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"Flavescence dorée" impacts growth, productivity and ultrastructure of *Vitis vinifera* plants in Portuguese "Vinhos Verdes" region

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Abstract

"Flavescence dorée" (FD) is a quarantine disease associated with the presence of a phytoplasma, transmitted by the insect vector *Scaphoideus titanus*. This disease affects grapevines and is of a great concern to the stability and sustainability of the wine industry, due to the harvest losses and death of infected plants. In Portugal, FD has seriously affected the "Vinhos Verdes" region, but so far the research on this topic is very limited. The current study confirmed that the FD phytoplasma strain involved in the outbreak in *Vitis vinifera* cv. Loureiro belongs to the 16SrV-D subgroup and shows very low RFLP variability in the *tuf* and *secY* genes. Transmission electron microscopy analysis of

leaf midribs from infected grapevine plants revealed that the shape and structure of the phloem cells were altered, presenting collapsed cells, callose accumulation in sieve plates and lipid accumulation in chloroplasts of phloem parenchyma cells. Moreover, data from two subsequent years showed that the FD presence was associated with an average delay of 10 to 15 days of the time to visible inflorescences and veraison compared to healthy plants. At veraison, FD also lead to a significant decrease in the budburst percentage (7% to 12%), fertility index (35% in 2015), leaf area (56% to 63%), and chlorophyll content (18% to 35% lower SPAD values). Hence, infected plants showed a drastic reduction in the yield, corresponding to a decrease between 51% and 92% ~~as~~ compared to healthy plants, which mostly resulted from a lower number of bunches (63% to 92% less), but also a decreased bunch weight (35% lower in 2015). Concerning berry quality, there were no significant differences in terms of total soluble solids and titrable acidity in both years. Here, this study concluded that the FD infection delays the grapevine development and leads to drastic production losses, which may be partly linked to the ultrastructural modifications observed in the phloem cells of infected plants.

Keywords: Berry quality; Bunch yield; Chlorophyll content; Grapevine; Phytoplasma; Transmission electron microscopy

1 Introduction

Phytoplasmas are prokariotic organisms without cell wall that derived from Gram-positive bacteria (Woese, 1987; Weisburg et al., 1989). They are phloem obligate parasites that circulate throughout the plant sieve tubes, preferentially accumulating in the leaves (Christensen et al., 2005). Phytoplasma presence may be associated with necrosis in the phloem elements, cell wall thickening, abnormal starch accumulation and high callose deposition in the sieve tubes (Musetti et al., 2013a; Santi et al., 2013; Ahmed et al., 2016). Other studies reported physiological alterations including stomatal closure, photosynthetic rate reduction, and carbohydrate accumulation in leaves (Endeshaw et al., 2012; Santi et al., 2013). These alterations may compromise the translocation of photoassimilates, reduce plant size and vigor, and consequently impare vine yield.

Currently, “flavescence dorée” (FD) is one of the most important diseases for grapevine in Europe, leading to severe losses in the grapevine production (Dermastia et al., 2017). It is a quarantine phytoplasma disease transmitted by the insect *Scaphoideus titanus* Ball (Schvester et al., 1963). The FD phytoplasmas belong to the 16SrV group, subgroups 16SrV-C and D (Bertaccini et al., 1995; Martini et al., 1999). The first outbreak of FD was reported in 1955, in Armagnac, France, and the disease has since dispersed to other European winegrowing regions such as Italy, Portugal, Spain, Serbia, Slovenia, Switzerland, Hungary, Croatia and Austria (Belli et al., 2010). In Portugal, FD was detected for the first time in 2006 in the “Vinhos Verdes” region (de Sousa et al., 2010), and since then it has spread throughout this region (DGAV, 2013). FD is listed in the European Plant Protection Organization (EPPO) List A2 and it is managed by the Community Directive No. 200/29 / EC (EPPO, 2007). Unfortunately, up to present there are no efficient methods to eradicate this disease (reviewed by Oliveira et al., 2019).

Plants infected with FD develop leaf rolling, leaf yellowing or reddening (depending on berry colour), stunted growth, unripen cane wood and shrivelled berries. In the shoots, one of the first FD symptoms in Portugal is a delayed or lack of apical and lateral bud break, that occurs in early summer (de Sousa et al., 2010). The shoots of susceptible grapevine cultivars often exhibit small black pustules, lower lignin content, are thinner, rubbery and hang pendulously (Roggia et al., 2014). The severity of these symptoms is dependent on the grapevine cultivar, plant vigor, presence of other pathogens and degree of infection (Zahavi et al., 2013).

To the best of our knowledge, no studies have been focused on the ultrastructural analysis of FD infected grapevines. Moreover, no quantitative information is avaliable on the impact of FD on plant development and productivity, whereas for other grapevine diseases, namely “bois noir” (BN) (Endeshaw et al., 2012; Zahavi et al., 2013) and mildew (Bertamini and Nedunchezhian, 2001; Bertamini et al., 2002; Jermini et al., 2010), this information is more readily available. About the impact of phytoplasma diseases on fruit quality the available information is even scarcer.

Portugal has the seventh largest vineyard area in the world, with over 200 000 ha, and an annual wine production of about 6.7 million hL (IVV, 2017). The country is ranked as the 11th world’s largest wine producer and the 10th wine exporter. The Minho region, also named “Vinhos Verdes” region, located in the northwest of the country, produces mostly white wines, known by their typical freshness and slightly higher acidity (Fraga et al., 2017). This is the third most representative wine producing region in Portugal, with 21 000 ha corresponding to 11% of the national wine area (CVRVV, 2015; IVV, 2017).
Given the economic impact of FD in the “Vinhos Verdes” region (Sousa et al, 2014), the most affected region in Portugal, it is important to study its effects on plant growth, productivity and grape quality. Therefore, this study aimed to: (i) conduct the molecular characterization of the FD phytoplasma infecting cv. Loureiro (one of the most relevant cultivar from “Vinhos Verdes” region) to identify the strain or strains involved, (ii) understand the effects of FD on the cellular ultrastructure of leaf midribs (using transmission electron microscopy - TEM), and (iii) evaluate the impact of FD on plant growth and physiology (budburst percentage, fertility rate, SPAD values, leaf area), yield (productivity, number of bunches and average bunch weight) and fruit quality (total soluble solids content and titrable acidity).

2 Material and methods

2.1 Plant material and growth conditions

This study was conducted in grapevine cv. Loureiro from a 20-year-old vineyard located at Quinta do Corvo (Fafe, Portugal; 41°31′00″N 8°12′56″W). Thirty-two grapevines were selected, of which 16 were healthy and 16 were infected with FD, as confirmed using the protocol described below. The vineyard is located in a granitic soil with a sandy-loam texture, a south solar exposure, an altitude between 300-400 m and a slope of 2 to 5%. Grapevines plants were trained in a single upward cordon system and spaced 3 m x 3 m apart. Fertilization and pest and disease control followed the integrated production system standards. FD was detected in 2009, and since then procedures for the elimination of the insect vector have been adopted following mandatory rules. This study was carried out in two subsequent years (2015 and 2016) starting in April (green shoot stage) until September (harvest time).

2.2 Identification and characterization of FD phytoplasmas

Symptomatic leaves were collected in July 2015 from the 16 FD infected plants and their midribs were powdered using liquid nitrogen. A similar processing was carried out in samples collected from ~~two~~ asymptomatic grapevines used as negative control. The extraction of total DNA was performed using 1 g of plant material according to Prince et al. (1993). After extraction, DNA was quantified with a spectrophotometer (BioRad) and stored at −20 °C for further analyses.

The extracted DNA was diluted to a final concentration of 20 ng/μL for PCR analyses using phytoplasma universal primer pair P1/P7 primer pair (Deng and Hiruki, 1991; Schneider et al., 1995) followed by nested-PCR on 1:29 dilution in sterile distilled water (SDW) of the obtained amplicons (1 μL) with primer pair 16R758f/23SR1804 (=M1/B6) (Gibb et al., 1995; Padovan et al., 1995). Samples lacking DNA and samples containing DNA from the healthy grapevines were used as negative controls. DNAs extracted from micropropagated periwinkles infected by aster yellows (AY, 16SrI-B), rubus stunt (RuS, 16SrV-E), and elm witches' broom (ULW, 16SrV-A) phytoplasmas (Bertaccini, 2014), together with DNAs extracted from grapevine infected by FD-C and FD-D phytoplasmas (Martini et al., 2002) and jujube witches' broom (JWB, 16SrV-B) phytoplasmas (Zhao et al., 2016), were used as positive controls. All the amplifications were performed in an automated thermal cycler (VWR, Belgium). Each reaction was performed in a total volume of 25 μL containing 2.5 μL of the 10× buffer, 200 μM of dNTP's, 0.625 U of *Taq* polymerase (Sigma Aldrich), and 0.4 μM of primer pair. The PCR conditions for the 35 cycles were: 1 min (2 min for the first cycle) denaturation at 94 °C, 2 min annealing at 55 °C (50 °C for the nested-PCR), and 3 min (10 min for the last cycle) at 72 °C for primer extension. Six microliters of each PCR product were separated by electrophoresis in 1% agarose gels, stained with ethidium bromide, and photographed under ultraviolet illumination (312 nm). Identification of detected phytoplasmas was done by RFLP analyses on 100 to 200 ng DNA of amplicons with *Taq*I restriction enzyme (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The digested DNA fragments were separated by electrophoresis in a 6.7% polyacrylamide gel, stained with ethidium bromide, and visualized as reported above.

Five positive samples were selected for the characterization of the FD strains on the ribosomal elongation factor (*tuf*) and the translocase (*secY*) genes. PCR analyses on the *tuf* gene were carried out using the primers and the conditions ~~as~~ described by Makarova et al. (2012) with annealing temperature of 50 °C. For the *secY* gene, nested PCR was performed using the primer pair FD9f/FD9r (Daire et al., 1997) followed by primer pair FD9f3/FD9r2 (Angelini et al., 2001). Each reaction was performed in a total volume of 25 μL as described above with the annealing temperature at 48 °C. All the PCR products were digested with restriction enzymes *Tru1*I, *Tsp509*I, *Taq*I (Fermentas, Vilnius, Lithuania) and the restriction products from the two genes were separated as described above. RFLP patterns were compared to those of positive control strains and of previously published strains (Martini et al., 1999, 2002; Contaldo et al., 2011).

2.3 TEM analysis of grapevine leaf midribs

The effect of phytoplasma infection on leaf ultrastructure anatomy was evaluated by TEM, using an adapted protocol from Santi et al. (2013). Four FD negative and four FD positive plants were selected, from which two medium-sized (approximately 15 cm wide) symptomatic leaves were collected in July of 2015. Sections with

7 mm length were cut in the central part of the main vein of the leaves. Samples were submerged and pressure infiltrated in a fixation solution consisting of 3% paraformaldehyde and 4% glutaraldehyde in 50 mM sodium cacodylate buffer with 2 mM $CaCl_2$, pH 7.2, where they remained for a total of 5 h, with a renewal of the fixation solution at 3 h. Samples were washed for 1 h at 4 °C in 50 mM sodium cacodylate buffer containing 2 mM $CaCl_2$ (pH 7.2), and maintained overnight in 2% (w/v) OsO_4 in the above-mentioned buffer at 4 °C. In the following day the dehydration step was carried out and samples were washed for 10 min with shaking following an alcohol dehydration panel: 25%; 50%; 70%, 80%, 90%; 96%; three times at 100%. Samples were then submerged in 100% propylene oxide for two periods of 15 min and Epon 812 resin (EMS)/araldite epoxy resin (Electron Microscopy Sciences, Fort Washington, PA, USA) infiltrations were performed and polymerized for two days at 60 °C. Semi-thin and ultra-thin sections were obtained with an Ultramicrotome RMC PowerTome PC = XL using diamond and glass knives. Semi-thin sections were treated as described by Richardson et al. (1960) and ultra-thin sections were stained with aqueous uranyl acetate and lead citrate (Reynolds method, Merk), and were observed under a JEM 1400 (Joel, Japan) electron microscope at 80 kV and photographed with an Orius Sc1000 Digital Camera.

2.4 Biometric analyses

The phenological stages were registered weekly, starting in April at green shoot (stage C) until flowering (stage I) and biweekly thereafter until harvest time (13rd and 22nd September in 2015 and 2016, respectively), following the Baggiolini scale (Baggiolini, 1952) which highlights budburst, flowering and veraison as the main stages (Carbonneau, 1981). Buds left on the shoots after winter pruning (i.e. pruning load) were counted to calculate budburst percentage and fertility index (Alonso et al., 2007), according to the formulas:

$$\%\,\text{of budburst} = \frac{\text{no. of burst buds}}{\text{pruning load}} \times 100$$

$$\text{Fertility index} = \frac{\text{no. of bunches}}{\text{pruning load}}$$

Chlorophyll content was assessed weekly using a Soil and Plant Analyzer Development (SPAD) meter (Konica Minolta SPAD - 502 Plus; Minolta Osaka, Japan). Five leaves were randomly selected from each plant and measurements were performed in triplicate in each leaf. Leaf area was determined using the method described by Lopes and Pinto (2005). In short, eight grapevines were selected at flowering (stage I) and at veraison (stage M) and a cane with a representative vigour of the plant was selected per grapevine. In each cane, the number of buds, main leaves and secondary leaves were counted. Additionally, the length of the right and left lateral midribs of major and minor main and secondary leaves were also measured.

2.5 Grape quality evaluation

At harvest time, the number of bunches, average bunch weight and bunch weight per plant were determined. From each grapevine, five berries per bunch (two at the top, two in the middle and one at the base) of six bunches were collected randomly. Those berries were used to calculate total soluble solids content - TSS (ºBrix) and titratable acidity - TA (g of tartaric acid/L). Samples were crushed, homogenized and centrifuged at 4000 rpm for 5 min. Supernatant was analysed in the refractometer (Atago, Japan). The pH of the supernatant was measured using a potentiometer (Crison, Barcelona) and the TA was determined according to Portuguese legislation (IPQ, 1999).

2.6 Statistical analysis

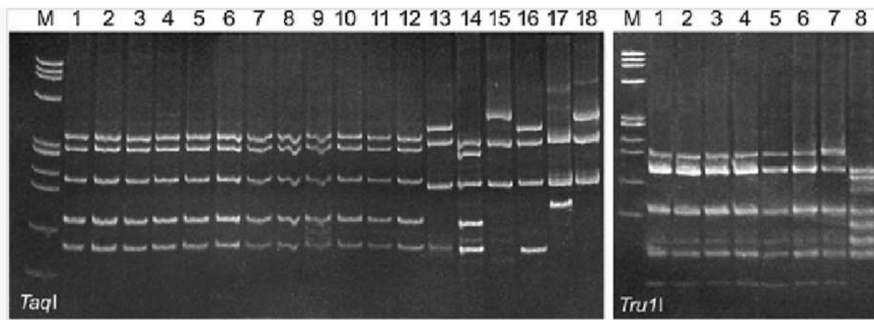
Data analysis was performed using GrafPad Prism software (version 6.0). The results correspond to the mean ± standard error of the mean (SEM). Differences between treatments were tested with parametric unpaired *t*-test and two-way ANOVA with a confidence level of 95% ($P < 0.05$).

3 Results and discussion

3.1 Phytoplasma molecular characterization

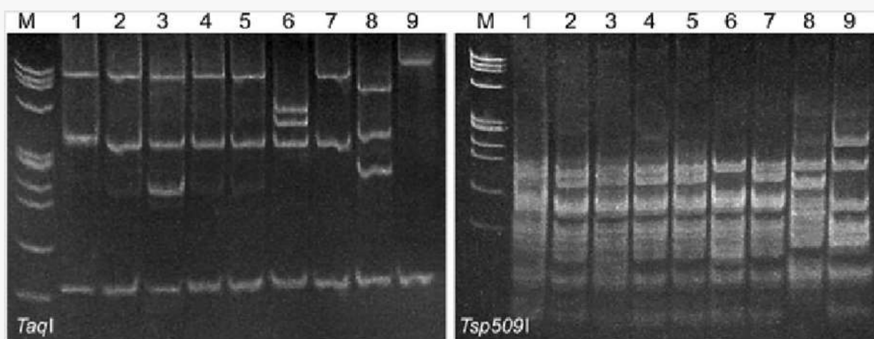
In this study all the 16 symptomatic grapevines tested resulted positive in PCR with primer pair M1/B6 and the restriction analyses with *Taq*I revealed the presence of a pattern referable to 16SrV-D phytoplasmas in all the samples (Fig. 1). The samples from the asymptomatic plants and from SDW as template resulted negative in all the PCR analyses with all the primers employed. Analysis on *tuf* and *secY* genes carried out for five of these strains indicated no variability in the RFLP profiles corroborating also the 16SrV-D subgroup affiliation (Figs. 1 and 2). This FD subgroup is strictly related with its vector *S. titanus*, and no alternative host plants or putative or proved insect vectors were reported, on the contrary of what was found for the FD strains in subgroup 16SrV-C (Dermastia et al., 2017). Here, only FD -D was identified in "Quinta do Corvo" vineyard, in agreement with the reported low varibility of this phytoplasma subgroup (Martini et al., 2002). Three FD clusters were described based on multigenic approaches: FD-1, which comprises strain FD70, not detected in field since the nineties; FD-2 (FD D), which comprises strains with no genetic variability; and FD-3 (FD C) reported as genetically variable and having both alternative plant hosts such as *Clematis vitalba*, and other insect vectors such as *Orientus ishidae* (Arnaud et al., 2007; Filippin et al., 2009; Lessio et al., 2016). FD D was detected in France, Italy and Spain associated with severe epidemics (Botti and Bertaccini, 2007). In Portugal there is limited information in this topic and until now, only this strain has been described after identification of the 16S ribosomal gene in the northern region (de Sousa et al., 2010; Sousa et al., 2011). The multigene characterization carried out on the strains from "Quinta do Corvo" vineyard further confirms the Portuguese phytoplasmas as FD D strains with no RFLP variability.

Fig. 1



On the left: RFLP patterns on polyacrylamide gel of some of the M1/B6 amplicons from: 1-12, symptomatic grapevine cv. Loureiro; 13, "flavescence dorée" (FD) strain 16SrV-C; 14, FD strain 16SrV-D; 15, elm witches' broom, ULW, (16SrV-A); 16, rubus stunt, RuS, (16SrV-E); 17, aster yellows, AY, (16SrI-B); 18, "stolbur", STOL, (16SrXII-A). On the right: RFLP patterns of some of the tuf amplicons from grapevine samples (1-5) compared with controls: 6, FD strain 16SrV-D; 7, FD strain 16SrV-D; 8, aster yellows (16SrI-B). Restriction enzymes are at the bottom of the figures. M, marker phiX174 DNA digested with *Hae*III length from top to bottom fragments in bp: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72.

Fig. 2

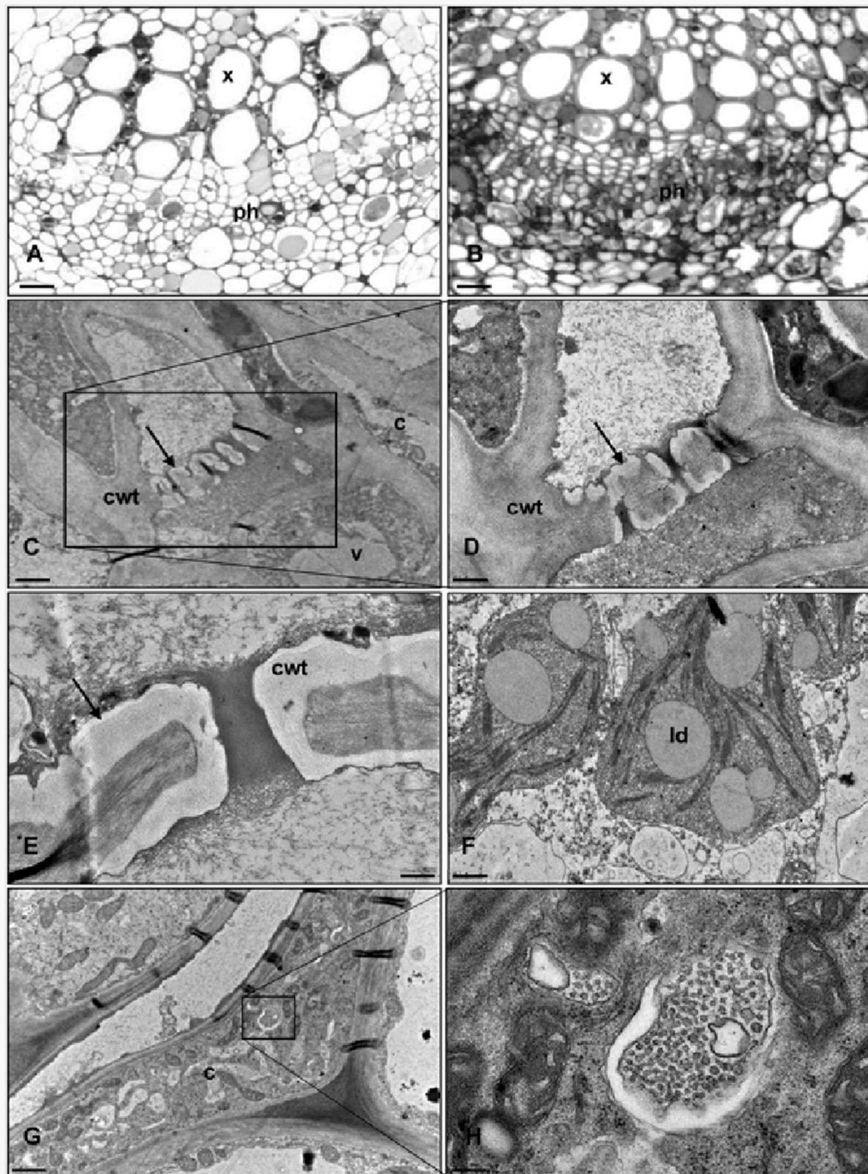


RFLP patterns on polyacrylamide gel of some of the Fd9f3/r2 amplicons from 1 to 5, symptomatic grapevine cv. Loureiro; 6, FD strain 16SrV-C; 7, FD strain 16SrV-D; 8, jujube witches' broom, JWB, (16SrV-B); 9, elm witches' broom, ULW, (16SrV-A); M, marker as in Fig. 1A.

3.2 Structural and ultrastructural analysis of infected grapevine leaf midribs

Optical microscopy images revealed structural modifications in the phloem tissues of symptomatic grapevine leaves (Fig. 3B), when compared with those of healthy plants (Fig. 3A). A more detailed image analysis at the phloem level of the FD infected plants using TEM (Fig. 3C-H) allowed to observe the presence of collapsed cells and vacuoles in phloem cells (Fig. 3C and G), cell wall thickening (Fig. 3C and E) and callose accumulation in sieve plates (Fig. 3C-E). These type of ultrastructural modifications have been described in other plant species infected by phytoplasmas including *Vicia faba* and *Solanum lycopersicum* (Musetti et al., 2013a; Santi et al., 2013; Ahmed et al., 2016), as well as in grapevine infected with other diseases, such as Pinot Gris Virus (Tarquini et al., 2018) and downy mildew (Farouk et al., 2017). However, this is the first study that analysed the leaf ultrastructure in FD infected grapevines.

Fig. 3



Images of sections of leaf midribs from healthy (A) and "flavescence dorée" infected (B to H) grapevines cv. Loureiro obtained by optical microscopy (A and B) and by transmission electron microscopy (C to H). A – xylem (x) and phloem (ph) tissues with cells presenting normal shape and structure; B – phloem tissue (ph) showing changes in cell shape and structure; C – phloem sieve plate/elements and phloem cells showing some level of ultrastructural disorganization, collapsed cell (cc) and cell wall thickening (cwt); v -vacuole; D – magnification of a different section of the region from the insert in C; E – high magnification of a sieve pore with extensive callose accumulation (arrow); F – chloroplasts with high lipid accumulation in large plastoglobuli (PG); G and F – collapsed cell (cc) with altered ultrastructure and abundance of multivesicular bodies. In (A) and (B) bars correspond to 10 µm; in (C) and (G) bars correspond to 1 µm; in (D) and (F) bars correspond to 0.5 µm; in (E) and (H) bars correspond to 200 nm.

Additionally, in the current work it was also found that FD infected grapevine plants had a higher accumulation of lipids in the chloroplasts of phloem parenchyma cells, as evidenced by the presence of large plastoglobuli (PGs) (Fig. 3F). The presence of large PGs in leaf chloroplasts has been associated with several abiotic stresses, and their analysis suggested that chloroplast PGs function in metabolism of prenyl lipids, recycling of phytol, remobilization of thylakoid lipids, and metabolism of jasmonate (Van Wijk

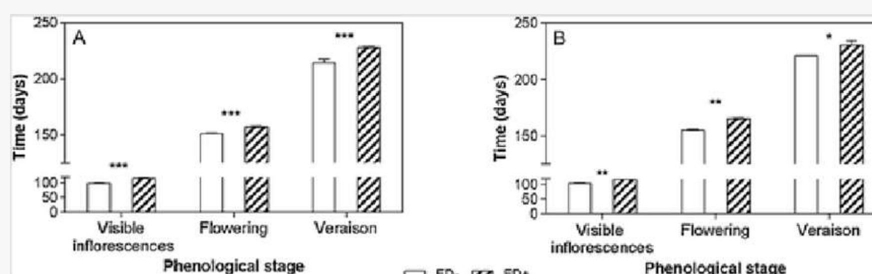
and Kessler, 2017). It is known that jasmonic acid, and its active compound methyl jasmonate, are endogenous plant hormones synthesized via the octadecanoic pathway (Creelman and Mullet, 1997). These plant growth regulators trigger the activation of plant defence mechanisms in several pathosystems (reviewed by Oliveira et al., 2019). Since the phytohormone jasmonic acid is biosynthesized from the fatty acid alpha-linolenic, in the chloroplasts, the observed high lipid concentration / PGs abundance could be related with FD infection and plant defence activation. This is in agreement with recent studies which have shown that other plant infections lead to an increase in total lipid content. This was the case of *Arabidopsis thaliana* Columbe ecotype, infected with *Botrytis cinerea* (Cela et al., 2018) and grapevine infected with *Plasmopara viticola*, responsible to downy mildew which produced lipids (ceramides and derivatives of arachidonic and eicosapentanoic acids) that are very important in the infection process (Negrel et al., 2018). Therefore, those authors suggested that the identification of unusual lipids by high-throughput techniques, such as LC-MS-based methods, in grapevine could be used for a biomarker of pathogens infection (Negrel et al., 2018). More studies are however needed in order to validate these results on FD infected grapevine and discriminate the type of lipids synthesized during the plant infection.

3.3 Impact of FD on grapevine growth and productivity

3.3.1 Biometric analyses

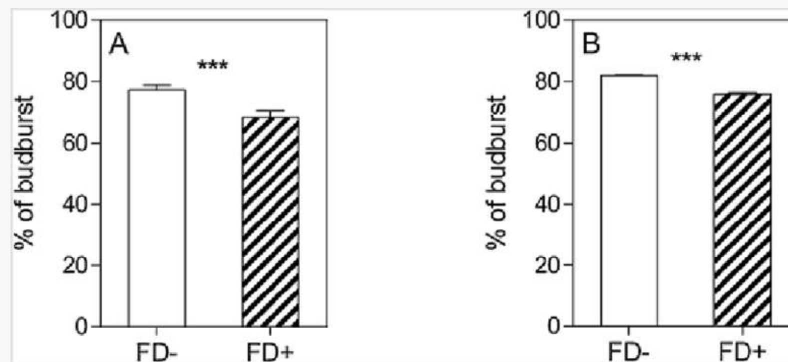
FD infected plants presented a significantly slower development than healthy plants (Fig. 4). In 2015, the visible inflorescences (stage F) was reached 15 days later in FD positive plants as compared to healthy ones (Fig. 4A). Moreover, at day 150, FD negative plants were at the flowering (stage I) while FD positive plants were, on average, 7 days delayed. From flowering stage till veraison (stage M), FD positive plants slowed down their development, showing again 15 days delay compared to FD negative grapevine plants (Fig. 4A). In 2016, this significant negative effect of FD on vine development was also observed but it was less pronouced. A delay on budburst was also previously reported as a characteristic symptom of the disease in this Portuguese winegrowing region (Sousa et al., 2011). In 2015, cv. Loureiro produced in "Vinhos Verdes" region had an atypical lower budburst rate (71%) (EVAG, 2015), which may be due to the dry winter (Morone et al., 2007; Fila et al., 2014). In this assay the FD positive plants showed, on average, a budburst rate of 68% (in 2015) and 76% (in 2016), which was significantly lower than the one of the healthy plants that presented 77% and 82% of budburst, in the same years respectively (Fig. 5).

Fig. 4



Evolution of the phenological stages (stage F: visible inflorescences; stage I: flowering; stage M: veraison, according to Baggiolini scale) registered in 2015 (A) and 2016 (B) in healthy (FD-) and "flavescence dorée" infected (FD+) grapevine cv. Loureiro. Time to reach the three studied phenological stages is expressed as day of the year (day 1 = 1 Jan). Data are means ± SEM of 4 biological replicates. Mean differences between FD- and FD + plants according to the two-way ANOVA have been denoted as *** ($P < 0.001$), ** ($P < 0.01$), * ($P < 0.05$).

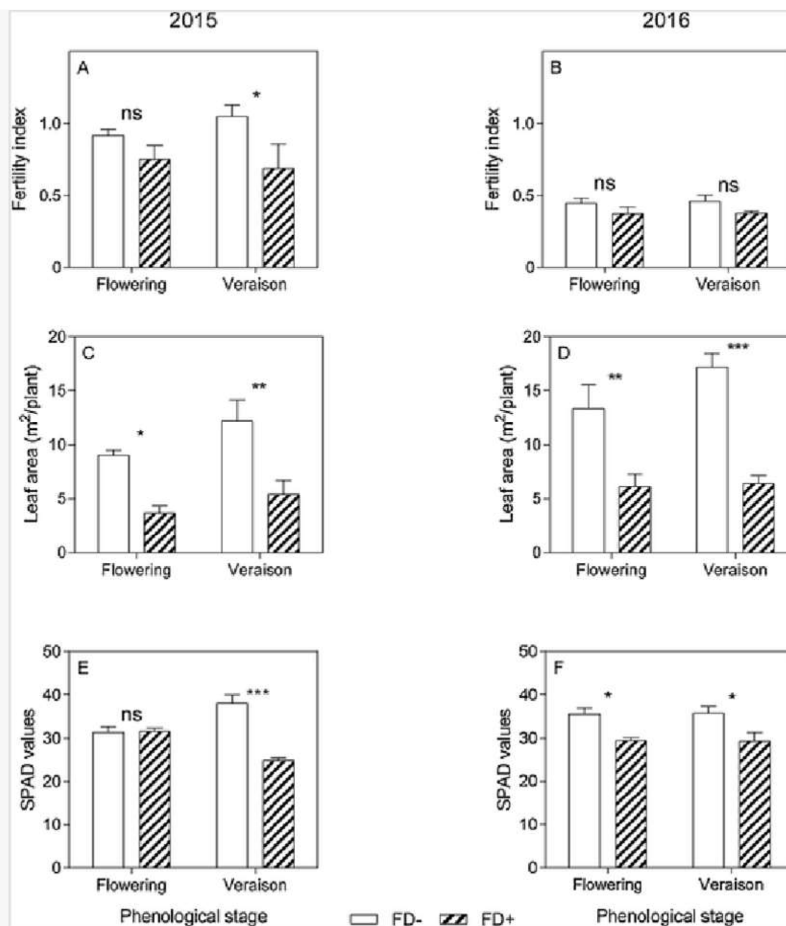
Fig. 5



Budburst percentage registered in 2015 (A) and 2016 (B) in healthy (FD-) and "flavescence dorée" infected (FD+) grapevine cv. Loureiro. Data are means ± SEM of 4 biological replicates. Mean differences between FD- and FD + plants according to the *t*-test have been denoted as *** ($P < 0.001$).

In 2015, FD positive plants presented a significantly lower fertility index only reaching 0.75 and 0.68 at flowering and veraison stages, respectively, while in FD negative plants it was of 0.92 and 1.05 for the same phenological stages (Fig. 6A). The year after, a similar trend was found, also with a more pronounced effect at veraison, but no significant differences were observed among healthy and diseased plants (Fig. 6B). These results are in accordance to the expected reduction of number of bunches per shoot in the FD positive plants (EPPO, 2007) and can be explained by the aforementioned reduction of the photoassimilate translocation (Matus et al., 2008). As phloem function is particularly important after veraison, the drastic reduction of carbohydrates supply to the bunches in FD infected plants, leads to bunch shrivelling and, consequently, to a higher decrease in the fertility index in the later stages of plant development.

Fig. 6



Fertility index (A and B), leaf area (C and D) and SPAD values (E and F) at flowering and veraison stages measured in 2015 and 2016 in healthy (FD-) and "flavescence dorée" infected (FD+) grapevine cv. Loureiro. Data are means ± SEM of 4 biological replicates. Mean differences between FD- and FD + plants according to the two-way ANOVA have been denoted as *** ($P < 0.001$), ** ($P < 0.01$), * ($P < 0.05$), ns (non-significant difference).

Leaf area is a fundamental parameter to understand grapevine responses to the environmental conditions and to crop management techniques (Lopes and Pinto, 2005). In this study, both at the flowering and at the veraison stages, there were significant differences in the leaf area between FD positive and negative plants in the two studied years (Fig. 6C and D)). In 2015, FD positive plants had lower total leaf area (3.63 m^2/plant at flowering and 5.40 m^2/plant at veraison) when compared to FD healthy plants (9.07 m^2/plant at flowering and 12.23 m^2/plant at veraison), showing a reduction of about 60% at both phenological stages. Similarly, in 2016, leaf area decrease reached 54% and 63% at flowering and veraison, respectively (Fig. 6D). This difference can be explained by the fact that FD positive plants were left with a lower pruning load (due to their stunted growth) and a lower budburst percentage (Fig. 5). Another study showed that mildew infected grapevine cv. Merlot can suffer up to 73% leaf area loss per plant (Jermini et al., 2010), affecting plant photosynthetic rate (Bertamini and Nedunchezhian, 2001; Bertamini et al., 2002).

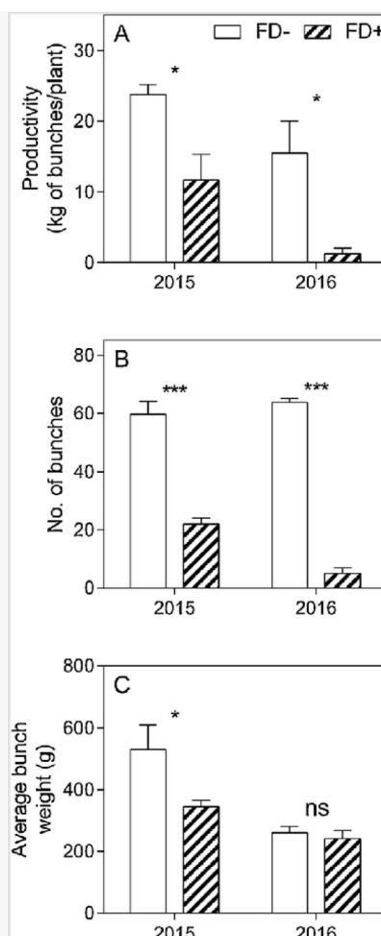
Leaf yellowing in one of the most common FD symptoms in white grapevine cultivars. Here, it was found that in general SPAD values in FD positive plants decreased significantly (up to 35%) when compared to healthy plants indicating a lower chlorophyll content of the leaves of diseased plants (Fig. 6E and 6 F). These findings are in agreement with a study conducted on cv. Chardonnay, which showed that the presence of BN decreased

the grapevine leaf chlorophyll content in about 22% at veraison (Endeshaw et al., 2012). In fact, Musetti et al. (2013b) found that during phytoplasma infection, the assimilate translocation in the host plant is severely affected, inducing massive changes in the phloem physiology. Additionally Santi et al. (2013) suggested that starch accumulation in the leaves from grapevine infected with BN, observed by electron microscopy, can result in phloem vessels blockage. Since the chloroplasts still photosynthesize, the accumulated photoassimilates are stored in the leaves in the form of starch, as they cannot be mobilized to other plant organs. This in turn is expected to further enhance the abnormal callose deposition, as observed in the present study (Fig. 3C-E), leadind to a negative feedback control on the photosynthetic rate (Stitt, 1991; Goldschmidt and Huber, 1992), which could have contributed to the leaf yellowing.

3.3.2 Productivity parameters

Although there are no studies that directly quantified the effects of FD phytoplasma infection on grapevine yield parameters, it has been described that in FD positive plants the inflorescences can become sterile, bunches more fragile, and consequently, berries fall easily (Bertaccini and Duduk, 2009). These findings can explain the negative impact of this disease on the fertility index (Fig. 6A) and, consequently, on the productivity (Fig. 7A). The later dropped from 23.8 kg of bunches per plant in healthy grapevines to 11.7 kg in FD positive plants in 2015, and from 15.5 kg to 1.25 kg in FD positive plants in 2016, representing a 51% and 90% yield decrease, respectively (Fig. 7A). This drastic yield reduction was mostly due to a significantly lower number of bunches showing, on average, a reduction of 63% and 92% in the two subsequent years (Fig. 7B). Moreover, in 2015 the average bunch weight was also significantly lower on FD positive plants, with a 35% decrease ~~as~~ compared to healthy plants (Fig. 7C). Previous studies on *Vitis vinifera* cvs. Cabernet Sauvignon and/or Chardonnay have shown that the presence of BN disease led to yield losses ranging from 68% to 85%, and around 55% lower number of bunches per plant (Endeshaw et al., 2012; Zahavi et al., 2013; Ember et al., 2018). Similar consequences on plant yield have also been described for grapevines affected by phloematic viruses (Komar et al., 2007; Alabi et al., 2016; Martínez et al., 2016). For example, *Grapevine Fanleaf Virus* infection caused a decrease of 40% in productivity in cv. Tempranillo, displaying both a significant reduction in the number of bunches and in the average bunch weight (Martínez et al., 2016), comparable with the one observed in the present study.

Fig. 7

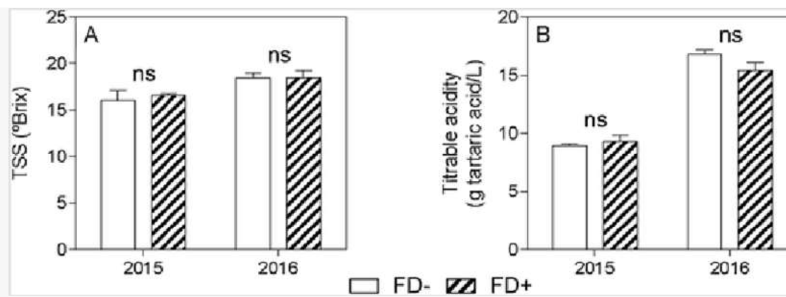


Productivity (A), number of bunches per plant (B) and average bunch weight (C) measured in 2015 and 2016 in healthy (FD-) and "flavescence dorée" infected (FD+) grapevine cv. Loureiro measured at harvest stage. Data are means ± SEM of 4 biological replicates. Mean differences between FD + and FD- plants according to the two-way ANOVA have been denoted as *** ($P < 0.001$), * ($P < 0.05$), ns (non-significant difference).

3.3.3 Impact of FD on grape berry quality

Sugars and organic acids are important metabolites that distinguish the quality of a wine (Boulton, 1980) and the studied cv. Loureiro is known for generally producing wines with high acidity. The values of acidity are related to the organic acids content in berries, which tend to decrease during maturation, while the sugar content increases (de Souza et al., 2005). The effect of the biotic stresses on berry quality parameters seem not to be consistent. A study conducted with cv. Chardonnay demonstrated that BN decreased 23% TSS and increased 52% TA (Endeshaw et al., 2012), whereas Zahavi et al. (2013) found no influence of this disease on cvs. Cabernet Sauvignon and Chardonnay. Concerning mildew infections it has been reported a significant effect on berry quality but to a lower degree, since powdery mildew decreased 6% on TSS in cv. Concord (Gadoury et al., 2001) and downy mildew infection led to an increase between 7-9% in TA in cv. Merlot (Jermini et al., 2010).. In the present study, there were no significant differences in TSS and in TA between FD positive and negative plants in both years (Fig. 8).

Fig. 8



Fruit total soluble solids (TSS; A) and titrable acidity (B) measured in 2015 and 2016 in healthy (FD-) and "flavescence dorée" infected (FD+) grapevine cv. Loureiro measured at harvest stage. Data are means ± SEM of 4 biological replicates. Mean differences between FD + and FD- plants according to the two-way ANOVA have been denoted as ns (non-significant difference).

4 Conclusions

In the current study only FD-D strain was identified in the analysed symptomatic cv. Loureiro grapevines from "Quinta do Corvo" vineyard of the "Vinhos Verdes" region. Infected plants showed a significant growth delay in both studied years. At veraison, FD also led to a significant decrease in the budburst rate, fertility index, leaf area, and chlorophyll content. Moreover, infected plants showed a drastic reduction in their productivity, which mostly resulted from a lower number of bunches. TEM analysis of leaf tissues of infected plants revealed ultrastructural modifications, abnormal callose accumulation in sieve plates and an increase in the content of lipids/plastoglobuli of chloroplasts from phloem parenchyma cells. These modifications compromise the phloem function and partly explain the negative impact of the disease on plant growth and yield. Nevertheless, further research is needed to better understand the molecular and biochemical mechanisms of FD phytoplasma interaction with grapevines that lead to the aforementioned impacts of the disease.

Uncited reference

Vitali et al. (2013).

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Highlights

- RFLP of infected grapevine cv. Loureiro identified a FD-D strain.
- FD infected leaves showed high callose and lipid accumulation in the phloem cells.
- FD lead to a delay in grapevine development and to drastic production losses.
- FD decreased berry quality resulting in higher acidity.
- Leaf ultrastructure modifications partly explain the FD impairment on vine growth.