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Developing molecular tools to improve grapevine resilience against pathogens: new insights on the role of chloroplasts lipids

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Abstract

Vitis vinifera is one of the most cultivated fruit crops in the world, being highly susceptible to *Plasmopara viticola,* the etiological agent of grapevine downy mildew, leading to heavy yield reductions and, consequently, to massive economic losses. To control this disease, preventive application of pesticides occurs throughout the season. Due to the environmental problems caused by pesticides and other chemicals, it is necessary to develop more sustainable control solutions.

Over time, several studies have demonstrated the importance of lipids in the grapevine's response to pathogens. This group of molecules comprises components of cell membranes, surface lipids, which are the first barrier that pathogens encounter, and signaling molecules, involved in the cellular changes that occur following infection. The importance of chloroplast lipids and fatty acids in the outcome of the infection, being either associated to the jasmonic acid hormone signaling pathway or through the modification of membrane fluidity has also been highlighted. However, knowledge on the main modifications related to fatty acid and lipid composition at the chloroplastidial level is still scarce. In this work we characterized for the first time, the gene families that code enzymes involved in chloroplast lipid biosynthesis. We have also determined both fatty acid and lipid modulation events during the interaction with *P. viticola*. We also exploited gene expression modulation in an infection context, using grapevine genotypes that harbour different resistance to *Plasmopara viticola loci* (RPVs) and characterized lipid modulation in the *Vitis vinifera* cultivars Chardonnay (susceptible), Calardis blanc (*Rpv3-1, Rpv3-2*), Regent (*Rpv3-1),* Sauvignac (*Rpv3-3, Rpv10*) and Solaris (*Rpv3-1, Rpv12)*.

Key-words:

Vitis vinifera, *Plasmopara viticola*, chloroplastidial lipids, lipid signaling, *Rpvs*

Resumo alargado

A videira *(Vitis vinifera)* é uma das culturas de fruto mais estudadas e cultivadas em todo o mundo, sendo que em Portugal é uma importante fonte económica através da indústria vitícola. No entanto, a videira é severamente afetada pela doença do míldio, provocada pelo oomicete biotrófico obrigatório *Plasmopara viticola*. Este patógeno provoca efeitos deletérios nas culturas de videira, podendo destruir culturas inteiras numa época, diminuir a qualidade das uvas e, consequentemente, a qualidade do vinho produzido, o que origina enormes perdas económicas. Para contornar os efeitos desta doença são aplicadas grandes quantidades de fitoquímicos, que possuem algumas desvantagens, como o desenvolvimento de estirpes com resistência aos fungicidas, aumento da poluição ambiental, impactos negativos ao nível da saúde humana e elevados custos económicos para a sua aplicação. A maioria das cultivares de *Vitis vinifera* cultivadas para a produção de uvas de mesa e vinho não possuem resistência ao *P. viticola*, pelo que têm sido desenvolvidos esforços no sentido de integrar *loci* de resistência de espécies de *Vitis* asiáticas e norte americanas em *V. vinifera*, criando híbridos de cruzamento resistentes. O *loci* de resistência mais utilizado nos programas de melhoramento da Europa é o *Resistance to Plasmopara viticola 3* (*Rpv3*) .

Estudos anteriores demonstraram que a sinalização pela hormona vegetal ácido jasmónico está envolvida na resposta de defesa de *V. vinifera* ao *P. viticola*. O ácido jasmónico é produzido por ação de uma série de enzimas, iniciando-se pela libertação de ácido α-linolénico (C18:3) da membrana do cloroplasto, pelas fosfolipases A. Vários estudos relacionados com o papel dos lípidos e dos ácidos gordos na resposta imune das plantas demonstraram que os lípidos e moléculas derivadas, como o ácido jasmónico e outras oxilipinas, participam na resposta da videira aos patógenos que a infetam, através da modulação do perfil lípido, de ácidos gordos e das vias de transdução de sinal. Além disso, os lípidos são os principais constituintes das membranas celulares, que juntamente com a cutícula são a primeira barreira que os patógenos têm de atravessar ao tentar infetar a planta, pelo que a composição lipídica da membrana da videira e a sua plasticidade e capacidade de modulação são de extrema relevância na resposta de defesa contra o *P. viticola*.

Vários estudos têm vindo a ser desenvolvidos no sentido de perceber de que forma ocorre a modulação dos ácidos gordos e lípidos durante a infeção e em que medida é que esta é diferente em variedades suscetíveis e tolerantes. Percebeu-se que a maioria das alterações nas variedade resistentes ocorrem ao nível dos lípidos dos cloroplastos, sendo que a quantidade de monogalactosildiacilglicerol (MGDG) e digalactosildiacilglicerol (DGDG) aumenta em condições de stresse biótico, assim como o conteúdo em C18:3, o que também se relaciona com o aumento da fluidez da membrana. Em contraste, percebeu-se que em variedades suscetíveis não ocorriam alterações significativas após a infeção quer na composição lipídica quer nos perfis de ácidos gordos.

Deste modo, o objetivo desta tese é contribuir para a elucidação do papel dos lípidos do cloroplasto na defesa de *V. vinifera* ao *P. viticola*. Para tal, foram avaliadas as alterações dos perfis lipídicos e de ácidos gordos, assim como a expressão dos genes envolvidos na síntese dos lípidos do cloroplasto em variedades de *V. vinifera* com diferentes *loci* de resistência (Chardonnay, suscetível; Regent e Calardis blanc, com genótipos *Rpv3-1* e *Rpv3-1/Rpv3-2*, respetivamente; Solaris e Sauvignac, com genótipos *Rpv3-3/Rpv10* e *Rpv3-1/Rpv12*, respetivamente). A membrana dos cloroplastos tem uma composição distinta das outras membranas celulares, sendo constituída essencialmente pelos galactolípidos MGDG e DGDG, contendo adicionalmente em menores quantidades um sulfolípido (sulfoquinovosildiacilglicerol

(SQDG)) e um fosfolípido (fosfatidilglicerol (PG)). Visto as famílias de genes responsáveis pela biossíntese dos lípidos do cloroplasto (*MGD1, MGD2, DGD1X2, DGD2, SQD1, SQD2, UGP3, PGPP1, PGP1* e *CDS*) não estarem ainda estudadas em videira, foi necessário proceder à sua caracterização antes da realização dos estudos de expressão génica. Adicionalmente, de modo a caracterizar as alterações em todos os glicerolípidos que compõem as membranas do cloroplasto, foi otimizado um protocolo de cromatografia em camada fina seguido de análise de cromatografia gasosa que permitiu isolar e caracterizar o SQDG pela primeira vez em videira.

Em relação ao conteúdo de ácidos gordos, percebeu-se que Sauvignac, uma variedade tolerante, demonstra um comportamento muito idêntico ao que se observa em Chardonnay, que é uma variedade suscetível, e as restantes variedades, todas com diferentes graus de tolerância, se comportam de maneira diferente e idêntica entre elas. Sabe-se que Sauvignac apresenta um background genético em pirâmide *Rpv3-1/Rpv12*, em que o papel do *Rpv12* se sobrepõe ao *Rpv3* e não parece estar associado às respostas lipídicas. Deste modo, Sauvignac não demonstra alterações significativas no seu conteúdo lipídico e em ácidos gordos. Em condições controlo, Sauvignac é a variedade que tem uma menor quantidade total de ácidos gordos, que não varia após a infeção. Em termos de quantidade de duplas ligações, Chardonnay é a variedade que apresenta os valores de índice de ligações duplas (DBI), ácidos gordos polinsaturados (PUFA) e ácidos gordos insaturados (UFA), que está de acordo com o conhecimento prévio.

Após a separação do SQDG dos restantes lípidos na placa de sílica, em Sauvignac, procedeu-se à análise do perfil lipídico desta variedade, assim como do seu conteúdo em ácidos gordos. A partir desta análise, podemos concluir que Sauvignac sofre uma grande alteração, após infeção, no conteúdo em SQDG, que apresenta uma acentuada diminuição. Sauvignac é também a variedade que tem a quantidade relativa mais elevada de SQDG sob condições controlo. Além disso, observa-se também uma diminuição na quantidade de ácido fosfatídico (PA), que é um precursor da biossíntese de PG e dos triacilgliceróis (TAGs), contidos nos lípidos neutros, que sofrem um grande aumento na sua percentagem após 4 horas de infeção pelo patógeno. Em contraste, os lípidos do cloroplastos, MGDG e DGDG, sofrem uma diminuição, o que demonstra que esta variedade apresenta barreiras de defesa focadas na utilização dos lípidos neutros (NL). Podemos confirmar esta informação ao analisar a percentagem de NL e MGDG em todas as variedades: nas variedades com a maior percentagem de MGDG (Chardonnay e Solaris), existe uma percentagem mais baixa de NL, assim como nas variedades que têm mais MGDG existem menos NL. Além disso, as variedades mais ricas em MGDG são também as que apresentam percentagens mais elevadas de C18:3, que se encontra presente em grande quantidade nos cloroplastos. As variedades com menor valor relativo de C18:3 apresentam percentagens mais elevadas de ácido palmítico (C16:0) e ácido oleico (C18:1). Em relação à análise da expressão génica, os resultados obtidos vão ao encontro dos resultados obtidos na análise lipídica, justificando em parte as modulações observadas nos lípidos.

Estes resultados demonstraram a importância do papel dos lípidos do cloroplasto na resposta de *V. vinifera* à infeção pelo *P. viticola*. Os lípidos sofrem diferentes modulações de acordo com o background genético da videira, promovendo alterações ao nível dos ácidos gordos. Além disso, a análise de expressão génica confirmou que existem realmente alterações na expressão dos genes envolvidos na biossíntese de lípidos presentes nos cloroplastos. Deste modo, podemos concluir que o background genético da videira determina a expressão diferencial dos genes envolvidos na síntese dos lípidos cloroplastidiais, fazendo com que estes sejam expressos em maior ou menor quantidade, de acordo com os mecanismos de defesa que cada variedade de *V. vinifera* adota para ultrapassar a infeção pelo *P. viticola*.

Palavras-chave:

Vitis vinifera, Plasmopara viticola, lípidos cloroplastidiais, sinalização lipídica, *loci* de resistência (*Rpvs*)

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Equation 2.1 - Calculation of double bound index.

Abbreviations:

12-OPDA – 12-oxophytodienoic Acid

AOC – Allene Oxide Synthase

AOS – Allene Oxide Cyclase

At – *Arabidopsis thaliana*

bp – Base Pairs

C16:0 – Palmitic Acid

C16:1t – Trans-hexadecanoic Acid

C17:0 – Heptadecanoic Acid

C18:0 – Stearic Acid

C18:1 – Oleic Acid

C18:2 – Linoleic Acid

C18:3 – α-linolenic Acid

CDP-DAG – Cytidine Diphosphate-Diacylglycerol

CDS - Cytidine Diphosphate-Diacylglycerol Synthase

COI1 – Coronative-insensitive 1

DAG – 1,2-diacylglycerol

DBI – Double Bond Index

DGD – Digalactosyldiacylglycerol Synthase

DGDG – Digalactosyldiacylglycerol

EF1α – Elongation Factor 1-alpha

ETI – Effector-triggered Immunity

FA – Fatty Acids

FAMEs - Fatty Acids Methyl Esters

Glc1P - α-D-glucose 1-phosphate

GC – Gas Chromatography

Hpi – Hours Post Infection

ILE - Isoleucine

JA – Jasmonic Acid

JAR1 – Jasmonates Resistant 1

LOX2 – Lipoxygenase 2

MAFFT - Multiple Alignment using Fast Fourier Transform

MAMPs – Microbe-associated Molecular Patterns

MGD – Monogalactosyldiacylglycerol Synthase

MGDG – Monogalactosyldiacylglycerol

ML – Maximum Likelihood

Mw – Molecular Weight

NL – Neutral Lipids

OPC:8 - 3-oxo-2(29[Z]-pentenyl)-cyclopentane-1-octanoic Acid

OPR3 - 12-Oxophytodienoic Acid Reductase 3

Os – *Oriza sativa*

PA – Phosphatidic Acid

PAMPs – Pathogen-associated Molecular Patterns

PC – Phosphatidylcholine

PE - Phosphatidylethanolamine

PG – Phosphatidylglycerol

PGP – Phosphatidylglycerol-phosphate

PGP1 - Phosphatidylglycerol-phosphate Synthase

PGPP1- Phosphatidylglycerol-phosphate Phosphatase

PI - Phosphatidylinositol

Pi – Inorganic Phosphate

pI – Isoelectric Point

PLA – Phospholipase A

PTI – PAMP-triggered Immunity

PUFA – Polyunsaturated Fatty Acids

qPCR – Quantitative Real-time Polymerase Chain Reaction

ROS – Reactive Oxygen Species

Rpv – Resistance to *Plasmopara viticola*

SA – Salicylic Acid

SAND – Proteins’ family **S**p100, **A**IRE-1, **N**ucP41/75, **D**EAF-1

Sl – *Solanum licopersicum*

SQD1 - UDP Sulfoquinovosyl Synthase

SQD2 - UDP-sulfoquinovose: DAG Sulfoquinovosyltransferase

SQDG – Sulfoquinovosyl Diacylglycerol

TAG - Triacylglycerol

TLC – Thin Layer Chromatography

UDP – Uridine Diphosphate

UFA – Unsaturated Fatty Acids

UGP - Uridine Diphosphate Glucose Pyro Phosphorylase

Zm – *Zea mays*

1. Introduction

The production of *Vitis vinifera* L., the most well-known and cultivated grapevine species, has been greatly impaired by the downy mildew disease, which is caused by *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni. Since *V. vinifera* is very susceptible to this biotrophic obligatory oomycete, it is essential to minimize crop losses through the application of several phytochemicals throughout the season (Koledenkova *et al,* 2022; Yin *et al*, 2016). Due to the appearance of fungicide resistance caused by the recurring application of pesticides (Massi *et al*, 2021), the cost of those pesticides and their application, environmental pollution and the impact in human health, the European Union (Directive 2009/128/EC) demands that non-chemical alternatives of controlling this disease must be developed in the future. Once *V. vinifera* cultivars do not present genetic resistance to downy mildew infections, efforts have been conducted on breeding programs to introgress resistance *loci* from wild North American and Asian *Vitis* species (resistant to this pathogen) into *V. vinifera*, resulting in new resistant grapevine crossing hybrids. The most used resistance *loci* in European breeding programs is the *Resistance to Plasmopara viticola 3* (*Rpv3*) (Eisenmann *et al,* 2019).

During infection by *P. viticola*, several defence processes are triggered in the plant. The first barriers that the microorganisms have to overtake are the cuticle and the cell wall. Once these microorganisms are inside the plant, they can secret conserved molecules (MAMPs or PAMPs, microbe- or pathogen-associated molecular patterns), leading to PAMP-triggered immunity (PTI) that they must overcome to successfully colonize the plant tissues. The second line of defence of plants, initiated due to pathogen effectors, relates to the production of resistance proteins, which provides immunity against microorganisms that overcome PTI, called Effector-triggered immunity (ETI) (Hein *et a*l, 2009). While these plant-pathogen interactions are occurring, the molecular environment of the plant is changing.

Jasmonic acid (JA) and salicylic acid (SA) signalling pathways are particularly important to grapevine defence responses, with the first pathway involved in the response to necrotrophic pathogens and the second associated with biotrophic pathogens infections (Glazebrook, 2005). JA is synthesized from α-linolenic acid (C18:3), and its biosynthetic pathway begins with the action of phospholipases A (PLAs), which release fatty acids from the chloroplast membrane to form a pool of free FA. Then, oxygenation of these FA occurs, transforming them into hydroperoxides, through lipoxygenase (13-LOX). Next, allene oxide synthase (AOS) will convert these hydroperoxides into 12,13-epoxylinolenic acid, which will be rapidly transformed into cis 12-oxo-phytodienoic acid (12-OPDA) by allene oxide cyclase (AOC). The enzymes involved in the reduction of this compound are found in the peroxisome, so it must be transported to the peroxisome, through an ABC transporter. In the peroxisome, the OPDA cyclopentenone component (12-oxo-phytodienoic acid) is reduced to cyclopentanone via 12-Oxophytodienoic Acid Reductase 3 OPDA reductase (OPR3). This latter compound undergoes three rounds of β-oxidation to form JA. After this, JA is conjugated with isoleucine (ILE) and transported to the cytoplasm, where it will be available for transport throughout the plant so that it can perform its functions in response to infections. (Ghorbel *et al,* 2021).

In 2015, it was proven that JA signalling is also active in the first hours of *P. viticola* infection. The expression of *LOX2, AOC, AOS* and *OPR3* underwent an early (6 and 12 hours post infection (hpi)) up-regulation, as well as genes coding for enzymes involved in activation (*JAR1*) and signalling (*COI1*) suffered a later (18 and 24 hpi) activation in a resistant genotype of *V. vinifera* against *P. viticola* (Figueiredo *et al,* 2015). Also, JA-ILE was shown to accumulate in the first hours of interaction in an *Rpv3* background (Guerreiro *et al*, 2016).

Studies regarding the role of lipids and FA in the immune response of plants (Cavaco *et al*, 2021) have shown that lipids and lipid associated molecules, as FA, glycerolipids and jasmonates, participate in grapevine resistance to pathogens, through the modulation of signal transduction pathways. In the crosstalk between plants and pathogens, lipids have very important roles in the pathogen life cycle completion, since the first barrier that microorganisms have to cross is the cuticle, which is mainly made up of cutin monomers and oligomers, composed of hydroxy or hydroxy/epoxy C16 and C18 fatty acids, produced in the chloroplast (Bakan *et al*, 2017; Cavaco *et al,* 2021). Furthermore, the membrane seems to be one of the most important factors in the progression of the life cycle of pathogens, since it is in the membrane that antimicrobial peptides are found, that are specific due to the different lipid composition between pathogen and host cells, mounting a first line of defense against pathogens (Teixeira *et al*, 2012).

In fact, the main components of plant cell membranes are galactolipids, lipids exclusively located in plastids, so these should be meaningful in plant defence responses (Canonne *et al*, 2011). When PTI is overcomed, the plant activates a second layer of defence, within this second layer, lipid composition may be modulated so plants are able to produce signaling and infection-fighting molecules (Cavaco *et al*, 2021). *Laureano et al* (2018) have shown that the main alterations in lipid composition take place at the chloroplast, where after pathogen challenge, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), the most abundant lipids in the chloroplast membranes, accumulated, and the content in C18:3 increased in the resistant genotype. In contrast, in the susceptible variety there were no significant changes in lipid and FA profiles. That increase could be associated with an increase in membrane fluidity, to prevent membrane damages, and/or related to lipid hydrolysis to release free C18:3 FA, through phospholipase A (PLA), for JA synthesis (Matos & Pham-Thi, 2009).

The main lipids present in the chloroplast are MGDG, DGDG, sulfoquinovosyl diacylglycerol (SQDG) and phosphatidylglycerol (PG), which biosynthetic pathways will be described in a synthetic way. In chloroplasts, 1,2-diacylglycerol (DAG) is converted in MGDG by monogalactosyldiacylglycerol synthase (MGD), and that lipid is used to produce DGDG by digalactosyldiacylglycerol synthase (DGD) (Figure 1.1). Regarding the synthesis of SQDG, it begins with the conversion of α-D-glucose 1-phosphate (Glc1P) in uridine diphosphate glucose (UDP-glucose) by UDP-glucose pyro phosphorylase (UGP), that is further converted in UDP-6-sulfoquinovosyl by UDP-sulfoquinovosyl synthase (SQD1), and then in SQDG by UDP-sulfoquinovose: DAG sulfoquinovosyltransferase (SQD2) (Figure 1.1). Finally, regarding PG, its synthesis begins with phosphatidic acid (PA) being transformed in cytidine diphosphate-diacylglycerol (CDP-DAG) by CDP-DAG synthase (CDS), that is transformed in phosphatidylglycerol-phosphate (PGP) by phosphatidylglycerol-phosphate synthase (PGP1), and then in PG by phosphatidylglycerol-phosphate phosphatase (PGPP) (Figure 1.1).

The plant-pathogen interaction is very dynamic and occurs in several cellular compartments. What is known is that signaling by pathogens leads to a desaturation of FA at the chloroplast level, for subsequent release of free FA, an important step for the formation of JA. What is not completely understood is how the increase in MGDG and DGDG occurs in the chloroplast, and how the interaction with the pathogen modulates the remaining lipids present in the chloroplast, such as SQDG and PG.

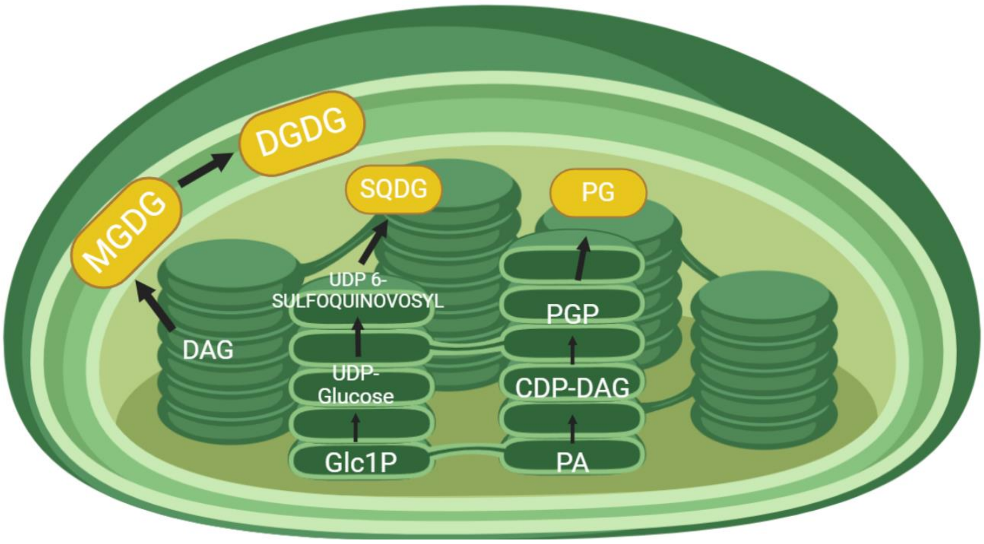


Figure 1.1 – MGDG, DGDG, SQDG and PG synthesis pathway in chloroplast. Regarding the synthesis of galactolipids, 1,2-diacylglycerol (DAG) is converted into MGDG by monogalactosyldiacylglycerol synthase (MGD), and into DGDG by digalactosyldiacylglycerol synthase (DGD). Regarding the synthesis of SQDG, α-D-glucose 1-phosphate (Glc1P) is converted in uridine diphosphate glucose (UDP-glucose) by UDP-glucose pyro phosphorylase (UGP), that is converted in UDP-6-sulfoquinovosyl by UDP-sulfoquinovosyl synthase (SQD1), and then in SQDG by UDP-sulfoquinovose: DAG sulfoquinovosyltransferase (SQD2). Regarding PG, phosphatidic acid (PA) is transformed in cytidine diphosphate-diacylglycerol (CDP-DAG) by CDP-DAG synthase (CDS), that is transformed in phosphatidylglycerol-phosphate (PGP) by phosphatidylglycerol-phosphate synthase (PGP1), and then in PG by phosphatidylglycerol-phosphate phosphatase (PGPP).

It is known that only genotypes that present the resistant genetic background (*Rpv3*) demonstrate modulation of FA and lipids, as well as overexpression of genes encoding enzymes involved in signaling through lipids, as was observed in Regent (*Rpv3-1*). Sauvignac (*Rpv3- 1/Rpv12*) and Chardonnay (susceptible) demonstrated an under expression of these genes. This demonstrates that the *Rpv3 loci* is associated with regulation by lipid signaling, while other *loci* appear not to be so related to lipids and their modulation (Laureano *et al*, 2023). The NW-10/16 of *P. viticola*, used in this present work, is an aggressive pathovar isolate capable of partially overcoming *Rpv3* resistance (some sporulation occurred in Regent) but not *Rpv3-1/Rpv12* pyramid resistance (no sporulation is seen in Sauvignac).

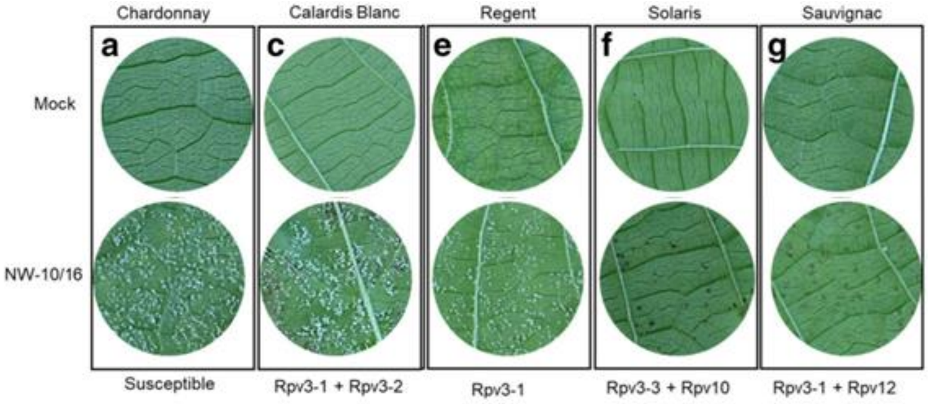


Figure 1.2 – Infection of *Plasmopara viticola* on *Vitis vinifera* susceptible cultivar 'Chardonnay' and tolerant cultivars 'Calardis Blanc', 'Regent', 'Solaris' and 'Sauvignac'. Pictures of representative leaves were taken at 6 days post inoculation (dpi). Chardonnay is susceptible to *P. viticola*, as sporulation can be seen when it is infected with NW-10/16. Calardis blanc and Regent are tolerant, presenting *loci Rpv3-1/Rpv3*-2 and *Rpv3-1*, respectively, however some sporulation occurs since this pathovar can overcome the resistance conferred by Rpv3. Solaris and Sauvignac have *loci Rpv3-3/Rpv10* and *Rpv3-1/Rpv12*, respectively, being resistant, so they do not present any sporulation when infected by this pathovar (adapted from Gouveia *et al*, 2020).

Within this thesis, we aim at understanding how *P. viticola* infection changes the modulation of chloroplastidial lipids, fatty acids and the genes responsible for their synthesis in grapevine. To achieve this goal, five *V. vinifera* genotypes harbouring different *Rpvs* (Figure 1.2) and thus, different tolerance levels to *P. viticola* were analysed. Chardonnay is a susceptible genotype; Calardis blanc and Regent are tolerant, with different Rpv backgrounds (*Rpv3-1/Rpv3-2* and *Rpv3-1*, respectively); and Sauvignac and Solaris are resistant (*Rpv3-1/Rpv12* and *Rpv3-3/Rpv10*, respectively) (Gouveia *et al*, 2020).

Four gene families coding for enzymes involved in the synthesis of chloroplastidial lipids were characterized in *V. vinifera,* for the first time. Gene expression was analysed at 4 hpi in the different grapevine genotypes. Accordingly, the modulation of fatty acids in total lipids and the relative amounts and fatty acid composition of MGDG, DGDG, SQDG and PG was analysed. This work provides a better understanding on how the four chloroplastidial lipids participate in the defense response of grapevine.

2. Material and Methods

2.1. Biological material

In this work, leaves of the 5 varieties of *Vitis vinifera* studied (Chardonnay, Calardis blanc, Sauvignac, Regent and Solaris) were used in the control and infected forms. All these leaves were harvested between the third and sixth node from the apex of the stem, since young and well-expanded leaves should be used for these trials. The leaves were infected using a suspension with *Plasmopara viticola* spores.

2.2. Identification of MGDG, DGDG, SQDG and PG Synthases in *Vitis vinifera*

The genes encoding MGDG, DGDG, SQDG and PG synthases in *Arabidopsis thaliana (*TAIR - https://www.arabidopsis.org/), *Oryza sativa (*NCBI - https://www.ncbi.nlm.nih.gov/), *Solanum lycopersicum* (SGN - https://solgenomics.net/) and *Zea mays* (NCBI) protein sequences were used as query organisms (databases accessed in October 2023). The coding sequences and the amino acid sequence of the respective proteins were retrieved. Subsequently, the protein sequences of these enzymes were aligned against the *V. vinifera* genome, using the NCBI tBLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi), followed by the retrieval of the coding sequences and respective protein sequences of *V. vinifera*. The position of the different *V. vinifera* genes on the chromosome, the number of base pairs (bp) and the size of the proteins were determined using the NCBI database (accessed in October 2023). Using Compute pI/Mw tool from Expasy (https://web.expasy.org/compute_pi/), it was possible to determine the molecular weight (Mw) and isoelectric point (pI) of *V. vinifera* proteins.

2.2 *Cis*-elements Analysis

Using the NCBI database, the 2000 bp sequences upstream of each gene coding region were obtained. Those sequences were used to identify the predicted *cis*-acting elements through the PlantCARE database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (accessed in October 2023).

2.3 Domain Structure Analysis, Putative Functions and Subcellular Location Prediction

Domain determination was performed using HMMER platform, through Pfam database (https://www.ebi.ac.uk/Tools/hmmer/) (accessed in October 2023). The putative functions of *V. vinifera* genes, represented by GO terms, were obtained using ProteInfer (https://google-research.github.io/proteinfer/) (accessed in October 2023). The subcellular location was predicted using TargetP-2.0 (https://services.healthtech.dtu.dk/services/TargetP-2.0/, accessed in October 2023), PredSL (http://aias.biol.uoa.gr/PredSL/, accessed in October 2023) and Localizer (https://localizer.csiro.au/, accessed in October 2023).

2.4 Construction of Phylogenetic Trees

To proceed with the construction of the phylogenetic trees of the galactolipids synthases family, SQDG synthases family and PG synthases family, the protein sequences obtained from *A. thaliana, O. sativa, S. lycopersicum, Z. mays* and *V. vinifera* were aligned using the *Multiple Alignment using Fast Fourier Transform* (MAFFT) (https://www.ebi.ac.uk/Tools/msa/mafft/) tool. Through RAxML-HPC v.8, on CIPRES Science Gateway (https://www.phylo.org/), a maximum likelihood (ML) phylogenetic analysis was performed, using the following parameters: protein substitution model PROTCAT, protein substitution model + BLOSUM62, bootstrap 1000 interactions with rapid bootstrap analysis (-fa). The phylogenetic trees for the three families were observed on FIGTree (http://tree.bio.ed.ac.uk/software/Figtree/) and edited on Inkscape (http://www.inkscape.org/, accessed January 2024).

2.5. Quantitative Real-Time PCR (qPCR)

The qPCR experiments were conducted using the Maxima™ SYBR Green qPCR Master Mix (2×) kit (Fermentas, Ontario, ON, Canada), according to the manufacturer's instructions, in a StepOne™ Real-Time PCR system (Applied Biosystems, Sourceforge, USA). Primers were produced by STAB VIDA, Caparica, Portugal. Primer sequences and melting curves of targeted genes are provided in Supplementary Data 1 and 2. For all genes analysed, the PCR cycling started with a denaturation step at 95 ºC for 10 min followed by 45 cycles of 95 ºC denaturation for 15 s and annealing/elongation at gene-specific temperatures (Supplementary data 1) for 30 s. Non-template controls were added for each gene and a dissociation curve analysis was performed to confirm single-product amplification and the existence of non-specific PCR products. Complementary DNA were already available in the lab. Those samples represent five *V. vinifera* cultivars, with different degrees of susceptibility to *P. viticola* that were inoculated with the pathogen (NW-10/16) and harvested at 4 hpi. Mock inoculated samples (water inoculation) were also harvested at 4 hpi as controls. In all samples, the expression of all genes (*MGD1, MGD2, DGD1X2, DGD2, SQD1, SQD2, UGP3, PGPP1, PGP1* and *CDS*) was analysed.

Gene expression was estimated according to *Hellemans et al* (2007). The expression of genes coding for Elongation Factor 1-alpha (*EF1α*) and SAND family protein (*SAND*) were used as reference genes (Monteiro *et al*, 2013). The gene expression estimated for inoculated samples consists of a balance between inoculated and mock inoculated samples, for each cultivar. Statistical significance ($p < 0.05$) of gene expression was determined by the Wilcoxon Signed rank test.

2.6. Lipid analysis

Samples of each variety were previously collected, reduced to powder and frozen at -80º C. Those samples were boiled in 8 mL of water for 3 minutes to inactivate lipolytic enzymes. The extraction of total lipids was performed using a mix of water/chloroform/methanol (1:1:1, v/v/v), as described in Matos *et al* (2008). After centrifugation of this mixture, the lower chloroformic phase was collected and dried under nitrogen at 37º C and stored in the freezer (-20ºC) with 500 µl of ethanol toluene (1:4 v/v) for later use. Fatty acids methyl esters (FAMEs) were prepared by trans-methylation of the lipid extract with methanol/sulfuric acid (97.5:2.5, v/v). Fatty acids quantitative analysis was performed using gas chromatography (GC) (430 Gas Chromatograph, Varian) at 210ºC, equipped with hydrogen flame ionization detector with heptadecanoic acid (C17:0) as an internal standard.

The separation of all the plastidial lipid classes, by thin layer chromatography (TLC) on silica plates (G-60, Merck, VWR), required an adaption of the protocols routinely used by our team (Esquível *et al*, 2017; Laureano *et al,* 2018) which use the solvent system developed by *Lepage* (1967), consisting of chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1), that separates the major polar lipids, whereas neutral lipids migrate in a single band in the solvent front. Since in this system the relatively abundant phosphatidylcholine (PC) co-migrates with SQDG, present in minute amounts, after visualisation of lipid bands with primuline (0.01% in 80% acetone) under UV light and scraping off the PC+SQDG containing band was extracted as above described for total lipids and the extract further subjected to another TLC using a solvent system described by Gardner and modified by *Grenier* (1972) (acetone/acetic acid/water (100:2:1)). Total lipid extracts from *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* (a microalgae without PC and containing higher amounts of SQDG than higher plants) (Matos *et al,* 2008; Esquível *et al,* 2017) were also spotted on TLC plates to serve as standards for band identification. FAMEs were prepared by trans-methylation with methanolic sulphuric acid (25:0.6, v/v). Several parameters were calculated, such as polyunsaturated fatty acids (PUFAs), unsaturated fatty acids (UFAs) and double bound index (DBI).

$$\text{DBI}= \frac{(\%monodienoic\ acids)+2(\%dienoic\ acids)+3(\%trienoic\ acids)}{100}$$

Equation 2.1 - Calculation of Double Bound Index (DBI)

Due to lack of normality and homogeneity, to evaluate statistical differences in FA composition among the different *V. vinifera* cultivars, a non-parametric test, namely the Krustal-Wallis test, was used through the IBS SPSS Statistics 29 (IBM, NY, USA). Significance was assumed when p-value ≤ 0.05.

3. Results and Discussion

3.1. Characterization of Grapevine Genes Encoding Chloroplastidial Enzymes Involved in Lipids Synthesis

In order to understand the role of lipids and fatty acids in grapevine's defence against *P. viticola*, a characterization of genes that encode enzymes that synthesize chloroplastidial lipids was performed. The members of those families were identified through comparison with the query organisms (*Arabidopsis thaliana*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays),* and a total of 14 genes encoding 10 predicted proteins were found in *V. vinifera* genome (Table 3.1).

MGDG synthases family, which comprises genes that encode enzymes involved in the synthesis of MGDG (Figure 1.2), has been previously characterized in other plant species, such as *Arabidopsis thaliana.* In *Arabidopsis thaliana,* three MGDG synthases have been identified and divided into two types differing in their N-terminal portion: type A (atMGD1 and atMGD3) and type B (atMGD2). In *Arabidopsis*, the enzymes of the first group are found on chromosomes 4 and 2, respectively, while atMGD3 is found on chromosome 5 (Awai *et al,* 2001). Two MGDs were found in *V. vinifera*, one of each group, named MGD1 and MGD2, which genes are localized in chromosome 4 (as in *Arabidopsis*) and 11, with 8 and 9 exons, respectively. Similarly, the genes coding for the enzymes of the first group of *Arabidopsis* also have 8 exons. Regarding the DGD family, which includes genes responsible for the synthesis of DGDG, 5 genes were found in *Zea mays*: two of these genes are found on chromosome 1, and the remaining on chromosomes 2, 4 and 5 (Wang *et al*, 2020). In *V. vinifera* two isoforms of DGD1, in chromosome 8, and one isoform of DGD2, in chromosome 15. *ZmDGD4* and *ZmDGD5* have 4 exons each, which differs from the homologue in *V. vinifera*, *DGD1*, which has 7 exons. *ZmDGD1*, *ZmDGD2* and *ZmDGD3* have 7 exons each, while the homologue in *V. vinifera*, *DGD2*, has 6 exons.

Table 3.1 - General features of *Vitis vinifera* genes and proteins analysed. Proposed grapevine enzymes nomenclature, gene *locus*, protein and nucleotide accessions (from NCBI), chromosome location, exon number, protein length, molecular weight (Mw) and isoelectric point (pI) are represented.

Enzymes	XM	XP	mW (Da)	pI	Bp	Cromossome	Exons	Protein length	LOC
MGD1	XM_002283374.5	XP_002283410.2	58195.34	8.89	2319	4 (6930876..6940274)	8	528	LOC100247378
MGD2	XM_002263407.4	XP_002263443.1	50415.45	7.15	1803	11 (5944998..5948427)	9	453	LOC100248939
DGD1 isoform X1	XM_002264623.5	XP_002264659.1	90307.46	8.33	2703	8 (8487726..8514922)	7	797	LOC100259167
DGD1 isoform X2	XM_059738923.1	XP_059594906.1	86609.32	8.49	2598	8 (8487726..8514922)	7	764	LOC100259167
DGD2	XM_002266280.5	XP_002266316.1	53098.47	6.82	1892	15 (23506832..23511799)	6	466	LOC100251641
SQD1	XM_002279049.5	XP_002279085.1	54361.89	8.81	1952	4 (986313..989112)	3	485	LOC100242408
SQD2	XM_002278832.5	XP_002278868.2	58556.4	9.13	2078	8 (17173525..17181609)	11	519	LOC100259616
UGP3v1	XM_002272984.5	XP_002273020.1	95701.09	5.94	3118	13 (831615..841789)	18	860	LOC100241552
UGP3v2	XM_010659945.3	XP_010658247.1	95772.17	5.94	3122	13 (831615..841789)	18	861	LOC100241552
UGP3v3	XM_010659944.3	XP_010658246.1	97031.61	5.99	3155	13 (831615..841789)	18	872	LOC100241552
UGP3v4	XM_010659943.3	XP_010658245.1	97102.69	5.99	3159	13 (831615..841789)	18	873	LOC100241552
PGPP1	XM_002283823.5	XP_002283859.3	39167.93	9.01	1717	12 (543752..550144)	5	344	LOC100252338
PGP1	XM_002281508.5	XP_002281544.1	37361.2	9.89	1932	13 (1923962..1928754)	7	348	LOC100245021
CDS	XM_010663378.3	XP_010661680.1	42836.17	9.72	1751	15 (17378950..17405345)	7	397	LOC100248919

Considering sulfolipids biosynthetic enzymes gene family, in *Arabidopsis* the genes *AtSQD1* and *AtSQD2* are described as located on chromosome 4 and 5, respectively (Wang *et al*, 2020). In *V. vinifera*, two enzymes responsible for SQDG synthesis were identified and named SQD1 and SQD2 for which the coding genes are located in chromosomes 4 and 8. *AtSQD1* has 2 exons and *AtSQD2* has 12 exons, different from what happens in *V. vinifera*, whose homologues have 3 and 11 exons, respectively. In *Arabidopsis thaliana* there is also an isoform of the *UGP3* gene, which has 17 exons. In *V. vinifera*, four isoforms of *UGP3* were found, located in chromosome 13, each one with 18 exons, which demonstrates some similarity in the gene structure (Okazaki *et al*, 2009).

Finally, for the PG biosynthesis associated gene family, one isoform of *CDS* was found in chromosome 15, with 7 exons, in *Vitis vinifera*. The homologous genes in *Arabidopsis* are located on chromosomes 1, 2, 3 and 4, with isoform *AtCDS4* also having 7 exons and *AtCDS5* having 6 exons (Haselier *et al*, 2010). Additionally, one isoform of *PGP1* and one isoform of *PGPP1*, associated with PG synthesis, were also found in chromosome 13 and 12, with 7 and 5 exons, respectively. The homologous genes found in *Arabidopsis* of *PGP1* are found on chromosomes 2 and 3, both with 6 exons (Tanoue *et al,* 2014).

3.2. Identification of *Cis*-Elements of Grapevine Chloroplastidial Enzymes Involved in Lipid Synthesis

An analysis of *cis*-regulatory elements in the promoters of grapevine genes encoding for chloroplastidial enzymes involved in lipid synthesis was conducted for a better knowledge regarding the possible transcription regulation and gene function of those grapevine genes (Supplementary Data 3). The conservative promoter elements, CAAT-box and TATA- box, were found in all gene sequences. In addition to the latter, the AT-TATA-box element was also identified in all genes involved in the synthesis of MGDG and PG, as well as in the genes encoding the SQD2 and UGP3 enzymes; this element was not detected in the promoter sequences of enzymes that synthesize DGDG. TATA-box was found in only a few of the coding sequences, in smaller numbers. Regarding the elements involved in wounding and pathogen/ defence response, 1 or 2 TC-rich repeats were found in 6 sequences analysed, 1 W-box was found in *MGD2* and in *DGD2*, while in the genes involved in PG biosynthesis up to 4 of these elements were found. The WUN-motif was found in 5 sequences, the WRE3 in 4 and the box S in 2. CGTCA- and TGACG-motifs, involved in JA response, were found equally in *DGD1*, *SQD2*, *PGP1* and *CDS*. The elements involved in the response to SA are fewer, but TCA-element was found once in *SQD2*, *UGP3* and *PGP1*. In the *AtSQD1* gene of *Arabidopsis* and in the *ZmSQD2*, *ZmSQD3* and *ZmSQD5* genes of *Zea mays*, elements involved in the response to SA are also found (Wang *et al,* 2020).

3.3 Protein Structure and Domain Analysis

Regarding protein size, enzymes involved in the synthesis of PG are the smaller, with the size of proteins varying between 344 and 397 amino acids. Proteins that participate in the synthesis of SQDG are those that have the greatest variation in their size, with the smallest having 485 amino acids and the largest having 873 aa. MGD1 has 528 amino acids and MGD2 has 453 amino acids. Finally, the two isoforms of DGD1 have an average length of 781 amino acids, and DGD2 is smaller, having only 466 amino acids. The enzymes responsible for the synthesis of galactolipids have a pI between 6.82 and 8.89; SQDG synthases between 5.94 and 9.13 and PG synthases are those with the highest pI, all of them above 9.00. These data agree with *Arabidopsis* and *Zea mays*, which also present pI for MGDG synthases between 6.3 and 9.5 and for DGDG synthases between 6,8 and 9 (Okazaki *et al*, 2009). Regarding Mw, PG synthases have the lowest values, between 37361.2 Da and 42836.1 Da. The enzymes involved in the synthesis of galactolipids have Mw between 50415 Da and 90307 Da. SQDG synthases have the highest Mw, with values up to 97102 Da (Table 3.1).

The recognition of domains is a crucial aspect in protein characterisation, enabling their classification and functional annotation. Each group of enzymes involved in chloroplastidial lipid synthesis has highly conserved domains which are remarkable hallmarks of each family (Figure 3.1).

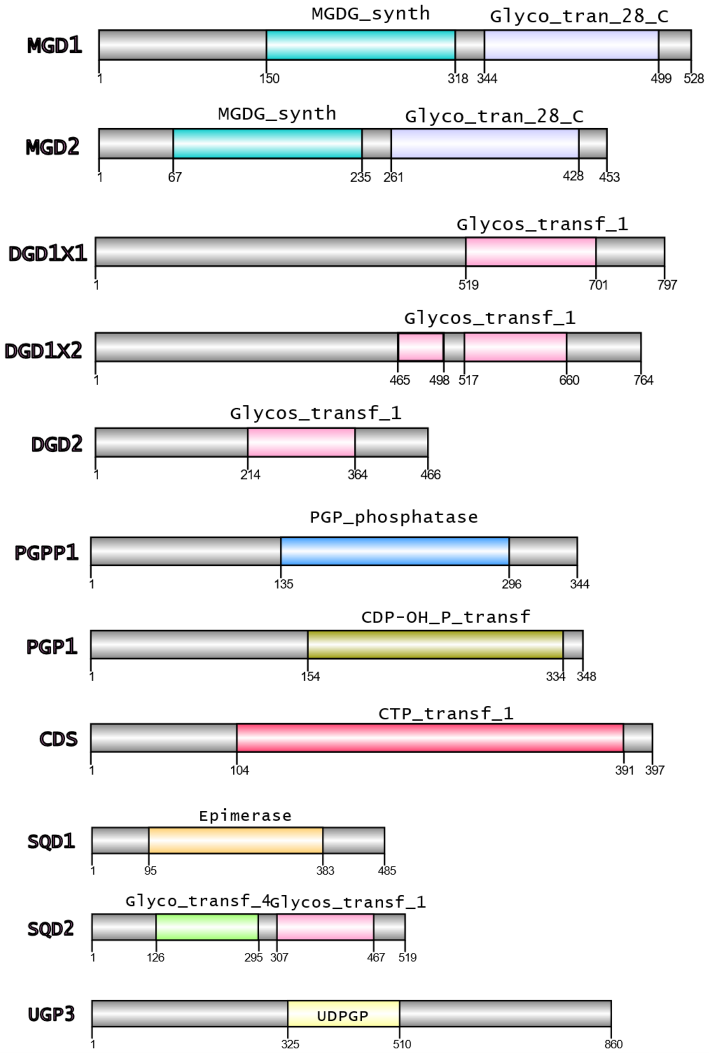


Figure 3.1 - Peptide domain structures of grapevine enzymes. Different domains are indicated by different colors with the respective start and end amino acids. The size of the protein is shown below the representation of each protein.

Both MGD enzymes have a MGDG synthase domain and a Glycosyltransferase family 28 C-terminal domain. All DGD enzymes have a Glycosyl transferases group 1 domain, with isoform 2 of DGD1 having two. Then, we can see that the two types of SQD enzymes have different domains. SQD1 has an Epimerase domain and SQD2 has a Glycosyl transferases group 1 domain and a Glycosyltransferase Family 4 domain. All domains mentioned so far are also present in homologous genes in *Arabidopsis thaliana* and *Zea mays* (Okazaki *et al*, 2009). UGP3 has a UTP-glucose-1-phosphate uridylyltransferase domain. PGPP1 has a Mitochondrial PGP phosphatase domain; PGP1 has a CDP-alcohol phosphatidyltransferase domain and CDS has a Cytidylyltransferase family domain.

3.4 Subcellular Targeting Prediction

Each enzyme acts in a specific cellular location that was predicted and summarized in Supplementary Data 4. The results shown that the majority of proteins were predicted to localise in the chloroplast, specifically in the thylakoids, which is in line with our hypothesis. Thus, as in *Arabidopsis*, UGP3 and SQD1 enzymes are predicted to be located in the chloroplast stroma (Okazaki *et al,* 2009), and CDS5 is located in chloroplast envelope (Haselier *et al,* 2010). However, MGD2, PGPP1 and PGP1 were predicted to localise in mitochondrion, although there is no agreement between all the tools used. In *Arabidopsis*, the homologous enzymes, AtPGPP1 and AtPGP1, are located in both the chloroplast and mitochondria (Zhou *et al,* 2017; Tanoue *et al*, 2014). Other proteins, as MGD1, DGD1X1, DGD1X2 and DGD2, do not seem to have an exact location in the cell, appearing as "other". According to previous knowledge, all of these proteins should be located in the chloroplast, although some are also present in other organelles, such as mitochondria. However, these tools only give us a prediction, so not all results are conclusive. For example, in the case of MGD1 and MGD2 it is known that these enzymes are found in the inner and outer envelope of the chloroplast, respectively (Kobayash *et al*, 2007).

3.5. Phylogenetic Analysis and Putative Functions of Grapevine Chloroplastidial Enzymes Involved in Lipid Synthesis

Phylogenetic analysis of grapevine enzymes involved in chloroplastidial lipid synthesis was performed in order to find evolutionary relationship between the predicted grapevine proteins and the proteins from the query organisms. Based on sequence identity with *Arabidopsis thaliana*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays,* these analysis allowed the proposal of a nomenclature for all the members found in *Vitis vinifera*. In Figure 3.2, we can see that the sequences corresponding to the genes that encode enzymes responsible for galactolipid synthesis are divided into two groups: the members of the MGDG synthase family are in one branch (represented in orange), while those belonging to the DGDG synthase family are in another (represented in red).

According to ProteInfer, these proteins have some putative functions. Grapevine MGDG synthases were predicted to be involved in 1,2-diacylglycerol 3-beta-galactosyltransferase activity (GO:0046509), glycolipid biosynthetic process (GO:0009247), galactolipid metabolic process (GO:0019374) and in chloroplast membrane (GO:0031969); grapevine DGDG synthases were predicted to be involved in primary metabolic process (GO:0044238), digalactosyldiacylglycerol synthase activity (GO:0046481), organic substance metabolic process (GO:0071704) and response to stimulus (GO:0050896).

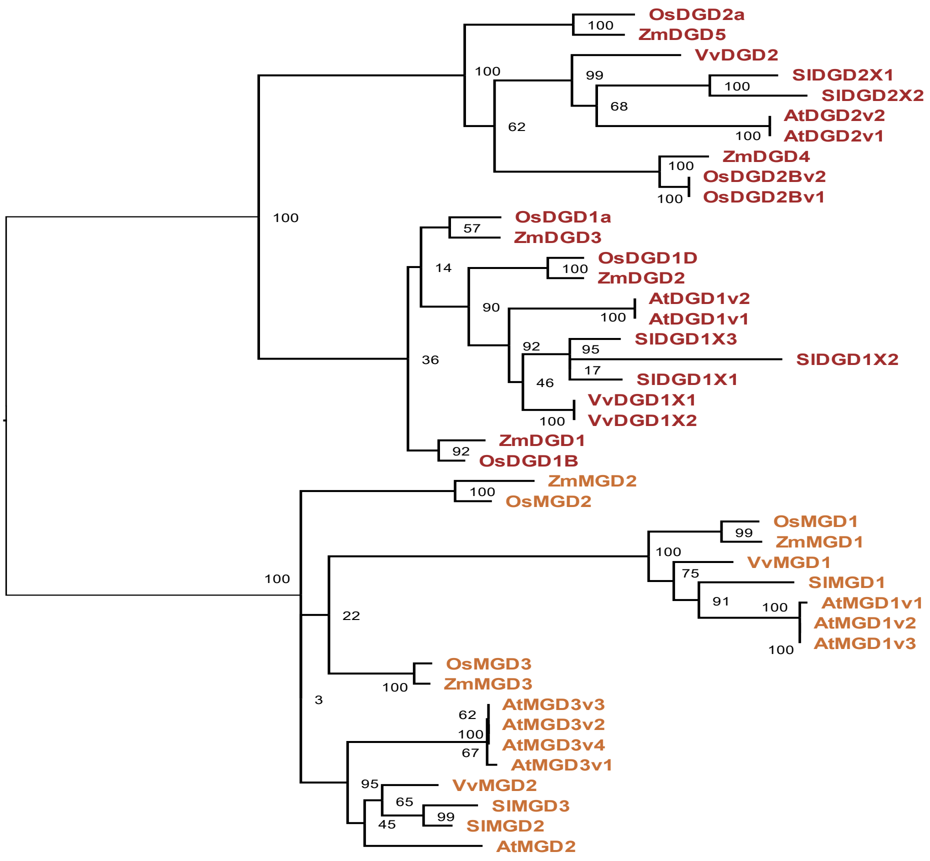


Figure 3.2 - Maximum likelihood phylogenetic tree based on MGDG synthases and DGDG synthases proteins from *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Zea mays* (Zm), *Solanum lycopersicum* (Sl) and *Vitis vinifera* (Vv). Distinct groups are represented by different colours: DGDG synthases in red and MGDG synthases in orange. The numbers above branches show bootstrap values.

In Figure 3.3, it is possible to see that the sequences corresponding to the genes that encode enzymes responsible for SQDG synthesis are divided into two groups: SQD2 enzymes in one branch (represented in pink), while SQD1 enzymes (dark purple) and UGP3 enzymes (represented in light purple) are in another branch. These enzymes, involved in the SQDG synthesis pathway, have some predictive functions: UDP sulfoquinovose synthase activity (GO:0046507), cellular lipid metabolic process (GO:0044255), lipid biosynthetic process (GO:0008610), regulation of cellular process (GO:0050794), thylakoid membrane organization (GO:0010027), regulation of photosynthesis (GO:0010109), chloroplast organization (GO:0009658), phospholipid biosynthetic process (GO:0008654), hormone-mediated signaling pathway (GO:0009755), glycolipid biosynthetic process (GO:0009247), cellular biosynthetic process (GO:0044249) and homeostatic process (GO:0042592).

Finally, regarding the genes encoding enzymes involved in PG synthesis, the phylogenetic tree is represented in Figure 3.4. In this phylogenetic analysis, it is possible to deduce that there are again two major groups: genes encoding PGPP1 enzymes (represented in purple) and genes encoding PGP1 enzymes (green) and CDS enzymes (blue). The predictive functions in which these enzymes are involved, according to ProteInfer, are CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase activity (GO:0008444), phosphatidylglycerol biosynthetic process (GO:0006655), phospholipid transporter activity (GO:0005548), plastid part (GO:0044435) and CDP-diacylglycerol biosynthetic process (GO:0016024).

From the analysis of these phylogenetic trees we can conclude that there are fewer isoforms in *Vitis vinifera* compared to the other reference organisms, which may be due to the fact that these organisms have already been better studied and that there are more detailed characterizations of these genes. In the case of *Vitis vinifera*, this is the first time that these gene families have been characterized, which means that more isoforms can still be discovered.

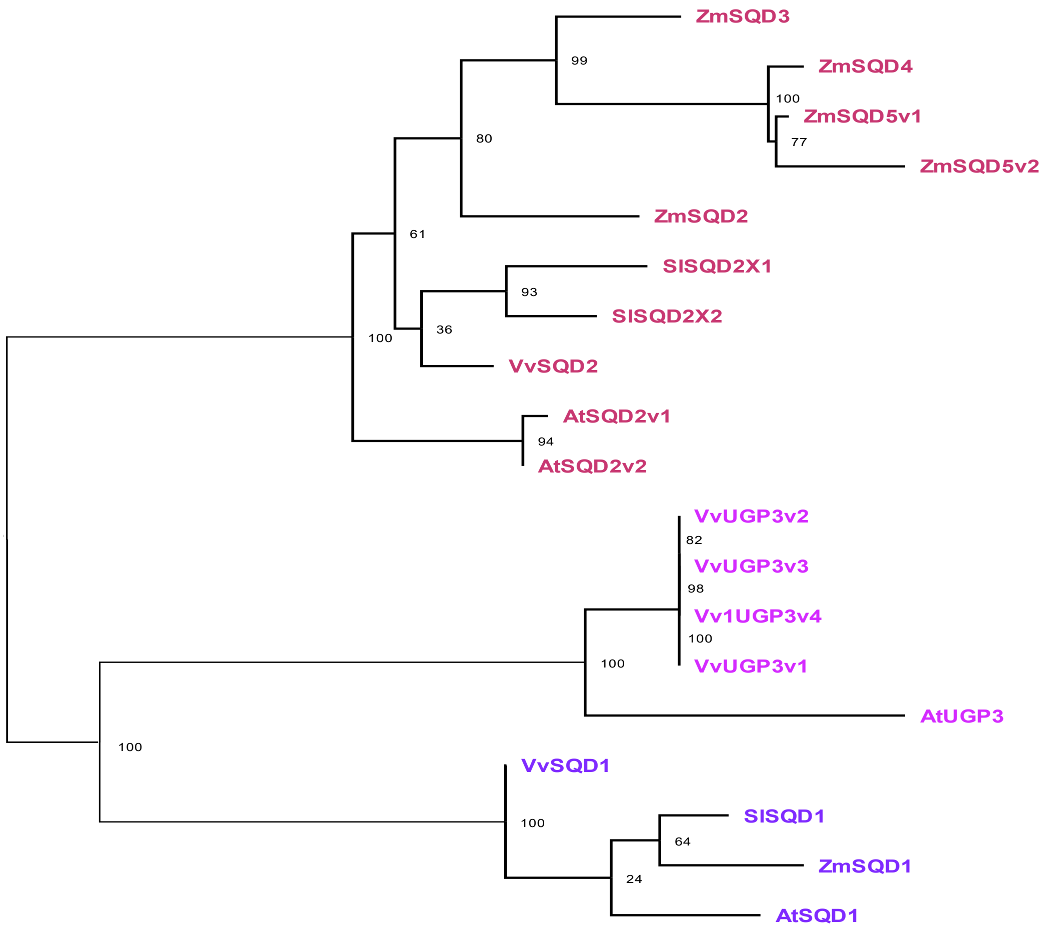


Figure 3.3 - Maximum likelihood phylogenetic tree based on SQDG synthases proteins from *Arabidopsis thaliana* (At), *Zea mays* (Zm), *Solanum lycopersicum* (Sl) and *Vitis vinifera* (Vv). Distinct groups are represented by different colours: SQD1 in dark purple, SQD2 in pink and UGP3 in light purple. The numbers above branches show bootstrap values.

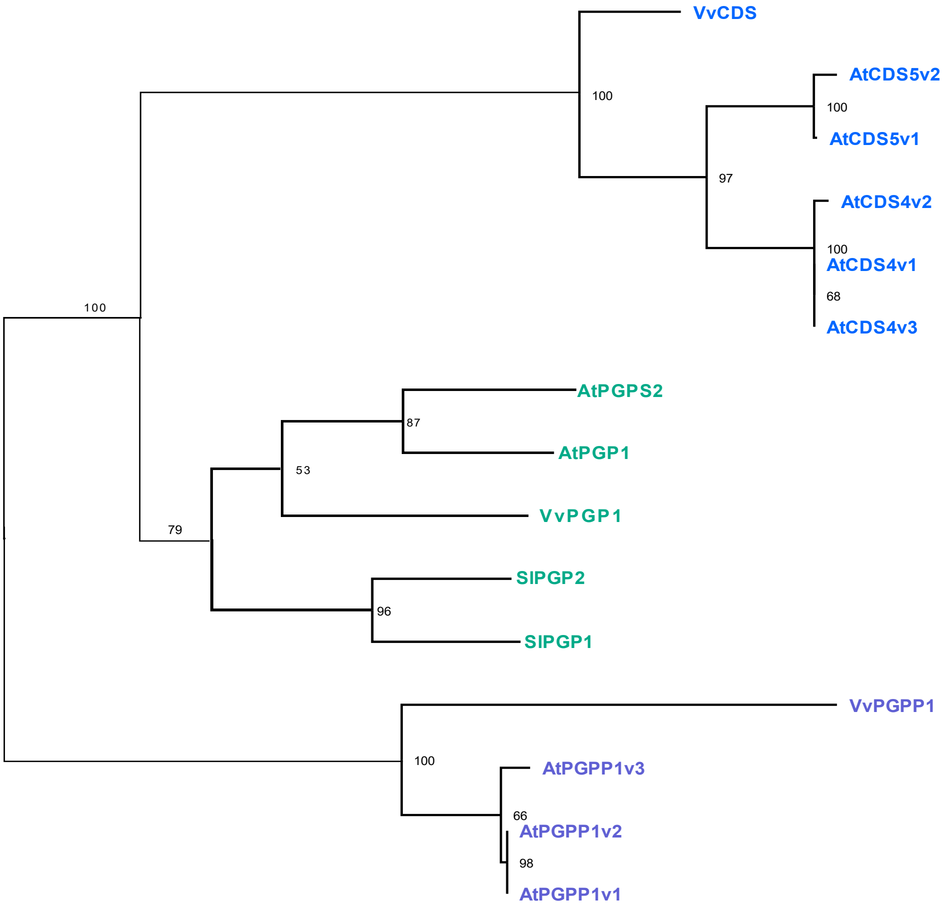


Figure 3.4 - Maximum likelihood phylogenetic tree based on PG synthases proteins from *Arabidopsis thaliana* (At), *Zea mays* (Zm), *Solanum lycopersicum* (Sl) and *Vitis vinifera* (Vv). Distinct groups are represented by different colours: PGPP1 in purple, PGP1 in green and CDS in blue. The numbers above branches show bootstrap values.

3.6 Lipid analysis

3.6.1 Analysis of Fatty Acids Composition of Total Lipids

In the first hours after the infection by a pathogen occurs, the fatty acid profile of the affected plant undergoes changes, which will impact the fluidity of the membrane and the enzymatic or non-enzymatic synthesis of bioactive lipid mediators, such as lipids, FA and oxylipins (Ludovici *et al*, 2014; Cavaco *et al*, 2021). Moreover, the total lipid content can be altered under stress conditions. Through an analysis carried out by gas chromatography the amounts and composition of fatty acids in total lipids was analysed using leaf samples collected four hours after infection (Figure 3.5).

When we compare the total amount of fatty acids present in the different cultivars under control conditions, we can observe significant differences with respect to the comparison between Sauvignac and Regent (p-value = 0.033) and Sauvignac and Solaris (p-value = 0.003), which despite being all tolerant to *P. viticola* show different fatty acids backgrounds, once that Sauvignac has a much smaller amount of fatty acids compared to the other two varieties.

In the susceptible genotype Chardonnay, the amount of total fatty acids remained stable after infection with the pathogen (Figure 3.5). These data agree with *Laureano et al* (2018), that showed that after infection, Trincadeira (susceptible to *P. viticola*) presents no significant changes in the fatty acid content. This demonstrates that in susceptible varieties there is a lower modulation of lipid and fatty acid content. In the case of Calardis blanc, Solaris and Regent, there is a large decrease in the total amount of fatty acids in the inoculated samples compared to the controls, which is less significant in Regent. These three varieties present a *Rpv3* background, and despite the fact that Solaris also have a *Rpv10*, lipid modulation occurs as in the other two *Rpv3* genotypes. Calardis blanc and Solaris present an *Rpv3-1*, and Regent presents *Rpv3-3*, therefore, this could be the explanation for the fact that the amount of total fatty acids decreases less in Regent in relation to the other varieties mentioned. Interestingly, in Sauvignac no decrease in total fatty acids is observed after infection even though this genotype also presents an *Rpv3* (Sauvignac harbours an *Rpv3-1/Rpv12 pyramid*). According to *Laureano et al* (2023), lipid associated signalling does not seem to occur in Sauvignac since *Rpv12* overlaps the action of *Rpv3*. It is noteworthy that this variety has already a very low lipid content under control conditions. The observed decrease in the quantity of FA may be due to reduced FA biosynthesis or degradation of FA to form other components important for plant defense, namely JA, whose precursor is C18:3 (Ghorbel *et al*, 2021).

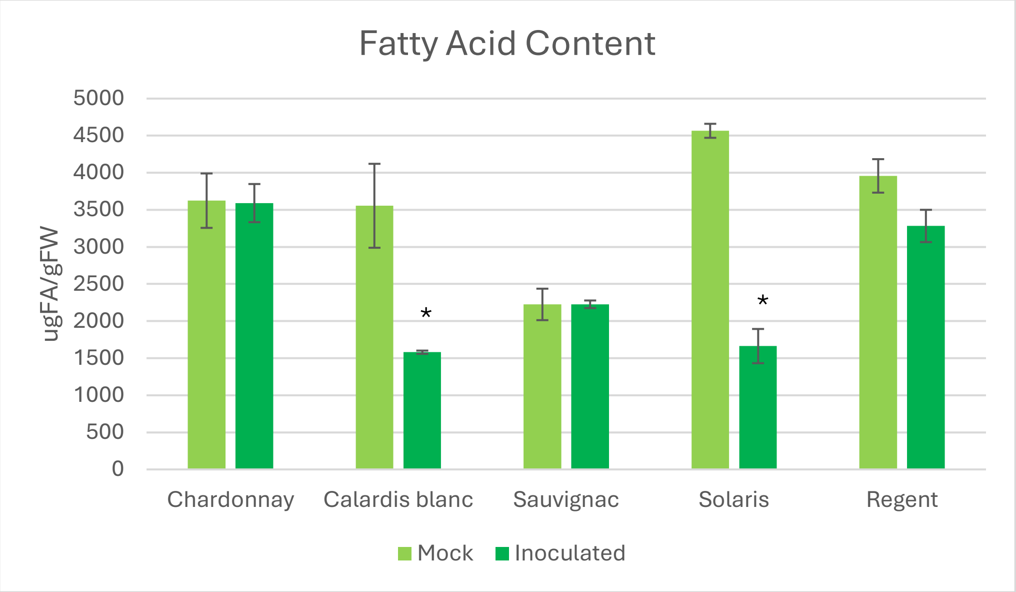


Figure 3.5 – Fatty acid content of Chardonnay, Calardis blanc, Sauvignac, Solaris and Regent varieties. Control samples are shown in light green, and samples inoculated with NW-10/16 for four hours are shown in dark green. A non-parametric test, namely the Krustal-Wallis test, was used through the IBS SPSS Statistics 29 (IBM, NY, USA) and significance was assumed when p-value $\leq$ 0.05.

Regarding the fatty acid distribution in total lipids, the most representative fatty acid is C18:3, followed by linoleic (C18:2) and palmitic (C16:0) acids. Fatty acids are synthesized in the chloroplast and the main molecules produced are C16:0 and stearic acid (C18:0), which is rapidly unsaturated by a soluble desaturase located in the stroma, giving rise to oleic acid (C18:1). Both C16:0 and C18:1 are incorporated in membrane lipids and can be further unsaturated by membrane associated desaturases giving rise to C18:2, which is converted in C18:3 (Kachroo *et al*, 2009). Another membrane desaturase, specific for PG desaturates palmitic acid into trans-hexadecanoic acid (C16:1t). As previously mentioned, the increase in C18:2 and C18:3 is important for the plant defense process, since these fatty acids increase membrane fluidity, making it less susceptible to damage. These FA can also be released from membrane lipids by the action of PLAs and serve as precursors of oxylipins such as JA, very important in the response of *V. vinifera* to *P. viticola* (Figueiredo *et al*, 2015).

As shown in *Laureano et al* (2018), susceptible varieties tend to not change their lipid profile in response to the pathogen attack, that is, they are not able to respond to this stress condition. In the susceptible variety, Chardonnay, we can observe an increase in the relative amount of C18:0, although not significant, as well as small increases in the amount of C18:1 and C18:2. These relative increases in C18:1 and C18:2 are not significant, however, according to *Laureano et al* (2023), it is only after 24 hpi that significant increases in these values are observed, so that future experiments would have to be carried out to corroborate these data. As observed in the work of *Laureano et al* (2023), the susceptible variety is the one with a higher relative amount of C18:3 compared to the more tolerant cultivars, allowing it to maintain membrane fluidity, plus it indicates that it has a greater amount of chloroplastidial lipids. From the analysis of these results, we can infer that Chardonnay has significantly higher percentage of C18:3 with respect to Sauvignac (p-value <0.001), Regent (p-value <0.001), and Calardis blanc (p-value = 0.014), which are tolerant to *P. viticola*. However, after infection, we can observe a small, although not significant, decrease in the amount of C18:3. This is in line with what would be expected, since Chardonnay does not have the capacity to adapt and does not significantly alter its lipid and FA profile. It is also important to emphasize that the susceptible variety, despite having the highest percentage of C18:3, is the one that has smaller percentages of C16:0 with respect to Calardis blanc, Regent and Sauvignac (p-value = 0.033; 0.007; 0.004, respectively), as are the smallest relative amount of C18:1, significantly smaller with respect to Sauvignac, Regent and Solaris (p-value = 0.038; 0.012; 0.003, respectively), which are tolerant. These results are in accordance with what was noted in Trincadeira, in *Laureano et al* (2018). The only significant lipid alteration detected in Chardonnay was an increase in the relative amount of C16:1t when inoculated with *P. viticola (*Figure 3.6).

In Solaris, which is resistant to *P. viticola*, the levels of C16:0 are the lowest and do not change significantly after infection, as with C16:1t. However, although these fatty acids do not undergo major changes, it is important to highlight the large decrease in C18:1 relative content, which is quite significant. The amount of C18:3 after infection does not appear to increase significantly, however, the amount of C18:2 shows a small but significant increase after infection, illustrating the increase in the amount of PUFAs (Figure 3.7B).

In Regent, the relative amount of C16:0 increases, although not significantly; however, the amount of C18:0 also increases significantly, so we can conclude that this plant produces more C16:0 to give rise to C18:0, so that it is subjected to the process of progressive desaturation, giving rise to C18:3, which is important in stress situations. In contrast, the results show a decrease in the relative amount of C18:2 and C18:3, also in accordance with Figure 3.7B, which shows a decrease in PUFAs. These results are not in agreement with what was observed in the work of *Laureano et al* (2018) made at 6hpi, in which the amount of C16:0 and C18:0 decreased, and the amount of C18:1, C18:2 and C18:3 increased. However, this may mean that Regent, which is tolerant to *P. viticola*, synthesized these fatty acids, which may have been subsequently used by the PLAs to release free FA, which will be used in the synthesis of JA. The final amount of the various components is not the result of their production alone, but rather of the balance between their production and their use as precursors of other molecules. Calardis blanc behaves in a similar way to Regent, as it is also a tolerant variety; however, it shows a small increase in the amount of C18:3 after infection, although not significantly.

Sauvignac does not show any variation in terms of C16:1t. The C16:0 content decreases slightly, which may be important for the synthesis of C18:3. We can observe a significant increase in the C18:0 content, which means that C16:0 is being transformed into C18:0. Finally, the amount of

C18:3 increases, although not significantly, which may result from a balance between C18:3 production and use in JA biosynthesis. In these results we can observe the same as in the results of *Laureano et al* (2023), where it was demonstrated that Sauvignac was the variety that presented a more constant FA profile during infection, with the exception of an increase in the amount of C18:1. In contrast, it is known that Sauvignac presents a *Rpv3-3/Rpv12* pyramid genetic background, in which the role of *Rpv12* overlaps *Rpv3* and does not seem to be associated with lipid responses. On the other hand, it is interesting that Sauvignac and Regent have the lowest amounts of C18:3, but also the highest amounts of C18:2 in comparison to Chardonnay (p-value <0.001), Solaris (p-value <0.001) and Calardis blanc (p-value =0.018; 0.006, respectively).

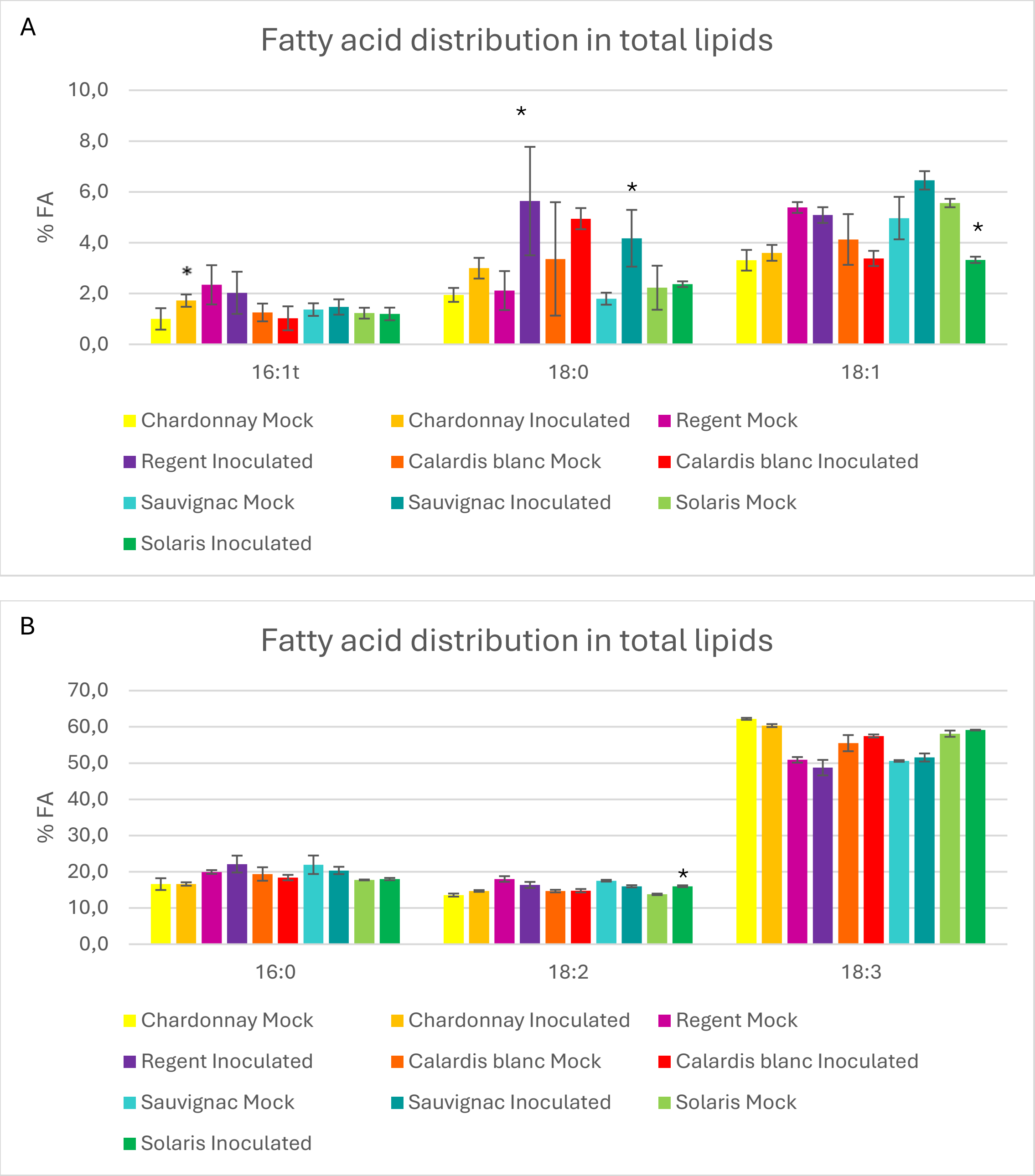


Figure 3.6 – Fatty acid distribution in total lipids in Chardonnay, Regent, Calardis blanc, Sauvignac and Solaris varieties. (A) Representation of fatty acids present in smaller quantities. (B) Representation of fatty acids present in larger quantities. In both graphs, the different control and inoculated varieties are represented in different colours. A non-parametric test, namely the Krustal-Wallis test, was used through the IBS SPSS Statistics 29 (IBM, NY, USA) and significance was assumed when p-value ≤ 0.05.

Membrane fluidity is very important for a plant to overcome stress and is affected by the degree of lipid unsaturation. A membrane with a high degree of unsaturation is more fluid than a membrane with few unsaturated fatty acids (Los *et al*, 2004). The degree of fluidity may be measured through the DBI, which is also related to membrane permeability. *Cavaco et al* (2023) demonstrated that susceptible varieties of *V. vinifera* present a higher proportion of PUFAs as well as higher DBI values compared to tolerant plants. Chardonnay is the variety that presents the highest DBI comparatively to Sauvignac, Regent and Calardis blanc (p-value = 0.002; 0.004; 0.025, respectively) (Supplementary Data 5), which is associated with greater fluidity of the cell membrane but could also reflect a higher content of chloroplast lipids, which are extremely rich in PUFAs, mainly C18:3, as previously observed in the susceptible variety Trincadeira (Laureano *et al*, 2018). However, when the plant is inoculated with the pathogen, the DBI decreases. The same happens with Regent, that is tolerant to *P. viticola.* The remaining varieties show a tendency to increase their double bonds when they are infected. The ability of these varieties to adjust membrane fluidity by altering the levels of saturated and unsaturated fatty acids is what allows them to acclimatize, where it is still possible, even after damage, for all proteins to function, particularly the photosynthetic apparatus and energy transduction pathways (Upchurch, 2008).

UFAs and PUFAs (Figure 3.7A and B) also present a different modulation in the genotypes studied. Chardonnay has significantly higher PUFA values than Sauvignac, Regent and Calardis blanc (p-value = 0.003; 0.003; 0.015, respectively), just as significantly higher values of UFAs (p-value = 0.013; 0.043; 0.016, respectively). Regent has a different behaviour from Calardis blanc and Solaris, since these last two varieties increase PUFA and UFA when inoculated with the pathogen, while Regent decreases. This could possibly be associated with the fact that Calardis blanc and Solaris have an *Rpv3-1* background and Regent *Rpv3-3*. These results are in line with DBI values and previous results presented in *Cavaco et al* (2023), that show that susceptible varieties tend to have a lower quantity of PUFAs when infected. This could possibly be associated with the fact that Calardis blanc and Solaris have an *Rpv3-1* background and Regent *Rpv3-3*. PUFAs are the main constituents of lipid membranes, allowing them to be quite fluid and thus less damaged. When a plant is infected by a pathogen, it will have to cross the lipid membrane, so it is important that it is flexible and contains PUFAs, which will be released by lipases, allowing them to act directly as free FA or as a substrate for molecules important in combating the pathogen, such as oxylipins (Kachroo *et al*, 2009; Pretorius *et al*, 2021). Sauvignac keeps PUFA levels stable, even after infection, just as Chardonnay, similarly to what was observed to the total fatty acid contents, in agreement with the hypothesis of a lack of lipid modulation after infection in these two genotypes.

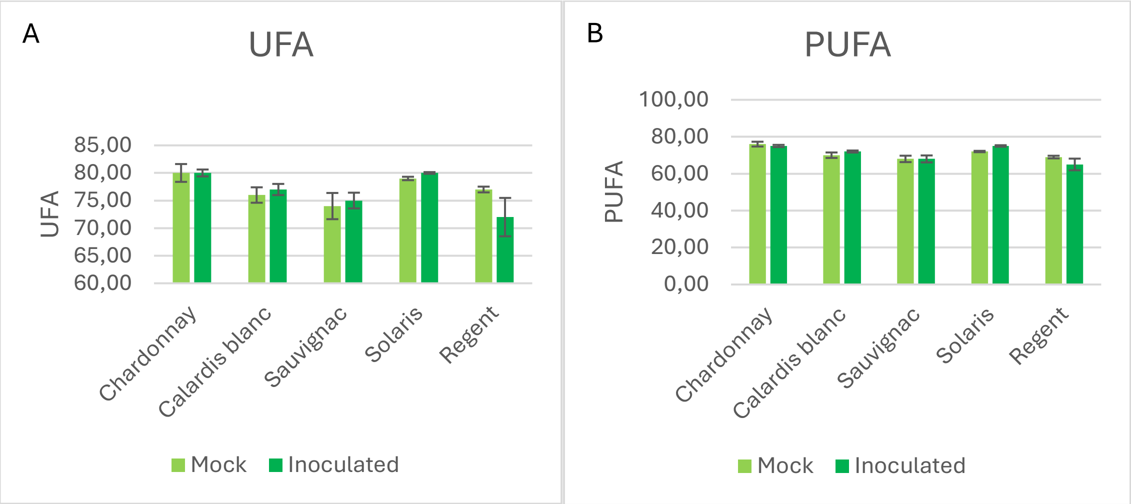


Figure 3.7 – (A) Unsaturated Fatty Acids (UFA) and (B) Polyunsaturated Fatty Acids (PUFA) of Chardonnay, Calardis blanc, Sauvignac, Solaris and Regent varieties. Control samples are shown in light green, and samples inoculated with NW-10/16 are shown in dark green. A non-parametric test, namely the Krustal-Wallis test, was used through the IBS SPSS Statistics 29 (IBM, NY, USA) and significance was assumed when p-value ≤ 0.05.

3.6.2 Analysis of Lipid Modulation of *V. vinifera* cv. Sauvignac during the first 4 hours of interaction with *P. viticola*

Through TLC it is possible to separate the different lipids present in the extract analysed, such as MGDG, DGDG, PG and SQDG, that are chloroplastidial lipids, extraplastidial lipids such as the phospholipids PC and phosphatidylethanolamine (PE), lipids involved in signalling such as PA and phosphatidylinositol (PI) and neutral lipids (NL) that include triacylglycerol (TAG), free FA and leaf surface lipids. From the analysis of the previous results, we can realize that Sauvignac is the tolerant variety that behaves more distinctly with respect to the other tolerant varieties, possibly due to its pyramidal *Rpv3-1/Rpv12* genetic background. This way, this was the selected variety to deepen the analysis of its lipid composition, including also the novelty of the SQDG isolation for further analysis through GC.

Regarding polar lipids, MGDG and DGDG are the most abundant lipids in leaves, followed by PG and PC. The signalling lipids PA and PI and membrane lipids as PE and SQDG are found in lower amounts (Figure 3.8). Galactolipids are present in large quantities in chloroplasts and cyanobacteria, but are absent in non-photosynthetic organisms, illustrating their importance for photosynthesis (Kobayashi *et al*, 2008). Some studies demonstrate that MGDG is associated with the core of the reaction centre of both photosystems I and II (Loll *et al*, 2007; Jordan *et al*, 2001). Also, studies carried out with *dgd1* mutants in *Arabidopsis*, indicated that DGDG is extremely important in the activity of photosystem II and in the stability and activity of photosystem I (Dormann *et al*, 1995; Guo *et al*, 2005).

MGDG and DGDG are mainly made up of C18:3 (Supplementary Data 6), with DGDG presenting a higher quantity of C16:0. These lipids present in the chloroplast have a high amount of C18:3 to allow the thylakoids to have high membrane fluidity and to allow PLAs to release FA in the chloroplasts to form a pool of free FA, available to act in the defense of the grapevine. Upon infection by *P. viticola*, the content of C18:3 in both MGDG and DGDG increases slightly, as well as the C16:0 content of the DGDG. The amount of C18:0, C18:1 and C18:2 decreases after infection, although the variations are very slight (Supplementary Data 6). This is in line with the hypothesis that this *P. viticola*-resistant variety, when infected by the pathogen, seeks to transform C16:0 into C18:0, so that it undergoes a chain of desaturations and transforms into C18:3, which is important for increasing membrane fluidity and the plant's resilience to stress (Kachroo *et al*, 2009). In the work of *Laureano et al* (2018), an increase in the amount of C18:3 is observed in both MGDG and DGDG. A decrease in the amount of C18:0, C18:1 and C18:2, which are the fatty acids that will give rise to C18:3, is also observed. However, the percentage of MGDG appears to decrease after 4 hours of infection, while the DGDG percentage remains more stable (Figure 3.8). In the work carried out by *Laureano et al* (2018), there is an increase in the amount of MGDG and DGDG after infection in Regent, so there are greater variations in Regent, which demonstrates that these varieties show different behaviours in response to infection by *P. viticola*.

When the plant is under stressful conditions hydrolysis of structural membrane phospholipids, such as PC and PE, give rise to PA, that is a second messenger in plant defence signalling. While PA is now regarded as a lipid signalling molecule implicated in cellular and physiological processes in response to abiotic and hormonal signals, it is also involved in plant-microbial interaction and plant defence response against fungal pathogens (Pleskot *et al*, 2013). Thus, PA is involved in several tasks in the plant defense process, regulating enzymatic activity, modulating proteins involved in Ca^{2+} signalling and oxidative burst, hormonal signaling pathways, chloroplast lipid metabolism, membrane-cytoskeleton interaction, targeting actin filaments and tubulin microfibers, participating in the perception of PAMPs, regulating reactive oxygen species (ROS) production in plant-microbe interactions and interacting with other lipid signal molecules, namely JA and oxylipins (Shah, 2005; Testerink & Munnik, 2011; Zhao, 2015). After inoculation, the amounts of PC and PE appears to decrease, however, the amount of PA also decreases significantly. This could mean that these lipids are being used to form PA, which is used by the plant to meet its needs under stressful conditions.

In addition to being a product of phospholipid degradation, PA is also an intermediate of the synthesis of some lipids like PG and TAGs. PG is the only phospholipid in thylakoids and is characterized by the presence of C16:1t (Supplementary Data 6), considered crucial to the evolution of chloroplasts, and to the photosynthetic transport of electrons (Wada & Murata, 2007; Kobayashi *et al*, 2024). The level of PG increases in Sauvignac after infection (Figure 3.8A). When changes in the lipid level of *V. vinifera* in response to *Erysiphe Necator* infection were studied, it was noticed that BC4 (a variety resistant to this pathogen), showed a large accumulation of PG (Ciubotaru *et al*, 2023). These data can be related to the data present here, in the sense that both varieties are resistant to the respective pathogen, and both present an increase in the amount of PG, which demonstrates an effort on the part of the grapevine's defense mechanisms to maintain stable photosynthesis functioning. After infection, C16:1t levels in PG increased, which is important for maintaining adequate levels of photosynthetic activity. Furthermore, the amount of C18:0 decreased significantly, while the amount of C18:2 and C18:3 increased (Supplementary Data 6), so the membrane became more fluid after infection so that the plant does not suffer such serious damage. Furthermore, the increase in C18:3 provides higher abundance of FA substrates for JA production, through PLAs. These data are the opposite of what was observed in the Regent variety at 6 hpi (Laureano *et al*, 2018*)*, in which the amount of C16:1t in PG decreased. However, the amount of C18:2 and C18:3 increased too.

SQDG is an extremely important lipid in photosynthetic organisms, since four SQDG molecules bind to each of the photosystem II monomers, maintaining its stability and activity (Nakajima *et al*, 2018). After inoculation, the total amount of SQDG decreased significantly in Sauvignac (Figure 3.8B), which may indicate that changes are occurring at the photosystem level, decreasing photosynthetic activity. In addition, the change in the percentage of SQDG appears to be the steepest with respect to all other lipids analysed. Regarding neutral lipids, it is known that these tend to increase in stressful situations, which is in line with the results presented. Neutral lipids include TAGs and free FA, for example, which are important in the defense response. We can observe that there is an increase in the percentage of neutral lipids after 4 hours of infection (Figure 3.8A), which demonstrates that Sauvignac is a variety that bears more on defense barriers, consisting essentially of neutral lipids. In addition, this increase may be correlated with the decrease in PA content, which, as stated earlier, is a precursor to the biosynthesis of TAGs.

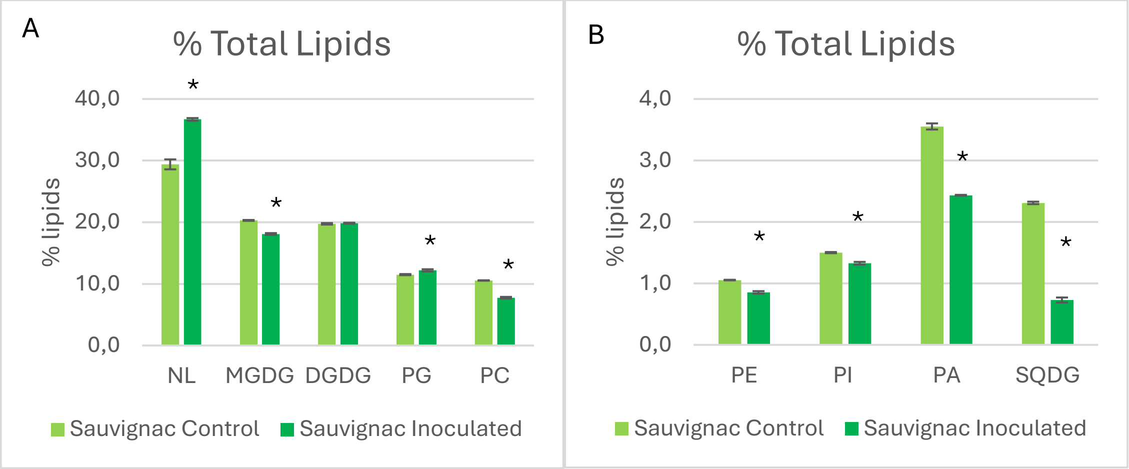


Figure 3.8 – Total Lipid Composition of *V. vinifera* cv. Sauvignac inoculated leaves with *P. viticola* at 4hpi. Control samples are shown in light green, and samples inoculated with NW-10/16 are shown in dark green. A non-parametric test, namely the Krustal-Wallis test, was used through the IBS SPSS Statistics 29 (IBM, NY, USA) and significance was assumed when p-value ≤ 0.05.

3.6.3. Fatty acid distribution in MGDG, SQDG and NL in five *V. vinifera* varieties with different degree of tolerance to *P. viticola*

Resulting from an experimental process of trying to separate SQDG from the remaining lipids, namely PC, which in TLC tends to be found in the same band as SQDG, a detailed description was obtained of the percentages of MGDG, NL and SQDG (Figure 3.9) and of the fatty acids that constitute these lipids (Supplementary Figure 7) of the varieties Chardonnay (susceptible), Calardis blanc and Regent (tolerant with different genetic backgrounds) and Sauvignac and Solaris (resistant with a pyramid of *Rpv3/Rpv12* and *Rpv3/Rpv10*, respectively).

When we observe the NL content of all the varieties analysed (Figure 3.9A), we can realize that the varieties that have more lipids of this type are Calardis blanc, Regent, and Sauvignac (all tolerant). The variety with a lower percentage of NL in control conditions is Chardonnay, demonstrating significant differences compared to Calardis blanc and Regent (p-value < 0.001; p-value = 0.012, respectively). In addition, the percentage of NL in Sauvignac is significantly lower than that of Calardis blanc (p-value = 0.012), which are both tolerant, which once again demonstrates the different Sauvignac behaviour compared to other tolerant varieties to the *P. viticola*. In contrast, when we observe the contents of MGDG, Chardonnay is the variety with a higher percentage of this lipid, with the varieties with a higher amount of NL (Calardis blanc, Regent and Sauvignac) showing the lower percentages of MGDG under control conditions (Figure 3.9A). Differences are quite significant when we compare Chardonnay with Calardis blanc (p-value = 0.007). In response to pathogen infection, Calardis blanc demonstrated a significant increase in the percentage of MGDG and Solaris demonstrated a significant decrease. Sauvignac, again, tends not to make significant changes in its lipid content, so MGDG levels don't vary much. Reversely, the percentage of NL in Calardis blanc decreases significantly, as the percentage of NL in Solaris increases significantly after pathogen infection. This demonstrates once again that when the leaves have a greater amount of MGDG, they have less NL, and the opposite is also valid.

MGDG always has a very high amount of C18:3 (Supplementary Data 7), since it is the most abundant lipid in plastidial membranes. These data are in accordance with those presented in Figure 3.6, as Chardonnay is the variety showing higher C18:3 values. This fatty acid is mainly found in the chloroplast lipids, namely in the MGDG, which is also significantly higher in

Chardonnay. These polyunsaturated fatty acids contribute to membrane fluidity, preventing damage to the photosynthetic machinery, and are mostly found in the MGDG and then in the DGDG. In addition, C18:3 is regarded as a precursor of JA biosynthesis, so it is important that it exists in large quantities (Laureano *et al*, 2023). It is also known that susceptible varieties have higher C18:3 ratios than resistant varieties. After infection, only Chardonnay and Sauvignac increased the amount of C18:3. Calardis blanc and Regent also show a significant increase in the amount of C18:1, which is involved in the biosynthesis process of C18:3. It is also interesting to note that Chardonnay is the variety that has the most C18:3, however, it is the one that has the least C18:2, which is also important for allowing membrane fluidity.

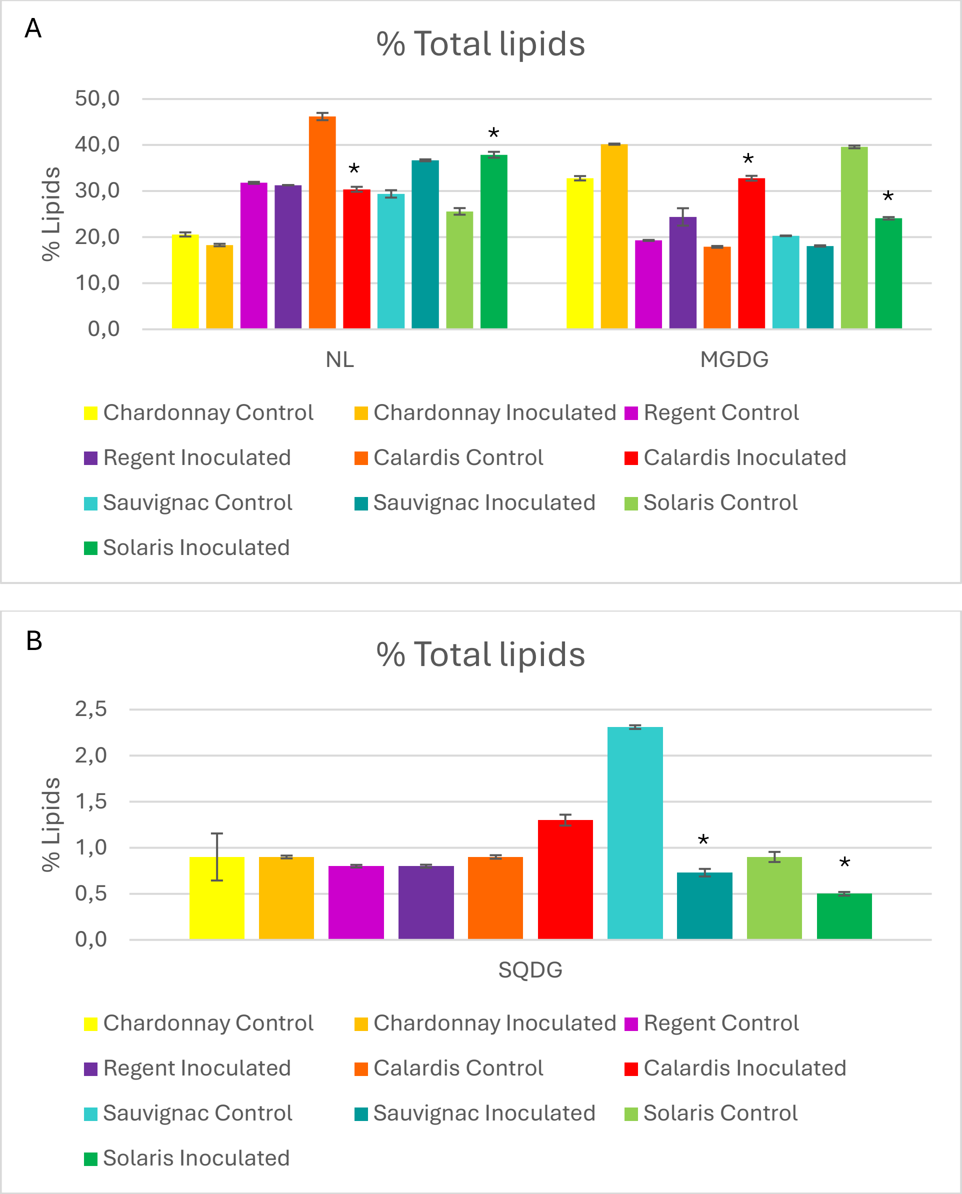


Figure 3.9 – Percentages of MGDG, SQDG and NL in five *V. vinifera* varieties with different degree of tolerance to *P. viticola*. (A) Representation of MGDG and NL, present in larger quantities. (B) Representation of SQDG, in smaller quantities. In both graphs, the different control and inoculated varieties are represented in different colours. A non-parametric test, namely the Krustal-Wallis test, was used through the IBS SPSS Statistics 29 (IBM, NY, USA) and significance was assumed when p-value ≤ 0.05.

SQDG is a plastidial lipid present in small quantities. Interestingly, the relative amount of this lipid in Sauvignac is significantly higher than in the other varieties (p-value = 0.001), under control conditions. In addition, after infection with *P. viticola*, Sauvignac demonstrates a significant decrease in the contents of this lipid, also observed in Solaris, but to a lower extent (Figure 3.9B). The contents of SQDG remained unchanged in Chardonnay and Regent and increased slightly in Calardis blanc. This lipid is mainly composed of C16:0 and C18:0, followed by C18:2. We can observe that in all varieties there is an increase in the amount of C18:3, except in Regent (Supplementary Data 7). Likewise, a decrease in the amount of C18:0 is observed in all varieties except Regent. It is noteworthy that due to its small amounts a less accurate (higher standard deviations) fatty acid composition was obtained, compared to the other lipids analysed.

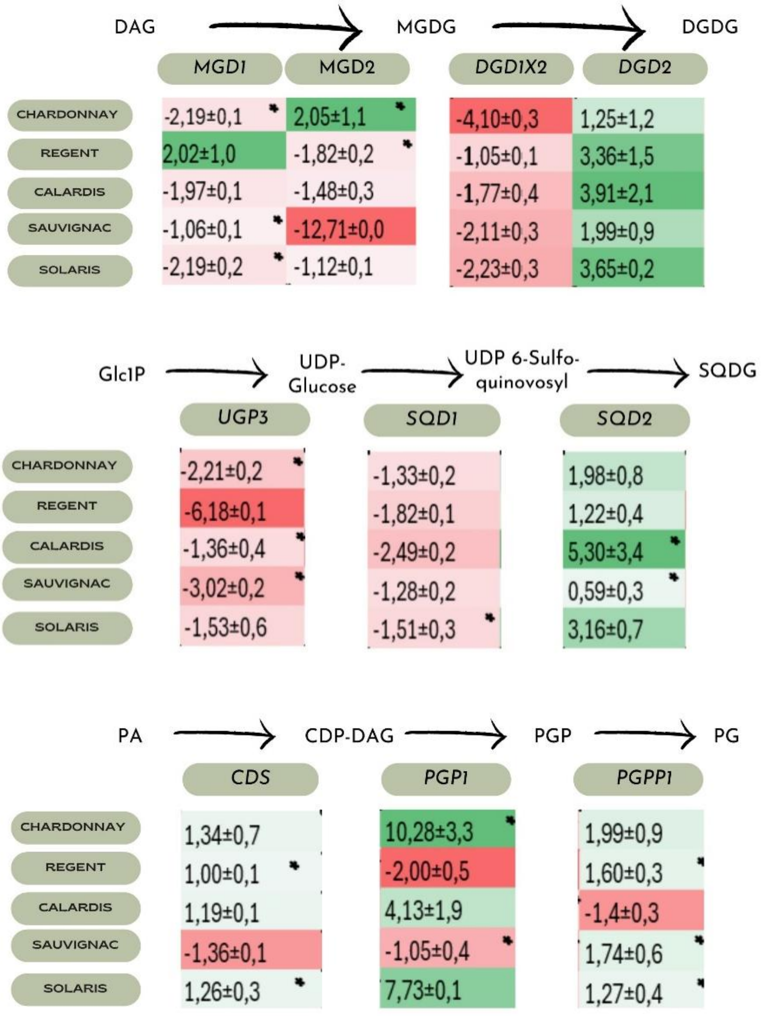
3.7 Gene Expression Analysis

In order to investigate if differential lipid modulation between the genotypes analysed is under transcriptional control, a gene expression analysis was carried (Figure 3.10). Regarding our data, only Regent presented an up-regulation of *MGD1* gene, which is the gene that most influences MGDG synthesis, despite *MGD2* gene being underexpressed. In Regent, our group previously reported an increase of MGDG levels after *P. viticola* infection, consistent with the expression profile observed for *MGD1*, thus suggesting the involvement of this grapevine gene in MGDG biosynthesis (Laureano *et al*, 2018). Chardonnay shows significant overexpression of the *MGD2* gene and significant underexpression of the *MGD1* gene. We can observe in the data in Figure 3.9A that the percentage of MGDG in Chardonnay increased after *P. viticola* infection. Sauvignac has a very significant down-regulation of *MGD1* gene, in addition to an underexpression of *MGD2*, so it is expected that this variety will suffer a decrease in MGDG synthesis. These data agree with what was observed in the analysis of lipid composition of Sauvignac (Figure 3.8), since in relation to total lipids the amount of MGDG decreased and agree with the data from Figure 3.9A, where it has been shown that MGDG increases in all varieties except Solaris (which has a significant decrease) and Sauvignac, which means that there is less production of this lipid. Solaris also appears to undergo a down-regulation in both genes involved in MGDG synthesis, being that down-regulation significant in the expression of *MGD1* gene.

In *Arabidopsis*, the *MGD1* gene has been shown to be involved in chlorophyll accumulation and thylakoid membrane development. In contrast, *MGD2* transcripts are present in smaller quantities in *Arabidopsis* and cannot compensate for the lack of *MGD1*. *DGD2* plays a very important role in the remodeling of membrane lipid composition under inorganic phosphate (Pi) scarcity conditions. Furthermore, it was also demonstrated that in shoots, the expression levels of *MGD1* increased when the plant was subjected to wounding stress (Kobayashi *et al*, 2008). These galactolipids are very important in several domains, protecting the thylakoid membrane from damage and maintaining membrane stability, through the regulation of the photosynthetic electron flow (Islam *et al*, 2020).

Regarding DGDG, we can see that all varieties present a positive modulation of *DGD2* at 4hpi. In all of the analysed genotypes, *DGD1X2* gene is downregulated. In Figure 3.8, we can see that there is an increase in the amount of DGDG in Sauviganc after infection, which allows creating the possibility that the *DGD2* gene is more important in the synthesis of DGDG in grapevine. We can also see that in the case of the *DGD2* gene, Sauvignac and Chardonnay inoculated present identical expression levels compared to the control samples, this is not unexpected as it was previously hypothesized that Sauvignac's resistance appears to be controlled by the *Rpv12 loci* and not by *Rpv3,* which puts Sauvignac on the same level as Chardonnay, which has no resistance *loci. (*Laureano *et al,* 2023*)*. The remaining genotypes, which contain *Rpv3 loci,* also behave in the same way, overexpressing the *DGD2* gene when infected but in a more pronounced way, which is in line with data from *Laureano et al* (2018), in which an increase in the amount of DGDG in Regent is observed.

SQD1 and *UGP3* genes, involved in the synthesis of SQDG, are down-regulated in all varieties analysed and *SQD2* gene is up-regulated in all of them, being that in Calardis blanc this overexpression is the highest. Since in Calardis blanc the percentage of SQDG tends to increase after infection (Figure 3.9B), *SQD2* could be behind this increase. Biotic stress causes oxidative stress in plants, which is characterized, in the first phase, by a relative increase in the amount of SQDG, as observed in broad bean leaves. However, in a second phase of oxidative stress, this lipid tends to decrease in quantity to serve as a source of cysteine, and so ends up decreasing in response to biotic stress. (Kosyk *et al*, 2009). In Sauvignac, the amount of SQDG after infection decreased significantly (Figure 3.8 and 3.9B), which may be due to significant underexpression of the *UGP3* gene associated with the small up-regulation od *SQD2* (Figure 3.10).



	MGD1	MGD2	DGD1X2	DGD2
CHARDONNAY	-2,19±0,1 *	2,05±1,1 *	-4,10±0,3	1,25±1,2
REGENT	2,02±1,0	-1,82±0,2 *	-1,05±0,1	3,36±1,5
CALARDIS	-1,97±0,1	-1,48±0,3	-1,77±0,4	3,91±2,1
SAUVIGNAC	-1,06±0,1 *	-12,71±0,0	-2,11±0,3	1,99±0,9
SOLARIS	-2,19±0,2 *	-1,12±0,1	-2,23±0,3	3,65±0,2

	UGP3	SQD1	SQD2
CHARDONNAY	-2,21±0,2 *	-1,33±0,2	1,98±0,8
REGENT	-6,18±0,1	-1,82±0,1	1,22±0,4
CALARDIS	-1,36±0,4 *	-2,49±0,2	5,30±3,4 *
SAUVIGNAC	-3,02±0,2 *	-1,28±0,2	0,59±0,3 *
SOLARIS	-1,53±0,6	-1,51±0,3 *	3,16±0,7

	CDS	PGP1	PGPP1
CHARDONNAY	1,34±0,7	10,28±3,3 *	1,99±0,9
REGENT	1,00±0,1 *	-2,00±0,5	1,60±0,3 *
CALARDIS	1,19±0,1	4,13±1,9	-1,4±0,3
SAUVIGNAC	-1,36±0,1	-1,05±0,4 *	1,74±0,6 *
SOLARIS	1,26±0,3 *	7,73±0,1	1,27±0,4 *

Figure 3.10 - Gene expression profile in Regent, Calardis blanc, Sauvignac, Solaris, and Chardonnay varieties. Strong red represents the minimum expression values (under expression), white represents no change in expression and strong green represents maximum expression (overexpression). The gene expression estimated for inoculated samples consists of a balance between inoculated and mock inoculated samples, for each cultivar. Asterisks indicate significant differences ($p < 0.05$).

Regarding the genes involved in PG synthesis, we can point out a small up-regulation of *PGPP1* in all varieties when infected, except in Calardis blanc. In *CDS* we can also observe a similar change in all varieties except Sauvignac. *PGP1* is a gene that is significantly overexpressed in

Chardonnay when infected, and is significantly down-regulated in Sauvignac. From this we can predict Regent and Solaris present an increase in the content of PG, since the *PGPP1* and *CDS* genes are significantly overexpressed in the context of infection. We can relate these data to what is observed in Figure 3.8, in which there is an increase in the amount of PG in Sauvignac, after infection. This increase may be due to overexpression of the *PGPP1* gene; however, there may be an increase because this lipid is not being used as a substrate of plant defense compounds, which could justify the underexpression of the *PGP1* gene. In this way, PG may be being produced less but also being used less by the grapevine.

4. Conclusion

Lipids are very important molecules for various biological processes in plants, therefore, their role in pathogen defense processes has obtained special attention. In this work, fatty acid composition and lipid profiles of five varieties of *V. vinifera* were analysed, with different *P. viticola* tolerance levels (Chardonnay is a susceptible genotype; Calardis blanc and Regent are tolerant, with different Rpv backgrounds (*Rpv3-1/Rpv3-2* and *Rpv3-1*, respectively); Sauvignac and Solaris are resistant (*Rpv3-1/Rpv12* and *Rpv3-3/Rpv10*, respectively)). In addition, the expression analysis of genes encoding enzymes involved in the synthesis of chloroplast lipids (MGDG, DGDG, PG and SQDG) was also performed, which demonstrated special relevance when it comes to the mechanisms developed by the grapevine to overcome mildew infection.

In terms of FA content, it was observed that Sauvignac has a very identical behaviour to what is observed in Chardonnay, which is a susceptible variety, whereas the remaining varieties (all tolerant of different levels) behave in the same way. In control conditions, Sauvignac is the variety that has the lower total FA content, which does not vary after infection. In terms of quantity of double bounds, Chardonnay is the variety that features higher DBI, PUFAs, and UFAs values, which is in accordance with prior studies. After the separation of the SQDG from the other lipids, in Sauvignac, we were able to make an analysis of the lipid profile of this variety. From this analysis we can realize that Sauvignac has the largest change after infection in SQDG, which undergoes a large decrease. Sauvignac is also the variety that has a higher relative amount of SQDG under control conditions. In addition, there is also a decrease in the amount of PA, which is a biosynthetic precursor of PG and TAGs, included in NLs, which increase after 4 h of infection. In contrast, chloroplast lipids seem to decline, which demonstrates that this variety, contrary to others previously analysed such as Regent, bears on defense barriers involving NLs. This information is corroborated when we analyse the percentage of NL and MGDG in all varieties. In varieties with the highest percentage of MGDG (Chardonnay and Solaris) there is a lower percentage of NL, as are the varieties that have more MGDG have less NL. In addition, the richest varieties in MGDG also have higher percentages of C18:3, the fatty acid present in chloroplasts in greater quantity. Varieties with less relative amount of C18:3 have higher percentages of C16:0 and C18:1. Regarding gene expression analysis, these results are generally in accordance with lipid analysis, justifying, at least in part, the modulations that are observed in lipids.

In conclusion, these results demonstrated that there is an important role of chloroplast lipids in the response of *V. vinifera* to *P. viticola* infection. The lipids undergo different modulations according to the genetic background of the vine, also promoting changes to the FAs level. In addition, gene expression analysis has confirmed that there are indeed changes in gene expression that are behind the biosynthesis of lipids present in chloroplasts. Importantly, a protocol was optimized that allowed, for first time in grapevine, the isolation of SQDG and to do the analysis of its FA composition.

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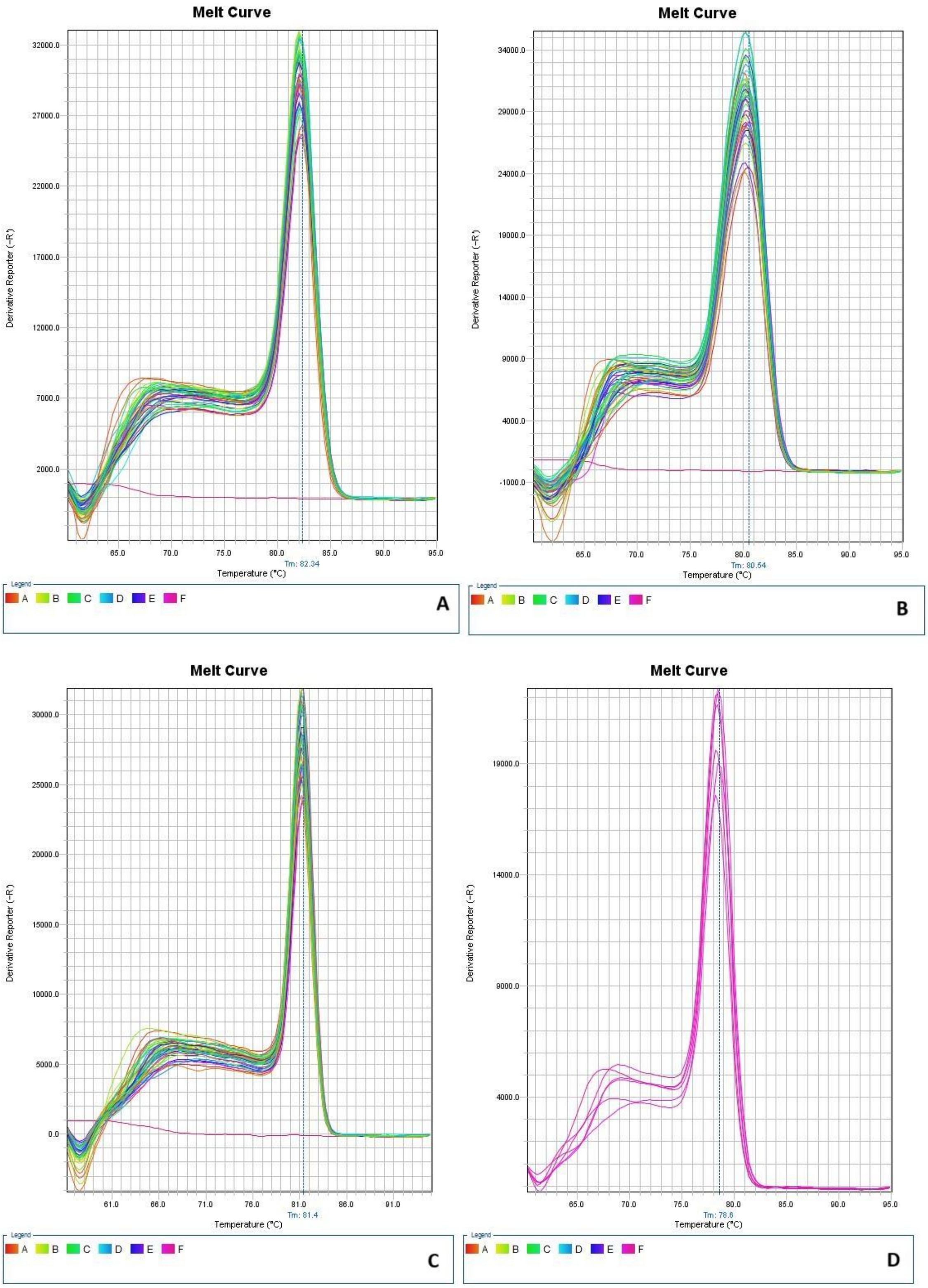
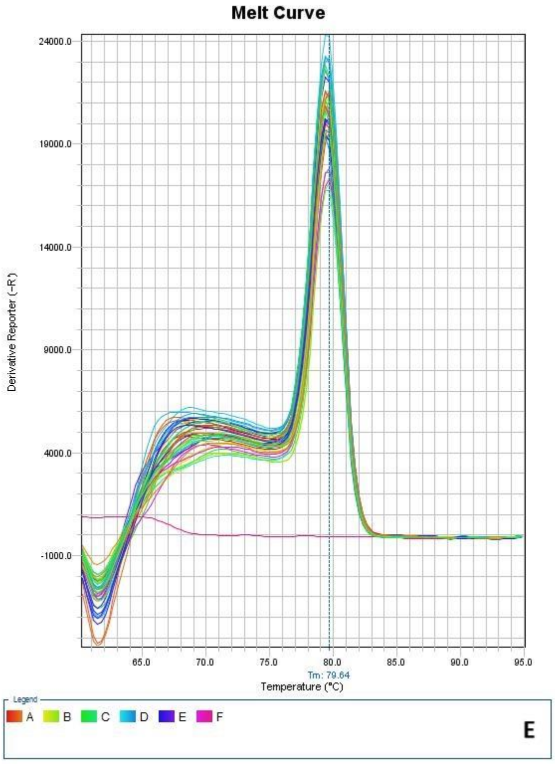
Suplementary Data 1 - Amplification Details Target genes transcripts primer sequences, amplicon length and primers annealing and melting temperatures.

Gene	Forward Primer (5’-3’)	Reverse Primer (3’-5’)	Annealing temperature (ºC)	Melting temperature (ºC)	Product length (amplicon)
MGD1	GCCTGTGATTGCATTATTAC	ATTTCCCACACCCATTTTC	56	81.4	145
MGD2	CCTCCCAATTATCCTCAAC	CGCCTGCACCATTATCTAC	60	78.6	80
DGD1X2	AGCCACAACCTCTTACTCCT	TGTGCATCATCCTTATTATTCA	60	79.6	115
DGD2	AGTGGGTTTGTGAAAGAGACA	TCCAACCCAGCAGCCTGTA	60	83.5	125
SQD1	AAGCAGAGGAGCATTACTACA	TGAGCAATGAATCCAGAAGAG	60	80.5	99
SQD2	CTACAATGGCTGACAAGAC	GAACATATCAATACAAAACAAT	56	79.2	145
UGP3	TCAAGTAATGATGAAAAGAGTT	TCCAGCCTACCACCAGAAA	60	79.3	110
PGPP1	TATTATGCACTGGTGATTCTCT	GATTTCCTTTTCCTTTCTTCTC	60	75.9	87
PGP1	CTGATCGGAAGAAGAGGAATA	GAGGAAGCATAGGTGTTGAG	60	79.5	142
CDS	AACCTTCCTGCCACTTTAT	ATTTCCCACTTTCATTTGTAT	56	76.6	83
EF1a	GAACTGGGTGCTTGATAGGC	ACCAAAATATCCGGAGTAAAAGA	60	80.5	164
SAND	CAACATCCTTTACCCATTGACAGA	GCATTTGATCCACTTGCAGATAAG	60	82.3	76

Suplementary Data 2 - Melting curves of targeted genes. (A) *SAND;* (B) *EF1α;* (C) *MGD1;* (D) *MGD2;* (E) *DGD1X2;* (F) *DGD2;* (G) *SQD1;* (H) *SQD2;* (I) *UGP3;* (J) *PGPP1;* (K) *PGP1;* (L) *CDS.*

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75.0
80.0
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90.0
95.0
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Legend
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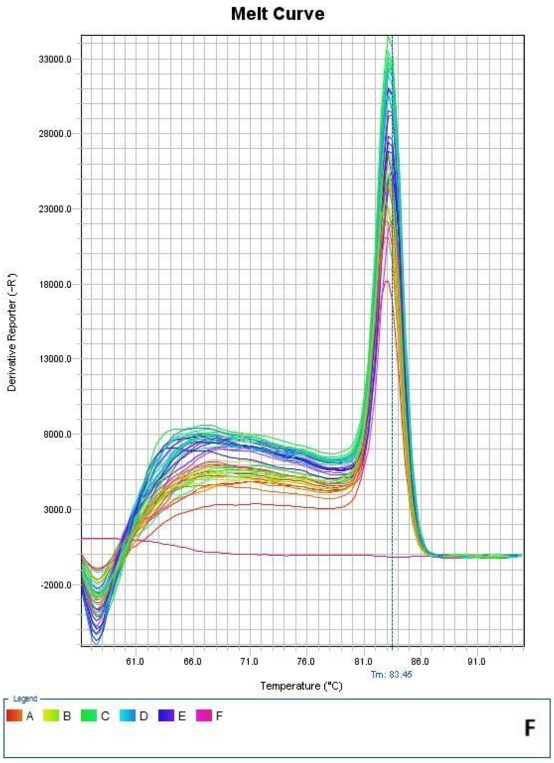
C
D
E
F
E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71.0
76.0
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Legend
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B
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E
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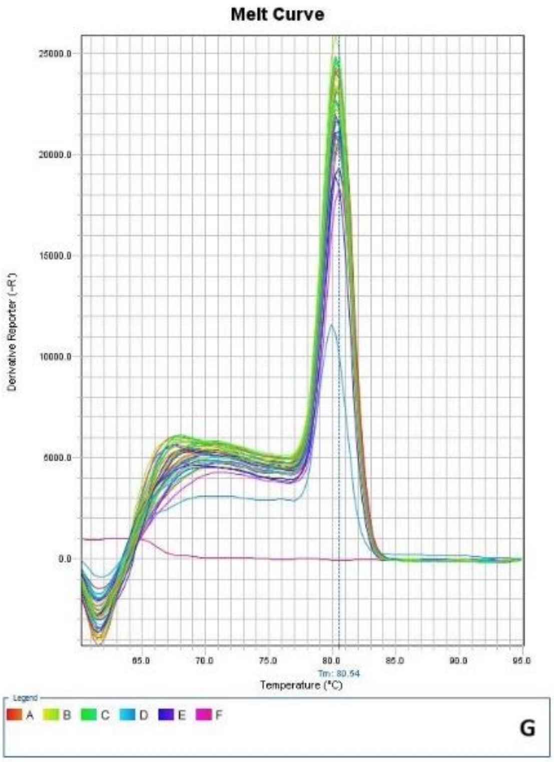
F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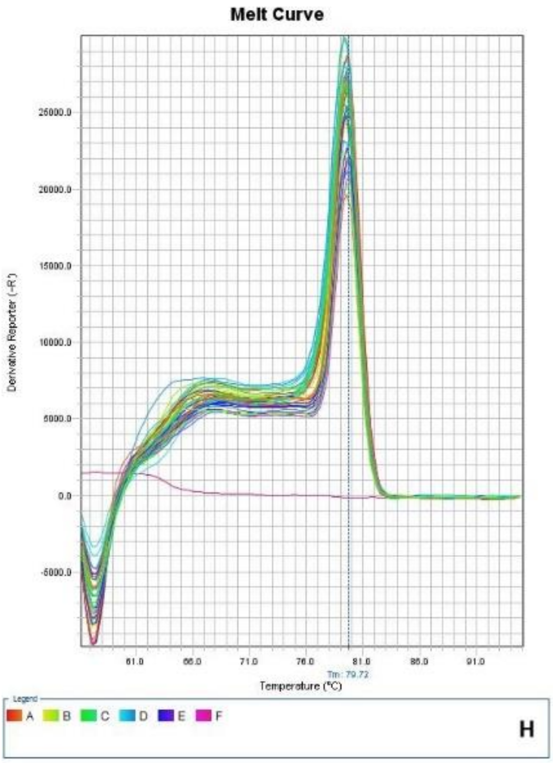
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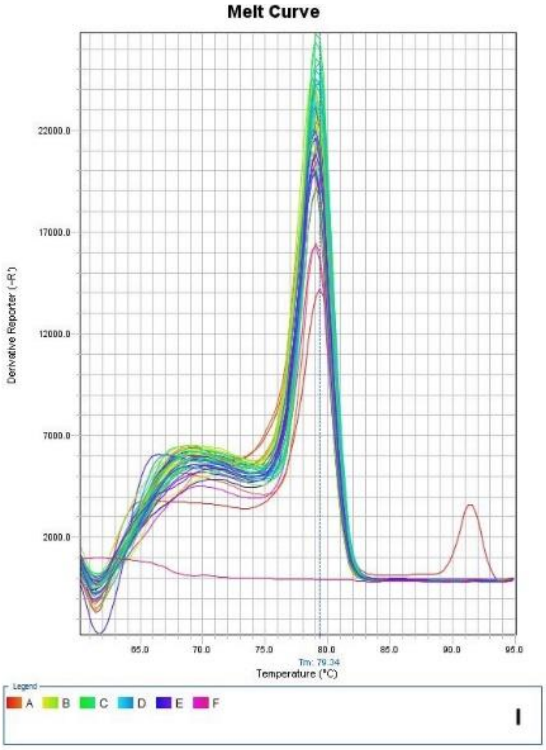
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C
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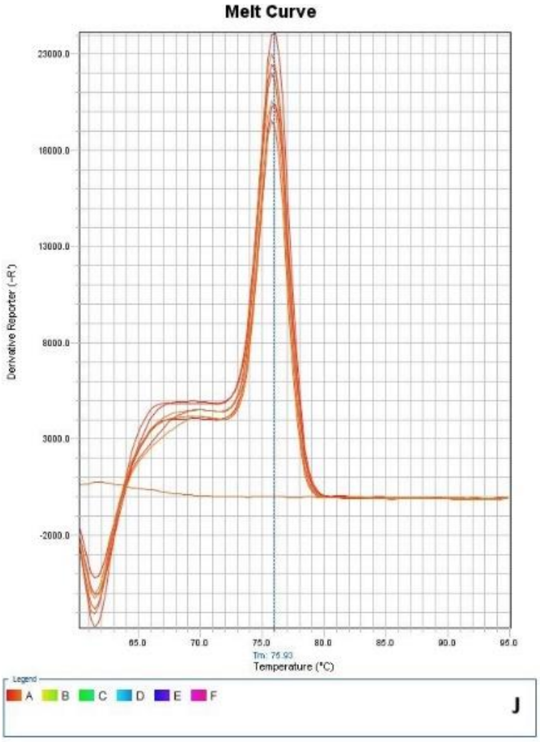
E
F

I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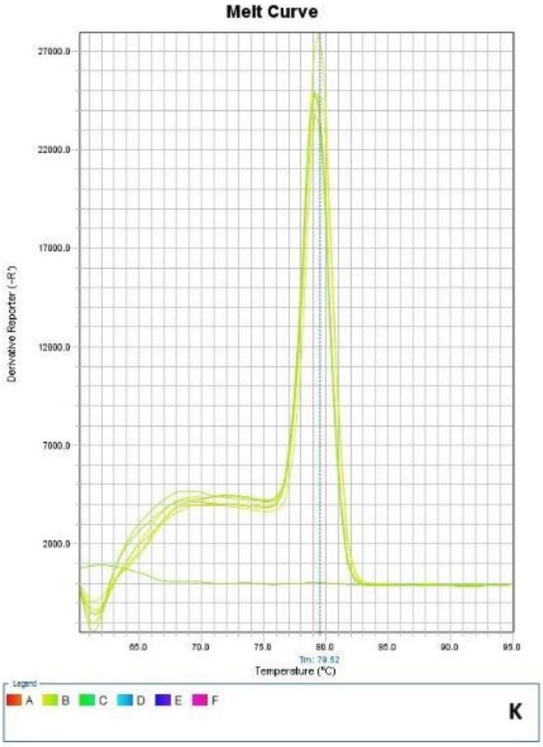
J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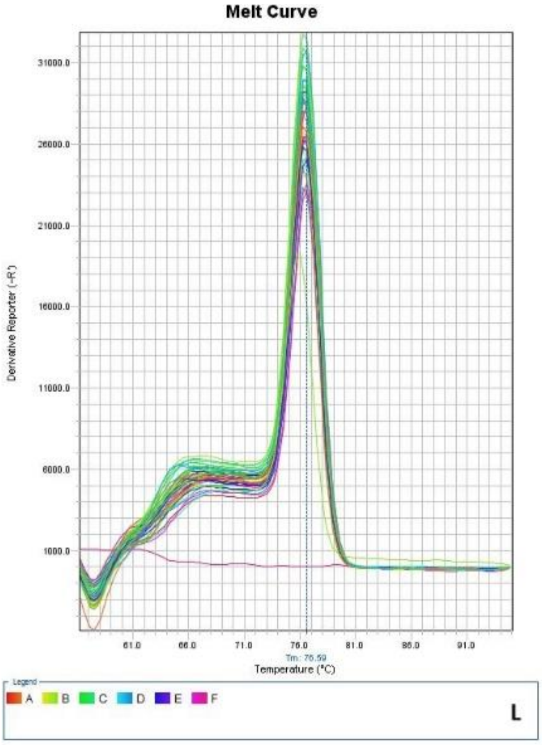
C
D
E
F

K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71.0
76.0
81.0
86.0
91.0
Tm: 76.59
Temperature (°C)
Legend
A
B
C
D
E
F

L

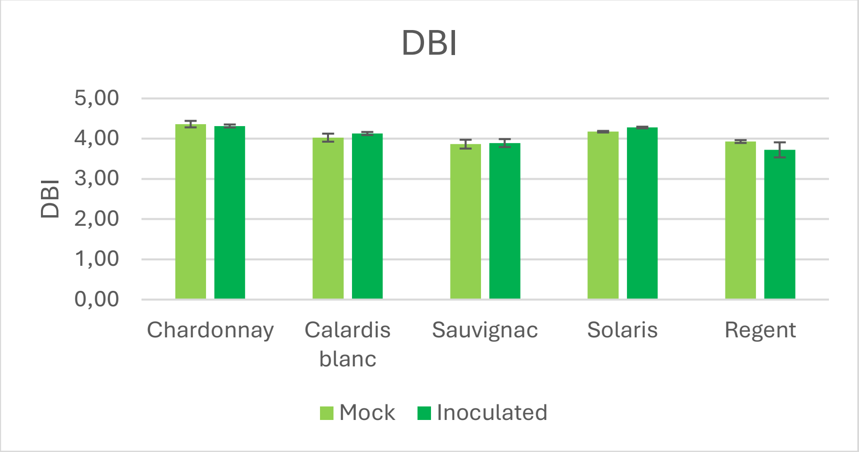
Suplementary Data 3 – *Cis*-elements of Grapevine Genes Encoding Chloroplastidial Enzymes Involved in Lipid Synthesis.

***Cis*-elements**		**MGDG**		**DGDG**		**SQDG**			**PG**		
		MGD1	***MGD2/3***	***DGD1***	***DGD2***	***SQD1***	***SQD2***	***UGP3***	***PGPP1***	***PGP1***	***CDS***
Core promoter / enhancer element	CAAT-box	46	41	1	48	41	27	46	28	46	32
	TATA-box	79	62	1	30	16	49	64	37	58	112
	AT-TATA-box	5	6				4	8	2	2	13
	TATA		2		2		2		2		2
Wounding and pathogen/ defence response	TC-rich repeats	2	1				1	1	1	2	
	W-box		1		1				2	3	4
	WUN-motif		3		4			1	2		1
	WRE3	2			3		1		2		
	box S	1			1						
JA response	CGTCA-motif			1			1			1	1
	TGACG-motif			1			1			1	1
SA response	TCA-element						1	1		1	

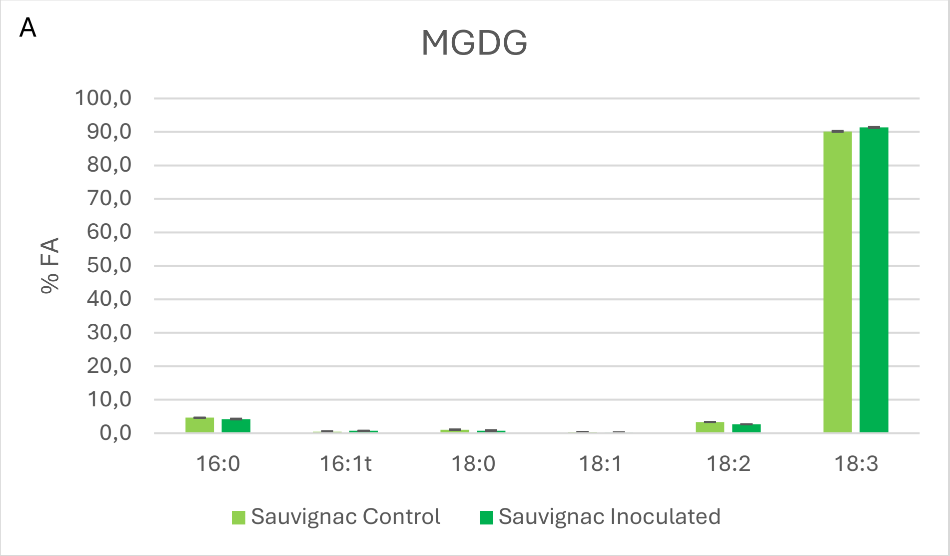
Suplementary Data 4 – Prediction of subcellular location of enzymes.

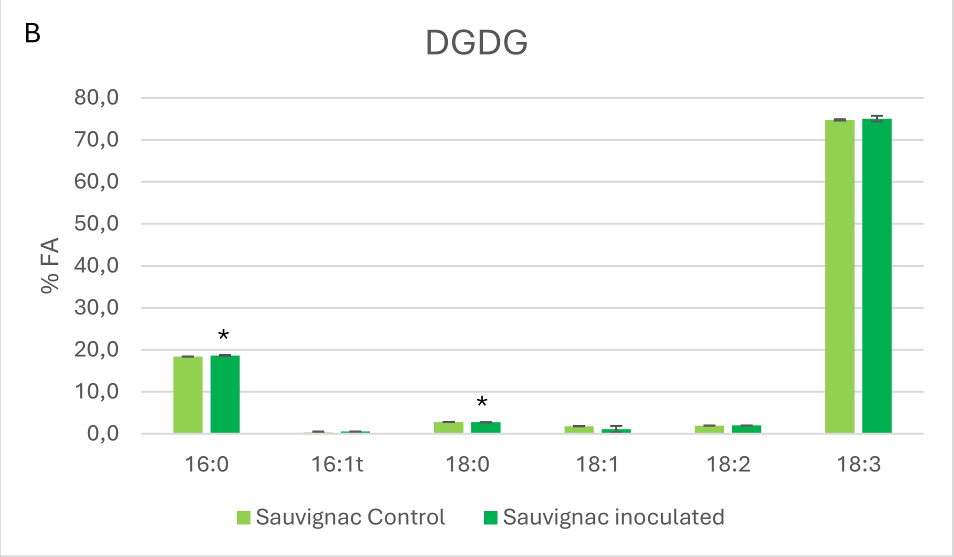
Protein	**Prediction**
MGD1	Other
MGD2	Other/mitochondrion
DGD1X1	Other
DGD1X2	Other
DGD2	Other
SQD1	(Chloroplast) Thylakoid luminal transfer peptide
SQD2	(Chloroplast) Thylakoid luminal transfer peptide
UGP3v1	(Chloroplast) Thylakoid luminal transfer peptide
UGP3v2	(Chloroplast) Thylakoid luminal transfer peptide
UGP3v3	(Chloroplast) Thylakoid luminal transfer peptide
UGP3v4	(Chloroplast) Thylakoid luminal transfer peptide
PGPP1	mitochondrion/chloroplast (thylakoid luminal transfer peptide)
PGP1	Other/mitochondrion
CDS	(Chloroplast) Thylakoid luminal transfer peptide

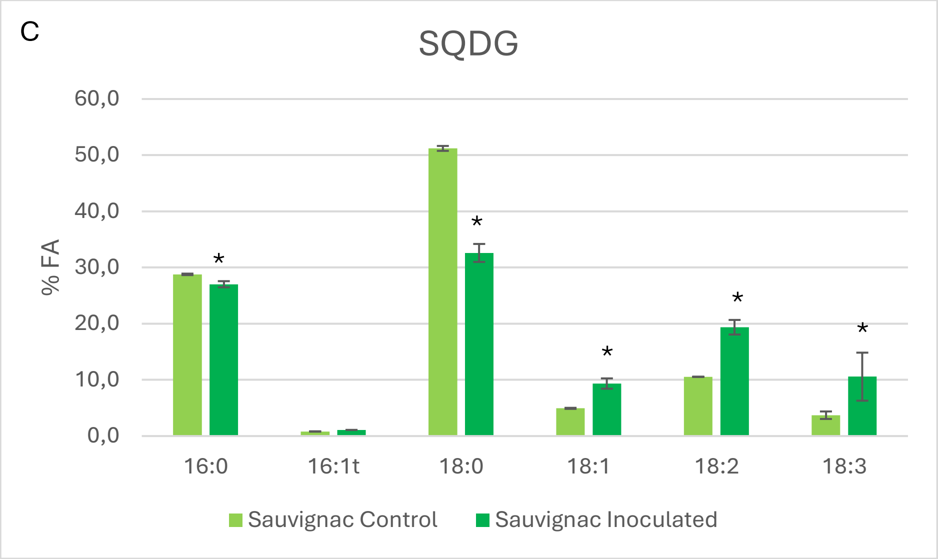
Supplementary Data 5 – Double Bond Index (DBI) of Chardonnay, Calardis blanc, Sauvignac, Solaris and Regent. Control samples are shown in light green, and samples inoculated with NW-10/16 are shown in dark green. A non-parametric test, namely the Krustal-Wallis test, was used through the IBS SPSS Statistics 29 (IBM, NY, USA) and significance was assumed when p-value ≤ 0.05.

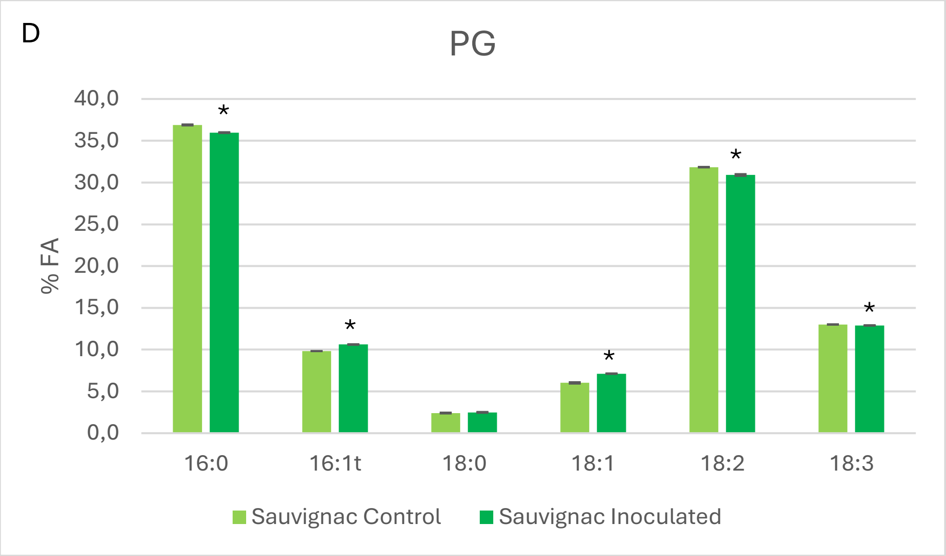


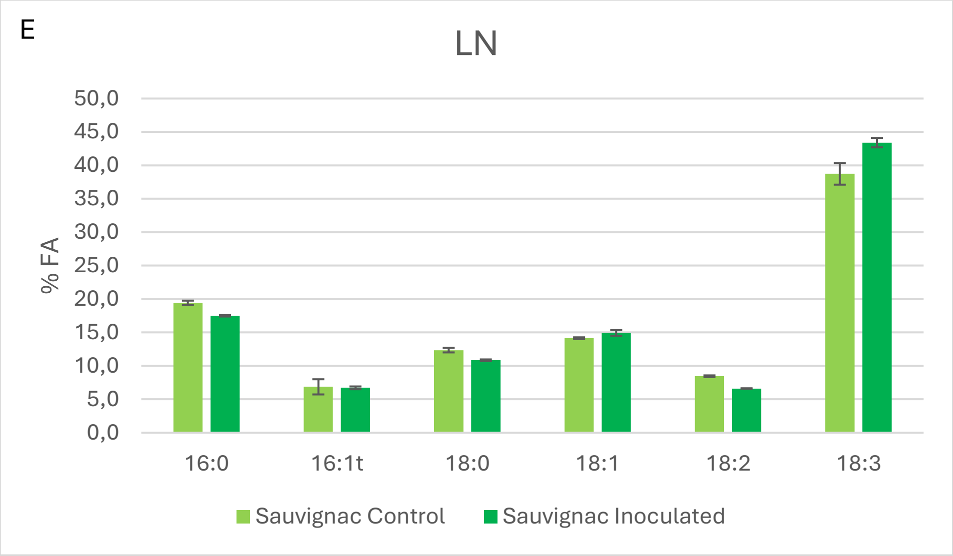
Supplementary Data 6 - Fatty acid composition of the different lipids present in Sauvignac leaves. Control samples are shown in light green, and samples inoculated with NW-10/16 are shown in dark green. A non-parametric test, namely the Krustal-Wallis test, was used through the IBS SPSS Statistics 29 (IBM, NY, USA) and significance was assumed when p-value ≤ 0.05.

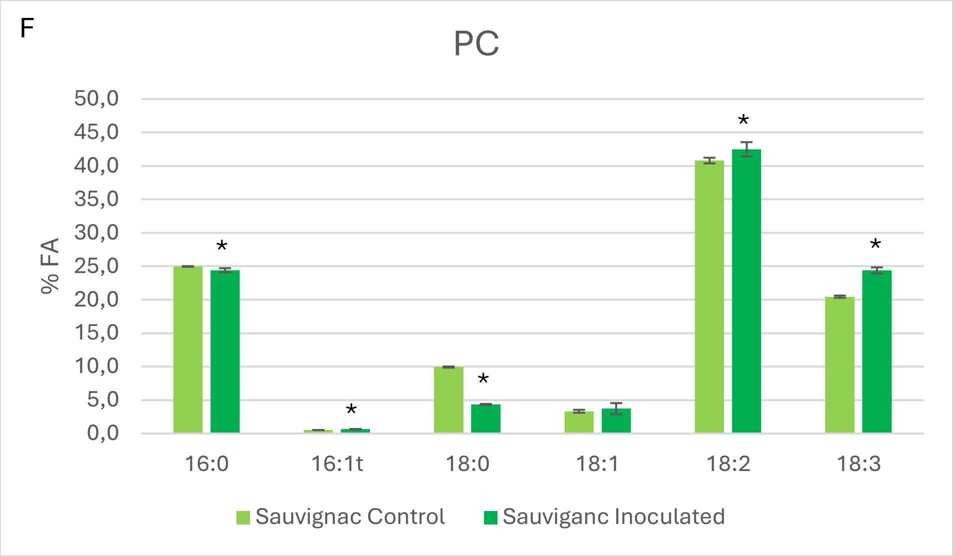


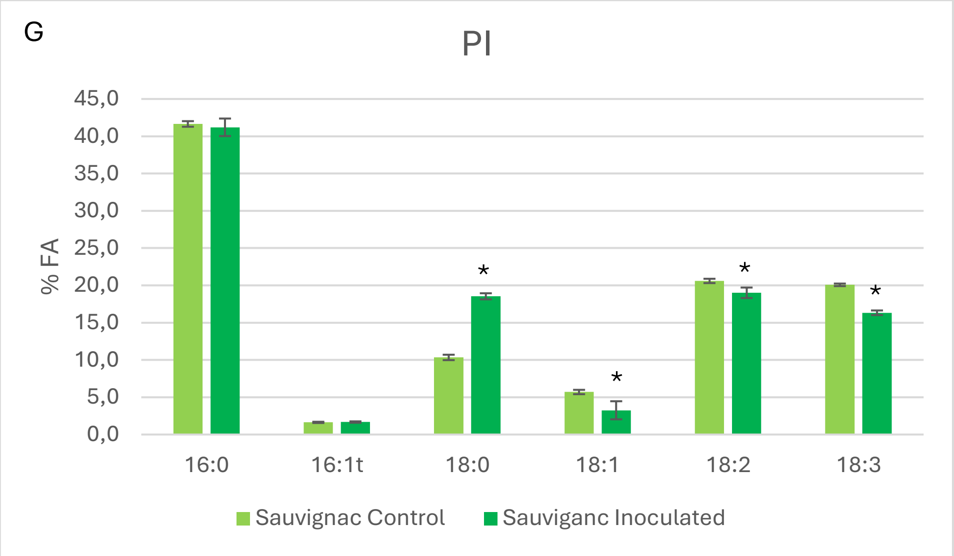


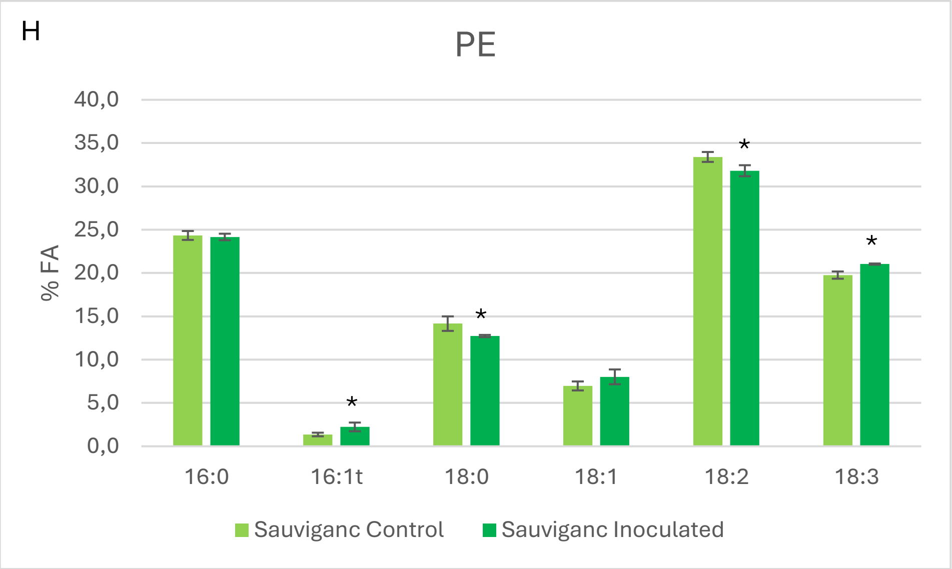

C
SQDG
60,0
50,0
40,0
30,0
20,0
10,0
0,0
% FA
*
*
*
*
*
16:0
16:1t
18:0
18:1
18:2
18:3
Sauvignac Control
Sauvignac Inoculated

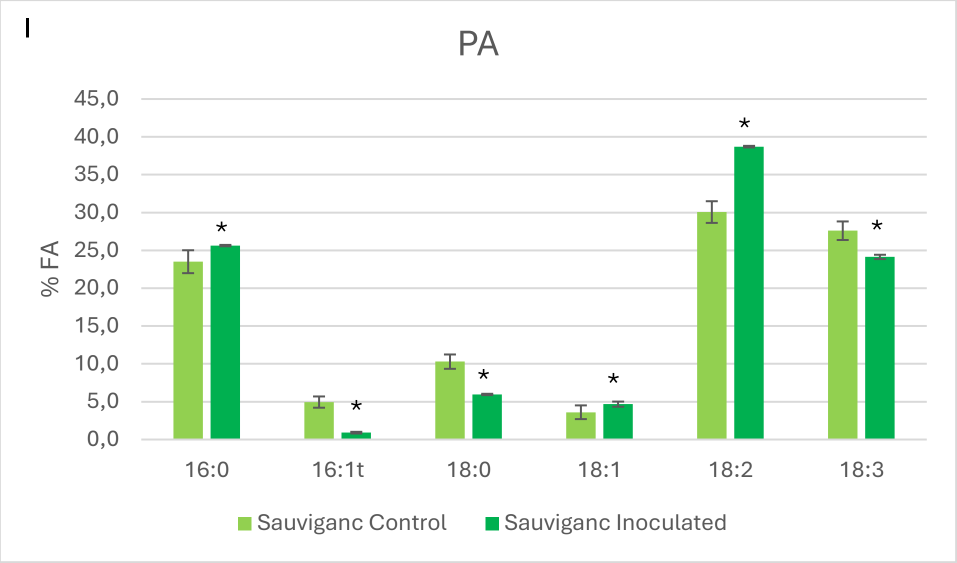

D
PG
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
% FA
*
*
*
*
*
16:0
16:1t
18:0
18:1
18:2
18:3
Sauvignac Control
Sauvignac Inoculated


E
LN
50,0
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
% FA
16:0
16:1t
18:0
18:1
18:2
18:3
Sauvignac Control
Sauvignac Inoculated

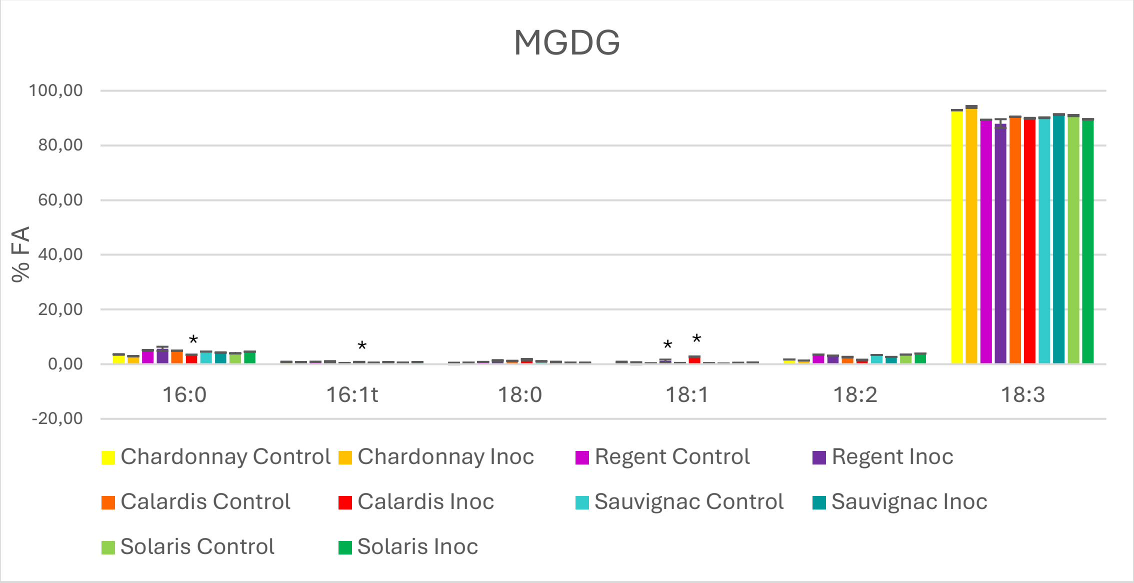

F
PC
% FA
50,0
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
*
*
*
*
*
16:0
16:1t
18:0
18:1
18:2
18:3
Sauvignac Control
Sauviganc Inoculated

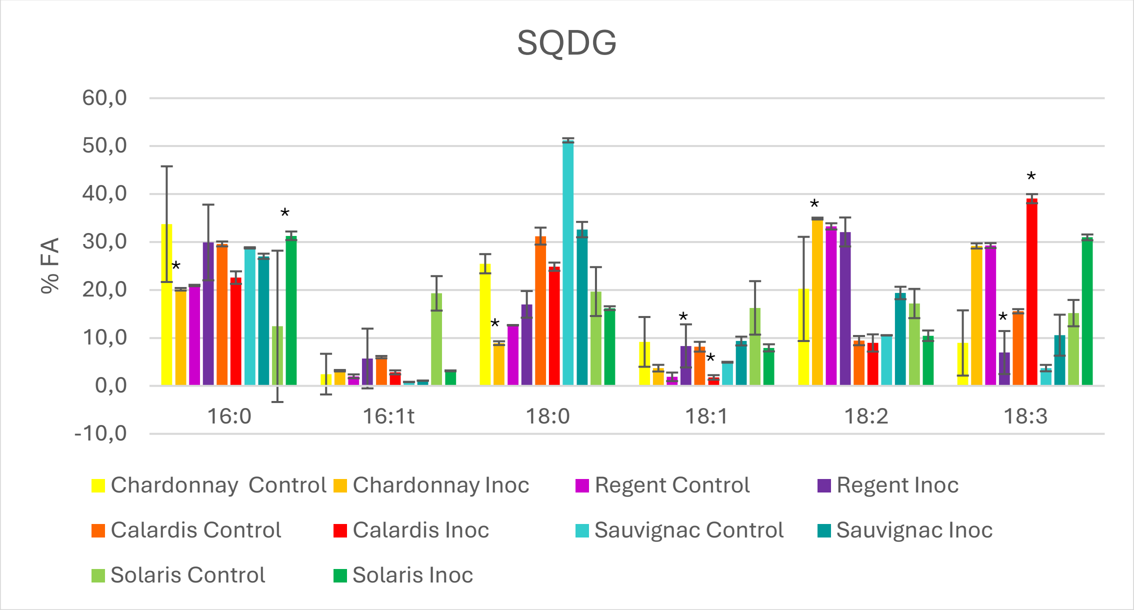

G
PI
% FA
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
*
*
*
*
16:0
16:1t
18:0
18:1
18:2
18:3
Sauvignac Control
Sauviganc Inoculated

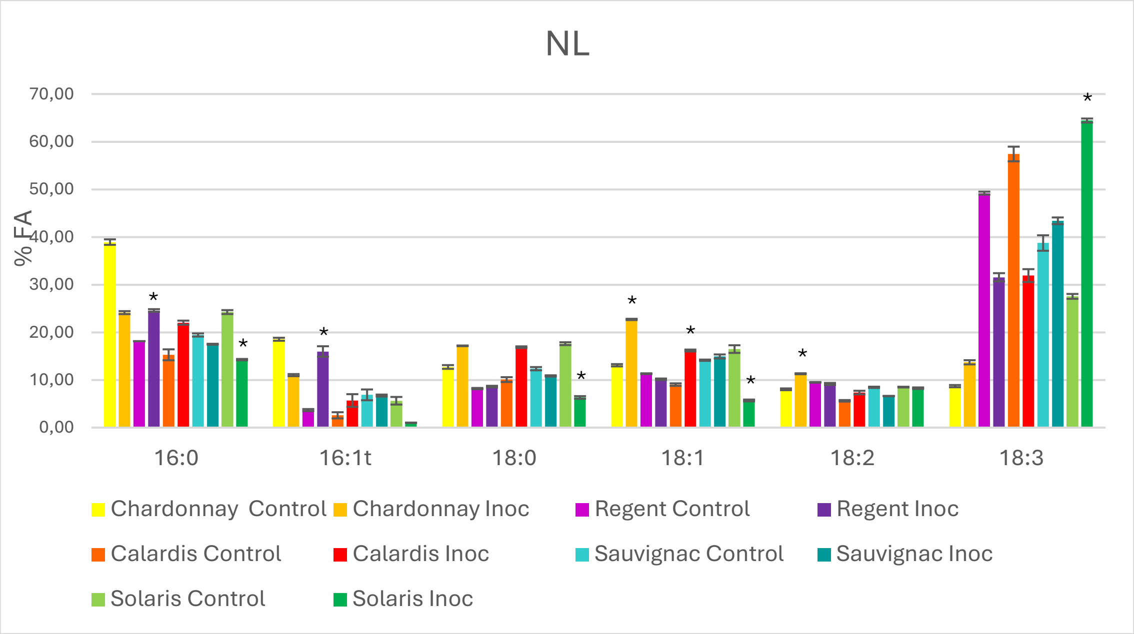

H
PE
% FA
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
*
*
*
*
16:0
16:1t
18:0
18:1
18:2
18:3
Sauviganc Control
Sauviganc Inoculated


I
PA
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
% FA
16:0
16:1t
18:0
18:1
18:2
18:3
*
Sauviganc Control
Sauviganc Inoculated

Supplementary Data 7 – Fatty acid composition of MGDG, SQDG and NL in five varieties of *V. vinifera* (Chardonnay, Regent, Calardis blanc, Sauvignac and Solaris); mock and inoculated with *P. viticola.* In all graphs, the different control and inoculated varieties are represented in different colours. A non-parametric test, namely the Krustal-Wallis test, was used through the IBS SPSS Statistics 29 (IBM, NY, USA) and significance was assumed when p-value ≤ 0.05.






NL
% FA
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
16:0
16:1t
18:0
18:1
18:2
18:3
*
Chardonnay Control
Chardonnay Inoc
Regent Control
Regent Inoc
Calardis Control
Calardis Inoc
Sauvignac Control
Sauvignac Inoc
Solaris Control
Solaris Inoc