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PORTO

New challenges on the control of “flavescence dorée” in grapevine: exploiting genetic resources and the use of elicitors

Thesis submitted to the Universidade Católica Portuguesa to attain the degree of PhD in Enology and Viticulture

By

Manuel João Rebelo de Araújo Oliveira

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Manuel João Rebelo de Araújo Oliveira

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July, 2021

Os únicos limites das nossas realizações de amanhã
são as nossas dúvidas e hesitações de hoje.
(Franklin Roosevelt)

A todos os que continuam a velar por mim…

ABSTRACT

"Flavescence dorée" (FD) is a grapevine quarantine disease associated with phytoplasmas and transmitted to healthy plants by insect vectors, mainly *Scaphoideus titanus* Ball. In Europe, FD was firstly reported in 1955, in France, and has dispersed through the central and southern European winegrowing regions, with a great impact in grapevine production. In Portugal, FD was identified for the first time in the "Vinhos Verdes" region (VVR) in 2006. Infected plants usually develop symptoms characterized by stunted growth, unriped cane wood, leaf rolling, yellowing or reddening (depending on berry colour), and shrivelled berries. Conventional management strategies rely mainly on the insecticide treatments, roguing infected plants and use of phytoplasma-free propagation material. However, these strategies are costly and could have undesirable environmental impacts. Development of sustainable approaches for FD control has been hampered by the lack of knowledge about phytoplasma biological properties and plant-pathogen interactions.

The main aim of this thesis was to study the use of elicitors – methyl jasmonate, MeJA, salicylic acid, SA and benzothiadiazole, BTH – against phytoplasma-associated disease in grapevine. Elicitors have been successfully applied as preventive and environmentally friendly treatments for several diseases, but there is limited information on their effect on phytoplasmas. Since phytoplasmas are difficult to cultivate, *Catharanthus roseus* (commonly known as periwinkle) was used as a model system to study phytoplasma: host interactions as this plant species could present different degrees of symptom' severity according with the phytoplasma strain. The specific goals of the present study were: (i) characterise FD phytoplasma strain in *Vitis vinifera* cv. Loureiro and study its effects on grapevine ultrastructure, growth, development, and productivity; (ii) understand the physiological and molecular responses triggered by the MeJA and SA application in FD-infected grapevines under field conditions; (iii) study the basal genomic and metabolomic profile of healthy and "aster yellows" phytoplasma-infected periwinkles and (iv) understand the role of MeJA and BTH in the induction of defence mechanisms against phytoplasmas.

Field trials revealed that the phytoplasmas infecting grapevine cv. Loureiro belong to the 16SrV-D subgroup and show very low RFLP variability in the *tuf* and *secY* genes. Moreover, a two-year study demonstrated that FD infection delays grapevine development, leading to drastic production losses (up to 51%) and a decrease in berry quality, which may be partly linked to ultrastructural modifications, such as collapsed cells in the phloem cell, cell wall

thickening and callose deposition, observed in the cells of infected plants. To understand grapevine responses to elicitors' application – MeJA e SA – plant growth, productivity and berry quality parameters, and the expression of defence related genes were evaluated comparing healthy and FD infected plants treated with MeJA and SA. The application of 25 mM SA at flowering stage showed a partial reduction of the FD symptoms, since at veraison these plants did not show significant differences when compared to healthy plants for those parameters. However, this elicitor did not show significant effects in enhancing the productivity parameters. On the other hand, MeJA application in two subsequent years showed that the timing of application as well as the elicitor concentration are crucial to the possible management of this disease. In fact, 12.5 mM MeJA seemed to reduce the symptomatology in FD-infected grapevines, especially with the improvement of grapevine yield, also demonstrating a tendency to improve plant growth, development, and productivity parameters. On the other hand, 25 mM MeJA triggered an upregulation of *Thau I*, *Thau II*, *Osm* and *PAL* (in 2015) and *Protα5s*, *CHIT4c*, *PIN*, *PGIP* and *GLU* (in 2016) genes. MeJA treatments also significantly increased saponin and proline synthesis in FD-infected plants.

To assess the impact of phytoplasmas on plant metabolism, three clones of micropropagated periwinkles were used: healthy and aster yellows infected shoots, showing mild (strain AY107) and severe (strain Hyd8) symptomatology degrees, respectively. In those trials it was possible to analyse the metabolomic profile of phytoplasma-infected periwinkles and the induction of defence mechanisms against these pathogens after the elicitation with MeJA and BTH. To that end samples were collected 1 and 4 days after elicitation (dae) treated with 12.5 or 25 mM MeJA and 3.5 or 7 mM BTH. The concentrations of 25 mM MeJA and 3.5 mM BTH showed the lowest malondialdehyde production four dae in infected shoots and an increase by 29% in flavonoid content in AY107 infected shoots one dae. This study also revealed that untreated-infected shoots of AY107 strain presented higher content of ABA, which could be an indication of active phytoplasma infection as well as both elicitors reduced the phytoplasma symptomatology decreasing the ABA content. In the Hyd8 infected shoots 7 mM BTH and 12.5 mM MeJA resulted the most efficient at 1 and 4 dae, respectively, at biochemistry and molecular levels.

Finally, a basal metabolomic analysis of the aforementioned periwinkle strain shoots identified 13 alkaloids and 11 phenolic compounds and showed the expression of genes related to alkaloid (AS, STR and PRX) and phenylpropanoid (PAL and CHS) biosynthesis pathways. Upregulation of *pal, chs*, *as* and *str* genes only in plants infected by both "aster yellows" strains

suggested that the phytoplasma infection influences both alkaloid and phenolic biosynthetic pathways at a metabolic and molecular levels.
Overall, the present PhD thesis put lights on the role of the studied elicitors as a novel and promising approach to manage phytoplasma diseases, developing and implementing new green practices towards a more sustainable and/or organic viticulture.

Keywords: elicitors, plant growth and productivity, gene expression, metabolomics, *Vitis vinifera*.

RESUMO

A flavescência dourada (FD) é uma doença de quarentena da videira causada por fitoplasmas e transmitida a plantas saudáveis por insetos vetor, principalmente o *Scaphoideus titanus* Ball. Na Europa, a FD foi reportada pela primeira vez em 1955, em França, dispersando-se pelas regiões vitivinícolas do centro e sul da Europa, com grande impacto na produção vitivinícola. Em Portugal, a FD foi identificada pela primeira vez, em 2006, na região dos "Vinhos Verdes" (RVV). As plantas infetadas desenvolvem geralmente sintomas caraterizados por um atraso no crescimento, mau atempamento e murchidão das varas, enrolamento foliar, amarelamento ou avermelhamento (dependendo da variedade) e murchidão dos cachos. As estratégias convencionais de gestão desta doença baseiam-se principalmente em tratamentos com inseticidas, no arranque das plantas infetadas e na produção de material de propagação saudável, livre de fitoplasmas. No entanto, estas estratégias são dispendiosas e podem ter impactos ambientais indesejáveis. O desenvolvimento de abordagens inovadoras e sustentáveis para o controlo da FD tem sido dificultado devido à falta de conhecimento sobre a biologia dos fitoplasmas bem como as interações planta-patógeno.

O objetivo principal desta tese foi estudar a utilização de elicitadores – metil jasmonato, MeJA, ácido salicílico, SA e benzotiadiazol, BTH – na luta contra as doenças provocadas por fitoplasmas na videira. Os elicitadores têm sido aplicados com sucesso como tratamentos preventivos e amigos do ambiente em várias doenças, mas a informação existente sobre o seu efeito em doenças provocadas por fitoplasmas é ainda limitada. Uma vez que os fitoplasmas são difíceis de cultivar, o *Catharanthus roseus* (vulgarmente conhecido como pervinca) foi utilizado no presente trabalho como um sistema modelo para estudar as interações fitoplasma:hospedeiro, uma vez que esta espécie coexiste com estes microrganismos de diferentes níveis de severidade dos sintomas de acordo com a estirpe de fitoplasma. Os objetivos específicos do presente estudo foram: i) caraterizar a estirpe de fitoplasma da FD presente em *Vitis vinifera* cv. Loureiro e o impacto no crescimento, desenvolvimento, produtividade e ultraestrutura celular da videira; (ii) compreender as respostas fisiológicas e moleculares desencadeadas pela aplicação de MeJA e SA em videiras infetadas com FD em condições de campo; (iii) estudar o perfil genómico e metabólico basal de *C. roseus* saudáveis e infetados com fitoplasmas "aster yellows" e (iv) compreender o papel do MeJA na indução dos mecanismos de defesa contra os fitoplasmas.

Os ensaios de campo revelaram que o fitoplasma presente nas videiras da casta Loureiro pertence ao subgrupo 16SrV-D, apresentando uma variabilidade no padrão de RFLP muito baixa nos genes *tuf* e *secY*. Mais ainda, um estudo de dois anos demonstrou que a infeção por FD atrasa o desenvolvimento da videira, levando a perdas drásticas de produção (até 51%) bem como a uma diminuição da qualidade das uvas, que pode estar parcialmente ligada às modificações ultraestruturais, tais como colapso de células e de vacúolos em células do floema, espessamento da parede celular e deposição de calose, observadas nas células das plantas infetadas. Para compreender as respostas da videira à aplicação de elicitadores – MeJA e SA – foram avaliados parâmetros de crescimento, produtividade das plantas, parâmetros de qualidade das uvas, assim como a expressão de genes relacionados com a defesa, comparando plantas saudáveis e infetadas com FD. A aplicação de 25 mM de SA na fase de floração mostrou uma redução parcial dos sintomas de FD, uma vez que, na fase do pintor, estas plantas não mostraram diferenças significativas quando comparadas com plantas saudáveis. No entanto, este elicitador não mostrou efeitos significativos sobre os parâmetros de produtividade. Por outro lado, a aplicação MeJA em dois anos subsequentes mostrou que o momento da aplicação, bem como a concentração de elicitador, são cruciais para o combate a esta doença. De facto, 12,5 mM de MeJA pareceu aliviar os sintomas da FD, demonstrando uma tendência para melhorar os parâmetros de crescimento, desenvolvimento e produtividade das plantas. Por outro lado, o tratamento com 25 mM de MeJA desencadeou um aumento da expressão dos genes *Thau I*, *Thau II*, *Osm* e *PAL* (em 2015) e *Protα5s*, *CHIT4c*, *PIN*, *PGIP* e *GLU* (em 2016). Os tratamentos com MeJA também aumentaram significativamente a síntese de saponinas e prolina em plantas infetadas com FD.

Por forma a avaliar impacto dos fitoplasmas no metabolismo das plantas, foram utilizadas três populações de *C. roseus* micropropagados: uma população de plantas saudáveis e 2 populações de plantas infetadas com "aster yellows", apresentando níveis de sintomatologia médio (estirpe AY107) e severo (estirpe Hyd8). Nesses trabalhos foi possível analisar pela primeira vez o perfil metabólico de *C. roseus* infetados com fitoplasmas e a indução dos mecanismos de defesa contra estes agentes patogénicos após a elicitação com MeJA e BTH. Para esse fim foram colhidas amostras 1 e 4 dias após a elicitação (dae) em plantas tratadas com 12,5 ou 25 mM de MeJA e com 3,5 ou 7 mM de BTH. As concentrações de 25 mM de MeJA e 3,5 mM de BTH mostraram a menor produção de malondialdeído 4 dae em plantas infetadas assim como um aumento do teor de flavonóides em 29% em plantas AY107, 1 dae. Este estudo revelou também que plantas não tratadas, da estirpe AY107, apresentavam maior concentração de ABA, o que poderá ser uma indicação da infeção ativa por fitoplasmas, assim como ambos os elicitadores

reduziram a sintomatologia provocada pelos fitoplasmas, diminuindo a concentração de ABA. Nas plantas da estirpe Hyd8, 7 mM de BTH e 12,5 mM de MeJA mostraram ser os compostos mais eficazes 1 e 4 dae, respetivamente, a nível bioquímico e molecular.

Por fim, através da análise metabólica basal das estirpes de *C. roseus* supramencionadas foi possível identificar 13 alcalóides e 11 compostos fenólicos, bem como a expressão dos genes relacionados com as vias biossintéticas dos alcalóides (*as*, *str* e *prx*) e dos fenilpropanóides (*pal* e *chs*). O aumento da expressão dos genes *pal*, *chs*, *as* e *str* apenas nas populações de plantas infetadas por "aster yellows" sugere que a infeção afeta tanto as vias biossintéticas dos alcalóides como dos fenilpropanóides, tanto a nível metabólico como molecular.

No geral, a presente tese de doutoramento apresenta os elicitadores como uma abordagem inovadora e promissora no combate a doenças causadas por fitoplasmas, desenvolvendo e implementando novas práticas verdes rumo a uma viticultura mais sustentável e/ou biológica.

Palavras-chave: crescimento e produtividade vegetal, elicitadores, expressão de genes, metabolómica, *Vitis vinifera*.

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LIST OF ABBREVIATIONS

as – *anthranilate synthase* gene

BF – before flowering stage

BTH – benzothiadiazole

CHIT4c – *acidic class IV chitinase* gene

chs – *chalcone synthase* gene

dae – days after elicitation

F – flowering stage

FD – "flavescence dorée"

FD- – healthy grapevine

FD+ – "flavescence dorée" infected grapevine

GLU – *β-1,3-glucanase* gene

ha – hectares

MeJA – methyl jasmonate

MIAs – monoterpenoid indole alkaloids

mM – millimolar

Osm – *Osmotin-like protein* gene

pal – *phenylalanine ammonia liase* gene

PBSP – *plant basic secretory protein* gene

PGIP – *polygalacturonase-inhibiting protein* gene

PIN – *serine protease inhibitor* gene

Protα5s – *proteasome α5 subunit* gene

prx – *peroxidase* gene

Rubisco – *AAA+ rubisco activase* gene

SA – salicylic acid

str – *strictosidine synthase* gene

STS – *stilbene synthase* gene

Thau I – *thaumatin I* gene

Thau II – *thaumatin II* gene

TIAs – terpenoid indole alkaloids

V – veraison stage

VVR – "Vinhos Verdes" region

CHAPTER 1

GENERAL INTRODUCTION

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1.1. Overview

Grapevine is a perennial plant, which belongs to the climbing *Vitaceae* family, to the genus *Vitis*, being the species *Vitis vinifera* (Lineu, 1753) the most traditionally grown for wine production. It is a phanerogamic species (with flower and seed) and angiosperm (seed protected by the fruit), whose bunch is made up of large and juicy berries with a high aptitude for the winemaking process (Magalhães, 2008). The life cycle of the grapevine is a succession of annual cycles, complementary to each other, which can be assessed by Baggiolini's scale. Each annual cycle presents five crucial phases for the development of the plant: germination of the seed, budburst, flowering, veraison, and ripening (Magalhães, 2008). From November to mid-March, the plant is in a latency/dormancy phase, showing no apparent vegetative activity. During this phase, the plant is preparing for budburst, which can only occur after the plant is exposed to a continuous period of cold (average daily temperatures below 10 ºC). When the temperature raises, budburst occurs, and the grapevine vegetative cycle begins by the growing of shoots and the constitution of fundamental organs such as sticks, leaves, flowers, tendrils, and fruits (Magalhães, 2008). The grapevine flowering stage occurs around May/June. By this time, the plant is ready to be pollinated (fecundation) and, in this way, to a berry setting that will develop until ripening (Magalhães, 2008).

Nowadays, the wine industry is an important economic driver since it has a high turnover and added value. Recent data show Portugal as the 11th largest wine producer in the world, with an annual production of 6 million hl. The year 2018 registered a positive balance of wine exports, representing around 804 million hl, where wine sector has grown 58% since 2000. In terms of economic value, 2018 also brooks records for wine exports to both the EU and non-EU countries, with around EUR 458 million and EUR 346 million, respectively, with growth in exports of around 21% to EU countries and about 145% to non-EU countries (IVV.IP, 2019), showing the vitality of this agricultural sector.

Portugal is the world leader in percentage of vineyard area, comparing to the total area of the country. In other words, 2.59% of the national territory is occupied by vineyards. Italy is the only country approaching, with 2.55%, followed by Spain with 2.01% and France even less, 1.45%. In 2018, according to the Portuguese Institute of Vine and Wine (IVV), Portugal had an area of 190,000 ha of vineyards, being the fourth country in the world with the largest area of planted grapevines, divided by the different regions. The “Vinho Verde” Region (VVR) is the third most representative viticulture region in terms of area, with 24 thousand ha which corresponds to 12.5% of the national viticultural area. Beiras Region (22.9%) is the largest one

followed by the Douro & Porto Region (22.8%) (IVV.IP, 2019). VVR is located in the Northwestern area of the country, traditionally called Entre-Douro-e-Minho (CVRVV, 2021a). The region presents a high total annual rainfall regime, averaging 1,500 mm, with irregular distribution throughout the year, but more concentrated in the winter and spring. The average yearly temperature expresses a mild climate, characterised by cold and rainy winters and hot and dry summers, and late spring. The topography of the VVR is quite irregular, being cut by a dense network of associated valleys, the appearance of which is stressed from the coast to the interior. Most of the soil is of granite origin, with little depth, predominantly sandy to franco-sandy textures, naturally high acidity, and phosphorus shortage. VVR native and most representative grapevine varieties, which are well adapted to this region are: "Alvarinho", "Arinto" ("Pedernã"), "Avesso", "Azal", "Loureiro", and "Trajadura" (white varieties), and "Borraçal", "Brancelho", "Espadeiro", "Padeiro de Basto", and "Vinhão" (red varieties) (CVRVV, 2021b). 'Loureiro' is a white grapevine variety, native to the VVR and Galicia, with the greatest expansion in the Minho region, with a total of 5851 ha of cultivated area in VVR, although it is also grown in the Beiras and Peninsula de Setúbal Regions. It is a very productive and fertile variety, with large but not compact bunches, with a yellowish or greenish colour. Its produced wines are characterised by high acidity and floral and fruity aromas, producing good monovarietal wines (Infovini, 2021).

In Portugal, "flavescence dorée" (FD) phytoplasma presence was firstly reported in 2006, in Amares (VVR) (de Sousa *et al.*, 2010). After that, the disease has spreaded to several counties of Minho, Douro and Beiras winegrowing regions and to the interior (northeast) and south, which means that, there are counties identified as outbreaks of FD (DGAV, 2013). When a vineyard is infected with FD, the immediate damage is the loss of production, with dry or unripen bunches, increasing bitterness, and consequent loss of quality. The grapevine loses vigour and in the medium term it dies (Sousa *et al.*, 2014). According to European rules, when infected plants are detected, it is mandatory to uproot them, as well as the grapevines that show similar symptoms to those infected and that are in the same plot or at least 1,000 m around. If a plot registers 20% of infected grapevines, it should all be uprooted. Uprooting operation should be carried out by 31 March of each year (DGAV, 2013). This measure has a high impact on the wine sector, affecting nurserymen, winegrowers, and wine producers. By the end of March 2016 around 226 ha of vineyards had been uprooted due to this disease, just in Portugal (DGAV, 2018).

1.2. Conventional and novel approaches for managing "Flavescence dorée" in grapevine: knowledge gaps and future prospects

Abstract

"Flavescence dorée" (FD) is a grapevine quarantine disease associated with phytoplasmas and transmitted to healthy plants by insect vectors, mainly *Scaphoideus titanus*. Development of efficient methods for its control has been hampered by the lack of knowledge about phytoplasma biologic properties, linked also to difficulties in its *in vitro* cultivation. Conventional management strategies rely mainly on the application of insecticide treatments, rouging of infected plants and production of phytoplasma-free propagation material. However, these strategies are costly and could have undesirable environmental impacts. Novel approaches are being investigated using transcriptomic and proteomic tools that can assist in identifying key regulators expressed by diseased, recovered and healthy plants. These studies allowed the identification of molecular profiles linked to the grapevine cultivar diverse susceptibility, that are of great interest for the development of FD tolerant plants by breeding programs. Other promising FD management strategies include the use of grapevine endophytic microorganisms with known biocontrol properties and endophytes living inside specialized insect cells, which can be potential candidates for FD vector control. Finally, the application of plant defence elicitors might be an interesting tool for FD containment, but more research is needed before they can be implemented. In this review, the methodologies used for detecting and confining FD diffusion are discussed, focusing mainly on conventional tools, current research perspectives and knowledge gaps.

1.2.1. Introduction

Phytoplasmas are phytopathogenic prokaryotes responsible for more than 700 different plant diseases worldwide (Bertaccini *et al.*, 2014); they belong to the Mollicutes class and are phylogenetically related to the Gram-positive bacteria (Weisburg *et al.*, 1989; EFSA, 2014).

The most important grapevine yellows diseases in the main viticultural areas of Europe are FD and Bois noir (BN) (Bertaccini and Duduk, 2013) associated to the presence of 16SrV-C/-D phytoplasmas (Martini *et al.*, 1999) and '*Candidatus* Phytoplasma solani' (Quaglino *et al.*, 2013), respectively. The FD phytoplasmas are the most dangerous, leading to drastic

grapevine yield losses and even to the death of the infected plants (Chuche and Thiéry, 2014). They are quarantine organisms enclosed in the EU2000/29 Council Directive on Harmful Organisms and the EPPO A2 list of pests (Prezelj *et al.*, 2013).

Although symptoms of FD had been observed in France since the beginning of the 20th century, it was only around 1950 that FD was named, after spreading in the vineyards of south-west France (especially in Gascogne and Armagnac) (Caudwell, 1957; 1983). Currently, FD is reported also in the other wine-producing European regions, including Austria, Croatia, Hungary, Portugal and Switzerland (CABI, 2015).

The molecular classification of FD occurred for the first time in Italy, being assigned to group 16SrV (Bertaccini *et al.*, 1995). FD phytoplasma strains are divided into two ribosomal subgroups, 16SrV-C and 16SrV-D, and are experimentally differentiated using polymorphisms in specific genes, such as *rpS3*, *secY,* amongst others (Angelini *et al.*, 2001; Martini *et al.*, 2002; Botti and Bertaccini, 2006; Arnauld *et al.*, 2007). Generally, FD types have different geographic distributions, although they may co-exist in the same geographic areas. For example, Angelini *et al.* (2001), showed that FD70 (from 16 SrV-C group), and FD88 and FD92 (from 16SrV-D group) are present in France. Still, strains of FD 16SrV-D are prevalent in Northern Italy (Martini *et al.*, 1999), France, Spain (Angelini *et al.*, 2001; Torres *et al.*, 2005) and Portugal (Sousa *et al.*, 2007), whereas FD 16SrV-C was detected in Northern Italy (Piedmont and Veneto regions), France (Caudwell, 1957). Martini *et al.*, 1999; Martini *et al.*, 2002), Serbia (Duduk *et al.*, 2004), Macedonia (Filippin *et al.*, 2009) and Slovenia (Foissac and Maixner, 2013).

Both FD strains are vectored by the leafhopper *Scaphoideus titanus* Ball (Schwester *et al.*, 1962; 1969; Mori *et al.*, 2002; EFSA, 2014), which acquires the phytoplasma during its first larval stage while feeding on infected plants (Boudon-Padieu *et al.*, 1989). The sap-feeding process of *S. titanus* was recently studied by electropenetrography technique (Chuche *et al.*, 2017a). Previously characterized as a phloem-feeding insect, this technique showed that *S. titanus* can ingest a mix of both xylem and phloem sap, thereby potentially also being able to spread *Xylella fastidiosa* in vineyards (Chuche *et al.*, 2017 b).

In Europe, *S. titanus* was monitored for the first time in the late 1950s in France (Bonfils and Schvester, 1960). FD dispersal is closely linked to this vector spread (Bertin *et al.*, 2007; Papura *et al.*, 2009), which was favoured by European climatic conditions (Bertin *et al.*, 2007; Chuche and Thiéry, 2014) and then potentially spread to northern Asia (Ge and Wen, 2006; Steffek *et al.*, 2007).

Although *S. titanus* is considered the main FD vector, recent studies showed that the polyphagous *Orientus ishidae* leafhopper (Mehle *et al.*, 2011) can also transmit the disease

(Lessio *et al.*, 2016; Trivellone *et al.*, 2016). Moreover, other leafhoppers like *Dictyophara europaea* (L.) (Filippin *et al.*, 2009) can harbour FD phytoplasma (EFSA, 2014).

The main host plant species for FD are *Vitis vinifera* and *V. riparia*, but the two FD strains differ in their host specificity. While for the FD-D strains no alternative plant host or insect vectors are reported, for FD-C alternative host plants such as *Clematis vitalba*, *Alnus glutinosa* (EFSA Journal, 2014; Malembic-Maher *et al.*, 2009), and *Ailanthus altissima* (Filippin *et al.*, 2011) have been identified (Angelini *et al.*, 2004).

Infected plants usually develop symptoms characterized by leaf rolling, yellowing or reddening (in agreement with berry color), stunted growth, unripe cane wood and shrivelled berries (Figure 1). The shoots of susceptible grapevine varieties also fail to lignify, are thin, rubbery, and hang pendulously (Caudwell, 1957).

Figure 1. Grapevine with FD symptoms: severely affected plants showing down word curling of the leaves that are red in a red cultivar (a and b). In (c) the cane has yellow leaves (white cultivar) and lack lignification.

FD has been subjected to mandatory control measures that over the last 15 years allowed reducing part of its impact in affected grapevine growing areas. However, FD phytoplasmas have the ability to differentiate into new strains in short periods of time, challenging the current disease management strategies (Bertaccini, 2015). The FD management strategies, which are currently based on insecticides against *S. titanus*, uprooting of symptomatic plants and utilization of disease-free propagation material (Marzorati *et al.*, 2006; Margaria *et al.*, 2014; Roggia *et al.*, 2014), in several circumstances, are not sufficient to contain this disease. As *S. titanus* and FD continue to expand to new areas it is of utmost importance to explore more sustainable, innovative and effective approaches to manage their dispersal.

The aim of this paper is to critically review the current knowledge of methodologies for FD detection and potential management of the disease and vector dispersal. To that end, this review will focus on describing the conventional approaches and current research perspectives for FD management. The knowledge gaps and future challenges related to the implementation of such methodologies are also discussed.

1.2.2. Detection and quantification of "flavescence dorée" phytoplasmas

Phytoplasma identification is based on its molecular detection in plant tissue samples or in insect vector specimens, and it can be done using low to high-throughput screening methodologies. In general, although the molecular techniques for FD detection are sensitive, there can be some problems due to the seasonal variation in the phytoplasma titre, since their irregular distribution within the plant and their low concentration in woody hosts, especially in young grafted plant material (Duduk and Mori, 2013). Therefore, visual inspection remains the basis for the routine practice.

Serology techniques, such as double-antibody sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA), have also been successfully applied to phytoplasma detection using polyclonal or monoclonal antibodies and molecular probes (Boudon-Padieu *et al.*, 1989; Daire *et al.*, 1992; Bertaccini *et al.*, 1993). Since the early 1990s, several PCR-based methodologies for phytoplasma detection and identification (Deng and Hiruki, 1991; Lee *et al.*, 1995; Lee *et al.*, 1998), have been optimized, allowing to gain new insights into the diversity and genetic interrelationships among these bacteria (Bertaccini and Duduk, 2009). Several quantitative PCR (q-PCR) protocols have been developed as fast routine detection methods for FD (Angelini *et al.*, 2007), with q-RT-PCR being five orders of magnitude more sensitive than

q-PCR (Margaria *et al.*, 2009; Salar *et al.*, 2013). Also, a triplex RT-PCR for FD phytoplasma detection has been tested with increased sensitivity (Pelletier *et al.*, 2009). Besides these approaches, LAMP based assays have been developed which are able to detect 16SrV group phytoplasma presence in grapevine in only about one hour (Kogovsek *et al.,* 2015).

1.2.3. Approaches to study FD pathogenicity and FD-host interactions

Having accurate genomic information regarding FD phytoplasma strains may be a very powerful tool. It not only gives the possibility to design more specific primers and better molecular probes, allowing strain discrimination and classification (Bertaccini, 2007), but it may also provide valuable information regarding FD pathogenicity mechanisms (Mehle *et al.*, 2014, Roggia *et al.*, 2014). A physical map of the 671 kbp chromosome of FD strain FD92 including the two rRNA operons, *tuf*, *uvrB-degV* and *secY-map* genes was produced (Malembic-Maher *et al.*, 2008). The 671 kbp chromosome of the same strain was later partially obtained by pyrosequencing (Carle *et al.*, 2011). Out of the 464 chromosomal coding sequences, 38% were involved in information transfer, 19% encoded metabolic enzymes, 9% corresponded to transporters, 1% were related to cellular processes, and 31% remained cryptic. It was shown that this FD phytoplasma strain possesses a complete glycolytic pathway and the authors suggested the presence of a prominent system for proteolysis that may have resulted from the adaptation to its woody hosts (Carle *et al.*, 2011). Once the full genome of FD will be sequenced, even more information will be available regarding other genes and molecular pathways which may contribute to the pathogenicity of FD. More recently, RNA-Seq technology was successfully applied for the first time to analyse the global transcriptome profile of an FD strain during grapevine infection (Abbà *et al.*, 2014). This work provided new insights into the transcriptional organization and gene structure of FD, generating about 8300 FD-mapped reads assembled in 347 sequences, corresponding to 215 annotated genes (Abbà *et al.*, 2014). Functional classification revealed that most of the expressed genes were either related to translation and protein biosynthesis or hypothetical proteins with unknown function. Some of the latter were predicted to be secreted, acting as effectors with a potential role in modulating the interaction with the host plant. Interestingly, qRT-PCR validation of the RNA-Seq expression values confirmed that a group II intron represented the FD genomic region with the highest expression during grapevine infection. This mobile element may contribute to the genomic plasticity increasing its fitness towards host-adaptive strategies (Abbà *et al.*, 2014).

Proteomics studies may also be very informative indicating different molecular level of

response to pathogen infection. But despite their potential, so far only a few studies have looked at the defence protein production triggered by FD infection. Margaria and Palmano (2011) monitored the effects of infection on the plant protein expression profile. Among the 576 analyzed spots, 33 proteins were differentially regulated in infected grapevines. In a posterior study, Margaria *et al.* (2013) uncovered novel aspects of grapevine response to phytoplasma infection using a proteomic and phospho-proteomic approach. They identified 48 proteins that differentially changed in abundance, phosphorylation, or both in response to FD infection. Amongst others, fifteen differentially phosphorylated proteins were identified in infected compared to healthy plants, including proteins involved in photosynthesis, response to stress and the antioxidant system. Further work will be necessary to assess whether the differences in proteome profiles are conserved among different grapevine cultivars showing similar levels of susceptibility to the disease, and under different environmental conditions.

Finally, metabolomics studies may also contribute to a better understanding of the molecules involved in plant susceptibility to FD. Recently, FD-grapevine interaction mechanisms were studied in infected grapevines of cv. Modra Frankinja under field conditions, and a non-targeted metabolomics analysis was conducted (Prezelj *et al.*, 2016). The analysis identified 22 significantly changed compounds with increased levels during infection. FD phytoplasma infection was shown to inhibit phloem transport, resulting in accumulation of carbohydrates and secondary metabolites that provoke a source-sink transition and a defence response status.

In order to better understand the role of the identified proteins in the infection pathway, a new system has been employed using spiroplasmas as the model organism. The construction of recombinant spiroplasmas exhibiting FD phytoplasma variable membrane proteins (Vmp), present in the midgut and salivary glands of *S. titanus*, provided a new biological approach for studying interactions of phytoplasma surface proteins with host cells (Renaudin *et al.*, 2015). A recent study showed that a *Spiroplasma citri* mutant G/6 for VmpA expression and used VmpA-coated fluorescent beads interacted with *Euscelidius variegatus* insect cells in culture and promoted the retention of VmpA-coated beads to the midgut of insect. Thus, VmpA acts as an adhesin that could be essential in the colonization of the insect by the FD phytoplasmas (Arricau-Bouvery *et al.*, 2018).

The abovementioned molecular studies are of great value in the comprehension of FD phytoplasma and how it interacts with its grapevine host. The discovery of the differential genomic, transcriptomic or metabolic profiles from different grapevine cultivars showing contrasting susceptibilities to the disease may help in understanding the underlying causes for

susceptibility and can assist in the development of breeding programs for FD tolerant grapevine genotypes. The knowledge obtained by looking at the pathogen, and at which genes, transcripts, proteins or metabolites are being expressed and secreted during its infection, will enhance the development of novel, more effective and specific control methodologies for FD management (Fig. 2; discussed in a section below).

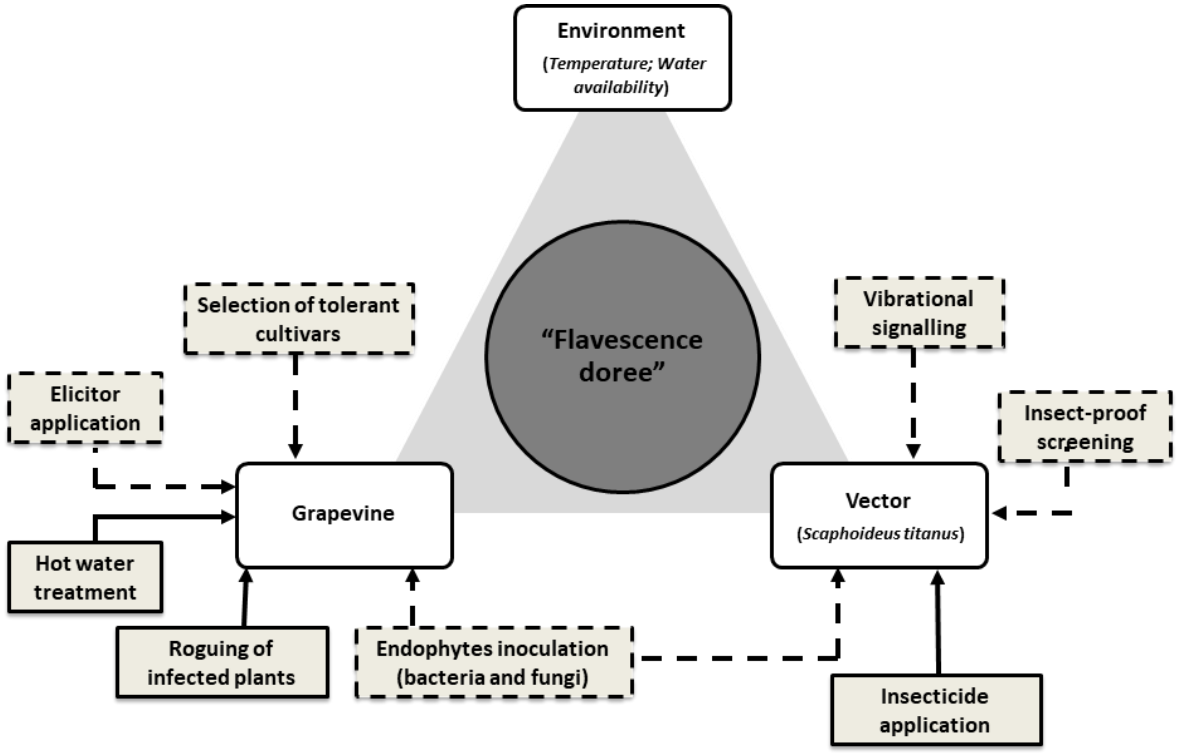

Figure 2. Schematic representation of the main factors influencing "flavescence doree" (FD) disease (white boxes) and the different control methodologies (grey boxes). Solid lines indicate conventional approaches and dashed lines indicate novel potential approaches for FD control.

1.2.4. Conventional approaches for managing FD

The treatment of infected vineyards with insecticides is mandatory in several countries and remains the most important tool for *S. titanus* elimination, and for significantly reducing disease pressure (Posenato *et al.*, 1996; Girolami *et al.*, 2002). Insecticide treatments are essentially directed to the insect mobile instars (nymphs and adults) and are applied to the vineyards up to two or three times a year in epidemically infected areas (Chuche and Thiéry, 2014). Nonetheless, because the effect of insecticides is not immediate, and phytoplasmas can be rapidly transmitted, this strategy has limited efficacy (Weintraub and Wilson, 2010). Moreover, also other important aspects, such as the grapevine production system (e. g. organic production) and the leafhopper density limit the possibility and the efficacy of insecticide treatments (Seljak, 2008; Zezlina *et al.*, 2013). Additionally, the intensive use of insecticides is

costly and has a negative impact on the environment (due to their soil persistence and water contamination); it may also have deleterious effects on human health (Compant *et al.*, 2012). Although there is a high number of diverse active substances used as insecticide against *S. titanus*, their intensive application can favour their persistence in soils and contamination of the environment, as well as the appearance of resistant strains of soil pathogenic microrganisms (Compant *et al.*, 2012). In a 2-year experiment, Zezlina *et al.* (2013) found that thiamethoxam, a substance that remains for a long time in the plants due to its low metabolization rate, produced the best results against *S. titanus* compared with other four neurotoxic substances. Also, chlorpyrifos-methyl (active compound of Reldan 22 EC) significantly reduced the leafhopper nymph numbers and had lower persistence in the environment compared with thiamethoxam (Zezlina *et al.*, 2013). In organic vineyards, the control of *S. titanus* is restricted to natural products, mostly relying on the use of pyrethrins. In a recent study, the efficacy of several natural products (e.g., pyrethrins, kaolin, orange oil, insecticidal soap and spinosad) has been analysed (Tacoli *et al.*, 2017). These authors have concluded that kaolin and pyrethrins are effective products for the management of FD. Nevertheless, their efficacy is lower than synthetic insecticides used in conventional agriculture.

The application of hot water treatment (HWT) to the propagation material (rootstocks, scions or grafted cuttings) is an important measure for preventing FD dissemination. The process consists in soaking dormant grafted cuttings in water at 45-50ºC for 40-45 minutes and is effective at eliminating FD phytoplasmas and *S. titanus* eggs (Caudwell *et al.*, 1997; Borgo *et al.*, 1999; Bertaccini *et al.*, 2001; Tassart-Subirats *et al.*, 2003). However, HWT may impose a severe stress to the treated plants if a homogenous temperature inside the treatment tanks is not achieved (Mannini, 2007; Waite and Morton, 2007).

The removal of infected plants (rouging) is also a regular practice for FD management. This procedure is mandatory in the cases of severe outbreaks of the disease, affecting more than 20% of the plants in the same vineyard. However, when the symptomatic plants have a spotted distribution, it is advisable to inspect and analyse also asymptomatic grapevines, since they may act as FD reservoirs contributing to the disease spread. In this context, abandoned vineyards should also be regularly inspected by the National Agricultural Departments and possibly uprooted or sprayed, because they are relevant FD sources of inoculum. Moreover, after rouging, chemical treatment against the vector must be applied to maintain low the incidence rate of FD (Pavan *et al.*, 2012).

The removal of wood from vineyards after winter pruning and the removal of suckers growing along the vertical trunk, which are abundantly colonized by nymphs that hatch from

the eggs laid into the bark of the trunk, can be used to reduce *S. titanus* populations (Trivellone *et al.*, 2015).

1.2.5. Alternative approaches for managing FD

The conventional strategies for FD management described above are costly, difficult to implement, and have a high environmental impact, therefore alternative management strategies must be developed. Current research perspectives with potential for managing FD spread are described below. It is expected that emerging tools may have the potential for managing FD if some of the knowledge gaps and limitations described below are properly addressed.

1.2.5.1. Use of tolerant cultivars

Empirical evidence has shown that it is possible to identify grapevine cultivars with differential susceptibility to FD (Jarausch *et al.*, 2013). Susceptibility may be linked to morphologic and physiological differences in the plant, which ultimately are determined by the genetic background of each cultivar, and on how they respond to the environment in which they are settled. It may also be linked to their response to specific FD effectors, and how these interact with the host plant (Eveillard *et al.*, 2016). Thus, in order to understand the differences in susceptibility to FD, it is important to look at the molecular aspects of the host as well as of the pathogen at a genomic, transcriptomic, proteomic and metabolomic level, as described above.

Jarausch *et al.* (2013) have defined grapevine´s field resistance to phytoplasmas as the absence of symptoms and of growth alterations combined with a low titre of the pathogen, whereas tolerance reflects the absence or mild symptoms, but with a high titre of the pathogen. The use of tolerant plant material is often referred to as a promising approach for phytoplasma-associated field disease control (Seemüller and Harries, 2010; Jarausch *et al.*, 2013). However, care must be taken since asymptomatic infected grapevines and rootstocks may contribute to disease dispersion (Eveillard *et al.*, 2016). Therefore, although tolerant plants might be an appealing complement to other FD management methodologies, it is important to guarantee that these plants would not represent phytoplasma reservoirs constituting a masked hotspot. Thus, ideally, resistant genotypes should be used, or at least genotypes with reduced susceptibility. In recent experiments, different susceptibilities were found in plants affected by FD. From Table 1 it is possible to observe that globally, most cultivars have been reported as

highly susceptible or intermediately susceptible and only a very limited number were referred as low susceptible cultivars. Nonetheless, many economically relevant cultivars have not yet been studied for tolerance/susceptibility to FD. The variability in FD susceptibility has hardly been addressed because of the difficulties in cultivating and transmitting phytoplasmas under controlled conditions of inoculation. Efforts at their *in vitro* culture and mutagenesis have been scarce since phytoplasmas are phloem limited (Maejima *et al.*, 2014) and an axenic cultivation method has only recently been characterized (Contaldo *et al.*, 2016, 2019). Additional studies need to be conducted before the physiological and molecular mechanisms of susceptibility to FD can be fully understood. Moreover, grapevines are produced in a vast range of environmental conditions, thus the interaction between genotype and the environment needs to be considered when studying such mechanisms. Also, the replacement of traditional European grapevine cultivars with less susceptible ones might not be compatible with the high-quality wines (which are usually obtained from old vineyards) nor with appellations (which traditionally use specific cultivars). To this end, a better option could be to use rootstocks as sources of field resistance, since natural infection and disease symptomatology has only been verified in a few genotypes (Jarausch and Torres, 2014). This source of resistance would have the great advantage that the commercial traits of the cultivar (scion) remain unchanged. However, a recent study has shown that rootstocks may carry high phytoplasma titres, potentially constituting a silent reservoir of infection (Eveillard *et al.*, 2016).

Table 1. Summary of different *Vitis vinifera* subsp. *vinifera* cultivars susceptibility to FD.

Degree of susceptibility	Grapevine cultivar	Source
Highly	Barbera	Belli *et al.*, 2000; Vercesi & Scattini, 2000; Vitali *et al.*, 2013
	Cabernet Sauvignon	Jarausch *et al.*, 2013; Eveillard *et al.*, 2016
	Chardonnay	Belli *et al.*, 2000; Vercesi & Scattini, 2000; Jarausch *et al.*, 2013
	Grenache	Jarausch *et al.*, 2013
	Pinot Blanc	Bressan *et al.*, 2005
	Pinot Gris	Belli *et el.*, 2000
	Plovdina	Kuzmanovic *et al.*, 2011
	Prosecco	Pavan *et al.*, 1997; Steffek *et al.*, 2007
	Sangiovese	Belli *et al.*, 2000
	Ugni Blanc	CABI, 2015

	Sauvignon B	Eveillard *et al.*, 2016
Intermediate	Pinot noir	Vercesi and Scattini, 2000
	Riparia Gloire de Montpellier	Eveillard *et al.*, 2016
	Cabernet Franc N	
	3309 Couderc	
	Grenache N	
	Chardonnay B	
	Sélection Oppenheim 4	
	41 B Millardet et de Grasset	
	110 Richter	
Low	Cortese	Belli *et al.*, 2000
	Erbamat	Belli *et al.*, 2000
	Moscato	Belli *et al.*, 2000
	Merlot	Belli *et al.*, 2000
		Jarausch *et al.*, 2013; Eveillard *et al.*, 2016
	Syrah	Jarausch *et al.*, 2013; Eveillard *et al.* 2016
	Magdeleine Noire des Charentes	Eveillard *et al.* 2016
	Pinot Noir N	
	Merlot N	
	Kober 5 BB	
	Nemadex Alain Bouquet	

1.2.5.2. Use of naturally recovered plants

The spontaneous remission of the disease symptoms, known as recovery, has been reported following the first year of symptom expression in grapevines infected by phytoplasmas (Caudwell, 1990; Morone *et al.*, 2007; Gambino *et al.*, 2013; Vitali *et al.*, 2013), but the mechanisms and the dynamics behind it are largely unknown (Gambino *et al.*, 2013; Vitali *et al.*, 2013). Recently, the seasonal time course of gas exchange rates in healthy, FD-infected and recovered grapevines from cultivars Barbera and Nebbiolo subjected to water stress and a control with no drought were studied (Vitali *et al.*, 2013). This study showed that in FD-infected plants, net photosynthesis and transpiration rate gradually decreased during the growing season and this effect was stronger when water availability was not a limiting factor. During recovery, plants that had been infected two years before (but not the ones infected the year before) regained the gas exchange performances, reaching values comparable to the ones measured

before FD infection. This could explain why the recovered grapevines can show higher yield than the symptomatic ones (Morone *et al.*, 2007). Vitali *et al.* (2013) concluded that metabolic, not stomata, leaf gas exchange limitation in FD-infected and recovered grapevines is the basis of the plant response to FD disease. Moreover, they also suggested that such response is dependent upon water availability, since water stress superimposes on FD infection in terms of stomata and metabolic non-stomata limitations to carbon assimilation. A study conducted by Margaria *et al.* (2014) provided a molecular and biochemical description of the flavonoid pathway in response to FD phytoplasma in infected and recovered plants. This study suggested that accumulation of proanthocyanidins, mainly in healthy and recovered grapevines, could help to minimize further infection by the insect vector (Margaria *et al.*, 2014). Some researchers have studied hydrogen peroxide role in the recovery phenomenon of FD infected plants using qRT-PCR to investigate the H_2O_2 expression in production related genes in 2-year-old-recovered plants (i.e., plants positive for FD phytoplasma in the past but FD-negative and symptomless for the last 2 years) of grapevine cultivars Barbera and Glera (Musetti *et al.*, 2007; Gambino *et al.*, 2013). In these studies, recovered plants produced larger amounts of hydrogen peroxide, compared with healthy and infected plants for the transcriptional activation of *gox* and *glp* genes involved in the H_2O_2 production and a downregulation of the *apx2* gene coding H_2O_2 scavenging was also observed.

1.2.5.3. Endophytes as elicitors of plant defence against FD

Exploiting the use of beneficial microorganisms with potential biocontrol properties is recently attracting the interest of several researchers (Pinto *et al.*, 2014). According to Petrini (1992) the term endophytes refers to all microorganisms that can colonize internal plant tissues without causing visible harm to their host. Several studies have shown that endophytes act as plant protectors against different pathogens and as elicitors of plant defences (Compant *et al.*, 2005). The effects of these organisms as promoters of plant growth due to a reduction of disease impact is well known (Bianco *et al.*, 2013). Their use for plant disease control offers numerous advantages: i) they do not interfere with the environment and human health; ii) they are targeted and efficient in low amounts; iii) they have a slow degradation rate as they can multiply and are less subjected to selection for resistance and, iv) their application can be extended to conventional and integrated pest management production systems (Berg, 2009). However, the success of these microorganisms as biocontrol agents depends on the study of endophyte-host interactions and on the effective production of chemical formulations with these organisms

(Hallmann *et al.*, 1997). Endophytes may be of bacterial or fungal nature, and they may be associated not only to the host plant, but also to the insect vector of FD. Below some of the most well-known endophytes having the potential to be used against FD are reported.

1.2.5.3.1. Rhizospheric and endophytic bacteria

Plant growth-promoting rhizobacteria (PGPR) are present in root surfaces and in the rhizosphere (Kloepper & Schroth, 1978; Kloepper *et al.*, 1999), and when these bacteria enter the roots, they are called endophytic bacteria. These are important for plant defence, being shielded from the competitive soil environment. Endophytic bacteria can also originate from the phyllosphere, anthosphere or spermosphere (Hallmann *et al.*, 1997), and can be found in the vascular system, intercellular space or cell cytoplasm (Bulgari *et al.*, 2009; Romanazzi *et al.*, 2009a) of several plant parts (roots, tubers, stem and leaves) (Hallmann *et al.*, 1997).

Several studies have suggested the involvement of endophytic bacteria in the recovery phenomenon against phytoplasma diseases in grapevine (Bulgari *et al.*, 2009; Romanazzi *et al.*, 2009a; Grisan *et al.*, 2011; Musetti *et al.*, 2011). Bulgari *et al.* (2011a; 2011b) showed that the diversity of the grapevine's endophytic bacterial community is greater in recovered grapevines previously infected with FD as compared to the one observed in diseased and healthy plants.

There is still only a limited number of studies addressing the biocontrol properties of endophytic bacteria against phytoplasmas. Gamalero *et al.* (2017) concluded that 1-aminocyclopropane-1-carboxylate (ACC) deaminase synthetized by *Pseudomonas migulae* can limit the damages by phytoplasmas in periwinkle. Endophytic bacteria have been studied also in BN infected grapevines (Bulgari *et al.*, 2011b), in apple proliferation disease associated with the presence of '*Candidatus* Phytoplasma mali' (Bulgari *et al.*, 2012), and in the chrysanthemum yellows phytoplasma (Gamalero *et al.*, 2010). Some of the mechanisms used to promote plant growth and to protect against plant pathogens are the same for free living PGPR and endophytic bacteria (Kloepper *et al.*, 1991; Höflich *et al.*, 1994). Competition for niches, carbon sources, production of inhibitory allelochemicals and triggering of induced systemic resistance are the main biocontrol mechanisms activated by the presence of these bacteria (Lugtenberg and Kamilova, 2009; Shoresh *et al.*, 2010; Bulgari *et al.*, 2011). The direct effects of these microorganisms on plants are related to the production of indole acetic acid, which stimulates the root development (Patten and Glick, 2002; Romanazzi *et al.*, 2009a), induces changes in the root architecture (Gamalero *et al.*, 2002; 2004; Romanazzi *et al.*, 2009a), improves the nutrient uptake by solubilization of phosphate (Gyaneshwar *et al.*, 2002;

Romanazzi *et al.*, 2009a) and increases the nitrogen fixation (Romanazzi *et al.*, 2009a; Vessey *et al.*, 2005).

The use of endophytic bacteria might be a promising tool for programs aiming at enhancing grapevine resistance against FD, however several studies are needed before these bacteria can be applied in a field scale.

1.2.5.3.2. Endophytic fungi

Endophytic fungi have also been isolated from FD recovered grapevines (Martini *et al.*, 2009; Bianco *et al.*, 2013); they are sources of secondary metabolites and antibiotics (Gimenez *et al.*, 2007) and are symbiotically associated with plants, improving their mineral nutrition (Smith and Read, 1997), protecting against pathogens (Azcon-Aguilar *et al.*, 2002) and improving plant resistance against environmental stresses (Turnau *et al.*, 2002; Bianco *et al.*, 2013). *Epicoccum nigrum* strains, isolated from infected *Catharanthus roseus*, have been reported as biocontrol agents against '*Ca.* P. mali'. Inoculation of *C. roseus*, colonized by *E. nigrum* resulted in significant reduction of disease symptoms and '*Ca.* P. mali' phytoplasma titre (Musetti *et al.*, 2011). In the study of Pinto *et al.* (2014) two other important protectors' agents were identified in *V. vinifera*: *Bulleromyces albus* and *Dioszegia* spp.. These fungi induce plant cell modifications indicative of plant cell defence activation, such as deposition of phloem protein plugs, deposition of callose in sieve tubes and synthesis of phenolic compounds in companion cells and sieve tubes (Musetti *et al.*, 2007). Plant inoculations with combinations of bacteria and fungi can enhance their beneficial effect (Russo *et al.*, 2012), as it has already been shown that rhizobacteria interact with arbuscular mycorrhizal fungi (Romanazzi *et al.*, 2009a). The inoculation of *Pseudomonas putida* S1Pf1Rif alone or in combination with the mycorrhizal fungus *Glomus mosseae* BEG12 effectively reduced chrysanthemum yellows phytoplasma infection in chrysanthemum (Gamalero *et al.*, 2010).

Fungi can thus be considered an interesting tool for FD biocontrol, but extensive research on this topic is still necessary. The study of the relationship between phytoplasmas, fungal endophytes and host plants is essential for the success of this application.

1.2.5.3.3. Endosymbiont microorganisms living inside the insect vector

The endosymbiontic microflora of insects is of extreme importance for their ecological and evolutionary success as they influence host growth, nutrition and fertility (Sachi *et al.*,

2008; Ishikawa, 2003). These microorganisms are present in specialized insect cells, like mycetocytes and bacteriocytes, and can colonize different insect organs or tissues or form aggregates (Sacchi *et al.*, 2008). Thus, the knowledge about the microbiological community that influences the insect vector ecological success is crucial for the search of biocontrol methodologies (Beard *et al.*, 1998; Chuche *et al.*, 2017). The use of microorganisms that are not pathogenic to the plant host but can interfere with the pathogen life cycle could be a good possibility (Baldridge *et al.*, 2004). The genetic manipulation of the insect microflora in order to select the microorganisms which have, for example, the capacity to spread to the insect progeny, is a promising approach since in this case, these microorganisms can block a specific genetic trait (Zabalou *et al.*, 2004). However, the chosen endophyte must: have a stable relationship with the insect vector, prevail within its population, cohabitate with the pathogen, be able to be manipulated in laboratory and be efficiently distributed within the insect community (Alma *et al.*, 2010). Also, it is important to keep in mind that a single symbiont strain can affect many vector species (Chuche *et al.*, 2017). Marzorati *et al.* (2006) identified an endosymbiont (ST1-C) belonging to the genus *Cardinium* in *S. titanus*. These bacteria are known to influence reproduction and behaviour of insect hosts (Zchori-Fein *et al.*, 2001; Kenyon and Junter, 2007). The FD acquired by the insect from infected plants was also found in the insect body by Marzorati *et al.* (2006), suggesting that if ST1-C and FD cohabit inside the insect and suggesting ST1-C as a possible FD control agent. Moreover, species of the acetic acid bacteria *Asaia* genus identified in *S. titanus* (Gonella *et al.*, 2012) may be involved in its nutritional metabolism (Crotti *et al.*, 2010), having the potential to control this insect vector.

1.2.5.4. Use of plant defence elicitors

Elicitors are signalling molecules that stimulate the plant's natural defence mechanisms against biotic and abiotic stresses (Belhadj *et al.*, 2008), without environmental impact and risk of selecting resistant pathogen strains, that is usually obtained with the use of conventional pesticides and antimicrobial compounds for pest and disease control (Ruiz-García and Gómez-Plaza, 2013). Current concerns about human health, food safety, and respect for the environment are stimulating the search for these alternative protection strategies. A variety of molecules can act as elicitors, including hormones, oligo- and polysaccharides, peptides, proteins and lipids (Côté *et al.*, 1998).

Jasmonates are endogenous plant hormones derived from fatty acids (Koda, 1992; Creelman and Mullet, 1997; Repka *et al.*, 2004). After their production, jasmonates, such as

jasmonic acid (JA), induce phytoalexin biosynthesis, which acts in the general plant response to pathogens (Gundlach *et al.*, 1992). The involvement of JA and its more active derivative methyl jasmonate (MeJA) in the signal transduction cascade has stimulated the use of this molecule as an inducer of plant defence mechanisms in a number of pathosystems (Belhadj *et al.*, 2008). The application of this compound has been studied due to its capacity to activate gene expression (e.g. *PAL*, *STS*, *PIN*, *PGIP*) and biosynthesis of secondary metabolites (e.g. phenolic compounds, alkaloids, pathogen-related proteins) in many plant species, including grapevine; it can also trigger significant protection against diverse pathogens such as *Erysiphe necator* and *Plasmopara viticola* (Pérez *et al.*, 1997; Belhadj *et al.*, 2006; Wen *et al.*, 2012; Thiruvengadam *et al.*, 2016). In grapevine, pre-harvest treatments with MeJA revealed that it activated chalcone synthase (CHS), stilbene synthase (STS), UDP-glucose: flavonoid-*O*-glucosyltransferase (UFGT), proteinase inhibitors and chitinase gene expression. Such activations triggered the cellular accumulation of both stilbenes and anthocyanins (Belhadj *et al.*, 2008). Treatment with MeJA has also been applied in leaves and suspension-cultured cells of grapevine *V. vinifera* L. cv. Limberger (Repka *et al.*, 2004). MeJA triggered a cascade of events which induced necrotic lesions, similar to those normally associated with resistance to avirulent pathogens, causing hypersensitive cell death, stimulating medium alkalinization accompanied by massive callose and phenolic compounds deposition (Repka *et al.*, 2004) and expression of *PR* genes (*PR-1*, *PR-2*, and *PR-3*) (Yang *et al.*, 1997; Repka, 2001). After exposure to a biotic or abiotic stress, the transcription of defence genes is activated in plants. Belhadj *et al.* (2008) used qRT-PCR to study the expression of eight genes related with defence mechanisms triggered by MeJA. Application of MeJA also triggered the activation of transcription pathways whose products act in grapevines against pathogens.

Besides MeJA, salicylic acid (SA) also attracted the attention of the researchers as a potential plant elicitor against several grapevine pathogens including: *Botrytis cinerea* (Renault *et al.* 1996), *Sphaceloma ampelinum* (Prakongkha *et al.*, 2013), *Plasmopara viticola* (Thiruvengadam *et al.*, 2016) and '*Candidatus* Phytoplasma solani' (Paolacci *et al.*, 2017). These studies have reported the acquisition of systemic resistance after SA application, which acts as a transcription factor of defence genes against pathogenesis. Moreover, some studies have been conducted in grapevines using other elicitors, such as chitosan (Romanazzi *et al.*, 2002) and benzothiadiazole (Iriti *et al.* 2004) against *B. cinerea*.

Although these findings indicate that elicitors might be good candidates for enhancing grapevine resistance to FD, further investigation is needed for better understanding of the

plants' genetic and physiological responses and to find the best timing, frequency and dosage of each elicitor, adjusted to different cultivars and environmental conditions.

1.2.5.5. Models to predict disease and vector spread

Prediction models that integrate information on climate change and its relation to disease and vector spread are scarce. Rigamonti *et al.* (2011) developed a phenology model to improve understanding of insect–vineyard dynamics and to improve the timing of applications of an insect growth regulator, considering the development of insect eggs, nymph instars and their transition to the adult stage. This model was improved after the discovery of the importance of temperature during oviposition (Rigamonti *et al.*, 2013a,b). Lessio *et al.* (2015) developed an epidemiological model for FD incorporating different parameters of the transmission process (acquisition of the disease, latency and expression of symptoms, recovery rate, removal and replacement of infected plants, insecticide treatments, and the effect of hotbeds). This model showed the risks of establishing new vineyards in locations where strong epidemics of FD are present.

Recently, Maggi *et al* (2016) suggested that the FD epidemic is multivariate, depending on infection incidence, vector population and flight behaviour as well as plant position in the vineyard. The researchers related these parameters with newly and recovered plants and presented a space-time epidemic model, where it was concluded that FD infection could come from outside (primary) or within (inside) of the vineyard and the spread of FD to healthy plants is more effective if they are nearby infected plants, due to the typically short-distance mobility of the vector.

1.2.5.6. Other possible FD management methodologies

Physical control methodologies include insect-exclusion screening for field coverage of mother plant vineyards (Mannini, 2007) and the use of reflective synthetic mulches covering the soil surrounding potential host plants; these have been used to repel insect vectors (Weintraub & Wilson, 2010), thus avoiding phytoplasma infections. However, the high cost associated with these techniques makes them impractical for a wide-scale application (Setiawen & Ragsdale, 1987). Moreover, there is the risk that use of screening in the vineyards where *S. titanus* eggs have potentially been laid could work counterproductively, restricting the insect habitat to the areas close to the crop.

Disruption of insect vector mating relies on breaking the mate recognition and localization process, which consists of substrate-borne vibrational signals, through emission of recorded calling songs (Mazzoni *et al.*, 2009; Eriksson *et al.*, 2012; Polajnar *et al.*, 2014). Possible limitations with implementation of this technique in the field include energy costs, the attenuation of vibrations with distance from the source of vibration, and the need for adjusting the timings and frequencies of the vibrational signal emissions according to the insect's natural activity pattern. Nevertheless, Polajnar *et al.* (2016) conducted a small-scale field trial and concluded that the approach appears robust enough to merit a scaled-up testing.

It has been suggested that the risk of exposure to pests and diseases in viticulture is a function of climate (Bois *et al.*, 2017) and that ecological control methods (e.g., pheromones, mating disruption) may possibly be affected by thermopluviometric, wind and hygrometry changes (Reineke & Thiéry, 2016). For example, Chuche *et al.* (2015) showed that *S. titanus* insect hatchings were synchronized with grapevine bud break, which varies in cold and mild winter vineyards. This illustrates that putative changes in the number of yearly insect generations are complex and difficult to predict for future global warming scenarios (Reineke & Thiéry, 2016). In a study looking at the impact of temperature increase on the establishment of *S. titanus*, Quiroga *et al.* (2017) concluded that a 3°C increase of average daily temperature increases the probability of insect establishment, due to a shorter diapause period following warmer winters (Chuche & Thiéry, 2009; Reineke & Thiéry, 2016). Also, climate change might increase population densities of alternative insect vectors of grapevine phytoplasmas (Reineke & Thiéry, 2016). Hence, research on the interactions between vector life cycle and climate fluctuations is needed before any strategy of disruptive mating can be applied.

Recently, there have been several developments in imaging technologies (multispectral, hyperspectral or thermal imaging) to support vineyard management; these may be used to enhance water use efficiency (e.g., Gutiérrez *et al.*, 2018; Poblete *et al.*, 2018), access optimal harvest time (Piazzolla *et al.*, 2013; Chen *et al.*, 2015) and improve disease detection (Bock *et al.*, 2008; Oerke *et al.*, 2016; Al-Saddik *et al.*, 2017; Buja *et al.*, 2021). Although the results obtained so far seem to be promising in terms of disease detection, identification and monitoring in precision agriculture (Al-Saddik *et al.*, 2017) more studies are needed before these noninvasive technologies can be satisfactory applied.

1.2.6. Applied perspectives to growers

Some of the aforementioned research perspectives are closer than others in terms of their practical implementation (e.g., vibrational signalling and imaging technologies compared to elicitor application). Until those research perspectives become real alternative methods for managing FD, and to prevent disease spreading, growers should continue applying the conventional approaches described above. These include hot water treatment of propagating material and applying the mandatory treatments against *S. titanus*. Prophylactic measures include the destruction of infected grapevine plants, pruning wood and uproot or severely prune abandoned vineyards. If there is a suspicion of infection, growers should perform a close visual monitoring of the disease symptoms (such as leaf rolling and changes in leaf colour, stunted growth of the putative infected plants and, at a later stage, the presence of shrivelled berries) and in case these symptoms are present samples should be sent to specialized laboratories for molecular analyses to confirm the presence of FD phytoplasmas.

1.2.7. Conclusions

The main advantages and drawbacks of methodologies for FD management are summarized in Table 2. The search for effective FD management measures has been hampered by several constrains including difficulties in cultivating phytoplasmas or in transmitting FD from plant to plant in controlled laboratory conditions. The recent findings on host-phytoplasma relationships and the evidence that phytoplasmas can be grown in a defined medium, open new gates for the development of powerful tools for FD management. Because FD has been spreading to new wine-growing regions, even with application of mandatory treatments against *S. titanus*, urgent measures for its effective containment are crucial. Nowadays, the best tool for FD management is still the mandatory application of insecticides to reduce *S. titanus* populations. Although these insecticides can be effective, they are clearly not sufficient as this epidemic is still expanding. Moreover, the increasing costs and the social pressure that are hazardous for health and the environment have led to a search of new ways to contain FD. The evaluation of the effect of FD infection at transcriptional and proteomic levels and the identification of FD defence-related genes will help to understand differential susceptibilities of grapevine cultivars. Identification of molecular traits associated with the susceptibility to FD would allow developing markers to be used in breeding assisted selection programmes.

Table 2. Summary of the main advantages and drawbacks of "flavescence dorée" control methodologies.

Method	Advantages	Drawbacks
Insecticide application	• Significant reduction of disease pressure	• Forbidden in organic grapevine production (efficacy depends on leafhopper density) • Cost (needs regular application) • Environmental contamination • Soil persistence, appearance of resistant strains • Possible deleterious effects on human health
Hot water treatment	• Effective elimination of phytoplasma and vector eggs	• Risk of reducing plant viability and productivity when treatment is not properly applied
Tolerant cultivars	• Harmless to the environment and human health	• Most cultivars have not yet been screened for susceptibility/resistance traits • Grapevine and wine production chain value is rather closed to the introduction of new cultivars • Interaction with new environment may influence susceptibility
Endophytes (bacteria and fungi)	• Harmless to the environment and human health • Efficient in low amounts • Slow degradation rate • Less susceptible to induce resistances • Can self-multiply • Can be applied in integrated pest management production systems	• More research is needed to: – Understand endophyte-host interaction; – Develop chemical formulations • Legislation hurdles
Elicitors application	• Harmless • Efficient induction of natural plant defences	• Reported antagonistic effects between jasmonates, salicylate and abscissic acid • Optimization of frequency and dosage application
Prediction models	• Harmless • More precision in FD control	• It takes a lot of time to develop a model
Vibrational signalling	• Harmless • Significant reduction of *S. titanus* populations • Affect the behaviour of other pests	• Does not eliminate vectors or FD • Proximity between males and females may still allow short-range chemical or visual cues which enable partner recognition • May have negative effects on beneficial fauna

Another promising alternative for FD containment is biocontrol using endophytic microorganisms, which have been shown to reduce disease pressure by directly or indirectly affecting pathogen or insect activity. Although the potential of endophytic microorganisms in disease protection is recognized, the plant-pathogen-endophytic microorganism interactions are not fully explored or understood. Furthermore, there is a growing evidence that exogenous application of elicitors (e.g., MeJA and SA) can trigger the defence mechanisms of several plant species, enhancing the level of defensive compounds. Thus, the application of these substances

in diseased plants might also become a good alternative for FD management, but more research is needed to explore the plant's physiological responses and to optimise elicitor application. These options are sustainable approaches to avoid the use of insecticides and roguing for FD containment and should be further evaluated at the experimental level.

1.3. Scope and outline of the thesis

So far there are no studies of the impact of the FD on Portuguese vineyards as well as effective treatments in the containment and eradication of this disease. Moreover, the information about the impact of phytoplasmas on plant metabolism is very scarce. On the other hand, although elicitor application has become a promising approach to manage plant diseases, their role on plant biochemical and molecular responses remains unravel. Building up this information, the overall aims of this thesis were therefore to study the impact of FD on Portuguese grapevine cv. Loureiro and the role of elicitors (methyl jasmonate, salicylic acid and benzothiadiazole) on plant defence (genomic and metabolomic) responses against phytoplasma presence. The specific goals of this thesis were to:

i) conduct the molecular characterization of the FD phytoplasma infecting cv. Loureiro (one of the most relevant cultivar from "Vinhos Verdes" region);
ii) evaluate the impact of FD on plant growth development, yield and fruit quality;
iii) understand the physiological and molecular responses triggered by the MeJA and SA application in FD-infected grapevines under field conditions;
iv) report the basal metabolomic profile of periwinkle shoots infected with two "aster yellows" phytoplasma strains associated with different symptomatology;
v) evaluate the role of MeJA and BTH on biochemistry and hormone profile in periwinkle shoots infected with two aster yellows phytoplasma strains associated with different symptomatology.

This thesis is organized into five Chapters, including a total of 6 Sub-chapters formatted as scientific articles. **Chapter 1** is composed of a General introduction followed by a detailed literature review covering the topics of the current knowledge of methodologies for FD detection and potential management of the disease and vector dispersal. To that end, this review focused on describing the conventional approaches and current research perspectives for FD management (**Chap 1.2**). In **Chapter 2** the impact of FD on growth, productivity and ultrastructure of grapevines cv. Loureiro was evaluated. For that, characterized the FD phytoplasma infecting grapevine cv. Loureiro was molecularly characterized, the effects of FD on the cellular ultrastructure of leaf midribs (using transmission electron microscopy - TEM) was assessed, and 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) was evaluated.

Chapter 3 investigates the role of elicitors to manage FD impacts on grapevine cv. Loureiro by studying anatomical, physiological, biochemical and molecular parameters, (i) evaluating the effect of different timings and concentrations of MeJA on the plant growth parameters, productivity indicators and berry quality (**Chap 3.1**); (ii) contributing to a better understanding of the molecular responses triggered by the MeJA application in FD-infected grapevines (**Chap 3.2**), and (iii) elucidating that SA application alleviate FD impact on development and productivity parameters (percentage of budburst, fertility index, time to veraison, and chlorophyll content), and grape quality parameters (degree Brix and titrable acidity) (**Chap 3.3**). **Chapter 4** unravels the impact of "aster yellows" phytoplasmas on genomics, metabolomics, and biochemistry of three cloned shoot strains of *in vitro* micropropagated periwinkles: healthy and "aster yellows" infected shoots, showing mild (strain AY107) and severe (strain Hyd8) symptomatology degrees. To this end, the role of MeJA and BTH on lipid peroxidation, and flavonoids were measured, and the phytohormone regulation via plant elicitation was evaluated (**Chap 4.1**). The basal metabolomic profile and gene expression were assessed and the effect of two MeJA concentrations on alkaloid and phenolic content and gene-related expression was evaluated (**Chap 4.2**). The main conclusions and future work of this study are presented in **Chapter 5**, which is followed by the general list of bibliographical references used throughout this thesis.

CHAPTER 2

"FLAVESCENCE DORÉE" IMPACTS GROWTH, PRODUCTIVITY AND ULTRASTRUCTURE OF *VITIS VINIFERA* PLANTS IN THE 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 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 FD presence was associated with an average delay of 10 to 15 days on the time to visible inflorescences and to veraison, compared to healthy plants. At veraison, FD also led 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% SPAD values). Hence, infected plants showed a drastic reduction in the yield, corresponding to a decrease between 51% and 92% 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.

2.1. Introduction

Phytoplasmas are prokaryotic organisms without cell wall that evolved from Gram-positive bacteria (Woese, 1987; Weisburg *et al.*, 1989). They are phloem obligate pathogens

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.*, 2013; Santi *et al.*, 2013; Ahmed *et al.*, 2016). Other studies reported physiological alterations include stomata closure, photosynthetic rate reduction, and carbohydrate accumulation in leaves (Endeshaw *et al.*, 2012; Santi *et al.*, 2013; Vitali *et al.*, 2013). These alterations may compromise the translocation of photoassimilates, reduce plant size and vigour, and consequently impair grapevine 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.*, 2019a).

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 available 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).

2.2. Material and Methods

2.2.1. 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; 41º31'00"N 8º12'56"W). Thirty-two grapevine plants 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%. Grapevine 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 identified 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).

2.2.2. Identification and characterization of FD phytoplasmas

Symptomatic leaves were collected in July 2015 from 16 FD symptomatic plants and their midribs were powdered using liquid nitrogen. A similar processing was carried out in samples collected from 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 (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 carried out 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. The PCR analyses on the *tuf*

gene were carried out using the primers and the conditions 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).

2.2.3. 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 after 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) OsO4 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 60ºC. 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 (Jeol, Japan) electron microscope at 80 kV and photographed with an Orius Sc1000 Digital Camera.

2.2.4. 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 (13th 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 the budburst percentage and fertility index (Alonso *et al.*, 2007), according to the formulas:

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

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

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.

2.2.5. 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. The 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 4,000 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).

2.2.6. 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% (0.05).

2.3. Results and Discussion

2.3.1. 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 showed the presence of a pattern referable to the ones of 16SrV-D phytoplasmas in all the samples (Fig. 3). 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. 3 and 4). 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 variability 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 fields 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 about 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.

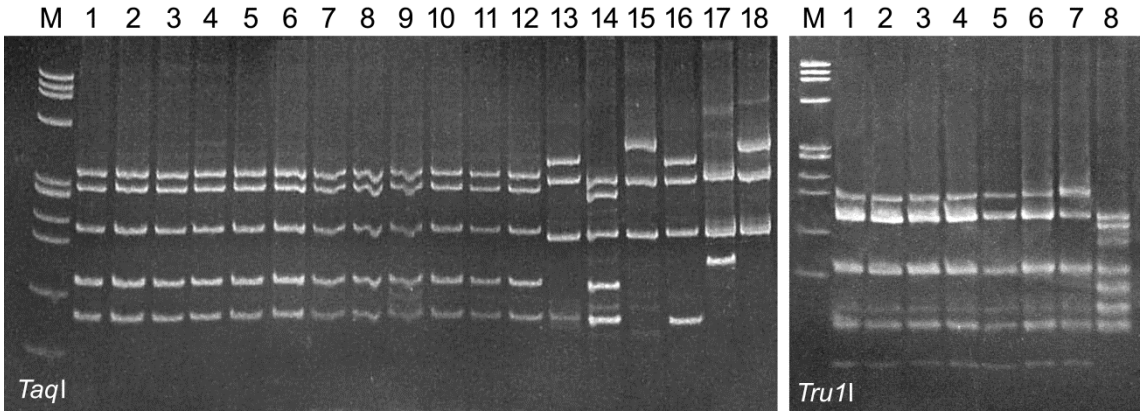

Figure 3. On the left: RFLP patterns on polyacrylamide gel of some of the M1/B6 amplicons from: 1-12, symptomatic grapevine cv. 'Loureiro'; 13, "flavescence dorée" (FD) strain 16SrV-C; 14, FD strain 16SrV-D; 15, elm witches' broom, ULW, (16SrV-A); 16, rubus stunt, RuS, (16SrV-E); 17, "aster yellows", 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.

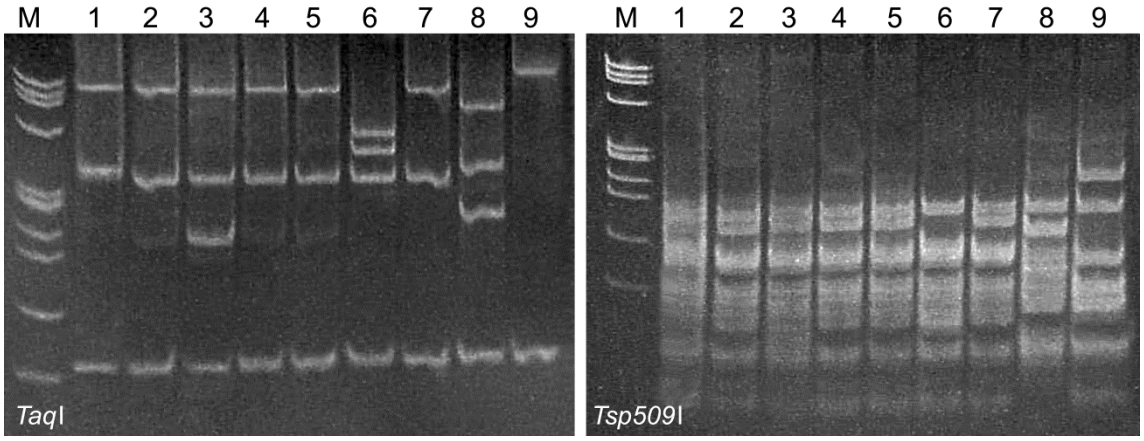

Figure 4. RFLP patterns on polyacrylamide gel of some of the Fd9f3/r2 amplicons from 1-5, symptomatic grapevine cv. 'Loureiro'; 6, FD strain 16SrV-C; 7, FD strain 16SrV-D; 8, jujube witches' broom, JWB, (16SrV-B); 9, elm witches' broom, ULW, (16SrV-A); M, marker as in Fig. 1A.

2.3.2. Structural and ultrastructural analysis of infected grapevine leaf midribs

Optical microscopy images revealed structural modifications in the phloem tissues of symptomatic grapevine leaves (Fig. 5B), when compared with those of healthy plants (Fig. 5A). A more detailed image analysis at the phloem level of the FD infected plants using TEM (Fig. 5C to 5H) allowed to observe the presence of collapsed cells and vacuoles in the phloem cells

(Fig. 5C and 5G), cell wall thickening (Fig. 5C and 5E) and callose accumulation in sieve plates (Fig. 5C, 5D and 5E). This type of ultrastructural modifications has been described in other plant species infected by phytoplasmas including *Vicia faba* and *Solanum lycopersicum* (Musetti *et al.*, 2013; Santi *et al.*, 2013; Ahmed *et al.*, 2016), as well as in grapevine infected with other pathogens, such as the Pinot Gris virus (Tarquini *et al.*, 2018) and the downy mildew agent (Farouk *et al.*, 2017). However, this is the first study that analysed the leaf ultrastructure in FD infected grapevines.

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. 5F). 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.*, 2019a). Since 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 pathogens 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 of 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, it was suggested that the identification of unusual lipids by high-throughput techniques, such as LC-MS-based methods, in grapevine could be used as a biomarker for pathogens infection (Negrel *et al.*, 2018). More studies are, however, needed to validate these results on FD infected grapevine and discriminate the type of lipids synthesized during the plant infection.

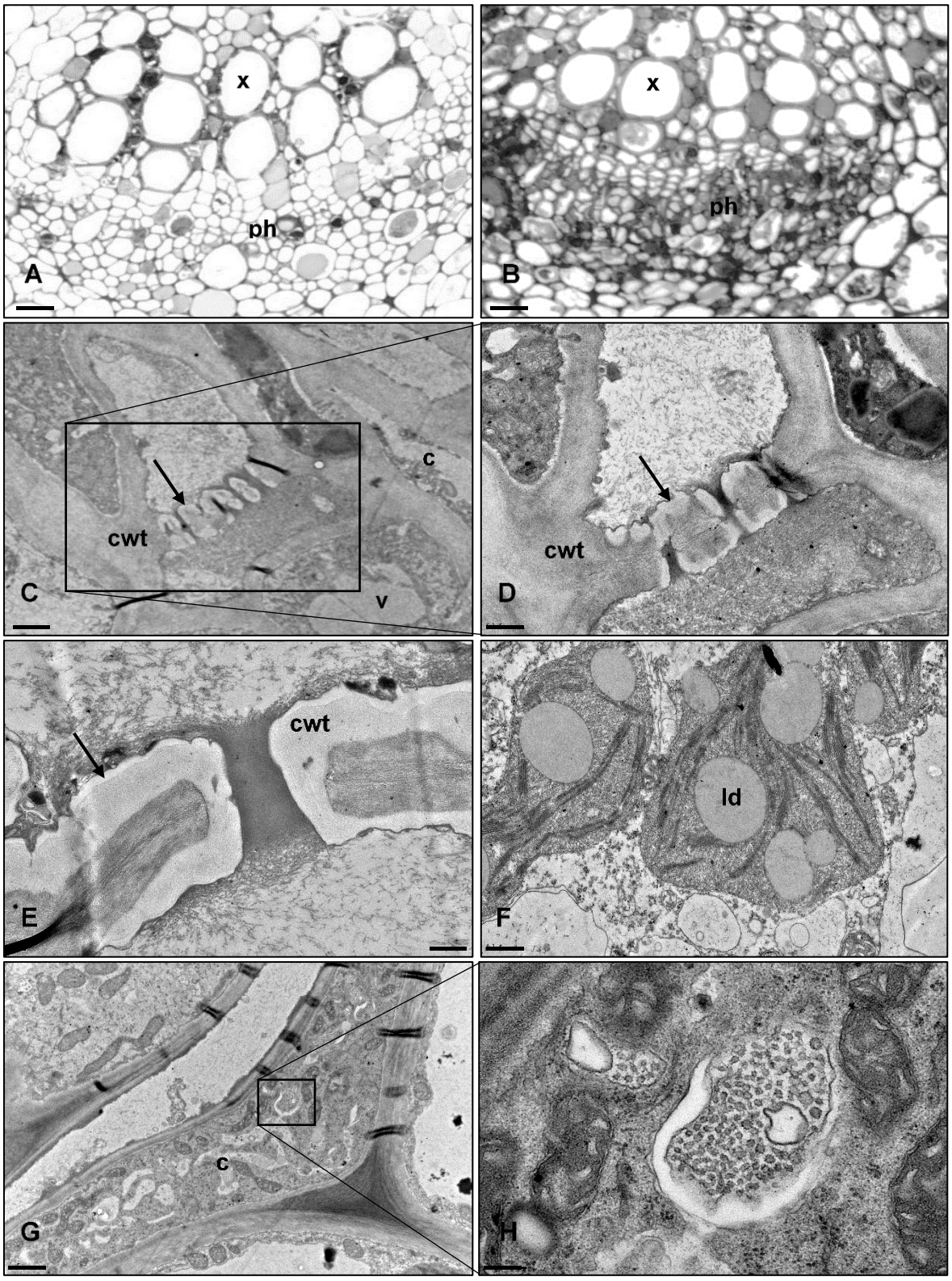

Figure 5. Images of sections of leaf midribs from healthy (A) and "flavescence dorée" infected (B to H) grapevines cv. Loureiro obtained by optical microscopy (A and B) and by transmission electron microscopy (C to H). A – xylem (x) and phloem (ph) tissues with cells presenting normal shape and structure; 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.

2.3.3. Impact of FD on grapevine growth and productivity

2.3.3.1. Biometric analyses

FD infected plants presented a significantly slower development than healthy plants (Fig. 6). In 2015, the visible inflorescences (stage F) was reached 15 days later in FD positive plants as compared to the healthy ones (Fig. 6A). 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 the FD negative grapevine plants (Fig. 6A). In 2016, this significant negative effect of FD on grapevine development was also observed, but it was less pronounced.

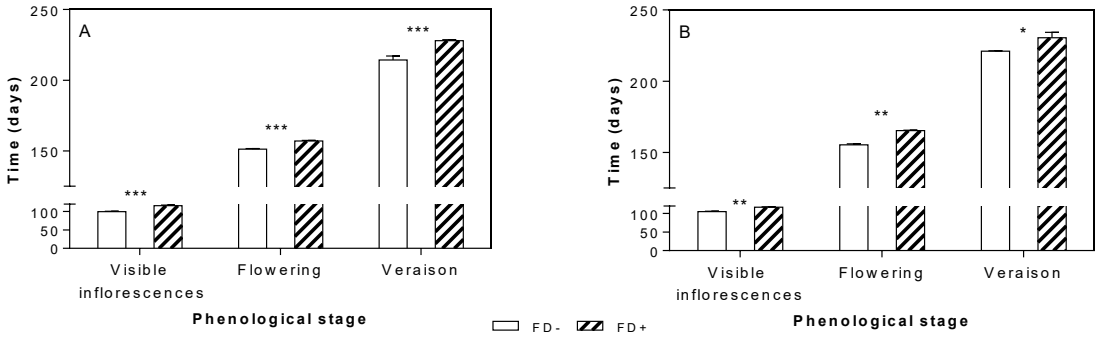

Figure 6. Evolution of the phenological stages (stage F: visible inflorescences; stage I: flowering; stage M: veraison, according to Baggiolini scale) registered in 2015 (A) and 2016 (B) in healthy (FD-) and "flavescence dorée" infected (FD+) grapevine cv. Loureiro. Time to reach the three studied phenological stages is expressed as day of the year (day1=1 January). Data are means ± SEM of 4 biological replicates. Mean differences between FD- and FD+ plants according to the two-way ANOVA have been marked as *** ($P < 0.001$), ** ($P < 0.01$), * ($P < 0.05$).

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, respectively, in the same years (Fig. 7).

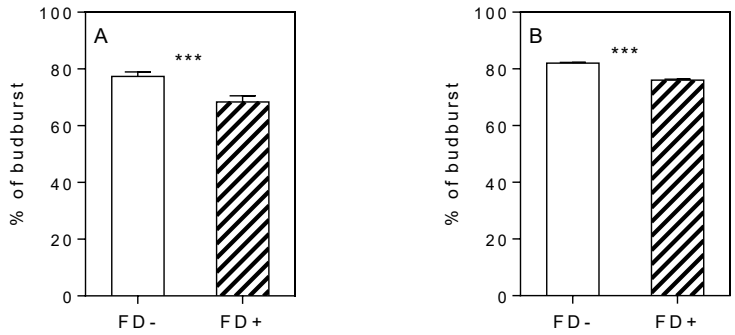

Figure 7. Budburst percentage registered in 2015 (A) and 2016 (B) in healthy (FD-) and "flavescence dorée" infected (FD+) grapevine cv. Loureiro. Data are means ± SEM of 4 biological replicates. Mean differences between FD- and FD+ plants according to the t-test have been marked as *** ($P < 0.001$).

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. 8A). The year after, a similar trend was found, also with a more pronounced effect at veraison, but no significant differences were observed between healthy and diseased plants (Fig. 8B). These results are in accordance with 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.

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 years (Fig. 8C and 8D). 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, the leaf area decrease reached 54% and 63% at flowering and veraison, respectively (Fig. 8D).

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 had a lower budburst percentage (Fig. 7). Another study showed that mildew infected grapevine cv. Merlot can suffer up to 73% of leaf area loss per plant (Jermini *et al.*, 2010), that is affecting plant photosynthetic rate (Bertamini and Nedunchezhian, 2001; Bertamini *et al.*, 2002).

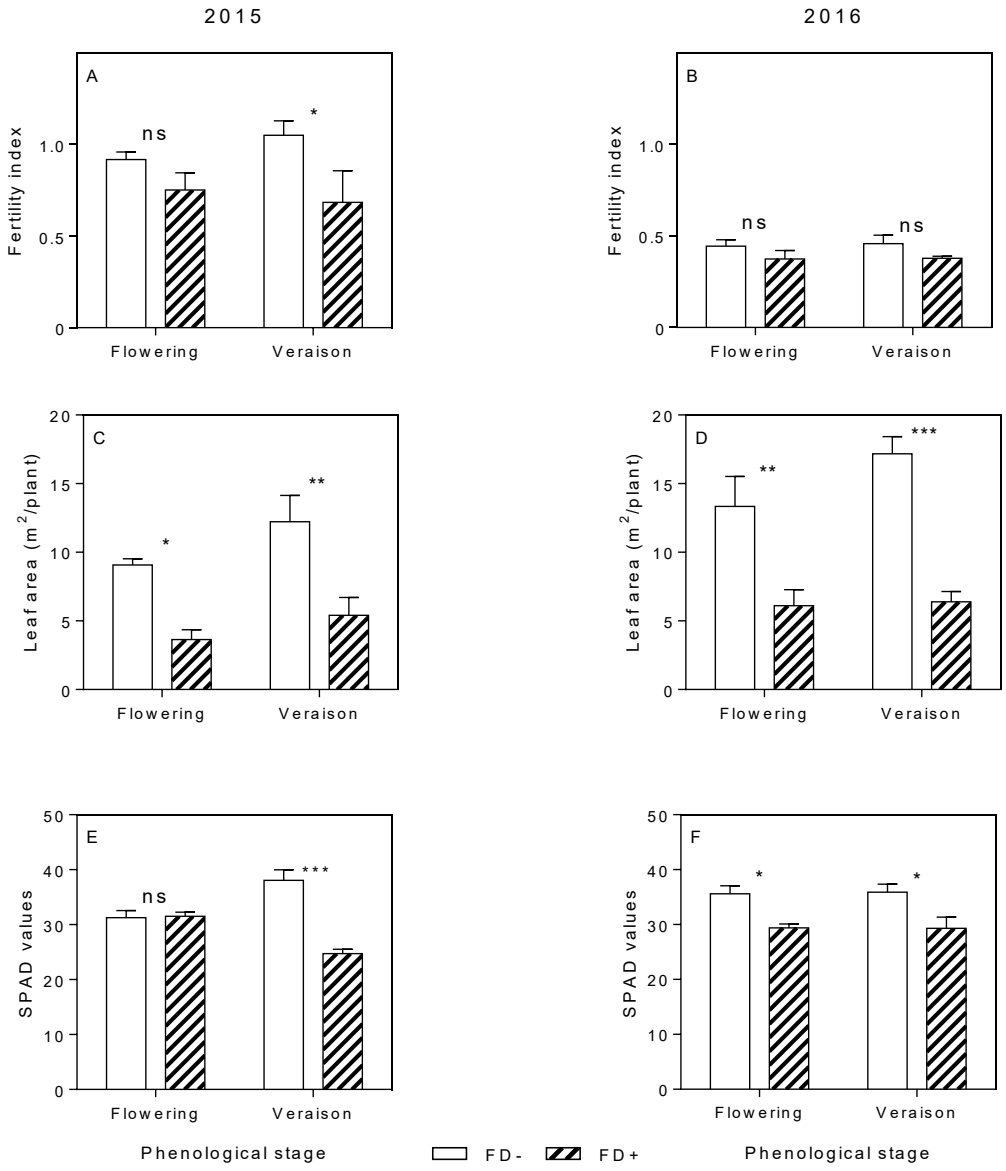

Figure 8. Fertility index (A and B), leaf area (C and D) and SPAD values (E and F) at flowering and veraison stages measured in 2015 and 2016 in healthy (FD-) and "flavescence dorée" infected (FD+) grapevine cv. Loureiro. Data are means ± SEM of 4 biological replicates. Mean differences between FD- and 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 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. 8E and 8F). 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 the 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. 5C to 5E), leading to a negative feedback control on the photosynthetic rate (Stitt, 1991, Goldschmidt and Huber 1992; Vitali *et al.*, 2013), which could have contributed to the leaf yellowing.

2.3.3.2. 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 (Caudwell, 1990). These findings can explain the negative impact of this disease on the fertility index (Fig. 8A) and, consequently, on the productivity (Fig. 9A). 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. 9A). 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. 9B). Moreover, in 2015 the average bunch weight was also significantly lower in FD positive plants, with a 35% decrease compared to healthy plants (Fig. 9C). 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.

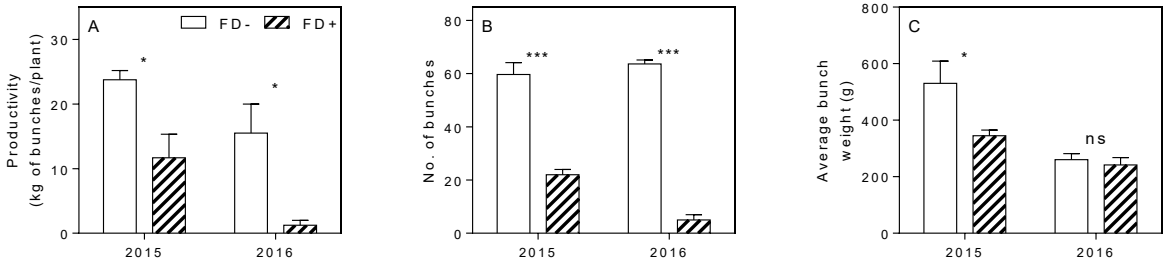

Figure 9. Productivity (A), number of bunches per plant (B) and average bunch weight (C) measured in 2015 and 2016 in healthy (FD-) and "flavescence dorée" infected (FD+) grapevine cv. Loureiro measured at harvest stage. Data are means ± SEM of 4 biological replicates. Mean differences between FD+ and FD- plants according to the two-way ANOVA have been denoted as *** ($P < 0.001$), * ($P < 0.05$), ns (non-significant difference).

2.3.3.3. 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 seems not to be consistent. A study conducted in the cv. Chardonnay demonstrated that BN presence 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 to 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. 10).

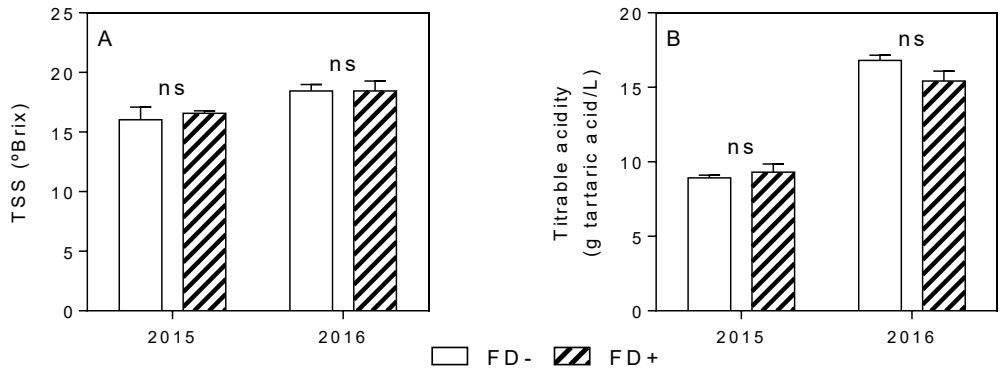

Figure 10. Fruit total soluble solids (TSS; A) and titrable acidity (B) measured in 2015 and 2016 in healthy (FD-) and "flavescence dorée" infected (FD+) grapevine cv. Loureiro measured at harvest stage. Data are means ± SEM of 4 biological replicates. Mean differences between FD+ and FD- plants according to the two-way ANOVA have been denoted as ns (non-significant difference).

2.4. Conclusions

In the current study only the FD-D strain was detected in the analysed symptomatic cv. Loureiro grapevines from "Quinta do Corvo" vineyard of the "Vinhos Verdes" region, in Portugal. Infected plants showed a significant growth delay in both years of the study. 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 in 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 presence in grapevines that lead to the described severe impacts of the disease.

Chapter 3

THE ROLE OF ELICITORS TO MANAGE FD IMPACTS ON GRAPEVINE ANATOMICAL, PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR PARAMETERS

The data presented in this chapter were published or submitted to publication:

Oliveira, M.J.R.A., Vasconcelos, M.W., Castro, S., Pinto, V., Bertaccini, A., Carvalho, S.M.P. 2021. Does methyl jasmonate application in *Vitis vinifera* cv. Loureiro improve plant resilience to "flavescence dorée" under field conditions? (submitted).

Oliveira, M.J.R.A., Vasconcelos, M.W., Lemos, I.C., Bertaccini, A., Carvalho, S.M.P. 2019b. Methyl jasmonate triggers metabolic responses and gene expression on *Vitis vinifera* cv. 'Loureiro' infected with "flavescence dorée". *Phytopathogenic Mollicutes* 9, pp 165-166. doi: 10.5958/2249-4677.2019.00083.5.

Oliveira, M.J.R.A., Vasconcelos, M.W., Castro, S., Bertaccini, A., Carvalho, S.M.P. 2019c. Does salicylic acid alleviate the impacts on growth, development and productivity of "flavescence dorée" in Portuguese "Vinhos Verdes" grapevines? *Phytopathogenic Mollicutes* 9, pp 167-168. doi: 10.5958/2249-4677.2019.00084.7.

3.1. Does methyl jasmonate application in *Vitis vinifera* cv. Loureiro improve plant resilience to "flavescence dorée" under field conditions?

Abstract

"Flavescence dorée" (FD) is a phytoplasma quarantine disease that affects grapevines, leading to high productivity losses or even the death of the infected plants. Currently, FD is a serious threat to the global wine industry due to its severity and its control is based on preventive methods to avoid disease dissemination. Methyl jasmonate (MeJA) has been suggested as a sustainable strategy to improve grapevine resilience to several diseases, but there is scarce information about the potential role of this elicitor in FD infected plants. This study aimed at contributing to a better understanding of the physiological and molecular responses triggered by the MeJA application in FD-infected grapevines under field conditions. Plant growth, productivity and berry quality parameters, and the expression of defense related genes were evaluated comparing healthy and FD infected plants treated with 12.5 and 25 mM MeJA in two subsequent years at different concentrations and timings of application. Application of 12.5 mM MeJA partly alleviated FD symptoms, with the tendency to improve plant growth and productivity parameters. Nonetheless, when 25 mM MeJA was applied to infected plants, it often revealed a negative impact on plant performance and productivity, regardless timing of application, but more pronounced when applied at flowering stage. High MeJA concentration also triggered an upregulation of genes related to primary metabolism (*Prot⍺5s*), pathogenesis-related proteins (*Thau I*, *Thau II, Osm*, *CHIT4c*, *PIN*, *PGIP* and *GLU*) and phenylpropanoids biosynthesis (*PAL*). Interestingly, MeJA application in healthy plants had a beneficial effect on the leaf area (with increases ranging between 50% and 1.1-fold), depending on MeJA concentration and time of application. Similarly, MeJA application in healthy grapevines increased plant productivity by 49% when applied twice at the lowest concentration. In general, this study concluded that the timing of application as well as elicitor concentration are crucial to manage this disease.

Keywords: berry quality, fertility index, gene expression, phytoplasma, SPAD values.

3.1.1. Introduction

"Flavescence dorée" (FD) is presently one of the most serious and economically worrisome diseases of the vineyards, because it causes drastic yield losses leading often to the death of the infected plants. It is a quarantine disease associated with the presence of phytoplasmas of groups 16SrV-C/-D (Martini *et al.*, 1999) that are in the A2 List of the European and Mediterranean Plant Protection Organization (EPPO) and Community Directive No. 200/29 / EC (Prezelj *et al.*, 2013). In Portugal so far only the FD-D subgroup was identified (Sousa *et al.*, 2010; Oliveira *et al.*, 2020). Phytoplasmas are phloem-limited bacteria and the phloem sap feeding leafhopper *Scaphoideus titanus* Ball is the most well-known vector of FD phytoplasmas (Chuche and Thiéry, 2014).

FD infected plants develop leaf rolling, yellowing or reddening (depending of berry colour), stunted growth, unripened cane wood and shriveled berries. In the shoots, one of the first FD symptoms, which appear in early summer, is a delayed or lack of apical and lateral budburst (Caudwell *et al.*, 1957; Sousa *et al.,* 2010; CABI, 2015). The shoots of susceptible grapevine varieties are thin and may exhibit small black pustules, reduced lignification and hang pendulously (Roggia *et al.*, 2014). The severity of these symptoms is dependent on the susceptibility of the grapevine varieties, plant vigor and presence of other pathogens (reviewed by Oliveira *et al.*, 2019a). The first focus of FD was reported in Armagnac (France), in 1955, and after that it has been dispersed to other European winegrowing regions such as Italy, Spain, Serbia, Switzerland and Portugal (Caudwell *et al.*, 1957; reviewed by Roggia *et al.*, 2014). In Portugal, it was detected firstly in 2006, in Amares, in the "Vinhos Verdes" region (Sousa *et al.*, 2010). From 2007 to 2012, about 194 hectars of vineyards were rougued, with a great socio-economic impact in the Portuguese wine-growing regions (Sousa *et al.*, 2011; DGAV, 2013).

Until now, the control strategies are limited to the use of healthy propagation material after hot water treatment (Bertaccini *et al.*, 2001; Mannini *et al.*, 2007), application of insecticides to the vector control (Chuche and Thiéry, 2014) and elimination of infected plants (Pavan *et al.*, 2012). As the current strategies for FD control are costly, difficult to implement and have a high environmental impact, alternative strategies should be developed preferably without pesticide application (Oliveira *et al.*, 2019a). Elicitors are compounds which trigger plant defences helping to develop and increase resistence to subsequent attacks by pathogens (Klarzynski *et al.*, 2000). These compounds are recognised by plant cell membrane and trigger

signal transduction pathways leading to the production of reactive oxygen species, polyphenols and phytoalexins, reinforcing plant cell wall, callose deposition, synthesis of stress enzymes and the accumulation of pathogenesis-related (PR) proteins (van Loon and van Strien, 1999).

Methyl jasmonate (MeJA), the derived ester of jasmonic acid (JA), is a phytohormone synthezised via the octadecanoic acid pathway from α-linolenic acid (Vick and Zimmerman, 1983; Hamberg and Gardner, 1992), and has a key role in many plant developmental processes and defence responses against stresses (Creelman and Mullet, 1997). In the last 20 years several studies have been conducted to understand the protective effects of MeJA in grapevine plants and in grapevine cultured cells with or without the presence of pathogens. A JA spray application in grapevines cv. Pinot noir showed a significant reduction in the numbers of phylloxera (*Daktulosphaira vitifoliae* Fitch) eggs and all nymph instars aproximately three-fold, and reduced Pacific spider mite fecundity (*Tetranychus pacificus* McGregor) (Omer *et al.*, 2000). Other studies conducted in grapevines cvs. Monastrell (syn. Mourvedre) and Tempranillo treated with MeJA decreased the grey mold (*Botrytis cinerea*) infection in treated grapevines (Ruiz-García *et al.*, 2012), increasing yield, berry quality and phenolic compounds on table grapes cvs. Magenta and Crimson (García-Pastor *et al.*, 2019). As aforementioned, MeJA activates transduction signal pathways leading to the expression of defence related genes, such as pathogenisis-related (PR) proteins and secondary metabolites (phenolics and flavonoids) (Repka, 2001; Delaunois *et al.*, 2014). MeJA also induces phenylalanine ammonia lyase (*PAL*) gene activation, which catalyses the phenylpropanoid biosynthesis. Cell culture has been often used as a model system to study the role of MeJA in gene expression activation. Repka *et al.* (2004) demonstrated that, in cultured cells of healthy grapevine cv. Limberger, MeJA increased the accumulation of PR proteins (PR-1, PR-2 and PR-8), peroxidase (PRX), chalcone isomerase and PAL (Repka *et al.*, 2004). Similarly, other studies have revealed that MeJA increased the expression of *PAL,* chalcone isomerase (Ahn *et al.*, 2014; Jiang *et al.*, 2015), chalcone synthase (*CHS*) and stilbene synthase (*STS*) genes in grapevine cvs. Campbell (Ahn *et al.*, 2014) and Kyoho (*Vitis vinifera* × *Vitis labrusca*) (Jiang *et al.*, 2015) against *B. cinerea*. Belhadj *et al.* (2006) showed for the first time that MeJA triggers the defence plant mechanisms against *Erysiphe necator*, the fungus responsible for powdery mildew through the expression of *PAL* and *STS* and defence-related genes encoding PR proteins, enhancing the tolerance of grapevine leaf cuttings in cvs. Cabernet Sauvignon and Merlot, both susceptible cultivars against this pathogen (Belhadj *et al.*, 2006). A further research in Chinese wild *Vitis pseudoreticulata* showed that MeJA enhanced retinoblastoma-related genes family, increasing the resistence against *E. necator* (Wen *et al.*, 2012). In spite of the demostrated MeJA

effectiveness as an elicitor of multiple defence responses in different grapevine diseases, to the best of our knowledge there is only a recent study that explored its potential role on FD control, focusing on its effect on the defence metabolites biosynthesis (proline and saponins) and the activation of defence-related genes (Oliveira *et al.*, 2019b). Those authors showed that MeJA applied before flowering and at veraison induced gene expression of Rubisco activase and PR proteins (PBSP, CHIT4c and PIN) 6 hours after elicitation. Building on that knowledge, this study aimed at: (i) evaluating the effect of different timings and concentrations of MeJA on the plant growth parameters, productivity indicators and berry quality; and (ii) contributing to a better understanding of the molecular responses triggered by the MeJA application in FD-infected grapevines. To this end, healthy and FD infected grapevines cv. Loureiro were treated with 0, 12.5 and 25 mM MeJA in two subsequent years. In 2015, these MeJA concentrations were applied at flowering, whereas in 2016 MeJA was applied before flowering and at veraison or just at veraison. We hypothesise that the timing and concentration of MeJA application might be relevant in the activation of grapevine defences and, therefore, in the mitigation of FD negative impact on plant growth.

3.1.2. Materials and Methods

3.1.2.1. Plant material and growth conditions

This study was carried out at "Quinta do Corvo" (41º31'03.52" N 8º12'45.59" O), Fafe, Portugal, in a twenty-year-old vineyard of *Vitis vinifera* cv. Loureiro. The vineyard has 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 were trained in a single upward cordon system and spaced 3 m x 3 m apart. The winter pruning was performed is single cordon. Fertilization and pest and disease control followed the integrated production system standards. FD phytoplasmas were detected in this farm in 2009 and the insect vector was eliminated. A total of 32 grapevines were selected, 16 healthy (FD-) and 16 infected with FD (FD+). FD screening was done by nested PCR as reported in (Oliveira *et al.*, 2020).

3.1.2.2. Elicitor application

To test the effect of different dosage and timings of MeJA application, 0 (non-elicited), 12.5 and 25 mM of MeJA was applied at flowering (stage I) in 2015, and just before flowering

and at veraison (stage M) in 2016, according to the Baggiolini scale (Baggiolini, 1952). Additionally, in 2016 a single MeJA application with 25 mM was performed at veraison. The treatments were performed on 18th May 2015 and in 2016 the applications were done on June 2nd and on August 25th. These concentrations were selected based on a previous study from our team where it was observed an activation of several plant defence genes (Oliveira *et al.*, 2019b). MeJA (Sigma-Aldrich, St. Louis, Missouri, EUA) was prepared by the dilution in a solution of 2.5% ethanol and 0.1% Tween 20 (Sigma-Aldrich, St. Louis, Missouri, EUA). Each treatment was applied on 4 biological replicates and the non-elicited plants were sprayed with solvent solution.

3.1.2.3. Biometric measurements

Leaf area (LA) was determined using the method described by Lopes and Pinto (Lopes and Pinto, 2005). In short, eight grapevine plants were selected at flowering (stage I) and at veraison (stage M) and a cane with a representative vigour of the plant was selected per each grapevine. In each cane, the number of buds, main leaves and secondary leaves were counted. The length of the right and left lateral midribs of major and minor main and secondary leaves were also measured.

Chlorophyll content was assessed 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 also at veraison stage.

Buds left on the shoots after winter pruning (*i.e.*, pruning load) were counted to calculate the fertility index (Alonso *et al.*, 2007), according to the formula:

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

3.1.2.4. Grape quality evaluation

At harvest (September 13th in 2015 and September 17th in 2016), productivity (bunches per plant), number of bunches and average bunch weight 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. These berries were used to calculate total soluble solids (TSS) and titrable acidity (TA) according to Oliveira *et al.* (2020). Samples were crushed,

homogenized, and centrifuged at 4,000 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 the Portuguese legislation (IPQ, 1999).

3.1.2.5. Gene expression

Six leaves per plant (with at least 10 cm width) of four biological replicates were collected just before (T0) and 24 hours after (T1) the elicitor application, frozen in liquid nitrogen and stored at -80°C. RNA extraction was performed using the RNeasy Plant Mini Kit (QIAGEN GmbH), accordingly to le Provost *et al.* (2007). RNA yield was measured by a spectrophotometry using a nano-photometer (Implen Isaza, Portugal). Synthesis of complementary DNA (cDNA) was performed using the iScrip cDNA Kit (BioRad, Hercules, California, EUA) according to the manufacturer instructions in a thermal cycler (VWR, Doppio, Belgium). RT-qPCR was performed using the NZY Taq 2x Green Master Mix Kit (Nzytech, Portugal). A total of 12 target genes were selected accordingly to previous studies related to grapevine diseases, (i) involved on primary metabolism (Rubisco activate – Rubisco Activase AAA^+ and Protα5s – Proteasome α5 subunit), (ii) encoding plant PR proteins (thaumatin I – Thau I, thaumatin II – Thau II, Osmotin-like protein – Osm, PBSP – Plant Basic Secretory Protein, CHIT4c – Acidic Class IV Chitinase, PIN – Inhibitors of Serine Protease, PGIP – Polygalacturonase-inhibiting Protein and GLU – *β*-1,3-glucanase) and (iii) encoding enzymes of phenylpropanoid biosynthesis (STS – Stilbene Synthase and PAL – Phenylalanine Ammonia Lyase) (Belhadj *et al.* 2006, 2008; Margaria and Palmano 2011). The primer sequences for each gene are listed in Table 3. The genes were amplified with initial denaturation of 120 sec at 95°C; 25 cycles of denaturation of 30 sec at 95°C, annealing of 30 sec at 55°C and extension of 60 sec at 72°C; final extension of 5 sec at 72°C. The 18S rDNA and ubiquitin were used as housekeeping reference genes. The Ct values of the target genes were normalized by the Ct values of the reference genes. Gene expression was calculated from the normalized Ct value.

3.1.2.6. Statistical analysis

Data analysis was performed using GraphPad Prism software (version 6.0). The results correspond to the mean ± standard error of the mean (SEM). Differences between treatments

were tested with a one-way ANOVA followed by Tukey's post-hoc test with a confidence level of 95% ($P < 0.05$).

3.1.3. Results

3.1.3.1. Role of MeJA on plant development and productivity

3.1.3.1.1. Biometric analyses

In both years, non-elicited FD infected plants showed a lower leaf area (LA) comparing with healthy ones (Fig. 11A and 11B). When analyzing the role of MeJA application on LA of infected plants, elicitation with 25 mM MeJA at flowering led to a significant increase of LA of about 2.8-fold (Fig. 11A), and 12.5 mM MeJA applied twice during the 2016 season showed a 38% increase of LA when compared with non-elicited infected plants (Fig. 11B). On the other hand, although infected grapevines treated twice with 25 mM MeJA did not show significant differences comparing with non-elicited plants, these plants showed a significant lower LA of 57% when compared with FD infected ones treated twice with 12.5 mM MeJA (Fig 11B). Interestingly, it was found that in healthy plants elicitation with 12.5 and 25 mM MeJA at flowering led to a significant increase of LA of about 0.7-fold and 1.1-fold, respectively (Fig. 1A). Moreover, plants elicited with a total of 25 mM MeJA [*i.e.* application of 12.5 mM before flowering and at veraison (BF+V) and 25 mM applied only at veraison (V)] showed a significant increase of 50% and 63%, respectively, when compared with healthy non-elicited plants (Fig. 11B).

Regarding SPAD analysis FD infected grapevines had consistently lower SPAD values when compared to healthy plants regardless of MeJA treatment, but this was more pronounced in 2015 than the years after (Fig. 11C and 11D). On the other hand, although FD infected plants treated with 25 mM (once or twice) did not show significant differences comparing with non-elicited plants, those plants had significantly lower SPAD values (up to 22%) when compared with grapevines treated with 12.5 mM MeJA (Fig. 1D). Looking at the effects of elicitation in healthy plants, double application of 25 mM MeJA increased significantly SPAD values by 22% when comparing with both non-elicited and 12.5 mM-treated grapevines (Fig. 11D).

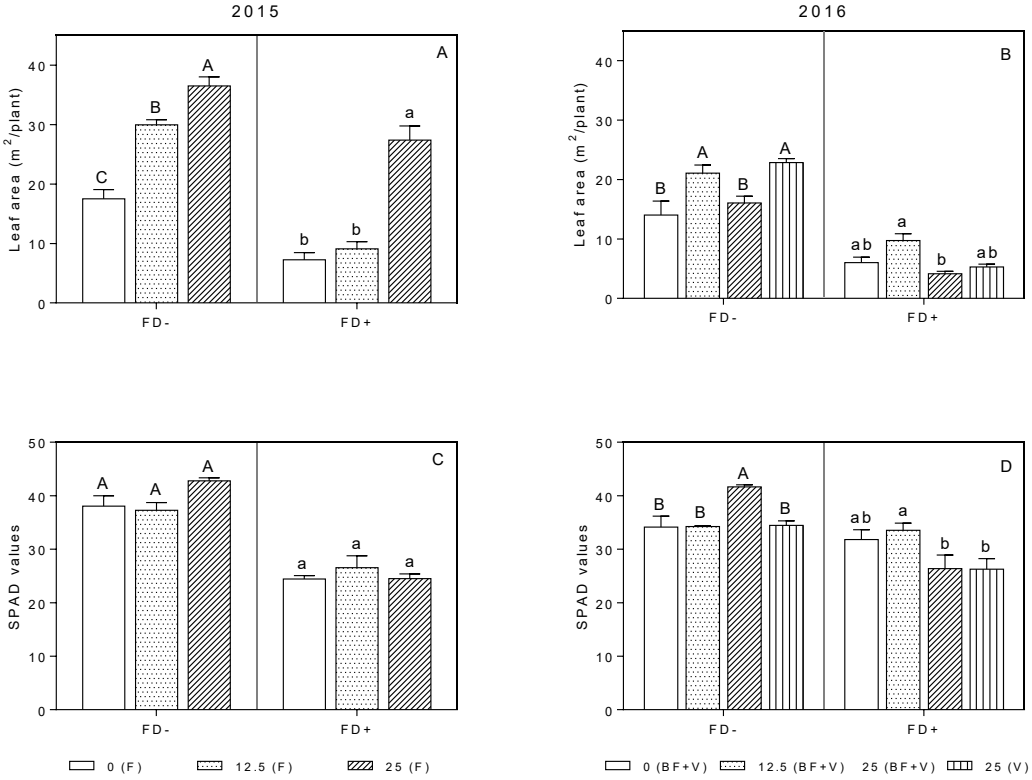

Figure 11. Leaf area (A and B) and SPAD values (C and D) measured at veraison (stage M) in healthy (FD-) and "flavescence dorée" infected (FD+) grapevines cv. Loureiro elicited with 0, 12.5 and 25 mM MeJA applied at flowering (F) in 2015, and applied twice (before flowering and at veraison, BF+V) or once (at veraison, V) in 2016. Data are means ± SEM of 4 biological replicates. Different letters above bars indicate significant statistical differences between treatments at $P \leq 0.05$, with uppercase letters representing significant statistical differences between different elicitor treatments among FD- plants, and different lowercase letters among FD+ plants, according to one-way ANOVA analyses followed by Tukey's post-hoc test.

"Flavescence dorée" had a clear negative effect on fertility index in 2015 and 2016. A reduction was observed in both years, and elicitor application was not successful in improving this parameter and in some circumstances, it even aggravates the induced stress on FD infected plants. That negative effect of MeJA elicitation occurred at higher doses. For instance, the application of 25 mM MeJA at flowering led to a significant reduction of 74% and 77% in fertility index in FD infected grapevines comparing with non-elicited infected plants and 12.5 mM MeJA-treated plants, respectively (Fig. 12A). Similarly, in 2016 plants elicited with the highest dose of MeJA (*i.e.* 25 mM applied twice) showed a significant decrease in fertility index by 80%, when compared to plants treated twice with 12.5 mM MeJA (Fig. 12B).

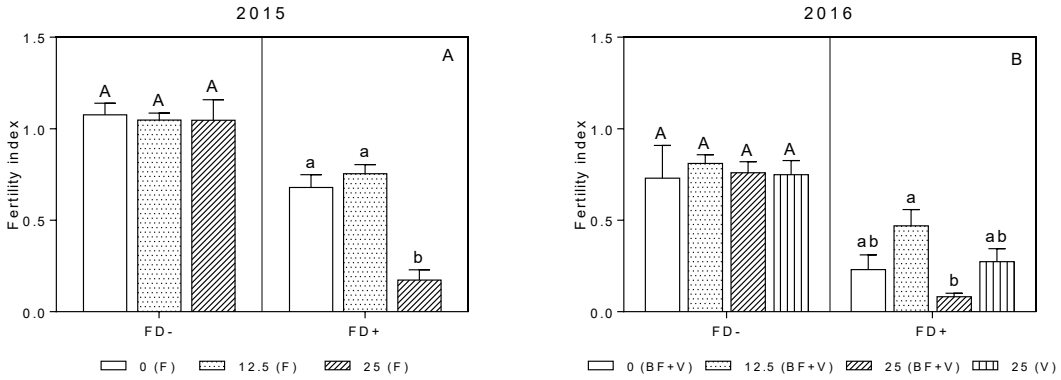

Figure 12. Fertility index measured at veraison (stage M) in healthy (FD-) and "flavescence dorée" infected (FD+) grapevines cv. Loureiro elicited with 0, 12.5 and 25 mM MeJA applied at flowering (F) in 2015, and applied twice (before flowering and at veraison, BF+V) or once (at veraison, V) in 2016. Data are means ± SEM of 4 biological replicates. Different letters above bars indicate significant statistical differences between treatments at $P \leq 0.05$, with uppercase letters representing significant statistical differences between different elicitor treatments among FD- plants, and different lowercase letters among FD+ plants, according to one-way ANOVA analyses followed by Tukey's post-hoc test.

3.1.3.1.2. Yield parameters

FD infection had a very negative effect on grapevine productivity in both years (Fig. 3A and 3B). When analysing the potential effect of MeJA elicitation to impair the disease impacts in infected plants, it was observed that only the double application of 12.5 mM MeJA (BF+V) resulted in a significant increase on plant yield comparing with non-elicited FD-infected plants (4-fold higher, still 4.7 kg/plant below the yield achieved in healthy plants non-elicited). This yield increase was mostly due to a higher number of bunches /1.7-fold increase) but also related to a higher average bunch weight (33% higher). Nonetheless, it was found that when MeJA was applied at higher concentration (25 mM), independently of the timing of and frequency, it clearly had a negative effect on plant yield, revealing to be an additional stress to the infected plants (Fig. 13). Concerning the healthy plants, although MeJA treated plants did not show significant differences in terms of yield, when compared with non-elicited plants, grapevines elicited twice with 12.5 mM MeJA had a significant higher yield (81%) as compared with a double application of 25 mM MeJA (Fig. 13B). Also, here, the increase yield was explained by a significantly higher number of bunches (46%).

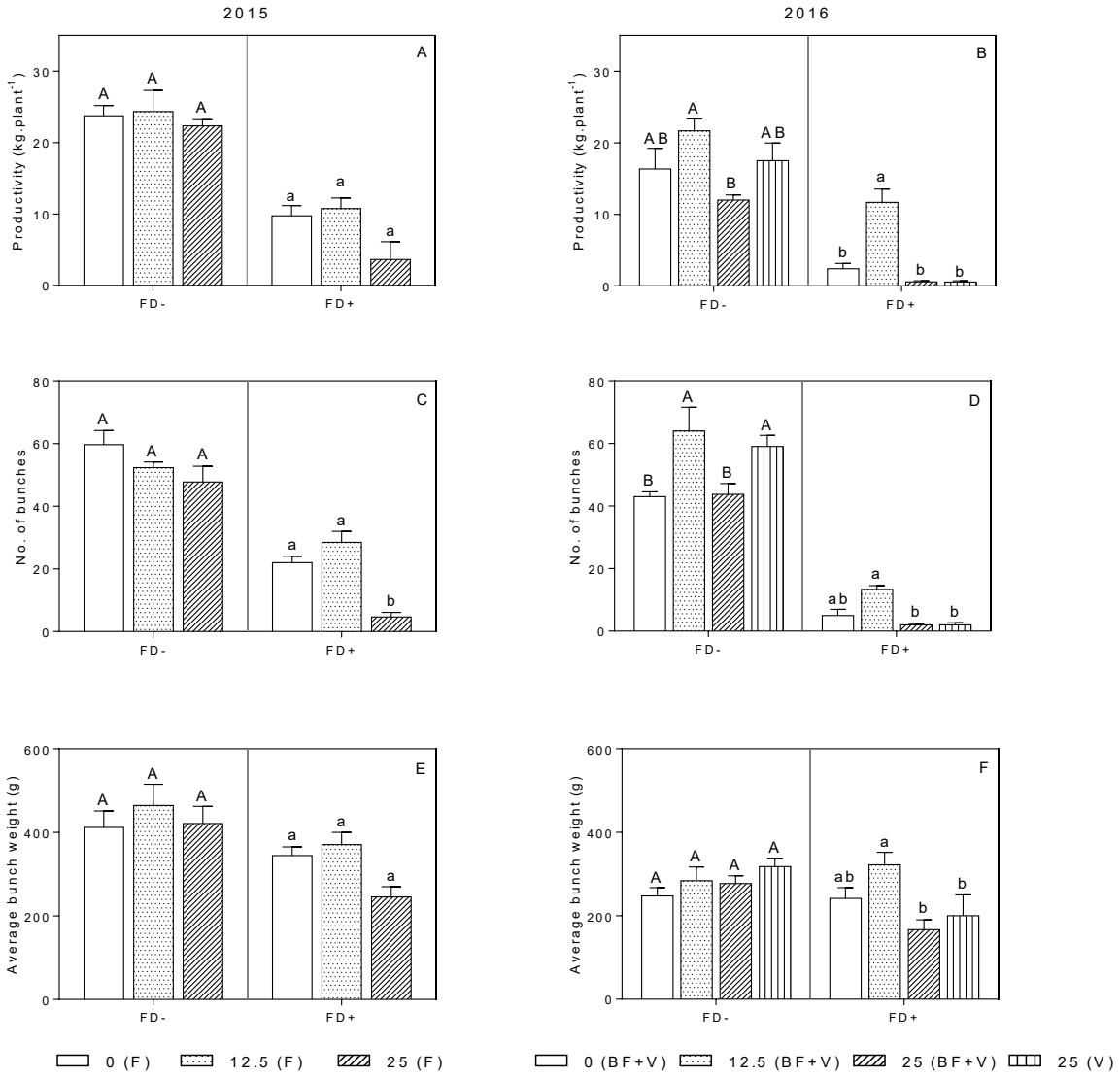

Figure 13. Productivity (A and B), number of bunches (C and D) and average bunch fresh weight (E and F) measured at harvest in healthy (FD-) and "flavescence dorée" infected (FD+) grapevines cv. Loureiro elicited with 0, 12.5 and 25 mM MeJA applied at flowering (F) in 2015, and applied twice (before flowering and at veraison, BF+V) or once (at veraison, V) in 2016. Data are means ± SEM of 4 biological replicates. Different letters above bars indicate significant statistical differences between treatments at $P \leq 0.05$, with uppercase letters representing significant statistical differences between different elicitor treatments among FD- plants, and different lowercase letters among FD+ plants, according to one-way ANOVA analyses followed by Tukey's post-hoc test.

3.1.3.1.3. Effect of MeJA on berry quality

Overall, FD infection did not have an impact on TSS nor TA regardless of elicitor treatment (Figs. 14A, 14C and 14D). In general, MeJA elicitation had no significant effect on berry quality of both healthy and FD-infected plants- However, the two treatments with 25 mM MeJA (*i.e.* twice at BF+V or once at V) led to a significant reduction in grape TSS up to 25% in FD-infected plants as compared with non-elicited ones (Fig. 14B).

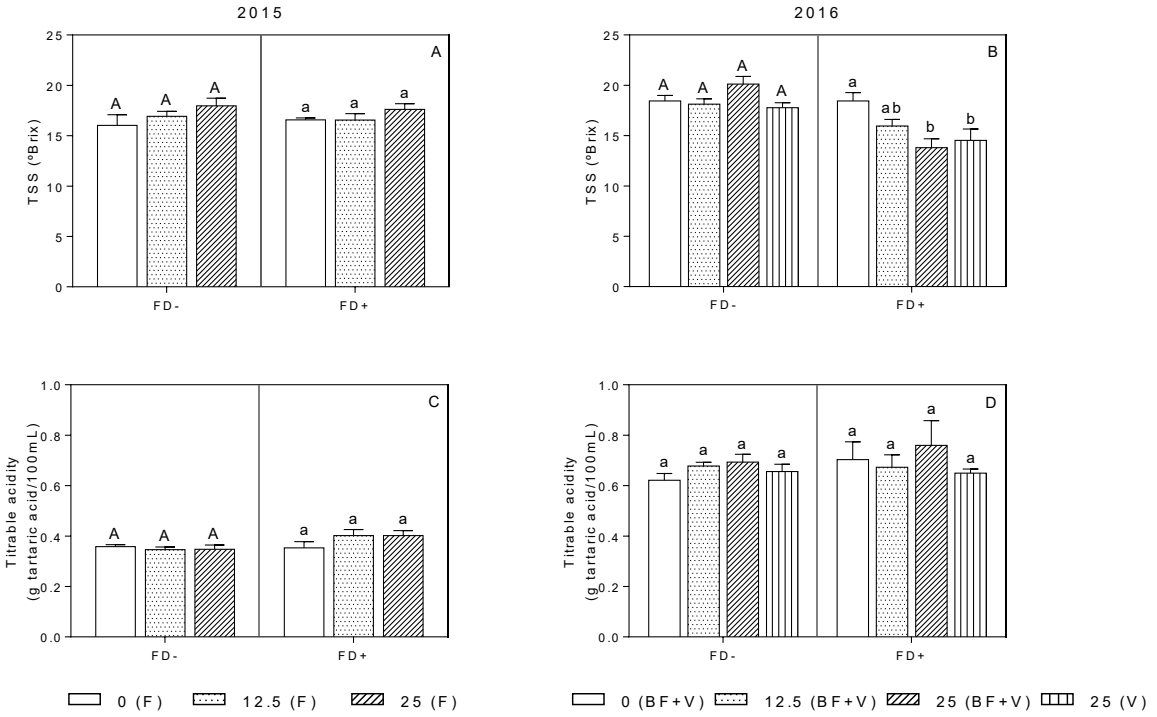

Figure 14. Fruit total soluble solids (TSS; A and B) and titrable acidity (C and D) measured in healthy (FD-) and "flavescence dorée" infected (FD+) grapevines cv. Loureiro elicited with 0, 12.5 and 25 mM MeJA applied at flowering (F) in 2015, and applied twice (before flowering and at veraison, BF+V) or once (at veraison, V) in 2016. Data are means ± SEM of 4 biological replicates. Different letters above bars indicate significant statistical differences between treatments at $P \leq 0.05$, with uppercase letters representing significant statistical differences between different elicitor treatments among FD- plants, and different lowercase letters among FD+ plants, according to one-way ANOVA analyses followed by Tukey's post-hoc test.

3.1.3.2. Gene expression

In the present study 12 target genes, divided into 3 categories, were selected according to previous studies related to grapevine diseases, involved in primary metabolism, encoding plant PR proteins, and encoding enzymes of phenylpropanoid biosynthesis.

In 2015, healthy grapevines (0 mM MeJA) did not change their gene expression pattern during the study, with exception of *Thau II* and *Osm* genes, which were upregulated at 24 h on non-elicited plants, while the FD infected plants before the elicitor application had an overexpression of those genes, which decrease 24 h later (Fig. 15A). On the other hand, in 2016, healthy and FD infected plants showed that *Protα5s*, *CHIT4c*, *PGIP* and *STS* genes were upregulated in T1 (Fig. 15B).

Although *Rubisco* gene did not change its expression during the study in both years, the FD infected plants showed a slight decrease of this gene expression. In the same way, *Protα5s*,

CHIT4c genes in both healthy and FD infected plants, and *PGIP*, *STS* and *PAL* genes only in FD infected grapevines, did not show any up/downregulation (Fig. 15A). In 2016, *Thau I*, *Thau II*, *Osm* and *PAL* genes did not change their expression pattern in both healthy and FD infected grapevines (Fig. 15B).

Generally, the MeJA treatments revealed a positive effect in the activation of gene expression, mainly on *Protα5s*, *Thau I*, *Thau II*, *Osm*, *CHIT4c*, *PIN*, *PGIP*, *GLU*, STS and *PAL* genes (Fig. 15A and 15B). FD infected plants elicited with 12.5 mM MeJA (F) showed an upregulation of *Thau I*, *Thau II* and *Osm* genes, while the same concentration induced a higher expression also of *STS, PIN*, *PGIP* and *PAL* genes (Fig. 15A). Application of 25 mM MeJA at flowering triggered *Thau I*, *Thau II* and *Osm* genes upregulation only on FD infected plants 24 h after the treatment (Fig. 15A).

Although all MeJA treatments seemed to activate *Protα5s*, *CHIT4c*, *PIN*, *PGIP*, *GLU* and *STS* genes expression, 25 mM (BF+V) and 25 mM (V) revealed the higher upregulation of these genes, while 12.5 mM MeJA did not show any effect in the expression of *STS*, *PGIP*, *GLU* and *CHIT4c* genes in FD infected plants (Fig. 15B).

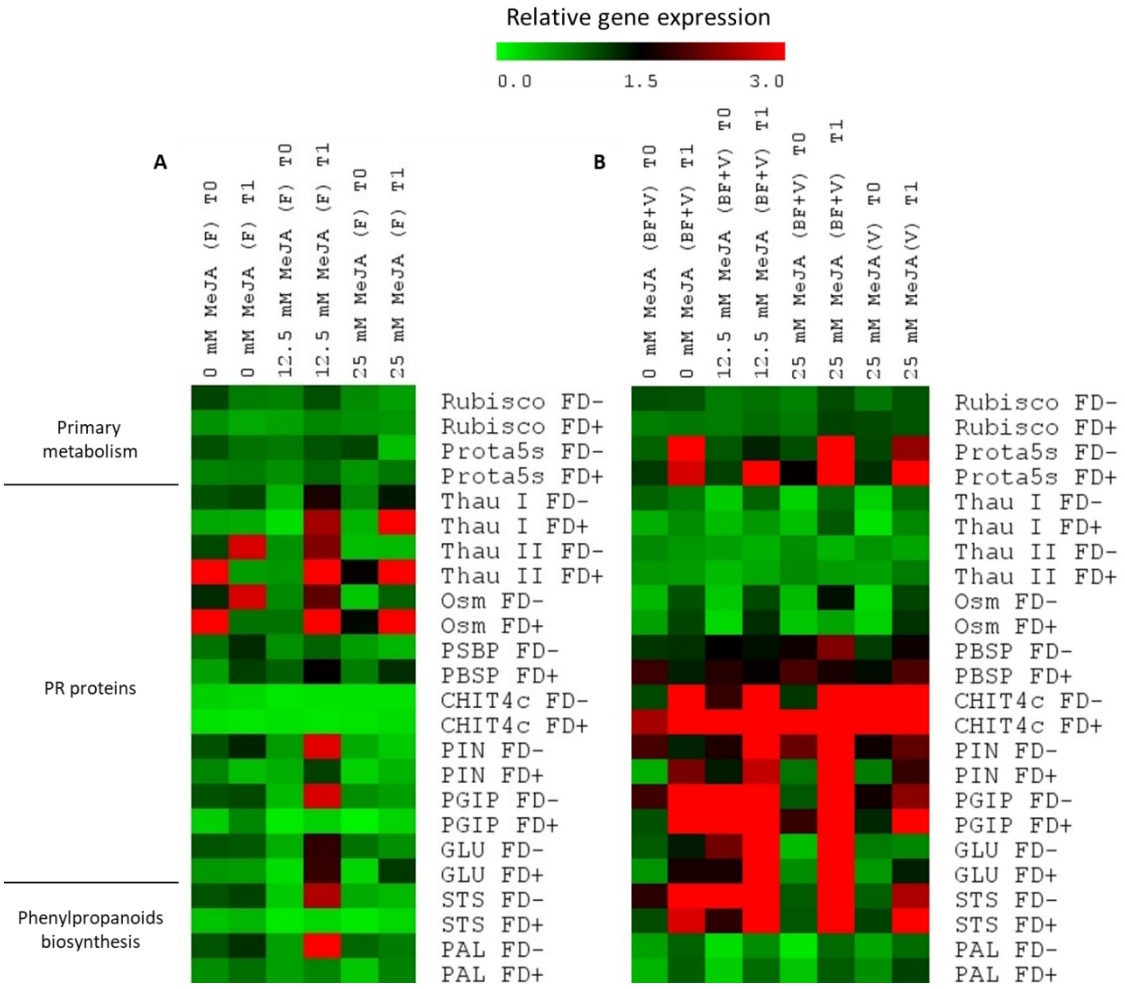

Figure 15. Heat map of relative fold expression of 12 target genes (AAA+ Rubisco activase – Rubisco; Proteasome α5 subunit - Protα5s; Thaumatin I - Thau I; Thaumatin II – ThauII; Osmotin-like protein – Osm; Plant Basic Secretory Protein – PBSP; acidic class IV chitinase - CHIT4c; serine protease inhibitor – PIN; polygalacturonase-inhibiting protein – PGIP; β-1,3-glucanase – GLU; Stilbene Synthase – STS; phenylalanine ammonia lyase - PAL) measured in healthy (FD-) and "flavescence dorée" infected (FD+) grapevines cv. Loureiro elicited with 0, 12.5 and 25 mM MeJA applied at flowering (F) in 2015 (A), and applied twice (before flowering and at veraison, BF+V) or once (at veraison, V) in 2016 (B) before elicitation (T0) and 24 hours (T1) after MeJA application. Data are means of 4 biological replicates.

3.1.4. Discussion

Literature about grapevine diseases management and their impact on plant growth and development parameters (such as fertility index, LA and chlorophyll content) is very scarce, therefore, this is the first study on MeJA application to alleviate FD symptoms and its impact on plant growth, development, productivity, and berry quality as well as the activation of plant defences against to the phytoplasmas. In 2015, 12.5 and 25 mM MeJA were applied at flowering stage, whereas in 2016, the same concentrations were applied before flowering followed by a second application at veraison, or only at veraison (25 mM (V)).

LA is one of the most important parameters that allows to understand grapevine responses to the environmental conditions and to the interventions made at the canopy level (Lopes and Pinto, 2005). The first symptoms of FD appeared between the end of May and beginning of July, consisting in leaf yellowing and this may be the main reason for the lower values of SPAD, reflecting the low chlorophyll content of infected plants when compared to healthy ones (Fig. 11). A previous study conducted in cv. Loureiro reported that FD infected plants registered a delay on growth and development compared with healthy grapevines, showing a significant decrease on LA and on chlorophyll content, which might be due to the FD infected plants having lower sprouting rates, causing a delay on plant growth, not reaching the canopy of the healthy plants (Oliveira *et al.*, 2020). Even more, although the LA of the infected grapevines had progressively increased (even though there was less comparison with healthy plants), leaf symptomatology had also developed, resulting in lower photosynthetic rates compared to healthy plants, with a consequent delay in plant development as previously reported for this disease (Vitali *et al.*, 2013). The development of the symptomatology reduces the photosynthetic capacity of the leaves of FD infected grapevines, resulting in lower chlorophyll content compared to the healthy plants. In this way, a continuous deficit of photoassimilates to the fruit could be expected (Santi *et al.*, 2013; Endeshaw *et al.*, 2014; Oliveira *et al.*, 2020). Regardless FD infection, MeJA application increased significantly LA up to 70% (Fig. 11A and 11B). Few studies have demonstrated the biostimulant effect of MeJA, increasing amino acid composition, grape and wine phenolic and volatile composition (Portu *et al.*, 2015; Garde-Cerdán *et al.*, 2016; Garde-Cerdán *et al.*, 2018; García-Pastor *et al.*, 2019). The present study showed for the first time that, besides improving the physiology of the plant, this elicitor has a significant impact on its anatomical characteristics and yield performance as well. Moreover, it was shown for the first time that MeJA application also improve the dimension of the LA, especially when applied once at higher concentration (25 mM) or even when applied twice at 12.5 mM. FD infected plants treated with 12.5 mM MeJA (BF+V) showed a slight lower increase on LA, while 25 mM MeJA (F) was significantly more effective, when it was applied at flowering. Regarding chlorophyll content, measurements showed that elicitor application in 2016 had better results on SPAD values than those carried out in 2015 (Fig. 11C and 11D). These responses in the second year after MeJA application could be related to a phenomenon designed as “priming” (Beckers and Conrath, 2007), which is characterized by the increased capacity of plants to show faster and stronger activation of defence responses in the next attack of the pathogen or even when the disease pressure increases. Cultured cell suspensions of *V. labrusca* previously infected *Streptomyces anulatus* S37 increased expression

of *PAL*, *STS* and *GLU* genes 9 to 24 hours post-inoculation with *Botrytis cinerea* (Vatsa-Portugal *et al.*, 2017). In another study, using *in vitro* plantlets of grapevine cv. Chardonnay the pre-inoculation of *Pseudomonas fluorescens* PTA-CT2 triggers the expression of defence-related genes related to salicylic and jasmonic acids, reducing *B. cinerea* colonization capacity (Gruau *et al.*, 2015). On the other hand, for the first time MeJA application (at 25 mM) revealed to be toxic to infected grapevines, which may be related to the presence of infection. A previous study also showed that application of 25 mM salicylic acid on grapevines cv. Loureiro did not alleviate FD symptoms and impacts on plant growth, development, and plant yield (Oliveira *et al.*, 2019c).

The fertility index was also affected by FD phytoplasma presence. Previous studies demonstrated that grapevines cv. Loureiro infected with FD showed a decrease of 35% on this plant development parameter (Oliveira *et al.*, 2019c, 2020). In addition to the differences recorded in the fertility index, it was also possible to verify that FD infected plants have shown shriveled bunches with bitter berries compared those of healthy plants. In the present study, fertility index of FD infected plants was notably lower than that one of healthy plants, except those treated with 12.5 mM MeJA in both years (Fig. 12). On the other hand, also in both years, 25 mM MeJA showed the higher decrease of fertility index, which may be related to the high elicitor concentration applied during inflorescence formation, causing some damages to the flowers as well as the increasing dosage (especially in 2016), that was toxic to grapevine and to the inflorescences. At harvest, in both years, although the FD infected plants had less bunches, this difference was not so evident in the average bunch weight between FD infected and healthy plants, except to the grapevines treated with 25 mM MeJA in 2015 (Fig. 13E and 13F). FD is usually associated with a decrease in productivity and sometimes the inflorescences become sterile, and the bunches are more fragile with berries more easily loose to the touch (Caudwell, 1990). A previous study has demonstrated that FD decreased cv. Loureiro productivity between 51% and 92% (Oliveira *et al.*, 2020). Although MeJA treatments applied at flowering did not seem to have any effect in yield increasing, the 25 mM MeJA concentration presented a tendency to be toxic, with a decrease of 62% comparing with FD non-elicited plants (Fig. 13B). Nevertheless, application of 12.5 mM MeJA (BF+V) presented a potential to increase the grapevine productivity, when compared with FD infected non-elicited plants and 25 mM MeJA treated plants. This is in agreement with the aforementioned toxic effect of high MeJA concentration as well as the "priming" capacity of elicitors, resulting in an improvement of productivity of FD infected grapevines in the second application of MeJA (Fig. 13B). In fact, the 12.5 mM MeJA (BF+V) registered the best results, whereby when it was applied twice it

resulted more effective in improving the plant yield, better than and one application of 25 mM MeJA (at flowering or at veraison) and/or two applications (before flowering and at veraison), which means that elicitor concentration is important to improve plant yield. Portu *et al.* (2015 and 2016) showed that 10 mM MeJA application at veraison and one week later did not affect plant productivity, berry weight as well as TSS and TA in cv. Tempranillo (Portu *et al.*, 2015; Portu *et al.*, 2016). A recent study demonstrated that MeJA effectiveness is strongly correlated with its concentrations (García-Pastor *et al.*, 2019). Indeed, although a range of concentrations between 0.01 and 10 mM MeJA were applied, the best results were registered with the concentration of 0.01 mM MeJA in the latter harvest for both studied cvs. Magenta and Crimson also concluded that 1, 5 and 10 mM MeJA delayed the ripening process for three weeks (García-Pastor *et al.*, 2019). This can be enhanced when grapevines are infected with pathogens, which increase the delay of ripening process, as observed in the present study (data not shown). Thus, the delay observed in the development of the FD infected plants, as well as the delay in the maturation of the bunches, caused significant differences on yield parameters, mainly in 2016, related with a significant decrease of number of bunches and of TSS of grapevines treated with 25 mM MeJA. In this way, a significant difference in TSS and TA values of FD infected plants due to the increase of berry acidity would be expected (Portu *et al.*, 2001; Jermini *et al.*, 2010). Nevertheless, this effect was only registered in plants treated with 25 mM MeJA (BF+V). According to a previous study, FD infected grapevines cv. Loureiro did not show differences in TSS between healthy and FD infected plants (Oliveira *et al.*, 2019c; Oliveira *et al.*, 2020). Previous studies conducted in cv. Tempranillo showed that 10 mM MeJA did not affect TSS and TA, which means that this concentration was not toxic to this grapevine cultivar. Nevertheless, the same concentration applied on cv. Monastrell (syn. Mourvedre) grapes in two years revealed an increase on TA in 2009 and a decrease on TSS in 2010 (Ruiz-García *et al.*, 2012). García-Pastor *et al.* (2019) also demonstrated that 1, 0.1 and 0.01 mM MeJA increased TSS while TA decreased in both cvs. Crimson and Magenta (García-Pastor *et al.*, 2019). In fact, lower MeJA concentrations increased the photosynthetic rate, stomatal conductance, and the sink strength of berry cells, leading to an increasing of sugar concentration, which enhances berry volume and weight (Wu *et al.*, 2012; García-Pastor *et al.*, 2019).

In the last 20 years many studies in grapevine have been conducted to understand the role of MeJA in the activation of defence mechanisms against pathogens, mainly by the activation of gene expression (Repka, 2001; Belhadj *et al.*, 2006; Wen *et al.*, 2012; Ahn *et al.*, 2014; Figueiredo *et al.*, 2015; Jiang *et al.*, 2015). Gene expression is activated in the following hours after pathogen attack or elicitor application, with a maximum between 10 h and 24 h,

decreasing after this time (Belhadj *et al.*, 2006). In the current study, RT-PCR analysis showed that, in general, after the application of two MeJA concentrations, gene expression was activated as reported in experiments performed in tobacco and tomato plants, in which a reduction of *B. cinerea* incidence was observed (Wen *et al.*, 2012; Ruiz-García and Gómez-Plaza, 2013). According to the study conducted by Margaria and Palmano (2011) the decrease of *Rubisco* gene expression in FD infected grapevines cv. Nebbiolo is a consequence of the reduction of the photosynthetic rate followed by an increasing of protein degradation conducted by the complex ubiquitin/26S proteasome (5α subunit) (Margaria and Palmano, 2011). However, in the present study the genes associated to photosynthesis and protein degradation were not significantly affected by the FD phytoplasma presence after elicitor treatments in 2015, independently of the application timing (Fig. 15A). In 2016, although *Rubisco* gene did not show any difference on its expression pattern, treated plants revealed an upregulation of *Protα5s* gene, meaning the enhancing grapevine metabolism events (Fig. 15B).

Concerning gene expression of PR proteins some differences were observed in the present two-year study. Indeed, 12.5 and 25 mM MeJA applied at flowering upregulated *Thau I*, *Thau II* and *Osm* genes in FD infected plants, while *PIN*, *PGIP*, *STS* and *PAL* genes were upregulated after the elicitation of healthy grapevines with 12.5 mM MeJA (Fig. 15A). The first studies conducted in grapevine cultured cells systems of cv. Limberger showed that MeJA induced gene expression of PR proteins GLU (PR-2), chitinase III (PR-8) and peroxidase (PR-9) 6 days after elicitor application (Repka, 2001). *Chitinases* (mainly *CHIT4c*) as well as *PIN* genes are upregulated by biotic stresses and exogenous MeJA application (Belhadj *et al.*, 2006; Belhadj *et al.*, 2008). MeJA-treated cells of cv. Monastrell revealed an upregulation of *thaumatin-like protein, CHIT4c* and *PIN* genes (Almagro *et al.*, 2014a). Belhadj *et al.* (2006) showed that the application of 5 and 15 mM MeJA in grapevines cv. Cabernet Sauvignon upregulated *CHIT4c*, *PIN*, *PGIP* and *GLU* genes between 10-24 h after the elicitor application, which contributed to the decrease of powdery mildew infection in about 75% (Belhadj *et al.*, 2006). Recent studies conducted by Margaria *et al.* (2011 and 2014) have demonstrated that FD infected grapevines cv. Nebbiolo strongly increased PR protein gene expression (*Thau I, Thau II, Osm* and *PBSP*) as well as flavonoid biosynthesis enzymes (Margaria and Palmano, 2011; Margaria *et al.*, 2014).

A few studies have looked at the role of MeJA in PR proteins' gene expression and on their role in the activation of the phenylpropanoid pathway, either as an elicitor (Repka *et al.*, 2004; Belhadj *et al.*, 2006; Ahn *et al.*, 2014) or as a biostimulant (Repka, 2001; Donnez *et al.*, 2011), *i.e.*, as a trigger of plant defence mechanisms or as a nutrient to improve plant

productivity, respectively. Concerning the role of MeJA on this secondary biosynthetic pathway, some studies have shown that MeJA upregulated *PAL* gene 4 h to 72 h in cvs. Monastrell (Lijavetzky *et al.*, 2008; Almagro *et al.*, 2014a) and Gamay-Fréaux (Belhadj *et al.*, 2008) after elicitor treatment. In the present study *PIN*, *PGIP*, *STS* and *PAL* genes were evaluated due to their important role on the activation of secondary metabolism and plant defences. Here, only 12.5 mM MeJA (F) treatment upregulated these genes in healthy plants (Fig. 15A). However, in 2016, both elicitor concentrations in both application timings led to *PIN*, *PGIP* and *STS* upregulation but not *PAL* overexpression (Fig. 15B). A study conducted by Ahn *et al.* (2014) demonstrated that MeJA upregulated *CHS* and *STS* during a 72 h study in grapevine cv. Campbell Early inoculated with *B. cinerea*. The same study observed that both *PAL* and *STS* genes had a maximum of transcription 6 h after the MeJA treatment (Ahn *et al.*, 2014), which is a shorter sampling time comparing with the present study, which could explain the lowest transcription rate of those genes in the present study. Other studies have shown different times for *PAL* gene transcription upregulation. A study conducted in potted grapevines cv. Cabernet Sauvignon inoculated with *B. cinerea* observed that both *PAL* and *STS* genes were upregulated 24 h after MeJA treatment (Belhadj *et al.*, 2006) and *STS* gene has showed expression 4-fold higher than *PAL* gene. This agrees with the results presented here, where *STS* gene had higher expression than *PAL* gene.

3.1.5. Conclusions

The present two-year study demonstrated the mitigation effect of the MeJA application on some grapevine growth, productivity and berry quality parameters, and genetic responses in FD infected grapevines. It also demonstrated for the first time that MeJA application seems to be a promising technique in FD management. However, its effect is dependent of concentration and timing of application. In fact, 12.5 mM MeJA applied twice (before flowering and at veraison) presented better results in grapevine yield, increasing productivity of about 4-fold, and a tendency to improve plant growth (LA and chlorophyll content) and productivity (fertility index, number of bunches and average of bunch weight) parameters. On the other hand, 25 mM MeJA applied in 2016 revealed to be toxic to infected grapevines, regardless timing of application. Application of 12.5 mM MeJA also triggered a great increase in gene expression with upregulation of *Thau I*, *Thau II*, *Osm* and *PAL* (in 2015) and *Protα5s*, *CHIT4c*, *PIN*, *PGIP* and *GLU* (in 2016) genes. Despite in this study gene expression showed a great variability,

Protα5s, *CHIT4c*, *PIN*, *PGIP*, *GLU* and *STS* genes resulted overexpressed 24 h after the application of both MeJA concentrations in both years. MeJA showed also a highly beneficial effect in healthy grapevines to LA values, with increases up to 1.1-fold in 2015, as well as in plant yield, increasing the number of bunches up to 94%, in 2016.

Altogether, this study allows to a have a first insight on the role of MeJA concentration, timing and frequency of application. Nonetheless, in spite this study was conducted in two years, some differences observed in terms of elicitor application (flowering *versus* before flowering followed by at veraison) could be confirmed. Even though the present study put lights on the role of MeJA either as a possible approach to manage FD and as a plant biostimulant, further studies are needed to better understand its molecular mechanisms, activated genes and secondary metabolites produced as well as other concentrations which could improve more accurately the plant responses to the pathogen presence.

3.1.6. Supplementary data

Table 3. Primer sequences of reference (housekeeping) and target genes analysed by RT-qPCR. Abbreviations: T_{ann} = annealing temperature.

		Primer sequence (5′-3′)		T_{ann}	Reference
		Forward	**Reverse**	**(ºC)**	
Housekeeping genes					
ACT	Actin	TCAGCACTTTCCAGCAGATG	TAGGGCAGGGCTTTCTTTCT	55.9	Belhadj *et al.*, 2006; 2008
18S rDNA	18S ribosomal DNA	GTGACGGAGAATTAGGGTTCG	CTGCCTTCCTTGGATGTGGTA	57.9	Osman *et al.*, 2007
Primary metabolism					
Rubisco	Rubisco Activase AAA^+	GCTCTTGGAGATGCGAACGT	GGGCTGCCTTGCCATAAA	58.7	Margaria and Palmano, 2011
Protein degradation					
Protα5s	Proteasome α5 subunit	GCACGAGTTGAAACTCAGAATCA TAG	GCAGGGCAAGGTCACAAAGA	60.6	Margaria and Palmano, 2011
Pathogen-related (PR) proteins					
Thau I	Thaumatin I	TGCCAGGGCTACGGTTCA	AGTCGAGGTTATTGGGCTGGTT	59.6	Margaria and Palmano, 2011
Thau II	Thaumatin II	CTTCACCCCCAGCTATGCA	GCCGCAGCCCAAACC	58.3	Margaria and Palmano, 2011
Osm	Osmotin-like protein	CTATTCTTCACCTCCAGCTATGCA	ACTGCCGCAGCCCAAAC	58.9	Margaria and Palmano, 2011
PBSP	Plant Basic Secretory Protein	GCGTCCGATTCACAAATGAA	CATATGAAGTCGGTGGCAGATA CT	57.5	Margaria and Palmano, 2011
CHIT4c	Acidic Class IV Chitinase	GGCGACGAATCCATTTATGTTA	CGGAACAAGGGTTTCATAATTC	56.3	Belhadj *et al.*, 2006; 2008
PIN	Inhibitors of Serine Protease	GCAGAAACCATTAAGAGGGAGA	TCTATCCGATGGTAGGGACACT	55.8	Belhadj *et al.*, 2006; 2008
PGIP	Polygalacturonase-inhibiting Protein	ACGGAACTTGTTCCAGTTTGAT	CGATTGTAACTCACGTTCAGGA	56.4	Belhadj *et al.*, 2006
GLU	β-1,3-glucanase	TCAGCACTTTCCAGCAGATG	TAGGGCAGGGCTTTCTTTCT	56.0	Belhadj *et al.*, 2006
Phenylpropanoids					
STS	Stilbene Synthase	GTGGGGCTCACCTTTCATT	CTGGGTGAGCAATCCAAAAT	55.9	Belhadj *et al.*, 2006; 2008
PAL	Phenylalanine Ammonia Lyase	TGCTGACTGGTGAAAAGGTG	CGTTCCAAGCACTGAGACAA	56.6	Belhadj *et al.*, 2006; 2008

Table 4. Relative fold of expression of 12 target genes (see legend of Figure 15) measured in healthy (FD-) and "flavescence dorée" infected (FD+) grapevines cv. Loureiro elicited with 0, 12.5 and 25 mM MeJA applied at flowering (F) in 2015, before elicitation (T0) and 24 hours (T1) after MeJA application. Data are means ± SEM of 4 biological replicates. For each gene, letters indicate statistical differences ($P < 0.05$) according to two-way ANOVA followed by Tukey's post-hoc test.

		Treatment					
Gene	**Plant**	**0 mM MeJA (F) T0**	**0 mM MeJA (F) T1**	**12.5 mM MeJA (F) T0**	**12.5 mM MeJA (F) T1**	**25 mM MeJA (F) T0**	**25 mM MeJA (F) T1**
Rubisco	FD-	1.085 ± 0.016^{a}	0.767 ± 0.119^{ab}	0.740 ± 0.057^{ab}	1.040 ± 0.071^{a}	0.680 ± 0.090^{ab}	0.550 ± 0.040^{b}
	FD+	0.633 ± 0.022^{b}	0.497 ± 0.053^{b}	0.538 ± 0.074^{b}	0.705 ± 0.059^{ab}	0.630 ± 0.019^{b}	0.610 ± 0.061^{b}
Prota5s	FD-	1.017 ± 0.103^{a}	0.827 ± 0.116^{ab}	0.777 ± 0.075^{ab}	1.003 ± 0.055^{a}	1.088 ± 0.058^{a}	0.370 ± 0.108^{b}
	FD+	0.737 ± 0.053^{ab}	0.770 ± 0.033^{ab}	0.650 ± 0.052^{ab}	0.890 ± 0.090^{ab}	0.607 ± 0.059^{ab}	0.790 ± 0.120^{ab}
Thau I	FD-	1.013 ± 0.095^{cd}	1.083 ± 0.087^{bd}	0.433 ± 0.020^{cd}	1.660 ± 0.288^{bc}	0.715 ± 0.087^{cd}	1.363 ± 0.056^{bd}
	FD+	0.510 ± 0.065^{cd}	0.477 ± 0.121^{cd}	0.177 ± 0.010^{d}	2.480 ± 0.490^{ab}	0.430 ± 0.180^{cd}	3.577 ± 0.450^{a}
Thau II	FD-	1.080 ± 0.253^{b}	2.790 ± 0.947^{b}	$0.640 \pm 0,135^{b}$	2.268 ± 0.230^{b}	0.375 ± 0.045^{b}	0.392 ± 0.123^{b}
	FD+	3.090 ± 1.002^{b}	0.567 ± 0.044^{b}	0.607 ± 0.152^{b}	16.65 ± 1.065^{a}	1.547 ± 0.296^{b}	12.88 ± 0.785^{a}
Osm	FD-	1.245 ± 0.143^{bd}	2.780 ± 0.923^{bcd}	0.683 ± 0.146^{d}	2.060 ± 0.410^{bd}	0.320 ± 0.071^{d}	0.918 ± 0.052^{d}
	FD+	3.647 ± 0.402^{bc}	0.823 ± 0.100^{cd}	0.820 ± 0.071^{cd}	14.21 ± 0.933^{a}	1.447 ± 0.257^{bd}	4.195 ± 0.417^{b}
PBSP	FD-	0.813 ± 0.138^{ac}	1.250 ± 0.035^{ac}	0.643 ± 0.124^{bc}	0.920 ± 0.071^{ac}	0.560 ± 0.066^{bc}	0.420 ± 0.085^{c}
	FD+	0.547 ± 0.097^{bc}	1.127 ± 0.191^{ac}	0.927 ± 0.064^{ac}	1.541 ± 0.146^{a}	0.750 ± 0.029^{bc}	1.233 ± 0.135^{ab}
CHIT4c	FD-	0.277 ± 0.092^{a}	0.225 ± 0.014^{a}	0.133 ± 0.027^{a}	0.163 ± 0.036^{a}	0.160 ± 0.025^{a}	0.160 ± 0.037^{a}
	FD+	0.167 ± 0.027^{a}	0.133 ± 0.027^{a}	0.167 ± 0.027^{a}	0.133 ± 0.027^{a}	0.153 ± 0.024^{a}	0.203 ± 0.050^{a}
PIN	FD-	1.023 ± 0.127^{b}	1.287 ± 0.361^{b}	0.583 ± 0.168b	2.848 ± 0.404^{a}	0.485 ± 0.059^{b}	0.310 ± 0.024^{b}

	FD+	0.707 ± 0.274^{b}	0.373 ± 0.145^{b}	0.463 ± 0.155^{b}	1.123 ± 0.166^{b}	0.257 ± 0.047^{b}	0.433 ± 0.118^{b}
PGIP	FD-	1.023 ± 0.122^{b}	1.073 ± 0.238^{b}	0.393 ± 0.049^{b}	2.780 ± 0.372^{a}	0.655 ± 0.078^{b}	0.563 ± 0.017^{b}
	FD+	0.277 ± 0.121^{b}	0.703 ± 0.205^{b}	0.110 ± 0.026^{b}	0.267 ± 0.071^{b}	0.063 ± 0.023^{b}	0.243 ± 0.039^{b}
GLU	FD-	1.003 ± 0.031^{b}	0.980 ± 0.087^{b}	0.467 ± 0.166^{bcd}	1.790 ± 0.086^{a}	0.823 ± 0.100^{bd}	0.643 ± 0.023^{bc}
	FD+	0.607 ± 0.050^{bd}	0.577 ± 0.166^{bd}	0.200 ± 0.045^{d}	1.833 ± 0.165^{a}	0.240 ± 0.062^{d}	1.170 ± 0.179^{abc}
STS	FD-	1.010 ± 0.073^{b}	1.110 ± 0.171^{b}	0.303 ± 0.058^{bc}	2.543 ± 0.318^{a}	0.485 ± 0.059^{bc}	0.405 ± 0.012^{bc}
	FD+	0.350 ± 0.022^{bc}	0.427 ± 0.046^{bc}	0.113 ± 0.024^{c}	0.227 ± 0.028^{c}	0.117 ± 0.023^{c}	0.220 ± 0.062^{c}
PAL	FD-	1.007 ± 0.066^{b}	1.220 ± 0.212^{b}	0.557 ± 0.121^{b}	3.435 ± 0.573^{a}	0.900 ± 0.108^{b}	0.770 ± 0.014^{b}
	FD+	0.670 ± 0.085^{b}	0.847 ± 0.105^{b}	0.557 ± 0.048^{b}	0.713 ± 0.105^{b}	0.327 ± 0.052^{b}	0.720 ± 0.130^{b}

Table 5. Relative fold of expression of 12 target genes (see legend of figure 15) measured in healthy (FD-) and "flavescence dorée" infected (FD+) grapevines cv. Loureiro elicited with 0, 12.5 and 25 mM MeJA applied twice (before flowering and at veraison, BF+V) or once (at veraison, V), in 2016, before elicitation (T0) and 24 hours (T1) after MeJA application. Data are means ± SEM of 4 biological replicates. For each gene, letters indicate statistical differences ($P < 0.05$) according to two-way ANOVA followed by Tukey's post-hoc test.

		Treatment							
Gene	**Plant**	**0 mM MeJA (BF+V) T0**	**0 mM MeJA (BF+V) T1**	**12.5 mM MeJA (BF+V) T0**	**12.5 mM MeJA (BF+V) T1**	**25 mM MeJA (BF+V) T0**	**25 mM MeJA (BF+V) T1**	**25 mM MeJA (V) T0**	**25 mM MeJA (V) T1**
Rubisco	FD-	1.030 ± 0.115 ^{a}	1.006 ± 0.106^{a}	0.767 ± 0.024^{a}	0.828 ± 0.042^{a}	0.728 ± 0.067^{a}	1.049 ± 0.084^{a}	0.793 ± 0.053^{a}	0.995 ± 0.109^{a}
	FD+	0.753 ± 0.018^{a}	0.791 ± 0.083^{a}	0.763 ± 0.083^{a}	0.866 ± 0.052^{a}	0.773 ± 0.079^{a}	1.103 ± 0.139^{a}	1.068 ± 0.074^{a}	0.995 ± 0.139^{a}
Prota5s	FD-	0.913 ± 0.021^{a}	3.528 ± 0.819^{a}	0.983 ± 0.123^{a}	1.300 ± 0.078^{a}	1.047 ± 0.056^{a}	3.315 ± 0.905^{a}	1.083 ± 0.066^{a}	2.373 ± 0.470^{a}
	FD+	1.165 ± 0.112^{a}	2.770 ± 0.910^{a}	1.118 ± 0.145^{a}	3.860 ± 0.611^{a}	1.533 ± 0.112^{a}	4.113 ± 0.864^{a}	1.213 ± 0.122^{a}	3.303 ± 0.785^{a}
Thau I	FD-	0.907 ± 0.084^{ab}	0.780 ± 0.098^{ac}	0.300 ± 0.081^{cd}	0.913 ± 0.051^{ab}	0.257 ± 0.036^{d}	0.898 ± 0.069^{ab}	0.275 ± 0.043^{d}	0.885 ± 0.031^{ab}
	FD+	0.465 ± 0.065^{cd}	0.667 ± 0.092^{ad}	0.388 ± 0.102^{cd}	0.600 ± 0.087^{ad}	0.380 ± 0.071^{cd}	0.983 ± 0.067^{a}	0.170 ± 0.035^{d}	0.700 ± 0.098^{a}
Thau II	FD-	0.690 ± 0.103^{a}	0.608 ± 0.190^{bd}	0.550 ± 0.100^{cd}	0.453 ± 0.099^{ab}	0.655 ± 0.086^{cd}	0.430 ± 0.084^{acd}	0.618 ± 0.160^{d}	0.525 ± 0.133^{a}
	FD+	0.610 ± 0.110^{ab}	0.580 ± 0.169^{bd}	0.400 ± 0.150^{d}	0.513 ± 0.096^{abc}	0.687 ± 0.124^{bcd}	0.580 ± 0.157^{a}	0.550 ± 0.221^{d}	0.760 ± 0.080^{a}
Osm	FD-	0.415 ± 0.036^{cd}	1.020 ± 0.110^{ab}	0.318 ± 0.068^{cd}	1.080 ± 0.077^{ab}	0.398 ± 0.106^{cd}	1.403 ± 0.101^{a}	0.228 ± 0.079^{cd}	1.089 ± 0.147^{ab}
	FD+	0.545 ± 0.066^{bd}	1.093 ± 0.142^{ab}	0.227 ± 0.040^{d}	1.233 ± 0.061^{a}	0.320 ± 0.045^{cd}	0.525 ± 0.078^{bd}	0.263 ± 0.074^{cd}	1.203 ± 0.112^{a}
PBSP	FD-	1.177 ± 0.105^{b}	1.220 ± 0.075^{ab}	1.504 ± 0.209^{ab}	1.402 ± 0.052^{ab}	1.610 ± 0.159^{ab}	2.260 ± 0,027^{a}	1.143 ± 0,137^{b}	1.583 ±0.130^{ab}
	FD+	1.847 ± 0.194^{ab}	1.323 ± 0.094^{ab}	1.720 ± 0.083^{ab}	1.550 ± 0.225^{ab}	1.930 ± 0.049^{ab}	1.653 ± 0.176^{ab}	1.427 ± 0.062^{ab}	1.957 ± 0.181^{ab}
CHIT4c	FD-	1.078 ± 0.232^{d}	3.380 ± 0.940^{cd}	1.830 ± 0.332^{cd}	7.200 ± 0.849^{bc}	1.175 ± 0.295^{d}	10.050 ± 0.489^{ab}	3.468 ± 1.022^{cd}	3.080 ± 0.615^{cd}
	FD+	2.488 ± 0.408^{cd}	3.663 ± 0.572^{cd}	5.325 ± 0.803^{c}	12.69 ± 0.583^{a}	4.107 ± 0.450^{cd}	9.280 ± 0.596^{ab}	3.187 ± 0.701^{cd}	13.755 ± 0.378^{a}
PIN	FD-	1.925 ± 0.423^{b}	1.300 ± 0.161^{b}	1.680 ± 0.332^{b}	4.653 ± 0.875^{ab}	2.120 ± 1.425^{b}	9.883 ± 0.570^{a}	1.595 ± 0.757^{b}	2.073 ± 0.606^{b}

	FD+	0.473 ± 0.072^{b}	2.218 ± 0.954^{b}	1.340 ± 0.527^{b}	2.648 ± 0.949^{b}	0.805 ± 0.234^{b}	3.028 ± 1.398^{b}	0.768 ± 0.425^{b}	1.835 ± 0.652^{b}
PGIP	FD-	1.873 ± 1.075^{c}	3.978 ± 1.816^{c}	3.547 ± 1.157^{c}	14.45 ± 1.794^{ab}	0.975 ± 0.381^{c}	22.83 ± 1.356^{a}	1.607 ± 0.074^{c}	2.319 ± 0.206^{c}
	FD+	1.013 ± 0.154^{c}	3.990 ± 1.410^{bc}	4.390 ± 1.626^{c}	17.55 ± 0.917^{a}	1.828 ± 0.376^{c}	7.197 ± 0.753^{c}	1.263 ± 0.355^{c}	4.823 ± 0.491^{bc}
GLU	FD-	0.967 ± 0.068^{c}	1.343 ± 0.262^{c}	2.165 ± 0.428^{bc}	3.503 ± 0.538^{b}	0.385 ± 0.101^{c}	6.913 ± 0.784^{a}	0.763 ± 0.105^{c}	0.703 ± 0.056^{c}
	FD+	0.627 ± 0.055^{c}	1.655 ± 0.352^{bc}	1.648 ± 0.247^{bc}	6.028 ± 0.572^{a}	0.653 ± 0.102^{c}	4.618 ± 0.509^{ab}	0.603 ± 0.076^{c}	1.315 ± 0.172^{c}
STS	FD-	1.760 ± 0.979^{cd}	3.415 ± 0.650^{bcd}	4.697 ± 0.304^{abc}	7.773 ± 0.651^{a}	0.965 ± 0.225^{d}	5.694 ± 1.221^{ab}	0.925 ± 0.264^{d}	2.520 ± 0.400^{bcd}
	FD+	1.050 ± 0.134^{d}	2.793 ± 0.202^{bcd}	1.785 ± 0.311^{cd}	5.115 ± 0.407^{ac}	0.983 ± 0.269^{d}	8.075 ± 0.642^{a}	1.108 ± 0.177^{d}	5.028 ± 0.570^{ac}
PAL	FD-	0.543 ± 0.107^{bcd}	0.923 ± 0.104^{ac}	0.198 ± 0.038^{d}	0.675 ± 0.100^{acd}	0.143 ± 0.055^{d}	0.878 ± 0.110^{abc}	0.523 ± 0.099^{bcd}	0.870 ± 0.036^{bc}
	FD+	0.445 ± 0.106^{cd}	0.948 ± 0.037^{ac}	0.283 ± 0.126^{d}	0.870 ± 0.094^{ab}	0.338 ± 0.068^{d}	0.965 ± 0.072^{ab}	0.658 ± 0.048^{bcd}	1.110 ± 0.031^{a}

3.2. Methyl jasmonate triggers metabolic responses and gene expression on *Vitis vinifera* cv. Loureiro infected with "flavescence dorée"

Abstract

"Flavescence dorée" (FD) is a grapevine epidemic quarantine phytoplasma disease associated with high yield losses. This study aimed to evaluate the effect of methyl jasmonate (MeJA) on the induction of defence mechanisms of grapevines cultivar Loureiro against FD, comparing defence metabolites production (proline and saponins) and gene expression patterns in healthy and infected grapevine plants. MeJA treatments significantly increased saponin and proline synthesis only in infected plants. Moreover, it induced gene expression of Rubisco activase and pathogen-related proteins (PBSP, CHITC4c and PIN) quantified 6 hours after the MeJA application.

Key words: "flavescence dorée", gene expression, grapevine, methyl jasmonate, proline, saponin

3.2.1. Introduction

"Flavescence dorée" (FD) is a grapevine quarantine disease widespread in Europe. During the last years, some studies have been developed to control FD impact and spread (Margaria and Palmano, 2011). Elicitors are becoming important tools in disease management, nevertheless studies testing their potential role to mitigate FD symptoms are scarce (Oliveira *et al.*, 2019a). This study aims to understand the effect of methyl jasmonate on the synthesis of defence metabolites and gene expression activation on FD-infected grapevines.

3.2.2. Materials and methods

3.2.2.1. Field treatments

The study was carried out between June (1 week before flowering) and August (veraison) 2016 at Fafe, Portugal, in a twenty-year-old vineyard cultivar 'Loureiro' where FD was detected in 2009. A selection of 16 healthy and 16 infected grapevines was divided in four groups: control plants (untreated) and plants treated with 0, 12.5 and 25 mM of methyl jasmonate (MeJA) dissolved in 2.5% ethanol, applied before flowering, and repeated at veraison.

3.2.2.2. Metabolites quantification

Saponins and proline were extracted and quantified in leaves collected in September (12 weeks after MeJA application), according to Patel *et al.* (2012) and Bates *et al.* (1973), respectively.

3.2.2.3. Gene expression

Leaf samples were collected before treatments (T0) and 6 hours after MeJA application (T1). RNA extraction was performed with a RNeasy Plant Mini Kit (QIAGEN GmbH), according to Le Provost *et al.* (2007) and RNA yield and quality were measured using a nano-photometer. Synthesis of complementary DNA was performed using the iScrip cDNA Kit in a thermal cycler. RT-qPCR was performed using the NZY Taq 2x Green Master Mix Kit with l denaturation of 120 seconds at 95°C followed by 25 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 60 seconds at 72°C; the final extension was of 5 seconds at 72°C. 18S rDNA and ubiquitin were used as housekeeping genes to normalize the Ct values. A total of 12 target genes were selected encoding: (i) plant PR proteins (thaumatin I – ThauI, thaumatin II – ThauII, Osmotin-like protein – Osm, PBSP – Plant Basic Secretory Protein, CHIT4c – Acidic Class IV Chitinase, PIN – Inhibitors of Serine Protease, PGIP – Polygalacturonase-inhibiting Protein and GLU – β-1,3-glucanase), (ii) enzymes involved in primary metabolism (RubAct – Rubisco Activase AAA+), (iii) protein degradation (Protα5 – Proteasome α5 subunit) and (iv) phenylpropanoid biosynthesis (STS – Stilbene Synthase and PAL – Phenylalanine Ammonia Lyase) (Belhadj *et al.*, 2006; 2008; Margaria and Palmano, 2011).

3.2.3. Results and Discussion

Untreated plants, when infected with FD showed 54% lower saponins' concentration compared with healthy plants (Fig. 16A). Interestingly, when FD plants were elicited with 12.5 and 25 mM MeJA the saponin concentration increased by 3.1-fold and 2-fold higher, respectively, as compared with untreated FD plants. Proline concentration was only significantly higher (2-fold) in infected plants treated with 25 mM MeJA (Fig. 16B).

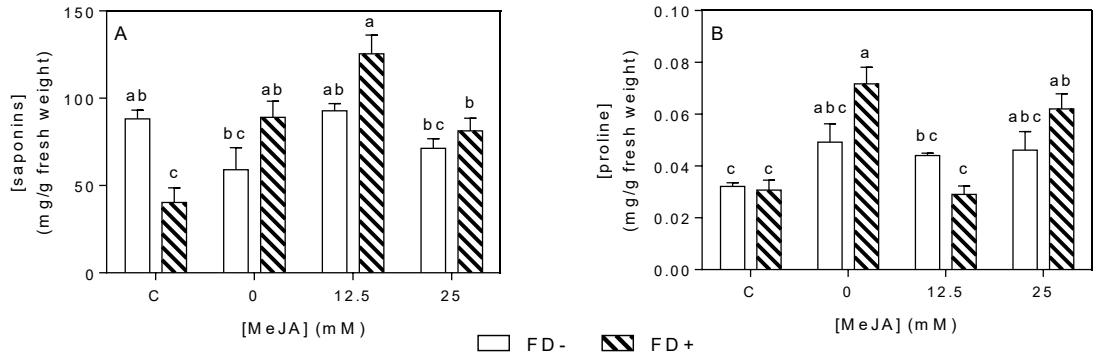

Figure 16. Saponins (A) and proline (B) concentration in healthy (FD-) and "flavescence dorée" infected (FD+) grapevine cultivar Loureiro untreated (C) and treated with 0 (solvent), 12.5 and 25 mM MeJA measured 12 weeks after the elicitor application. Data are means ± SEM of 4 biological replicates. Different letters indicate significant differences ($P < 0.05$) according to two-way ANOVA.

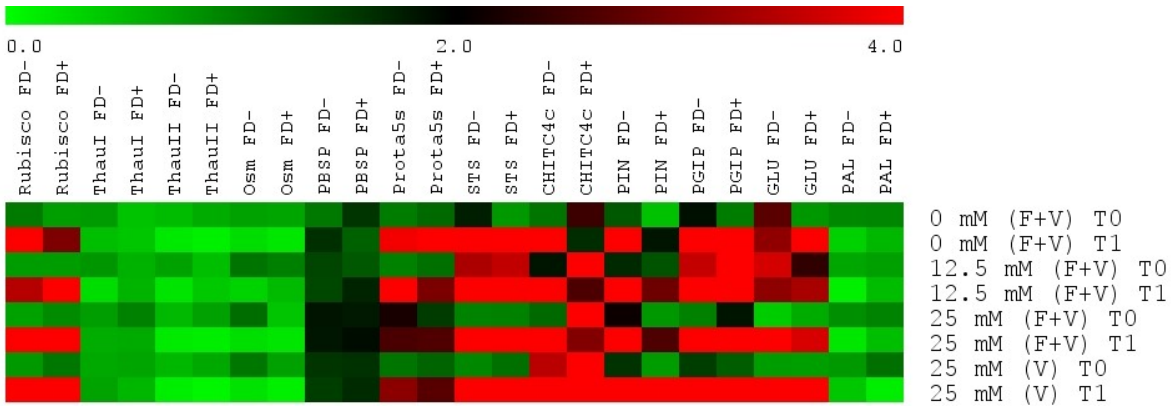

Figure 17. Heat map obtained by RTqPCR of leaf samples from healthy (FD-) and infected (FD+) grapevines cv. Loureiro before (T0) and 6 hours after (T1) MeJA application performed at flowering and at veraison (F+V) or only at veraison (V).

This study showed that both MeJA concentrations upregulated *Rubisco*, *Protα5s*, *STS*, *CHITC4c*, *PIN* and *PGIP* genes in healthy grapevines (Figure 17). In diseased plants a single MeJA application at veraison lead to an upregulation of *Rubisco*, *PIN*, *PGIP* and *GLU* genes in FD plants, whereas a double application of this elicitor additionally induced an overexpression of *CHITC4c* gene 6 hours after treatment. An under expression of *ThauI*, *ThauII*, *Osm* and *PAL* genes was found when applying 25 mM MeJA in FD plants (single or double application). The elicitation with both MeJA concentrations in FD infected grapevines highly enhanced the saponins concentration, whereas the proline concentration was only significantly increased at 25 mM MeJA. Interestingly, in healthy plants no effects were found on these metabolites' concentration in response to MeJA. It is however important to stress out that part of the MeJA elicitation effect on the described metabolic responses seem to be related to the solvent *per se*, since its application (0 MeJA treatment) in FD plants induced a significant increase of the concentration of saponins and proline. Therefore, more studies are needed to clarify these responses. The *Rubisco* gene was downregulated in untreated FD infected plants as compared to healthy in agreement with a previous study reporting that FD repression of photosynthetic genes (Margaria and Palmano, 2011). In contrast with the present work, other authors have shown an increase in expression of *ThauI*, *ThauII* and *Osm* genes in grapevines infected with FD (Margaria and Palmano, 2011) and "bois noir" (Albertazzi *et al.*, 2009), but their study did not evaluate gene expression pattern following MeJA treatments. Here, MeJA concentrations upregulated *CHITC4c*, *PIN* and *PGIP* genes in FD infected plants, which in turn is in accordance with earlier studies conducted in healthy grapevines that demonstrated a higher expression of *STS*, *CHIT4c*, *PIN* and *PGIP* genes following MeJA application (Belhadj *et al.*, 2006; Martinez-Esteso *et al.*, 2009). This study contributes to a better understanding of the defence mechanisms triggered by the MeJA application in FD-infected grapevines under field conditions.

3.3. Does salicylic acid alleviate the impacts on growth, development, and productivity of "flavescence dorée" in Portuguese "Vinhos Verdes" grapevines?

Abstract

"Flavescence dorée" (FD) is a quarantine disease of great concern to the stability and sustainability of the wine industry, due to drastic harvest losses and death of infected plants. Previous studies have suggested that salicylic acid (SA) may improve plant resistance against grapevine diseases, but no studies have been carried out in FD. The effect of 25 mM SA applied at flowering stage to healthy and FD infected grapevines cultivar Loureiro was evaluated in a field trial. The evaluation of development and productivity parameters (percentage of budburst, fertility index, time to veraison, and chlorophyll content) and grape quality parameters (degree Brix and titrable acidity) was carried out. Fertility index and chlorophyll content were significantly lower in infected plants and SA partly reduced the FD symptoms, since at veraison these plants did not show significant differences when compared to healthy plants for those parameters. However, the productivity was also significantly lower in infected plants with no significant effect of SA application. Concerning berry quality was not significantly different comparing to healthy and infected plants or SA-treated and untreated plants.

Key words: berry quality, fertility index, salicylic acid, yield.

3.3.1. Introduction

"Flavescence dorée" (FD) is a quarantine disease transmitted by insects (*Scaphoideus titanus* and *Orientus ishidae*) and by the commercialization of infected vegetative propagation material. The current management strategies for FD control are costly, difficult to implement and have a high environmental impact (Oliveira *et al.*, 2019a). Elicitors such as salicylic acid (SA) could be exploited since they activate multiple plant defence systems against several pathogens (Dodds and Rathjen, 2010). Other studies with an SA analogue (benzothiadiazole) showed in grapevine plants induced resistance towards *Plasmopara viticola*, *Erisyphe necator*

(Dufour *et al.*, 2013) and *Botrytis cinerea* (Iriti *et al.*, 2005), reducing the disease symptomatology. This study evaluated the potential of SA on the mitigation of the negative effects of FD on grapevine growth, development, and productivity.

3.3.2. Materials and methods

3.3.2.1. Field treatments

The study was carried out between April (budburst) and September (harvest) 2015 in Fafe, Portugal, in a twenty-year-old vineyard of cultivar Loureiro. A total of 16 grapevines were selected: 8 healthy and 8 FD-infected. Salicylic acid was diluted in Milli-Q water and was applied to the plants at the flowering stage at 0 mM (control) and 25 mM SA (n=4).

3.3.2.2. Biometric measurements

Phenological stages were registered biweekly until flowering and then weekly according to the Baggiolini (1952) scale. The buds left on the shoots after winter pruning were used to calculate the percentage of budburst and fertility index (Alonso *et al.*, 2007), according to the formula:

$$\%\ \text{of budburst} = \frac{\text{no. of sprouted buds}}{\text{pruning load}} \times 100 \qquad \text{Fertility index} = \frac{\text{no. of bunches}}{\text{pruning load}}$$

Chlorophyll content was assessed weekly using a SPAD meter on five leaves randomly selected from each plant. Leaf area was determined using the method of Lopes and Pinto (2005) and both assays were performed in triplicate.

3.3.2.3. Productivity

The number of bunches and the average bunch weight were determined using five berries per bunch collected randomly in six bunches. The total soluble solids (ºBrix) and titrable acidity were measured and statistical analysis was carried out with two-way ANOVA.

3.3.3. Results

Phenological stages evolution revealed that FD infected plants showed a significant ($P < 0.05$) delay in development, resulting on extra 23 days in time to veraison compared to healthy plants and the 25 mM SA application at flowering had no effect on this parameter (Table 6). However, both groups of plants did not show differences in the budburst rate. Fertility index and chlorophyll content were significantly lower, 39% and 36% lower, respectively, in infected plants however at veraison these plants did not differ significantly from the healthy ones on these parameters (Table 6). The FD infected plants had a 65% lower yield due to a significant reduction on the number of bunches while the average bunch weight was not affected (Table 7). No significant differences were observed between healthy and infected or between SA-treated and untreated plants in the berry quality.

Table 6. Growth and development parameters evaluated at veraison in healthy (FD-) and "flavescence dorée" infected (FD+) grapevines cv. Loureiro. Data are means ± SEM. Identical letters indicate statistically identical values.

Plant	[SA] (mM)	Time to veraison (days)*	Budburst (%)	Fertility index	Chlorophyll content (SPAD values)	Leaf area increase (%)**
FD$^-$	0	201.5 ± 1.3 ^{b}	81.0 ± 3.2 ^{a}	1.05 ± 0.07 ^{a}	38.1 ± 1.7^{a}	52.9 ± 7.2 ^{a}
	25	203.0 ± 1.2 ^{b}	79.4 ± 2.7 ^{a}	0.85 ± 0.08 ^{ab}	37.3 ± 0.9^{a}	112.8 ± 32.3 ^{a}
FD$^+$	0	225.3 ± 1.5 ^{a}	78.2 ± 2.7 ^{a}	0.65 ± 0.06 ^{b}	24.4 ± 0.5^{b}	86.7 ± 0.7 ^{a}
	25	225.8 ± 1.1 ^{a}	67.8 ± 4.6 ^{a}	0.79 ± 0.04 ^{ab}	30.5 ± 3.1^{ab}	136.7 ± 4.4^{a}

*days of the year (1 jan = day 1)

**relative increase in leaf area from flowering to veraison

Table 7. Productivity in healthy (FD-) and FD infected (FD+) grapevines cv. Loureiro at harvest stage. Data are means ± SEM. Identical letters indicate statistically identical values.

Plant	[SA] (mM)	Productivity (kg.plant^{-1})	No of bunches	Average bunch weight (g)	TSS (ºBrix)	TA (g tartaric acid.L^{-1})
FD$^-$	0	23.8 ± 1.2 ^{a}	52.8 ± 6.6 ^{a}	480.0 ± 64.3 ^{a}	16.9 ± 0.7 ^{a}	8.9 ± 0.2 ^{a}
	25	23.3 ± 2.0 ^{a}	43.8 ± 6.7 ^{ab}	565.2 ± 60.1 ^{a}	16.7 ± 0.9 ^{a}	7.7 ± 0.3 ^{a}
FD$^+$	0	8.3 ± 0.3 ^{b}	17.3 ± 1.0 ^{b}	488.5 ± 45.9 ^{a}	16.6 ± 0.2 ^{a}	9.0 ± 0.5 ^{a}
	25	6.0 ± 0.7 ^{b}	19.0 ± 0.7 ^{ab}	313.9 ± 25.5 ^{a}	16.8 ± 0.3 ^{a}	8.8 ± 0.8 ^{a}

3.3.4. Discussion

FD presence is associated with unripen cane wood (Caudwell, 1957), leading to a delay in reaching veraison as observed in the present study. In spite the positive effect of SA application on growth and development parameters in FD infected plants, it had no significant effect on grapevine productivity. This result is likely due to the lower pruning load left on FD plants reflecting their lower vigour. This is the first study on SA application to grapevines infected with FD and it is concluded that this elicitor seems to have a beneficial effect on plant growth and development, but only for a restricted number of parameters. This result opens doors for the development of more effective and environmentally friendly tools for phytoplasma disease control. However, more studies should be carried out in order to further verify if diverse elicitor concentrations or other time of application would induce a better plant responses.

Chapter 4

THE ROLE OF ELICITORS ON GENOMICS, METABOLOMICS AND BIOCHEMISTRY OF *IN VITRO* SHOOTS OF *CATHARANTHUS ROSEUS* INFECTED BY PHYTOPLASMAS

The data presented in this chapter was submitted/in preparation to publication:

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Oliveira, M.J.R.A., Carvalho, S.M.P., Gonçalves, P.A.F., Monforte, A.R., Ferreira, A.C., Bertaccini, A., Vasconcelos, M.W. 2021. Methyl jasmonate enhances gene expression and metabolomic profile of alkaloids and phenolic compounds in *Catharanthus roseus* infected with "aster yellows" phytoplasmas. (submitted)

4.1. The role of methyl jasmonate and benzothiadiazole on biochemical responses of *Catharanthus roseus* shoots infected with aster yellows phytoplasmas

Abstract

Phytoplasmas are wall-less bacteria responsible for worldwide devastating diseases having great economic impacts. So far, there are no effective and sustainable treatments against phytoplasma diseases, being insecticides against vectors, hot water treatment and roguing the infected plants the most used strategies. Elicitors have been successfully applied as preventive and environmentally friendly treatments in several diseases, but there is limited information on their effect in phytoplasmas. In the present work two elicitors – methyl jasmonate (MeJA) and benzothiadiazole (BTH) – were applied to three cloned shoot strains of *in vitro* micropropagated *Catharanthus roseus*: healthy, aster yellows infected with mild symptomatology (strain AY107) and infected with severe symptomatology (strain Hyd8). Lipid peroxidation and contents of total flavonoids, jasmonic, salicylic, and abscisic acids were measured in shoots treated with 0, 12.5 and 25 mM MeJA and with 0, 3.5 and 7 mM BTH, one and four days after elicitation (dae). The concentrations of 25 mM MeJA and 3.5 mM BTH showed the lowest malondialdehyde production 4 dae, with a decrease of 56% (in Hyd8 infected shoots) and 61%, (in AY107 infected shoots) respectively. However, the MeJA application did not have a significant effect in the total flavonoids content, while following 3.5 mM BTH application this has been increased by 29% in AY107 infected periwinkle shoots 1 dae. The high content of abscisic acid in the untreated shoots of the strain AY107 is an indication of active phytoplasma infection as well as both elicitors reduced the phytoplasma symptomatology decreasing the abscisic acid content. In the shoots infected with Hyd8 strain 12.5 mM MeJA and 7 mM BTH resulted the most efficient at 4 and 1 dae, respectively.

Keywords: *Catharanthus roseus*, elicitors, flavonoids, lipid peroxidation, phytohormones, phytoplasmas.

4.1.1. Introduction

Phytoplasmas are wall-less prokaryotes which are restricted to phloem tissues, and are amongst the most recently discovered plant pathogens. They are associated with over 600 diseases worldwide affecting several hundreds of plant species, including relevant agronomic crops (*e.g.*, "flavescence dorée" and "bois noir" in grapevine, apple proliferation in apple trees and European stone fruits yellows in apricot trees) (Musetti *et al.*, 2004, 2005; Fiore *et al.*, 2018; Oliveira *et al.*, 2019a). These pathogens are responsible for plant decline and severe yield losses leading to a large economic impacts (Kumari *et al.*, 2019).

Although, until now, there are no effective and environmentally friendly strategies against phytoplasma infections, some novel approaches to symptomtology mitigation have been suggested and developed, such as elicitors application, endophytes inoculation, mating disruption for insect vectors and new imaging technologies (Lacava *et al.*, 2007; Perazzolli *et al.*, 2008; Musetti *et al.*, 2011; Oliveira *et al.*, 2019a; Buja *et al.*, 2021). Elicitors are a class of compounds with an important biological role in plants and that may improve resistance against plant pathogens. These compounds have several advantages, such as ecological safety, long-drawn systemic effects, induction of multiple defence systems and of non-specific resistance to several pathogens (Zhao *et al.*, 2005; Dodds and Rathjen, 2010), increasing plant growth and development and often having a positive effect on crop yield and fruit quality (Portu *et al.*, 2016; Paladines-Quezada *et al.*, 2018; García-Pastor *et al.*, 2019).

Jasmonic acid (JA) is an endogenous phytohormone, derived from fatty acids, that acts against pathogen attack and wounding, triggering defence responses locally and systemically (Ramirez-Estrada *et al.*, 2016). Methyl jasmonate (MeJA) is a JA-derived compound with an important role in signal transduction processes, which regulate the induction of plant defence mechanisms in several pathosystems (Repka *et al.*, 2004; Belhadj *et al.*, 2008). The latter is one of the most studied chemical elicitors, and together with salicylic acid (SA) and ethylene, it has been applied to a significant number of plant species mostly in laboratory culture systems, to study phytohormones and secondary metabolites production (flavonoids, phenolic acids, and alkaloids) (reviewed by Giri and Zaheer, 2016). This review also reported that MeJA, beyond other chemical elicitors, has shown a multitasking role in triggering an array of cellular activities, allowing to understand the biosynthesis of secondary metabolism as well as gene expresion and hormones production (Giri and Zaheer, 2016). Patt *et al.* (2018) demonstrated that its application to *Citrus sinensis* (L.) Osbeck (cv. Valencia sweet orange) increased the volatile emission levels of signalling compounds against the psyllid *Diaphorina citri*, the vector of '*Candidatus* Liberibacter asiaticus' agent of "huanglongbing" citrus disease.

MeJA-treated plants showed aggregations of nine or more psyllids on them, indicating that exogenous applications of MeJA could be used to influence Asian citrus psyllid settling behavior and attract its natural enemies. Moreover, a trial conducted in "flavescence dorée" (FD) field-infected grapevine cv. Loureiro has shown that MeJA application increased the secondary metabolites content. Indeed, when FD plants were elicited with 12.5 and 25 mM MeJA the saponin concentration increased by 3.1-fold and 2-fold higher, respectively, when compared with untreated FD ones. Also proline concentration was significantly higher (2-fold) in infected plants treated with 25 mM MeJA (Oliveira *et al.*, 2019b). However, despite the aforementioned study, to the best of our knowledge there are no studies which have determined whether MeJA application has the potential to be used to trigger defence mechanisms in phytoplasma infected plants and to contribute to phytoplasma diseases management in commercial plants.

Benzothiadiazole (BTH) is a synthetic functional analogue of salicylic acid (SA) that acts similarly to SA, activating plant systemic aquired resistance (SAR) in several crop species (Iriti *et al.*, 2011; Gozzo and Faoro, 2013). Since 2005 some studies were carried out to study BTH efficacy on the induction of SAR in phytoplasma-infected plants. Bressan and Purcell (2005) showed that BTH demonstrated a protective effect in *Arabidopsis thaliana* against X-disease phytoplasma, as well as a repellent effect to its leafhopper vector *Collodonus montanus* (van Duzee). BTH also delayed the symptoms expression and reduced the '*Candidatus* Phytoplasma asteris' (strain CY) multiplication in chrysanthemum (D'Amelio *et al.*, 2007) and daisy (D'Amelio *et al.*, 2010). A recent study conducted in "bois noir"-infected grapevines demonstrated that, although BTH did not induce symptom reduction in cv. Barbera, it had a strong effect against the insect vector *Scaphoideus titanus*, reducing its capacity to transmit FD phytoplasmas grapevine to grapevine (Miliordos *et al.*, 2016). In contrast, two other studies showed that two consecutive years' applications of BTH increased the recovery rate of grapevines cv. Chardonnay, increasing the number of recovered plants up to 130% (Romanazzi *et al.*, 2009b; 2009c).

Although salicylate- and jasmonate-mediated responses against pathogen infection have been reported to be antagonistic (Paolacci *et al.*, 2017), both have been applied to activate plant defence mechanisms. Even more recent studies conducted in grapevine cv. Monastrell (syn. Mourvèdre), showed that the combined application of MeJA and BTH improved grape and wine quality as well as grapevine protection against fungal pathogens such as *Botrytis cinerea* (Ruiz-García *et al.*, 2012; Ruiz-García *et al.*, 2013). MeJA and BTH application were shown to be dependent from the grapevine variety and meteorological conditions since their application improved berry quality (skin cell walls, phenolic compounds, proteins, and sugars) in cvs. Monastrell (syn. Mourvèdre) and Cabernet Sauvignon, maintaining berry integrity, while in cv. Merlot the elicitor treatment reduced the

parameters aforementioned, decreasing also berry integrity (Paladines-Quezada *et al.*, 2018). In general elicitors, in particular, MeJA and BTH, have been successfully applied in several crops, either as an elicitor or as a biostimulant.

Infected plants usually respond by increase cell oxidation status, triggering secondary metabolism pathways (*e.g*., flavonoids biosynthesis), leading to plant hormonal imbalance (Prezelj *et al.*, 2016). Malondialdehyde (MDA) is a biomarker of oxidative damage stress in cells, resulting in lipid peroxidation, caused by the susceptibility of membranes to reactive oxygen species (ROS) presence (Hodges *et al.*,1999). Some studies reported that, despite MDA can also be considered as an indicator of oxidative damage, its concentration was decreased after treatments against plant diseases (Lou *et al.*, 2011; Abdollahi *et al.*, 2012). Thus, a study conducted on tomato plants treated with oligandrin before *B. cinerea* infection showed that in the elicited plants the MDA content was reduced by 26% (Lou *et al.*, 2011). Abdollahi *et al.* (2012) described that lime trees treated with electromagnetic field show a reduction of the witches' broom intensity associated with the presence of '*Ca.* P. aurantifolia' without showing lipid membrane damages, and having a significant increase of the antioxidant enzymes activity.

Concerning the difficulty to cultivate phytoplasmas (Contaldo *et al.*, 2012; 2016; 2019), and thus to test potential antiphytoplasma compounds, *Catharanthus roseus* (L.) G. Don., known as periwinkle has been used as a model system to study phytoplasma-plant interactions and for maintaining in *in vitro* culture phytoplasma strains (Bertaccini *et al.*, 1992). Moreover, this specie has also been used to study defence mechanisms against several other pathogens, in which MeJA and SA concentrations have been reported to increase due to trigger induced systemic resistance (ISR) and SAR, stimulating the terpenoid indole alkaloids, flavonoids, and phenolic compounds production (reviewed by Mustafa and Verpoorte, 2007). Polyamines were the first applied compounds that induced a symptom reduction in periwinkles (Musetti *et al.*, 1999), while treatments carried out with tetracyclines showed to have a bactericidal effect on phytoplasmas (Ishiie *et al.*, 1967; Singh *et al.*, 2007). The first report of remission of phytoplasma symptoms in micropropagated shoots was shown by Perica *et al.* (2007) demonstrating that aster yellows infected *C. roseus* shoots growing in Murashige and Skoog (1962) medium supplemented with different concentrations of auxins indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) induced the undetectability of '*Ca.* P. asteris' by nested PCR, increasing also shoots elongation, fresh weight and photosynthetic performance (Ćurković-Perica *et al.*, 2007; Ćurković-Perica, 2008), in contrast with β-aminobutyric acid (BABA), no curative effects were observed (Ćurković-Perica and Seruga-Music, 2005).

Despite there are many studies that have been reported the role of elicitors, this is the first study which study the role of MeJA and BTH on phytoplasma-infected plants. Moreover, although

several evidences have demonstrated that elicitors might be an important tool to phytoplasma diseases management, there is little knowledge on their role on plant responses in terms of lipid peroxidation protection, secondary metabolites and phytohormones production contributing to a better understanding of their mode of action. Building up the aforementioned information, we hypothesize that MeJA and BTH have an elicitor effect on aster yellows phytoplasma and that plant protection mechanisms are partly explained by their role on phytohormones profile (namely ABA, JA and SA) and on MDA and secondary metabolites content. This study therefore aimed to evaluate the role of MeJA and BTH on lipid peroxidation (MDA production), flavonoids, and phytohormones in periwinkle shoots infected with two aster yellows phytoplasma strains associated with different symptomatology.

4.1.2. Material and Methods

4.1.2.1. Plant material and growth conditions

The work was carried out using three groups of periwinkles [*Catharanthus roseus* (L.) G. Don.] clones micropropagated: healthy (HV) and infected with aster yellows phytoplasmas strains (16SrI-B) showing mild symptoms (strain AY107) and severe symptoms (Hyd8, hydrangea phyllody) from the EPPO-Q-Bank collection maintained at the University of Bologna (Italy) (Bertaccini *et al.*, 1992; Bertaccini, 2014). Periwinkle shoots were grown in Murashige and Skoog (MS) (1962) medium supplemented with 0.12 mg/l 6-benzylaminopurine (BAP) (Bertaccini *et al.*, 1992), and were maintained in a growth chamber (F600 Aralab, Sintra, Portugal), with a photoperiod of 16 h, light intensity of 180 $\mu mol.s^{-1}.m^{-2}$, 85% of relative humidity, and a range of temperature between 20-22 ºC.

4.1.2.2. Preparation of elicitors' solutions

MeJA (Sigma-Aldrich, St. Louis, Missouri, EUA) was prepared from a solvent stock solution of 2.5% ethanol and 0.1% Tween 20 (Sigma-Aldrich, St. Louis, Missouri, EUA). To optimize the efficacy and dosage of MeJA application, four treatment groups were used per strain: control, with no treatment was done, 0 mM MeJA (MeJA solvent, ethanol 2.5 % (v/v)), 12.5 and 25 mM MeJA.

BTH (Sigma-Aldrich, St. Louis, Missouri, EUA) at the concentrations of 3.5 and 7 mM, dissolved in Milli-Q water. These concentrations have been selected based on a preliminary assay (data not shown), in which several concentrations were tested to choose the ones that were not toxic. Control shoots were not sprayed.

4.1.2.3. Shoot elicitation

Micropropagated shoots were sprayed with the elicitor solutions, applied to three independent biological replicates (each consisting of three shoots per *in vitro* flask). Sampling was carried out in two time points: 1 day after elicitation (1 dae) and 4 days after elicitation (4 dae). After sampling, plant material was powdered with liquid nitrogen and stored at -80ºC.

4.1.2.4. Spectrophotometric analyses

4.1.2.4.1. Malondialdehyde (MDA)

An adapted protocol from Health and Packer (1968) was used for MDA quantification. Fifty milligrams of sample were added to 500 µL of 0.1% trichloroacetic acid (w/v) and mixed vigorously for 90 sec. After sample centrifugation for 5 min at 10,000 g, 250 µL of the supernatant were transferred to a new microcentrifuge tube and mixed with 1 mL of 0.5% thiobarbituric acid in 20% trichloroacetic acid. The mixture was incubated at 100°C for 30 min, after which the reaction was stopped by rapidly transferring the samples to ice. Samples were centrifuged at 10,000 g for 10 min and the supernatant was used to measure absorbances spectrophotometrically at 532 and 600 nm in a nanophotometer (Implen GmbH, München, Germany). MDA (nmol.g^{-1} fresh weight) was determined by the following formula:

$$\mathrm{MDA} = \frac{(\mathrm{Abs}_{532} - \mathrm{Abs}_{600}) \times \mathrm{volume}}{\varepsilon = 155\mathrm{mM/cm} \times \mathrm{biomass}}$$

4.1.2.4.2. Total flavonoids content

Lyophilized plant tissues (50 mg) were extracted with 1.5 mL of 80% aqueous methanol (v/v) in an ultrasonic bath for 30 min. Samples were centrifuged for 15 min at 15,000 g and the supernatant was transferred to a new microcentrifuge tube kept in ice. For total flavonoids content quantification 2 mL of ultrapure water and 150 µL of 5% sodium nitrite were added to 100 µL of sample. After a 5 min incubation period at room temperature, 150 µL of 10% aluminium chloride, 1 mL of 1 M sodium hydroxide and 1.2 mL ultrapure water were added to each sample. Absorbance was recorded in a nanophotometer (Implen GmbH, München, Germany) at 510 nm and flavonoids concentration was determined considering a catechin standard curve (Chang *et al.*, 2002).

4.1.2.5. Phytohormones quantification

Abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) were quantified following an adapted protocol from de Ollas *et al.* (2013). Fifty milligrams of dried sample were mixed with 100 ng of $ABAd_6$, 100 ng of SAd_6 and 100 ng of dihydrojasmonic acid and homogenized with 2 mL of distilled water. After vigorous stirring with glass beads for 10 min, samples were centrifuged for 10 min at 12,000 g at 4°C and the supernatant was recovered to a new centrifuge tube. Extract pH was adjusted to 3 with 80% acetic acid, after which samples were added 2 mL of diethyl ether, mixed vigorously and the organic upper layer recovered and vacuum evaporated in SpeedVac (Jouan, Saint Herblain, France). The dry residue was resuspended with 1 mL of 10% methanol by sonication for 10 min and the resulting solution was filtrated through a 0.2 µm PTFE filter and injected into an HPLC system (Waters Alliance 2695, Waters Corp., Milford, MA, United States). Separations were carried out on a C18 column (Kromasil 100, 5 mm particle size, 100 2.1 mm, Scharlab, Barcelona, Spain) using a linear gradient of methanol and ultrapure water supplemented with 0.1% acetic acid at a flow rate of 300 $mL.min^{-1}$. Hormones were quantified with a Quattro LC triple quadrupole mass spectrometer (Micromass, Manchester, United Kingdom) connected online to the output of the column through an orthogonal Z-spray electrospray ion source. Phytohormones were quantified after external calibration against the standards using Mass Lynx v4.1 software (Waters Corporation, Massachusetts, USA).

4.1.2.6. 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 a two-way ANOVA followed by Tukey's post-hoc test with a confidence level of 95% ($P < 0.05$).

4.1.3. Results

4.1.3.1. Plant material

In vitro periwinkle shoots infected with phytoplasmas maintained the phytoplasma-associated symptoms such as leaf curling, abnormal proliferation, internode shortening, and stunting (Fig. 18). HV shoots showed lower micropropagation rates since these explants had a low branching capacity in this culture medium. During the growth period it could also be observed that periwinkle shoots infected with aster yellow phytoplasmas presented a different morphology when compared with healthy ones. HV shoots showed larger leaves and a greater elongation of internodes than Hyd8 and smaller than AY107. In the present work, the shoots of the strain AY107 showed stunted growth with etiolation and in some cases leaf yellowing. On the other hand, Hyd8 strain shoots showed witches' broom and dwarfism, with a shortening of the internodes, high adventitious sprouting and reduced number of leaves compared with healthy shoots showing regular internode distance without leaf yellowing and/or curling.

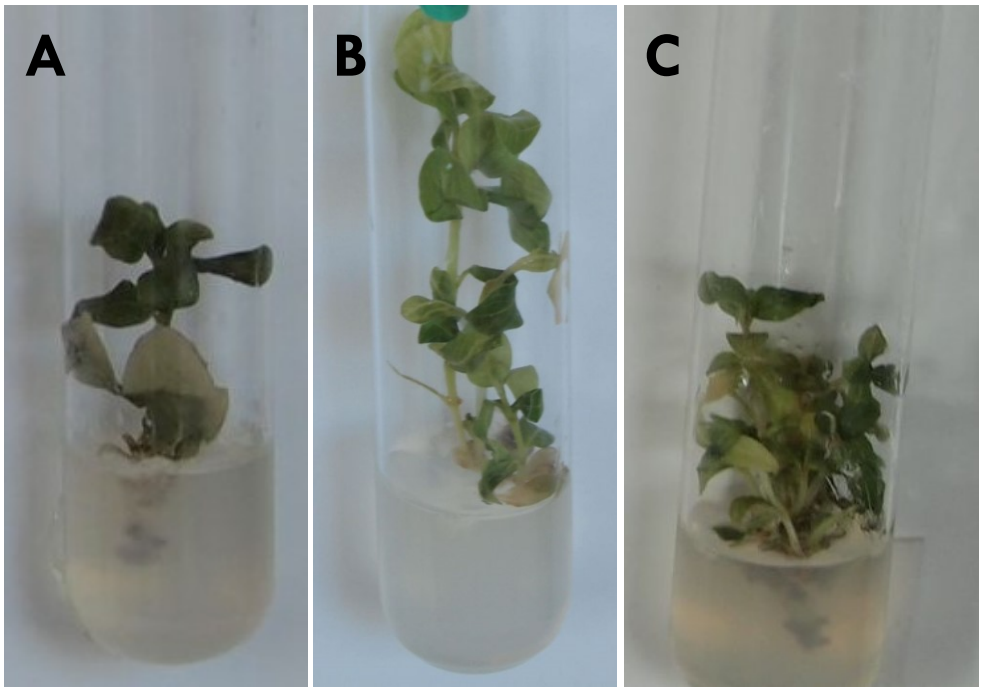


Figure 18. Periwinkle shoots micropropagated: healthy (HV) and infected with aster yellows phytoplasmas strains (16SrI-B) showing mild symptoms (strain AY107) and showing severe symptoms (Hyd8, hydrangea phyllody) (from left to right).

4.1.3.2. Spectrophotometric analyses

4.1.3.2.1. Malondialdehyde (MDA)

The MDA production in untreated *C. roseus* shoot group (control) was significantly higher in the aster yellows infected shoots comparing with the untreated ones (Figs. 19A, B and C). The shoots with severe symptoms showed a significantly higher MDA (Figs. 19A and B). At 1 dae, the shoots of AY107 and Hyd8 strains revealed 1.4-fold and 1.5-fold higher MDA concentration, respectively, compared with healthy shoots (Fig. 19A). Four days after elicitation (4 dae), this difference was higher and the shoots of strains AY107 and Hyd8 revealed an MDA concentration increase of 1.6-fold and 2.0-fold, respectively (Fig. 19B).

Plant elicitation with different concentrations of both elicitors significantly reduced the MDA concentration in infected shoots. Interestingly, with MeJA application, even the solvent (ethanol 2.5%) decreased the MDA concentration of about 63% and 60% in the shoots of AY107 and Hyd8 strains, respectively (Figs. 19A and B). The application of 12.5 mM MeJA decreased the lipid peroxidation, 1 dae, independently from the solvent. Indeed, the shoots of AY107 and Hyd8 strains

showed a decrease of 48% and 45%, respectively, of MDA concentration comparing with the respective untreated shoots (Fig. 19A). The untreated healthy shoots 4 dae showed the same pattern than 1 dae, while the AY107 and Hyd8 infected shoots showed an MDA concentration increase of 1.55-fold and 2.0-fold, respectively compared with healthy shoots (Fig. 19A).

Concerning the BTH application, 1 dae the shoots infected by AY107 and Hyd8 strains revealed 53% and 38% higher MDA concentration compared with healthy shoots (Fig.19C). In both time-points the MDA concentration decreased significantly ($P < 0.05$) after BTH treatments in shoots infected by both phytoplasma strains. In particular, 1 dae 3.5 mM BTH showed a 32% decrease in the shoots of the Hyd8 strain and a 41% decrease in healthy shoots (Fig. 19C). The application of 7 mM BTH showed a significant decrease of MDA concentration in all treated shoot groups, compared with the untreated, despite this decrease was not statistically significant compared with the one measured in the shoots treated with 3.5 mM BTH. Four days after elicitation, the periwinkle shoot groups treated with BTH revealed the same pattern than 1 dae: both BTH concentrations decreased significantly ($P < 0.05$) the MDA concentration up to 61% in the shoots of the AY107 strain (Fig. 19D).

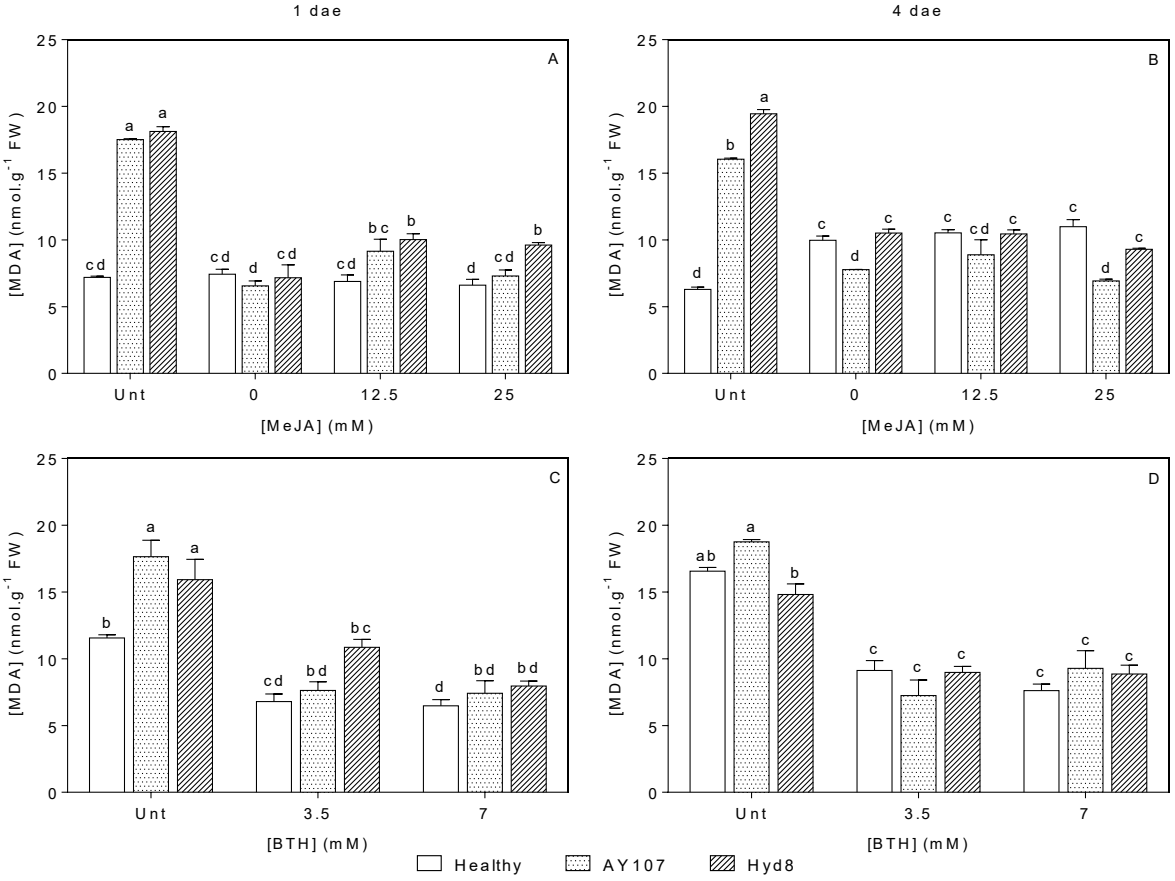


Figure 19. Malondialdehyde (MDA) concentration measured in *C. roseus* infected with aster yellows phytoplasmas with different symptom severity, including healthy, mild (strain AY107) and severe (strain Hyd8) symptoms, untreated (Unt),

treated with 0 (solvent, ethanol 2.5%), 12.5 and 25 mM methyl jasmonate (MeJA) (A and B), and with 3.5 and 7 mM benzothiadiazole (BTH) dissolved in water (C and D), measured 1 day (A and C) and 4 days (B and D) after elicitation (dae). Each value is the mean of three biological replicates resulting from the pool of three shoots ± SEM. Bars with different letters are statistically different at $P < 0.05$.

4.1.3.2.2. Total flavonoid content

One day after elicitation (1 dae), periwinkle shoots treated with MeJA did not exhibit effect on flavonoid content, compared with the untreated shoot group (Fig. 20A). Nevertheless at 4 dae, untreated shoots of the Hyd8 strain revealed a significant high flavonoid content than healthy shoots and the shoots of the AY107 strain (32% and 26%, respectively) (Fig. 20B). Although, at the same time point MeJA application increased flavonoids content significantly in HV and AY107 strain periwinkles shoots, this effect however seems to be related to the solvent *per se*. With regards to BTH, 1 dae, in the untreated shoot group, the shoots of the Hyd8 strain showed 23% lower flavonoid content compared with the untreated healthy shoots (Fig. 20C). At this time point, the shoots of the strain AY107 treated with 3.5 mM BTH revealed an increase of 29% on flavonoid content compared with those of the AY107 untreated strain. Four days after elicitation, untreated shoots of the Hyd8 strain showed 36% lower flavonoid content compared with untreated healthy shoots (Fig. 20D). Nevertheless, at 4 dae, BTH treatments did not result in any effect on all the shoot groups.

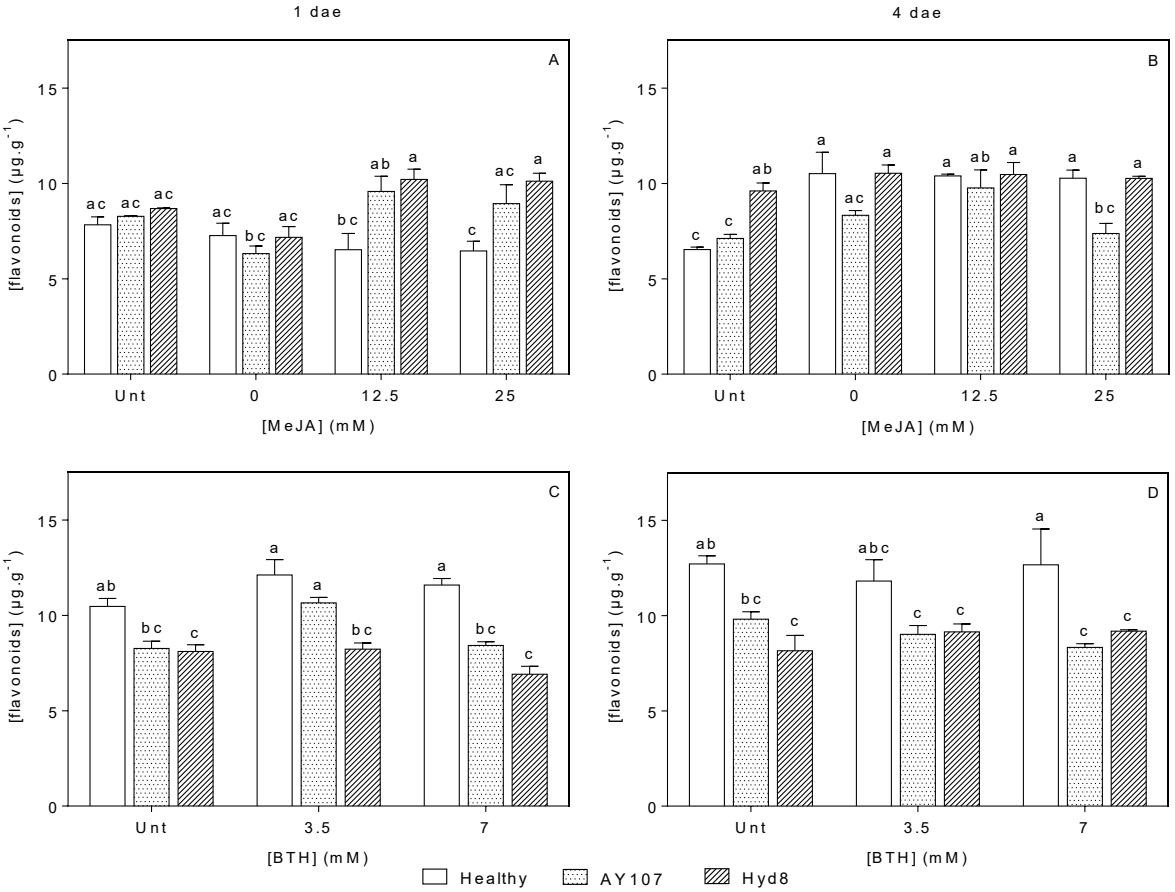


Figure 20. Flavonoid content measured in *C. roseus* infected with aster yellows phytoplasmas with different symptom severity, including healthy, mild (strain AY107) and severe (strain Hyd8) symptoms, untreated (Unt), treated with 0 (solvent (ethanol 2.5%), 12.5 and 25 mM methyl jasmonate (MeJA) (A and B), and with 3.5 and 7 mM benzothiadiazole (BTH) dissolved in water (C and D), measured 1 day (A and C) and 4 days (B and D) after elicitation (dae). Each value is the mean of three biological replicates resulting from the pool of three shoots ± SEM. Bars with different letters are statistically different at $P < 0.05$.

4.1.3.3. Phytohormones quantification

The quantification of phytohormones showed that periwinkle shoots infected with the phytoplasma strain associated with severe symptoms (Hyd8) had a significantly lower concentration of phytohormones comparing with the healthy and/or AY107 strain groups (Figs. 21, 22 and 23). In fact, 1 dae, healthy untreated plants had 2.7-fold and 45.5-fold higher JA concentration when compared to AY107 and Hyd8 strains, respectively (Fig. 21A). AY107 and Hyd8 untreated strain groups, showed 82% and 97% lower [JA] comparing with untreated HV plant strain. However, when the MeJA solvent was applied, these differences disappear, as there is a strong inhibition of JA synthesis by the healthy plants.

The MeJA application at both concentrations significantly increased the JA concentration in all shoots in both time points, regardless solvent effect. Shoots of strains AY107 and Hyd8 untreated 1 dae, showed 82% and 97% low JA concentration compared with untreated healthy shoots (Fig. 21A), at 4 dae the JA concentration was similar to the one registered at 1 dae (Fig. 21B).

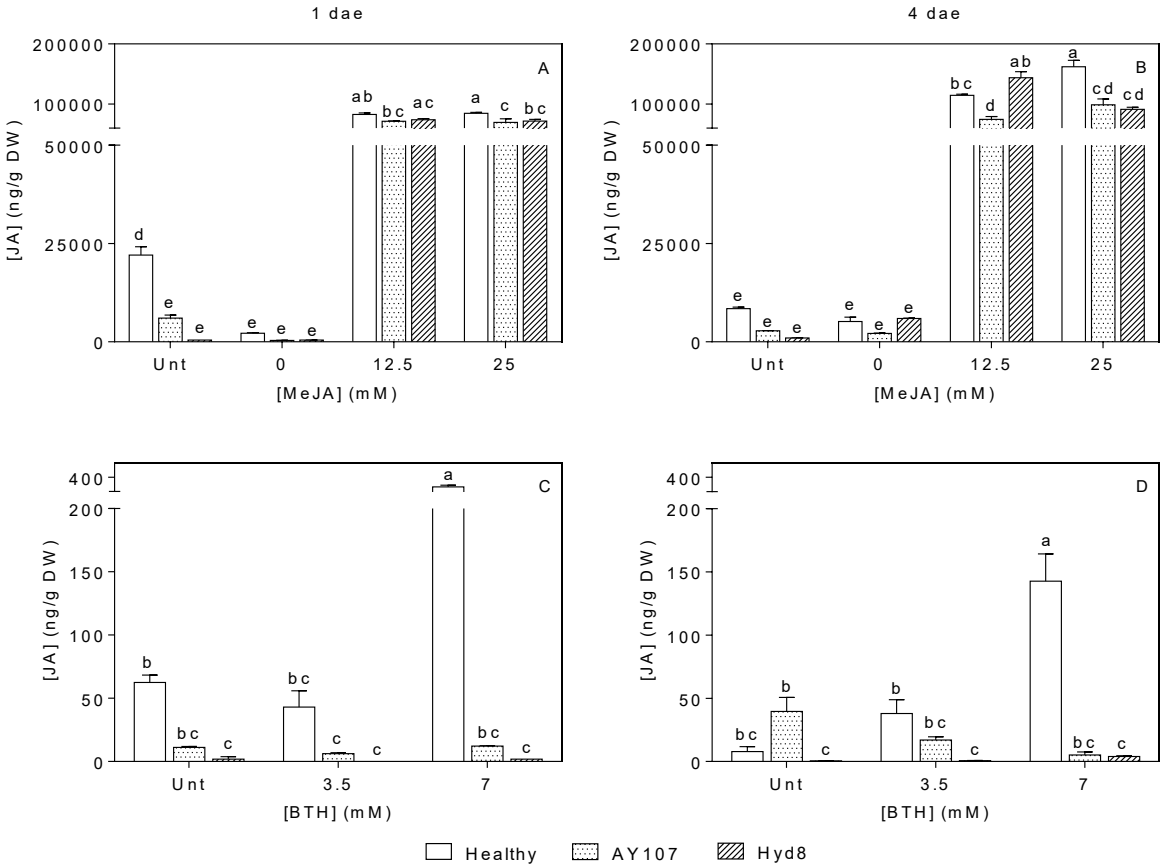


Figure 21. Jasmonic acid (JA) concentrations measured in *C. roseus* infected with aster yellows phytoplasmas with different symptom severity, including healthy, mild (strain AY107) and severe (strain Hyd8) symptoms, untreated (Unt), treated with 0 (solvent, ethanol 2.5%), 12.5 and 25 mM methyl jasmonate (MeJA) (A and B), and with 3.5 and 7 mM benzothiadiazole (BTH) dissolved in water (C and D), measured 1 day (A and C) and 4 days (B and D) after elicitation (dae). Each value is the mean of three biological replicates resulting from the pool of three shoots ± SEM. Bars with different letters are statistically different at $P < 0.05$.

Concerning BTH assay, 1 dae in untreated shoot groups, untreated healthy periwinkle shoots showed a significantly higher JA concentration compared with AY107 and Hyd8 strains (1.6-fold and 32-fold, respectively) (Fig. 21C). At this time point, only the treatment of 7 mM BTH increased the JA concentration in healthy shoots (2.2-fold) comparing to those untreated. In the same way, 4 dae, only the treatment of 7 mM BTH increased the JA concentration in healthy shoots (16.2-fold) compared to the untreated ones (Fig. 21D). At 1 dae, the accumulation of SA in the Hyd8 strain untreated shoot group was 61% and 59% significantly lower, compared with untreated healthy and

AY107 strain infected shoots, respectively (Fig. 22A), while the solvent treatment (2.5% ethanol) increased significantly this phytohormone in 61% in healthy shoots and significantly reduced the SA concentration of 40% in the shoots of the AY107 strain. At the same time point (1 dae) the effect of MeJA treatments on SA concentration seemed to be related to the solvent *per se*. Four days after elicitation, in the untreated shoot group, AY107 and Hyd8 strains showed 16% and 64% lower concentration of SA compared with the untreated healthy shoots (Fig. 22B). The solvent treatment reduced the concentration of SA of 25% in the shoots of strain AY107 while did not affect the shoots of healthy and Hyd8 strains. At the same time point, 12.5 mM MeJA increased of the 52% the SA concentration in the shoots of the Hyd8 strain compared with those untreated, while 25 mM MeJA decreased SA concentration of 38% in healthy shoots, of 68% in shoots of strain AY107 and of 49% in shoots of Hyd8 strain compared with respective untreated shoots (Fig. 22B). Untreated shoot group of AY107 strain showed 51% lower SA concentration 1 dae compared with untreated healthy shoots, while 3.5 mM BTH increased significantly (70%) the SA concentration in the healthy shoots (Fig. 22C). Four days after elicitor application, 7 mM BTH increased significantly (32%) the SA concentration compared with untreated shoots (Fig. 22D).

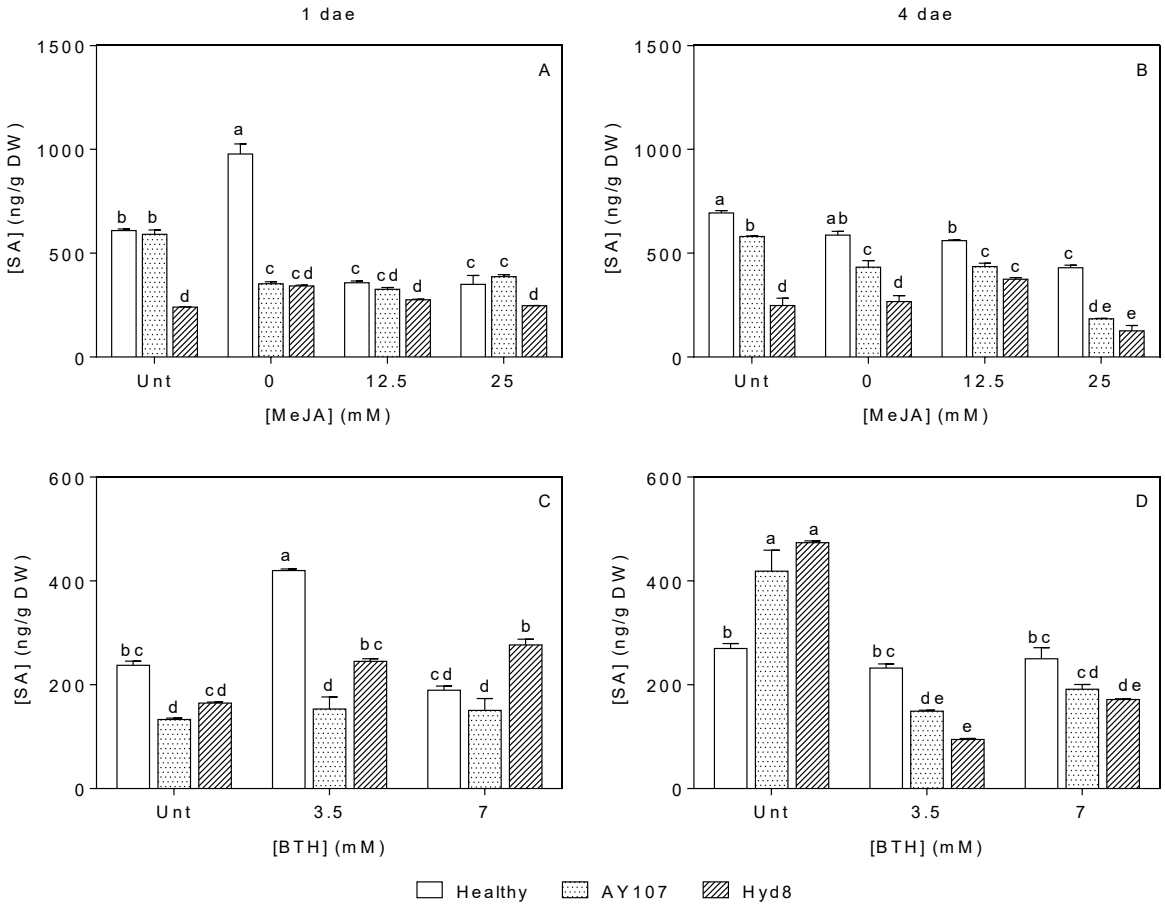


Figure 22. Salicylic acid (SA) concentrations measured in *C. roseus* infected with aster yellows phytoplasmas with different symptom severity, including healthy, mild (strain AY107) and severe (strain Hyd8) symptoms, untreated (Unt), treated with 0 (solvent, ethanol 2.5%), 12.5 and 25 mM methyl jasmonate (MeJA) (A and B), and with 3.5 and 7 mM benzothiadiazole (BTH) dissolved in water (C and D), measured 1 day (A and C) and 4 days (B and D) after elicitation (dae). Each value is the mean of three biological replicates resulting from the pool of three shoots ± SEM. Bars with different letters are statistically different at $P < 0.05$.

Regarding the accumulation of ABA, MeJA application, 1 dae, the untreated shoots of AY107 strain showed 41% and 62% high concentration ($P < 0.05$) of ABA compared with untreated healthy shoots and shoots of Hyd8 strain (Fig. 23A). The solvent reduced the ABA concentration of 54% in healthy shoots; of 98% in shoots of AY107 and 100% in shoots of Hyd8 strains in both time points. Contrarily of 1 dae, at 4 dae only untreated Hyd8 revealed a significantly low concentration of ABA than untreated healthy shoots and shoots of AY107 strain (51% and 55%, respectively) (Fig. 23B). Thus, at this time point, although healthy periwinkles seemed to not be affected by MeJA solvent, this treatment reduced significantly ($P < 0.05$) ABA concentration in infected periwinkle strains (78% in AY107 strain and 77% in Hyd8 strain). While concentration of 12.5 mM MeJA reduced of 41%

ABA concentration in infected shoots of AY107 strain, 25 mM MeJA reduced of 37% the ABA concentration of healthy periwinkle shoots.

In BTH assay, 1 dae, AY107 and Hyd8 untreated shoots showed 38% and 52% less ABA concentration, respectively compared with untreated healthy shoots (Fig. 23C). BTH application decreased the ABA concentration in all shoots: 3.5 mM decreased it of 86%, 69% and 95% in healthy, AY107 and Hyd8 shoots, respectively; 7 mM decreased of 97%, 70% and 93% in healthy, AY107 and Hyd8 shoots, respectively. Four days after elicitation, untreated shoots of the Hyd8 strain revealed a significantly ($P < 0.05$) low ABA concentration compared to healthy and AY107 (57% and 51%, respectively) shoots (Fig. 23D). Similarly, at 4 dae, BTH decreased the ABA concentration in all shoots, except those healthy treated with 3.5 mM: 3.5 mM in with a decrease of 45% and 53% was registered in shoots of the strains AY107 and Hyd8, respectively; 7 mM BTH decreased of 62%, 47% and 66% the ABA concentration in the shoots of healthy, AY107 and Hyd8 strains, respectively.

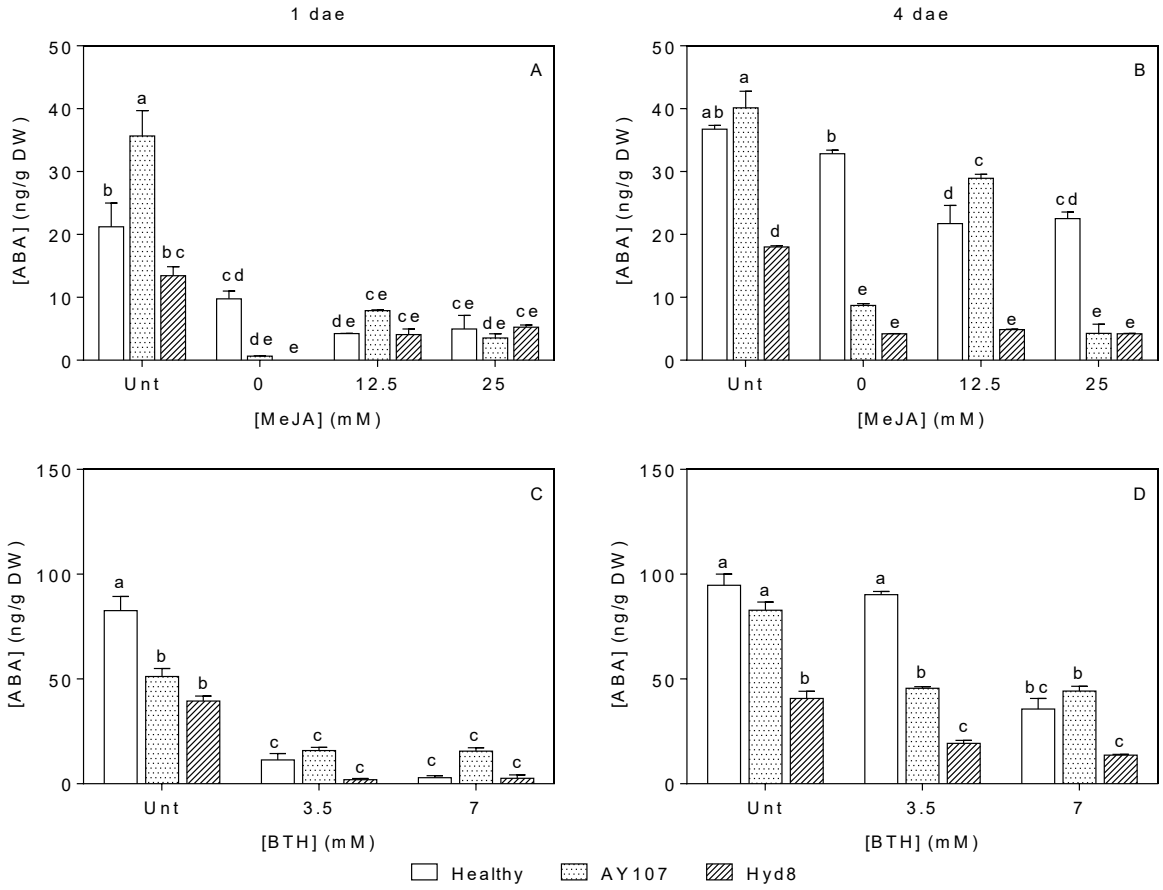


Figure 23. Absisic acid (ABA) concentrations measured in *C. roseus* infected with aster yellows phytoplasmas with different symptom severity, including healthy, mild (strain AY107) and severe (strain Hyd8) symptoms, untreated (Unt), treated with 0 (solvent, ethanol 2.5%), 12.5 and 25 mM methyl jasmonate (MeJA) (A and B), and with 3.5 and 7 mM benzothiadiazole (BTH) dissolved in water (C and D), measured 1 day (A and C) and 4 days (B and D) after elicitation

(dae). Each value is the mean of three biological replicates resulting from the pool of three shoots ± SEM. Bars with different letters are statistically different at $P < 0.05$.

4.1.4. Discussion

Since it is difficult to maintain phytoplasmas in culture, the study of these microorganisms is only possible by studying their interaction with host plants (Choi *et al.*, 2004). However, working with field-FD-diseased plants also presents difficulties and limitations, since growers must uproot infected plants and, in the field, there are many factors (temperature, humidity, rainfall, and other infections) which interfere with the studies limiting the possibility of data interpretation (Oliveira *et al.*, 2020). Thus, *in vitro* culture becomes an important alternative tool for studying diseases associated with the phytoplasma presence and their interaction with the hosts. Periwinkle is a model plant that could be used to study mechanisms of phytoplasma infection, since it is easily maintained under laboratory conditions, and coexists with phytoplasmas of different virulence, exhibiting several symptoms, which suggest a deep disturbance in plant's normal metabolism (Choi *et al.*, 2004; Bertaccini, 2007; Perica, 2008). In the present study, periwinkles were infected by phytoplasmas of two different virulence – intermediate (AY107) and high virulence (Hyd8). AY107 strain showed abnormal elongation of internodes (etiolation) and stunted growth without axillary sprouting (Fig. 18). On the other hand, Hyd8 strain presented dwarfism, increase of axillary sprouting (witch's broom) with small and deformed leaves and virescence. In contrast to what it is here reported for strain AY107, previous studies observed that 16SrI-B (HYDB), 16SrI-F (AY-A), 16SrIII-F (KVI), 16SrV-A (EY-C) and 16SrXII-A (SA-I) phytoplasma strains observed phytoplasmas induced proliferations, leaf curling and internode shortening in *in vitro* micropropagated periwinkle shoots (Bertaccini *et al.*, 1992; Perica and Music, 2005; Perica *et al.*, 2007; Perica, 2008). This fact suggests that phytoplasmas present different virulence degrees, which affect differentially periwinkles' phenotype and consequently other plant species.

Plant models have been fully characterized and used to study either physiological, biochemical and molecular responses in controlled conditions in order to further interpolate to more complex plant systems. In the present study, periwinkle shoots infected with two phytoplasma strains – AY107 and Hyd8 – were used to study biochemical responses to treatments with different elicitors. As mentioned before, MDA is a biomarker of oxidative damage stress in cells, resulting in lipid peroxidation, caused by the susceptibility of membranes to ROS attack (Hodges *et al.*, 1999). Phytoplasma-infected periwinkles showed higher MDA content than healthy ones in agreement with

Kiprovski *et al.* (2018) studies in leaves of *Oenothera biennis* plants infected by '*Ca.* P. solani', which presented 2-fold higher MDA content than leaves of healthy plants, likewise what was found in tomato plants infected with *B. cinerea* (Lou *et al.*, 2011). Nevertheless, studies conducted in apple and apricot trees infected with apple proliferation and European stone fruits yellows phytoplasmas, respectively have shown that recovered trees presented a significant higher MDA content than infected and healthy ones (Musetti *et al.*, 2004, 2005) concluding that the MDA concentration can be linked to the regulation of the recovery phenomenon (Musetti *et al.*, 2005) that may or may not involve the elimination of the pathogen from the host plant.

When infected, plants increase cell oxidation status, triggering secondary metabolism pathways, leading to plant hormonal imbalance (Prezelj *et al.*, 2016). Despite MDA being related to the recovery phenomenon, some studies reported that this metabolite can also be considered as an indicator of oxidative damage after treatments against plant diseases (Lou *et al.*, 2011; Abdollahi *et al.*, 2012). Lou *et al.* (2011) showed that tomato plants elicited with oligandrin before *B. cinerea* infection reduced MDA content by 26%. Moreover, lime trees treated with electromagnetic field show a reduction of the witches' broom intensity associated with the presence of '*Ca.* P. aurantifolia' without damages on lipid membrane, and with a significant increase of the antioxidant enzymes activity (Abdollahi *et al.*, 2012). In the present study both elicitors application decreased the MDA content in both time points for both phytoplasma strains and the concentration of 12.5 mM MeJA was the most effective. Similar results were obtained also with BTH application where both concentrations decreased the MDA content.

In the present study the flavonoid content showed different responses to the treatments. In general, one day after MeJA application there was no effect in treated shoots and 4 dae only healthy shoots showed an increase in flavonoid content, but very likely related to the solvent *per se* (Fig. 20B). Only 4 dae the shoots of Hyd8 strain showed an increase of 32% in flavonoid. Contrastingly, in the treatment with BTH, untreated healthy *C. roseus* shoots presented a significantly higher flavonoid content than in the shoots of the Hyd8 strain in both time points (23% and 36%, respectively) (Fig. 20C and 20D). This is in agreement with a previous study which showed that *O. biennis* presented approximately 50% higher flavonoids content than plants infected with '*Ca.* P. solani' (Kiprovski *et al.*, 2018); it was suggested also that phytoplasmas inhibit the flavonoid biosynthetic pathway and increase the biosynthesis of caffeic, chlorogenic and cinnamic derivatives in phytoplasma-infected *C. roseus* (Musetti, 2010).

To the best of our knowledge, this is the first study on the impact of elicitors application on the flavonoid content in periwinkles infected with phytoplasmas. One day after elicitation the shoots of the Hyd8 strain presented a significant increase of 36% and 38% compared with healthy shoots

when treated with 12.5 mM and 25 mM MeJA, respectively. The same effect was registered in AY107 periwinkle shoots treated with 12.5 mM at 4 dae, which increased their flavonoid content by 27% compared with untreated AY107 plants. The BTH application on AY107 shoots 1 dae with 3.5 mM showed a significant increase of flavonoids (22%); this increase could indicate that these compounds activate the gene expression machinery for these molecules production. Flavonoids have been reported as the main phenolic compounds produced in the leaves of healthy *O. biennis* (86.2% of total polyphenols content), considered as antioxidant compounds (Kiprovski *et al.*, 2018). In summary, the overall lower MDA production with the concomitant increase of flavonoid content shown by Hyd8 strain treated with both MeJA concentrations and by AY107 strain treated with 3.5 mM BTH indicated that both elicitors activate systemic acquired resistance, inducing both SA and JA signal pathways, activating phenylpropanoid pathway and decreasing the cellular damage, but in a different way according to the diverse virulence of the phytoplasma strain.

Several studies have been shown that phytoplasmas infection triggered both MeJA and SA transduction pathways, activating plant defence mechanisms (reviewed by Dermastia, 2019). Nevertheless, in the present study the untreated periwinkles infected Hyd8 strain (inducing severe symptoms) revealed lower concentration of all studied phytohormones JA, SA and ABA in both time points (Figs. 21-23). Indeed, Sugio *et al.* (2011) showed that "aster yellows" witches' broom phytoplasma strain produced the protein SAP11 that binds to the transcription factors of lipoxygenase gene (*LOX*), blocking JA biosynthesis. Moreover, the apple proliferation phytoplasma was demonstrated to not only block *LOX* expression but also allene oxide cyclase (*AOC*) gene, which are responsible for MeJA biosynthesis (Tai *et al.*, 2013). On the other hand, untreated plants infected with phytoplasmas inducing mild symptoms (AY107 strain) did not show a similar pattern, especially in ABA and SA presence, and this is probably linked to the mild symptom expression of this phytoplasma strain possibly activating SAR leading to an overproduction of SA. Untreated periwinkle shoots of strain AY107 showed an increase of ABA responsible for stomata closure, therefore avoiding water losses and consequently leaf yellowing, associating the ABA production to the plant response to phytoplasma presence as reported for the apple proliferation phytoplasma (Zimmermann *et al.*, 2015).

Some variations in phytohormone concentrations were highlighted after elicitors' treatments 1 and 4 dae; both MeJA concentrations greatly increased JA content while in general the same concentrations significantly decreased SA and ABA concentrations. This is in agreement with previous studies that concluded that exogenous application of MeJA activated the octadecanoic pathway, triggering positive feedback, which leads to its biosynthesis (Cheong and Choi, 2003; Goossens *et al.*, 2016). Indeed, in the present study MeJA application increased the JA concentration

from 82% in infected plants, without solvent effect, in both time points, compared with respective untreated infected plants. Moreover, at 4 dae, 12.5 mM MeJA showed a significant increase of about 147-fold JA content in Hyd8 plant strain. In contrast, at the same time point, healthy plants treated with 25 mM showed 44% higher JA content when compared with the same plant strain treated with 12.5 mM MeJA. Although, MeJA concentrations a positive effect on JA increase, it was highlighted that MeJA solvent (2.5% ethanol) significantly decrease JA concentrations in all periwinkle shoots, in contrast with healthy periwinkles in which ethanol increased SA content. This fact explain that the aggression caused by ethanol activates SAR, increasing SA content, in healthy shoots. Since infected periwinkles are already under a high level of oxidation, ethanol did not affect the SA content.

The present study also demonstrated that MeJA application negatively influenced SA and ABA production. However, in Hyd8 periwinkle shoots, MeJA application seemed to be concentration-dependent, since 25 mM MeJA decreased significantly by 49% SA content, while 12.5 mM MeJA increased its concentration on 34%, comparing with Hyd8 untreated plants. Although it is well documented that JA and SA pathways are antagonistics (Tai *et al.*, 2013; Zimmermann *et al.*, 2015; Goossens *et al.*, 2016), in the present work 12.5 mM MeJA concentration significantly increased SA concentration (34%) in Hyd8 comparing with untreated plants, while in general MeJA reduced ABA production, in both infected plant strains and in both time points, up to 89%. In the case of phytoplasma infection, Zimmermann *et al.* (2015) demonstrated that ABA content increased stomatal conductance in apple proliferation disease. The accumulation of phytoplasmas in the sieve tubes blocks the sink-source translocation of carbohydrates, from the leaves to the bunches or young leaves. Moreover, phytoplasmas infection could also affect xylem translocation to the leaves. Thus, ABA synthesis is activated due to the water deficit in the leaves, leading to stomata closure. As aforementioned, MeJA application activates JA pathway synthesis and consequently the phenylpropanoid biosynthesis pathway, inhibiting SA pathway (Goossens *et al.*, 2016), while the decrease of ABA production could be related to the downregulation of genes involved in photosynthetic pigments (chlorophylls a/b and carotenoids) pathway (Cheong and Choi, 2003). Nevertheless, in the present study, there was no evidence that MeJA inhibited SA production and ABA decrease production as well.

In the present work the SA content was mainly lower in infected shoots (AY107 and Hyd8 strains), allowing the coexistence of the pathogen (phytoplasmas) and host (periwinkle plants) (Sugio *et al.*, 2011; Dermastia, 2019). BTH is a functional synthetic analogue of SA which in the present study did not affect JA content in phytoplasmas-infected periwinkles, despite in healthy shoots 7 mM BTH has significantly increased that phytohormone 3- and 17-fold, at 1 dae and 4 dae, respectively. SA has been reported to increase its concentration in plants infected with phytoplasmas due to the

activation of SAR defence mechanism (Tai *et al.*, 2013; Zimmermann *et al.*, 2015). However Patui *et al.* (2012) reported that, whereas apple proliferation-infected trees showed high SA content, recovered plants presented high JA content. Only 7 mM BTH significantly increased by 68% SA 1 dae, however, Wani *et al.* (2016) reported that SA (or its analogues) dosage application depends on varieties of plants and type of stresses. In this way, the concentration of 7 mM BTH is the better to increase SA content in phytoplasmas-infected periwinkles, inducing SAR mechanism.

The reported interaction between ABA and SA is intriguing since it depends on the branches of the pathways that interact, and also in function of the plant species (Nawrath *et al.*, 2006). For instance, ABA blocks the SA-dependent defence in tomato, increasing its susceptibility against *B. cinerea* (Audenaert *et al.*, 2002), while in many other plant species both elicitors have shown to induce protective antioxidant molecules against several abiotic stresses (reviewed by Syeed and Khan, 2010). Likely to auxins the ABA content significantly increased in phytoplasma infected apple trees (Zimmermann *et al.*, 2015), that is in contrast with the results of in the present study, where BTH was demonstrated to have a potential to decrease ABA concentrations in the shoots of all the phytoplasma strains, in both time points (Fig. 23C and 23D). ABA is biosynthesized by isoprenoid pathway, the same pathway of chlorophylls. Chlorosis is one of the most important symptoms of phytoplasmas-infected plants, which means that chlorophylls are degraded, as reported by Teixeira *et al.* (2020). The decrease of ABA content by BTH could be directed by chlorophylls synthesis, and reduce the yellowing symptoms, increasing the photosynthetic rate of the infected plants.

4.1.5. Conclusions

This study allowed to understand the role of elicitors MeJA and BTH on shoots infected by phytoplasmas in lipid peroxidation, flavonoid content, and phytohormones production. Both elicitors revealed the potential to treat plants infected with phytoplasmas, demonstrating lower MDA production. Moreover, MeJA application did not have a relevant/significant effect in flavonoid content, while 3.5 mM BTH significantly increased flavonoid content, especially 1 dae.

Also, high ABA concentration could be an indication of phytoplasma infection as shown for untreated AY107 infected periwinkle shoots. Elicitors application increased the phytohormones production and Hyd8 phytoplasma-infected periwinkles responded better to 12.5 mM MeJA and 7 mM BTH. Moreover, both elicitors decreased the ABA contents revealing that these compounds could be an important tool to reduce the phytoplasma symptoms.

Even though the use of elicitors in crop protection and pest management is still in the very early stages it should be tested as management method.

4.2. Elicitation with methyl jasmonate enhances gene expression and metabolomic profile of alkaloids and phenolic compounds in *Catharanthus roseus* infected with aster yellows phytoplasmas

Abstract

Periwinkle is a perennial plant used as a model system to study phytoplasma-plant host interactions. Alkaloids and phenolic compounds are secondary metabolites involved in plant defence responses, and methyl jasmonate (MeJA) is often used as an elicitor for enhancing plant defence responses. However, little is known about the effect of elicitors application in plants infected with phytoplasmas. Here the MeJA possible elicitation induced in periwinkles defence responses against aster yellows via alterations in gene expression and plant´s metabolomics profile was studied. To test this hypothesis, *C. roseus* plants infected with phytoplasma strains with diverse virulence (AY107-intermediate, and Hyd8-high) were elicited with two MeJA concentrations (12.5 and 25 mM) and metabolomics and gene expression analysis were conducted one and four days after elicitor application. Healthy plants revealed 13 alkaloids and 11 phenolic compounds whose metabolomics pattern was severely altered due to phytoplasma presence and MeJA treatment. Infected plants exhibited high abundance of serpentine, pleiocarpanine, alstonine, aricine, syringaresinol and orientin, while the MeJA application increased significantly deoxy elenoic acid and hydroxytyrosol. The expression of genes related to alkaloid (*as, str* and *prx*) and phenylpropanoid (*pal* and *chs*) biosynthesis pathways was also modified by the presence of phytoplasmas and the elicitation with MeJA. The *pal, chs, as* and *str* genes were upregulated in plants infected by both "aster yellows" strains, suggesting that the infection acts on both alkaloid and phenolic biosynthetic pathways at a metabolic and molecular level.

Keywords: aster yellows, elicitors, gene expression, metabolomics, periwinkle.

4.2.1. Introduction

Phytoplasmas are phytopathogenic bacteria associated with more than 300 diseases in more than 700 different plant species including fruit trees, vegetables, and ornamentals (Bertaccini *et al.*, 2014). They are phloem-limited pleomorphic bacteria, mainly transmitted by leafhoppers or propagation material (Kumari *et al.*, 2019), and are classified based on their differences in the 16S

ribosomal RNA gene sequence (Lee *et al.*, 1998). '*Candidatus* Phytoplasma asteris', also known as aster yellows (AY) phytoplasma, belongs to the 16SrI group and is infecting diverse plant species worldwide (Lee *et al.*, 2004). AY phytoplasmas are associated with over 100 economically important diseases and represent one of the most diverse and widespread phytoplasma ribosomal group leading to important economic losses, affecting important horticultural crops such as lettuce, carrots, celery, and ornamental plants such as gladiolus, hydrangeas, China aster, and purple coneflower (Bertaccini and Duduk, 2009). In addition, AY phytoplasmas can infect woody plants, namely lemon, peach, apple, cherry, and grapevine (Bertaccini, 2007). These phytoplasmas affect plant growth and development, modifying their hormonal balance and leading to several physiological, anatomical, and metabolic changes (Dermastia, 2019).

Catharanthus roseus (L.) G. Don, commonly named Madagascar periwinkle, is a perennial plant commonly grown for its ornamental and medicinal value. Thus, this specie is frequently used as a model system for medical, biotechnological, and secondary metabolism studies, since it produces over 130 alkaloids (van der Heijden *et al.*, 2004; Verma *et al.*, 2012), which have many applications in the treatment of human cancers and circulatory diseases (Aslam *et al.*, 2010; Moudi *et al.*, 2013). In addition, periwinkle also produces a high number of phenolic compounds (Mustafa and Verpoorte, 2007; Ferreres *et al.*, 2008; Demkura *et al.*, 2010). Combined, these two families of compounds are known to have important roles against biotic and abiotic stresses (Mustafa and Verpoorte, 2007; Liu *et al.*, 2016). Moreover, *C. roseus* is very susceptible to phytoplasma infection, being commonly used in laboratories as a host plant to maintain and study phytoplasma-plant interactions (Bertaccini *et al.*, 1992). Infection of *C. roseus* with phytoplasmas are known to increase alkaloid (Choi *et al.* 2004; Favali *et al.*, 2004; Chen *et al.* 2017) and phenolic compounds (Choi *et al.*, 2004) concentrations' as well as upregulating the expression of genes related to their biosynthetic pathways (Wang *et al.* 2018; Srivastava *et al.*, 2014).

Current control methods of phytoplasmas rely on eradication of the insect vectors and propagation of healthy material, which are not practically applicable due to their high cost and environmental impact; novel strategies have therefore been explored to halt phytoplasma infections by using environmentally friendly methodologies, such as the application of elicitors (Ruiz-García *et al.*, 2012; Oliveira *et al.*, 2018; García-Pastor *et al.*, 2019). Methyl jasmonate (MeJA) is an elicitor widely used to enhance plants responsiveness to pathogens since it activates different plant defence mechanisms, reinforcing plant cell wall and inducing the synthesis of secondary metabolites (Goossens *et al.*, 2016; Zhao *et al.*, 2005; Wasternack *et al.*, 2013). Several studies have suggested that MeJA is able to modulate gene expression to increase the content of phenolic compounds and alkaloids, including the phenylalanine ammonia-lyase (*pal*) (Mustafa and Verpoorte, 2007; Liu *et al.*,

2016), anthranilate synthase (*as*) and strictosidine synthase (*str*) (Almagro *et al.*, 2014b; Liu *et al.*, 2016; Pan *et al.*, 2018) genes.

Despite recent advances, the effect of phytoplasma infection and the effect of MeJA elicitation on *C. roseus* plant defence responses is not yet elucidated. To the best of our knowledge there are no studies answering the question: is there a defence-inducing effect of MeJA application in phytoplasma-infected periwinkles and does it modulate the alkaloid and phenylpropanoid pathways? To answer this question, the present work aimed to report the metabolomic profile of cloned shoot strains of *in vitro* micropropagated periwinkles; and to understand the effect of MeJA elicitation on alkaloid and phenolic metabolomics profile and on the expression of genes related to their biosynthetic pathway. This in-deep and complementary analysis provides new insights into signaling and metabolic pathways, transcriptional control, and defence responses affecting phytoplasma disease virulence and the role by which MeJA triggers those responses.

4.2.2. Materials and Methods

4.2.2.1. Plant material and growth conditions

This work was carried out using three periwinkle micropropagated unrooted clones. These included: healthy shoots (HV) and shoots infected with AY phytoplasma strains (16SrI-B) having mild (AY107) or severe (Hyd8, hydrangea phyllody) symptoms. AY107-infected shoots showed etiolation and stunted growth without axillary sprouting, while Hyd8-infected shoots presented witches' broom, dwarfism, small and deformed leaves, and virescence (Bertaccini *et al.*, 1992). The strains used are from the EPPO-Q-Bank collection (Bertaccini *et al.*, 1992; 2012). Periwinkle shoots were grown in a Murashige and Skoog-based medium (1962) according to Bertaccini *et al.* (1992). Micropropagated shoots were maintained in a growth chamber (F600 Aralab, Sintra, Portugal), with a photoperiod of 16 hours, light intensity of 180 $\mu mol.s^{-1}.m^{-2}$, a range of temperature between 20 and 22ºC, and 85% of relative humidity.

4.2.2.2. Plant elicitation and sampling

Solutions of 0, 12.5 and 25 mM MeJA (Sigma-Aldrich, St. Louis, Missouri, USA) were prepared in 2.5% (v/v) ethanol and 0.1% (v/v) Tween 20 (Sigma-Aldrich, St. Louis, Missouri, USA).

The MeJA solutions were applied as a foliar spray to the in vitro shoots and the solution remained in contact with the plants until the end of the experiment. Periwinkle shoots were collected at three timepoints: before MeJA application (T0), 24 hours after application (T1), and 4 days after application (T2). These samples were collected in triplicate and each biologic replicate consisted of a pool of three individual plants. After collection, the plant material was powdered with liquid nitrogen and stored at -80ºC.

4.2.2.3. Metabolomic analyses

Metabolite extraction was performed according to de Vos et al. (2007). Briefly, 600 µL of ice-cold extraction solution (99.875% (v/v) methanol acidified with 0.125% (v/v) formic acid) was added to 200 mg of powdered frozen tissue. Fifteen µL of internal standards (gallic acid and hydroxytyrosol) were added to each sample, vortexed for 10 seconds and sonicated continuously for 25 minutes at maximum frequency (35 kHz) in a water bath at room temperature. Samples were then centrifuged for 20 minutes at maximum speed (10,000 g) and the supernatant was filtered through a 0.2 µm PTFE filter using a disposable syringe into a 1.8 mL glass vial and stored at -20 °C until chromatographic analysis.

Metabolomic analysis was performed by LC-ESI-UHR-QqTOF-MS according to Monforte et al. (2017). For this, the UltiMate 3000 Dionex UHPLC (Thermo Scientific, USA) equipment was used, connected to a Qq-time-of-flight (UHR-QqTOF) ultra-light resolution mass spectrum with 50000 full-sensitivity resolution (FSR) (Impact II, Bruker Daltonics, Bremen, Germany). The separation of metabolites was performed using an Acclaim RSLC 120 C18 column (100 mm x 2.1 mm, 2.2 µm) (Dionex, CA, USA). Mobile phases consisted of 0.1% aqueous formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The gradient started with solvent B at 5%, it was increased to 95% in 7 minutes, kept constant for 2 minutes, returned to 5% in 1 minute and maintained at 5% for an additional 5 minutes at a flow rate of 0.25 mL/min. The injection volume was 1 µL and each sample was injected in duplicate. Parameters for MS analysis of alkaloids and phenolic compounds were set using positive and negative ionization modes, respectively, with spectra acquired over a range from m/z 20 to 1,000. The parameters were as follows: capillary voltage, 4.5 kV; drying gas temperature, 200ºC; drying gas flow, 8.0 L/min; nebulizing gas pressure, 2 bar; collision RF, 300 Vpp; transfer time, 120 µs and prepulse storage, 4 µs. Post-acquisition internal mass calibration used sodium formate clusters with the sodium formate delivered by a syringe pump at the beginning of each chromatographic analysis. The element composition of the compounds was confirmed according

to accurate mass and isotope rate calculations designated as mSigma (Bruker Daltonics), which was within 5 mDa of the assigned element composition and mSigma values of < 20 provided confirmation.

4.2.2.4. Gene expression analyses

RNA extraction was performed using the RNeasy Plant Mini Kit (QIAGEN GmbH, Germany), accordingly to le Provost *et al.* (2007). RNA yield was determined spectrophotometrically using a Nanophotometer (Implen Isaza, Portugal). Synthesis of complementary DNA (cDNA) was performed using the iScrip cDNA Kit (BioRad, California, USA) according to manufacturer instructions in a thermal cycler (VWR, Doppio, Belgium). Quantitative reverse transcription PCR (RT-qPCR) was performed using the NZY Taq 2x Green Master Mix Kit (Nzytech, Portugal). The expression of five target genes encoding (i) enzymes of alkaloids biosynthesis (*as* – anthranilate synthase, *str* – strictosidine synthase and *prx* – peroxidase) and (ii) enzymes of phenylpropanoid biosynthesis (*pal* – phenylalanine ammonia lyase and *chs* – chalcone synthase) was studied according to periwinkle secondary metabolism (Kaltenbach *et al.*, 1999; Liu *et al.*, 2016). The primers used are listed in Table 1. The cycling conditions after a denaturation step (95°C; 120 seconds), were: 25 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 60 seconds at 72°C, with a final extension of 5 seconds at 72°C. The *40S ribosomal protein S9* (*rsp*) and *actin* genes were used as reference genes (He *et al.*, 2011; Peebles *et al.,* 2009) to normalize and calculate the Ct values.

4.2.2.5. Statistical analysis

Data analysis was performed using GraphPad Prism software (version 6.0). The results correspond to the mean ± standard error of the mean (SEM). To assess metabolomic profile and gene expression, before elicitor application, differences between periwinkle strains were analysed using one-way ANOVA followed by Tukey's post-hoc test with a confidence level of 95% ($P < 0.05$). To evaluate the effect of MeJA application in secondary metabolites production and targeted genes triggering, differences between treatments were analysed using a two-way ANOVA followed by Tukey's post-hoc test with a confidence level of 95% ($P < 0.05$).

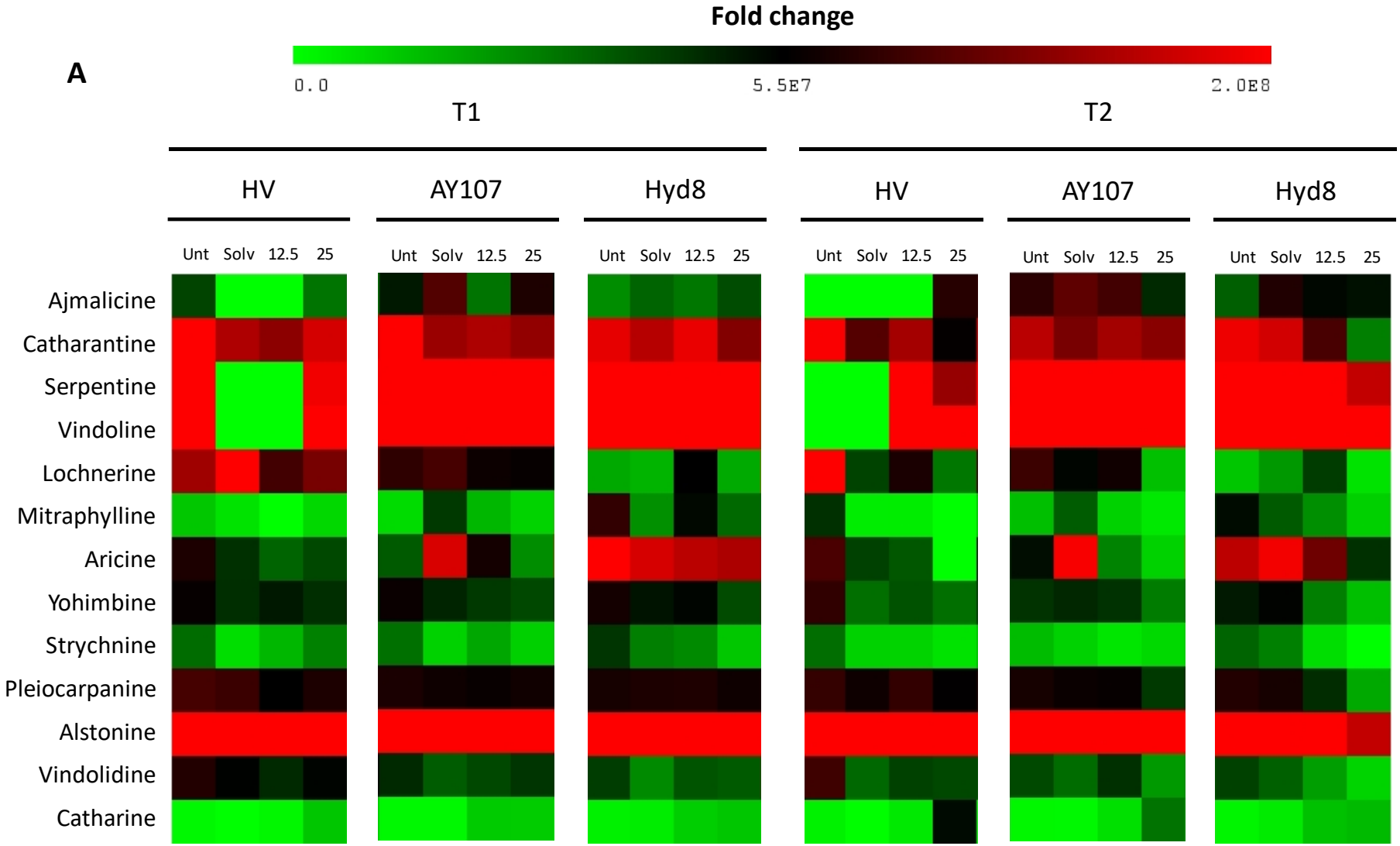
4.2.3. Results

4.2.3.1. Effect of phytoplasma presence and MeJA elicitation on alkaloids and phenolic compounds content

Metabolomic analysis by LC-ESI-UHR-QqTOF-MS was directed to alkaloids, through the positive mode, and to phenolic compounds, through the negative mode. The analysis identified 24 compounds: 13 alkaloids (ajmalicine, catharantine, serpentine, vindoline, lochnerine, mitraphylline, aricine, yohimbine, strychnine, pleiocarpanine, alstonine, vindolidine and catharine) and 11 phenolic compounds (hydroxytyrosol, deoxy elenoic acid, elenoic acid, syringaresinol, maslinic acid, caffeic acid, lawsone, *p*-coumaric acid, quinic acid, orientin and hydroxybenzoic acid) (Fig. 24). Non-elicited periwinkle shoots infected with AY phytoplasmas showed a distinct metabolomic pattern when compared with healthy shoots (Fig. 24; Table 9 of supplementary data). While HV plants presented high vindoline content, AY107 and Hyd8 plants showed high levels of serpentine and pleiocarpanine (Fig. 24). When compared to HV plants, AY107-infected shoots presented significant higher levels of about 31% of serpentine and alstonine content while Hyd8-infected shoots presented significantly higher levels of mitraphylline (52%), aricine (68%), catharine (74%), elenoic acid (36%), syringaresinol (58%) and orientin (60%) (Fig. 24). When looking at phenolic compounds, HV plants presented a significantly higher content of hydroxytyrosol, deoxy elenoic acid, maslinic acid, caffeic acid, lawsone and *p*-coumaric acid, comparing to infected periwinkle shoots (from 37%, 70%, 60%, 65%, 72% and 37%, respectively) (Fig. 24).

To understand the effect of MeJA on the synthesis of alkaloids and phenolic compounds, a time course analysis was performed in plants treated with 12.5 mM and 25 mM MeJA (Fig. 25). Except for deoxy elenoic acid, the MeJA treatments did not influence phenolic compounds relative abundance. Moreover, in both timepoints, the treatment with MeJA solvent (2.5% ethanol) decreased the relative abundance of alkaloids, except in Hyd8 shoots at T2. Furthermore, in general, the alkaloids relative abundance was not significantly altered at different timepoints. The exception was for HV shoots treated with 12.5 mM MeJA at T2, which showed increased levels of serpentine and vindoline (Fig. 25A). At the same timepoint, shoots elicited with 25 mM MeJA showed higher synthesis of deoxy elenoic acid and hydroxytyrosol, independently from periwinkle strain when compared to untreated plants (Fig. 25; Table 10 of supplementary data).

Figure 24. Metabolomic profile of alkaloids and phenolic compounds measured in *C. roseus* shoots healthy (HV) and infected with AY phytoplasma strains with mild (AY107) and high (Hyd8) virulence, before MeJA treatment (T0). Each value is the mean of fold change of three biological replicates resulting from the pool of three plants ± SEM. Different letters are statistically different at $P < 0.05$ according to two-way ANOVA followed by Tukey's post-hoc test.


A
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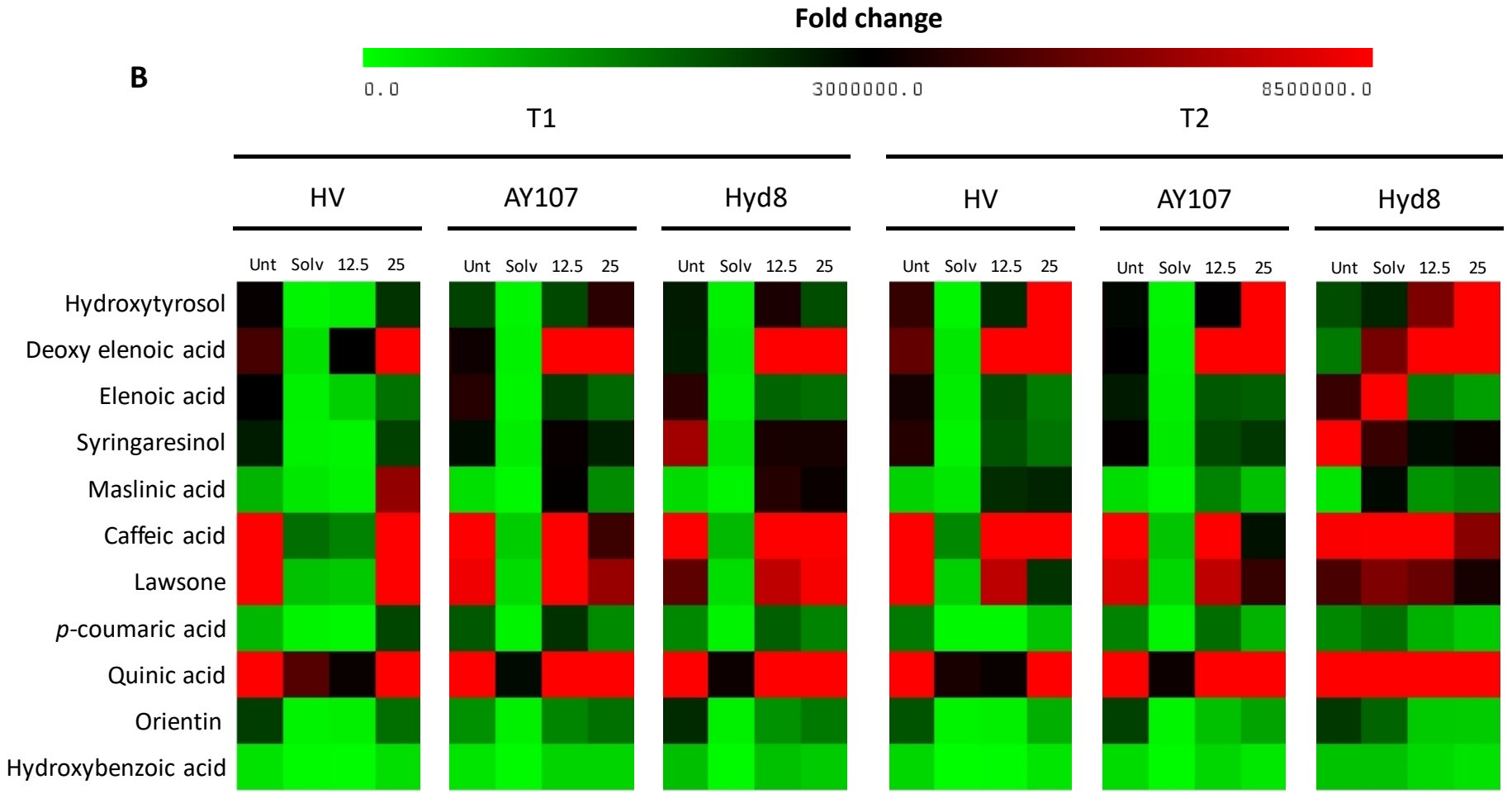

Figure 25. Metabolomic profile of alkaloids (A) and phenolic compounds (B) in *C. roseus* shoots healthy (HV) and infected with AY phytoplasma strains with mild (AY107) and high (Hyd8) virulence, 1 (T1) and 4 (T2) days after elicitor application. Shoots were non-elicited (Unt), or treated with 0 (solvent, solv. - 2.5% ethanol), 12.5 and 25 mM MeJA. Each value is the mean of fold changes of three biological replicates, each resulting from the pool of three shoots.

4.2.3.2. Effect of phytoplasma presence and MeJA elicitation on gene expression

The relative gene expression of alkaloid and phenylpropanoid pathway encoding genes was analysed to understand the effect of phytoplasma presence at the molecular level. The results showed that shoots infected with the most virulent phytoplasma strain (Hyd8) presented a significant upregulation of *as*, *str*, *prx* and *chs* genes (Fig. 26A, 26B, 26C and 26E), while no differences were found on the expression of the *pal* gene (Fig. 26D). In fact, Hyd8 strain showed a significant upregulation of 1.3-fold and 0.8-fold on *as* gene compared with HV and AY107 shoots, respectively. Gene expression of *str* and *chs* genes was altered to the same extend in AY107 infected shoots (Fig. 26B and 26E). The expression of *as* gene was significantly lower in AY107 (1.5-fold) than in Hyd8 (2.3-fold) infected shoots (Fig. 26A). Moreover, the expression of the *prx* gene was unchanged in plants infected with AY107 (Fig. 26C).

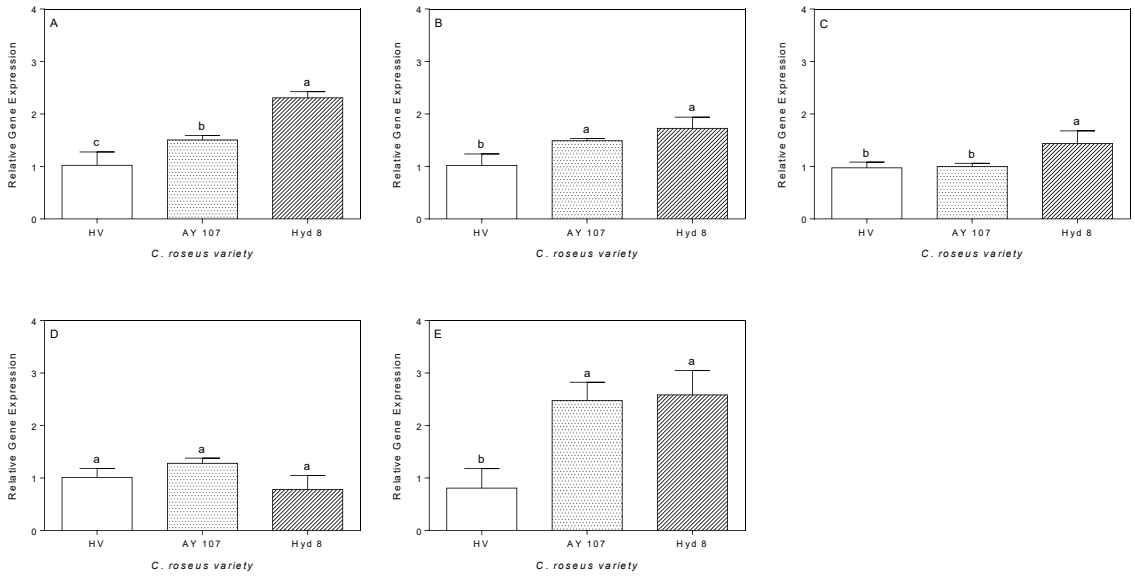

Figure 26. Relative gene expression of alkaloid pathway encoding genes (*anthranilate synthase*, *as* – A, *strictosidine synthase*, *str* – B, and *peroxidase*, *prx* – C) and phenylpropanoid pathway encoding genes (*phenylalanine ammonia liase*, *pal* – D, and *chalcone synthase*, *chs* – E) determined in *C. roseus* shoots healthy (HV) and infected with AY phytoplasma strains having mild (AY107) and high (Hyd8) virulence, before MeJA treatment. Each value is the mean of three biological replicates resulting from the pool of three shoots ± SEM. Different letters are statistically different at $P < 0.05$ according to two-way ANOVA followed by Tukey's post-hoc test.

The MeJA treatment on alkaloid and phenylpropanoid pathway encoding genes showed that 24h after elicitation (T1) there was an overexpression of *pal*, *chs*, *str* and *as* genes in AY107-infected periwinkles treated with 12.5 mM MeJA and in Hyd8 plants treated with 25 mM MeJA (Fig. 27; Table 12 of supplementary data). Four days after elicitation (T2) with 12.5 or 25 mM MeJA, there was an upregulation of *as, str, pal* and *chs* in HV and AY107 plants, while in Hyd8 plants this only

occurred at the highest MeJA concentration (Fig. 27). In general, all MeJA treatments downregulated the *prx* gene expression at both timepoints (Table 12 in the supplementary data), whereas in T1 the effect of treatments seemed to be related only to the solvent *per se* (Fig. 27).

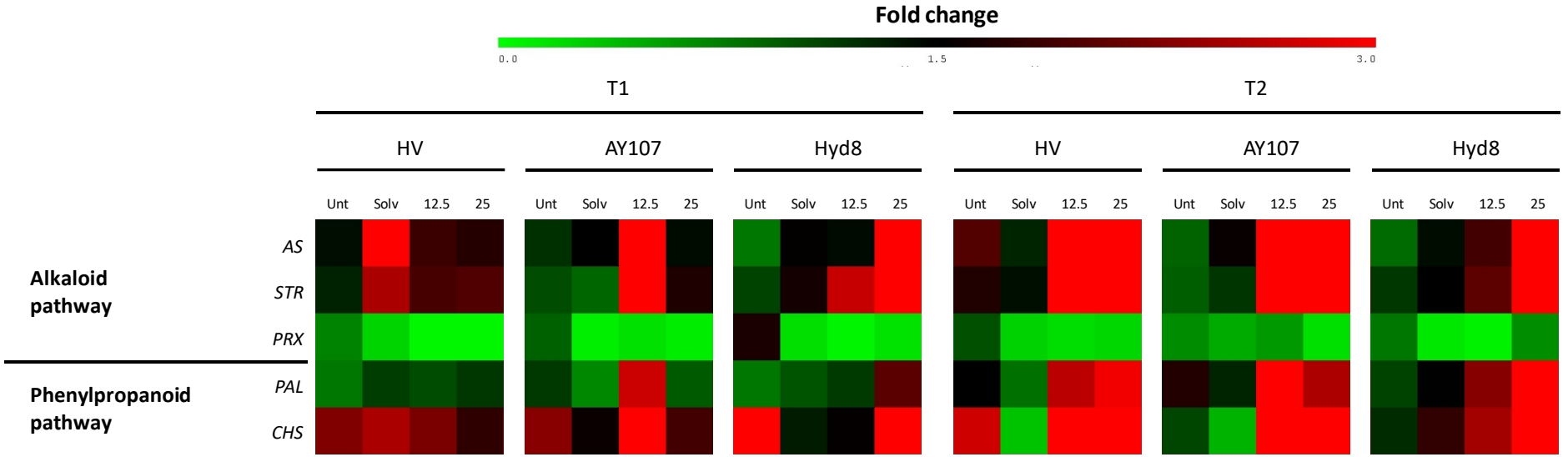

Figure 27. Relative gene expression of alkaloid pathway encoding genes (*anthranilate synthase*, *as*, *strictosidine synthase*, *str*, and *peroxidase*, *prx*) and phenylpropanoid pathway encoding genes (*phenylalanine ammonia liase*, *pal*, and *chalcone synthase*, *chs*) determined in *C. roseus* shoots healthy (HV) and infected with AY phytoplasma strains with mild (AY107) and high (Hyd8) virulence, 1 (T1) and 4 (T2) days after elicitor treatments. Shoots were non-elicited (Unt), or treated with 0 (solvent, solv. - 2.5% ethanol), 12.5 and 25 mM MeJA. Each value is the mean of three biological replicates resulting from the pool of three shoots.

4.2.4. Discussion

Contrary to bacteria, only recently it became possible to obtain phytoplasmas cultures (Contaldo *et al.*, 2012; 2016; 2019) and, therefore, *in vitro* shoot propagation is still an important tool for studying phytoplasma-associated diseases (Bertaccini *et al.*, 1992). Periwinkle is a model plant being used to study mechanisms of phytoplasma infection, since it is easily maintained under laboratory conditions, and hosts phytoplasmas exhibiting distinct symptoms (Choi *et al.*, 2004; Perica and Music, 2005; Bertaccini, 2007; Perica *et al.*, 2007; Perica, 2008).

In the current work the alkaloid and phenolic metabolomic profile of periwinkles infected with AY phytoplasmas showing different symptoms was studied. Moreover, it was determined the effect of two MeJA concentrations on gene expression and metabolites production. Metabolomic analysis allowed identifying the main alkaloids and phenolic compounds affected by the presence of phytoplasmas and/or MeJA elicitation. The alkaloids mitraphylline, aricine, yohimbine, strychnine, pleiocarpanine, alstonine, vindolidine and catharine, were detected for the first time in periwinkle shoots. Other alkaloids here detected had been previously identified in *C. roseus* plants, such as ajmalicine (Lee-Parsons *et al.*, 2006; Zhou *et al.*, 2015), catharantine, vindoline (Zhou *et al.*, 2015), serpentine (Lee-Parsons *et al.*, 2006) and lochnerine (Chen *et al.*, 2013). The phenolics deoxy elenoic acid, syringaresinol, maslinic acid, lawsone were identified for the first time in this specie, whereas hydroxytyrosol, caffeic and quinic acids, *p*-coumaric and hydroxybenzoic acids were reported in healthy periwinkles (Proestos *et al.*, 2005; Chen *et al.*, 2017; Liu *et al.*, 2016).

Before MeJA treatment, healthy periwinkles presented a high abundance of vindoline, hydroxytyrosol, deoxy elenoic acid, caffeic acid, lawsone and quinic acid. On the other hand, AY107-infected shoots exhibited high abundance of serpentine, pleiocarpanine and alstonine, while serpentine, pleiocarpanine, aricine, syringaresinol and orientin were more abundant in Hyd8 periwinkle shoots, showing for the first time that phytoplasma strains associated with different symptoms influence the metabolomic profile of periwinkle shoots. Similarly, Favali *et al.* (2004) reported that *C. roseus* infected with clover phyllody phytoplasma showed an increase of ajmalicine by 2-fold and serpentine by 4-fold. Moreover, it was shown that leaves of phytoplasma-infected periwinkles had an increase by 2- to 4-fold of vindoline as well as of its precursors secologanin and loganic acid (Choi *et al.*, 2004), while this was observed in healthy periwinkle shoots. Srivastava *et al.* (2014) have demonstrated that the effect of

phytoplasmas on alkaloid concentration is dependent on the plant organ: ajmalicine and serpentine content decreases in infected flowers of *C. roseus*, whereas a slight increase was observed in infected leaves. Increased serpentine levels may be due to an indirect feedback mechanism resulting from a reduction of vinblastine biosynthesis (Srivastava *et al.*, 2014). This could justify the dramatic increase of serpentine content measured in presence of AY phytoplasma strains, where a decrease on vindoline levels was also observed. The increased levels of serpentine may also lead to be accomplished by a significant increase of pleiocarpanine, aricine and alstonine. Infected shoots exhibited lower levels of vindoline when compared to healthy ones, suggesting that phytoplasma infection could inhibit vindoline biosynthesis, as vincristine and vinblastine, activating the biosynthetic pathway of monoterpenoid indole alkaloids (MIAs).

Several studies have analysed the production of phenolic compounds in phytoplasma infected periwinkles and other crops, such as mulberry, maize and apple (Choi *et al.*, 2004; Junqueira *et al.*, 2004; Musetti *et al.*, 2004; Gai *et al.*, 2014). A previous study in *C. roseus* reported that phytoplasma infections activates the phenylpropanoids biosynthetic pathway increasing chlorogenic acid and polyphenols (gallic acid derivatives) (Choi *et al.*, 2004). However, here periwinkles with AY phytoplasmas had a decrease in the concentration of several phenolic compounds, especially those with reported anti-microbial activity, such as lawsone, deoxy elenoic, caffeic and quinic acids, when compared with healthy shoots. These differences could be explained by the virulence of the strain, the different phenolic compounds analysed and different experimental setups. Hyd8 also led to an increase of syringaresinol and orientin. Syringaresinol is a lignan, which was recently associated with low susceptibility of *Fraxinus excelsior* to the highly destructive ash dieback disease (Nemesio-Gorriz *et al.*, 2020). Orientin, also called luteolin-8-C-glucoside, is a water-soluble flavonoid-like compound with pharmaceutical properties (Lam *et al.*, 2016) and for the first time an increased concentration of orientin in phytoplasma infected shoots is reported in this work. Syringaresinol and orientin compounds appear to be related to the strain virulence, since higher levels were only detected in shoots infected with the most virulent strain. Overall, this study revealed a new metabolic profile induced by phytoplasmas related to strain virulence that include production of serpentine, pleiocarpanine or other MIAs (alstonine and aricine), syringaresinol and orientin.

In fact, the presence of alkaloids and phenolics with therapeutic properties in infected *C. roseus* may provide a pharmaceutical relevance to these plants. Therefore, *in vitro* culture of phytoplasma-infected periwinkles could be interesting for the extraction of therapeutic

metabolites, despite that infected periwinkle, especially AY107 strain, have shown stunted growth and consequently lowest biomass production.

The effect of MeJA elicitation on alkaloids and phenolic compounds profile was found to trigger an increase serpentine and vindoline presence in healthy shoots. On the other hand, MeJA solvent (2.5% ethanol) itself increased aricine in AY107 shoots in both timepoints, while catharantine and alstonine showed high production throughout the study. El-Sayed and Verpoorte (2002, 2004) have shown that MeJA induced catharanthine, ajmalicine and strictosidine accumulation in *C. roseus* cell suspension cultures. When treated with 10 and 100 µM MeJA these cells enhanced the ajmalicine production 9 days after treatment (Lee-Parsons and Estürk, 2005). Elicitor application to *in vitro* plants is an interesting finding in the study of plant physiology and development. Liu *et al.* (2016) reported that healthy *C. roseus* elicited with a mixture of ethylene (ET) and MeJA (significantly increased the content of phenolic compounds and alkaloid intermediates (Liu *et al.*, 2016). Studies using *in vitro* cell cultures of healthy *C. roseus* have also reported that exogenous application of MeJA to *in vitro* cell cultures increases ajmalicine, catharanthine, serpentine and vindoline (Hernández-Dominguez *et al.*, 2004; Lee-Parsons *et al.*, 2004, 2006; Vásquez-Flota *et al.*, 2009; Almagro *et al.*, 2014b; Zhou *et al.*, 2009, 2015). The current study shows for the first time that the biosynthesis of phenolic compounds was also affected by phytoplasma presence, decreasing organic acids, such as deoxy elenoic, maslinic, caffeic, *p*-coumaric and quinic, and increasing flavonoids, namely orientin, as well as syringaresinol, a lignan. MeJA application increased significantly deoxy elenoic acid. However, the MeJA solvent *per se* is also able to decrease the relative abundance of all compounds, with exception of Hyd8-shoots 4 days after MeJA elicitation.

The expression modulation of five genes of the phenylpropanoid and alkaloid pathways was studied comparing expression patterns between periwinkle infected with phytoplasma strains having different virulence. Phytoplasma presence triggered both biosynthetic pathways by the upregulation of the *as*, *str*, *prx* and *chs* genes, especially in Hyd8-shoots, suggesting that both alkaloid and phenylpropanoid pathways are involved in the plant´s response against phytoplasmas. Moreover, the upregulation of *as* and *str* genes, responsible for TIA biosynthesis (Almagro *et al.*, 2014b; Pan *et al.*, 2018), corroborate the observed increases in alkaloids content, especially ajmalicine and serpentine. A positive correlation was previously described between the expression of terpenoid pathway genes and the accumulation of related alkaloids in phytoplasma-infected periwinkle flowers (Srivastava *et al.*, 2014). The expression of MIA genes related to strictosidine, such as lochnerine and alstonine, was also increased, suggesting that in phytoplasma-infected periwinkles this pathway, producing vinblastine and vincristine,

is activated instead of the TIA. In agreement with these findings, the *str* gene was significantly upregulated in periwinkles infected with *Spiroplasma citri*, indicating this gene as a potential biomarker of infection (Nejat *et al.*, 2012). However, Srivastava *et al.* (2014) observed that the expression level of *pal*, *str* and *prx* genes was not significantly different between healthy and phytoplasma-infected periwinkles.

Although the PRX enzyme has been related to the production of vincristine and vinblastine by the polymerization of catharanthine and vindoline (Costa *et al.*, 2007), the results obtained strongly suggest that *prx* gene might be associated with production of serpentine, since PRX also catalyses this reaction. Studies aiming to identify peroxidase isoforms will help to understand the biological function of PRXs in the alkaloid biosynthesis. Previous studies have also shown that *prx* gene expression was induced by wounding, UV exposition and cold (Kumar *et al.*, 2005; Costa *et al.*, 2007), suggesting a wide role of this gene in plant stress control.

Phenylpropanoid biosynthesis pathway-encoding genes such as *chs* was increased in coconut, grapevine, and tomato plants infected by phytoplasmas (Matjaz *et al.*, 2009; Ahmad *et al.*, 2013; Nejat *et al.*, 2015). In the present study AY phytoplasmas also upregulated the expression of the *chs* gene, confirming that these pathogens trigger phenylpropanoid biosynthesis pathway as previously reported (Ahmad *et al.,* 2013). A study on tomato plants infected with two strains of "stolbur" phytoplasmas demonstrated that different phytoplasma strains affected differentially the plant defence metabolism. Furthermore, Wang *et al.* (2018) showed that '*Ca.* P. *ziziphi*' associated with jujube witches' broom, modulate the phenylpropanoid biosynthesis genes in jujube tree upregulating *pal* and *chs* genes 5.34-fold and 5.57-fold, respectively. The 12.5 and 25 mM MeJA upregulated the expression of *as*, *str*, *pal* and *chs* genes in AY107 and Hyd8 infected shoot just one 1 day after elicitation, furthermore, 4 days after MeJA application the upregulation of the same group of genes in the three types of studied periwinkle shoots was observed. A previous study revealed that 6 µM MeJA upregulated *str* and *tdc* (tryptophan decarboxylase) genes in healthy *C. roseus* 6 hours after elicitation while the *pal* gene was downregulated (Bahieldin *et al.*, 2018), suggesting that MeJA triggered indole alkaloids pathway instead of phenylpropanoids biosynthesis. In the work done here although *pal* gene did not show difference among the studied periwinkle clones before MeJA application, in T2 both MeJA concentrations upregulated both genes of the phenylpropanoid pathway, especially in phytoplasma-infected shoots. The MeJA application also downregulated *prx* gene, which corroborate the fact that bisindole alkaloids, such as vincristine and vinblastine, were not affected by the elicitor treatment. Other studies have shown that MeJA in combination with cyclodextrins or ethylene upregulated *as* and *str* genes

in periwinkle cultured cells, increasing the alkaloid content, especially ajmalicine, serpentine and catharanthine (Almagro *et al.*, 2014b; Pan *et al.*, 2018). Further, application of MeJA and benzothiadiazole (BTH), a synthetic analogue of salicylic acid, before phytoplasma infection, led to a pre-activation of defence pathways by upregulating the *pal*, *chs* and *pr* genes, enhancing the plants secondary metabolism and the tolerance to phytoplasma infections (Ahmad *et al.*, 2013).

4.2.5. Conclusions

The present study is an integrated approach on the effects of "aster yellows" phytoplasmas presence in metabolomic profiles and gene expression of *in vitro* healthy and infected periwinkle shoots with two differentially virulent strains and the role of MeJA in the modulation of defence mechanisms. Among the 24 compounds (13 alkaloids and 11 phenolics) identified, 8 new alkaloids (mitraphylline, aricine, yohimbine, strychnine, pleiocarpanine, alstonine, vindolidine and catharine) and 4 new phenolic compounds, (deoxy elenoic acid, syringaresinol, maslinic acid, and lawsone) were detected. Gene expression revealed that phytoplasma infection activated both alkaloid and phenylpropanoid biosynthesis pathways, upregulating *as*, *str*, *prx* (alkaloid pathway) and *chs* (phenylpropanoid pathway) genes. The presence of these alkaloids and phenolics in infected *C. roseus* may provide a pharmaceutical relevance to these plants, since *in vitro* culture of phytoplasma-infected periwinkles could be interesting for the extraction of therapeutic metabolites. Moreover, the present study sheds lights on the role of MeJA either as a novel and promising approach to manage phytoplasma diseases. The MeJA elicitation triggered early upregulation of genes in AY107 and Hyd8 infected shoots indicating that in presence of a virulent strain, a higher elicitor concentration is necessary to activate plant defences. On the other hand, at a later timepoint, all targeted genes were upregulated, suggesting that with time, in the infected plants the gene expression to the elicitor application dosage can be adjusted. Thus, understanding how elicitors can enhance plant´s responsiveness will be a step forward towards establishing new strategies to control phytoplasma infections and to also verify, in *C. roseus*, their possible interest for the commercial production of alkaloids as anti-cancer drugs.

4.2.6. Supplementary data

Table 8. Primer sequences of housekeeping and target genes analysed by RT-qPCR. Abbreviations: T_{ann} = annealing temperature.

	GenBank Accession number	Primer sequence (5′-3′)		T_{ann} (ºC)	Reference
		Forward	Reverse		
Housekeeping genes					
act (*Actin*)	XM_006597211.3	GGC GGA TGC TGA GGA TAT TC	TCC AGA GTC CAG AAC AAT ACC A	56.8	He *et al.*, 2011
rsp9 (*40S ribosomal protein S9*)	AJ749993	GAG GGC CAA AAC AAA CTT GA	CCC TTA TGT GCC TTT GCC TA	56.3	Peebles *et al.*, 2009
Phenylpropanoids					
pal (*Phenylalanine Ammonia Lyase*)	AB042520.1	GGC CAC CAA GAT GAT CGA	CAA TGG CCA ATC TTG CAT TG	56.8	Liu *et al.* 2016
chs **(***Chalcone Synthase***)**	AJ131813.1	GGC AAA GAA GCC GCT CAA AA	ACG CTT AAC GGA AGA GCG AA	59.6	Kaltenbach *et al.*, 1999
Alkaloids					
as (*Anthranilate Synthase*)	AJ250008.1	GCG AAC ATT TGC AGA TCC AT	GGC CGA TTT GTT ATT GTT CC	56.2	Liu *et al.* 2016
str (*Strictosidine Synthase*)	KU214864.1	AAC CAT ACC CCA TAG CTG CC	CGG AGC ATA GGA AGG GCT TT	57.9	This study
prx (*Peroxidase*)	N809932.1	AGG GAT TCC GTT GTT GCC TT	GCC GGA TAG GGC TAC CAT TT	60.3	This study

Table 9. Metabolomic profile determined by LC-ESI-UHR-QqTOF-MS in MS/MS Mode in *C. roseus* healthy (HV), and with mild (AY 107) and high (Hyd 8) virulent strains of aster yellows phytoplasmas, before elicitor application. Each value is the mean of fold change of three biological replicates resulting from the pool of three plants ± SEM. Gallic acid and hydroxytyrosol were used as internal standard to negative and positive modes, respectively. Different letters are statistically different at $P < 0.05$ according to one-way ANOVA followed by comparisons for each pair using Student's *t* test.

Metabolite	Retention time (min)	*Catharanthus roseus* population		
		HV	AY107	Hyd8
Ajmalicine	5.9	$9.17 \times 10^{7} \pm 5.02 \times 10^{7\,a}$	$8.54 \times 10^{7} \pm 2.45 \times 10^{5\,a}$	$2.77 \times 10^{7} \pm 1.91 \times 10^{6\,b}$
Catharantine	6.8	$1.70 \times 10^{8} \pm 3.54 \times 10^{6\,a}$	$1.89 \times 10^{8} \pm 1.06 \times 10^{7\,a}$	$2.21 \times 10^{8} \pm 2.75 \times 10^{6\,a}$
Serpentine	9.3	$3.46 \times 10^{8} \pm 1.44 \times 10^{6\,c}$	$5.04 \times 10^{8} \pm 5.74 \times 10^{6\,a}$	$3.82 \times 10^{8} \pm 4.77 \times 10^{6\,b}$
Vindoline	9.8	$6.40 \times 10^{8} \pm 2.99 \times 10^{7\,a}$	$4.82 \times 10^{8} \pm 6.42 \times 10^{6\,b}$	$4.67 \times 10^{8} \pm 6.50 \times 10^{6\,b}$
Lochnerine	3.7	$9.95 \times 10^{7} \pm 5.24 \times 10^{6\,a}$	$1.14 \times 10^{8} \pm 1.79 \times 10^{6\,a}$	$1.42 \times 10^{7} \pm 4.00 \times 10^{5\,b}$
Mitraphylline	3.9	$1.95 \times 10^{7} \pm 1.24 \times 10^{6\,b}$	$1.64 \times 10^{7} \pm 2.01 \times 10^{5\,b}$	$4.05 \times 10^{7} \pm 5.34 \times 10^{5\,a}$
Aricine	6.9	$5.63 \times 10^{7} \pm 5.55 \times 10^{6\,b}$	$3.84 \times 10^{7} \pm 2.30 \times 10^{6\,b}$	$1.74 \times 10^{8} \pm 1.04 \times 10^{7\,a}$
Yohimbine	4.2	$7.54 \times 10^{7} \pm 1.74 \times 10^{6\,a}$	$6.07 \times 10^{7} \pm 2.50 \times 10^{6\,a}$	$6.65 \times 10^{7} \pm 7.92 \times 10^{6\,a}$
Strychnine	8.3	$3.73 \times 10^{7} \pm 3.03 \times 10^{6\,a}$	$2.23 \times 10^{7} \pm 8.06 \times 10^{5\,b}$	$2.97 \times 10^{7} \pm 6.33 \times 10^{5\,ab}$
Pleiocarpanine	8.8	$0.00 \pm 0.00^{\,b}$	$8.15 \times 10^{7} \pm 9.57 \times 10^{5\,a}$	$7.60 \times 10^{7} \pm 7.28 \times 10^{6\,a}$
Alstonine	9.3	$3.48 \times 10^{8} \pm 7.13 \times 10^{6\,b}$	$5.06 \times 10^{8} \pm 5.74 \times 10^{6\,a}$	$3.82 \times 10^{8} \pm 5.21 \times 10^{6\,b}$
Vindolidine	6.4	$6.21 \times 10^{7} \pm 2.00 \times 10^{6\,a}$	$5.00 \times 10^{7} \pm 1.04 \times 10^{5\,b}$	$2.97 \times 10^{7} \pm 1.08 \times 10^{6\,c}$
Catharine	12.8	$4.11 \times 10^{5} \pm 5.28 \times 10^{3\,c}$	$7.56 \times 10^{5} \pm 2.46 \times 10^{4\,b}$	$1.58 \times 10^{6} \pm 9.71 \times 10^{3\,a}$
Hydroxytyrosol	5.5	$2.96 \times 10^{6} \pm 1.21 \times 10^{5\,a}$	$1.50 \times 10^{6} \pm 1.16 \times 10^{5\,b}$	$1.85 \times 10^{6} \pm 4.66 \times 10^{4\,b}$
Deoxy elenoic acid	3.1	$5.53 \times 10^{6} \pm 2.72 \times 10^{5\,a}$	$1.40 \times 10^{6} \pm 6.83 \times 10^{4\,b}$	$1.61 \times 10^{6} \pm 9.26 \times 10^{5\,b}$
Elenoic acid	4	$2.70 \times 10^{6} \pm 2.72 \times 10^{5\,c}$	$1.40 \times 10^{6} \pm 6.83 \times 10^{4\,b}$	$1.61 \times 10^{6} \pm 9.26 \times 10^{5\,a}$
Syringaresinol	1.1	$2.48 \times 10^{6} \pm 7.43 \times 10^{4\,a}$	$2.59 \times 10^{6} \pm 8.20 \times 10^{4\,a}$	$5.94 \times 10^{6} \pm 2.82 \times 10^{4\,a}$
Maslinic acid	3.7	$8.95 \times 10^{5} \pm 9.39 \times 10^{4\,a}$	$3.61 \times 10^{5} \pm 5.33 \times 10^{4\,a}$	$5.37 \times 10^{5} \pm 1.80 \times 10^{4\,a}$
Caffeic acid	4.3	$3.02 \times 10^{7} \pm 1.56 \times 10^{6\,a}$	$1.05 \times 10^{7} \pm 9.24 \times 10^{5\,b}$	$1.37 \times 10^{7} \pm 4.97 \times 10^{5\,b}$

Lawsone	4	$1.06 \times 10^{7} \pm 1.06 \times 10^{6}$ [a]	$2.93 \times 10^{6} \pm 1.68 \times 10^{6}$ [b]	$4.69 \times 10^{6} \pm 2.52 \times 10^{4}$ [ab]
***p*-coumaric acid**	3.5	$1.75 \times 10^{6} \pm 0.00$ [a]	$1.11 \times 10^{6} \pm 1.50 \times 10^{4}$ [c]	$1.39 \times 10^{6} \pm 3.61 \times 10^{4}$ [b]
Quinic acid	5	$5.05 \times 10^{7} \pm 0.00$ [a]	$2.08 \times 10^{7} \pm 1.28 \times 10^{7}$ [a]	$3.93 \times 10^{7} \pm 4.19 \times 10^{5}$ [a]
Orientin	3.5	$1.90 \times 10^{6} \pm 0.00$ [b]	$1.23 \times 10^{6} \pm 9.80 \times 10^{4}$ [c]	$4.81 \times 10^{6} \pm 1.29 \times 10^{5}$ [a]
Hydroxybenzoic acid	3.6	$5.15 \times 10^{5} \pm 0.00$ [a]	$3.55 \times 10^{5} \pm 4.42 \times 10^{4}$ [a]	$6.27 \times 10^{5} \pm 1.39 \times 10^{5}$ [a]

Table 10. Metabolomic profile determined by LC-ESI-UHR-QqTOF-MS in MS/MS Mode in *C. roseus* healthy (HV), and with mild (AY 107) and high (Hyd8) virulent strains of aster yellows phytoplasmas, 1 and 4 days after elicitor treatments (T1 and T2, respectively). Each value is the mean of fold change of three biological replicates resulting from the pool of three plants ± SEM. Gallic acid and hydroxytyrosol were used as internal standard to negative and positive modes, respectively. For each timepoint, different letters are statistically different at $P < 0.05$ according to two-way ANOVA followed by Tukey's post-hoc test.

Metabolite	*Catharanthus roseus* population	Treatment / Timepoint							
		T1				T2			
		Unt	0 mM MeJA	12.5 mM MeJA	25 mM MeJA	Unt	0 mM MeJA	12.5 mM MeJA	25 mM MeJA
Ajmalicine	**HV**	$4.02 \times 10^{7} \pm 1.40 \times 10^{6\ cd}$	$0.00 \pm 0.00^{\ f}$	$0.00 \pm 0.00^{\ f}$	$2.99 \times 10^{7} \pm 3.16 \times 10^{6\ de}$	$0.00 \pm 0.00^{\ c}$	$0.00 \pm 0.00^{\ c}$	$0.00 \pm 0.00^{\ c}$	$7.76 \times 10^{7} \pm 5.73 \times 10^{5\ a}$
	AY107	$4.98 \times 10^{7} \pm 3.06 \times 10^{6\ c}$	$1.03 \times 10^{8} \pm 2.20 \times 10^{3\ a}$	$2.99 \times 10^{7} \pm 4.39 \times 10^{5\ de}$	$7.23 \times 10^{7} \pm 5.83 \times 10^{5\ b}$	$8.07 \times 10^{7} \pm 5.61 \times 10^{6\ ab}$	$1.10 \times 10^{8} \pm 9.99 \times 10^{5\ a}$	$9.32 \times 10^{7} \pm 4.05 \times 10^{6\ ab}$	$4.60 \times 10^{7} \pm 1.24 \times 10^{4\ abc}$
	Hyd8	$2.45 \times 10^{7} \pm 9.47 \times 10^{4\ e}$	$3.30 \times 10^{7} \pm 9.69 \times 10^{4\ de}$	$2.90 \times 10^{7} \pm 4.68 \times 10^{6\ de}$	$3.86 \times 10^{7} \pm 1.11 \times 10^{5\ cde}$	$3.43 \times 10^{7} \pm 7.66 \times 10^{5\ bc}$	$7.40 \times 10^{7} \pm 7.44 \times 10^{5\ ab}$	$5.33 \times 10^{7} \pm 9.10 \times 10^{5\ abc}$	$5.14 \times 10^{7} \pm 3.12 \times 10^{7\ abc}$
Catharantine	**HV**	$3.21 \times 10^{8} \pm 8.71 \times 10^{7\ a}$	$1.55 \times 10^{8} \pm 1.02 \times 10^{6\ a}$	$1.36 \times 10^{8} \pm 4.28 \times 10^{6\ a}$	$1.77 \times 10^{8} \pm 1.82 \times 10^{7\ a}$	$1.98 \times 10^{8} \pm 6.82 \times 10^{6\ a}$	$1.04 \times 10^{8} \pm 1.38 \times 10^{6\ ac}$	$1.49 \times 10^{8} \pm 0.00^{\ ab}$	$5.82 \times 10^{7} \pm 4.11 \times 10^{7\ bc}$
	AY107	$2.13 \times 10^{8} \pm 1.12 \times 10^{7\ a}$	$1.43 \times 10^{8} \pm 1.62 \times 10^{6\ a}$	$1.54 \times 10^{8} \pm 4.30 \times 10^{6\ a}$	$1.39 \times 10^{8} \pm 1.11 \times 10^{6\ a}$	$1.62 \times 10^{8} \pm 1.56 \times 10^{7\ ab}$	$1.24 \times 10^{8} \pm 1.23 \times 10^{5\ ac}$	$1.49 \times 10^{8} \pm 1.61 \times 10^{6\ ab}$	$1.33 \times 10^{8} \pm 5.76 \times 10^{5\ ac}$
	Hyd8	$1.87 \times 10^{8} \pm 6.43 \times 10^{5\ a}$	$1.59 \times 10^{8} \pm 1.04 \times 10^{6\ a}$	$1.89 \times 10^{8} \pm 1.82 \times 10^{7\ a}$	$1.30 \times 10^{8} \pm 6.21 \times 10^{5\ a}$	$1.92 \times 10^{8} \pm 8.39 \times 10^{6\ a}$	$1.75 \times 10^{7} \pm 1.69 \times 10^{6\ a}$	$9.69 \times 10^{7} \pm 1.82 \times 10^{5\ ac}$	$2.76 \times 10^{7} \pm 1.95 \times 10^{7\ c}$
Serpentine	**HV**	$3.14 \times 10^{8} \pm 9.10 \times 10^{6\ a}$	$0.00 \pm 0.00^{\ b}$	$0.00 \pm 0.00^{\ b}$	$1.94 \times 10^{8} \pm 1.24 \times 10^{8\ ab}$	$0.00 \pm 0.00^{\ b}$	$0.00 \pm 0.00^{\ b}$	$3.28 \times 10^{8} \pm 0.00^{\ a}$	$1.41 \times 10^{8} \pm 9.99 \times 10^{7\ ab}$
	AY107	$4.08 \times 10^{8} \pm 1.20 \times 10^{7\ a}$	$4.05 \times 10^{8} \pm 2.30 \times 10^{6\ a}$	$4.12 \times 10^{8} \pm 9.37 \times 10^{6\ a}$	$4.72 \times 10^{8} \pm 3.33 \times 10^{6\ a}$	$4.11 \times 10^{8} \pm 2.13 \times 10^{7\ a}$	$3.78 \times 10^{8} \pm 5.14 \times 10^{5\ a}$	$4.09 \times 10^{8} \pm 1.02 \times 10^{7\ a}$	$3.59 \times 10^{8} \pm 2.34 \times 10^{6\ a}$
	Hyd8	$4.18 \times 10^{8} \pm 3.63 \times 10^{5\ a}$	$3.55 \times 10^{8} \pm 4.27 \times 10^{6\ a}$	$3.51 \times 10^{8} \pm 1.60 \times 10^{7\ a}$	$3.24 \times 10^{8} \pm 2.33 \times 10^{6\ a}$	$3.91 \times 10^{8} \pm 8.21 \times 10^{6\ a}$	$3.21 \times 10^{8} \pm 1.27 \times 10^{6\ a}$	$3.17 \times 10^{8} \pm 1.65 \times 10^{6\ a}$	$1.67 \times 10^{8} \pm 9.14 \times 10^{7\ ab}$

Vindoline	**HV**	$6.78 \times 10^{8} \pm 1.67 \times 10^{7}$ [a]	0.00 ± 0.00 [e]	0.00 ± 0.00 [e]	$5.60 \times 10^{8} \pm 6.35 \times 10^{6}$ [b]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	$5.15 \times 10^{8} \pm 0.00$ [a]	$5.21 \times 10^{8} \pm 1.52 \times 10^{7}$ [a]
	AY107	$5.48 \times 10^{8} \pm 9.81 \times 10^{6}$ [b]	$4.37 \times 10^{8} \pm 3.30 \times 10^{6}$ [d]	$4.48 \times 10^{8} \pm 5.36 \times 10^{6}$ [cd]	$4.56 \times 10^{8} \pm 1.75 \times 10^{6}$ [cd]	$2.16 \times 10^{8} \pm 1.15 \times 10^{8}$ [a]	$4.62 \times 10^{8} \pm 2.06 \times 10^{6}$ [a]	$4.52 \times 10^{8} \pm 5.96 \times 10^{6}$ [a]	$3.54 \times 10^{8} \pm 1.48 \times 10^{6}$ [a]
	Hyd8	$5.19 \times 10^{8} \pm 1.51 \times 10^{6}$ [bc]	$4.44 \times 10^{8} \pm 4.28 \times 10^{6}$ [d]	$5.33 \times 10^{8} \pm 2.31 \times 10^{7}$ [b]	$4.50 \times 10^{8} \pm 3.22 \times 10^{6}$ [cd]	$4.79 \times 10^{8} \pm 1.22 \times 10^{7}$ [a]	$3.06 \times 10^{8} \pm 1.77 \times 10^{8}$ [a]	$4.33 \times 10^{8} \pm 1.86 \times 10^{6}$ [a]	$2.17 \times 10^{8} \pm 1.47 \times 10^{8}$ [a]
Lochnerine	**HV**	$1.46 \times 10^{8} \pm 4.13 \times 10^{6}$ [a]	$4.95 \times 10^{9} \pm 3.43 \times 10^{9}$ [a]	$9.40 \times 10^{7} \pm 6.41 \times 10^{5}$ [a]	$1.24 \times 10^{8} \pm 9.84 \times 10^{5}$ [a]	$6.19 \times 10^{8} \pm 3.45 \times 10^{8}$ [a]	$4.04 \times 10^{7} \pm 5.19 \times 10^{5}$ [a]	$7.04 \times 10^{7} \pm 0.00$ [a]	$2.90 \times 10^{7} \pm 7.73 \times 10^{5}$ [a]
	AY107	$8.18 \times 10^{7} \pm 1.74 \times 10^{6}$ [a]	$9.56 \times 10^{7} \pm 1.92 \times 10^{6}$ [a]	$6.26 \times 10^{7} \pm 1.58 \times 10^{6}$ [a]	$5.93 \times 10^{7} \pm 4.11 \times 10^{5}$ [a]	$8.95 \times 10^{7} \pm 2.56 \times 10^{7}$ [a]	$5.38 \times 10^{7} \pm 8.68 \times 10^{5}$ [a]	$6.53 \times 10^{7} \pm 5.74 \times 10^{5}$ [a]	$1.34 \times 10^{7} \pm 4.06 \times 10^{4}$ [a]
	Hyd8	$1.84 \times 10^{7} \pm 4.73 \times 10^{5}$ [a]	$1.61 \times 10^{7} \pm 4.67 \times 10^{5}$ [a]	$5.51 \times 10^{7} \pm 2.00 \times 10^{6}$ [a]	$1.82 \times 10^{7} \pm 3.13 \times 10^{4}$ [a]	$1.22 \times 10^{7} \pm 3.92 \times 10^{5}$ [a]	$2.20 \times 10^{7} \pm 4.87 \times 10^{4}$ [a]	$4.23 \times 10^{7} \pm 1.23 \times 10^{5}$ [a]	$6.19 \times 10^{6} \pm \times 0.00$ [a]
Mitraphylline	**HV**	$1.19 \times 10^{7} \pm 3.36 \times 10^{5}$ [f]	$6.07 \times 10^{6} \pm 3.01 \times 10^{5}$ [fg]	0.00 ± 0.00 [g]	$8.31 \times 10^{6} \pm 1.89 \times 10^{5}$ [fg]	$4.47 \times 10^{7} \pm 2.21 \times 10^{6}$ [ab]	$3.95 \times 10^{6} \pm 6.80 \times 10^{4}$ [de]	$3.74 \times 10^{6} \pm 0.00$ [de]	0.00 ± 0.00 [e]
	AY107	$7.46 \times 10^{6} \pm 4.89 \times 10^{5}$ [fg]	$4.26 \times 10^{7} \pm 7.98 \times 10^{5}$ [c]	$1.54 \times 10^{7} \pm 1.15 \times 10^{5}$ [ef]	$9.38 \times 10^{6} \pm 5.72 \times 10^{4}$ [f]	$1.39 \times 10^{7} \pm 3.66 \times 10^{6}$ [cd]	$3.49 \times 10^{7} \pm 2.22 \times 10^{5}$ [b]	$9.67 \times 10^{6} \pm 2.52 \times 10^{5}$ [de]	$4.61 \times 10^{6} \pm 2.94 \times 10^{4}$ [de]
	Hyd8	$8.45 \times 10^{7} \pm 3.08 \times 10^{6}$ [a]	$2.38 \times 10^{7} \pm 1.54 \times 10^{5}$ [de]	$5.33 \times 10^{7} \pm 2.02 \times 10^{6}$ [b]	$3.23 \times 10^{7} \pm 1.83 \times 10^{5}$ [d]	$5.25 \times 10^{7} \pm 1.34 \times 10^{6}$ [a]	$3.55 \times 10^{7} \pm 1.87 \times 10^{5}$ [b]	$2.41 \times 10^{7} \pm 3.22 \times 10^{4}$ [c]	$1.02 \times 10^{7} \pm 0.00$ [de]
Aricine	**HV**	$7.16 \times 10^{7} \pm 7.44 \times 10^{6}$ [c]	$4.49 \times 10^{7} \pm 6.63 \times 10^{6}$ [c]	$3.32 \times 10^{7} \pm 1.57 \times 10^{6}$ [c]	$3.95 \times 10^{7} \pm 5.90 \times 10^{6}$ [c]	$9.80 \times 10^{7} \pm 3.91 \times 10^{6}$ [c]	$4.12 \times 10^{7} \pm 3.21 \times 10^{6}$ [de]	$3.60 \times 10^{7} \pm 0.00$ [de]	0.00 ± 0.00 [f]
	AY107	$3.56 \times 10^{7} \pm 5.94 \times 10^{6}$ [c]	$1.79 \times 10^{8} \pm 8.15 \times 10^{5}$ [ab]	$6.80 \times 10^{7} \pm 3.93 \times 10^{6}$ [c]	$2.42 \times 10^{7} \pm 4.96 \times 10^{4}$ [c]	$5.21 \times 10^{7} \pm 1.30 \times 10^{7}$ [d]	$1.97 \times 10^{8} \pm 1.29 \times 10^{6}$ [a]	$2.66 \times 10^{7} \pm 2.62 \times 10^{5}$ [df]	$9.60 \times 10^{6} \pm 2.80 \times 10^{5}$ [ef]
	Hyd8	$2.10 \times 10^{8} \pm 8.70 \times 10^{5}$ [a]	$1.78 \times 10^{8} \pm 5.22 \times 10^{6}$ [ab]	$1.62 \times 10^{8} \pm 1.86 \times 10^{7}$ [ab]	$1.54 \times 10^{8} \pm 1.70 \times 10^{5}$ [b]	$1.63 \times 10^{8} \pm 1.89 \times 10^{6}$ [b]	$1.94 \times 10^{8} \pm 2.20 \times 10^{6}$ [ab]	$1.19 \times 10^{8} \pm 9.98 \times 10^{5}$ [c]	$4.44 \times 10^{7} \pm 0.00$ [d]
Yohimbine	**HV**	$5.97 \times 10^{7} \pm 2.03 \times 10^{6}$ [abc]	$4.50 \times 10^{7} \pm 6.83 \times 10^{4}$ [de]	$4.98 \times 10^{7} \pm 1.45 \times 10^{6}$ [bde]	$4.50 \times 10^{7} \pm 1.62 \times 10^{6}$ [de]	$8.34 \times 10^{7} \pm 8.12 \times 10^{6}$ [a]	$3.10 \times 10^{7} \pm 2.08 \times 10^{6}$ [bc]	$3.73 \times 10^{7} \pm 0.00$ [bc]	$3.09 \times 10^{7} \pm 6.44 \times 10^{5}$ [bc]
	AY107	$6.16 \times 10^{7} \pm 1.66 \times 10^{6}$ [ab]	$4.72 \times 10^{7} \pm 1.43 \times 10^{6}$ [cde]	$4.26 \times 10^{7} \pm 4.32 \times 10^{5}$ [de]	$3.96 \times 10^{7} \pm 1.89 \times 10^{6}$ [e]	$4.39 \times 10^{7} \pm 6.64 \times 10^{6}$ [bc]	$4.64 \times 10^{7} \pm 7.00 \times 10^{5}$ [b]	$4.44 \times 10^{7} \pm 1.96 \times 10^{5}$ [bc]	$2.80 \times 10^{7} \pm 1.19 \times 10^{6}$ [bc]
	Hyd8	$6.78 \times 10^{7} \pm 1.66 \times 10^{6}$ [a]	$5.08 \times 10^{7} \pm 6.77 \times 10^{5}$ [bde]	$5.37 \times 10^{7} \pm 3.88 \times 10^{6}$ [bd]	$3.87 \times 10^{7} \pm 3.93 \times 10^{5}$ [e]	$4.96 \times 10^{7} \pm 2.20 \times 10^{6}$ [b]	$5.43 \times 10^{7} \pm 1.06 \times 10^{6}$ [ab]	$2.76 \times 10^{7} \pm 3.57 \times 10^{5}$ [bc]	$1.38 \times 10^{7} \pm 8.34 \times 10^{6}$ [c]

Strychnine	**HV**	$3.15 \times 10^{7} \pm 1.04 \times 10^{6}$ ab	$7.02 \times 10^{6} \pm 4.96 \times 10^{6}$ de	$1.54 \times 10^{7} \pm 1.01 \times 10^{6}$ ce	$2.69 \times 10^{7} \pm 2.88 \times 10^{5}$ bc	$3.12 \times 10^{7} \pm 4.84 \times 10^{6}$ a	$9.59 \times 10^{6} \pm 5.80 \times 10^{5}$ bc	$9.43 \times 10^{6} \pm 0.00$ bc	$6.14 \times 10^{6} \pm 3.42 \times 10^{5}$ c
	AY107	$3.07 \times 10^{7} \pm 1.88 \times 10^{6}$ b	$9.68 \times 10^{6} \pm 2.03 \times 10^{5}$ de	$1.89 \times 10^{7} \pm 9.38 \times 10^{5}$ be	$1.03 \times 10^{7} \pm 2.33 \times 10^{4}$ e	$1.44 \times 10^{7} \pm 6.33 \times 10^{6}$ abc	$9.73 \times 10^{6} \pm 1.01 \times 10^{5}$ bc	$5.35 \times 10^{6} \pm 3.78 \times 10^{6}$ c	$8.05 \times 10^{6} \pm 1.08 \times 10^{5}$ bc
	Hyd8	$4.37 \times 10^{7} \pm 2.54 \times 10^{5}$ a	$2.75 \times 10^{7} \pm 7.10 \times 10^{4}$ bc	$2.52 \times 10^{7} \pm 2.01 \times 10^{5}$ bc	$1.20 \times 10^{7} \pm 3.02 \times 10^{5}$ e	$3.32 \times 10^{7} \pm 6.85 \times 10^{5}$ a	$2.72 \times 10^{7} \pm 1.39 \times 10^{5}$ ab	$7.30 \times 10^{6} \pm 1.44 \times 10^{5}$ bc	0.00 ± 0.00 c
Pleiocarpanine	**HV**	$9.49 \times 10^{7} \pm 9.91 \times 10^{5}$ a	$8.87 \times 10^{7} \pm 3.68 \times 10^{6}$ a	$5.64 \times 10^{7} \pm 3.77 \times 10^{6}$ a	$7.18 \times 10^{7} \pm 1.29 \times 10^{7}$ a	$8.55 \times 10^{7} \pm 4.82 \times 10^{6}$ a	$6.39 \times 10^{7} \pm 5.65 \times 10^{6}$ ab	$8.49 \times 10^{7} \pm 0.00$ a	$5.73 \times 10^{7} \pm 2.65 \times 10^{6}$ ac
	AY107	$7.07 \times 10^{7} \pm 7.57 \times 10^{5}$ a	$6.40 \times 10^{7} \pm 1.66 \times 10^{6}$ a	$6.06 \times 10^{7} \pm 4.95 \times 10^{6}$ a	$6.55 \times 10^{7} \pm 3.11 \times 10^{5}$ a	$6.82 \times 10^{7} \pm 1.00 \times 10^{7}$ ab	$6.21 \times 10^{7} \pm 3.16 \times 10^{5}$ ab	$5.90 \times 10^{7} \pm 3.30 \times 10^{6}$ ac	$4.26 \times 10^{7} \pm 9.01 \times 10^{5}$ bc
	Hyd8	$6.83 \times 10^{7} \pm 9.36 \times 10^{5}$ a	$7.16 \times 10^{7} \pm 1.25 \times 10^{6}$ a	$7.29 \times 10^{7} \pm 1.20 \times 10^{7}$ a	$6.40 \times 10^{7} \pm 1.71 \times 10^{6}$ a	$7.52 \times 10^{7} \pm 3.20 \times 10^{6}$ ab	$6.82 \times 10^{7} \pm 1.04 \times 10^{6}$ ab	$4.53 \times 10^{7} \pm 1.11 \times 10^{5}$ ac	$1.85 \times 10^{7} \pm 1.21 \times 10^{7}$ c
Alstonine	**HV**	$3.15 \times 10^{8} \pm 6.36 \times 10^{6}$ d	$2.93 \times 10^{8} \pm 3.66 \times 10^{6}$ d	$3.29 \times 10^{8} \pm 4.10 \times 10^{6}$ d	$3.48 \times 10^{8} \pm 1.56 \times 10^{7}$ cd	$3.90 \times 10^{8} \pm 2.67 \times 10^{7}$ ab	$3.32 \times 10^{8} \pm 2.06 \times 10^{6}$ ab	$3.29 \times 10^{8} \pm 0.00$ ab	$2.84 \times 10^{8} \pm 5.79 \times 10^{6}$ ab
	AY107	$4.09 \times 10^{8} \pm 1.15 \times 10^{7}$ ac	$4.03 \times 10^{8} \pm 8.30 \times 10^{5}$ bc	$4.07 \times 10^{8} \pm 9.38 \times 10^{6}$ ac	$4.70 \times 10^{8} \pm 2.26 \times 10^{6}$ a	$4.10 \times 10^{8} \pm 2.02 \times 10^{7}$ a	$3.79 \times 10^{8} \pm 2.36 \times 10^{5}$ ab	$4.09 \times 10^{8} \pm 1.02 \times 10^{7}$ a	$3.62 \times 10^{8} \pm 3.34 \times 10^{6}$ ab
	Hyd8	$4.18 \times 10^{8} \pm 8.11 \times 10^{5}$ ab	$3.52 \times 10^{8} \pm 3.83 \times 10^{6}$ cd	$3.50 \times 10^{8} \pm 1.65 \times 10^{7}$ cd	$3.24 \times 10^{8} \pm 1.33 \times 10^{5}$ d	$4.30 \times 10^{8} \pm 2.28 \times 10^{7}$ a	$3.19 \times 10^{8} \pm 1.26 \times 10^{6}$ ab	$3.15 \times 10^{8} \pm 2.10 \times 10^{6}$ ab	$1.67 \times 10^{8} \pm 9.06 \times 10^{7}$ b
Vindolidine	**HV**	$7.59 \times 10^{7} \pm 3.94 \times 10^{6}$ a	$5.45 \times 10^{7} \pm 2.41 \times 10^{6}$ b	$4.60 \times 10^{7} \pm 1.04 \times 10^{6}$ bc	$5.40 \times 10^{7} \pm 1.46 \times 10^{6}$ b	$9.14 \times 10^{7} \pm 1.63 \times 10^{6}$ a	$3.25 \times 10^{7} \pm 6.68 \times 10^{5}$ bc	$4.09 \times 10^{7} \pm 0.00$ b	$3.93 \times 10^{7} \pm 9.41 \times 10^{5}$ b
	AY107	$4.61 \times 10^{7} \pm 6.35 \times 10^{4}$ bc	$3.49 \times 10^{7} \pm 1.72 \times 10^{5}$ cd	$3.93 \times 10^{7} \pm 2.01 \times 10^{5}$ c	$4.36 \times 10^{7} \pm 4.28 \times 10^{5}$ bc	$3.92 \times 10^{7} \pm 8.63 \times 10^{6}$ b	$3.17 \times 10^{7} \pm 2.59 \times 10^{5}$ bc	$4.48 \times 10^{7} \pm 1.15 \times 10^{6}$ b	$2.17 \times 10^{7} \pm 6.63 \times 10^{4}$ bc
	Hyd8	$4.16 \times 10^{7} \pm 6.08 \times 10^{5}$ c	$2.51 \times 10^{7} \pm 8.10 \times 10^{4}$ d	$3.68 \times 10^{7} \pm 4.92 \times 10^{5}$ c	$3.53 \times 10^{7} \pm 2.04 \times 10^{5}$ cd	$4.08 \times 10^{7} \pm 7.88 \times 10^{5}$ b	$3.41 \times 10^{7} \pm 8.39 \times 10^{4}$ b	$2.09 \times 10^{7} \pm 1.10 \times 10^{5}$ bc	$9.14 \times 10^{6} \pm 5.87 \times 10^{6}$ c
Catharine	**HV**	$1.22 \times 10^{6} \pm 4.72 \times 10^{4}$ e	0.00 ± 0.00 e	$1.64 \times 10^{6} \pm 1.16 \times 10^{6}$ cd	$1.23 \times 10^{7} \pm 4.72 \times 10^{4}$ a	$2.59 \times 10^{6} \pm 2.40 \times 10^{4}$ c	$2.11 \times 10^{5} \pm 1.49 \times 10^{5}$ c	$4.61 \times 10^{6} \pm 0.00$ c	$5.27 \times 10^{7} \pm 5.55 \times 10^{5}$ a
	AY107	$1.38 \times 10^{6} \pm 2.36 \times 10^{4}$ de	$1.10 \times 10^{6} \pm 2.59 \times 10^{3}$ e	$1.13 \times 10^{7} \pm 4.54 \times 10^{5}$ ab	$1.09 \times 10^{7} \pm 3.36 \times 10^{5}$ ab	$1.72 \times 10^{6} \pm 6.08 \times 10^{5}$ c	$1.15 \times 10^{6} \pm 6.58 \times 10^{4}$ c	$6.60 \times 10^{6} \pm 4.27 \times 10^{4}$ c	$3.00 \times 10^{7} \pm 1.41 \times 10^{5}$ bc
	Hyd8	$2.89 \times 10^{6} \pm 1.14 \times 10^{5}$ cd	$3.75 \times 10^{6} \pm 6.23 \times 10^{4}$ c	$1.05 \times 10^{7} \pm 2.28 \times 10^{5}$ b	$1.23 \times 10^{7} \pm 2.68 \times 10^{5}$ a	$1.75 \times 10^{6} \pm 1.37 \times 10^{5}$ c	$4.14 \times 10^{6} \pm 1.66 \times 10^{5}$ c	$1.33 \times 10^{7} \pm 2.92 \times 10^{5}$ bc	$1.48 \times 10^{7} \pm 9.80 \times 10^{6}$ bc

Hydroxytyrosol	**HV**	$3.16 \times 10^{6} \pm 4.98 \times 10^{4\ b}$	$1.07 \times 10^{5} \pm 3.98 \times 10^{4\ e}$	$1.83 \times 10^{5} \pm 3.64 \times 10^{2\ e}$	$2.40 \times 10^{6} \pm 1.81 \times 10^{4\ cd}$	$4.15 \times 10^{6} \pm 1.02 \times 10^{5\ d}$	$1.02 \times 10^{5} \pm 2.92 \times 10^{3\ h}$	$2.51 \times 10^{6} \pm \times 0.00^{\ fg}$	$1.15 \times 10^{6} \pm 8.59 \times 10^{4\ a}$
	AY107	$2.21 \times 10^{6} \pm 5.34 \times 10^{4\ d}$	$1.13 \times 10^{5} \pm 4.46 \times 10^{3\ e}$	$2.15 \times 10^{6} \pm 1.61 \times 10^{3\ d}$	$3.95 \times 10^{6} \pm 8.27 \times 10^{4\ a}$	$2.92 \times 10^{6} \pm 3.05 \times 10^{4\ ef}$	$1.11 \times 10^{5} \pm 1.67 \times 10^{2\ h}$	$3.08 \times 10^{6} \pm 4.36 \times 10^{4\ e}$	$9.40 \times 10^{6} \pm 1.40 \times 10^{5\ b}$
	Hyd8	$2.68 \times 10^{6} \pm 5.04 \times 10^{4\ c}$	$1.63 \times 10^{5} \pm 4.21 \times 10^{3\ e}$	$3.62 \times 10^{6} \pm 7.58 \times 10^{4\ a}$	$2.08 \times 10^{6} \pm 1.43 \times 10^{4\ d}$	$2.09 \times 10^{6} \pm 3.49 \times 10^{4\ g}$	$2.55 \times 10^{6} \pm 0.00^{\ g}$	$5.72 \times 10^{6} \pm 3.25 \times 10^{4\ c}$	$1.14 \times 10^{7} \pm 9.26 \times 10^{4\ a}$
Deoxy elenoic acid	**HV**	$4.55 \times 10^{6} \pm 1.23 \times 10^{5\ e}$	$3.53 \times 10^{5} \pm 2.13 \times 10^{3\ e}$	$3.02 \times 10^{6} \pm 3.65 \times 10^{4\ e}$	$4.28 \times 10^{7} \pm 2.88 \times 10^{6\ bc}$	$5.14 \times 10^{6} \pm 5.07 \times 10^{4\ de}$	$2.67 \times 10^{5} \pm 2.47 \times 10^{3\ e}$	$2.25 \times 10^{7} \pm 0.00^{\ cd}$	$2.79 \times 10^{7} \pm 1.33 \times 10^{5\ c}$
	AY107	$3.34 \times 10^{6} \pm 2.43 \times 10^{4\ e}$	$1.43 \times 10^{5} \pm 6.58 \times 10^{2\ e}$	$3.42 \times 10^{7} \pm 2.14 \times 10^{5\ cd}$	$5.85 \times 10^{7} \pm 2.66 \times 10^{6\ a}$	$3.05 \times 10^{6} \pm 1.95 \times 10^{5\ de}$	$1.43 \times 10^{5} \pm 1.92 \times 10^{3\ e}$	$2.00 \times 10^{7} \pm 2.74 \times 10^{5\ ce}$	$1.61 \times 10^{8} \pm 2.83 \times 10^{2\ a}$
	Hyd8	$2.65 \times 10^{6} \pm 1.62 \times 10^{4\ e}$	$2.55 \times 10^{5} \pm 7.56 \times 10^{3\ e}$	$5.11 \times 10^{7} \pm 1.63 \times 10^{6\ ab}$	$2.97 \times 10^{7} \pm 1.28 \times 10^{5\ d}$	$1.56 \times 10^{6} \pm 2.31 \times 10^{3\ de}$	$5.58 \times 10^{6} \pm 0.00^{\ de}$	$8.18 \times 10^{7} \pm 9.08 \times 10^{6\ b}$	$1.15 \times 10^{8} \pm 2.12 \times 10^{6\ b}$
Elenoic acid	**HV**	$2.99 \times 10^{6} \pm 2.60 \times 10^{5\ ab}$	$1.58 \times 10^{5} \pm 5.22 \times 10^{3\ e}$	$5.45 \times 10^{5} \pm 3.26 \times 10^{5\ de}$	$1.66 \times 10^{6} \pm 1.20 \times 10^{5\ cd}$	$3.45 \times 10^{6} \pm 9.59 \times 10^{4\ bc}$	$2.10 \times 10^{5} \pm 3.30 \times 10^{3\ f}$	$2.10 \times 10^{6} \pm \times 0.00^{\ de}$	$1.54 \times 10^{6} \pm 2.02 \times 10^{5\ e}$
	AY107	$3.86 \times 10^{6} \pm 5.14 \times 10^{4\ a}$	$1.15 \times 10^{5} \pm 9.34 \times 10^{3\ e}$	$2.30 \times 10^{6} \pm 2.66 \times 10^{4\ bc}$	$1.77 \times 10^{6} \pm 4.05 \times 10^{4\ bd}$	$2.69 \times 10^{6} \pm 3.75 \times 10^{4\ cd}$	$1.88 \times 10^{5} \pm 2.92 \times 10^{4\ f}$	$1.96 \times 10^{6} \pm 6.03 \times 10^{3\ de}$	$1.87 \times 10^{6} \pm 2.61 \times 10^{5\ de}$
	Hyd8	$3.94 \times 10^{6} \pm 2.24 \times 10^{5\ a}$	$1.57 \times 10^{5} \pm 1.80 \times 10^{4\ e}$	$1.82 \times 10^{6} \pm 1.76 \times 10^{5\ bc}$	$1.70 \times 10^{6} \pm 1.43 \times 10^{5\ cd}$	$4.23 \times 10^{6} \pm 5.28 \times 10^{4\ b}$	$2.35 \times 10^{7} \pm 0.00^{\ a}$	$1.56 \times 10^{6} \pm 2.35 \times 10^{5\ e}$	$1.13 \times 10^{6} \pm 5.43 \times 10^{4\ ef}$
Syringaresinol	**HV**	$2.67 \times 10^{6} \pm 3.43 \times 10^{4\ ce}$	$1.53 \times 10^{5} \pm 1.01 \times 10^{4\ f}$	$1.02 \times 10^{5} \pm 7.58 \times 10^{3\ f}$	$2.23 \times 10^{6} \pm 2.95 \times 10^{4\ e}$	$3.83 \times 10^{6} \pm 1.66 \times 10^{5\ bc}$	$1.24 \times 10^{5} \pm 4.62 \times 10^{3\ h}$	$2.01 \times 10^{6} \pm 0.00^{\ fg}$	$1.63 \times 10^{6} \pm 2.01 \times 10^{5\ g}$
	AY107	$2.85 \times 10^{6} \pm 3.21 \times 10^{4\ cd}$	$2.02 \times 10^{5} \pm 4.84 \times 10^{3\ f}$	$3.20 \times 10^{6} \pm 1.16 \times 10^{4\ bc}$	$2.62 \times 10^{6} \pm 1.27 \times 10^{5\ de}$	$3.13 \times 10^{6} \pm 6.62 \times 10^{4\ d}$	$2.46 \times 10^{5} \pm 3.90 \times 10^{3\ h}$	$2.15 \times 10^{6} \pm 5.62 \times 10^{3\ fg}$	$2.34 \times 10^{6} \pm 5.55 \times 10^{4\ ef}$
	Hyd8	$6.52 \times 10^{6} \pm 1.87 \times 10^{5\ a}$	$3.08 \times 10^{5} \pm 5.17 \times 10^{3\ f}$	$3.57 \times 10^{6} \pm 4.39 \times 10^{4\ b}$	$3.51 \times 10^{6} \pm 7.33 \times 10^{4\ bd}$	$8.50 \times 10^{6} \pm 8.13 \times 10^{4\ a}$	$4.24 \times 10^{6} \pm 0.00^{\ b}$	$2.85 \times 10^{6} \pm 9.00 \times 10^{4\ de}$	$3.24 \times 10^{6} \pm 1.06 \times 10^{4\ cd}$
Maslinic acid	**HV**	$8.78 \times 10^{5} \pm 3.80 \times 10^{4\ e}$	$2.80 \times 10^{5} \pm 1.38 \times 10^{4\ fh}$	$1.48 \times 10^{5} \pm 1.49 \times 10^{3\ gh}$	$6.17 \times 10^{6} \pm 7.24 \times 10^{3\ a}$	$5.09 \times 10^{5} \pm 8.43 \times 10^{4\ de}$	$2.80 \times 10^{5} \pm 2.28 \times 10^{3\ f}$	$2.48 \times 10^{6} \pm \times 0.00^{\ b}$	$2.59 \times 10^{6} \pm 5.44 \times 10^{4\ ab}$
	AY107	$3.64 \times 10^{5} \pm 4.54 \times 10^{4\ fg}$	$7.61 \times 10^{4} \pm 6.22 \times 10^{2\ h}$	$3.09 \times 10^{6} \pm 3.54 \times 10^{4\ c}$	$1.36 \times 10^{6} \pm 7.41 \times 10^{4\ d}$	$3.95 \times 10^{5} \pm 7.20 \times 10^{3\ df}$	$7.78 \times 10^{4} \pm 3.97 \times 10^{3\ f}$	$1.45 \times 10^{6} \pm 1.01 \times 10^{5\ c}$	$7.18 \times 10^{5} \pm 2.60 \times 10^{3\ d}$
	Hyd8	$4.21 \times 10^{5} \pm 2.15 \times 10^{4\ f}$	$1.16 \times 10^{5} \pm 5.49 \times 10^{3\ gh}$	$3.84 \times 10^{6} \pm 6.94 \times 10^{3\ b}$	$3.26 \times 10^{6} \pm 4.59 \times 10^{4\ c}$	$2.98 \times 10^{5} \pm 5.96 \times 10^{4\ ef}$	$2.89 \times 10^{6} \pm 0.00^{\ a}$	$1.25 \times 10^{6} \pm 1.07 \times 10^{4\ c}$	$1.46 \times 10^{6} \pm 6.19 \times 10^{4\ c}$

Caffeic acid	**HV**	$2.51 \times 10^{7} \pm 6.91 \times 10^{5}$ [a]	$1.70 \times 10^{6} \pm 2.17 \times 10^{4}$ [e]	$1.45 \times 10^{6} \pm 4.58 \times 10^{4}$ [e]	$1.77 \times 10^{7} \pm 9.08 \times 10^{4}$ [b]	$3.50 \times 10^{7} \pm 4.20 \times 10^{6}$ [a]	$1.38 \times 10^{6} \pm 4.16 \times 10^{4}$ [de]	$1.49 \times 10^{7} \pm 0.00$ [b]	$1.22 \times 10^{7} \pm 6.67 \times 10^{5}$ [bc]
	AY107	$1.84 \times 10^{7} \pm 4.21 \times 10^{4}$ [b]	$5.83 \times 10^{5} \pm 9.15 \times 10^{4}$ [e]	$1.10 \times 10^{7} \pm 2.31 \times 10^{5}$ [c]	$4.31 \times 10^{6} \pm 1.98 \times 10^{6}$ [de]	$1.73 \times 10^{7} \pm 2.76 \times 10^{5}$ [b]	$6.73 \times 10^{5} \pm 3.34 \times 10^{4}$ [e]	$1.16 \times 10^{7} \pm 1.60 \times 10^{5}$ [bd]	$2.79 \times 10^{6} \pm 1.46 \times 10^{6}$ [cde]
	Hyd8	$1.14 \times 10^{7} \pm 1.13 \times 10^{4}$ [c]	$8.13 \times 10^{5} \pm 3.58 \times 10^{4}$ [e]	$1.66 \times 10^{7} \pm 2.29 \times 10^{5}$ [b]	$1.15 \times 10^{7} \pm 4.56 \times 10^{5}$ [c]	$8.42 \times 10^{6} \pm 1.87 \times 10^{3}$ [bd]	$1.29 \times 10^{7} \pm 0.00$ [bc]	$9.47 \times 10^{6} \pm 1.38 \times 10^{5}$ [bde]	$5.96 \times 10^{6} \pm 4.08 \times 10^{5}$ [cde]
Lawsone	**HV**	$8.85 \times 10^{6} \pm 1.28 \times 10^{5}$ [a]	$7.16 \times 10^{5} \pm 3.37 0\times 10^{4}$ [e]	$6.26 \times 10^{5} \pm 8.12 \times 10^{3}$ [e]	$9.10 \times 10^{6} \pm 1.85 \times 10^{5}$ [a]	$9.54 \times 10^{6} \pm 2.68 \times 10^{4}$ [a]	$5.51 \times 10^{5} \pm 4.76 \times 10^{3}$ [i]	$7.08 \times 10^{6} \pm 0.00$ [c]	$2.39 \times 10^{6} \pm 7.77 \times 10^{4}$ [h]
	AY107	$8.20 \times 10^{6} \pm 1.07 \times 10^{5}$ [ab]	$4.12 \times 10^{5} \pm 7.27 \times 10^{3}$ [e]	$9.13 \times 10^{6} \pm 1.66 \times 10^{5}$ [a]	$6.31 \times 10^{6} \pm 1.01 \times 10^{5}$ [c]	$7.84 \times 10^{6} \pm 1.33 \times 10^{5}$ [b]	$4.66 \times 10^{5} \pm 1.21 \times 10^{4}$ [i]	$7.12 \times 10^{6} \pm 1.20 \times 10^{5}$ [c]	$4.18 \times 10^{6} \pm 5.26 \times 10^{4}$ [f]
	Hyd8	$5.07 \times 10^{6} \pm 1.31 \times 10^{5}$ [d]	$3.98 \times 10^{5} \pm 1.62 \times 10^{4}$ [e]	$7.15 \times 10^{6} \pm 2.34 \times 10^{5}$ [bc]	$8.34 \times 10^{6} \pm 2.32 \times 10^{5}$ [ab]	$4.61 \times 10^{6} \pm 6.53 \times 10^{4}$ [f]	$5.77 \times 10^{6} \pm 0.00$ [d]	$5.25 \times 10^{6} \pm 5.60 \times 10^{4}$ [e]	$3.51 \times 10^{6} \pm 3.11 \times 10^{4}$ [g]
***p*- coumaric acid**	**HV**	$8.32 \times 10^{5} \pm 3.02 \times 10^{4}$ [c]	$1.21 \times 10^{5} \pm 1.96 \times 10^{4}$ [d]	$8.23 \times 10^{4} \pm 6.02 \times 10^{3}$ [d]	$2.16 \times 10^{6} \pm 1.68 \times 10^{5}$ [a]	$1.56 \times 10^{6} \pm 4.75 \times 10^{4}$ [ab]	$6.75 \times 10^{4} \pm 1.73 \times 10^{3}$ [d]	$7.38 \times 10^{4} \pm 0.00$ [d]	$6.78 \times 10^{5} \pm 3.60 \times 10^{4}$ [c]
	AY107	$1.96 \times 10^{6} \pm 5.70 \times 10^{4}$ [ab]	$1.26 \times 10^{5} \pm 7.08 \times 10^{3}$ [d]	$2.41 \times 10^{6} \pm 1.44 \times 10^{5}$ [a]	$1.37 \times 10^{6} \pm 9.73 \times 10^{4}$ [bc]	$1.44 \times 10^{6} \pm 9.94 \times 10^{4}$ [ab]	$9.70 \times 10^{4} \pm 9.36 \times 10^{3}$ [d]	$1.71 \times 10^{6} \pm 1.51 \times 10^{4}$ [a]	$8.73 \times 10^{5} \pm 4.24 \times 10^{4}$ [c]
	Hyd8	$1.40 \times 10^{6} \pm 7.32 \times 10^{3}$ [bc]	$1.32 \times 10^{5} \pm 1.79 \times 10^{4}$ [d]	$1.88 \times 10^{6} \pm 5.36 \times 10^{4}$ [ab]	$1.45 \times 10^{6} \pm 2.50 \times 10^{4}$ [b]	$1.39 \times 10^{6} \pm 3.00 \times 10^{3}$ [b]	$1.67 \times 10^{6} \pm 0.00$ [ab]	$9.03 \times 10^{5} \pm 2.09 \times 10^{4}$ [c]	$6.20 \times 10^{5} \pm 2.17 \times 10^{4}$ [c]
Quinic acid	**HV**	$5.55 \times 10^{7} \pm 3.77 \times 10^{6}$ [ab]	$4.84 \times 10^{6} \pm 7.45 \times 10^{4}$ [c]	$3.22 \times 10^{6} \pm 8.43 \times 10^{3}$ [c]	$5.33 \times 10^{7} \pm 8.97 \times 10^{5}$ [ab]	$6.26 \times 10^{7} \pm 5.07 \times 10^{6}$ [a]	$3.57 \times 10^{6} \pm 2.34 \times 10^{3}$ [e]	$3.23 \times 10^{6} \pm 0.00$ [e]	$1.52 \times 10^{7} \pm 7.97 \times 10^{5}$ [de]
	AY107	$5.84 \times 10^{7} \pm 1.09 \times 10^{5}$ [a]	$2.88 \times 10^{6} \pm 1.07 \times 10^{3}$ [c]	$6.34 \times 10^{7} \pm 1.59 \times 10^{5}$ [a]	$5.99 \times 10^{7} \pm 6.96 \times 10^{5}$ [a]	$5.28 \times 10^{7} \pm 9.14 \times 10^{5}$ [ab]	$3.31 \times 10^{6} \pm 5.54 \times 10^{3}$ [e]	$6.41 \times 10^{7} \pm 4.00 \times 10^{5}$ [a]	$3.47 \times 10^{7} \pm 2.84 2.84 \times 10^{5}$ [bc]
	Hyd8	$4.53 \times 10^{7} \pm 5.30 \times 10^{5}$ [b]	$3.31 \times 10^{6} \pm 4.99 \times 10^{4}$ [c]	$5.27 \times 10^{7} \pm 3.48 \times 10^{6}$ [ab]	$5.72 \times 10^{7} \pm 6.86 \times 10^{5}$ [ab]	$4.51 \times 10^{7} \pm 7.07 \times 10^{5}$ [b]	$5.60 \times 10^{7} \pm 0.00$ [ab]	$4.38 \times 10^{7} \pm 2.38 \times 10^{6}$ [b]	$2.67 \times 10^{7} \pm 1.85 \times 10^{5}$ [cd]
Orientin	**HV**	$2.28 \times 10^{6} \pm 4.05 \times 10^{4}$ [a]	$1.41 \times 10^{5} \pm 1.89 \times 10^{3}$ [d]	$1.70 \times 10^{5} \pm 2.11 \times 10^{3}$ [d]	$1.68 \times 10^{6} \pm 3.79 \times 10^{4}$ [b]	$2.00 \times 10^{6} \pm 3.17 \times 10^{3}$ [ab]	$1.23 \times 10^{5} \pm 2.75 \times 10^{2}$ [f]	$1.73 \times 10^{5} \pm 0.00$ [ef]	$9.37 \times 10^{5} \pm 9.18 \times 10^{4}$ [cd]
	AY107	$1.29 \times 10^{6} \pm 6.36 \times 10^{3}$ [c]	$1.59 \times 10^{5} \pm 1.10 \times 10^{4}$ [d]	$1.43 \times 10^{6} \pm 2.10 \times 10^{4}$ [bc]	$1.67 \times 10^{6} \pm 2.14 \times 10^{4}$ [b]	$2.24 \times 10^{6} \pm 1.45 \times 10^{4}$ [a]	$1.17 \times 10^{5} \pm 3.73 \times 10^{2}$ [f]	$7.25 \times 10^{5} \pm 1.03 \times 10^{4}$ [cd]	$1.07 \times 10^{6} \pm 7.78 \times 10^{4}$ [c]
	Hyd8	$2.50 \times 10^{6} \pm 7.18 \times 10^{2}$ [a]	$1.53 \times 10^{5} \pm 8.76 \times 10^{2}$ [d]	$1.28 \times 10^{6} \pm 3.90 \times 10^{4}$ [c]	$1.58 \times 10^{6} \pm 1.35 \times 10^{5}$ [bc]	$2.33 \times 10^{6} \pm 4.30 \times 10^{4}$ [a]	$1.81 \times 10^{6} \pm 0.00$ [b]	$6.11 \times 10^{5} \pm 1.24 \times 10^{5}$ [d]	$5.89 \times 10^{5} \pm 4.17 \times 10^{4}$ [de]

Hydroxybenzoic acid	**HV**	$3.40 \times 10^5 \pm 3.60 \times 10^4$ cd	$4.38 \times 10^4 \pm 0.00$ e	$3.18 \times 10^4 \pm 2.62 \times 10^3$ e	$3.73 \times 10^5 \pm 8.26 \times 10^3$ cd	$4.59 \times 10^5 \pm 3.63 \times 10^4$ b	$1.90 \times 10^4 \pm 3.21 \times 10^3$ d	$2.81 \times 10^4 \pm 0.00$ d	$2.93 \times 10^5 \pm 2.33 \times 10^4$ c
	AY107	$3.04 \times 10^5 \pm 1.25 \times 10^4$ d	$3.77 \times 10^4 \pm 9.19 \times 10^2$ e	$4.19 \times 10^5 \pm 1.73 \times 10^4$ bc	$4.87 \times 10^5 \pm 6.60 \times 10^3$ bc	$4.10 \times 10^5 \pm 2.75 \times 10^4$ bc	$4.27 \times 10^4 \pm 1.33 \times 10^3$ d	$4.73 \times 10^5 \pm 9.47 \times 10^3$ b	$2.79 \times 10^5 \pm 3.91 \times 10^4$ c
	Hyd8	$7.57 \times 10^5 \pm 1.55 \times 10^4$ a	$5.19 \times 10^4 \pm 1.37 \times 10^3$ e	$7.26 \times 10^5 \pm 4.93 \times 10^4$ a	$6.01 \times 10^5 \pm 1.75 \times 10^4$ ab	$6.95 \times 10^5 \pm 1.05 \times 10^4$ a	$7.14 \times 10^5 \pm 0.00$ a	$4.38 \times 10^5 \pm 7.36 \times 10^3$ bc	$3.23 \times 10^5 \pm 3.68 \times 10^3$ bc

Table 11. Metabolomic profile determined by LC-ESI-UHR-QqTOF-MS in MS/MS Mode in *C. roseus* healthy (HV), and with mild (AY 107) and high (Hyd8) virulent strains of aster yellows phytoplasmas, 1 and 4 days after elicitor treatments (T1 and T2, respectively). Each value is the mean of fold change of three biological replicates resulting from the pool of three plants ± SEM. Gallic acid and hydroxytyrosol were used as internal standard to negative and positive modes, respectively. For each timepoint, different letters are statistically different at $P < 0.05$ according to two-way ANOVA followed by Tukey's post-hoc test.

***Gene* / Shoots**	**HV**	**AY107**	**Hyd8**
as	1.02 ± 0.12^{c}	1.51 ± 0.04^{b}	2.31 ± 0.05^{a}
str	1.02 ± 0.10^{b}	1.49 ± 0.02^{a}	1.73 ± 0.10^{a}
prx	0.97 ± 0.05^{b}	1.00 ± 0.03^{b}	1.44 ± 0.11^{a}
pal	1.01 ± 0.08^{a}	1.28 ± 0.04^{a}	0.78 ± 0.11^{a}
chs	0.81 ± 0.17^{b}	2.47 ± 0.17^{a}	2.58 ± 0.22^{a}

Table 12. Relative fold of expression of 5 target genes (*phenylalanine ammonia liase*, *pal*, and *chalcone synthase*, *chs*) and TIAs pathway encoding genes (*anthranilate synthase, as*, *strictosidine synthase*, *str*, and *peroxidase*, *prx*) determined in healthy (HV) and *C. roseus* shoots infected with AY phytoplasma strains with mild (AY107) and high (Hyd8) virulence, before elicitation (T0), and 1 and 4 days after elicitor treatments (T1 and T2, respectively) treated with 12.5 and 25 mM MeJA. Each value is the mean of three biological replicates resulting from the pool of three shoots ± SEM. One-fold change represents no relative change in gene expression, as compared to untreated (Unt) healthy shoots in each time point. For each gene, letters indicate statistical differences ($P < 0.05$) according to two-way ANOVA followed by Tukey's post-hoc test.

		Treatment							
Gene	**Shoots**	**Unt_T1**	**0 mM MeJA_T1**	**12.5 mM MeJA_T1**	**25 mM MeJA_T1**	**Unt_T2**	**0 mM MeJA_T2**	**12.5 mM MeJA_T2**	**25 mM MeJA_T2**
as	**HV**	1.421 ± 0.480^{c}	3.918 ± 0.500^{b}	1.850 ± 0.320^{c}	1.728 ± 0.480^{c}	1.992 ± 0.410^{cd}	1.283 ± 0.100^{d}	4.023 ± 0.750^{b}	5.261 ± 0.952^{b}
	AY107	1.215 ± 0.590^{c}	1.500 ± 0.180^{c}	6.200 ± 1.140^{a}	1.430 ± 0.220^{c}	0.913 ± 0.340^{d}	1.558 ± 0.490^{d}	4.406 ± 0.900^{b}	3.614 ± 0.830^{bc}
	Hyd8	0.800 ± 0.190^{c}	1.523 ± 0.160^{c}	1.437 ± 0.130^{c}	4.606 ± 0.740^{b}	0.867 ± 0.160^{d}	1.429 ± 0.220^{d}	1.895 ± 0.320^{cd}	7.600 ± 0.870^{a}
str	**HV**	1.296 ± 0.480^{bcd}	2.510 ± 0.500^{bc}	1.927 ± 0.320^{bd}	1.979 ± 0.480^{bcd}	1.701 ± 0.410^{d}	1.412 ± 0.100^{d}	3.965 ± 0.950^{bc}	4.965 ± 1.520^{b}
	AY107	1.048 ± 0.590^{d}	0.900 ± 0.040^{d}	5.700 ± 0.940^{a}	1.689 ± 0.220^{bcd}	0.943 ± 0.340^{d}	1.187 ± 0.490^{d}	4.017 ± 0.900^{bc}	4.842 ± 0.830^{b}
	Hyd8	1.100 ± 0.300^{cd}	1.631 ± 0.160^{bcd}	2.672 ± 0.130^{b}	4.697 ± 0.740^{a}	1.174 ± 0.160^{d}	1.509 ± 0.220^{d}	2.052 ± 0.320^{cd}	7.000 ± 0.670^{a}
prx	**HV**	0.718 ± 0.090^{b}	0.251 ± 0.040^{c}	0.043 ± 0.010^{c}	0.045 ± 0.020^{c}	1.025 ± 0.120^{a}	0.267 ± 0.110^{de}	0.196 ± 0.030^{e}	0.233 ± 0.070^{de}
	AY107	0.924 ± 0.130^{b}	0.100 ± 0.040^{c}	0.173 ± 0.070^{c}	0.104 ± 0.020^{c}	0.674 ± 0.100^{bc}	0.493 ± 0.160^{cd}	0.592 ± 0.140^{bc}	0.182 ± 0.060^{e}
	Hyd8	1.667 ± 0.190^{a}	0.186 ± 0.030^{c}	0.050 ± 0.010^{c}	0.167 ± 0.050^{c}	0.790 ± 0.100^{ab}	0.135 ± 0.020^{e}	0.076 ± 0.010^{e}	0.662 ± 0.090^{bc}
pal	**HV**	0.800 ± 0.060^{c}	1.141 ± 0.200^{c}	1.050 ± 0.110^{c}	1.180 ± 0.270^{c}	1.506 ± 0.170^{ce}	0.830 ± 0.020^{e}	2.605 ± 0.720^{bc}	2.931 ± 0.620^{b}
	AY107	1.160 ± 0.190^{c}	0.700 ± 0.030^{c}	2.700 ± 0.370^{a}	0.960 ± 0.140^{c}	1.714 ± 0.170^{ce}	1.290 ± 0.400^{de}	3.075 ± 0.550^{ab}	2.519 ± 0.340^{bc}
	Hyd8	0.800 ± 0.050^{c}	1.002 ± 0.100^{c}	1.155 ± 0.130^{c}	2.047 ± 0.270^{b}	1.111 ± 0.030^{e}	1.494 ± 0.060^{ce}	2.303 ± 0.310^{bcd}	3.800 ± 0.310^{a}
chs	**HV**	2.260 ± 1.070^{b}	2.511 ± 1.240^{b}	2.227 ± 1.060^{b}	1.779 ± 0.860^{b}	2.722 ± 0.850^{d}	0.352 ± 0.020^{d}	3.072 ± 2.250^{d}	16.800 ± 1.930^{b}
	AY107	2.308 ± 1.530^{b}	1.572 ± 0.950^{b}	12.300 ± 2.600^{a}	1.895 ± 0.580^{b}	1.093 ± 0.590^{d}	0.446 ± 0.180^{d}	3.100 ± 1.500^{d}	12.300 ± 0.400^{c}
	Hyd8	3.300 ± 0.860^{b}	1.340 ± 0.580^{b}	1.532 ± 0.720^{b}	10.302 ± 2.020^{a}	1.241 ± 0.440^{d}	1.796 ± 0.420^{d}	2.466 ± 1.220^{d}	21.300 ± 1.910^{a}

CHAPTER 5

GENERAL CONCLUSIONS AND FUTURE WORK

5.1. General conclusions

The search for effective FD management measures has been hampered by several constraints including difficulties in cultivating phytoplasmas and in transmitting FD from plant to plant under controlled laboratory conditions. Moreover, with the application of mandatory treatments against *S. titanus* FD has been spreading to new wine-growing regions, demanding urgent measures for its effective containment. Although insecticides can be effective, they are clearly not sufficient to contain this expanding epidemic. Moreover, the increasing costs and social pressure against treatments that are hazardous for health and the environment have led to a search for new ways to contain FD.

Based on the previous literature review, the main objectives of the present thesis were to study the impact of FD on Portuguese grapevine cv. 'Loureiro' and the role of elicitors – MeJA, SA and BTH – on plant defence responses against phytoplasmas in order to study the new challenges on the control of FD in grapevine. Thus, this work aimed to (i) study the impact of FD on grapevine growth, development, yield and fruit quality, (ii) understand the effect of elicitors (MeJA, SA) on morphology, physiology and molecular parameters of grapevine infected with FD, and (iii) unravel the effect of elicitors (MeJA and BTH) on the metabolomic profile and gene expression of periwinkle infected with "aster yellows" phytoplasmas.

In the current study, molecular characterization of FD phytoplasmas revealed that FD-D was the only strain present in the analysed symptomatic cv. 'Loureiro' grapevines from "Quinta do Corvo" vineyard of the "Vinhos Verdes" Region.

It was clear that FD has negative impacts in plant growth, development and yield. In the two-year study (2015 and 2016), infected plants revealed a significant growth delay in both years of the study, with a delay of 10 days, approximately in visible bunches, flowering and veraison phenological stages. At veraison, FD also led to a significant decrease in the budburst rate, fertility index, leaf area, and chlorophyll content. Furthermore, infected plants showed a drastic reduction in bunch yield, which resulted from a lower number of bunches as well as a decreased bunch weight.

We also concluded that the impacts of FD infection are not only visible at a macro but also at cellular level. 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.

The study conducted on the role of MeJA and SA in alleviating disease severity provided new information on the potential usefulness (or lack thereof) of these elicitors. An in-depth analysis was carried out to better understand the defence mechanisms triggered by SA on grapevines infected with FD under field conditions. In this study we observed that SA had a beneficial effect on plant growth and development, but only for fertility index and chlorophyll content. However, SA application had no significant amelioration effect on grapevine productivity, which could be due to the lower pruning load left on FD plants reflecting their lower vigour. Still, because this is the first study on SA application to field-grown grapevines infected with FD, broad conclusions must be taken with caution.

A two-year study which was focused on the mitigation effect of MeJA application on FD-infected grapevine demonstrated for the first time that this elicitor is a promising tool in FD management. Altogether, this study allows to a have a first insight on the role of MeJA concentration, timing, and frequency of application. We concluded that 12.5 mM MeJA applied twice (before flowering and at veraison) presented better results in grapevine yield, increasing productivity of about 4-fold, with a tendency to improve plant growth (leaf area and chlorophyll content) and productivity (fertility index, number of bunches and average of bunch weight) parameters, when compared with untreated infected plants. Regarding genetic responses, we concluded that MeJA application upregulated different genes in each studied year, triggering a great increase in gene expression of *Thau I*, *Thau II*, *Osm* and *PAL*, in 2015, while in 2016 *Protα5s*, *CHIT4c*, *PIN*, *PGIP* and *GLU* were the upregulated genes. Moreover, MeJA application had a beneficial effect in healthy grapevines with regards to leaf area values, with increases up to 1.1-fold in 2015, as well as in plant yield, increasing the number of bunches up to 94%, in 2016. Still, we concluded that a special caution must be taken with regards to the applied concentrations, since higher MeJA concentrations (*e.g.,* 25 mM) were toxic to infected grapevines, regardless of the timing of application.

Bearing in mind the obvious limitations of comparing a field grown grapevine with an *in vitro* grown periwinkle plant, in this PhD thesis we concluded that periwinkle (*C. roseus*) is a good model plant to study the molecular and metabolomics responses of phytoplasma-host interactions. In the present work the role of MeJA and BTH was investigated on biochemical responses and the activation of plant genomic and metabolomic defence mechanisms on healthy and phytoplasma-infected periwinkle shoots with two symptomatology degrees. In this study we concluded that both elicitors had the potential to reduce MDA, with a differential effect on flavonoid content. Although flavonoids are secondary metabolites overproduced under infection conditions, MeJA application did not have a relevant/significant effect in flavonoid

content, while 3.5 mM BTH significantly increased it, especially 1 day after elicitation (dae). Moreover, this work showed that AY107 periwinkle strain had higher ABA concentrations when compared to healthy ones, which could be an indication of phytoplasma infection development as shown for untreated AY107 infected periwinkle shoots. Elicitors' application increased phytohormone production and Hyd8 phytoplasma-infected periwinkles responded better to 12.5 mM MeJA and 7 mM BTH. Moreover, both elicitors decreased the ABA contents revealing that these compounds could be an important tool to alleviate phytoplasma symptoms. A deep study of MeJA application on metabolomic responses on healthy and infected periwinkles identified 13 alkaloids and 11 phenolic compounds, of which alkaloids mitraphylline, aricine, yohimbine, strychnine, pleiocarpanine, alstonine, vindolidine and catharine, and the phenolics deoxy elenolic acid, syringaresinol, maslinic acid, lawsone were identified for the first time in periwinkle shoots. Gene expression revealed that phytoplasma infection activated both periwinkle alkaloid and phenylpropanoid biosynthesis pathways, upregulating AS, STR, PRX (alkaloid pathway) and CHS (phenylpropanoid pathway) genes. The fact that phytoplasma infection is able to modulate the alkaloid and phenolic profile of infected periwinkles may have a therapeutic relevance since periwinkle alkaloids have a well-known medical importance.

Summing up, this thesis concluded that infection by phytoplasmas has negative effects on grapevine growth, development and productivity as well as at the cellular level. The application of elicitors in the vineyard must be considered with many safeguards, namely the type of elicitor, the concentration to be used, and its timing of application, keeping in mind that there is always the environmental factor (climate) that can influence plant responses. Moreover, periwinkle can be a model to consider for fundamental studies on the treatment of diseases caused by phytoplasmas, but the extrapolation of findings to grapevines (or other crops) should be done carefully.

5.2. Future work

The present thesis opens new doors for the development of more effective and environmentally friendly tools for phytoplasma disease control. However, further studies should be carried out in order to:

- exploit genetic resources of Portuguese grapevines in order to identify the more tolerant cultivars and the mechanisms associated with that tolerance;

- better understand the impact of FD on berry maturation and quality;

- assess if elicitors modify berry and wine properties (polyphenols, acidity, antioxidants);

- verify if other elicitor concentrations or time of application would induce more accurately plant responses in phytoplasma-infected plants;

- better understand the role of elicitors in plant genomic, transcriptomic, and metabolic defence activation against phytoplasmas as well as their role in the symptom expression in field grown plants;

- unravel the role of elicitors on FD recovery phenomenon;

- consider the potential of multispectral and hyperspectral technologies (*e.g.*, drone imaging) for the early detection of disease and disease vector, for better field management.

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ANNEXES

Annex 1. Poster: Impact of "Flavescence dorée" on plant growth, productivity and fruit quality of *Vitis vinifera* cv. Loureiro from the "Vinho Verde" region.

Impact of Flavescence dorée on plant growth, productivity and fruit quality of *Vitis vinifera* cv. 'Loureiro' from the 'Vinho Verde' region

M.J. Rebelo[1], S.C.A. Castro[2], A. Bertaccini[3], M.W. Vasconcelos[1] and S.M.P. Carvalho[1,2*]

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Introduction

Flavescence doreé (FD) is a quarantine disease caused by a phytoplasma. FD is transmitted to healthy plants by an insect vector, *Scaphoideus titanus* Ball. FD symptoms are characterized by plant decline, stunted growth and abnormal lignification, flower abortion, leaf rolling and discoloration, unripened shoots and shriveled grapes.

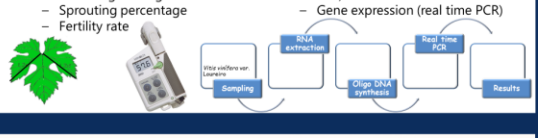
This disease is a serious problem to the stability and sustainability of 'Vinho Verde' region, where FD is present since 2008 leading to severe harvest losses or even the death of the infected plants.

This study, conducted in 'Vinho Verde' region, aimed to:

(i) quantify the impact of FD on plant growth and development;
(ii) evaluate the impact of FD productivity and fruit quality;
(iii) evaluate the expression of PAL, STS, PIN and PGIP genes linked to the secondary metabolism and plant defense.

Materials and Methods

- **Plant material:** 20 year-old vineyard (Fafe; Portugal)
- **Treatments:** Healthy (**FD**$^-$) and FD infected (**FD**$^+$) grapevines cv. *'Loureiro'*
- **Measurements:**
  - Leaf area,
  - Chlorophyll content (SPAD values)
  - Phenological stage
  - Sprouting percentage
  - Fertility rate
  - Productivity
  - Fruit quality (total acidity, degree brix)
  - Gene expression (real time PCR)

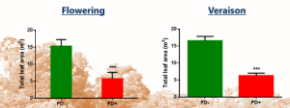


Results

FD- FD+

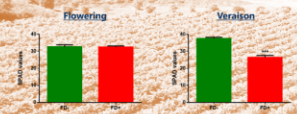
Plant growth and development

Leaf area



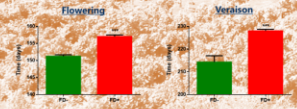
Diseased plants had lower leaf area (40%)

SPAD values



FD$^+$ plants had lower chlorophyll content (9%), in veraison

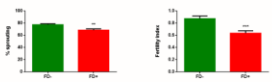
Phenological stage



FD+ plants took longer to reach flowering and veraison stage (~15 days)

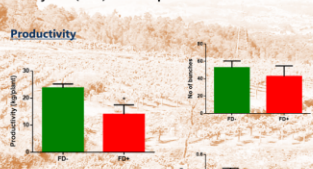
Productivity and fruit quality

% of sprouting and fertility index



FD$^+$ plants had lower % of sprouting (15%) and fertility rate (20%) than FD$^-$ plants

Productivity



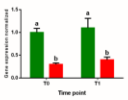
FD$^+$ had lower productivity (FD$^-$ = 23.8 ± 1.4 kg/plant and FD+ = 14.0 ± 3.5 kg/plant)

Titrable acidity and Total soluble sugars

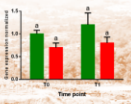
FD$^+$ plants didn't have significant differences to FD$^-$ plants about fruit quality parameters

Gene expression

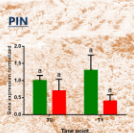
STS



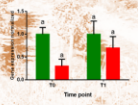
PAL



PIN



PGIP



FD$^+$ plants signaling pathways were activated, with an upregulation of STS gene.

Conclusions

- ✓ FD delayed plant development when compared to healthy plants (on average 15 days) and FD infected plants presented less leaf area and chlorophyll content;
- ✓ FD led to a significant production loss. Although, fruit quality (titrable acidity and total soluble sugars) is not influenced by disease;
- ✓ FD triggers gene expression of secondary metabolism pathways, particularly STS gene.

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Annex 2. Poster: Impact of "flavescence dorée" on plant growth, productivity and fruit quality of *Vitis vinifera* cv. Loureiro from the "Vinho Verde" region.

Impact of Flavescence dorée on plant growth, productivity and fruit quality of *Vitis vinifera* cv. 'Loureiro' from the 'Vinho Verde' region

M.J. Oliveira[1], V. Pinto[2], A. Bertaccini[3], M.W. Vasconcelos[1] and S.M.P. Carvalho[1,2*]

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Introduction

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This disease is a serious problem to the stability and sustainability of 'Vinho Verde' region, where FD is present since 2008 leading to severe harvest losses or even the death of the infected plants.
This study, conducted in 'Vinho Verde' region, aimed to:
(i) quantify the impact of FD on plant growth and development;
(ii) evaluate the impact of FD productivity and fruit quality;
(iii) evaluate the impact of FD on cellular structure using transmission electron microscopy (TEM).

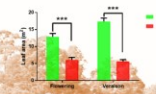
Materials and Methods

- **Plant material:** 20 year-old vineyard (Fafe; Portugal)
- **Treatments:** Healthy (**FD⁻**) and FD infected (**FD⁺**) grapevines cv. *'Loureiro'*
- **Measurements:**
  - Leaf area,
  - Chlorophyll content (SPAD values)
  - Phenological stage
  - Sprouting percentage
  - Fertility rate
  - Productivity
  - Fruit quality (total acidity, degree brix)
  - Cellular ultrastructure (TEM)

Results

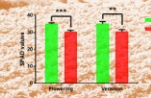
Plant growth and development

Leaf area



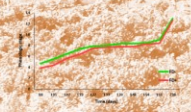
Diseased plants had lower leaf area (52% in flowering and 68% in veraison)

SPAD values



FD⁺ plants had lower chlorophyll content (13% in flowering and in veraison)

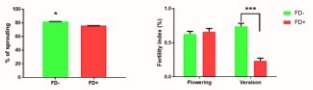
Phenological stage



FD⁺ plants took longer to reach flowering and veraison stage (~7 days)

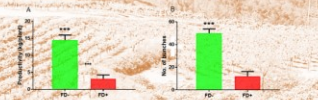
Productivity and fruit quality

% of sprouting and fertility index



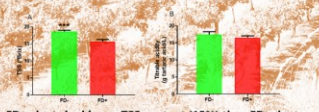
FD⁺ plants had lower % of sprouting (7%) and fertility rate (68% in veraison) than FD⁻ plants

Productivity



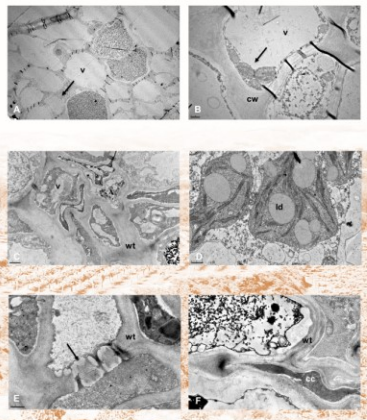
FD⁺ had lower productivity (FD⁻ = 14.5 ± 1.5kg/plant and FD⁺ = 3.1 ± 1.1 kg/plant)

Titrable acidity and Total soluble sugars



FD⁺ plants had lower TSS content (15%) than FD⁻ plants

TEM



FD⁺ plants (C-E), displayed ultrastructural alterations: collapsed vacuoles (V) and cells (C), necrosis of some cells, cell wall (cw) thickening and callose accumulation (E), large accumulation of starch and lipids (ld) in the plastids (D).

Conclusions

- ✓ FD delayed plant development when compared to healthy plants (on average 15 days) and FD infected plants presented less leaf area and chlorophyll content;
- ✓ FD led to a significant production loss. Although, fruit quality (titrable acidity and total soluble sugars) is not influenced by disease;
- ✓ Disorganization of phloem cells, an accumulation of callosis in the sieve elements and necrotic cells.

Acknowledgements

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M. J. Oliveira was supported financially through FCT doctoral scholarships SFRH/BD/103895/2014.

FCT

Annex 3. Poster: Does salicylic acid alleviate the impacts on growth, development and productivity of "flavescence dorée" in Portuguese "Vinhos Verdes" grapevines?

Does salicylic acid alleviate the impacts on growth, development and productivity of "flavescence dorée" in Portuguese "Vinhos Verdes" grapevines?

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Introduction

- "Flavescence dorée" (FD) is a grapevine quarantine disease associated with phytoplasma presence. It is transmitted by insects (*Scaphoideus titanus* and *Orientus ishidae*) and by the commercialization of infected vegetative propagation material. FD symptoms are characterized by plant decline, stunted growth and abnormal lignification, flower abortion, leaf rolling and discoloration, unripened shoots and shriveled grapes.
- This disease is a serious problem to the stability and sustainability of "Vinhos Verdes" region, where FD is present since 2008 leading to severe harvest losses or even the death of the infected plants.
- The current management strategies for FD control are costly, difficult to implement and have a high environmental impact. Elicitors such as salicylic acid (SA) could be exploited since they activate multiple plant defense systems against several pathogens.
- This study, conducted in 'Vinhos Verdes' region, aimed to evaluate the potential of SA on the mitigation of the negative effects of FD on grapevine growth, development and productivity.

Materials and Methods

- **Plant material:** 20 year-old vineyard (Fafe; Portugal)
- **4 Treatments** (n=4):
  - Healthy (**FD⁻**) and FD infected (**FD⁺**) grapevines cv. Loureiro
  - Elicitation with 0 mM SA (control) and 25 mM SA at flowering
- **Measurements:**
  - Phenological stage
  - Budburst percentage
  - Fertility rate
  - Chlorophyll content (SPAD values)
  - Leaf area
  - Productivity
  - Fruit quality (total acidity, degree brix)

Results and Discussion

Plant growth and development

Table 1 – Growth and development parameters evaluated at veraison in healthy (FD⁻) and "flavescence dorée" infected (FD⁺) grapevines cv. Loureiro. Data are means ± SEM. Different letters indicate statistically different means (comparison in columns).

Plant	[SA](mM)	Time to veraison (days)*	Budburst (%)	Fertility index	Chlorophyll content (SPAD values)	Leaf area increase (%)**
FD⁻	0	201.5 ± 1.3 [b]	81.0 ± 3.2 [a]	1.05 ± 0.07 [a]	38.1 ± 1.7 [a]	52.9 ± 7.2 [a]
	25	203.0 ± 1.2 [b]	79.4 ± 2.7 [a]	0.85 ± 0.08 [ab]	37.3 ± 0.9 [a]	112.8 ± 32.3 [a]
FD⁺	0	225.3 ± 1.5 [a]	78.2 ± 2.7 [a]	0.65 ± 0.06 [b]	24.4 ± 0.5 [b]	86.7 ± 0.7 [a]
	25	225.8 ± 1.1 [a]	67.8 ± 4.6 [a]	0.79 ± 0.04 [ab]	30.5 ± 3.1 [ab]	136.7 ± 4.4 [a]

*days of the year (1 january = day 1)
**relative increase in leaf area from flowering to veraison

- **Non-elicited FD⁺ plants showed: 23 days delay in time to veraison; 39% lower fertility index and 36% lower chlorophyll content**
- **Application of 25 mM SA at flowering: no effect on phenological evolution, budburst rate and leaf area increase…**
- **… but FD infected plants treated with SA did not differ significantly from the healthy ones on fertility index and chlorophyll content**

Productivity and fruit quality

Table 2 – Productivity in healthy (FD⁻) and "flavescence dorée" infected (FD⁺) grapevines cv. Loureiro. Data are means ± SEM. Different letters indicate statistically different means (comparison in columns).

Plant	[SA] (mM)	Productivity (kg/plant)	No of bunches	Average bunch weight (g)	TSS (ºBrix)	TA (g tartaric acid.L^{-1})
FD-	0	23.8 ± 1.2 [a]	52.8 ± 6.6 [a]	480.0 ± 64.3 [a]	16.9 ± 0.7 [a]	8.9 ± 0.2 [a]
	25	23.3 ± 2.0 [a]	43.8 ± 6.7 [ab]	565.2 ± 60.1 [a]	16.7 ± 0.9 [a]	7.7 ± 0.3 [a]
FD+	0	8.3 ± 0.3 [b]	17.3 ± 1.0 [b]	488.5 ± 45.9 [a]	16.6 ± 0.2 [a]	9.0 ± 0.5 [a]
	25	6.0 ± 0.7 [b]	19.0 ± 0.7 [ab]	313.9 ± 25.5 [a]	16.8 ± 0.3 [a]	8.8 ± 0.8 [a]

- **Non-elicited FD⁺ plants had: 65% lower yield (due to a significant reduction on the number of bunches; average bunch weight was not affected)**
- **No significant differences were observed between healthy and infected or between SA-treated and untreated plants in the berry quality**

Conclusions

- ✓ **FD presence delayed plant development, and also led to a decrease of fertility index, chlorophyll content and production loss when compared to healthy plants**
- ✓ **Fruit quality (titrable acidity and total soluble sugars) was not influenced by the disease**
- ✓ **SA application seems to have a beneficial effect on plant growth and development, but only for a restricted number of parameters, namely fertility index and chlorophyll index**
- ✓ **More studies should be carried out in order to further verify if diverse elicitor concentrations' or other time of application would induce better plant responses**

Acknowledgements

This work was supported by FCT- Portuguese Foundation for Science and Technology through projects EXPL/AGR-PRO/1155/2013, UID/Multi/50016/2013 and UID/Multi/50016/2019. M.J.R.A. Oliveira was supported through FCT doctoral scholarship SFRH/BD/103895/2014.

Annex 4. Poster: The role of methyl jasmonate on genomic and metabolomic profile of *Catharanthus roseus* infected with "aster yellows" phytoplasmas with different virulence degrees.

The role of methyl jasmonate on genomic and metabolomic profile of *Catharanthus roseus* infected with "aster yellow" phytoplasmas with different virulence degrees

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Introduction

Phytoplasmas are phloem-bound anaerobic microorganisms, evolved from Gram-positive bacteria, which cannot be maintained in axenic culture. They are usually transmitted through insect vectors or vegetative propagation material. Periwinkle (*Catharanthus roseus*) is a susceptible host and maybe used as a model plant to study phytoplasma:host interactions. The main symptoms of infection include leaf etiolation, plant dwarfism, and a greater number of axillary shoots and small leaves. Methyl jasmonate (MeJA) is an elicitor with potential to increase plant defense. The goal of this work was to understand the impact of the infection of "aster yellow" phytoplasmas with different virulence degrees in *in vitro*-grown periwinkles, specifically on the modulation of the expression of pathogen-resistance genes and on the induction of the synthesis of secondary metabolites.

Materials and Methods

- **Plant material:** healthy (**HV**) and infected *C. roseus* with "aster yellow" of different virulence degrees (**AY107**, intermediate virulence, and **Hyd8**, high virulence)
- **Treatments:** untreated (Ctrl); treated with 0 (2.5% ethanol, solvent), 12.5 and 25 mM MeJA
- **Time points:** before application (T0), 1 and 4 days (T1 and T2, respectively) after application
- **Measurements:**
  - Metabolomic profile (LC-ESI-UHR-QqTOF-MS/MS)
  - Gene expression (*PAL*, *CHS*, *AS*, *STR*, *PRX*)

Results

Metabolomic profile

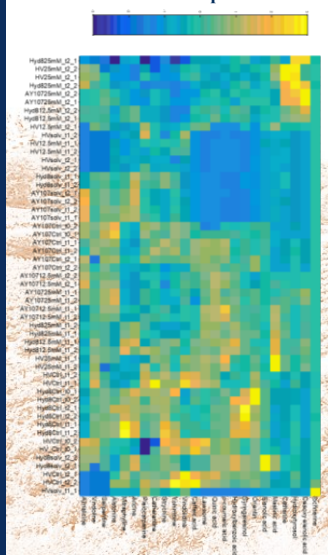

Figure 1. Metabolomic profile of *Catharanthus roseus* healthy (HV), intermediate (AY 107) and high (Hyd 8) infected with "Aster yellow" phytoplasmas, untreated (Ctrl), treated with 0 (solv.), 12.5 and 25 mM MeJA, before elicitor treatment (T0) and 1 and 4 days after elicitor treatments (T1 and T2, respectively). Each value is the mean of two biological replicates (numbers 1 and 2) resulting from the pool of three plants.

- **Metabolomic analysis identified 24 metabolites: 13 alkaloids and 11 phenolics.**
- **Untreated Hyd8 plants presented higher abundance of aricine, catharantine, mitraphylline, catharine, orientin, syryngarenisol and elenoic acid, comparing with untreated HV and AY107 plant strains.**
- **Periwinkles elicited with 25 mM showed higher accumulation of deosyelenoic acid, hydroxytyrosol and catharine, in T2.**
- **AY107 strain elicited with 12.5 mM MeJA presented higher synthesis of compounds comparing with plants elicited with 25 mM MeJA of each strain.**

Genomic profile

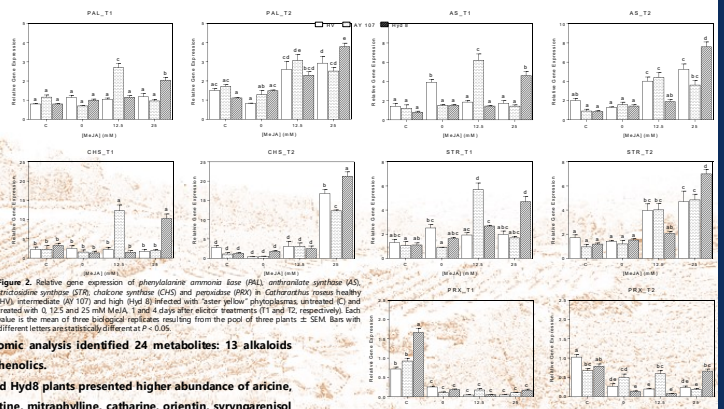

Figure 2. Relative gene expression of *phenylalanine ammonia lase* (*PAL*), *anthranilate synthase* (*AS*), *strictosidine synthase* (*STR*), *chalcone synthase* (*CHS*) and *peroxidase* (*PRX*) in *Catharanthus roseus* healthy (HV) intermediate (*AY 107*) and high (Hyd 8) infected with "aster yellow" phytoplasmas, untreated (C) and treated with 0, 12.5 and 25 mM MeJA, 1 and 4 days after elicitor treatments (T1 and T2, respectively). Each value is the mean of three biological replicates resulting from the pool of three plants ± SEM. Bars with different letters are statistically different at $P < 0.05$.

Data are means ± SEM. Different letters indicate statistically different means (comparison in columns).

- **In T1, AY107 strain treated with 12.5 mM MeJA and Hyd8 treated with 25 mM MeJA upregulated *PAL*, *CHS*, *AS* and *STR* genes up to 10-fold.**
- **In T2, 25 mM MeJA treatment upregulated *PAL*, *CHS*, *AS* and *STR* genes in all periwinkle strains, although Hyd8 has been more responsive.**
- **In T2, 12.5 mM upregulated *PAL*, *AS* and *STR* genes in HV and AY107 periwinkle strains.**
- **MeJA treatment downregulated *PRX* gene.**

Conclusions

- ✓ Infected plants showed higher alkaloid content comparing with healthy ones.
- ✓ Treatment with 25 mM MeJA increased deosy elenoic acid, hydrixytyrosol and catharine synthesis.
- ✓ MeJA upregulated *PAL*, *CHS*, *AS* and *STR* genes and downregulated *PRX* gene.
- ✓ AY107 periwinkle strain was more responsive to 12.5 mM MeJA, while Hyd8 strain was more responsive to 25 mM MeJA.

Acknowledgements

This work was supported by FCT- Portuguese Foundation for Science and Technology through projects EXPL/AGR-PRO/1155/2013 and UID/Multi/50016/2013. M.J.R.A. Oliveira was supported through FCT doctoral scholarship SFRH/BD/103895/2014.

Annex 5. Poster: Benzothiadiazole enhances metabolic responses in *Catharanthus roseus* infected with "aster yellows" phytoplasmas.

Benzothiadiazole enhances metabolic responses in *Catharanthus roseus* infected with "aster yellow" phytoplasmas

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Introduction

- Phytoplasmas are phloem-bound anaerobic microorganisms, evolved from Gram-positive bacteria, which cannot be maintained in axenic culture. They are usually transmitted through insect vectors or vegetative propagation material.
- So far, no effective treatments against phytoplasma diseases exists, being the application of insecticides, hot water treatment and roguing the infected the commonly used strategies to mitigate diseases' effects. Nevertheless, elicitors have been pointed as an important tool to manage phytoplasma diseases since they are harmless and environmentally friendly.
- Periwinkle (*Catharanthus roseus*) is a susceptible host and maybe used as a model plant to study phytoplasma:host interactions. The main symptoms of infection include leaf etiolation, plant dwarfism, and a greater number of axillary shoots and small leaves.
- This study aimed to evaluate the role of three different concentrations of benzothiadiazole (BTH) on periwinkle biochemical responses (cellular oxidative damage, flavonoid content and phytohormones content) against "aster yellow" phytoplasmas with different symptom degrees.

Materials and Methods

- **Plant material:** healthy (**HV**) and infected *C. roseus* with "aster yellow" of different symptom degrees (mild, strain **AY107**, and severe, strain **Hyd8**)
- **Treatments:** untreated and treated 3.5 and 7.5 mM BTH
- **Time points:** 1 and 4 days after application (1 and 4 dae, respectively).
- **Measurements:**
  - Lipid peroxidation (malondialdehyde – MDA – content)
  - Flavonoid content
  - Phytohormones content (jasmonic acid – JA, salicylic acid – SA, and abscisic acid – ABA).

Results and Discussion

Lipid peroxidation

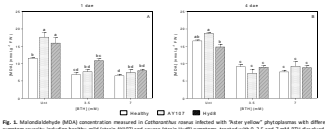

Fig. 1. Malondialdehyde (MDA) concentration measured in *Catharanthus roseus* infected with "hoter yellow" phytoplasmas with different symptom severity, including healthy, mild (strain AY107) and severe (strain Hyd8) symptoms, treated with 0, 3.5 and 7 mM BTH dissolved in water, measured 1 and 4 days after elicitation (dae). Each value is the mean of three biological replicates resulting from the pool of three shoots ± SEM. Bars with different letters are statistically different at P < 0.05.

- **Untreated** AY107 and Hyd8 strains revealed 53% and 38% higher [MDA] compared with healthy shoots (**Fig.1A**).
- **1 dae**, 3.5 mM BTH decreased MDA content by 41% in healthy and 32% in Hyd8 shoots (**Fig. 1A**).
- **1 dae**, 7 mM BTH decreased a significantly [MDA] in all treated shoot groups, compared with the untreated ones (**Fig. 1A**).
- **4 dae:** both [BTH] decreased significantly [MDA] up to 61% in AY107 shoots (**Fig. 1B**).

Flavonoid content

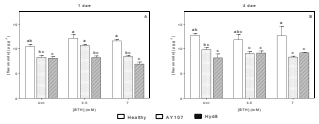

Fig. 2. Flavonoid content measured in *Catharanthus roseus* infected with "hoter yellow" phytoplasmas with different symptom severity, including healthy, mild (strain AY107) and severe (strain Hyd8) symptoms, treated with 0, 3.5 and 7 mM BTH dissolved in water, measured 1 and 4 days after elicitation (dae). Each value is the mean of three biological replicates resulting from the pool of three shoots ± SEM. Bars with different letters are statistically different at P < 0.05.

- **BTH** did not affect [JA] in **infected periwinkle** shoots (**Fig. 3A** and **3B**)
- **1 dae**, **untreated** AY107 shoots showed 51% lower [SA] compared with untreated healthy shoots (**Fig. 3C**).
- **1 dae**, 3.5 mM BTH increased significantly (70%) the [SA] in the healthy shoots (**Fig. 3C**).
- **4 dae:** 7 mM BTH decreased significantly [SA] up to 32% in infected shoots compared with untreated shoots (**Fig. 3D**).
- **1 dae**: untreated infected shoots showed 38% and 52% less [ABA], respectively compared with untreated healthy shoots (**Fig. 3E**). BTH decreased the [ABA]: 3.5 mM decreased of 86%, 69% and 95% in healthy, AY107 and Hyd8 shoots, respectively; 7 mM decreased of 97%, 70% and 93% in healthy, AY107 and Hyd8 shoots, respectively.
- **4 dae**, untreated Hyd8 shoots revealed a significantly low [ABA] compared to healthy and AY107 (57% and 51%, respectively) shoots (**Fig. 3F**). BTH decreased [ABA]: 3.5 mM decreased of 45% and 53% in AY107 and Hyd8 shoots, respectively; 7 mM BTH decreased of 62%, 47% and 66% the [ABA] in healthy, AY107 and Hyd 8 shoots, respectively.

Phytohormones content

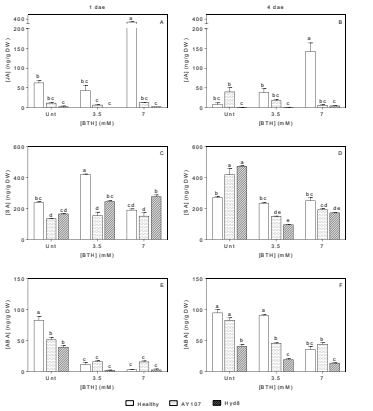

Fig. 3. Jasmonic acid (JA) (A and B), salicylic acid (SA) (C and D) and abscisic acid (ABA) (E and F) concentrations measured in *Catharanthus roseus* infected with "hoter yellow" phytoplasmas with different symptom severity, including healthy, mild (strain AY107) and severe (strain Hyd8) symptoms, treated with 0, 3.5 and 7 mM BTH dissolved in water, measured 1 and 4 days after elicitation (dae). Each value is the mean of three biological replicates resulting from the pool of three shoots ± SEM. Bars with different letters are statistically different at P < 0.05.

- **Untreated** Hyd8 strain showed 23% and 36% lower flavonoid content, **1 dae** and **4 dae**, respectively, compared with the untreated healthy shoots (**Fig. 2A** and **2B**).
- **1 dae:** 3.5 mM BTH increased flavonoids by 29% in AY107 shoots compared with untreated ones (**Fig. 2A**).

Conclusions

- ✓ **In both timepoints** the [MDA] decreased significantly after BTH treatments in both **infected shoots**.
- ✓ Application of **3.5 mM BTH** increased flavonoids in **AY107** shoot strain.
- ✓ BTH increased [JA] and [SA] in **healthy** periwinkles and did not affect phytohormones in **infected shoot strains**.
- ✓ Both BTH concentrations decreased [ABA] of **infected periwinkle shoots** in both timepoints.

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