vectors: *H. obsoletus*, *R. panzeri*; potential vectors: *R. quinquecostatus*, *R. cuspidatus* and non-vectors*: Euscelidius variegatus*, *Circulifer haematoceps* were tested in dot-blot analyses. In addition different ecotypes of *H. obsoletus* populations from bindweed (HoCa), stinging nettle (HoUd) and lavender (HoLa) were compared. As preliminary results, differences in the binding intensity between STAMP cluster II (fp_ST4 and fp_ST9) and protein of insect species and *H. obsoletus* ecotypes were obtained. It was demonstrated that *stamp* cluster II has the capability to interact with protein(s) of *R. quinquecostatus*, as well as with *H. obsoletus* bindweed and lavender ecotypes. However, interaction of cluster II with *R. panzeri* and *H. obsoletus* stinging nettle ecotype was remarkably weaker and interaction with non-vector species, i.e. *C. haematoceps*, *E. variegatus* and *R. cuspidatus*, was not detected (Figure 45, 46). *In vitro* interaction between stolbur phytoplasma antigenic membrane protein and insect protein was

demonstrated for the first time. However, further experiments are required to confirm these results. In order to complete this study investigation all the representatives of four *stamp* groups are needed. Production of recombinant protein of cluster III (ST6), cluster IV (ST13) were accomplished and cluster I (ST1) is ongoing.

Origin		Insect species	Host plant	Intensity of interaction		
				ST4	ST9	Neg.
FR, Charente	+	***H. obsoletus***	bindweed	●	●	-
FR, Champlong		***H. obsoletus***	lavender	●	●	-
FR, Elsas	+	***H. obsoletus***	stinging nettle	●	●	-
DE, Kesten		***H. obsoletus***	stinging nettle	●	●	●
HU, Lovászi		***H. obsoletus***	stinging nettle	●	●	-
SR	+	***R. panzeri***	corn	●	●	-
FR, (Triazol)		***R. panzeri***	weeds	●	●	-
HU, Andornaktalya	+	***R. quinquecostatus***	common knotgrass, myrobalan plum,	●	●	-
FR, Gironde J-L		***R. quinquecostatus***	wild plant	●	●	○
HU, Etyek	-	***R. cuspidatus***	common hawthorn	○	○	
insectarium		***C. haematoceps***	wheat	-	○	-
insectarium		***E. variegatus***	wheat	○	○	○
Recombinant protein		pf_ST4		●	●	●
Recombinant protein		pf_ST9		●	●	●

Figure 46. Summary of interaction experiment between STAMP cluster II and different insect total protein

Legend:
● very strong signal, ● strong signal, ● medium signal, ● mild signal, ○ no signal (non-specific signal), red + in red box: strong interaction, orange + in orange box: mild interaction, green + in green box: no interaction.

7.2. Effects of Bois noir disease on performance of *V. vinifera* L. cv. Chardonnay in Eger wine region

7.2.1. Meteorology and vintage

Meteorological data for every year of the experiment (2012, 2013 and 2014) and the preceding year (Figure 47, 48, 49) was as follows: the year of 2011 was an average year without extremity (rainfall 944 mm). In 2012 the precipitation was extremely low (rainfall 443 mm), especially over the period of active vegetative growth (pre-veraison) and veraison; these conditions may have affected the bud differentiation and yield of the next year. After the relatively dry years, a more balanced 2013 followed (rainfall: 731 mm) with an unusually cold spring that delayed flowering. Weather conditions in 2014 (rainfall: 768 mm) were normal until veraison, during which 50 % of the annual rainfall occurred. Due to the wet conditions, severe fungal infection affected the harvest of 2014.

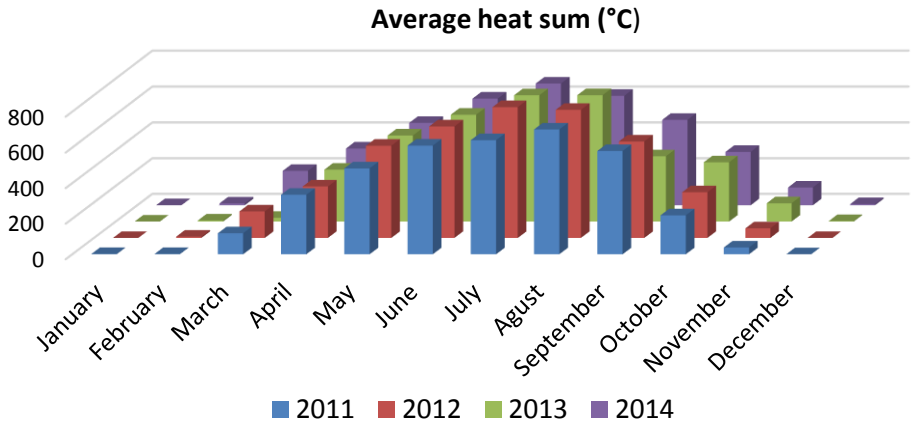

Figure 47. Yearly heat summation of the experimental plot, Eger, Kőlyuktető

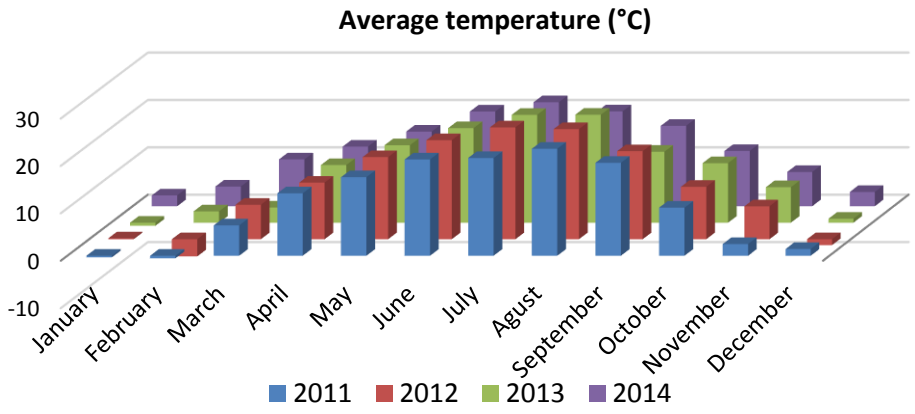

Figure 48. Yearly temperature of the experimental plot, Eger, Kőlyuktető

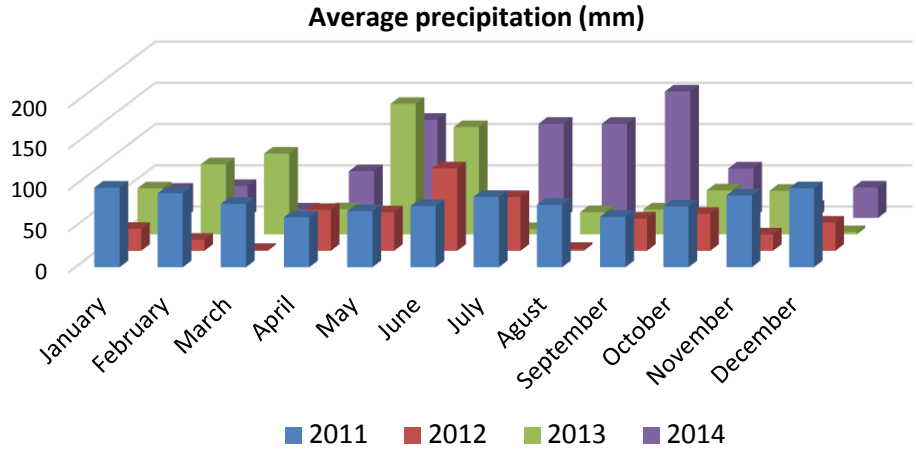

Figure 49. Yearly precipitation of the experimental plot, Eger, Kőlyuktető

7.2.2. Vegetative performance measurements

A summaries of the effects of BN disease on all measured parameters and those that showed significant differences are presented in Table 16, 17, 18 and Figure 57, 58, respectively.

Pruning mass and cane lignification. Although consistently lower values in pruning mass were found for BN-affected (BNA) vines, differences between healthy (H) and BNA grapevines were not significant (Table 16). The number of canes per vine was similar between H and BNA vines. However, a significant influence of the year surfaced for both parameters ($P < 0.001$, $P < 0.05$). Differences in number of lignified canes between H and BNA were detected in 2012 and 2013 ($P < 0.05$, $P < 0.01$), and a vintage effect was also observed ($P < 0.05$). Significant lack of lignification of BN infected plants occurred in each year (2012 $P < 0.001$, 2013 and 2014 $P < 0.01$; Table 16, Figure 50). The number of immature canes per BNA vine decreased by an average of 26.4 % over the 3-year period. An interaction between vintage effect and BN regarding the number of non-lignified canes per vine was not observed [$F(2,84) = 0.22$; $P = 0.80$], suggesting that the effect of BN on cane maturation had the same tendency every year. Buds on non-lignified canes were not viable (data not shown). A significant difference in the pith:wood ratio of H and BNA vines was only detected in the second and fourth internodes [H: 1.34 mm, BNA: 1.12 mm; $F(2,42) > 3.3$; $P < 0.05$ in both cases].

Leaf rolling. Phytoplasma-induced leaf rolling significantly decreased ($P < 0.001$) the total leaf surface by an average of 28 % over 2 years (Table 16, Figure 21).

Leaf fresh and dry mass. Leaf fresh and dry matter contents (expressed as mg/cm^2) of BNA plants were 11.2 % and 19.5 % higher, respectively, than those of healthy plants. Significantly higher leaf water content in BNA plants was observed only in 2013 ($P < 0.01$; Table 16).

Figure 50. Canes and cross section of asymptomatic (left) and GY symptomatic (right) 'Chardonnay'

Figure 51. Asymptomatic (left) and BN symptomatic (right) bunches often appeared on the same shoot

Leaf chlorophyll. Measurements of the relative Chl index (i.e. SPAD values) revealed a significant decrease in the BNA symptomatic leaves (collected at the middle shoot position) (2012 and 2014 $P < 0.001$; Table 16). Values showed high variations within the different measure points per leaf. The relationship between SPAD values and Chl content according to pigment extraction and spectrophotometric detection (data not shown) indicated a significant decrease in foliar Chl content in infected plants. The 2-year average SPAD values for H and BNA symptomatic plants were 39.5 (2,276 µg/g Chl $a + b$) and 27.5 (909 µg/g Chl $a + b$), respectively (Table 16). According to measurements taken at the basal, middle and top leaf positions, the SPAD values in BNA vines significantly (2012 $P < 0.001$) decreased from the top to the basal shoot positions (Figure 52), where the symptoms are usually greater and a drop in SPAD indicated severe pigment degradation.

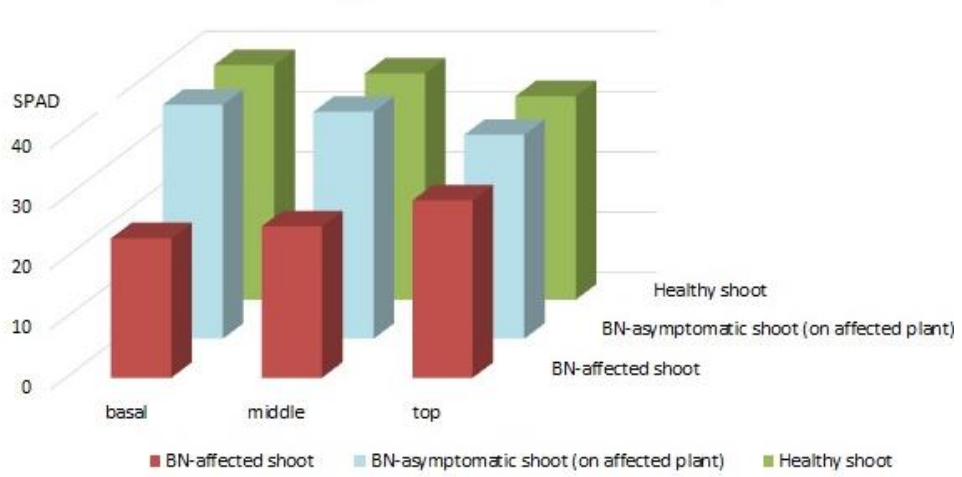

Figure 52. Chlorophyll content at basal,middle and top shoot position

Table 16. Growth, yield and fruit composition of Bois noir-affected and healthy grapevines with the applied statistical methods and their results

Measured parameter	2012			2013			2014			3-year average			Average decrease§ (-) increase (+) %	Inter-action	Year effect F(df1;df2)	Symptom Wilk's λ	Year effect Wilk's λ	Stat. method
	H	BNA	F(df1;df2)	H	BNA	F(df1;df2)	H	BNA	F(df1;df2)	H	BNA	F(df1;df2)						
Vegetative performance																		
Pruning mass§§/vine(g)	630.7	557.3	F(1;28)=0.73 ns	375.3	324.7	F(1;28)=0.51 ns	717.3	588.0	F(1;28)=1.65 ns	574.4	490.0	F(1;84)=2.86 +	**-14.7**	ns	F(2;84)=13.75 ***	-	-	2ANOVA
Total canes§§§/vine (pc)	11.4	12.9	F(1;22)=0.50 ns	16.3	12.7	F(1;17)=3.16 +	16.1	18.6	F(1;21)=1.45 ns	14.6	14.7	F(1;60)=0.01 ns	**NA**	ns	F(2;60)=6.57 **	-	-	2ANOVA
Lignified canes/vine (pc)	11.1	7.8	F(1;22)=3.57 *	15.6	8.7	F(1;17)=9.39 **	15.6	14.3	F(1;21)=0.29 ns	14.0	10.3	F(1;60)=10.23 **	**-26.4**	ns	F(2;60)=7.58 **	-	-	2ANOVA
Non-lignified canes/vine (pc)	0.3	5.0	F(1;22)=29.51 ***	0.7	4.0	F(1;17)=9.13 **	0.5	4.3	F(1;21)=10.31 **	0.5	4.4	F(1;60)=42.45 ***	**+11.3**	ns	F(2;60)=0.09 ns	-	-	2ANOVA
Rolling-caused decrease of leaf surface (%)	-	-	NA	0.0	29.5	t(9)=6.56 ***	0.0	26.4	t(9)=7.40 ***	0.0	28.0	t(19)=9.92 ***	**-28**	-	t(18)=0.54 ns	-	-	1s and 2s Student's t
Leaf fresh mass (mg/cm^2)	30.66	32.12	F(1;18)=3.33 +	31.97	37.31	F(1;34)=16.27 ***	29.61	34.45	F(1;38)=14.99 ***	30.75	34.63	F(1;90)=24.98 ***	**+11.2**	ns	F(2;90)=7.00 **	0.76 ***	0.13 **	2MANOVA
Leaf dry mass (mg/cm^2)	8.58	9.08	F(1;18)=1.89 ns	8.53	11.18	F(1;34)=18.42 ***	12.16	16.14	F(1;38)=20.93 ***	9.76	12.13	F(1;90)=26.18 ***	**+19.5**	*	F(2;90)=54.10 ***			
Leaf water content (mg/cm^2)	22.07	23.04	F(1;18)=3.58 +	23.44	26.12	F(1;34)=10.58 **	17.45	18.31	F(1;38)=2.31 ns	20.99	22.49	F(1;90)=12.60 ***	**+6.8**	+	F(2;90)=119.01 ***	-	-	2ANOVA
Relative chlorophyll index (SPAD)	38.5	22.5	F(1;19)=44.51 ***	-	-	-	40.6	30.0	F(1;38)=58.39 ***	39.5	27.5	F(1;22)= 105.41 ***	**-30.4**	+	F(1;22)=15.64 ***			2ANOVA
Regenerative performance																		
Yield/vine (kg)	1.394	0.651	F(1;26)=16.73 ***	2.0967	0.517	F(1;26)=85.68 ***	2.739	0.798	F(1;26)=39.33 ***	2.077	0.656	F(1;82)=112.64 ***	**-68.4**	**	F(2;82)=10.52 ***	57.48 ***	13.55 ***	2MANOVA
Bunch mass (g)	84.38	96.93	F(1;26)=1.59 ns	71.09	39.36	F(1;26)=32.22 ***	98.57	61.65	F(1;26)=11.93 **	84.65	65.98	F(1;82)=12.63 ***	**NA**	***	F(2;82)=15.95 ***			
100 berry mass (g)	130.19	105.96	F(1;26)=15.22 ***	166.09	85.28	F(1;26)=142.48 ***	167.47	121.58	F(1;26)=50.05 ***	154.59	104.27	F(1;82)=168.30 ***	**-32.5**	***	F(2;82)=16.44 ***			
Bunch number/vine (pc)	16.33	6.73	F(1;26)=30.51 ***	29.87	12.53	F(1;26)=53.96 ***	27.67	12.73	F(1;26)=30.44 ***	24.62	10.67	F(1;82)=102.99 ***	**-56.7**	+	F(2;82)=19.93 ***			
Symptomatic bunch/vine (pc)	0.00	4.67	t(14)=24.97 ***	0.00	9.47	t(14)=7.60 ***	0.67	8.93	t(14)=22.56 ***	0.22	7.69	t(44)=14.29 ***	**+97.2**	-	F(2;42)=11.91 ***	-	-	1ANOVA and 1sStudent's t
Dry bunch/vine (pc)	0.00	1.60	t(14)=4.41 ***	0.40	2.33	t(14)=3.70 **	0.00	0.13	t(14)=1.00 ns	0.13	1.36	t(44)=4.89 ***	**+90.4**	-	F(2;42)=6.89**	-	-	
Fruit composition																		
Titratable acidity (g/L tartaric acid)	6.72	7.92	F(1;28)=16.63 ***	8.96	11.16	F(1;28)=25.99 ***	9.73	11.30	F(1;28)=8.66 **	8.40	10.13	F(1;82)=43.84 ***	**+16.4**	ns	F(2;82)=63.47 ***	0.59 ***	0.21***	2MANOVA
pH	3.44	3.35	F(1;28)=9.56 **	3.21	3.09	F(1;28)=46.53 ***	3.24	3.18	F(1;28)=3.25 +	3.30	3.21	F(1;82)=31.92 **	**-2.7**	ns	F(2;82)=85.95 ***			
Soluble solids (Brix°)	23.3	22.5	F(1;28)=4.33 *	21.3	18.9	F(1;28)=18.00 **	19.9	19.1	F(1;28)=2.49 +	21.5	20.1	F(1;82)=23.22 **	**-6.2**	ns	F(2;82)=58.40 ***			

Legend: BNA: BN-affected grapevine cv. 'Chardonnay'. H: healthy grapevine cv. 'Chardonnay'. **§**: average changes of BN-affected vine performance compared to those of healthy plants based on a 3-year average (%). **§§**: pruning mass refers to the annual growth of the previous vegetation. **§§§**: calculated as sum of lignified and non-lignified canes. -: no data. NA: not applicable. Asterisks refer to statistically significant differences, at *P* values: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{+}P < 0.1$. ns: no significant difference. df: degrees of freedom. Box of dotted line: variables involved in MANOVA model. n(M)ANOVA: n-way (M)ANOVA model. 1s(2s)Student's t: one sample (two-sample) Student's t test.

Table 17. Proportion of flowers and berries on Bois noir-affected and healthy bunches with the applied statistical methods and their results

Parameters/bunch	2013			2014			2-year average			Inter-action	Year's effect F(df1;df2)	Symptom Wilk's λ	Year's effect Wilk's λ	Stat. method
	H	BNA	F(df1;df2)	H	BNA	F(df1;df2)	H	BNA	F(df1;df2)					
Flowers (pc)	274.4	234.4	F(1;18)=2.07 ns	160.0	144.5	F(1;18)=0.65 ns	217.2	189.4	F(1;36)=2.69 ns	ns	F(1;36)=36.49 ***			2ANOVA
Bunch mass (g)	56.1	22.4	F(1;18)=13.00 **	97.2	85.4	F(1;18)=0.48 ns	76.6	53.9	F(1;36)=5.50 *	ns	F(1;36)=28.75 ***			
Rachis mass (g)	2.4	1.9	F(1;18)=1.32 ns	4.4	4.02	F(1;18)=0.36 ns	3.4	2.9	F(1;36)=1.40 ns	ns	F(1;36)=28.18 ***	0.81 +	0.39 ***	2MANOVA
Total berries§ (pc)	86.6	71.8	F(1;18)=0.65 ns	71.0	78.5	F(1;18)=0.49 ns	78.8	75.2	F(1;36)=0.19 ns	ns	F(1;36)=0.18 ns			
Normal berries (pc)	21.6	4.4	F(1;18)=14.11 **	60.6	50.6	F(1;18)=1.22 ns	41.9	27.5	F(1;36)=7.16 *	ns	F(1;36)=70.39 ***			
Abnormal berries (pc)	46.7	35.0	F(1;18)=0.68 ns	8.0	20.0	F(1;18)= 3.34 +	27.3	27.5	F(1;36)= 0.00 ns	ns	F(1;36)=11.78 **	0.78 *	0.29 ***	2MANOVA
Dry berries (pc)	18.3	32.4	F(1;18)=2.62 ns	2.4	7.9	F(1;18)=7.07 *	10.4	20.2	F(1;36)= 4.79	ns	F(1;36)=20.53 ***			
Set flowers/total berries (%)	31.6	30.6	NA	44.4	54.3	NA	36.0	39.7	NA	NA	NA	NA	NA	NA

Legend Table 17:
BNA: BN-affected grapevine cv. 'Chardonnay'. H: healthy grapevine cv. 'Chardonnay'. **§**: calculated as sum of normal, abnormal and dry berries. NA: not applicable. Statistical differences were evaluated by year, asterisks refer to statistically significant differences at *P* values: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{+}P < 0.1$. ns: no significant difference. df: degrees of freedom. Box of dotted line: variables involved in MANOVA model. n(M)ANOVA: n-way (M)ANOVA model.

Legend Table 18:
BNA: wine of total yield of BN-affected grapevine cv. 'Chardonnay'. H: wine of total yield of healthy grapevine cv. 'Chardonnay'. BNS: wine of yield of BN-affected shoots (shriveled bunches only) cv. 'Chardonnay'. **§**: average changes of BN-affected vine performance compared to those of healthy plants based on a 3-year average (%). -: no data. NA: not applicable. BDL: below detection limit. †: measurements of organic acids were performed by spectrophotometry (tartaric acid) and Boehringer Mannheim enzyme test (malic and lactic acids). Statistical differences were evaluated by year, asterisks refer to statistically significant differences at *P* values: $^{*}P < 0.05$, $^{+}P < 0.1$. ns: no significant difference. Grey letters: statistical analyses were not applicable.

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Table 18. Results of wine analysis of Bois noir-affected and healthy vines (vintage 2012, 2013 and 2014)

Parameters with year' effect significance	2012			2013				2014				3 year mean				Average increase§ (+) /decrease (-) %
	H	BNA		H	BNA	BNS		H	BNA	BNS		H	BNA	BNS		
Basic analysis																
Alcohol (v/v %)*	13.15	12.84	*	12.87	11.78	10.58	*	11.87	11.26	9.72	-	12.63	11.96	10.15	*	**-5.30**
Glycerol (g/L)*	8.595	8.877	+	5.68	5.48	5.56	*	-	-	-	-	7.14	7.18	NA	*	**NA**
Total extract (g/L)ns	24.10	23.20	ns	20.30	22.83	22.70	ns	20.60	21.90	24.50	-	21.67	22.64	23.60	ns	**+4.28**
Residual sugar (g/L)ns	2.95	1.75	ns	2.37	3.7	3.0	*	1.2	1.2	1.4	-	2.17	2.22	2.20	ns	**-0.04**
Fructose (g/L)ns	2.66	1.96	ns	1.89	2.69	1.74	*	-	-	-	-	2.27	2.32	NA	ns	**+2.00**
Glucose (g/L)*	0.89	0.83	ns	0.52	0.63	0.84	*	-	-	-	-	0.70	0.73	NA	ns	**+3.08**
Total polyphenols (mg/L)*	309.0	308.5	ns	222.3	254.3	282.0	ns	221.0	242.0	259.0	-	250.8	268.3	270.5	ns	**+6.52**
Colour (OD 420nm)*	0.105	0.102	ns	0.058	0.09	0.99	*	0.053	0.068	0.77	-	0.07	0.09	0.88	*	**+22.22**
Organic acids																
TA (g/L tartaric acid)*	6.80	7.15	*	7.17	8.07	9.10	*	7.70	8.30	9.80	-	7.22	7.84	9.45	*	**+7.91**
pH*	3.08	3.04	*	3.42	3.43	3.35	ns	2.92	2.93	2.72	-	3.14	3.13	3.04	ns	**-0.32**
Tartaric acid (g/L)*	3.144	3.190	ns	3.667	4.076	4.795	*	2.29†	2.29†	3.37†	-	3.03	3.19	9.45	ns	**+4.76**
Malic acid (g/L)*	2.268	2.345	ns	4.101	4.355	4.490	*	3.75†	3.89†	4.01†	-	3.37	3.53	3.04	*	**+4.45**
Citric acid (g/L)*	0.361	0.371	*	0.476	0.519	0.542	*	-	-	-	-	0.42	0.45	4.08	*	**+5.96**
Succinic acid (g/L)*	1.348	1.407	*	0.618	0.586	0.662	ns	-	-	-	-	0.98	1.00	4.25	ns	**+1.35**
Lactic acid (g/L)*	0.347	0.381	ns	0.212	0.217	0.248	ns	0.01†	0.04†	0.01†	-	0.19	0.21	0,54	+	**+10.82**
Acetic acid (g/L)*	0.185	0.190	ns	0.407	0.265	0.238	*	-	-	-	-	0.30	0.23	0,66	+	**NA**
Elements																
Aluminum (mg/L)*	0.50	0.74	ns	1.41	1.27	1.30	ns	0.827	0.978	1.52	-	0.91	1.00	1.41	ns	**+9.00**
Boron (mg/L)ns	3.49	3.44	ns	3.40	3.55	3.33	ns	3.11	4.07	3.33	-	3.33	3.69	3.33	ns	**+9.76**
Calcium (mg/L)*	203.0	213.50	ns	94.68	102.92	128.20	*	55.72	69.29	87.37	-	117.80	128.57	107.79	*	**+8.38**
Copper (mg/L)*	2.05	2.70	*	0.18	0.13	BDL	ns	BDL	BDL	BDL	-	NA	NA	NA	ns	**NA**
Iron (mg/L)*	0.56	0.54	ns	0.96	0.74	0.94	+	0.17	0.30	0.45	-	0.56	0.53	0.70	*	**-5.36**
Potassium (mg/L)*	742.00	721.00	ns	454.80	457.83	437.40	ns	397.3	392.8	371.70	-	531.37	523.88	404.55	ns	**-1.41**
Magnesium (mg/L)*	131.00	131.00	ns	80.79	86.73	90.42	+	36.59	41.89	48.89	-	82.79	86.54	69.66	*	**+4.33**
Sodium (mg/L)*	29.50	25.50	ns	17.74	9.57	8.85	ns	8.06	13.90	17.84	-	18.43	16.32	13.35	+	**NA**
Zinc (mg/L)*	0.41	0.39	ns	0.50	0.57	0.57	ns	0.55	0.63	0.49	-	0.49	0.53	0.53	ns	**+7.55**
Simple phenols																
Caftaric acid (mg/L)*	77.60	80.06	*	26.89	28.27	36.71	ns	-	-	-	-	52.24	54.17	NA	*	**+3.56**
Caffeic acid (mg/L)NA	BDL	BDL	NA	2.21	3.46	3.29	*	-	-	-	-	NA	NA	NA	NA	**+36.13**
Catechin (mg/L)*	19.85	19.58	*	11.51	8.99	14.42	*	-	-	-	-	15.68	14.29	NA	ns	**-8.86**
Epicatechin (mg/L)NA	0.97	0.83	+	BDL	BDL	BDL	NA	-	-	-	-	0.97	0.83	NA	NA	**-14.43**
Protocatechuic acid (mg/L)NA	BDL	0.84	NA	1.07	1.03	0.95	ns	-	-	-	-	1.07	0.93	NA	NA	**-13.08**

7.2.3. Reproductive performance measurements

Fruit set. Fruit set was investigated in 2013 and 2014 (Table 17). No significant differences in the number of flowers between H and BNA vines were observed. In 2013 bunch mass and the number of normal berries per bunch of H plants were significantly higher ($P < 0.01$) than those of BNA plants, but the vintage also influenced these parameters ($P < 0.001$, Table 17).

Morphological investigation of berries did not show differences between bunches from H and BN plants, but a significantly higher number of dry berries (2014 $P < 0.05$) frequently occurred in bunches of infected vines (Table 17).

Yield. In all experimental years, the yield of BNA grapevines was significantly lower than that of H vines (in all years $P < 0.001$; Table 16; Figure 57, 58). The berry mass, bunch mass, and number of bunches per vine collectively resulted in 53.3 % to 75.3 % (depending on the year) crop loss of diseased plants compared to healthy plants. Bunches with shrivelled berries and dry bunches were frequently observed on symptomatic vines, averaging 7.6 and 1.36 bunches/vine, respectively (in all years $P < 0.001$; Figure 51). Because of the dry conditions the production from H plants was more than 40 % lower in 2012 compared to the other experimental years.

Fruit composition. Individually studied, 15-15 H and BNA plants resulted in a significant increase in titratable acidity of the juice of BNA vines, ranging between 1.2 and 2.2 g/L higher in all 3 years (2012 and 2013 $P < 0.001$, 2014 $P < 0.01$), whereas the pH was lower (2012 $P < 0.01$, 2013 $P < 0.001$, 2014 $P < 0.1$; Table 16). The concentration of soluble solids was significantly lower in BNA vines (2012 $P < 0.05$, 2013 $P < 0.01$, 2014 $P < 0.1$; Table 16), implying a lower potential wine alcohol.

7.2.4. Neural network model and discriminant analysis of vegetative and reproductive parameters

Based on a neural network model, the significance of berry mass, number of bunches per vine, yield, and TA was 0.29, 0.17, 0.15 and 0.13, respectively. Less significant, but still characteristic features, were bunch mass, must pH, and °Brix, with importance values of 0.08, 0.09, and 0.09, respectively (Figure 58). The training set success was 100 %, whereas the testing set success was 92 %. Discriminant analysis revealed a significant separation effect for berry mass, yield, number of bunches per vine, and TA with a significant Wilk's lambda ($P < 0.05$). Canonical correlation was as high as 0.81 and the correlations between discriminating variables and standardized canonical discriminant functions were 0.79, 0.69, 0.62 and 0.29, respectively, resulting in 100 % cross-validated separation success.

7.2.5. Small-scale wine production

The timing of harvest was based on the maturation index (°Brix:TA), which was the most optimal in 2013, according to weather conditions (Figure 47, 48, 49). During wine production, fermentation of must from BNA grapes (BNS in particular) was slower than that from H grapes, finishing 5-7 days later.

7.2.6. Wine analyses

In wines fermented to dryness, significant differences in residual sugar content of batches were not observed [infection: $F(1,5) = 0.03$, $P = 0.88$; year: $F(1,5) = 0.99$, $P = 0.37$; Table 18]. Alcohol content of wines from H plants was higher than that of BNA plants in all 3 years [infection: $F(1,5) = 13.33$, $P < 0.05$; year: $F(1,5) = 10.22$; $P < 0.05$; Table 18].

Wines from BNA plants had significantly higher tartaric acid content and slightly higher malic acid content compared to those of H plants [TA, infection: $F(1,5) = 17.78$, $P < 0.01$; TA, year: $F(1,5) = 15.24$, $P < 0.05$; tartaric acid, infection: $F(1,5) = 12.28$, $P < 0.05$; tartaric acid, year: $F(1,5) = 0.25$; $P = 0.65$; malic acid, infection: $F(1,5) = 2.97$; $P = 0.1$; malic acid, year: $F(1,5) = 485.6$, $P < 0.001$; Table 18].

Oxidation-reduction conditions were similar in each batch (adjusted to 40 mg/L free sulphur). Wines produced from infected plants in 2013 and 2014 exhibited a pinkish discolouration, which was most prominent in 2013. The pink discolouration was observed in each of the three replicates of wine from BNA and BNS grapes, but not in wine produced from H grapes (Figure 55). The HPLC measurements of phenolic compounds, which most likely contributed to the pink colouration (Lutter *et al*. 2007), revealed a 36.31 % increase in caffeic acid of BNA wines [$F(1,4) = 56.64$, $P < 0.01$; Table 18]. On the contrary, (+)-catechin and (-)-epicatechin concentrations were 8.86 % and 14.43 % lower, respectively, in wines from BNA grapes [catechin: $F(1,4) = 56.64$, $P < 0.01$; epicatechin: $F(1,4) = 0.25$, $P = 0.65$; Table 18].

Minerals that were above the detection limit are provided in Table 18. The calcium content of wines from BNA grapes was elevated in every year [$F(1,5) = 4.87$, $P < 0.1$], with the highest concentrations observed in 2012, which was the driest year. Magnesium content in wine from H grapes was lower in 2013 and 2014 than in 2012 [$F(1,5) = 15.4$, $P < 0.05$]. Iron concentrations in 2013 showed contradictory results to other years, with lower concentrations in wines from BNA grapes [$F(1,5) = 4.69$, $P < 0.1$]. Mineral contents were significantly affected by the year [$F(1,5) > 11.00$, $P < 0.05$), except for Zn ($P < 0.1$) and B ($P = 0.6$)].

7.2.7. Sensory analyses

Differences among the wines were evident, the extent of which varied among the years. Panellists consistently determined that wine from BNA grapes was of a lower quality than wine from healthy grapes (Figure 53, 54, 56). However, this was not statistically supported in each experimental year. In 2012, the difference was slightly significant only for overall quality (Mann-Whitney's U = 31.5, $P = 0.056$). Differences were most significant in 2013 (Mann-Whitney's U < 156.5, $P < 0.03$ for colour shade, aroma intensity, taste intensity, taste length, fruitiness/floral/citrus aroma or taste default, bitterness, harmony, and overall quality; and Mann-Whitney's U > 162.5, $P > 0.06$ for acidity, body, and varietal aroma). There were no significant differences in 2014 ($P > 0.2$). Wines from the grapes of healthy plants were light yellow and light bodied, and evaluated as crisp in all years. They also had a fruity bouquet with dominant citrus aromas of a moderate length and intensity. Wines from BNA grapes had reduced aroma and flavour. However, fruity aromas were distinctive, the wines tasted flat, and were characterized by a deeper colour (pinkish in some years) (Figure 55), pronounced acidity and bitterness.

All of these characteristics were most noticeable in wines from BNS grapes in both years (2013 and 2014), the wines of which failed to produce acceptable quality (statistical analysis of BNS wines was not applicable). Overall, wines from H grapes were preferred, followed by wines from BNA grapes and wines from BNS grapes. During the three years of winemaking all of the oenological replicates of all batches were subjected to profile analysis and no faulty wines were identified.

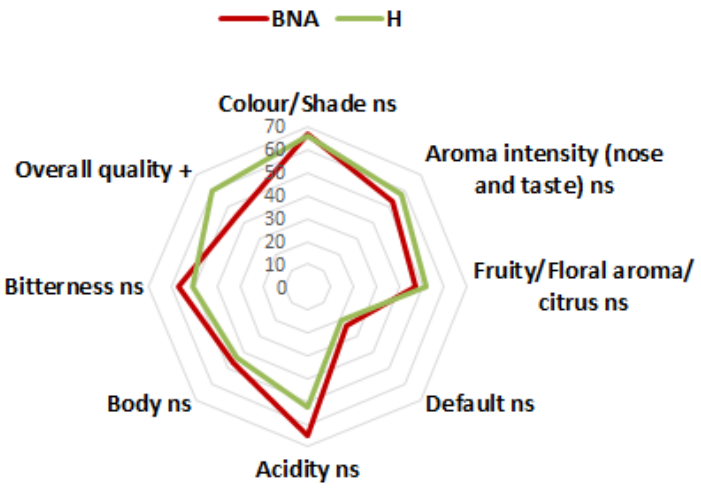

Figure 53. Wine profile analysis of year 2012

Legend: H: yield of healthy vines, BNA: yield of BN-affected vines, BNS: shrivelling bunches of BN-affected shoots, ns: no significant difference, $^{+}$ $P < 0.1$. Statistical analysis of BNS wines was not applicable.

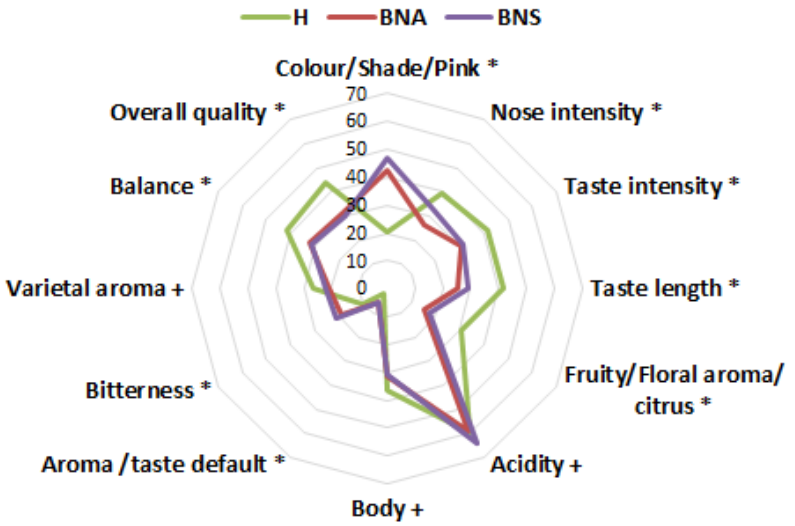

Figure 54. Wine profile analysis of year 2013

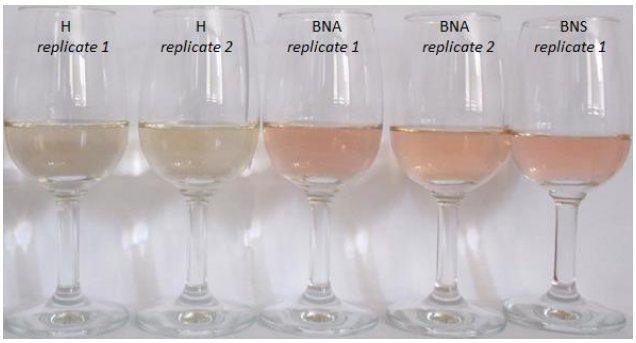

Figure 55. Wines of year 2013

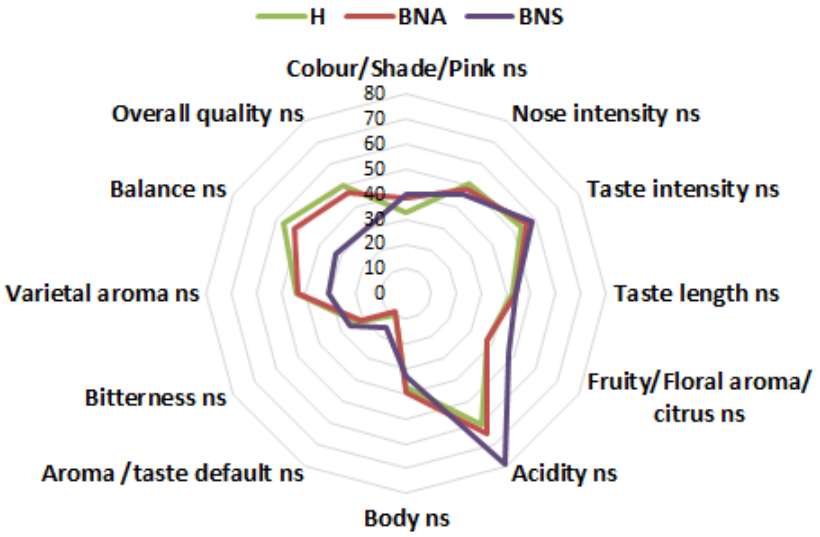

Figure 56. Wine profile analysis of year 2014

Legend: H: yield of healthy vines, BNA: yield of BN-affected vines, BNS: shrivelling bunches of BN-affected shoots. Asterisks refer to statistically significant differences between H and BNA at values: * $P < 0.05$, $^{+}$ $P < 0.1$, ns: no significant difference. Statistical analysis of BNS wines was not applicable.

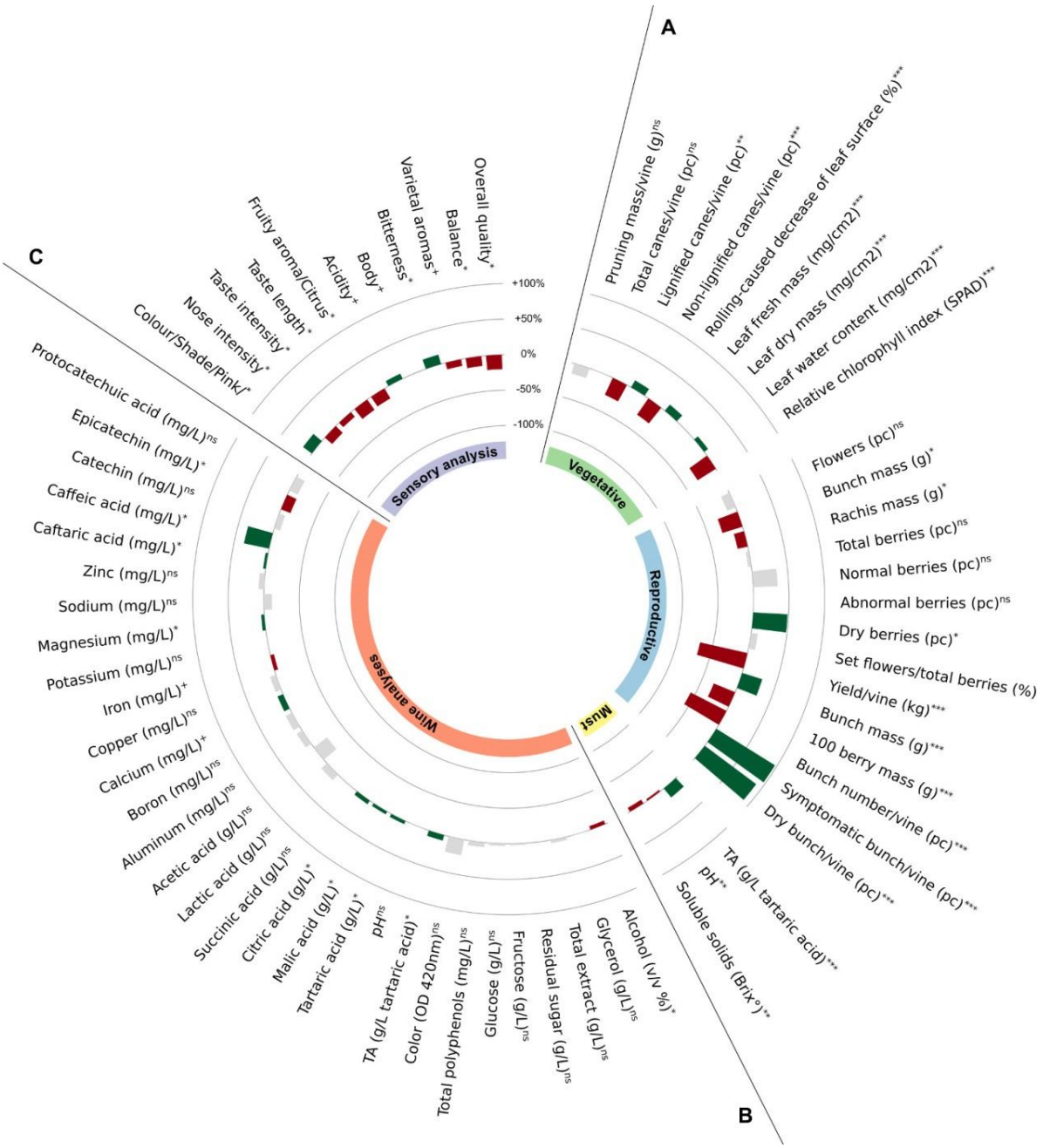

Figure 57. Bois noir (BN) disease in *Vitis vinifera* L. cv. 'Chardonnay': summary of all measured parameters related to vegetative growth, reproduction, and wine quality

Legend: Internal circle represents the groups of parameters: vegetative (green arch) and reproductive (blue arch) performance, fruit quality (yellow arch), wine (orange arch) and sensory (violet arch) analyses. Median circles in grey: scale of average changes of BN-affected vine performance compared to those of healthy plants based on a 3-year average (%). The green and red columns refer to performance increases (+) and decreases (-), respectively. The grey column refers to parameters with no significant differences. External circle: list of measured parameters. Asterisks refer to significant difference at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{+}P < 0.1$ values; ns: no significant difference. A: vegetative and reproductive performance and must quality of 15 healthy and BN-affected wines analysed by two-way ANOVA. B: analyses of wines of healthy and BN-affected plants by two-way MANOVA. C: sensory analysis (year 2014 only) of wines of healthy and BN-affected plants by Mann-Whitney's U test.

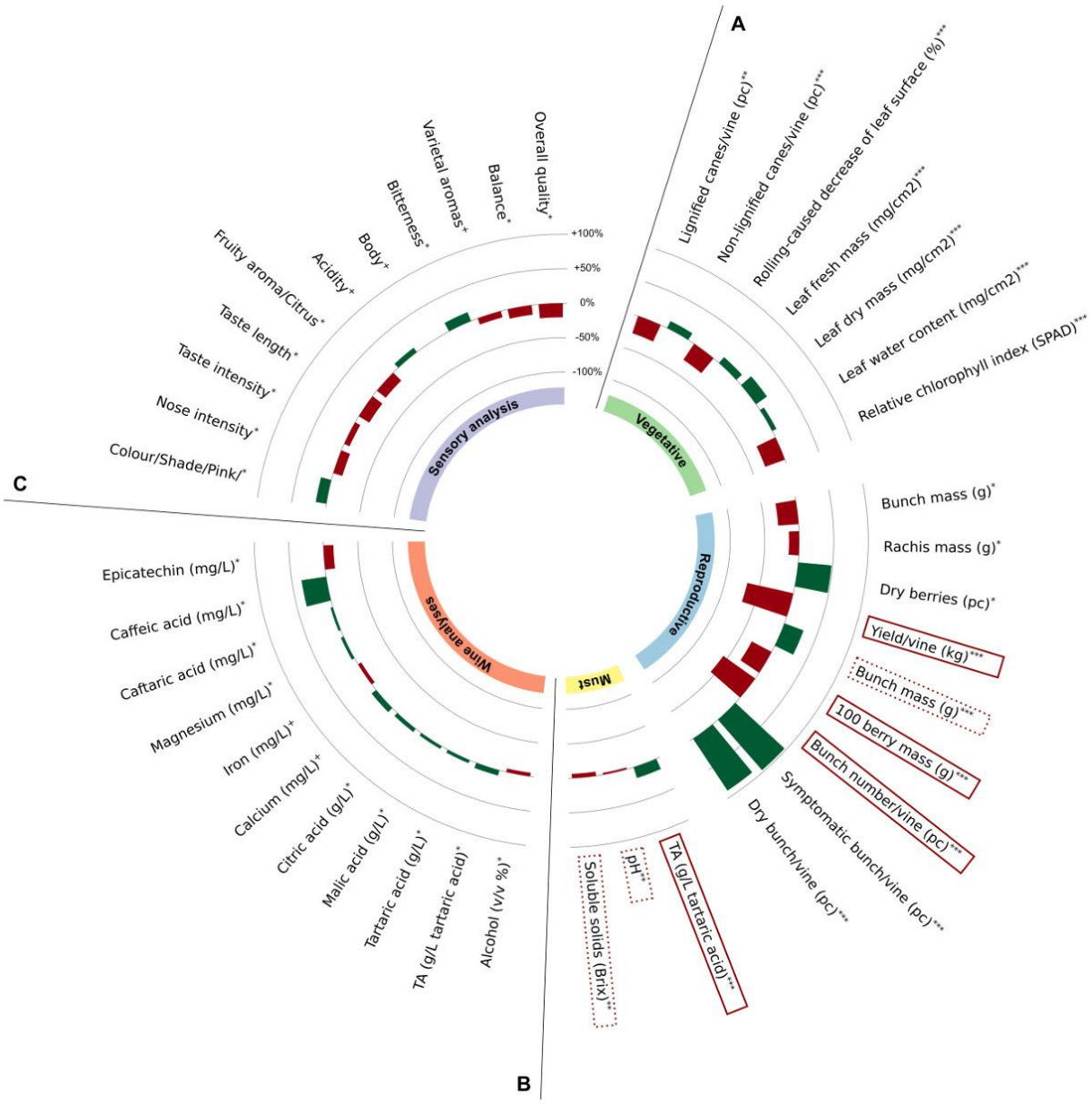

Figure 58. Summary of parameters related to the decrease or increase in vegetative growth, reproduction, and wine quality caused by Bois noir (BN) disease in *V. vinifera* L. cv. 'Chardonnay' that show a significant difference

Legend: Internal circle represents the groups of parameters: vegetative (green arch) and reproductive (blue arch) performance, fruit quality (yellow arch), wine (orange arch) and sensory (violet arch) analyses. Median circles in grey: scale of average changes of BN-affected vine performance compared to those of healthy plants based on a 3-year average (%). The green and red columns refer to performance increases (+) and decreases (-), respectively. External circle: list measured parameters that showed significant differences. Asterisks refer to significant difference at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{+}P < 0.1$ values. Red line box: most significant parameters characterise BN (neural network model); red dotted box: parameters less significant characterize BN (neural network model). Neural network was applied only for vegetative (green arch) and reproductive (blue arch) parameters. A: vegetative and reproductive performance and must quality of 15 healthy and BN-affected wines analysed by two-way ANOVA. B: analyses of wines of healthy and BN-affected plants by two-way MANOVA. C: sensory analysis (year 2014 only) of wines of healthy and BN-affected plants by Mann-Whitney's U test.

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

Curative treatments were performed using commercial products Kendal (Valagro) and Bion (Syngenta) with active agents of glutathione-oligosaccharine and benzothiadiazole, respectively. From 2011 to 2014 incidence and severity of the symptoms were recorded and marked with different colours. Figure 59 shows the three-year evolution of the symptoms on each individual plant.

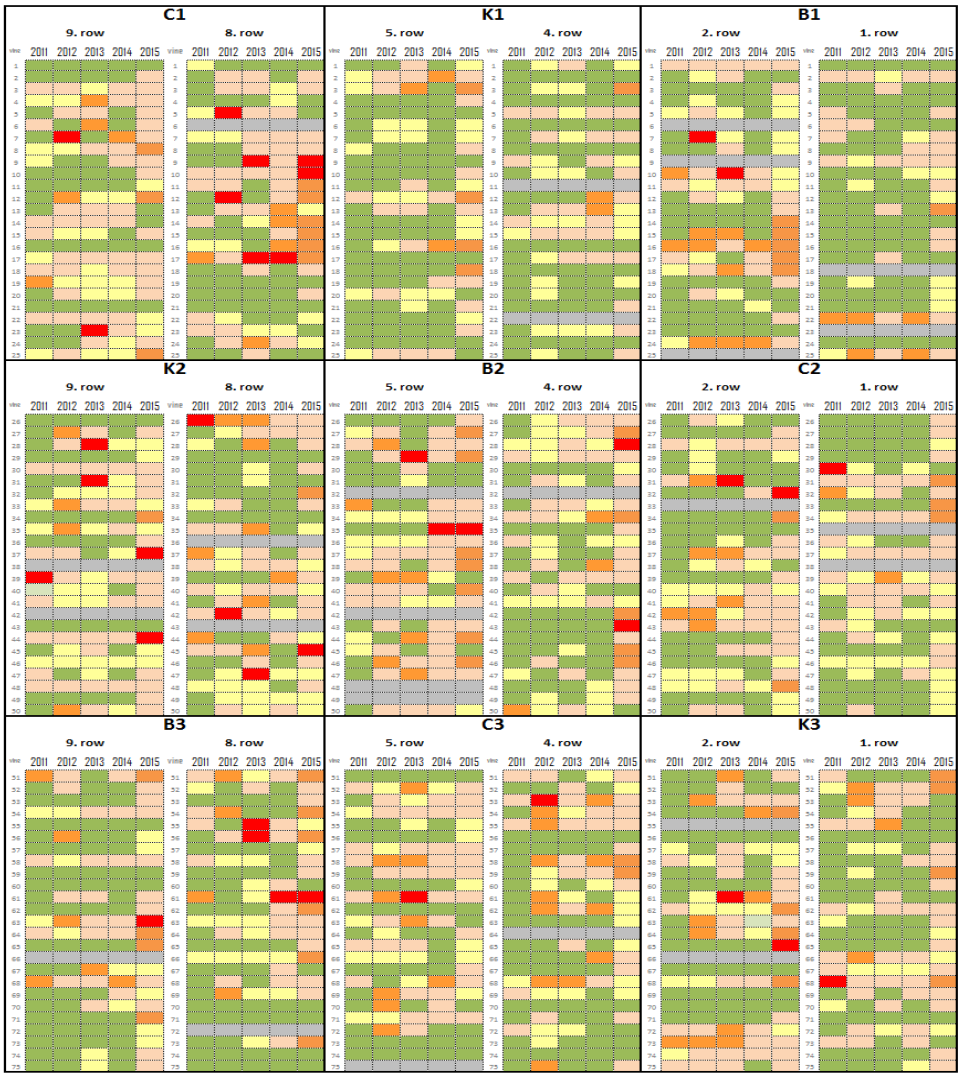

Figure 59. Result of 4-year BN monitoring: symptom evolution from 2011 to 2014

Legend: C1-C3: control blocks, B1-B3: Bion treated block, K1-K3: Kendal treated blocks; each block contains 50 vins (25 vine/row).

Code	Symptom
	Died plant
	Asymptomatic vine (-)
	Mild (+): leaf roll, mild yellowing; incidence of the affected shoots/vine below 15 %
	Moderate (++): leaf roll, yellowing, necrosis, uneven shoot lignification, berry shrivelling and/or drying; incidence of the affected shoots/vine between 15-25 %
	Severe (+++): leaf roll, yellowing, necrosis, uneven shoot lignification, berry shrivelling and/or drying;incidence of the affected shoots/vine between 25-35 %
	Extreme (++++): leaf roll, yellowing, necrosis, uneven shoot lignification, berry shrivelling and/or drying; incidence of the affected shoots/vine between 40-100 %.

Effectiveness of the treatments Control (C), Kendal (K) and Bion (B) were determined by the means of R values, i.e. R0, R1, R2, R3 and R4 calculated for all blocks (Table 10, 11). Results of both two-way MANOVA/ANOVA (treatment + year) and Marascuillo's procedure gave simlair results.

For R0 and R1, MANOVA overall test was significant for years (Wilk's lambda = 0.116; $P < 0.001$) and not significant for treatment (Wilk's lambda = 0.861; P = 0.476). Interaction was not significant, either ($P > 0.05$). In case of R2 and R3, ANOVA resulted in not significant results (R2: $F_{treatm}(2;18) = 0.841$, $P = 0.448$; $F_{year}(2;18) = 1.801$, $P = 0.194$; R3: $F_{treatm}(2;12) = 0.639$, $P = 0.545$; $F_{year}(1;12) = 0.384$, $P = 0.547$).According to one-sample Student's t-test, R4 does not differ significantly from zero ($t(2) = 1.0$; $P = 0.423$).

i) The year comparison of group/treatment revealed significant differences between groups in R1 value only in 2013 (Table 19). According to Marascuillo's procedure, the effectiveness of Kendal (K) treatment on recovery was the lowest, untreated control (C) was in beetwen and Bion (B) was the highest. However, based on Tukey's post-hoc test, B and C showed similary higher recovery than K.

ii) When R values were compared after different treatments, significant result was found only in the case of R0 and R1. The very same result was obtained for R0, separately evaluating for C, B and K. Year 2015 resulted in significantly lower values than the others.

Table 19. Results of Tukey's post-hoc test of R1 value in year 2012-2015 ($P < 0.05$)

Year	Treatment		
	C	**B**	**K**
2012	a	**ab**	*a*
2013	ab	**b**	*a*
2014	b	**b**	*b*
2015	a	**a**	*a*

Tukey's post-hoc test

Year	Treatment		
	C	**B**	**K**
2012	a	**ab**	*a*
2013	ab	**b**	*a*
2014	b	**b**	*b*
2015	a	**a**	*a*

Marascuillo's procedure

Legend: letters correspont to significant differences, a < b, different types belongs to treatment C (control), B (Bion), K (Kendal).

Results of spraying with resistance inducers revealed limited extents of symptom remission of the BN-affected plants. Unfortunately, remarkable differences between control and treatments with the active agent could not be observed. Only R0 (stayed asymptomatic in year n compared to year n-1) and R1 (recovered in year n compared to year n-1) showed significant differences between tretaments, and the best short-term recovery effect was found in the case of Kendal with glutathione-oligosaccharine active agent. Considering that the remission rate of untreated control was nearly as high as in the case of the treatments, we may state that the used resistance inducer had no short term effect on BN symptom remission in Eger wine region, Hungary.

10.14751/SZIE.2016.070

8. DISCUSSION

8.1. Epidemiology of Bois noir disease in Hungary

8.1.1. Genetic diversity of '*Ca.* P. solani' strains in Hungarian wine regions

Multi locus sequence typing is a molecular tool to characterize pathogenic bacteria for identification purposes, as well as for tracing infection source to arrest/prevent disease outbreaks. Typing of multiple loci is based on nucleotide data of housekeeping genes where the majority of the variations are selectively neutral (Jolley *et al*. 2001). This could be combined with sequence data of virulence genes, which are more polymorphic than housekeeping genes, thus providing us relevant epidemiological information.

For '*Ca*. P. solani genotyping *tuf* and *secY* housekeeping and *vmp1* and *stamp* variable functional markers are used routinely (Fialová *et al*. 2009, Cvrkovic *et al*. 2013, Aryan *et al*. 2014, Murolo *et al*. 2014, Pacifico *et al*. 2015, Plavec *et al*. 2015, Kosovac *et al*. 2016). In this study, new housekeeping markers were developed in order to improve genotyping tools. Variability of the markers was different. *YidC* showed the highest variability and was therefore applied in MLST. Based on five markers, i.e. *tuf, secY, yidC, vmp1* and *stamp* the biodiversity of '*Ca.* P. solani' isolates was investigated and ecosystems of diverse viticultural areas of Hungary were characterised.

Tuf. In our experiment, *tuf*-b1 and *tuf*-b2 types were found in Hungary. Genotype *tuf*-b1 is related to bindweed, harbouring a unique epidemiological cycle namely a phytoplasma exchange between bindweed (main host for populations of '*Ca*. P. solani' *tuf*-b1 type) and *H. obsoletus* bindweed ecotype (main host for populations of *H. obsoletus*), whereas *tuf*-b2 (variant of tuf-*a* nettle type) is propagated from infected stinging nettle (Langer and Maixner 2004). The *H. obsoletus* bindweed ecotype propagates *tuf*-b1 and nettle ecotype *tuf*-b2 to grapevine or to other crops. Based on recent studies *H. obsoletus* populations adapted to different plant hosts (ecotypes) such as *C. arvensis* (bindweed), *U. dioica* (stinging nettle), *Salvia sclarea* (clary sage), *Crepis foetida* (stinking hawksbeard), *Vitex agnus-castus* (vitex, chaste tree) and *L. angustifolia* (lavender) (Chuche *et al*. 2013, 2016b, Kosovac *et al*. 2015). Populations found on these hosts showed morphological (i.e. lower size), behavioural (i.e. feeding preference) and genetic (i.e. COI haplotypes) differences, as well as varying endosymbiont populations (Johannesen *et al*. 2012, Imo *et al*. 2013, Chuche *et al*. 2016b). Despite the fact that *H. obsoletus* is polyphagous, it shows strong preference to their native plant (i.e. bindweed or stinging nettle), which is the elemental and original infection source of the given phytoplasma genotype (Imo *et al*. 2013). *Tuf*-b1 (previously named *tuf*-b) is more prevalent in South-eastern and Eastern Europe, while and *tuf*-b2 (variant of *tuf*-a) are present in Western

Europe (Johannesen *et al*. 2008, 2012). The presence and spreading of nettle *tuf* types (*tuf*-a, *tuf*-b2) are correlate to the vector's host shift, namely the movement of *H. obsoletus* from bindweed to stinging nettle which led to an increased spread of stinging nettle-specific *tuf* strain in Germany, France and Switzerland (Imo *et al*. 2013). In recent years, appearance of nettle-type (*tuf*-a) have been reported in neighbouring countries of Hungary, although bindeweed-type (*tuf*-b) still seems predominant in Eastern Europe (Table 20). It is noticeable that in Austrian vineyards between 2003 and 2008, the '*Ca*. P. solani' infection level of stinging nettle was very low and the *H. obsoletus* population was observed mainly on bindweed (Riedle-Bauer *et al*. 2006). In the past few years, high *H. obsoletus* populations have appeared on stinging nettle (Aryan *et al*. 2014). In Hungary, despite the attempt to detect phytoplasma on stinging nettle, *tuf*-a type has not been found yet, neither on stinging nettle, nor on grapevine. In fact, phytoplasma-infected stinging nettle has not been found at all. However, *tuf*-b2 (type related to stinging nettle) was identified on grapevine and *Solanaceous* crops in Hungary. This suggests that stinging nettle is a potential infection source of '*Ca*. P. solani' also in Hungary.

Vmp1. Investigation of *vmp1* gene revealed the presence of V2, V9, V13 and V18 genotypes in Hungary. Results of this study correspond to the high variability of '*Ca*. P. solani' found in the Czech Republic, Italy, Serbia, Macedonia, Croatia and Montenegro; however the proportion of *vmp1* genotypes highly differed amongst the countries (Fialová *et al*. 2009, Pacifico *et al*. 2009, Murolo *et al*. 2010, Cvrkovic *et al*. 2013, Kosovac *et al*. 2015, Plavec *et al*. 2015, Atanasova *et al*. 2015) (Table 20). The V18 genotype, which can be found in North-west, North-east and Central Hungary, was also detected in high abundance in Croatia and Macedonia (Plavec *et al*. 2015, Atanasova *et al*. 2015). In general, in the neigbouring countries, this genotype is present both on grapevine and stinging nettle, which also confirm our suspicion that stinging nettle acts as a '*Ca*. P. solani' source in Hungarian vineyards.

SecY. Variability of *secY* in Hungary was nearly the same than found in Central Europe, where S1, S4 and S6 genotypes are dominant (Table 20). In general S1 and S4 are present on bindweed and on grapevine, and S6 is mostly reported on stinging nettle and on grapevine (Foissac, personal communication). Sporadic occurrence of the S7 genotype in grapevine was observed only in the southern part of Hungary, close to the Croatian border. The S7 genotype was also reported in Croatia, suggesting its introduction from the southern part of Europe to Hungary (Fabre *et al*. 2011b). Based on limited dispersion of S7, it can hypothesized that it might have been introduced to Hungary recently, or this genotype might require specific interaction with a local vector.

Stamp. The diversity of the *stamp* gene was significant and dispersal of *stamp* genotypes showed a moderate geographical pattern. Cluster II and IV were present in north-west, north-east, south and central parts of the country, with a high dominance of genotypes belonging to cluster II.

Genotypes of cluster III were found only sporadically. On grapevine, ST6 (cluster IV) was dominant, followed by a lower incidence of ST4 and ST9 (cluster II). As only ST4 and ST9 were detected on bindweed, we may be stated that bindweed is the main source of these genotypes. Based on international results ST6 is a frequently found genotype on stinging nettle and on grapevine (Foissac, personal communication). Results of this study, i.e. ST6 dominance on grapevine, are in agreement with finding of Elekes *et al*. (2006), namely that there was a low population density of *H. obsoletus* on bindweed, but instead it was presenting on stinging nettle. Although information is very limited on *stamp* genotypes of Hungarian *H. obsoletus* populations and of stinging nettle, the dominance of ST6 on grapevine and support that this genotype may have been introduced from stinging nettle or newly find reservoir red deadnettle to grapevine. From the aspect of BN disease of grapevine, stinging nettle seems the most important reservoir of the pathogen in Hungary, although its role has to be confirmed.

Table 20. Occurrence of *tuf/vmp1/secY/stamp* '*Ca*. P. solani' genotypes in central Europe

Country	Genotype				Reference
	tuf	***vmp1***	***secY***	***stamp***	
Hungary	***tuf*-b*** ***tuf*-b1, *tuf*-b2****	**V2**, V9, V14, **V18**	**S1**, S4, **S6**	ST4, **ST6, ST9**, ST9D, ST11, ST13, ST21, ST22, , ST52	Result of this study
Czech republic	***tuf*-b**, *tuf*-a*	high variabilty***	S1	-	Fialová *et al*. 2009
Austria	***tuf*-a**, *tuf*-b* tuf-b1 and ***tuf*-b2****	**V17**	same as Charante, MOL (S1) in France ***	same as Rqg50 (ST9), STOL(ST13) in Serbia ***; At9 (ST9D)	Aryan *et al*. 2014
Croatia	***tuf*-b**, *tuf*-a*	**V2**, V3, V4, **V18**	S4, S6	same as CPsM4_At1 in Austria ***	Plavec *et al*. 2015
Serbia	***tuf*-b***	V2-TA, V4,V7-A, V14	S1, S4, S5	ST9(Rqg50), ST9D(Rqg31=At9), ST11(BG4560), ST13(STOL), ST22(Rpm35), ST29(Vv24)	Cvrkovic *et al*. 2013
Montenegro	***tuf*-b**, *tuf*-a* *tuf*-b1, *tuf*-b2**	V2-TA,**V3**, V4, **V14**, V17	-	ST9, ST9D, **ST13**, **ST22**, ST29	Kosovac *et al*. 2015
Macedonia	***tuf*-b*** *tuf*-b2** *(tuf*-ab)	V2-TA,V3, V18	-	ST9, ST13	Atanasova *et al*. 2015
Italy	***tuf*-a**, *tuf*-b*	V2-TA, **V3**, V4, V5, V6, V7, V8	-	-	Pacifico *et al*. 2009 Murolo *et al*. 2010

Legend: bold indicate the most abundant genotype(s); * *tuf* type based on PCR/RFLP: *tuf*-a and *tuf*-b; ** *tuf* type based on sequence analysis: *tuf*-b1, *tuf*-b2 (Figure 8, Table 3); *** genotypes could not been give due to the lack of unique denomination.

In Hungary, the sporadic presence of cluster III (ST13 and ST22) on both plant and on the insect (i.e. *R. quinquecostatus)*, which genotypes are widespread in south Balkan, suggests their diffusion from the south to the north. According to results of this study, it is colcluded that the most prevalent genotypes on grapevines in Hungary are S6/V18/ST6, S1/V2/ST4 and S1/V2/ST9, which correspond to genotypes found on the main hosts i.e. bindweed and stinging nettle of '*Ca*. P. solani'. First report of '*Ca*. P. solani' infection of lavender (*L. angustifolia*) in Hungary, as well on red deadnettle (*Lamium purpureum*) and field elm (*Ulmus minor*) may be considered new '*Ca.* P. solani' host plants.

8.1.2. Insect transmission of Hungarian '*Ca*. P. solani' strains

Experimental transmission was conducted with planthoppers of the *Cixiidae* family collected in four locations. Strain ST4 and ST13 of *stamp* clusters II and III were transmitted to Madagascar periwinkle by *H. obsoletus* and *R. quinquecostatus,* respectively. Insects *H. obsoletus* and *R. quinquecostatus* were the most abundant species at the collection sites. This is in agreement with the finding of Elekes *et al*. (2006). *R. melanochaetus* was first reported in the Tokaji wine region, and *R. cuspidatus* in the Etyek-Budai region, with no evidence that this species could act as vector of '*Ca*. P. solani'. In accordance with Elekes *et al*. (2006) a high population of *H. obsoletus* was found on stinging nettle and very few individuals on bindweed.

8.1.3. Insect-pathogen protein interaction

Phytoplasma surface proteins certainly play an important role in the phytoplasma life cycle by interacting with specific receptor proteins of the insect, which makes the species act as a competent vector (Fabre *et al*. 2011a, Suzuki *et al* 2006). The antigenic membrane protein (AMP) of '*Ca*. P. asteris' has been shown to interact with the insect microfilaments containing actin (Suzuki *et al*. 2006). Galetto *et al*. (2011) demonstrated interaction between ATP-synthase (a mitochondrial protein in the plasma membrane of the midgut and salivary glands of '*Ca*. P. asteris' vector *Euscelidius variegatus*) and AMP. An ortolog of AMP was identified recently, named STAMP (Fabre *et al* 2011a). STAMP is under positive selection and might have a function in the interaction between insect and phytoplasma. According to the extensive studies on the main known '*Ca*. P. solani' vector (*H. obsoletus*) and biological cycle of the pathogen, BN has been stated as non-epidemic on grapevine as it does not propagate from grapevine to grapevine (Maixner 1994, Johannesen *et al*. 2012). Monitoring of '*Ca*. P. solani' vectors in Hungarian vineyards revealed a low abundance of *H. obsoletus* (Elekes *et al*. 2006). A similar conclusion was drawn in Serbia, where the frequency of BN-affected grapevines did not correlate with low population density of *H. obsoletus* (Cvrkovic *et al*. 2013). Nevertheless, BN disease is steadily spreading in Hungarian as well as European vineyards. This suggests that other factors such as further competent vector or specific insect-pathogen interaction might be involved in this process. To detect '*Ca*. P. solani' monoclonal antibody 2A10 MAb were produced (Garnier *et al*. 1990, Fos *et al*. 1992). *In situ* immunofluorescence detection demonstrated that the MAb 2A10 recognizes STAMP of strains belonging to cluster I (Fos *et al*. 1992, Fabre *et al*. 2011a). In our experiment we first demonstrated that MAb 2A10 is able to recognise *stamp* cluster II, III and IV, therefore a very useful tool for the investigation of vector protein – '*Ca*. P. solani' protein interaction. Additionaly, we demonstrated that STAMP cluster II interact with proteins of *H. obsoletus* bindweed and lavender ecotypes, and with *R. quinquecostatus*. Interaction with *H. obsoletus* stinging nettle ecotype and *R. panzeri*

showed less intensity. This suggests that *H. obsoletus* bindweed ecotype might be the most competent vector of *stamp* genotypes of cluster II. The '*Ca*. P. solani' vectoring ability of *R. quinquecostatus* to grapevine has not been demonstrated so far (Cvrkovic *et al*. 2013). Although, based on the results of this study (including transmission trial, detection of *stamp* ST13 genotype on grapevine and interaction experiment) it can be hypothesized that *R. quinquecostatus* could be a competent '*Ca*. P. solani' vector.

8.2. Effects of Bois noir disease on performance of *V. vinifera* L. cv. Chardonnay in Eger wine region

Impact of BN on grapevine physiology and vegetative development. Pathogenic organisms secrete effector molecules, which induce various physiological changes in the host to provide a competitive advantage for themselves and their vector(s) (Hogenhout *et al*. 2008, Sugio *et al*. 2011a, MacLean *et al*. 2014). The altered phloem transport in mollicute-infected plants leads to imbalances in carbohydrate distribution, i.e. accumulation in source leaves and lack in sink organs, including grapes (Lepka *et al*. 1999, Bové *et al*. 2003, Santi *et al*. 2013). Regarding photosynthesis, it had been shown that photosynthetic activity is impaired in BN-affected plants (Guthrie *et al*. 2001, Bertamini *et al*. 2002b). Also, BN-diseased 'Chardonnay' and 'Primitivo' cultivars showed decreased transpiration (Endeshaw *et al*. 2012). In agreement with these earlier reports, a 30 % reduction in chlorophyll content and a 28 % decrease in photosynthetically active leaf surface (due to leaf rolling) were observed. Under relatively normal circumstances, the whole, vertically positioned canopy of the grapevine is almost never light saturated, because of light patterns and density of the canopy (Hunter *et al*. 1994, Hunter and Visser 1988, Hunter and Visser 1989). The '*Ca*. P. solani' affected shoots are not vigorous enough or have a horizontal growth habit, especially with lack of lignification (Boudon-Padieu 2003). Thus, the exposure of the affected shoots to sunlight under general (vertical trellis) cultivation conditions may be even less and grapes further shaded. The disruption of phloem transport of phytoplasma infected plants is certainly the most significant physiological phenomenon. It may act negatively through feedback inhibition and would lead to a further decline performance of diseased vines (Hunter and Ruffner 2001, Hren *et al*. 2009). Because of incomplete maturation, an average of 30 % of canes of BN-diseased vines showed uneven lignification. During winter, non-lignified canes turned black and did not carry any latent or active buds that are necessary for re-initiation of production in the following season. Shoots that were heavily infected by '*Ca*. P. solani' in summer never recovered, and, whatever the pruning practice in winter, the canes did not carry any shoots or bunches, resulting in an unpredictable yield. The results point the importance of the age of the plant, indicating that young vines and young shoots would not recover when heavily infected.

The productivity of buds is determined within three months after bud break and poor fruit set can be caused by physiological and environmental stresses (Keller 2010). No significant differences in the number of flowers and berries between healthy and BN-affected plants were observed. These results suggest that the negative effects of '*Ca*. P. solani' infection may have commenced only after flowering, i.e. dry berries or bunches being visible at the berry set stage, and were enhanced during the grape ripening stage. Yield and fruit composition of grapevines depend on the seasonal photosynthetic capacity of the canopy (Hunter and Visser 1988). Bois noir infection leads to a decrease in bunch weight, berry weight, and number of bunches per vine resulting in a 70 % yield loss (Endeshaw *et al*. 2012), which is in accordance with the findings of other studies (Garau *et al*. 2007, Zahavi *et al*. 2013, Romanazzi *et al*. 2013). In this study, the decrease in the number of bunches and yield of infected vines averaged between 56.7 % and 68.4 %, respectively. The extent and proportion of the loss (i.e. yield, bunch mass and berry mass) varied in years and there was a significant year × infection interaction ($P < 0.05$), indicating a strong dependence of infection on the environmental factors of different years. According to Panassiti and colleagues (2015), the environmental conditions and grapevine cultivars influenced BN incidence. Phytoplasmas virulence depends on temperature, plant age and genetic backgrounds of phytoplasma and plant host (Foissac and Wilson 2010, Sugio *et al*. 2011b, Jarausch *et al*. 2013). 'Chardonnay' is considered one of the most sensitive grapevine cultivars to BN disease (Martelli and Boudon-Padieu 2006). Despite the high susceptibility of this cultivar, BN-affected vines still produced grapes. However, the presence of bunches with shrivelled berries on BN-diseased shoots would most likely lead to a lower quality wine.

Impact of BN on 'Chardonnay' wine quality. Phloem-limited pathogens are known to affect grapevine performance as well as fruit quality (Boudon-Padieu 2003). Decreases in yield and fruit soluble solids caused by Grapevine leaf roll and GVA viruses are well described (Mannini *et al*. 2012b). Interestingly, Fleck virus positively changed grape maturity (i.e. colour intensity) of cv. 'Nebbiolo', but the yield drop was a remarkable 40 % (Mannini *et al*. 2012a). Premature berry dehydration that occurred in 'Merlot' cultivars was associated with phytoplasma infection, suggesting that the phytoplasma-caused partitioning between the nutrient source and berries results in inhibited sugar transport, poor synthesis of anthocyanins, and the lack of organic acid degradation (Matus *et al*. 2008). Based on results of this study, i.e. elevated TA and lower pH and °Brix, similar effects on fruit composition may have occured in BN-affected vines. The amount of plant origin organic acids was elevated in wine produced from BN-affected grapes, suggesting that their breakdown was affected during berry maturation. Biosynthesis of these compounds takes place before veraison and their concentrations decrease considerably in berries during maturation because of increase in berry volume (e.g. tartaric acid) and degradation (mainly malic acid)

through respiration (Hunter and Ruffner 2001). It is important to note that a more pronounced negative effect on fruit composition was observed in 2013, in the year among the three experimental years when the weather conditions were the most optimal for grapevine production. In fact, negative effects were slightly masked in the years with unfavourable weather, i.e. lower heat sumnation and higher precipitation during veraison and ripening. It could be hypothesized that the highest impact of '*Ca*. P. solani' can only be seen in warm and dry years, when the grapevines experience higher environment-induced stress. However, further studies are needed to support this hypothesis. The elevated calcium content of wines produced from BN-diseased grapes may have led to the crystallisation of calcium-tartrate which compromises wine stability. Calcium-tartrate deposition in the bottle frequently occurs with calcium content over 60 mg/L, leading to crystallisation in champagne-based wines even at low pH (Ribéreau-Gayon *et al*. 2006). To overcome problems related to stability, wines with higher calcium contents require intensified stabilisation steps before bottling and marketing.

Phenolic compounds in grapevines considerably influence the organoleptic properties of wine (Pinelo *et al*. 2006). The amount of phenolic compounds of BN-diseased 'Chardonnay' berries was investigated and changes to secondary metabolites in the berry skin were described (Rusjan *et al*. 2012). The amount of flavonols decreased while flavanol and hydroxycinnamic acid contents increased in BN-affected shrivelled berries (Rusjan *et al*. 2012). Consistent with these previous findings, this study revealed elevated hydroxycinnamic acid (i.e. caffeic acids) and decreased flavonoid contents (catechin and epicatechin) in wines produced from BN-diseased grapes. Lutter *et al*. (2007) determined that caffeic acid forms dihydroxybenzaldehyde [in the presence of iron (II)], which reacts with (+)-catechin and leads to a discolouration of wine-mimicking solutions. We observed pink discolouration in wines from BN-affected and shrivelled grapes produced in 2013 and 2014 and detected elevated phenolic contents. This suggested that the reaction described by Lutter *et al*. (2007) may have occured occur in the wines produced from BN-affected and shrivelled grapes. However, higher acidity, i.e. lower pH of must of BN-affected vines, might have caused the pigments in BNA and BNS wines to change colour. The decrease in flavonoid content may address the question whether wine produced from BN-affected grapes lack the health beneficial effects ascribed to grape antioxidants. It would be necessary to clarify the role of phenolic compounds in the wine pinking phenomenon. However, it was recently shown that in the context of model plants, phytoplasma infection led to an over-expression of anthocyanin, but in the context of anthocyanin defective mutants, programmed senescence is taking over (Himeno *et al*. 2014). White cultivars of *V. vinifera* L. are considered ancient natural mutants that had been selected by humans. Thus, the presence of any phytoplasmas and possibly many other virus-like

parasites could had a major effect on pigment biosynthesis and therefore on wine colour (Roggia *et al*. 2014).

8.3. Curative field treatments of BN infected grapevines applying resistance inducers

BN has a complex disease cycle, which includes alternative host plants as sources of inoculum and non-ampelophagous vectors, it is very difficult to control (Constable 2010, Maixner 2011). Due to the remission, i.e. temporary recovery and recovery events, i.e. permanent disappearing of symptoms and phytoplasma from the plant) the number of symptomatic plants is fluctuating in the vineyard (Osler *et al*. 1993, Belli *et al*. 2010, Romanazzi *et al*. 2013). In the cured plants -compared to originally healthy plants- elevated H_2O_2, jasmonic acid and NAD(P)H peroxidases activity were observed, and decrease in salicylic acid content occurred. This refers to systemic acquired resistance (SAR), which in the plant could be triggered by certain molecules in the plant (Musetti *et al*. 2013, Santi *et al*. 2013). Based on the disease status of the plant (i.e. severity and incidence), which also depends on cultivar, BN results in yield and quality losses of various proportions (Pavan *et al*. 2012, Ember *et al*. 2014, 2016). The effects of GY diseases also depend on the virulence of the pathogen and other environmental factors, such as temperature (Foissac and Wilson 2010, Danet *et al*. 2011). Measures applied against insect vectors or prophylactic actions could be applied to reduce the damage. However, it may not be effective enough to protect our vineyards from BN outbreaks. An innovative treatment strategy was developed applying elicitors such as chitosan, glutathione-oligosaccharines, benzothiadiazole, and phosetyl-Al to induce symptom remission and temp to achieve recovery of BN-affected grapevines (Romanazzi *et al*. 2009). In our three-year spraying experiment with Bion (benzothiadiazole) and Kendal (glutathione-oligosaccharines) moderate symptom remission was observed in both treatments, however, the extent of remission in the case of the control was nearly in the same scale. The most intensive curative effect, in short term remission, was observed in the case of Kendal treatment, followed by untreated control, then Bion. The extents of remissions in the case of treatments were lower than we expected, and long term differences have not yet been observed (i.e. 2, 3 or 4 year symptomless status after remission). Side effects from the treatments were not observed. However, the remission rates in our experiment were lower than those reported by Romanazzi *et al* (2013). It is important to note that the extent of remission/recovery may be different between years, and influenced by climatic and growth factors, such as temperature, precipitation and the conditions of the individual vine. The long-term effect of the applied treatments should therefore be monitored.

9. NEW SCIENTIFIC RESULTS / ÚJ TUDOMÁNYOS EREDMÉNYEK

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 marker 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.

9. ÚJ TUDOMÁNYOS EREDMÉNYEK

1. Bizonyítottuk a ’*Candidatus* Phytoplasma solani’ okozta Bois noir betegség szignifikánsan negatív hatásait a szőlő vegetatív és reproduktív teljesítményére, valamint a termés beltartalmi mutatóira, Chardonnay fajtán az Egri borvidéken (Magyarország).

2. Elsőként bizonyítottuk, hogy a ’*Ca*. P. solani’ okozta Bois noir betegség negatívan befolyásolja a bor minőségét Chardonnay fajta esetében az Egri borvidéken (Magyarország).

3. Bizonyítottuk, hogy a BN fertőzéshatásra kialakuló tőkénkénti termésmennyiségben, fürtszámban és a bogyótömegben bekövetkezett terméscsökkenés és az évjárat között szignifikáns összefüggés van.

4. ‘*Ca*. P. solani’ törzsek genetikai diverzitásának vizsgálatára alkalmas két új háztartási géneket kódoló markert fejlesztettünk: a *yidC* (sejt membrán fehérje integrációs rendszer egyik fehérjéét kódoló gén) és *ligA* (NAD(+)-függő DNS ligázt kódoló gén) markerek megfelelő variabilitásuk miatt alkalmazhatóak a multi lókusz szekvencia-alapú molekuláris jellemzésre (MLST).

5. Elvégeztük a Magyarországi ‘*Ca*. P. solani’ izolátumok diverzitásának vizsgálatát *tuf, secY, yidC, vmp1* and *stamp* gének alapján. Vizsgálatainkkal bizonyítottuk a következő genotípusok hazai borvidékeken való jelenlétét. *Tuf*: *tuf*-b1 and és *tuf*-b2; *secY*: S1, S4 és S6; *vmp1*: V2, V9, V13 és V18; *stamp*: ST6 (IV csoport), ST4 és ST9, ST9D (II csoport), és szórványosan az ST13 és ST22 (III csoport). A szőlő növényen leggyakrabban előforduló genotípusok az S6/V18/ST6, S1/V2/ST4 és S1/V2/ST9 voltak.

6. Magyarországon elsőként bizonyítottuk a ‘*Ca.* P. solani’ fertőzést levendulán (*Lavandula angustifolia*). Elsőként mutattunk ki ‘*Ca*. P. solani’ fertőzést árvacsalánból (*Lamium purpureum*) és mezei szilből (*Ulmus minor*).

7. Átviteli kísérlettel elsőként bizonyítottuk, hogy a *Hyalesthes obsoletus* képes ‘*Ca*. P. solani’ ST4 (II csoport) *stamp* genotípust, a *Reptalus quinquecostatus* kabóca pedig az ST13 (III csoport) *stamp* genotípus Madagaszkári rózsameténgre táplálkozása során átvinni.

8. Elsőként bizonyítottuk, hogy a ‘*Ca*. P. solani’ *stamp* I csoportba tartozó törzs ellen előállított 2A10 monoklonális antitest alkalmas a *stamp* II, III és IV csoportok szerológiai kimutatására.

9. Elsőként bizonyítottuk a *Ca*. P. solani’ STAMP és kabóca közötti *in vitro* interakciót. Rovar fehérje és ’*Ca*. P. solani’ membránfehérje (STAMP fp_ST4 és fp_ST9) közötti kapcsolatok vizsgálat során bizonyítottuk a STAMP II csoport (fp_ST4 és fp_ST9) a *H. obsoletus* (apró szulák ökotípus), valamint a *R. quinquecostatus* fajok között létrejövő kapcsolatot.

10. CONCLUSIONS AND PERSPECTIVES

Multi locus sequence typing (MLST) is used to investigate epidemiological properties and population genetics of prokaryotes and is based on sequence analyses of different genetic loci. To increase robustness and precise characterization of bacterial 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. 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 (Murolo and Romanazzi 2015). 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 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 vector 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 economical sustainability. Regarding the vectors themselves, climate changes can alter in the short or longer term the distribution and behaviour of several insect species, including those of the *Heteroptera*

and *Homoptera* orders (Boudon-Padieu and Maixner 2007, Musolin and Fujisaki 2006). At higher temperature, 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'. Bois noir disease in a vineyard of cv. 'Chardonnay' in the Eger wine region of Hungary heavily decreased yield and adversely affected grape composition, which resulted in variable wine quality. More pronounced negative effects on fruit composition and wine quality occured 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 studied 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. Genetic characterisation of '*Ca.* P. solani' strains infecting the plants tested in this study is underway.

Also important could be the masking effect of BN over other GY disease management. Indeed, BN and FD, as any other GY, induce identical symptoms, and BN cases lead to a masking of early FD outbreaks. Therefore further studies need to be undertaken in Hungary or elsewhere on cv. 'Chardonnay' and other cultivars in order to better evaluate the economic impact of the disease in terms of yield and quality. Moreover continuation of '*Ca*. P. solani' genotyping, as well as STAMP-insect protein interaction studies would be crucial, in order to develop a new effective pest management strategies against Bois noir disease.

11. SUMMARY / ÖSSZEFOGLALÁS

Grapevine Yellows diseases caused by phytoplasmas induce chronic damages worldwide. In European and Hungarian vineyards '*Candidatus* Phytoplasma solani' causing Bois noir disease is common. '*Ca.* P. solani' is endemic to the Euro-Mediterranean area and is of wild plant origin. It is transmitted from bindweed and stinging nettle to grapevine and to other crops by different planthoppers of the *Cixiidae* family. In Europe, at least four cixids are vectoring '*Ca*. P. solani', Among them, only *H. obsoletus* and *R. panzeri* are proven vectors of '*Ca*. P. solani' to grapevine, however vectoring ability of others species cannot be excluded. Ecotypes of *H. obsoletus* are vectoring specific '*Ca*. P. solani' genotypes and are related to plant hosts bindweed or stinging nettle. However the role of the infected propagation material is also important in spreading. Apart from hot-water treatment of planting material, there is no effective treatment against phytoplasma diseases; only prophylactic measures can be taken. As BN-vector insects are not feeding permanently on grapevine the insecticide treatments against them are not efficient, thus rendering hard to control the spreading of BN disease.

11.1. Epidemiology of Bois noir disease in Hungary

11.1.1. 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 ecosystem of BN disease in five geographically representative wine regions of Hungary was surveyed. Multi locus sequence typing (MLST) 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. To increase the predictive value regarding epidemic properties, markers linked to interaction with insect vectors can also be sequenced. 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 new housekeeping markers were developed. 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) was the most variable.

Based on PCR/RFLP and sequence analyses of five genetic loci, namely *tuf, secY, yidC, vmp1* and *stamp* biodiversity of '*Ca.* P. solani' isolates of viticultural areas of Hungary: Egri, Tokaji, Kunsági, Villányi, Soproni and Etyek-Budai were investigated. In this experiment *tuf*-b type (PCR/RFLP) and *tuf*-b1 and *tuf*-b2 (by sequencing) genotypes were found. Presence of *tuf*-a is typical in Western Europe, and *tuf*-b is most common in Central and Eastern Europe (Langer and

Maixner 2004, Johannesen *et al*. 2012). Presence of *tuf*-a is in correlation with the vector's host shift, namely the movement of *H. obsoletus* from field bindweed to stinging nettle which led to the increased spread of the stinging nettle-specific *tuf*-a strain in Western Europe (Imo *et al*. 2013). Differentiation of *tuf*-a and *tuf*-b genotypes was based on PCR/RFLP, which provides limited sequence information (Langer és Maixner 2004). Recent information obtained based on sequence analysis of *tuf* fragments revealed further genotypes which were renamed. The nettle-related genotypes are *tuf*-a, *tuf*-b2 and *tuf*-b3; and the bindweed related is *tuf*-b1 (Foissac personal communication, 4th European BN Workshop, 2016). In Hungary, despite the attempt to detect phytoplasma from stinging nettle *tuf*-a type has not been detected yet neither on stinging nettle, nor on grapevine, however *tuf*-b2 (nettle type) was found on grapevine. Investigation of *vmp1* gene revealed the presence of V2, V9, V13 and V18 genotypes in Hungary. 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. Variability of *yidC* was nearly similar to *secY*. The *secY* S1, S4 and S6 genotypes are dominant in Hungary, similar than 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 results of this study. 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.

We can conclude that in Hungary the most prevalent genotypes on grapevine are S6/V18/ST6, S1/V2/ST4 and S1/V2/ST9. The role of bindweed in spreading of S1/V2/ST4 and S1/V2/ST9 genotypes to grapevine was confirmed. Based on European and also on our results, the importance of stinging nettle and/or red deadnettle as main dissemination source of S6/V18/ST6 to grapevine can be suggested. However, the roles of these plants in BN ecosystem in Hungary have to be confirmed. The presence of *stamp* ST13 genotype on grapevine -the genotype transmitted by *Reptalus quinquecostatus* to periwinkle- suggesting that this planthopper could be a competent vector of '*Ca*. P. solani' to grapevine. 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. Infection of '*Ca.* P. solani' on lavender is reported for the first time in Hungary. In addition, '*Ca.* P. solani' infection is reported for first time on red deadnettle (*Lamium purpureum*) and field elm (*Ulmus minor*).

11.1.2. Insect transmission of Hungarian '*Ca.* P. solani' strains

In Hungary experimental transmission were done in 2013. Planthoppers of the *Cixiidae* family: *H. obsoletus, R. panzeri, R. quinquecostatus, R. cuspidatus* were collected from four locations and an experimental host Madagascar periwinkle (*C. roseus*) was 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. The *R. cuspidatus* was not proven as vector. Species *H. obsoletus* and *R. quinquecostatus* were the most abundant in the examined regions, which is in agreement with the findings of Elekes *et al.* (2006). *R. melanochaetus* was first reported in the Tokaji wine region and *R. cuspidatus* in the Etyek-Budai region. In accordance with Elekes *et al*. (2006) a high population of *H. obsoletus* was found on stinging nettle and very few individuals on bindweed.

11.1.3. 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 the 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 were obtained. New generation genome sequencing of the strains was achieved. Insightful information has not been obtained yet, as the *de novo* assembling and annotation is in progress.

11.1.4. Insect-pathogen protein interaction

Phytoplasma surface proteins play an important role in the phytoplasma life cycle and polymorphism of these protein might determine the transmission ability of different insect species (Suzuki *et al*. 2006, Fabre *et al*. 2011a). Based on variable surface protein gene (*stamp*) strains of '*Ca.* P. solani' from several European countries were classified into four distinct phylogenetic clusters with a geographical distribution exhibiting geographical patterns. This could be explained by the distribution of known insect vectors and the adaptation of '*Ca*. P. solani' strains to a specific vector. In order to better understand the vector specificity an interaction study was initiated. To perform this experiment, we tested and demonstrated that 2A10 momoclonal antibody -raised against STAMP of cluster I (Garnier *et al*. 1990, Fos *et al*. 1992)- is able to recognise *stamp* clusters II, III and IV, and is therefore a very useful tool for further vector protein - '*Ca*. P. solani' protein interaction studies.

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. Therefore heterologous expression of STAMP fp_ST4 and fp_ST9 recombinant proteins in *E. coli* was carried out. Interaction ability of the purified 16 kDa STAMP(s) (fp_ST4 and fp_ST9) with various insects' protein was tested in dot-blot analysis usin 2A10 MAb. BN vectors: *H. obsoletus*, *R. panzeri;* potential vectors: *R. quinquecostatus*, *R. cuspidatus* and non-vectors*: Euscelidius variegatus Circulifer haematoceps,* 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 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 (including transmission trial and interaction experiment) it can be hypothesized that *R. quinquecostatus* could be a competent vector of '*Ca*. P. solani' ST13 *stamp* genotype to other herbaceous hosts. *In vitro* interaction between '*Ca*. P. solani' STAMP and insect proteins was demonstrated for the first time in this study.

11.2. Effect of Bois noir disease on performance of *V. vinifera* L. cv. Chardonnay in Eger wine region

Damage of phytoplasma-induced BN has not been comprehensively investigated, especially with regards to wine attributes. In a three-year field experiment the impact of BN on vegetative, reproductive, and wine characteristics of *V. vinifera* L. cv. 'Chardonnay' was investigated in Eger wine region of Hungary. Measurements included vegetative and reproductive growth, berry must and wine composition and wine sensory profiles. The most significant factors characterising BN infection were identified using a neural network model and discriminant analysis.

Significant increases/decreases in vegetative and reproductive parameters 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 %).

It was demonstrated for the fist 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 %), lower flavonoids (catechin -8.9 % and epicatechin -14.4 %). 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. 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.

There were noticeable differences in analytical parameters among wines produced from healthy and BN-affected grapes. These differences were partly 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.

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

Several studies reported the remission and recovery phenomenon on several phytoplasma infected grapevine cultivars. Remission (i.e. temporary disappearance of BN symptoms) and recovery (i.e. permanent disappearance of the symptoms and phytoplasma from the plant) are associated with systemic acquired resistance which could be induced by certain molecules (Musetti *et al*. 2013, Santi *et al*. 2013). An innovative field treatment was developed to control BN, herein, elicitors such as chitosan, glutathione-oligosaccharines, benzothiadiazole and phosetyl-Al were applied to induce recovery of BN-affected grapevines (Romanazzi *et al*. 2013). Similarly to this study, a field experiment was set up in a '*Ca*. P. solani' infected 'Chardonnay' plot in the Eger wine region. In the three-year experiment (2012-2014) commercial products Kendal (Valagro, Italy) and Bion (Syngenta, Switzerland), 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 Kendal treatment followed by untreated control, then Bion. The extents of remissions were moderate in all cases, and differences in long term (2, 3 or 4 year symptomless status after remission) has not yet observed. However, long term positive effect on symptom remission of the performed tretaments may be expected. Side effects from the treatments were not observed.

10.14751/SZIE.2016.070

10.14751/SZIE.2016.070

A BOIS NOIR BETEGSÉG JÁRVÁNYTANA, ÉS HATÁSA A SZŐLŐ ÉS A BOR MINŐSÉGÉRE MAGYARORSZÁGON

11. ÖSSZEFOGLALÁS

A fitoplazmák okozta Szőlő Sárgaság betegségek világszerte károkat okoznak a szőlő- és borágazatnak. Az európai és így a magyarországi ültetvényekben a Bois noir (BN) betegség melynek kórokozója a ’*Candidatus* Phytoplasma solani’ igen gyakori. Az Euro-Mediterrán térségben a ’*Ca*. P. solani’ endemikus. A BN betegség kórokozója vad rezervoárokról, mint az aprószulák és a nagy csalán növényekről terjed szőlőre és más termesztett növényre. Európában minimum négy faj (*Cixiidae*) a ’*Ca*. P. solani’vektora. Közöttük kettő, a *Hyalesthes obsoletus* és a *Reptalus panzeri*, amely képes a kórokozót szőlőre átvinni, azonban további *Cixiidae* családba tartozó kabócafajok vektroátviteli képessége sem kizárható. *H. obsoletus* ökotípusok képesek különböző ’*Ca*. P. solani’ genotípusokat átvinni, amely genotípusok gazdanövényhez is köthetők, mint az aprószulák és a nagy csalán. A betegség terjedésében a fertőzött szaporítóanyagnak jelentős szerepe lehet. A fitoplazmás megbetegedések ellen hatékony védekezés technológia nem áll rendelkezésre, ezért a károk csökkentése érdekében nagy hangsúly kerül a megelőző intézkedésekre. Mivel a BN betegség kórokozójának vektorai nem tartózkodnak/táplálkoznak huzamosan a szőlőn, az ellenük való rovarölőszeres kezelések nem hatékonyak.

11.1. Bois noir betegség járványtani vonatkozásai Magyarországon

11.1.1. ‘*Ca*. P. solani’ törzsek genetikai diverzitása a magyarországi borvidékeken

Annak érdekében, hogy megállapítsuk mely fitoplazma gazdanövények a ’*Ca*. P. solani’ kórokozó fertőzési forrásai és melyek jelentősek a szőlő növények fitoplazmával történő megfertőződésében, öt magyarországi borvidéken mértük fel a BN ökológiai rendszereit. Vizsgálatainkba több genetikai lókuszt (multi lókusz szekvencia analízis MLST) vontunk be. Az MLST a baktériumok járványtani és populáció genetikai jellemzésre gyakran alkalmazott módszer. E módszer a különböző gének szekvencia analízisén alapszik. A precíz és megbízható eredmények érdekében általában alacsonyabb variabilitású, neutrális (szelekciós nyomástól mentes) háztartási géneket vonnak be az MLST vizsgálatba. Azonban variábilis genetikai markerek alkalmazása további fontos információkat hordoz. Amely gének alkalmasak különböző járványtani tulajdonságok feltárására, mint pl. a vektor kórokozó kapcsolatok. A ‘*Ca*. P. solani genotípus meghatározásra *tuf* és *secY* háztartási, valamint *vmp1* és *stamp* funkcionális markerek széles körben alkalmazottak. Annak érdekében, hogy szélesítsük a ‘*Ca*. P. solani’ diverzitásvizsgálatra alkalmazható markerek számát, munkánk során öt, eddig még nem vizsgált, háztartási géneket kódoló lókuszt választottunk ki, és vizsgáltuk alkalmazhatóságukat a törzsek

jellemzésére. A markerek variabilitását reprezentatív '*Ca*. P. solani' törzseken teszteltük. Az öt marker közül a *yidC* (sejt membrán fehérje integrációs rendszer egyik fehérjéét kódoló gén) bizonyult a legvariabilisebbnek.

PCR/RFLP teszttel valamint szekvencia analízissel elvégeztük az Egri, Tokaji, Kunsági, Villányi, Soproni és Etyek-Budai borvidékekről származó '*Ca*. P. solani' izolátumok diverzitásának vizsgálatát *tuf, secY, yidC, vmp1* és *stamp* gének alapján. Vizsgálatainkkal bizonyítottuk a következő genotípusok hazai borvidékeken való jelenlétét. *Tuf*-a, a nagy csalánhoz, mint fő rezervoár növényhez köthető törzs genotípus, elsősorban nyugat Európában; míg *tuf*-b, az aprószulákhoz, mint fő rezervoár növényhez köthető törzs, kelet és Közép-Európában van jelen (Langer and Maixner 2004, Johannesen *et al*. 2012). A *tuf*-a terjedésében jelentős szerepet játszott a *H. obsoletus* vektor aprószulákról a nagy csalánra történt gazdanövény váltása és ezt követő elterjedése Nyugat-Európában (Imo *et al*. 2013). Az elmúlt években a *tuf*-a törzset megtalálták Kelet-Európában is, azonban *tuf*-b jelenléte még mindig domináns. Fontos megjegyezni, hogy *tuf-a* és *tuf-b* elkülönítés PCR/RFLP módszerrel történik (Langer és Maixner 2004), amely módszer limitált mértékben ad információt szekvencia-beli eltérésekre. A tuf gén újabb szekvencia vizsgálatai alapján további tuf genotípus kimutatása vált lehetővé (*tuf*-b1 aprószulákhoz, *tuf*-b2 nagy csalánhoz, és *tuf*-c sövényszulákhoz köthető haplotípusok) (Foissac személyes közlés, 4. Európai BN Munkaértekezlet, 2016). Számos mintavétel és kísérletünk ellenére a *tuf*-a genotípust sem csalánon, sem szőlőn még nem tudtuk kimutatni hazánkban. A szekvencia-alapú szigálatok szerint azonban a *tuf*-b1 és *tuf*-b2 típusokat azonosítottunk hazai szőlő, és *Solanaceae* családba tartozó növényeken. A *vmp1* gén vizsgálata a V2, V9, V13 és V18 genotípusok leggyakoribb előfordulását igazolta Magyarországon. A V18 gyakori genotípus mind szőlőn mind nagy csalánon Európában. Ez a tény arra utalhat, hogy e genotípus hazánkban is nagy csalánról terjed a szőlőre. A *yidC* gén variabilitás és törzsek közötti hasonlóságok jelentős összhangban voltak a *secY* gén vizsgálati eredményeivel. Európa többi országához hasonlóan a *secY* S1, S4 és S6 genotípusok gyakoriak hazánkban. Általában az S1 és S4 apró szulákon és szőlőn gyakori, míg az S6 genotípust elsősorban nagy csalánon és szőlőn írták le (Foissac személyes közlés). Ez összhangban áll a mi hazai eredményeinkkel is miszerint az S1 és S4 szőlőn és aprószulákon volt gyakori, míg az S6 genotípust csak szőlőn találtuk meg. A *stamp* gén nagyfokú diverzitását hazai viszonylatban is igazoltuk. Szőlőn dominánsan a *stamp* ST6 (IV *stamp* csoport) fordult elő, ezt követték a ST4 és ST9, ST9D (I *stamp* csoport) genotípusok. Apró szulákon az ST4 és ST9 fordult elő, ami arra utal, hogy e genotípusok az apró szulákról, mint fitoplazma rezervoárról kerülnek a szőlőre.

Összefoglalásként elmondhatjuk, hogy a szőlő növényen leggyakrabban előforduló genotípusok az S6/V18/ST6, S1/V2/ST4 és S1/V2/ST9 voltak Az aprószulák szerepét a S1/V2/ST4 és S1/V2/ST9 genotípusok szőlőre történő terjesztésében igazoltuk. Európai és a hazai eremények

alapján is feltételezhető, hogy a S6/V18/ST6 szőlőre történő terjedésében a nagy csalán és a piros árvacsalán -mint fertőzési forrás- fontos lehet. Azonban e növények járványtanban betöltött szerepét hazai körülmények között is meg kell erősíteni. A *stamp* ST13 genotípus jelenléte szőlőn, amelyet genotípus *R. quinquecostatus* általi átvitelét bizonyítottuk Madagaszkári rózsameténgre, arra utal, hogy e kabócafaj is kompetens vektora lehet a '*Ca*. P. solani'-nak szőlőn. A hazai '*Ca*. P. solani' törzsek magas fokú variabilitása a polifág vektorok és különböző fitoplazma rezervoár növények szerepét és jelentőségét támasztja alá a fertőzés terjedésében. Magyarországon elsőként bizonyítottuk a '*Ca*. P. solani' jelenlétét levendulában (*Lavandula angustifolia*). Továbbá elsőként mutattunk ki '*Ca*. P. solani' fertőzést árvacsalánból (*Lamium purpureum*) és mezei szilből (*Ulmus minor*).

11.1.2. Magyarországi '*Ca*. P. solani' törzsek rovarátviteli kísérletei

2013-ban, Magyarország négy helységéből gyűjtött *Cixiidae* családba tartozó kabócafajokkal: *H. obsoletus, R. panzeri, R. quinquecostatus* és *R. cuspidatus* végeztünk rovarátviteli kísérleteket. A begyűjtött rovarokat madagaszkári rózsameténgre (*C. roseus*) helyeztük táplálkozni és a tünetek megjelenést megfigyeltük. A növényeket, melyen tünetek jelentek meg molekuláris vizsgálatnak vetettük alá, és az átvitt fitoplazma törzseket *tuf, secY* és *stamp* gének alapján jellemeztük. Átviteli kísérleteinkkel elsőként bizonyítottuk, hogy a *H. obsoletus* képes a '*Ca*. P. solani' ST4 (II csoport) *stamp* genotípust, a *R. quinquecostatus* kabóca pedig az ST13 (III csoport) *stamp* genotípus madagaszkári rózsameténgre átvinni. *R. cuspidatus* fitoplazma átviteli képességét nem tudtuk bizonyítani. Elmondható, hogy a *H. obsoletus* és *R. quinquecostatus* fajok voltak a leggyakrabban csapdázott rovarok, amely megállapításunk megerősíti az Elekes és mtsai. (2006) által leírtakat. *R. melanochaetus* faj megjelenéséről elsőként számoltunk be a Tokaji borvidéken, míg a *R. cuspidatus* első leírása történt meg az Etyek-Budai régióban. Hasonlóan Elekes és mtsai. (2006) megfigyeléséhez a *H. obsoletus* populációkat nagy csalánon gyűjtöttük, apró szulákon csak néhány egyed fordult elő.

11.1.3. Magyarországi '*Ca*. P. solani' törzsek új generációs szekvencia meghatározás

Sikeres átviteli kísérletünk, és az átvitt törzsek (HO11, REP2) molekuláris jellemzése után, elvégeztük a ST4 (*stamp* II csoport) és ST13 (*stamp* III csoport) genotípusokat hordozó magyarországi '*Ca*. P. solani' törzsek új generációs genom szekvenálását. Az ST4 genotípus az apró szulákról gyűjtött *H. obsoletus* által átvitt HO11 kódú törzsben, az ST13 genotípust pedig a vad gazdanövényről gyűjtött *R. quinquecostatus* kabócák által átvitt REP2 kódú törzsben mutattuk ki. A sikeres NSG érdekében a fitoplazma DNS-t sűrűség-gradiens ultracentrifugálással különítettük el a növényi DNS-től. A frakcionálás cézium klorid oldatban, sűrűség alapján történt. Részletes genom információk még nem állnak rendelkezésre, mivel a genomok *de novo* összeállítása még folyamatban van.

11.1.4. Rovar-kórokozó fehérje kapcsolatok vizsgálata

A fitoplazma membránfehérjék nagy jelentőségűek a kórokozó biológiai ciklusában, és e fehérjék polimorfizmusa feltehetően meghatározza egy adott rovar fitoplazma átviteli képességét (Suzuki *et al*. 2006, Fabre *et al*. 2011a). A Stolbur antigén membrán fehérjét kódoló *stamp* gén szekvenciája alapján az európai '*Ca.* P. solani' törzseket négy genetikai csoportba sorolták (Fabre *et al*. 2011b), amely törzsek elterjedése földrajzi mintázatot mutatott (Foissac *et al*. 2013). A STAMP fehérje kimutatásra monoklonális antitest (2A10 MAb) áll rendelkezésre, amelyet a *stamp* I csoportba tartozozó '*Ca*. P. solani' törzs ellen állítottak elő (Fos *et al*. 1992, Fabre *et al*. 2011a). Vizsgálatainkkal bizonyítottuk, hogy a '*Ca*. P. solani' *stamp* I csoportba tartozó törzs ellen előállított 2A10 monoklonális antitest felismeri, ezért alkalmas a *stamp* II, III és IV csoportok szerológiai kimutatására is.

A specifikus '*Ca*. P. solani' - vektor kapcsolatok vizsgálatához nagy segítséget nyújtott a magyar törzsek molekuláris jellemzése, amely alapján a hazai törzseket túlnyomó részben a *stamp* II csoportba tartoztak. A két leggyakoribb ST4 és ST9 genotípust kiválasztva rekombináns STAMP fehérjéket (His6xtag-el ellátott) *E.coli* BL21* törzsében expresszáltattuk. Dot-western-blot kísérletben a 16 kDa méretű STAMP fehérjék (fp_ST4 és fp_ST9) interakciós kepeségét vizsgáltuk különböző kabóca fajok fehérjéivel. A vizsgált rovar fajok és/vagy ökotípusok a következők voltak: BN vektorok: *H. obsoletus* és *R. panzeri;* potenciális vektorok: *R. quinquecostatus* és *R. cuspidatus*, nem-vektor fajok: *Euscelidius variegatus* és *Circulifer haematoceps*, valamint *H. obsoletus* faj ökotípusai (apró szulákról, nagy csalánról és levenduláról származó populációk). Bizonyítottuk, a STAMP II csoport (fp_ST4 és fp_ST9) a *H. obsoletus* (apró szulák ökotípus), valamint a *R. quinquecostatus* kabóca fajok között létrejövő kapcsolatot. Míg a *H. obsoletus* (nagy csalán ökotípus) és a *R. panzeri* fajokkal az interakció alacsonyabb intenzitást mutatott. Eredményeink arra utalnak, hogy a *H. obsoletus* apró szulák ökotípusa a *stamp* II csoport kompetens vektora. *R. quinquecostatus* vektorátviteli képességeit szőlő esetében még nem sikerült bizonyítani (Cvrkovic *et al*. 2013). Azonban az eredményeink alapján feltételezhetjük, hogy a *R. quinquecostatus* lehetséges vektora a *stamp* ST13 genotípusnak. Vizsgálatainkkal elsőként bizonyítottuk *in vitro* interakciót '*Ca*. P. solani' STAMP és *H. obsoletus, R. quinquecostatus* kabócák között.

11.2. Bois noir betegség hatása a *V. vinifera* L. Chardonnay fajta teljesítményére az Egri borvidéken

A '*Ca*. P. solani' okozta BN betegség okozta károkat eddig csak részben tárták fel, a borra gyakorolt hatását ez idáig nem vizsgálták. Három éves kísérletben vizsgáltuk a BN betegség hatását *V. vinifera* L. Chardonnay fajtán, az Egri borvidéken. Vizsgáltuk a *'Ca*. P. solani'-nak a szőlő vegetatív és reproduktív paramétereire, valamint termésminőségre gyakorolt hatásait.

Továbbá a fertőzött és tünetmentes tételekből készült kísérleti borokat rutin és analitikai, valamint érzékszervi vizsgálatoknak vettetük alá. A BN betegség által előidézett legtipikusabb faktorokat/hatásokat neural network és diszkriminancia analízissel értékeltük.

A vegetatív és reproduktív tulajdonságokban, valamint a termés beltartalmában mért értékek szignifikáns növekedése/csökkenése, az egészséges tőkékhez viszonyítva, 3 év átlágában a következők voltak: sodródás okozta levélfelület csökkenés (-28.0 %), level friss- és száraztömeg (+11.2 and +19.5 %), relatív klorofil index (-30.4 %), tőkénkénti össztermés (-68.4 %), 100 bogyó tömeg (-32.5 %), fürtszám/tőke (-56.7 %), tünetes fürt/tőke (+97.2 %), száraz fürt/tőke (+90.4 %), titrálható savtartalom (+16.4 %), pH (-2.7 %), és vízben oldható szárazanyag-tartalom (Brix°) (-6.2 %). Méréseinkkel megerősítettük, hogy a BN betegség hatására csökken a fotoszintetikusan aktív lombfelület, a fertőzött hajtások nem érnek be, rajtuk a rügyek életképtelenek, elfagynak. Igazoltuk, hogy a betegség hatására a termésmennyiség jelentősen csökken, amelynek mértéke mind a három vizsgált évben meghaladta az 53 %-ot.

A fertőzött tőkék terméséből készült borok kedvezőtlen érzékszervi jellemzőit (magas sav- és keserűérzet, rózsaszín elszíneződés) a rutin és analitikai vizsgálatokkal is alátámasztottuk. Elsőként bizonyítottuk, hogy a ’*Ca*. P. solani’ okozta Bois noir betegség negatívan befolyásolja a bor minőségét a ‘Chardonnay’ fajta esetében az Egri borvidéken (Magyarország). A mért paratméterek szignifikáns növekedése/csökkenése, az egészséges tőkék terméséből készült borokhoz viszonyítva, 3 év átlagában a következők voltak: alacsonyabb alkoholtartalom (-5.3 %), mélyebb szín (egyes években rózsaszín/pink elszíneződés) (+22.2 %), emelkedett titrálható savtartalom (+7.9 %), emelkedett az alma- és citromsav mennyisége (+4.5 %, +6.0 %), emelkedett kálcium és magnézium szint (+8.4 %, 4.4 %), csökkent a vas (-5.4 %), emelkedett hidroxifahéjsavak mennyisége (kaftársav +3.6 %, kávésav +36.1 %), csökkent a flavonoid tartalom (katechin -8.9 %, epikatechin -14.4 %).

A 2012 (jellemzően meleg és extrém száraz) és 2013 (kiegyenlített viszonyok, kiváló évjárat) valamint 2014 (rendkívül gyenge évjárat az érési időszak rendkívül csapadékos időjárása miatt) évjáratok termésadatai és a borminőségek közötti szignifikáns különbségeket a vizsgálati eredményeink jól demonstrálják. Jelentős különbségek a fertőzött tőkék terméséből készült és az egészséges tőkék termésének borai között az analitikai paraméterekben mutatkoztak. A borok érzékszervi bírálata során tapasztalt negatív hatások a 2013-as évben voltak a legmarkánsabbak.

A borok emelkedett szerves sav és polifenol tartalma volt felelős a BN fertőzött termés borának markánsan savas valamint keserű ízérzetért. Ezen bor tételek alacsonyabb cukortartamú mustból készültek, így alkohol tartalmuk is alacsonyabb volt. A rózsaszín (pink) elszíneződés, főként a 2013-as év BN fertőzött boraiban, súlyos borhibának tekinthető, amely jelentősen csökkentheti ezen borok paci értékét. Bizonyítottuk, hogy a BN fertőzés hatására kialakuló tőkénkénti

termésmennyiségben, fürtszámban és a bogyótömegben bekövetkezett terméscsökkenés és az évjárat között szignifikáns összefüggést van. Azaz a BN okozta negatív hatás mértéke erősen függ az adott év hőmérsékleti és csapadékbeli viszonyaitól. A szőlő számára kedvezőtlen években (erősen csapadékos és hűvösebb) a BN betegség hatásai maszkírozódnak. A BN kórokozója által fertőzött tőkék vegetatív és reproduktív teljesítménye, valamit a borminőség csökken, ezáltal a szőlőültetvény gazdaságos fenntarthatósága számottevően visszaesik.

11.3. A '*Ca*. P. solani'-fertőzött szőlő tőkék gyógyítására irányuló kezelések

A BN okozta súlyos károk ellenére, több fajtán gyakori jelenség a fitoplazmás szőlőnövények spontán végbemenő gyógyulása, regenerálódása, amely lehet időszakos vagy végleges. E természetes folyamat a tőkék csak egy részénél megy végbe és ezek hátterében álló okok kevéssé ismertek. A gyógyult tőkékben -az egészségeshez viszonyítva- magasabb reaktív H_2O_2, jázmonsav szintet, és emelkedett NAD(P)H peroxidáz aktivitást mértek, ez csökkenő szalicilsav mennyiséggel párosult, ami szisztemikus szerzett rezisztenciára (SAR) utal (Musetti *et al*. 2013, Santi *et al*. 2013). A Szőlő sárgaságot okozó fitoplazmás megbetegedések, de elsősorban a BN betegség kezelésére, eddig még nem alkalmazott rezisztencia-fokozó szerekkel végeztek permetezési kísérleteket, amely során öt szerrel (chitosan, két glutathione-oligosaccharine formula, benzothiadiazole, és phosetyl-Al) permeteztek '*Ca*. P. solani' fertőzött Chardonnay tőkéket és idéztek elő gyógyulást (Romanazzi *et al*. 2013). Hasonlóan a fenti kísérlethez, Bois noir fertőzésben szenvedő szőlőtőkék gyógyításra irányuló kísérletek állítottunk be egy Chardonnay ültetvényben az Egri borvidéken. A vizsgálatban glutathione-oligosaccharine (Kendal, Valagro, Olaszország) és benzothiadiazole (Syngenta, Svájc) hatóanyagokat alkalmaztunk 3 éven át (2012-2014). Rövid távú gyógyulás volt tapasztalható mind a két szer alkalmazása során, azonban a kezeletlen kontrolnál hasonló méretben tapasztaltunk természetes gyógyulást. A legmagasabb gyógyulási százalék rövid-távon (1 év) a Kendal készítménnyel való kezelés eredményezte, ezt követte a kezeletlen kontrol, majd a Bion. A szerek hosszabb távú (a gyógyulás utáni 2, 3, és 4 tünetmentes év) hatása nem volt kimutatható. Pozitívum, hogy a szerek fitotoxikus hatást nem figyeltünk meg. Mivel e kezelések hosszabb távon fejthetik ki gyógyító hatásukaz, ezért fontos feladatunk a következő években az alkalmazott kezelések hatásának a megfigyelése.

ANNEX 1. REFERENCES

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10.14751/SZIE.2016.070

ACKNOWLEDGEMENTS

During the course of my research many people have assisted both directly and indirectly.

I would like to thank my supervisors Prof. György Dénes Bisztay and Prof. László Palkovics for their help and everlasting support during my work.

I would like to extend gratitude for their intensive guidance to my external supervisors Dr. Xavier Foissac in molecular epidemiological studies and Prof. Jacobus J. Hunter in grapevine physiology and viticultural work. I am grateful for them receiving me in their lab and providing me devotional help and resources for my experiments.

I would like to emphasise my gratefulness for Dr. Péter Bodor for his interest and guidance in the viticultural work, and hours of help and discussion he provided during the three years.

I would like to thank Dr. Márta Ladányi for her outstanding contribution and work that she provided during the analyses of my data.

I thank our colleagues in the Department of Viticulture: Dr. István Fazekas, Attila Nagy, Tamás Varga, Dr. Gyrörgy Lukácsy, Dr. Tünde Kupi, Andrea Szekszárdi, Nikoletta Kellner, Géza Győrffy, Ágnes Bujna, Ágnes Zikhar, Eszter Pájer (student), Bence Czigány (student) and Rita Elek (Genlogs Ltd.) for their help with measurements. I also thank Dr. Mária Kölber, Dr. Rita Lózsa, Dr. Borbála Bálo, Dr. Tamás Deák and Dr. László Gáspár for their support and valuable comments during the experimental phase, as well as in preparation of publications and the thesis.

Additionally, I would like to thank colleagues the Department of Oenology: Dr. György Pásti, Dr. Diana Sárdi Nyitrainé, Dr. Annamária Leskó, Balázs Nagy, Prof. Miklós Kállay, Lászlóné Géher, Zsuzsanna Kiss Hegyiné, Judit Horváth for their valuable comments and for preforming the analytical measurements of the experimental wines. As well as to Dr. András Szekeres and Dr. Ottó Bencsik (University of Szeged) for HPLC measurements of the experimental wines.

I am indebted Dr. Zsolt Zsófi for providing experimental site, for Zita Pálfi for producing experimental wines, to Dr. Szabolcs Villangó and Xenia Pálfi for vineyard and meteorological data, as well as to Zoltán Lóska, István Patai, Zsolt Pálmai, Tamás Lénárd, Tamás Vincze, and Zoltán Tik for their help in the microvinification process and treatments.

I am very thankful to colleagues in the Mollicutes team at INRA, Bordeaux: Delphin Desque, Jean-Luc Danet, Dr. Sylvie Malembic-Maher, Pascal Salar, Kaylig Guionneaud, Martine, Patricia Carle, Marie-Pierre Dubrana, Dr. Laure Beven, Sybille Duret, Dr. Nathalie Bouvery, Dr. Colette Saillard,

Dr. Jöel Renaudin, Dr. Alain Blanchard, Dr. Sandrine Eveillard, Dr. Cristophe Garcion, Camille Jollard, and all colleagues for their always selfless support during my internship, where I felt home.

Also thanks to the team of Prof. Hunter: Neels Volschenk, Estelle Rhode, Alwina Marais, Stawie Fouché and Christopher Paulse, for their kindness and assistance in viticultural measurements.

I wish to recognise the Hungarian representatives of Syngenta and Valagro companies, who kindly provided material for field-trials.

I am indebted to Stolbur-Euromed Consortium allowing me to access to reference sequences of '*Ca*. P. solani' strains: Fabre A., Ember I., Della Bartola M., Plavec J., Avramov Z., Mortada C., Eroglu S., Balakishiyeva G., Acs Z., Baric S., Batlle A., Bouyahia H., Carle P., Chireceanu C., Choueiri E., Cvrkovic T., Danet J.L., Ertunc F., Guionneaud K., Huseynova I, Jreijiri F., Jovic J., Katis N., Kostadinovska E., Krizanac I., Krjanjic S., Lavina A., Maliogka V., Mammadov A.C., Materazzi A., Murolo S., Oancea F., Omar A. F., Pacifico D., Romanazzi G., Sabate J., Safarova D., Sahin F., Salar P., Skoric D., Valova P., Viorel f., Zahavi T., Johannesen J., Kolber M., Maixner M., Marzachi C., Navratil M., Seruga-Music M., Tosevski I., and Foissac X.

As well as thanks to Dr. Xavier Foissac, Dr. Michael Maixner, Dr. Jelena Jovic, Dr. Assunta Bertaccini and Dr. Ing-Ming Lee for kindly providing phytoplasma reference controls and insects.

This PhD project was funded by the National Research, Development and Innovation Fund of the Hungarian Government (KTIA_AIK_12-1-2013-0001). I received financial support from Erasmus and Campus Hungary Grants for visiting the laboratories of my external supervisors.

I am grateful to Lisa Bóha for English editing.

I also wish to extend my thanks to the many wonderful people I met during my work, colleagues, owners and employees of the vineyards or laboratories I visited.

My success during the PhD period would not have been possible if it were not for the personal sacrifice of my husband Tamás Szabó, and my family. Many thanks for their never ending patience and support.