



Recovery of *Vitis vinifera* L. cv. ‘Kékfrankos’ from ‘bois noir’ disease

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Abstract Investigation of diseases caused by phytoplasmas, a group of cell-wall-less gram-positive bacteria has received significant attention in plant pathology. Grapevine is a host of two, genetically distinct phytoplasmas: Line Flavescence dorée (FD) phytoplasma associated to ‘flavescence dorée’ and ‘*Candidatus* Phytoplasma solani’ responsible for ‘bois noir’ (BN) disease. In the current study, we focused on BN diseased grapevines (*Vitis vinifera* L. cv. ‘Kékfrankos’), measured their photosynthetic performance and leaf hydrogen peroxide (H_2O_2) concentration. The latter is generally considered as a key molecule in the process of ‘recovery’ which is a spontaneous and unpredictable long-term remission of disease symptoms. This phenomenon also occurred during the time of our experiment. Infection resulted in reduced gas exchange performance and maximum quantum efficiency of PSII with an increased regulated non-photochemical quenching of PSII and H_2O_2 concentration. Changes in gas exchange seem to be a systemic response, while reduced photochemistry is a local response to ‘*Ca. P. solani*’ infection. H_2O_2 accumulation in BN phytoplasma infected plants, unlike in FD disease, was found to

be a typical response to the appearance of a biotic stressor.

Keywords ‘*Candidatus* Phytoplasma solani’ · ‘Bois noir’ · Recovery · Hydrogen peroxide · *Vitis vinifera* L

Phytoplasmas are pleomorphic, cell-wall-less prokaryotes with a cell size under 1 μm in diameter. Their genome is extremely small among bacteria (0.6–1.6 Mbp) and lacking many pathways synthesizing important metabolites (Kube et al. 2012), which makes them obligate parasites of their plant hosts and insect vectors (Bertaccini and Duduk 2009). They are grouped in the genus ‘*Candidatus* Phytoplasma’ and further divided in subgroups based upon their gene sequence coding 16S rRNA. The two most important ones infecting European grapevines are from distinct subgroups. Flavescence dorée (FD) phytoplasmas are part of subgroups 16SrV-C and 16SrV-D and are associated to ‘flavescence dorée’ disease, while ‘*Candidatus* Phytoplasma solani’ belongs to subgroup 16SrXII-A and is associated with ‘bois noir’ (BN) disease (Lee et al. 2000). As these phytoplasmas induce identical symptoms, they cannot be differentiated by visual inspection. Shoots become partially non-lignified and have shorter internodes. Leaves roll downwards and change their colour during vegetation: white varieties become yellow to golden, red varieties become reddish to purple. Flowers and berries wither and may also die (Kölber 2011), resulting in great economic loss. Despite the alterations in essential functions, the infection may disappear spontaneously. This

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phenomenon is called ‘recovery’ and it has been highly investigated but mostly in connection with FD disease (Musetti et al. 2007; Gambino et al. 2013). Different models predicting infection and recovery fluencies were suggested in the past few years (Panassiti et al. 2015, 2017; Rotter et al. 2018; Tomkins et al. 2018). In the case of FD and other phytoplasma-diseases in different cultivated plants the key element of recovery is hydrogen peroxide (H_2O_2) accumulation which serves as a signal to induce defense responses in plants, such as the Systemic Acquired Resistance (SAR) (Musetti et al. 2004, 2005, 2007; Gambino et al. 2013). In our study, H_2O_2 concentration and photosynthetic activity of BN phytoplasma infected grapevine (*Vitis vinifera* L. cv. ‘Kékfrankos’) leaves were measured in midsummer, when interfering effects of seasonal physiological changes do not occur.

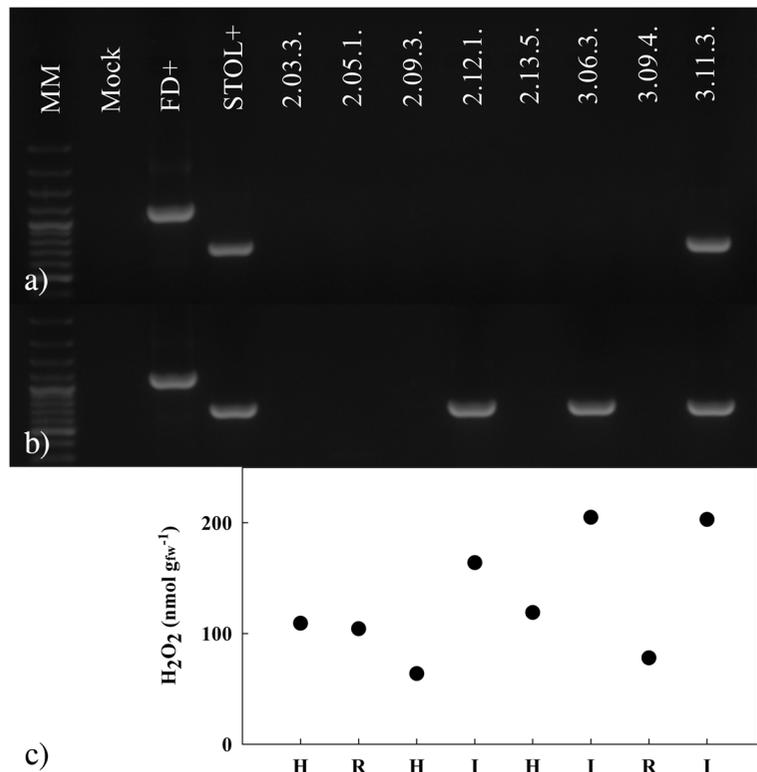
The study was conducted over a 2-year period, 2014–2015, in a commercial vineyard located near Villány (Villány Wine District, South-Hungary; latitude 45°51' N, longitude 18°26'E; elevation 120 m asl). Twenty-six-year-old vines of *V. vinifera* L. cv. ‘Kékfrankos’ (syn. ‘Blaufr nkisch’) were studied under non-irrigated conditions. ‘Kékfrankos’ vines were grafted on a commonly used rootstock variety ‘TK5BB’ (*V. berlandieri* x *V. riparia*). The soil was a Ramann-type brown forest soil mixed with clay. Vines were grown with 3 × 1 m vine spacing with North-South row direction. Vines were cordon-trained and hand-pruned in umbrella system (vertically shoot-positioned trellis). The site is situated within the Praellyricum (plant geographical district), which on average receives 680 mm of precipitation per year, 2010 h of sunshine annually and with an annual mean temperature of 10.8 °C (Dövényi 2010).

Previous studies from other research groups mention different BN incidence in cv. ‘Kékfrankos’. Starý et al. 2013 examined 1380 vines from 2006 to 2010 and found a constant disease incidence of about 5%. On the other hand, Riedle-Bauer et al. 2010 reported BN incidence about 20% evaluated from 486 plants. Phytoplasma infection occurred naturally in the vineyard described above where about 30% of the vines (equals to a 2500 plants/ha incidence) showed symptoms of BN. ‘*Ca. P. solani*’ infection was confirmed by our laboratory in 2014 (data not shown). For the infection test, whole leaves were frozen in liquid nitrogen and ground with a mortar and pestle. A purification step was carried out (Xu et al. 2004) followed by a standard CTAB based total DNA extraction protocol. Multiplex nested PCR

was performed to detect the phytoplasmas associated to FD or BN, respectively FD phytoplasma or ‘*Ca. P. solani*’, following the protocol suggested by the European and Mediterranean Plant Protection Organisation (EPPO 2007). Amplicons were visualised with 1.2% agarose gel electrophoresis stained with ECO Safe Nucleic Acid Staining Solution 20,000x (Avegene Life Science, Taipei, Taiwan) fluorescent dye. Plants were retested for infection in July and September 2015 (Fig. 1A and B). FD phytoplasma was not detected in any of the examined grapevines. Although ‘*Ca. P. solani*’ was detected only in one plant in July (despite the rolling and discoloration symptoms) but its presence was verified in three grapevines in September, proving that DNA based methods for phytoplasma detection are more reliable from samples collected in autumn. Some of the examined grapevines went through remission and the pathogen was not detected from leaf samples collected in 2015 (Fig. 1.).

Physiological measurements were performed in July 2015 (Table 1). Photosynthesis was assessed by measuring gas exchange of leaves (same ones were used for pathogen detection) with an LCA-4 type open system IRGA (ADC LCA-4 Bioscientific Ltd., Hoddesdon, UK). Photosynthetic rate (A , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), transpiration rate (E , $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), stomatal conductance to H_2O (g_s , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), intercellular CO_2 concentration (C_i , $\mu\text{mol CO}_2 \text{ mol}^{-1}$) was measured and water use efficiency (WUE, $\mu\text{mol CO}_2 / \text{mmol H}_2\text{O}$) was calculated. Three records (three technical repetitions) were taken from one leaf and means were used in statistical analyses. Then five 0.8 cm diameter discs were cut from the leaves (including veins), placed in a falcon tube filled with 6% TCA (trichloroacetic acid) and kept on ice during transportation to the laboratory to measure H_2O_2 concentrations. The assay was performed as described earlier (Mátai and Hideg 2017). Leaves from the next node showing the same symptoms were removed from plants, stored in water during transportation from field to laboratory and kept in darkness for 45 min before chlorophyll fluorescence measurements with the MAXI-version of the Imaging-PAM (Heinz Walz GmbH, Effeltrich, Germany). Maximum (F_v/f_m) and effective ($Y(II)$) PSII quantum yields were calculated according to Genty et al. 1989. Yields of regulated ($Y(NPQ)$) and non-regulated ($Y(NO)$) non-photochemical quenching were determined according to Klughammer and Schreiber 2008. Differences between healthy, recovered and infected group of leaves

Fig. 1 Leaf H₂O₂ concentration and phytoplasma infection. A) Results of multiplex nested PCR of samples collected in July 2015. B) Results of multiplex nested PCR of samples collected in September 2015. C) H₂O₂ concentrations measured in the same leaves as in A). For A) and B) MM, Molecular weight Marker; Mock, PCR performed without template DNA; FD+, Flavescence dorée positive control; STOL+, Bois noir positive control; lane 5–12, studied grapevines. For C) circles represent the means of measured H₂O₂ concentrations in nmol g_{fw}⁻¹; H, healthy plant; R, recovered plant; I, infected plant



were assessed with two-sample Student's t-tests for either equal or unequal variances, depending on results of F-tests using MS Excel Analysis ToolPak (Version 2007, Microsoft Corporation, Redmond, WA, USA). Significantly different ($p < 0.05$) means are marked with different letters in Table 1.

To our knowledge, effect of '*Ca. P. solani*' infection on gas exchange performance of grapevines cv. 'Kékfrankos' was examined for the first time in this study. As it was expected from the visual symptoms and the economic impact of the disease, infected plants had impaired CO₂ assimilation (79% of healthy plants), transpiration (86% of healthy plants), stomatal conductance (61% of healthy plants) and intercellular CO₂ concentration (90% of healthy plants). In contradiction with the results of a previous study examining *V. vinifera* L. cv. 'Chardonnay' (Endeshaw et al. 2012) water use efficiency was unaffected which may indicate that 'Kékfrankos' is possibly a less sensitive cultivar to '*Ca. P. solani*' infection. Another study with BN phytoplasma infected *V. vinifera* L. cv. 'Chardonnay' plants reported that infection decreases the maximum quantum efficiency of PSII (Bertamini et al. 2002). However, these authors found a more intense effect than in the

Table 1 Physiological characteristics of studied grapevine plants

	Healthy	Recovered	Infected
A	14.78 ± 1.34 ^a	11.62 ± 0.62 ^b	11.72 ± 0.96 ^b
E	2.51 ± 0.29 ^a	1.92 ± 0.26 ^b	2.15 ± 0.21 ^b
g _s	0.31 ± 0.10 ^a	0.18 ± 0.04 ^b	0.19 ± 0.03 ^b
C _i	237.2 ± 16.1 ^a	215.5 ± 9.3 ^b	214.4 ± 14.8 ^b
WUE	5.92 ± 0.39 ^a	6.10 ± 0.55 ^a	5.49 ± 0.54 ^a
Fv/fm	0.821 ± 0.027 ^a	0.821 ± 0.024 ^a	0.756 ± 0.068 ^b
Y(II)	0.285 ± 0.022 ^a	0.273 ± 0.018 ^a	0.257 ± 0.036 ^a
Y(NPQ)	0.568 ± 0.024 ^a	0.570 ± 0.025 ^{ab}	0.596 ± 0.030 ^b
Y(NO)	0.147 ± 0.005 ^a	0.158 ± 0.019 ^a	0.147 ± 0.010 ^a
H ₂ O ₂	97.4 ± 29.6 ^a	91.2 ± 18.7 ^a	190.8 ± 23.0 ^b

Results are shown as means ± standard deviations. Parameters are expressed as follows: photosynthetic rate (A, μmol CO₂ m⁻² s⁻¹), transpiration rate (E, mmol H₂O m⁻² s⁻¹), stomatal conductance to H₂O (g_s, mol H₂O m⁻² s⁻¹), intercellular CO₂ concentration (C_i, μmol CO₂ mol⁻¹) water use efficiency (WUE, μmol CO₂ / mmol H₂O), maximum yield of PSII (Fv/fm), effective yield of PSII (Y(II)), regulated non-photochemical quenching (Y(NPQ)), non-regulated non-photochemical quenching (Y(NO)), hydrogen peroxide content (H₂O₂, nmol g_{fw}⁻¹). Different letters represent significant ($p < 0.05$, two-sample Student's t test) difference between means of groups

case of our study (92% of healthy plants). This also may suggest that the examined cultivar is different in susceptibility or it could be due to the different sampling time. Moreover, we further analysed the light acclimated photochemical and non-photochemical yields of examined grapevines, that are missing from the literature, and found a significant increase in Y(NPQ) (105% of healthy plants). Accumulation of H₂O₂ in infected leaves (196% of healthy plants) is a typical biotic stress response described in many papers (e.g. Apel and Hirt 2004; Foyer and Noctor 2005). Recovered plants showed the same or even more decreased gas exchange characteristics as the BN phytoplasma infected plants (A, 78% and 79%, respectively; E, 76% and 86%, respectively; g_s, 58% and 61%, respectively; C_i, 91% and 90%, respectively) but the same photochemistry (Fv/fm, 100%; Y(NO), 107%) and H₂O₂ concentration (94%) as the healthy ones. This shows that recovery is not equal to the total disappearance of physiological changes induced by phytoplasma infection but the maintenance of systemic responses.

In conclusion, effects of ‘*Ca. P. solani*’ infection on gas exchange performance, photochemical processes and leaf H₂O₂ content of *V. vinifera* L. cv. ‘Kékfrankos’ plants were examined first time in this study. The presence of the pathogen resulted in decreased photosynthesis and elevated H₂O₂ concentration which is a typical biotic stress response. Among these, only low gas exchange parameters were maintained after the plants went through recovery. H₂O₂ levels in the examined recovered plants were the same as in the healthy ones. In the literature, one may find that plants recovered from other phytoplasma diseases had much higher leaf H₂O₂ concentration than healthy plants (Musetti et al. 2005). In other case the level of H₂O₂ in the leaves rather correlated with the severity of the symptoms (Pitino et al. 2017). Our findings are more concordant with the latter observations. However, our results are based on a small number of cases. Therefore, further research is necessary on a bigger group of plants to verify these results and to understand the mechanisms of recovery in ‘Kékfrankos’ grapevines.

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Author contributions AM and GJ conceived and designed the study. AM and PT collected samples and performed measurements. AM carried out data analysis. AM wrote the manuscript with the participation of PT and GJ.

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Compliance with ethical standards

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

Informed consent All authors read and approved the final manuscript.

Human studies and participants There were no involvement of human participants and/or animals in the present study.

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