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Bois noir affects the yield and wine quality of *Vitis vinifera* L. cv. 'Chardonnay'

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Abstract The Bois noir (BN) disease induced by '*Candidatus* Phytoplasma solani' (CPs) is common in European vineyards. Its damage has not been fully investigated, especially with regards to wine attributes. The impact of BN on yield, berry composition and wine characteristics of *Vitis vinifera* L. cv. 'Chardonnay' was therefore comprehensively characterized in a 3-year field experiment in Hungary, Eger winegrowing region. Additionally, the bindweed-related *tuf*-b1 genotype was identified to be involved in the BN pathosystem in the experimental vineyard. Infection of CPs *tuf*-b1 genotype resulted in severe yield loss, the average decrease in number of bunches and total yield per vine was 56.7% and 68.4%, respectively. Analyses of wines produced from grapes of BN infected vines revealed decreased alcohol, epicatechin and iron contents; and increased organic acids, titratable acidity, catechin and calcium contents. Sensory evaluation of these wines confirmed

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unfavourable characteristics, i.e. higher acidity, bitterness, and usually pinkish discolouration. Negative impact on berry composition and wine quality were pronounced in the vintage with favourable weather conditions for grapevine production, whereas the negative effects of BN infection were less prominent, even masked, in the vintages with unfavourable weather (wet and cool). To reduce BN-caused damage, the need for improved preventative and curative measures for BN disease is highlighted.

Keywords '*Candidatus* Phytoplasma solani' · Bindweed · *Tuf* · Grapevine yellows · Phenolic compounds · Yield loss · Wine quality

Introduction

European grape production is affected by phytoplasma-induced Grapevine yellows (GY). One of the major GY is Bois noir (BN) caused by '*Candidatus* Phytoplasma solani' (CPs) (Foissac and Maixner 2013; Quaglino et al. 2013). Given its non-quarantine status, management of BN disease is not a primary focus and significance of the disease-caused damage is underestimated.

The pathogen CPs is endemic to Europe where it infects several crops, including grapevines, vegetables and natural vegetation (Lee et al. 2000). During non-epidemic spread (i.e. crops like *solanaceous* plants and grapevines that are dead-end hosts for the pathogen), CPs is transmitted from bindweed (*Convolvulus arvensis* L.) and stinging nettle (*Urtica dioica* L.) to grapevine (*Vitis vinifera* L.) by different phloem-feeding planthoppers of the *Cixiidae* family (Maixner 1994; Sforza et al. 1998; Cvrković et al. 2013).

The economic importance of a pathogen (e.g. CPs) 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). Although BN is considered less damaging than the epidemic Flavescence dorée (FD), the only GY classified as a quarantine pathogen in Europe, its disease cycle is more complex because of the biology of the polyphagous vectors *Hyalesthes obsoletus* Signoret and *Reptalus panzeri* Löw (Foissac and Maixner 2013). Different CPs strains/genotypes might affect a given cultivar differently. Determination of the prevalent CPs genotype at vineyard level is decisive in order to apply accurate disease management. In Europe, genotyping of phytoplasma genes, i.e. *tuf* encoding TU elongation factor, allows us to trace infection sources of CPs, as weed hosts are associated with specific genotypes to propagate on. For example, *tuf*-a, *tuf*-b2 and *tuf*-b3 are related to stinging nettle, and *tuf*-b1 related to bindweed (Langer and Maixner 2004; Kosovac et al. 2016; Foissac personal communication in accordance with 4th Bois noir Workshop, Klosterneuburg, Austria). In Hungary, the *tuf*-a type has not been detected yet on stinging nettle or on grapevine. However, *tuf*-b1 was found on grapevine, suggesting that bindweed could be the main infection source of this genotype in Hungary (Ember, unpublished data).

As with all GY, BN is associated with a complex of symptoms, which include leaf rolling, leaf yellowing or reddening (depending on the cultivar), uneven shoot lignification, berry shrivelling, and bunch drying. The incidence and severity of the symptoms vary among cultivars: 'Chardonnay', 'Riesling', 'Cabernet Sauvignon', 'Barbera', 'Sauvignon blanc' and 'Sémillon' are among the most sensitive cultivars (EFSA PLH Panel 2014; Panassiti et al. 2015), while cv. 'Merlot' and *V. simpsonii* are of low susceptibility (Eveillard et al. 2016).

Phytoplasma infection causes sieve-tube occlusion which leads to the disturbance of the phloem function (Musetti et al. 2007, 2009, 2013). The energy demands of phytoplasmas regarding growth, induce physiological changes in the infected plants (Lepka et al. 1999; Hren et al. 2009; Landi and Romanazzi 2011). Photosynthesis and hormone metabolism are heavily affected, both in diseased model plants and grapevines. Changes in expression of genes involved in carbohydrate metabolism and glycolysis certainly impact on the flow of assimilates to the grapes (Jagoueix-Eveillard et al. 2001; Pracros et al. 2006). BN infection significantly reduced the performance of certain grapevine cultivars in many aspects, such as at a physiological, yield and fruit quality level (Garau et al. 2007; Matus et al. 2008; Endeshaw et al. 2012; Rusjan et al. 2012; Zahavi et al. 2013; Romanazzi et al. 2013; Rusjan and Mikulic-Petkovsek 2015).

The damage in BN diseased vineyards is undervalued, as fluctuation in infection status, i.e. severity and incidence, results in an extended range of yield losses. Additionally, the decline of CPs infected vines in terms of wine quality has not yet been determined. The aim of the current 3-year field experiment was to describe the impact of BN on *V. vinifera* L. cv. 'Chardonnay' berry and wine quality, in the Eger wine region of Hungary. In addition, the *tuf*-type of the damaging CPs strain present in the experimental vines was identified.

Materials and methods

Experimental site and plant material

The experimental vineyard is situated in the Eger wine region of Hungary (47°86′N, 20°38′E, 173 m), belonging to Eszterházy Károly University, Research Institute for Viticulture and Enology. The climatic conditions of the region are humid continental with a mean annual temperature of 10.5 °C and average annual precipitation of 600 mm (Peel et al. 2007). Meteorological data for every year of the experiment (2012, 2013, and 2014) and the preceding year (2011) were recorded (Appendix 1, Fig. 3).

Measurements of berry composition and yield 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 and 3.0 m between rows. The experimental vineyard was cordon trained with 4-bud spurs (18–20 buds/vine), shoots were vertically positioned and managed according to common practices applied to a commercial vineyard.

Three random blocks (50 vines per block) were selected (Appendix 1, Fig. 4) in which the phytoplasma infection status of the individual vines was visually evaluated before harvest in each year of the experiment (2011–2014). Severity and incidence of phytoplasma disease were recorded individually and scored from mild to severe according to number of symptomatic shoots, i.e. mild, moderate and severe, when symptoms were present on ≤15%, 16–25%, and 26–35% of the shoots, respectively. In each block 5–5 healthy (H) and BN-affected (BNA), in total 15–15 grapevines were assigned for measurements for vine analyses, with each replicate consisting of a single vine. Plants that received a score of moderately infected were analysed in the experiment of yield and berry composition. Grape yields from the remaining H and BNA (mild, moderate, and severe) plants of the experimental blocks were used for micro-vinification.

CPs detection and genotyping

The incidence of CPs infection as well as the health status of the 15–15 selected plants were confirmed at molecular level, using 16S rDNA phytoplasma-specific primers, according to protocol detailed in Ember et al. (2011). The 15 CPs infected samples were subjected to molecular typing of *tuf* gene according to the protocol described in Plavec et al. (2015) in nested PCR. Prior to direct sequencing, purification of *tuf* amplicon was carried out using Zymoclean Gel DNA recovery Kit (USA, CA) according to manufacturer instructions. Purified 15 ng per 100 bp of product/sample were sent for sequencing (both strands) (Base-Clear, Leiden, The Netherlands). Staden Package Version 3.3 and CLUSTAL W were used for assembling and alignments. A neighbour joining (NJ) method with Tamura-Nei model was applied to construct phylogenetic trees using MEGA 6 software. *Tuf* references DE_1925 and DE_30003 were kindly provided by Dr. Michael Maixner (Germany), and all *tuf* reference sequences were provided by Dr. Xavier Foissac (France).

Yield and berry composition

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

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

Microvinification

Grapes of three vintages (2012, 2013, and 2014) were hand-harvested at technological maturity on the 31th August 2012, the 17th September 2013, and the18th September 2014. Musts were fermented in the winery of Eszterházy Károly University, Research Institute for Viticulture and Enology. In all three experimental blocks, total yield was gathered and processed separately for H and BNA vines. Grapes (60 kg per treatment) from H (total yield) and BNA vines (total yield) were fermented in three oenological replicates for each treatment in 2012 and 2013. In 2014, only one replicate of each treatment was processed because of a limited quantity of grape yield. Additional experimental wine was made (BNS) in 2013 and 2014, when selectively only affected bunches (BN-shrivelled, BNS) were gathered

from infected shoots. Due to the limited number of symptomatic bunches, one oenological replicate of BNS wine (40 kg per treatment) was made in each year. It is important to note that dry bunches were avoided during harvesting. Quick crushing and destemming were followed by the addition of sulphite (0.2 mL/L Sterisol) and treatment with pectolytic 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 (250 mg/L) (Uvaferm; Lallemand S.A.S, Saint Simon, France). Complex yeast nutrients (Uvavital Komplex; Danstar Ferment AG, Switzerland) were added three times. The free sulphur concentration of the fermented batches was adjusted to 30 mg/L. Wines were fined with calcium/natrium bentonite, and the final concentration of free sulphur was adjusted to 40 mg/L in each treatment by adding sulphur dioxide. Wine was bottled in February in all years.

Wine analyses

The following parameters were measured for each wine replicate: alcohol content (Gibertini distiller), total extract (densimetry using hydrostatic balance), residual sugar (Luff-Schoorl method), titratable acidity (after titration), pH, tartaric acid (spectrophotometry), malic and lactic acids (Boehringer Mannheim enzyme test), total polyphenols (Folin-Ciocalteu reagent calibrated for gallic acid), colour intensity (spectrophotometrically, 420 nm), and element 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).

To separate the different flavonoid compounds, wine samples (10 μl) were analysed without sample pretreatment using a modular Shimadzu HPLC system (Shimadzu, Japan) including LC20-AD pump, DGU-14A degasser, SIL10-ADvp autosampler, CTO-10ASvp column oven and SPD-10Avp UV-VIS detector and equipped with a Kinetex 2.6 μ XB-C18 100A (100 × 4.6 mm, Gen-Lab, Hungary) column at a flow rate of 1 ml/min at 35 °C according to the method described by Schwarz et al. (2012) with some minor modifications. Eluents A and B were water and acetonitrile, respectively, both supplemented with 1% acetic acid. During HPLC analysis the following solvent gradient was used: 0% B, 16.3% B at 16.40 min, 18.4% B from 16.90 min to 20.30 min, 19.4% B at 24.90 min, 20.4% B at 27.50 min, 100% B from 27.51 min to 30.40 min, and 0% B from 30.41 min to 37.0 min. Flavonoid content was quantified using a standard reference compound of caftaric acid (8.0 min) and *t*-caffeic acid (11.7 min) at 320 nm and gallic acid (3.5 min), protocatechuic acid (6.04 min), (+)-catechin (10.7 min) and (−)-epicatechin (15.1 min) at 280 nm. In the case of organic acid, ethanol and sugar analyses in wine samples (20 μl), the same HPLC system was used with a RID-10A (Shimadzu, Japan) refractive index detector. The separations were carried out on a Hi-Plex H (300 × 7.7 mm, 8 μ, Agilent, USA) column applying 1.7 mM H_2SO_4 as mobile phase at a flow rate of 0.4 ml/min at 70 °C. Citric acid (12.7 min), tartaric acid (13.3 min), glucose (14.2 min), malic acid (14.8 min), fructose (15.2 min), succinic acid (18.1 min), lactic acid (19.3 min), glycerol (20.1 min), acetic acid (22.9 min) and ethanol (31.9 min) contents were measured.

Sensory analysis

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/smell (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 of all three replicates per treatment were evaluated as parallel test on an unmarked line scale from 0 (poor) to 100 (prominent).

Statistical analyses

Two-way MANOVA models with affection/disease factors (BNA vs. H) and years (2012, 2013, and 2014) were used to detect the differences in the following variables: yield, number of bunches, bunch weight, and 100-berry weight. The same method was used to analyse the dependent berry 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. Results of wine analyses were evaluated with a two-way MANOVA model with disease factors (BNA vs. H) and years (2012 and 2013) for basic parameters, organic acids and elements, and simple phenols. When

normality of the residuals was required, the absolute values for skewness and kurtosis of the distribution proved to be below 1. Homogeneity of variances was assessed 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 completed to detect the factor effects regarding each variable. The sensory analysis evaluations of the 11 panellists were analysed using the Mann–Whitney U test. For statistical analyses IBM SPSS version 22 (IBM Corp., Armonk, NY, USA) were used. In the case of BNS wines, due to the limited yield, only one replicate was made, thus statistical analysis for BNS wines was not applicable.

Results

CPs detection and genotyping

Presence of phytoplasma belonging to 16SrXII-A subgroup (CPs) was confirmed in all 15 BN-symptomatic grapevines. No phytoplasma was detected in asymptomatic plants. The *tuf* genotyping of 15 CPs positive grapevines was completed for 11 isolates. Lack of amplification in the case of four samples were due to lower sensitivity of *tuf* nested-PCR protocol. All characterised samples proved to be *tuf*-b1 bindweed genotype (GenBank accession number: KY678899).

Yield and berry composition

In all experimental years, the yield of BNA grapevines was significantly lower than that of H vines (in all years $P < 0.001$; Table 1). The berry weight, bunch weight, and number of bunches per vine collectively resulted in 53.3–75.3% (depending on the year) yield loss of BNA plants compared to H plants. Bunches with shrivelled berries and bunches with dry berries were frequently observed on symptomatic vines, averaging 7.6 and 1.36 bunches/vine, respectively (in all years $P < 0.001$; Appendix 1, Fig. 5). Due to the dry weather conditions the production from H plants was more than 40% lower in 2012 than in the other vintages. The extent and share of the loss (i.e. yield, bunch and berry weight) varied in years and there was a significant year × infection interaction. Berry composition of 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, whereas the pH was lower (Table 1). The content of soluble solids was significantly lower in BNA juice (Table 1), implying a lower potential for alcohol content in wine.

Vintage and microvinification

Meteorological data for vintages of the experiment are shown in Appendix 1, Fig. 3. Vintages 2011 and 2012 were extremely dry, especially over the period of intensive vegetative growth of the vine (before veraison) and at veraison. After these dry years, a more balanced 2013 followed with unusually cold days in spring that delayed flowering. However, the weather conditions during the rest of the growing period were suitable for grapevine production. 2014 the weather was without extremity until veraison, during which 50% of the annual rainfall occurred resulting in severe fungal infection that affected the harvest in terms of quantity and quality.

The timing of harvest was based on the technological maturity determined by the maturation index of °Brix: TA. In all three vintages fermentation of must from BNA grapes was slower than that from H grapes, finishing 5–7 days later.

Wine analyses

In wines fermented to dryness, no significant differences in residual sugar content were observed [infection: $F(1,5) = 0.03$, $P = 0.88$; year: $F(1,5) = 0.99$, $P = 0.37$; Table 2]. However, alcohol content of wines from H vines was significantly higher than that of BNA vines in all three vintages [infection: $F(1,5) = 13.33$, $P < 0.05$; year: $F(1,5) = 10.22$; $P < 0.05$; Table 2], and wines from BNA vines had significantly higher tartaric acid content compared to those of H vines [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: $F(1,5) = 0.25$; Table 2]. Although, oxidation-reduction conditions were adjusted to 40 mg/L free sulphur in each treatment, wines produced from infected vines 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 (Fig. 1; Appendix 1, Fig. 6). The HPLC measurements of phenolic compounds revealed a 36.31% increase in caffeic acid content of BNA wines [$F(1,4) = 56.64$, $P < 0.01$; Table 2].

Table 1 Yield and fruit composition of Bois noir-affected and healthy grapevines

Measured parameter	2012			2013			2014			3-year average			Average decrease§ (-) increase (+) %	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)	H	BNA	F(df1;df2)						
Yield																		
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 ***	**-22,1**	***	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)=-7.497 ***	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 ***			
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)=3.75 +	3.30	3.21	F(1;82)=31.92 **	**-2.7**	ns	F(2;82)=85.95 ***	0.59 ***	0.21***	2MANOVA
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 ***			

BNA, BN-affected grapevine cv. 'Chardonnay'. *H*, healthy grapevine cv. 'Chardonnay'. §: 3-year average changes of BN-affected vine performance compared to those of healthy plants based on a 3-year average (%).

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. 1sStudent's t: one sample (two-sample) Student's t test

On the contrary, (+)-catechin and (−)-epicatechin contents were 8.86% and 14.43% lower, respectively, in wines from BNA grapes compared to H [catechin: $F(1,4) = 56.64$, $P < 0.01$; epicatechin: $F(1,4) = 0.25$, $P = 0.65$; Table 2]. Elements that were above the detection limit are provided in Table 2. The Ca 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. Mg content in wine from H grapes was lower in 2013 than in 2012, nevertheless, according to the results of years 2012 and 2013 in H grapes was significantly lower than in BNA samples [$F(1,5) = 15.4$, $P < 0.05$]. Fe concentrations was lower in wines from BNA grapes in both vintages 2012 and 2013 [$F(1,5) = 4.49$, $P = 0.08$], the difference was significant in 2013 [$F(1,4) = 27.49$, $P < 0.01$]. Element contents were significantly affected by the vintage [$F(1,5) > 11.00$, $P < 0.05$), except for Zn ($P < 0.1$) and B ($P = 0.6$)].

Sensory analysis

Differences between H and BNA wines were pronounced in each experimental vintages. Panellists unanimously and consistently determined that wine from BNA grapes was of a lower quality than wine from H grapes. In 2013 differences were significant for colour, smell intensity, taste intensity, taste length, smell reminiscent of citrus, aroma/taste fault, bitterness, harmony, and overall quality (Mann-Whitney's $U < 156.5$, $P < 0.03$); for acidity, body, and varietal aroma (Mann-Whitney's $U > 162.5$, $P > 0.06$) (Fig. 1). Between BNA and H wines of vintages 2012 and 2014 there were no significant differences in any of the sensory parameters ($P > 0.05$, $P > 0.2$).

Wines from the grapes of H vines were light yellow and modest bodied, and evaluated as crisp in all years. They also had a fruity bouquet with prevalent smell reminiscence of citrus of a moderate length and intensity. Wines from BNA grapes had reduced aroma and flavour, fruity aromas were distinctive, the wines tasted flat, and were characterized by intense colour (deep yellow, and pinkish discolouration in some years), and pronounced acidity and bitterness were perceived (Fig. 1; Appendix 1, Fig. 6). 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.

Discussion

Impact of CPs strain on yield

Yield and berry composition of grapevines depend on several factors, among them the seasonal photosynthetic capacity of the canopy (Hunter and Visser 1988). In many cases CPs infection causes severe symptoms, i.e. leaf

Table 2 Results of wine analysis of Bois noir-affected and healthy grapevines (vintage 2012, 2013, and 2014)

Parameters with year' effect significance	2012			2013				2014				Multi 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 420 nm)*	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																
Aluminium (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

Table 2 (continued)

Parameters with year' effect significance	2012			2013			2014			Multi year mean			Average increase[§] (+) /decrease (−) %
	H	BNA		H	BNA	*BNS*	H	BNA	*BNS*	H	BNA	*BNS*	
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

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 (shrivelled 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, comparison between H and BNA, asterisks refer to statistically significant differences at P values: $^{*}P < 0.05$, $^{+}P < 0.1$. ns: no significant difference. Italic letters: statistical analyses were not applicable

rolling, chlorophyll degradation, and incomplete lignification of shoots which affect grapevine production and result in a 70% yield loss (Endeshaw et al. 2012; Garau et al. 2007; Zahavi et al. 2013; Romanazzi et al. 2013; Rusjan and Mikulic-Petkovsek 2015). In this study, the yield loss of infected vines was in the above mentioned range, averaged between 56.7% and 68.4%, respectively, indicating a strong dependence of infection on the environmental factors of different vintages. The environmental conditions and grapevine cultivars influence BN incidence, and virulence depends on temperature, plant age and genetic backgrounds of phytoplasma and plant host (Foissac and Wilson 2010; Sugio et al. 2011; Panassiti et al. 2015). Genotyping of CPs causing severe yield damage of experimental grapevines revealed bindweed-type *tuf*-b1 genotype alone, supporting the hypothesis that bindweed was the main infection source of CPs in the experimental vineyard. Knowledge on stolbur antigenic membrane protein (*stamp)* genotype could provide further insight to CPs epidemiology, as it draws information not only on the host, but on the vector itself (Foissac and Maixner 2013).

Impact of BN on berry and wine quality

'Chardonnay' is considered as one of the most sensitive grapevine cultivars to BN disease (Martelli and Boudon-Padieu 2006). Despite the high susceptibility of this cultivar, grape production still eventuated on BNA vines in Eger, which led to a remarkable decline in berry composition. Phloem-limited pathogens are known to affect fruit quality (Boudon-Padieu 2003). 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). In our study, similar effects (higher TA, and lower pH and soluble solids) on berry composition occurred in BNA 'Chardonnay' (Fig. 2). The amount of organic acids (i.e. tartaric, malic and citric acids) was higher in wine produced from BNA grapes compared to that of wine produced from healthy grapes, demonstrating that their breakdown was affected during berry maturation. More pronounced differences in berry composition were observed in 2013, the year among the three experimental years in which the weather conditions were the most favourable for grapevine production. In fact, negative effects were less evident in the years with unfavourable

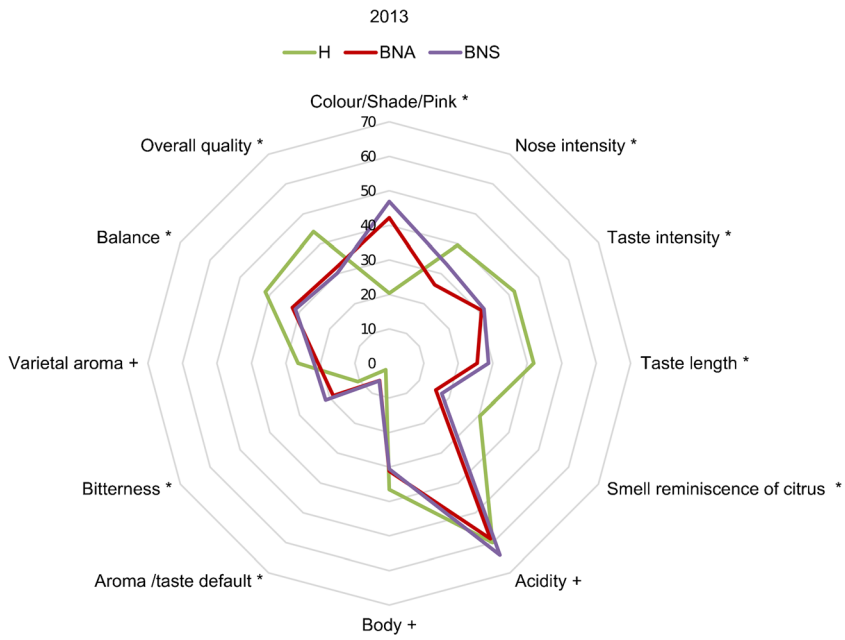

Fig. 1 Wine profile analysis of year 2013. *Legend*: H: yield of healthy vines. BNA: yield of BN-affected vines. BNS: shrivelling bunches of BN-affected shoots. Asterisks refer to statistically significant differences between H and BNA at values: * P 0.05, + $P < 0.1$. Statistical analysis of BNS wines was not applicable

weather, i.e. lower heat sum and higher precipitation during veraison and ripening. It is a general presumption that the highest impact of CPs can only be seen in warm and dry years, when the grapevines experience higher environment-induced stress, which was experienced in the cases of other grapevine diseases, i.e. ESCA (Borgo et al. 2016). However, further studies are needed to support this hypothesis for CPs.

In response to phytoplasma infection, massive callose and structural proteins (i.e. phloem protein, sieve-element occlusion protein) accumulation occur in plants, i.e. tobacco, broad bean, and grapevine. These defence mechanisms are directed at restricting phytoplasma colonisation as well as reducing phloem photo-assimilate transport. Deposition of callose and phloem protein is a Ca^{2+} dependent event, provoked by Ca^{2+} influx into sieve elements (Musetti et al. 2011, 2013; Santi et al. 2013). The BNA wines had elevated Ca contents, implying that the phytoplasma infection had detrimental effects on berry composition and may compromise wine stability. High Ca contents in wines lead to the crystallisation of calcium-tartrate, which deposition in the bottle frequently occurs with Ca content over 60 mg/L (Ribéreau-Gayon et al. 2006). To overcome problems related to stability, wines with higher Ca contents require intensified stabilisation steps before bottling and marketing.

Beside phloem occlusion, thickening of the cell wall and concomitant increased phenolics were observed in phytoplasma-infected plants (reviewed in Musetti et al. 2013). These phenolic compounds in grapevines may considerably influence the organoleptic properties of wine. Changes in secondary metabolites in the berry skin of BN-diseased 'Chardonnay' were described, the amount of flavonols decreasing while flavanol and hydroxycinnamic acid contents increased in BN-affected shrivelled berries (Rusjan et al. 2012). Consistent with these findings, our study revealed elevated hydroxycinnamic acids (caftaric and caffeic acids) along with decreased flavonoid contents, i.e. (+)-catechin and (−)-epicatechin, in wines produced from BNA grapes. Additionally, in 2013 and 2014, pink discolouration was observed and elevated phenolic contents were detected in wines from BNA and BNS grapes. Lutter et al. (2007) determined that caffeic acid forms dihydroxybenzaldehyde [in the presence of Fe (II)], which reacts with (+)-catechin and leads to a discolouration of wine-mimicking solutions. The results suggest that this reaction may occur in wines produced from BNA and BNS grapes. 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. Thus, it would be necessary to clarify the role of phenolic compounds in the wine pinking phenomenon and consequently identify their proportion in BNA grape and wine.

Flavonoids, including anthocyanin are also playing role in plant defences against pathogens and plant-microbe interactions, as also reported for grapevine (Kortekamp 2006). Boss and his co-workers (1996)

Fig. 2 Summary of parameters related to the decrease or increase in yield, must and wine quality caused by Bois noir disease in *V. vinifera* L. cv. 'Chardonnay'. *Legend:* Yield and must quality of 15 healthy and BN-affected vines have been analysed by two-way ANOVA. Wines of healthy and BN-affected plants have been analysed by the two-way MANOVA method, while the sensory analysis by the Mann-Whitney U test. The internal circle depicts the four groups of measured parameters: yield, must, wine and sensory analyses. Median circles in grey represent a logarithmic (log10) scale of average changes of BN-affected vine performance compared to those of healthy plants based on a multi-year average (%); red and blue columns refer to performance increases (+) and decreases (−), respectively. External circle: measured parameters showing significant differences (+$P < 0.1$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

found that white grape cultivars appear to lack anthocyanins because they lack UDP glucose-flavonoid 3-o-glucosyl transferase (UFGT) expression, and also had decreased expression of other flavonoid pathway genes. However, they did not investigate the expression of these genes under elevated stress conditions. It was recently shown that in the context of model plants, phytoplasma infection led to the over-expression of the anthocyanin pathway causing purple top symptom and escaping – at least temporary – leaf senescence. This suggests that anthocyanin might be responsible for the suppression of leaf cell death. In the context of anthocyanin defective mutants, appearance of yellowing symptoms were indeed followed by programmed senescence (Himeno et al. 2014). Anthocyanin synthesis does not occur in white cultivars, but genes involved in this process i.e. *chs* (responsible for the first step of flavonoid synthesis) and *dfr* (responsible for synthesis of anthocyanin derivatives) are however expressed even in the white cultivars 'Chardonnay', 'Semillon' and 'Reisling', although at lower level than in red cultivars (Boss et al. 1996; Kortekamp 2006). In our experiment anthocyanins were not detected (data not shown) neither in BNA, nor in BNS wines. Further studies are therefore needed to investigate whether or not the lack of anthocyanin synthesis in white cultivars is leading to earlier senescence when compared to red cultivars. How far these processes are involved in BN pathogenesis will certainly remain an open scientific question in the coming years.

The Eger region in Hungary belongs to the northern wine growing areas of Europe where the quality of wine is strongly influenced by the climatic conditions of the year. Differences among vintages were observed in this

study, e.g. between the 2012 vintage, marked by warm and extremely dry weather, and the 2013 vintage, where 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 BNA 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 BNA and shrivelled grapes. These wines, due to the lack of sufficient sugar accumulation in berries, resulted in lower alcohol contents. Although a lower pH ameliorates the flatness of the wine, the higher malic acid content alters the balance among the remaining organic acids (Ribéreau-Gayon et al. 2006). The pink discolouration of BNA wines was considered a wine fault that would decrease the market value of these wines. Thus, the importance of sulphur treatments and oxygen exclusion to maintain reductive conditions of wines must be emphasised in cases where yields contain considerable amounts of BN-affected bunches.

Impact of BN and consequences

Bois noir is common in European vineyards, but because of its complex disease cycle that includes alternative host plants as sources of inoculum and non-ampelophagous vectors, it is very difficult to control (Constable 2010; Maixner 2011). 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.

The impacts 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). 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). These factors together influence the economic damage caused by CPs. According to Pavan et al. (2012), factors like lifetime of the cultivar, planting density and proportion of symptomatic plants affect the productivity of a BN diseased vineyard, and influence the decision to replace the CPs infected vines. In the case of 'Chardonnay', the maintenance of BN-diseased plants appeared to be more profitable than their elimination, even though BN is a chronic disease (Pavan et al. 2012). On the other hand, because of significant yield and quality losses, the economic sustainability of BN-affected vineyards is compromised enough to suggest replanting (Garau et al. 2007; Endeshaw et al. 2012; Rusjan et al. 2012).

Our results show high yield and quality losses for 'Chardonnay' in Hungarian pedo-climatic conditions. This will certainly have a negative effect on economical sustainability and supports removal of affected vines. Exclusion of BNA bunches during harvesting is highly advisable. The masking effect of BN on the visibility of other GY diseases and consequently the efficacy of control measures could also be important. Indeed, BN and FD induce identical symptoms, and BN cases result in masking early FD outbreaks. In south-east France, after 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. Since then, BN incidence has regularly decreased in France (X. Foissac personal communication, Maixner 2011; Kuntzmann et al. 2014). Recent detection of FD in Hungarian vineyards (Kriston et al. 2013) may create a similar situation.

Conclusion

Bois noir disease was caused by bindweed-related *tuf*-b1 genotype of CPs in a vineyard of cv. 'Chardonnay' in the Eger wine growing region. The disease heavily decreased yield and adversely affected grape composition, resulting in variable wine quality. Negative effects on berry composition and wine quality were prominent in the year with suitable/favourable weather conditions, whereas the negative effects were less evident in the years with unfavourable weather (wet and cool). BN disease is an emerging problem in Hungary. According to our results, bindweed is the main CPs reservoir in the examined vineyard and measures should be focused on this. The most important factor in viticulture is the maintenance of yield, in balance with quality, and integrated pest management strategies through additional mitigating measures are acutely required.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This research does not include any animal and/or human trials.

Ethical approval The authors bear all the ethical responsibilities of this manuscript.

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