UNIVERSIDADE DE LISBOA
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# Lipid Signalling in Grapevine Resistance against Fungal Pathogens

*"Documento Definitivo"*

**Doutoramento em Biologia**
Especialidade de Biologia de Sistemas

Ana Rita Sebastião Mendes Cavaco

Tese orientada por:
Professora Doutora Andreia Figueiredo
Professora Doutora Ana Rita Matos

Documento especialmente elaborado para a obtenção do grau de doutor

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Júri:

Presidente:

- Doutor Rui Manuel dos Santos Malhó, Professor Catedrático e Presidente do Departamento de Biologia Vegetal da Faculdade de Ciências da Universidade de Lisboa

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- Doutor Yariv Brotman, Senior Lecturer, Faculty of Natural Sciences of the Bem-Gurion da University of the Negev, Israel;
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Para a elaboração da presente tese de Doutoramento foram usados integralmente como capítulos, 4 artigos científicos publicados e 2 submetidos para publicação em revistas científicas internacionais indexadas. No decorrer deste doutoramento foram ainda publicados 4 trabalhos colaborativos paralelos ao projeto da candidata:

- Vidigal P, Duarte B, Cavaco AR, Caçador I, Figueiredo A, Matos AR, Viegas W, Monteiro F (2018) Preliminary diversity assessment of an undervalued tropical bean (Lablab purpureus (L.) Sweet) through fatty acid profiling. Plant Physiol Biochem 132 508–514 https://doi.org/10.1016/j.plaphy.2018.10.001
- Laureano G, Figueiredo J, Cavaco AR, Duarte B, Caçador I, Malhó R, Sousa Silva M, Matos AR, Figueiredo A (2018) The interplay between membrane lipids and phospholipase A family members in grapevine resistance against *Plasmopara viticola*. Sci Rep 8:14538. https://doi.org/10.1038/s41598-018-32559-z
- Laureano G, Cavaco AR, Matos AR, Figueiredo A (2021) Fatty Acid Desaturases: Uncovering Their Involvement in Grapevine Defence against Downy Mildew. Int J Mol Sci 22 (11), 5473. https://doi.org/10.3390/ijms22115473
- Maia M, Cavaco AR, Laureano G, Cunha J, Eiras-Dias J, Matos AR, Duarte B, Figueiredo A (2021) More than Just Wine: The Nutritional Benefits of Grapevine Leaves. Foods 10 (10), 2251. https://doi.org/10.3390/foods10102251

De acordo com o disposto no ponto 1 do artigo nº31 do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, deliberação n.º 7024/2017, publicada em Diário da República, 2.ª série – N.º 155 – 11 de agosto de 2017, a Autora da presente dissertação declara ter sido a principal executante do trabalho, tendo ativamente participando na conceção do desenho experimental, na interpretação dos resultados obtidos e na redação dos manuscritos.

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"And now, for something completely different…"

-John Cleese, in Monty Python´s Flying circus

# Resumo

Parte deste resumo foi incluído na seguinte publicação: Cavaco AR, Matos AR, Figueiredo A (2021) As moléculas lipídicas e o seu impacto na sanidade da vinha: em busca de uma viticultura mais sustentável. Agrotec. 38:62-64

A sustentabilidade da agricultura é um tema emergente que tem um lugar de destaque na investigação que se faz atualmente em patologia vegetal e fitossanidade. De modo a caminhar para uma agricultura sustentável é necessária a redução da aplicação de pesticidas. No entanto, a viticultura é uma das práticas agrícolas em que a aplicação de fitoquímicos é mais intensiva, dado que doenças como o míldio ameaçam devastar a maior parte das culturas em cada época de cultivo. Para desenvolver estratégias mais sustentáveis de controlo das doenças é importante compreender os processos moleculares que estão por trás da tolerância ou suscetibilidade a estas. Os lípidos têm um papel importante nos processos de defesa da videira. O estudo destas moléculas em diferentes frentes da interação videira-patógeno tem vindo a preencher lacunas no conhecimento nesta área e a permitir desvendar aos poucos este complicado sistema de interação. Apesar dos avanços que têm sido feitos no papel dos lípidos na defesa da videira ao míldio, assim como noutras interações planta-patógeno, existem ainda muitas áreas por explorar e muitas perguntas por responder.

Os lípidos são importantes moléculas constituintes das células. Esta importância deve-se principalmente a: 1) terem um papel estrutural nas membranas celulares e superfície protetora dos tecidos, 2) constituírem reserva de energia e por último 3) serem elementos-chave nos processos se sinalização celular. Na interação planta-patógeno, foram já identificadas alterações relacionadas com a composição lipídica membranar e identificados lípidos e seus derivados que participam e regulam diversos processos de defesa.

A primeira linha de defesa apresentada pela planta à entrada de patógenos consiste na cutícula, camada presente na superfície das folhas que serve de barreira física. Esta estrutura é composta principalmente por cutina, uma biomolécula formada por ácidos gordos. Quando o patógeno consegue ultrapassar esta barreira, entra em contacto com o espaço extracelular (ie apoplasto). Este compartimento, um dos mais importantes na defesa das plantas, é o local onde ocorre o reconhecimento de moléculas específicas do patógeno (metabolitos ou efetores) pelo hospedeiro e onde são despoletadas as primeiras reações de defesa da planta. Existem evidências de que os lípidos presentes no apoplasto têm um papel importante no estabelecimento da resposta sistémica da planta ao patógeno (*systemic acquired resistance*, SAR). Nos processos de defesa das plantas, ocorrem alterações ao nível de diferentes lípidos nas suas células. Estas alterações estão relacionadas, por um lado, com a regulação da fluidez das membranas celulares (um processo importante para a manutenção das suas funções essenciais durante o combate a patógenos) e por outro lado com a produção de compostos derivados dos lípidos membranares que se formam por ação de enzimas do metabolismo lipídico. Um exemplo deste tipo de metabolitos são as oxilipinas, moléculas resultantes da oxidação dos ácidos gordos, que são responsáveis pelo despoletar de diversos processos de resposta imunitária nas primeiras horas de interação entre planta e o patógeno na reação hipersensível. De entre as oxilipinas destaca-se o ácido jasmónico. Esta molécula é derivada do ácido gordo ω-3 ácido α-linolénico, (um ácido gordo polinsaturado que é um dos ácidos gordos maioritários dos lípidos de membrana) e é parte integrante de uma via de sinalização que leva à expressão de diferentes genes que são responsáveis por reações de defesa da planta.

Outra das formas através da qual as moléculas lipídicas e os seus derivados desempenham um papel importante na resposta das plantas a doenças é a eliciação da resposta imunitária. Ou seja, a aplicação de moléculas lipídicas, como é o caso do ácido jasmónico e do seu derivado – metil-jasmonato que causam uma resposta semelhante à que ocorre quando a planta entra em contacto com um patógeno. Vários estudos comprovam que quando as plantas entram em contacto com um patógeno após eliciação com estas moléculas a resposta imunitária é mais rápida e mais intensa. O ácido jasmónico é uma molécula importante no combate a doenças como o míldio da videira. Já foi observado durante a infeção com esta doença que a produção de ácido jasmónico pela planta é induzida, assim como os processos de sinalização celulares mediados por esta molécula que conduzem à resposta imunitária. A aplicação foliar de metil-jasmonato em vinha (*V. vinifera* cv Cabernet sauvignon) já foi associada ao aumento das defesas naturais da planta contra o agente etiológico do oídio, através do aumento da expressão de genes associados a processos de defesa (*pathogenesis related proteins*, quitinases, beta-1,3-glucanases) e à ativação do metabolismo secundário (stilbenoides).

Para além de possuírem um papel ativo na defesa das plantas, os lípidos também podem ser utilizados como biomarcadores. A sua presença ou abundância podem ser utilizadas como ferramenta para prever a resistência ou suscetibilidade de uma planta a determinada doença. Por exemplo, na interação do milho com o fungo *Fusarium verticillioides*, agente etiológico da podridão da raiz, foi detetada a acumulação de 25 moléculas lipídicas incluindo oxilipinas. De facto, neste sistema de interação planta-patógeno, entre as vias metabólicas significativamente alteradas após infeção encontram-se vias do metabolismo lipídico, incluindo da síntese de ácidos gordos. As moléculas acumuladas nesta interação constituem marcadores de defesa e resistência à doença.

Por outro lado, alguns lípidos podem também constituir marcadores de doença. Este papel pode ser atribuído a moléculas lipídicas específicas do patógeno detetadas durante a infeção. Um exemplo do possível uso de lípidos específicos do patógeno como marcadores de doença foi reportado em videiras infetadas com míldio, sendo a deteção destas moléculas anterior ao aparecimento dos primeiros sintomas visíveis. Neste estudo, a maior acumulação de lípidos específicos do patógeno ocorreu na variedade 'Syrah', a mais suscetível ao míldio entre as variedades contempladas, tendo a menor acumulação sido observada na casta 'Bianca', parcialmente resistente ao míldio.

A videira é uma das plantas mais importantes economicamente a nível mundial devido à produção do seu fruto principalmente para vinho ou uva de mesa. Em 2019 a área de vinha cultivada era de cerca de 7,4 milhões de hectares a nível mundial. O mercado de exportação de vinho produziu cerca de 31,8 mil milhões de euros. Portugal foi neste ano o 9º maior exportador de vinho, produzindo cerca de 800 milhões de euros.

*Vitis vinifera* L., é a espécie de videira mais usada na agricultura intensiva. Contudo, esta espécie apresenta um elevado grau de suscetibilidade a diversas doenças, que representam ainda uma ameaça à viticultura moderna. Entre as doenças mais devastadoras encontra-se o míldio, causado pelo oomicete obrigatório *Plasmopara viticola* (Berk. E Curt.) Berl. & de Toni. Na ausência de tratamento e em condições atmosféricas favoráveis ao desenvolvimento do *P. viticola* (humidade relativa >90% e temperaturas amenas) infeção com este patógeno pode levar à destruição de até 75% de uma cultura.

A estratégia de controlo das doenças da vinha passa pela aplicação preventiva de elevadas quantidades de fitoquímicos desde o surgimento das primeiras folhas, sendo a viticultura uma das atividades agrícolas em que a aplicação de produtos fitossanitários é mais intensiva. Esta estratégia de controlo de doenças acarreta consequências como o aumento da poluição ambiental, a toxicidade residual dos

produtos fitossanitários que permanecem nos solos, frutos e produtos transformados e a evolução dos próprios patógenos culminando no aparecimento de isolados mais resistentes aos tratamentos aplicados.

A agenda de objetivos de desenvolvimento sustentável das nações unidas para 2030 contempla diferentes áreas prioritárias. Vários destes objetivos estão relacionados com a sustentabilidade da agricultura. Para além disso, em 2009 foi lançada uma diretiva do Parlamento Europeu (2009/128/CE) que estabelece um quadro de ação a nível comunitário para uma utilização sustentável dos pesticidas. De modo a alcançar estes objetivos e caminhar para a sustentabilidade da agricultura, nomeadamente da viticultura, é primordial a redução da aplicação de fitoquímicos. Uma vez que o controlo de doenças é vital para a produtividade da viticultura, é necessário o desenvolvimento de diferentes estratégias que permitam uma menor aplicação de produtos fitossanitários. A compreensão da interação entre videira e patógeno, dos processos moleculares de defesa da planta assim como os eventos que levam ao seu desencadeamento tornam-se, portanto, fatores chave para a construção de estratégias mais sustentáveis de controlo de doença e para alcançar os objetivos da agenda 2030 da Nações Unidas e da União Europeia.

Uma das principais abordagens para a redução da utilização de fitoquímicos na viticultura baseia-se em programas de melhoramento. Através destes programas promove-se a introgressão de características de resistência em variedades elite de *V. vinifera*. Atualmente são utilizadas ferramentas moleculares, nomeadamente a seleção assistida por marcadores, de forma a promover uma maior rapidez e eficácia na seleção das plântulas que adquirem a característica de resistência. No entanto é necessário aprofundar o conhecimento da interação da videira com patógenos de forma a identificar novas moléculas que possam ser utilizadas como biomarcadores. Neste sentido, estudos pós infeção de cultivares de videira com o *P. viticola*, demonstraram que diversas moléculas lipídicas, como é o caso do ácido oleanólico, são moduladas e podem ser consideradas marcadores de infeção. A nível constitutivo, o estudo da composição em ácidos gordos de diferentes variedades de videira com diferentes graus de suscetibilidade ao míldio permitiu detetar diferenças no conteúdo de algumas moléculas entre as variedades tolerantes e suscetíveis. Por exemplo, as variedades suscetíveis parecem apresentar constitutivamente uma tendência para um maior conteúdo em ácidos gordos polinsaturados. Estas variedades também apresentam uma maior expressão de genes que codificam enzimas responsáveis pela formação de ácidos gordos mono- e polinsaturados, as dessaturases de ácidos gordos. Estas diferenças de conteúdo de ácidos gordos e de expressão de genes relacionados com o metabolismo dos lípidos, poderão potencialmente ser utilizadas para auxiliar na seleção híbridos obtidos em programas de melhoramento com características de tolerância ao míldio (discutido nos capítulos II e III desta tese).

Recentemente foi também observado que o mecanismo de defesa da videira mediado por lípidos é regulado de forma diferente dependendo do estilo de vida do seu patógeno (biotrófico – mantendo a planta viva para concluir o seu ciclo de vida – ou necrotrófico – causando a morte da planta para concluir o seu ciclo de vida). Após a inoculação da cultivar tolerante *V. vinífera* cv Regent com *P. viticola* (biotrófico, invadindo o tecido vegetal através da abertura estomática), *Erisiphe necator* (biotrófico, invadindo o tecido vegetal através de aberturas causadas por feridas) e *Botrytis cinerea* (necrotrófico), o despoletar dos processos de regulação dos níveis de ácidos gordos na defesa da planta revelou ser mais tardio e mais duradouro na interação com o patógeno necrotrófico (discutido no capítulo IV desta tese).

Nos últimos anos têm ocorrido avanços significativos na compreensão dos processos moleculares de defesa da videira mediados por lípidos. Estudos recentes nesta área permitiram concluir que durante a interação entre o agente causador do míldio e duas castas de videira, 'Trincadeira' (cultivar portuguesa suscetível ao míldio) e 'Regent' (cultivar obtida em programas de melhoramento, tolerante ao míldio), apenas na interação com a casta tolerante ocorreram alterações no conteúdo de alguns ácidos gordos.

De facto, na interação com a cultivar suscetível, o processo de oxidação lipídica e stress oxidativo que ocorre apenas nos primeiros instantes da infeção não é suficiente para a combater. Entre ácidos gordos cujos níveis sofrem alterações destaca-se o ácido α-linolénico, cujo conteúdo aumenta nas primeiras horas de interação entre o patógeno e a casta tolerante. Esta molécula é precursora na síntese do ácido jasmónico, cuja acumulação fora observada também no processo de defesa da cultivar 'Regent' contra o míldio. Na mesma interação ocorreu ainda o aumento da expressão nas primeiras horas de genes que codificam enzimas responsáveis pela libertação de ácidos gordos dos lípidos membranares, que têm o nome de fosfolipases A, cuja ação é essencial para que os ácidos gordos possam entrar em processos de sinalização celular. Outros resultados indicaram ainda que durante a interação 'Regent' – *P. viticola* o processo de defesa mediado por esta molécula é induzido nas primeiras horas. Foi verificado também que o ácido jasmónico, pode ser aplicado como eliciador da resposta imune, de forma a agir como "vacina" para a planta. Esta molécula foi aplicada em videiras com diferentes graus de suscetibilidade ao míldio: a espécie *Vitis riparia* (tolerante) e *Vitis vinifera* cultivar 'Pinot noir'(suscetível). Foi possível observar que após a eliciação ocorreu uma alteração dos conteúdos dos ácidos gordos em ambos os genótipos tolerante e suscetível, indicando que o ácido jasmónico pode iniciar a resposta imune antes do contacto com o patógeno em ambas as variedades (discutido no capítulo V desta tese).

Por último, vários estudos demonstraram que o espaço extracelular é um dos compartimentos mais importantes na comunicação entre a planta e o patógeno. Em videira, não foram conduzidos estudos que promovam uma melhor compreensão da dinâmica deste compartimento ao nível do reconhecimento do agente invasor ou dos processos de sinalização subsequentes. Como último objetivo desta tese, foi otimizado um protocolo de extração do fluido que compõe o espaço extracelular. Pela primeira vez, foi caracterizada a sua composição constitutiva em ácidos gordos e lípidos e foi estabelecida uma nova metodologia para auferir a pureza (não contaminação com citoplasma) do fluído extraído (estes resultados são discutidos no capítulo VI desta tese).

Em suma, neste trabalho, com o patossistema videira-*P. viticola* como ponto de partida, começámos por avaliar o papel de lípidos e ácidos gordos como potenciais marcadores moleculares de tolerância ou suscetibilidade. Um grau mais elevado de insaturação dos ácidos gordos assim como níveis mais elevados de algumas classes lipídicas como os lípidos plastidiais foram encontrados nos genótipos de videira suscetíveis ao *P. viticola*. Por outro lado, os genótipos tolerantes apresentam um grau mais baixo de insaturação dos ácidos gordos, o que se pode refletir, por exemplo numa maior rigidez membranar que possivelmente dificulta a invasão pelo patógeno e respetivo desenvolvimento. Neste trabalho foi ainda demonstrada a capacidade do ácido jasmónico de eliciar eventos de modulação de ácidos gordos, semelhantes aos que ocorrem após inoculação com um patógeno, em genótipos tolerantes e suscetíveis. Para além disso, de modo a perceber se o mecanismo observado na interação incompatível entre videira e *P. viticola* está conservado na interação com patógenos com diferentes ciclos de vida e/ou estratégias de invasão do hospedeiro, a modulação dos ácidos gordos e a sua regulação pelos genes FAD (do português desaturase de ácidos gordos) foram estudados. Com este estudo foi possível provar que a modulação de ácidos gordos segue um padrão distinto na interação com patógenos biotróficos e necrotróficos. Finalmente, foi estudado o primeiro campo de batalha na interação planta-patógeno: o apoplasto. O nosso grupo desenvolveu uma metodologia para extração do fluido apoplástico de folhas de videira que permite a análise de metabolitos e proteínas a partir de uma única amostra. Na análise do metaboloma, os lípidos e derivados revelaram ser a classe molecular mais abundante.

Os resultados deste trabalho reafirmam a importância da sinalização mediada por lípidos na defesa da videira contra doenças geradas por fungos e oomycetes e abrem o caminho para identificar e validar lípidos como biomarcadores para serem usados em programas de melhoramento. No que diz respeito ao

apoplasto da folha da videira, é possível dizer que apenas a “ponta do iceberg” foi revelada, ainda com muito para explorar. O desenvolvimento do método de extração do fluido apoplástico pelo nosso grupo abre as portas para que seja revelado todo o potencial deste compartimento e para a total compreensão dos primeiros momentos da interação videira-patógeno.

**Palavras-chave:** *Vitis vinifera*, lípidos, viticultura, marcadores, míldio

## Abstract

Grapevine (*Vitis vinifera* L) is one of the most economically important crops worldwide, mostly due to its uses for wine and table grape production. However, it is prone to several diseases. Downy and powdery mildews and grey mold, caused by *Plasmopara viticola*, *Erisiphe necator* and *Botrytis cinerea*, respectively, are among the most devastating ones. Disease control strategies include phytochemical applications every growing season, jeopardizing the sustainability of viticulture. Understanding the molecular processes behind disease resistance or susceptibility is vital to define alternative control strategies and select new disease resistance traits for breeding programs. The identification of molecular markers that allow discriminating tolerant and susceptible grapevine genotypes to their pathogens is an important step to help breeders select genotypes for crossings to produce hybrids with good winemaking and disease tolerance traits.

Lipids and lipid-derived metabolites are not only major structural and metabolic constituents of the cell, but they also function as modulators of a multitude of signal transduction pathways evoked by biotic stresses. It has been proposed that specific fatty acids (FA) may be involved in plant resistance against pathogens with different colonization strategies (biotroph, hemibiotroph and necrotroph). Previous results indicate that the content of several FA suffers alterations at early time-points after grapevine inoculation with the biotrophic oomycete *Plasmopara viticola*. These alterations are linked with reactive oxygen species and Jasmonic acid (JA) associated signalling. Moreover, lipid molecules and their derivatives, including JA, when applied externally, can cause a modulation of the lipid and FA signalling mechanisms in a similar manner to the pathogen challenge. Plants that are exposed to these elicitor molecules show a quicker and more intense defence response upon contact with a pathogen.

The extracellular matrix (ie apoplast) is the first battlefield where pathogen recognition occurs and secretion of both defence molecules and pathogen effectors take place. Therefore, the apoplast is one of the most important cell compartments in plant-pathogen interaction. Nonetheless, despite our knowledge on apoplast involvement on several processes from cell growth to stress responses, its dynamics is still poorly known due to the lack of efficient extraction processes adequate to each plant system. Because apoplastic fluid extraction from woody plants is a challenging task, studies regarding grapevine apoplast are still scarce to this day. There are two published studies on the grapevine leaf apoplast proteome and none on its metabolome.

In this work, the problems raised above were addressed. The analysis of the constitutive lipid and FA composition of tolerant and susceptible grapevine genotypes to *P. viticola* was carried out, along with the expression analysis of FA desaturase (FAD) genes. These studies allowed to identify lipids and FA as potential biomarkers for tolerance or susceptibility to *P. viticola*. The saturated FA, mainly in monogalactosyldiacylglycerol and phosphatidyl choline are candidate tolerance biomarkers and the polyunsaturated linoleic acid (C18:2) as well as the plastidial lipids are candidate susceptibility biomarkers. Moreover, the higher expression levels of FAD4, FAD6 and FAD8 in susceptible genotypes suggest that they might also be considered as candidate biomarkers for susceptibility. The analysis of the total leaf FA composition revealed corroborating results in terms of FA saturation degree and FAD expression, and it is a more rapid and less costly approach (discussed in the chapters II and III).

Due to the relevance of the JA mediated lipid signalling in the grapevine-*P. viticola* interaction, another question that raised was whether this mechanism would be conserved in the interaction with other pathogens with different invasion and/or lifestyles. Therefore, the FA modulation events, crucial for JA synthesis and signalling, were also addressed in the grapevine interaction with *E. necator* (biotroph, invading the plant leaf in the adaxial page from wound apertures) and *B. cinerea* (necrotroph). While

the interaction with the biotrophs may trigger a higher synthesis of polyunsaturated FA (PUFA) at early time-points with a tendency to return to basal levels, the interaction with *B. cinerea* may trigger a later and more durable induction of PUFA synthesis. In all interactions, membrane fluidity modulation occurred, which may be crucial to maintain cellular function during infection (discussed in chapter IV).

Since lipid molecules and JA showed previously to play important roles in the grapevine defence responses to *P. viticola*, the potential role of this molecule as a FA signalling trigger was studied. In fact, FA modulation after JA elicitation is similar to that described previously after *P. viticola* inoculation even in a susceptible cultivar, highlighting the potential of this molecule as an alternative to prevent grapevine diseases (discussed in chapter V).

To uncover the lipid signalling events of the first moments of plant pathogen interaction, a thorough analysis of the apoplastic fluid is necessary. A new methodological approach to isolate grapevine leaf apoplast compatible with proteomic and lipidomic based studies was defined. The constitutive metabolome was assessed by FTICR-MS, which allowed the identification of 514 unique putative compounds revealing a broad spectrum of molecular classes. Among them, lipids are the most abundant molecular class. This methodology represents an optimization to the existing protocols and opens the way to study the lipid signalling events in the first battlefield of the grapevine-pathogen interaction (discussed in chapter VI).

This work allowed to bring us a few steps closer to the complete disclosure of the grapevine lipid mediated defence mechanisms highlighting also candidate molecules to be used in future breeding programs for disease tolerance.

**Keywords:** *Vitis vinifera*, lipids, viticulture, biomarkers, downy mildew

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## Abbreviations

12-OH-JA - 12-hydroxyjasmonic acid
12-OPDA - 12-oxo-phytodienoic acid
2-HOT - 2(R)-Hydroxy-9(Z),12(Z),15(Z)-octadecatrienoic acid
4-HNE - 4-Hydroxy-2-Nonenal
ABA - Abscisic acid
ABC - ATP-binding cassette
ACP - Acyl carrier protein
acp – acyl carrier protein
AOC - Allene oxide cyclase
AOS - Allene oxide synthase
APF - Apoplastic fluid
APF - Apoplastic fluid
AzA - Azelaic acid
BSA - Bovine serum albumin
C16:0 - Palmitic acid
C16:1*t* - Trans-3-hexadecanoic acid
C17:0 - Margaric acid
C18:0 - Stearic acid
C18:1 - Oleic acid
C18:2 - Linoleic acid
C18:3 - α-linolenic acid
C20:4 - Arachidonic acid
C20:5 - Eicosapentanoic acid
CAP – canonical analysis of principal coordinates
CHAPS - 3-cholamidopropyl dimethylammonio 1-propanesulfonate
CJ - cis-jasmone
DAD - Defective in anther dehiscence
DAG – Diacylglycerol
DAMP - Damage associated molecular pattern
DBI – double bond index
DGDG – Digalactosyldiacylglycerol
DGK - Diacylglycerol kinase
dn-OPDA - Deoxymethylated vegetable dienic acid
ECS - Extracellular space
EF1α – Elongation factor-1alpha
EIHM - Extra-invasive hyphal membrane
ER - Endoplasmic reticulum
ESI - Electrospray ionization
ETI - Effector Triggered Immunity
EV - Extracellular vesicles
FA - Fatty acid
FAD – Fatty acid desaturase
FAE – Fatty acid elongase
FAME – Fatty Acid Methyl Esters
FB1 - Fumonisin B1

FFA - Free fatty acids
FT-ICR-MS - Fourier transform-ion cyclotron resonance-mass spectrometry
G3P - Glycerol-3-phosphate
GC - Gas chromatography
gDNA - Genomic DNA
HODE - Hydroxyoctadecadienoic acid
hpe - hours post elicitation
hpi – hours post inoculation
IEF - Isoelectric focusing
IP - inositol phosphate
IP3 - Inositol trisphosphate
JA – Jasmonic acid
JA-Ile - JA conjugated with isoleucine
JAZ - Jasmonate ZIM-domain
LACS - Long-chain acyl-coenzyme A synthetase
LCB - Long-chain base
LCB-P - Long-chain base phosphate
LOS - Lipooligosaccharides
LOX – Lipoxygenase
LPS - Lipopolysaccharides
LTP - Lipid transfer protein
MDA - Malondialdehyde
MDH - Malate dehydrogenase
MeJA - Methyl jasmonate
MGD1 - Monogalactosyldiacylglycerol synthase 1
MGDG – monogalactosyldiacylglycerol
MS - Mass spectrometry
NAD - Nicotinamide adenine dinucleotide
NBT - Nitroblue tetrazolium
NO - Nitric oxide
nsLTP - nonspecific lipid transfer protein
PA – phosphatidic acid
PAMP - Pathogen-Associated Molecular Patters
PC – phosphatidylcholine
PCD - Programmed cell death
PE – Phosphatidylethanolamine
PG – Phosphatidylglycerol
PI – phosphatidylinositol
PI(4,5)$P_2$ - Phosphatidylinositol 4,5-bisphosphate
PI-PLC - Phosphatidylinositol-specific phospholipase C
PL - Phospholipases
PLA – Phospholipase A
PLD - Phospholipase D
PMS - Phenazine methosulphate
PR - Pathogenesis-related
PS - Phosphatidylserine
PUFA – polyunsaturated fatty acids

PVP – Polyvinylpyrrolidine
ROS - Reactive oxygen species
Rpv - *P. viticola* resistance associated loci
SA – Salicylic acid
SAR – Systemic Acquired Resistance
SFA – saturated fatty acids
SNAP33 - N-ethylmaleimide-sensitive factor adaptor protein 33
SP - Signal peptide
SQDG - Sulfoquinivosyl diacylglycerol
SSI2 - Suppressor of SA-inducible
TGD - Trigalactosyldiacylglycerol
TOF - Time of flight
UBQ – Ubiquitin
UFA – unsaturated fatty acids
UPLC - Ultra-performance liquid chromatography
VIC - Vacuum-infiltration-centrifugation
VLCFA - Very-long-chain fatty acid
α-DOX - α-dioxygenase

# CHAPTER I

## 1. General Introduction

The first part of the Chapter I, (sections 1-5) was published as a review article in Cellular and Molecular Life Sciences

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Through the different types of plant immunity, including Pathogen-Associated Molecular Patters (PAMP) triggered immunity (PTI) [1] and Effector Triggered Immunity (ETI) [2], lipids and fatty acids (FA) play a crucial role, which has been brought to light over the past few decades [3]. The first barrier found by pathogens when encountering the host is the cuticle. This structure is mainly composed by cutin monomers and oligomers, consisting of hydroxy and epoxy-hydroxy C16 and C18 FA [4]. After entering the plant tissue, pathogens find one of the most important cellular compartments in defence, the apoplast. This compartment includes the extracellular matrix and the apoplastic fluid (APF) [5]. In plant-microbe interaction, pathogens secret molecular effectors into the apoplast, triggering a broad modulation on this compartment [6]. Protein composition alterations of the apoplast were reported to occur both qualitatively and quantitatively [7–9], but the modulation of lipids in the apoplast during plant-pathogen interaction remains a black box [10]. Nonetheless, there are a few evidences of the importance of apoplastic lipids in plant-pathogen interactions in systemic acquired resistance (SAR) establishment [11].

Considering the whole cell, it is known that upon pathogen challenge the plant's lipidic profile may suffer alterations often associated with modification of membrane fluidity, and enzymatic and non-enzymatic synthesis of bioactive lipid mediators such as lipid and FA oxidation products, oxylipins [12].This modulation was pointed out as a key factor to trigger plant immunity [13–15]. Lipids may be also considered as possible biomarker tools for susceptibility or resistance [16, 17] or even for disease, before the first visual symptoms appear [18]. Some lipids interact with defence associated proteins in order to exert their role in plant-pathogen interaction, namely with lipid transfer proteins (LTP) and fibrilins [19–23].Upon pathogen challenge, the synthesis and hydrolysis of different lipid species is necessary to trigger several defence mechanisms. These reactions are necessary so that lipids such as phosphatidic acid (PA) and free fatty acids (FFA) exert signalling roles and activate the jasmonic acid (JA) signalling pathway, programmed cell death (PCD), among others. Phospholipases A, C and D [24] are activated and contribute to release signalling lipids and FA from membranes. Recently, it was shown that pathogen-induced accumulation of nitric oxide (NO) and reactive oxygen species (ROS) promotes the production of azelaic acid (AzA), a lipid derivative that primes plants for salicylic acid (SA)-dependent defences [25]. The oxidation leads to the formation of oxylipins, which participate in a myriad of signalling pathways. These oxidation reactions can be either enzymatic or ROS mediated [26]. Most plant oxylipins are formed via enzymatic activity of the lipoxygenase (LOX) pathway. LOX enzymes catalyse the oxidation of linoleic acid (C18:2) and α-linolenic acid (C18:3) at the carbon position 9 or 13, resulting in the formation of 9- and 13-hydroperoxides, respectively [27]. Oxylipins play an important role in a variety of functions including growth, aging, development, and defence responses to environmental stimuli [28].

In the cross-talk between plants and pathogens, lipids play an important role mainly in: 1) pathogen development and life cycle completion [29, 30]; 2) pathogen recognition and defence response triggering by the host (Thevissen et al., 2003b; Sagaram et al., 2013; Iizasa et al., 2017; Järvå et al., 2018) and 3) hindering host defence systems and overcoming resistance [35–38]. During plant-pathogen interaction, some lipid metabolism alterations occur also in pathogens including in FA biosynthesis, elongation and degradation and glycerophospholipid metabolism, which are necessary for the pathogen's development and lifecycle completion [29]. In the case of pathogenic fungi and oomycete that form invasive hyphae, surrounded by an extra-invasive hyphal membrane (EIHM), the enrichment of this structure with Phosphoinositides (PI) is essential to build a conductive environment [30]. There is increasing evidence that lipids are part of a language that is transversal to all life kingdoms, which opens new insights into the studies of lipid metabolism and signalling both in plant and pathogen. Understanding lipid dynamics in the field of plant-pathogen interactions is arguably essential to complete the knowledge brought up to light by proteomics and transcriptomics. The analysis of lipids and their derivatives enables the possibility of describing the cross-talk between plants and pathogens and the

discovery of pathogen combined strategies targeting lipid pathways. Ultimately, a thorough and complete uncover of the role of lipids and their signalling pathways may allow finding new control strategies and therapeutic targets for plant diseases.

### 1.1. Fatty acids and lipids' role in plant-pathogen interaction

In order to survive, plants must perceive and transduce signals to elicit appropriate responses to environmental stimuli. Plant defence responses require energy and activation of signalling molecules, primarily supplied by primary metabolism of carbohydrates, organic acids, amines, amino acids, and lipids [39].

In plant-pathogen interactions, the first barrier found by pathogens before entering the host is the cuticle. This structure protects plants against drought, extreme temperatures, UV radiation, chemical attack, mechanical injuries, and biotic stress [40].It is mainly made up of cuticular wax and cutin (Table 1.1) [41], C16 (C16:0- palmitic acid) and C18 (eg C18:1- oleic acid) FA, produced in the chloroplast are exported to the endoplasmic reticulum (ER) [42] as Acyl-CoA esters and extended to form very-long-chain FA (VLCFA; C>20). The acyl chain extension is catalysed by the FA elongase (FAE) complex, on the ER membrane [43]. In the process of FA elongation with the FAE complex, malonyl-CoA is the two-carbon donor [44] contrarily to plastidial FA biosynthesis where this role is played by malonyl-acyl carrier protein (ACP) [42]. The VLCFA are then converted into cuticular waxes either by deactivation of acyl-CoA thioesters to release free acids, by conversion of aliphatic esters via the condensation of an acyl moiety with a primary alcohol, or via reductive pathways that convert acyl-CoAs to primary alcohols or aldehydes. The other component of the cuticle, cutin, is a polyester of C16 and C18 diacids, and ω- and mid-chain hydroxy FA [45]. Cutin is formed by the polymerization of the hydroxy group of C16 and C18 ω-hydroxy FA [41]. Cutin biosynthesis requires the activity of FA oxidases, acyl-activating enzymes (long-chain acyl-coenzyme A synthetase (LACS)) and acyltransferases (glycerol-3-phosphate (G3P) acyltransferase (GPAT)) [41]. Plant cuticle may have multiple roles during plant-pathogen interactions, which can be affected by its thickness, permeability, or specific cuticular components in different tissues [40]. Increasing evidence indicates that the cuticle is actively involved in plant defence [46]. Xia and collaborators observed that Gibberellin-treated Arabidopsis plants respond with increased levels of cuticular wax and cutin components, in association with improved plant immunity responses against *Pseudomonas syringae* [47]. A number of studies have been associating the plant cuticle with PTI, including from PAMP and damage associated molecular pattern (DAMP) and ETI. Therefore, the plant cuticle seems to have a role in the activation of both local and systemic defence [46, 48]. During plant-pathogen interactions, the composition of the plant cuticle may be affected by pathogens. Plant leaf wax components, such as very-long-chain C26 aldehydes of *Zea mays* could affect spore germination and penetration of *Blumeria graminis* f.sp. hordei in barley [49]. Upon barley inoculation with *Fusarium graminearum*, the causal agent of Fusarium head blight, the regulation of genes involved in FFA biosynthesis by the WAX INDUCER1 (HvWIN1) transcription factor occurs. As a result, part of the FFA are channelled to the reinforcement of the cuticle, leading to disease resistance [50]. In addition to wax and cutin, plant cuticle contains terpenoids and flavonoids, which have antifungal activities [51, 52]. Although structural lipids drawn from primary metabolism limit pathogen entry, in some situations the basal defence mechanisms are overcame by pathogens. Therefore, plants also reshape their composition of lipids in response to biotic stress to produce metabolites that function as signals or antimicrobial agents.

Sphingolipids (Table 1.1) are present in cell membranes and have both structural and regulatory roles [53]. These molecules are key players in signalling pathways related to development and responses to abiotic and biotic stresses and are vital for pathogen recognition [54].These nonglycerol lipids contain a ceramide backbone and a FA attached to a long-chain amino alcohol [53]. The long-chain base (LCB) of the sphingolipid may vary in length, which is usually between 16 and 20 carbons. The balance

between sphingolipid bioactive molecules, including LCB and its phosphate (LCB-P, as further discussed) are determining for the regulation of the cell survival death equilibrium [54]. Ceramide is the basic component of sphingolipids and can be modified, forming more complex sphingolipids, for instance glucosyl-ceramide and inositol-phosphorylceramide [53]. Ceramide can also be converted to inositol-phosphorylceramide by transfer of inositol phosphate (IP) from PI [53]. A high degree of sphingolipid variety is also due to long-chain base and acyl chain modifications [53]. Highly hydroxylated sphingolipids increase membrane stability and decrease membrane permeability, providing a higher tolerance to fungal pathogens [53]. Sphingolipids are known to have a role in pathogen associated PCD [55]. This defence process could be associated either with increased levels of long-chain bases or the ratio of long-chain bases to ceramides [56]. Moreover, the transfer of sphingosine between membranes also plays a key role in PCD [55]. However, König and collaborators observed that double mutants for fatty acid hydroxylase1/2 (*Atfah1/Atfah2*) (responsible for the hydroxylation of ceramide FA on theα position) that accumulate SA and ceramides are more tolerant to the biotrophic fungus *Golovinomyces cichoracearum* but do not display a PCD-like phenotype. These observations indicate that ceramides alone are not involved in the induction of PCD, being hydroxylation of the ceramides FA in the α position important for this process [57]. Moreover, a sphingoid base hydroxylase *sbh1/sbh2* double mutant completely lacking trihydroxy- LCBs showed enhanced expression of PCD marker genes [58], suggesting that hydroxylated forms of LCB are primary mediators for LCB-induced PCD.

Glycerolipids play a critical role in plant defence against pathogens. Particularly, PA, one of the central molecules in lipid defence signalling, induces defence responses like ROS production, expression of defence genes and PCD [59]. PA can derive from several glycerophospholipids, including the hydrolysis of Phosphatidylcholine (PC), Phosphatidylethanolamine (PE) and PI [59]. This lipid facilitates transport of lipids across membranes [60], as binding of PA to the enzyme monogalactosyldiacylglycerol synthase 1 (MGD1) stimulates monogalactosyldiacylglycerol (MGDG) biosynthesis in the chloroplasts [61], and binding of PA to trigalactosyldiacylglycerol proteins (TGD), facilitates the import of lipids from ER into chloroplasts [62]. Due to its small head group and bulky acyl chains, PA forms a conical shape and induces negative curves in membranes [63, 64]. A local increase in membrane PA levels may impact membrane structure and charge, thereby affecting protein or cofactor docking, vesicle formation, and membrane fusion [65]. PA also presents a regulatory role in abscisic acid (ABA)-mediated stomatal closure [66]. Upon pathogen challenge in *Nicotiana benthamiana*, increased amounts of PA induce immune responses including programmed cell death, accumulation of ROS, and induction of PR-4 expression [67].

PI (Table 1.1) also plays an important role in plant defence. This lipid hydrolysis catalysed by PI-specific phospholipase C (PI-PLC) originates the signalling molecules IP and diacylglycerol (DAG) [68]. PI may also be processed by kinases and phosphatases, originating deferent phosphoinositide species. As an example, PI 4,5-bisphosphate ($PI(4,5)P_2$), often called $PIP_2$, is the principal substrate of PLC [69]. The hydrolysis of glycerophospholipids catalysed by phospholipases A (PLA) generates FFA and lysophospholipids. These molecules include, for example, Lyso-PA and lyso-PC (Table 1.1) [68]. The signalling activity of lysophospholipids is dependent on the length and position of acyl chain, degree of saturation, and presence of the phosphate head group [70]. FFA can exert different roles in plant-pathogen interaction, from anti-fungal activity to signalling towards the octadecanoic pathway that leads to the formation of JA [71].

Galactolipids also play a major role in plant defence, namely in modulation of the JA pathway. An increased MGDG:digalactosyldiacylglycerol (DGDG) ratio induces JA overproduction and changes chloroplast shape [72]. Mutations in DGD1, the major DGDG-synthesizing enzyme, severely reduce DGDG content and induce JA overproduction, resulting in stunted growth [72]. MGDG and DGDG also regulate SA levels and SAR [73]. While DGDG is responsible for NO and SA accumulation during

SAR, MGDG (Table 1.1) regulates the biosynthesis of AzA (Figure 1.1) and G3P that function downstream of NO [73].

## 1.2. The major players in Lipid metabolism and lipid-associated signalling pathways

Upon pathogen challenge, the synthesis and hydrolysis of different lipid species is activated, which is necessary for the triggering of several defence mechanisms. These reactions are necessary so that lipid molecules such as PA (Table 1.1) and FFA exert signalling roles and activate defence related genes, the JA signalling pathway, PCD, among others. The first step to trigger lipid and FA signalling is the activation of the enzymes phospholipases A, C and D [24].

Phospholipases A (PLA), which comprehend the patatin-like, defective in anther dehiscence (DAD)-like and secretory PLA, catalyse the hydrolysis of phospholipids and glycolipids for the formation of lysophospholipids and FFA (Figure 1.1) [74]. FFA, namely C18:3 (Table 1.1) may act as second messengers or as precursors of various oxylipins such as JA (Table 1.1) [75] (Figure 1.1). In pepper leaves, a patatin-like PLA, CaPLP1 is strongly up-regulated during *Xanthomonas campestris* p*V.* vesicatoria infection, especially in the incompatible interactions. In this interaction, CaPLP1 is involved in PCD mediated defence signalling in response to infective microbial pathogens [76]. Also, in Arabidopsis patatin-like PLAs were shown to be involved in pathogen response [77]. Upon inoculation of *N. benthamiana* with *Phytophtora parasitica*, higher transcription levels of $PLA_2$ as well as higher levels of Lysophosphatidylcholine (LysoPC) are observed [78]. Up-regulation of several PLA-encoding genes including patatin-like, DAD-like and secretory PLA were up-regulated in grapevine leaves infected with *Plasmopara viticola* [13].

Phospholipase C catalyses, among other, the hydrolysis of PI, mainly $PIP_2$, to produce $Ca^{2+}$, inositol trisphosphate (IP3), a mobilizing second messenger, and DAG (Table 1.1), which is further phosphorylated in a reaction catalysed by DAG kinase (DGK) to produce PA [79, 80] (Figure 1.1). In the presence of the fungal effector xylanase, there is an activation of the enzyme Phosphatidylinositol-phospholipase C, SlPLC2. This enzyme is required for xylanase-induced expression of the SA-defence gene marker Pathogenesis Related1 (SlPR1) and the HR tomato gene marker Hypersensitive Response 203J [81]. Also, pathogen-induced lysoPC production is mediated by PLA hydrolysis of oxidized phospholipids, which are the products of free radical damage to unsaturated acyl chains of glycerophospholipids in response to pathogen infestation. The increased levels of this lipid lead to the expression of defence related genes, like pathogenesis-related (PR) and LOX. Moreover, this LysoPC leads to a higher ROS production and contributes to cell death [78].

Phospholipase D (PLD) catalyses the hydrolysis mainly of PC and PE to form PA [59] (Figure 1.1). Some PLD can act as positive or negative regulators of plant immunity [59, 82]. PLDs (α, β, γ, δ, ε and ζ) can be differentiated depending on their requirements and/or affinities for $Ca^{2+}$, PIP2 and FFA [83]. The predominant isoenzyme is the α- type PLD, which can be detected in both the leaves and seeds of plants and is responsible for the majority of the baseline PLD activity found therein. PLDα does not require phosphoinositides for its activity when assayed in the presence of mM levels of $Ca^{2+}$ ions. In contrast, the β, γ, δ and ε PLD isoenzymes from Arabidopsis show their highest activity at μM Ca2+ concentrations and require the presence of PIP2 to be fully active [84]. Recently, Schlöffel and coworkers observed that Arabidopsis knock-out mutants for the PLDγ1 (but not PLDγ2 or 3) gene showed a higher resistance to *P. syringae* pv DC3000 (biotrophic) and *Botrytis cinerea* (necrotrophic) [85]. Since the immune response to pathogens with different infection strategies involves antagonistic signalling cascades, SA and JA pathways [86], PLDγ1 may act as a central signalling hub that modulates plant immune responses to different pathogens, working as a negative modulator of the plant immune system [85]. Upon elicitation with the flagellin flg22, mutant plants respond with a 2-fold increase in ROS production, which indicates that PLDγ1 acts as a negative regulator of plant immunity. This PLD functions independently of SA and JA and is not related to PA production [85].

FA desaturation is also a highly important process for plant defence [3]. The unsaturation of newly formed FA is carried out by the stromal enzyme SACPD (or Δ9 desaturases), which introduces a cis double bond into the acyl-ACP at C9 position [87]. The substrate specificity of the different SACPD isoforms depends on the acyl chain length and the position of the double bond [88]. Among them, the suppressor of SA-inducible (SSI2)-SACPD shows higher specific activity and preference towards C18:0 than for C16:0 [89]. Reactions catalysed by SACPD originate the monounsaturated FA C18:1 and the unsaturated palmitic acid, C16:1 (Figure 1.1). Hydrolysis of unsaturated and saturated FA-ACP is preferentially catalysed by the FA acyl- ACP-thioesterases FATA and FATB, respectively [90]. FFA are activated as CoA esters by acyl-CoA synthetase and exported to the cytoplasm. These lipid species are then processed in the ER [91].

The desaturation of FA present in membrane lipids is catalysed by membrane-bound FA desaturase (FAD) enzymes present in the chloroplast or ER membranes [92]. FAD2 and FAD3 catalyse the desaturation of C18:1 and C18:2, respectively, esterified both at sn-1 and sn-2 positions of glycerolipids in the ER [93, 94] (Figure 1.1). Desaturation of C18:1 and C18:2 in plastidial membranes is catalysed by FAD6 and FAD7/FAD8, respectively. The FAD6 and FAD7/FAD8 enzymes can catalyse FA desaturation in glycerolipids containing either C16 or C18 FAs at sn-1 or sn-2 positions [95] (Figure 1.1). Two other plastidial desaturases, FAD4 and FAD5 specifically catalyse the synthesis of trans C16:1 or Δ7 C16:1 on phosphatidylglycerol (PG) or MGDG, respectively [73, 96] (Figure 1.1). Soria-García and co-workers observed that the Arabidopsis desaturase AtFAD8 showed a JA-dependent response both at the gene expression and protein levels, suggesting that this enzyme is coordinated in defence responses [97]. Moreover, ABA induced the decreasing of AtFAD7 mRNA and protein levels, controlling AtFAD7 desaturase activity. This result suggests a higher specialization of FAD7 on biotic and defence responses (as supplier of JA biosynthesis precursors), that could be blocked antagonically by ABA [97].

In early plant-pathogen interaction, lipid and FA oxidation is one of the most important processes. The oxidation process leads to the formation of oxylipins, which participate in a myriad of signalling pathways. These oxidation reactions can be either enzymatic or ROS mediated [26]. Most plant oxylipins identified until now are formed via enzymatic activity of the LOX pathway. LOX catalyses the oxidation of C18:2 and C18:3 at the carbon position 9 or 13, resulting in the formation of 9- and 13-hydroperoxides, respectively [27]. Recently, it was shown that pathogen-induced accumulation of NO and ROS promotes the production of Aza, a lipid derivative that primes plants for SA-dependent defences [25]. Oxylipins play an important role in a variety of functions including growth, aging, development, and defence responses to environmental stimuli [28]. For instance, the 7,8,9-, 9S,10S,11R-, and 12,13,17-trihydroxy-octadecenoic acids (Table 1.1) showed to inhibit the growth of the plant pathogens *B. graminis*, *Phytophthora infestans*, and *B. cinerea* [98]. Interestingly, the oxylipin 2(R)-Hydroxy-9(Z),12(Z),15(Z)-octadecatrienoic acid (2-HOT) was also found to be produced in Arabidopsis leaf oil bodies upon inoculation with *Colletotrichum higginsianum* via α-dioxygenase (α-DOX) [99].

The LOX pathway has been proposed to act directly in plant defence by producing antimicrobial compounds [100] or by signalling molecules such as JA that regulates gene expression in plant defence and cell death [101]. Jasmonic acid (JA) is one of the most studied plant oxylipins. Its biosynthesis occurs through different pathways including the octadecanoid pathway starting from C18:3 and the hexadecanoid pathway starting from hexadecatrienoic acid (C16:3) (Table 1.1) in 16:3 plants such as Arabidopsis [102]. The sequential steps of these pathways take place in different cellular compartments: chloroplasts, peroxisome, and cytoplasm (Figure 1.1). The synthesis of 12-oxo-phytodienoic acid (12-OPDA) or deoxymethylated vegetable dienic acid (dn-OPDA) from the oxidation unsaturated FA occurs in the chloroplast in reactions catalysed by LOX, allene oxide synthase (AOS) and allene oxide cyclase (AOC) (Figure 1.1). JA is formed as a result of subsequent β-oxidation reactions that occur in the peroxisome. In the cytoplasm, JA is metabolized to different structures, such as methyl jasmonate (MeJA), the bioactive form of JA conjugated with isoleucine (JA-Ile), cis-jasmone (CJ), and 12-hydroxyjasmonic acid

(12-OH-JA). The bioactive form of JA will then induce the expression of resistance related genes [103]. Recent results show that JA-signalling pathway may be suppressed by uncharacterized factors derived from virulent *Xanthomonas oryzae* p*V.* oryzae [104]. A transcriptomic study on rice leaves infected with black streaked dwarf virus showed that the expression of JA synthesis-related genes, OsLOX, OsAOS and jasmonate O methyltransferase (OsJMT1) was significantly increased while the hydroperoxidelyase (OsHPL3), a competitor of AOS for the same substrate, was down-regulated [105]. Moreover, in response to *Aspergillus parasiticus* infection in peanut seeds, accumulation of free fatty acids and induction if LOX activity and gene expression was also observed. This signalling mechanism operates rapidly in resistant cultivars [14]. Recent results show that after wheat inoculation with pathogens of the *Fusarium* genus, there is a transcript accumulation mainly of TaLox2, TaJAZ9 and the putative PR genes TaPR-4b [106]. The activation of these genes showed that the JA pathway occurs in the defence responses in wheat-fusarium pathosystems. The enzyme LOXd showed to be highly up regulated in tomato plants inoculated with *Fusarium solani* [107].

Also, lipid peroxidation products, such as Malondialdehyde (MDA) or 4-Hydroxy-2-Nonenal (4-HNE), were reported to regulate stress associated transcription factors [108]. After grapevine inoculation with *P. viticola*, an increase of the lipid peroxidation was observed, including an increase of the MDA levels in a resistant cultivar [21]. In contact with the bacterial effector AvrRpm1, during HR, oxidized derivatives of MGDG, DGDG, sulfoquinivosyl diacylglycerol (SQDG), PG and PI were identified in Arabidopsis [109]. Among these lipid oxidized forms were the OPDA containing lipids. Despite the fact that the function of the OPDA-containing lipids remains uncertain, it had previously been proposed that Arabidopsis OPDA-containing galactolipids (arabidopsides) might act as chemical defensive compounds against microorganisms as well as function for delayed release of OPDA [110].

Lipid signalling is a key process for the long distance communication of several stimuli and, therefore, for the establishment of SAR [reviewed in 108]. A nonspecific LTP (nsLTP) have been described to participate in SAR through the interaction with lipid-derived molecules like JA [112]. A nsLTP from *Brassica rapa* displayed both antifungal and antibacterial activity [112]. Moreover, a nsLTP from *Arabidopsis thaliana* has been implicated in the AzA dependent development of SAR [20]. In Arabidopsis transgenic lines expressing wheat LTP4, it was observed that this protein induced a higher resistance to the fungi *B. cinerea* and *Alternaria solani*. TdLTP4 showed to be implicated in JA signalling since it is responsive to this oxylipin and upon its expression there is downregulation of Jasmonate ZIM-domain (JAZ) encoding genes [113]. Another lipid associated protein, fibrillin, belongs to a family called plastid lipid associated proteins. It can be found at higher levels in plants during defence responses. Upon grapevine inoculation with *P. viticola*, this protein was observed at higher levels in a resistant cultivar [21]. These proteins act as scaffolds for building lipid droplets that contain FFA, pigments and other lipophilic compounds [19]. There is a correlation between the levels of fibrillin and JA synthesis. Plastoglobules may function as a specialized platform for the synthesis of early JA precursors, storing enough FA (with a prevalence of C18:3) to trigger its synthesis after local oxidative stress [22]. Moreover, upon inoculation of *N. benthamiana* with the bacteria *Ralstonia solanacearum the protein SEC14, a phospholipid transfer protein is induced* [114]. This protein exhibits phospholipid transfer activities [115], and may be involved in plant immune response via phospholipid-turnover.

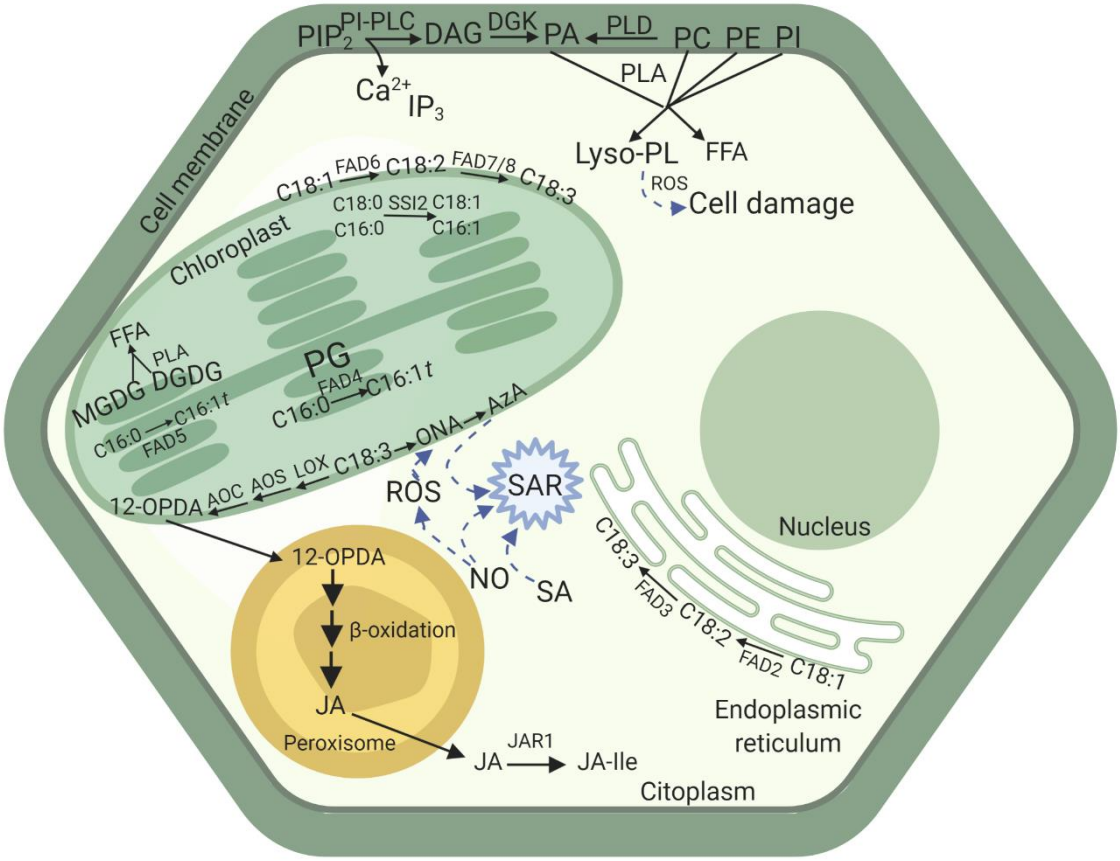


**Figure 1.1.** Lipid signalling events in plant-pathogen interactions Lipid signalling events in plant-pathogen interactions. $PIP_2$: phosphatidylinositol 4,5-bisphosphate; PLA: phospholipase A; PI-PLC: phosphatidylinositol specific phospholipase C; DAG: diacylglycerol; DGK: diacylglycerol kinase; PA: phosphatidic acid; PLD: phospholipase D; PC: phosphatidylcholine; PE; phosphatidylethanolamine; PI: phosphatidylinositol; $IP_3$: inositol-3-phosphate; Lyso-PL: lysophospholipid; FFA: free fatty acid; ROS: reactive oxygen species; MGDG: monogalactosyldiacylglycerol; DGDG: digalactosyldiacylglycerol; FAD: fatty acid desaturase; PG: phosphatidylglycerol; 12-OPDA: 12-oxo-phytodienoic acid; AOC: allene oxide cyclase; AOS: allene oxide synthase; LOX: lipoxygenase; ONA: 9-oxononanoic acid: AzA: azelaic acid; JA: jasmonic acid; JA-Ile: jasmonic acid conjugated with isoleucine; JAR1: jasmonates-amide synthetase; NO: nitric oxide; SA: salicylic acid; SAR: systemic acquired resistance; SSI2: SA-inducible 2; C16:0: palmitic acid; C16:1*t*: trans-hexadecanoic acid; C18:0: stearic acid; C18:1: oleic acid; C18:2: linoleic acid; C18:3: α-linolenic acid. The blue arrows indicate induction

### 1.3. Important discoveries on the role of lipids in defence and disease

The importance of lipids and FA in plant-pathogen interactions has been previously revised [3, 122]. From 2013, an increasing number of studies have shown that plant lipids and FA play key roles in the interaction with pathogens.

Pathogen lipids may act as PAMP and PAMP recognition by the host trigger immunity responses associated with the alterations in the composition of the plant lipid membrane, modification of membrane fluidity, and enzymatic and non-enzymatic genesis of bioactive lipid mediators such as lipid and FA oxidation products, oxylipins [12]. These molecules also integrate tailored defence mechanisms against a diverse array of pathogens with different lifestyles.

Necrotrophic pathogens extract nutrients from dead cells killed prior to or during colonization [123]**.** On the other hand, plant biotrophic pathogens establish a long-term feeding relationship with the living cells of their hosts, rather than killing the host cells as part of the infection process [124]. Hemibiotrophic pathogens start by having a biotrophic lifestyle and then change to a necrotrophic mode [125]. Saprophytes obtain nutrients from dead and decaying organic matter [126]. Müler and co-workers

observed that in the incompatible interaction between peanut seed and the necrotrophic fungus *A. parasiticus* there is an activation of the LOX pathway [14]. This result reinforces that the production of oxylipins is an important process in in the defence mechanisms against necrotrophs [127, 128]. Recently, several works have shown strong evidences that grapevine tolerance to the biotrophic *oomycete P. viticola* could also, in the first hours, be mediated by JA and lipid associated signalling. After pathogen challenge, JA biosynthesis, JA-Ile synthesis, $H_2O_2$ accumulation, and lipid peroxidation were observed [21, 129, 130]. FA and lipids were shown to be modulated upon inoculation of the tolerant grapevine *Vitis vinifera* cv Regent with *P. viticola*, particularly at 6 and 12 hours post inoculation (hpi). Both C16:0 and C18:0 relative content decreased when compared to mock-inoculated samples, while the relative content of the unsaturated FA, C18:1, C18:2 and C18:3 increased [13]. Since C18:3 is a biosynthetic precursor of JA [131], the increase of its levels in the first stages of plant-pathogen incompatible interaction might be associated to the triggering of JA signalling pathway. Furthermore, this alteration might be related to the protection of the photosynthetic machinery during the invasion [13]. In fact, leaf C18:3 is mostly present in the galactolipids, MGDG and DGDG, which account for more than 85% of thylakoid lipids [132]. The ability to adjust membrane lipid fluidity by changing the levels of unsaturated fatty acids is a feature of stress response, which allows to maintain the function of integral proteins, such as the photosynthetic machinery [133]. During grapevine-*P. viticola* incompatible interaction, a significant increase of the levels of MGDG and DGDG as well as the double bound index, which reflects membrane fluidity, occurs [13].

Leaf lipids from *V. vinifera* cv Bianca, tolerant to *P. viticola*, showed the greatest differences among the differently accumulated metabolites. Among the accumulated lipid compounds, were arachidic acid, oleanolic acid, and uvaol [16]. Additionally, a decrease in some unsaturated fatty acids after *P. viticola* infection was observed, which may be linked to the activation of JA pathway [16]. JA signalling was also found to be activated in rice defence against the hemibiotrophic fungus *Magnaporthe oryzae* [134].

Sphingolipids are key players in the induction of PCD [135]. These lipids are structurally characterized by a sphingoid base acyl chain amide linked to a FA, forming ceramide. The different physical properties of sphingolipids are due to the different structures that can present in plants. The LCB of the sphingolipid may vary in length, which is usually between 16 and 20 carbons. The amide-linked FA or VLCFA can also undergo modifications, varying in length from 16 to 30 carbons and can be hydroxylated at the C2 position and desaturated at the ω-9 position leading to high variability of sphingolipids [53]**.** The balance between sphingolipid bioactive molecules, including LCB and its phosphate (LCB-P) are determining for the regulation of the cell survival death equilibrium [54]. Magnin-Robert and co-workers observed in Arabidopsis that when this equilibrium was disrupted by knocking down the gene encoding for LCB-P lyase, a survival/death imbalance occurs favouring cell survival. As a result, the mutant plant showed higher susceptibility to the hemibiotrophic pathogen *P. syringae* pv tomato in comparison to wild-type plants [54]. Liu and co-workers also observed that there is an activation of PCD upon *N. benthamiana* inoculation with *P. syringae* p*V.* maculicola. Furthermore, the authors observed that accompanying the induction of PCD there is a synergistic coordination of JA and SA signalling, which may also leave the plant less vulnerable to necrotrophic pathogens [136].

In the interaction between citrus and the bacteria *Candidatus Liberibacter asiaticus*, causal agent of Huanglongbing disease, metabolites including four FA and two lipid oxidation products including of C18:3 and PA were reliably decreased. In this case, the pathogen may cause the altered metabolism of long-chain fatty acids, possibly leading to the manipulation of the host FA associated defence, including the synthesis of JA [15]. The results described above show that lipids and FA not only play central roles in defence and disease but also may be considered as candidates for resistance/susceptibility molecular biomarkers.

**Table 1.1.** Plant lipid molecules involved in plant-pathogen interactions that contributed to major break troughs in the last years

| Molecule | Function | Reference | Example of pathosystem |
|---|---|---|---|
| Cutin | Physical barrier | [116] | *Solanum lycopersicum - Botrytis cinerea* |
| C16 and C18 fatty acids | Structural and signalling lipid constituents<br>Signalling | [13] | *Vitis vinifera - Plasmopara viticola* |
| α-Linolenic acid (C18:3) | JA synthesis precursor | [13] | *Vitis vinifera - Plasmopara viticola* |
| 7,8,9-, 9S,10S,11R-octadecenoic acid<br>2,13,17-trihydroxy-octadecenoic acid | Pathogen growth inhibition | [117] | *Boehmeria nivea - Phytophthora capsici* |
| Hexadecatrienoic acid | JA synthesis precursor (C16:3 plants) | [118] | *Arabidopsis thaliana - Pseudomonas syringae* |
| Sphingolipids | Structure<br>Regulation of membrane permeability | [53] | *Arabidopsis thaliana - Pseudomonas syringae* |
| | PCD | [53] | *Arabidopsis thaliana - Botrytis cinerea* |
| | | [55] | *Arabidopsis thaliana - Pseudomonas syringae* |
| α-Hydroxylated ceramides | PCD | [57] | *Arabidopsis thaliana - Golovinomyces cichoracearum* |
| Trihydroxy-LCB | LCB-induced PCD | [57, 58] | *Arabidopsis thaliana - Golovinomyces cichoracearum* |
| PA | Signalling | [80] | *Arabidopsis thaliana - Botrytis cinerea* |
| | ROS production | | *Triticum aestivum - Puccinia striiformis* |
| | Defence gene expression | | *Arabidopsis thaliana - Botrytis cinerea* |
| | PCD | | *Triticum aestivum - Puccinia striiformis* |
| PI eg PI 4,5-bisphosphate | Signalling | [119] | *Arabidopsis thaliana - Pseudomonas syringae* |
| Lyso-Phospholipids | Signalling | [120] | *Arabidopsis thaliana - Botrytis cinerea* |
| | PR and LOX gene expression<br>ROS production<br>PCD | [78] | *Nicothiana benthamiana - Phytophtora parasitica* |
| JA, active form JA-Ile | Signalling<br>Defence gene expression | [121] | *Arabidopsis thaliana - Pseudomonas syringae* |
| DAG | Signalling<br>PA synthesis | [114] | *Nicothiana benthamiana - Ralstonia solanacearum* |

Untargeted metabolite analysis of tomato leaves inoculated with the biotrophic fungus *Cladosporium fulvum* revealed that falcarindiol, a diacetylenic FA possessing two triple bonds, is among three major metabolites present. After incubation with bacterial effectors, falcarinidol synthesis was also induced, indicating that it is involved in both bacterial and fungal interactions with tomato [137]. This unusual FA had been reported to be biosynthesized in response to pest and pathogen stress [reviewed in 129].
Regarding saprophyte pathogens as the case of *A. parasiticus*, after inoculation of peanut seeds Müller and collaborators observed significant differences of FFA contents between infected and control seeds [14]. Lipids and FA were also identified as defence markers in maize grain inoculated with the fungus *Fusarium verticillioides* [17]. In this pathosystem, a metabolome analysis revealed that several lipid compounds correlated with the mycotoxin fumonisin accumulation. 25 discriminant metabolites, all belonging to lipid classes, have been putatively identified. Moreover, the most significantly altered pathways upon infection with *F. verticillioides* are involved in lipid synthesis, such as phospholipid and FA biosynthesis, glycerophospholipid metabolism, and linoleic acid metabolism [17]. Furthermore, Ludovici et al. reported a significant increase of oxylipins in maize ears after *F. verticillioides* infection, suggesting the triggering of defence responses [12].
The enrichment of subcellular regions in certain phospholipids, mainly Phosphatidylserine (PS) may also be important for an efficient defence response against viruses. This process was observed in the interaction between *A. thaliana* and cucumber mosaic virus. It is vital for the formation vesicle-like membrane invaginations and the recruitment of the molecular machinery to form viral and host siRNA. Arabidopsis mutants lacking the lipid flipases ALA1 and ALA2 were not able to form vesicle-like membrane invaginations and showed and enhanced susceptibility to the cucumber mosaic virus [139].
Over the last years, the role of the plant cuticle in plant-pathogen interactions as more than just a physical barrier has been gaining attention. DAMP, such as cutin monomers have shown to serve as signals that activate plant defences against pathogens [40]. In response to infection with *Colletotrichum gloeosporioides*, tomato fruit cuticle was remodelled, and fruit cuticle biosynthesis was up-regulated during appressorium formation even before penetration [140]. In another study, inoculation of citrus petals with *Colletotrichum acutatum*, caused the epidermal cells to increase lipid synthesis, which altered the cuticle structure [141].

### 1.4. Pathogen Lipids, a different perspective

Lipid metabolism in plant pathogens during infection plays an important role either in: 1) development and life cycle completion; 2) pathogen recognition and defence response triggering in the host; 3) hindering host defence systems and overcoming resistance.
Botero and collaborators (2018) observed, in the context of the hemibiotrophic *P. infestans* infection of potato leaves, the importance of FA biosynthesis, elongation, and degradation pathways. FA elongation pathways showed active fluxes during the early biotrophic phase and changed to a null flux at later time points. On the other hand, in the necrotrophy phase of infection, the glycerophospholipid metabolism was altered and their metabolic fluxes changed. [29]**.** Upon plant infection, an accumulation of pathogen specific lipids and FA molecules may also occur, which indicate that these molecules can be used as molecular biomarkers for infection. One example of this was reported by Negrel and co-workers during the interaction between grapevine and *P. viticola* [18]. In this work, *P. viticola* specific lipids and FA were detected from very early stages of the infection process before the first external symptoms. *P. viticola* specific lipids, which include Eicosapentanoic acid (C20:5) and arachidonic acid (C20:4)-containing lipids and C16:1 ceramides including Cer(d16:1/16:0) were identified [18]. C20:5 and C20:4 had been previously detected in oomycetes [142]. *P. viticola* specific lipid accumulation in the fully susceptible variety *V. vinifera* cv Syrah was significantly higher than in *V. vinifera* cv Bianca [18] which is partially resistant [143]. The pattern of lipid accumulation was modified along the infection process.

At early stages, C20:4 and C20:5 were more accumulated as FFA, whereas in later stages, the triacylglycerols containing these FA, and especially trieicosapentaenoyl-glycerol (TEPG), were more accumulated. Lipid accumulation pattern may therefore be used as an indicator of the infection developmental stage [18].

Recently published reports on the mechanisms of the entry of oomycete RxLR effectors have revealed that these effectors bind to phosphoinositol-3-phosphate (PI3P) known as an intracellular molecule [144].

One of the ways by which some pathogens (mainly biotrophs) overcome plant defence is the arresting of PCD trough the action of effectors. Elicitation of *A. thaliana* plants with the mycotoxin fumonisin B1 (FB1) resulted not only in the accumulation of LCB and of C16 FA-containing sphingolipids, but also in a decrease in the sphingolipid content containing VLCFA [145]. Furthermore, studies of Arabidopsis mutants with disruptions in gene loci governing sphingolipid metabolism confirmed a link between sphingolipid homeostasis and PCD associated with plant defence [146].

Defensins constitute an ancient and diverse set of natural antimicrobial proteins [147]. Different pathogen lipids bind to plant defensins, which causes the permeabilization of fungal membranes [148]. The engagement with specific fungal membrane phospholipids affects the ability of certain plant defensins to kill fungal cells [149, 150]. Furthermore, different phospholipids trigger the formation of discrete defensin–phospholipid complexes with unique topologies [149, 150]. Sphingolipids like glycosylceramides and mannosyl diinositolphosphoryl ceramides also bind to plant defensins forming complexes necessary for these proteins anti-fungal activity, although these interactions are still poorly understood [34]. Saragram and co-workers observed that a fungal PA interacts with a *Medicago truncatula* defensin (MtDef4) [33]. When fungal membrane lipids interact with defensin oligomers, a combination of curvature stress and lipid sequestration occurs, resulting in complete structural destabilization and subsequent permeabilization of the membrane [32].

In bacteria, PAMP are conserved cell-surface structures including flagellin, lipopeptides, peptidoglycans and lipopolysaccharides (LPS) [151]. LPS, with a major role in bacterial growth and survival [152], can trigger PTI [153]. LPS from plant pathogenic bacteria could induce a PCD in Arabidopsis leaves in a dose dependent manner, depending on an early ROS production. Moreover, these molecules were able to induce PR1 gene expression [154]. LPS are composed of a hydrophilic heteropolysaccharide (comprising the core oligosaccharide and O-specific polysaccharide or O-chain) covalently linked to a lipophilic moiety termed lipid A, which anchors these macromolecules to the outer membrane. LPS without the O-chain are lipooligosaccharides (LOS) [155]. During Arabidopsis infection with *X. campestris* p*V.* campestris (Xcc), LOS promoted pathogen recognition. LOS induced the upregulation of the PR1 and PR2 genes in Arabidopsis leaves [156]. The LOS lipid A moiety was found to be active in a later phase of the interaction, contrarily to the oligosaccharide, which induced gene upregulation early in the interaction [156]. Although both Xcc lipid A and core oligosaccharide are active in defence gene induction, it is possible that they are recognized by different plant receptors [155]. Xcc LOS interacts with two members of the F-box protein family involved in pathogen recognition, namely F-box and F box- LRR. F box-LRR might be involved in the recognition of Xcc LOS by activating a proteasome-mediate hydrolysis of repressor proteins that negatively regulate target genes with a role in plant defence. LOS also interacts with two protein kinases involved in cellular signal transduction pathways and nsLTP1 [156]. Furthermore, LOS was found to promote the activation of ROS signalling [156].

Fungal lipases seem to play an important role in the establishment of their virulence [157]. It was reported that FFA analyses during wheat infection with the *F. graminearum* revealed that there was an enrichment in unsaturated FA, namely C18:1, C18:2 and C18:3 derived by the fungal secreted lipase FGL1 activity and that they could inhibit callose synthase [35]. It is likely that the FFA resulting from the fungal lipase activity have a plant lipid source. By promoting the inhibition of callose synthase, these pathogens induced FFA inhibit the deposition of callose, allowing the fungi to overcome this layer of

type II resistance. The growth of the fungus in the host implicates a challenge to lipid integrity due to the generation of ROS via mitochondrial activity [158]. The bacterial effector RipAL from *R. solanacearum* has lipase activity (containing a putative lipase domain that shared homology with the Arabidopsis PLA DAD1, which contributes to JA formation) and is among the type III effector proteins called Rips (*Ralstonia*-injected proteins) [159]. This effector induced the expression of marker genes for JA signalling in *N. benthamiana* and suppressed SA-mediated signalling [38]. Moreover, RipAL targets chloroplast lipids and causes chlorosis accompanied by the reduction of chlorophyll content when expressed in plant leaves [38]**.** Therefore, this effector might induce a disorder in chloroplasts by catalysing its lipids hydrolysis. RipAL contributes to the development of disease symptoms caused by *R. solanacearum* in pepper leaves through its putative lipase activity [38].

Fungal Phospholipases (PL) have also been shown to counteract oxidative damage by removing oxidized fatty acids from phospholipids in membranes [37]. Corn seedlings infected with the biotrophic fungi *Ustilago maydis* mutant with depletion on the PL lip2 gene exhibited a reduction in the severity of disease symptoms. It is possible that Lip2 plays a protective role against the oxidative stress encountered on host by removing detrimental oxidized polyunsaturated fatty acids from the cell membrane, mitigating the damage caused by plant ROS-triggered lipid peroxidation. It seems that Lip2 is important for supporting lipid homeostasis during *U. maydis* to proliferation in the host tissue. Given the increased susceptibility of the lip2 mutant to inhibitors of respiration, it is also likely that Lip2 supports mitochondrial function by influencing the integrity of the mitochondrial specific lipid cardiolipin, in the mitochondrial inner membrane [37].

Pathogenic fungi can form invasive hyphae, which are surrounded by an extra-invasive hyphal membrane (EIHM) (Figure 1.2). This structure is a plant-cell-derived membrane and continuous with the plant plasma membrane [160]. The fungi induces an enrichment of PI in the EIHM, which is crucial for the pathogen development [30]. During *C. higginsianum* infection in Arabidopsis, an enrichment of $PI(4,5)P_2$ in the EIHM occurs. Since the exocytic factor EXO84b also accumulated at the EIHM, but not endocytic factors, the enrichment of $PI(4,5)P_2$ may associated with an exocytic trafficking event rather than with endocytosis. The enrichment of $PI(4,5)P_2$ in the EIHM might reflect the general importance of this phosphoinositide moiety in rapid secretion [30]. The enrichment of $PI(4,5)P_2$ was also found in EIHM upon inoculation with *Colletotrichum orbiculare*, but not with *Golovinomyces orontii* or *Hyaloperonospora arabidopsidis*, which indicates a pathogen-specific strategy for the modulation of the phospholipid contents of the interfacial membrane to generate an environment conducive to the pathogen [30].

As discussed above, oxylipin production is a vital process in plant defence mechanisms. Nonetheless, oxylipin production showed also to be important for the development of some pathogens. In fact, in the interaction between maize and *F. Verticillioides*, fungal oxylipin production, including 9- and 13- Hydroxyoctadecadienoic acid (HODE) showed to be important during pathogenesis [12]. In another work focusing on the same pathosystem, different fungal oxylipins, namely 9S-DOX-AOS products showed to be pathogenicity promoters by inducing the expression of maize pathogenicity-promoting LOX3 (ZmLOX3) [161]. Moreover, 10-HOME and 7,10-DiHOME also showed do play an important role in the establishment of virulence of *P. aeruginosa* in lettuce [162]

### 1.5. Apoplast – an important compartment with still much left to unveil

One of the most important cellular compartments in the first moments of plant-pathogen interaction is the apoplast. This compartment includes the extracellular matrix and the APF [5]. It is involved in several functions during normal growth and under biotic and abiotic stress conditions, including pathogen interaction, pollutants, drought, salinity and temperature [10, 163, 164]. In plant-microbe interaction, upon pathogen secretion of molecular effectors to the apoplast that trigger the host immune system, a

metabolism modulation occurs [6]. It was already observed that stress conditions lead to the alteration of the protein composition of the apoplast both qualitatively and quantitatively [7, 9]. However, to this day, studies concerning the modulation of lipid in the apoplast during plant-pathogen interaction are very scarce, causing the picture of the role of lipids in the apoplast to be rather blurry [10]. Nonetheless, at a constitutive level, lipids were already identified in the grapevine leaf apoplast [165]. Moreover, different studies have been evidencing the importance of apoplastic lipids in plant-pathogen interactions. Lipids, extremely hydrophobic molecules, need to pass through the apoplastic compartment or the highly hydrophilic cell wall. This transference process is mediated by nsLTP. As already discussed above, these proteins are associated with diverse plant functions and may be upregulated in response to infection and exhibit antimicrobial activity [112, 113, 166]. Maldonado and collaborators identified a putative LTP protein in Arabidopsis and hypothesized that the protein may bind a lipid molecule and suggested that a plasma membrane receptor may also play a role in the LTP-mediated long distance signalling during SAR [167].

PLA, a vital protein family in plant lipid signalling, was also described to be translocated to the apoplast during pathogen infection. Translocation of the secretory $PLA_2\alpha$ to the apoplast was rapidly enhanced in response to inoculation of Arabidopsis leaves with *P. syringae* p*V.* tomato DC3000 carrying the effector avrRpm1. This result suggests that $PLA_2\alpha$ secretion to apoplast and lipid signalling upon bacterial infection may play a role in host defence responses, where host cells first encounter invading pathogens [168].

Another evidence of the importance of this compartment in lipid signalling during plant-pathogen interactions is that it was previously observed that, in response to JA, a modulation of phospholipids levels of the sunflower apoplast occurs. In this study, JA treatment resulted in significant changes in the phospholipid profile, showing the accumulation of PG and a decrease in PI [11]. Considering the role of phosphoinositides in plant signalling, the modulation of its levels by JA supports the participation of apoplastic phospholipids in intercellular communication events.

Within the apoplast, extracellular vesicles (EV) contribute to innate immunity and may mediate intercellular communication in plants as well as in animals. EV can be defined as spherical particles enclosed by a phospholipid bilayer that are released from cells into their environment and are composed of bioactive molecules, including RNAs, DNAs, proteins, and lipids [169]. To this day, there are only a few studies on the EV role in plant-pathogen interactions. However, EV are reported to be mobilized in response to pathogen infection and enriched in defence related proteins. The secretion of EV was observed to be enhanced during Arabidopsis infection with a virulent strain of the bacterial pathogen *P. syringae* and in response to SA treatments [170].

In the past years, a few evidences that EV may be important for lipid signalling arose. Furthermore, being EV lipid bilayer structures, their lipid composition is likely pivotal to their function.

Many fungal and oomycete pathogens enter plant cells by penetrating the host cell wall and differentiating specialized intracellular feeding structures, haustoria, by invagination of the plant plasma membrane [171]. As a result, the plant host may promote the formation of a cell wall thickening structure, the papillae and haustorial encasements in order to limit pathogen development [172] (Figure 1.2). In order to form these structures, the defined components, including the proteins syntaxin AtSYP121/PENETRATION1 (PEN1) and soluble N-ethylmaleimide-sensitive factor adaptor protein 33 (SNAP33), the ATP-binding cassette (ABC) transporter PEN3, callose and membrane lipids are transported through exosomes [173] (Figure 1.2). Meyer and co-workers observed that upon powdery mildew infection in Arabidopsis, not only integral membrane proteins such as PEN1 but also membrane lipids become incorporated into haustorial encasements. This phenomenon is not restricted to powdery mildew fungi, since membrane lipids were also detected in oomycete haustorial encasements [173] (Figure 1.2). Recently, Regente and collaborators found that some protein families are enriched in EV upon fungal inoculation, including lipase, acyl hydrolases and LTPs [174]. lipoxygenases were also found in the EV

of Turnip mosaic virus 1 infected *N. benthamiana* leaves [175]. The enrichment of these proteins in EV is indicative that these particles and lipids have an important role in the establishment of SAR.

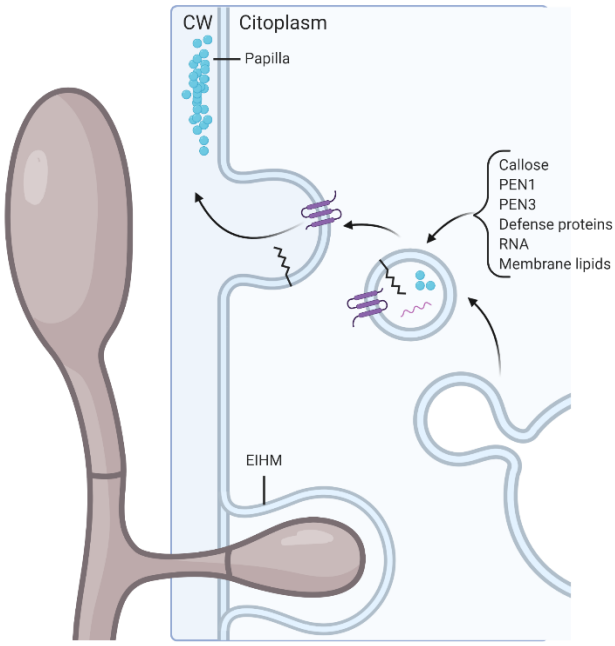


**Figure 1.2.** Extracellular vesicle secretion in the interaction between plant and fungal or oomycete pathogens. CW: cell wall; EIHM: extra invasive haustorial membrane; PEN1/3: syntaxin /PENETRATION1/3. Blue circles indicate callose

As lipid structures, it is possible that extracellular vesicles contain different lipids that confer membrane fluidity and can compress as they move through pores in the cell wall [175].
A thorough study of the apoplast, particularly concerning EV lipids may allow completely complete unveiling the role of this structure in plant-pathogen interactions, being a key element to uncover molecules that participate in intercellular communication and transport.

### 1.6. The case of grapevine

Grapevine (*Vitis spp*) is one of the most valuable crops worldwide due to its various uses, from table grape to wine production, with around 7.4mHa of estimated cultivated area in 2019. Wine industry plays a key role in several countries' economy, with revenue around 31.8 billion euros [176]. The domesticated *V. vinifera* is the only grapevine species used in intensive agriculture [177, 178]. This species is prone to several diseases. The most studied grapevine diseases are caused by viruses, bacteria and by fungi and oomycete [179], which represent the greatest threats to modern viticulture [180]. Among the most economically important diseases are downy and powdery mildews and grey mold, caused by *P. viticola* (Berk. and Curt.) Berl. and de Toni [181], *Erysiphe necator* (Schweinf.) Burrill [182] and *B. cinerea* [183], respectively. *P. viticola* and *E. necator* are obligatory biotrophs [180, 184]. While *P. viticola* invades the plant from the stomatal aperture [185], *E. necator* relies on the secretion of lytic enzymes such as lipases, esterases, and cutinases in order to promote wounds in the tissue and enter the plant [184]. *B. cinerea*, a necrotrophic pathogen, invades the host tissue by active penetration, involving the release of lytic enzymes or by passive ingress [186]. Being an opportunistic pathogen, it can initiate infection at the stomatal cavity, at wound sites, or at sites that have previously been infected by other pathogens [186].

Several works have highlighted the importance of lipid modulation events in the grapevine interaction with its pathogens. Ali and coworkers observed an accumulation of C18:3 in *V. vinifera* cv Regent, a tolerant cultivar to *P. viticola*, which could be due to the induction of the octadecanoid biosynthesis by the pathogen [187]. Recent results also showed that after pathogen challenge, only in the tolerant genotype a modulation of several lipid classes occurs. A decrease of the levels of saturated FA and increase on the levels of unsaturated FA occurred mainly of C18:3, which can be associated to the modulation of the galactolipids. Hence, the reported lipid modulation events may be associated to photosynthetic membranes protection, or lipid hydrolysis to obtain a free C18:3 fatty acid content for JA synthesis [13]. Moreover, the increase in the levels of unsaturated FA may also help to counteract the oxidative burst occurring in the resistant genotype after *P. viticola* challenge [188]. These observations suggest that the tolerant genotype triggers lipid-associated signalling [13]. Chitarrini and collaborators also observed lipid modulation events in *V. vinifera* cv 'Bianca' at 24 hpi of the levels of FA including C18:1, C18:2 and C18:3 [189]. These FA are essential as signalling molecules in the activation of defence related programmed cell death [190]. In the interaction between grapevine and *Botryosphaeria* dieback an alteration of the levels of lipids and FA also occur, mostly in the white area of the trunk [191]. Recently, in a study concerning grapevine defence to another trunk disease, Esca, lipid modulation events were also observed. The incompatible interaction caused a decline in free FA as well as AzA. Systemic changes in FA flux also occurred in distal organs of the grapevine, which is important for SAR establishment. The most obvious changes in lipid metabolism occurred on the galactolipids and JA, highlighting the importance of this molecule [192].

One of the most important processes in lipid signalling is FA desaturation. In grapevine, its importance in the disease defence mechanisms has been highlighted not only by the unsaturated FA modulation events that occur in incompatible interactions [13, 189, 192] but also by the modulation of the expression levels of the FAD genes. Recent works show that during grapevine incompatible interaction with *P. viticola*, there is a modulation of the expression levels of FAD, mainly *FAD8*, which catalyses the formation of C18:3 from C18:2 in the chloroplast, while expressive FA modulation in the galactolipids is also observed in the first moments of infection. These observations show the importance of FAD in the lipid modulation events in defence, including the formation of the JA precursor, C18:3 (unpublished data). Furthermore, the modulation of FAD expression (and, accordingly, FA levels) has been proven to follow a distinct pattern in the incompatible interaction between grapevine and pathogens with different lifestyles and invasion strategies: biotrophs (*P. viticola* and *E. necator*) and a necrotroph (*B. cinerea*). In all the interactions, a progressive desaturation of stearic acid (C18:0) to C18:3 occurred, which was observed for a longer period against *B. cinerea*. While the interaction with the biotrophs may trigger a higher synthesis of polyunsaturated FA at early time-points with a tendency to return to basal levels, the interaction with *B. cinerea* may trigger a later and more durable induction of polyunsaturated FA synthesis. In all interactions, membrane fluidity modulation also occurred, which may be crucial to maintain cellular function during infection [193].

Lipid oxidation pathways and their products, oxylipins, namely JA, have proven to play an important role in grapevine defence. In a study that compared the interaction between *P. viticola* and two *V. vinifera* cultivars (one tolerant, 'Regent' and one susceptible, 'Trincadeira'), lipid peroxidation increased in both but expression of key genes for JA synthesis and the accumulation of its precursor was higher in the tolerant genotype [188]. Prior to these observations, the first clues of the involvement of the JA and lipid associated signalling in the grapevine defence against *P. viticola* started to arise from studies from the same group [129, 130]. Altogether, these data suggest that the JA pathway is induced not only in the defence mechanisms against necrotrophic pathogens, but also against biotrophs like *P. viticola*. In a transcriptome analysis of the incompatible interaction between *V. amurenesis* and *P. viticola*, the JA signalling pathway was also shown to be upregulated [194]. Moreover, in the interaction

between grapevine and *Candidatus Phytoplasma solani* jasmonate was accumulated and it was suggested that this oxylipin pathway may have a role in the establishment of disease recovery [195]. The importance of JA signalling was also highlighted in the defence mechanisms against the esca complex [192].

Another evidence of the importance of lipid signalling in grapevine-pathogen interaction is the involvement of lipid associated proteins in the defence processes. A plastid lipid associated protein, fibrillin, presented higher levels in an incompatible interaction with *P. viticola* [188]. In the incompatible interaction between *V. amurenesis* and *P. viticola*, the LTP PR14 was shown to be upregulated [21]. Moreover, grapevine LTP1 is overexpressed in response to ergosterol or *Botrytis*-derived elicitor treatments [196]. This protein showed to have affinity to bind JA and might be involved in JA signal transduction [197].

Some of the grapevine pathogens inflict wounds in order to invade its tissues, as, for instance, *E. necator* [198]. Upon wounding, lipid modulation events also occur. Chitarrini and collaborators observed an accumulation of FA-specific compounds such as C18:3, C18:2, and C16:0, having the differences occurred mostly in free fatty acids, glycerolipids and glycerophospholipids. [199]

Besides the importance of FA and lipids as signalling molecules, they are genetically determined and evolutionarily conserved, being potentially useful as chemotaxonomic tools and biomarkers of susceptibility and tolerance/resistance to pathogens [200]. Recent FA and lipid profiling results from our group allowed to differentiate the tolerant from the susceptible grapevine genotypes and species to *P. viticola*. The observed differences reflect mainly on the membrane fluidity, which arises as the core differentiating feature between tolerant and susceptible *V. vinifera* cultivars and *Vitis* species. At the lipid level, the molecules that allow the separation between the grapevine genotypes at study were the galactolipids (co-related with susceptibility), as well as the signalling lipid PA and neutral lipids (co-related with tolerance) (unpublished data). It was previously observed that when comparing the content in saturated and unsaturated FA in *V. vinifera* cvs Regent and Trincadeira, that the unsaturated/saturated FA ratio is higher in 'Trincadeira' than in 'Regent', together with a high double bound index. Differences regarding the abundance of several lipid classes were also observed: 'Regent' presents lower content of both MGDG and DGDG and higher content of all other lipid classes (PG, PC, PE, PI, PA, FFA, triacylglycerol and other lipids) when compared to 'Trincadeira' [13].

Lipids and FA molecules are key players in the different processes of plant-pathogen interaction. Studying lipid metabolism and signalling both in plant and pathogen is arguably essential to completely uncover the knowledge brought up to light by proteomics and transcriptomics. The analysis of lipids and their derivatives enables the possibility of describing the crosstalk between plant and pathogen and the discovery of pathogen combined strategies targeting lipid pathways.

An increasing number of studies have described lipid modulation events that are important in defence and disease processes. Plant lipids play important roles from the first physical barrier against pathogens, the cutin, to signalling pathways that trigger different immune responses and defence related genes. Lipids were also shown to be candidate biomarkers of resistance or susceptibility to different pathogens. Furthermore, studies on the apoplast and EV have highlighting the possible role of lipids in the intercellular communication and the establishment of SAR during plant-pathogen interactions. From the pathogen perspective, it is evidenced that lipid molecules and lipid metabolism play a pivotal role in the pathogen's life cycle completion, triggering of recognition (ie bacterial LPS and LOS) and in evading the host immune system and potentiating infection.

The latest studies summarized here indicate that it is highly important to continue pointing the research direction towards the lipid signalling processes in plant-pathogen interaction to completely unveil the molecular mechanisms behind plant disease susceptibility and resistance. Concerning the apoplast, a deep knowledge of the lipid modulation events that take place in this compartment may allow unencrypting the first moments of pathogen perceiving and immune response. In what concerns the grapevine, a complete insight on the role of lipid molecules in grapevine resistance mechanisms may allow the understanding of the molecular machinery behind the processes of resistance/susceptibility to fungal pathogens and, ultimately, the definition of new disease control strategies.

### 1.7. Thesis outline

With this thesis, we aim to address the role of lipid molecules in the grapevine's defence mechanisms to fungal and oomycete associated diseases.

With the grapevine-*P. viticola* pathosystem, well studied in our group, as a starting point, we searched for lipid and FA molecules as biomarkers for disease tolerance or susceptibility. Also, knowing the importance of lipid and JA mediated signalling pathways in this interaction, the potential of JA to elicit defence-like lipid modulation events was evaluated in tolerant and susceptible grapevines. Furthermore, in order to understand whether the observed mechanism in the grapevine-*P. viticola* incompatible interaction is conserved in the interaction with pathogens with different lifestyles and/or invasion strategies, fatty acid modulation and its regulation by fatty acid desaturates (FAD) was also studied. Finally, we also aimed at studying the first battlefield of the grapevine-pathogen interaction, the apoplast.

In chapter II, we analysed the constitutive leaf lipid and FA composition of *Vitis* species and *V. vinifera* cultivars tolerant and susceptible to *P. viticola* as well as the gene expression of different FAD, responsible for the synthesis of unsaturated FA. Our results indicate that FA and lipids are suitable chemotaxonomic tools to predict grapevine susceptibility to *P. viticola*. Susceptible genotypes present higher relative amounts of plastidial lipids and unsaturated FA, displaying also higher expression levels of FAD genes, whereas tolerant genotypes have higher contents of neutral lipids. Different molecules arose as tolerance and susceptibility to *P. viticola* biomarker candidates, as the saturated FA, mainly in monogalactosyldiacylglycerol and phosphatidyl choline (tolerance biomarker) and the polyunsaturated linoleic acid as well as the plastidial lipids (susceptibility biomarkers). Moreover, the higher expression levels of FD4, FAD6 and FAD8 in susceptible genotypes suggest that they might also be considered as candidate biomarkers for susceptibility.

In Chapter III, we assessed the constitutive total leaf FA composition of tolerant and susceptible grapevine genotypes to *P. viticola* to search for biomarkers for tolerance and susceptibility. We also analysed the gene expression of FAD genes, responsible for the synthesis of unsaturated FA. Our results indicate that FA are suitable chemotaxonomic tools to study grapevine. Analysing the total FA content also revealed to be a less costly and less time-consuming alternative methodology in comparison to the analysis of the different lipid classes. Susceptible cultivars present a higher expression of FAD genes and higher unsaturated FA levels. Different molecules arose as candidate tolerance and susceptibility biomarkers to *P. viticola*. While palmitic acid may be a tolerance biomarker and potential resistance trait for breeding programs, α-linolenic acid may be a susceptibility biomarker.

In chapter IV, we addressed the question of whether the observed lipid-mediated defence mechanism against *P. viticola* is conserved in the interaction with *E. necator* (also a biotroph, with a different invasion strategy) and *B. cinerea* (necrotroph). We characterized FA modulation in *V. vinifera* cv Regent (a tolerant cultivar) during the first 24h of interaction with *P. viticola*, *E. necator* and *B. cinerea* and

correlated the FA modulation events with the expression profiles of genes encoding the FA desaturases FAD6 and FAD8. In all the interactions, a progressive desaturation of stearic acid to α-linolenic acid, precursor of JA, occurred, which was observed for a longer period against *B. cinerea*. Our results provide evidence of a distinct FA-meditated signalling pattern in grapevine interaction with biotrophs and necrotrophs. While the interaction with the biotrophs seems to trigger a higher synthesis of polyunsaturated FA at early time-points with a tendency to return to basal levels, the interaction with *B. cinerea* may trigger a later and more durable induction of polyunsaturated FA synthesis.

In chapter V, we addressed the potential of JA as an elicitor of defence-like signalling events in tolerant and susceptible grapevine genotypes. We have assessed the link between JA elicitation and both FA and immunity-related subtilase expression modulation. Our results show that FA modulation events after JA elicitation is similar to the described previously after *P. viticola* inoculation and that immunity-related subtilase expression also increases in the tolerant genotype, particularly the subtilase VviSBT5.3a, thus suggesting a shared mechanism.

In chapter VI grapevine leaf apoplast was isolated. This is a key element to completely unveil the molecular mechanisms in the grapevine-pathogen interaction. As grapevine is a woody plant, the extraction of its leaf apoplastic fluid is rather challenging. Therefore, there are very few studies on grapevine leaf apoplast: until today, two articles were published on its proteome and none on its metabolome. We described, for the first time, an optimized vacuum-infiltration-centrifugation method that allows a simultaneous extraction of grapevine apoplastic proteins and metabolites from leaves on a single sample, compatible with high-throughput mass spectrometry analyses. The extracted apoplast from two grapevine cultivars, *V. vinifera* cv Trincadeira and *V. vinifera* cv Regent, was directly used for proteomics and metabolomics analysis. The proteome was analysed by nanoLC-MS/MS and more than 700 common proteins were identified, with highly diverse biological functions. The metabolome profile through FT-ICR-MS allowed the identification of 514 unique putative compounds revealing a broad spectrum of molecular classes, with lipids as the most abundant class. This extraction method opens the way to study the lipid signalling mechanisms in the first moments of grapevine-pathogen interaction in the apoplast.

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# CHAPTER II

## 2. Lipid and fatty acid molecules as biomarkers of grapevine tolerance and susceptibility to *Plasmopara viticola*

Chapter II is based on the manuscript to be submitted to Plant Science
*Cavaco AR, Laureano G, Duarte B, Marques da Silva J, Gameiro C, Cunha J, Eiras-Dias J, Matos AR and Figueiredo A. Lipid and fatty acid molecules as biomarkers of grapevine tolerance and susceptibility to Plasmopara viticola*

## 2.1. Abstract

Grapevine (*Vitis vinifera* L) is one of the most important crops in the world but it is prone to several diseases. Downy mildew, caused by *Plasmopara viticola*, is among the most devastating ones. Disease control strategies include phytochemical applications every growing season, jeopardizing the sustainability of viticulture. Understanding the molecular processes behind resistance or susceptibility to *P. viticola* is vital to define alternative control strategies and select new disease resistance traits for breeding programs. The identification of molecular markers that allow discriminating tolerant and susceptible grapevine genotypes to *P. viticola* is an important step. Lipid and fatty acid (FA) molecules, previously shown to be important for grapevine defence responses to *P. viticola*, may be considered as chemotaxonomic tools. In this work, we made a preliminary assessement of the constitutive lipid and FA composition of two *Vitis* species and two *V. vinifera* cultivars tolerant and susceptible to *P. viticola* as well as characterized gene expression of fatty acid desaturases (FAD), responsible for the synthesis of unsaturated FA. Our results indicate that FA and lipids are suitable chemotaxonomic tools to predict grapevine susceptibility to *P. viticola*. Susceptible cultivars present higher relative amounts of plastidial lipids and unsaturated FA, displaying also higher expression levels of FAD genes, whereas tolerant genotypes have higher contents of neutral lipids. Different molecules arise as tolerance and susceptibility to *P. viticola* biomarkers candidates, as the saturated FA, mainly in monogalactosyldiacylglycerol and phosphatidyl choline (tolerance biomarker) and the polyunsaturated linoleic acid (C18:2) as well as the plastidial lipids (susceptibility biomarkers). Moreover, the higher expression levels of FD4, FAD6 and FAD8 in susceptible genotypes suggest that they might also be considered as candidate biomarkers for susceptibility.



**Keywords:** chemophenotyping; fatty acid desaturases; galactolipids; palmitic acid; linoleic acid, plastidial lipids, *Vitis vinifera*; membrane fluidity; molecular markers; *Plasmopara viticola* susceptible; tolerant


**Abbreviations:**

C16:0 – palmitic acid

C16:1*t* - trans-hexadecanoic acid

C18:0 – stearic acid

C18:1 – oleic acid

C18:2 – linoleic acid

C18:3 – α-linolenic acid

CAP – canonical analysis of principal coordinates

DBI – double bond index

DGDG – digalactosyldiacylglycerol

ER – endoplasmic reticulum

FA – fatty acids

FAD – fatty acid desaturase

FAME – fatty acid methyl esters

MGDG – monogalactosyldiacylglycerol

PA – phosphatidic acid

PC – phosphatidylcholine

PE – phosphatidylethanolamine

PG – phosphatidylglycerol

PI – phosphatidylinositol

PUFA – polyunsaturated fatty acids

SFA – saturated fatty acids

UFA – unsaturated fatty acids

## 2.2. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most valuable crops worldwide due to its various uses, from table grape to wine production. In 2019, it represented 7.4 mHa of the estimated cultivated area contributing to a wine industry revenue of around 31.8 billion euros [1]. The domesticated *V. vinifera* is the only grapevine species used in intensive agriculture [2, 3]. However, this species is prone to several oomycete and fungal-associated diseases, which represents one of the greatest threats to modern viticulture [4]. Among them, downy mildew, caused by the obligatory biotrophic oomycete *Plasmopara viticola* (Berk. & Curt.) Berl. & de Toni, is one of the most devastating diseases [4, 5]. As a control measure for this disease, several fungicide applications are necessary every growing season [6, 7], thus, viticulture is one of the agricultural activities which makes the most intensive use of plant protection products [8]. This harbours heavy environmental costs such as pollution, an increase in the number of resistant fungi strains and residual toxicity on products for human consumption, with a great number of the commercialized wines containing trace amounts of phytopharmaceutical products [9, 10]. The United Nations objectives for sustainable development for 2030 as well as a directive from the European Parliament (2009/128/CE) urge the reduction of pesticides usage in agriculture. This highlights the need to deepen our understanding of the molecular processes behind resistance or susceptibility of some *Vitis* species and *V. vinifera* genotypes to *P. viticola* in order to define new disease control strategies and to be able to select new disease resistance traits for breeding programs aiming at sustainable viticulture. Breeding for resistance is one of the most promising approaches to overcome pesticide over-usage. Several *P. viticola* partially resistant grapevine crossing hybrids are currently being commercialized e.g., 'Regent', 'Calardis Blanc', 'Solaris' (*Vitis* International Variety Catalogue, www.vivc.de). In these programs, the selection process is long and time-consuming, thus the establishment of tolerance/susceptibility biomarkers may aid to shorten this selection time leading to a more efficient breeding process. So far, genetic markers are available [11] allowing marker-assisted selection but metabolic markets have only recently been pointed out [12].

Membrane lipids are not only important structural molecules, but they also play key roles in plant defence, providing substrates for signalling molecules [13]. Recent works from our group highlighted the importance of lipids in the grapevine defence mechanisms against downy mildew. Distinct lipid modulation events in the first hours after inoculation of *V. vinifera* cv Trincadeira (susceptible) and *V. vinifera* cv Regent (tolerant) with *P. viticola* were reported. After pathogen challenge, modulation of the relative amounts of individual lipid classes as well as in their FA composition was observed only in the tolerant cultivar 'Regent' suggesting that this genotype may trigger lipid-associated signalling mechanisms [14]. The unsaturation degree of membrane lipids fatty acids directly affects membrane fluidity and permeability [15]. This feature is one of the factors that affect plant-pathogen interaction and the plant defence mechanisms [16].

Since FA are metabolites of the highly conserved acetyl-CoA pathway, these molecules are suitable to perform phenotyping studies. The FA and lipid profiles were previously used as a chemophenotyping tool for studies including the propensity of humans to develop coronary heart disease and ion compartmentation in cells of higher plants [17, 18]. Usually, phenotyping studies employ well-known techniques such as imaging, sensors [19] and polyacrylamide gel electrophoresis of protein extracts [20]. Lipid analysis is a straightforward approach and relatively less expensive than molecular methods and may therefore complement other methodologies and dissolve some uncertainties that arise from the results obtained with these approaches.

In the present work, we tested the potential of lipid class and FA profiling as a tool to discriminate between tolerant and susceptible *V. vinifera* cultivars and *Vitis* species to *P. viticola*. We have characterized the lipid and FA profiles of 4 grapevine genotypes: 2 tolerant (*V. riparia* and *V. vinifera* cv Regent) and 2 susceptible (*V. vinifera* cv Trincadeira and cv Pinot noir). We were able to discriminate between tolerant and susceptible grapevine genotypes through the levels of plastidial lipids, mainly monogalactosyldiacylglycerol (MGDG), phosphatidic acid (PA) and neutral lipids. Also, FA unsaturation degree was considered discriminating between tolerance and susceptibility. Furthermore, we analysed the expression of four FA desaturase coding genes: *FAD3.1*, *FAD4*, *FAD6* and *FAD8*, which corroborated our FA unsaturation results. Our results highlight new candidate biomarkers for grapevine tolerance and susceptibility to *P. viticola* and the potential use of lipid and FA and profiles as chemophenotyping tools.

## 2.4. Materials and Methods

### 2.4.1. Plant Material

Two tolerant (*V. riparia* and *V. vinifera* cv Regent) and two susceptible (*V. vinifera* cv Trincadeira and cv Pinot noir) grapevine genotypes to *P. viticola* were used in this study.

*Vitis riparia Michaux* cv Riparia Gloire de Montpellier (VIVC 4824), presents high tolerance to *P. viticola* (9 in the OIV descriptor 452); *V. vinifera* cv Regent (VIVC 4572), is a crossing hybrid with tolerance to *P. viticola* (OIV descriptor 452 – 7); *V. vinifera* cv Trincadeira (VIVC 15685) and cv Pinot noir (VIVC 9279) present high susceptibility to *P. viticola* (OIV descriptor 452 – 1/3). Also, field observed behaviour in different seasons at into the Portuguese Ampelographic Collection (Colecção Ampelográfica Nacional, CAN), the international reference for Portuguese *Vitis* germplasm (international code PRT051). CAN is property of INIAV-Estação Vitivinícola Nacional (Dois Portos), located at Quinta da Almoinha, 60 km north of Lisbon (9º 11′ 19″ W; 39º 02′ 31″ N; 75 m above sea level). CAN maintenance conditions are homogeneous modern alluvial soils (lowlands) as well as well-

drained soil; rootstock of a unique variety (Selection Oppenheim 4–SO4) was used for all genotypes including other *Vitis* species and other rootstocks represented in the field; each accession comes from one unique plant; temperate with dry and mild summer, climate. For plant material collection, the best possible health status was guaranteed for all genotypes was confirmed as plants were tested for the principal grapevine fungal/oomycetes diseases as well as grapevine viruses (healthy genotypes and synonym genotypes were planted in a continuous line for didactic proposes); same trailing system (bilateral cordon, Royat), canopy maintenance and agricultural management. Three leaves (3rd to 5th from the shoot apex) were harvested from 5 different plants per biological replicate and immediately frozen in liquid nitrogen. Five biological replicates were used in this study.

### 2.3.2 Lipid analysis

Liquid nitrogen-homogenized leaves were boiled in water for 5 min to inactivate lipolytic enzymes. The extraction of lipophilic compounds was performed using a mixture of chloroform/methanol/water (1:1:1, v/v/v), as previously described [14]. Separation of lipid classes was performed by thin-layer chromatography (TLC) on silica plates (G-60, Merck, VWR) using the following solvent system: Chloroform/methanol/acetone/acetic acid/water (100/20/40/20/8, v/v/v/v/v). Lipids bands were visualized with 0.01% primuline in 80% acetone (v/v) under UV light and scraped off. Fatty acid methyl esters (FAME) were prepared by trans-esterification in methanol:sulphuric acid solution (39:1 v/v) for 1h at $70^0$C. The reaction was stopped by cooling. The methyl esters were recovered by adding petroleum ether and ultrapure water (3:2, v/v), the organic phase was collected, at 37 °C under nitrogen atmosphere and resuspended in hexane. One µL of sample was injected for each analysis. FAME quantitative analysis was performed by gas chromatography (Varian 430-GC gas chromatograph) equipped with a hydrogen flame ionization detector using a fused silica 0.25 mm i.d.×50m capillary column (WCOT Fused Silica, CP-Sil 88 for FAME, Varian), according to [14]. The double bond index (DBI) was calculated as follows:

DBI = (% monodienoic acids) + 2 (% dienoic acids) + 3 (% trienoic acids)/100.

### 2.3.3. RNA extraction and cDNA synthesis

Total RNA was isolated from the *V. vinifera* cultivars and *Vitis* species ground leaves using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. On-Column DNase I Digestion (Sigma-Aldrich, USA) was used to hydrolyse residual genomic DNA, as described by the manufacturer. RNA purity and concentration were determined using a NanoDrop-1000 spectrophotometer (Thermo Scientific). The presence of gDNA in RNA samples was assessed by qPCR analysis [21] on crude RNA samples by targeting the Elongation Factor 1-alpha (EF1α) gene. Complementary DNA (cDNA) was synthesized from 2.5 µg of extracted RNA using RevertAid®H Minus Reverse Transcriptase (Fermentas, Ontario, Canada) anchored with Oligo(dT)23 primer (Sigma-Aldrich, USA), as described in [12].

### 2.3.4. Quantitative Real Time PCR (qPCR)

qPCR experiments were performed in a StepOne™ Real-Time PCR system (Applied Biosystems, Sourceforge, USA) using the Maxima™ SYBR Green qPCR Master Mix (2×) kit (Bio-Rad, USA), following manufacturer's instructions. Each reaction contained 2.5 mM MgCl2 and 2 µM of each primer

were used in 10 µL volume reactions, with 1 µL of cDNA (diluted 1:10) as a template. A control without cDNA template was included in each set of reactions. Primer sequences and reaction details are provided in Table 2.1. For all genes, thermal cycling started with a 95 °C denaturation step for 10 minutes followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing at gene-specific temperature for 30 seconds. Three biological replicates and two technical replicates were used for each sample. Elongation Factor 1-alpha (EF1α) and Ubiquitin-conjugating enzyme (UBQ) coding genes were used for expression data normalization as previously described [22]. For each gene, standard curve efficiency was calculated according to [23]. The quantification cycle (Cq) values of the genes of interest in the *Vitis* species and *V. vinifera* cultivars at study were extracted and normalized by the geometric mean of the Cq of *UBQ* and *EF1α*, as described in [12]

**Table 2.1.** Target and reference gene oligonucleotide sequences and reaction details

| Identifier/NCBI Assession Number | Primer sequence (5´-3´) | Amplicon length (bp) | Amplification Efficiency (E) | Ta (ºC) | Tm (ºC) |
|---|---|---|---|---|---|
| Reference genes | | | | | |
| *EF1α* | F: AACCAAAATATCCGGAGTAAAAGA | 150 | 1.99 | 60 | 80.66 |
| XM_002284888.2 | R: GAACTGGGTGCTTGATAGGC | | | | |
| *UBQ* | F: GTGGTATTATTGAGCCATCCTT | 182 | 2.08 | 60 | 81.4 |
| XM_002284161.3 | R: AACCTCCAATCCAGTCATCTAC | | | | |
| Target genes | | | | | |
| *FAD3.1* | F: ATAGAAGCCCAGGGAAGAAG | 135 | 1.94 | 60 | 90.19 |
| XM_002277537.4 | R: CAAAGGATACGCAAACAAGCA | | | | |
| *FAD4* | F: TGTTCAGCCAGCAGTTCCAT | 97 | 1.98 | 60 | 83.48 |
| XM_002280947.4 | R: CTCGACACTAGCAGTCCAG | | | | |
| *FAD6* | F: CATGGTTGGGTTATCACTTCT | 147 | 1.94 | 60 | 79.02 |
| XM_003634815.2P48 | R: CTATCCAACGAGGGTAATCAC | | | | |
| *FAD8* | F: GGCACTTTCCCTCCTCCTT | 151 | 1.92 | 58 | 79.77 |
| XM_002264314.4 | R: GGGCCTTATGCCACATTCT | | | | |

### 2.3.5. Statistical analysis

To evaluate the differences in the lipid and fatty acid profile of the tolerant and susceptible genotypes, a multivariate approach was applied. Canonical analysis of principal coordinates (CAP), using Euclidean distances, was used to visualize differences in multivariate space regarding lipid and fatty acid relative composition as described in [24] Multivariate statistical analyses were conducted in Primer 6 software [25]. Significance of the differences between groups concerning the lipid and FA levels as well as the gene expression was assessed using IBM® SPSS® Statistics software (version 26.0; SPSS Inc., USA) with a Wilcox-Mann–Whitney's U test when comparing two groups (tolerant and susceptible) and with a Kruskal-Wallis test when comparing 4 groups (*V. riparia*, *V. vinifera* cv Regent,

*V. vinifera* cv Trincadeira and *V. vinifera* cv Pinot noir). After Kruskal-Wallis test, p-values were adjusted with the Bonferroni method as described in [24]. Results yielding a p value < 0.05 were considered statistically significant.

## 2.4. Results

### 2.4.1. The content in plastidial lipids, neutral lipids and phosphatidic acid and the FA composition of the different lipid classes allow the discrimination between susceptible and tolerant genotypes

An approach assessing the lipid composition of two susceptible (*V. vinifera* cv Trincadeira and *V. vinifera* cv Pinot noir) and two tolerant (*V. riparia* and *V. vinifera* cv Regent) grapevine genotypes to *P. viticola* was conducted. Our results indicate that some molecules allow the discrimination between tolerant and susceptible genotypes to *P. viticola*, namely the galactolipid MGDG that shows much higher levels in the susceptible cultivars and digalactosyldiacylglycerol (DGDG), that presents the same tendency. When grouped plastidial lipids are also more abundant in susceptible cultivars, whereas the tolerant genotypes have increased amounts of extraplastidial lipids (Figure 2.1D). In the tolerant genotypes, higher levels of neutral lipids may be highlighted, being the most significant difference observed between *V. riparia* and *V. vinifera* cv Pinot noir (Figure 2.1A). In addition, the amounts of PA, a key lipid biosynthetic precursor as well as an important signalling molecule, are also higher in tolerant genotypes (Figure 2.1A). Another difference found between genotypes is the MGDG/DGDG ratio, higher in susceptible plants.

Furthermore, the CAP analysis (91.7 % classification efficiency) confirms that galactolipids, mainly MGDG, contribute to the grouping of the susceptible variants (Figure 2.1B). In addition, this analysis also highlighted *V. vinifera* cv Regent, which stands out for its higher content on phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylcholine (PC). It is noteworthy that this tolerant *V. vinifera* cultivar forms its group in the centre of CAP second axis, between *V. riparia* (tolerant to *P. viticola*) and the susceptible genotypes, also *V. vinifera* cultivars (Figure 2.1B).

Besides the observed separation concerning the lipid composition, different lipid classes showed differences in their FA compositions between susceptible and tolerant genotypes, namely MGDG, PG, PC, phosphatidylethanolamine (PE), and neutral lipids (Figure 2.2-2.6).

The FA composition of MGDG allows the grouping of the susceptible *V. vinifera* cultivars together, while *V. riparia* and *V. vinifera* cv Regent can be highlighted on their own (Fig. 2). *V. riparia* presents a higher content of α-linoleic acid (C18:3) and 'Regent' is highlighted by its content of the remaining FA (Fig. 2). Nonetheless, in the C18:2 content, this cultivar is closer to the susceptible genotypes (Fig. 2). Due to its higher content of C18:3, *V. riparia* presents a higher DBI. This species also presents a higher oleic acid (C18:1)/stearic acid (C18:0) ratio, which tends to be higher in the tolerant genotypes at study (Fig. 2C). The CAP analysis (91.7 % classification efficiency) also highlights the fact that *V. vinifera* cv Regent separates from the susceptible cultivars considering the CAP1 but not considering CAP2 (Fig. 2B).

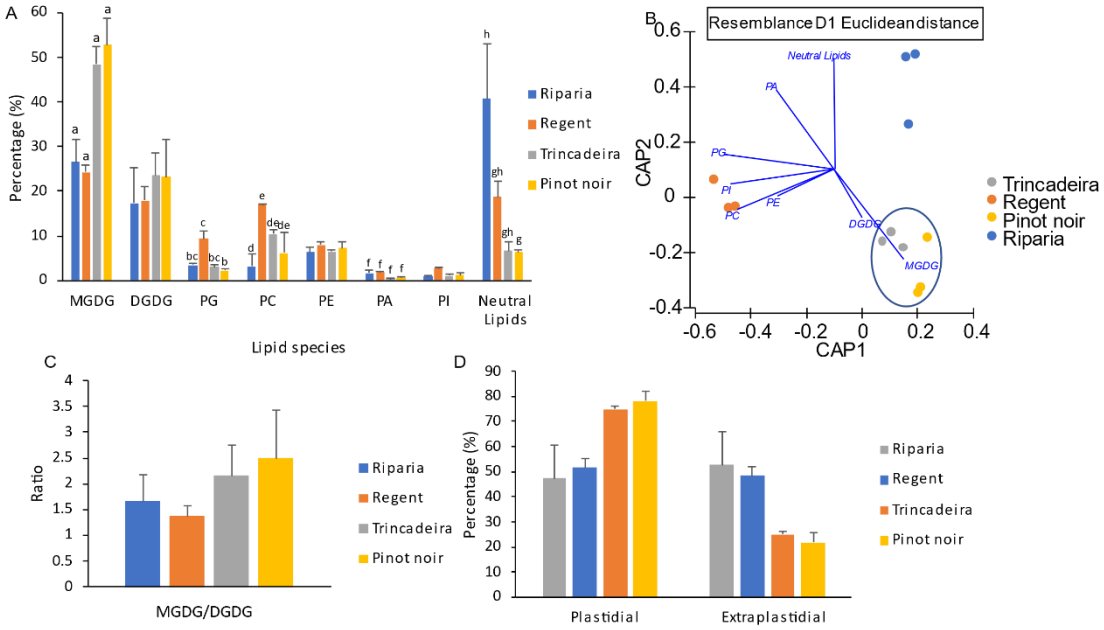


**Figure 2.1.** Constitutive leaf lipid profiles of *V.* riparia, *V. vinifera* cv Regent, *V. vinifera* cv Trincadeira and *V. vinifera* cv Pinot noir (A) Lipid classes composition; (B) CAP analysis plot based in the Euclidean distances between samples considering the relative amount of each lipid class; (C) MGDG/DGDG ratio; (D) relative amounts of plastidial and extraplastidial lipids. The grey circle highlights groups. Average ± standard deviation, N = 3, different letters indicate significant differences at $p < 0.05$.

Taking into account the FA composition of PG (Figure 2.3), the four genotypes under study present different distributions of the levels of C18:2 but not in pairwise comparisons (Figure 2.3A). Furthermore, the tolerant genotypes show a tendency to present higher levels of C18:1 (Figure 2.3A). Concerning the FA degree of unsaturation, the genotypes at study present differences in the levels of polyunsaturated FA (Figure 2.3B), with a highlight to *V. riparia* which shows the higher levels, being the most significant difference observed between this variety and 'Regent' (Figure 2.3B)

Regarding FA composition of PC (Figure 2.4) different distributions of the levels of C18:1 and C18:3 were observed, although a distinct pattern between tolerant and susceptible genotypes was not detected. Nonetheless, it was possible to observe a tendency for higher levels of saturated FA, palmitic acid (C16:0) and C18:0, in the tolerant genotypes and C18:2 in the susceptible (Figure 2.4A). CAP analysis (58.3 % classification efficiency) allowed to highlight 'Trincadeira' (100% classification efficiency for this group) due to its higher levels of C18:2 and 'Regent' for its levels of C18:1 (Figure 2.4B). The overall classification of the CAP analysis is lowered by mostly by a misclassification of Pinot noir and *V. riparia* samples taking into account their PC fatty acid profile.

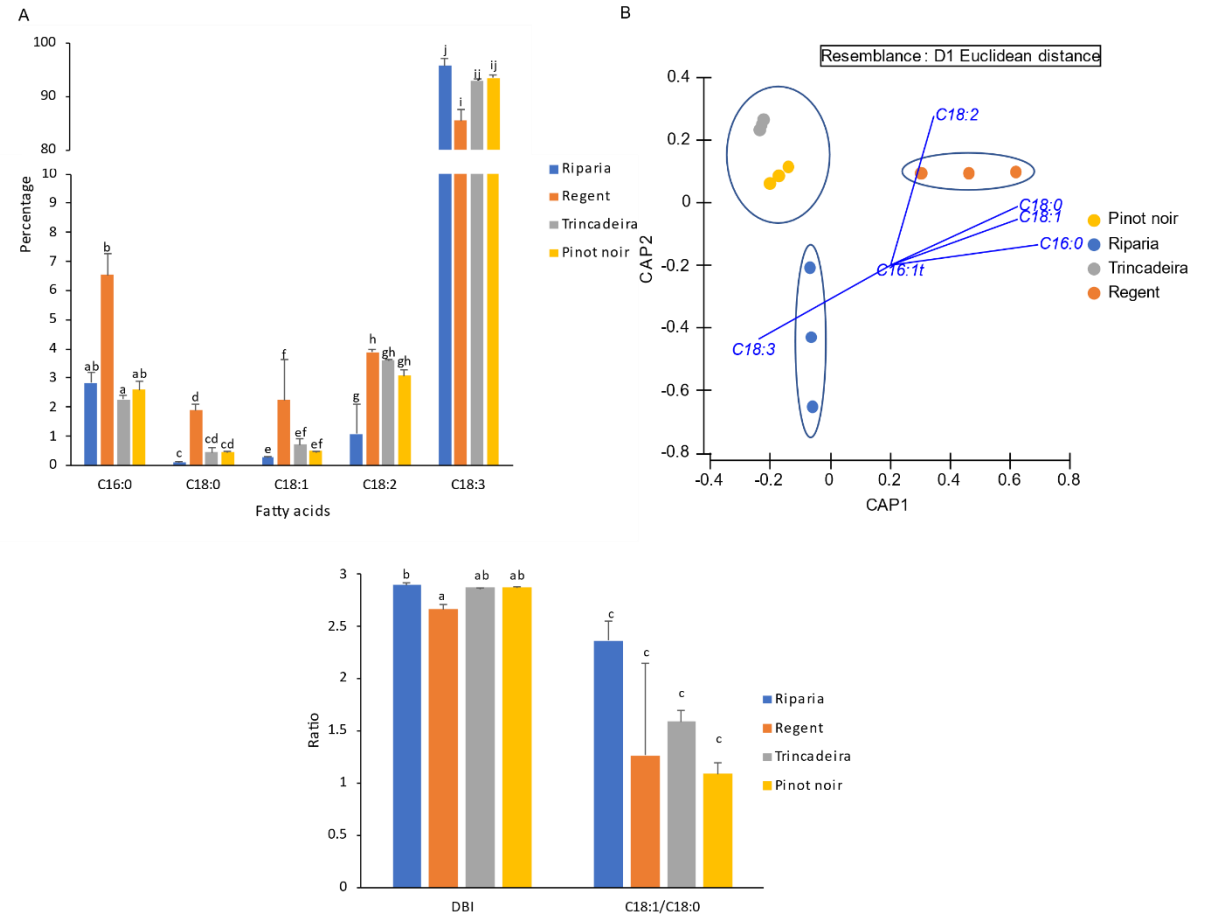


**Figure 2.2.** Constitutive fatty acid profile of leaf monogalactosyldiacylglycerol of *V.* riparia, *V. vinifera* cv Regent, *V. vinifera* cv Trincadeira and *V. vinifera* cv Pinot noir (A) FA percentage; (B) CAP analysis plot based in the Euclidean distances between samples considering the FA percentage; (C) Double bond Index (DBI) and C18:1/C18:0. The grey circle highlights groups. Average ± standard deviation, N = 3, different letters indicate significant differences at $p < 0.05$.

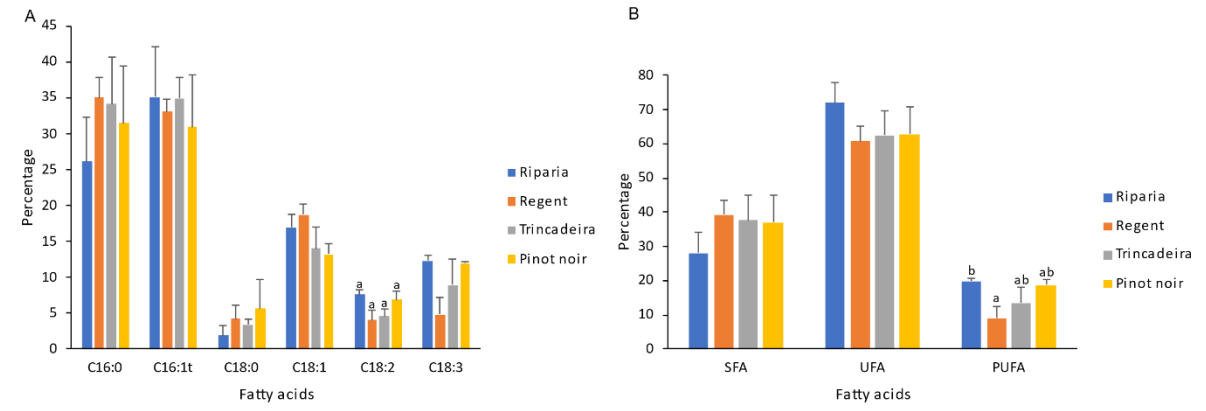


**Figure 2.3.** Constitutive Fatty acid profile of leaf Phosphaditylglycerol of *V.* riparia, *V. vinifera* cv Regent, *V. vinifera* cv Trincadeira and *V. vinifera* cv Pinot noir (A) FA percentage and (B) saturated, unsaturated and polyunsaturated FA (SFA, UFA and PUFA). Average ± standard deviation, N = 3, different letters indicate significant differences at $p < 0.05$.

The four genotypes also present a significantly different distribution of the C18:2/C18:1 ratio, with a highlight on 'Trincadeira'. The susceptible genotypes show a tendency for a predominance of UFA and PUFA (in relation to the SFA contents), as well as a higher DBI (Figure 2.4C).

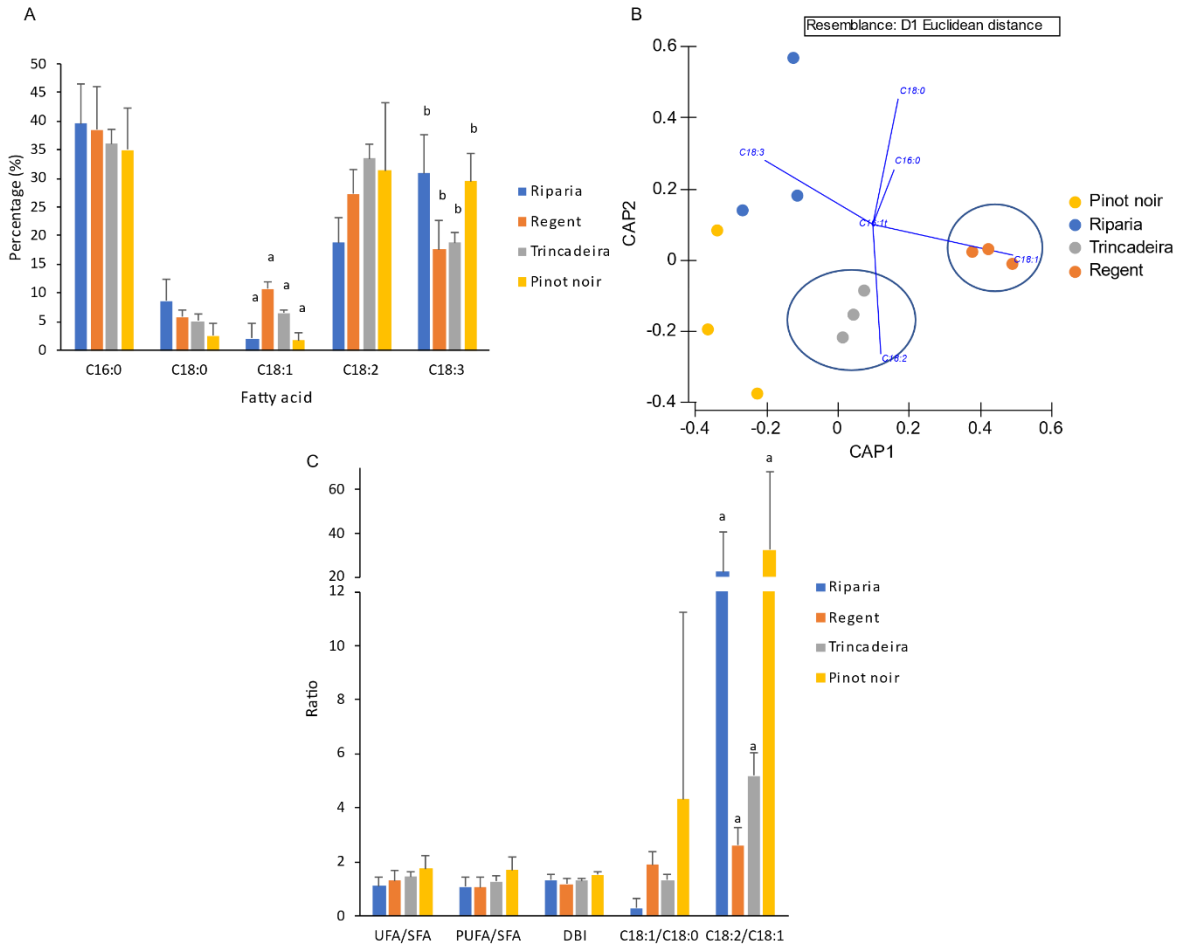


**Figure 2.4.** Constitutive fatty acid profile of leaf Phosphaditylcholine of *V.* riparia, *V. vinifera* cv Regent, *V. vinifera* cv Trincadeira and *V. vinifera* cv Pinot noir (A) FA percentage; (B) CAP analysis plot based in the Euclidean distances between samples considering the FA percentage; (C) Double bond index (DBI), unsaturated to saturated FA (UFA/SFA), polyunsaturated to saturated FA (PUFA/SFA), C18:1/C18:0 and C18:2/C18:1 ratios. The grey circle highlights groups. Average ± standard deviation, N = 3, different letters indicate significant differences at $p < 0.05$.

Concerning the FA composition of PE, significant differences of the C18:3 content were observed, with emphasis on *V. riparia*, whose separation from the other genotypes is explained by the highest content on this FA (Figure 2.5A and B). *V. vinifera* cv Trincadeira and Pinot noir show a tendency for higher levels of C18:2 than the tolerant assessments. The CAP analysis (66.7% classification efficiency) also showed that it is possible to separate the susceptible from the tolerant genotypes based on their C18:2 content in PE (Figure 2.5A and B). The genotypes at study also showed different DBI values, despite no significant differences in the pairwise comparisons were found, (Figure 2.5C) and this feature follows a similar tendency to the C18:3 levels.

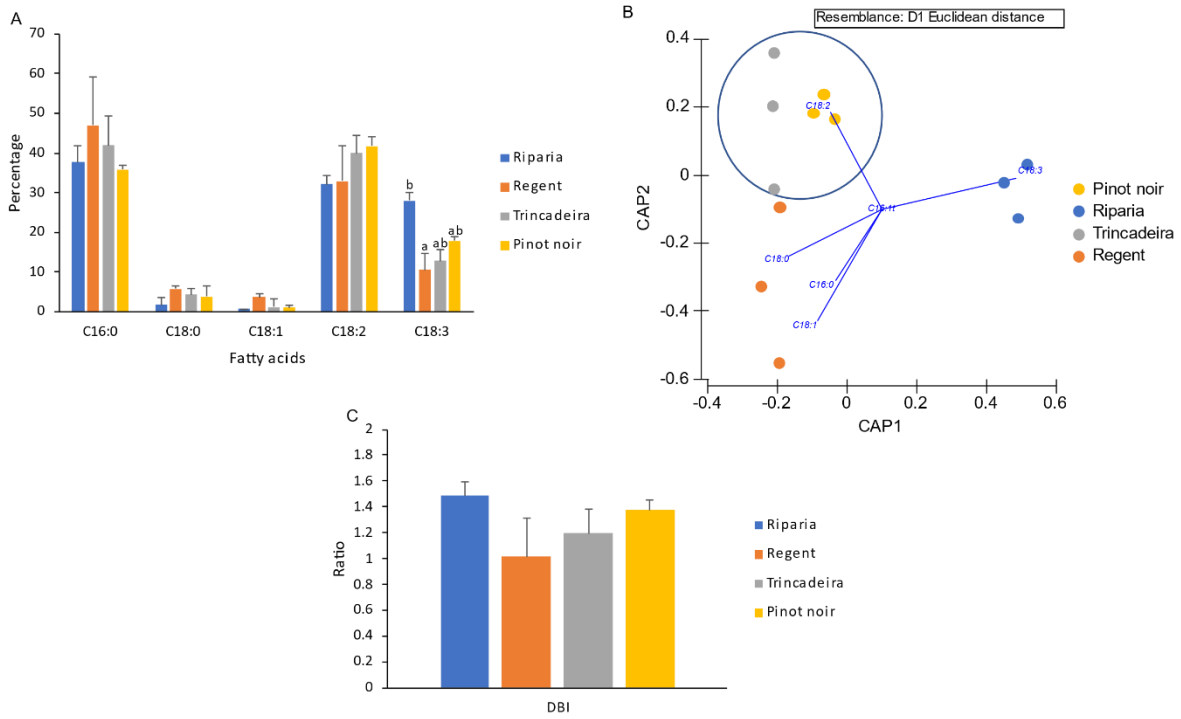


**Figure 2.5.** Constitutive fatty acid profile of leaf Phosphaditylethanolamine of *V.* riparia, *V. vinifera* cv Regent, *V. vinifera* cv Trincadeira and *V. vinifera* cv Pinot noir (A) FA percentage and (B) CAP analysis plot based in the Euclidean distances between samples considering the FA percentage; (C) Double bond index (DBI). The grey circle highlights groups. Average ± standard deviation, N = 3, different letters indicate significant differences at $p < 0.05$.

Concerning the FA composition of neutral lipids, which include diacylglycerols (DAG), triacylglycerols (TAG), and free FA (FFA) and wax esters [26], different distributions were observed mainly in the levels of C16:0, C18:0 and C18:3 (Figure 2.6). Taking into account the SFA, 'Regent' and 'Trincadeira' cultivars present higher levels of these FA in this lipid class (Figure 2.6A, B). Concerning the PUFA, mainly C18:3, *V. riparia* presents the higher content. The four genotypes at study showed different distributions of the levels of SFA, UFA and PUFA (Figure 2.6B) and DBI follows a similar tendency to UFA (Figure 2.6C). Moreover, different distributions of the C18:3/C18:2 were also observed, following the same tendency as DBI, which highlights mainly the differences between *V. riparia* and the remaining genotypes (Fig. 6D).

The FA compositions of DGDG, PA and PI did not show distinguishable patterns between tolerant and susceptible grapevine genotypes to *P. viticola.*

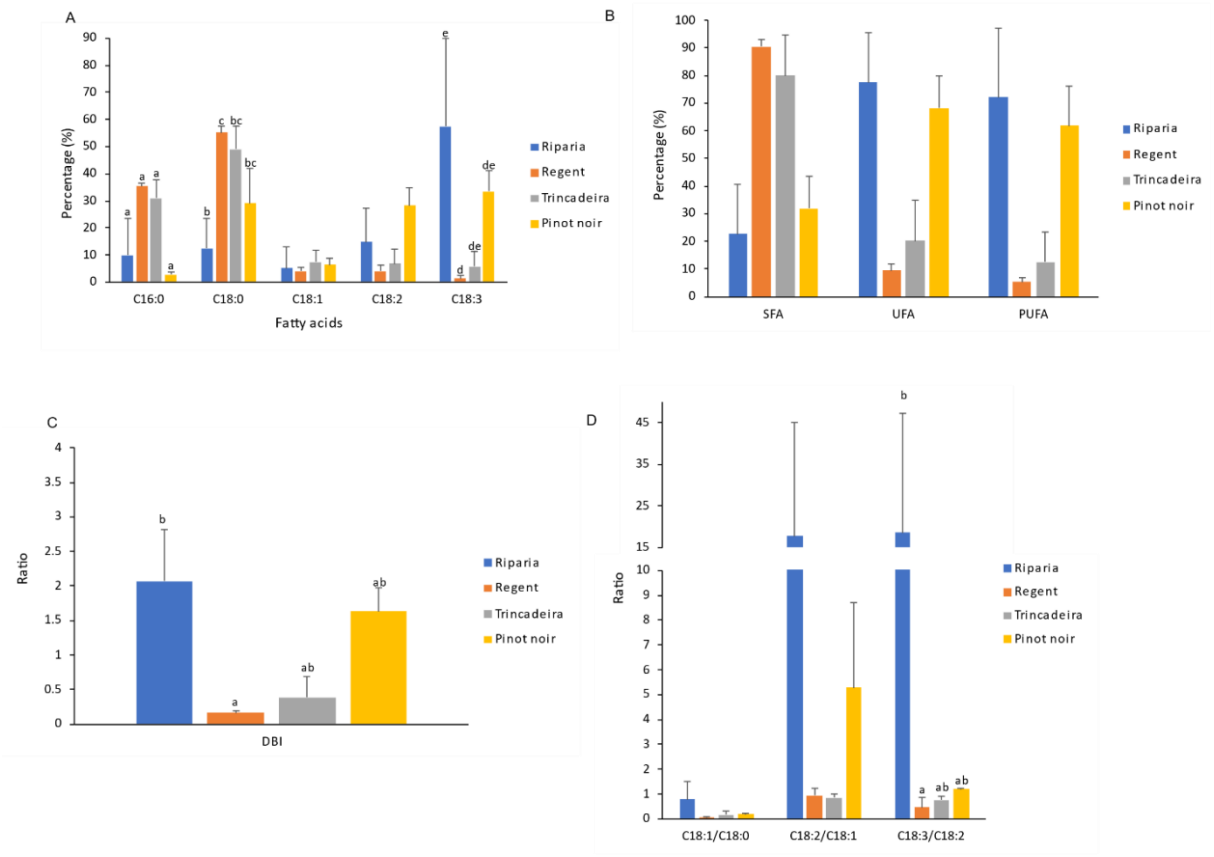


**Figure 2.6.** Constitutive Fatty acid profile of leaf Neutral lipids of *V.* riparia, *V. vinifera* cv Regent, *V. vinifera* cv Trincadeira and *V. vinifera* cv Pinot noir (A) FA percentage; (B) saturated, unsaturated and polyunsaturated FA (SFA, UFA and PUFA); (C) Double bond index (DBI); C18:1/C18:0, C18:2/C18:1 and C18:3/C18:2 ratios. Average ± standard deviation, N = 3, different letters indicate significant differences at $p < 0.05$.

When analyzing the genotypes at study as two populations (tolerant and susceptible), different lipid classes and the FA composition of some of these classes allowed to distinguish between tolerance and susceptibility (Figure 2.7). Susceptible genotypes presented higher abundances of plastidial lipids (although the differences concerning individual contents of MGDG and DGDG were not statistically significant, they also presented a tendency to be higher in the susceptible cultivars (Figure A.1)), while the tolerant genotypes presented higher levels of PG and extraplastidial lipids including PA and mostly neutral lipids (Figure 2.7). Regarding the FA which showed significant differences, they were found in the plastidial lipids MGDG and PG, and in the extraplastidial PC and PE (Figure 2.7B). Tolerant genotypes showed higher levels of C16:0 in MGDG, C18:1 in PG and C18:0 in PC. On the other hand, the susceptible genotypes showed higher levels of C18:2 in PC and PE (Figure 2.7B).

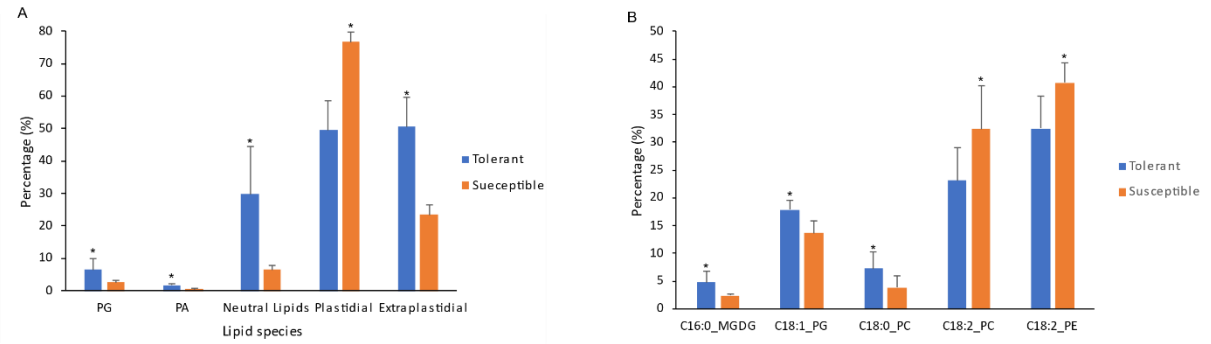


**Figure 2.7.** Constitutive Leaf lipid and fatty acids composition of grapevine genotypes tolerant (*V.* riparia and *V. vinifera* cv Regent) and susceptible (*V. vinifera* cv Trincadeira and *V. vinifera* cv Pinot noir) to *P. viticola* (A) lipid classes composition; (B) FA percentage. Average ± standard deviation, N = 6. Asterisk indicates significant differences at $p < 0.05$

In sum, the relative amounts of the different lipid classes contribute to the separation between susceptible and tolerant grapevine genotypes to *P. viticola*, with emphasis on the plastidial MGDG and neutral lipids (Figure 2.1Figure 2.7). The FA composition of MGDG also contribute for this separation, as well as the relative amounts of C18:2 and C18:3 of PC and PE as well as C18:1 of PC (Figure 2.4Figure 2.5). Regarding the analysis of the genotypes at study as two populations, saturated FA, namely C16:0 of MGDG and C18:0 of PC, also allowed the separation between tolerance and susceptibility (Figure 2.7B).

### 2.4.2. Susceptible grapevine cultivars present higher expression of plastidial FAD genes

The FA desaturation of membrane lipids is catalysed by membrane-bound FAD enzymes present in the chloroplast or the endoplasmic reticulum (ER) [27]. The activity of these enzymes affects the FA composition of membrane and storage lipids. Taking into account the results of lipid analyses, indicating a general trend for a higher degree of FA unsaturation in susceptible genotypes, we hypothesised that the constitutive expression of specific FAD genes could also allow the separation between tolerant and susceptible grapevine genotypes.

Therefore, we analysed the expression of *FAD3.1*, responsible for the synthesis of C18:3 from C18:2 in the ER [28] and impacting the FA composition of extraplastidial phospholipids (PC, PE, PI and PA) and neutral lipids, *FAD4*, that catalyses the desaturation of C16:0 into trans-hexadecaenoic acid (C16:1*t*) in the plastidial phospholipid PG [29], *FAD6* and *FAD8* responsible for the formation of C18:2 and C18:3 in plastidial lipids (MGDG, DGDG and PG), respectively [27].

When comparing the FAD genes expression in the four grapevine genotypes, the genes encoding plastidial enzymes tended to present higher expression in the susceptible *V. vinifera* cultivars, although not statistically significant (Figure 2.8). Nonetheless, when the samples were analysed as two populations, *FAD8* showed a significantly higher expression in the susceptible cultivars (Figure 2.8). On the other hand, no distinguishable pattern was observed concerning *FAD3.1* expression (Figure 2.8).

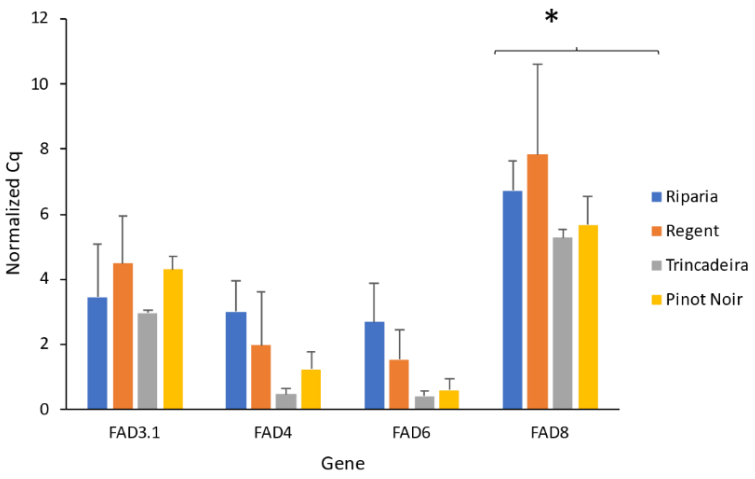


**Figure 2.8.** Constitutive leaf expression of Fatty acid desaturase (FAD) genes. Normalized quantification cycles (Cq) values for FAD genes – FAD3.1, FAD 4, FAD6 and FAD8 in leaves of *V.* riparia, *V. vinifera* cv Regent, *V. vinifera* cv Trincadeira and *V. vinifera* cV. Cq values were normalized by the geometric mean of the Cq of UBQ and EF1α. Average ± standard deviation, N = 3. Asterisk indicates significant differences regarding the comparison of the genotypes at study as two populations, tolerant (*V.* riparia and *V. vinifera* cv Regent) and susceptible (*V. vinifera* cvs Trincadeira and Pinot noir) using the Mann-Whitney test at $p < 0.05$

## 2.5. Discussion

The FA composition has been recently used as a chemophenotyping tool for studies including the propensity of humans to develop coronary heart disease and ion compartmentation in cells of higher plants [17, 18].

Our results show that the lipid and FA profiles, as well as the expression levels of FAD coding genes, may be potential chemophenotyping tools for grapevine genotypes. Moreover, some molecules can be highlighted as candidate biomolecules for tolerance and susceptibility to *P. viticola*.

The relative abundance of the different lipid classes, as well as their FA composition, allowed the separation between the grapevine genotypes at study. Plastidial lipids, with emphasis on the galactolipid MGDG, showed higher levels in the susceptible cultivars to *P. viticola*. Moreover, the MDGD/DGDG ratio also showed a tendency to be higher in the susceptible cultivars (Figure 2.1). Other lipid classes showed higher levels in the tolerant genotypes, including PA and neutral lipids (Figure 2.1Figure 2.7). Neutral lipids include wax esters, the storage lipid TAG, its precursor DAG as well as FFA [26, 30]. The higher neutral lipid levels in the tolerant genotypes, with an emphasis on *V. riparia* might be an important candidate biomarker, since the storage lipids, which normally accumulate in leaves under stress conditions (Gigon et al., 2004) may be used as transient storage of FA, which will be used to form galactolipids once the stress is alleviated [31]. Previous results from our group showed that the increase in galactolipids contents during the incompatible interaction (in tolerant genotypes) between grapevine and *P. viticola* may be an important feature to maintain the normal cell function during pathogen attack [14]. Furthermore, neutral lipids are components of the leaf surface wax, which is part of the first barrier against pathogens [32]. FFA are also important signaling molecules in stress conditions [33]. This reinforces the hypothesis that the higher levels of neutral lipids in tolerant genotypes may represent important candidate biomarkers for tolerance to *P. viticola.*

Besides being an intermediate in lipid synthesis PA is also an important signaling lipid, namely under stress conditions, such as biotic stress [34]. The higher levels of this lipid in the tolerant genotypes may be an important feature for a prompter defence signaling cascade triggering upon the challenge by a

pathogen. Furthermore, this lipid is the starting point for the formation of other more complex phospholipids, such as PC, PE and PI which also have important roles in defense signaling [34, 35]. Therefore, higher PA levels may also be an important feature and a candidate biomarker for tolerance.

The levels of C16:0 showed a tendency to be higher in the tolerant grapevine genotypes in PC as well as in MGDG. The C16:0 is a major FA involved in the control of membrane permeability. It was previously proposed as a biomarker resistance of the Bulgarian *V. vinifera* cv Storgozia to *P. viticola* [36]. Moreover, another SFA, C18:0, also showed a tendency to have higher levels in the same lipid classes. On the other hand, the susceptible *V. vinifera* cultivars showed a tendency to present higher levels of C18:2 in PC and PE, which arise as a candidate biomarker for grapevine susceptibility to *P. viticola*. Taking into account that the FA degree of unsaturation is a key factor for the regulation of membrane fluidity [37], this feature may arise as an important differentiating trait between tolerant genotypes, which seem to have less fluid membranes, and the susceptible genotypes which may present a higher constitutive membrane fluidity. A higher membrane rigidity might be an important feature to hinder the pathogen infiltration in the grapevine leaves.

Interestingly, the obtained results allowed the highlighting of *V. vinifera* cv Regent in the FA composition of some lipid species. The CAP analysis positioned 'Regent' closer to the susceptible *V. vinifera* cultivars Trincadeira and Pinot noir or between them and *V. riparia*. These observations may be due to the fact that despite being a tolerant cultivar 'Regent', is a crossing hybrid with the last crossing being made between *V. vinifera* (*V. vinifera* c*V.* Diana X c*V.* Chambourcin). This common genetic background may be associated with the fact that *V. vinifera* cv Regent stands out regarding the FA composition of several lipid classes, between the susceptible cultivars and the tolerant *V. riparia*. Hence, it is also noteworthy that the obtained results allowed to highlight differentiating features between the two tolerant cultivars, which belong to two different species.

The results obtained from the qPCR gene expression analysis of FAD genes agrees in general with the results of lipid analyses, since a higher expression of plastidial desaturases (FAD4, Fad6 and FAD8) was observed in susceptible cultivars in agreement with their higher content of plastidial lipids and a higher degree of unsaturated FA. In contrast, no significant differences were observed for the FAD gene encoding the ER enzyme (FAD3) (Figure 2.8). *FAD3.1* catalyses the formation of C18:3 in the ER [28], *FAD4*, *FAD6* and *FAD8* are responsible for the formation of C16:1*t*, C18:2 and C18:3 in the chloroplast, respectively [27, 29]. Calvo and collaborators had observed that plant derived PUFA function as sporogenic factors for fungi of the genus *Aspergillus* [38], which corroborate the hypothesis that higher basal levels of PUFA may be a characteristic of pathogen susceptible genotypes. The observed tendency for higher expression levels of *FAD4*, *FAD6* and *FAD8* in the susceptible cultivars could constitute candidate biomarkers for susceptibility to *P. viticola*.

Taken together, our results corroborate the hypothesis that the FA and lipid composition is suitable to be used as a chemophenityping tool. Different molecules arise from this study as candidate constitutive biomarkers for tolerance and susceptibility to *P. viticola*. Higher levels of PA and neutral lipids as well as saturated FA, mainly in MGDG and PC may be biomarkers for tolerance and a potential resistance trait to be used in breeding programs. On the other hand, MGDG and higher UFA levels in membrane extraplastidial lipid classes may be highlighted as biomarkers for susceptibility. Moreover, higher expression levels of *FD4*, *FAD6* and *FAD8* might be considered as candidate biomarkers for susceptibility.

## 2.6. Acknowledgements

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## 2.7. Author contributions

AF, ARM and ARC conceived the study and planned the experiment; JED and JC were responsible for the plant material; GL and ARC performed the experiments; AF, ARM, ARC, GL and BD performed data analysis. All the authors have revised the manuscript. All authors have read and approved the manuscript.

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# CHAPTER III

## 3. Grapevine leaf fatty acid profiling: a straitghtforward tool to determine tolerance/susceptilibity to *P. viticola*

Chapter III is based on the manuscript in preparation
*Cavaco AR, Laureano G, Duarte B, Cunha J, Eiras-Dias J, Matos AR and Figueiredo A. Unveiling grapevine leaf fatty acids as eficient biochemical markers of grapevine tolerance/susceptibility towards P. viticola*

### 3.1. Abstract

Grapevine (*Vitis vinifera* L) is prone to several diseases. Downy mildew, caused by *Plasmopara viticola*, is among the most devastating. Disease control strategies include phytochemicals application every growing season, jeopardizing the sustainability of viticulture. Understanding the molecular processes behind resistance or susceptibility to *P. viticola* is vital to define alternative control strategies and select new disease resistance traits for breeding programs. Identifying molecular markers for discriminating tolerant and susceptible grapevine genotypes to *P. viticola* is an important step. Fatty acid (FA) molecules, important for grapevine defence responses to *P. viticola*, were previously used as chemophenotyping tools. In this work, we analysed the total FA content of tolerant and susceptible *Vitis* species and *V. vinifera* cultivars to *P. viticola*. We also analysed the gene expression of fatty acid desaturases (FAD), responsible for the synthesis of unsaturated FA. Our results indicate that FA are suitable chemophenotyping tools to study grapevine. Moreover, analyzing to total FA continent is a less costly and less time-consuming alternative methodology in comparison to the analysis of the different lipid classes. Susceptible cultivars present a higher expression of FAD genes and higher unsaturated FA levels. Different molecules arise as candidate tolerance and susceptibility biomarkers to *P. viticola*. While C16:0 may be considered a tolerance biomarker and potential resistance trait for breeding programs, C18:2 may be considered a susceptibility biomarker.



**Keywords:** chemophenotyping; fatty acid desaturases; palmitic acid; linoleic acid; *Vitis vinifera*; membrane fluidity; molecular markers; susceptible; tolerant; unsaturated fatty acids


**Abbreviations:**

C16:0 – palmitic acid

C16:1*t* - trans-palmitoleic acid

C18:0 – stearic acid

C18:1 – oleic acid

C18:2 – linoleic acid

C18:3 – α-linolenic acid

CAP – canonical analysis of principal coordinates

DBI – double bond index

DGDG – digalactosyldiacylglycerol

ER – endoplasmic reticulum

FA – fatty acids

FAD – fatty acid desaturase

FAME – fatty acid methyl esters

MGDG – monogalactosyldiacylglycerol

PA – phosphatidic acid

PC – phosphatidylcholine

PE – phosphatidylethanolamine

PG – phosphatidylglycerol

PI – phosphatidylinositol

PUFA – polyunsaturated fatty acids

SFA – saturated fatty acids

UFA – unsaturated fatty acids

## 3.2. Introduction

Fatty acids (FA) are metabolites of the highly conserved acetyl-CoA pathway and due to their importance as structural components of cells were shown to be suitable to perform phenotyping studies. The FA profile was previously used as a chemophenotyping tool for studies including the propensity of humans to develop coronary heart disease and ion compartmentation in cells of higher plants [1, 2]. Usually, phenotyping studies employ well-known techniques such as imaging, sensors and polyacrylamide gel electrophoresis of protein extracts [3, 4] and polyacrylamide gel electrophoresis of protein extracts [3]. This approach may complement other methodologies and dissolve some uncertainties that arise from the results obtained with these approaches. The use of the total FA composition, as an alternative approach, may allow a less costly and less time-consuming methodology for chemophenotyping studies and to assess the grapevine tolerance or susceptibility to *P. viticola*.

Grapevine (*Vitis vinifera* L.) is one of the most important crops worldwide with around 7.4 mHa of estimated cultivated area in 2019 [5]. The wine industry plays a central role in several countries economy, with a revenue around 31.8 billion euros [5]. The domesticated *V. vinifera* is the only grapevine species used in intensive agriculture [6, 7]. However, this species is prone to several oomycete and fungal-associated diseases, which represents one of the greatest threats to modern viticulture [8]. Grapevine downy mildew, caused by the obligatory biotrophic oomycete *Plasmopara viticola* (Berk. & Curt.) Berl. & de Toni, is one of the most devastating diseases [8, 9]. As a control measure for this disease, several fungicide applications are necessary every growing season [10, 11], with heavy environmental costs such as pollution, increase in the number of resistant fungi strains and residual toxicity on products for human consumption [10, 12, 13]. In a great number of the commercialized wines, trace amounts of phytopharmaceutical products can be detected [14]. Viticulture is the agriculture activity which makes the most intensive use of plant protection products [15]. The United Nations objectives for the sustainable development for 2030 as well as a directive from the European Parliament (2009/128/CE) urge the reduction of pesticides usage in agriculture. This highlights the need to deepen our understanding on the molecular processes behind resistance or susceptibility of some *Vitis* species and *V. vinifera* cultivars to *P. viticola* to define new disease control strategies and to be able to select new disease resistance traits for new hybrid crossings aiming at a sustainable viticulture. Therefore, it is important to identify molecular markers that allow the discrimination between tolerant and susceptible grapevine genotypes to *P. viticola*. Grapevine leaf fatty acids have been shown to play an important role

in the activation of defence mechanisms during *P. viticola* infection. We observed FA modulation events in the first hours after inoculation of *V. vinifera* cv Regent (tolerant) with *P. viticola*, but no significant changes in the FA profiles occurred in *V. vinifera* cv Trincadeira (susceptible). These results suggest that the tolerant genotype may trigger lipid-associated signalling mechanisms [16]. The unsaturation degree of fatty acids (FA) that compose membrane lipids directly affects membrane fluidity and permeability [17]. This feature is one of the factors that affects plant-pathogen interaction and the plant defence mechanisms [18].

Since the plant leaf FA composition has proven to be suitable to be used as a chemophenotyping tool, it can also be considered to study grapevine and to evaluates the potential of FA molecules as biomarkers for resistance or susceptibility to *P. viticola*. Furthermore, the use of grapevine leaf total FA composition may a less costly and less time-consuming alternative methodology for chemophenotyping studies and to assess the grapevine tolerance or susceptibility to *P. viticola*.

In the present work we used the FA profile as a tool to discriminate tolerant and susceptible *V. vinifera* cultivars and *Vitis* species to *P. viticola*. We have characterized the FA profile of 6 *Vitis* species and 5 *V. vinifera* cultivars with phenotypes of tolerance or susceptibility to *P. viticola*. Furthermore, we analysed the expression of 4 FA desaturase coding genes: *FAD3.1, FAD4, FAD6* and *FAD8*. We were able to discriminate the tolerant and susceptible grapevine genotypes according to the FA profiles and expression of specific FAD genes. The observed differences concern mainly the FA unsaturation degree, which was corroborated by the expression levels of FAD genes. These differences in FA composition, are likely related to differences in the proportions of individual lipid classes and their fatty acids compositions, recently reported by our team for a subset of the genotypes studied in the present work. Despite the differences in the fatty acid profiles no significant differences were observed in the double bond index, which reflects membrane fluidity. The differentiating features between tolerant and susceptible *V. vinifera* cultivars and *Vitis* species here identified, highlight new candidate biomarkers for grapevine tolerance and susceptibility to *P. viticola* and the potential use of FA and profile as fast and straightforward a chemophenotyping tool.

## 3.3. Materials and Methods

### 3.3.1. Plant Material

Five *Vitis* species (*V. riparia, V. candicans, V. rotundifolia, V. labrusca and V. rupestris*), one *V. vinifera* subsp. *sylvestris* (from now on refered to as *V. sylvestris,* wild plants that grow into Portuguese river basins) and four *Vitis vinifera* cultivars (´Regent', 'Trincadeira', 'Pinot noir' and 'Riesling'), indicated in Table 3.1, exhibiting different tolerance levels to *P. viticola,* were used in this study.

*Vitis riparia Michaux* cv Riparia Gloire de Montpellier (VIVC 4824) and *V. rotundifolia* (*Muscadinia Rotundifolia Michaux cV. Rotundifolia*, VIVC 13586) present high tolerance to *P. viticola* (9 in the OIV descriptor 452); *V. vinifera* cv Regent (VIVC 4572), is a crossing hybrid with tolerance to *P. viticola*. With the same degree of resistance there are *V. labrusca* cv Isabella (VIVC 5560), *V. rupestris Scheele* cv *Rupestris* du lot (VIVC 10389) and *V. candicans Engelmann* (VIVC 13508) (OIV descriptor 452 - 7); *V. vinifera* cv Trincadeira (VIVC 15685), cv Pinot noir (VIVC 9279) and *cv* Riesling (VIVC 10077) present high susceptibility to *P. viticola* (OIV descriptor 452 – 1/3). Also, field observed behavior in different seasons at into the Portuguese Ampelographic *Vitis* Collection (Colecção Ampelográfica Nacional, CAN). CAN is property of INIAV-Estação Vitivinícola Nacional (Dois Portos), located at Quinta da Almoinha, 60 km north of Lisbon (9º 11′ 19″ W; 39º 02′ 31″ N; 75 m above sea level). CAN

maintenance conditions are homogeneous modern alluvial soils (lowlands) as well as well drained soil; rootstock of a unique variety (Selection Oppenheim 4–SO4) was used for all genotypes including other *Vitis* species and other rootstocks represented in the field; each accession comes from one unique plant; temperate with dry and mild summer, climate. For plant material collection, the best possible health status was guaranteed for all genotypes was confirmed as plants were tested for the principal grapevine fungal/oomycetes diseases as well as grapevine viruses (healthy genotypes and synonym genotypes were planted in continuous line for didactic proposes); same trailing system (bilateral cordon, Royat), canopy maintenance and agricultural management.

Three leaves (3rd to 5th from the shoot apex) were harvested from 5 different plants per biological replicate and immediately frozen in liquid nitrogen. Five biological replicates were used in this study.

### 3.3.2. Lipid analysis

Fatty acid methyl esters (FAME) were prepared by trans-esterification with 50mg of ground leaf and 20µg of Margaric acid as an internal standard in Methanol:Sulphuric acid solution (39:1 v/v) for 1h at $70^0$C. The reaction was stopped by cooling. The methyl esters were recovered by adding petroleum ether and ultrapure water (3:2, v/v) and the organic phase was collected. The organic phase was dried at 37°C under nitrogen atmosphere and resuspended in hexane. 1µL of sample was injected for each analysis. FAME quantitative analysis was performed using gas chromatography (Varian 430-GC gas chromatograph) equipped with a hydrogen flame ionization detector using a fused silica 0.25 mm i.d.×50m capillary column (WCOT Fused Silica, CP-Sil 88 for FAME, Varian), according to [16]. The double bond index (DBI) was calculated as follows:

DBI = (% monodienoic acids) + 2 (% dienoic acids) + 3 (% trienoic acids)/100.

### 3.3.3. RNA extraction and cDNA synthesis

Total RNA was isolated from the *V. vinifera* cultivars and *Vitis* species ground leaves using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. On-Column DNase I Digestion (Sigma-Aldrich, USA) was used to hydrolyse residual genomic DNA, as described by the manufacturer. RNA purity and concentration were determined using a NanoDrop-1000 spectrophotometer (Thermo Scientific). The presence of gDNA in RNA samples was assessed by qPCR analysis [20] on crude RNA by targeting the Elongation Factor 1-alpha (EF1α) gene. Complementary DNA (cDNA) was synthesized from 2.5 µg of extracted RNA using RevertAid®H Minus Reverse Transcriptase (Fermentas, Ontario, Canada) anchored with Oligo(dT)23 primer (Sigma-Aldrich, USA), as described in [21].

**Table 3.1.** Wild *Vitis* species, *V. vinifera* subsp. *sylvestris* and grapevine cultivars analyzed. Species and cultivar names, type of accession, origin and response to *P. viticola* are indicated (information adapted from [19] and https://www.vivc.de/). Classification of resistance: 1—very low; 3—low, 5—medium, 7—high, 9—very high or total. T—Tolerant; S—Susceptible.

| ***Vitis* species** | **Subspecies (subsp.) or cultivar (c*V.*)** | **VIVC variety number** | **Abbreviation** | **Type of accession** | **Origin** | **Degree of resistance according to OIV descriptor 452** | **Overall response to fungi/oomycete pathogens** |
|---|---|---|---|---|---|---|---|
| *V. labrusca* | Isabella | 5560 | LAB | Wild species | United States of America | 7 | T |
| *V. rotundifolia* | Muscadinia *Rotundifolia* Michaux c*V. Rotundifolia* | 13586 | ROT | Wild species | United States of America | 9 | T |
| *V. riparia Michaux* | Riparia Gloire de Montpellier | 4824 | RIP | Wild species | United States of America | 9 | T |
| *V. candicans Engelmann* | *Vitis Candicans* Engelmann | 13508 | CAN | Wild species | United States of America | 7 | T |
| *V. rupestris Scheele* | *Rupestris* du lot | 10389 | RU | Wild species | United States of America | 7 | T |
| *V. vinifera* | Subsp. *sylvestris* | | SYL | Wild plant | Portugal | 3 | T |
| | Subsp. *sativa* c*V.* Regent | 4572 | REG | Cultivated hybrid (crossing *V. vinifera* c*V.* Diana X c*V.* Chambourcin) | Germany | 7 | T |
| | Subsp. *sativa* c*V.* Riesling Weiss | 10077 | RL | Cultivated grapevine | Germany | 3 | S |
| | Subsp. *sativa* c*V.* Pinot Noir | 9279 | PN | Cultivated grapevine | France | 3 | S |
| | *Subsp. sativa cV. Cabernet Sauvignon* | 1929 | CS | Cultivated grapevine | France | 1/3 | S |
| | Subsp. *sativa* c*V.* Trincadeira | 15685 | TRI | Cultivated grapevine | Portugal | 1/3 | S |

### 3.3.4. Quantitative Real Time PCR (qPCR)

qPCR experiments were performed in a StepOne™ Real-Time PCR system (Applied Biosystems, Sourceforge, USA) using the Maxima™ SYBR Green qPCR Master Mix (2×) kit (Bio-Rad, USA), following manufacturer's instructions. Each reaction contained 2.5 mM MgCl2 and 2 μM of each primer were used in 10 μL volume reactions, with 1 μL of cDNA (diluted 1:10) as template. A control without cDNA template was included in each set of reactions. Primer sequences and reaction details are provided in Table 3.2. For all genes, thermal cycling started with a 95 °C denaturation step for 10 minutes followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing at gene specific temperature for 30 seconds. Three biological replicates and two technical replicates were used for each sample. Elongation Factor 1-alpha (EF1α) and Ubiquitin-conjugating enzyme (UBQ) coding genes were used for expression data normalization as previously described [22]. For each gene, standard curve efficiency was calculated according to [23]. The quantification cycle (Cq) values of the genes of interest in the *Vitis* species and *V. vinifera* cultivars at study were extracted and normalized by the geometric mean of the Cq of *UBQ* and *EF1α*, as described in [21]

**Table 3.2.** Target and reference gene oligonucleotide sequences and reaction details

| **Identifier/NCBI Assession Number** | **Primer sequence (5´-3´)** | **Amplicon length (bp)** | **Amplification Efficiency (E)** | **Ta (ºC)** | **Tm (ºC)** |
|---|---|---|---|---|---|
| Reference genes | | | | | |
| *EF1α* | F: AACCAAAATATCCGGAGTAAAAGA | 150 | 1.99 | 60 | 80.66 |
| XM_002284888.2 | R: GAACTGGGTGCTTGATAGGC | | | | |
| *UBQ* | F: GTGGTATTATTGAGCCATCCTT | 182 | 2.08 | 60 | 81.4 |
| XM_002284161.3 | R: AACCTCCAATCCAGTCATCTAC | | | | |
| Target genes | | | | | |
| *FAD3.1* | F: ATAGAAGCCCAGGGAAGAAG | 135 | 1.94 | 60 | 90.19 |
| XM_002277537.4 | R: CAAAGGATACGCAAACAAGCA | | | | |
| *FAD4* | F: TGTTCAGCCAGCAGTTCCAT | 97 | 1.98 | 60 | 83.48 |
| XM_002280947.4 | R: CTCGACACTAGCAGTCCAG | | | | |
| *FAD6* | F: CATGGTTGGGTTATCACTTCT | 147 | 1.94 | 60 | 79.02 |
| XM_003634815.2P48 | R: CTATCCAACGAGGGTAATCAC | | | | |
| *FAD8* | F: GGCACTTTCCCTCCTCCTT | 151 | 1.92 | 58 | 79.77 |
| XM_002264314.4 | R: GGGCCTTATGCCACATTCT | | | | |

### 3.3.5. Statistical analysis

To evaluate the differences in the lipid and fatty acid profile of the tolerant and susceptible genotypes, a multivariate approach was applied. Canonical analysis of principle coordinates (CAP), using Euclidean distances, was used to visualize differences in multivariate space regarding lipid and fatty acid relative composition as described in [24] Multivariate statistical analyses were conducted in Primer 6 software [25]. The ability for each target gene to discriminate between tolerant and susceptible grapevine genotypes was assessed by testing the homocedasticity of groups with Bartlett's test and by assessing significance of the differences between groups with a Wilcox-Mann–Whitney's U test, as described in [21]. All p-values were adjusted for false discovery rate using the Benjamini–Hochberg procedure. Bartlett's and Wilcoxon–Mann–Whitney tests were performed in R [26], using the 'bartlett.test', 'wilcox.test' and 'p.adjust' functions, respectively. Significance of the differences between groups concerning the FA levels was assessed using IBM® SPSS® Statistics software (version 26.0; SPSS Inc., USA) with a Kruskal-Wallis test. After Kruskal-Wallis test, p-values were adjusted with the Bonferroni method as described in [24]. Results yielding a p value $< 0.05$ were considered statistically significant.

## 3.4. Results

### 3.4.1. Susceptible grapevine cultivars present higher contents of UFA

The FA composition has been recently used as a chemophenotyping tool for studies including the propensity of humans to develop coronary heart disease and ion compartmentation in cells of higher plants [1, 2]. To understand whether the FA composition could be used to discriminate susceptible and tolerant grapevine genotypes to *P. viticola*, we analysed the FA content of 7 tolerant genotypes (*V. riparia, V. sylvestris, V. candicans, V. rupestris, V. labrusca, V. rotundifolia* and *V. vinifera* cv Regent) and 4 susceptible *V. vinifera* cultivars ('Trincadeira', 'Riesling', 'Cabernet sauvignon' and 'Pinot noir') (Figure 3.1).

Palmitic acid (C16:0) shows a tendency to be more abundant in tolerant genotypes and linoleic acid (C18:2) showed statistically significant differences, being more abundant in susceptible genotypes (Figure 3.1). The observed FA profile shows that while the tolerant genotypes present a tendency for a higher content of C16:0, the majority of the susceptible cultivars present a higher content of C18:2. In general, it is observable that the tolerant genotypes have a tendency to present higher levels of saturated FA (SFA), while susceptible cultivars seem to present higher levels of unsaturated FA (UFA) (Figure 3.2A). The ratios of unsaturated to saturated FA seem to be higher in the susceptible cultivars, which also present a tendency for a higher DBI (Figure 3.2B).

Some ratios between different FA presented statistically significant differences, also allowing to distinguish between the tolerant and susceptible genotypes (Figure 3.3). Among them, C18:3/C18:2 showed a more distinguishable patterns with a tendency to be lower in the susceptible genotypes (Figure 3.3A). The C18:2/C16:0 ratio was also calculated because these FA showed to be predominant in susceptible and tolerant genotypes, respectively (Figure 3.1). This ratio sowed to be higher in the susceptible genotypes, being the most significant differences observed between *V. vinifera* cv Cabernet sauvignon and the tolerant genotypes *V. vivinifera* cv Regent and *V. rupestris* (Figure 3.3B).

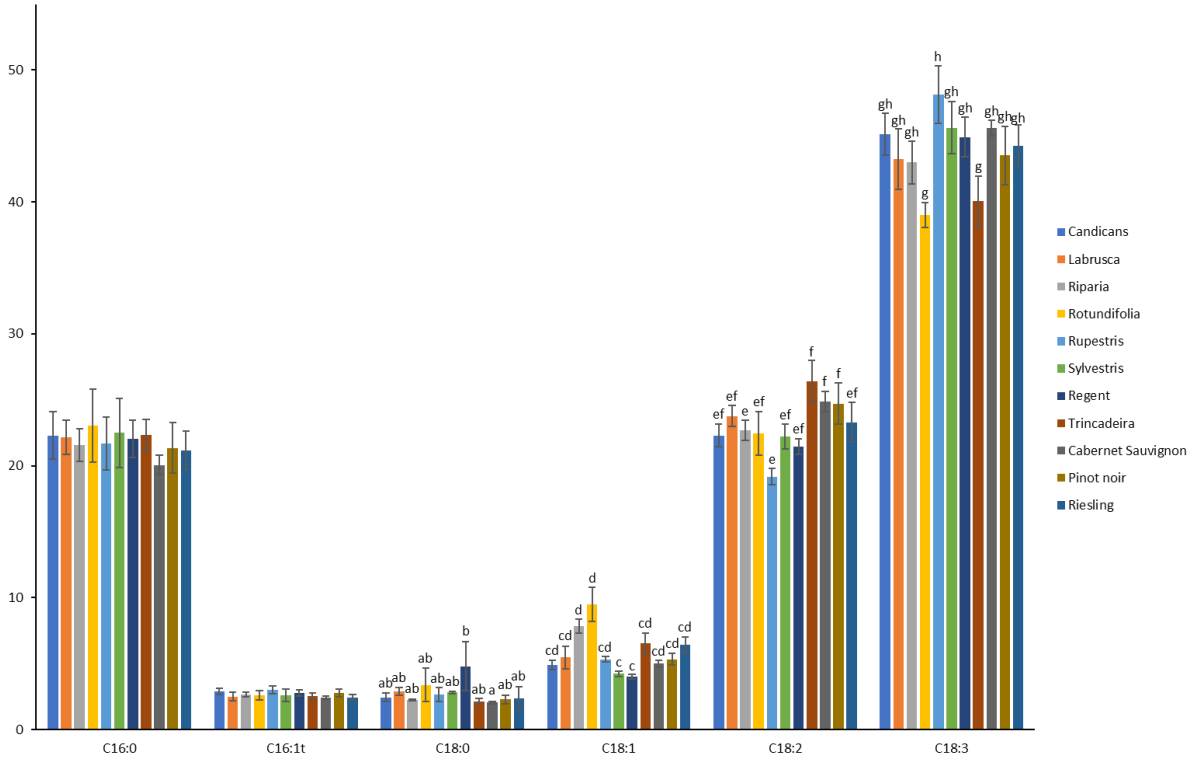


**Figure 3.1.** Fatty acid profile of 7 tolerant *Vitis* species to *P. viticola* (*V. candicans*, *V. labrusca*, *V.* riparia, *V. rotundifolia*, *V. rupestris* and *V. vinifera* cv Regent) and 4 susceptible *V. vinifera* cultivars ('Trincadeira'. 'Cabernet sauvignon', 'Pinot noir'). Average ± standard deviation, N = 3, different letters indicate significant differences at $p < 0.05$.

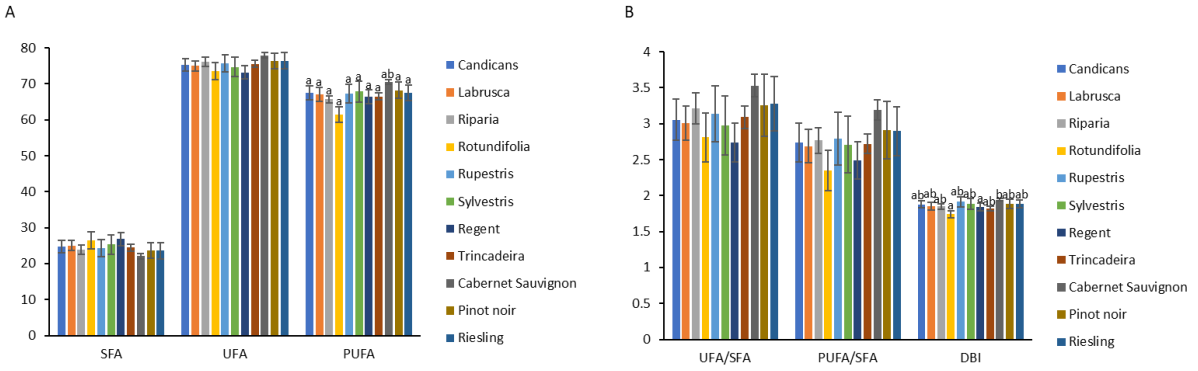


**Figure 3.2.** (A) Percentage of saturated FA (SFA), unsaturated FA (UFA) and polyunsaturated FA (PUFA) (B) Ratios between UFA and SFA (UFA/SFA) and between PUFA and SFA (PUFA/SFA) and Double bond index (DBI) 7 tolerant *Vitis* species to *P. viticola* (*V. candicans*, *V. labrusca*, *V. riparia*, *V. rotundifolia*, *V. rupestris* and *V. vinifera* cv Regent) and 4 susceptible *V. vinifera* cultivars ('Trincadeira'. 'Cabernet sauvignon', 'Pinot noir' and 'Riesling'). Average ± standard deviation, N = 3, different letters indicate significant differences at $p < 0.05$.

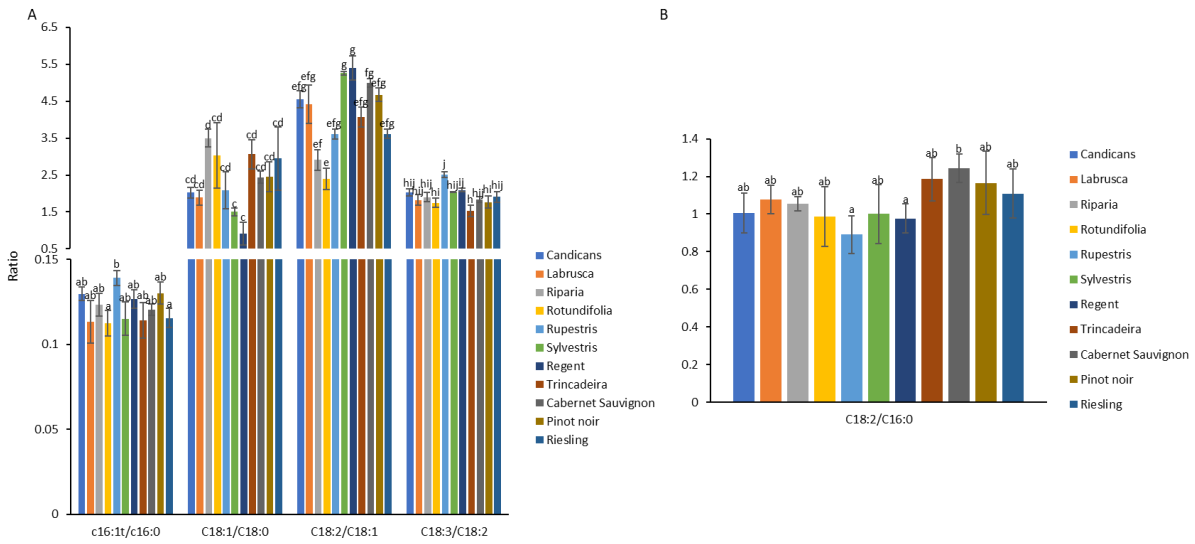


**Figure 3.3.** (A) C16:1t/C16:0, C18:0/C18:0, C18:2/C18:1 and C18:3/C18:2 ratios, (B) C18:2/C16:0 ratio of 7 tolerant *Vitis* species to *P. viticola* (*V. candicans*, *V. labrusca*, *V.* riparia, *V. rotundifolia*, *V. rupestris* and *V. vinifera* cv Regent) and 4 susceptible *V. vinifera* cultivars ('Trincadeira'. 'Cabernet sauvignon', 'Pinot noir' and 'Riesling'). Average ± standard deviation, N = 3, different letters indicate significant differences at $p < 0.05$.

### 3.4.2. Susceptible grapevine gultivars present higher expression of plastidial FAD genes

One of the most important processes for membrane lipid homeostasis as well as in stress conditions is FA desaturation [27]. The desaturation of FA present in membrane lipids is catalysed by membrane-bound FA desaturase (FAD) enzymes present in the chloroplast or the endoplasmic reticulum (ER) [28].

Since the activity of these enzymes affect the FA composition of membrane lipids, which showed to be able to separate grapevine genotypes, we hypothesised that the basal expression of some FAD genes could also allow the separation between tolerant and susceptible grapevine genotypes to *P. viticola*. Therefore, we analysed the expression of *FAD3.1*, responsible for the synthesis of C18:3 from C18:2 in the ER [29], *FAD4*, that catalyses the desaturation of C16:0 into trans-palmitoleic acid (C16:1*t*) [30], *FAD6* and *FAD8* responsible for the synthesis of C18:2 and C18:3 in the chloroplast, respectively [31].

The gene expression analysis by qPCR revealed that the genes encoding the plastidial enzymes *FAD4*, *FAD6* and *FAD8* showed a significantly higher expression in the susceptible grapevine cultivars to *P. viticola* at study compared to the tolerant genotyes (Fig. 4B-D). On the other hand, there were no significant differences concerning the expression of the ER enzyme *FAD3.1* coding gene (Figure 3.4A).

## 3.5. Discussion

Our results show that the total FA profile as well as the expression levels of FAD coding genes may be potential chemophenotyping tools for grapevine genotypes. Moreover, some molecules can be highlighted as candidate biomolecules for tolerance and susceptibility to *P. viticola*.

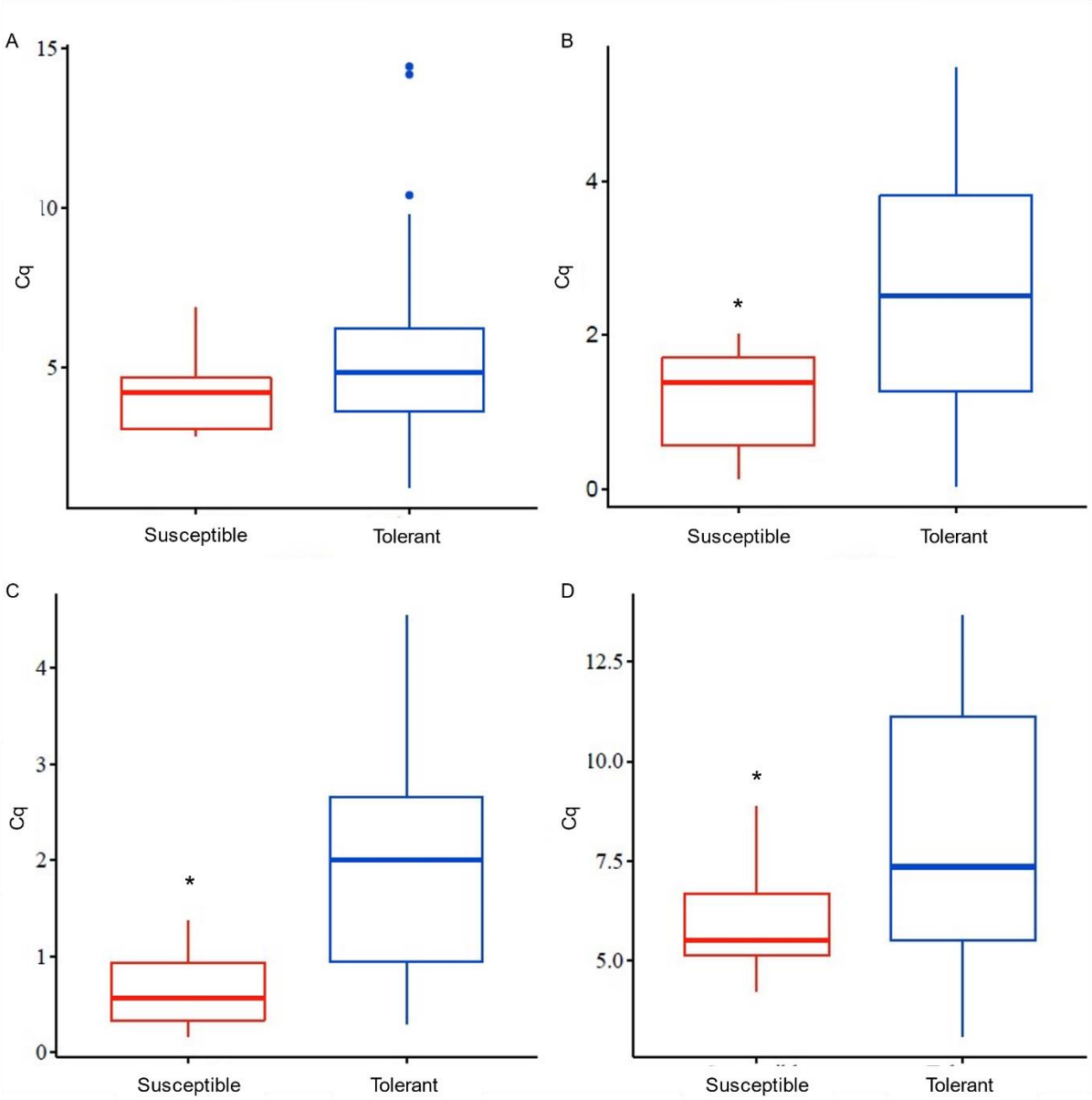


**Figure 3.4.** Boxplot of quantification cycles (Cq) values for the FAD genes at study in susceptible (*V. vinifera* cv Trincadeira, Cabernet sauvignon, Pinot noir and Riesling) and tolerant (*V. candicans*, *V. labrusca*, *V.* riparia, *V. rotundifolia*, *V. rupestris* and *V. vinifera* cv Regent) genotypes. (A) *FAD3.1*, (B) *FAD4*, (C) *FAD6* and (D) *FAD6*. Cq values were normalized by the geometric mean of the Cq of *UBQ* and *EF1α*. Data for susceptible genotypes is represented in red and data for tolerant genotypes is represented in blue. Asterisks indicate significant difference between susceptible and tolerant genotypes ($p < 0.05$)

The total FA profile of the 11 grapevine genotypes at study allowed to observe that the tolerant genotypes to *P. viticola* (*V. riparia, V. sylvestris, V. rotundifolia, V. candicans, V. labrusca, V. rupestris* and *V. vinifera* cv Regent) show a tendency for higher levels of C16:0 in comparison to the susceptible *V. vinifera* cultivars ('Trincadeira', 'Pinot noir', 'Cabernet sauvignon' and 'Riesling') (Figure 3.1). C16:0 is a major FA involved in permeability control of lipid membranes. This FA was also previously proposed as a biomarker resistance of the Bulgarian *V. vinifera* cv Storgozia to *P. viticola* [32]. On the other hand, the susceptible *V. vinifera* cultivars presented a tendency for higher levels of C18:3, which arises as a candidate biomarker for grapevine susceptibility to *P. viticola*. Also, C18:2/C16:0 showed to be able to distinguish between susceptible and tolerant genotypes, with a tendency to present higher

levels in the latter group (Figure 3.3B). In fact, while the tolerant genotypes showed higher levels of SFA, the susceptible cultivars presented higher levels of UFA and higher DBI (Figure 3.2), which is in accordance with the FA that were highlighted in tolerant and susceptible assessments. Taking into account that the FA degree of unsaturation is a key factor for the regulation of membrane fluidity [33], this feature may arise as an important differentiating trait between tolerant genotypes, which seem to have more rigid membranes, and the susceptible cultivars which may present a higher constitutive membrane fluidity. A higher membrane rigidity might be an important feature to hinder pathogen colonization of grapevine leaves.

Since the FA unsaturation degree showed to be a critical feature to separate between the tolerant and susceptible grapevine genotypes to *P. viticola*, we analyzed the expression of several FAD genes encoding for enzymes responsible for the synthesis of unsaturated FA. We hypothesized the constitutive expression profile of these genes could also allow the discrimination between tolerant and susceptible grapevine genotypes to *P. viticola*. The results obtained from the qPCR gene expression analysis of *FAD3.1*, *FAD4*, *FAD6* and *FAD8* (Figure 3.4) are in accordance with the observed FA profiles. While *FAD3.1* catalyses the synthesis of C18:3 in the ER [29], *FAD4*, *FAD6* and *FAD8* are responsible for the synthesis of C16:1*t*, C18:2 and C18:3 in the chloroplast, respectively [30, 31]. We observed that, although *FAD3.1* did not show a significant difference of expression between tolerant and susceptible genotypes, *FAD4*, *FAD6* and *FAD8* showed a higher expression the in susceptible *V. vinifera* cultivars (Figure 3.4). Indeed, in these cultivars, there may be a higher basal mobilization of the cellular machinery for the synthesis of UFA (including PUFA) in the chloroplast. Calvo and collaborators had observed that plant derived PUFA function as sporogenic factors for fungi of the genus *Aspergillus* [34], which corroborate the hypothesis that higher basal levels of PUFA may be a characteristic of pathogen susceptible genotypes.

Considering that the susceptible cultivars also showed higher levels of PUFA (Figure 3.2) and *FAD6* and *FAD8* expression (Figure 3.4), responsible for the formation of PUFA in the galactolipids [30], these results complement and corroborate each other.

Taken together, our results corroborate the hypothesis that the FA and lipid composition is suitable to be used as a chemophenotyping tool. Different molecules arise from this study as candidate constitutive biomarkers for tolerance and susceptibility to *P. viticola*. While C16:0 may be a biomarker for tolerance and a potential resistance trait to be used in breeding programs, C18:3 may be highlighted as a biomarker for susceptibility.

### 3.6. Acknowledgements

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### 3.7. Author contributions

AF, ARM and ARC conceived the study and planned the experiment; JED and JC were responsible for the plant material; GL and ARC performed the experiments; AF, ARM, ARC, GL and BD performed

data analysis. ARM and AF are co-senior authors in this work. All authors have read and approved the manuscript.

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# CHAPTER IV

## 4. Fatty acid modulation and desaturase gene expression are differentially triggered in grapevine incompatible interaction with biotrophs and necrotrophs

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## 4.1. Abstract

Grapevine (*Vitis vinifera* L.) is prone to fungal and *oomycete* diseases. Downy and powdery mildews and grey mold, are caused by *Plasmopara viticola, Erisiphe necator* and *Botrytis cinerea*, respectively. *P. viticola* and *E. necator* are obligatory biotrophs whereas *B. cinerea* is a necrotroph. In tolerant grapevine cultivars, plant-pathogen interaction induces defence responses, including metabolite and protein accumulation and hypersensitive reaction. Lipid and lipid-derived molecules may have a key role in the activation of defence mechanisms. Previous results suggest that *V. vinifera* cv Regent tolerance to *P. viticola* may be mediated in the first hours post inoculation by fatty acid (FA) associated signalling. In the present study we characterized FA modulation in *V. vinifera* cv Regent leaves upon inoculation with *P. viticola*, *E. necator* and *B. cinerea* and correlated FA modulation with the expression profiles of genes encoding the FA desaturases FAD6 and FAD8. In all the interactions, a progressive desaturation of stearic acid to α-linolenic acid, precursor of jasmonic acid, occurred, which was observed for a longer period against *B. cinerea*. Our results provide evidence of a distinct FA meditated signalling pattern in grapevine interaction with biotrophs and necrotrophs. While the interaction with the biotrophs may trigger a higher synthesis of polyunsaturated FA (PUFA) at early time-points with a tendency to return to basal levels, the interaction with *B. cinerea* may trigger a later and more durable induction of PUFA synthesis. In all interactions, membrane fluidity modulation occurred, which may be crucial to maintain cellular function during infection.

**Highlights:**

- Fatty acid signalling is differentially modulated in the tolerant *V. vinifera* cv Regent defence response to downy and powdery mildews and grey mold, according to the pathogen's life style
- α-linolenic acid synthesis is induced in the first hours against biotrophs, and later against the necrotroph.
- The interaction with biotrophs (*P. viticola* and *E. necator*) and the necrotroph (*B. cinerea*) triggers different FAD6 and FAD8 expression patterns
- An adjustment of the fatty acids degree of unsaturation occurs during the infection

**Key-words:** *Vitis vinifera*; Downy mildew; Powdery mildew; Grey mold; Biotrophy; Necrotrophy; Fatty acid desaturases

**Abbreviations**

acp – acyl carrier protein

C16:0 – Palmitic Acid

C16:1t – Trans-3-hexadecanoic Acid

C17:0 – Margaric Acid

C18:0 – Stearic Acid

C18:1 – Oleic Acid

C18:2 – Linoleic Acid

C18:3 – α-Linolenic Acid

DBI – Double Bond Index

EF1α – Elongation factor-1alpha

ER – Endoplasmic Reticulum

FA – Fatty Acid

FAD – Fatty Acid Desaturase

FAME – Fatty Acid Methyl Esters

GC – Gas Chromatography

hpi – hours post inoculation

JA – Jasmonic Acid

PG – Phosphatidylglycerol

PLA – Phospholipase A

PR – Pathogenesis related

RPV – Resistant to *Plasmopara viticola*

SA – Salicylic acid

SAR – Systemic Acquired Resistance

UBQ - Ubiquitin

## 4.2. Introduction

Grapevine (*Vitis vinifera* L.) is susceptible to several pathogens that affect fruit yield and wine quality due to the direct infection of berries or loss of plant vigour caused by the infection of the green tissues [1]. Among the most economically important diseases are downy and powdery mildews and grey mold, caused by *Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni [1], *Erisiphe necator* (Schweinf.) Burrill [2] and *Botrytis cinerea* [3], respectively. *P. viticola* and *E. necator* are obligatory biotrophs [1, 4]. While *P. viticola* invades the plant from the stomatal aperture [5], *E. necator* relies on the secretion of lytic enzymes such as lipases, esterases, and cutinases in order to promote wounds in the tissue and enter the plant [4] (Figure 4.1). The necrotrophic pathogen, *B. cinerea*, invades the host tissue by active penetration, involving the release of lytic enzymes or by passive ingress [6]. Being an opportunistic pathogen, it can initiate infection at wound sites, or at sites that have previously been infected by other pathogens. This necrotroph can also enter the host via the substomatal cavity [6].

Due to these and other pathogens, viticulture is one of the agricultural activities which makes the most intensive use of plant protection products [7]. As a control measure, several preventive fungicide applications are performed every growing season (Blum et al., 2010). This measure leads to serious risks concerning environmental pollution, raising the number of resistant pathogen strains and increasing residual toxicity [10]. In the past decades, resistance associated breeding programs contributed for diminishing the application of phytochemicals. Successful examples are the commercially available crossing lines *V. vinifera* cv Regent, Bianca and Solaris [11]. However, it has been described that some *P. viticola* isolates are able to overcome their resistance mechanisms, particularly the resistance mediated by the *rpv3* loci [9, 12]. This highlights the need to deepen our understanding on grapevine resistance mechanisms in order to define new disease control strategies towards a more sustainable viticulture.

In grapevine, pathogen invasion induces defence reactions, as the accumulation of metabolites such as stilbenes and pathogenesis-related proteins [13] and often hypersensitive reaction [14]. In the incompatible interaction between grapevine and biotrophic pathogens, the hypersensitive cell death is often activated to restrict the growth of the pathogen and stimulate specific defence reactions near the infected area as well as the systemic acquired resistance (SAR) mechanisms [15]. In the case of powdery mildew, grapevine reactions also include the inhibition of pathogen entrance either with constitutive physical barriers (such as leaf surface wax), the production of antimicrobial secondary metabolites [16] or the recruitment of proteins that mediate membrane fusion events [17]. Concerning defence mechanisms against *P. viticola*, the incompatible interaction can also involve the deposition of callose around the stomata to inhibit zoospore germination or around haustoria to stop pathogen progression [18] (Figure 4.1A). The establishment of the incompatible interaction between grapevine and *B. cinerea*, a necrotrophic pathogen, supports the activation of the signalling pathway of the α-linolenic derived hormone jasmonic acid (JA) [17]. Important grapevine basal immunity features in this pathosystem include the quantity and density of stomata and leaf trichomes [17]. Also, an oxidative burst followed by and a timely elevation of antioxidative capacity [19] and phytoalexin accumulation seem to be necessary for defence activation [20]. In order to degrade the fungus cell wall, grapevine is also able to secrete a number of chitinases and other hydrolytic enzymes [21]. Moreover, synthesis and secretion of pathogenesis-related proteins (PR) [22] and the activation of defensin-like genes [23], are part of the inducible defence mechanism deployed.

Over the past decade, several studies have also shown that lipid and lipid-derived molecules, may play an important role in the activation of plant defence mechanisms against pathogens [24, 25]. Thus, lipids are not only the major structural and energy storage constituents of the cell but they also function as modulators of a multitude of signal transduction pathways, during normal cellular function and under stress conditions [26]. It has been proposed that specific fatty acids (FA) may be involved in plant tolerance to pathogens with different colonization strategies (biotroph, hemibiotroph and necrotroph) [26]. [27] described the putative involvement of JA, polyamines and auxins, and reprogramming of carbohydrate and lipid metabolisms toward the synthesis of secondary metabolites involved in grapevine defence against *B. cinerea*. The alteration of lipid metabolism has also been observed in *Vitis vinifera* c*V.* Cabernet Sauvignon interaction with *Eutypa lata* [28]. Chitarrini and collaborators also observed lipid modulation events in *V. vinifera* c*V.* 'Bianca' during the interaction with *P. viticola* at 24 hours post inoculation (hpi) of the levels of oleic, linoleic and α-linolenic acids (C18:1, C18:2 and C18:3) [29] (Figure 4.1B). These FA are essential as signalling molecules in the activation of defence related programmed cell death [30]. The process of FA desaturation is necessary for the availability of unsaturated FA and is highly important for plant defense [26]. The desaturation of FA present in membrane lipids is catalyzed by membrane-bound FA desaturase (FAD) enzymes present in the chloroplast or the endoplasmic reticulum (ER) (Dong et al., 2016). FAD2 and FAD3 catalyze the

desaturation of C18:1 and C18:2, respectively, in the ER [32, 33]. Desaturation of C18:1 and C18:2 in plastidial membranes is catalyzed by FAD6 and FAD7/FAD8, respectively [34]. Another plastidial desaturase, FAD4 specifically catalyzes the synthesis of trans C16:1 on phosphatidylglycerol (PG) [35]. Soria-García and co-workers observed that the Arabidopsis desaturase AtFAD8 showed a JA-dependent response both at the gene expression and protein levels, suggesting that this enzyme is coordinated in defence responses [36].

Previous works from our group have demonstrated that grapevine tolerance to *P. viticola* might be mediated in the first hours of interaction by the oxylipin JA and lipid associated signalling [24, 37]. We have shown that the content of C18:3, JA precursor, is higher in tolerant genotypes, compared to susceptible ones, and increases upon *P. viticola* inoculation [25, 38]. Moreover, the expression of key genes for JA synthesis is higher after inoculation and both JA and JA-Ile (the active form of JA) content increase at 6 and 12hpi [24] (Figure 4.1B) presenting the first clues on the importance of JA and lipid associated signalling in grapevine tolerance mechanisms against a biotrophic pathogen. More recently we have shown that upon inoculation with *P. viticola,* the tolerant cultivar Regent displays modulation of its lipid and fatty acid composition, which results in increased amount of plastidial lipids, enriched in C18:3 [25]. The concomitant up-regulation of phospholipase A (PLA) genes encoding plastidial enzymes favours the activation of the JA-pathway, since these enzymes provide the fatty acid precursor of the hormone by cleaving it from chloroplast membrane lipids.

In the present work, the characterization of FA modulation in the first hours (6 and 24hpi) of *P. viticola, E. necator* and *B. cinerea* interaction with *V. vinifera* c*V.* Regent, a tolerant cultivar, was conducted. The FA modulation events were also correlated with the expression profiles of FAD6 and FAD8, responsible for the synthesis of C18:2 and C18:3 in the chloroplast. The obtained results provide new insights on the role of FA in the establishment of the incompatible interaction with these pathogens with different colonisation and/or invasion strategies.

## 4.3. Material and Methods

### 4.3.1. Plant Material

*Vitis vinifera* c*V.* Regent is a crossing line, bred for both downy and powdery mildew resistance at Julius Kuhn Institute (JKI, Germany). Woody shoots were taken in winter, from Portuguese National Ampelographic Collection (international code - PRT 051) and were kept to freezer at 4ºC to break dormancy. In the beginning of springtime, cuttings were planted in pots containing a mixture of soil and humus (2:1), grown in a greenhouse under a 16/8 (light/dark) and normal humidity at 12 to 25 C. [39]. Prior to inoculation, the 3-5$^{th}$ leaves from the shoot apex were detached, washed in distilled water by dipping, sterilized in 2% hypochlorite (90s), washed in distilled water (3 times), dried and placed on agar plates (8% agarose).

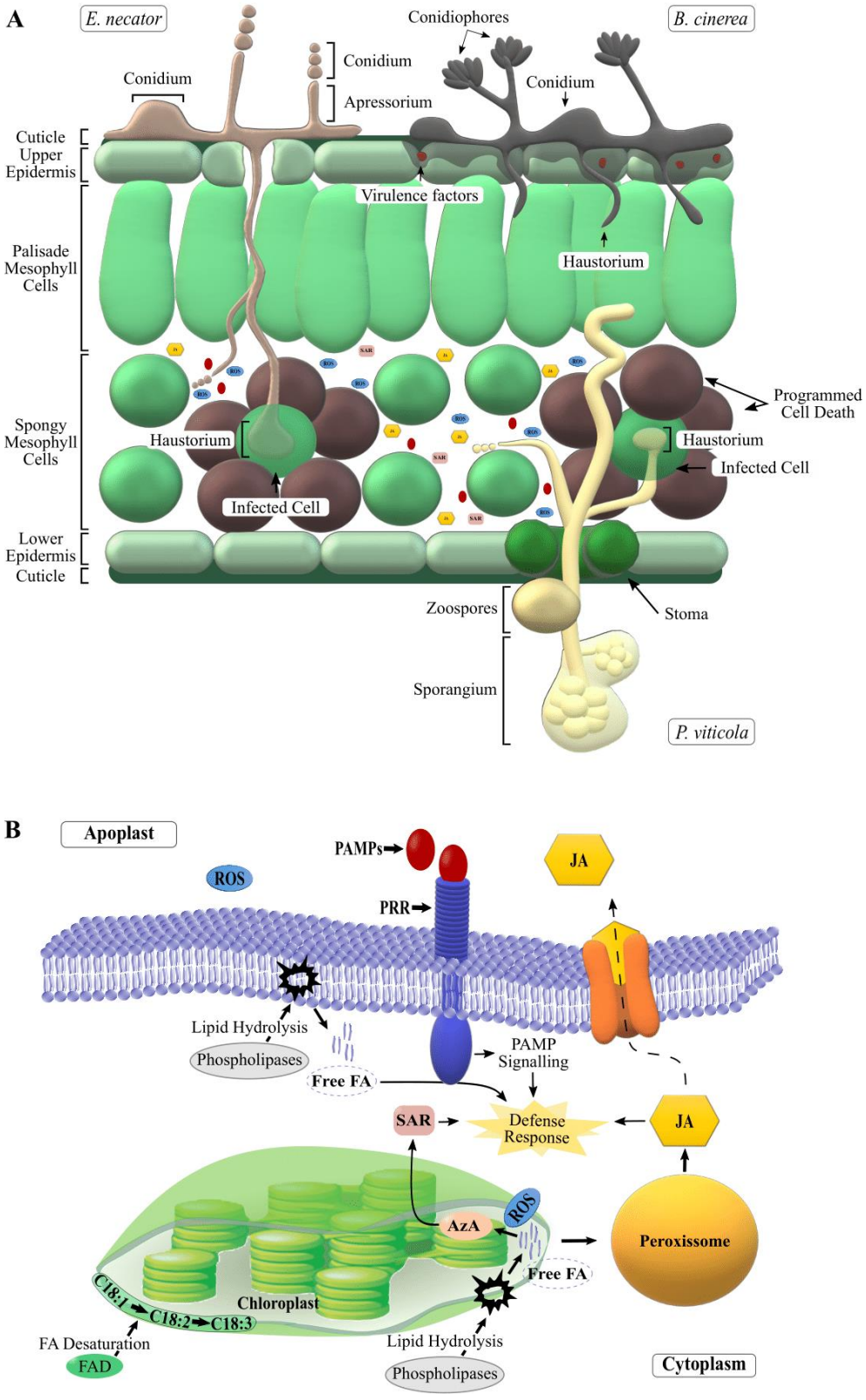


**Figure 4.1.** Defence response of a tolerant grapevine variety to *Plasmopara viticola*, *Erisiphe necator* and *Botrytis cinerea*. (A) *Plasmopara viticola*, *E. necator* and *B. cinerea* infection mechanism (B) Lipid mediated signalling pathway**.** FA – Fatty acid, C18:1 – Oleic acid, C18:2 – Linoleic acid, C18:3 – α-Linolenic acid, FAD – Fatty acid desaturase, ROS – Reactive oxygen species, PAMP – Pathogen associated molecular patterns, PRR – Pattern recognition receptor, SAR – Systemic acquired resistance, AzA – Azelaic acid, JA – Jasmonic acid

### 4.3.2. Inoculation Experiments

*Plasmopara viticola* sporangia were collected from symptomatic leaves from field infected plants after an overnight incubation in a moist chamber at room temperature, as previously described [39]. Sporangia were carefully collected by brushing, dried and stored at −20 °C. *P. viticola* sporangia vitality was confirmed by microscopy [40]. An aqueous suspension containing $10^4$ sporangia $ml^{-1}$ was used to spray the detached leaves on the abaxial surface, while controls were made by spraying the leaves with water (mock inoculations).

*E. necator* conidia were collected from symptomatic leaves from field infected plants. Inoculations with *E. necator* were performed by brushing the conidia from the leaf surface of the infected leaves directly to the adaxial surface of detached leaves. Mock inoculations were performed by brushing the leaf surface without the pathogen.

*B. cinerea* was cultured in Petri dishes on potato dextrose agar (PDA) medium (Merck, Germany) at 20 °C for 8 days. Conidial suspension was obtained by flooding the fungal culture with sterile distilled water and rubbing the mycelium. An aqueous conidial suspension was prepared with sterile distilled water up to $10^4$ conidia $ml^{-1}$. Detached grapevine leaves were dipped in the suspension and placed on the agar plates. Mock inoculations were performed by dipping the leaves in water.

After inoculation, the leaves were kept in a phytotron (S600 PHL from ARALAB) at 16/8 light/dark and 25ºC of temperature conditions. Leaves were deep frozen in liquid nitrogen at 6 and 24hpi. Mock inoculations were collected at the same time points. Five independent biological replicates were collected for each condition.

### 4.3.3. Fatty acid analysis

The *V. vinifera* cv Regent leaves inoculated and mock treated with *P. viticola, E. necator* or *B. cinerea* were ground in liquid nitrogen. Twenty micrograms of the internal standard margaric acid (C17:0) were added to each sample of 50 mg of grinded leaves, followed by the addition of 3 mL of methanol-sulfuric acid solution (39:1 v/v). The methylation reaction occurred for 1 h at 70 °C and was stopped by cooling. The methyl esters were recovered by adding petroleum ether and ultrapure water (3:2, v/v) and the organic phase was collected. The organic phase was dried at 37 °C under nitrogen atmosphere and resuspended in hexane. 1µL of sample was injected for each analysis. FA methyl esters (FAME) quantitative analysis was performed using gas chromatography (Varian 430-GC gas chromatograph) equipped with a hydrogen flame ionization detector using a fused silica 0.25 mm i.d.×50m capillary column (WCOT Fused Silica, CP-Sil 88 for FAME, Varian), according to [41]. The double bond index (DBI) was calculated as follows:

DBI = (% monodienoic acids) + 2 (% dienoic acids) + 3 (% trienoic acids)/100.

### 4.3.4. RNA extraction and cDNA synthesis

RNA was extracted from grinded leaves using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. Residual genomic DNA (gDNA) was digested with DNase I (On-Column Dnase I Digestion Set, SigmaAldrich, USA. RNA purity and concentration were measured at 260/280 nm in a spectrophotometer (Nanodrop-1000, Thermo Fisher Scientific, USA). The presence of gDNA in RNA samples was assessed by qPCR analysis [42] on crude RNA by targeting the Elongation Factor 1-alpha (EF1α) gene. Complementary DNA (cDNA) was synthesized from 1.5 µg of

extracted RNA using RevertAid® H Minus Reverse Transcriptase (Thermo Fisher Scientific, USA), anchored with Oligo(dT)23 primer (Thermo Fisher Scientific, USA), as previously described [43].

### 4.3.5. Gene expression analysis

Quantitative real-time PCR (qPCR) experiments were performed in a StepOne™ Real-Time PCR system (Applied Biosystems, Sourceforge, USA) using the iTaq™ Universal SYBR Green Supermix (Bio-Rad, USA), following manufacturer's instructions. Each reaction contained 2.5 mM MgCl2 and 2 µM of each primer were used in 10 µL volume reactions, with 1 µL of cDNA (diluted 1:10) as template. A negative control without cDNA was included in each set of reactions. For all genes, thermal cycling started with a 95 °C denaturation step for 10 minutes followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing at gene specific temperature for 30 seconds. Three biological replicates and two technical replicates were performed for each experimental set. Gene expression (fold change) was calculated as described by [44]. EF1α and Ubiquitin-conjugating enzyme (UBQ) coding genes were used as reference genes as described in [45]. Primer sequences and reaction details are provided in Table 4.1.

**Table 4.1.** Target and reference gene oligonucleotide sequences and reaction details

| Identifier/NCBI Assession Number | Primer sequence (5´-3´) | Amplicon length (bp) | Amplification Efficiency (E) | Ta (ºC) | Tm (ºC) |
|---|---|---|---|---|---|
| Reference genes | | | | | |
| *EF1α* | F: AACCAAAATATCCGGAGTAAAAGA | 150 | 1.99 | 60 | 79.91 |
| XM_002284888.2 | R: GAACTGGGTGCTTGATAGGC | | | | |
| *UBQ* | F: GTGGTATTATTGAGCCATCCTT | 182 | 1.91 | 60 | 84.23 |
| XM_002284161.3 | R: AACCTCCAATCCAGTCATCTAC | | | | |
| Target genes | | | | | |
| *FAD6* | F: CATGGTTGGGTTATCACTTCT | 147 | 1.99 | 60 | 78.72 |
| XM_003634815.2P48 | R: CTATCCAACGAGGGTAATCAC | | | | |
| *FAD8* | F: GGCACTTTCCCTCCTCCTT | 151 | 1.91 | 58 | 79.77 |
| XM_002264314.4 | R: GGGCCTTATGCCACATTCT | | | | |

### 4.3.6. Statistical analysis

In order to analyse the statistical significance of FA profile and gene expression modulation, statistical analysis was performed by the Mann-Whitney U test using IBM® SPSS® Statistics software (version 23.0; SPSS Inc., USA). Results yielding a p value $< 0.05$ were considered statistically significant.

## 4.4. Results and Discussion

### 4.4.1. Fatty acid modulation events and expression of FAD6 and FAD8 genes show a differential pattern in the incompatible interaction with *P. viticola*, *E. necator* and *B. cinerea*

JA signalling occurs mainly in the incompatible interaction with necrotrophs [17]. Nonetheless, it was previously shown that the grapevine incompatible interaction with *P. viticola*, a biotrophic oomycete, leads also to JA biosynthesis and signalling events [24]. It was also shown that the levels of the JA precursor C18:3, are constitutively higher in tolerant cultivars and altered in both tolerant and susceptible cultivars after *P. viticola* challenge [25]. Taking into account that *E. necator* is also a biotrophic pathogen, we hypothesize that JA and FA associated signalling might be part of a conserved mechanism occurring in the first hours of the establishment of grapevine incompatible interactions with both biotrophic and necrotrophic pathogens. In order to unveil how pathogens with different life cycles and invasion strategies trigger FA associated signalling, we have analysed the FA profile of grapevine leaves in the first hours after inoculation with two biotrophs - *P. viticola* (Figure 4.2) and *E. necator* (Figure 4.3) – and a necrotroph - *B. cinerea* (Figure 4.4).

At 6hpi with *P. viticola*, *V. vinifera* c*V.* Regent presents a decrease of the levels of both palmitic and stearic acids (C16:0 and C18:0 respectively) and an increase of the levels of C18:1, C18:2 and C18:3 (Figure 4.2). In plant leaves FA biosynthesis, which takes place in the chloroplast, the saturated FA C16:0 and C18:0 are formed. In turn, C18:0 is desaturated into C18:1 in a reaction catalysed by Δ9-stearoyl-ACP desaturase [46]. Both FA are incorporated in membrane glycerolipids and C18:1 undergoes progressively desaturation catalysed by plastidial or ER membrane desaturases into C18:2 and C18:3 [47]. C16:0, once incorporated in membrane lipids, can also be converted in C16:1*t* (trans-3-hexadecanoic acid), but exclusively in a specific chloroplast phospholipid, PG [48].

C18:1 was previously shown to participate in plant defence mechanisms by binding to proteins with anti cell death effect [49], increase of endogenous nitric oxide [50] or azelaic acid biosynthesis, contributing to SAR [51]. This result suggests that there is a lower biosynthesis or incorporation of C16:0 in membrane glycerolipids and C18:1 is likely being successively desaturated, leading to the formation of C18:3. This FA, once hydrolysed from membrane glycerolipids mediated by PLA [25], might be channelled for the production of JA, which will trigger JA associated defence mechanisms. Upon inoculation with *P. viticola*, we observed higher levels of unsaturated FA including an increase of the levels of C16:1t suggesting protection of the photosynthetic machinery upon pathogen challenge, given the importance of PG in this process. In sum it appears that the lipid synthesis machinery may be oriented towards the formation of C18:3 favouring the biosynthesis of JA and also to a higher production of chloroplast membrane lipids [25].

At 24hpi no significant differences were observed in the levels of C16:0 and C18:0 compared to mock treated leaves (Figure 4.2). C18:1 and C18:2 show a decreasing of their levels whereas C18:3 content increases. The modulation of the FA levels observed at 24hpi suggest that the formation of C18:1 and C18:2 tended to return to basal levels but the desaturation reaction leading to the production of C18:3 is still higher and the JA associated defence mechanisms are still being triggered at this time point. Nonetheless, the FA levels are returning to homeostasis.

Previous works report C18:3 accumulation in Regent after inoculation with *P. viticola* [38]. Furthermore, an increase of the levels of unsaturated FA had already been associated to tolerance to fungal and bacterial pathogens (reviewed in Walley et al., 2013). These results, together with the observations from the present work, highlight the importance of 18 carbons' FA in plant defence mechanisms.

Considering the interaction between grapevine and *E. necator*, at 6hpi there is a decrease of the levels of C16:0 and a similar tendency for C18:0 and C16:1t (Figure 4.3). Furthermore, it is observable that C18:2 levels decrease and those of C18:1 and C18:3 increase after pathogen challenge. The observed alterations might suggest that the formation of C18:3 is stimulated, despite the apparent lack of up-regulation of the desaturation reactions leading to the formation of 18:1 and 18:2, resulting in the increase of these FA, as also seen for *P. viticola.* At 24hpi, the only significant alterations compared to mock treated leaves are the increase of C18:2 accompanied by the decrease of the C18:3 content. This result suggests that at this time point, the signalling cascades leading to the triggering of JA related defence mechanisms may have slowed down and the synthesis of its precursor, C18:3, is no longer stimulated.

Although both pathogens trigger common alterations regarding the overall trends of FA modulation, it seems that in *P. viticola* there is a sharper distinction between the two time points studied, with bigger changes taking place at 6hpi. In addition, the fold changes in the FA relative amounts are higher in plants infected with *P. viticola* compared to those treated with *E. necator.*

There is a decrease in the levels of C18:3, after an increase at 6hpi. During powdery mildew infection process, the fungal carbohydrate metabolism machinery suffers a contraction [52]. In this situation, plant FA may arise as an alternative carbon source for *E. necator*. Keymer and collaborators had previously observed in arbuscular mycorrhiza that reduced plant FA biosynthesis impairs pathogenic fungal infection [53].

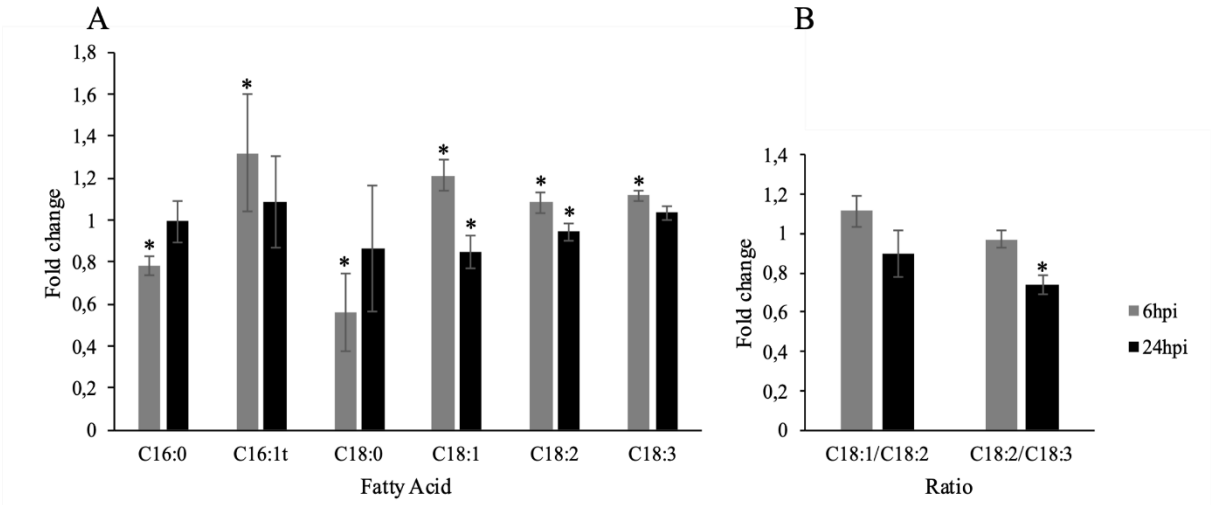


**Figure 4.2.** (A) FA profile (B) C18:1 to C18:2 (C18:1/C18:2) and C18:2 to C18:3 (C18:2/C18:3) ratios of *V. vinifera* cv Regent leaves mock treated and inoculated with *P. viticola* at 6 and 24hpi. hpi – hours post inoculation. Values correspond to relative amounts fold changes between inoculated and mock treated samples. N=5. Error bars represent the standard deviation, calculated as follows: Fold change sd = $\left|\frac{A}{B}\right| \times \sqrt{\left(\left(\frac{sdA}{A}\right)^2 + \left(\frac{sdB}{B}\right)^2\right)}$. A: inoculated group; B: mock treated group; sd: standard deviation. Asterisks indicate significant differences ($p < 0.05$).

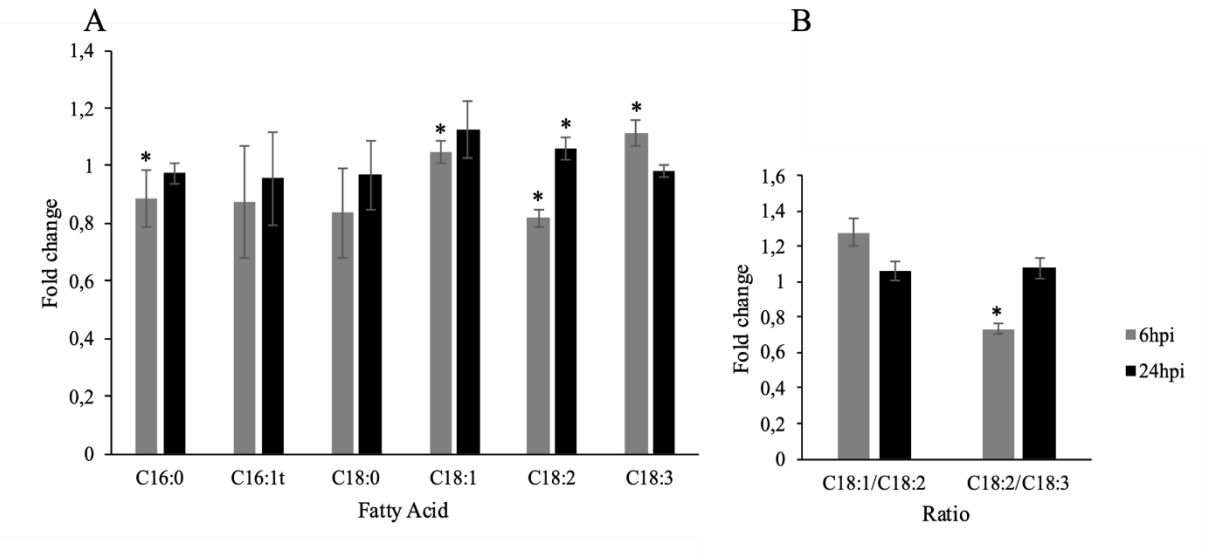


**Figure 4.3.** (A) FA profile (B) C18:1 to C18:2 (C18:1/C18:2) and C18:2 to C18:3 (C18:2/C18:3) ratios of *V. vinifera* cv Regent leaves mock treated and inoculated with *E. necator* at 6 and 24hpi. hpi – hours post inoculation. Values correspond to relative amounts fold change between inoculated and mock treated samples. N=5. Error bars represent the standard deviation, calculated as follows: Fold change sd = $|\frac{A}{B}| \times \sqrt{(\left(\frac{sdA}{A}\right)^2 + \left(\frac{sdB}{B}\right)^2)}$. A: inoculated group; B: mock treated group; sd: standard deviation. Asterisks indicate significant differences ($p < 0.05$).

Considering the incompatible interaction between grapevine and *B. cinerea* (Figure 4.4), at 6hpi there is an increasing of the levels of C16:0, C16:1t and C18:2, while the levels of C18:3 decrease. Similarly, to what is observed in the interaction with *P. viticola* an enrichment in C16:1t occurs upon inoculation with *B. cinerea*, which may also suggest a modulation of the chloroplast membrane in order to protect the photosynthetic apparatus during the plant response to this pathogen. At 24hpi an increasing of the levels of C18:3 occurs, while the levels of the remaining C18 FA present a tendency to decrease. This observation is indicative that progressive desaturation of C18 FA is still occurring and that the synthesis of C18:3 is being highly stimulated.

Contrarily to what is observed in the interactions with the biotrophic pathogens, where C18:3 tends do decrease back to basal levels, in the interaction with *B. cinerea* the FA mediated signalling seems to be prolonged for a longer period. Since *B. cinerea* is a necrotrophic pathogen JA signalling will be the main activated pathway [17], which corroborates the suggestion that the FA modulation events that occur during the grapevine-*B. cinerea* interaction are oriented to the biosynthesis of JA. Considering the FA modulation events during the interaction with the biotrophic pathogens at study, they seem to suggest that in the first hours post inoculation the JA pathway may be induced. Later in the interaction, other pathways may be induced to overcome the infection, including the antagonistic salicylic acid (SA) mediated pathway, which is also known to be activated during grapevine interaction with biotrophic pathogens [54].

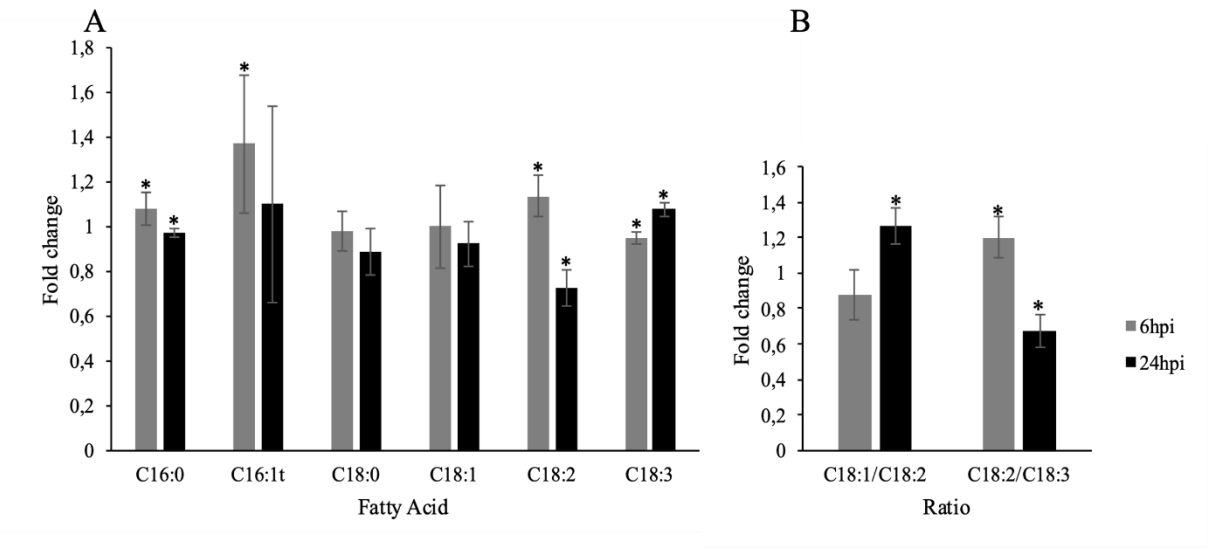


**Figure 4.4.** (A) FA profile (B) C18:1 to C18:2 (C18:1/C18:2) and C18:2 to C18:3 (C18:2/C18:3) ratios of *V. vinifera* cv Regent leaves mock treated and inoculated with *B. cinerea* at 6 and 24hpi. hpi – hours post inoculation. Values correspond to relative amounts fold change between inoculated and mock treated samples. N=5. Error bars represent the standard deviation, calculated as follows: Fold change sd = $\left|\frac{A}{B}\right| \times \sqrt{\left(\left(\frac{sdA}{A}\right)^2 + \left(\frac{sdB}{B}\right)^2\right)}$. A: inoculated group; B: mock treated group; sd: standard deviation. Asterisks indicate significant differences ($p < 0.05$).

Considering the three interactions at study, differences regarding the evolution of the C18:1/C18:2 and C18:2/C18:3 ratios along the infection were observed (Figure 4.2Figure 4.3Figure 4.4B). Upon inoculation with *P. viticola* there was an increase of the C18:1/C18:2 ratio at 6hpi, while at 24hpi no significant alteration occurred. Regarding the C18:2/C18:3 ratio there was a trend for a decrease at 24hpi (Figure 4.2B). Concerning the interaction with *E. necator*, the major alterations occurred at 6hpi. While 18:1/C18:2 increased, returning to near basal levels at 24hpi, C18:2/C18:3 decreased at the early time point, showing a slight increase in comparison to mock treated leaves at 24hpi (Figure 4.3B). During the interaction with the necrotrophic pathogen the C18:1/C18:2 ratio showed a tendency to decrease at 6hpi and increased at 24hpi. On the other hand, C18:2/C18:3 increased at 6hpi and decreased at 24hpi, due to a seemingly higher induction of C18:3 biosynthesis in the later time point of the interaction.

Since the analysed ratios showed differences along the infection process in the three pathosystems, and the bulk of these FA in leaves are present in plastidial lipids we analysed the expression of the genes encoding for the plastidial enzymes responsible for the formation of C18:2 (FAD6) and C18:3 (FAD8) (Figure 4.5). The C18:1/C18:2 ratio may reflect the activity of FAD6 in the chloroplast [55], which is where a number of pathways are induced in stress conditions, including the JA synthesis [56]. The C18:2/C18:3 ratio may be related to the activity of FAD8 in the chloroplast [57]. The activity of this enzyme is crucial for the formation of C18:3 and consequently can impact the formation of JA.

Concerning the expression profile of FAD6, while in the interaction with *P. viticola* and *E. necator* there was an up-regulation at 6hpi and a down-regulation at 24hpi, the opposite scenario was observed in the interaction with *B. cinerea*. Furthermore, FAD8 suffered a down-regulation at both time points in the interaction with the biotrophs while in the interaction with the necrotrophic pathogen a tendency similar to the FAD6 expression profile was observed. Therefore, an expression pattern can be distinguished between the interaction with biotrophs and the necrotroph.

The FA mediated signalling events can be partly corroborated by the FAD expression profiles. Concerning the incompatible interaction with *B. cinerea*, there is an up-regulation of both genes at 24hpi

after a down-regulation at the earlier time-point. These results suggest that there may be a more enduring stimulation of polyunsaturated FA (PUFA) biosynthesis that may lead to the induction of the JA pathway. This is in accordance with the higher levels of the JA precursor observed at 24hpi in this interaction (Figure 4.4A).

Concerning the grapevine incompatible interaction with the biotrophic pathogens at study, *FAD6* suffered an up-regulation at 6hpi followed by a down-regulation. This result is also in accordance with the observed FA profile along the inoculation, since in the interaction with the biotrophic pathogens there were major alterations at the earlier time-point, with a tendency to return to basal levels at 24hpi. *FAD8* expression profile showed some differences between *P. viticola* and *E. necator*. Although in both pathosystems *FAD8* was down-regulated, in the interaction with *P. viticola* the expression showed to be closer to mock treated leaves. On the other hand, in the interaction with *E. necator* this gene was down-regulated at 6hpi, followed by a lower expression at 24 hpi. This result also agrees with the observed FA profile (Figure 4.3A) since the C18:3 levels decreased in later time-points.

Taken together these results indicate that FAD6 and FAD8 play an important role in the incompatible interaction between grapevine and the pathogens at study and their expression might be differently triggered according to the pathogen's lifestyle.

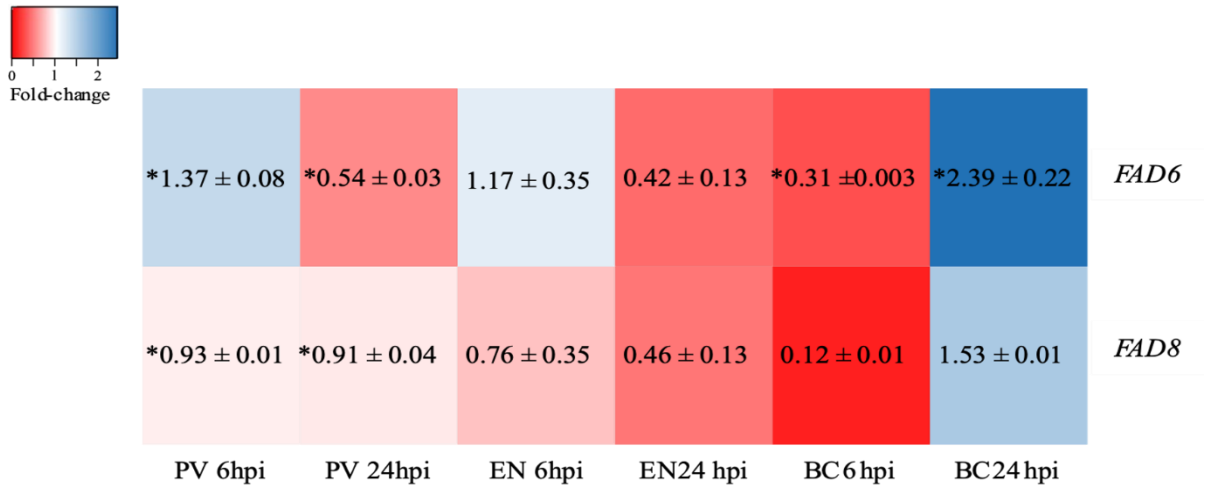


**Figure 4.5.** Expression profile of FAD6 and FAD8 in *V. vinifera* cv Regent leaves inoculated with *P. viticola*, *E. necator* and *B. cinerea* at 6 and 24hpi. hpi – hours post inoculation. PV – *Plasmopara viticola*. EN – *Erysiphe necator*. BC – *Botrytis cinerea.* Fold-change values are relative to expression in mock treated leaves. N=6. Asterisks indicate significant differences ($p < 0.05$).

### 4.4.2. Fatty acid modulation impacts membrane fluidity during plant-pathogen interaction

The observed alterations in FA profiles affected the number of double bounds, impacting the double bond index (DBI), that reflects membrane fluidity [58]. In the interaction with the biotrophic pathogens, 6hpi is the time point where major FA modulation occurs. At 6hpi, DBI increased, to a higher extent under *P. viticola* inoculation, while at 24hpi no significant differences were observed (Figure 4.6). In the interaction with *B. cinerea*, where the major FA modulation events occurred at 24hpi, there was a decrease in the DBI followed by an increase (Figure 4.6). The adjustment of the membrane fluidity consists in a plant stress response that allows the cell to keep a sustainable environment to maintain its normal function [59]. Therefore, the increase of the DBI (reflecting a higher membrane fluidity) that

was observed in all the pathosystems in a time wise accordance with the FA modulation events, may avoid membrane damage and, consequently, impairment of the energy transduction pathways and primary metabolism [14].

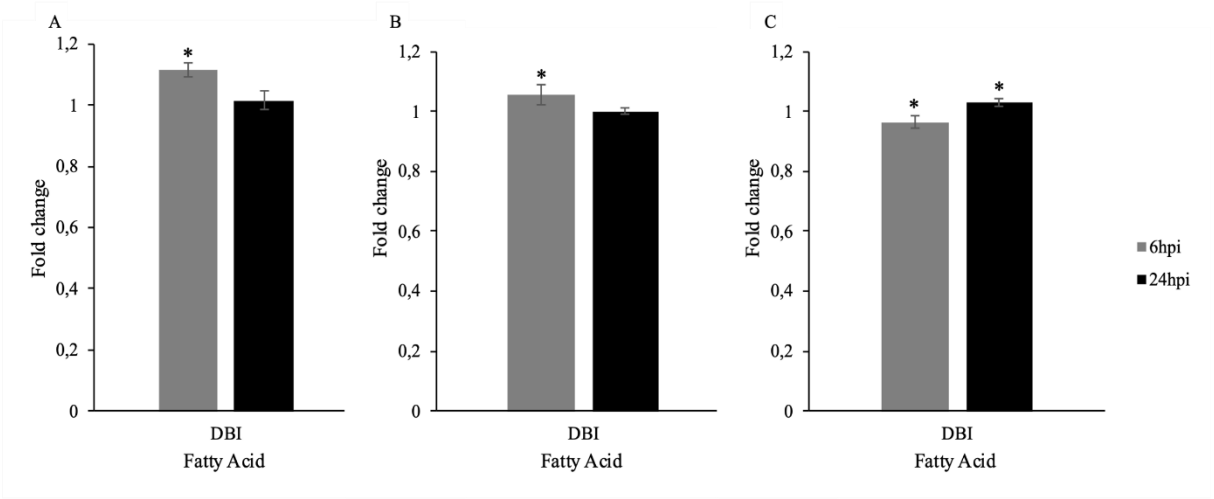


**Figure 4.6.** Double bond index (DBI) of *V. vinifera* cv Regent leaves mock treated and inoculated with *P. viticola* (A), *E. necator* (B) and *B. cinerea* (C) at 6 and 24hpi. hpi – hours post inoculation. Values correspond to DBI fold change between inoculated and mock treated samples. N=5. Error bars represent the standard deviation, calculated as follows: Fold change sd = $|\frac{A}{B}| \times \sqrt{(\left(\frac{sdA}{A}\right)^2 + \left(\frac{sdB}{B}\right)^2)}$. A: inoculated group; B: mock treated group; sd: standard deviation. Asterisks indicate significant differences ($p < 0.05$).

### 4.5. Conclusions

Our results provide new insights on the FA associated signalling in grapevine defence against two biotrophs (*P. viticola* and *E. necator*) and a necrotroph (*B. cinerea*). The relevance of FA signalling in the incompatible interaction with these pathogens is shown. At 6hpi, the observed FA modulation events during the incompatible interaction with the biotrophs suggest that C18:0 is being progressively desaturated to C18:3, a precursor of JA and at the later time point tend to return to basal levels. On the other hand, during the interaction with *B. cinerea*, the induction of PUFA synthesis continues to occur at 24hpi. Previous results showed that JA-mediated pathways are activated against *P. viticola*. In the present work the observed FA modulation upon inoculation with *E. necator* suggests that the JA-mediated mechanism may be conserved between both biotrophic pathosystems. The results obtained in this work may suggest that in the response against biotrophs the JA-mediated signalling may be activated in the first hours of the infection. This is consistent with the fact that in these pathosystems the antagonistic SA-mediated pathway is activated along the incompatible interaction. On the other hand, the FA modulation events observed during the interaction with *B. cinerea* are consistent with the fact that the JA-mediated pathway is mainly activated against necrotrophs. *FAD6* and *FAD8* expression profiles are generally corroborated by the FA modulation events. Moreover, these results indicate that these enzymes play an important role in the incompatible interaction between grapevine and the pathogens at study and their expression might be differently modulated according to the pathogen's lifestyle. Alterations of the DBI were also observed in all the pathosystems, which reflects membrane fluidity. An adjustment of this feature may be crucial to maintain cellular function during the infection. Taken together, the results obtained in the present work provide evidence that a distinct FA meditated

signalling pattern is detectable between grapevine interaction with biotrophs (*P. viticola* and *E. necator*) which, despite have different invasion strategies seem to generally trigger a similar process and necrotrophs (*B. cinerea*). While the interaction with the biotrophic pathogens may trigger a higher synthesis of PUFA at early time-points with a latter tendency to return to basal levels, the interaction with *B. cinerea* may trigger a later and more durable induction of PUFA synthesis.

### 4.6. Author contribution

AF, ARM and ARC conceived the study and planned the experiment; JED and JC were responsible for the plant material; GL and ARC performed the experiments; AF, ARM, ARC and GL performed data analysis. AF, ARM, ARC and GL wrote the manuscript. ARM and AF are co-senior authors in this work. All authors have read and approved the manuscript.

### 4.7. Acknowledgements

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# CHAPTER V

## 5. Subtilisin-like proteins and lipid signalling events: the missing links in grapevine resistance to *Plasmopara viticola*

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## 5.1. Abstract

The reduction of phytochemicals used to control pests and diseases is one of the demands on modern viticulture. Grapevine is one of the most important crops grown in temperate climates where Europe's wine industry represents 40% of the world production. The cultivated grapevine, *Vitis vinifera,* is prone to several diseases, being downy mildew one of the most devastating. Preventive fungicide applications are used on each growing season to control disease incidence with major environmental and economic constrains. A deeper knowledge on the grapevine-*P. viticola* interaction is needed to define alternative disease control strategies.

We have shown that, during the first hours of interaction with *P. viticola,* the modulation of chloroplast-associated lipids is important for protection of the photosynthetic machinery and biosynthesis of jasmonic acid (JA). We have also identified subtilisin-like proteases as strong resistance-associated candidates. In the present study, we have accessed the link between JA elicitation and both fatty acid (FA) and immunity-related subtilase expression modulation. Our results show that FA modulation after JA elicitation is similar to the previously described after *P. viticola* inoculation and that immunity-related subtilase expression also increases in the tolerant genotype, particularly the subtilase VviSBT5.3a, thus suggesting a shared mechanism.

**Keywords:** subtilase, downy mildew, elicitation, fatty acids, *Vitis vinifera,* jasmonic acid

## 5.2. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important fruit crops worldwide, with high economic importance particularly in Portugal, where it accounted for 680 million euro of exports in 2016 [1]. Of the fungal diseases affecting grapevine production, downy mildew, caused by the biotroph oomycete *Plasmopara viticola* (Berk. et Curt.) and De Toni, is one of the most important and widely studied (reviewed in [2]). Our previous studies on this pathosystem have shown that, during the first hours of interaction, several events occur on tolerant genotypes, involving lipid modulation, jasmonic acid (JA) production and activation of serine proteases from the subtilase family [3–5].

Subtilases are the second largest family of serine peptidases that, despite presenting a wide range of biological functions in plants, have been shown to participate actively on defence responses against abiotic and biotic stresses (reviewed in [6]). In grapevine, the first clues highlighting subtilase participation in defence mechanisms were reported by our group [7, 8]. Gindro et al. (2012) have also reported that serine protease inhibitors influenced grapevine tolerance to *P. viticola* [9]. We have characterized the subtilase gene family in grapevine and showed that some subtilases sharing sequence similarity with Arabidopsis SBT3.3 and tomato P69 (associated to immune priming events) are located near *P. viticola* resistance associated loci (*Rpv*) and are strongly induced as soon as 6h after inoculation with the downy mildew pathogen [5].

It was shown previously that the signalling pathways associated to grapevine resistance to *P. viticola* are linked to lipid signaling, namely through JA [3, 4, 10–12]. We have shown that *Vitis vinifera* cv 'Regent' presents an early up-regulation of enzymes involved in JA biosynthesis and later up-regulation for JA signalling enzymes [4], together with a greater accumulation of both JA, JA-Isoleucine and salicylic acid in the first hours of interaction [4]. Non-specific lipid transfer proteins as well as plastid lipid-associated proteins (also named fibrillins) were also accumulated in 'Regent' suggesting lipid transport [12]. We have also shown that, after *P. viticola* inoculation, lipid content is altered, leading to membrane lipid modulation events that lead to accumulation of C18:3 (JA precursor) [3].

The first clue into a link between subtilases and JA signaling was reported in *Sorghum bicolor* where, after elicitation with methyl jasmonate (MeJA), subtilase expression increased [13]. Also, in cotton

plants, studies have described GbSBT1 subtilase activation after inoculation with *Verticillium dahliae* and plant elicitation with JA [14]. One of the links between JA signalling and subtilases may be associated to one of the few known subtilase substrates, prosystemin [15]. After cleavage by subtilase, prosystemin generates systemin, which plays a fundamental role in response to herbivore damage or mechanical wounding through the activation of the octadecanoid pathway for JA biosynthesis [16]. Very recently, it was found that prosystemin is processed into systemin by a specific type of subtilase named phytaspase [17]. Phytaspases are a group of subtilases associated with programmed cell death (PCD) in plants exposed to biotic and abiotic stresses [18].

The present work provides a preliminary investigation of the possible link between grapevine immunity-related subtilase activation and JA. Based on our previous results of gene expression of grapevine subtilases upon *P. viticola* inoculation, we have selected the four more expressed genes (*VviSBT3.20, VviSBT3.21 Isoform X1, VviSBT4.*19 *Isoform X1* and *VviSBT5.3a*). Modulation of fatty acid (FA) profile and subtilase expression were assessed in two grapevine genotypes with different tolerance to *P. viticola* (*Vitis riparia,* tolerant and *V. vinifera* cv 'Pinot noir', susceptible) after plant elicitation with JA.

## 5.3. Material and methods

### 5.3.1. Plant Material and elicitation experiments

*Vitis riparia* and *Vitis vinifera* c*V.* Pinot Noir genotypes were selected for this study. *Vitis Riparia* presents a high degree of resistance against several grapevine pathogens. *V. vinifera* c*V.* Pinot Noir is one of the most widely used cultivars for wine production, but very susceptible to pathogens such as *P. viticola*. Plants from both genotypes were obtained from the commercial nursery *Vitis* Oeste, Portugal, and kept under controlled conditions in a growth chamber (natural day/night rhythm, relative humidity 60% and a photosynthetic photon flux density of 300 µmol $m^{-2}$ $s^{-1}$). Grapevine leaves were elicited with 1 mM JA (Sigma Aldrich) in 0.05% (v/v) TWEEN 20 solution, by spraying the entire plant. Control plants were sprayed with a 0.05% (v/v) TWEEN 20 alone. The second and third fully expanded leaves beneath the shoot apex were harvested at 6 and 24 h post elicitation (hpe), immediately frozen in liquid nitrogen and stored at −80°C. Three biological replicates were collected, being each biological replicate a pool of three leaves from three different plants.

### 5.3.2. Fatty acid analysis

Direct acid transesterification was performed in order to determine FA composition. Twenty micrograms of internal standard (margaric acid) was added to 50 mg of each sample of ground *V. riparia* and *V. vinifera* cv 'Pinot Noir' leaves, followed by the addition of Methanol/Sulfuric acid solution (39:1 v/v). Methylation was run for 1h at 70°C and was stopped by cooling. The fatty acid methyl esters (FAME) obtained were rescued by adding petroleum ether and ultrapure water (3:2), followed by briefly vortexing and centrifugation (3000 rpm, 5min). The upper phase was collected and evaporated to dryness at 37°C under nitrogen atmosphere. The samples were re-suspended in hexane prior to FAME separation in a gas chromatograph (3900 Gas Chromatograph, Varian, Palo Alto, CA, USA) equipped with a hydrogen flame ionization detector using a fused silica 0.25 mm i.d. × 50 m capillary column (WCOT Fused Silica, CP-Sil 88 for FAME, Varian).

The double-bond index (DBI), a measure of the unsaturation degree of the FA pool was calculated according to the following equation [19]:

$$DBI = \frac{\%C16{:}1t + \%C18{:}1 + 2 \times \%C18{:}2 + 3 \times \%C18{:}3}{100}$$

### 5.3.3. Quantitative Real-Time PCR

Total RNA was isolated from frozen leaves with the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA), according to manufacturer's instructions. Residual genomic DNA was digested with DNase I (On-Column DNase I Digestion Set, Sigma-Aldrich, USA). RNA purity and concentration were measured at 260/280 nm using a spectrophotometer (NanoDrop-1000, Thermo Scientific) while RNA integrity was verified by agarose gel electrophoresis (1.2% agarose in TBE buffer). Genomic DNA (gDNA) contamination was checked by qPCR analysis of a target on the crude RNA (Vandesompele *et al*., 2002). cDNA was synthesized from 2.5 µg of total RNA using RevertAid®H Minus Reverse Transcriptase (Fermentas, Ontario, Canada) anchored with Oligo$(dT)_{23}$ primer (Fermentas, Ontario, Canada), according to manufacturer's instructions.

qPCR experiments were carried out using Maxima™ SYBR Green qPCR Master Mix (2×) kit (Fermentas, Ontario, Canada), according to manufacturer's instructions, in a StepOne™ Real-Time PCR system (Applied Biosystems, Sourceforge, USA). Primer sequences and reaction details are provided in Table 5.1

**Table 5.1.** Thermal cycling for all genes started with a denaturation step at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing (Table 5.1) for 30 s. Each set of reactions included a control without cDNA template. Dissociation curves were used to analyse non-specific PCR products. Three biological replicates and two technical replicates were used for each sample. Gene expression (fold change) was calculated as described by [20]. The reference genes used for the normalization were the previously described by [21]. Statistical significance ($p < 0.05$) of gene expression was determined by the Mann–Whitney U test using IBM® SPSS® Statistics version 23.0 software (SPSS Inc., USA).

## 5.4. Results and discussion

In both genotypes, JA elicitation promoted FA modulation (Figure 5.1), mostly that of the polyunsaturated FAs (PUFAs). The contents of oleic (C18:1) and linoleic (C18:2) acids decreased, while α-linolenic acid (C18:3) levels increased after elicitation in both genotypes. The decrease of both C18:1 and C18:2 may be due to desaturation reactions, leading to the synthesis of C18:3 [22]. In our previous work, similar FA-modulation events were observed after inoculation with *P. viticola* only in the tolerant *V. vinifera* 'Regent', with no significant modulation occurring in the susceptible genotype *V. vinifera* 'Trincadeira' [3]. Elicitation with JA seems to promote an FA modulation in both genotypes similar to that triggered by *P. viticola* in tolerant plants; thus, it may elicit immunity of both tolerant and susceptible genotypes prior to pathogen challenge. Comparison of the two genotypes studied shows that the variation of FA levels, including PUFAs, saturated and mono-unsaturated acids, is more intense at 24 hpe in *V. vinifera* 'Pinot Noir' (Figure 5.1), suggesting that JA elicitation may lead to a more intense immune priming response in the susceptible cultivar.

**Table 5.1.** Target and reference gene oligonucleotide sequences, amplicon length, annealing, and melting temperature and amplification efficiency

| Gene name | NCBI Accession Number | Primer sequence | Amplicon length (bp) | Ta (°C) | Tm (°C) | Amplification efficiency (E) |
|---|---|---|---|---|---|---|
| **Reference genes [21]** | | | | | | |
| ***EF1α* (elongation factor 1-alpha)** | *XM_002284888.3* | F: GAACTGGGTGCTTGATAGGC<br>R: ACCAAAATATCCGGAGTAAAAGA | 164 | 60 | 79.59 | 1,82 |
| **Subtilases target genes** | | | | | | |
| ***VviSBT3.20*** | *XM_002273159.3* | F: CAAGCCCCATTAGCACAC<br>R: TTAGAATCAAGATCAAAGAAG | 87 | 56 | -[1] | -[1] |
| ***VviSBT3.21 Isoform X1*** | *XM_010649370.1* | F: GGGATATGGCCTGAGTCTGA<br>R: CAACGCGCACCGATTATTTT | 134 | 60 | 79.30 | 2.08 |
| ***VviSBT4.19 Isoform X1*** | *XM_010660203.1* | F: AATCCTGGTGTTCTTGTGG<br>R: ATTAGGTAAAATGTTGTGCTTG | 73 | 58 | 71.96 | 1.88 |
| ***VviSBT5.3a*** | *XM_010659200.1* | F: CAGCGAGTTTTAGTGATGAAG<br>R: GGGGTATGGAAGGAAGAGT | 172 | 58 | 79.77 | 2.08 |

1 Discarded due to low transcript abundance

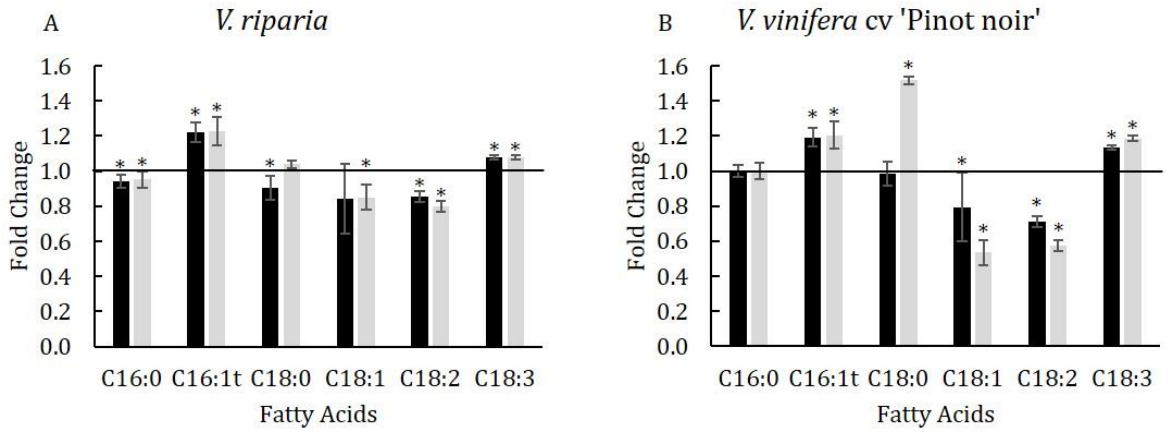


**Figure 5.1.** Changes in fatty acid variation of *V.* riparia (A) and *V. vinifera* 'Pinot Noir' (B) at 6 (black columns) and 24 (grey columns) h post elicitation with jasmonic acid. Values correspond to the ratio between the control and the elicited samples ± SEM, n=3. Asterisks indicate significant differences ($p<0.05$).

The observed alterations at the PUFA level led to an increase of the DBI (Figure 5.2). A higher DBI reflects an increase of membrane fluidity and consequently membrane permeability [23]. Under biotic and/or abiotic stress, plants have the ability to adjust lipid membrane fluidity in order to maintain a suitable environment for the function of integral proteins [24]. In our previous work, we observed a strong modulation of the levels of the chloroplast membrane lipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) [3], which are very rich in C18:3. The content of these fatty acids increased in plastidial lipids, leading to an increased DBI. This chloroplast membrane lipid modulation suggests a mechanism for protection of the photosynthetic membranes. After elicitation, similar mechanisms may be triggered, increasing plant protection to subsequent biotic challenges.

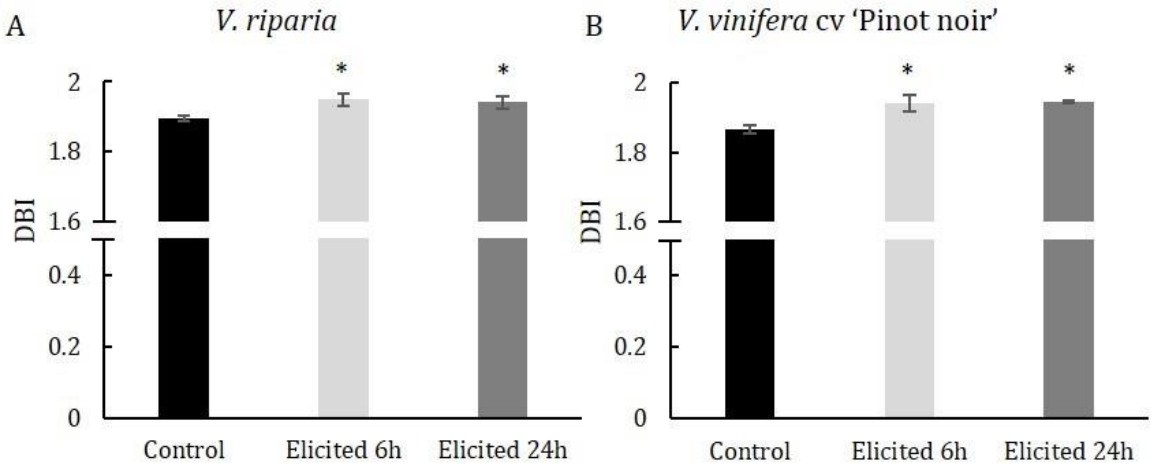


**Figure 5.2.** Double-bond index (DBI) of *V.* riparia (A) and *V. vinifera* 'Pinot Noir' (B) at 6 and 24 hpe with jasmonic acid. Values correspond to mean relative percentages ± SEM, n=4. Asterisks indicate significant differences ($p<0.05$).

We have previously assessed the expression of 14 subtilase genes after *P. viticola* challenge and, although the expression profile increased for the majority of the tested subtilases, a delay was observed when comparing susceptible and tolerant grapevine genotypes [5], suggesting that an early increase in expression is a feature of resistant grapevines. The four grapevine subtilases that were shown to present the higher expression increase after *P. viticola* challenge were selected and analysed prior to and after JA elicitation (Figure 5.3).

After elicitation with JA, the gene expression profile was altered in both genotypes. VviSBT4.19 isoform X1 expression was downregulated in *V. vinifera* 'Pinot Noir' with JA at both 6 (0.54±0.29) and 24 (0.04±0.06) hpe and upregulated in *V.* riparia (6 hpe, 3.34±2.96; 24 hpe, 4.16±2.60). VviSBT5.3a presented a very large increase in expression after JA signalling in *V.* riparia (6 hpe, 90.20±39.94; 24 hpe, 81.13±20.29). Our results are in agreement with those described elsewhere for *V. vinifera* 'Regent' [25], thus suggesting that both subtilases may be linked to JA signalling pathways in *P. viticola*-tolerant genotypes. Moreover, despite the fact that lipid modulation is similar in the two genotypes studied (susceptible and tolerant to *P. viticola*), subtilases were only positively modulated in the tolerant genotype, suggesting that their action may be crucial for the establishment of the incompatible interaction.

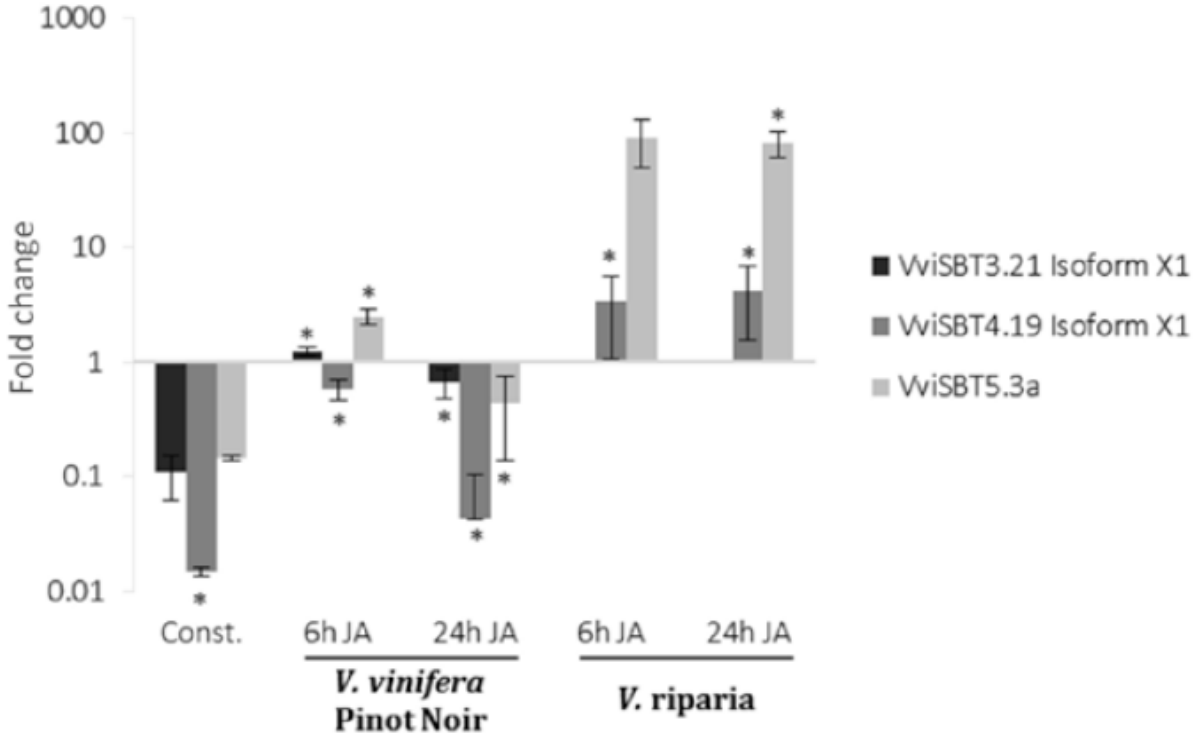


**Figure 5.3.** Constitutive and jasmonic acid-elicited subtilase gene expression profiles in *V. vinifera* 'Pinot noir' and *V.* riparia. Values below 1 correspond to downregulation, values of 1 to mean basal expression, and values greater than 1 to upregulation. Const., Constitutive subtilase gene expression in *V. riparia* compared with *V. vinifera* 'Pinot Noir'; JA, jasmonic acid. Asterisks (*) represent significant differences ($p \leq 0.05$).

## 5.5. Conclusion

Our results suggest that subtilases may be linked to JA signalling pathways in *P. viticola*-tolerant genotypes. After JA elicitation, lipid modulation occurs in both genotypes, leading to a higher availability of the JA substrate C18:3. However, considering that subtilases may be involved in activation of the octadecanoid pathway only in the tolerant genotype, subtilase modulation occurred, particularly of VviSBT5.3a. More studies have to be conducted to prove this hypothesis and the molecular link between subtilase and FA modulation.

## 5.6. Acknowledgements

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# CHAPTER VI

## 6. An optimized apoplastic fluid extraction method for the characterization of grapevine leaves proteome and metabolome from a single sample

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*Figueiredo J*[*]*, Cavaco AR*[*]*, Guerra-Guimarães L, Leclercq C, Renaut J, Cunha J, Eiras-Dias J, Cordeiro C, Matos AR, Sousa Silva M and Figueiredo A. (2021) An apoplastic fluid extraction method for the characterization of grapevine leaves proteome and metabolome from a single sample. Physiol Plantarum, 171: 343-357.* https://doi.org/10.1111/ppl.13198
[*]*These authors contributed equally to this work*

## 6.1. Abstract

The analysis of complex biological systems keeps challenging researchers. The main goal of systems biology is to decipher interactions within cells, by integrating datasets from large scale analytical approaches including transcriptomics, proteomics and metabolomics and more specialized 'OMICS such as epigenomics and lipidomics. Studying different cellular compartments allows a broader understanding of cell dynamics. Plant apoplast, the cellular compartment external to the plasma membrane including the cell wall, is particularly demanding to analyse. Despite our knowledge on apoplast involvement on several processes from cell growth to stress responses, its dynamics is still poorly known due to the lack of efficient extraction processes adequate to each plant system. Analysing woody plants such as grapevine raises even more challenges. Grapevine is among the most important fruit crops worldwide and a wider characterization of its apoplast is essential for a deeper understanding of its physiology and cellular mechanisms. Here, we describe, for the first time, an optimized vacuum-infiltration-centrifugation method that allows a simultaneous extraction of grapevine apoplastic proteins and metabolites from leaves on a single sample, compatible with high-throughput mass spectrometry analyses.

The extracted apoplast from two grapevine cultivars, *Vitis vinifera* cv 'Trincadeira' and 'Regent', was directly used for proteomics and metabolomics analysis. The proteome was analysed by nanoLC-MS/MS and more than 700 common proteins were identified, with highly diverse biological functions. The metabolome profile through FT-ICR-MS allowed the identification of 514 unique putative compounds revealing a broad spectrum of molecular classes.

Abbreviations: APF, apoplastic fluid; BSA, bovine serum albumin; C16:0, palmitic acid; C16:1*t*, trans-3-hexadecanoic acid; C17:0, margaric acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, α-linolenic acid; CHAPS, 3-cholamidopropyl dimethylammonio 1-propanesulfonate; ECS, extracellular space; ER, endoplasmic reticulum; ESI, electrospray ionization; FA, fatty acid; FT-ICR-MS, fourier transform-ion cyclotron resonance-mass spectrometry; GC, gas chromatography; IEF, isoelectric focusing; MDH, malate dehydrogenase; MS, mass spectrometry; NAD, nicotinamide adenine dinucleotide; NBT, nitroblue tetrazolium; PG, phosphatidylglycerol; PMS, phenazine methosulphate; PVP, polyvinylpyrrolidine; SP, signal peptide; TOF, time of flight; UPLC, ultra-performance liquid chromatography; VIC, vacuum-infiltration-centrifugation

**Keywords:** *Vitis vinifera*, extracellular space, nanoLC-MS/MS, FT-ICR-MS, proteins, metabolites

## 6.2. Introduction

The apoplast, referred to as a synonym of extracellular space (ECS), is formed by the whole compartment external to the plant plasma membrane that includes the cell wall, the free space between cells (or intercellular space) and the apoplastic fluid (APF) [1]. In the beginning of the past century, due to the lack of methods and experimental approaches for the correct study of this compartment *in vivo* [2], there was a firm belief that the extracellular space contained mainly water, minerals and low-molecular weight compounds involved in metabolism [3]. The difficulty in obtaining sufficient apoplastic material without damaging the plant cell and, thus, avoiding potential contamination with cytoplasmic contents also contributed to the delay in APF characterization. Over the last decade, technological advances (e.g.; mass spectrometry analysis and database information availability) allowed a thorough characterization of this highly dynamic compartment highlighting its role in cellular metabolism [4, 5]. The ECS participates in plant signalling, growth, physiology, cell wall maintenance and reproduction and is affected by environmental conditions [6, 7]. In the case of roots, microorganisms that colonize this compartment contribute to the nutrition of higher plants through its ability to fix di-

nitrogen (reviewed in [8]. Apoplast is also responsible for water vapor and gas exchange involved in transpiration and photosynthesis, and for amino acid trafficking to the phloem [9].

APF studies have also uncovered plant's secretome that comprises the set of all proteins and small molecules that are exported out of the symplast. Several roles were assigned to secreted proteins through APF proteomics approaches [1, 4, 10–12]. In contrast, transcriptomic [13] and metabolomics [14–16] studies of plant apoplast are rare and in what concerns lipidomics it remains a black box [17].

The most commonly used technique for plant APF extraction is the vacuum infiltration- centrifugation (VIC), described by Klement and co-workers in 1965 [18] consisting on vacuum-infiltration with appropriate extraction buffer and centrifugation [5]. The composition of the extraction buffer and both infiltration and centrifugation procedures may vary significantly depending on the plant species and the downstream goal of the APF extraction. Several detailed centrifugation-based methods for extracting APF from plant leaves were described for herbaceous plants as Arabidopsis [14, 19], common bean [5, 16], maize [5, 9]; faba bean, peas, spinach [5], among others. For woody plants, leaf infiltration raises more challenges due to the morphology of the leaves that impairs infiltration buffer accessibility, but several VIC based studies were also described for poplar, eucalyptus, prunus, bitter orange tree, apricot, peach, grapevine and coffee [20–24].

Grapevine (*Vitis vinifera* L.) is one of the most valuable fruit crops worldwide, representing 7.4 kHa of the cultivated area in 2018 (data from the 2019 Statistical Report on World Vitiviniculture, International Organization of Vine and Wine). In the last year, 292 million hectolitres (mhL) of wine were produced all over the world, making the wine industry one of the most important economic sectors in several countries. In this study, two *Vitis vinifera* cultivars 'Trincadeira' and 'Regent' were used as plant models. 'Trincadeira' is one of the most widely planted red grape cultivars in Portugal and 'Regent' is a dark-skinned crossing line grape cultivar created in Germany. In Portugal, 'Regent' is not authorized for wine production [25] and its plantation is restricted to the germplasm bank. It presents resistance loci to *Plasmopara viticola* (loci RPV3.1) and to *Erysiphe necator* (loci RUN3 and RUN9), the downy and powdery mildews causing agents, respectively, and thus it is useful as donor of tolerance genes to cryptogamic disease whenever included in breeding programs of Portuguese autochthonous cultivars.

In the last decade, almost 10000 studies were conducted on grapevine (NCBI, https://www.ncbi.nlm.nih.gov/, accessed in May, 2020), encompassing studies in all its tissues, at constitutive level as well as under environmental and human-induced stimuli, using different technologies and analysing diverse OMIC's. Genomics, transcriptomics, proteomics and metabolomics have been the most explored to unravel the different physiological processes and response mechanisms that are activated and repressed under several conditions. However, only two papers focusing on grapevine leaf APF proteomics were published so far [20, 26] and none on APF metabolomics. A more comprehensive analysis of this compartment in grapevine leaf may uncover molecules that participate in intercellular communication, transport and plant-environment interaction. Here, we developed an improved apoplast extraction method to obtain a simultaneous extraction of proteins and metabolites from the same sample, compatible with direct analysis of the proteome and metabolome by mass spectrometry (MS). This study will open new possibilities for a more comprehensive study of grapevine leaf APF.

## 6.3. Materials and Methods

### 6.3.1. Plant material

Two grapevine cultivars *V. vinifera* 'Trincadeira' and 'Regent' with different phenological characteristics were used. Young fully expanded leaves, from both cultivars, grown in greenhouse at the Portuguese Grapevine Germplasm Bank (PRT051) at INIAV — Estação Vitivinícola Nacional (Dois Portos, Portugal), were harvested. Leaves from the 3$^{th}$ to 5$^{th}$ position from the shoot apex were collected from 15-20 plants. Plant growth conditions were standardized for both cultivars, growing at the same temperature, light cycle and humidity. Leaves' harvest occurred 2-3h into the light period and plants were watered in the previous day. Hydration conditions were the same for both 'Regent' and 'Trincadeira'. Leaves were washed in distilled water, disinfected in a bleach solution (5.5% sodium hypochlorite) for 1 min and 30 s and then rinsed three times in distilled water. Leaves were dried with sterile filter paper and immediately used for apoplast extraction. Leaf material was also collected and immediately frozen in liquid nitrogen and kept at -80 ºC for extraction of total soluble proteins and fatty acids.

### 6.3.2. Apoplastic fluid extraction

Apoplastic fluid was extracted according to Guerra-Guimarães et al., 2015 [22] with minor modifications. Twenty-five grams of fresh material (corresponding to approximately 30 young fully expanded leaves) from both grapevine cultivars were used. Leaves were cut in small fragments (approximately 2 cm$^2$) and vacuum infiltrated (25 kPa) with 0.1 M Tris-HCl buffer (pH 8.0) solution containing 0.5 M KCl, 0.006 M CHAPS, and 2 % (w/v) $Na_2SO_3$ (at 4 ºC), for six cycles of 30 s. After infiltration, fragments were washed in cold distilled water and centrifuged at 5000 *g*, during 15 min at 4 ºC, the APF was collected and stored at -20 ºC (Figure 6.1A). All steps of the protocol were performed at 4 ºC, including leaf cutting, lab material and buffers to minimize the impact of the technical procedure and preserve the biological samples.

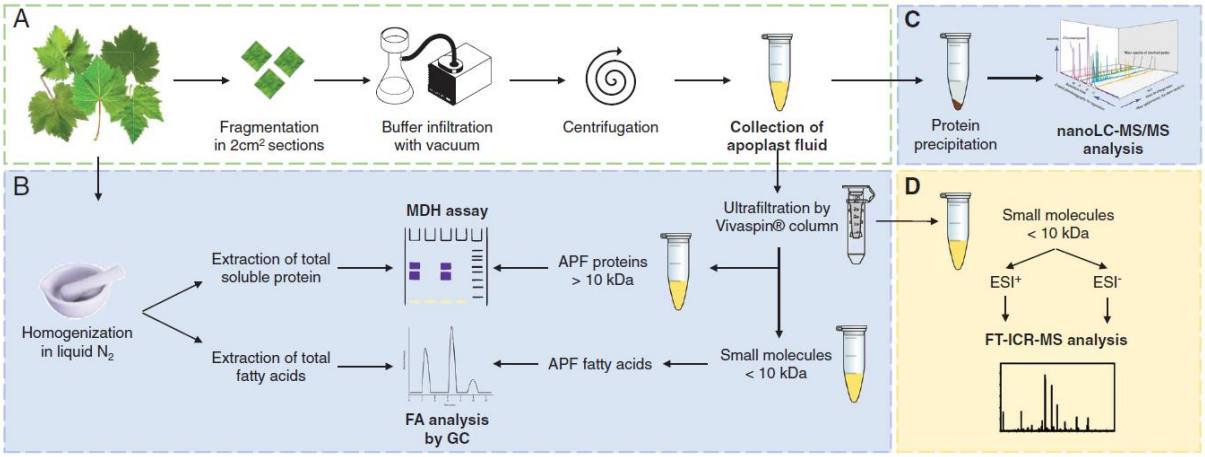


**Figure 6.1.** Experimental workflow. A) Grapevine leaves apoplast fluid extraction; B) Purity assessment of apoplastic fluid based on Malate Dehydrogenase (MDH) assay and Fatty Acids (FA) identification by Gas Chromatography (GC); C) Proteomic analysis by Nanoscale Liquid Chromatography coupled to Tandem Mass Spectrometry (nanoLC-MS/MS); D) Metabolomic analysis by Fourier Transform-Ion Cyclotron Resonance-Mass Spectrometry (FT-ICR-MS), in positive (ESI+) and negative (ESI-) ionization modes.

### 6.3.3. Extraction of total soluble proteins

Leaves from both grapevine cultivars were grinded with liquid nitrogen. A volume of 1.2 mL of 0.2 M Tris-HCl buffer (pH 8.2) solution containing, 0.14 M NaCl, 0.05% (v/v) Tween 20, 0.2% (w/v) bovine serum albumin (BSA), 2% (w/v) $Na_2SO_3$, 2% (w/v) polyvinylpyrrolidone (PVP) K25, insoluble PVP (1:1, w/w) was added to 150 mg of leaves. Samples were vortexed and incubated on ice during 30 min with agitation. The homogenate was centrifuged at 14000 *g*, during 15 min at 4 ºC [27], and the supernatant was collected and store at -20 ºC.

The protein content of the extracts was quantified based on the Bradford method using the Bio-Rad protein assay protocol for microtiter plates (according to manufacturer's instructions). A bovine serum albumin (BSA) was used as standard and absorbance was measured at 595 nm.

### 6.3.4. Malate dehydrogenase assay

The APF extracts were desalted and concentrated on Vivaspin® columns with a membrane ultrafiltration of 10 kDa (Sartorius, Germany), according to manufacturer's instructions. Malate dehydrogenase (MDH) identification, used as a cytosolic biomarker, was performed in both APF (protein present in the upper fraction > 10kDa) and total soluble protein extracts. Proteins were analysed by isoelectric focusing electrophoresis (IEF) on 5% polyacrylamide gel with 2% ampholyte pH 3-10 (Servalyt of SERVA), performed according to Loureiro et al., 2011 and Robertson et al., 1987. After protein migration, the gel was incubated in 0.1 M Tris-HCl buffer pH 8.5 containing 0.025% (w/v) nicotinamide adenine dinucleotide (NAD), 10% (v/v) 1 M sodium hydrogen malate, 0.02% (w/v) nitroblue tetrazolium (NBT), 0.001% (w/v) phenazine methosulfate (PMS), in the dark at 40 ºC. The appearance of purple-blue bands indicates the presence of MDH [30] (Figure 6.1B).

### 6.3.5. Fatty acid analysis

The detection of the trans-3-hexadecenoic acid (C16:1t), a plastidial fatty acid (FA), was performed in both APF and total grapevine leaf extracts to assess apoplastic fluid quality. For FA analysis, 150 µL of the APF fraction below <10 kDa, obtained after ultrafiltration, were used. For total extracts, 50 mg of grinded leaves were used per cultivar. Twenty micrograms of margaric acid (C17:0), used as internal standard, were added to each sample (APF and total extracts), followed by the addition of 3 mL of methanol-sulfuric acid solution (39:1 v/v). The methylation reaction occurred for 1 h at 70 ºC and was stopped by cooling. The methyl esters were recovered by adding petroleum ether and ultrapure water (3:2, v/v) and the organic phase was collected. The organic phase was dried at 37 ºC under nitrogen atmosphere and resuspended in 20 µL hexane. 1 µL of sample was injected for each analysis. FAs quantitative analysis was performed using gas chromatography (430GC Gas Chromatograph, Varian, Palo Alto, CA, USA) as described before [31] (Figure 6.1B). Five technical replicates were done.

### 6.3.6. Proteomic analysis by nanoLC-MS/MS

APF protein precipitation: The proteins were precipitated using 5 volumes of 0.1 M ammonium acetate in methanol and kept overnight at -20 ºC. The samples were centrifuged at 4000 *g*, during 30 min at -10 ºC and the pellets were recovered. The pellets were washed once with 0.1 M ammonium acetate in 100% methanol, twice with 80% (v/v) acetone and twice with 70% (v/v) ethanol [32]. The pellets were dried and resuspended in 0.03 M Tris-HCl buffer (pH 8.8) solution containing, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS [22] (Figure 6.1C). Protein quantification was performed with RC DC™ protein assay kit (Bio-Rad) according to the manufacturer's procedure [33].

MS-based protein identification: Twenty micrograms of protein were separated on a precast gel (Criterion™ XT precast 1D gel 4–12% Bis-Tris, Bio-Rad) and then stained with Instant Blue (Gentaur BVBA, Kampenhout, Belgium). Proteins were reduced, alkylated then digested by trypsin enzyme (sequencing mass grade, Promega) [34]. Protein identification was achieved by nanoLC-MS/MS using a nanoLC-425 Eksigent system coupled to high-resolution MS TripleTOF® 6600 (SCIEX, Darmstadt, Germany). Extracted peptides were solubilized and loaded on a C18 pre-column (C18 PepMap™, 5 µm, 5 mm × 300 µm, Thermo scientific) for 10 minutes at a flow rate of 2 µL/min using loading buffer (2% (v/v) acetonitrile, 0.05% (v/v) trifluoroacetic acid). Then, peptides were separated with a C18 reverse-phase column (C18 PepMap™ 100, 3 µm, 100 Å, 75 µm × 15 cm, Thermo scientific) using a linear binary gradient (solvent A: 0.1% (v/v) formic acid ; solvent B: 0.1% (v/v) formic acid in acetonitrile) at a flow rate of 300 nL/min. Peptides were eluted from 3 to 30% solvent B over 60 min, increased to 40% B during 10 minutes then to 80% B until 5 min. The column was regenerated by washing for 7 min at 80% B and re-equilibrated for 18 min at 3% B.

The data was acquired in positive nano-electrospray (nano-$ESI^+$) mode set to obtain a high resolution TOF-MS scans over a mass range 300-1250 m/z. The 30 most intense precursors were selected for fragmentation in high sensitivity mode (MS/MS scans range 100-1500 m/z) using the automatically adjusted system of rolling collision energy voltage. The ion accumulation time was set to 250 ms (MS) and to 50 ms (MS/MS). The MS data were imported into Progenesis QI for Proteomics software (*V*.4.1, Nonlinear Dynamics, Waters). The protein and peptide identification searching NCBIprot *'Vitis vinifera'* database released on 20th of February 2019 (208304 sequences) via Mascot Daemon (*V*.2.6.0. Matrix Science, UK) were imported to Progenesis QIP and matched to peptide spectra. The Mascot research parameters were: a peptide tolerance of 20 ppm, a fragment mass tolerance of 0.3 Da, carbamidomethylation of cysteine as fixed modification and oxidation of methionine, N-terminal protein acetylation and tryptophan to kynurenine as variable modifications. Only the proteins identified with a significance MASCOT-calculated threshold p-value < 0.05, at least two significant peptides per proteins and one unique peptide per proteins were accepted.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [35] via the PRIDE [36] partner repository with the dataset identifier PXD015558 and 10.6019/PXD015558.

Further proteomic data processing: The identified proteins were classified based on Enzyme Commission number using Blast2GO software (version 5.2.5, https://www.blast2go.com/, Conesa et al., 2005). The functional annotation of the identified proteins was performed based on MapMan "Bincode" ontology (http://mapman.gabipd.org/web/guest/mapman) using Mercator Automated Sequence Annotation Pipeline (http://mapman.gabipd.org/web/guest/app/mercator, [38] and Gene Ontology annotation using Blast2GO software. The subcellular localization prediction of the proteins was performed using SignalP 5.0, TargetP 1.1 and SecretomeP 2.0 servers (http://www.cbs.dtu.dk/services/, [39–41], ApoplastP (http://apoplastp.csiro.au/, [42], BUSCA (http://busca.biocomp.unibo.it/, [43], LocTree3 (https://rostlab.org/services/loctree3/, [44], Mercator (https://www.plabipd.de/portal/mercator-sequence-annotation, [38] and Blast2GO. The default parameters were used for all the programs.

### 6.3.7. Metabolomic analysis by FT-ICR-MS

The APF extracts were filtered through the Vivaspin® columns and the fraction below <10 kDa was analysed by direct injection in a Fourier Transform-Ion Cyclotron Resonance (FT-ICR) mass spectrometer, operating in positive ($ESI^+$) and negative ($ESI^-$) ionization modes (Figure 6.1D). The samples were diluted 1000-fold in methanol before injection [45]. Formic acid (final concentration 0.1% (v/v), Sigma Aldrich, MS grade) was added for samples analysed in positive ion mode. Leucine-enkephalin (YGGFL, Sigma Aldrich) was added to all samples at a concentration of 0.5 µg/mL and used

as a standard for control and quality assessment of analytical precision ($[M+H]^+$ = 556.276575 Da or $[M-H]^-$ = 554.260925 Da) and for internal online calibration during sample acquisition. Samples were analysed by direct infusion on the 7-Tesla SolariX XR FT-ICR-MS equipped with ParaCell (Bruker Daltonics). Mass spectra were recorded in a mass range 100-1000 m/z and 200 scans were accumulated. Five technical replicates were analysed for each grapevine cultivar.

Data Analysis 4.1 software package (Bruker Daltonics, Bremen, Germany) was used to analyse the spectra and generate the peak list further exported as ASCII files with a signal-to-noise ratio of 4. The mass list for each sample was submitted to MassTRIX 3 server (http://masstrix.org, accessed in March 2019, [46]. The parameters considered were the following: scan mode was either positive or negative; the adducts $M+H^+$, $M+K^+$ and $M+Na^+$ were chosen for positive scan mode; the adducts $M-H^+$ and $M+Cl^-$ were selected for negative scan mode; 1 ppm was the maximum m/z deviation considered; '*Vitis vinifera'* was selected as the organism; the search was performed in the databases "KEGG (Kyoto Encyclopedia of Genes and Genomes)/HMDB (Human Metabolome Database)/LipidMaps without isotopes". Masses that existed in at least 3 out of the 5 replicates were considered.

For each ionization mode, the obtained mass lists of 'Trincadeira' and 'Regent' were merged and the common compounds were analysed. Compound classification was performed as previously described [47]. Briefly, for each putatively identified metabolite, an initial conversion of HMDB (http://www.hmdb.ca/, [48] to KEGG (http://www.genome.jp/kegg/, [49] identifiers was performed using the "Linked entries option" option in the KEGG REST Service (http://rest.genome.jp/link/compound/hmdb). For metabolites putatively assigned as lipids, the ones with KEGG identifiers with LIPID MAPS (Lipidomics Gateway, http://www.lipidmaps.org/, [50] correspondence were also converted using the "Linked entries option" option (http://rest.genome.jp/link/compound/lipidmaps) and all were classified using LIPID MAPS classification. The remaining compounds with KEGG identifiers were classified using KEGG database classification. To assess the presence of this compounds in the Plantae Kingdom, a final conversion of LIPID MAPS identifiers to KEGG was performed and all KEGG identifiers were searched in the KNApSAcK database (http://kanaya.naist.jp/KNApSAcK/, [51]. For compounds with multiple annotations a manual curation was performed.

## 6.4. Results

### 6.4.1. Optimization of the apoplastic fluid extraction

The APF extraction protocol based on the vacuum infiltration (VIC) method was optimized in order to extract simultaneously proteins and metabolites compatible with direct analysis by mass spectrometry. The infiltration buffer consisted of 0.1 M Tris-HCl buffer (pH 8.0) solution containing 0.5 M KCl, 0.006 M CHAPS, and 2 % (w/v) $Na_2SO_3$ (at 4 ºC). The vacuum pressure used during leaf infiltration was of 25 kPa and the total infiltration time to 3 minutes (6 periods of 30 seconds) to minimize cell integrity damage. By using reduced infiltration and centrifugation times, the overall duration of the extraction protocol was shortened, which is a highly critical issue in metabolomics to minimise the turnover and reactivity of cellular metabolites.

For the same grapevine starting material, 'Trincadeira' showed a slightly higher APF volume comparatively to 'Regent', 13mL and 8 mL, respectively. Regarding protein yield, 1.7 mg of protein per gram of fresh weight (mg/gFW) was obtained for 'Trincadeira' and 1.2 mg/gFW for 'Regent'.

### 6.4.2. Purity assessment of apoplastic fluid

The quality of the apoplastic fluid (absence of cytoplasmic content) was assessed by the malate dehydrogenase (MDH) enzymatic analysis by isoelectric focusing (IEF) gel electrophoresis. In addition, we have applied a fatty acid (FA) analysis by gas chromatography (GC) to determine APF purity.

MDH activity was evaluated in the APF proteins and compared with total protein extracts. The appearance of purple-blue bands in total soluble protein extracts of both cultivars indicates the presence of MDH, a cytoplasmic biomarker. No MDH was detected in the APF extracts (Figure 6.2).

The abundance of trans-3-hexadecenoic acid (C16:1*t*) was evaluated in total leaf extracts and APF extracts to assess the absence of cytosol contamination, since the C16:1-trans fatty acid containing phosphatidylglycerol (PG) molecular species is specific to chloroplast membranes [52]. FA composition of the APF extracts was assessed by GC (Figure 6.3) and several FA were identified. C16:1*t* was detected in whole leaf extracts but not detected in the APF extracts.

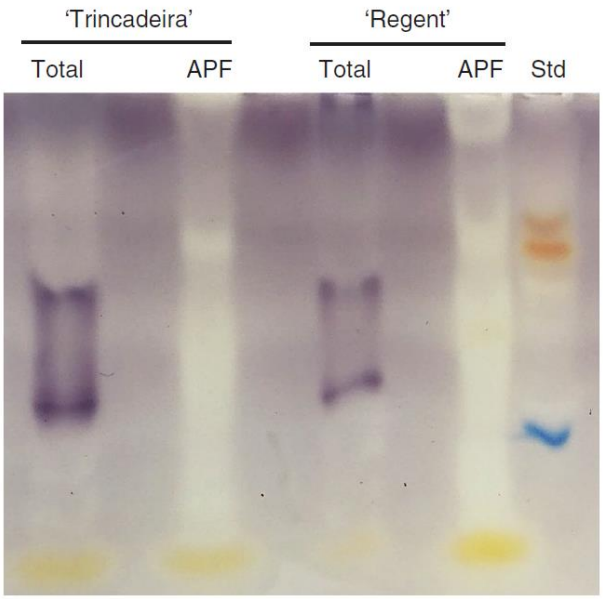


**Figure 6.2.** Malate dehydrogenase isoenzyme activity identification after isoelectric focusing (IEF) on polyacrylamide gels (pH 3-10) stained with malate, NAD+ and nitroblue tetrazolium. MDH activity was evaluated on leaf extracts of total soluble proteins and APF proteins of *V. vinifera* cV. 'Trincadeira' (Tri) and *V. vinifera* cV. 'Regent' (Reg). Fifteen micrograms of protein were loaded per lane; Std - IEF pre-colour standard (BIO RAD 161-0310). The appearance of purple-blue bands indicates the presence of MDH

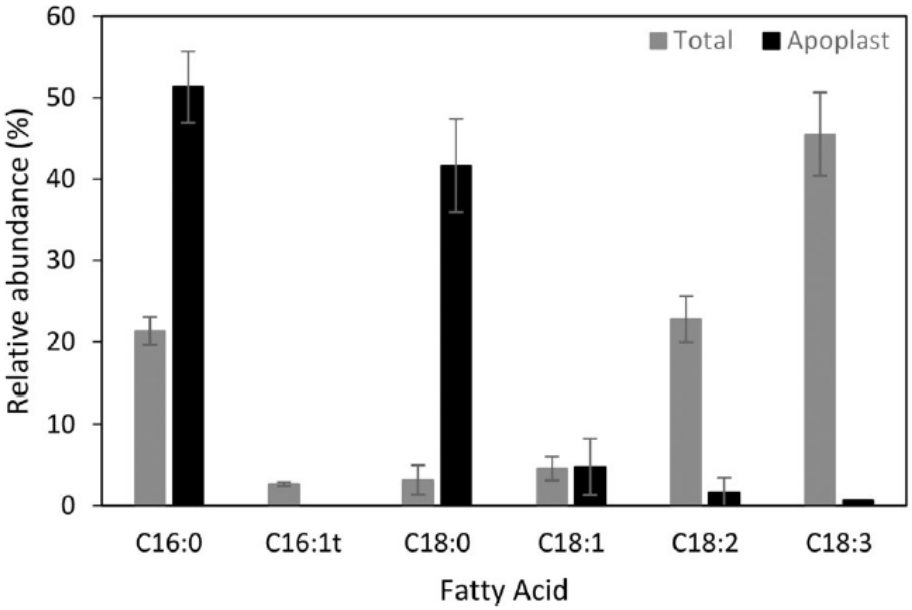


**Figure 6.3.** Percentage of the total fatty acids (FAs) identified in extracts of leaves and apoplastic fluids considering both grapevine genotypes. The values refer to the average of the FAs relative abundance in *V. vinifera* cvs 'Trincadeira' and 'Regent'. C16:0 – Palmitic Acid; C16:1t –trans-3-hexadecenoic acid; C18:0 – Stearic Acid; C18:1 – Oleic Acid; C18:2 - Linoleic Acid; C18:3 - α-Linolenic Acid

### 6.4.3. Proteomic analysis by nanoLC-MS/MS

APF proteins were analysed by nanoLC-MS/MS followed by homology search in NCBI*prot 'Vitis vinifera'* database, allowing the identification of 721 proteins common to both cultivars (Table D.1). To further validate the extracellular nature of the identified proteins, several bioinformatic tools suitable for predicting protein secretion were used. Secretion of proteins can be predicted through the classical secretory pathways (SignalP 5.0, TargetP 1.1, ApoplastP, BUSCA, LocTree3 and Mercator) or through unconventional secretory pathways (SecretomeP) (Table D.2). Based on the obtained results, the 721 identified proteins were grouped in 4 different classes according to the following criteria: i) proteins with a predicted signal peptide (SP) by SignalP (Class I, 44%); ii) proteins predicted to be secreted through classical secretory pathways but, by other software than SignalP 5.0 (Class II, 36%); iii) proteins predicted to be secreted by unconventional secretory pathways (USP) based on SecretomeP (Class III, 7%), and proteins with no predicted secretion (Class IV, 13%). The proteins from the Class IV could eventually be a result of symplastic co-extraction with APF (although no MDH was detected) or unknown leaderless secreted proteins that were not predicted by SecretomeP. The functional categorization of the 629 proteins belonging to Classes I, II and III, based on MapMan "Bin" and GO annotation, indicated that those proteins are mostly involved in: cell wall metabolism, protein metabolism and response to biotic and abiotic stress (Figure 6.4).

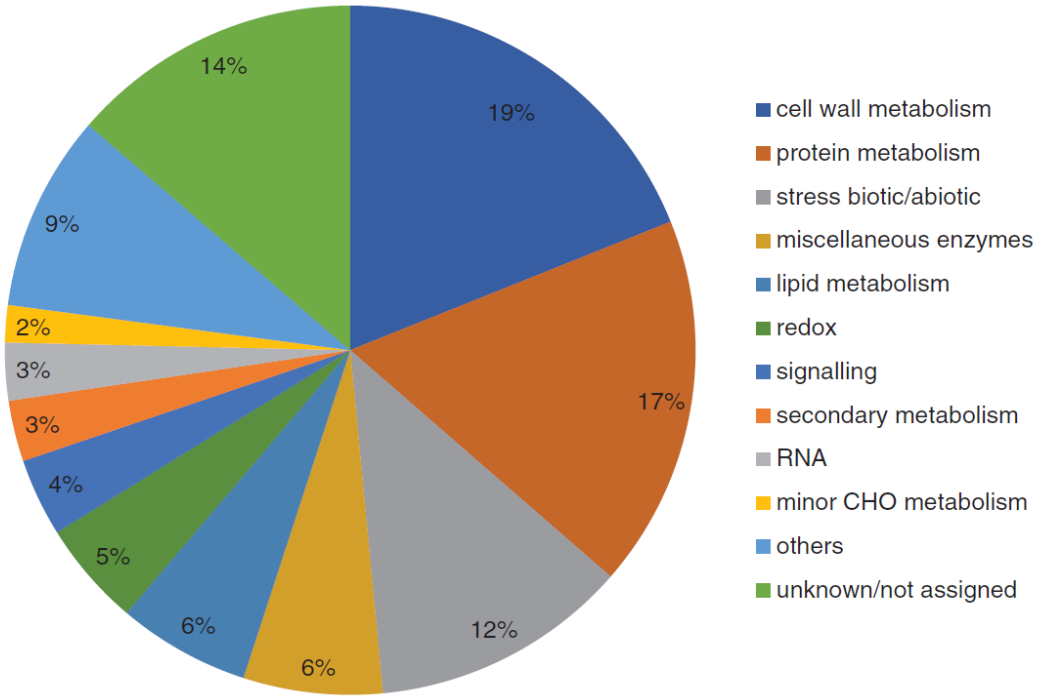


**Figure 6.4.** Biological process of the identified proteins commonly assigned in the APF of *V. vinifera* cvs 'Trincadeira' and 'Regent', based on Blast2GO and MapMan 'Bin' annotation.

### 6.4.4. Metabolomic analysis by FT-ICR-MS

After the ultrafiltration of the APF extracts on the Vivaspin® columns, the metabolites present in the APF lower fraction (< 10kDa) were analysed by Fourier-Transform Ion-Cyclotron-Resonance Mass Spectrometry (FT-ICR-MS) through an untargeted approach. To increase metabolome coverage, samples were analysed in both positive ($ESI^+$) and negative ($ESI^-$) ionization modes (a representative spectrum of an $ESI^+$ analysis is shown in Figure D.1).

A total of 15096 and 15432 ion peaks were detected for 'Trincadeira' and 'Regent', respectively, in both ionization modes. The obtained mass lists were submitted to MassTRIX and the metabolite search for annotation was performed in the databases KEGG, HMDB and LipidMaps. A total of 1100 putative metabolites were annotated for 'Trincadeira' and 1657 for 'Regent' (Table 6.1). Of the putative annotated metabolites, 514 were common to both grapevine genotypes. The main metabolic classes represented were 'Lipids', followed by 'Phenolic compounds' and 'Carbohydrates' (Figure 6.5, Table D.3).

**Table 6.1.** Number of obtained peaks after analysis of the *V. vinifera* cvs. 'Trincadeira' (Tri) and 'Regent' (Reg) APF samples by FT-ICR-MS and number of annotated masses in ESI+ and ESI- ionization modes.

| Ionization mode | Cultivar | Number of peaks (m/z) | Number of annotated masses |
|---|---|---|---|
| ESI+ | Tri | 7071 | 846 |
| | Reg | 6309 | 1189 |
| ESI- | Tri | 8025 | 254 |
| | Reg | 9123 | 468 |
| | Total | 30528 | 2757 |

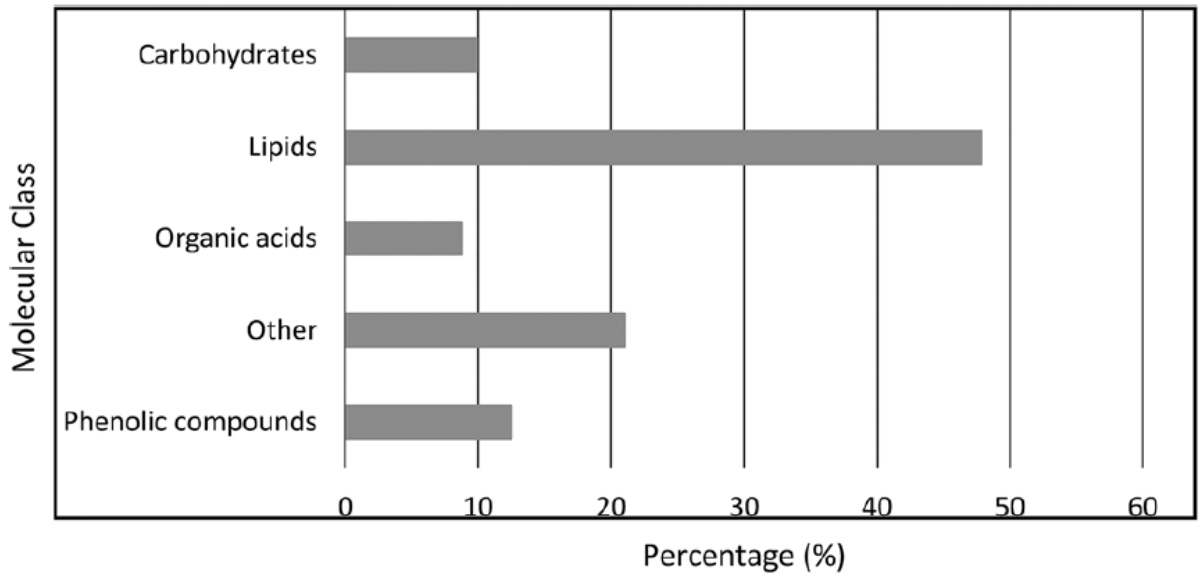


**Figure 6.5.** Major metabolic classes of the compounds commonly assigned in the APF of *V. vinifera* cvs 'Trincadeira' and 'Regent'.

### 6.4.5. Proteins and metabolites that participate in the same biochemical pathways

Ten representative proteins and metabolites were selected based on their participation in the same biochemical pathways (substrates, reaction products or regulation of protein function). All of the selected proteins belong to Classes I and II (proteins with a predicted signal peptide and predicted to be secreted through classical secretory pathways), being involved in cell wall metabolism (beta-xylosidase/alpha-L-arabinofuranosidase, heparanase-like protein 3, L-ascorbate oxidase-like, D-glucuronate, luteolin 7-o-glucoronide and L-ascorbate), protein metabolism and defence (cucumisin isoform X1, chitotriosidase-1, acidic phosphatase 1, jasmonic acid, linoleic acid, N-acetyl-D-glucosamine, sinapyl-alcohol, riboflavin); lipid metabolism (non-specific lipid-transfer protein, hexadecanoic acid, octadecanoic acid, sn-glycero-3-phospho-1-inositol), polyamine metabolism (polyamine oxidase, spermidine) and carbohydrate metabolism (alpha-amylase, sucrose, gibberellin) (Table 6.2).

## 6.5. Discussion

Understanding apoplast dynamics is essential to elucidate how plants respond to the surrounding environment, how these components interact between themselves, with the plasma membrane and the cell wall. These interactions are crucial for a comprehensive overview of cell communication, transport and how plant performance is affected. In grapevine, leaf ECS has been overlooked with only two studies published so far [20, 26]. The present study describes an optimized VIC protocol for *V. vinifera* leaves for the simultaneous extraction of apoplastic proteins and metabolites from a single sample. The VIC protocol involves two critical steps. The first step is the vacuum pressure applied together with the composition of the infiltration solution, and the second step is the centrifugation force used [53]. The optimization performed allowed us to use a small amount of grapevine leaves as well as a short extraction time, while maintaining cellular integrity and a good protein yield.

**Table 6.2.** Most representative proteins and metabolites identified in APF grapevine leaves.

| Proteins | | | | | | Metabolites | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Biological process** | **BIN code** | **Description** | **Accession** | **Enzyme codes** | **Class** | **Name** | **Kegg cid** | **Class** | **Pathway** |
| cell wall metabolism | 'cell wall.degradation.mannan-xylose-arabinose-fucose' | Beta-xylosidase/alpha-L-arabinofuranosidase 2 [*Vitis vinifera*] | RVW84640.1 | EC:3.2.1.55; EC:3.2.1.37 | I | D-Glucoronate | C00191 | Carbohydrates | Pentose and glucoronate interconvertions |
| | 'cell wall.degradation' | Heparanase-like protein 3 [*Vitis vinifera*] | RVX05222.1 | EC:3.2.1.31 | I | D-Glucoronate | C00191 | Carbohydrates | Pentose and glucoronate interconvertions |
| | | | | | | Luteolin 7-o-glucoronide | C03515 | Phenolic compounds | Flavone and flavonol biosynthesis |
| | 'cell wall.pectin*esterases.PME' | L-ascorbate oxidase-like [*Vitis vinifera*] | RVW50968.1 | | I | L-Ascorbate | C00072 | Other | Ascorbate and aldarate metabolism |
| Protein metabolism | 'protein.degradation.subtilases' | PREDICTED: cucumisin isoform X1 [*Vitis vinifera*] | XP_010658505.1 | EC:3.4.21 | I | Jasmonic acid | C08491 | Lipids | Octadecanoic Pathway |
| | | | | | | Linoleic acid | C01595 | | |
| stress biotic/abiotic | 'stress.biotic' | PREDICTED: chitotriosidase-1 [*Vitis vinifera*] | XP_002270579.1 | EC:3.2.1.14 | I | N-acetyl-D-glucosamine | C00140 | Carbohydrates | Aminosugar and nucleotide sugar metabolism |
| | stress.abiotic.cold'/'misc.peroxidases' | Peroxidase 5 [*Vitis vinifera*] | RVW41243.1 | EC:1.11.1.7 | II | Sinapyl-alcohol | C02325 | Phenolic compounds | Phenylpropanoid biosynthesis |
| miscellaneous enzymes | 'misc.acid and other phosphatases' | Acid phosphatase 1 [*Vitis vinifera*] | RVW14589.1 | EC:3.1.3.2 | I | Riboflavin | C00255 | Other | Riboflavin metabolism |
| Lipid metabolism | ‘lipid metabolism.lipid transfer proteins’ | Non-specific lipid-transfer protein [*Vitis vinifera*] | RVW40993.1 | | I | Hexadecanoic acid | C00249 | Lipids | Lipid transport |
| | | | | | | Octadecanoic acid | C01530 | | |
| | | | | | | Sn-glycero-3-phospho-1-inositol | C01225 | | |
| Others | 'polyamine metabolism.degradation.polyamin oxidase' | Polyamine oxidase [*Vitis vinifera*] | RVX10496.1 | | I | Spermine | C00750 | Other | Arginine and proline metabolismo; β-alanine metabolism |
| | 'major CHO metabolism.degradation.starch. starch cleavage.alpha amylase' | Alpha-amylase [*Vitis vinifera*] | RVW61137.1 | | I | Sucrose | C00089 | Carbohydrates | Carbohydrate metabolism |
| | | | | | | Gibberellin | C01699 | Lipids | Response to gibberellin |

The reduction of the initial amount of leaves achieved by our team can be highly important for several experimental studies (e.g. biotic or abiotic stress imposition) where the available biological material can be a critical point. The infiltration solution, a Tris-HCl buffer containing KCl, $Na_2SO_3$ and CHAPS, allowed the simultaneous extraction of proteins and metabolites, without sample oxidation. The presence of KCl in the APF infiltration buffer of different woody plant species, has been extensively used [23, 54]. The KCl led to the additional extraction of glycoproteins and guaranteed weakly bound cell wall proteins solubilization with low cytoplasm leaking [55]. The sodium sulphite ($Na_2SO_3$) minimizes the damage caused by oxidation (avoiding browning reactions) [56], being equally efficient as 2-mercaptoethanol or DTT but with less health and environmental hazards and safer for handling. On the other hand, CHAPS, a zwitterionic detergent, help in protein and lipid solubilisation [57, 58] while preserving the native functional state of the extracted proteins. So, enzymatic assays can be performed in the APF extracts. Comparing to other published studies in grapevine [20, 59], a reduction in the overall duration of the extraction protocol was achieved which is highly important to mitigate the proteolytic activity and minimize the turnover and reactivity of cellular metabolites, fundamental for the success of proteomic and metabolomics analysis [60] (Figure 6.1A).

After APF extraction the volume obtained for each genotype varied, 13 mL and 8 mL were obtained for ‘Trincadeira’ and ‘Regent’, respectively. In fact, both genotypes present contrasting mature leaves morphology reflecting variations in the histological structure which may influence the effectiveness of vacuum infiltration and APF extraction. According to the descriptors of the *Vitis* International Variety Catalogue [61], ‘Trincadeira’ presents a medium density of prostate hairs between the main veins on the lower side of the blade (OIV084) while ‘Regent’ presents a low density. The goffering of the blade is also contrasting with ‘Trincadeira’ presenting a very strong goffering and ‘Regent’ an absent or very weak (OIV072) (Figure 6.6). Also, considering mesophyll organization and thickness, ‘Trincadeira’ presents a denser mesophyll than ‘Regent’. Even though different volumes were obtained, in terms of protein yield, the values obtained were 1.7 mg/gFW for ‘Trincadeira’ and 1.2 mg/gFW for ‘Regent’, thus highlighting the robustness of this protocol.

The cytosolic biomarker malate dehydrogenase commonly used by several authors in different plant species [22, 23, 62] was applied for purity assessment of the extracted apoplastic fluid. Furthermore, we have also used C16:1t, a chloroplast membrane lipid FA [52] as a new cytosolic marker to access apoplast integrity. Neither malate dehydrogenase activity nor the C16:1*t* were detected in the APF extracts confirming cell integrity maintenance in both grapevine cultivars studied.

Functional annotation of the 700 identified proteins covered several biological processes as “cell wall metabolism”, “protein metabolism” and “response to stress”. These findings are in accordance with previous data obtained for leaf APF proteins not only in grapevine [20] but also for other plants, despite the different analytical techniques used [16, 22, 23, 54, 63].

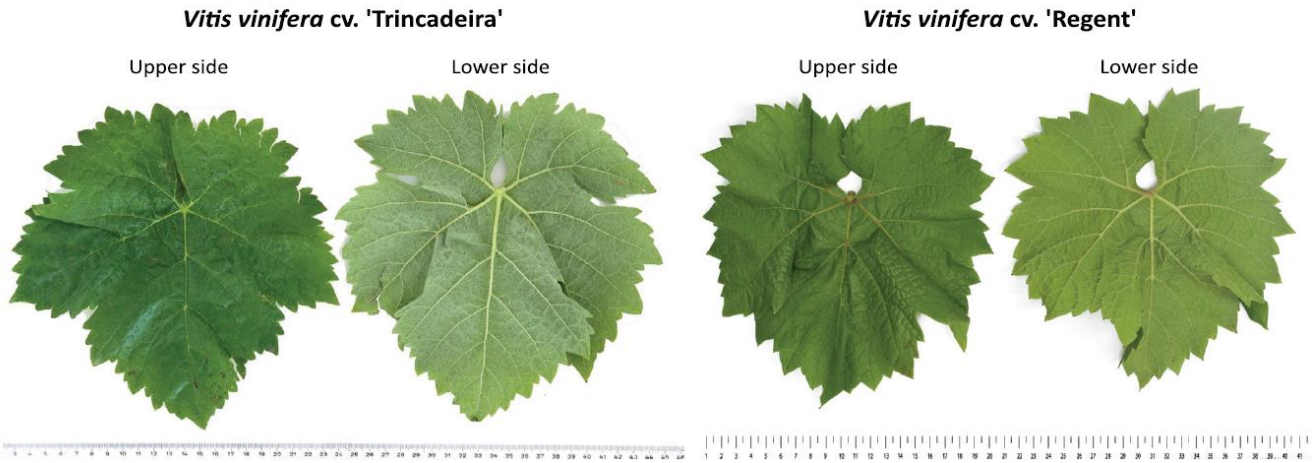


**Figure 6.6.** Differences between mature leaves: upper side and lower side of blade from in *V. vinifera* cvs. 'Trincadeira' and 'Regent'. * Photo credit: Doris Schneider, Ursula Brühl, Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for Grapevine Breeding Geilweilerhof - 76833 Siebeldingen, Germany

Several bioinformatic tools were used to predict cellular localization of the identified peptides. Around 80% of the identified APF proteins carried an N-terminal signal peptide reaching their destination by the classical secretory pathway. Data from other research groups also indicate a high percentage of N-terminal signal peptide in APF proteins, e.g., 90% for coffee [54], 66% for grapevine [20] and 65% for soybean [64]. However, the presence of proteins lacking signal peptide, leaderless secreted proteins (LSP) or non-classically secreted proteins [10] were about 7% of the identified proteins in this work. These LSPs have been also referred in other APF studies [16, 54, 65]. According to Rabouille (2017), unconventional protein secretion is complex and comprises many issues such as; proteins without a signal peptide or a transmembrane domain that can translocate across the plasma membrane, and proteins that reach the plasma membrane by bypassing the Golgi despite entering the endoplasmic reticulum [66].

The untargeted metabolomics approach allowed the identification of 514 unique putative compounds and revealed that APF was enriched in lipids, carbohydrates and phenolic compounds. Moreover, while assessing APF purity through fatty acid profiling by GC, we were also able to identify saturated (C16:0 and C18:0) and unsaturated FAs oleic, linoleic and α-linolenic acids (C18:1, C18:2 and C18:3). These FA were previously identified on grapevine leaves [67, 68]. In French bean, both α-linolenic and linoleic acids were detected in the APF [69]. While in the APF the saturated FA was in higher abundance than unsaturated FA, in the total cell extract the opposite was observed (Figure 6.3). This difference may be due to the fact that the total extract includes cell membranes, where the unsaturated FA are the major components of their lipids [70, 71].

The few studies concerning APF metabolomics focus mainly on plant-pathogen interactions [14–16]. GC-MS analysis of *Phaseolus vulgaris* APF after infection with the halo blight pathogen *Pseudomonas syringae pV. phaseolicola* revealed 60 compounds being the most abundant metabolites organic acids and carbohydrates [16]. Floerl and co-workers also performed a metabolic fingerprinting by UPLC-MS of *Arabidopsis thaliana* APF after inoculation with *Verticillium longisporum* and identified 17 infection markers belonging to lipids, organic acids, bioactive fatty acids and phenolic compounds [14].

We have selected a set of 10 representative proteins and metabolites which might be interconnected in different biochemical pathways, like cell wall and protein metabolism (Table

6.2). In cell wall metabolism, beta-xylosidase/alpha-L-arabinofuranosidase, previously identified in the APF of grapevine [20], coffee [22], poplar [23] and tobacco [72], is an enzyme responsible for the hydrolysis of 1,4-β-D-xylan and arabinan in D-xyl and L-ara [73]. D-xyl metabolite is the precursor of the pentose and glucuronate interconversions pathway. One of the metabolites involved in this pathway is D-glucuronate, which was found in the APF metabolome sequencing performed. Heparanase is a protein related to cell wall metabolism already described in both grapevine and poplar APFs [20, 23]. This enzyme catalyses the conversion of luteolin 7-O-[β-D-glucuronosyl-(1→2)-β-D-glucuronide]-4'-O-β-D-glucuronide in luteolin 7-O-[β-D-glucuronosyl-(1→2)-β-D-glucuronide], releasing D-glucuronate from the reaction [74]. The luteolin-7-o-glucoronide, found in the grapevine leaf APF, is a metabolite precursor of this reaction. L-ascorbate oxidase belongs to the ascorbate and aldarate metabolism pathway using L-ascorbate as substrate [75]. Both enzyme and metabolite were identified in our grapevine APF. Having a role in protein and lipid metabolisms, cucumisin, a serine protease from the subtilase family, was identified. Serine proteases, previously identified in grapevine, coffee and poplar APFs [20, 22, 23] were described to be involved in the octadecanoic pathway catalysing the maturation of prosystemin into systemin [76]. Systemin activates a lipase in receptor cell membranes resulting in the release of α-linolenic acid, the jasmonic acid precursor. Both α-linolenic acid and jasmonic acid were found in APF metabolome. α-Linolenic acid was already detected in French bean apoplast [69]. In the biochemical pathways associated to plant defence to both biotic and abiotic stresses, we have identified a chitotriosidase, an enzyme that participates in the amino sugar and nucleotide sugar metabolism pathway through the hydrolysis of chitin in N-acetyl-D-glucosamine [77]. This last metabolite was also detected in the grapevine APF metabolome. Peroxidase 5, as well as sinapyl alcohol, was also identified in our analysis. This enzyme is involved in the phenylpropanoid biosynthesis pathway through the conversion of sinapyl into syringyl lignin [78, 79]. Peroxidase was already detected in poplar and tobacco APFs [23, 72]. Acid Phosphatase 1, previously reported in the apoplast of pea root nodules [80], catalyses reactions in the thiamine and riboflavin metabolism pathways [81, 82]. Riboflavin, detected in the grapevine APF, is the reaction product of riboflavin-5-phosphate dephosphorylation, catalysed by this enzyme [82]. Riboflavin was previously identified in *Hyoscyamus albus* APF [83].

Considering lipid metabolism, non-specific lipid-transfer proteins interact with several lipid molecules [84] namely hexadecanoic, octadecenoic acids and phospholipid sn-glycero-3-phospho-1-inositol, also found in our APF. In poplar apoplast, these non-specific lipid-transfer proteins have been already reported [23]. Belonging to polyamine metabolism, we found the polyamine oxidase, previously reported in APF of oat [85] and tobacco [86]. This enzyme participates in arginine and proline [87] as well as β-alanine [88] metabolic pathways. One of the reactions catalysed by this enzyme is the conversion of spermine in spermidine [88]. Spermidine was found in our metabolome characterization. Alpha-amylase participates in carbohydrate metabolism [89] and is responsive to gibberellin [90]. Alpha-amylase, sucrose, sucrose derivatives and gibberellin were found in the grapevine APF.

In this work, we developed a method for the simultaneous extraction of proteins and metabolites from the same APF sample and improved the amenability for MS analysis and compatibility with several OMIC's technologies. As a proof of concept, the methodology was tested in two different grapevine cultivars, with contrasting leaf morphology, and over 700 proteins and 500 metabolites were identified by MS, common to both grapevine cultivars. The major grapevine fatty acids were also analysed by GC.

The proposed methodology opens new insights for global characterization of plant APF, searching compartment complexity and paving the way to uncover signalling networks and interactions within a systems biology approach.

### 6.6. Author contributions

AF and LGG conceived the study; JED and JC were responsible for the plant material; JF, LGG and ARC optimized the APF extraction method; MSS, CC, JF and ARC performed the metabolome profiling by FT-ICR-MS; ARC and ARM performed the fatty acid profiling through GC; CL and JR performed the proteome profiling by nanoLC-MS/MS; JF, ARC, AF and LGG analysed the data and wrote the manuscript. All of the authors have read and corrected the manuscript.

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### 6.9. Data availability statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD015558 and 10.6019/PXD015558. The metabolomics data that support the findings of this study are available in figshare data repository through https://doi.org/10.6084/m9.figshare.12341216.v1

# CHAPTER VII

## 7. Conclusions and Perspectives

This thesis has contributed to unveil some mechanisms towards the complete disclosure of the grapevine lipid mediated defence mechanisms and allowed to highlight candidate molecules to be used in future breeding programs for disease tolerance.

Taking the grapevine-*P. viticola* pathosystem as a starting point, we first searched for lipid and FA molecules as biomarkers for disease tolerance or susceptibility. A higher FA degree of unsaturation and higher levels of different lipid classes including the plastidial lipids seem to be features of the susceptible grapevine cultivars to *P. viticola*. On the other hand, tolerant genotypes showed to present a lower degree of FA unsaturation, which may reflect on more rigid membranes that possibly hinder the pathogen´s invasion and development. In this work we also showed the the ability of JA to elicit defence-like lipid modulation events in tolerant and susceptible grapevines alike. Furthermore, to understand whether the observed mechanism in the grapevine-*P. viticola* incompatible interaction is conserved in the interaction with pathogens with different lifestyles and/or invasion strategies, fatty acid modulation and its regulation by FAD genes was also studied. With this we proved that FA modulation follows a distinct pattern in the interaction with biotrophs and necrotrophs. Finally, we also aimed at studying the first battlefield of the grapevine-pathogen interaction: the apoplast. Our group developed a straightforward methodology for grapevine leaf apoplastic fluid extraction that allows the analysis of metabolites and proteins from a single sample. In the metabolome analysis, lipids revealed to be the most abundant molecular class.

Our findings reaffirm the importance of lipid-mediated signalling in grapevine defence to fungal and oomycete diseases and lead to way to choose lipid biomarkers to be used in breeding programs. Concerning the grapevine leaf apoplast, we can state that a "tip of the iceberg" was unveiled, with still much to be explored. The apoplastic fluid extraction methodology developed by our group opens the way to unleash this compartment's full potential and to completely understand the first moments of grapevine-pathogen interaction

Here, I provide a brief summary of the key results from this thesis and discussion concerning potential future directions.

## 7.1. Key results

In this work, the grapevine leaf lipid and FA profiles showed to be suitable tools for grapevine chemotaxonomy studies. While higher levels of PA and neutral lipids as well as saturated FA, mainly in MGDG and PC may be biomarkers for tolerance and a potential resistance trait to be used in breeding programs, MGDG and higher UFA levels in membrane extraplastidial lipid classes may be highlighted as biomarkers for susceptibility. Concerning the total FA profile, while tolerant genotypes to *P. viticola* showed higher levels of saturated FA, mainly C16:0, susceptible genotypes presented higher levels of UFA, with a predominance of higher levels of C18:2. The lipid profile, besides allowing the separation between tolerant and susceptible genotypes, also allowed to highlight *V. vinifera* cv Regent, which reinforces the fitness of lipid profiles to be used as chemotaxonomy tools. It is noteworthy that this cultivar is highlighted in several CAP analyses because it has features that connect it to both the susceptible and tolerant groups. 'Regent' is a hybrid bread for resistance to downy and powdery mildew [1] and has a *V. vinifera* genetic background like most of the susceptible genotypes.

JA is an important player in the defence signalling mechanisms during plants interaction with pathogens with different lifestyles [2], including the grapevine-*P. viticola* interaction [3, 4]. Moreover, JA and its derivatives have well studied properties as elicitors of defence like reactions in plants, resulting in a

more prompt and intense reaction upon further contact with the pathogen [5]. Here, the exogenous application of JA showed to induced defence-like FA modulation events, in a similar manner to what was previously observed in the incompatible interaction between grapevine and *P. viticola* [6]. This effect was observed in both tolerant and susceptible grapevine genotypes to *P. viticola,* showing the potential on JA to elicit defence against this pathogen.

After our group uncovered FA modulation events in the grapevine incompatible interaction with *P. viticola*, highlighting the induction the C18:3 synthesis, a JA biosynthesis precursor [6], the following question arose: is this FA modulation mechanism conserved in the incompatible interaction with pathogens with different lifestyles and tissue invasion strategies? In order to answer this question, grapevine leaf FA composition was studied along the first hours of the incompatible interaction between *V. vinifera* cv Regent and *P. viticola* (biotroph, invading from the stomatal aperture), *E. necator* (biotroph, invading from wounds) and *B. cinerea* (necrotroph and capable of different invasion strategies) [7]. In all the studied interactions, a successive FA unsaturation leading to the formation of C18:3 occurred. However, while in the interaction with both the biotrophs (which, despite having different invasion strategies seem to generally trigger a similar process) there was an earlier induction of PUFA formation that tended to return to basal levels as 24hpi, the interaction with the necrotroph resulted in a later and more durable induction PUFA synthesis. FA modulation events during the interaction with the biotrophic pathogens at study, they seem to suggest that in the first hours post inoculation the JA pathway may be induced. Later in the interaction, other pathways may be induced to overcome the infection, including the antagonistic SA-mediated pathway, which is also known to be activated during grapevine interaction with biotrophic pathogens [8]. On the other hand, the FA modulation events observed during the interaction with *B. cinerea* are consistent with the fact that the JA-mediated pathway is mainly activated against necrotrophs [7]. In all interactions, membrane fluidity modulation occurred, which may be crucial to maintain cellular function during infection.

In different chapters of this thesis, FAD genes expression was assessed. Given the importance of FA and lipid signalling in the grapevine interaction with its pathogens, studying the process of FA desaturation, hence the FAD gene family is crucial to understand the molecular mechanisms behind defence reactions. FAD2 and FAD3 catalyse the desaturation of C18:1 and C18:2, respectively, in the ER [9, 10]. Desaturation of C18:1 and C18:2 in plastidial membranes is catalysed by FAD6 and FAD7/FAD8, respectively [11]. Another plastidial desaturase, FAD4 specifically catalyses the synthesis of trans C16:1 on phosphatidylglycerol (PG) [12]. When studying the grapevine incompatible interaction with *P. viticola*, *E. necator* and *B. cinerea*, the expression of *FAD6* and *FAD8* genes, responsible for the formation of PUFA in the chloroplast (where the bulk amount of PUFA is present) was analysed. *FAD6* and *FAD8* expression profiles are generally corroborated by the FA modulation events. Moreover, these results indicate that FAD enzymes play an important role in the incompatible interaction between grapevine and the pathogens at study and their expression might be differently modulated according to the pathogen's lifestyle. Their expression profiles corroborate the FA modulation events contrarily to what was observed in the interaction with the biotrophs, the interaction with the necrotroph triggered the induction of both *FAD6* and *FAD8* expression in the later time-point. The expression of *FAD3.1, FAD4, FAD6* and *FAD8* was also analysed at the constitutive level when studying the potential of lipid and FA profiles as biomarkers for tolerance and susceptibility to *P. viticola*. FAD expression profiles showed to corroborate the lipid and FA profiles of the different grapevine genotypes. The susceptible cultivars presented a tendency for higher expression levels of *FAD4*, *FAD6* and *FAD8*, responsible for the formation of UFA, mostly the latter. A higher expression of these genes might be considered as possible markers for grapevine susceptibility to *P. viticola*.

Understanding apoplast dynamics is essential to elucidate how plants respond to the surrounding environment, including in biotic stress. These interactions are crucial for a comprehensive overview of cell communication, transport and how plant performance is affected. In grapevine, leaf apoplastic fluid has been overlooked with only two studies published so far [13, 14]. The lack of studies on the grapevine leaf apoplast might be mostly since extracting the apoplastic fluid from woody plants was a rather challenging process with low leaf infiltration efficiency and a great sample amount demand. We optimized a VIC protocol for *V. vinifera* leaves for the simultaneous extraction of apoplastic proteins and metabolites from a single sample. The VIC protocol involves two critical features: the vacuum pressure applied together with the composition of the infiltration solution, and the centrifugation force used [15] which were optimized. Moreover, the optimization performed translated in a less time and sample consuming process in comparison to pre-existing protocols [13, 16], while maintaining cellular integrity. The infiltration solution, a Tris-HCl buffer containing KCl, Na2SO3 and CHAPS, allowed the simultaneous extraction of proteins and metabolites, without sample oxidation. The presence of KCl in the infiltration buffer of different woody plant species, has been extensively used [17, 18]. The KCl led to the additional extraction of glycoproteins and guaranteed weakly bound cell wall proteins solubilization with low cytoplasm leaking [19]. The sodium sulphite (Na2SO3) minimizes the damage caused by oxidation (avoiding browning reactions) [20], being equally efficient as 2-mercaptoethanol or DTT but with less health and environmental hazards and safer for handling. On the other hand, CHAPS, a zwitterionic detergent, help in protein and lipid solubilisation [21, 22] while preserving the native functional state of the extracted proteins. The untargeted metabolomics approach allowed the identification of 514 unique putative compounds. Several molecular classes were detected, being lipids the most abundant. The gas chromatography analysis of the extracted apoplastic fluid served two purposes: on the one hand, it revealed to be a novel a rapid way to assess the extract purity, because the FA C16:1*t* is exclusive of the chloroplast [23] and hence should so appear in the apoplast. On the other hand, it allowed to identify saturated (C16:0 and C18:0) and unsaturated FA (C18:1, C18:2 and C18:3). These FA were previously identified on grapevine leaves [6]. While in the apoplastic fluid the saturated FA was in higher abundance, in the total cell extract the opposite was observed. This difference may be due to the fact that the total extract includes cell membranes, where the unsaturated FA are the major components of their lipids [24, 25].

This work allowed to bring us a few steps closer to the complete disclosure of the grapevine lipid mediated defence mechanisms and to the discovery of new and more sustainable alternatives to fight grapevine diseases.

### 7.2. Future perspectives

The conclusions taken from the work developed in this thesis led to new questions to be explored.

To validate the molecules identified as candidate biomarkers for tolerance and susceptibility to *P. viticola*, an upscaling of the lipid and FA analysis can be made. The analysis can be extended to a whole ampelographic camp and the lipid and FA composition can be corelated with the different degrees of disease tolerance according to the 2nd Edition of the OIV Descriptor List for Grape Varieties and *Vitis* species [26]. This way a better screening of the candidate biomarkers can be made to guide the breeding process for disease resistance.

Since JA showed to elicit defence-like FA modulation events in both tolerant and susceptible genotypes to *P. viticola*, a thorough lipidomic analysis should also be performed to understand whether the lipid-

mediated signalling occurs in a more rapid and intense manner upon contact with the pathogen. To unveil the full potential of JA, this molecule can be applied before inoculation with different pathogens, including *P. viticola, E. necator* and *B. cinerea,* since they cause some of the most devastating grapevine diseases.

The FAD gene family has proved to be highly important in grapevine defence responses in biotic stress. Further studies regarding these genes, including knock-out mutants can be made in order to completely understand its role in grapevine-pathogen interaction. Moreover, to understand the regulation of FA desaturation at the enzyme level, FAD enzyme activity assays can be performed.

Grapevine leaf apoplast is one of the most promising fields of this work with yet so much to be unveiled. Previous studies already showed the importance of apoplast lipids in SAR. This was mostly evidenced by the detection of lipid associated proteins in this compartment during plant-pathogen interaction, including LTP and $PLA_2$ [27, 28]. Concerning the grapevine-*P. viticola* interaction, an inoculation assay was performed in tolerant and susceptible pants (*V. vinifera* cv Regent and *V. vinifera* cv Trincadeira, respectively) and leaf apoplastic fluid was extracted in several time-points. The next step is to do a thorough lipidome screening of the apoplast samples to uncover the lipid signalling events that take place in the first moments of the meeting between grapevine and *P. viticola* and what is behind tolerance and susceptibility.

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# APPENDIX A

## A. Supplementary Materials - Chapter II

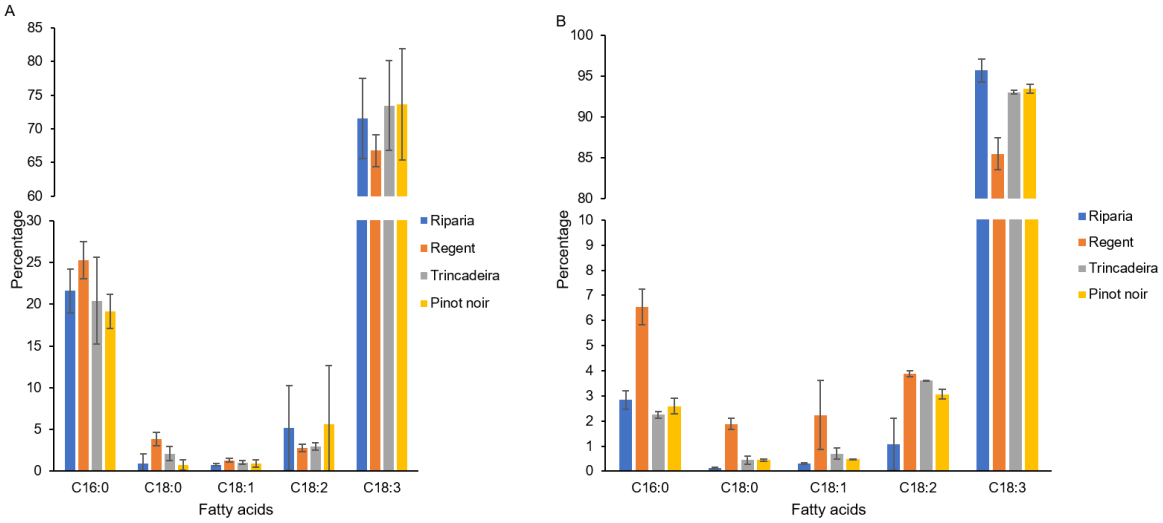


**Figure A.1.** Constitutive Fatty acid profile of leaf digalactosyldiacylglycerol (A) and monogalactosyldiacylglycerol (B) of *V.* riparia, *V. vinifera* cv Regent, *V. vinifera* cv Trincadeira and *V. vinifera* cv Pinot noir.

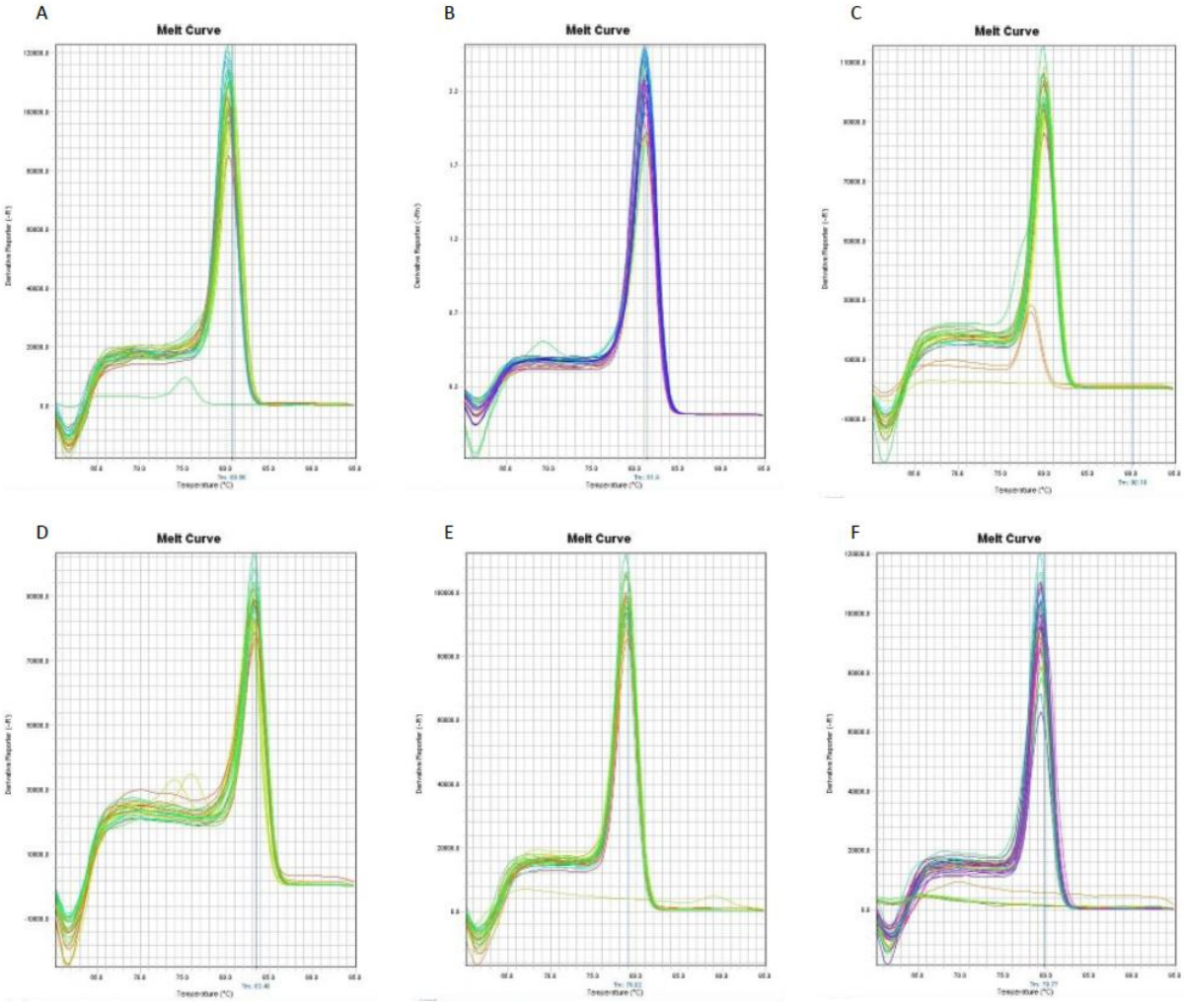


**Figure A.2.** Melting curves of the reference and target genes: EF1α (A), UBQ (B), FAD3.1 (C) FAD4 (D), FAD6 (E) and FAD8 (F)

# APPENDIX B

## B. Supplementary Materials - Chapter III

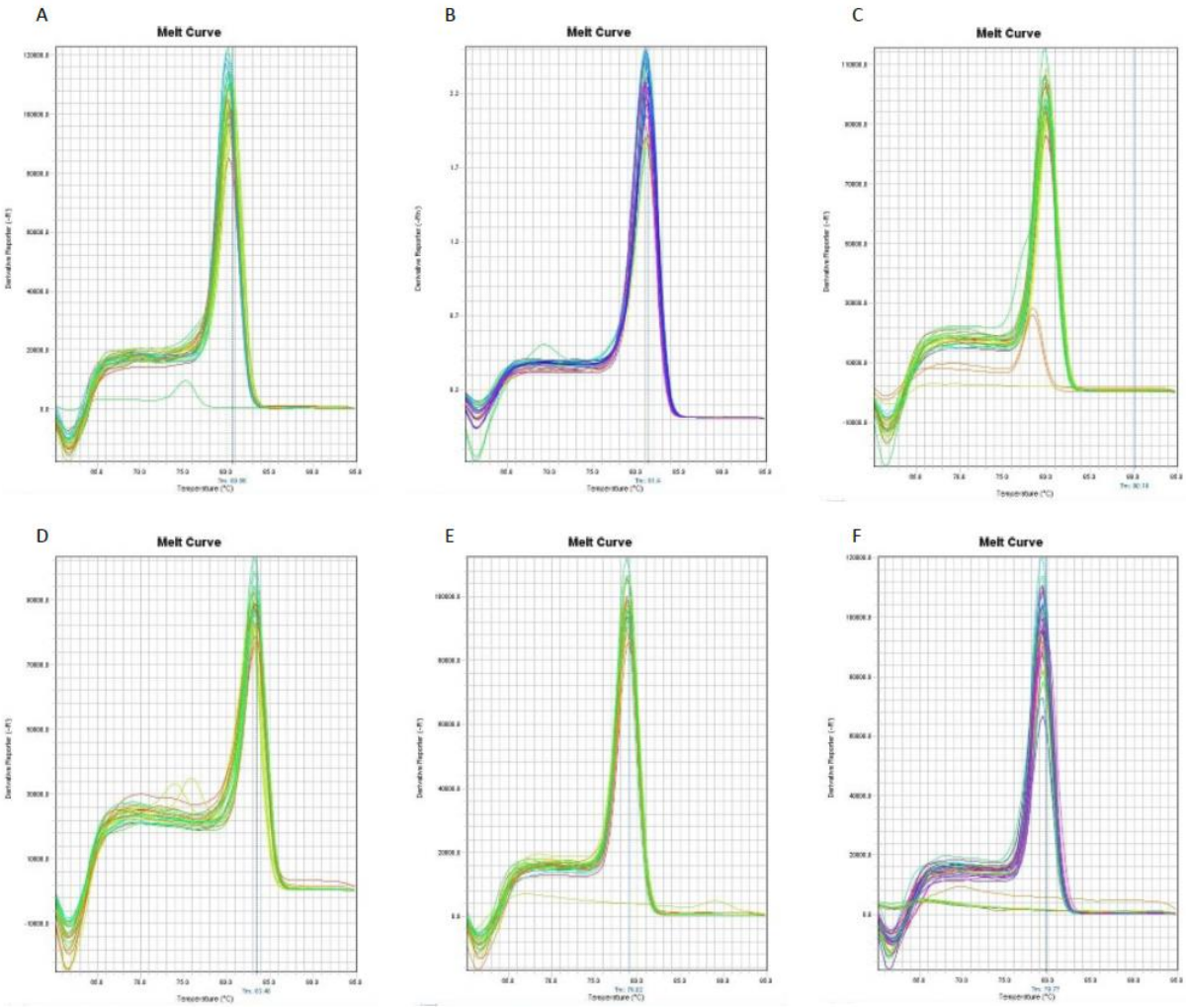


**Figure B.1.** Melting curves of the reference and target genes: EF1α (A), UBQ (B), FAD3.1 (C) FAD4 (D), FAD6 (E) and FAD8 (F)

# APPENDIX C

## C. Supplementary Materials - Chapter V

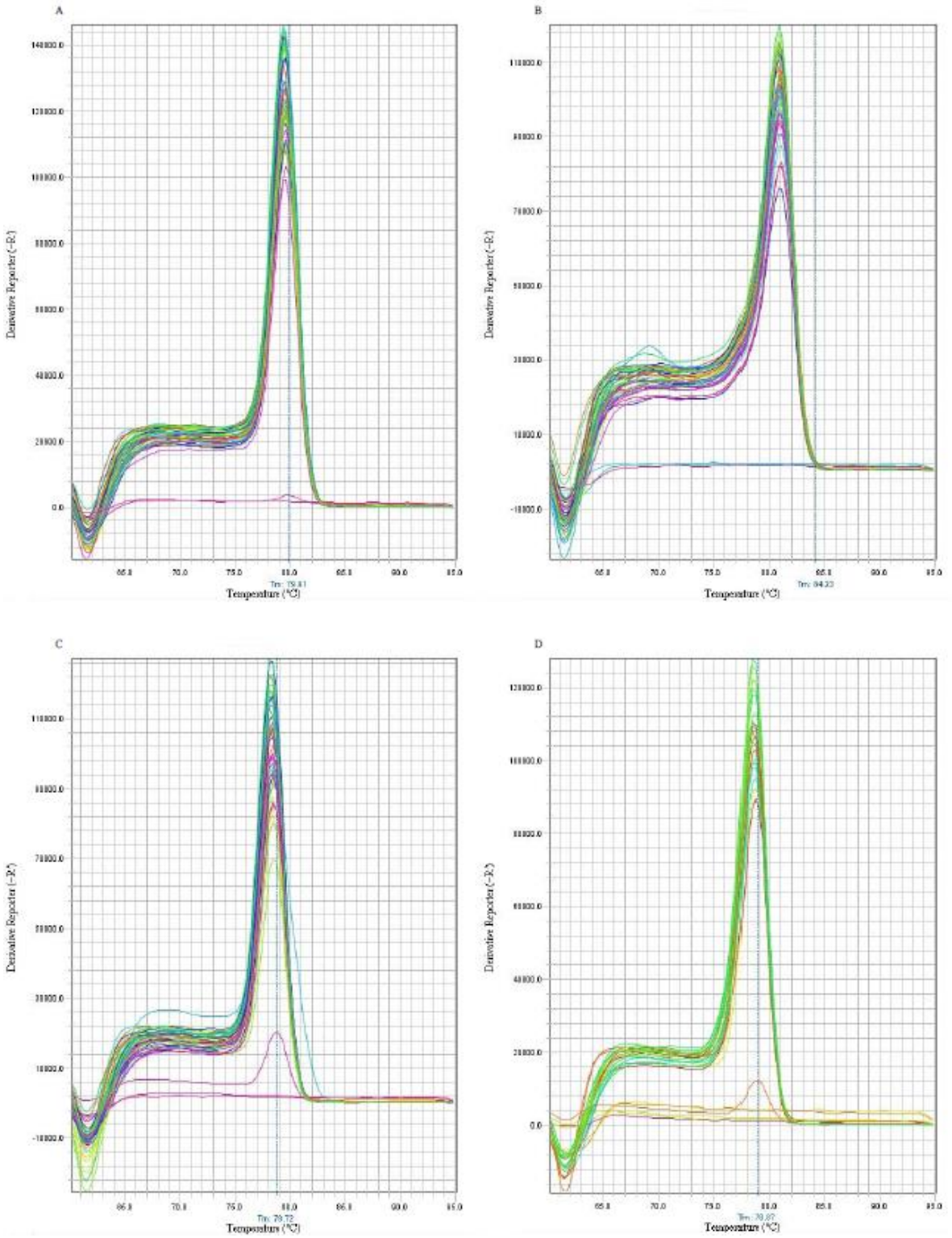


**Figure C.1**. Melting curves of the reference and target genes: EF1α (A), UBQ (B), FAD6 (C) and FAD8 (D)

# APPENDIX D

## D. Supplementary Materials - Chapter V

**Table D.1.** Chemical composition of tested buffers.

| Buffer | Composition |
|---|---|
| **A** | 0.1 M Tris-HCl pH 7.0, 0.5 M KCl, 0.02% BSA, 2% Na2SO3 |
| **B** | 0.1 M Tris-HCl pH 7.6, 0.05 M L-ascorbic acid, 0.5 M KCl, 0.25 M β-mercaptoethanol |
| **C** | 0.1 M Tris-HCl pH 8.0, 0.5 M KCl, 0.006 M CHAPS |
| **D** | 0.1 M Tris-HCl pH 8.0, 0.5 M KCl, 0.006 M CHAPS, and 2% Na2SO3 |

**Table D.2.** LC-MS/MS data obtained for the grapevine leaf apoplastic proteins identified using NCBI *Vitis* database

The table is not included in this document because of its dimentions. This material is available at https://onlinelibrary.wiley.com/doi/abs/10.1111/ppl.13198

**Table D.3.** Annotation of the grapevine leaf apoplastic proteins based on MapMan 'BIN' categories and Blast2GO, and subcellular localization prediction using SignalP 5.0, TargetP 1.1, ApoplastP, BUSCA, LocTree3, Mercator and SecretomeP

The table is not included in this document because of its dimentions. This material is available at https://onlinelibrary.wiley.com/doi/abs/10.1111/ppl.13198

**Table D.4.** Putative identification and classification of the detected metabolites in the APF of *V. vinifera* cvs Regent and Trincadeira. Raw mass is the mass detected by the FTICR-MS; KEGG_mass corresponds to the mass on the KEGG database; ppm is the error in part-per-milion relative to the database mass; KEGG_cid corresponds to the putative database identifier; KEGG_formula is the chemical formula of the coumpounds; KEGG_name correspond to the putative identification; Class is obtained as depicted in Materials and methods; KNApSAcK indicates if the compound is described in plants on the KNApSAcK database.

The table is not included in this document because of its dimentions. This material is available at https://onlinelibrary.wiley.com/doi/abs/10.1111/ppl.13198

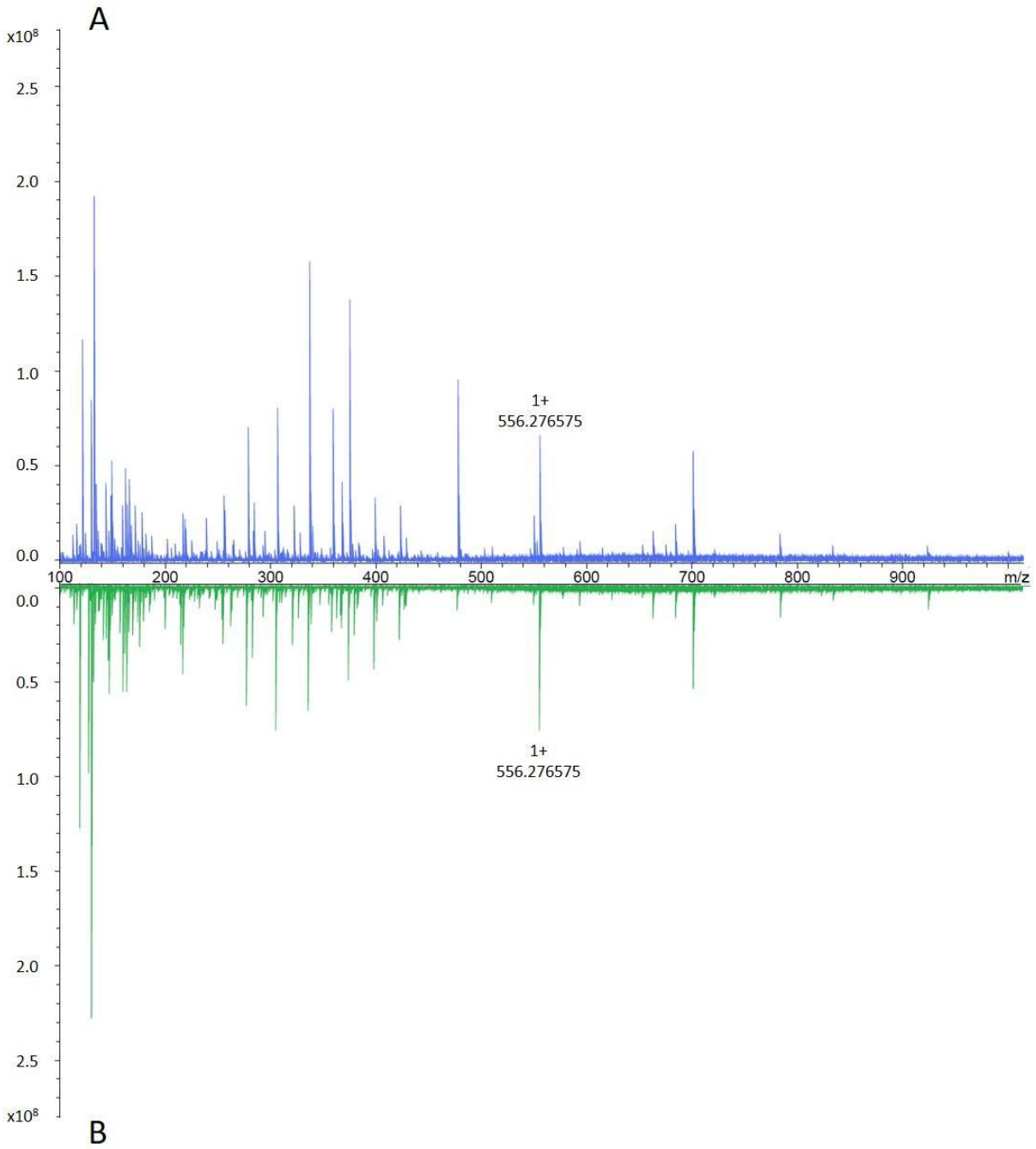


**Figure D.1.** Representative mass spectra of *Vitis vinifera* low molecular mass APF. Data were acquired in positive (ESI+) electrospray mode by direct infusion FT-ICR-MS in the range 100–1000 m/z. Leucine-enkephalin mass ([M+H]+): 556.276575 Da (A) *V. vinifera* cv Trincadeira; (B) *V. vinifera* cv Regent.