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THÈSE EN COTUTELLE PRÉSENTÉE

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ÉCOLE DOCTORALE SCIENCES DE LA VIE ET DE LA SANTÉ

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SPÉCIALITÉ ŒNOLOGIE / SCIENCES BIOLOGIQUES

Par Aleksandra BURDZIEJ

Effect of selected elicitors on grapevine (*Vitis vinifera*) primary and secondary metabolism: focus on stilbenes and triterpenoids

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Sous la direction de Stéphanie CLUZET
et de Anna SZAKIEL

Soutenue le 17 décembre 2020

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Effet d'éliciteurs sélectionnés sur le métabolisme primaire et secondaire de la vigne (*Vitis vinifera*) : focus sur les stilbènes et les triterpénoïdes

Dans le cadre de la promotion d'une viticulture durable, le développement d'alternatives écologiques à l'utilisation de pesticides de synthèse pour le contrôle des maladies de la vigne (*Vitis vinifera*) gagne en importance. Une des méthodes de bio-contrôle proposée est l'induction de l'immunité des plantes par l'application de substances biodégradables, non toxiques pour la santé et l'environnement, appelées éliciteurs ou stimulateurs de défense des plantes (SDP). La résistance conférée contre divers agents pathogènes peut être obtenue grâce à l'emploi de molécules utilisant le plus fréquemment les voies de signalisation de l'acide jasmonique (JA), de l'acide salicylique (SA) et/ou de l'éthylène (ET). De telles voies peuvent déclencher l'induction de gènes liés à la défense tels ceux codant des enzymes responsables de la biosynthèse des stilbènes, des métabolites phénoliques antimicrobiens parmi les plus importants des Vitacées. Au vignoble, pour contrôler les maladies, les éliciteurs peuvent être appliqués en complément des pesticides de synthèse et non en remplacement total car leur efficacité est souvent variable selon les agents pathogènes et les conditions environnementales. Afin de développer leur utilisation, des études supplémentaires qui pourraient notamment élucider leur mécanisme d'action sont nécessaires. L'objectif de cette thèse était d'étudier les réponses de la vigne à des éliciteurs de différents modes d'action, comme le jasmonate de méthyle (MeJA), impliqué dans la voie de signalisation du JA, l'acide *S*-méthyl ester 2,1,3-benzothiadiazole-7-carbothioïque (BTH), un analogue synthétique du SA, des phosphonates (PHOS), molécules à double action stimulateur-fongicide. Le profil des stéroïdes et des triterpénoïdes pentacycliques a été caractérisé par des analyses en chromatographie en phase gazeuse couplée à la spectrométrie de masse (GC-MS). Dans un premier temps, l'effet éliciteur du MeJA a été évalué dans des suspensions cellulaires (in vitro) de plusieurs cultivars de *V. vinifera.* Une surproduction de triterpénoïdes pentacycliques a été observée avec des différences selon la variété considérée : induction de l'accumulation de la bétuline et de l'acide oléanolique ou des phytostérols pour le Petit Verdot, le Gamay Teinturier) et le Cabernet Sauvignon, respectivement. Puis les élicitations ont été effectuées au niveau des feuilles de boutures de serre de *V. vinifera* cv. Cabernet Sauvignon. Un effet stimulant sur les triterpénoïdes pentacycliques liés à la défense a été démontré au détriment de la biosynthèse des stérols, composants structurels essentiels des membranes cellulaires. Par l'utilisation de puces NeoVigen et la chromatographie liquide à ultra haute performance couplée à la spectrométrie de masse (UHPLC-MS), l'induction de l'expression de gènes liés à la défense et l'accumulation de polyphénols (stilbènes, flavanols et flavonols) ont été notées suite aux trois traitements éliciteurs. La protection de la vigne conférée par les éliciteurs a été confirmée par des biotests sur disques foliaires inoculés par l'oomycète biotrophe *Plasmopara viticola,* l'agent responsable du mildiou. Par ailleurs, il est important d'avoir connaissance de l'impact des éliciteurs sur le métabolisme général afin d'obtenir l'effet optimal entre croissance, rendement et défense. Ainsi, une approche métabolomique utilisant la spectroscopie de résonance magnétique nucléaire du proton (RMN 1H) a été menée. Une reprogrammation similaire et/ou spécifique selon l'éliciteur considéré a été notée en particulier au niveau des glucides, des acides aminés et de certains intermédiaires du cycle de Krebs. Les recherches présentées dans cette thèse, démontrent que la compréhension approfondie de l'interaction entre l'éliciteur, les réponses moléculaires et métaboliques de la plante et le pathogène, est cruciale pour le développement de stratégies de protection efficaces basées sur l'utilisation des SDP pour contrôler les maladies de la vigne.

Mots clés : *Vitis* spp., stimulateurs de défense des plantes, benzothiadiazole, jasmonate de méthyle, phosphonates, mildiou

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Effect of selected elicitors on grapevine (*Vitis vinifera*) primary and secondary metabolism: focus on stilbenes and triterpenoids

In the frame of promoting sustainable vitiviniculture, the development of eco-friendly alternatives to synthetic chemical products for phytosanitary treatments against grapevine (*Vitis vinifera*) pests is gaining importance. One of the bio-control methods that can be proposed is the induction of plant immunity by using elicitors, also called plant defense stimulators (PDS), as these substances are biodegradable and, non-toxic to health and environment. A conferred resistance against various pathogens can be obtained with natural molecules acting most frequently through jasmonic acid (JA), salicylic acid (SA), and/or ethylene (ET) signaling pathways. These pathways are involved in the induction of defense-related genes such as those encoding enzymes responsible for the biosynthesis of stilbenes, which are the most important polyphenolic antimicrobial metabolites (phytoalexins) in Vitaceae. For vineyard protection, PDS can be applied as a complement for pesticides and not as a full replacement since their effectiveness is often variable according to pathogens and environmental conditions. In order to develop the strategies based on PDS use, more studies which could elucidate their mechanism of action are needed. The aim of this thesis was to examine the responses of grapevine to elicitors of different mode of action, as methyl jasmonate (MeJA), implicated in JA signaling pathway, 2,1,3-benzothiadiazole-7-carbothioic acid *S*-methyl ester (BTH), a synthetic analogue of SA, and phosphonates (PHOS), molecules of a double stimulator-fungicide action. Due to scarce information about steroids and pentacyclic triterpenoids in grapevine, their profile after PDS treatment were characterized in different grapevine experimental models using gas chromatography-mass spectrometry (GC-MS) analyses. Firstly, the effect of elicitation with MeJA was evaluated in cell suspension cultures (in vitro) of *V. vinifera*. An overproduction of bioactive pentacyclic triterpenoids occurred with differences according to the cultivar studied, i.e., acumulation of betulin and oleanolic acid or phytosterols was noted in respectively Petit Verdot, Gamay Teinturier and Cabernet Sauvignon cell suspension cultures. Then, elicitations were effectuated on the leaves of *V. vinifera* cv. Cabernet Sauvignon greenhouse cuttings. A stimulatory effect on the potentially defense-related pentacyclic triterpenoids at the expense of the biosynthesis of sterols, which are essential structural components of cell membranes, was shown. By the use of NeoVigen microarrays, and ultra-performance liquid chromatography-mass spectrometry (UHPLC-MS), the accumulation of defense-related transcripts and polyphenols (stilbenes, flavanols and flavonols) were noted after the three elicitors treatments. Grapevine protection conferred by these elicitors was confirmed on foliar discs against the biotrophic oomycete *Plasmopara viticola*, the causal agent of downy mildew. Furthermore, the impact of PDS on primary metabolism should be evaluated in order to ensure, in the longer term, the best trade-off between growth, yield and defense. Thus, a thorough metabolomic approach using proton nuclear magnetic resonance spectroscopy (1H-NMR) was performed. A reprogramming similar and/or specific to the elicitor applied was noted, particularly within carbohydrates, amino acids, and some of the Krebs cycle intermediates. The research presented in the current dissertation revealed that the thorough comprehension of the interaction between elicitor, plant molecular and metabolic responses and pathogen, is crucial for the development of effective protection strategies based on the use of PDS for grapevine diseases control.

Keywords: *Vitis* spp., plant defense stimulators, benzothiadiazole, methyl jasmonate, phosphonates, downy mildew

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Wpływ wybranych elicytorów na metabolizm pierwotny i wtórny winorośli właściwej (*Vitis vinifera*): ze szczególnym uwzględnieniem stilbenów i triterpenoidów

Rozwój przyjaznych dla środowiska alternatywnych metod ochrony winorośli (*Vitis vinifera*) przeciw szkodnikom zyskuje na znaczeniu w kontekście zapobiegania nadmiernemu użyciu pestycydów i promowania zrównoważonego rolnictwa. Indukcja naturalnej obrony roślin poprzez działanie elicytorów (stymulatorów odporności roślin) jest jedną z najbardziej obiecujących metod kontroli biologicznej, jako że opiera się na zastosowaniu substancji biodegradowalnych i nietoksycznych dla środowiska i zdrowia ludzi. Elicytory zaaplikowane na roślinę wyzwalają w niej mechanizmy obronne, prowadząc do rozwijania odporności na kolejne ataki patogenów. Percepcja elicytora uruchamia szlaki sygnałowe, z których najbardziej kluczowe są te związane z kwasem salicylowym (SA), kwasem jasmonowym (JA) lub etylenem (ET). Konsekwencje aktywowania kaskady reakcji odpornościowych to m.in. indukcja ekspresji genów związanych z obroną, np. kodujących białka związane z patogenezą (PR), czy enzymy odpowiedzialne za biosyntezę wyspecjalizowanych metabolitów o właściwościach przeciwdrobnoustrojowych (przede wszystkim stilbenów u roślin z rodziny Vitaceae). Stymulatory odporności roślin są obecnie stosowane jako uzupełnienie pestycydów, a nie jako środek zastępczy, ponieważ ich skuteczność jest często zmienna w zależności od konkretnego patogena i warunków środowiskowych. W celu opracowania strategii ochrony winorośli przed chorobami całkowicie opartych na elicytorach, potrzeba jest wielu badań, które pozwolą m.in. wyjaśnić mechanizm działania tych środków. Celem niniejszej pracy było zbadanie odpowiedzi winorośli na elicytory o różnej aktywności biologicznej: jasmonian metylu (MeJA), związany ze szlakiem sygnałowym JA, benzotiadiazol (BTH), syntetyczny analog SA oraz sole fosforanowe (PHOS), o działaniu stymulującym, jak i grzybobójczym. Profil steroidów i triterpenoidów pentacyklicznych winorośli scharakteryzowano za pomocą chromatografii gazowej sprzężonej ze spektrometrią mas (GC-MS). Potencjalny wpływ MeJA na profil triterpenoidów oceniono w hodowlach zawiesin komórkowych *in vitro V. vinifera*. W zależności od badanej odmiany, zaobserwowano wzmożoną biosyntezę triterpenoidów pentacyklicznych, takich jak betulina (Petit Verdot) i kwas oleanolowy (Gamay Teinturier), a także fitosteroli (Cabernet Sauvignon). W elicytowanych liściach szklarniowych sadzonek Cabernet Sauvignon, wykazano konkurencyjność szlaków biosyntezy triterpenoidow, tzn. zwiększona akumulacja triterpenoidow pentacyklicznych, związanych z chemiczną obroną roślin, odbyła się kosztem biosyntezy steroli, związków niezbędnych dla budowy i funkcjonowania błon komórkowych. Dzięki analizom z wykorzystaniem mikromacierzy NeoVigen i ultrasprawnej chromatografii cieczowej ze spektrometrią mas (UHPLC-MS), w liściach poddanych elicytacji stwierdzono akumulację transkryptów związanych z obroną oraz zwiększoną zawartość polifenoli (stilbenów, flawanoli i flawonoli). Biotesty na krążkach liściowych wykazały, że badane elicytory nadały odporność liściom przeciwko *Plasmopara viticola,* tj. patogenowi wywołującemu mączniaka rzekomego. Ponadto, badanie wpływu elicytorów na metabolizm pierwotny jest istotny w celu zapewnienia roślinie swoistego kompromisu między aktywowaniem kosztownych energetycznie mechanizmów obronnych, a utrzymaniem prawidłowego funkcjonowania podstawowych procesów fizjologicznych. Badania metabolomiczne z wykorzystaniem spektroskopii protonowego jądrowego rezonansu magnetycznego (1H-NMR) wykazały szereg istotnych zmian w profilu węglowodanów, aminokwasów i niektórych substratów cyklu Krebsa w liściach poddanych elicytacji. Wyniki badań przedstawione w niniejszej rozprawie wykazują, iż dokładne zrozumienie interakcji między elicytorem, odpowiedzią molekularną i metaboliczną rośliny a patogenem, ma kluczowe znaczenie dla rozwoju skutecznych strategii obrony winorośli przed chorobami, opartych na stymulatorach odporności roślin.

Słowa kluczowe: *Vitis* spp., stymulatory odporności roślin, benzotiadiazol, jasmonian metylu, fosforany, mączniak rzekomy

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Table of contents

ABBREVIATIONS

[Ca^{2+}]$_{cyt}$, cytosolic calcium ions;
13-HPOT, 13-hydroperoxylinolenic acid;
1H-NMR, proton Nuclear Magnetic Resonance
4CL, 4-coumarate-coenzyme A ligase;
ABA, abscisic acid;
ACC, 1-aminocyclopropane-1-carboxylic acid;
ACO1, 1-aminocyclopropane-1-carboxylic acid oxidase;
ACS, aminocyclopropane-1-carboxylic acid synthase;
ADH, alcohol dehydrogenase;
AGPs, arabinogalactan proteins;
AIM1, abnormal inflorescence meristem1;
Alli, alliinase;
ANOVA, analysis of variance;
ANR, anthocyanidine reductase;
ANS, nthocyanidin synthase;
ANTS, anthranilate synthase;
AOC, allene oxide cyclase;
AOS, allene oxide synthase;
APOX, ascorbate peroxidase;
APX, ascorbate peroxidase;
ArMV, Arabic Mosaïc Virus;
ASM, acibenzolar-*S*-methyl;
AT, acetoacetyl-CoA thiolase;
ATP, adenosine triphosphate;
Avr, avirulence gene product;
B.C., Before Christ;
B.P., Before Present;
BA2H, benzoic acid-2-hydroxylase;
BABA, *β*-aminobutyric acid;
BAK1, brassinosteroid-insensitive 1-associated receptor kinase 1;
BcPG, *Botrytis cinerea* polygalacturonase;
BcPG1, endopolygalacturonase 1;
BDA, Black Dead Arm;
BTH, benzothiadiazole;
C3H, *p*-coumarate 3-hydroxylase;
C4H, cinnamate-4-hydroxylase;
Ca^{2+}, calcium ions;
CAD, cinnamyl alcohol dehydrogenase;
CAGR, Compound Annual Growth Rate;
CALS, callose synthase;
cAMP, cyclic adenosine monophosphate;
CAT, catalase;
CCR, cinnamoyl-CoA reductase;
cDNA, complementary deoxyribonucleic acid;
CDPKs, calcium-dependent protein kinases;
CERK1, chitin elicitor receptor kinase 1;
cGMP, cyclic guanosine monophosphate;
CHI, chalcone isomerase;
CHORM, chorismate mutase;
CHORS, chorismate synthase;
CHS, chalcone synthase;
Cl^-, chloride ion;
CM, chorismate mutase;
CNIV, Le Comité National des Interprofessions des Vins;
CoA, Coenzyme A;
COI1, CORONATINE INSENSITIVE1;
COMT, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase;
CS6, Cabernet Sauvignon strain 6;
CWDE, Cell Wall Degrading Enzymes;
CWPs, cell wall associated non-enzymatic proteins;
CYP, cytochrome P450;
CYP710A, cytochrome P450, family 710;
D.W., Dry Weight;
DAG, diacylglycerol;
DAMPs, damage-associated molecular patterns;
DFR, dihydroflavonol 4-reductase;
DIR, dirigent protein (enzyme un-known);
DMAPP, dimethylallyl diphosphate;
DNA, deoxyribonucleic acid;
DOXP, 1-deoxy-D-xylulose 5-phosphate;
dpi, days post-inoculation;
dpt, days post-treatment;
DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase;
DXS, 1-deoxy-D-xylulose-5-phosphate synthase;
EC, European Commission
ECH, enoyl-CoA hydratase;
EDS1, lipase 3/ENHANCED DISEASE SUSCEPTIBILITY 1;
EDS5, ENHANCED DISEASE SUSCEPTIBILITY 5;
EF1γ, elongation factor eEF1 gamma chain;
EGF, Epidermal Growth Factor;
EGFV, Ecophysiologie et Génomique Fonctionnelle de la Vigne laboratory
EIN3, ethylene insensitive 3-Binding F Box Protein 1;
eLRR, extracellular leucine-rich repeat;
EPS1, ENHANCED PSEUDOMONAS SUSCEPTIBILITY 1;
EST, Expressed Sequence Tag;
ET, ethylene;
ETS, Effector Triggered Susceptibility;
F.W., Fresh Weight;
F3H, flavanone-3-hydroxylase;
FAR, ent-kaurene synthase;
FID, Flame Ionization Detector;
Fig., Figure;
FLS, flavonol synthase;
FLS2, flagellin sensing 2;
FNS, flavone synthase;
FPP, farnesyl diphosphate;
FPPS, farnesyl pyrophosphate synthase;
G proteins, GTP-binding proteins;
GA, gibberellin;
GABA, *γ*-aminobutyric acid;
GAP, 3-phosphoglyceraldehyde;

GAPDH, glyceraldehyde 3-phosphate dehydrogenase;
GC-MS, Gas Chromatography-Mass Spectrometry;
GFLV, Grapevine Fanleaf Virus;
GGPP, geranylgeranyl diphosphate;
GH3-6, (+)-7-*iso*-jasmonoyl-L-isoleucine synthase;
GPP, geranyl diphosphate;
GPX, guaiacol peroxidase;
GRLaV, Grapevine leafroll-associated virus;
GRPs, Glycine-rich proteins;
GST-phi, glutathione S-transferase class-phi;
GST-tau, glutathione S-transferase Tau class;
GT3, Gamay Fréaux var. Teinturier strain 3;
GTD, Grapevine Trunk Diseases;
GTF, glycotransferase;
GTP, guanosine-5'-triphosphate;
GT_T, Gamay Fréaux var. Teinturier;
h, hours;
H^+, hydrogen ion;
H_2O_2, hydrogen peroxide;
ha, hectares;
HCA, hydroxycinnamic acids;
HCPC, Hierarchical Clustering on Principal Components;
HDS, hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase;
hl, hectoliters;
HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase;
hpt, hours post-treatment;
HR, Hypersensitive Response;
HRGPs, hydroxyproline-rich glycoproteins;
IC_{50}, the half maximal inhibitory concentration;
ICS, isochorismate synthase;
IDI, isopentenyl diphosphate isomerase;
IDS, isopentenyl diphosphate: dimethylallyl diphosphate synthase;
IGGP, International Grape Genomics Program;
INA, 2,6-dichloroisonicotinic acid;
INRAE, French National Research Institute for Agriculture, Food and Environment;
IP_3, 1,4,5-trisphosphate;
IPP, isopentenyl diphosphate;
ISR, Induced Systemic Resistance;
ISVV, Institute of Vine and Wine Science of University of Bordeaux;
JA-Ile, (+)-7-*iso*-jasmonoyl-L-isoleucine;
JA, Jasmonic Acid;
JAR1, jasmonate-resistant 1;
JAs, jasmonates;
JAZ, JASMONATE ZIM DOMAIN;
JMT, jasmonate methyl transferase;
K^+, potassium ion;
KEGG, Kyoto Encyclopedia of Genes and Genomes;
L3, 3rd leaves from the apex;
L4, 4th leaves from the apex;
L5, 5th leaves from the apex;
LAR, leucoanthocyanidine reductase;
LAR, Local Acquired Resistance;
LDOX, leucoanthocyanidin synthase;
LORE, lipooligosaccharide-specific reduced elicitation;
LOX, lipoxygenase;
LPS, lipopolysaccharide;
LPSs, lipopolysaccharides;
LYK, lysin motif-containing receptor-like kinase;
MAMPs, Microbe-Associated Molecular Pattern;
MAPKs, Mitogen-Activated Protein Kinases;
mc-3-OH-FAs, medium chain 3-hydroxy fatty acids;
MEcPP, 2,4-cyclodiphosphate-2-methylerythritol;
MeJA, methyl jasmonate;
MEP, 2-*C*-methyl-D-erythritol 4-phosphate;
MeSA, methyl salicylate;
mha, milions of hectares;
MIB, Molecules with Biological Interest laboratory;
MPK4, Mitogen-activated protein kinase 4;
MRL, Maximum Residue Level;
MRM, Multiple Reaction Monitoring;
MTI, MAMPs-Triggered Immunity;
MVA, mevalonate;
MYB, R2R3-type V-myb myeloblastosis viral oncogene homolog transcription;
MYCs, MYC transcription factors;
NADPH, nicotinamide adenine dinucleotide phosphate;
NB-LRR, nucleotide-binding and leucine-rich repeat;
NDS, Natural Defense Stimulators;
NHR, non-host resistance;
NO, nitric oxide;
NO_3^-, nitrate ion;
NPR1, non expressor of pathogenesis related protein 1;
O_2^-, superoxide anion;
OCH_3, methoxy group;
OGs, oligogalacturonides;
OH, hydroxyl group;
OIV, International Organisation of Vine and Wine;
OPDA, (9*S*, 13*S*)-12-oxo-*cis*-10,15-phytodienoic acid;
OPR3, oxophytodienoate redutase 3;
OSCs, oxidosqualene cyclase;
PA, Phosphatidic Acid;
PAMPs, Pathogen-Associated Molecular Patterns;
PCA, Principal Component Analysis;
PDS, Plant Defense Stimulators;
PECT, pectin methyl esterase;
PEP, phosphoenolpyruvate;
PepMV, Pepino Mosaic Virus;
PGIP, Polygalacturonases-Inhibiting Proteins;
PGPF, Plant Growth Promoting Fungi;
PGPR, Plant Growth Promoting Rhizobacteria;
PHOS, phosphonates;
PINs, proteinase inhibitors;

PK, Protein Kinase;
PLA_1, phospholipase 1;
PP, protein phosphatase;
PP1, protein kinase 1;
PP2A, 2A protein phosphatase;
PR proteins, pathogenesis-related proteins;
PR1, pathogen-related protein 1;
PR10, ribonuclease;
PR11, chitinase class V;
PR12, defensin-like protein-oxalate oxidase;
PR13, thionins;
PR14, lipid transfer protein;
PR15, germin-like protein-oxalate oxidase;
PR16, oxalate oxidase-like protein;
PR2, beta-1,3-glucanase;
PR3, endochitinase class;
PR4, chitinase class IV;
PR5, thaumatin-like protein;
PR6, serine protease inhibitor;
PR7, subtilisin-like endoprotease;
PR8, acidic endochitinase-like;
PR9, cationic peroxidase 1;
PRRs, pattern recognition receptors;
PS3, sulfated laminarin;
PTI, PAMPs-Triggered Immunity;
PUFA, polyunsaturated fatty acid;
PV, Petit Verdot;
qPCR, quantitative Polymerase Chain Reaction;
QTL, Quantitative Trait Loci;
R, resistance genes;
RBPG1, responsiveness to *Botrytis cinerea* polygalacturonase (BcPG);
R_F, retardation factor;
RLKs, receptor-like kinases;
RLPs, receptor-like proteins;
RNA, ribonucleic acid;
ROMT, resveratrol *O*-methyl-tranferase;
ROS, Reactive Oxygen Species;
RT, Room Temperature;
***S*-AdoMet**, *S*-adenosylmethionine;
SA, Salicylic Acid;
SABP2, salicylic acid-binding protein 2;
SAMT, salicylic acid methyl transferase;
SAR, Systemic Acquired Resistance;
SAVE, Santé et Agroécologie du Vignoble laboratory;
SCF^{COI1}, F-box protein CORONATINE INSENSITIVE1 of Skp1/Cullin/F-box E3 ubiquitin ligase complex;
SD, standard deviation;
SH, sulfhydryl group;
SMTs, C24-sterol methyltransferases;
SOBIR1, suppressor of BIR1;
spp., several species;
STS, stilbene synthase;
TE, thiol esterase;
THIORYLS8, catalytic thioredoxin-like protein 4A;
TIP41, TIP41-like protein;
TLC, Thin Layer Chromatography;
TMV, Tobacco Mosaic Virus;
TPS, terpenes synthase;
t_R, retention time;
TUA, tubulin alpha;
UAA, Utilised Agricultural Areas;
UDP, uridine diphosphate;
UFGT, UDP-glucose: flavonoid 3-*O*-glucosyltransferase;
UHPLC-MS, Ultra-Performance Liquid Chromatography-Mass Spectrometry;
UMR, Joint Research Unit;
UNESCO, United Nations Educational, Scientific and Cultural Organization;
UV, ultraviolet;
var., variety;
VOCs, Volatile Organic Compounds;
Vv, *Vitis vinifera*;
WAK1, wall-associated kinase 1;
WRKY, WRKY transcription factor;
α-LeA, α-linolenic acid;

I. INTRODUCTION

1. GRAPEVINE (VITACEAE)

1.1. Taxonomy and diversity

Grapevines (*Vitis* L.) are dicotyledonous angiosperm plants belonging to the order Rhamnales and to the family of Vitaceae (Ampelidaceae), which comprises 14 genera (Christenhusz and Byng, 2016). The most agronomically important is *Vitis* genus subdivided into two subgenera, *Muscadinia*, and *Vitis* (formerly *Euvitis*), distributed in the temperate and intertropical zones of Northern Hemisphere: Europe, Asia, and America (Table 1). The *Muscadinia* subgenus comprises only three species, including *M. rotundifolia*, remarkably resistant to the main cryptogamic diseases, cultivated for wine in the south-east of North America. Most of the cultivated species belong to the subgenus *Vitis* and are classified into three groups according to their geographic origin.

Table 1. Taxonomy of Vitaceae family. Species of economic importance (*) (according to Bosak, 2018).

Family	Genus	Subgenus	Species	Origin
Vitaceae	*Vitis*	*Vitis (formerly Euvitis)*	*Vitis vinifera**	West Asia and Europe
			Vitis amurensis, Vitis davidii*, Vitis coignetiae*, Vitis flexuosa*, Vitis pentagona*, Vitis pseudoreticulata, Vitis piasezkii, Vitis adenoclada, Vitis hancockii, Vitis erytrophylla, Vitis bellula, Vitis chunganensis*	East Asia
			Vitis labrusca, Vitis aestivalis*, Vitis lincecumii*, Vitis rupestris*, Vitis riparia*, Vitis berlandieri*, Vitis candicans, Vitis longii, Vitis champinii, Vitis mustangensis, Vitis californica*	North America
		Muscadinia	*Muscadinia rotundifolia*, Muscadinia munsoniana, Muscadinia popenoei*	North and Central America

The East Asian species are currently considered to be of limited importance to vitiviniculture, contrarily to the unique Euro-Asian species, *Vitis vinifera* L., which acquired significant economic interest over time (for details see subsec. 1.5). It includes two subspecies: the wild *Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi, and the cultivated *Vitis vinifera* L. subsp. *vinifera* (formerly *sativa*, referred to as *V. vinifera* hereafter). The latter one is present in the form of the very large majority of grapevine varieties or cultivars, from both natural crosses and varietal selection, of which the number is estimated at around 10 000 (OIV, consulted on September 2020). In France, 250 of varieties for table and wine grapes can be cultivated, however, only 10 of them (Merlot, Trebbiano Toscano/Ugni blanc, Garnacha Tinta/Grenache, Syrah, Chardonnay, Cabernet Sauvignon, Cabernet Franc, Carignan Noir/Mazuela, Pinot Noir/Blauer Burgunder, Sauvignon Blanc) represent 70.2% of the total surface area of vineyards (806 000 ha) (OIV, 2020).

A great majority of grapevines classified as *V. vinifera* L. derive from hybridisation by grafting of different *Vitis* species which took place from the 19th century, when the introduction to Europe of the American species led to the importation of previously unknown diseases – powdery mildew in 1845, phylloxera in 1868, downy mildew in 1878, and black-rot in 1885 (Galet, 2000). The North American species, in particular *V. berlandieri*, *V. riparia* and *V. rupestris* have been used as breeding rootstock in order to associate their resistance with the quality of European varieties, and to cope with the high susceptibility to diseases of the latter. Over the years, the process of hybridisation, further assisted by genetic mutations, ongoing vegetative propagation, as well as sexual reproduction has led to a significant increase in genetic diversity of *Vitis* plants, and creation of a number of varieties, estimated today at 21 045 (OIV, 2020). Polish vineyards are an example of those which are mainly based on hybrids resistant to low temperatures or to various diseases. They include Solaris, Hibernal, Johanniter, Seyval Blanc, or Maréchal Foch, as well as Regent, or Rondo for red wines (Bosak, 2018).

In order to maintain wine grape productivity and quality in the face of climate fluctuations, anticipation in agricultural adaptation is required. In this context, Vitadapt project can be cited (Destrac and van Leeuwen, 2016) as designed in 2009 for research for varieties more adapted to changing environment, by National Research Institute for Agriculture, Food and Environment (Institut national de recherche pour l'agriculture, l'alimentation et l'environnement, INRAE) and Science Institute of Vine and Wine (Institut des Sciences de la Vigne et du Vin, ISVV) in Bordeaux, France.

1.2. Botany and biology

The members of the Vitaceae family are lianas and shrubs with herbaceous or sarmentous stems. The shoots possess nodes that generate tendrils or flower clusters opposite the alternate leaves. The flowers are small and green, and the leaves differ in shape and size depending on the species and cultivar (Jackson, 2000; Keller, 2015). The ovary of a flower is composed of two carpels, partially enclosed by a receptacle which becomes a two-compartment berry, containing from 2 to 4 seeds. Plants from *Vitis* genus are distinguished from related genera primarily by floral characteristics – petals remain fused into the structure called calyptra which falls as a cap during flowering (Fortes and Pais, 2016). The *Muscadinia* and *Vitis* subgenera differ in chromosome composition, which are respectively 2n=40, and 38, as well as in anatomical characteristics of internal cane morphology, tendrils, seed morphology, and bark shredding (Jackson, 2000). The wild *Vitis* species are dioecious, whereas the most of cultivated *V. vinifera* varieties have perfect (bisexual) flowers, thanks to which they are more likely to self-pollinate and produce fruit (Keller, 2015). Figure 1 presents some botanical features of a cultivated *V. vinifera*.

The wild *Vitis* vines generally grow in shady deciduous forests, naturally climbing on trees and bushes, or if without support, spreading above the ground, where shoots easily take root and create new plants through vegetative propagation. However, sexual reproduction remains a fundamental survival strategy of grapevine. Thus, in principle it is the heliophilous plant, that is why vineyards are often placed at sunny hills. The growth of cultivated grapevine is maintained with the trellises in order to control the quantity and quality of the grapes. The cultivated *V. vinifera* are essentially obtained by cloning and grafting, i.e., multiplication system which consists in fixing a graft (a clone coming from a cane of a cultivated *Vitis*) on a selected rootstock to increase crop yield and resistance. In the vineyard, the upper part of grapevine (graft or scion) consists of the trunk, canes, and shoots, intended to produce the leaves, tendrils, flowers, and fruits, while the rootstock or lower part produces the root system and serves as support (Fig. 2).

Figure 1. *Vitis vinifera* L. 1, shoot; 2, leaf with 5 main veins; 3, tendril; 4, inflorescence; 5, flower bud (young closed flower); 6, opening of the flower's corolla (dehiscent flower); 7, hermaphrodite flower with both stamens (n=5) and a pistil; 8, longitudinal section of an ovary; 9, cross section of an ovary; 10, stamen; 11, pollen; 12, bunch of grapes; 13, longitudinal section of a berry; 14, cross section of a berry; 15, grape seed; 16 and 17, longitudinal sections of a seed; 18, cross section of a seed (https://pl.wikipedia.org/; modified).

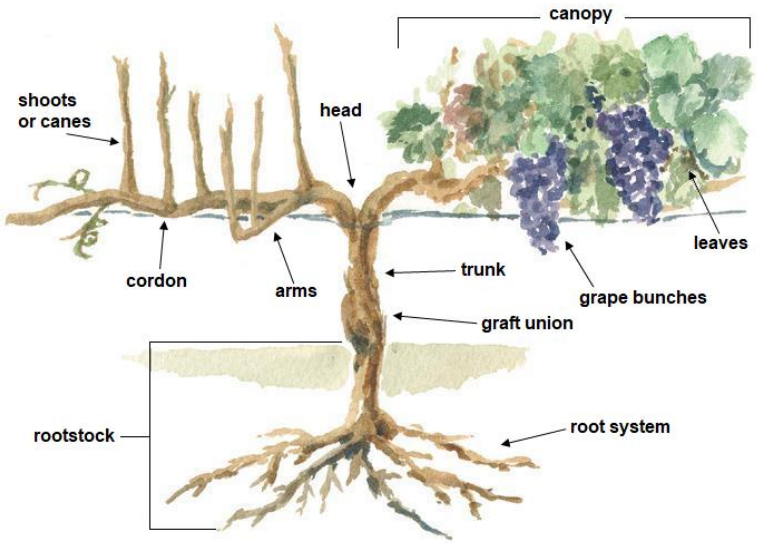

Figure 2. Diagram of a cultivated grapevine (https://www.evineyardapp.com/; modified).

1.3. Phenology and growth cycle

Grapevine is a perennial plant of which the longevity can reach a century. The growth cycle takes place over a year according to phenological stages of different length depending on climate and variety. Phenology is regarded as the first biological indicator of climate change, and described as one of the main factors to be considered for varietal adaptation (Wolkovich et al., 2017). The development of mature, fruiting grapevines follows two distinct cycles: the vegetative cycle, including the reproductive one, and the winter cycle (Jones, 2003) (Fig. 3). In the Northern Hemisphere from November to March, grapevine is in dormancy. When the soil begins to warm, osmotic forces pushes the xylem sap up from the root system, and the appearance of so-called bleeding on the surface of pruning wounds indicates the resumption of plant activity. In spring (between March and April), budburst (fr. *débourrement*) marks the onset of vegetative growth and future reproductive organs. The unfolding leaves and the emerging inflorescences are often reddish in many cultivars as a mechanism of protection from the excess of light through the transient accumulation of anthocyanins, replaced by the time by chlorophylls and carotenoids (Keller, 2015). Leaf growth and shoots elongation take place until July. Between May and June, it is the beginning of flowering with the release of pollen and fertilization, quickly followed by fruit set (fr. *nouaison*): transformation of the flower's ovaries into fruit. In August, veraison (the onset of ripening) occurs and is marked visually by the change of grapes color, due to gradual replacement of the chlorophyll in the skin by anthocyanins (red varieties), and carotenoids (white varieties). The skin and seed tissues also enrich themselves with tannins which are nearly absent in the flesh. During maturation, the composition of the grape undergoes major changes: decrease in the organic acids content (such as malic acid), and strong accumulation of sugars, phenolic and aromatic compounds (Coombe, 1992). The chemical structures of the tannins alter, and their content tends to decline in the flesh, leading to less astringency of berries and resulting wine. The end of the veraison phase of the fruit will be determined by the harvest date, generally around September, but it depends on cultivar, the subjective determination of the optimal fruit maturity, and other factors such as the threat of detrimental weather and diseases. If the berries are not harvested, there may occur an over-ripening characterized by withering of the fruits. Veraison is accompanied by cold hardening (fr. *aoûtement*), i.e., ripening and lignification of herbaceaous shoots which become canes. The shoot growth cycle is completed by leaf senescence, the recycling of foliar nutrients to the permanent woody parts of the vine, and leaf fall (abscission) in November (Dard, 1994).

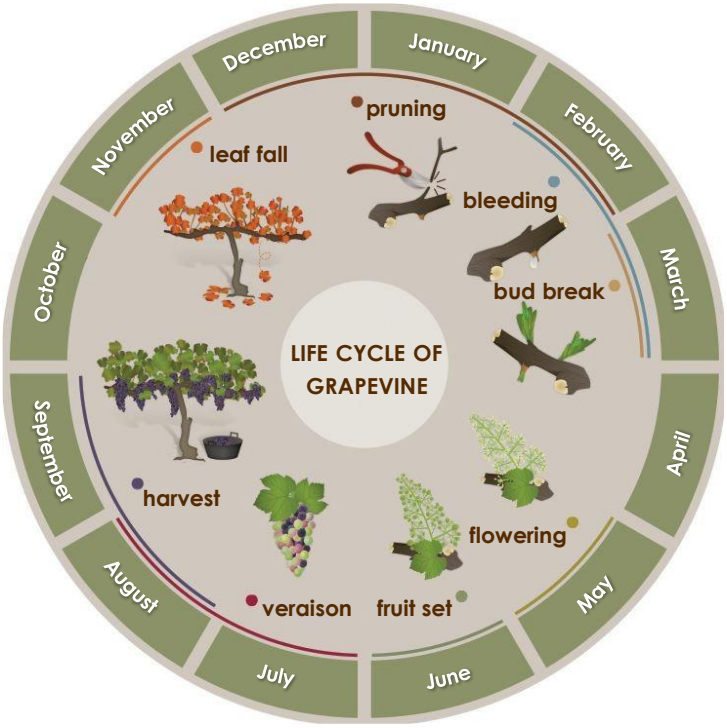

Figure 3. Annual growth cycle of grapevines (based on Dard, 1994).

1.4. History, evolution and spread

Two groups of fossil remains of vines have been distinguished: *Vitis ludwigii*, found in Europe from the Pliocene deposists (2 to 10 milion years B.P.), and *V. teutonica* types, discovered as far back as the Eocene (40 to 55 million years B.P.) (Jackson, 2000). The first form is presumed to be the ancestor of American vines of the *Muscadinia* order, from the second one probably all modern grapevines belonging to the *Vitis* order are derived. Other types of vines from the tertiary period disappeared in the Pleistocene, i.e., quaternary glacial period (~8000 B.C.), which markedly affected the evolution of *Vitis*. Grapevines southward displacement, as well as the existence of favorable sites (refuges) allowed their continued existence (Cattonaro et al., 2013).

Typical characteristics of the progenitor of *Vitis* probably evolved as part of the forest adaptation during the Eocene era. The adaptive features included replacement of some floral clusters into tendrils (homologous structures) at the terminal nodes, in order to improve climbing ability. Nowadays, winegrowers manage vines to promote differentiation of fruitful buds. The vines also developed an extensive, strong and efficient root system, allowing it to compete with dense clusters of trees and other plants. Moreover, birds significantly

contributed to the vine evolution through an effective seed propagation and rapid expansion to distant areas (zoochory). Indeed, small, sweet, aromatic, and caloric berries can be swallowed by these animals without damaging the seeds, allowing the growth of new plants. For this reason, most of wild wines have dark-colored fruits that can be easily perceived by birds (Bosak, 2018).

The first traces of grapevine domestication predate written history and date back more than 7000 years B.C. in Eurasia (Rowley and Ribaut, 2003). Selection of hermaphrodite (self-pollinating) plants which give a more regular crop is considered as the crucial event of grapevine cultivation, as well as size and high sugar content of grapes, from the beginning used for wine making (Fortes and Pais, 2016). The discovery of vases filled with grape seeds in Zagros mountains in Iran, dating from 7500 years B.C. evidences a very old practice of vinification (Michel et al., 1993). However, the earliest representation of such processes, e.g., an amphorae containing white wine dating from the 3rd millennium B.C., was found in Egypt (Huetz de Lemps, 2001). In ancient Greece, grapevine, along with olive and wheat, became one of the three pillars of the Mediterranean triad. Grapevine was established throughout the Mediterranean Basin and in Celtic Gaul at the time of the creation of Marseille by the Greek Phocaeans (600 years B.C.) (Terral et al., 2010). The expansion of viticulture followed the legionaries during the Romans conquests. Systematic extension across Gaul took place from the 5th century B.C. in Languedoc, Aquitaine and Burgundy regions, to reach northwards Champagne, Rhône, Loire, Moselle, and Seine valleys (Terral et al., 2010). The strengthening of the value attached to wine appeared following the deployment of Christianity after the fall of the Roman Empire during the 5th century. Ecclesiastical viticulture allowed to maintain and increase the wine tradition until the 13th century (Unwin, 2005). In the Middle Ages, the spread of Christian civilization was at the origin of the expansion of vitiviniculture in the world, for exemple in Poland, where the oldest archaeological discoveries of such practices date back to the 10th and 11th centuries (Wawro, 2018). After the official discovery of America on October 12, 1492 by the Italian-born navigator Christopher Columbus, the European civilization of wine set out to conquer a new world. Produced and appreciated in North and South America, cultivated vines and wine were gradually gaining new territory: South Africa, Australia, New Zealand, the Pacific Northwest (Johnson, 2016). During the last years vineyards have stabilized at around 7.5 milions of hectares (mha), and a half of them are represented by Spain (13%), China (12%), France (11%), Italy (9%), and Turkey (6%) (OIV, 2020) (Figure 4).

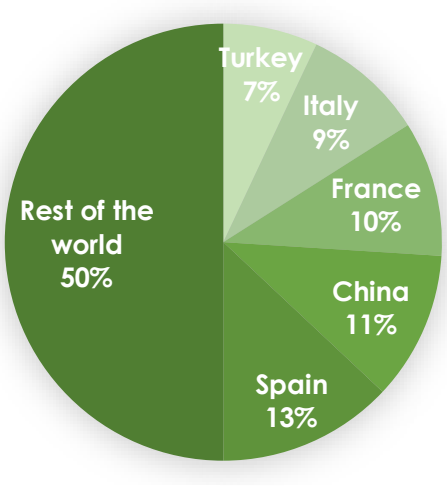

Figure 4. Area under vines worldwide (based on OIV, 2020).

1.5. Importance of the vitiviniculture sector

Grapevine plays today a primary role in agriculture and global economics. For this reason, a better knowledge of its biology such as interaction with environment is essential in order to improve vineyard management, so the yield and quality in the context of environment preservation. The economic importance of the vine can be conceived from the fact that it is the fourth plant whose genome has been sequenced (Jaillon et al., 2007; Velasco et al., 2007) after thale cress (*Arabidopsis thaliana*), rice (*Oryza sativa*), and poplar (*Populus trichocarpa*), in the frame of The International Grape Genomics Program (IGGP) established in 2001. The sequencing was carried out on a Pinot Noir clone, homozygous, PN40024 and revealed the existence of 30 434 genes. The transfer of knowledge acquired from model plants to grapevine allowed to predict many resistance genes and to decipher mechanisms of tolerance to stresses (Velasco et al., 2007; Fortes and Pais, 2016).

Depending on variety, grapevine is mainly cultivated for fruits which can be consumed fresh (table grapes), or be transformed into products with high added value such as cosmetics, grapeseed oil, vinegar, and wine, the last purpose having the greatest economic value. Recently, the recycling of stilbenes-rich by-products from vineyards (roots, wood or canes), has been prosposed one of a solution for plant protection against diseases (Gabaston et al., 2017). In France, vineyards with 0.8 mha cover only 3% of the utilised agricultural areas (UAA), yet, wine and liquors are the 2nd largest contributor to the national commercial balance (the 1st for the food industry), behind aeronautics and ahead of cosmetics with € 12.7 billion (CNIV, 2019). France is ranked as 2nd wine producer in the world after Italy, with a

production amounting to 4.2 billion liters, and the 1st exporter with value of € 9 billion (OIV, 2019). Globally, wine market is predicted to grow with a Compound Annual Growth Rate (CAGR) of 5.8%, during the forecast period (2019 – 2024) (https://www.marketresearchfuture.com/reports/wine-market-1655). The main reason is a gaining demand for wine in developed countries, such as Poland, where in addition, viticulture is dynamically growing. At the moment, there are 294 vineyards in Poland, which cover approximately 470 ha (https://www.kowr.gov.pl/interwencja/wino, consulted on September 2020), distributed particularly in the west and the south regions of the country such as those of Zielona Góra, Wrocław, Kraków, Podkarpacie, Kazimierz Dolny, and lately, even in the north, in Pomorskie, despite more exigent weather conditions. In 2019, about 14 361 hl of wine were produced in Poland.

Besides, vitiviniculture has been a part of human culture for thousands years and constitute societal richness in many countries. From the outset, grapevine and wine have been not only an indicator of civilization, but also a carrier of important religious symbols, and an inseparable element of culture, traditions, food habits, that have contributed, over time, to form a type of society and an art of living. The notoriety of wine regions and enotourism are strongly promoted in France where some vineyards are listed as a UNESCO World Heritage Site, such as the territory of the Jurisdiction of Saint Emilion, near Bordeaux, or the hills, houses and cellars of Champagne, and the climates of the Burgundy (https://whc.unesco.org/).

2. GRAPEVINE DISEASES

Cultivated grapevine (*V. vinifera* L.) has been selected since ancient times for the production of quality wine grapes, thus, like all cultivated plants, its susceptibility to diseases is high. The large number of damaging pests includes fungi and oomycetes, insects, mites, bacteria, viruses, and their vectors, such as nematodes, attacking the aerial and the underground parts of the plant (Table 2).

Table 2. The main diseases of grapevine (non-exhaustive list) (http://ephytia.inra.fr/fr/C/6045/Vigne-Index-des-maladies-ravageurs-vecteurs-et-auxiliaires).

Pest	Disease	Pathogen
Fungi and oomycetes	Anthracnose	*Elsinoë ampelina*
	Black-rot	*Guirgnardia bidwellii*
	Downy mildew	*Plasmopara viticola*
	Excoriosis	*Phomopsis viticola*
	Gray mold	*Botrytis cinerea*
	Powdery mildew	*Erysiphe necator*
	Rot brenner	*Pseudopezicula tracheiphila*
	Esca (Grapevine Leaf Stripe Disease and Apoplexy)	*Phaeomoniella chlamydospora, Fomitiporia punctata, Stereum rirsutum, Eutypa lata, Phaeoacremonium aleophilum*
	BDA (Black Dead Arm)	*Botryosphaeria obtusa, Neofusicoccum parvum, Lasidiplodia theobromae, Clonostachys rosea, Diplodia seriata, Diplodia mutila, Spencermartinsia viticola, Botryosphaeria dothidea, Neofusicoccum luteum*
	Eutypiosis	*Eutypa lata*
Insects	Phylloxera	*Daktulosphaira vitifoliae*
	Cluster moths	eudemis (*Lobesia botrana*), cochylis (*Eupoecilia ambiguella*), pyralid (*Sparganothis pilleriana*)
	Leafhoppers	*Scaphoïdeus titanus* (Ball), *Empoasca vitis, Metcalfa pruinosa*
	Flea beetle	*Altica* sp. (Coleoptera)
Mites	Acariasis	*Calipitrimerus vitis*
	Erinosis	*Eriophyes vitis*
	Yellow mites	*Eotetranychus carpini* (Oudemans)
	Red mites	*Panonychus ulmi* (Koch)
Bacteria/ vector	Pierce's disease	*Xyllela fastidiosa* / *Homalodisca* spp.
	Flavescence dorée	*Phytoplasma* spp. / *Scaphoïdeus titanus* (Ball)
	Bois noir	*Phytoplasma* spp. / *Hyalesthes obsoletus*
	Crown gall	*Agrobacterium vitis*
Viruses/ vector	Court-noué disease	GFLV (Grapevine Fan Leaf Virus) / *Xiphinema index* ArMV (Arabic Mosaïc Virus) / *Xiphinema diversicaudatum*
	Leafroll disease	GRLaV (Grapevine leafroll-associated virus) / *Pseudococcus viburni*

2.1. Overview of major cryptogamic diseases

Phytopathogenic fungi and oomycetes represent the majority of grapevine pests and can be divided into three groups, according to the plant parts attacked: (i) aerial part: downy mildew, powdery mildew, gray mold, black-rot, excoriosis, rot brenner, anthracnose; (ii) grapevine trunk diseases (GTD), developing in the xylem vessels, e.g., eutypiosis, Esca, black dead arm (BDA); (iii) root diseases: rots, e.g., Armillaria root rot. The cryptogamic diseases of grapevine have currently the biggest economic impact since they lead to the most devastating yield losses and affect harvest quality, or can threaten the sustainability of the vitiviniculture heritage through the infestation of long-lasting organs (GTD) (Bois et al., 2017). The most important cryptogamic diseases are presented below, with emphasis on downy mildew, studied in the frame of this thesis.

2.1.1. Downy mildew

Downy mildew is caused by an obligatory parasite, the biotrophic oomycete *Plasmopara viticola* (Berk. & M. A. Curtis) Berl. & De Toni., belonging to kingdom Chromista, family of Peronosporaceae. It is phylogenetically close to diatoms and brown algae due to among others sensitivity to the presence of water in the environment. It was accidentally introduced to Europe, and discovered in Coutras (Gironde, France) in 1878, probably due to the importation of cuttings from American vineyards in order to fight against phylloxera (Gessler et al., 2011). Currently downy mildew is widespread in almost all of the world's vineyards and causes huge yield and quality losses of the crop. In the first half of the 20th century, significant damages in European viticultures due to *P. viticola* were reported, e.g., the loss of 70% of the grape crop and of 20 million hl of wine per year in France (Gessler et al., 2011).

All the herbaceous parts of the vine may be affected. The leaves are most often the first organs of the plant where infection occurs. The first visible symptoms appear on the upper side of the youngest leaves, in the form of yellow, oily spots (Fig. 5). In favorable conditions (warm and humid) on the underside of the oily stains, on the lower side of the leaf, there is a white coating formed by sporangia, where the spores are produced and can spread down at very rapid rates. In adverse conditions, the spots dry up and brown. With the return of moisture, sporulation can appear again, on the edges of the spots. On the older leaves, the symptoms appear as small, irregular, necrotic spots. Severe infections can lead to defoliation. Infected inflorescences and young berries turn brown and dry up.

Figure 5. Symptoms of downy mildew caused by the oomycete *Plasmopara viticola* on grapevine leaves (Wikipedia).

Infection of flowers and young grapes clusters is particularly detrimental due to its direct impact on the size and quality of the future crop and organoleptic properties of wines. Indeed, due to defoliation the chemical composition of the berries changes following the deregulation of metabolism of carbohydrates. A decrease in sugar content in the berries occurs among with an increase in acidity (Jermini et al., 2010). Recent studies highlighted that wine grapes attacked by downy mildew are responsible for odors reminiscent of cooked fruits (prune, cooked peach, fig), and herbs (ivy, geranium) in red wines. The cooked fruit notes were identified as being due to 3-methyl-2,4-nonanedione, γ-nonalactone and γ-decalactone, while the herbaceous and green aromas were identified as (*Z*)-1,5-octadiene-3-one and 3-isobutyl-2-methoxypyrazine (Pons, 2018).

2.1.1.1. Pathogenesis of *Plasmopara viticola*

P. viticola development cycle includes primary and secondary infection steps according to alternate sexual and asexual phases. The coexistence of the latter ensures the preservation and dissemination of this pathogen. The sexual cycle begins in the autumn at the time of leaf senescence and is intended to perpetuate the species during the cold season until the return of the grapevine vegetation cycle. The sexual reproduction occurs in the leaf mesophyll and is preceded by the formation of the sexual organs, arised from the filaments of mycelium, where meiosis takes place and results in the creation of gamets, the only parts of the *P. viticola* life cycle that are haploid (Fig. 6). The oogonia contain the female gametes (oospheres), and an antheridium (or spermatocyst) contains the male gametes. Heterothallic crossing occurs, i.e., the transfer of male gamete to the oogonium through gametangial contact, migration of male nuclei into oogonia and the fertilization of the oospheres. Diploid zygote develops into oospore that germinates and gives rise to vegetative diploid hyphae (Gessler et al., 2011).

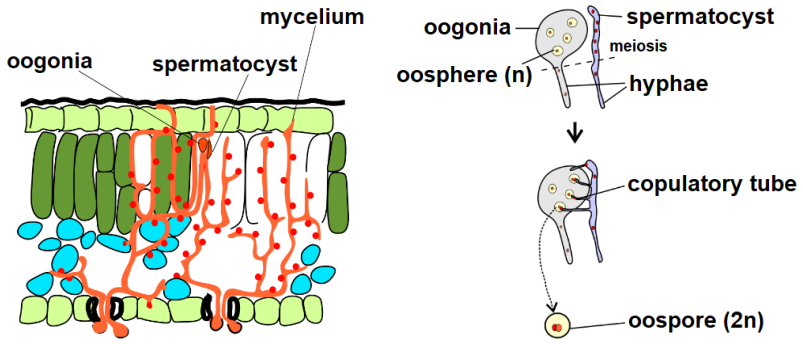

Figure 6. Sexual reproduction of *Plasmopara viticola*: formation of gametocysts inside the grapevine leaf. Formation of a copulatory tube between gametocysts of different types and production of oospores (Wikipedia).

The oospores (also called winter eggs) ensure the survival of *P. viticola* during the winter by staying dormant in the leaves or on the ground. There, the stock of oospores can vary from 100 to 30,000 spores per square meter (Rossi et al., 2009). The presence of a double wall allows these structures to withstand extreme temperature conditions (down to -20 °C) (Vercesi et al., 1999). In addition, even under optimal conditions of temperature (20 °C) and humidity (> 95%), the oospores do not germinate before January (Burruano, 2000).

At the arrival of the spring, after a maturation phase, when the average temperature is higher than 11 °C, and during the cumulus of rains exceeding 10 mm, the oospores germinate (Caffi et al., 2009). They begin to produce dozens of diploid nuclei through mitosis before a germ tube emerges (Burruano, 2000). The nuclei then migrate into the tube and to one or more piriform cavities, called sporocysts (also called macrosporangia, sporangia, or macroconidia). The oospores retain their ability to germinate even for several years (Caffi et al., 2011).

Inside the macrosporangia, the nuclei multiply through mitotic division, so that each of them can release 60 to 200 mononuclear spores, called zoospores, responsible for the primary contaminations of the asexual phase. These are mainly ensured by the splashing of raindrops loaded with zoospores, which reach the canopy and the stomata, the vast majority of which are on the underside of the leaves. The zoospores can move through the water thanks to their two flagella, thus, the infection can endure only in the presence of continuous moisture. Unlike oospores, the lifespan of zoospores is very short – deposited on a surface that dries quickly, do not survive.

Once present on their target tissues, the zoospores lose their flagella and will come to cling to the level of the stomata. Due to infection by *P. viticola*, the grapevine leaves stomata

remain abnormally open, and unresponsive to abscisic acid (ABA). Two *V. vinifera* glycoproteins (a phototropin and a lysophospholipase) were found to be induced by downy mildew, thus, proposed as biomarkers of stomatal deregulation (Guillier et al., 2015). The phenomenon of very rapid encystation (<10 min) is followed by the formation of a germ tube from the zoospores which will grow through the ostiole to the internal tissues (Kiefer et al., 2002). In the substomatic cavity the formation of a substomatic vesicle occurs, and gives rise to the primary hyphae and mycelium.

During the incubation period, i.e., between the infection and the onset of disease symptoms, *P. viticola* colonizes tissues by penetrating the interior of the cell and intercellular spaces. *P. viticola* establishes its biotrophic parasitism by developing haustoria, i.e., structures arised from a part of hyphea, which breach the plasma membrane of the parenchyma cells and allow the nutrition of the pathogen at the expense of the host (Unger et al., 2007). In nonhost tissues, haustoria developpment of *P. viticola* are stopped by cell wall-associated defense responses, which suggest that formation of these structures is a key stage evidencing of host specifity of this pathogen (Díez-Navajas et al., 2008). Moreover, haustoria allow the transfer of pathogenicity effector proteins into the plant cells. Apoplastic or cytoplasmic effectors of *P. viticola* trigger the host cell susceptibility, manipulate physiological and biochemical events, and contribute to counter-defense by inhibiting host enzymes (e.g., proteases and glucanases) accumulating in response to infection (Dodds et al., 2009; Yin, 2017). Analysis of expressed sequence tag (EST) from germinated zoospores has highlighted other factors of *P. viticola* pathogenesis in grapevine, such as a protein similar to fungal laccases probably involved in stilbene detoxification, or an invertase-coding gene allowing the uptake of carbohydrates from the host (Mestre et al., 2012; Luis et al., 2013).

A substomatal body develops and peels off the epidermal tissue from the parenchymal tissue, which gives the first characteristic symptoms of a primary infection in the form of greasy patches, initially translucent and then yellowing due to loss of chlorophyll pigment (from the palisade parenchyma), visible on the adaxial side of the leaf (Allègre et al., 2007). Putative virulence factors present at the oil spot stage have been identified, such as hydrolytic enzymes, protein inhibitors, elicitor-like proteins, members of the RXLR family of effectors, an INL11B-like elicitin, and a protein with Kazal-like protease inhibitor fold (Mestre et al., 2012; Polesani et al., 2010).

The incubation phase can last from 5 to 12 days depending on environmental conditions, i.e., temperature and humidity. The optimum temperature for *P. viticola* varies between 19 and 26 °C. Generally, the primary infection is not large and only a small

percentage of the leaves show disease symptoms. Mycelial growth depends on the variety, temperature, age and tissue anatomy. Lesions have larger diameters in young tissues, where mycelium develops intensively. On older leaves, the spots have a mosaic shape, because the pathogen is not able to breach the lumen of the vascular tissue. In addition, on older leaves, sporulation takes place only on the edges of the spots, not on the entire surface. Primary infections may continue throughout the epidemic, any time between May and August. At this stage, two ways of reproduction open – sexual reproduction (at the time of leaf senescence in the autumn), or asexual one, promoting the spread of the infection on different organs of grapevine (Fig. 7).

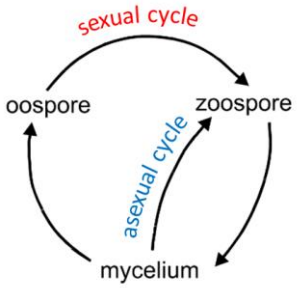

Figure 7. The two development cycles of *Plasmopara viticola*.

The asexual phase begins at the end of the incubation period, when the mycelium emerges from the limbus through the stomata, producing sporangia carried by sporangiophores, which are visible as a whitish felting on the lower surface of the leaves. Sporulation takes place in 7 hours, in the dark (it is inhibited by light). Under favorable conditions, carried away by wind and rain, sporangia are at the origin of secondary infections, i.e., they germinate and release the zoospores which are transferred from primary infection sites to new leaves, shoots, inflorescences and berries. Development cycle of *P. viticola* is resumed in Figure 8.

A comprehension of grapevine resistance mechanisms against downy mildew is required, as a result of a great evolutionary potential of its causal agent, and a need of development of new strategies to control it. Indeed, several isolates of *P. viticola* were reported to be able to break down grapevine resistance of interspecific hybrids, or to develop resistance to phytosanitary means of control (Chen et al., 2007a; Blum et al., 2010; Peressotti et al., 2010; Casagrande et al., 2011). Several studies performed in an attempt to decipher biochemical biomarkers of *V. vinifera* resistance to downy mildew can be cited (Billet et al., 2020; Chitarrini et al., 2017a; Guerreiro et al., 2016).

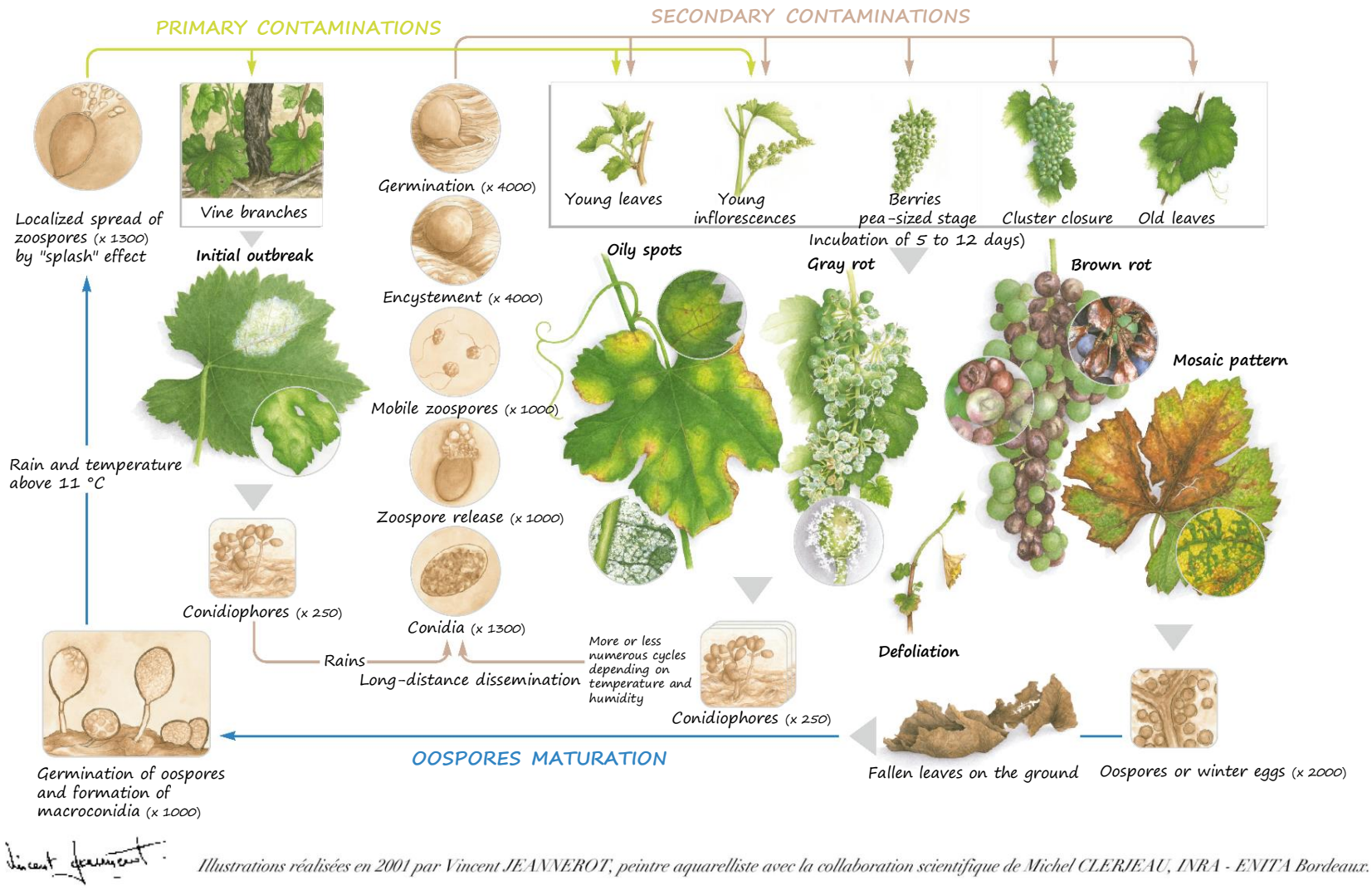

Figure 8. Development cycle of *Plasmopara viticola* on grapevine (image by Vincent Jeannerot, adapted from https://www.bayer-agri.fr/; modified).

2.1.2. Powdery mildew

Powdery mildew is caused by an ascomycete fungus, *Erysiphe necator* (Schwein.) Burrill (syn. *Uncinula necator*), belonging to the Erysiphaceae family. *E. necator* is host-specific, exclusively a parasite of Vitaceae, of which the most susceptible are *Vitis* plants. It originates from North America, where it initially coexisted on the wild forms of *V. labrusca*, which was the starting material for many varieties spread worldwide (Mazurek, 2018). At first, powdery mildew was introduced to Europe in 1845 in France. In 1854, it caused its maximum damage, resulting in the French crop losses up to even 80% (Bioletti, 1907). Today, powdery mildew concerns all the vineyards in the world with different intensities depending on the regions and grape varieties. Optimal conditions for infection and development of the disease occur in the range of 20-27 °C. Powdery mildew can cause significant production losses and alter the quality of grapes and wine.

E. necator is an obligate parasite capable of attacking all the green organs of grapevine. In spring, buds contaminated the previous season develop 'flag shoots' with short internodes, and curled or distorted leaves. The surfaces of the infected organs are covered with a white-gray coating (Fig. 9). Flower infection usually leads to a worsening of fruit formation, whereas direct infestation of grapes before or shortly after flowering contributes to a strong reduction in the value of wine due to a decrease in the content of sugars, phenols, an increase of acidity, and a loss of colour (Gadoury et al., 2007). Furthermore, fruits attacked in the early stages of development most often dry up and fall off. On berries infected later, but even before they reach their maximum size, the mycelium causes destruction of the epidermis walls and inhibits skin growth. Because the pulp continues to grow, the berries burst and split under internal pressure. In addition, these berries are then likely to be colonized by *Botrytis cinerea*, the causal agent of gray mold.

Gadoury et al. (2012) gives a complete review of the strategies of pathogenesis of *E. necator*. Briefly, this pathogen can overwinter as a mycelium inside the dormant buds or in the form of fruiting bodies, sexual forms chasmothecia (formerly cleistothecia), on the surface of infected organs, and in the crevices of the bark (Gadoury et al., 2012). Asexual reproduction is carried out through conidiophores producing a chain of conidia. Their germination terminates in the differentiation of mycelium into specialized infectious structures called appressoria, and formation of haustoria, which sink into epidermal cells of the host to take up nutrients, and to secrete proteins suppressing host defenses (Armijo et al., 2016). For the successful infection, *E. necator* modulates host components. Like for other

obligate biotrophic pathogens, nutrient acquisition from the host is critical for *E. necator* development due to the loss of genes related to metabolic pathways involved in essential and stress-related processes (Jones et al., 2014). Secondary hyphae spread along the infected tissue, eventually forming conidiophores with conidia. In the case of unfavourable environmental or nutritional conditions, the fusion of an antheridia and an oogon leads to the formation of chasmothecia, containing the asci which release the ascospores. Under optimal conditions ascospores germinate and form appressoria. By germinating, ascospores also cause mycelia growing on the herbaceous parts to initiate new infections (Gadoury et al., 2012; Armijo et al., 2016).

Figure 9. Symptoms of powdery mildew on grapevine (left: 'flag shoot'; right: symptoms on the underside of the leaf) (Photos: P. Cartolaro, https://www6.inrae.fr/).

2.1.3. Gray mold

Gray mold is a disease known since Antiquity. It is caused by an ascomycete fungus belonging to the family of Sclerotiniaceae, *Botryotinia fuckeliana*, most often observed in its imperfect form, *Botrytis cinerea* (Pers.). It affects many plant species, although its most notable hosts are grapes, as the term *botrytis* suggests (from Greek *Botrus*, βοτρυς – "bunch of grapes"). *B. cinerea* has a relation to the phenomena both of saprophytism and parasitism since it uses organic debris as a nutritional base to contaminate healthy tissue. Moreover, *B. cinerea* development exemplifies the complex interactions within the so-called disease triangle, i.e., a mutual relationship between the host plant, the environment and the pathogen. In certain regions and under specific environmental (moist nights, foggy mornings, dry days) and edaphic (low nutrient and well-drained soils) conditions, *B. cinerea* can cause noble rot (Fig. 10), which is highly sought-after for the creation of unique, sweet wines referred to as botrytized wines (Magyar, 2011). Thus, in such conditions, this fungus is appreciated in

Bordeaux for the manufacture of Sauternes, or in Tokaj-Hegyalja wine region in Hungary and Slovakia for the production of Tokaj Aszú.

The frequency of infestation lesions on leaves and shoots is relatively low. As a result of infections caused by spores, brown spots of varying size appear on the surface of the leaves, on the underside a grayish network develops. The infection on leaves does not have a greater impact on grapevine growth and yield. However, the present mycelia on these leaves are a potential source of infection for growing flowers, and then berries which acquire their sensitivity to the fungus around veraison and throughout maturation. During this stage, an increase of sugar content in grapes, as well as rainfall that may occur and mechanically damage the skin, are the factors leading to the epidemic development of the disease. Gray mold can lead to a drastic decrease in the berry yield, or to a negative impact of the quality of resulting wine. For exemple, grapes infected by *B. cinerea* leads to the appearance in the wine of octen-1-ol-3 (Ribereau-Gayon, 2006), which confers an odor reminiscent of mushrooms. In addition, *B. cinerea* can secrete laccase enzymes which lead to a risk of oxydasic casse in grapes; thus, a deterioration in the quality of the musts through polyphenols oxidation with a consequent color degradation (browning for red wines, and yellowing for white ones) (Ribereau-Gayon, 2006).

Figure 10. Semillon grapes in the Sauternes (Bordeaux) showing prized noble rot (Wikipedia).

Information about *B. cinerea* life cycle is described in the review of Williamson et al. (2007). Briefly, during the winter *B. cinerea* is preserved in the form of aggregates of hyphae called sclerotia that endure on shoots infected the previous season or plant debris remained on soil surface. In the spring, the intact mycelia or sclerotia grow and produce asexual fruiting bodies (conidiophores) carrying asexual spores (conidia) (Williamson et al., 2007). Conidia are carried by the wind or the rain, settle on other tissues, and the mycelium then develops on the contaminated organ. From the end of flowering until the beginning of veraison, the infection of the fruits remains latent, due to a high resistance of unripe berries conditioned by,

among others, the production of defense-related compounds and a low amount of carbohydrates. Mycelium can resume its growth right at the beginning of the veraison period. The infection begins with the germination of conidia in the presence of water and nutrients received from the surface of the host. The germinating conidium is capable of perforating the plant cuticle thanks to a penetration peg, a part of newly formed infective structure called appressorium (Armijo et al., 2016). The latter secretes cell wall degrading enzymes (CWDE) as pectinases, laccases, cellulases, and hemicellulases, cutinases and lipases, which allow to breach cuticule and outer epithelial wall. Moreover, during infection *B. cinerea* secretes substances involved in pathogenesis, such as oxalic acid, which acidifies the infected region facilitating the activation of CWDE, and favoring hyphal growth. Toxins (e.g., botrydial, botcinolides) are also important virulence factors in host tissues (AbuQamar et al., 2017). All these substances promote sporulation and host cell death. Finally, *B. cinerea* alters plant metabolism by using the carbon sources, thus, deprives the host sugars (hexoses) through activation of hexokinase, and degradation of sucrose (Rui and Hahn, 2007).

3. PLANT IMMUNITY

Plants thrive in a complex environment where they are constantly exposed to various stresses, whether abiotic (e.g., drought, UV-radiation, high and low temperature, salinity, heavy metals), or biotic (attack of living agents, allelopathy) (Redondo-Gómez, 2013). In order to control the harmful factors, plants have established multicomponent signalling that can be shared, or specific depending on the origin of the stimulus (Fujita et al., 2006). Also, the plant has the own capacity to deal with stresses, due to its genetic background leading to a certain level of resistance. Unlike abiotic stresses, which differ only in the intensity and duration of the stimuli, interactions of plant with biotic components seem to be more complex and dynamic due to the continuous so-called coevolutionary arms race between hosts and pathogens. In consequence, the reciprocal adaptive changes in parasites counter-defense and host-defense incessantly occur and ensure the survival of the both.

The plant immune system has fine-tuned to repel the pressure of the multitude of potentially pathogenic agents. Disease development is relatively infrequent thanks to non-host resistance (NHR), referred to as heterologous plant-microbe interaction or basic incompatibility, which provides the immunity of an entire plant species to all genetic variants of a given pathogen species (Nürnberger and Lipka, 2005). NHR is primarily conferred by the

first line of defense, i.e., passive (constitutive) barriers, which prevents the entry of pathogens through anatomical structures, anti-microbial enzymes and metabolites toxic to aggressors (phytoanticipins) (see subsec. 3.2.).

In the case of a successful pathogen invasion by breaching these preformed barriers, active (inducible) defenses are triggered (see subsec. 3.3.). It occurs following the perception of more or less generic, slowly evolving molecular patterns associated with non-pathogenic microorganisms (MAMPs), pathogens (PAMPs), or components released by the plant during tissue damage (DAMPs – damage-associated molecular patterns). These molecular patterns are recognized by plant transmembrane pattern recognition receptors (PRRs). Non-specific MAMPs- or PAMPs-triggered immunity (MTI or PTI, respectively) is then established and helps to ward off most invasions. Local acquired resistance (LAR), activated at the site of infection, is generally a signal for a mobilization throughout the plant and the emergence of the systemic acquired resistance (SAR). Induced defenses are deployed *de novo*, and include among others the formation of defensive molecules (phytoalexins), preceded by early reactions. The protective effect conferred by SAR is phenotypically similar to induced systemic resistance (ISR), triggered by interaction with a non-pathogenic microorganism, most of all rhizobacteria (Jourdan et al., 2008). This most durable form of plant disease resistance is assured by an interplay of both constitutive barriers and inducible reactions.

3.1. Coevolution between plants and pathogens

The robust nature of NHR is indicated by infrequent historical host range shifts. On the other hand, most of pathogens display a high degree of host-specificity, and lead to the development of disease symptoms within a plant species, due to adaptation to the host and establishment of basic compatibility. These pathogens are able to overcome NHR, to annihilate the plant defense mechanisms, and to take advantage of the host cell functions for their own benefit. Pathogen can colonize the plant and reproduce thanks to the expression of essential pathogenicity genes and the production of effector molecules (virulence factors), leading to the effector triggered susceptibility (ETS) of the host (Dodds and Rathjen, 2010). Therefore, some plants have developed the immunity strategy based on the recognition of effectors, hence called effector triggered immunity (ETI), directed against specific breeds, varieties, isolates or biotypes of intruders. We can thus speak of another type of an incompatible interaction, host-resistance (or cultivar-specific resistance), which confers to a cultivar or an accession of a plant species the immunity to some of strains of a pathogen

species (Cheng et al., 2012). ETI is established via the receptors encoded by resistance genes (R), in particular the polymorphic proteins NB-LRR (nucleotide-binding and leucine-rich repeat), acting on specific avirulence gene product (Avr) of the pathogen (gene-for-gene resistance). Following the recognition, a signaling cascade leads to the triggering of an immunity reaction in the plant. ETI is very often accompanied by hypersensitive response (HR), i.e., a form of genetically programmed cell death (Dodds and Rathjen, 2010; Yang et al., 2015). HR is implemented very quickly at the invasion site, leading to the cell necrosis, and thus allowing to block the spread of infection by limiting pathogen access to water and nutrients (Jones and Dangl, 2006).

Contrarily to NHR which is believed to be a multi-gene trait, ETI is generally controlled by single resistance genes, which makes this kind of immunity rather short-lasting in the term of evolution (Gill et al., 2015). Plant resistance conditioned by the presence of R factors can be suppressed by the appearance of subsequent effectors in the pathogen or elimination of Avr factors. Expression of subsequent series of effectors goes hand in hand with recognition by new R factors. During plant-pathogen interaction, the ETS and ETI phases are constantly interwoven. The phenomenon in which after the primary PTI phase a periodic change in the level of plant resistance to a given pathogenic microorganism and cyclic ETS-ETI transitions is observed, has been represented as a zigzag model (Fig. 11) (Jones and Dangl, 2006). A plant resistance linked to an R gene is often overcome after a few years of agricultural use (Collmer et al., 2009).

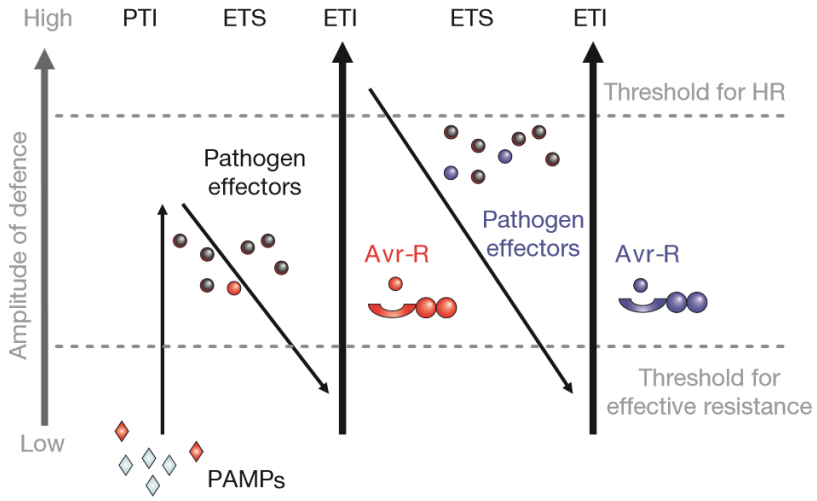

Figure 11. A zigzag model in plant-pathogen coevolving interaction (Jones and Dangl, 2006; modified). Avr, avirulence gene product; ETI, effector triggered immunity; ETS, effector triggered susceptibility; HR, hypersensitive response; PAMPs, pathogen associated molecular patterns; PTI, PAMPs-triggered immunity; R, resistance genes.

3.2. Constitutive defenses

At the cellular level, the cell wall is a major constitutive defense of the plant that provides a physical barrier to both pathogen ingress and mechanical stresses (Malinovsky et al., 2014). In response to different strategies that pathogens have evolved in order to breach this first obstacle (such as the CWDE secretion), plants have rearranged the cell wall structures and three dimensional architectures based on the same main components. The skeletal elements of the cell wall are made of cellulose microfibrils, cross-linked with a heterogenous matrix of hemicelluloses, proteins, and pectins. The latter are abundant in the primary cell walls and are partly replaced by lignins in the secondary mature cell walls, making the cells highly impermeable. Despite the protective effect of the rigid anatomical structure of the cell wall, it also incorporates a wide variety of chemical defenses. In the case of an attack, deposition of reinforcing polymers, in particular callose, as well as phenolic complexes, is one of the induced basal defense strategies. Moreover, the release of degradation fragments of the cell wall, such as oligogalactutonides, can act as elicitors that trigger further defenses mechanisms. Some of the signal molecules, such as nitric oxide (NO) and reactive oxygen species (ROS), are generated in the cell wall (O'Brien et al., 2012).

Essential plant constitutive defense barrier is the dermal tissue, i.e., the outermost tissue of all plant cells of the primary growth and in the herbaceous plant stem (the epidermis) or roots (the rhizodermis). In perennial plants, the dermis of roots and stems undergoes the secondary growth and is replaced by the periderm. Both the epidermis and periderm are saturated with fatty polymeric macromolecules, respectively cutin and suberin, embedded in, or associated with a complex mixture of low-polar lipids called waxes (Kolattukudy, 1980). All these substances form a protective layer on the dermis (outer bark or phellem for stems, and cuticle for the epidermis), and their production can be additionally induced by abiotic or biotic stimuli. They provide the strength and protection to the plant through water loss inhibition, gas exchange regulation, and thermal insulation. Epidermis waxes protect the plant from intense sunlight and wind. Moreover, the dermis can produce on its surface specialized appendages (trichomes, root hairs, thorns), providing both physical and chemical protection. However, the suberized and cutinized tissues can be overcome by pathogens either passively, taking advantage of micro-injuries (viruses and bacteria), or through enzymatic activity of suberinases and cutinases (some fungi and oomycetes) (O'Gorman et al., 2009; Reina-Pinto and Yephremov, 2009; Belbahri et al., 2008).

Mechanical barriers are accompanied by accumulation of preformed secondary metabolites (phytoanticipins) and anti-microbial proteins which represent a chemical form of constitutive defenses. Phytoanticipins belong to one of the three large chemical classes: alkaloids (i), phenolics (ii), and terpenoids (iii), which act on herbivores, pathogens and parasites. In grapevine, examples of phytoanticipins are flavonols which are constituent compounds contributing to the resistance of the leaves to *P. viticola* (Latouche et al., 2013), as well as mono- and sesquiterpenoids that are highly volatile organic compounds (VOCs), contributing to the insect repellent fragrances (War et al., 2019), and triterpenoids of different functions (Szakiel et al., 2011) (see chapter 4).

Plants preformed defensive proteins can efficiently impede a pathogen attack by disrupting cellular ion balance in fungi or bacteria (defensins) (Andersen et al., 2018), or by acting as digestive enzyme inhibitors of vertebrate and invertebrate herbivores (α-amylase inhibitors, lectins, ricin) (Freeman, 2008; Andersen et al., 2018). Some phytopathogenic fungi and bacteria have acquired an ability to obviate the plant chemical defenses through detoxification of phytoanticipins and of antimicrobial proteins (VanEtten et al., 1995; Lee et al., 2010; Mason et al., 2014).

Regardless of the pathogen strategy to subvert plant constitutive defenses, microorganisms that manage to enter cells directly are called necrotrophic agents (e.g., *B. cinerea*), and those which develop tissue penetration systems without degrading cells are called biotrophic agents (e.g., *P. viticola*, *E. necator*). Hemibiotrophs, on the other hand, have a biotrophic character at the start of the infectious cycle, and necrotrophic at the end of the cycle (e.g., *Guignardia bidwellii*). In any case, plants have developed systems for recognizing biotic attacks (Glazebrook et al., 2005).

3.3. Induced defenses

Induced defenses are established in the frame of both non-host MTI/PTI and specific ETI strategies, and require the recognition of elicitors, i.e., substances expressed by pathogens themselves (MAMPs, PAMPs, virulence factors), or plant endogenous signals generated under pathogen pressure (DAMPs), which provoke defense reactions through cell signaling cascade in attacked cells (Henry et al., 2012). The induced reactions are deployed *de novo*, act in a synergistic and coordinated manner both in time and space, and result in the overall expression of resistance (LAR, SAR or ISR) (Henry et al., 2012).

3.3.1. Elicitors and their receptor-mediated recognition

The activation of specific transmembrane plant PRRs is conditioned by the appearance of elicitors of different origin. In the case of ETI, effectors (proteins Avr) are so-called race specific elicitors and recognized by the R proteins of which the most are represented by NB-LRR intracellular protein family, and by extracellular LRR domain membrane proteins (eLRR) (Marone et al., 2013; Zhang and Thomma, 2013). NB-LRR proteins recognize effectors either through direct physical interaction between the receptor and its ligand, or through an indirect interaction mediated by other proteins (DeYoung and Innes, 2006). In turn, MAMPs and PAMPs, involved in MTI and PTI, are generic elicitors of various nature including lipids (e.g., fungal ergosterol); glycolipids (bacterial rhamnolipids, lipopolysaccharides (LPSs) from Gram-negative bacteria); oligosaccharides (fungal chitin); polysaccharides (*β*-glucans of oomycetes *Phytophtora* spp., fungi *Pyricularia oryzae* and brown algae); peptides (elicitins of oomycetes *Phytophtora* spp. and *Pythium* spp., harpins from Gram-negative bacteria); glycopeptides (bacterial peptidoglycans); and proteins (flagellin from Gram-negative bacteria, enzymes such as xylanase from fungi *Trichoderma* spp. or endopolygalacturonase) (Mishra et al., 2012; Henry et al., 2012; Boller and Felix, 2009; Nürnberger and Lipka, 2005). These eliciting components are produced in a constitutive way by microorganism because they are generally essential for its proper functioning but do not necessarily play a role in pathogenicity (PAMPs) or in the establishment of symbiosis (MAMPs) (Jourdan et al., 2008). Molecular patterns of DAMPs, involved in an indirect perception of pathogens, can be oligogalacturonides (OGs) (originated from degraded plant cell wall), cutin monomers (derived from damaged cuticle), or peptides (e.g., systemin in Solanaceae released in response to the wound) (Boller and Felix, 2009). Since the discovery of plant induced resistance through the action of elicitors, their use for agricultural purposes has been tested and proposed for many crops (see chapter 6, subsec. 6.2.4.) (Walters et al., 2007).

MAMPs, PAMPs, and DAMPs recognition occurs through PRRs generally belonging to receptor-like kinases (RLKs) or receptor-like proteins (RLPs). Most of those consist of three distinct domains: an extracellular domain interacting with an elicitor, a transmembrane domain and an intracellular domain, responsible for signal transmission (Marone, 2013; Nürnberger and Lipka 2005). Figure 12 presents the most important elicitors and the corresponding receptors that have been identified in grapevine and/or Arabidopsis (Héloir et al., 2019).

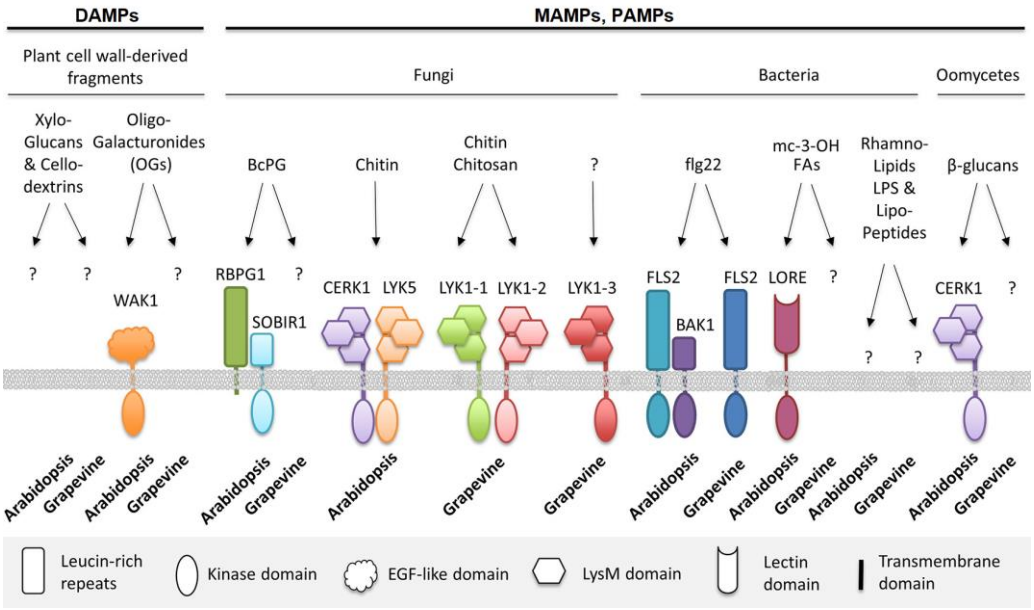

Figure 12. Elicitors recognized by corresponding grapevine and/or Arabidopsis PRRs (Pattern recognition receptors) (Héloir et al., 2019; modified). BAK1, brassinosteroid-insensitive 1-associated receptor kinase 1; BcPG, *Botrytis cinerea* polygalacturonase; CERK1, chitin elicitor receptor kinase 1; DAMPs, Damage-associated molecular patterns; EGF, epidermal growth factor; flg22, N-terminal conserved epitope of 22 amino acids of flagellin perceived by plant receptor; FLS2, flagellin sensing 2; LORE, lipooligosaccharide-specific reduced elicitation; LPS, lipopolysaccharide; LYK, lysin motif-containing receptor-like kinase; MAMPs, Microbe-Associated Molecular Patterns; mc-3-OH-FAs, medium chain 3-hydroxy fatty acids; PAMPs, Pathogen-Associated Molecular Patterns; RBPG1, responsiveness to *Botrytis cinerea* polygalacturonase (BcPG); SOBIR1, suppressor of BIR1; WAK1, wall-associated kinase 1.

3.3.2. Signalling cascade and early events

The mechanisms ensuring signal transduction and further immune responses are found in the two types of immunity (MTI/PTI and ETI). However, in contrast to PTI, ETI is associated with more intense local responses (speed, quantity of compounds produced) and generally terminates with HR (Jones and Dangl, 2006). Elicitor perception results in the activation of an intracellular signalling cascade by causing changes in conformation of receptor, or activation of coupled effectors, such as GTP-binding proteins (G proteins), protein kinases or phosphatases, lipases, and ion channels (Zhao et al., 2005). Down-stream cell responses include phosphorylation and dephosphorylation processes, ion fluxes through the plasma membrane, with the mobilization of diverse signalling molecules, in particular calcium ions (Ca^{2+}), NO, ROS, and other second messengers, such as inositol 1,4,5-trisphosphate (IP_3) and cyclic nucleotides (cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate) (Mishra et al., 2012; Zhao et al., 2005). The action of these multiple components mediates an amplification of the stress-induced signal and leads to

changes in the expression of specific nuclear genes and the initiation of defense reactions, in particular the production of pathogenesis-related (PR) proteins, protein phosphorylation, callose deposits, and phytoalexin biosynthesis. Depending on the type of elicitor and plant species, the early events are more or less combined, differ in kinetics and intensity, and the further (hormonal) signaling molecules take part in parallel or cross-linking pathways, leading to different responses (Fig. 13) (Mishra et al., 2012; Pieterse et al., 2006).

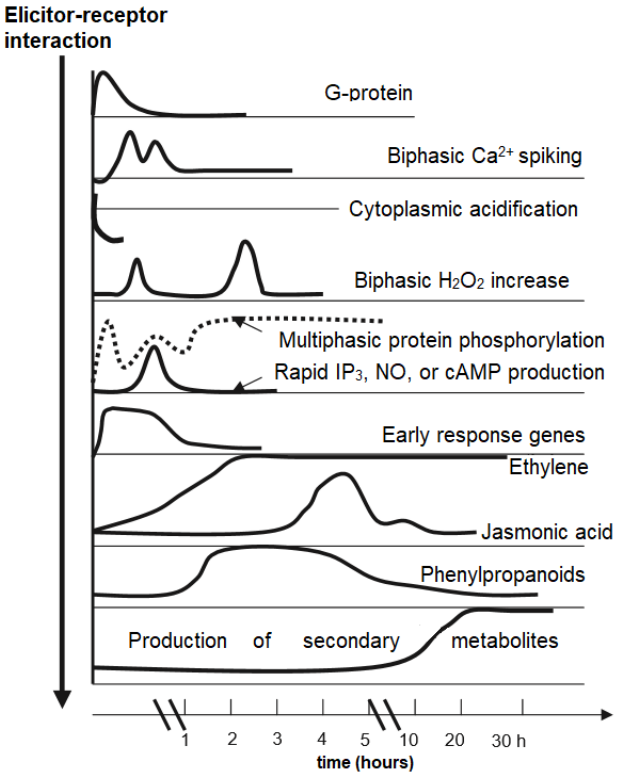

Figure 13. Illustration of successive and parallel elicitor-induced reactions. In particular cases, only some of these events can occur (Zhao et al., 2005; modified). Ca^{2+}, calcium ions; H_2O_2, hydrogen peroxide; IP_3, inositol 1,4,5-trisphosphate; cAMP, cyclic adenosine monophosphate; NO, nitric oxide; G-protein, guanine nucleotide-binding protein.

In grapevine, signalling network induced by elicitation was elucidated on cell suspensions treated with endopolygalacturonase 1 from *B. cinerea* (BcPG1) (Fig. 14) (Poinssot et al., 2003; Vandelle et al., 2006). In this study, the early events preceding the activation of defense genes were similar to those described in tobacco cells in response to cryptogein, but seemed to be differently regulated. The key early events resulting from signal transduction and their implication on further defense reactions are described in the following subsections.

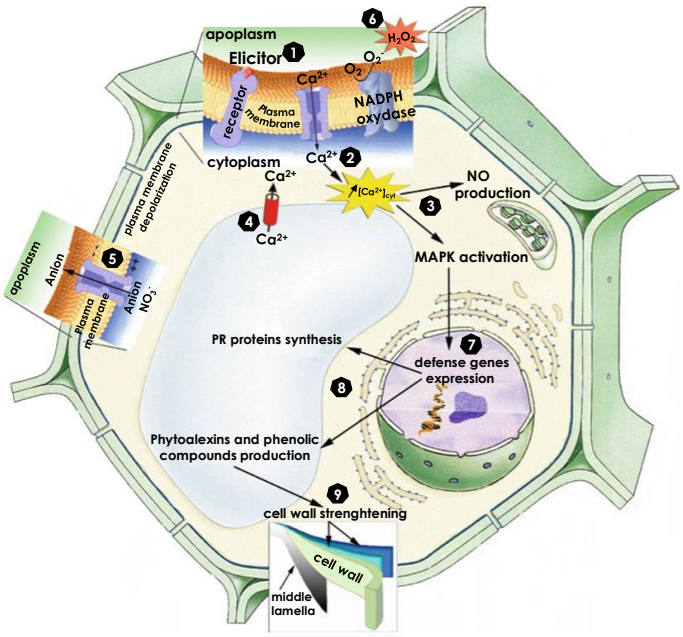

Figure 14. Signalling and cascade of events induced in grapevine by endopolygalacturonase 1 (BcPG1) from *Botrytis cinerea* (Adrian et al., 2012; modified). 1, perception of the elicitor; 2, calcium (Ca^{2+}) influx; 3, activation of protein kinases and nitric oxide (NO) production due to an increase of cytoplasmic calcium ($[Ca^{2+}]_{cyt}$) concentration; 4, calcium (Ca^{2+}) efflux from intracellular pools induced by nitric oxide (NO); 5, anion efflux; 6, reactive oxygen species (ROS) production (O_2^-, superoxide anion; H_2O_2, hydrogen peroxide); 7, expression of defense genes; 8, production of pathogenesis-related (PR) proteins and phytoalexins (e.g., phenolic compounds); 9, cell wall strengthening.

3.3.2.1. Activation of protein kinases

Elicitor-induced signal transduction, throughout downstream early events, but also further defense reactions, is accompanied by protein phosphorylation and dephosphorylation processes via the activity of respectively protein kinases (PK) and protein phosphatases (PP). The central and most studied are calcium-dependent PKs (CDPKs) and mitogen-activated PKs (MAPKs) (Zhao et al., 2005; Garcia-Brugger et al., 2006; Mishra et al., 2012), which are believed to regulate the signal transduction positively, while type 1 and/or 2A PP (PP1 and PP2A) are likely to regulate it negatively and prevent a constitutive activation under normal conditions (Lecourieux-Ouaked et al., 2000). Transient changes in protein phosphorylation allow the cross-talk of various stress-related signalling pathways by regulating the protein or enzyme activity, and the consequent cellular reactions. Upon elicitor recognition, plasma membrane proteins are reversibly phosphorylated which mobilizes ions fluxes and generates subsequent signalling components, such as ROS, ensuring the continuation and the amplification of further reactions (Zhao et al., 2005; Garcia-Brugger et al., 2006). Phosphorylation and dephosphorylation processes are then required in ABA, ET, and JA

biosynthesis and/or signalling, the activation of defense-related genes, through the regulation of transcription factors, secondary metabolites production, the stomatal closure, and HR (Meng and Zhang, 2013). In grapevine, the activation of MAPK cascade was demonstrated during the establishment of its resistance against downy mildew, for example following foliar treatment of casein hydrosylates (Lachhab et al., 2014), or laminarin (Gauthier et al., 2014).

3.3.2.2. Modifications of ion fluxes and oxygen burst

The direct consequences of an elicitor recognition by the plant cell are rapid reactions occurring mainly on the periphery of the cell and not requiring gene expression. These are changes in permeability leading to ion exchanges between the intra and extracellular media, and an oxygen burst, i.e., a succession of oxygen reduction reactions leading to the ROS formation, which occur within the first minutes to a few hours upon stimuli perception, and in long-term lead to a whole transcriptome reprogramming (Garcia-Brugger et al., 2006; Zhao et al., 2005).

Modifications of ion fluxes across the plasma membrane are induced within 5 minutes after the recognition of the signal, and include efflux of potassium (K^+), chloride ions (Cl^-) and nitrate ions (NO_3^-) to the outside of the cell and a concomitant influx of hydrogen (H^+) and calcium ions (Ca^{2+}) into the interior of the cell (Garcia-Brugger et al., 2006; Zhao et al., 2005). These ion flows cause a difference in electrochemical potential on either side of the membrane, then leading to its depolarization that can act upstream of cell death (Gauthier et al., 2014). Ca^{2+} ions are particularly determining for the continuation of the signaling cascade, because this ion is necessary for an array of downstream responses (Lecourieux et al., 2002). The elevated cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) concentration occurs due to an uptake from the extracellular medium and/or by mobilization of organelles, and following to accumulation of cAMP or cGMP (Zhao et al., 2005). The accumulated Ca^{2+} ions become themselves second messengers that initiate various processes directly or via Ca^{2+} sensors, e.g., calmodulin which is activated by Ca^{2+} binding (Verma et al., 2016). Ca^{2+} and activated calmodulin further activate Ca^{2+}/calmodulin-dependent PK and PP, membrane-bound enzymes, or transcriptional factors (Zhao et al., 2005; Lecourieux et al., 2002). The $[Ca^{2+}]_{cyt}$ spiking induces extracellular alkalinization (by ATPase inhibition), and corresponding intracellular acidification, which mediates other cellular responses such as biosynthesis of plant secondary metabolites. It has been shown, that artificial acidification of the cytoplasm in tobacco cell culture induced the transcription of phenylalanine ammonia-lyase (PAL) and 3-hydroxy-3-methylglutaryl-CoA

reductase (HMGR), the key genes of respectively phenylpropanoid and isoprenoid pathways (Lapous et al., 1998). Nuclear Ca^{2+} among with the MAPKs trigger the expression of defense-related genes (Jourdan et al., 2008). Ca^{2+} ions regulate also phospholipases responsible for biosynthesis of jasmonates or other messengers, such as IP_3, phosphatidic acid (PA) and diacylglycerol (DAG) (Mishra et al., 2012). The $[Ca^{2+}]_{cyt}$ spiking is a variable parameter depending on the type of infection and thus, the type of elicitor. In grapevine, an increase of $[Ca^{2+}]_{cyt}$ was shown as a result of BcPG1 from *B. cinerea*, oligogalacturonides or methyl jasmonate (MeJA) elicitation (Vandelle et al., 2006; Faurie et al., 2009; Poinssot et al., 2003). Furthermore, Ca^{2+} initiates the production of ROS through the activation of NAD(P)H oxidase. In grapevine, following the elicitation with BcPG1, $[Ca^{2+}]_{cyt}$ spiking activated NO production which induced a leakage of Ca^{2+} ions from intracellular pools, activating the ROS production, the expression of defense-related genes, and the production of phytoalexins (Adrian et al., 2012).

In many plant systems, including grapevine, biphasic ROS generation is observed: the first occurs at about 10-30 min and the second at 1-3 h after fungal elicitation (Zhao et al. 2005; Aziz et al., 2003; Poinssot et al., 2003). Superoxide anion (O_2^-) can be generated by the activity of NADPH oxidase in the plasma membrane or by the ribonucleotide nucleases and xanthine oxidases (Gadjev et al., 2008). Apoplastic, mitochondrial or chloroplastic peroxidases are also involved in the synthesis of O_2^-. O_2^- is rapidly disproportionated to hydrogen peroxide (H_2O_2) by superoxide dismutase or in by the non-enzymatic route (Sharma et al., 2012). In order to reduce the oxidative activity in cells, H_2O_2 can then be transformed into H_2O by three enzymes: catalase (CAT), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) (Sharma et al., 2012). Protective effect against oxidative stress is also ensured by antioxidant enzymes including ascorbate-peroxidases, glutathione-peroxidases, catalases and non-enzymatic antioxidants such as ascorbic acid, glutathione and riboflavin (Delledone et al., 1998). On grapevine plants cultivated under controlled conditions, sulfated laminarin (PS3) potentiated the production of H_2O_2 at the level of infection sites (Dubreuil-Maurizi et al., 2010; Trouvelot et al., 2008).

ROS perform several roles in defenses: a direct antimicrobial effect, a strengthening of the cell walls by promoting lignification (Hückelhoven, 2007) and a role of secondary messengers sometimes leading to HR and cell death, as well as to defensive gene and phytoalexin biosynthesis induction, depending on plant species (Torres et al., 2006; Zhao et al., 2005; Boller and Felix, 2009). For example, sesquiterpene cyclases and PAL were reported to be induced directly by ROS (Xing et al., 2013; Maldonado-Bonilla et al., 2008).

Otherwise, H_2O_2 can mediate non-enzymatic or enzymatic lipid peroxidation that activate the octadecanoid pathway leading to biosynthesis of JA and derivatives, which play a role in the stimulation of secondary metabolites (Zhao et al., 2005).

3.3.3. Secondary signals of a hormonal nature

Secondary signals of hormonal nature provide signal amplification and specificity of further reactions. The major phytohormones that besides being involved in the plant growth and development, play a role in defense, are salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), which perform major function in responses to biotic stress, but also to some environmental or mechanical stimuli (Bari and Jones, 2009; Janda et al., 2007). The production of JA, SA, ET varies greatly in timing and according to the type of pathogen (Koornneef et al., 2008). In addition to their role in the local response, these three signals contribute to intercellular transmission, which allows the signal to be spread toward uninfected tissues of the plant, i.e., the induction of systemic immunity. It has been presumed that SAR is acquired in distant tissues as a result of local infection of the pathogen and is mediated by SA, while JA and ET synergistically act as signaling molecules of ISR, acquired from roots to above-ground parts of the plant (Weber et al., 2016). However, ongoing researches suggest a redefinition of this paradigm. For example, it was noted that jasmonates regulate root-stem interaction in response to herbivorous attacks and foliar application of these phytohormones reduced formation of galls of nematodes in the roots of tomato, oats, rice, soy and *Arabidopsis thaliana* (Machado et al., 2018). In order to counteract the host phytohormones production, pathogens can interfere these modifications or produce themselves plant hormones as a component of the invading strategy (Robert-Seilaniantz et al., 2007).

3.3.3.1. Ethylene

Under normal conditions, ET plays an important role in plant growth and development, including seed germination, root hair development, root nodulation, fruit ripening, senescence, leaf abscission (Kwak and Lee, 1997; Wang et al., 2002). ET production appears upon either pathogen attack or abiotic stress, in particular exposure to ozone and wounding (Zhao et al., 2005). Increased ET amount in the plant cell can be considered as a marker of one of the earliest elicitor-induced response of phytohormonal nature, occurring

even within 10 min after the signal perception (Boller and Felix, 2009; Ecker and Davis, 1987).

ET is a gaseous molecule biosynthesised in two step reaction from *S*-adenosylmethionine (*S*-AdoMet) via 1-aminocyclopropane-1-carboxylic acid (ACC), through the activity of ACC synthase (ACS), which transforms *S*-AdoMet into ACC, and ACC oxidase into ET (Wang et al., 2002). ACS is the rate-limiting enzyme and the major target of regulation in ET biosynthesis, and its induced activity appeared to depend on, among others, the $[Ca^{2+}]_{cyt}$ spiking (Wang et al., 2002; Kwak and Lee, 1997; Cho and Yoo, 2009). ET constitutes the core signalling molecule in numerous stress-related processes, such as the ROS generation (Zhang et al., 2016), and mediates the responses on the CDPK and MAPK signalling pathways (Wang et al., 2002; Ludwig et al., 2005).

The function of ET in resistance to diseases is ambivalent – deficiency of this hormone can either enhance defense responses, or in some cases, increase a susceptibility of the plant to a pathogen and promote the pathogenesis (Wang et al., 2002). In cell cultures, the critical factor for the effect of ET was the dose – at high concentration ET inhibited the production of secondary metabolites, and promoted it at low concentration (Mishra et al., 2012). ET is produced abundantly during HR and is a strong inducer of certain PR genes (Carr et al., 2010). Anyway, only several plant species require ET as a signal for a production of phytoalexins (Mishra et al., 2012). In grapes, ET treatment led to simultaneous accumulation of flavonoids and anthocyanins and up-regulation of genes regulating their biosynthesis (El-Kereamy et al., 2003). Besides, ET is necessary for anthocyanins accumulation in berries during ripening phase (Chervin et al., 2004). Grapevine foliar cuttings sprayed with ethylene-releasing ethephon (2-chloroethylphosphonic acid) triggered the protection against *E. necator* by 70%, as a consequence of induction of several defense-related reactions, such as accumulation of certain PR-proteins and stilbenes (Belhadj et al., 2008a).

The activity of ET is tightly interconnected with that of other phytohormones, in particular of JA (Zhao et al., 2005). The cross-talk JA/ET is implicated in numerous processes of plant development and defense responses. However, ET and MeJA can act antagonistically stimulating different sets of stress-related genes (Zhao et al., 2005).

3.3.3.2. Jasmonic acid

In healthy tissues, signaling mediated via JA along with other jasmonates (JAs) regulates an array of endogenous processes related to plant growth and development. These molecules act through either inhibition of cell growth and differentiation, e.g., of root and seedling, or stimulation of final stages of plant ontogenesis, such as leaf aging. JAs also contribute to the regulation of generative development, i.e., stamen development, fruit ripening, and flowering (Huang et al., 2017). Stress factors that activate JA signaling pathway, generally with the cooperation of ET, include herbivore attack, pathogen infection, wounding, as well as extreme environmental conditions, such as freezing, drought, and exposure to ozone, UV radiation, or high temperature (Huang et al., 2017; Browse, 2009).

JAs belong to the family of oxylipins, structurally deriving from the cyclic ketone cyclopentanone. JAs are biosynthesised in the octadecanoid pathway, as a result of oxidation of α-linolenic acid (α-LeA), a major polyunsaturated fatty acid (PUFA) in plant membranes (Fig. 15) (Turner et al., 2002; Pieterse et al., 2012). α-LeA is generated from galactolipids and released from chloroplast membrane through the activity of phospholipase 1 (PLA_1) (Ryu, 2004; Wasternack et Hause, 2013), which is induced by a complex of early events (mainly ROS generation), that follow the elicitor recognition (Ryu, 2004). α-LeA is then oxygenated by 13-lipoxygenase (13-LOX). The formed peroxide, 13-hydroperoxylinolenic acid (13-HPOT), is cyclised through the activity of allene oxide synthase (AOS) into 12, 13(*S*)-epoxylinolenic acid which is cyclised by allene oxide cyclase (AOC) into (9*S*, 13*S*)-12-oxo-*cis*-10,15-phytodienoic acid (OPDA). Recently, a protein called JASSY was characterized as responsible for the export of OPDA from chloroplast to peroxisome, where further modifications occur (Wasternack and Hause, 2019). These are catalyzed by oxophytodienoate redutase 3 (OPR3) (Dave and Graham, 2012) and a cycle of three β-oxidations leading to the formation of the predominant, active isomer of JA, (3*R*, 7*S*)-JA (Browse, 2009). JA undergoes various enzymatic transformations which generate many derivatives of different biological activities. For example, methyl esterification of JA by JA-methyl transferase (JMT) leads to the production of methyl jasmonate (MeJA), which mediates defense responses and is responsible for interplant communication (Seo et al., 2001). The core signaling molecule of JA pathway is (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile), a product of conjugation catalyzed by jasmonate-resistant 1 (JAR1) (Browse, 2009). JA-Ile binds to JASMONATE ZIM DOMAIN (JAZ) proteins which are targeted by F-box protein CORONATINE INSENSITIVE1 (COI1) of Skp1/Cullin/F-box E3 ubiquitin ligase complex (SCF^{COI1}) for

degradation by the ubiquitin/26S proteasome pathway (Pauwels and Goossens, 2011). The released transcription factors (MYCs) induce the expression of defense-related genes, ensuring the activation of downstream responses (Pauwels and Goossens, 2011). The most of studies elucidating JA signaling were conducted on Arabidopsis cultures, but the key components were also characterized in other species, such as grapevine (Pieterse et al., 2012; Figueiredo et al., 2015).

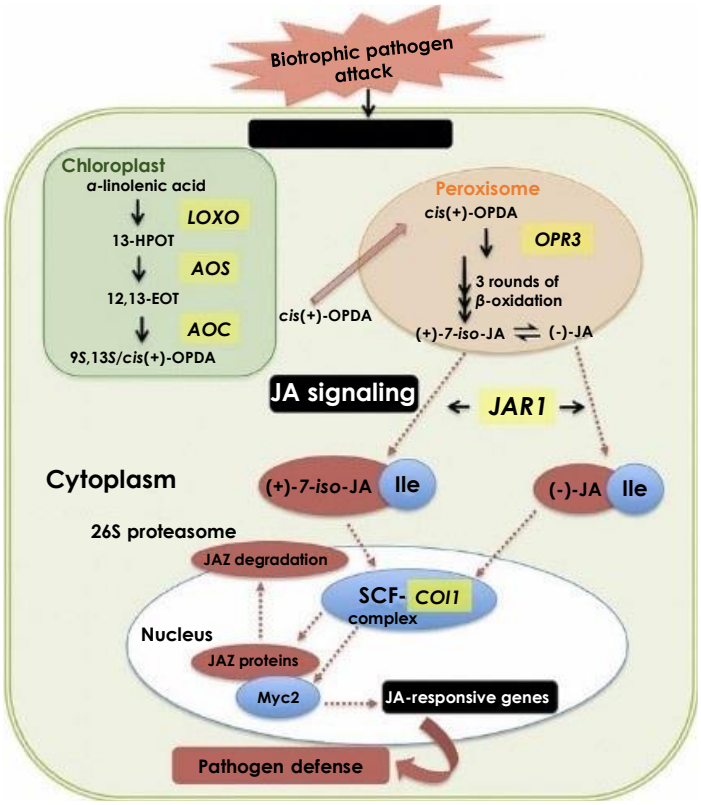

Figure 15. Jasmonic acid (JA) biosynthesis, early steps of its activation and signalling pathways upon biotrophic fungi attack in grapevine (Figueiredo et al., 2015; modified). 13-HPOT (13*S*-hydroperoxy-(9*Z*,11*E*,15)-octadecatrienoic acid); AOC, allene oxide cyclase; AOS, allene oxide synthase; COI, CORONATINE INSENSITIVE1; JA-Ile, (+)-7-*iso*-jasmonoyl-L-isoleucine; JAR1, jasmonate-resistant 1; JAZ, JASMONATE ZIM DOMAIN proteins; LOXO, 13- lipoxygenase (LOX) enzyme; MYC2, MYC2 transcription factor; OPDA, (9*S*, 13*S*)-12-oxo-*cis*-10,15-phytodienoic acid; OPR3, oxophytodienoate redutase 3; SCF^{COI1} complex, F-box protein CORONATINE INSENSITIVE1 (COI1) of Skp1/Cullin/F-box E3 ubiquitin ligase complex.

JA-responsive genes include those encoding PR proteins (and more particularly PR3, PR4, and PR12) (Ali et al., 2018) and enzymes implicated in the biosynthesis of a wide variety of secondary metabolites, including terpenoids, alkaloids, and phenylpropanoids (Browse, 2009; Zhao et al., 2005). Moreover, it seems that the induced biosynthesis of some compounds is specifically mediated by JA. For example, in liquorice (*Glycyrrhiza glabra*) cell culture, soyasaponin biosynthesis along with the expression of *β*-amyrin synthase, squalene synthase, and UDP-glucuronic acid: soyasapogenol B glucuronosyltransferase were

up-regulated upon treatment with MeJA, but down-regulated by yeast elicitor. In turn, both elicitors stimulated 5-deoxyflavonoid biosynthesis and the genes encoding polyketide reductase enzymes (Hayashi et al., 2003). In different experimental models of grapevine, numerous studies have demonstrated polyphenols and PR-proteins accumulation, with enhanced resistance against pathogens, following an exogenous application of MeJA (Belhadj et al., 2006, 2008b; Faurie et al., 2009; Ruiz-García et al., 2012; Larronde et al., 2003; Repka et al., 2004; Lijavetzky et al., 2008; Vezzulli et al., 2007).

The spread of JA-mediated immune signals to other tissues was well described in tomato leaves upon wounding stress (Narváez-Vásquez et al. 1999, Hause et al. 2003, Wasternack et al., 2007). The key transmitters in this process are systemins, i.e., 18-amino acid peptides which induce proteinase inhibitors (PINs) responsible for the plant resistance against herbivore attack (Hause et al., 2003).

In below-ground part of plants, JAs along with ET are critical for beneficial microbe-root interactions. Resistance of roots as well as of above-ground tissues is established through ISR by non-pathogenic plant growth promoting rhizobacteria or fungi (PGPR and PGPF, respectively), or compounds derived from them, recognized by the plant as MAMPs (Basso and Veneault-Fourrey, 2020; Pieterse et al., 2014). In grapevine, ISR conferred by *Pseudomonas* spp., *Bacillus subtilis*, and *Pantoea agglomerans* bacteria was shown to be effective against *B. cinerea* (Aziz et al., 2016; Verhagen et al., 2010; Gruau et al., 2015). The effect of ISR consists on priming, i.e., enhancement of responsiveness of the plant to a subsequent pathogen attack. ISR does not involve direct transcriptomic reprogramming, but leads to an increase in sensitivity of the cells to JA and ET and accelerated expression of genes induced by these hormones, in particular PR-genes (phytoalexins are characteristic rather for local responses) (Pieterse et al., 2009). ISR is regulated by redox-sensitive transcriptional regulator, NPR1 (Non-expressor of PR Gene 1), which is also implicated in LAR and SAR, thus consists as a mediator of JA/ET-SA cross-talk (Pieterse et al., 2009). The role of NPR1 in JA signalling is associated to a cytosolic function, while in SA signalling it is related to a function in the nucleus (Romera et al., 2019).

3.3.3.3. Salicylic acid

SA regulates a wide variety of physiological events occurring in plants throughout their whole lifecycle (Rivas-San Vicente and Plasencia, 2011). In coordination with other phytohormones, SA is required for both vegetative growth and generative development,

including seed germination, root system and leaf growth, flowering, and senescence. Moreover, SA is implicated in important biochemical processes, such as photosynthesis (by, among others, co-regulating stomatal closure), and cellular respiration, as well as thermogenesis in thermogenic plants (Janda et al., 2007; Vlot et al., 2009; Popova et al., 1997). In the interaction with external factors, SA has been recognized as regulatory signal in responses to abiotic stress, mainly UVs radiation, ozone, drought, salinity, chilling, heat, and heavy metals (Janda et al., 2007). In biotic stress, SA is responsible for local and systemic signalling leading to the expression of the plant defenses (Vlot et al., 2009; Verma et al., 2016; Pieterse et al. 2012).

SA (2-hydroxy benzoic acid) belongs to phenolic acids, and contains one hydroxyl group and one carboxyl group attached to the benzene ring in ortho-substitution. It is biosynthesised from chorismate, originating from shikimate pathway, via two possible distinct routes: the phenylpropanoid, or the isochorismate (ICS) pathway, both initiated in chloroplast, and finalized in cytosol (Fig. 16) (Lefevere et al., 2020). Depending on plant species, these two pathways can equally contribute to SA biosynthesis, or one of them can prevail. In the ICS pathway, SA can be synthesised directly from ICS, resulting from the activity of IC synthase (ICS) on chorismate. Recently, two other enzymes responsible for the alternative SA biosynthesis were discovered and named after the phenotype of knock-out plants – avrPphB SUSCEPTIBLE3 (PBS3) enzyme, responsible for the formation of a conjugate isochorismate-9-glutamate, which can spontaneously decompose into SA, or through the activity of ENHANCED PSEUDOMONAS SUSCEPTIBILITY 1 (EPS1) acyltransferase (Torrens-Spence et al., 2019). ICS is likely to be transported from chloroplast to cytosol by the ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5) protein (Lefevere et al., 2020).

In the phenylpropanoid pathway, chloroplastic and/or cytosolic chorismate mutase (CM), transforms chorismate into prephenate, which is conversed to *trans*-cinnamic acid by phenylalanine ammonia-lyase (PAL), the key branch point in the biosynthesis of other compounds of defensive properties, i.e., polyphenols and phenolic acids. Two last steps in PAL pathway for SA biosynthesis are catalysed by abnormal inflorescence meristem1 (AIM1) enzyme and probably by benzoic acid-2-hydroxylase (BA2H), which respectively transforms *trans*-cinnamic into benzoic acid, and this latter into SA (Lefevere et al., 2020).

SA can be subjected to various modifications, such as conjugation of amino acids, glycosylation, methylation, or hydroxylation of the aromatic ring, leading to the formation of its derivatives which active or inactive SA-signaling (Pandey, 2017; Dempsey et al., 2011). The volatile molecule methyl salicylate (MeSA), formed by the activity of carboxyl

methyltransferase plays an essential role in the establishment of SAR in the plant-insect interaction (Rivas-San Vicente and Plasencia, 2011; Lefevere et al., 2020). Recently, MeSA glucoside formed from SA by a glycosyltransferase, was shown to negatively regulate salicylates (SAs) homeostasis and SAR (Chen et al., 2019). Another mobile metabolite inducing SAR is lately discovered *N*-hydroxy-pipecolic acid (Chen et al., 2018).

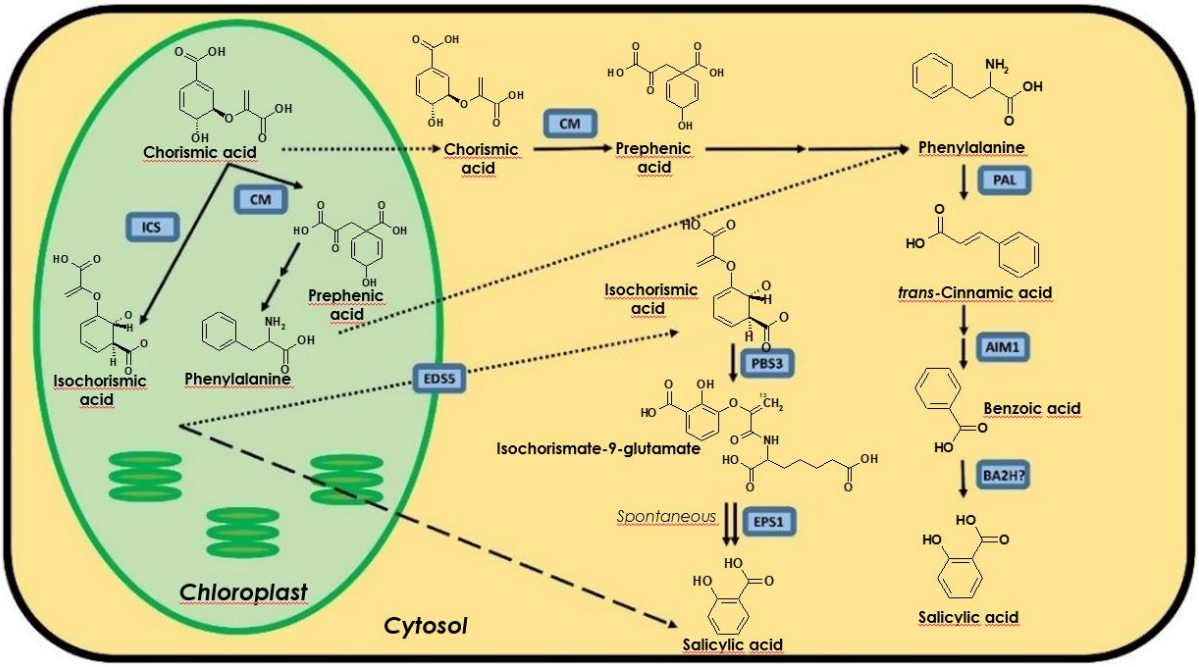

Figure 16. Possible pathways of salicylic acid (SA) biosynthesis in plants (Lefevere et al., 2020; modified). ICS, isochorismate synthase; CM, chorismate mutase; PAL, phenylalanine ammonia-lyase; AIM1, abnormal inflorescence meristem1; BA2H, benzoic acid 2-hydroxylase; EDS5, ENHANCED DISEASE SUSCEPTIBILITY 5; PBS3, avrPphB SUSCEPTIBLE3; EPS1, ENHANCED PSEUDOMONAS SUSCEPTIBILITY 1.

In local responses, elicitor-induced SA accumulation, along with ROS, NO, and MAPKs cascade, triggers cell death and the development of HR (Vlot et al., 2009; Alvarez, 2000). The most specific genes induced following SA-signaling are those encoding PR-proteins, of which the certain are commonly used as defense markers for the SA-dependent pathways (PR1 and PR5) (Ali et al., 2018; Kombrink and Somssich, 1997). SA also regulates the expression of genes responsible for the oxidative burst (Clemente-Moreno et al., 2012; Pandey, 2017) and the biosynthesis of phytoalexins (Pandey, 2017). These latter, however, are induced rather only in local responses (Pandey, 2017). The expression of defense responses is regulated both in LAR and SAR by NPR1 protein, which interacts with transcription factors (e.g., the family of WRKY), leading to the induction of SA-responsive genes related to defense (Pieterse and Van Loon, 2004). Following tobacco mosaic virus (TMV) infection,

SAR establishment was possible due to changes in cell redox status and the activation of SA-binding protein 2 (SABP2) (Pokotylo et al., 2019), which allows the conversion of biologically inactive MeSA, a phloem-mobile signal, to SA, thus, the expression of defenses in distant tissues (Vlot et al., 2009; Romera et al., 2019). As in the case of ISR, SAR is associated with priming (Conrath et al., 2002). SAR appears relatively late (several hours to a few days) and gives the plant resistance to a large number of microorganisms which may persist for several weeks (Ryals et al., 1996; Delaney, 1997).

Exogenous application of SA or its functional synthetic analogues, in particular 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole-*S*-methylester (BTH), induced local and/or systemic expression of various defense reactions and conferred the resistance to pathogens in many plants, including grapevine (Perazzolli et al., 2008; Liu et al., 2016; Bellée et al., 2018; Lawton et al., 1996). Interestingly, in cultures of some plants species, it was shown that SA or BTH, dependently on concentration, seem to either prime the induction of some defense genes, or directly stimulate another set of genes providing resistance (Conrath et al., 2006).

3.3.3.4. Cross-talk between the different phytohormonal pathways

Induction of defense reactions in plant challenged by an environmental cue can entail the so-called fitness cost, i.e., the allocation of energy in the establishment of resistance (Heil, 2002). Thus, the plant needs to fine-tune mechanisms to generate a suitable response and minimize the energy costs (Pieterse et al., 2009; Thomma et al., 2001). One of the strategies employed can be priming, when the costly defense responses are expressed only in case of actual need (Conrath et al., 2002). However, the fundamental role in optimization of defenses and their integration to physiological processes, as well as to cope with simultaneous invasion of bioagressors of different lifestyles, plays the cross-talks among different phytohormonal pathways (Koornneef et al., 2008; Vos et al., 2015a). Although long attributed to different physiological processes in the plant, ABA, gibberellins (GA), cytokinins (CKs), and auxin are also involved in the response to biotic stresses (Verma et al., 2016; López et al., 2008). These phytohormones are produced differentially by plants in response to infections. Some pathogens are also capable of produce analogs of these hormones to disrupt the hormonal balance of the plant and therefore suppress the ability to defend itself (Robert-Seilaniantz et al., 2007). A number of molecular components implicated in interconnections among CKs,

ABA, GA, auxin, SA, JA, and ET has been recognized so far, mainly in the model plant Arabidopsis (see for review: Verma et al., 2016). The cross-talk among all these hormones modulates plant development processes, integrating them in relation to various stresses.

Plants seem to employ a specific hormonal pathway adapted to the type of parasite (Garcia-Brugger et al., 2006). It has been classically thought that JA/ET play a crucial role in the response to necrotrophic pathogens and herbivorous insects attacks whereas SA is determined for the defense against biotrophic and hemi-biotrophic pathogens (Thomma et al., 2001; Pieterse et al. 2009). These two pathways have been believed to be antagonistic. An increase in SA concentration results in inhibition of JA/ET pathway and vice versa, while JA and ET pathways may act synergistically. Meanwhile, recent studies suggest implication of JA-signaling in the resistance of grapevine to *P. viticola* (Figueiredo et al., 2015; Guerreiro et al., 2016; Marchive et al., 2013). The regulation of defenses by SA, JA/ET is complex and to date, the signaling pathways seem to interact with each other, either positively or negatively (Garcia-Brugger et al., 2006; Koornneef et al., 2008; Yang et al., 2015).

These cross-regulations are dependent on the action of particular proteins acting as component of signal transduction. For example, MAP kinase 4 (MPK4) identified in Arabidopsis is a positive regulator of the JA pathway and an inhibitor of SA pathway (Kong et al., 2012). NPR1 (non expressor of PR1) has been extensively studied in Arabidopsis (for review see Backer et al., 2019). It is a key protein in the SA-dependent signaling pathway, as well as in inhibition of the JA pathway (Durrant and Dong, 2004). A multigene family homologous to the Arabidopsis NPR1 family was identified in grapevine and was shown to play a major role in the pathway regulated by SA (Bergeault et al., 2010; Velasco et al., 2007). The overexpression of the functional ortholog of NPR1 in grapevine was shown to enhance its resistance against *E. necator* (Le Henanff et al., 2011), while the overexpression of a WRKY-type transcription factor activated JA-signaling and the expression of defense genes, leading to an improved resistance against *P. viticola* (Marchive et al., 2013). A better comprehension of phytohormonal cross-talk mechanisms and a discover of additional ones, in responses to different kind of stresses, could be useful in the development of strategies of plant protection, such as elicitation.

3.3.4. Expression of resistance

Consequences of activating the cascade of immune responses include strengthening of the cell wall, induction of defense-related genes, e.g., encoding proteins related to

pathogenesis (PR proteins) and enzymes responsible for the biosynthesis of phytoalexins (Hammond-Kosack and Jones, 1996; Dixon et al., 1996; Benhamou and Nicole, 1999; Andersen et al., 2018; Garcion et al., 2014).

3.3.4.1. Strengthening of the cell wall

Strengthening of the cell wall in response to a pathogen attack, or wounding, occurs through the deposition of reinforcing material at the site of the contact with the stimuli and formation of callose-rich occlusive papillae, which physically block the progression of infection (Brown et al., 1998; Voigt et al., 2014). The integrity of the cell wall can be also maintained through the activity of polygalacturonases-inhibiting proteins (PGIP) synthetized by the plant (Federici et al., 2006). ROS are strongly involved in the cell wall strengthening, in particular by promoting bridging reactions between proteins rich in proline residues or by allowing cross-linking of phenolic compounds via peroxidases (Hückelhoven, 2007). One of the earliest events is the activation of plant cell wall associated non-enzymatic proteins (CWPs), which exert different mechanisms of action (Rashid, 2016). For example, hydroxyproline-rich glycoproteins (HRGPs) (e.g., extensins) are accumulated and cross-linked in the cell wall architecture, which ensures the mechanical strength and impenetrability of the cell wall, and provides sites for lignin deposition (Dixon et al., 1996; Showalter, 1993). Also, arabinogalactan proteins (AGPs) are secreted at the site of infection and serve as a soluble molecular signal for the activation of PR proteins gene expression. Glycine-rich proteins (GRPs) bind to RNA of the pathogen leading to its degradation (Rashid, 2016). Impregnation of the cell wall with lignins and suberins is also ROS-promoted and takes place via polymerization of phenolic precursors of the phenylpropanoid pathway (Dixon et al., 1996). The adjacent cells receive the signal to respond by synthetizing callose and lignin between their walls and those of infected cells (papillae), which limit the spread of infection, and allow to contain the parasite to eliminate it through the action of ROS and toxic metabolites produced by the plant in response to infection (O'Brien et al., 2012; Asselbergh et al., 2007).

In grapevine, lignin and callose deposits were noted upon treatment with *β*-aminobutyric acid (BABA) (Hamiduzzaman et al., 2005), sulfated laminarin (PS3) (Trouvelot et al., 2008) or thiamine (Boubakri et al., 2012), and was one of the mechanisms that contributed to an enhanced resistance to *P. viticola* in these studies.

3.3.4.2. Pathogenesis-related proteins accumulation

Pathogenesis-related (PR) proteins, as indicated by the name, are a group of proteins strongly accumulated during pathogenesis, not detectable (or at very low levels) in healthy plant tissue (Stintzi et al., 1993). Thus, they are generally used as biomarkers of both local and systemic induced resistance. PR proteins have specific physico-chemical properties: they are very stable in an acid medium and resist the action of proteases produced by the plant itself or by pathogenic microorganisms. These properties give them a great stability in unfavorable environments where they accumulate, especially in the vacuole, and in the intercellular spaces occupied by pathogens (Kauffmann et al., 1987).

The presence of PR proteins upon biotic stress or elicitation was reported in many plant species. Thus, their role in plant defenses has been highlighted. PR proteins were first described in tobacco plant leaves following infection with the tobacco mosaic virus (Gianinazzi et al., 1970). The current classification includes 17 families of PR proteins (van Loon and van Strien, 1999), mostly characterized in tobacco, tomato, Arabidopsis and grapevine (Bézier et al. 2002; Kortekamp, 2006). The identified PR proteins possess antimicrobial, or even toxic activities. Some of them (such as β-1,3-glucanases (PR2) and chitinases (PR3, PR4, PR8 and PR11)) are directly capable of degrading the cell walls of phytopathogenic fungi (Giannakis el al., 1998). Moreover, PR2 proteins have also an indirect effect, via the release of oligosaccharides (for example from the walls of the pathogen) which can act as elicitors themselves and induce defense mechanisms (Giannakis et al., 1998). Others have been identified as osmotins belonging to the PR5 family (thaumatin-like) of antifungal activity and the capacity of permeabilizing microbial membranes (Kombrink and Somssich, 1997; van Loon et al., 2006); endoproteases (PR6); protease inhibitors (PR7); peroxidases (PR9); PR10 with some of which have a possible ribonuclease activity; defensins (PR12), small peptides rich in cysteines whose mode of action is very varied; thionins (PR13) of antibacterial and antifungal activity; lipid transfer proteins (PR14); germins (oxalate oxidases) and germin-like proteins (oxalate-oxidase-like proteins) (PR15 and PR16, respectively) with superoxide dismutase activity (Hu and Reddy, 1997). The function of the PR17 family members remains elusive but their accumulation in pathogen-induced wheat and tobacco plants was reported (Christensen et al., 2002).

The most abundantly produced PR proteins in response to an attack are those of PR1 family (Breen et al., 2017), which are known in particular for their involvement in SA-dependent defense responses. At present, their exact role has not been clearly established.

Some authors have demonstrated their antimicrobial properties in vitro (Niderman et al., 1995) and others have shown the involvement of a C-terminal peptide carried by the protein in signaling plant immunity, facilitating defense responses against microbial and herbivorous agents (Breen et al., 2017; Chen et al., 2014).

In grapevine, numerous studies have permitted to identify PR proteins implicated in the expression of defenses either upon pathogen inoculation or elicitation. For example, Renault et al. (1996) detected an induction of PR proteins, including a chitinase and several *β*-1,3-glucanases, in grapevine leaves, after application of SA or infection with *B. cinerea*. Busam et al. (1997) reported differential expression of the two grapevine chitinase genes in response to SAR activators and infection with *P. viticola*. Giannakis et al. (1998) described a correlation between the combined activities of a chitinase and a *β*-1,3-glucanase from several grapevine cultivars and resistance observed in the field against powdery mildew. Furthermore, the proteins purified from the leaves of a resistant cultivar inhibited the growth of powdery mildew in vitro. In addition, the activity levels of two chitinases and of a *β*-1,3-glucanase noticeably increased in the leaves and berries of susceptible grapevine cultivars infected with powdery mildew associated with the expression of the corresponding defense genes (PR2, PR3 and PR5) (Jacobs et al., 1999). A family of defensins (PR12) allowed to inhibit the germination of conidia of *B. cinerea* in vitro (Giacomelli et al., 2012). Besides, the expression of certain genes encoding PR proteins in grapevine is particularly important during veraison; chitinase (PR3) and thaumatin-like (PR5) are the main representatives. Their presence at advanced stages of the ripening of berries explains the resistance to certain pathogens despite the increase in sugar contents (Tattersall et al., 1997).

3.3.4.3. Activation of secondary metabolism

Protection provided by the activation of secondary metabolites is prominently developed in plants. In 1891, the German biochemist Albrecht Kossel separated secondary metabolites from primary metabolites, such as phytosterols, acyl lipids, nucleotides, amino acids, and organic acids (Komives, 2017). Unlike them, secondary metabolites appear to be dispensable for plant growth and development, but play variety of roles in response to abiotic and biotic stresses, allowing the plant not only to deter the pathogen attacks, but also to adapt to its environment, by among others, acting as allelopathic agents, and to improve reproduction by attracting pollinating insects or seed-dispersing animals (Vogt, 2010;

Harborne, 2001). The chemical diversity of these natural substances represents the result of a process of biochemical evolution often imposed by the phenomena of coevolution between plants and other organisms with which they interact. Besides, secondary metabolites make plant an immense source of bioactive molecules of pharmaceutical or nutritional value.

It is estimated that to date plant secondary metabolites include approximately 200 000 defined structures (Croteau et al., 2007; Hartmann, 2007). Based on the biosynthetic origin they are classified in to the one of the three major chemical family representatives: (i) the terpenoids, derived from the five-carbon precursor isopentenyl diphosphate (IPP), (ii) the alkaloids (non-protein nitrogen compounds) and sulfur-containing compounds, biosynthesized principally from amino acids, and (iii) the phenylpropanoids, including phenolic and polyphenolic compounds, derived from the shikimic acid pathway or the malonate/acetate pathway (Herrmann, 1995; Croteau et al., 2007). In the most of the cases, plant secondary metabolites are biosynthesised in a specific organ and/or cell compartment, and transported to their site of storage (Wiermann, 1981; Isah, 2019). Hydrophilic compounds (e.g., alkaloids, glucosinolates, and tannins) are deposited in vacuoles or idioblasts, while lipophilic metabolites (e.g., terpene-based essential oils) are stored in thylakoid membranes or cuticles, resin ducts and trichomes (Wiermann, 1981; Isah, 2019)

Some of secondary metabolites involved in the plant defense are defined as phytoalexins (from Greek *alexein*, to defend) i.e., 'low molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms' (Paxton, 1981). Other group, phytoanticipins, are referred to as 'low molecular weight, antimicrobial compounds that are present in plants before challenge by microorganisms or are produced after infection solely from pre-existing constituents' (VanEtten et al., 1995). The difference between these two types of compounds is not very obvious because it is based on their function and not their structure. In some cases, compounds can belong to both classes (VanEtten et al., 1995).

Secondary metabolites can be involved in plant defense in a number of ways. They can act directly against the pathogen, and due to their toxicity will inhibit or block the pathogen at the site of infection (mainly phytoalexins) (Raynal et al., 1980; Walters et al., 2007). For example, their antifungal action can prevent germination, penetration into the host or colonization of vascular vessels of the plant (Jeandet et al., 2002; Adrian et al., 1997), some secondary metabolites are also bactericidal or virucide (Francis et al., 2002; Chan et al., 2013). Direct toxic activity of secondary metabolites has also been reported in insects and mammals. For example, some molecules are able to interfere with the molting process in

insects (Sláma, 1980), others are able to bind to proteins and disrupt their action (Bennick, 2002), and some are irritating to the skin (Jassbi, 2006). In addition, certain secondary metabolites allow plants to protect themselves by acting as a repellent against birds or rodents (Fischer et al., 2013). They can also be involved in defense as signal molecules, such as JA or SA (Edreva et al., 2008). Finally, some of secondary metabolites are capable of reacting with ROS, which therefore gives them an antioxidant role and allows the plant to maintain cellular redox homeostasis (Pourcel et al., 2007; Foyer and Noctor, 2005).

For the remainder of this bibliographical review, two families of compounds are going to be detailed: phenylpropanoids (from which stilbenes, the main phytoalexins of grapevine), and terpenoids (both secondary and primary metabolites, i.e., triterpenoids and sterols, respectively).

4. TERPENES

To date more than 25 000 of terpenes (isoprenoids) have been identified, making them the largest and the most structurally diverse group of plant metabolites (Croteau et al., 2007; Wink, 2003; Hill and Connolly, 2015). The name "terpene" originates from the word turpentine ("terpentin" in German), which was discovered to be a mixture of hydrocarbons with a carbon-to-hydrogen ratio of 5:8 (Croteau, 1998). Since then, several essential oils have been identified with the same carbon-to-hydrogen ratio and classified as terpenes. Although the structures of terpenoids are organized as derivatives of isoprene (2-methyl-1,3-butadiene) (hence the name isoprenoids), the latter compound is not involved in the biosynthesis, but is a product of thermal decomposition of many terpenoid substances (Croteau et al., 2007). In fact, the structure of all terpenoids is obtained by repetitive fusion of branched five-carbon units based on isopentane skeleton (Croteau et al., 2007). The chemist Ruzicka proposed a nomenclature for triterpenes groups according to the number of isoprene units that constitute them: monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterpenes (C_{25}), triterpenes (C_{30}) and carotenoids (C_{40}) (Ruzicka, 1953).

4.1. Biosynthesis

Biosynthesis of terpenes is compartmentalized, as is that of their biological precursor, isopentenyl diphosphate (IPP) (Fig. 17). The mevalonate (MVA) pathway, located in cytoplasm, had been long regarded as the sole pathway for isoprenoid biosynthesis in living organisms (Seigler, 1998). It begins with the condensation of three molecules of acetyl

coenzyme A, resulting in the formation of 3-hydroxy-3-methylglutaryl-CoA (3-hydroxy-3-methylglutaryl CoA), and mevalonic acid. The latter is then converted to IPP and its isomer, dimethylallyl diphosphate (DMAPP) due to phosphorylation and decarboxylation reactions. A key enzyme in this pathway is HMG-CoA reductase (HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase), which catalyzes the transformation of HMG to mevalonic acid (Stermer et al., 1994).

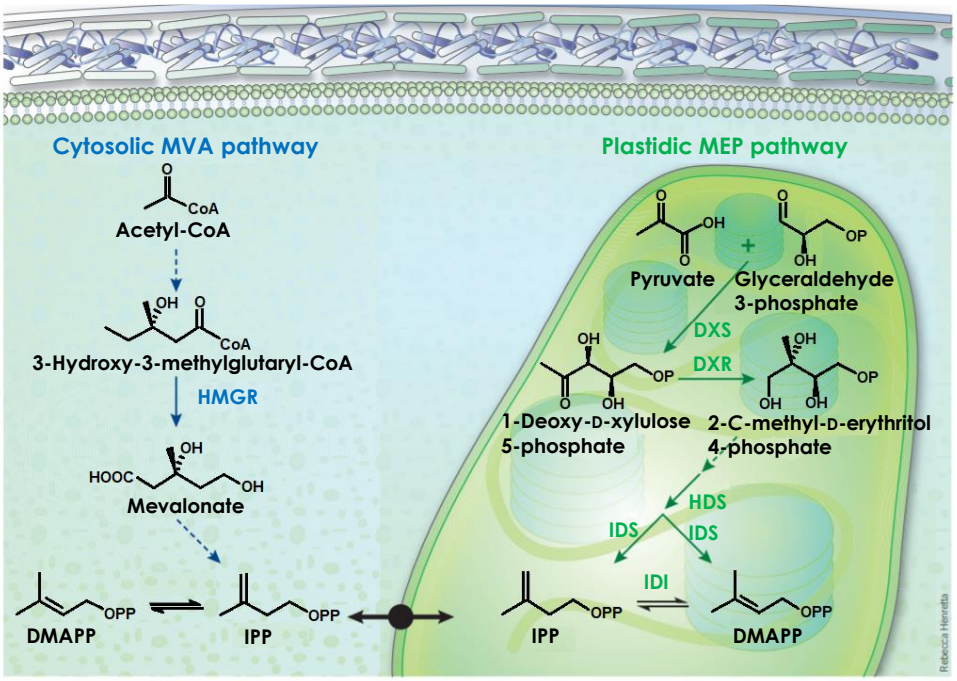

Figure 17. Alternative pathways of isopentenyl diphosphate (IPP) formation in plant cell (Roberts, 2007; modified). DMAPP, dimethylallyl diphosphate; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; HDS, hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase; HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; IDI, isopentenyl diphosphate isomerase; IDS, isopentenyl diphosphate: dimethylallyl diphosphate synthase; MVA, mevalonate.

Like the animal HMGR enzyme, that controls cholesterol biosynthesis, the plant one, associated with the ER membrane, is also highly regulated. A large increase in the HMGR activity following an exposure to pathogens or pathogen-derived elicitors was reported in plants synthesizing isoprenoid phytoalexins (Stermer et al., 1994). Indeed, HMGR is encoded by small gene families and the specific ones can be induced by mechanical or biotic stress which lead to posttranslational regulation of the enzyme (Weissenborn et al.,1995). Different mechanisms can be responsible for the HMGR regulation in the direction of the biosynthesis of different terpene families. It is suggested that topology of the enzyme facilitates either the elicitor-induced production of phytolexins, or primary metabolites (sterols) (Weissenborn et al.,1995).

However, MVA pathway is not a unique route leading to terpenes. An alternative route independent to mevalonate, called the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway/1-deoxy-D-xylulose 5-phosphate (DOXP) has been discovered in several eubacteria, algae and in chloroplasts of higher plants (Rohmer et al., 1996; Rohmer, 1999; Rodríguez-Concepción and Boronat, 2002). This pathway starts with a condensation of pyruvic acid with 3-phosphoglyceraldehyde (GAP) to form 1-deoxyxylulose 5-phosphate. This process is catalyzed by 1-deoxyxylulose 5-phosphate synthase (DXS). In the next step, 5-phosphate 1-deoxyxylulose is converted to 2-methylerythritol 4-phosphate (MEP), Dxr reductoisomerase (1-deoxy-D-xylulose 5-phosphate reductoisomerase, DXR). The MEP is then converted to cyclic forms of 2,4-cyclodiphosphate-2-methylerythritol (2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate, ME-cPP), in which the enzymes IspD, IspE and IspF are involved. MEcPP is converted to hydroxymethylbutenyl 4-diphosphate, and this compound can be directly converted to IPP and DMAPP using appropriate synthases (Eisenreich et al., 2001).

Most of organisms (with few exceptions as *Streptomyces* spp.) employs only one of the isoprenoid biosynthetic pathways. Plants are unique in maintaining both of them parallel. It has been hypothesised that the existence of two alternative, spatially separated pathways is beneficial for plant's interaction with environment. Influenced by various stresses, plants are subjected to a rapid demand for defense compounds, and the cross-flow of intermediates in the biosynthesis pathways of terpenes can be considered as one of the strategy of survival (Bick and Lange, 2003). Indeed, it has been proved that in the case of a temporal block or down-regulation of one of isoprenoid pathway, compounds with mixed origin can be produced. Generally, the biosynthesis of carotenoids and phytyl chains of chlorophyll occurs in plastids by MEP pathway; of triterpenoids – in cytosol by MVA pathway; some monoterpenes, sesquiterpenes and polyterpenes can be formed from units of both origin, which deliver a "mosaic" structure (Laule et al., 2003; Hemmerlin et al., 2003).

The immediate precursors of terpenes subclasses are prenyl diphosphate homologs formed by repetitive addition of five-carbon units (Fig. 18). These elongation reactions are carried out by cytosolic and plastidic prenyltransferases (McGarvey and Croteau, 1995; Liang et al., 2002). IPP and DMAPP isomers undergo a series of successive condensation reactions in the head-to-tail orientation to form geranyl diphosphate (GPP, precursor of monoterpenes), farnesyl diphosphate (FPP, precursor of sesquiterpenes), and geranylgeranyl diphosphate (GGPP, at the origin of diterpenes). Other patterns of condensation of isoprene units are possible in the biosynthesis of terpenoids, such as head-to-head joining of two FPP units (at

the origin of triterpenes), or of two GGP units (precursor of tetraterpenes) (Croteau et al., 2007).

The different prenyl diphosphates serve as substrates for the specific terpenes synthases (TPSs) to yield the terpenes skeletons of a vast molecular diversity, such as multiple ring systems (Tholl, 2006; Croteau et al., 2007). The subsequent arrangement of terpenes structures is due to secondary enzymatic modifications, including oxidation, reduction, isomerization, and conjugation reactions that bring the functional properties of these compounds. Terpenoids are thus derivatives of terpenes, which in addition to the terpene hydrocarbon backbone, contain additional functional groups, such as carboxyl or hydroxyl (Hill and Connolly, 2015; Ludwiczuk et al., 2017).

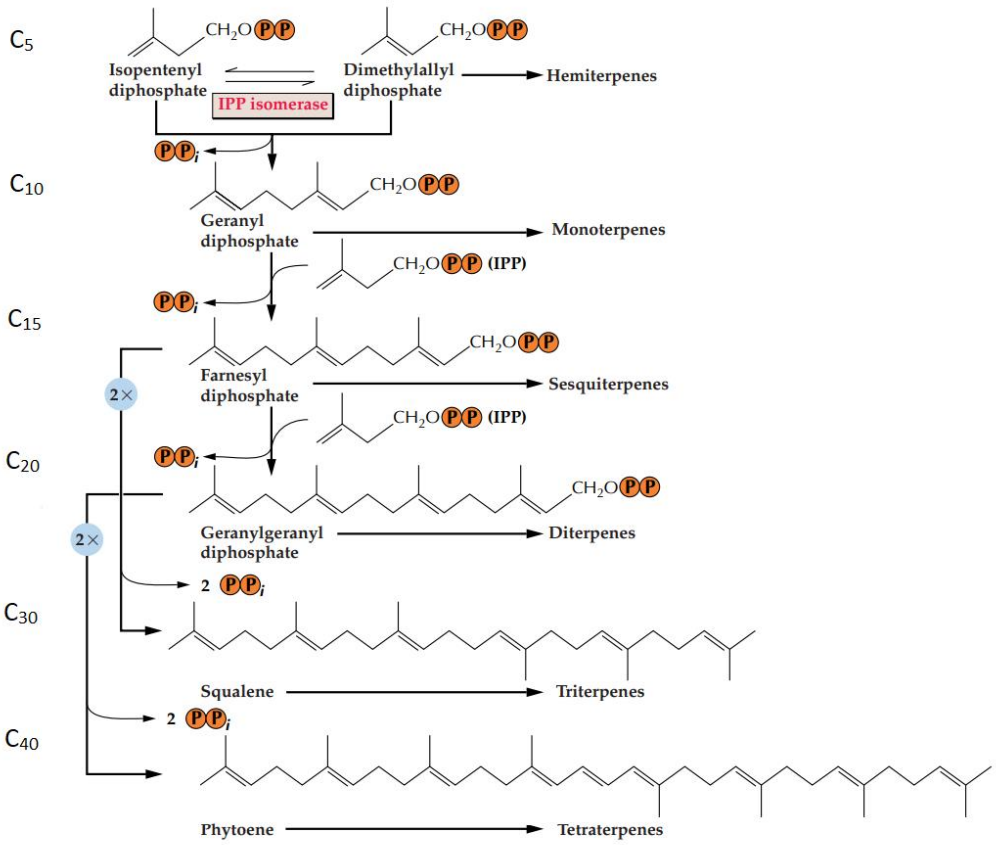

Figure. 18. The major subclassed of terpenoids biosynthesized from the basic five-carbon unit, IPP (isopentenyl diphosphate) and its isomer DMAPP (dimethylallyl diphosphate) (Croteau et al., 2007).

4.2. Functions

Terpenoids are involved in both primary and secondary metabolisms. The essential functions include those performed by phytohormones (gibberellic acid, abscisic acid, cytokinins, brassinosteroids), the components of electron carriers (cytochrome a, quinones, chlorophylls), and elements directly implicated in photosynthesis (carotenoids), or

indispensable for membrane permeability and fluidity (sterols) (Wink, 2003). Numerous terpenic secondary metabolites are recognized as crucial plant chemical defense and/or the interaction with environment, serving as pollinator attractants or herbivore repellents, antibiotics or toxins (Tholl, 2015; Cheng et al., 2007; Singh and Sharma, 2015).

The role of mono-, sesqui-, and diterpenes is the most studied in plant defense. Mono- and sesquiterpenes are the main components of volatile organic compounds (VOCs) so they constitute signaling molecules as attractants for pollinators (Muhlemann et al., 2014). Examples of fragrant substances are menthol or thymol, derived from essential oils extracted respectively from the mint leaves and the thyme flowers, or geraniol and linalool, essential constituents of the typicality of muscat wines (Marais, 2017; Noble et al., 1988). Certain diterpenes have also been studied for their involvement in plant defense as insecticide or repulsive agents (Scheffler and Romano, 2008). The biosynthesis, accumulation, emission or secretion of terpenoid secondary metabolites occur in anatomically highly specialized structures, which sequester these products away and prevent autotoxicity. Due to their nonphotosynthetic nature, the carbon and energy necessary for terpenoid biosynthesis is supplied by adjacent cells. For example, the glandular trichomes and secretory cavities of leaves and the glandular epiderms of flower petals (essential oils); the resin ducts and blisters of conifer species (a defensive resin composed of a monoterpene turpentine and a diterpenoid acid, i.e., rosin); specialized epidermis for triterpenoid surface waxes formation; laticifers (triterpenes and polyterpenes, such as rubber) (Croteau et al., 2007).

Many terpenoids exhibit biological properties, which is why they found application in pharmacy and medicine. Several of them are used on an industrial scale (cosmetics and perfumes, rubber, and food industries), but the yield of their synthesis is nowadays generally accelerated by transgene expression to obtain phytopharmaceuticals (e.g., taxol, artemisinin), insecticides (e.g., pyrethrins, azadirachtin), or industrial intermediates for essential oils used in flavors and perfumes (Croteau et al., 2007; Roberts, 2007; Ludwiczuk et al., 2017; Abbas et al., 2017).

4.3. Steroids and triterpenoids

4.3.1. Biosynthesis

Biosynthesis of triterpenes (C_{30}) requires a head-to-head condensation of two molecules of FPP (C_{15}), catalysed by a prenyltransferase squalene synthase, leading to the

production of a linear hydrocarbon, squalene. The latter is oxidized into the 2,3-epoxide, oxydosqualene, and then cyclized by oxidosqualene cyclases (OSCs). The resulting 2,3-oxydosqualene is converted, for example, into tetracyclic structure of cycloartenol (precursor of phytosterols and brassinosteroids), or pentacyclic triterpenoids (of 5 carbon skeletons) of various arrangement (e.g., surface wax components, such as oleanolic acid) (Haralampidis et al., 2002) (Fig. 19).

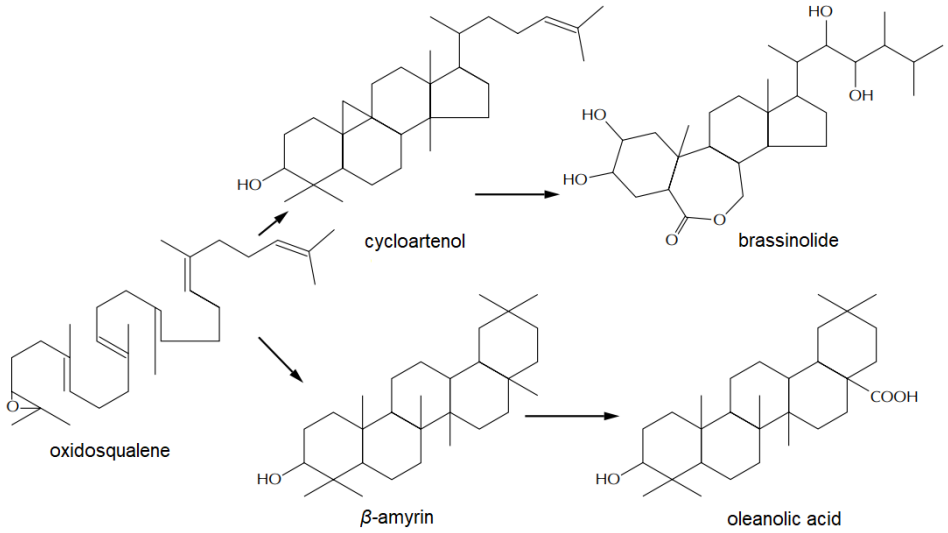

Figure 19. Structures of certain triterpenoids (Corteau et al., 2007; modified).

The action of OSCs depends on three criteria: protonation of 2,3-oxidosqualene by a catalytic acid (initiation of cyclization), the presence of a catalytic cavity which guides the cyclization of the oxidosqualene, the protection of intermediates during cyclization to prevent other reactions (Augustin et al., 2011). The large number of possibilities of internal connections occurring during cyclization leads to a great variability of structures. Over 100 different triterpene structures have been found in nature and derive from the activity of various OSCs (CYP, cytochrome P450 enzymes) (Fukushima et al., 2011). In the biosynthesis of pentacyclic triterpenes, the main feature of the cyclization cascade catalyzed by OSCs is the *chair-chair-chair*-conformation, unlike the *chair-boat-chair*-conformation catalyzed by steroidal OSCs. Protosteryl cation is the intermediate in the cyclization of sterols. In the biosynthesis of pentacyclic triterpenes, the first intermediate is the dammarenyl cation, which, if deprotonated, is the source of dammarane-type triterpenes (Augustin et al., 2011; Haralampidis et al., 2002). Other rearrangements lead to tirucallane-type triterpenes or to the baccharenyl cation. The transformation of the latter leads to the pentacyclic cation lupanyl, the precursor of lupane-type triterpenes. The reopening of the 5-carbon cycle of lupanyl and

its expansion into a 6-carbon ring transforms it into a germanicyl cation, converted then in oleanyl cation (triterpenes of the oleane-type), in taraxasterenyl cation (triterpenes of the taraxasterane), or in ursanyl cation (ursane-type triterpenes) (Fig. 20) (Augustin et al., 2011; Vincken et al., 2007; Haralampidis et al., 2002). The two main deciding enzymes the cyclization process is related to lupeol synthase and α- or β-amyrin synthase. Ursolic acids, oleanol and betulin are formed from α-amyrin, β-amyrin and lupeol, respectively by gradual oxidation of C_{28} (Haralampidis et al., 2002). Pentacyclic triterpens exist in plants either in free or bound forms (esters or glycosides, i.e., saponins) (Vincken et al., 2007).

Steroids are the tetracyclic compounds based on perhydro-1,2-cyclopentano-phenantren moiety. Plant steroids involve phytosterols, characterized by a hydroxyl group at C3, biosynthesised from cycloartanol, generated from the cyclization of 2,3-oxidosqualene, which is by the way also the precursor of fungal and mammalian lanosterol (Brown, 1998).

Main enzymes involved in post-oxidosqualene cyclization of phytosterols are C24-sterol methyltransferases (SMTs) and C22-sterol desaturase belonging to cytochrome P450, family 710 (CYP710A) (Rogowska and Szakiel, 2020). The side chain of phytosterols usually possesses an additional functional group (methyl or ethyl) at C24. The primary secondary methylation occurs through the activity of two isoforms of SMTs (SMT1 and SMT2), localized in the endoplasmic reticulum. The ratio of 24-methyl- and 24-ethylsterols is determined by the activity of SMT2. This feature is crucial during plant ontogenesis and responses to stress factors (Valitova et al., 2016). The final step in sterols biosynthesis is the secondary methylation resulting the formation of 24-ethylsterols, such as sitosterol. The latter is conversed into stigmasterol in the reaction catalyzed by C22-sterol desaturase which leads to the introduction of a double bond at the position C22 (Valitova et al., 2016; Benveniste, 2004).

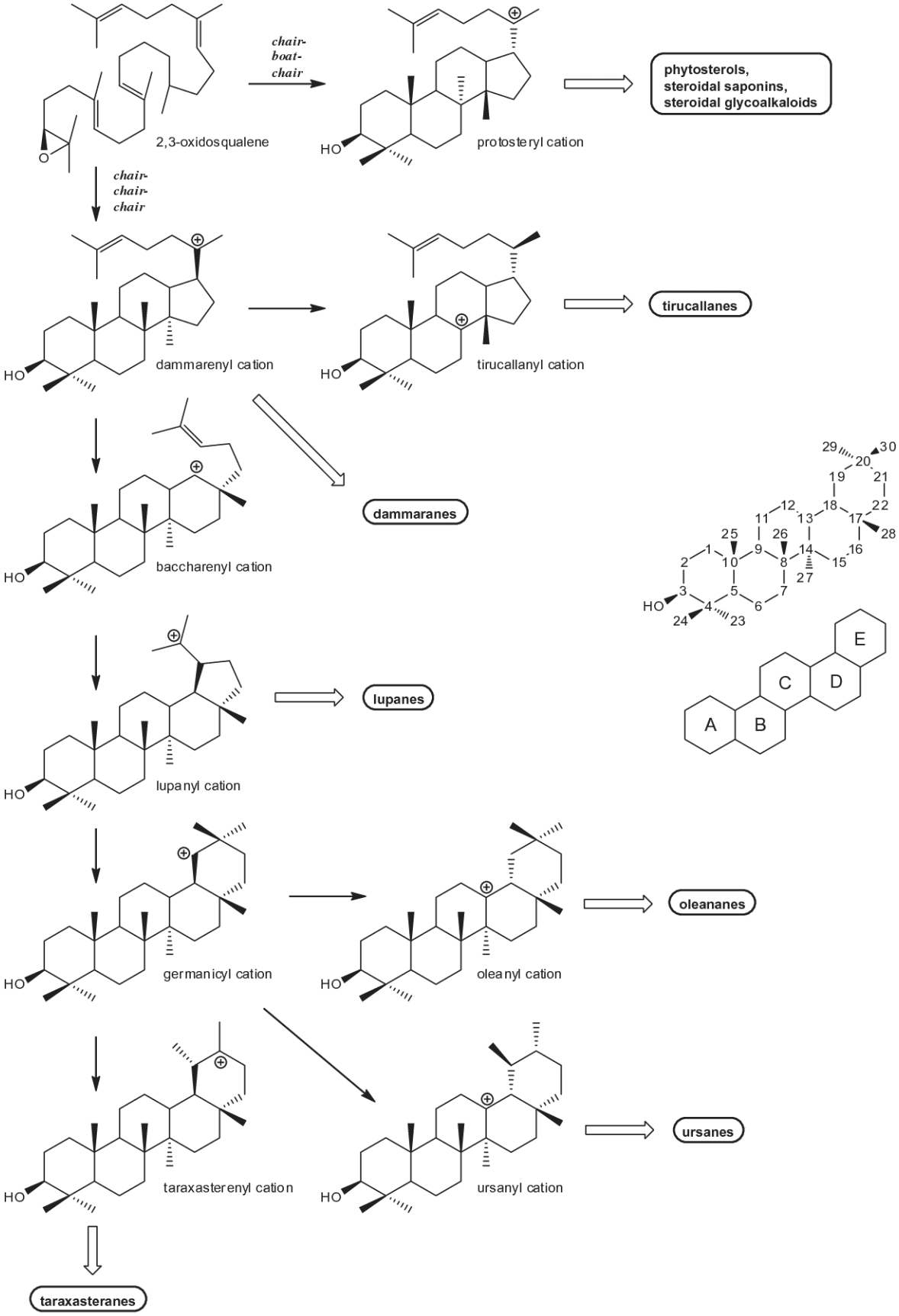

Figure 20. Cyclization cascade of 2,3-oxidosqualene and the resulting triterpenes (Augustin et al., 2011; modified)

Over 200 different plant sterols structures have been identified in various plant species. Contrarily to fungi and mammals, of which the cell contains one sterol (i.e., ergosterol and cholesterol, respectively), plant cells synthesize a mixture of tetracylic triterpenoids. They can be classified into the most abundant 4-desmethylsterols (e.g., sitosterol, campesterol, and stigmasterol) (Fig. 21), as well as 4-methylsterols, or 4,4'-dimethylsterols groups (Moreau et al., 2002). Cholesterol can be also found at significant amount in some plant species, e.g., those of the Solanaceae family. In plants, the presence of a mixture of sterols is suggested to have a role in certain processes related to growth and development and stress (Rogowska and Szakiel, 2020). The modifications in the phytosterol composition, including changes in the ratio between individual compounds, have been suggested to be essential for processes related to stress compensation (Schaeffer et al., 2001; Aboobucker and Suza, 2019). The diversity of sterols may be thus related to the broad spectrum of vial functions in plants. The most common phytosterols have a double bond at C5 of the B-ring (they are referred to as Δ5-phytosterols), or at C7 (Δ7-phytosterols). Phytosterols exist in a free or bound forms (esters with fatty or phenolic acids, glycosides and acylated steryl glycosides) (for review see Rogowska and Szakiel 2020; Ferrer et al., 2017).

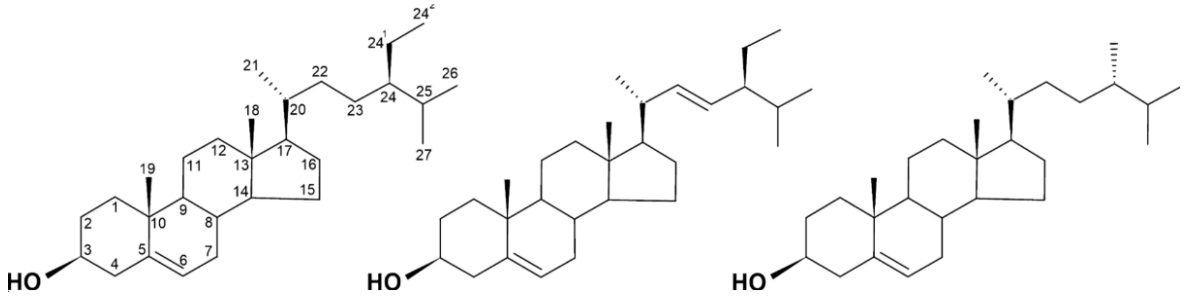

Figure 21. Structures of the most common phytosterols: sitosterol, stigmasterol, campesterol (from left to right) (Rogowska and Szakiel, 2020).

4.3.2. Role in grapevine responses to stresses

Triterpenoids are generally considered as constitutive plant defense molecules (i.e., phytoanticipins) rather than phytoalexins (Pensec et al., 2016). The biosynthetic pathways of sterols and pentacyclic triterpenoids pass through several branching points that can be regulated at various levels, thus, can be important for plant response to stresses. The study of this class of compounds allows to follow both primary and secondary metabolisms.

Sterols, along with glycerolipids and sphingolipids are the main components of the cell membranes, where they perform structural functions, regulating their fluidity and

permeability (Hartmann, 1998; Clouse, 2002). Some sterols may take part in controlling metabolic processes by creating nanodomains with sphingolipids, the so-called lipid rafts, i.e., areas with different protein-lipid composition than other areas of the membrane, that are involved in intracellular signalling or protein transport across cell membranes (Dufourc et al., 2008). Sterols are primary metabolites, but are very important precursors in the biosynthesis of secondary metabolites, e.g., glycoalkaloids and cardenolides, as well as brassinosteroids, which are essential for the regulation of plant development and morphogenesis (Hartman 1998; Moreau et al. 2002). Changes in sterol ratios have been demonstrated to be induced by environmental factors in several plant species (cold, drought, UV radiation) (Posé et al., 2009; Wagatsuma et al., 2015; Manzano et al., 2016). This feature influences the proprieties of the cell membranes; thus, the role of sterols in abiotic stress has been be suggested. In *V. vinifera*, low intensity UV-B treatment (16 h at 8.25 μW cm^{-2}) led to an increase of phytosterols (sitosterol and stigmasterol), as well as a pentacyclic triterpene lupeol, as an indirect consequence of ABA signalling, suggesting the role of these membrane-related triterpenes in a grapevine acclimation (Berli et al., 2009; Gil et al., 2012). Several studies have shown the involvement of phytosterols in response to biotic stresses in some plant species (for review see Ferrer et al., 2017). A sitosterol derivative, stigmasta-3,5-diene-7-one identified in the grapevine leaves of a Bulgarian cultivar (Seyve Villard) has been proposed as biomarker of resistance towards *P. viticola* and *E. necator* (Batovska et al., 2008; 2009).

Among pentacyclic triterpenoids, saponins, i.e., triterpenoid glycosides, are well recognized to play an important role in plant chemical defense and interactions with environment (Szakiel et al., 2011). Indeed, the presence of saponins at high levels in healthy plants indicates that the content of plant saponins determine their ability to protect themselves against pathogens (Morrissey and Osbourn, 1999). Saponins, most likely through their action on plasma membranes, are capable of affecting animals, bacteria, nematodes, fungi and lipids of viruses (Francis et al., 2002). More and more studies highlight a possible role of these latter in plant defense in different species, including *V. vinifera* (Batovska et al., 2008, 2009; Özer et al., 2017). However, only several studies have been conducted in order to associate triterpenoids composition in the fruit and the leaves of grapevine and its resistance towards different stresses. Some pentacyclic triterpenoids of toxic effects, such as α-amyrin, lupeol and oleanolic acid have been proposed as biomarkers of grapevine resistance to *P. viticola* or *E. necator* (Batovska et al., 2009; Chitarrini et al., 2017a). A recent study reported an increased amount of ursolic, oleanolic, and betulinic acids in grapes (*V. vinifera* cv. Montepulciano) following treatment of bunches with chitosan (Lucini et al., 2018).

4.3.2.1. Triterpenoids in cuticular waxes

The presence of unconjugated triterpenoids in the leaves and fruits epicuticular waxes (Buschhaus and Jetter, 2011), which constitute the first line of defense, ensure the plant resistance to mechanical and biotic stresses (Müller and Rieder, 2005). The occurrence of triterpenoids in grapes cuticle has been known for a long time (Radler and Horn, 1965). The first studies of the composition of grape cuticle waxes were carried out in 1892, and in 1938 two triterpenoids were identified: oleanolic acid and *β*-sitosterol (Radler and Horn, 1965). Furthermore, other triterpenoids have been identified in grape berry cuticular wax, such as oleanolic aldehyde (Dagna et al., 1982; Zhang et al. 2004), erythrodiol (Dagna et al., 1982), phytosterols (e.g., lanosterol (Le Fur et al. 1994)), betulin, betulinic acid, lupeol (Rivero-Cruz et al., 2008). This layer is particularly rich in oleanolic acid (the content of this compound reaches between 50 and 80% of the total weight of the wax) (Radler and Horn, 1965; Commenil et al., 1997; Pensec et al., 2014).

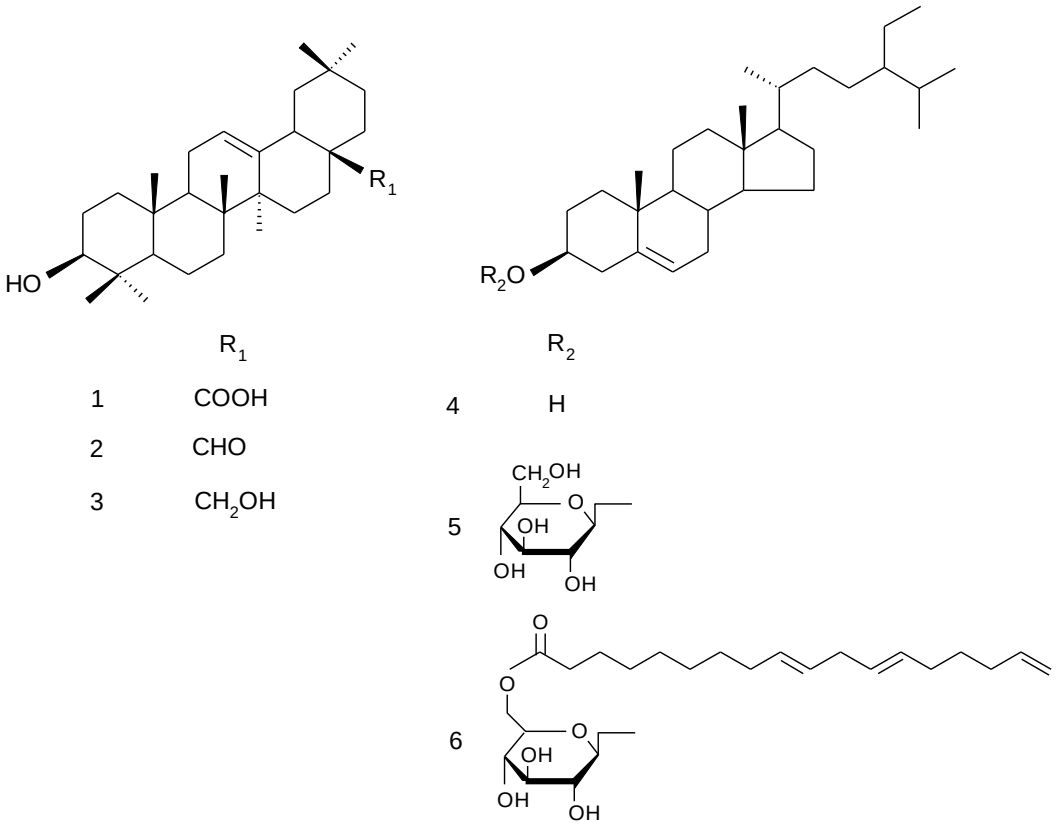

Figure 22. Chemical structures of some triterpenoids occurring in cuticular waxes of grape berries (*Vitis vinifera*). 1, oleanolic acid; 2, oleanolic aldehyde; 3, erythrodiol; 4, *β*-sitosterol; 5, *β*-sitosterol-3-*O*-*β*-D-glucoside; 6, *β*-sitosterol-6'-linolenoyl-3-*O*-*β*-D-glucopyranoside (Szakiel et al., 2012a).

The organization of such a high abundance of a single molecule is explained by a molecular model of the three dimensional arrangement of this triterpenoid acid with the main aliphatic constituent of cuticular wax, n-hexacosanol (Casado and Heredia, 1999). Oleanolic acid probably forms dimers by hydrogen bonds between the hydroxyl group of one molecule and the carboxyl group of the other. The remaining functional groups can interact with other molecules of oleanolic acid, and thus arrange spatially aliphatic alcohols (Casado and Heredia, 1999) (Fig. 23). The chemical characteristics and molecular arrangement of grapes cuticular waxes have been suggested to play a key role of this layer not only as support to maintain fruit integrity, but also as a protection barrier against different stresses (Comménil et al., 1997; Pensec et al., 2014). Changes in the triterpenoid content in cuticular waxes of grapes were demonstrated to occur throughout different phenological stages of fruits, which could explain modulations in their susceptibility to *B. cinerea* (Pensec et al., 2014; Comménil et al., 1997; Bard and Olson, 1994). Grape pomace, which is estimated to represent 13% of the grape weight, has been considered as an interesting by-product of vinification that could be used in order to obtain biologically active triterpenoids (Szakiel et al., 2012a).

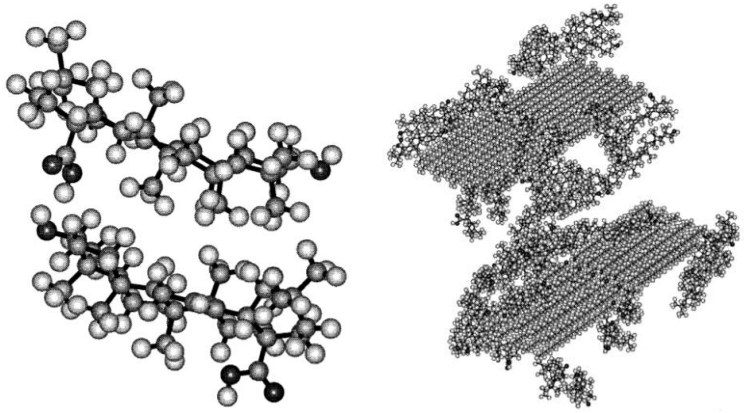

Figure 23. Molecular model of oleanolic acid dimer (left), and of the arrangement of oleanolic acid and n-hexacosanol in the the cuticular wax of grape berry (right) (adapted from Casado and Heredia, 1999).

There is a little data available on triterpenoids in wine, and their presence in grape cuticle waxes suggests that most of these compounds are lost in the manufacturing process. Recent findings provided evidence about the origin of triterpenoids in Bordeaux wine aged in oak barrels made of *Quercus petraea* (Matt.) and *Quercus robur* L. The identified molecules contributed to the sweetness of the wines and were proposed as the molecular markers of taste changes during aging due to the oak used (Marchal et al., 2011; Gammacurta et al., 2019; Gammacurta et al., 2020).

5. POLYPHENOLS

Polyphenols represent a very heterogeneous group of secondary metabolites, including approximately 8 000 compounds, widely distributed in plant kingdom. Structure of polyphenols contains at least one phenol function, i.e., a benzene ring, or aromatic ring linked to at least one modified (or not) hydroxyl group. The biosynthesis of most plant phenolics emerges from the phenylpropanoid and phenylpropanoid-acetate pathways (Croteau et al., 2007) (Fig. 24).

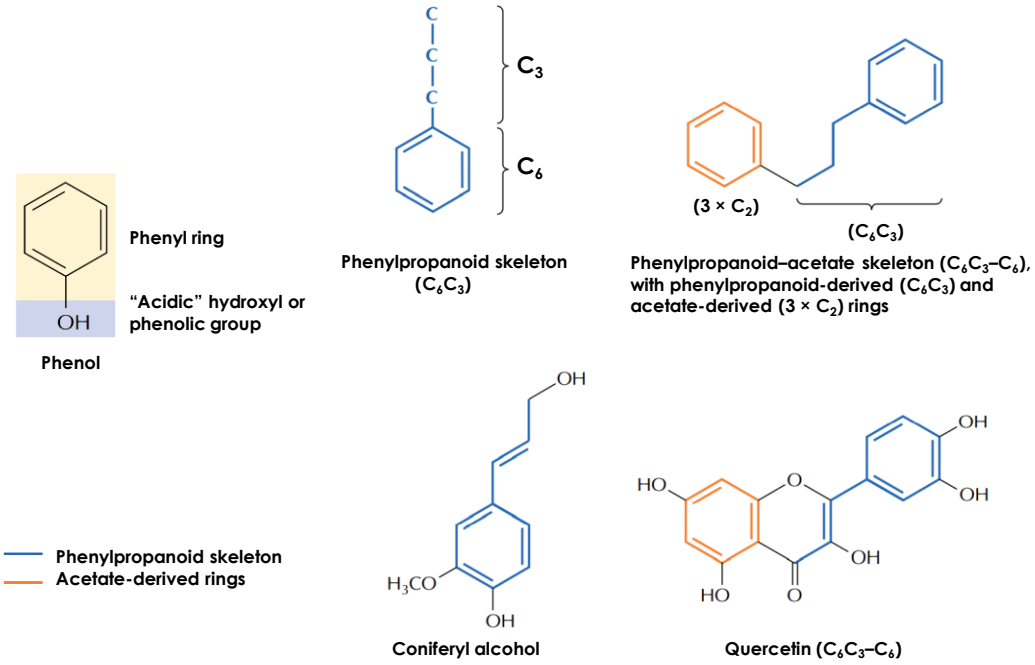

Figure 24. Structure of phenol. Phenylpropanoid and phenylpropanoid-acetate skeletons with examples (Croteau et al., 2007; modified).

5.1. Biosynthesis

Most of phenolic defense-related molecules are phenylpropanoids, and include such classes as flavonoids, stilbenes, coumarins, phenolic acids, and lignans. Some phenolics are biosynthesised through alternative pathways; however, they share many common features. For example, proanthocyanidins are generated by the phenylpropanoid-acetate pathway; hydrolyzable tannins are copolymers of carbohydrates and gallic and ellagic acids deriving from the shikimate pathway; tetrahydrocannabinoids from cannabis are phenolic compounds derived from polyketide (acetate) and terpenoid routes (Croteau et al., 2007).

The phenylpropanoid pathway derives by way of shikimate which gives arise of certain aromatic amino acids. It begins with the condensation of phosphoenolpyruvate (PEP)

and erythrose-4-phosphate, followed by different reaction mechanisms of phosphoprylations, dehydratations, decarboxylations and condensations leading in particular to the formation of phenylalanine (Lattanzio, 2013; Seigler, 1998). The biosynthesis of phenylpropanoids is initiated by the action of phenylalanine ammonia-lyase (PAL) which converts phenylalanine into *trans*-cinnamic acid. PAL is one of the most extensively studied enzymes involved in secondary metabolism. It is suggested, that this enzymatic step had been crucial for the colonization of land by plants. The addition of an OH function to *trans*-cinnamic acid by cinnamate-4-hydroxylase (C4H) converts it into *p*-coumaric acid. The reaction of the latter with coenzyme A (CoA) is carried out via the action of 4-coumarate-CoA ligase (4CL) and results in the formation of coumaroyl-CoA (Ehlting et al., 2006; Ferrer et al., 1999; MacDonald and D'Cunha, 2007). This compound is a crossroad for distinct metabolic pathways leading to different groups of polyphenols, which according to their structures can be classified into two categories – flavonoids and non-flavonoids (Fig. 25, 26).

Flavonoids are compounds with a common structure formed by two benzene rings linked together by an oxygenated heterocycle. For this, chalcones have to be obtained by the action of chalcone synthase (CHS) responsible for condensation of coumaroyl-CoA with three malonyl-CoA units (Fig. 25). The key step of flavonoids pathway is the isomerization of chalcones by chalcone isomerase (CHI), leading to the formation of flavanones, and then flavonols, flavones and leucoanthocyanidins (flavan-3,4-diols). The latter are the precursor of flavan-3-ols (2*R*, 3*R*), such as epicatechin, or anthocyanidins which generate glycosylated forms called anthocyanins. Anthocyanidins can also form flavan-3-ols (2*R*, 3*S*), e.g., catechin. The condensation of several flavan-3-ols units generates polymerized forms known as proanthocyanidins or condensed tannins (Lattanzio, 2013).

Non-flavonoids correspond to other classes of polyphenols, among which are found stilbenes, phenolic acids and lignans (Fig. 26). The condensation of coumaroyl-CoA with three units of malonyl-CoA by stilbene synthase (STS) results in the formation of resveratrol, the basic unit of stilbenes. Resveratrol can subsequently undergo different reaction mechanisms, such as glycosylation, methylation, oligomerization or isomerization resulting in a diversity of stilbenic compounds (see subsec. 5.3.3) (Sáez et al., 2018). Phenolic acids, and more particularly benzoic acids, are obtained from coumaroyl-CoA through several enzymatic reactions carried out by enoyl-CoA hydratase (ECH), alcohol dehydrogenase (ADH), acetoacetyl-CoA thiolase (AT) and thiol esterase (TE). This way is also interfered by the precursor of coumaroyl-CoA, *p*-coumaric acid, which is at the origin of cinnamic acid derivatives, formed by the activity of *p*-coumarate 3-hydroxylase (C3H) and caffeic acid/5-

hydroxyferulic acid *O*-methyltransferase (COMT) (Goleniowski et al., 2013). The phenolic acids pathway is followed by that of lignans, initiated by the reduction of ferulic acid by cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) to coniferyl alcohol. The condensation of two units of coniferyl alcohol results in the formation of pinoresinol, the precursor of the lignan pathway (Satake et al., 2015).

A competition for precursors between STS and CHS was reported in transgenic plants, spruce and tobacco (Fischer et al., 1997; Hammerbacher et al., 2011). In grapevine, such effect could potentially influence the balance between the biosynthesis of polyphenols contributing to organoleptic properties of grapes and wine, as well as conferring the protection against environmental challenges (flavonoids), and defense related molecules (stilbenes) (Teixeira et al., 2013). However, such effect was tested in cell suspension of *V. vinifera* cv. Gamay Fréaux var. Teinturier, and it did not occur. Elicitation with MeJA led to the expression of defense responses, including the up-regulation of STS with the accumulation of stilbenes, and the simultaneous induction of CHS and UDP glucose: flavonoid-3-*O*-glucosyltransferase (UFGT), resulting in the accumulation of anthocyanins (Belhadj et al., 2008b).

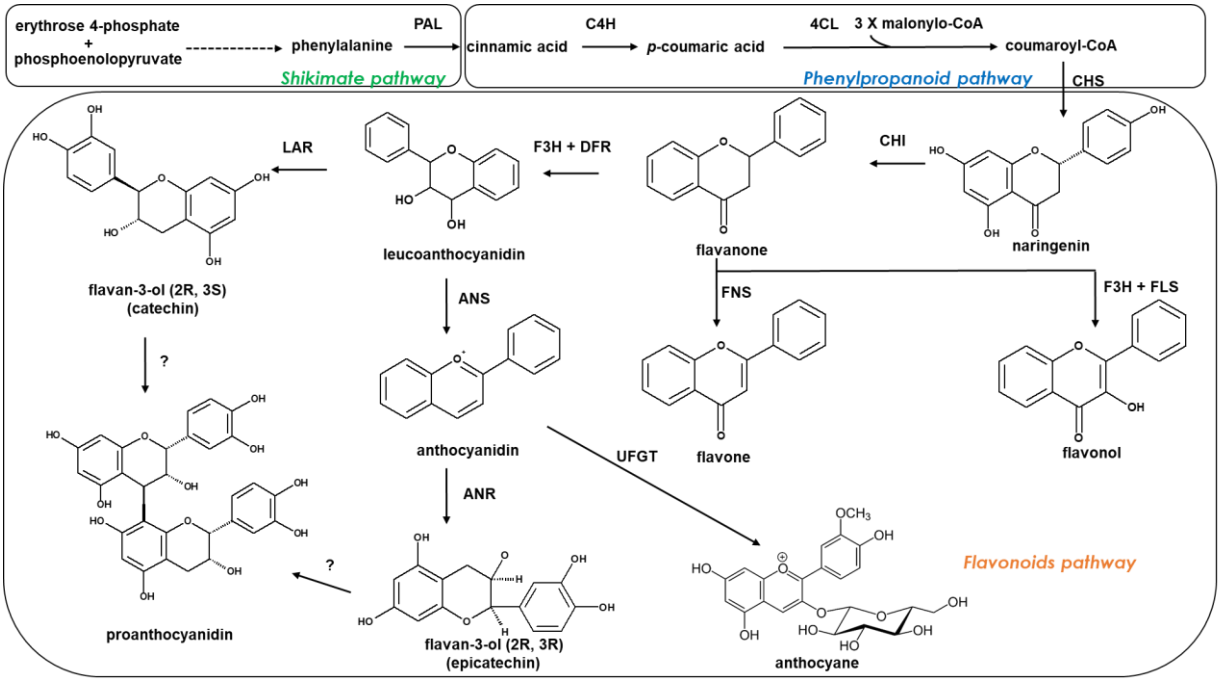

Figure 25. Biosynthesis pathway of flavonoids. (Marè et al., 2013; KEGG). 4CL, 4-coumarate-CoA ligase; ANR, anthocyanidine reductase; ANS, anthocyanidin synthase; C4H, cinnamate-4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; LAR, leucoanthocyanidine reductase; PAL, phenylalanine ammonia-lyase; UFGT, UDP-glucose: flavonoid 3-*O*-glucosyltransferase.

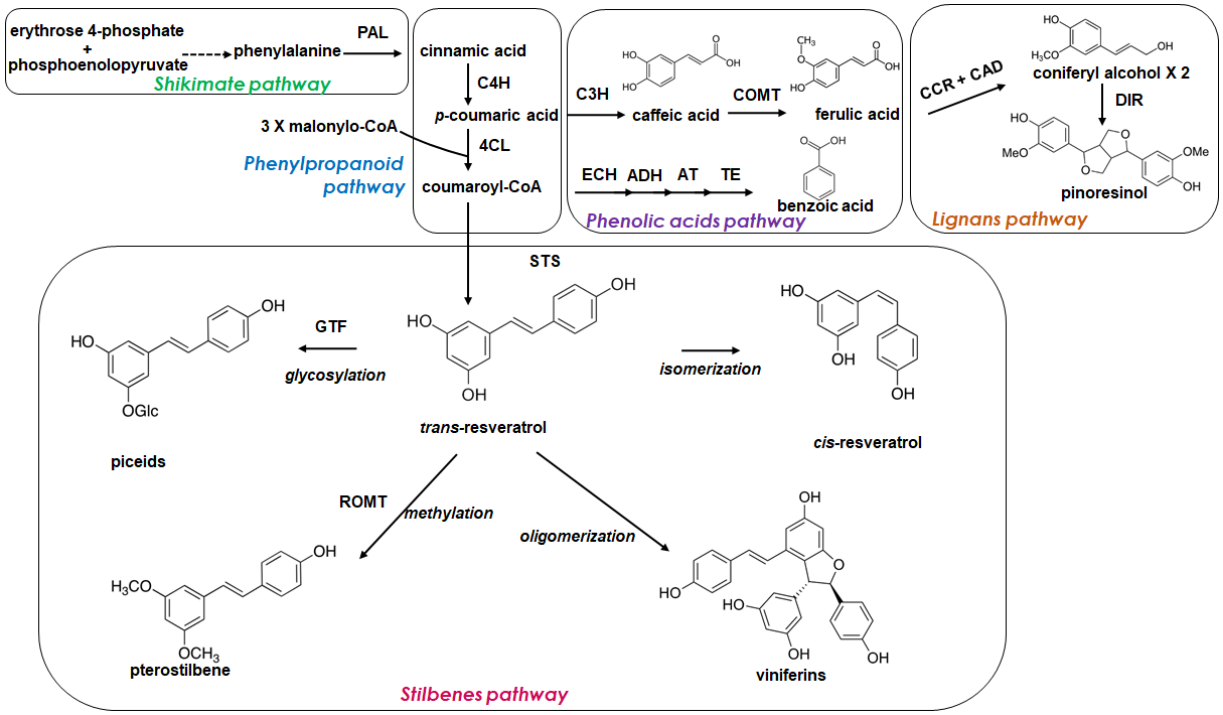

Figure 26. Biosynthesis pathway of non-flavonoids (Marè et al. 2013; Mérillon and Ramawat, 2020; KEGG). 4CL, 4-coumarate-CoA ligase; ADH, alcohol dehydrogenase; AT, acetoacetyl-CoA thiolase; C3H, *p*-coumarate 3-hydroxylase; C4H, cinnamate-4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl-CoA reductase; COMT, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase; DIR, a dirigent protein (enzyme un-known); ECH, enoyl-CoA hydratase; GTF, glycotransferase; PAL, phenylalanine ammonia-lyase; ROMT, resveratrol *O*-methyl-tranferase; STS, stilbene synthase; TE, thiol esterase.

5.2. Functions

Plants seem to have developed the capacity of synthetizing phenolic compounds during their adaptation from aquatic to terrestrial environment. Indeed, polyphenols are essential for the continued survival of all types of vascular plants. They fulfil a very broad range of physiological functions, including as follows: the attraction of pollinating insects via the synthesis of pigments (anthocyanins) or the emission of volatile compounds; the synthesis of structural compounds such as lignin or suberin; the synthesis of defense-related compounds, such as phytoalexins against pathogens or herbivores (coumarins, furanocoumarins, and stilbenes), signaling molecules (isoflavonoids), UV protection molecules (flavonoids), feeding deterrents and wood protectants (proanthocyanidins, condensed tannins); induction of nodulation at the level of roots of legumes for symbiosis with bacteria of the genus Rhizobium (Ferrer et al., 2008; Dixon and Paiva, 1995; Hahlbrock and Scheel, 1989). Phenolic compounds are also extensively studied in human health for their

anti-oxidant and anti-inflammatory properties and their beneficial effect in the prevention of cancer, cardiovascular and neurodegenerative diseases (Fremont 2000; Iriti and Faoro, 2009).

Polyphenols play a crucial role in grapevine defense against biotic and abiotic stresses. In response to pathogens attack, studies have mainly focused on the influence of stilbenes as defensive molecules. However, some phenolic phytoanticipins also have an important role in limiting diseases. For example, flavonols, constitutively biosynthesized in the grapevine leaves, could slow infection by *P. viticola* (Latouche et al., 2013); phenolic acids, such as hydroxycinnamic acids (HCA) have a role of UV-screening epidermal compounds (Kolb and Pfündel, 2005); certain phenolic acids have an antifungal role, particularly in young berries where they are constitutively synthesized from fruit set to veraison (Pezet et al., 2003). The most important phenolic compounds of grapevine are presented below.

5.3. Grapevine polyphenols

5.3.1. Flavonoids

Flavonoid compounds have a C6-C3-C6 structure: two benzene rings are linked by an oxygenated heterocycle to form a flavan unit (Fig. 27). Depending on the degree of oxidation of heterocycle C, flavonoids are divided into several subgroups. The most representive in grapevine are anthocyanins, flavanols and flavonols.

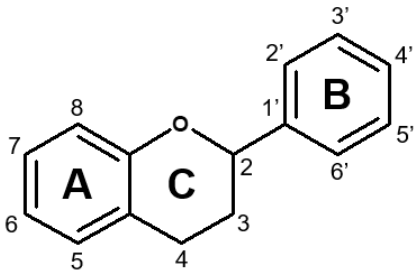

Figure 27. General structure of flavonoids (C6-C3-C6).

5.3.1.1. Anthocyanins

Anthocyanins are pigments of which the hue varies from blue to red depending on the environment (pH, light, temperature), and structure. They are chemically characterized by the presence of a positive charge on the C-ring oxygen also called the flavylium ion.

Anthocyanins are soluble in water and accumulate in vacuoles, in the epidermis of the leaves and in the grape berry skin, where they have a protective role against UV radiation and

oxidative stress, and can attract disseminators (Guidoni et al., 1997). Moreover, an inhibitory effect of cyanidin and of its glycosylated form, on the growth of *B. cinerea* has been established suggesting that the constituent anthocyanins of the grape berry may play a role in the resistance to this fungus (Nyerges et al., 1975). However, anthocyanins are generally not considered as defense molecules in grapevine challenged to biotic stress.

The anthocyanin content is an important quality factor for red wines (200 to 800 mg/l). Currently, more than fifteen anthocyanins have been identified in the grape berry. In most grape varieties, five anthocyanidins are in the majority: cyanidin, delphinidin, petunidin, peonidin and malvidin. Generally, in the form of 3-*O*-*β*-monoglucosides (Fig. 28), they can also be esterified by different acid groups (acetic, coumaric and caffeic) (Spranger, 1993; Archier, 1992). It has been shown that peonidin is the major anthocyanidin of *Teinturier* grape varieties such as Gamay (Ribereau-Gayon, 1982; Archier, 1992; Eder et al., 1994), while the other red grape varieties (Cabernet Sauvignon, Merlot, etc.) contain mainly malvidin 3-*O*-*β*-glucoside. Attempts are made to improve the quantitative and qualitative content of anthocyanins in grape berries through elicitation in order to enhance their pigmentation and thus, the commercial value (Shahab et al., 2020).

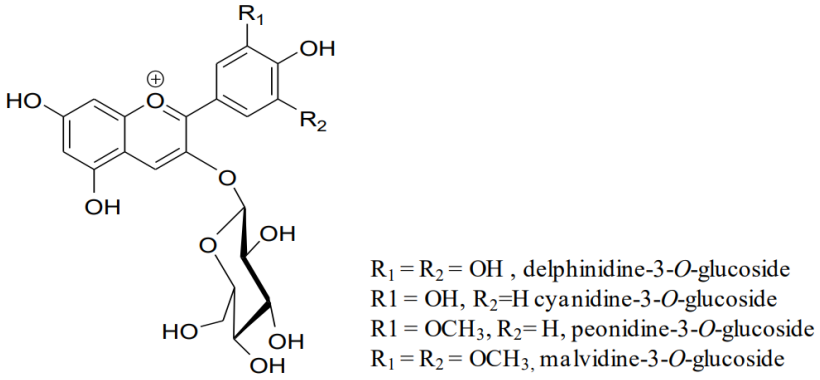

Figure 28. Structures of major anthocyanosides in grapevine.

5.3.1.2. Flavanols

Flavanols are monomeric flavonoids composed of three different classes: flavan-3-ols, flavan-4-ols and flavan-3,4-diols. The former one is the most present in nature. Flavan-3-ols have a general C6-C3-C6 structure with a benzopyran unit (rings A and C) and an aromatic ring (ring B) linked to the C_2 carbon of the pyranic ring (ring C). The difference between each of the flavanol subclasses mentioned above, lies in the model hydroxylation of the pyran ring (cycle C). In the case of flavan-3-ols, the hydroxyl group is found at the C_3 (Fig. 29).

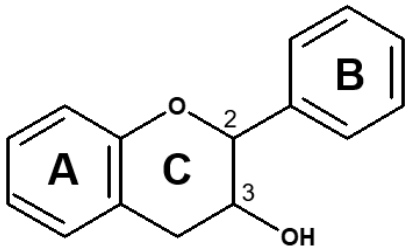

Figure 29. General structure of flavan-3-ols.

Eight flavan-3-ols monomers can be found in grapevines: catechin, gallocatechin, epigallocatechin, epigallocatechin gallate, epicatechin, gallocatechin gallate, epicatechin gallate, and catechin gallate, depending on the tissue (Goufo et al., 2020). From monomeric structures will in particular result oligomerized/polymerized forms called proanthocyanidins (condensed tannins). The best known are the procyanidins, oligomers resulting from the condensation of 2 to 5 monomeric units of catechin, epicatechin or epigallocatechin type.

The parts of the plant that contain most flavanols, especially tannins, are the seeds and the berry skin, with a maximum accumulation at the time of veraison (Souquet et al., 1996; Gagné, 2009; Lacampagne, 2010). These molecules are very studied from an oenological point of view – extracted during winemaking play a large part in the taste properties of wines, in particular red ones (Waterhouse and Teissèdre, 1997; Castellari et al., 2002). More recently, epicatechin vanillate has been identified in the grape seed and red wine (Ma et al., 2018). Among vegetative organs of grapevine, flavanols have been identified in leaves, stems and canes, in different quantities depending on grapevine variety. Apart catechin, epicatechin, and other flavanol monomers present in all these tissues, proanthocyanidins have been also identified. Procyanidin A1, B1, B2 are biosynthesized in leaves, stems, and canes, procyanidin B2, B3 and B4 – in leaves and stems, while procyanidin C1, procyanidin T2, prodelphinidin A-type, and a procyanidin dimer gallate were detected in stems and canes (Goufo et al., 2020).

Flavanols are part of grapevine's defense arsenal. Catechin, epicatechin-3-*O*-gallate, and some of proanthocyanidins inhibit the activity of *B. cinerea* stilbene oxidases (Goetz et al., 1999; Iriti et al., 2005). This is also the case of tannins which seem to contribute to the resistance of certain grapevine varieties to gray mold (Pezet et al., 2003). Several studies have evidenced a role of gallic derivatives and catechetical tannins in grapevine resistance to downy mildew (Calderon et al., 1992; Dai et al., 1995). Certain flavanolic polymers are capable of complexing with proteins or polysaccharides in the plant cell wall. Their location

in the palisade parenchyma and the lower epidermis in resistant grapevine varieties has been partly correlated with an increased tolerance to *P. viticola* (Dai et al., 1995; Mondolot-Cosson et al., 1997). Moreover, catechins negatively affected mycelial growth of *P. viticola* (Dai et al., 1995; Kortekamp and Zyprian, 2003). In the leaves of susceptible cultivar Riesling an enhanced expression of genes encoding enzymes involved in flavanols biosynthesis (CHS, CHI, DFR, F3H, LAR) and their products, was reported after inoculation with *Pseudoperonospora cubensis* and *P. viticola* (Kortekamp, 2006). These genes were not expressed in the leaves of resistant cultivar Gloire due to the native high content of flavonoid compounds. However, rapid activation of the flavanol biosynthetic pathway may indicate increased or induced resistance (Kortekamp, 2006).

5.3.1.3. Flavonols

Flavonols are yellow pigments characterized by the presence of a hydroxyl group (-OH) at the C_3 and a carbonyl group (C=O) at the C_4 of the central heterocyclic ring (Fig. 30). Their diversity is due to the location of additional -OH groups in the molecule, and sometimes additional methyl groups ($-CH_3$).

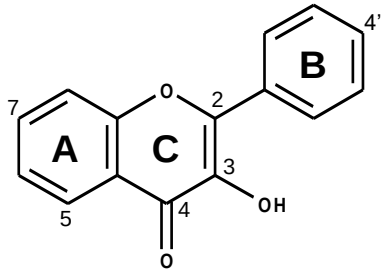

Figure 30. General structure of flavonols.

Flavonols are present in leaves, stems, flowers and berries of all grapevine varieties. Their content in grapes increases from veraison, in parallel with the increased expression of flavonol synthase genes (Downey et al., 2003). Main flavonols in berries are quercetin, myricetin, kaempferol (Waterhouse and Teissèdre, 1997), some quercetin heterosides (rutin, quercitrin, isoquercitrin) (Frankel et al., 1995) as well as dihydroflavonols (astilbine, dihydro-myricetin-3-*O*-rhamnoside) (Vitrac et al., 2001). In the leaves, quercetin, kaempferol, myricetin, apigenin and luteolin are found in aglycon or glycosylated form (Hmamouchi et al., 1996; Monagas et al., 2006). Luteolin, quercetin-3-*O*-glucoside and dihydroquercetin, a flavanonol, have been identified in the grapevine shoots (Püssa et al., 2006). Additionally,

quercetin 3,7,4'-tri-*O*-glucoside, quercetin 3,7-di-*O*-glucoside and quercetin 4'-*O*-glucoside were identified in Polish grapes in such varieties as Ortega, Auxerrois, or Siegerrebe (Kapusta, 2016).

Like anthocyanins, flavonols play a role in UV protection and have an antioxidant activity (Zhang et al., 2013). In wine, flavonols importantly influence the copigmentation by binding with anthocyanins. They serve also as markers useful in grape taxonomy (Flamini et al., 2013). Certain flavonols inhibit the growth of pathogenic fungi and are sometimes considered as phytoalexins (Grayer and Harborne, 1994). For example, quercetin-3-*O*-glucuronide was shown to act as a potential inhibitor of *B. cinerea* stilbene oxidase (Goetz et al., 1999)

5.3.2. Phenolic acids

Phenolic acids (or phenolcarboxylic acids) are compounds structurally formed from a phenolic ring and at least one organic carboxylic acid function. Depending on the carbon units of the side chain attached to the phenolic ring, phenol acids can be divided into compounds C6-C1, C6-C2 and C6-C3. The two most important subclasses are C6-C1 (hydroxybenzoic acid) and C6-C3 (hydroxycinnamic acid) (Fig. 31) (Goleniowski et al., 2013).

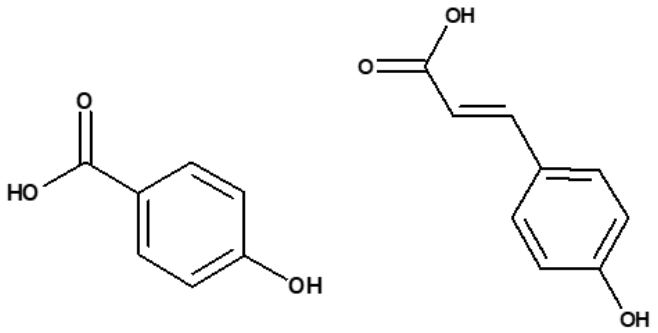

Figure 31. General structure of most important subclasses of phenolic acids. Left: hydroxybenzoic acid, right: hydroxycinnamic acid.

In grapes, major hydroxybenzoic acids are gallic and vanillic acids, and those belonging to the hydroxycinnamic acids are present in free form or esterified with tartaric acid or glycosides (Waterhouse and Teissèdre, 1997). In the roots, *p*-coumaric, ferulic and caffeic acids have been identified (Weidner et al., 2009). Grapevine shoots contain gallic, vanillic,

protocatechic, syringic, ferulic, caftaric and coutaric acids (Goufo et al., 2020). Caffeic and caftaric acids are also present in leaves (Monagas et al., 2006; Hmamouchi et al., 1996).

Phenolic acids play an important role in response to phytopathogens attack. Studies have shown an increase in their biosynthesis during infection, used in their simple forms as signaling molecules, or associated with lignin to strengthen the structure of the cell wall (Cvikrová et al., 2006). These molecules have also shown antifungal activities against *B. cinerea* or the fungi from the Botryosphaeriaceae family, responsible for wood diseases (Lambert et al., 2012; Patzke and Schieber, 2018). Certain phenolic acids in grapes have been shown to inhibit the growth of *B. cinerea* in vitro (Nyerges et al., 1975) and the activity of stilbene oxidases of this pathogen (Goetz et al., 1999). In planta, phenolic acids seem to play a role in the protection against *B. cinerea* in the flower and berry before veraison (Keller, 2002; Pezet et al., 2003).

5.3.3. Stilbenes

Stilbenes (1,2-diphenylethylene) are formed from two phenyl rings linked together by an ethylene bridge generating a C6-C2-C6 structure. This double bond makes it possible to generate stilbenes in one of the two forms: the *trans* (*E*) and the *cis* (*Z*) (Pawlus et al., 2012) (Fig. 32). The aromatic rings are generally substituted by different functions such as hydroxyl, methyl, methoxyl, prenyl or geranyl groups. Monomeric units can also couple resulting in the construction of dimers, trimers, tetramers, up to octamers (Mérillon and Ramawat, 2020). The basic unit of stilbenes is *trans*-resveratrol (*trans*-3,5,4-trihydroxystilbene), the first stilbene identified in Vitaceae, in grapevine leaves subjected to UV or inoculated with *B. cinerea* (Langcake and Pryce, 1976).

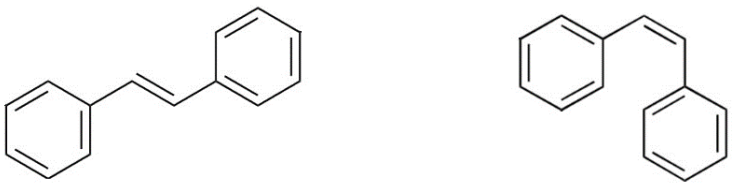

Figure 32. General structure of stilbenes (left: *trans*- form, right: *cis*- form).

Over a thousand natural stilbenes have been described in the literature. Despite this chemical diversity, only a limited number of plant families produce these secondary metabolites, for example Polygonaceae, Cyperaceae, Pinaceae or Vitaceae (Rivière et al.,

2012). In grapevine, stilbenes can be induced or constitutive and are present in all parts of the plant (Roubelakis-Angelakis, 2009) where their synthesis varies depending on the tissue, organ and grapevine variety, as well as on environmental conditions (Creasy and Coffee, 1988). Apart resveratrol, monomeric examples of grapevine stilbenes are its glycosylated (piceid) and methylated (pterostilbene: 3,5-dimethoxy-4'-hydroxy-trans-stilbene) forms, as well as viniferins: dimers, trimers, tetramers and other more complex compounds obtained by oligomerization of resveratrol (Langcake and Pryce, 1976) (Fig. 26, subsec. 5.1).

As it was previously mentioned, the key enzyme of the stilbenes biosynthesis pathway is STS, which catalyzes the formation of resveratrol. The cloning of genes encoding STS has shown that they form a multigenic family (Melchior and Kindl, 1990), and genome analysis of grapevine has allowed to estimate their number between 20 and 40 genes (Velasco et al., 2007; Jaillon et al., 2007). The glycosylation and methoxylation of resveratrol are carried out by respectively glucosyltransferase and *O*-methyltransferase. Interestingly, in *V. labrusca* cv. Concorde one glucosyltransferase produces both glucosides of stilbenes and esters of hydroxycinnamic acids in berries (Hall and De Luca, 2007). A resveratrol *O*-methyl-tranferase (ROMT) and its corresponding gene have been identified in grapevine. Transient expression of *ROMT* in *Nicotiana benthamiana* induced the accumulation of pterostilbene in its tissues (Schmidlin et al., 2008). The expression of this *ROMT* gene was also induced by *P. viticola*. The process of oligomerization could be carried out either via peroxidases or non-enzymatically (Chong et al., 2009).

Stilbenes are present in greater or lesser quantity in all organs of *Vitis* spp. plants (Pawlus et al., 2012). Generally, they are constitutively accumulated in lignified organs, and are particularly inducible in leaves and berries (Bavaresco et al., 2007; Mattivi et al., 2011). They can be thus considered as markers of grapevine defense (Dercks and Creasy, 1989a). In *V. vinifera*, at least 23 monomers, 30 dimers, 8 trimers and 16 tetramers have been identified (Goufo et al., 2020). Morever, in stems of *V. vinifera*, Papastamoulis et al. (2014) identified such oligomers as ampelopsin C, davidiol A, leachianol F, leachianol G, maackin A, as well as a new hexameric stilbene viniphenol A. Around twenty stilbenes have been reported in wines, in larger amount in red ones (Waffo-Téguo et al., 2013; Guerrero et al., 2020). These are in particular resveratrol, piceid, and derivatives, in their free or glycosylated forms, but a tetramer hopeaphenol was also identified (Guebailia et al., 2006). Table 3 provides a non-exhaustive summary of stilbenes identified in different grapevine (*V. vinifera*) organs, expressed constitutively, or upon a stress factor.

The chemical structure of stilbenes is responsible for a blue/purple fluorescence observed on the surface of leaves or berries after exposure to UV radiation. Therefore, beyond quantification, stilbenes can be localized *in situ* (Jeandet et al., 2002).

Table 3. Major stilbenes identified in different organs of diverse *Vitis vinifera* cultivars. (1), monomer; (2), dimer; (3); trimer; (4), tetramer.

Compound (Degree of polymerization)	Organ						References
	LEAVES	GRAPES	STEMS	CANES	WOOD	ROOTS	
***trans*-Resveratrol (1)**	√	√	√	√	√	√	Langcake and Pryce, 1976; Creasy and Coffee, 1988; Pawlus et al., 2013
***trans*-Pterostilbene (1)**	√	√	√				Langcake and McCarthy, 1979; Pezet and Pont, 1988
Piceatannol (1)	√	√	√	√	√	√	Bavaresco et al., 2002; Zga et al., 2009; Pawlus et al., 2013
***trans*-Piceid (1)**	√	√	√	√	√	√	Larronde et al., 2003; Yan et al., 2001; Kim et al., 2014; Lambert et al., 2012; Pawlus et al., 2013
***trans*-Astringin (1)**	√	√	√		√		Landrault et al., 2002; Rusjan et al., 2017; Cantos et al. 2002; Püssa et al., 2006
***trans*-ε-Viniferin (2)**	√	√	√	√	√	√	Langcake and Pryce, 1977; Bavaresco et al., 1997; Pawlus et al., 2013; Boukharta et al., 1996; Reniero et al., 1996
***trans*-δ-Viniferin (2)**	√	√	√	√		√	Pezet et al., 2003; Wei et al., 2016
Pallidol (2)	√	√	√	√	√	√	Landrault et al., 2002; Mattivi et al., 2011; Kim et al., 2014; Amalfitano et al., 2011; Gorena et al., 2014; Wallis and Chen, 2012
Ampelopsin A (2)			√	√	√	√	Jean-Denis, 2005; Reniero et al., 1996
Ampelopsin D (2)	√		√	√	√		Mattivi et al., 2011; Martin et al., 2009; Sáez et al., 2018; Püssa et al., 2006
***trans*-ω-Viniferin (2)**	√	√		√	√	√	Mattivi et al., 2011; Guerrero et al. 2016; Pawlus et al., 2013; Gabaston et al., 2017
Ampelopsin F (2)			√	√	√		Yan et al., 2001
Malibatol (2)			√	√	√		Yan et al., 2001
Parthenocissin A (2)		√	√	√	√	√	Flamini et al., 2013; Gabaston et al., 2019
***trans*-Miyabenol C (3)**	√	√	√	√	√	√	Barjot et al., 2007; Mattivi et al., 2011; Flamini et al., 2016; Esatbeyoglu et al., 2016
α-Viniferin (3)	√		√		√		Langcake and Pryce, 1977; Amalfitano et al., 2011; Wallis and Chen, 2012
Ampelopsin H (4)	√	√	√	√	√		Amalfitano et al., 2005; Mattivi et al., 2011; Flamini et al., 2016
Vitisin A (r2-viniferin)			√	√	√	√	Reniero et al., 1996; Korhammer et al., 1995; Gabaston et al., 2017; Macke et al., 2012; Yan et al., 2001
Vitisin C (4)			√	√	√		Ito and Niwa, 1996
Vitisin B (r-viniferin) (4)			√	√	√	√	Ito and Niwa, 1996; Fujii et al., 2005; Pawlus et al., 2013
Hopeaphenol (4)	√	√	√	√	√	√	Yan et al., 2001; Flamini et al., 2013; Reniero et al., 1996
Isohopeaphenol (4)	√	√	√	√	√	√	Yan et al., 2001; Mattivi et al., 2011; Flamini et al., 2013; Lambert et al., 2012; Gabaston et al., 2017
Viniferol A (4)			√	√	√		Yan et al., 2001

5.3.3.1. Role in grapevine disease resistance and defense

The production of stilbenes is one of the major responses of grapevine following infection by a phytopathogenic fungus (Langcake and Pryce, 1977; Langcake, 1981; Pezet and Pont, 1995). Different pathogens have been reported to induce the production of these phytoalexins in several grapevine experimental models, including *B. cinerea* (Langcake and Pryce, 1976; Langcake and McCarthy, 1979; Langcake, 1981; Bavaresco et al., 1997), *P. viticola* (Langcake and Pryce, 1976; Adrian et al., 1997; Langcake, 1981), *E. necator* (Schnee et al., 2008; Fung et al., 2008), and *Ph. viticola* (Hoos and Blaich, 1990). Certain abiotic stresses are also capable of inducing the biosynthesis of stilbenes, for example, the stress generated by UV radiation (Creasy and Coffee, 1988; Douillet-Breuil et al., 1999), aluminum ions (Adrian et al., 1996), or ozone (Sarig et al., 1996). Finally, various plant defense stimulators (PDS) (elicitors), such as phytohormones and their analogues (e.g., MeJA, BTH, the analog forms of JA and SA, respectively), or plant extracts, are powerful inducers of stilbenes synthesis (Delaunois et al., 2014) (see chapter 6). The enhanced accumulation of stilbenes is generally correlated with an upstream increase in the transcription of the genes involved. Two R2R3-type V-myb myeloblastosis viral oncogene homolog transcription (*MYB14* and *MYB15*) factors regulating the genes of the stilbenes biosynthesis pathway have been identified and reported to be strongly co-expressed with *STS* in response to biotic and abiotic stresses (Höll et al., 2013; Vannozzi et al., 2018). The induction of *STS* in grapevine occurs in two stages: a rapid induction of *STS* following stress, and a later induction. The two-stage induction of *STS* has been observed in *V. vinifera* cv. Pinot noir and Chardonnay after UV treatment, and resveratrol synthesis is correlated with the level of *STS* transcripts (Borie et al., 2004).

Transgenesis experiments with *STS* genes have greatly contributed to the demonstration of the role of stilbenic phytoalexins. The transfer of a grapevine *STS* gene under the control of a strong promoter in tobacco confers an increased resistance to *B. cinerea* in transformed plants compared to control plants (Fischer and Hain, 1994). Grapevines transformed by a construction of a *STS* (*Vst1*) gene under the control of an inducible promoter were produced (Coutos-Thévenot et al., 2001). In these plants, the accumulation of *trans*-resveratrol was markedly increased (by a factor of 200) with correlation to their very high resistance to *B. cinerea*.

5.3.3.1.1. Antifungal activity

Antifungal activity of stilbenes has been extensively studied due to the high susceptibility of *V. vinifera* to cryptogamic diseases. In fact, stilbenes have long been known for their antifungal properties (Erdtman, 1939). The inhibitory effect of resveratrol was tested for the first time on *B. cinerea* and *P. viticola*, but it showed a low inhibitor activity (IC_{50}>200 µg/ml) (Langcake and Pryce 1977, 1979). It has to be noted that tested at high concentrations, resveratrol is not soluble. Other experiences showed that resveratrol decreases the germination of *B. cinerea* spores with an IC_{50} of 90 µg/ml (Adrian et al., 1997) and that of *P. viticola* with an IC_{50} of 110 µg/ml (Dercks and Creasy, 1989). Resveratrol also inhibits the growth of *Ph. viticola* (Hoos and Blaich, 1990), however, its activity remains relatively low (Pezet et al., 2004). Among the stilbenes already tested, *α*- and *ε*-viniferins give better results: IC_{50} at 100 and 97 µg/ml, respectively, on the germination of *B. cinerea* spores and IC_{50} at 35 and 19 µg/ml, respectively, on the germination of *P. viticola* spores. Pterostilbene was revealed to be even more effective than viniferins: for example, its IC_{50} was between 18 and 24 µg/ml for the inhibition of germination of *B. cinerea* spores (Langcake and Pryce, 1979). *δ*-Viniferin, discovered in 2003 in leaves, was just as active as pterostilbene on *P. viticola* (Pezet et al., 2003). Piceid, the glucoside of resveratrol, does not show an efficacy on *P. viticola* (Pezet et al., 2004): glycosylation decreases the activity and might be necessary for the plant to ensure the storage and/or transport of stilbenes. Beyond the simple observation of the direct antifungal effect of some of these compounds, the concentrations at which they are active are compared with the concentrations recorded *in planta*. In the case of pterostilbene, *α*-, *ε*-, and *δ*-viniferins, the inhibitory concentrations are of the same range as those found in the infected plant by *P. viticola*, but the comparison is less accurate in the case of infection with *B. cinerea* (Roubelakis-Angelakis, 2009).

Mechanisms of action of stilbenes responsible for their multiple fungistatic and fungicidal effects generally rely on their capacity of causing inhibition of germination and mycelial growth, disorganization of the membrane system of mitochondria, inhibition of respiratory activity of conidia, destruction of ribosomes, endoplasmic reticulum and membranes (Sbaghi, 1996). It has also been shown that stilbenes could inactivate certain fungal enzymes containing –SH groups at their active site, such as cellulases, xylanases or pectinases. The effects of stilbenes on fungal cells are described in a review of Pezet and Pont (1995). Not all stilbenes have the same efficiency. It depends on their structure and in particular on the electronic effects (Pont and Pezet, 1990). It seems that the more the

substituents of the rings are electron-withdrawing, like the groups –OH and –OCH_3, the more polarized the molecule, the stronger is its antifungal activity. The polarization of the molecule could promote the interaction of stilbenes with proteins membranes leading to depolarization of the membrane and its alteration. The effects of substituents are accentuated by the presence of several conjugate systems present in all stilbenes which correspond to benzene rings, characteristic of polyphenols and to the double bond between these rings which is specific to stilbenes. The activity of the molecule also depends on its lipophilic nature, and thus, on its ability to interact with biological membranes (Schultz et al., 1990). The low lipophilicity of resveratrol would explain its limited activity. On the contrary, the strong activity of pterostilbene would come from the capacity of the –OCH_3 groups to create hydrogen bonds with the membrane proteins. Finally, it seems that the size of the molecule and its isomerization can play a role in its level of activity since the *cis* forms and some dimers have an inhibitory effect higher than that of *trans*-resveratrol (Pezet and Pont, 1990; Gabaston et al., 2017). Canes, woods, and roots are parts of grapevine with a high content of biologically active complex stilbenes. Thus, it is proposed to use extracts from these by-products as a biological plant protection measure. Indeed, complex stilbenes from grapevine shoots can inhibit in vitro the growth of *P. viticola* and several fungi involved in wood diseases in vines (Lambert et al., 2012; Schnee et al., 2013; Gabaston et al., 2017).

Detoxification of stilbenes by phytopathogens, as a part of their pathogenesis, has been studied. For example, *B. cinerea* is able to degrade resveratrol and pterostilbene through the activity of a laccase-like stilbene oxidase (Hoos and Blaich, 1990). The strains capable of oxidizing resveratrol are more virulent than those which do not have this ability, thus, the detoxification process is an important factor in the pathogenicity of these strains (Sbaghi et al., 1996). It was demonstrated that *P. viticola* can detoxify ε-viniferin (Dercks and Creasy, 1989), however, the mechanism of such effect has not been elucidated so far.

5.3.3.1.2. Role in native resistance of *Vitis* plants

Correlation of constitutive stilbenes content and the disease resistance level of *Vitis* plants can be hypothesized from all the observations mentioned above. Indeed, the presence of stilbenes *in planta* plays a crucial role in plant defense mechanisms, for example, in triggering the hypersensitive reaction. A rapid accumulation of resveratrol and δ-viniferin was shown in cell lines of *V. rupestris* (a resistant variety) in response to harpin, which would be

at the origin of the initiation of the hypersensitive response inducing cell death (Chang et al., 2011). These reactions were not observed in cell lines of Pinot noir, a susceptible variety. Resveratrol induces a cascade of reactions leading to cell death, including rapid alkalinization, accumulation of transcripts encoding PR proteins, oxidative burst, and microtubule disorganization with actin filament aggregation (Chang et al., 2011). In addition to acting as a phytoalexin, resveratrol could therefore be considered as a signal molecule.

Constitutively synthesized stilbenes are considered to be important agents of passive defenses (Chong et al., 2009). For example, the *cis*- and *trans*- forms of resveratrol accumulate in the skin (exocarp) of ripening berries (Jeandet et al., 1991), and they are thought to form a natural constitutive barrier against fungi and bacteria which preferentially develop at this stage of berry development (Gatto et al., 2008). The leaves and berries are naturally low in stilbenes, but these compounds are strongly induced in the case of a fungal infection: for example, the inoculation of *B. cinerea* on leaf, flower or berry induces a strong accumulation of stilbenes (Langcake and Pryce, 1976).

Comparison of the production of stilbenes in susceptible and resistant grapevine varieties, especially other species of the genus *Vitis* and interspecific hybrids, is widely effectuated in order to assess the defense potential of different cultivars in a variety improvement program. The production of these phytoalexins can be used as selection criterion for resistant grapevine varieties (Gindro et al., 2006). Indeed, the sensitive and resistant varieties of grapevines are essentially differentiated by the nature of the stilbenes produced.

The comparison of grapevine varieties with different sensitivity to *B. cinerea* showed that the higher the concentration of resveratrol in the tissues, the lower the fungus causes lesions (Langcake and McCarthy, 1979). Other studies have been able to demonstrate a positive correlation between the presence of resveratrol and ε-viniferins in the leaves of certain grapevine genotypes (including the resistant species *V. rupestris* and *V. cinerea*), and simultaneous resistance against *B. cinerea*, the stilbenes concentrations exceeding the concentrations necessary for the inhibition of the growth of the fungus in vitro (Douillet-Breuil et al., 1999).

Similar results were obtained for *P. viticola* (Dercks and Creasy, 1989). The resistant grape varieties accumulated more ε- and δ-viniferin, compounds that are strongly antimicrobials, than susceptible grapevine varieties (Pezet et al., 2004; Alonso-Villaverde et al., 2011). Other oligomers of unknown structures, described as resveratrol dimers, dimethylated resveratrol dimers and a resveratrol trimer were produced in lower quantities in downy mildew-infected grapevine leaves (Jean-Denis, 2006). However, their effect on this

pathogen is not known. In another study, stilbenes accumulated at high concentrations at sites of infection of resistant grape leaves (*V. vinifera* cv. Solaris) infected with *P. viticola*, compared to a susceptible cultivar (*V. vinifera* cv. Chasselas) (Pezet et al., 2004). In *V. vinifera* cv. Solaris, *P. viticola* does not grow, suggesting that stilbenes derived from this cultivar are toxic to mycelial growth. In fact, in the susceptible Chasselas variety infected with downy mildew, a large quantity of relatively non-toxic piceid is synthesized, whereas in the resistance variety Solaris, resveratrol is converted into toxic ε- and δ-viniferins. Similar observations were made for grapevine and *E. necator* interaction (Schnee et al., 2008). The production of ε- and δ-viniferins is positively correlated with the resistance of the plant to this pathogen. In addition, along with the induction of PR genes, the elicitation of the production of these compounds by MeJA protected the grapevine against *E. necator* (Belhadj et al., 2006).

Moreover, the location of stilbenes accumulation varies depending on the stress applied to the leaves. For example, after inoculation of *P. viticola*, stilbenes content increased specifically in the mesophyll and more particularly in the stomata, which are the entry points of the mycelium to the mesophyll and at which the sporangiophores develop. Following a stress by irradiation with UV radiation, stilbenes are homogeneously synthesized over the entire surface of the leaf (Hamm et al., 2010). A study on sensitive and partially resistant grapevine leaves showed a correlation between the presence of the resistance allele *Rpv1* and the accumulation of stilbenes in the mesophyll, where downy mildew mycelium grows. Susceptible plants lacking the *Rpv1* resistance allele showed synthesis of stilbenes only in the epidermis (Bellow et al., 2012). Thus, leaf resistance may be related to the location profile of stilbenes in leaf tissue.

6. STRATEGIES OF GRAPEVINE PROTECTION AGAINST DISEASES

6.1. Conventional farming

Since the 1850s, the increase in world trade has resulted in the dispersion of grapevine diseases and the emergence of certain pathogens in areas that were free from them. Today, the most significant damages in vineyard are engendered by cryptogamic diseases caused by *B. cinerea*, *E. necator*, and *P. viticola*. The solution that has been widely used against these agents in grapevine since the 19th century is the application of copper-based fungicides, then synthetic, altogether classified as conventional methods. Protection against fungal diseases is

generally well controlled, but have non-negligible financial, environmental and health costs because the treatments are repeated regularly during the period of sensitivity to certain pathogens, such as downy mildew. Moreover, due to the development of fungal resistant strains (Toffolatti et al., 2020), the efficiency of fungicides has to be maintained through the alteration of treatments with different mechanisms of action or with new active substances (Corio-Costet et al., 2011; Dufour et al., 2011).

6.1.1. Copper-based fungicides

The antimicrobial effect of copper-based solution (Bordeaux mixture, in reference to the wine region) was accidentally discovered on downy mildew in 1882 by Alexis Millardet (Gessler et al., 2011). Copper-based fungicides are applied today in organic viticulture against crown gall (*A. vitis*), excoriose (*Ph. viticola*), and downy mildew. The copper formulations are preventive, non-systemic methods which remain on the surface of the leaf without acting inside the vascular system. The nature of the Bordeaux mixture as a contact, and not as a penetrant product, explains its propensity to be leached away by rain leading to the loss of effectiveness, thus, the augmentation of number of treatments may be necessary in the case of heavy rains (Anatole-Monnier, 2014). Copper-based products form a physical barrier on the leaf inhibiting the development of the pathogen before its penetration to the tissue (Gisi and Sierotzki, 2008), acting as a broad spectrum biocide due to its interaction with nucleic acids, interference with active enzymatic sites and transport system energy, as well as disruption of the integrity of cell membranes (Lamichhane et al., 2018).

Unfortunately, the intensive use of copper-based fungicides for over a century has led to a certain number of consequences related to human health and biodiversity. These products have direct and indirect negative effects, such as phytotoxicity, development of resistance (e.g., *Xanthomonas campestris*, a bacteria responsible for necrosis on the grapevine leaves (Marques et al., 2009)), accumulation in the soil (Wang et al., 2009), or impact on fruit quality. The repeated use of Bordeaux mixture on grapevine (four treatments in three weeks) leads to a modification of content of sugars, organic acids, lipids and polyphenols in grapes, similar to metabolic deregulation (Martins et al., 2014). Moreover, content of copper close to the maximum residue level (MRL) was identified in grape products as well as in water sources which may be potentially harmful for human health (García-Esparza et al., 2006; Mackie et al., 2012; Mirlean et al., 2005; Tariba, 2011).

6.1.2. Synthetic fungicides

The development of synthetic chemicals to replace copper-based fungicides dates already back to the period of World War II, which had led to a shortage of copper. The research focused on developing contact chemicals including sulphur in their formulations (Gessler et al., 2011). These products, like Bordeaux mixture, form a barrier between the leaf and the pathogen with inhibitory activities at enzyme sites, interference with transport systems or further modulation of nucleic acid synthesis. The diversity of action of these surface products have allowed to characterize these products as "multisite fungicides" which impedes the development of pathogen resistance (Corio-Costet et al., 2011). However, these contact fungicides are sensitive to rain washout and require several applications depending on climatic conditions. In addition, these products are generally not very selective and may have negative effects on non-target organisms which are beneficial for the ecosystem. Over the past 30 years, research has given rise to more specific fungicides, based on targeted activity of pathogen metabolism (single-site fungicides), thus, having few side effects on other non-target processes or organisms. Unlike contact products, most single-site fungicides have the ability to penetrate into the leaf and are thus protected against washing away by rain (penetrating fungicide). Some of them are also systemic and move to untreated parts of the plant thanks to vascular networks (systemic fungicide) (Gisi and Sierotzki, 2008). However, the most single-site inhibitors have a high risk of generating resistance in the fungi. Indeed, the targeted activity can more easily lead to adaptation of the pathogen.

The use of synthetic fungicides is not without risk to human health (especially for farmers who are directly exposed to these products (Flamini and De Rosso, 2006)), and environment (Wightwick et al., 2010). Ecological consequences concern soil, earthworm populations, as well as surface and groundwater quality (Komárek et al., 2010). Crops, such as grapevine, are directly affected (Petit et al., 2009), as well as the production of wine (Jermini et al., 2010), in which pesticide residues have been identified (Basa Cesnik et al., 2008). Pesticides can affect natural yeast communities (Milanovic et al., 2013), and wine aroma (González Álvarez et al., 2012).

The progressive discovery of the toxicity of both copper-based and synthetic pesticides for humans and environment is gradually leading to the withdrawal of these active substances from the market. The European Union introduced a Directive on Sustainable Use of Chemical Pesticides (2009/128/EC) which aims to enhance the use of eco-friendly alternatives (Czaja et al., 2014; Kvakkestad et al., 2020). The possible approaches include

agronomic management of phytosanitary risks (prophylaxis, cultural practices, reasoned control) and alternative strategies, such as the improvement of resistance by genetic methods (production of hybrids or transgenesis), biological control, the use of antimicrobial plant products, and stimulation of natural defenses through the use of elicitors. Some of them are described below, with emphasis on elicitation, which is the subject of the current thesis.

6.2. Alternative or complementary methods

6.2.1. Varietal improvement

Grapevine genetic improvement programs generally target two main diseases: downy and powdery mildews. New varieties have to combine durable resistance and a berry quality suitable for the production of high quality wine. Genetic programs and crossing strategies have emerged to identify resistance traits (called quantitative trait loci, QTL) allowing lasting resistance in grapevine (Merdinoglu et al., 2018; Poltronieri et al., 2020). The main players in grapevine variety creation are European countries (France, Germany, Italy, and Switzerland). In France, since 1974, INRAE has been developing a program that aim to incorporate original resistance factors, carried by *Muscadinia rotundifolia*, a species very resistant to powdery and downy mildews, into the European *V. vinifera*. This program resulted in a series of genotypes (called "Bouquet"), which were then, in a new breeding program, crossed with wild species of American and Asian grapevines. The generated material, called "ResDur", must reinforce the durability of resistance by pyramiding several resistance genes. Four of the "ResDur" grapevine varieties (Artaban, Vidoc, Floreal and Voltis), combining resistance genes, were registered in 2018, and a series of 20 additional varieties will be released by 2024 (Schneider et al., 2019).

Although this technique allows to carry out introgressions of resistance genes while keeping organoleptic qualities intact, the process of creating resistant grapevine varieties is long. Merdinoglu et al. (2018) report that performing a genetic program based on a pyramidalization of seven genetic resistance traits requires 16 years of work. The rapid evolution and variable in consumer demands in terms of sensorial quality of wine can be a barrier of development of this method which requires nearly two decades of experimentation. Moreover, the large-scale cultivation of these varieties does not exclude the ability of pests to circumvent the obtained resistance. Indeed, the powdery and downy mildews populations of grapevine have a strong evolutionary potential, illustrated by the speed at which these two

pathogens have already responded to pressure from fungicides by developing resistance to almost all products. Several cases of bypass to QTL have been already described in some of the newly obtained varieties (Andrivon and Savini, 2019).

6.2.2. Microbiological biocontrol agents

The term biocontrol is defined as the use of living organisms and/or substances as methods of crop protection. Microorganisms that can be used for biocontrol against pathogenic agents belong to three major biological groups: i) fungi, yeasts, oomycetes, and eukaryotic organisms; ii) bacteria and actinomycetes; iii) viruses (Gwynn, 2014). Three main modes of action of biocontrol agents have been described: direct destruction of pathogen or inhibition of its development, competition with the pathogen, and interaction with the pathogenesis process (Köhl et al., 2019).

Direct inhibition or destruction of the pathogen can mobilize two mechanisms: antibiosis (the antagonist organism produces secondary metabolites toxic to the pathogen) and hyperparasitism (the antagonist penetrates the tissues of the pathogen, and results in its destruction via the colonization of its organs). Substances responsible for antibiosis have been characterized in various species of bacteria and fungi, in particular *Bacillus subtilis*, *Pseudomonas fluorescens*, *Streptomyces* sp., or *Trichoderma* sp. (Yendyo et al., 2017). A disadvantage of the use of these microbiological agents is the risks of toxicity of molecules produced for environment, users and consumers, and the emergence of resistant strains in the target pathogen. An example of hyperparasitism is the fungus *Ampelomyces quisqualis* which parasitizes the grapevine's *E. necator* (Legler et al., 2016). The use of hyperparasites presents constraints, such as the need for direct contact with the pathogen itself and swift action to ensure its destruction.

Certain microorganisms (bacteria, yeasts, filamentous fungi) can inhibit the germination of conidia in the pathogenic through competition for nutrients such as nitrogen, carbon or macro- and microelements. This mode of action is particularly effective against pathogenic fungi that the spores need a source of nutrients to initiate their germination. Reducing the rate of germinated spores and slowing down the mycelial growth of the pathogen limits the number of infections and the expansion of lesions. Competition for nutrients has for example been established for the antagonist fungus *T. harzianum* T39 for control of *B. cinerea* (O'Neill et al., 1996).

Interaction with the process of pathogenesis can mobilize three distinct mechanisms: (i) interference with the pathogenicity of the pathogen, in particular by degrading certain hydrolytic enzymes, or by reducing their effectiveness (via for example a modification of the pH of the medium); (ii) a modification of the surface properties of the organs of the host culture, for example, of the wettability of the leaves that hamper the adhesion process and growth of pathogen; (iii) induction of natural resistance in the host plant by the production of elicitors of various chemical natures, e.g., *Bacillus subtilis* QST 713, the active bacteria of the product Serenade®, currently available in the market for example in France and Poland to fight against diseases of several crops, including respectively grapevine or fruit trees (Rahman, 2016).

The effectiveness of biocontrol agents is governed by complex factors. Under cash crop conditions, their survival, activity and stability will depend on: the environmental context (the fluctuation of microclimatic conditions, variations in nutrient availability on the host surface); cultural practices (the variety cultivated, fertilization of the plant or other phytosanitary treatments applied); the quality of the biocontrol product (e.g., formulation) and the method of application (the establishment of induced resistance in the plant requires a certain delay); characteristics of the target pathogens (rapid-onset diseases are more difficult to restrain than slow-onset monocyclic diseases).

Several authors underline the potential interest of the use of microbiological agents against downy mildew. For example, a suspension of *Fusarium proliferatum* spores is able to reduce infection due to *P. viticola* on leaf discs but also in the vineyard (Falk et al., 1996). *T. harzianum* T39, which can induce the defenses of grapevine, gives it a local protection of more than 80% compared to the control (Perazzolli et al., 2008). *Bacillus subtilis* KS1 has proved its effectiveness on leaves and berries, which can probably act by antibiosis, i.e., production of antifungal antibiotic iturin A, that showed toxic activities against several fungi of grapevine including *P. viticola* (Furuya et al., 2011). Recently characterized *B. subtilis* GLB191 and GLB197 also demonstrated the capacity of inhibition of *P. viticola* colonization (Zhang et al., 2017; Li et al., 2019).

During the last decade, biocontrol was the category of non-chemical alternatives that increased the most around the world (Robin and Marchand, 2019; Kvakkestad et al., 2020). In France, several products based on microorganisms have been marketed for grapevine protection. Beside those mentioned above, *Bacillus amyloliquefaciens* FZB24 (Taegro®) can be applied to fight against downy and powdery mildews, and gray mold, through antibiosis, and induction of natural resistance. Against grey mould several other products are available of

which the active substance employ different modes of action, i.e. antibiosis and/or induction of natural resistance, competition for space and/or nutrition, combined. These are *Bacillus amyloliquefaciens* (Amylo-X WG®), *Aureobasidium pullulans* DSM 14940 -14941 (Botector), *Saccharomyces cerevisiae* LAS02 (Julietta/Hiva), *Metschnikowia fructicola* NRRL Y-27328 (Noli), *B. subtilis* QST 713 (Rhapsody or Serenade Max), *Bacillus amyloliquefaciens* MBI600 (Serifel), *Trichoderma atroviride* SC1 (Vintec). Three products are marketed against eutypiosis, Esca, and BDA, i.e., *Trichoderma asperellum* and *gamsii* ICC080 and ICCO12, *Trichoderma atroviride* l-1237, *Trichoderma atroviride* SC1. These microorganisms act by colonization of wounds, antibiosis, and competition for space and nutrition (https://ecophytopic.fr/proteger/liste-des-produits-de-biocontrole). Several of these products are also available in Poland (https://www.gov.pl/web/rolnictwo/rodki-ochrony-roslin-spelniajace-wymogi-produkcji-ekologicznej).

6.2.3. Antimicrobial plant products

The active substances and organic biocidal preparations are derived from plant extracts and consist either of crude extracts comprising a mixture of compounds, or of purified molecules, able to exhibit direct toxicity toward phytopathogens. Most of these biologically active substances inhibit either the growth of colonies or hyphae, or the formation or germination of spores (Stojković et al., 2013). Plant extracts can also contain eliciting components, such as polysaccharides, that induce the natural defenses in host plant (Eder and Cosio, 1994). Natural products of antimicrobial activity have the advantage to be biodegradable, respectful of the environment and potentially little or no harmful for the human health.

There is an abundant literature about antimicrobial properties of plant natural products (for review, see Shuping and Eloff, 2017). First approaches consisted on the study of plants widely used in folk medicine. A lot of research has been focused on the grapevine protection against downy mildew by the use of crude extracts. A pioneering study on *Inula viscosa* (Asteraceae) was initiated to control *P. viticola* in vineyard conditions (Cohen et al., 2006). For an inhibition of 90% of downy mildew, a relatively high concentration (between 3 and 3.7 g/l) of *Inula viscosa* extract was required. These following plant extracts have also given encouraging results against downy mildew: sage (*Salvia officinalis*) (Dagostin et al., 2011), neem (*Azadirachta indica*) (Rajeswari et al., 2008) willow (*Salix alba*), wormwood

(*Artemisia absinthium*) (Andreu et al., 2018), common rush (*Juncus effusus*), *Verbesina lanata* (Thuerig et al., 2016), larch (*Larix decidua*) (Thuerig et al., 2018), grapevine (*V. vinifera*) (Schnee et al., 2013; Richard et al., 2016), or common spruce (*Picea abies*) (Gabaston et al. 2017b). Total inhibition of downy mildew on leaf discs and *in planta* was estimated at concentrations around 1000 mg/l. The passage in the vineyard tends to require higher applied doses but the use of formulations would limit this problem as seems to show the work of Thuerig et al. (2018). The anti-oomycidal activity is due to compounds with antimicrobial properties, usually produced by the plant as phytoalexins. In the forestry and grapevine by-products, phenolic compounds were demonstrated as responsible for anti-mildew activities (Thuerig et al., 2018; Richard et al., 2016). In grapevine canes, these include in particular stilbenes, especially tetramers, such as r-viniferin (vitisin B) and hopeaphenol, as well as *ε*-viniferin dimer (Schnee et al., 2013).

Recently, a strategy based on the valuation of plant waste or by-products from agriculture or industrial operations is considered as a promising alternative to conventional methods of protection. Indeed, these low cost abundant residual plant materials contain antimicrobial compounds respectful of nature. The utility of stilbenes extracts obtained from grapevine waste tissues, i.e., canes, wood, and roots, as a tool of disease control has been proposed (Guerrero et al., 2016; Pawlus et al., 2013). An anti-mildew activity (IC_{50}) was particularly high for wood and roots extracts, due to the presence of high content of tetrameric stilbenes (r-viniferin (vitisin B), hopeaphenol, r2-viniferin (vitisin A), and isohopeaphenol) (Gabaston et al., 2017a; Gabaston et al., 2019).

An original approach consisting on the production of highly antimicrobial oligomeric stilbenes from natural resveratrol through a hemisynthesis has been developing (El Khawand et al., 2020). The obtained reaction mixture containing *δ*-viniferin, parthenostilbenin B, oxistilbenins A and B, exhibited a higher (of 8 fold, in regard to canes extracts) inhibitory effect against *P. viticola* on leaves as well as antimicrobial activity against *B. cinerea* in vitro (El Khawand et al., 2020).

A recent study evidenced the ability of a commercial plant extract of a confidential composition (Arysta Lifesciences-Laboratoires Goëmar, Saint-Malo, France) to protect grapevine from *P. viticola* (Krzyzaniak et al., 2018). A strong direct effect on the release and motility of *P. viticola* zoospores was revealed. The activity of the plant extract applied consisted also on the activation of several defense-related responses, such as the production of H_2O_2, the up-regulation of genes encoding PR proteins and STS, as well as the accumulation of resveratrol and piceid (Krzyzaniak et al., 2018).

Examples of natural products tested against other grapevine pathogens can be cited. Saponins from *Quillaja saponaria* were demonstrated to limit the growth of the mycelium as well as germination of spores from different strains of *B. cinerea*, and inhibit the nematode vectors of GFLV (Fischer et al., 2011). Lethal effects on the nematodes *X. index, Meloidogyne incognita, Globodera rostochiensis* and *Heterodera carotae,* were obtained through the application of saponins from *Medicago* spp. (Argentieri et al., 2008). Grapevine canes extracts containing stilbenes (*E*-piceatannol, *E*-resveratrol, isohopeaphenol, *E*-ε-viniferin and *E*-vitisin B) showed insecticidal effects against *Spodoptera littoralis* larvae, one of the most destructive phytopathogen attacking several crops, including grapevine (Pavela et al., 2017)

Despite numerous purified molecules or natural extracts showing antimicrobial effect and/or inducing defense reactions have been widely reported (for review see Delaunois et al., 2014), only few of them ensure an actual resistance against pathogens in vineyard conditions (Walters et al., 2013; Delaunois et al., 2014). In France, biocidal preparations that have been approved for grapevine protection, include eugenol, geraniol, and thymol (Mevalone/Nirka/Yatto) of fungicide activity, designed for the treatment of gray mold, and sweet orange oil (Limocide/Essen'ciel/Prev-AM/Prev-AM plus) acting as fungicide and elicitor (PDS), for treatment of mainly downy and powdery mildews (https://ecophytopic.fr/proteger/liste-des-produits-de-biocontrole). The latter product is also permitted in the Polish market (Limocide) for the protection of several crops, such as apple and pear trees, cabbages, or strawberry (https://www.gov.pl/web/rolnictwo/rodki-ochrony-roslin-spelniajace-wymogi-produkcji-ekologicznej).

6.2.4. Elicitation (stimulation of plant natural defense)

Elicitation (from Latin *elicere*: excite, arouse, trigger) is a promising strategy of plants protection that has been extensively developing in order to reduce the synthetic pesticides inputs. It consists on the use of biodegradable, non-invasive to human health factors of both biotic and abiotic origins, called elicitors, Plant Defense Stimulators (PDS), or Natural Defense Stimulators (NDS). The two latter terms are imposed for those used in crop protection, as for instance in France and Poland. Elicitors, when applied on the plant, induce its natural defense reactions, and lead to the development of resistance to a subsequent attack by a pathogen (Eder and Cosio, 1994; Klarzynski and Fritig, 2001).

The discovery of elicitors is linked to that of the components of plant immunity (see chapter 3). The existence of plants defense mechanisms against bioaggressors was foreboded at the beginning of the 20th century by researchers studying the phenomenon of plant resistance to diseases (Andrivon and Savini, 2019). The study of these mechanisms with biochemical methods, in the 1970s, allowed to highlight molecules of microbial origin or derived from phytopathogenic fungus capable of triggering the production of antimicrobial metabolites by the plant (Keen, 1975; Angelova et al., 2006). One of the earliest, prominent examples is the research of Albersheim et al. (1977) who first isolated oligosaccharides capable of activating plant defense genes.

Today the term "elicitor" more broadly refers to any product that triggers defense reactions of any type (Eder and Cosio, 1994; Boller and Felix, 2009). In some cases, elicitors lead to an enhanced ability of plant to mobilize faster and more specific defense reaction to the subsequent pathogen attack (priming) (Conrath et al., 2006). It seems, that most induced resistance phenomena are a result of a combination of direct stimulation and priming (Ahmad et al., 2010). The exogenous application of elicitors aims to mimic an attack by a pest and to trigger preventively the plant defenses. Elicitors can induce either local and/or systemic disease resistance (Vallad and Goodman, 2004).

A great diversity of biotic elicitors is known today, of different chemical nature, including purified molecules or crude natural extracts. They can directly originate from plants, microorganisms, minerals (exogenous elicitors), or be released from a part of the subjected plant as a consequence of the action of pathogen's enzymes (endogenous elicitors) (Eder and Cosio, 1994; Boller and Felix 2009; Dodds and Rathjen, 2010). Pathogen-secreted effectors are classified as elicitors as well (Dodds and Rathjen, 2010). Phytohormones, such as SA, JA, ET and their natural or synthetic derivatives also act as elicitors when applied exogenously on plant (Iriti et al., 2005; Repka et al., 2004). Most non-hormonal elicitors are considered to be PAMPs, and therefore are supposed to elicit the PTI. It is recognized that a PAMP is recognized by many plant species, but many exceptions exist. Hormonal elicitors are likely to activate a response similar to that mobilized during ETI, which is a more powerful type of plant immunity response; however, it was confirmed only in some cases. It has to be mentioned, that the microorganisms used in biological control of plant diseases often have a mode of action involving the induction of resistance through the release of molecules acting as elicitors (see section 6.2.2.) (Walters et al., 2013). Some abiotic stress factors can also be considered as elicitors. For example, the induction of stilbenes accumulation was reported in

the grapevine leaves (*trans*-resveratrol and *trans*-*δ*-viniferin) and berries (*trans*-resveratrol) upon UV-C irradiation (Larronde et al., 2003; Pezet et al., 2003).

The use of elicitors for plant protection was proposed in the 1980s, and soon (the 1990s), a first synthetic PDS, acibenzolar-*S*-methyl (ASM)/benzothiadiazole (BTH) (Bion®, Ciba-Geigy, today Novartis) was approved as a phytosanitary product (Walters et al., 2013). In France, ASM/BTH (Bion®, Syngenta) is currently approved against wheat powdery mildew, bacterial diseases of tomato, or chrysanthemum rust. Since then, many publications reported other elicitors, of various chemical natures (saccharides, proteins, etc.), origins (the cell wall of plants, fungi, bacteria, phytopathogenic oomycetes, and others), and the mode of action in studied plant (for review see: Bektas and Eulgem, 2015). Between the end of the 1990s and the beginning of the 2000s, the research in this subject significantly developed and aimed to increase the efficiency of PDS under crop production conditions. Several natural PDS of variable efficacy based on laminarin, chitosan, harpin or plant extract were marketed. Today, numerous scientific publications on PDS and their effects, continue to appear. However, the commercial offer of PDS remains restrained, and practical applications are still limited. Detailed description of development of induced resistance as a crop protection, including a commercial perspective, can be found in the chapter of Walters et al. (2013).

6.2.4.1. Natural and synthetic elicitors tested on grapevine

The latest review about the state of research on elicitors as a mean of controlling grapevine pathogens in the laboratory and in the field is that of Delaunois et al. (2014). Several PDS products are available on the market in France for grapevine protection, or for other crop plants in Poland (see section 6.2.4.2.). Many unauthorized substances are the subject of numerous studies against various diseases in grapevine. The elicitors tested are most often of natural or synthetic origin. Description of a non-exhaustive list of example molecules which have shown an eliciting action in different experimental models of grapevine, in particular against downy and powdery mildews, or gray mold, is presented below.

The study of early defense mechanisms in grapevine, was mainly carried out via the application of endopolygalacturonase 1 (T4BcPG1) of *B. cinerea* T4, and laminarin, a linear polymer of *β*-1,3-glucan extracted and purified from the brown algae *Laminaria digitate* (Aziz et al., 2003; Poinssot et al., 2003). In cell cultures these elicitors caused a quick and

temporal oxidative burst, and an increase of intracellular calcium ion concentration. Laminarin also protected grapevine leaves from infections with *B. cinerea* and *P. viticola* (Aziz et al., 2003), and it has been commercialized to protect grapevine against powdery mildew. The application of a sulphated laminarin (PS3) on the leaves of a susceptible grapevine variety has allowed to elucidate defense reactions that occur at the site of elicitor's application. PS3 acted through JA and led to the generation of H_2O_2, callose production, deposition of phenolic compounds, and in response to *P. viticola*, caused a hypersensitive type reaction (Trouvelot et al., 2008; Adrian et al., 2017). Furthermore, biomarkers of PS3-induced grapevine resistance have been proposed, such as erythritol phosphate, a derivative of 2-*C*-methyl-D-erythritol 4-phosphate involved in the terpenes biosynthesis pathway (MEP) (Adrian et al., 2017); sesquiterpene VOCs, (*E*,*E*)-α-farnesene (Chalal et al., 2015); proteins 12-oxophytodienoate reductase (OPR-like) related to the JAs biosynthesis pathway (OPDA), and an arsenite-resistance protein (Serrate-like protein) (Lemaître-Guillier et al., 2017). Other plant extracts such as those of rhubarb root and buckthorn bark are able to significantly reduce the development of *P. viticola*, with induction of δ-viniferin synthesis (Gindro et al., 2007).

An example of an endogenous elicitor are oligogalacturonides (OG), i.e., oligomers of α-1,4-linked galacturonosyl residues, released on partial degradation of the plant cell wall homogalacturonan. OG function as DAMPs, and have shown their efficacy in protection against *B. cinerea* (Aziz et al., 2003) and against *P. viticola* (Allègre et al., 2009). More recently, a complex of oligochitosan and oligopectates (a PAMP and a DAMP) called COS-OGA has been shown to be effective against powdery mildew in grapevine, with a protection rate of nearly 80% in the vineyard (van Aubel et al., 2014). COS-OGA is currently available on the French market to be used as grapevine protection against downy and powdery mildews.

Among biotic elicitors of fungal origin, chitosan (deacetylated derivative of chitin), has been shown to be effective against many fungal diseases such as gray mold, downy and powdery mildews through the induction of accumulation of phytoalexins in the grapevine leaves (Trotel-Aziz et al., 2006; Iriti et al., 2011). Ergosterol, a component of the membrane of fungi, is able to stimulate the expression of a gene encoding a STS and to increase the level of resistance against *B. cinerea* (Laquitaine et al., 2006). A microbiological biocontrol agent, *T. harzianum* T39, induced resistance of a susceptible grapevine cultivated in greenhouse against downy mildew by priming effect, through the activation of JA/ET signaling pathways (Perazzolli et al., 2008).

Bacterial rhamnolipids have been described as elicitors of non-specific immunity of grapevine recognized by the plant as MAMPs (Varnier et al., 2009). Due to their property as

surfactants, they quickly penetrate into cells and therefore do not require formulation for the application. In addition to a direct action on the spores of *B. cinerea*, rhamnolipids provided a better protection of grapevine against this pathogen thanks to the stimulation of early defense responses, such as calcium ion influx, ROS production and the phosphorylation cascade (Varnier et al., 2009).

Vitamins B1 and B2 have been described as inducers of resistance against *P. viticola* (Boubakri et al., 2013). Thiamine (vitamin B1) also allowed the activation of early defense reactions, induced a HR and consequently triggered leaves resistance to infection by *P. viticola* (Boubakri et al., 2012).

Extracts of a protein nature have also been reported as capable of inducing resistance against downy mildew. Protein hydrolysates from soybean and casein provided a protection of grapevine against *P. viticola* by 76 and 63%, respectively (Lachhab et al., 2014). The mechanisms of action of these elicitors tested on grapevine cell suspensions involved the up-regulation of PR-proteins genes and *STS*, along with the accumulation of resveratrol, δ- and ε-viniferins (Lachhab et al., 2014).

Among elicitor of phytohormonal nature, MeJA, a JA analogue, was shown to reduce the severity of powdery mildew by 70% (Belhadj et al., 2006). It was the result of the induction of a large number of defense responses, such as up-regulation of transcript levels and/or proteins as PR-proteins (chitinase, β-1,3-glucanase, serine protease inhibitor, polygalacturonase-inhibiting protein), peroxidase, cell wall extension and enzymes involved in the phytoalexin biosynthetic pathway such as PAL and STS, in correlation with the accumulation of stilbenes (Repka et al., 2004; Belhadj et al., 2006). Similar results were obtained in the study of the effect of ethephon (ET releasing substance) on grapevine. Respectively 64 and 70% of protection was obtained against *E. necator* on detached leaves and foliar cuttings, due to the accumulation of certain PR-proteins and stilbenes (Belhadj et al., 2008a).

One of the most common synthetic elicitor is β-aminobutyric acid (BABA), a non-protein amino acid. BABA was shown to induce local and systemic resistance in the grapevine leaves against downy mildew (Reuveni et al., 2001; Harm et al., 2011). It is considered as to be able of potentiating defenses against this pathogen via the production of NADPH oxidase dependent ROS (Dubreuil-Maurizi et al., 2010) or accumulation of pterostilbene (Slaughter et al., 2008). However, the effectiveness of BABA decreases on grapevines, when climatic conditions are more favorable to downy mildew (Reuveni et al., 2001), in addition, it is slightly phytotoxic at high concentrations (Hodge et al., 2012).

The most encouraging results of synthetic elicitors tested provides already mentioned ASM/BTH, as well as mono- or diphosphate salts (phosphonates or phosphites), both of them being registered for crop protection in many countries, to fight mainly against mildews. BTH and phosphonates act as priming compounds, allowing a more rapid, efficient, and/or intense activation of defense responses upon secondary biotic or abiotic stress (Conrath, 2009).

As a SA analogue BTH induces the onset of SAR with the up-regulation of typical SA defense reactions. In grapevine, BTH treatment have been shown to stimulate the up-regulation of many PR-protein genes, especially those coding for glucanase and chitinases, as well as genes encoding *VvSTS and VvROMT*, two enzymes implicated in the stilbene biosynthesis, or *VvLOX* (lipoxygenase) and *VvGST* (glutathione *S*-transferase) genes expression (Bellée et al., 2018). It was demonstrated as capable of protecting against gray mold, with approximately 30% reduction in the severity of grapes (Iriti et al., 2005; Bellée et al., 2018), or against powdery or downy mildews with growth inhibitions of 60 to 98% depending on the isolates (Dufour et al., 2013; Harm et al., 2011). Analysis tools ("BioMolChem") have been developed to highlight and characterize the defensive state of grapevine in response to a pathogen or to elicitors such as BTH, giving an account of, among others, the expression of more than 20 genes involved in defense (Corio-Costet et al., 2013).

Phosphonates display a complex mode of action: (i) direct inhibition of pathogen development, mainly against oomycetes, (ii) indirect action as inducer of the release of stress metabolites from the pathogen that elicit defense responses, (iii) direct stimulation of plant defense responses (Lobato et al., 2010). Moreover, phosphites can be considered as biostimulants that improve plant yield and quality (Gómez-Merinoa and Trejo-Téllez 2015). Phosphonates have been used for the control of *Phytophthora* diseases in several plants for more than 30 years (Hardy et al., 2001). These products have remarkable efficacy against oomycetes, at least 70-80% (Andrivon and Savini, 2019). Phosphite-induced resistance has been proposed to be dependent to SA and JA/ET pathways (Eshraghi et al., 2011), or to be SA and JA-independent (Dhar Burra et al., 2014). Consequently, a wide range of host defenses can be activated following phosphite treatment. In grapevine, phosphonates induced local and systemic resistance against mildew, through both the stimulation of defense responses and direct antifungal activity (Smillie et al., 1989; Dufour et al., 2013, Massoud et al., 2012). However, their mode of action on a specific immune signaling pathway remained unclear. Despite being fungicides, phosphonates' favorable ecotoxicological profile makes them very interesting products to limit the use of copper-based or synthetic fungicides. However, field trials show that in general they are almost completely effective in case of moderate disease

pressure, but are insufficient in case of strong infection and must then be associated with a low dose fungicide. Moreover, classification of phosphonates as SDP is the subject of controversy. Certainly, their eliciting properties are proven, but at the doses used their fungicidal effect seems to be predominant (Andrivon and Savini, 2019). Another debate concerns their classification as synthetic products. Phosphonates are not authorized in organic farming, contrarily to salts of copper, however, both types of products are minerals. Finally, accumulation of phosphonates in harvested products needs to be mentioned (Forrer et al., 2017).

6.2.4.2. The use of elicitors: from the laboratory to the vineyard

Most of the PDS studied are experimental products or not yet approved in France, or in Poland, as phytosanitary products. In France at the moment (September 2020), ten PDS type of products of synthetic or natural origin (some with additional fungicidal properties, or acting by antibiosis and/or through space competition) obtained a marketing authorization for their use in viticulture (Table 4). Some of the active substances are formulated by several different companies (not mentioned). All of them target downy mildew, powdery mildew, and/or gray mold. In Poland, only three types of PDS are available on the market, i.e., COS-OGA (Fytosave SL), laminarin (Laminone, Nutivax, Plantivax, Vaxiplant SL), and mild strains of Pepino mosaic virus (PepMV, V10®) (https://www.gov.pl/web/rolnictwo/rodki-ochrony-roslin-spelniajace-wymogi-produkcji-ekologicznej, consulted on September 2020). These products are destined for the protection of respectively strawberry or grapevine against powdery mildew; pear and apple trees against, inter alia, fire blight, and grapevine against powdery mildew; tomato against severe strain of PepMV.

Unlike laboratory tests, few elicitor efficacy tests have indeed been performed in the open field. The results are often disappointing, because the products tested, although fully effective in the laboratory, show partial effectiveness in the vineyard (Trouvelot et al., 2016; Walters et al., 2013). For example, in the screening of alternative products that could be applied against *P. viticola,* elicitors have been shown to confer resistance in the range of 40 to 80% compared to untreated controls, while conventional products allowed to achieve resistance at 90 to 100% (Dagostin et al., 2011). However, PDS can help to minimize the quantity of fungicides used. The benefit of combinations of PDS and fungicides at reduced doses has been known for at least 15 years (for example, association between BTH and

synthetic fungicides, synergy between BABA and mancozeb and others (Andrivon and Savini, 2019). Some tests associations of PDS and copper products give encouraging results against the grapevine downy mildew (Romanazzi et al., 2016).

Table 4. Elicitors (Plant Defense Stimulators, PDS) authorized in France for the use on grapevine. According to https://ephy.anses.fr/ (consultation on September 2020), and https://ecophytopic.fr/proteger/liste-des-produits-de-biocontrole.

Origin	Active substance	Targeted disease	Mode of action	Commercial name (®)
Synthetic	disodium phosphonate	downy mildew	fungicide, PDS	Ceraxel/BCPC358FC/Fosika/ Redeli/Sirius/Fructial
	potassium phosphonate	downy mildew	fungicide, PDS	LBG-01F34/Etonan/Pertinan/ Phytosarcan/Savial Forte/Epatan/Kutaisi
Natural	COS-OGA	downy mildew, powdery mildew	PDS	Bastid/Messager/Blason/Bstim/ Fytosave/Esdeaine/Galopin+ Performer SL/Vacazoteli
	Cerevisane (yeast cell walls)	downy mildew, powdery mildew, gray mold	PDS	Romeo/Actileaf/Caromait/Cervitis/ Cezane 941 PM/Romeo Garden
	Bacillus amyloliquefaciens FZB24	downy mildew, powdery mildew, gray mold	antibiosis, PDS	Taegro
	laminarin	powdery mildew	PDS	Vacciplant/Iodus 2
	fenugreek extract	powdery mildew	PDS	Stifenia
	Bacillus amyloliquefaciens	gray mold	antibiosis, PDS	Amylo-X WG
	Bacillus subtilis QST 713	gray mold	space competition, antibiosis, PDS	Rhapsody, Serenade Max
	Bacillus amyloliquefaciens MBI600	gray mold	antibiosis, PDS	Serifel

New PDS of various origins are regularly revealed in scientific publications: metabolites or extracts of microorganisms, fatty acids (such as hexanoic acid, promising on citrus fruits), secondary metabolites of plants (methyl salicylate), plant extracts more or less characterized (Andrivon and Savini, 2019). However, most of these elicitors, even with efficiency proven in the field, seem to be abandoned. For example, an extract of *Penicillium chrysogenum* ("Pen") was shown to be effective, among others, against *P. viticola* of grapevine, but has never been commercialized (Thuerig et al., 2006). This fact highlights the existence of obstacles to the development of PDS, which requires the control of many factors: impact of environmental conditions, grapevine variety effect, age of the plants, persistence, impact of pressure from disease bioavailability within the plant, the influence of the formulation, as well as commercial profitability (Walters et al., 2013).

6.1.4.3. Factors influencing the effectiveness of elicitors

Unlike a fungicide which acts directly on the pathogen, the induction of resistance through elicitation solicits the plant. Therefore, the effectiveness of PDS is conditioned by, obviously, the three protagonists alone, i.e., elicitor, plant and pathogen, but also by more global factors such as environmental conditions that also can modulate the effectiveness themselves, as well as influence the relationships between these three players (Walters et al., 2013; Héloir et al., 2019).

The effectiveness of elicitors in protecting a plant against disease depends first of all on the doses employed. For example, extracts of green algae (*Ulva armoricana*) used at dilutions of 1:9 to 1:36 induced protection against powdery mildew of grapevine, but the effectiveness decreased for the strongest dilutions of this extract (Jaulneau et al., 2011). In contrast, chitosan induced protection of grapevine against *B. cinerea* at 50 µg/ml, but higher concentrations (up to 300 µg/ml) did not lead to further reduction of the lesions size (Trotel-Aziz et al., 2006). Moreover, formulation of PDS is essential, since it must cross the cuticle and then the cell wall in order to be perceived by the plant. For example, adjuvant in the formulation of PS3 allows to increase its bioavailability in grapevine by facilitating the penetration into leaf tissues, thus, increasing the level of resistance induced against downy mildew (Paris et al., 2016). The chemical structure of elicitors is also important. For example, chitooligosaccharides have a capacity to induce defenses (PAL, H_2O_2, cell death), depending on their degree of polymerization and acetylation (Cabrera et al., 2006). On the other hand, even if no difference in protection has been revealed with acetylated or non-acetylated

oligogalacturonides against wheat powdery mildew, the types of defenses induced may differ (Randoux et al., 2010). Beyond the active principle alone, it is also within the agronomic context that the effectiveness of an elicitor can be variable. Frequency of treatments can be a key factor as well: weekly applications of BTH have shown better protection against *Xanthomonas* sp. on tomato, compared to bi-weekly applications (Huang et al., 2012). Finally, the combination of elicitors with reduced doses of conventional fungicides (or other control methods) within a phytosanitary program can increase, or conversely decrease or have no effect on the overall efficacy, depending on the pathosystem considered (Delaunois et al., 2014).

In addition, the variability in PDS efficacy may be due to disease pressure. It has been shown that, in apple trees, harpin-induced resistance to *Penicillium expansum* was inoculum dose-dependent (Capdeville et al., 2003). Likewise, the product Milsana® (knotweed extract) did not protect tomatoes against powdery mildew in the field when parasite pressure was high (Konstantinidou-Doltsinis et al., 2006). Moreover, for the same dose of inoculum, variations in effectiveness can also occur and are linked to the degree of aggressiveness of the strains. Indeed, a study carried out on 41 strains of *B. cinerea* presenting different levels of severity, revealed that the efficacy of tomato protection provided by a biocontrol agent was the more marked, the less aggressive the strain was (Bardin et al., 2013). Finally, for the same host plant, a given elicitor protects against one, or two pathogens, but rarely against a broad spectrum of different genera. However, it is difficult to evidence it with the help of bibliographic references, since scientific articles generally more readily report positive efficacy results in a given pathosystem.

The influence of the genotype on the plant's response to elicitors was also reported (for review Bruce, 2014; Walters et al., 2013; Dufour et al., 2011; Pagliarani et al., 2020). For example, the effectiveness of protection provided by *T. harzianum* T39 against downy mildew depended on the grapevine variety. However, defenses induced by BTH, provided nearly 90% protection for the fourteen grapevine varieties tested (Banani et al., 2014). The age of the organs treated is also to be considered, because for certain plants such as grapevines, treatments with an elicitor such as PS3 will be more effective on older leaves (Steimetz et al., 2012), while for others like BABA, they will be on younger leaves (Sharma et al., 2010). Finally, elicitation may entail a metabolic and energetic cost in plants (Heil, 2002). Several studies have proven a reduced level of both defense-related responses and protection against pathogens provided by elicitors, in plant cultivated under conditions of limited water or nutrients supply (Aljabal et al., 2015). Balance between growth and defense needs to be

therefore ensured to keep the plant in the good physiological state, and as a consequence, increase its responsiveness to elicitors. One of the solutions is the use of biostimulants, i.e., fertilizer-like substances, that have been gaining interest for the use on grapevine (Krzyzaniak et al., 2017; Corio-Costet et al., 2018).

Environmental conditions can act on the different elements of the interaction elicitor-plant-pathogen in very complex ways. For example, relative humidity and temperature can create favorable conditions for the penetration of the active molecules into plant organs (Baur, 1999). However, under these same conditions, the development of the pathogen can also be promoted. Reduced water inputs (e.g., little rainfall, soil drainage) can result in the attainment of water stress, which can reduce the plant's response to an elicitor in certain cases (Aljabal et al., 2015). Other parameters, such as light (quantity, quality, duration), thermal stress (cold or hot), certain soil characteristics (salinity, heavy metals, pH), precipitation, UV radiation, cultural practices and human activities, could potentially be factors influencing the efficiency of elicitors, especially since plants are exposed to several of them simultaneously under field conditions (Atkinson and Urwin, 2012).

II. AIMS AND OBJECTIVES OF THE RESEARCH

Innovative protection strategies for replacement or complementation to reduce synthetic pesticide inputs is a priority in vitiviniculture. Stimulation of plant defenses, referred to as elicitation, may represent a promising pest control method. Exploiting the mechanisms of natural resistance is at the basis of this strategy in which plants are treated with elicitors (PDS, Plant Defense Stimulators). Elicitors have the advantage of being biodegradable and non-toxic compounds to the environment. Through the activation of signaling pathways, these substances induce the expression of different defense reactions. These are PR proteins accumulation, the cell wall strengthening, and phytoalexins biosynthesis, of which the most studied in grapevine are polyphenols, in particular stilbenes. In this context, information about other classes of grapevine secondary (specialized) metabolites, such as pentacyclic triterpenoids is more limited.

In the contemporary agriculture, PDS are included as a biocontrol strategy. However, these products are never applied as a unique control solution, but as a complement to synthetic pesticides. Indeed, their effectiveness is often variable according to different factors, in particular to the pathogen and environmental conditions. In order to develop more reliable elicitor-based strategies for vineyard protection, studies elucidating the mechanism of action of PDS, are strongly required. In particular, it will be essential to propose biological control methods aiming to fight against the pathogens responsible for major grapevine diseases having a high economic impact and decreasing berry and wine quality, as *Plasmopara viticola* (downy mildew). PDS induce defense responses, based mainly on specialized metabolism. However, such activation may occur at the expense of other metabolic pathways functioning. Thus, in grapevine treated with PDS, primary metabolism, including those of sterols (essential structural components of cell membrane) should be followed in order to ensure the plant vigor, important for the quality of grape berries and grape-derived products.

The aim of the undertaken research was to examine the responses of grapevine *V. vinifera* to selected elicitors of different modes of action: methyl jasmonate (MeJA), implicated in jasmonic acid (JA) signaling pathway, benzothiadiazole/acibenzolar-*S*-methyl (BTH/ASM), a synthetic analog of salicylic acid (SA), and phosphonates (PHOS), substances of a double stimulator-fungicide action. Broadly, the responses that were studied included the evaluation of the plant defense status and the follow-up of global metabolic changes in order

to assess an eventual so-called fitness-cost due to elicitation. Mechanisms of action of the applied elicitors were compared to each other in the context of interconnections of the signaling pathways employed.

In an attempt to achieve the mentioned purpose of this thesis, four main objectives were established. The following studies were performed:

1. Triterpenoids were firstly characterized by GC-MS in different experimental models of grapevine. The data on triterpenoids occurring in grapevine are rather scarce, therefore this part of the research is particularly valuable.

1.1. Profiling of triterpenoids was initially performed in the cells of in vitro suspensions cultures of different *V. vinifera* cultivars: Cabernet Sauvignon, Gamay Teinturier and Petit Verdot.

1.2. The profile of triterpenoids was then assessed in the greenhouse foliar cuttings (Cabernet Sauvignon). The leaves, stems, wood, and roots were analyzed.

1.3. Triterpenoid content was additionally assessed in the leaves of both domesticated and wild grapevines growing in the vineyard. The wild *Vitis aestivalis* Michx., *Vitis labrusca* L., *Vitis riparia* Michx., and *Vitis vinifera* subsp. *sylvestris* (Gmelin) Hegi were studied. These grapevines have been used by winegrowers as progenitors or rootstocks of domesticated vines. Their triterpenoid profiles were compared with those of cultivated *V. vinifera* (Alvarinho, Cabernet Sauvignon, Gamay, Marselan, Mauzac and Merlot).

2. The impact of the studied elicitors on grapevine triterpenoids was evaluated by GC-MS analyses. The follow-up of this class of compounds allow to monitor the elicitor-induced changes within both primary and secondary metabolisms. The possibility of an enhancement of pentacyclic triterpenoid production, as compounds bearing several biological activities, with the use of the applied elicitors was investigated.

2.1. In order to decipher the effect of elicitation on triterpenoids as grapevine defense responses, kinetics of changes in the content of these compounds was determined in the cells of *V. vinifera* cv. Cabernet Sauvignon, Gamay Teinturier and Petit Verdot from in vitro suspensions cultures treated with MeJA.

2.2. The changes in triterpenoids were followed in the whole leaves and leaf cuticular waxes of greenhouse *V. vinifera* cv. Cabernet Sauvignon elicited with MeJA and BTH.

Responsiveness of grapevine to elicitation at the level of triterpenoids accumulation has not been reported in literature. In order to precise, among others, the time of sampling, the data concerning other plant species were considered and preliminary assays were firstly

performed. Then different time points of plant material collection after treatments was performed in these two studies.

3. The potential of MeJA, BTH and PHOS on conferring resistance to greenhouse *V. vinifera* cv. Cabernet Sauvignon leaves against *P. viticola* was measured. The aim of this study was to provide an insight into the persistence of the PDS effect on grapevine leaves at relatively long period of time after elicitation (6 days). Common and specific defense responses were studied after elicitation +/- downy mildew inoculation using approaches based on biological, molecular and chemical measurements. The "BioMolChem" method developed by Corio-Costet et al. (2013) was used.

3.1. The protection conferred by the three studied elicitors was estimated on foliar discs generated from the leaves previously subjected to elicitation and inoculated with *P. viticola*.

3.2. The plant defense responses were monitored as changes in the content of polyphenols (stilbenes, flavonols and flavanols) by UHPLC-MS.

3.3. Further analyses concerned the transcript levels of 48 genes implicated in different mechanisms of plant defenses: PR proteins accumulation, secondary metabolites (including polyphenols), indole, and oxylipins biosynthesis, redox status, cell wall reinforcement, hormone signaling. The RT-qPCR (Fluidigm) technique and NeoVigen microarrays were used.

Molecular and chemical measurements were effectuated on the leaves treated by the elicitors, collected after 6 days, and inoculated with *P. viticola*. The leaves inoculated with the pathogen were sampled after 48 h (i.e., 8 days after elicitation and 48 h after inoculation).

4. A thorough metabolomic approach with the use of 1H-NMR spectroscopy was performed. The evaluation of elicitor effect on the plant general metabolism is required in order to obtain the best trade-off between growth, yield and defense. Early (24 h post-elicitation) and long-term (6 days post-elicitation) responses, mainly at the level of primary metabolites including carbohydrates, organic acids, amino acids, amines, as well as some phenolics and their precursors, were studied in the leaves of greenhouse *V. vinifera* cv. Cabernet Sauvignon.

The described objectives are presented in the following chapters of the part IV of this dissertation, i.e., results and discussion, consisting of the corresponding four axes defined above.

III. MATERIALS AND METHODS

All biological experiments were carried out in Villenave d'Ornon, France, at MIB laboratory, UR Oenology, ISVV, University of Bordeaux (in vitro cultures), and at the laboratory and the infrastructure of UMR SAVE (Joint Research Unit, Vineyard Health and Agroecology, INRAE, ISVV) (plants treatments, pathogen inoculation, molecular analyses). Biochemical analyses were performed at MIB laboratory (polyphenols extractions and their analyses; NMR experiments), and in Department of Plant Biochemistry at Faculty of Biology of University of Warsaw, Poland (triterpenoids extractions and their analyses).

1. BIOLOGICAL MATERIAL

Depending on the experiment, the plant material included three experimental models of grapevine: cell suspensions; leaves, stems, wood and roots of foliar cuttings from the greenhouse; leaves of plants from the vineyard. The pathogen studied in one of the experiments was the causal agent of the grapevine downy mildew, the oomycete *P. viticola*.

1.2. Plant material

1.2.1. In vitro cultures

Cell suspension cultures were obtained from calli (maintained on solid culture media) of the following *V. vinifera* cultivars: Cabernet Sauvignon (CS6), Gamay Fréaux var. Teinturier (GT_T strain), Gamay Fréaux var. Teinturier (GT3 strain) and Petit Verdot (PV) (Fig. 33). GT cultures have been initiated from the pulp of young fruits, at the ENSA of Toulouse, in 1978, and were then supplied to the MIB (GESVAB) laboratory in the form of callus, in 1994. The callus of CS6 and PV were established in 1994 in the MIB laboratory from grapevine petioles (Bordeaux, Château Cabannieux and Château Dillon, respectively).

Figure 33. Cell suspensions of *Vitis vinifera* cv. Cabernet Sauvignon (CS6), Petit Verdot (PV) and Gamay Teinturier (GT3) (from left to right).

Cell suspension cultures were cultivated as described by Decendit and Mérillon (1996) and Belhadj et al. (2008b) in the maintenance medium containing B5 macroelements (Gamborg et al., 1968), microelements (Murashige and Skoog, 1962), vitamins (Chupeau and Morel, 1970) with an addition of 58 mM sucrose, and 250 mg/l casein hydrolysate. It was supplemented with either 0.5 µM 1-naphthaleneacetic acid and 1 µM kinetin (for GT strains), or 2.5 µM 1-naphthaleneacetic acid and 0.5 µM 6-benzylaminopurine (for CS6 and PV) (Table 5). The pH of the media was adjusted to 5.8 with KOH before autoclavage. Cell suspensions were weekly subcultured by inoculating cells into fresh medium at a ratio of 1/5 (v/v) in 250 ml Erlenmeyer flasks containing 50 ml of medium. All handling was done aseptically in a laminar flow hood with the use of sterilized materials (erlenmeyers, graduated cylinders...). The cultures were maintained under continuous fluorescent light (5000 lux) at 25±1 °C on an orbital shaker (100 rpm). These culture conditions were previously developed in the laboratory (Larronde et al., 1998).

Table 5. Composition of the maintenance medium for cell suspension cultures of *Vitis vinifera* cv. Gamay Teinturier, Cabernet Sauvignon and Petit Verdot.

MACROELEMENTS (Gamborg et al., 1968; modified)	mg/l
KNO_3	2500
$(NH_4)_2SO_4$	134
$CaCl_2,2H_2O$	150
$NaH_2PO_4,2H_2O$	150
$MgSO_4,7H_2O$	250
MICROELEMENTS (Murashige and Skoog, 1962; modified)	mg/l
$MnSO_4,H_2O$	16,9
$ZnSO_4,7H_2O$	8,6
H_3BO_3	6,2
KI	0,83
$Na_2MoO_4,2H_2O$	0,25
$CuSO_4,5H_2O$	0,025
$CoCl_2,6H_2O$	0,025
$FeSO_4,7H_2O$	27,8
VITAMINS (Chupeau and Morel, 1970)	g/l
myo-inositol	100
nicotinic acid	1
calcium D(+)-panthotenate	1
(+)-biotin	0,01
pyridoxal hydrochloride	1
thiamine dichloride	1
ORGANIC COMPOUNDS	g/l
sucrose	20
casein hydrolyzate	0,25
GROWTH SUBSTANCES	mg/l
kinetin / benzylaminopurine	0,2 / 0,12
naphthalene acetic acid 0,5 µM / 2,5 µM	0,1 / 0,5

1.2.2. Plants

1.2.2.1. Greenhouse cuttings

Grapevine *V. vinifera* cv. Cabernet Sauvignon foliar cuttings (Fig. 34) were supplied by D.Sc. Marie-France Corio-Costet and cultivated by Sébastien Gambier (UMR SAVE, INRAE). The plants have been propagated from the single-eyed wood cuttings provided by Château Couhins (Gironde, France), clone 191. They were placed in sand terrines and after 3 to 4 weeks, the rooted vines were potted in sandy soil. The plants were grown under controlled conditions: 25/20 °C day/night air temperature, 75% relative humidity and a 16 h photoperiod (350 µmol/m^2/s). Two-month-old cuttings with approximately 10 leaves, with no disease or stress symptoms, were generally used for the different experiments.

Figure 34. Foliar cuttings of *Vitis vinifera* cv. Cabernet Sauvignon cultivated in the greenhouse.

1.2.2.2. Vineyard plants

Vineyard grapevines originated from experimental fields in Villenave d'Ornon, France (Fig. 35). Wild *Vitis* spp. studied, i.e., *Vitis aestivalis* Michx., *Vitis labrusca* L., *Vitis riparia* Michx. and *Vitis vinifera* subsp. *sylvestris* (Gmelin) Hegi, were introduced from North America and France between 1963 and 2000 at St Louis experimental field which belongs to INRAE germplasm collection and is managed by the UMR EGFV (Joint Research Unit of Ecophysiology and Grape Functional Genomics). The *V. vinifera* varieties studied, i.e., Cabernet Sauvignon, Gamay, Marselan and Merlot, Alvarinho, Mauzac were planted in 2009 in the "Vitadapt" experimental field and cultivated by UMR 1287 EGFV (INRAE) laboratory team (Villenave d'Ornon, France). All plants were similarly grafted and have been managed by conventional methods. For each grapevine, 10 mature fully expanded leaves (approximately from the sixth position from the apex, and located in the same orientation in the vines) were collected from plants with no evidence of disease or stress symptoms.

Figure 35. Grapevines from the experimental field, Villenave d'Ornon, France.

1.3. Pathogen material

P. viticola isolate (ORG) of the laboratory collection (UMR SAVE, INRAE) has been collected from a *V. vinifera* plant in a vineyard located in Gironde (France) in 2014. It was multiplied as previously described by Corio-Costet et al. (2011). The isolate was subcultured weekly on fresh grapevine leaves (*V. vinifera* cv. Cabernet Sauvignon from the greenhouse) by depositing on their abaxial face 15 µl drops of a 5000 sporangia/ml spore suspension, collected and suspended in sterile water at 4 °C. The inoculated leaves were incubated on a Whatman paper soaked with 3.5 ml of sterile water, in sealed Petri dishes overnight at 22 °C in the dark for stomata opening and zoospore penetration. Twenty-four hours later the water droplets were removed by aspiration with a vacuum pump. The inoculated leaves were incubated for 7 days at 22±2 °C with a 16 h day/8 h night photoperiod (25 µE/m^2/s) for the sporangia production used for the experiments (Fig. 36).

Figure 36. Sporangia of *Plasmopara viticola* (ORG isolate) on *Vitis vinifera* cv. Cabernet Sauvignon.

2. CHEMICALS

2.1. Elicitors

Three elicitors were used for the experiments. Methyl jasmonate (MeJA 95%, methyl (1*R*,2*R*)-3-oxo-2-(2*Z*)-2-pentenyl-cyclopentaneacetate, Sigma®, USA) (Fig. 37) is a natural analog of jasmonic acid, widely tested for its potential for inducing grapevine resistance. In our experiments, it was used formulated with a wetting agent Triton X-100 (Triton) (Sigma®, USA).

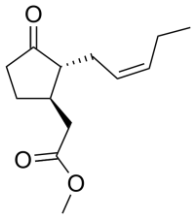

Figure 37. Chemical structure of methyl jasmonate (MeJA).

Benzothiadiazole (BTH, *S*-methyl benzo[1,2,3,]thiadiazole-7-carthioate), also named acibenzolar-*S*-methyl (ASM)) (Fig. 38) was purchased from Syngenta, Switzerland (Bion®, 50WG). It is a synthetic analogof salicylic acid, registred as a plant defense stimulator (PDS) used for protection of several crops.

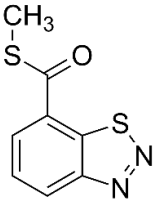

Figure 38. Chemical structure of benzothiadiazole/acibenzolar-*S*-methyl (BTH/ASM).

Phosphonates (PHOS) (LBG-01F34®, De Sangosse, France, formulated, 730 g/l) are mono- and dipotassium salts of phosphorous acid (Fig. 39). It is a registered product against downy mildew on grapevine acting as fungicide and PDS.

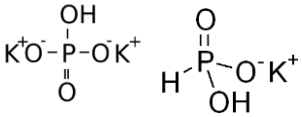

Figure 39. Chemical formulas of mono- and dipotassium salts of phosphorous acid – phosphonates (PHOS).

2.2. Standards and chemical reagents

In the MIB laboratory (UR Oenology, ISVV, University of Bordeaux)

Reagents:

- dipotassium phosphate; monopotassium phosphate; sodium chloride – Sigma-Aldrich;

Solvents:

- chloroform – Sigma-Aldrich;
- ethanol – Merck, Sigma-Aldrich;
- isoamyl alcohol – Merck, Sigma-Aldrich;
- methanol – Prolabo, France;
- methanol-d_4 (99.80% D) – Euriso-Top, St-Aubin and Gif-sur-Yvette, France;

Standards:

Polyphenol analyses:

- all polyphenols were produced and purified in the MIB laboratory of UR Oenology;

Metabolomics:

- acetic acid – Fisher Scientific, UK;
- adenine, alanine, γ-aminobutyric acid (GABA), ascorbic acid, calcium formate, choline, fumaric acid, gallic acid, glutamic acid, glutamine, *myo*-inositol, malic acid, proline, pyruvic acid, succinic acid, syringic acid, threonine, trigonelline, tyrosine, valine – Sigma-Aldrich, St Louis, USA;
- quercetin-3-*O*-glucoside – BioChemika, Sigma-Aldrich, Germany;
- tartaric acid – Fluka, Sigma-Aldrich, Germany;

Other:

- 3-(Trimethylsilyl) propanoic-2,2,3,3-d_4 acid sodium salt (TMSP, 98% D); deuterated water (D_2O) – Euriso-Top, St-Aubin and Gif-sur-Yvette, France;
- Milli-Q® ultrapure water – ElgaLabWater, USA;
- Sep-Pak® C18 cartridge – Sigma-Aldrich;

In the laboratory of Department of Plant Biochemistry, Faculty of Biology, University of Warsaw:

Reagents:

- acetic acid; sodium nitrite; sulfuric acid – Chempur, Piekary Śląskie, Poland;
- hydrochloric acid – Stanlab, Lublin, Poland;
- *N*-nitroso-*N*-methylurea – synthetized at the laboratory of Department of Plant Biochemistry;
- potassium hydroxide; sodium hydroxide – POCH S.A., Gliwice, Poland;

Solvents:

- chloroform; diethyl ether; methanol – Chempur, Piekary Śląskie, Poland;

Standards:

- α-amyrin; β-amyrin; campesterol; faradiol; lupeol; sitosterol; stigmasterol; uvaol – Sigma-Aldrich (Saint Louis, USA);
- oleanolic acid – extracted and purified from the marigold *Calendula officinalis* flowers at the laboratory of Department of Plant Biochemistry
- oleanolic acid methylester – Roth, Karlsruhe, Germany;
- ursolic acid – MB Biomedicals, Germany;

Other:

- silica gel 60 G, Merck, Darmstadt, Germany.

3. TREATMENTS

3.1. Elicitation – design of experiments

Depending on the experiment, distinct treatments were performed. The plant material was harvested at different time points: "0" (for triterpenoids profiling, part IV, chapter 1); hours or days post-treatment (hpt or dpt), frozen in liquid nitrogen, and stored at -80 °C until lyophilisation and/or further analyses. In the subsections below, proceeding and corresponding chapters for the results and discussion (part IV of the dissertation) of each experiment are specified.

3.1.1. In vitro cultures

Grapevine cell suspensions subjected for treatment with MeJA were in the middle of their exponential growth phase, i.e., they aged 7 days. According to the procedure described by Belhadj et al. (2008b), MeJA was dissolved in EtOH, sterilized by filtration (0.22 µm) and added at 50 or 100 µM final concentration to cell suspensions. Control cultures received only the vehicle solvent.

GT_T cell suspensions were subjected to treatment with MeJA at 50 and 100 µM, and the cells were harvested at 24 hpt, 72 hpt and 7 dpt (part IV, chapter 2). The second experiment was performed on GT3, PV and CS6 cell suspensions. They were treated with MeJA at 50 µM, and the cells were harvested at 48 hpt and 7 dpt. Cells (Fig. 40) were harvested by vacuum filtration on a nylon screen cloth (porosity 30 µm).

Figure 40. Cells harvested from grapevine suspension cultures. Left picture: fresh cells of Gamay Teinturier GT3 (above) and GT_T (below). Right picture: dried cells of Petit Verdot (PV), Gamay Teinturier (GT3), Cabernet Sauvignon (CS6) (from left to right).

3.1.2. Greenhouse grapevines

Foliar cuttings were treated separately with either MeJA (dissolved in 1% EtOH), at a final concentration of 5 mM (1.09 g/l), in an aqueous solution containing Triton (0.1% final concentration); or BTH at 2 g/l; or PHOS at 1.5 g/l. The control plants were treated with distilled water or Triton (0.1%). All solutions were sprayed on grapevine leaves using a micro-diffuser (Ecospray®). For each experiment the leaves of entire plants were treated on their adaxial and abaxial surface, in a homogeneous manner (approximately 1 ml of each elicitor solution was sprayed per leaf). The level of harvested leaves on the plant and the time point of harvest depended on the experiment performed. The 3rd, 4th and 5th leaves from the apex are referred as ‘young’ or ‘L3’, ‘medium’ or ‘L4’, and ‘old’ or ‘L5’, respectively (Fig. 41).

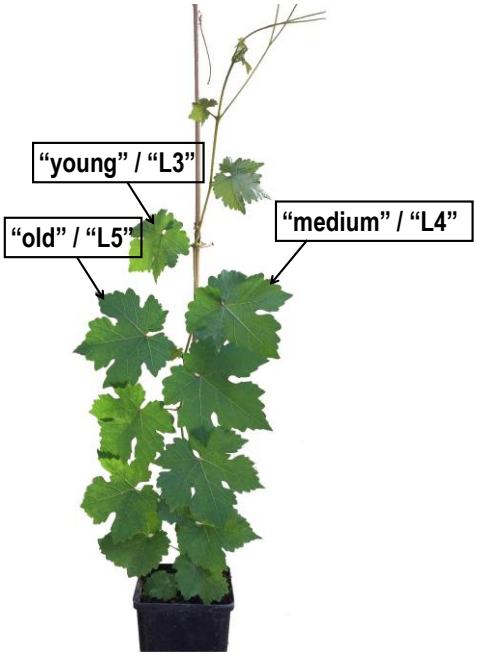

Figure 41. Scheme of leaf levels of greenhouse *Vitis vinifera* cv. Cabernet Sauvignon.

For the experiment aiming to follow the effect of MeJA on triterpenoids content (part IV, chapter 1), 6 plants per modality were used, and the L3, L4 and L5 were harvested after at 7 and 14 dpt. For the study of triterpenoid composition of cuticular waxes upon elicitation with MeJA and BTH, 9 plants per modality were used and the L5 were harvested at 14 dpt.

Fourteen cuttings per condition were used for the study of metabolomics, polyphenols and the level of resistance conferred to the leaves by elicitation with MeJA, BTH and PHOS towards *P. viticola* (disease intensity measurements and gene expression) (part IV, chapters 3 and 4). The L3 and L4 were harvested after 24 hpt and 6 dpt. Metabolomic analyses were performed on the leaves collected at 24 hpt and 6 dpt. Polyphenols and gene expression analysis along with *P. viticola* assays were performed on the leaves harvested at 6 dpt. Design of the latter experiment is resumed in Figure 42. For disease measurement, foliar discs were excised from 8 collected leaves of 4 cuttings sampled per condition. The remaining parts of these leaves were cut in half and subjected for gene expression and polyphenol analyses. Twenty whole leaves of 10 cuttings per condition were thoroughly rinsed with water, inoculated, or not, with *P. viticola*, and collected after 48 h (8 dpt – un-inoculated or 8 dpt-2 dpi – inoculated leaves) for gene expression and polyphenol analyses. Foliar discs were generated on the day of the harvest from these 20 leaves for estimation of *P. viticola* disease intensity.

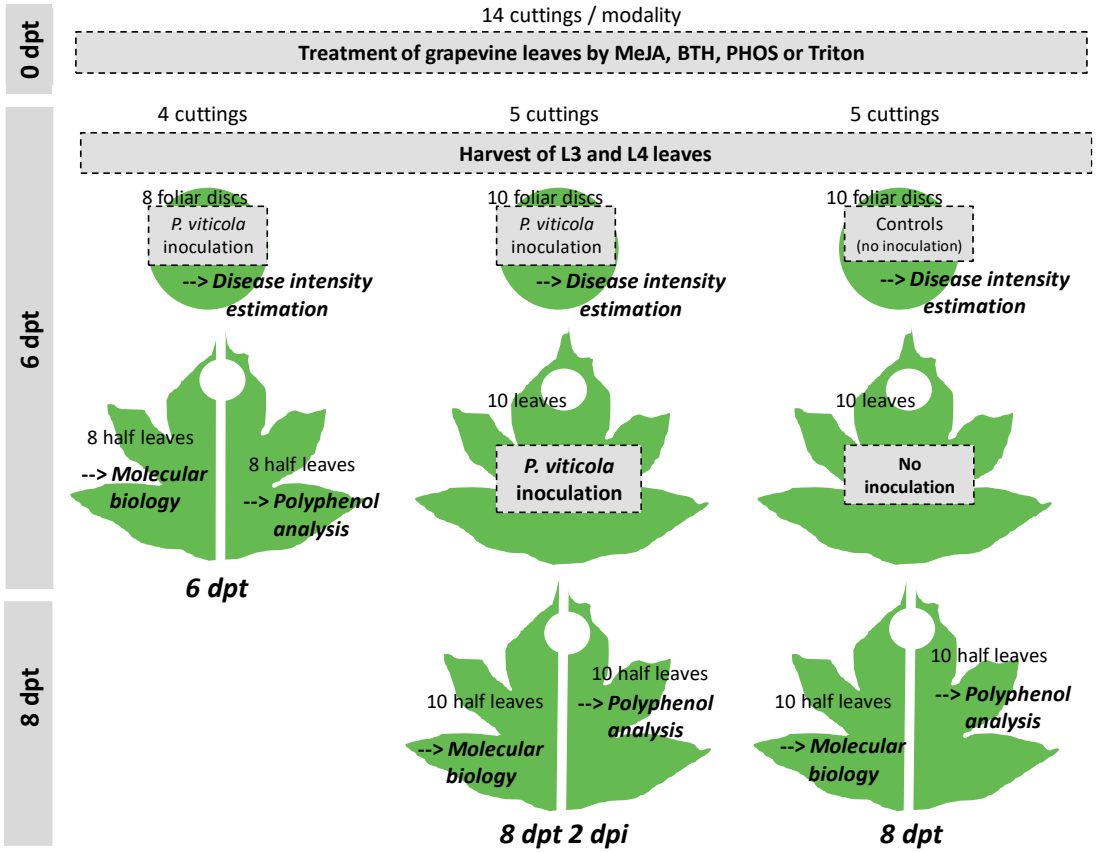

Figure 42. Design of the experiment aiming to investigate defense responses of grapevine leaves differently elicited and/or inoculated with downy mildew.

3.2. Inoculation of leaves

The inoculation was performed using the expertise of UMR SAVE, INRAE (Villeanve d'Ornon), according to the procedure described by Corio-Costet et al. (2011). Each of the detached leaves per modality was placed with the abaxial surface uppermost on a moist Whatman paper in Petri dishes. The leaves were inoculated with 15 droplets of 15 µl of 8500 sporangia/ml spore suspension, and, along with controls (not inoculated leaves), they were incubated overnight at 22 °C in darkness. One day after, the residual droplets were aspirated using a vacuum pump, and all the leaves were placed in the growth chamber under the following controlled conditions: temperature 22 °C and a 16 h day/8 h night photoperiod (25 µE/m^2/s) until sampling.

3.2.1. Disease intensity measurement

Disease intensity measurement was proceeded as described previously by Corio-Costet et al. (2011). From each detached leaf, one 25 mm-wide disc was excised with a pastry cutter and disposed in Petri dishes, on a moist Whatman paper. Inoculation was done as described above. Leaf discs were inoculated with 3 droplets of spores/disc (Fig. 43). After 7 days of incubation, the contamination level was assessed with a visual scale from 0 to 100% of sporulation, as previously described by Dufour and Corio-Costet (2013).

Figure 43. Inoculation of grapevine leaf discs with *Plasmopara viticola* for disease intensity measurement.

4. EXTRACTIONS AND SAMPLE PREPARATION

4.1. Triterpenoids

The preparation of samples for triterpenoid analyses was performed according to the method used by Szakiel et al. (2012b).

4.1.1. Extraction from cell cultures or tissues of whole leaves

The quantity of the freeze-dried plant material taken for triterpenoids analyses depended on availability of material of each experimental model, i.e., cells – 300 mg; leaves, stems, wood, roots from greenhouse plants – 500 mg; leaves from vineyard plants – 1 g. The plant material was grounded to a fine powder in a grinding mortar and extracted with 100 ml of diethyl ether in Soxhlet apparatus for 8 h. The obtained extracts were evaporated to dryness at 40 °C under reduced pressure and stored at RT until fractionation.

4.1.2. Extraction from leaf cuticular waxes

The detached intact fresh leaves (1 g) were dipped holding them by the petioles in 100 ml of chloroform, and gently stirred for 30 sec at RT. The extraction was repeated twice with a new portion of solvent (100 ml). The obtained extracts were concentrated using a rotary evaporator, transferred in a small amount of chloroform into glass vials, evaporated to dryness and stored at RT until fractionation.

4.2. Fractionation of extracts

Whole tissue or cell extracts were redissolved in diethyl ether, while leaf cuticular waxes extracts were redissolved in a Folch solution, i.e., the mixture chloroform: methanol, 2:1 (v/v). All the extracts were fractionated by preparative thin layer chromatography (TLC) on 20 cm × 20 cm glass plates coated with a 0.25 mm layer of silica gel 60 G, previously activated at 120 ºC for 30 min. The solvent system chloroform: methanol 97:3 (v/v) was applied for developing. Three main fractions designed to further analysis were obtained: free (non-esterified) neutral steroids and triterpenes (alcohols, aldehydes and ketones) (R_F 0.3-0.9), triterpene acids (R_F 0.2-0.3) (Fig. 44). The individual fractions were localized on plates by comparison with standards of oleanolic acid, faradiol, sitosterol and α-amyrin. Standards were visualized by spraying the relevant part of the plate with 50% H_2SO_4, followed by heating with a hot-air stream. The gel containing the fractions of interest was scrubbed off the plate, placed in elution columns and eluted in 10 volumes of diethyl ether compared to the volume of gel removed. The fractions containing free neutral steroids and triterpenes were directly analyzed by GC-MS, the fractions containing triterpenes acids were first methylated with diazomethane, and the fraction of triterpenoid esters was subjected to alkaline hydrolysis.

Figure 44. Diethyl ether extract of grapevine leaves fractionated by preparative thin layer chromatography.

4.2.1. Methylation of triterpenoid acids

N-Nitroso-*N*-methylurea (2.06 g) was added to a mixture of 20 ml of diethyl ether and 6 ml of 25% aqueous KOH in a cooled glass separatory funnel. The organic phase containing diazomethane was washed with cold distilled water (3 x 50 ml) to obtain neutral pH, and separated from the aqueous layer. Triterpene acids fractions placed in glass vials were dissolved in 10 ml of the obtained solution of diazomethane in diethyl ether, sealed and incubated at RT for 48 h. Then, the samples were opened and left under a fume cupboard until the evaporation of the solvent.

4.3. Polyphenols

Polyphenols were extracted following the protocol described previously by Belhadj et al. (2006). The freeze-dried grapevine leaves and roots were finely powdered in a grinding mortar and 50 mg of the material was placed in a hemolysis tube. Extraction was made by agitation of the samples with 2.5 ml of methanol overnight at 4 °C. After centrifugation (3500 *g*, 10 min), the procedure was repeated twice at RT over 1.5 h with new portions of MeOH (2.5 ml). The obtained supernatants of each sample were pooled and concentrated using a Speed Vac (Savant, USA). Extracts were redissolved in 1 ml of 30% MeOH and purified through solid phase extraction (SPE) on a Sep-Pak® C18 cartridge using Visiprep™ SPE Vacuum Manifold (Sigma-Aldrich) (Fig. 45). Previously, the columns were conditioned by successive washings with pure MeOH (2 ml), 50% MeOH (2 ml), and distilled water (6 ml). After depositing the sample, washing with 2 ml of distilled water was carried out. Polyphenols extracts were eluted with 4 ml of 90% MeOH, and evaporated to dryness. Samples were redissolved in 1 ml of 50% MeOH, filtered through 0.45 µm polytetrafluoroethylene (PTFE) membrane filters, and stored at -20 °C until analysis. During preparation, samples were protected from light and high temperature.

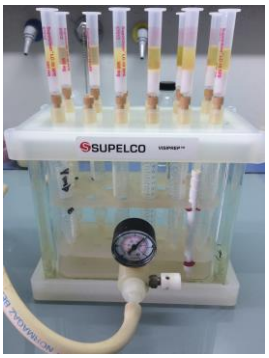

Figure 45. Purification of polyphenols extracts through solid phase extraction (SPE).

4.4. Primary metabolites

For this metabolomic study, we developed a protocol of extraction according to Kim et al. (2010), with a modification for the addition of internal quantification standard (calcium formate) and a NMR chemical shift reference (TMSP, 3-(Trimethylsilyl) propanoic-2,2,3,3-d_4). Extraction buffer was composed of methanol-d_4 and of pH 6.0 KH_2PO_4 buffer (0.1 M). The latter was made according to DeAngelis (2007), i.e., 86.8 ml of 1M KH_2PO_4 13.2 ml of 1M K_2HPO_4 were prepared in deuterated water (D_2O) and mixed in the ratio indicated. For each sample, the buffer contained 750 µL methanol-d_4, 750 µL of phosphate buffer, 0.3 mM TMSP, and 4 mM calcium formate, and was centrifuged at 13 500 *g* for 5 min. The supernatant (1000 µl) served for extraction of freeze-dried and grounded to a fine powder leaves (50 mg). Samples were vortexed at RT for 1 min, ultrasonicated for 20 min and centrifuged at 17 500 rpm for 10 min. The extract (600 µl of the supernatant) was transferred in a 5 mm NMR tube and analyzed by NMR the same day (Fig. 46).

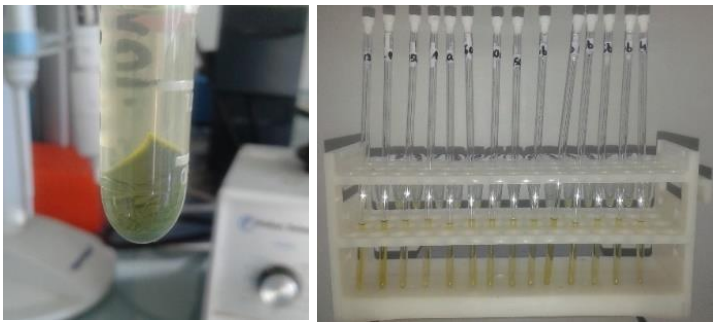

Figure 46. Extracts of grapevines leaves for NMR analyses.

4.5. RNA extraction and preparation for RT-qPCR analyses

Total RNA was extracted from frozen plant material by Dr. Anthony Bellée (UMR SAVE, INRAE), partly with the use of Spectrum™ Plant Total RNA (Sigma-Aldrich) solutions, following the manufacturer's instructions. RNA extraction from leaves was made as it was described by Dufour et al., (2016). RNAase free material and DEPC water was used while working with RNA. The plant material was grounded to a fine powder in liquid nitrogen using a grinding mortar. For each condition, three batches of 200 mg of leaves were done. The plant material was placed in screw cap test tubes. The buffer for RNA extraction from the leaves was composed of 300 mM Tris HCl, pH 8.0; 25 mM EDTA; 2 mM NaCl; 2% cetyl trimethyl ammonium bromide (CTAB); 2% polyvinyl polypyrrolidone (PVPP); 0.05% spermidine trihydrochloride; and 2% β-mercaptoethanol added extemporaneously. The buffer

was preheated to 56 °C and added to the plant material. The mixture was stirred vigorously by vortex and incubated in a water bath at 56 °C for 10 min under regular agitation. Then, an equal volume of a cool mixture of chloroform/isoamyl alcohol (24:1, v/v) was added and the samples were centrifuged (3500 *g*, 15 min). The following steps were conducted using the Spectrum^{TM} Plant Total RNA kit according to one of the protocols proposed by the manufacturer. The proceeding included: filtration of samples on columns from the kit, RNA precipitation with cool 70% EtOH, binding of the obtained nucleic acids on a column, a series of washings with between each step 1 min centrifugation (13 000 *g*) at 4 °C. Before elution of RNA with the "Elution solution" from the kit, the total RNA extracts were treated with DNase I (10 units) on a Nucleospin® RNA plant column (Macherey-Nagel), in order to remove all traces of contaminating genomic DNA. This enzymatic treatment was carried out using the supplier's instructions.

The integrity of the ribosomal subunits was checked by agarose (1.2%, in 1X TAE buffer) gel electrophoresis in the presence of GelRed^{TM} (0.01%, v/v) for the revelation of nucleic acids. One microgram of total RNA was deposited in the presence of a RNA loading dye (Sigma-Aldrich). The migration took place in 1X TAE at a voltage of 100 mV. The observation of two major bands, corresponding to the 28S and 18S ribosomal RNAs (the most abundant in the extracts), indicated that the RNAs were not degraded. The absence of the band corresponding to genomic DNA was noted excluding the contamination.

The concentration of total RNA was estimated by measuring the absorbance at 260 nm which corresponds to their maximum absorption (Biophotometer, Eppendorf). The optical densities (ODs) were also measured at 230 and 280 nm to assess the presence of sugars and proteins. The purity of the RNAs was estimated by calculating the A_{260}/A_{280} ratio, which, if it was close to 2, the extracted nucleic acids were considered as of good quality. Also, the A_{260}/A_{230} had to be between 2.0-2.2.

The reverse transcription was performed with the kit of Invitrogen® using 10 µg of total RNA, following the manufacturer's instructions. The obtained cDNAs were diluted 1:10 with sterile water and stored at -20 °C until analyses.

5. ANALYSES

5.1. Triterpenoids

Identification and quantification of triterpenoids was performed using an Agilent Technologies 7890A gas chromatograph (Perlan Technologies, Warsaw, Poland) coupled to a 5975C mass spectrometric detector and a flame ionization detector (FID) (GC-MS/FID). The samples were dissolved in a mixture of diethyl ether: methanol (5:1) and a volume of 1-4 µl was applied by split injection 1:10. The GC separation was made on a HP-5MS UI column of 30 m × 0.25 mm, 0.25 µm (Agilent Technologies). The temperature programme was applied: initial temperature of 160 °C for 2 min, 280 °C at 5 °C/1 min and the final temperature of 280°C held for further 44 min with constant helium flow rate of 1 ml/min. The parameters for GC-MS/FID detection were as followed: inlet and a FID temperature 290 ºC, MS transfer line temperature 275 ºC, quadrupole temperature 150 ºC, ion source temperature 230 ºC, EI 70eV, *m/z* range 33-500; FID gas (H_2) flow 30 ml/min (hydrogen generator), air flow 400 ml/min. The compounds were identified by comparing their mass spectra with the data base (Wiley 9th ED. & NIST 2008 Lib. SW, Version 2010) or data from the literature, and by comparison of the retention times and corresponding mass spectra with those of authentic standards: α-amyrin, β-amyrin, campesterol, sitosterol, and stigmasterol. The quantification was carried out using external standard method based on calibration curves determined for typical representatives of each triterpenoid group analyzed, i.e., α-amyrin for monohydroxyalcohols, stigmasterol for steroids, oleanolic acid methyl ester for triterpene acid methyl esters. Standards were injected in the concentration ranging from 0.002 to 2.0 mg/ml. Chromatograms were processed with the use of Agilent G1701EA GC/MSD ChemStation software.

5.2. Polyphenols

Polyphenol analysis was performed by ultra-performance liquid chromatography with the use of a 1260 Infinity UHPLC (Agilent Technologies, Courtaboeuf, France) coupled to a 6430 triple quadrupole mass spectrometer (Agilent Technologies, Courtaboeuf, France), equipped with a Gerstel MPS2 autosampler. Samples were injected at 4 µl into a SB-C18 column of 100 mm × 2.1 mm, 1.8 mm (Agilent Zorbax) thermostated at 40 °C. Compounds were separated at a flow rate of 0.4 ml/min. The mobile phase was made of solvent A (distilled water, 0.1% formic acid) and solvent B (acetonitrile, 0.1% formic acid). The run was performed as follows: 0 to 3.5 min, 18% B; 3.5 to 6.5 min from 18% B to 33% B; 6.5 to 12

min from 33% B to 40% B; 12 to 13 min 40% B to 95% B; 13 to 16 min, 95% B; 16 to 16.5 min, from 95% B to 18% B. The parameters used for the source were as followed: capillary voltage, capillary voltage, 3000 V; nebulizer pressure, 15 psi; nitrogen flow rate, 11 l/min; gas temperature, 350 °C. Measurements were made with a multiple reaction monitoring (MRM) method in positive or negative mode according to the compounds. Quantities of all analyzed compounds were determined from calibration curves of pure corresponding standards, of injected concentrations ranging from 0.004 to 20 mg/ml. Data were analyzed by the Agilent Mass Hunter Quantitative Analysis Software (Agilent Technologies, Courtaboeuf, France). Concentrations were expressed in µg/mg of pure phenolic compound. The linearity of the response of the standard molecules was checked by plotting the peak area versus the concentration of the compounds.

5.3. Metabolomics (1H NMR)

Proton nuclear magnetic resonance spectroscopy (1H NMR) was applied in order to measure the changes within primary metabolites in the leaves upon different treatments. We developed a 1H NMR-based method for a quantification of metabolites in grapevine leaves, based on the method applied for wine analyses as described by Gougeon et al. (2018). All the spectra acquisitions were performed on a 600 MHz AVANCE III spectrometer (Bruker, Wissembourg, France) operating at a proton frequency of 600.27 MHz using a 5-mm TXI probe with Z-gradient coils. Post-acquisition treatments were performed in Topspin software version 3.2 (Bruker Biospin, Germany). Methanol-d_4 was used as the internal lock. A simple presaturation sequence (zg) with pulse width of 9.9 µs (90 ° tip angle) was applied to suppress the residual water signal, with an integration of 50 Hz (25 Hz on both sides of the signal centre). 1D 1H spectra were then acquired using zgesgp pulse sequence program with the same parameters for all experiments: 32 scans requiring 5 min measuring time and acquisition time (AQ) of 3.408 s, time domain (TD) of 65536 real data points, 16.0183 ppm (9615.385 Hz) spectral width (SW), and 5 s relaxation delay (RD). The free induction decays (FIDs) were multiplied by an exponential weighting function corresponding to line broadening (LB) of 0.3 Hz, before applying Fournier transformation. The spectra were aligned to TMSP signal (δ = 0 ppm). For the greatest reproducibility, phase correction was performed manually (Bharti and Roy, 2012).

The resulting spectra were manually baseline-corrected using the Whittaker Smoother method with the MestReNova NMR software version 11.0.3 (Mestrelab Research, Spain).

The spectral peaks were assigned by comparing chemical shifts and multiplicity with the literature and by metered addition of the various standards (specified in subsec. 2.2.) in the samples. Classical 2D-NMR experiments including COSY, TOCSY, ROESY, HSQC and HMBC were used for compound identification if necessary.

A semi-automatic quantification with Simple Mixture Analysis plugin (SMA, MestReNova) was performed. Peak deconvolutions were performed using the Global Spectral Deconvolution (GSD) method (Cobas et al., 2011). An example of applying the GSD function is presented in Figure 47.

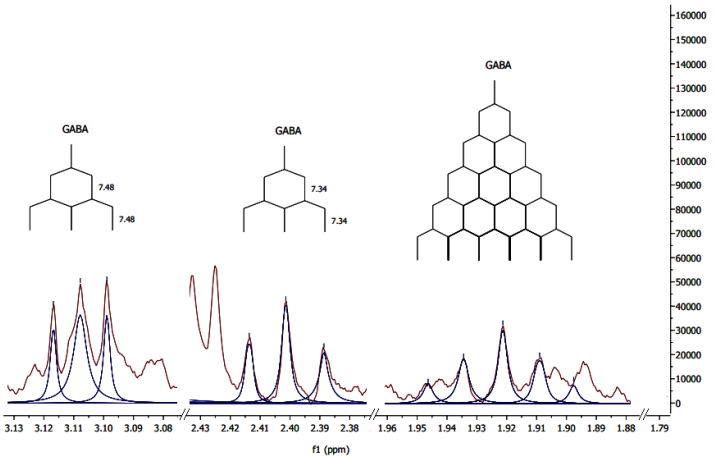

Figure 47. Example of the resolution power of Global Spectral Deconvolution (GSD). Deconvolved GSD peaks in the region of interest are shown in blue and the original raw spectrum in red.

Semi-quantification of metabolites was performed by utilizing calcium formate at the known concentration as the internal standard method. Compounds were quantified by the relative ratio of the intensities of their peak integrals and those of the internal standard (calcium formate). The analyte signals were evaluated according to the formula developed by Godelmann et al. (2016) presented below.

$$m_x = \frac{MW_x}{MW_{std}} \times \frac{n_{std}}{n_x} \times \frac{A_x}{A_{std}} \times m_{std} \times CF$$

m_x and m_{std} – masses (g) of the analyte and the standard, respectively;
MW_x and MW_{std} – molecular weights (g/mol) of the analyte and the standard;
n_x and n_{std} – numbers of protons of the analyte and the standard;
A_x and A_{std} – integral values of the analyte and the standard.

5.4. High-throughput gene expression

Real-time quantitative polymerase chain reaction (RT-qPCR) analyses on a microarray was done by D.Sc. Marie-France Corio-Costet and Dr. Anthony Bellée (UMR SAVE, INRAE) with the use of microfluidic dynamic array (Fluidigm) technology available on GenoToul platform (Toulouse, France). Genes studied (48) are implicated in different mechanisms of plant defense, i.e., PR proteins accumulation, secondary metabolites, indole, and oxylipins biosynthesis, redox status, cell wall reinforcement, hormone signaling. Five reference genes were used. Specific primers sets of the "NeoViGen96" chip designed previously were applied (Dufour et al., 2016). Details of the genes studied along with the primers used are presented in Table 6.

The proceeding employed for the high-throughput gene expression quantification was described by Bellée et al., 2018. Briefly, cDNA (5 ng/µl) was first preamplified by adding the reaction mixtures containing primers pool (50 mM) and the the TaqMan PreAmp Master Mix (1:2, Applied Biosystems), with 14 cycles of 95 °C for 15 s and 60 °C for 4 min. The cDNA obtained was used diluted (1:5 with TE buffer) for qPCR analysis in a reaction mixture (TaqMan Gene Expression Master Mix, Applied Biosystems; DNA Binding Dye Sample Loading Reagent, Fluidigm, Issy-les-Moulineaux, France; EvaGreen, Interchim, Montlucon, France). Real-time qPCR was performed with the use of a BioMark HD system (Fluidigm Corporation). The 96.96 dynamic array was used according to the manufacturer's protocol (http://www.fluidigm.com/user-documents). Each sample (5 µl) contained 1× TaqMan Universal Master Mix (without UNG), 1× GE sample loading reagent (Fluidigm PN 85000746), and diluted preamplified cDNA. The loaded chip was subjected to following cycles: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Melting curves were determined to confirm the specificity of the products. The levels of expression were calculated based on a multiple gene normalization method and by the use of the principles and formulas of Vandesompele et al. (2002).

Table 6. (to be continued) Sequences of the primers of the different genes studied (full names below the table on the following page) in grapevine leaves with "NeoVigen96" chip analyzed in the Biomark HD system.

Family	Name	Accession number	Forward 5' > 3'	Reverse 3' > 5'
Reference genes	*VvEF1γ*	AF176496	GAAGGTTGACCTCTCGGATG	AGAGCCTCTCCCTCAAAAGG
	VvTIP41	XM_002270674	CAGCGGGCAGCGATCGAAGA	CATTTCCGCTCCGGCAGCCTT
	VvTHIORYLS8	XM_002283586	TCACTCTGGATGGGCCGTCG	TCCCAATCGTGGCCGAACCG
	VvTuA	XM_002285685.1	GTCGGCGCTGAAGGTGTGGA	GAGGTGGCGGGCAAACCCTC
	VvGAPDH	CB973647	TTCTCGTTGAGGGCTATTCCA	CCACAGACTTCATCGGTGACA
PR proteins	*VvPR1*	AJ536326	CCCAGAACTCTCCACAGGAC	GCAGCTACAGTGTCGTTCCA
	VvPR2	AF239617	GGGGAGATGTGAGGGGTTAT	TGCAGTGAACAAAGCGTAGG
	VvPR3	U97522.1	ACTACGGCGCTGCTGGAAACA	TGGCACCGAAACCTTGGCTTAG
	VvPR4	XM_002264684.1	CCCAGAGCGCCAGCAATGTGA	TTGCTGCGCCATGCCAAGGG
	VvPR5	XM_002282874.1	CCCCGGCACCACCAATGCTC	TGGGGGAGAACCGTAGCCCTG
	VvPR6	XM_002284418	ACGAAAACGGCATCGTAATC	TCTTACTGGGGCACCATTTC
	VvPR7	XM_002275435.1	CGTTAAGCAGCTGGAAAGGAGCA	TCCTCCGTCAGTCTGGCTGCAA
	VvPR8	Z68123	AATGATGCCCAAAACGTAGC	ATAAGGCTCGAGCAAGGTCA
	VvPR9	XM_002285687.1	ACTGCACCAAGAAAGAGCACCAG	AGCTGTGCATGTGCCATCCCC
	VvPR10	AJ291705	GCTCAAAGTGGTGGCTTCTC	CTCTACATCGCCCTTGGTGT
	VvPR11	XM_002270543.1	CTCCACTGCGCAAACCGTGGT	TTTGCGTTTTCGGAGGAAATCGTGA
	VvPR12	XM_002281153	GTGCAAGAACTGGGAGGGTGCC	GCAGAAGCATGCAACTCCCGGG
	VvPR14	XM_002270934	CGCCACCACACAAGACCGCA	AGGGAGGCCAGCAGCCAGAC
	VvPR15	XM_002284176.1	GTTTCCTGGCCCTCATGGAATTGGC	GTGTCCTGCAGTGGGCTTGGA
Secondary metabolites biosynthesis	*VvPAL*	X75967	ACAACAATGGACTGCCATCA	CACTTTCGACATGGTTGGTG
	VvSTS	X76892.1	ATCGAAGATCACCCACCTTG	CTTAGCGGTTCGAAGGACAG
	VvROMT	FM178870	TGCCTCTAGGCTCCTTCTAA	TTTGAAACCAAGCACTCAGA
	VvCHS	X75969.1	CCAACAATGGTGTCAGTTGC	CTCGGTCATGTGCTCACTGT
	VvCHI	X75963	AGAAGCCAAAGCCATTGAGA	CCAAGGGGAGAATGAGTGAA
	VvDFR	XM_002281822.1	GGCCACCGTTCGCGATCCAA	GAAGACGCCGGTGCAGCCTT
	VvLDOX	X75966	TGGTGGGATGGAAGAGCTAC	CCCACTTGCCCTCATAGAAA
	VvF3H	X75965.1	TGACTCGCTCTCTTCAAGCA	CACCTTGGGACGTTCATCTT
	VvHMGR	XM_002275791.1	AACGCACACTCCGCTCCACG	GCGGCGGCGATCTTCATCGA
	VvFPPS	XM_002272605.1	TCGCCAATGGGTCGAGCGTA	TGCCTGCCTTGCAGCAACTTGT
	VvFAR	XM_002281343.1	GCCATGGCACTCCACCTCTCCTAA	AGGCGGGCTGGTAATGCGCT
Indole	*VvANTS*	XM 002281597	AAAAATCCAAGAGGGGTGCT	AAGCTTCTCCGATGCACTGT
	VvCHORM	FJ604854	TCATTGAGAGGGCCAAATTC	AGGAGGCAGAAAAAGCATCA
	VvCHORS	FJ604855	GCCTTCACATGCAGATGCTA	CTGCAACTCTCCCAATGGTT
Redox status	*VvGST1-Tau*	AY156048.1	GGGATCTCAAAGGCAAAACA	AAAAGGGCTTGCGGAGTAAT
	VvGST2-Phi	AY156049	CATGAAGGCCGGCCAGCACA	CGCGAAGAATTCGCTCTGGCCA
	VvGST3-Tau	XM_002283178	TGTTTGGCCGCAAACGGGGT	TCCCCAGCCAGGTACTTGCTCT
	VvGST4-Tau	XM_002271673	AGCTGGAATGGCGCACTTGGT	TGGAAAGGTGCATACATGGCCACG
	VvGST5-tau	XM_002283173	CCTTGAGCTCTACCCTGCCCCA	AGCAGCCAGCCCTAGACATGGA

Table 6. (continued) Sequences of the primers of the different genes studied in grapevine leaves with "NeoVigen96" chip analysed in the Biomark HD system.

Family	Name	Accession number	Forward 5' > 3'	Reverse 3' > 5'
Oxylipins	***VvLOX13***	XM_002285538	AAACCGTGCATTCCCGGCCC	GGCAGGGACGTAGCCAACCC
	VvLOX9	AY159556	GACAAGAAGGACGAGCCTTG	CATAAGGGTACTGCCCGAAA
Cell wall reinforcement	***VvAlli***	XM_002266017.1	AGCCCTTCTGGATGCAGCATGC	TGTAGCTTGCGGATGAGCTTCACT
	VvAPOX/APH1	XM_002284731.1	AGCTCAGAGGCCTCATCGCTGA	TACCGGCAGAGTGCCATGCG
	VvCAD/CCR	XM_002285332.1	AGTCCGATTGGAAGACGGCAGT	TGCCCCTGTCACACACACCA
	VvCALS	AJ430780.1	TGGAAATGCAATTCAAACGA	CGAATGCCATGTCTGTATGG
	VvPECT	XM_002283905.1	GGGTTGCGCCCTGAGGACAC	CAATCACCCGAGCCGCCTGG
Hormone signaling	***VvEDS1***	XM_002281059.1	CAGGTCACAGCCTGGGTGCG	TCGGGCGGGACGATCTCGTT
	VvWRKY2	AY596466	AGAGGCAAGGCGATGTAGAA	CTGGGGAACAAGCCTTCATA
	VvJAR1/GH3-6	XM_002283193.1	GCAACGGGGCACGACTACTGT	GCCGTGGCGGTGCAAGTACT
	VvACO1	XM_002273394.1	GCCGGTTTGAAGTTCCAGGCCA	ACTCAAACTGTGGCAATGGGACCC
	VvACC	AF424611	GAAGGCCTTTTACGGGTCTC	CCAGCATCAGTGTGTGCTCT
	VvEIN3	XM_02285213.1	CCTCGCAAGCGGTCTCGCAT	TGGAGACCCGAGCGCAGGAG
	VvSAMT	XM_002262982.1	AATCCTTGCCCAAGTTCCAG	GAGACAACCATTGGAGACTG
	VvICS	XM_002267645.2	TCTTCCCCGCTGTTTCTTCT	CTAAACCGTTGCCATCTCCG

References genes: *VvEF1γ,* elongation factor eEF1 gamma chain; *VvTIP41*, TIP41-like protein; *VvTHIORYLS8*, catalytic thioredoxin-like protein 4A; *VvTUA*, tubulin alpha; *VvGAPDH*, glyceraldehyde 3-phosphate dehydrogenase; **PR proteins:** *VvPR1*, pathogen-related protein 1; *VvPR2*, beta-1,3-glucanase; *VvPR3*, endochitinase class; *VvPR4*, chitinase class IV; *VvPR5*, thaumatin-like protein; *VvPR6*, serine protease inhibitor; *VvPR7*, subtilisin-like endoprotease; *VvPR8*, acidic endochitinase-like; *VvPR9*, cationic peroxidase 1; *VvPR10*, ribonuclease; *VvPR11*, chitinase class V; *VvPR12*, defensin-like protein-oxalate oxidase; *VvPR14*, lipid transfer protein; *VvPR15*, germin-like protein-oxalate oxidase; **Secondary metabolites biosynthesis:** *VvPAL*, phenylalanine ammonia lyase; *VvSTS*, stilbene synthase; *VvROMT*, resveratrol *O*-methyltransferase; *VvCHS*, chalcone synthase; *VvCHI*, chalcone isomerase; *VvDFR*, dihydroflavonol 4-reductase; *VvLDOX*, leucoanthocyanidin synthase; *VvF3H*, flavanone-3-hydroxylase; *VvHMGR*, 3-hydroxy-3-methylglutaryl coenzyme A reductase; *VvFAR*, ent-kaurene synthase; *VvFPPS*, farnesyl pyrophosphate synthase **Indole:** *VvANTS*, anthranilate synthase; *VvCHORM*, chorismate mutase; *VvCHORS*, chorismate synthase; **Redox status:** *VvGST-phi*, glutathione *S*-transferase class-phi; *VvGST-tau*, glutathione *S*-transferase Tau class; **Oxylipins:** *VvLOX*, lipoxygenase; **Cell wall reinforcement:** *VvAlli*, alliinase; *VvAPOX*, ascorbate peroxidase; *VvCAD*, cinnamyl alcohol dehydrogenase; *VvCALS*, callose synthase; *VvPECT*, pectin methyl esterase; **Hormone signalling:** *VvEDS1*, lipase 3/enhanced disease susceptibility gene; *VvWRKY*, WRKY transcription factor; *VvJAR1/GH3-6*, JA-Ile-synthase; *VvACO1*, 1-aminocyclopropane-1-carboxylic acid oxidase; *VvACC*, 1-aminocyclopropane-1-carboxylate synthase; *VvEIN3*, ethylene insensitive 3-Binding F Box Protein 1; *VvSAMT*, salicylic acid methyl transferase; *VvICS*, isochorismate synthase.

5.5. Statistics and software used

Samples were prepared in three and five biological replicates in biochemical and molecular experiments, respectively. The results presented on graphs and tables consist of the mean values ± standard deviation (S.D.). Three technical repetitions were also employed for the chromatographic and RT-qPCR analyses.

Statistical significance of the effect of treatments performed was determined by one-way analysis of variance (ANOVA). Tukey's or Dunnet's multiple range test was applied to compare simultaneously the means of every sample. Statistical significance was considered at $p \leq 0.05$. All these statistics were carried out in R software (version 3.4.3) using the FactoMineR plugin or in Prism® 7.04 (GraphPad software, Inc.). For the disease intensity measurement are (subsec. 3.2.1), the mean values for sporulation inhibition conferred by each elicitor were subjected to statistical analyses by a nonparametric test (Kruskal-Wallis) and significant differences were determined by Tukey's test at the 5% significance level.

Multivariate analyses, i.e., hierarchical clustering on principal components (HCPC) and principal component analyses (PCA) were performed in R software version 3.4.3 using the FactoMineR plugin.

IV. RESULTS AND DISCUSSION

1. PROFILING OF TRITERPENOIDS IN GRAPEVINE

The identification of steroids and triterpenoids present in the analyzed extracts was made with the use of GC-MS method as described in part III (chapter 4, sec. 4.1). The relative retention times of peaks associated with the identified triterpenoids and the characteristic ions in their mass spectra are summarized in Table A1 (Appendix). In MS spectra of free, non-derivatized sterols a molecular ion is usually easily observed, and it can have significant intensity (e.g., ion 414 in mass spectrum of *β*-sitosterol, 412 of stigmasterol or 386 of cholesterol) (Table A1). Another typical ion is $[M-18]^+$, which is generated after the loss of 3*β*-hydroxy group as water. This ion can be quite strong (relative intensity of 50-60%), particularly in sterols with double bond in position 5 (Δ^5 sterols). Further fragmentation by a retro-Diels-Alder reaction leads to other diagnostic ions (Abidi, 2001). The example of such fragmentation of *β*-sitosterol is presented in Figure 47.

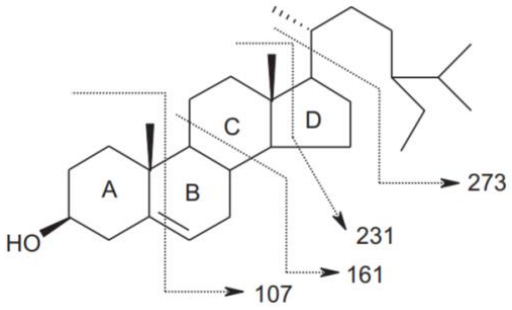

Figure 47. Main fragmentation pattern of *β*-sitosterol (adapted from Pant et al., 2013).

The main fragmentation pattern of oleanolic acid and other pentacyclic triterpenoids that contain a C12-C13 double bond, follows a retro-Diels-Alder cleavage of the C-ring, leading to the generation of fragments consisting of the ABC-rings (dienophile) and the CDE-rings (diene). The fragment containing CDE-ring is one of the most characteristic ions observed at *m*/*z* 262. This ion is then subjected to further fragmentation, by losing its methyl-carboxy group, which leads to an ion at *m*/*z* 203, the most abundant in the spectrum (Pollier and Goossens, 2012). Other characteristic ions, observed at *m*/*z* 410, 39, 191 or 189 have a very low abundance (Table A1). The generation of the main ions during MS fragmentation of oleanolic acid is presented in Figure 48.

The chemical structures of the major identified compounds are shown in Figure A1. Besides free forms of steroids and pentacyclic alcohols, aldehydes and ketones, some low molecular esters, i.e. methyl and acetate esters, co-fractionated with free neutral triterpenoids

due to a similar chromatographic mobility. Lupeol and α-amyrin were identified as a mixture associated with one common peak, since they are not separated well on the applied GC-MS column, as described earlier (Pensec et al., 2016). The supplementary HPLC analysis (Szakiel et al., 2012b) revealed that lupeol was highly dominating in this mixture obtained from grapes (with the ratio lupeol: α-amyrin approximately 10:1). In the present study these compounds were quantified together.

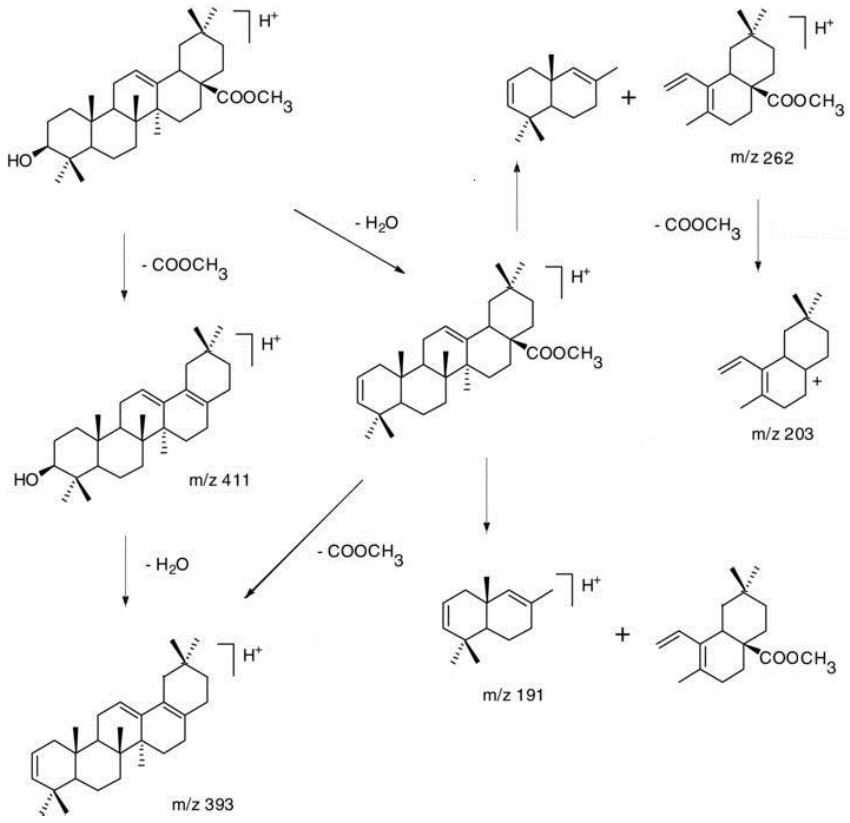

Figure 48. Main fragmentation pattern of oleanolic acid methyl ester (adapted from Pollier and Goossens 2012, modified).

1.1. In vitro cultures

The profile of triterpenoids was studied in the diethyl ether extracts of the cells from in vitro suspensions cultures of three *V. vinifera* cultivars: Cabernet Sauvignon (CS6), Gamay Fréaux var. Teinturier (GT_T strain), Gamay Fréaux var. Teinturier (GT3 strain) and Petit Verdot (PV) (Table 7).

The total content of identified and quantified steroids and pentacyclic triterpenoids reached approximately 3.2, 5.14, 3.04, 4.3 mg/g D.W. in the cells from PV, CS6, GT3 and GT_T cultures, respectively. The content of total steroids ranged from approximately 1.12, 4.85, 3.04, 3.93 mg/g D.W. in these samples, respectively. The common qualitative feature in

steroid profile of these three cultivars was the presence of four typical phytosterols: campesterol ((24*R*)-ergost-5-en-3*β*-ol), stigmasterol ((22*E*)-stigmasta-5,22-dien-3*β*-ol), sitosterol (stigmast-5-en-3*β*-ol) accompanied with its fully hydrogenated derivative, sitostanol (stigmastan-3*β*-ol), as well as isofucosterol (stigmasta-5,24(28)-dien-3-ol); as well as a steroid ketone, tremulone (stigmasta-3,5-dien-7-one). However, the amount of these compounds differed across the cultivars studied. Sitosterol occurred at predominant quantity in all the cultivars and amounted to 0.44, 4.1, 2.3 and 3.03 mg/g D.W. in PV, CS6, GT3 and GT_T, respectively. The cells of PV contained the lowest quantity of campesterol, stigmasterol, sitosterol, and sitostanol, around 4.1-, 6.2-, 7.1- and 2.7-fold less, respectively, in comparison with the remaining cultivars. In turn, the content of tremulone was the highest in this modality (around 2.2-fold in regard to other cultures). Also, among steroids two tetracyclic alcohols were identified, cycloartanol (9*β*,19-cyclo-lanostan-3*β*-ol) and 24-methylenecycloartanol (24-methylene-9*β*,19-cyclo-lanostan-3*β*-ol). The first of these compounds was detected in PV and GT_T (approximately 139.73 and 99.57 µg/g D.W., respectively), while the second one was detected in CS6, and also in GT_T (approximately 106.37 and 46.70 µg/g D.W., respectively). Moreover, PV was the only cultivar which contained spinasterone (stigmasta-7,22-dien-3-one) and 9,19-cyclolanost-23-ene-3,25-diol (cycloart-23-ene-3*β*,25-diol), in the quantity of approximately 39.01 and 26.74 µg/g D.W., respectively. Two other tetracyclic ketones were detected: stigmastane-3,6-dione ((22*E*,24*R*)-stigmasta-4,22-diene-3,6-dione) and sitostenone (stigmast-4-en-3-one), exclusively in CS6 (12.55 ± 1.8 µg/g D.W.) and GT3 (4.64 ± 0.46 µg/g D.W.), respectively.

Pentacyclic triterpenoids detected in grapevine cells from suspension cultures belong to oleanane-, ursane- and lupane-type skeletons. Ten pentacyclic triterpenoids were identified: *α*-amyrin (urs-12-en-3*β*-ol), *β*-amyrin (olean-12-en-3*β*-ol), lupeol (lup-20(29)-en-3*β*-ol) and its ester, lupeol acetate; two ketones, *α*- and *β*-amyrenones (urs-12-en-3-one and olean-12-en-3-one), betulin (lup-20(29)-ene-3*β*,28-diol), and uvaol (urs-12-ene-3*β*,28-diol). Among triterpene acids two isomers: oleanolic acid (3*β*-hydroxy-olean-12-en-28-oic acid) and ursolic acid (3*β*-hydroxy-urs-12-en-28-oic acid) were detected both in free form and it the form of methyl esters. Additionally, betulinic acid ((3*β*)-3-hydroxy-lup-20(29)-en-28-oic acid) was detected in extracts from PV cells. The total pentacyclic triterpenoids content accounted for approximately 2.07 mg/g D.W. in the cells of PV cultures. This content was around 7-fold lower in CS6 and GT_T, reaching 253.66 and 369.86 µg/g D.W., respectively. In GT3 only one pentacyclic triterpenoid was detected, oleanolic acid, in the amount of 3.01 ± 0.23 µg/g D.W. PV contained all of the identified pentacyclic triterpenoids, except ursolic acid, which

occurred only in GT3 in the quantity of 91.38 ± 7.99 µg/g D.W. However, PV accumulated the methyl ester of this compound, which along with uvaol and betulinic acid occurred only in this cultivar, in the amount of approximately 209.62, 9.62, and 28.98 µg/g D.W., respectively. Moreover, PV cells were particularly abundant in *α*-amyrenon/lupenon which reached 991.21 ± µg/g D.W. The content of the remaining pentacyclic triterpenoids detected in PV was approximately 40.92, 391.97, 11.39, and 148.64 µg/g D.W., respectively of *β*-amyrin, *α*-amyrin/lupeol, oleanolic acid, and of the methyl ester of the latter. In CS6 only 3 pentacyclic triterpenoids were identified, *β*-amyrin, *α*-amyrin/lupeol, and betulin (approximately 24.97, 148.26, and 80.42 µg/g D.W., respectively). In GT_T *α*-amyrin/lupeol, ursolic acid, oleanolic acid and its methyl ester were recognized (80.93, 91.37, 114.75, and 82.8 µg/g D.W., respectively).

Table 7. The content of triterpenoids identified in the cells from in vitro suspension cultures of *Vitis vinifera* cv. Petit Verdot, Cabernet Sauvignon (strain 6), Gamay Fréaux var. Teinturier (GT_T and GT3 strains). Results are referenced to cells dry weight and expressed in µg/g as the means ± S.D. of samples analyzed in triplicate.

Steroids	**Petit Verdot**	**Cabernet Sauvignon "6"**	**Gamay Teinturier "3"**	**Gamay Teinturier "T"**
campesterol	71.92 ± 5.51	259.72 ± 18.19	292.21 ± 22.18	342.04 ± 24.93
stigmasterol	19.28 ± 2.48	170.49 ± 12.05	62.51 ± 4.25	123.58 ± 9.86
sitosterol	440.29 ± 33.8	4089.72 ± 310.1	2298.15 ± 195.92	3033.14 ± 242.85
sitostanol	18.19 ± 1.3	43.40 ± 3.35	56.80 ± 4.45	49.72 ± 4.84
isofucosterol	262.85 ± 19.5	141.37 ± 10.82	306.34 ± 23.85	167.48 ± 13.58
cycloartanol	139.73 ± 9.7	n.d.	n.d.	99.57 ± 7.25
tremulone	111.38 ± 8.43	62.0 ± 4.8	20.03 ± 1.66	70.58 ± 6.4
24-methylenocycloartanol	n.d.	106.36 ± 8.15	n.d.	46.70 ± 3.65
stigmastane-3,6-dione	n.d.	12.55 ± 1.8	n.d.	n.d.
sitostenone	n.d.	n.d.	4.64 ± 0.46	n.d.
spinasterone	39.01 ± 3.0	n.d.	n.d.	n.d.
9,19-cyclolanost-23-ene-3,25-diol	26.73 ± 2.8	n.d.	n.d.	n.d.
Sum	**1129.41 ± 60.8**	**4885.65 ± 266.23**	**3040.71 ± 172.85**	**3932.84 ± 214.25**
Pentacyclic triterpenoids				
β- amyrin	40.92 ± 3.18	24.97 ± 1.84	n.d.	n.d.
α- amyrenone/lupenone	991.21 ± 75.25	n.d.	n.d.	n.d.
α- amyrin/lupeol	391.97 ± 31.25	148.26 ± 12.35	n.d.	80.93 ± 7.2
betulin	238.4 ± 19.25	80.42 ± 7.16	n.d.	n.d.
uvaol	9.62 ± 1.74	n.d.	n.d.	n.d.
oleanolic acid	11.39 ± 1.89	n.d.	3.01 ± 0.23	114.75 ± 9.87
betulinic acid	28.98 ± 2.23	n.d.	n.d.	n.d.
ursolic acid	n.d.	n.d.	n.d.	91.37 ± 7.99
oleanolic acid methyl ester	148.64 ± 12.38	n.d.	n.d.	82.8 ± 6.35
ursolic acid methyl ester	209.62 ± 15.05	n.d.	n.d.	n.d.
Sum	**2070.81 ± 115.08**	**253.66 ± 15.34**	**3.01 ± 0.15**	**369.86 ± 22.91**
Total	**3200.23 ± 180.94**	**5139.31 ± 390.25**	**3043.72 ± 173.09**	**4302.7 ± 245.38**

1.2. *Vitis vinifera* cv. Cabernet Sauvignon from the greenhouse

1.2.1. Whole leaf tissue

Some features of the main triterpenoid profile were similar in the leaves and in the cells of the same cultivar (CS6) described above in the chapter 1.1., i.e., the presence of several steroids (with dominating sitosterol, campesterol, cholesterol, stigmasterol, 24-methylenecycloartanol, tremulone) and a group of oleanane, lupane and ursane pentacyclic triterpenes (α-, β-amyrins, lupeol). However, some qualitative differences were also revealed, including the occurrence of pentacyclic taraxerene alcohol (i.e., taraxerol, (D-friedooleanan-14-en-3β-ol)), lupeol acetate, and two ketones: lupenone and α-amyrenone as well as oleanolic and ursolic acids, along with their methyl esters, exclusively in the leaves, and the presence of betulin, isofucosterol and stigmastane-3,6-dione only in the cells from in vitro cultures. The ratio of steroids to pentacyclic triterpenes differed significantly between the two experimental models tested, equaling approximately 19:1 in suspension cultures, and 3:1 in leaves. Representative GC chromatograms of the fraction containing steroids and neutral triterpenopids obtained from the extracts of the cells and the leaves are shown in Figure 49.

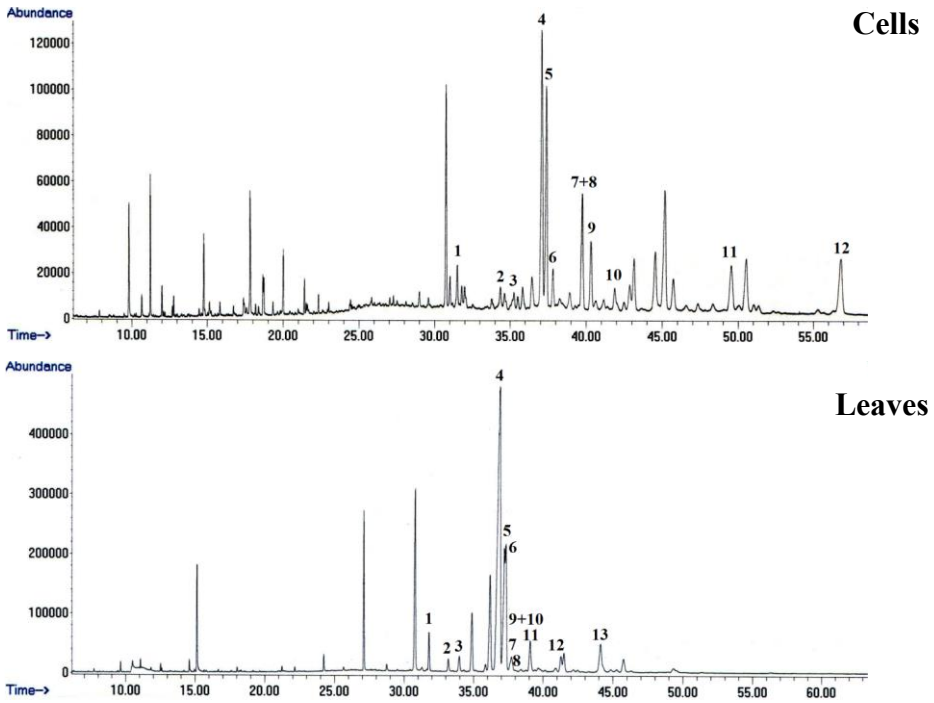

Figure 49. Representative GC-FID chromatograms of the fractions containing sterols and neutral triterpenes from diethyl ether extracts of *Vitis vinifera* cv. Cabernet Sauvignon leaves from the greenhouse and cells from in vitro suspension cultures. 1, cholesterol; 2, campesterol; 3, stigmasterol; 4, sitosterol; 5, sitostanol; 6, taraxerol (leaves)/isofucosterol (cells); 7, β-amyrin (leaves)/α-amyrin (cells); 8, α-amyrenone/lupenone (leaves)/lupeol (cells); 9, α-amyrin (leaves)/tremulone (cells); 10, lupeol (leaves)/24-methylenocycloartanol (cells); 11, tremulone (leaves)/stigmasta-3,6-dienon (cells); 12, 24-methylenocycloartanol (leaves)/betulin (cells); 13, lupeol acetate.

The content of triterpenoids in the grapevine leaves is presented in Table 8. The qualitative and quantitative profile of triterpenoids was very similar in the leaves of different stages of ontogenesis. Thus, the results are presented as the means of the contents of individual compounds across the 'young', 'medium' and 'old' leaves. The content of steroids was approximately 1.2 mg/g D.W., with the domination of sitosterol of which the amount reached 732.55 ± 45.88 µg/g D.W. The total quantity of pentacyclic triterpenoids was 414.34 ± 29.15 µg/g D.W., and taraxerol was a prevailing compound (256.93 ± 20.09 µg/g D.W.). The sum of all the triterpenoids studied was approximately 1.62 mg/g D.W.

Table 8. The content of major triterpenoids identified in the leaves of *Vitis vinifera* cv. Cabernet Sauvignon from the greenhouse. Results are the means of triterpenoids occurring in 'young', 'medium' and 'old' leaves, and are referenced to leaf dry weight and expressed in µg/g as the means ± S.D. of samples analyzed in triplicate.

Steroids	***Vitis vinifera* cv. Cabernet Sauvignon**
cholesterol	49.72 ± 3.04
campesterol	92.77 ± 5.13
stigmasterol	104.91 ± 11.75
sitosterol	732.55 ± 45.88
sitostanol	89.33 ± 8.29
tremulone	66.51 ± 3.43
24-methylenocycloartanol	66.95 ± 5.6
Sum	**1202.74 ± 110.74**
Pentacyclic triterpenoids	
taraxerol	256.93 ± 20.09
β- amyrin	31.74 ± 4.22
α- amyrenone/lupenone	4.76 ± 1.58
α- amyrine/lupeol	43.49 ± 3.72
lupeol acetate	53.12 ± 4.26
oleanolic acid	16.45 ± 1.12
ursolic acid	7.85 ± 0.33
Sum	**414.34 ± 29.15**
Total	**1617.08 ± 153.03**

1.2.2. Leaf cuticular waxes

The analysis of triterpenoids in leaf cuticular waxes revealed the similar capacity of the biosynthesis of these compounds in the leaf epiderm layer as the whole leaf tissue, however, with some modifications. The main steroids were phytosterols: cholesterol, campesterol, sitosterol, stigmasterol, tremulone, and steroids: 24-methylenocycloartanol, as well as cycloartenol (3*β*)-9,19-cyclolanost-24-en-3-ol). The latter is an important precursor of all plant steroids, generated through the cyclization of 2,3-oxidosqualene. Cycloartenol was

not detected in any other tissue studied in this research. The profile of pentacyclic triterpenoids in cuticular waxes also revealed interesting specific features, such as the occurrence of oleanolic acid derivatives, i.e., 3-oxo-oleanolic acid and olean-2,12-dien-28-oic acid, as well as betulinic acid, which was not detected in the whole tissue. The content of triterpenoids in leaf cuticular waxes of grapevine is presented in Table 9. The total amount of triterpenoids reached 189.67 µg/g ± 1.03 F.W. The ratio of steroids and pentacyclic triterpenoids was approximately 1:1, which is different from the one observed in the leaves (3:1). In the vast majority of the tissues studied here, sitosterol was the prevailing compound. In leaf cuticular waxes its content reached approximately 16.32 µg/g F.W. making it the second dominating compound, after the pentacyclic triterpene alcohol, taraxerol (50.77 µg/g F.W.).

Table 9. The content of triterpenoids identified in leaf cuticular waxes of *Vitis vinifera* cv. Cabernet Sauvignon from the greenhouse. Results are the means of triterpenoids occurring in 'young', 'medium' and 'old' leaves, and are referenced to leaf fresh weight, expressed in µg/g as the means ± S.D. of samples analyzed in triplicate.

Steroids	**Leaf cuticular waxes**
cholesterol	41,6 ± 0,25
campesterol	12,78 ± 0,08
sitosterol	16,32 ± 0,1
stigmasterol	5,35 ± 0,08
cycloartenol	3,85 ± 0,05
tremulone	6,55 ± 0,07
24-methylenocycloartanol	12,05 ± 0,08
Sum	**98,53 ± 0,56**
Pentacyclic triterpenoids	
taraxerol	50,77 ± 0,33
β- amyrin	13,28 ± 0,19
α- amyrin/lupeol	0,43 ± 0,01
α- amyrenone/lupenone	5,45 ± 0,05
lupeol acetate	2,09 ± 0,03
oleanolic acid	9,68 ± 0,04
3-oxo-oleanolic acid	1,622 ± 0,01
olean-2,12-dien-28-oic acid	1,13 ± 0,01
betulinic acid	1,09 ± 0,0
ursolic acid	3,31 ± 0,03
oleanolic acid methyl ester	1,68 ± 0,02
ursolic acid methyl ester	0,55 ± 0,0
Sum	**91,14 ± 0,5**
Total	**189,67 ± 1,03**

1.2.2. Stems, wood and roots

The main profile of stems, wood and roots of *V. vinifera* cv. Cabernet Sauvignon from the greenhouse is presented in Table 10. Contrarily to the cells from in vitro suspension cultures and the leaves, in these organs more pentacyclic triterpenoids were detected than steroids. Among steroids, 24-methylenocycloartanol and three phytosterols were recognized (campesterol, sitosterol and stigmasterol). The identified pentacyclic triterpenoids included taraxerol, α-, β-amyrins, lupeol, betulin, oleanolic and ursolic acids along with their methyl esters, as well as betulinic acid. The total content of triterpenoids was the highest in the roots, reaching approximately 6.22 mg/g D.W., while in the stems and the wood was around 2.62 and 3.15 mg/g D.W., respectively. Consequently, the roots were the organ that contained the most steroids, reaching 5.14 ± 691.74 mg/g D.W., i.e., around 2.4 times more than in the stems and the wood. The content of pentacyclic triterpenoids in the wood and the roots was much higher than in other grapevine material studied, reaching approximately 1.2 mg/g D.W. It is, for example, almost 3 times more than in the leaves. In the stems, an average amount of pentacyclic triterpenoids occurred (approximately 177.54 µg/g D.W.). Taraxerol was the most abundant pentacyclic compound in this tissue (72.28 ± 3.98 µg/g D.W.), followed by α-amyrin/lupeol, β-amyrin, oleanolic acid, and ursolic acid, which reached approximately 38.28, 35.77, 18.89, 12.30 µg/g D.W., respectively. The roots contained approximately 211. 99 and 73.86 µg/g D.W. of taraxerol and α-amyrin/lupeol, i.e., about 2 times more than the wood. In contrast, the amount of betulin, oleanolic, and ursolic acid was 2.1, 20, and 3.3 times higher in the wood than in the roots. Moreover, betulinic acid was found only in the wood, in the quantity of 96.69 ± 5.82 µg/g D.W. The wood contained 2 times more of oleanolic methyl ester, while the roots 9.2 times more of ursolic acid methyl ester.

Table 11. The content of triterpenoids identified in the stems, leaves and roots of *Vitis vinifera* cv. Cabernet Sauvignon from the greenhouse. Results are referenced to leaf dry weight and expressed in μg/g as the means ± S.D. of samples analyzed in triplicate.

Steroids	Stems	Wood	Roots
campesterol	234.32 ± 12.81	156.75 ± 8.96	396.13 ± 61.23
sitosterol	1686.26 ± 98.16	1135.75 ± 65.08	3287.52 ± 541.31
stigmasterol	412.56 ± 23.56	461.93 ± 26.28	1247.82 ± 155.55
24-methylenocycloartanol	112.18 ± 6.13	116.32 ± 6.35	205.64 ± 11.43
Sum	**2445.33 ± 122.38**	**1870.76 ± 95.54**	**5137.11 ± 691.74**
Pentacyclic triterpenoids			
taraxerol	72.28 ± 3.98	113.32 ± 6.23	211.99 ± 32.76
β- amyrin	35.77 ± 2.05	35.91 ± 1.99	73.86 ± 9.95
α- amyrin/lupeol	38.28 ± 3.09	485.09 ± 27.58	496.58 ± 27.61
betulin	n.d.	195.68 ± 12.69	92.68 ± 15.26
oleanolic acid	18.89 ± 1.23	236.11 ± 12.98	11.82 ± 1.83
betulinic acid	n.d.	96.63 ± 5.82	n.d.
ursolic acid	12.30 ± 0.78	12.26 ± 0.88	3.7 ± 0.42
oleanolic acid methyl ester	n.d.	88.32 ± 4.78	48.72 ± 8.02
ursolic acid methyl ester	n.d.	15.42 ± 0.98	141.53 ± 19.06
Sum	**177.54 ± 8.98**	**1278.45 ± 65.82**	**1080.9 ± 113.12**
Total	**2622.87 ± 130.16**	**3149.21 ± 167.48**	**6218.01 ± 339.84**

1.3. Wild and domesticated grapevines *Vitis* spp. from the field

1.3.1. Identification of triterpenoids occurring in the leaves

The extracts of the leaves of four wild grapevines (*V. aestivalis* Michx., *V. labrusca* L., *V. riparia* Michx., *V. vinifera* subsp. *sylvestris* (Gmelin) Hegi), and six domesticated *V. vinifera* cultivars (Alvarinho, Cabernet Sauvignon, Gamay, Marselan, Mauzac, and Merlot) were subjected to GC-MS/FID analysis. The fractions containing steroids and neutral pentacyclic triterpenoids obtained from diethyl extracts revealed rather similar qualitative profile of triterpenoids in the plants studied. Representative chromatograms are shown in Figure 50. The peaks in triterpenoid range, i.e., of retention time higher than 32 min in the applied program, were associated with several sterols: cholesterol, campesterol, stigmasterol, sitosterol, as well as pentacyclic compounds of ursane-, oleanane-, lupane- and friedooleanane (taraxerane)-type skeletons, i.e., *α*-amyrin, *β*-amyrin, lupeol and taraxerol, respectively. One of the intermediates of sterol biosynthetic pathway, 24-methylenecycloartanol, was also found in detectable amounts in all analyzed extracts. Two esters, which co-fractionated with free triterpenoids, i.e., sitosterol and lupeol acetates, were identified in the majority of analyzed extracts. In turn, a sitosterol *γ*-isomer, clionasterol, was found exclusively in *V. vinifera* subsp.

sylvestris (Gmelin) Hegi. In some extracts, traces of ursane-type ketone, α-amyrenone, were detected, however, in amounts too low for FID quantitation. Other numerous peaks of retention times up to 30 min were associated with aliphatic or phenolic compounds. Two peaks of t_R of 30.03 and 32.05 min were identified as typical phenol/diterpenoid antioxidants: γ- and α-tocopherol, respectively.

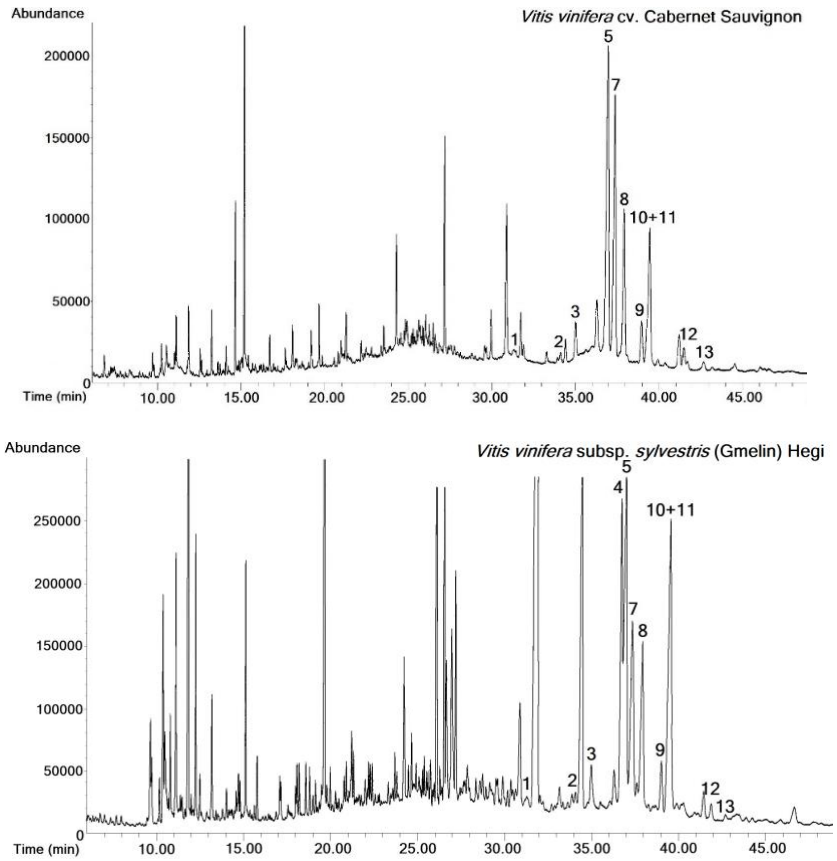

Figure 50. Representative GC-FID chromatograms of the fraction containing steroids and neutral triterpenoids from diethyl ether leaf extracts of grapevines. 1, cholesterol; 2, campesterol; 3, stigmasterol; 4, clionasterol; 5, sitosterol; 6, sitostanol; 7, taraxerol; 8, β-amyrin; 9, sitosterol acetate; 10+11, lupeol/α-amyrin; 12, 12, 24-methylenocycloartanol; 13, lupeol acetate.

1.2.2. Quantitative analysis of triterpenoids occurring in the leaves

Although the main triterpenoid profile was similar in all grapevines studied, the existence of significant differences in quantitative profiles among the analyzed species and cultivars was noted. The average total content was lower in the leaves of domesticated cultivars (approximately 1.7 mg/g, ranging from 1.0 to 2.4 mg/g) than in wild grapevines (approximately 2.1 mg/g, ranging from 1.6 to 2.7 mg/g). The contents of triterpenoids in foliar extracts of studied domesticated and wild grapevines are presented in Table 12A and 12B.

Table 12. Triterpenoid content in the leaves of different *Vitis* spp. plants. A, domesticated *Vitis vinifera* cultivars; B, wild grapevines: *Vitis aestivalis* Michx., *Vitis labrusca* L., *Vitis riparia* Michx., *Vitis vinifera* subsp. *sylvestris* (Gmelin) Hegi). Results are referenced to leaf dry weight and expressed in μg/g as the means ± S.D. of samples analyzed in triplicate.

A

	V. vinifera cv.					
Steroids	**Alvarinho**	**Cabernet Sauvignon**	**Gamay**	**Marselan**	**Mauzac**	**Merlot**
Cholesterol	31,34 ± 4,70	20,17 ± 3,02	33,91 ± 3,05	50,50 ± 7,57	67,53 ± 10,13	31,90 ± 4,78
Campesterol	15,76 ± 1,58	11,72 ± 1,17	13,60 ± 1,22	23,42 ± 2,34	19,63 ± 1,96	12,47 ± 1,25
Stigmasterol	54,28 ± 4,89	54,77 ± 4,93	50,74 ± 4,57	77,15 ± 6,94	61,21 ± 5,51	45,66 ± 4,11
Clionasterol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sitosterol	758,51 ± 75,85	537,24 ± 53,72	643,68 ± 64,37	1 141,06 ± 114,11	920,51 ± 92,05	486,77 ± 48,68
Sitostanol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sitosterol acetate	n.d.	58,45 ± 2,92	11,43 ± 0,86	156,56 ± 14,09	48,96 ± 7,34	16,41 ± 1,64
24-Methylenocycloartanol	58,65 ± 7,04	33,48 ± 4,02	52,45 ± 4,72	267,11 ± 8,16	93,14 ± 11,18	49,03 ± 5,88
Sum	918,54	715,83	805,8	1 715,80	1 210,97	642,24
Pentacyclic triterpenoids						
Taraxerol	330,36 ± 49,55	366,49 ± 54,97	145,77 ± 14,58	384,14 ± 57,62	37,69 ± 5,65	226,66 ± 34,00
β-Amyrin	160,24 ± 14,42	193,28 ± 17,40	168,84 ± 16,88	145,03 ± 13,05	139,85 ± 12,59	105,15 ± 9,46
Lupeol/α-Amyrin	340,44 ± 37,45	220,14 ± 24,22	651,78 ± 65,18	91,65 ± 10,08	1 315,18 ±	23,83 ± 2,62
Lupeol acetate	4,30 ± 0,34	16,42 ± 1,31	n.d.	46,48 ± 3,72	4,46 ± 0,36	10,18 ± 0,81
Sum	835,33	796,34	966,4	667,3	1 497,19	365,82
Total	1 753,87	1 512,17	1 772,20	2 383,09	2 708,15	1 008,06

B

	wild *Vitis*			
Steroids	***V. aestivalis* Michx.**	***V. labrusca* L.**	***V. riparia* Michx.**	***V. sylvestris***
Cholesterol	37,51 ± 5,63	44,41 ± 6,66	29,31 ± 4,40	49,12 ± 7,37
Campesterol	45,14 ± 4,51	42,90 ± 4,29	24,62 ± 2,46	38,25 ± 3,82
Stigmasterol	134,58 ± 12,11	85,74 ± 7,72	90,70 ± 8,16	106,93 ± 9,62
Clionasterol	n.d.	n.d.	n.d.	310,14 ± 46,52
Sitosterol	735,69 ± 73,57	714,83 ± 71,48	822,07 ± 82,21	419,73 ± 41,97
Sitostanol	n.d.	55,02 ± 8,25	32,77 ± 4,92	n.d.
Sitosterol acetate	10,30 ± 0,67	n.d.	21,01 ± 3,15	70,66 ± 10,60
24-Methylenocycloartanol	49,57 ± 5,95	179,46 ± 21,53	52,00 ± 6,24	20,94 ± 2,51
Sum	1 012,79	1 122,35	1 072,48	705,63
Pentacyclic triterpenoids				
Taraxerol	49,70 ± 7,45	14,23 ± 2,13	26,33 ± 3,95	269,35 ± 40,40
β- Amyrin	143,23 ± 12,89	363,23 ± 32,69	546,48 ± 49,18	218,82 ± 19,69
Lupeol/*α* -Amyrin	369,16 ± 40,61	1 172,94 ± 129,02	88,37 ± 9,72	397,19 ± 43,69
Lupeol acetate	3,04 ± 0,24	8,00 ± 0,64	3,75 ± 0,30	6,95 ± 0,56
Sum	565,13	1 558,39	664,93	892,31
Total	1 577,92	2 680,75	1 737,41	1 597,94

The total content of sterols ranged from 0.6 mg/g D.W. in extract from Merlot to 1.7 mg/g in Marselan. The average sterol content was similar in the leaves of wild and domesticated grapevines and equaled approximately 1 mg/g D.W. However, the content of sterols in wild grapevines seemed to be more stable and uniform among species, since the fluctuation between the lowest and the highest content did not exceed 0.2 mg, i.e., 16% of the highest amount. On the contrary, the difference between the lowest and the highest content of

sterols in domesticated grapevines exceeded 1 mg, that is, 63% of the highest amount. Sitosterol was the most abundant compound in the sterol profile of all analyzed grapevines, constituting from 63% of the total sterol content in *V. labrusca* L. to 82% in Alvarinho cultivar. The smallest amount of sitosterol was detected in *V. vinifera* spp. *sylvestris* (Gmelin) Hegi (only 41% of the total sterol content), however, as it was described above, in this species also the sitosterol *γ*-isomer, clionasterol, was found, and together these two isomers constituted 72% of the total sterol content. The second abundant sterol in the wild species was stigmasterol, with the exception of *V. labrusca* L. in which 24-methylenecycloartanol was prevailing. In Mauzac, the amount of stigmasterol was exceeded by cholesterol. The content of the third dominant phytosterol, campesterol, was in average more than twice higher in wild grapevines (approx. 0.34 mg/g) than in domesticated cultivars (0.15 mg/g).

The total content of pentacyclic triterpenoids ranged from 0.36 mg/g D.W. in Merlot cultivar to 1.5 mg/g in wild *V. labrusca* L., and it was generally much lower in the domesticated grapevines (approximately 0.7 mg/g D.W.) than in the wild species (approximately 1 mg/g D.W.). In the majority of the wild grapevines analyzed in this study, lupeol was the predominant pentacyclic compound, constituting (as a mixture with *α*-amyrin) from 45% of total pentacyclic triterpenoids in *V. vinifera* spp. *sylvestris* Gmelin (Hegi) to 75% in *V. labrusca* L. The only exception among wild grapevines was *V. riparia* Michx., in which *β*-amyrin was the prevailing pentacyclic compound (82% of total pentacyclic triterpenoids).

Among the domesticated grapevines, lupeol dominated in three cultivars, Alvarinho, Gamay and Mauzac (the mixture of lupeol and *α*-amyrin constituting 40, 67 and 89% of total pentacyclic triterpenoids, respectively). However, in Alvarinho the content of the mixture of lupeol and *α*-amyrin was only a little lower than that of taraxerol. In Cabernet Sauvignon, Marselan and Merlot, taraxerol was predominating (46, 58 and 62% of the sum of pentacyclic triterpenoids, respectively). The content of *β*-amyrin ranged from approximately 0.11 mg/g D.W. in Merlot to 0.19 mg/g D.W. in Cabernet Sauvignon, and it was not the dominant compound in any of domesticated cultivars. Interestingly, a similar level of taraxerol was found in domesticated *V. vinifera* cultivars and in their oldest wild ancestor *V. vinifera* subsp. *sylvestris* (Gmelin) Hegi. The profiles of major pentacyclic triterpenoids occurring in the leaves of grapevines studied are presented in Figure 51.

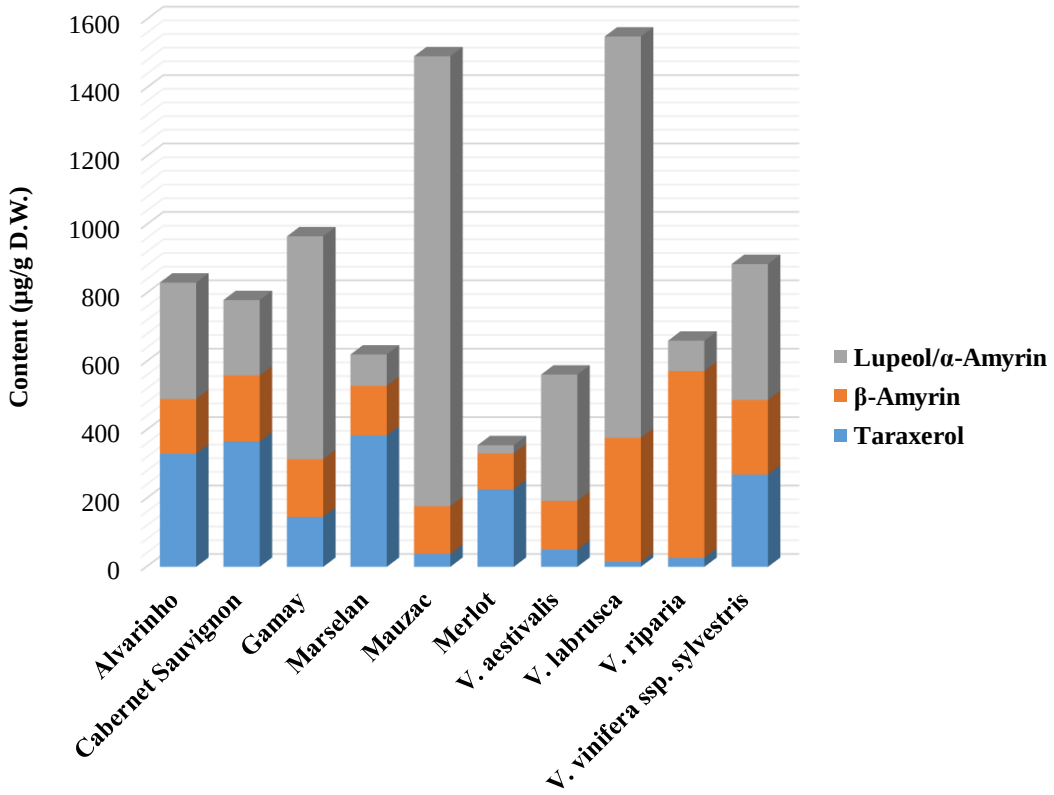

Figure 51. The profile of pentacyclic triterpenoids occurring in the leaves of domesticated *Vitis vinifera* cultivars (Alvarinho, Cabernet Sauvignon, Gamay, Marselan, Mauzac, and Merlot) and wild *Vitis* spp. (*Vitits aestivalis* Michx., *Vitis labrusca* L., *Vitis riparia* Michx., *Vitis vinifera* subsp. *sylvestris* (Gmelin) Hegi). Results are referenced to leaf dry weight and expressed in µg/g.

1.4. Discussion

As it was described in the introduction of this thesis (part I, chapter 4, sec. 4.3), triterpenoids represent a large group of natural products synthesized from isopentenyl diphosphate via the C_{30} precursor squalene. This class comprises tetracyclic steroids and pentacyclic compounds of high structural and functional diversity. The roles of triterpenoids are ascribed both to primary (e.g., participation of sterols in the structure and fluidity regulation of cellular membranes) (Rogowska and Szakiel, 2020) and to secondary metabolism, since some of these compounds participate in diverse strategies of plant chemical defense (González-Coloma et al., 2011). In grapevine, for example, the regulation of different OSCs catalyzing the biosynthesis of various pentacyclic triterpenoids was observed upon UV-C irradiation or downy mildew and gray mold infection, suggesting a role of these compounds in the response to both abiotic and biotic stresses. Furthermore, different expression patterns of induction were revealed by biotrophic and necrotrophic fungi (Pensec et al., 2016). Still, in

contrast to polyphenols, the biosynthesis and functions of triterpenoids have been much more scarcely studied in grapevine.

In order to provide more knowledge about triterpenoids occurring in grapevine, the current work aimed to characterize their profile in different experimental models of this plant. Free steroids and pentacyclic triterpenoids were studied, since the saponins do not seem to be produced in grapevine (Pensec, 2013). Steroid and triterpenoid profiles were investigated in cells from in vitro suspension cultures of different cultivars of *V. vinifera*; leaves (both whole tissues and cuticular waxes), stems, wood and roots of *V. vinifera* cv. Cabernet Sauvignon from the greenhouse; leaves of various *Vitis* spp. from the vineyard. Plant material was extracted with diethyl ether in Soxhlet apparatus. The obtained extracts were fractionated by preparative TLC and analyzed directly by GC-MS/FID (neutral steroids and pentacyclic triterpenes) or subjected to derivatization (methylation or alkaline hydrolysis of respectively triterpene acids and esters), prior to this analysis. This approach allows a good efficacy (more than 94% recovery of triterpenoid constituents) and relative selectivity precluding contamination with more polar compounds (triterpenoid glycosides and phenolics) (Szakiel et al., 2012b). The study revealed the capacity of the biosynthesis of several steroids, including typical phytosterols, as well as pentacyclic triterpenoids of oleanane-, ursane-, lupane- and friedooleanane (taraxerene)-type skeletons in grapevine. However, the profile differed according to the experimental model and cultivar.

Cells from in vitro suspension cultures produced tetra- and pentacyclic triterpenoids, including steroids (mainly campesterol, cholesterol, sitosterol, stigmasterol, cycloartanol, 24-methylenecycloartanol, tremulone) and compounds of oleanane-, ursane-, lupane-type skeletons. Important quantitative and qualitative differences could be observed in the profile of these compounds among the cultivars studied. The cells of PV contained the lowest amount of steroids, but spinasterone and 9,19-cyclolanost-23-ene-3,25-diol were present only in this cultivar. In turn, PV biosynthesized 2-times more of pentacyclic triterpenoids than steroids, and the content and diversity of these compounds was the highest among the three cultivars. Betulinic acid and uvaol were specific for PV. CS6 and GT_T were quite similar in term of the ratio steroids: pentacyclic triterpenoids, with the prevailing amount of steroids. The profile of steroids was similar in these two cultivars, but they differed in the pentacyclic triterpenoids profile. CS6 accumulated α-, β-amyrins, lupeol and betulin, while GT_T biosynthesized α-amyrin/lupeol, oleanolic acid along with its methyl ester, as well as, ursolic acid, which was specific for this cultivar in this study. GT_3 accumulated a similar amount of steroids as the

other strain of this cultivar, GT_T. However, in GT_3 only one pentacyclic triterpenoid, oleanolic acid, was detected at very low quantity.

Due to the wide spectrum of various biological activities, triterpenoids are the subject of numerous phytochemical and pharmacological studies. Thus, the research for new, triterpenoids-rich sources of plant origin is carried out and in vitro cultures are an interesting option for effective, sustainable and profitable production of these compounds that can be used for the formulation of drugs, cosmetics or insecticides (Biswas and Dwivedi, 2019). Encouraging results have been obtained for the effects of pentacyclic triterpenoids as anti-tumor, anti-inflammatory, antibacterial, antiviral (including anti-HIV), hepatoprotective, or anti-atherosclerotic agents (for review see Dzubak et al., 2006; Singh and Sharma, 2015). These properties, combined with relative low toxicity, were more than once confirmed in in vivo assays (for review see Agra et al., 2015; Dzubak et al., 2006). The most extensively studied is oleanolic acid which displays a wide range of biological activities and has been used in clinical field as an anti-hepatitis drug in China for over 30 years (Lin et al., 2016; Sultana and Ata, 2008). Besides, in Uyghurs folk medicine, grape berries of *V. vinifera* (suosuo grapes) are efficient in the prevention and treatment of liver disorders due to, among others, the great amount of oleanolic acid in cuticular waxes of these fruits (see introduction, sec. 4.3, subsec. 4.3.2.1) (Liu et al., 2012). Potential medicinal properties have also other free pentacyclic triterpenoids identified in the current study (Singh and Sharma, 2015). For example, lupeol, which was obtained from grape pomace (Amico et al., 2004), is recognized for its anticancer, antiprotozoal, chemopreventive and anti-inflammatory activities (Gallo and Sarachine, 2009). Betulin is used for the treatment of cancer and at the National Center of Cancer in China (Yin et al., 2013) and betulinic acid can be applied in the phase of infectious disease drug treatment for AIDS (Smith et al., 2007). Some studies demonstrated anti-tumor activities of ursolic acid in vitro, but this molecule is rather used for synthesis of more active derivatives (Ma et al., 2005).

The current study provides first information about triterpenoids in grapevine in vitro cell suspensions. In the laboratory of the Department of Plant Biochemistry, several types of in vitro cultures of marigold (*Calendula officinalis* cv. Persimmon Beauty) have been successfully induced for the production of bioactive saponins (Szakiel et al., 2003; Wiktorowska et al., 2010; Długosz et al., 2013). Oleanolic acid and its glycosylated forms showed an inhibitory effect towards several Gram-positive or Gram-negative bacteria and *Heligmosomoides polygyrus* nematode, an intestinal parasite of mice (Szakiel et al., 2008). Further investigation could be worthwhile if saponins biosynthesis in grapevine cell cultures

is possible since these forms of triterpenoids have not been detected in native plant. Indeed, a number of plant in vitro cultures biosynthesize new compounds, not detected in native plant of the same species. For example, as a result of the study performed in the collaboration between the Department of Plant Biochemistry and University of Lorraine, France, a new oleanane-type saponine was identified in *C. officinalis* hairy roots subjected to JA-elicitation (Markowski et al., 2019). Influence of elicitation with MeJA of grapevine cell suspension cultures is presented and discussed in the chapter 2 of the current part of this dissertation.

In native grapevines, the most important qualitative difference in regard to the cells from suspension cultures was the presence of a pentacyclic triterpenoid of friedooleanane (taraxerane)-type skeleton, taraxerol. Moreover, organ specialization in grapevines could be observed. In previous studies, taraxerol was detected only in the leaves of several *V. vinifera* cultivars grown in the Upper Rhine Valley (Alsace), and not found in the grape berries (Pensec et al., 2014, 2016). In turn, grapes biosynthesized another pentacyclic triterpene, erythrodiol ((3*β*)-olean-12-ene-3,28-diol) (Pensec et al., 2014, 2016), which consequently was not detected in any of the studied organs in the current work. Also, in this study betulin was present only in the wood and the roots, and betulinic acid in the wood and the leaf cuticular waxes. The leaf cuticular wax was also the unique to accumulate derivatives of oleanolic acid (3-oxo-oleanolic acid and olean-2,12-dien-28-oic). The current study provides first information about triterpenoids occurring in grapevine stems, wood and roots. The contribution of bioactive triterpenoids in grapevine resistance towards biotic and abiotic stresses would be worth to investigate.

Indeed, plant defense based on the activity of pentacyclic triterpenoids has been recognized (González-Coloma et al., 2011). For example, lupeol, betulinic and ursolic acids from *Curtisia dentata* (Burm.f) C.A. Sm. leaves showed antifungal and antibacterial activities against several pathogens (Shai et al., 2008). Betulin and betulinic acid are abundantly present in the bark of *Betula* spp. (birch) (hence their name) and are suggested to participate in plant defense through, for example, an insecticidal activity against larvae of maize, potato and tobacco pests (González-Coloma et al., 2011). Besides, betulin and betulinic acids attract an interest due to their wide range of biological and pharmacological activities (Yogeeswari and Sriram, 2005). The toxicity of *α*-amyrin, lupeol, and taraxerol towards insects was reported and it is supposed to result from the ability of these molecules to inhibit acyl chain packing in lipid bilayers (Rodríguez et al., 1997).

The components of cuticular waxes (terpenoids, fatty acids, heterocyclic compounds) are suggested to constitute a chemical barrier against pathogens and may participate in plant

stress response to harmful abiotic factors (for review see Reina-Pinto and Yephremov, 2009). In this study, the composition in triterpenoids of leaf cuticular waxes of grapevine cultivated in the greenhouse was investigated. Several studies were performed in order to characterize triterpenoids present at grapevine leaf surface (Pensec et al., 2016; Batovska et al., 2008; Özer et al., 2017; Berli et al., 2010). In the current work, wider range of triterpenoids was detected in leaf waxes than in the literature. Taraxerol was the prevailing compound, and several pentacyclic triterpene acids were identified: oleanolic acid along with its derivatives (3-oxo-oleanolic acid and oleana-2,12-dien-28-oic), ursolic and betulinic acids. Moreover, almost an equal ratio of steroids and pentacyclic compounds was noted, contrarily to the whole leaf tissue, where steroids generally dominate 3:1 over pentacyclic triterpenoids. These results may suggest the participation of pentacyclic triterpenoids occurring in cuticular waxes in chemical defense.

The content of lupeol and *β*-amyrin in waxes from leaves of field grown seedling of the Bulgarian *V. vinifera* cv. Storgozia, increased in the autumn (Batovska et al., 2009). The authors suggest that the accumulation of these triterpenoids could be associated with the repulsion of microorganisms attacking the grapevine leaves in this season (Batovska et al., 2009). In grape berries of *V. vinifera* cultivars from the Upper Rhine Valley, triterpenoids of cuticular waxes decreased during fruit ripening, which could partly explain the susceptibility to pathogens of mature grapes (Pensec et al., 2016). Triterpenoids present in cuticular waxes may have a potential role in the resistance of these plants to fungal and bacterial infections. In grapevine, *α*-amyrin, lupeol, taraxerol, and oleanolic acid, as well as a sitosterol derivative, stigmasta-3,5-diene-7-one, have been proposed as biomarkers of grapevine resistance to *P. viticola* or *E. necator* (Batovska et al., 2009; Chitarrini et al., 2017a, Özer et al., 2017). Concerning abiotic stresses, important modifications of membrane-related phytosterols (sitosterols and stigmasterol) and pentacyclic triterpene lupeol, were observed after low UV-B treatment (Gil et al., 2012). Some studies show a role of lupeol in lipid stabilization and its involvement in maintaining the redox state by scavenging free radicals (Nagaraj et al., 2000). These latter can be generated as instance by UV radiation. It could be worth to study a correlation of the high abundance of lupeol in the leaves of grapevine cultivars (e.g., *V. vinifera* cv. Pinot Noir grown in the Upper Rhine Valley, as reported by Pensec et al., 2016) with the resistance to UV stress factor.

However, in order to make conclusions about triterpenoid-type biomarkers of grapevine resistance, extensive studies of triterpenoids profiles in many grapevine varieties grown in different regions are necessary. It seems that individual varieties or cultivars may

differ significantly in the composition of triterpenoids, and this differentiation is probably due to a greater extent to genetic factors related to the inheritance of traits from parent varieties (an example of which may be a similar triterpenoids profile characteristic of all Pinot varieties tested in the work of Pensec et al. (2016)) and resulting from subsequent breeding selection rather than natural selection for resistance to pathogenic infections.

In an attempt to verify an importance of triterpenoids in the determination of grapevines origins, the leaves of wild and domesticated grapevines were compared in term of the profile of these compounds. Four wild grapevine crops used as progenitors or rootstocks of domesticated vines (*V. aestivalis* Michx., *V. labrusca* L., *V. riparia* Michx., *V. vinifera* subsp. *sylvestris* (Gmelin) Hegi; and six domesticated *V. vinifera* cultivars: Alvarinho, Cabernet Sauvignon, Gamay, Marselan, Mauzac, and Merlot were studied. The average total content of triterpenoids was generally higher and more uniform in wild grapevines. Although the main profiles of steroids and triterpenoids were similar in all analyzed extracts, remarkable quantitative differences in the content of these compounds in *Vitis* spp. leaves were demonstrated. The predominance of pentacyclic triterpenoids of ursane-, oleanane-, lupane- and friedooleanane (taraxerane)-type skeletons depended on the studied cultivar/variety. In the majority of the analyzed wild grapevines, lupeol dominated. Among the domesticated grapevines, lupeol prevailed only in two cultivars, Alvarinho and Gamay, whereas in Cabernet Sauvignon, Marselan and Merlot, the predominating pentacyclic triterpenoid was taraxerol. The obtained data supplement information about biochemical diversity of *Vitis* genus and allow species discrimination considering phylogenetic relationships confirmed by hierarchical clustering on principal components (HCPC) (Fig. 52). Two main types of clusters emerged: one of them grouped the domesticated grapevines together, and the other one showed strong similarities of triterpenoid profiles among the wild grapevines. The results seemed to predict some phylogenetic relationships of domesticated *V. vinifera* cultivars. For example, Cabernet Sauvignon and Merlot were clustered together which may be due to their common parent Cabernet Franc (Boursiquot et al., 2009). Unexpectedly, *V. vinifera* cv. Marselan was grouped apart from the cultivar involved in its parentage, Cabernet Sauvignon. Perhaps, the genetic background of Grenache, which is another parent of Marselan, prevails in terms of steroids and triterpenoids against the remaining cultivars of its origin (http:// www.vivc.de). In turn, Gamay (Pinot Noir × Heunisch Weiss) was grouped along with Cabernet Sauvignon and Merlot, even if a close kinship of these cultivars was not revealed (Terral et al., 2010). The obtained results suggest that triterpenoids may be an important phenotypic trait of *Vitis* plants. However, an advanced

study of hybrid domesticated grapevines varieties and their respective parents is needed in order to verify an importance of triterpenoid profile in the determination of grapevines origin.

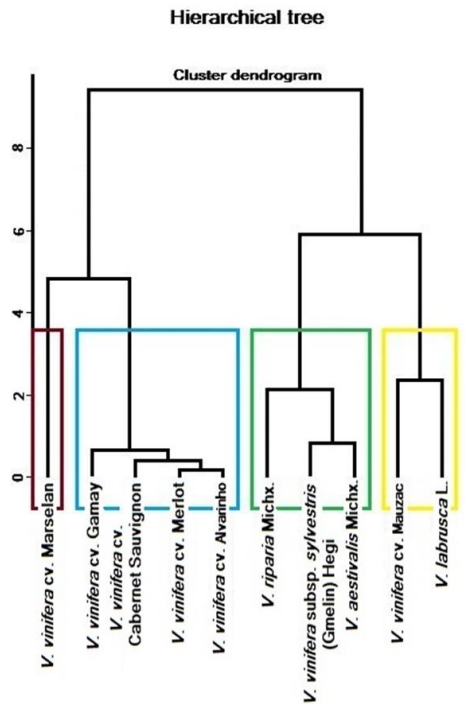

Figure 52. Cluster dendrogram performed by hierarchical clustering on principal components (HCPC) showing the discrimination of wild and domesticated grapevines *Vitis* spp. based on the composition of triterpenoids in extracts from whole leaves.

To summarize, the results presented in the current chapter supplement existing data about triterpenoids occurring in grapevine. Information about biosynthetic capacity of triterpenoids by grapevine in vitro cell suspensions cultures, as well as the characterization of these compounds in stems, wood and roots are provided for the first time by this study. Based on their pharmacological effects, triterpenoids attract a particular attention and information about new molecules within this class are sought-after. Further investigation is needed in order to elucidate a potential link between grapevine resistance to biotic and abiotic stresses with the triterpenoid profile.

2. EFFECT OF ELICITATION ON METABOLISM OF TRITERPENOIDS IN GRAPEVINE

2.1. In vitro cultures

2.1.1. Influence of the elicitor concentration

Cell suspension cultures of Gamay Fréaux var. Teinturier (GT_T) were subjected to elicitation with MeJA at two concentrations: 50 µM and 100 µM, and the cells were collected after 24 hpt, 72 hpt and 7 dpt. In this experiment, the profile of main phytosterols, i.e., campesterol, stigmasterol and sitosterol, along with their precursor 24-methylenocycloartanol, was followed. Among pentacyclic triterpenoids, oleanolic and ursolic acids, as well as the sum of α-amyrin and lupeol were considered. The content of these triterpenoids is presented in Table 13. In the control cells, the total amount of triterpenoids reached approximately 4.88, 5.13, and 5.33 mg/g D.W. at 24 hpt, 72 hpt and 7 dpt, respectively. The amount of all steroids and pentacyclic triterpenoids was, respectively, 4.54 mg/g and 334.36 µg/g D.W. (24 hpt), 4.95 mg/g and 172.72 µg/g D.W. (72 hpt), 5.19 mg/g and 143.58 µg/g D.W. (7 dpt).

Upon MeJA elicitation, the three phytosterols shared a similar pattern of changes, shown on the example of sitosterol in Figure 53A. At 24 hpt the treatment with 100 µM MeJA did not impact the amounts of phytosterols. The elicitor applied at the concentration of 50 µM led to an important accumulation of campesterol and stigmasterol (1.6- and 3-fold, respectively). With time, i.e., at 72 hpt and 7 dpt, the content of phytosterols was negatively impacted by MeJA at both concentrations, generally decreasing their contents around 1.2-fold. An inverse effect was observed for the metabolism of 24-methylenocycloartanol. Its content declined at 24 hpt upon elicitation with 100 µM MeJA (1.7-fold), while in the longer term, the level of this compound significantly increased after treatment with the elicitor at both concentrations, at 72 hpt around 2.3-fold, and particularly at 7 dpt, around 7.6-fold.

The effect of MeJA-treatment on the content of the analyzed pentacyclic triterpenoids (Fig. 53B) was specific for each of the modalities – time-point and the concentration of the elicitor. At 24 hpt, 50 µM MeJA led to a significant accumulation of oleanolic acid (2.6-fold), and a decrease of the content of ursolic acid (1.8-fold). This latter was even more impacted upon elicitation with 100 µM MeJA, along with α-amyrin/lupeol (a 12- and 3.6-fold decrease, respectively). Conversely, at 72 hpt the amount of oleanolic acid notably declined after the treatment with MeJA at 50 µM and 100 µM (7.8- and 3.6-fold, respectively). Like at 24 hpt, the content of ursolic acid decreased (3.5-fold upon elicitation with 100 µM; not detected in the sample 50 µM). In turn, the amount of α-amyrin/lupeol increased 5.9- and 1.9-fold after

the elicitation with 50 µM and 100 µM MeJA, respectively. At 7 dpt, 50 µM MeJA led to an accumulation of oleanolic acid and even its isomer, ursolic acid (around 3-fold). A remarkable increase of the content of oleanolic acid occurred after the treatment with 100 µM MeJA (6.6-fold).

Table 13. The profile of major triterpenoids in the cells from suspension cultures of Gamay Fréaux var. Teinturier (GT_T) subjected to elicitation with 50 µM or 100 µM methyl jasmonate (MeJA), and collected after 24 h, 72 h and 7 days post-treatment. Results are referenced to cells dry weight and expressed in µg/g as the means ± S.D. of three independent samples. The significance levels at $p \leq 0.05$ were established with comparison to the control samples: 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*), by one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's multiple comparison test.

	24 hpt		
Steroids	**Control**	**50 µM MeJA**	**100 µM MeJA**
campesterol	342.04 ± 17.15	530.54 ± 25.36 *	335.28 ± 17.96
stigmasterol	123.58 ± 7.18	372.87 ± 19.54 **	136.48 ± 8.82
sitosterol	4033.14 ± 421.72	4321.02 ± 538.24	2977.48 ± 281.56
24-methylenocycloartanol	46.7 ± 4.47	37.03 ± 2.02	26.87 ± 2.06 *
Sum	**4545.47 ± 227.37**	**5261.48 ± 283.09 ***	**3476.13 ± 183.99**
Pentacyclic triterpenoids			
oleanolic acid	125.88 ± 9.39	331.81 ± 28.34 ***	82.59 ± 8.64
ursolic acid	110.25 ± 10.43	60.0 ± 4.47 *	9.17 ± 0.41 **
α- amyrin/lupeol	98.23 ± 12.24	139.46 ± 4.82	27.37 ± 2.86 **
Sum	**334.36 ± 17.72**	**531.28 ± 27.86 ***	**119.14 ± 6.96 ***
Total	**4879.83 ± 253.99**	**5792.76 ± 299.64 ***	**3595.27 ± 179.69 ***

	72 hpt		
Steroids	**Control**	**50 µM MeJA**	**100 µM MeJA**
campesterol	396.99 ± 18.85	287.04 ± 16.36 *	299.17 ± 15.96 *
stigmasterol	149.79 ± 7.55	131.22 ± 7.56 *	152.14 ± 8.64 *
sitosterol	4381.77 ± 239.09	3039.16 ± 257.0 *	3007.13 ± 230.05 *
24-methylenocycloartanol	25.14 ± 1.35	54.55 ± 4.17 **	59.42 ± 5.57 **
Sum	**4953.70 ± 258.96**	**3511.99 ± 156.36 ***	**3517.88 ± 156.89 ***
Pentacyclic triterpenoids			
oleanolic acid	132.39 ± 13.84	16.87 ± 2.29 ****	36.38 ± 7.08 ***
ursolic acid	21.3 ± 2.63	n.d. ****	6.14 ± 0.79 *
α- amyrin/lupeol	19.03 ± 2.07	112.66 ± 15.16 ***	36.72 ± 2.0 *
Sum	**172.72 ± 9.64**	**129.54 ± 6.98 ***	**79.25 ± 4.96 ****
Total	**5126.43 ± 266.32**	**3641.54 ± 182.07 ***	**3597.13 ± 189.86 ***

	7 dpt		
Steroids	**Control**	**50 µM MeJA**	**100 µM MeJA**
campesterol	410.25 ± 20.52	320.45 ± 16.02 *	290.82 ± 14.55 *
stigmasterol	194.95 ± 8.74	161.29 ± 7.08	138.99 ± 7.95 *
sitosterol	4530.89 ± 433.33	3107.72 ± 169.57 *	3326.54 ± 254.63 *
24-methylenocycloartanol	50.51 ± 3.48	374.16 ± 32.38 ***	390.08 ± 40.0 ***
Sum	**5186.61 ± 265.33**	**3963.63 ± 178.96 ***	**4146.44 ± 208.33 ***
Pentacyclic triterpenoids			
oleanolic acid	72.89 ± 3.33	215.91 ± 22.76 **	483.89 ± 55.44 ***
ursolic acid	33.19 ± 2.05	109.15 ± 9.77 *	48.94 ± 2.7
α- amyrin/lupeol	37.48 ± 9.04	69.61 ± 9.04	76.77 ± 12.18
Sum	**143.58 ± 6.18**	**394.68 ± 18.76 ***	**609.61 ± 31.58 ****
Total	**5330.19 ± 246.85**	**4358.32 ± 218.93 ***	**4756.06 ± 245.81 ***

A **Steroids**

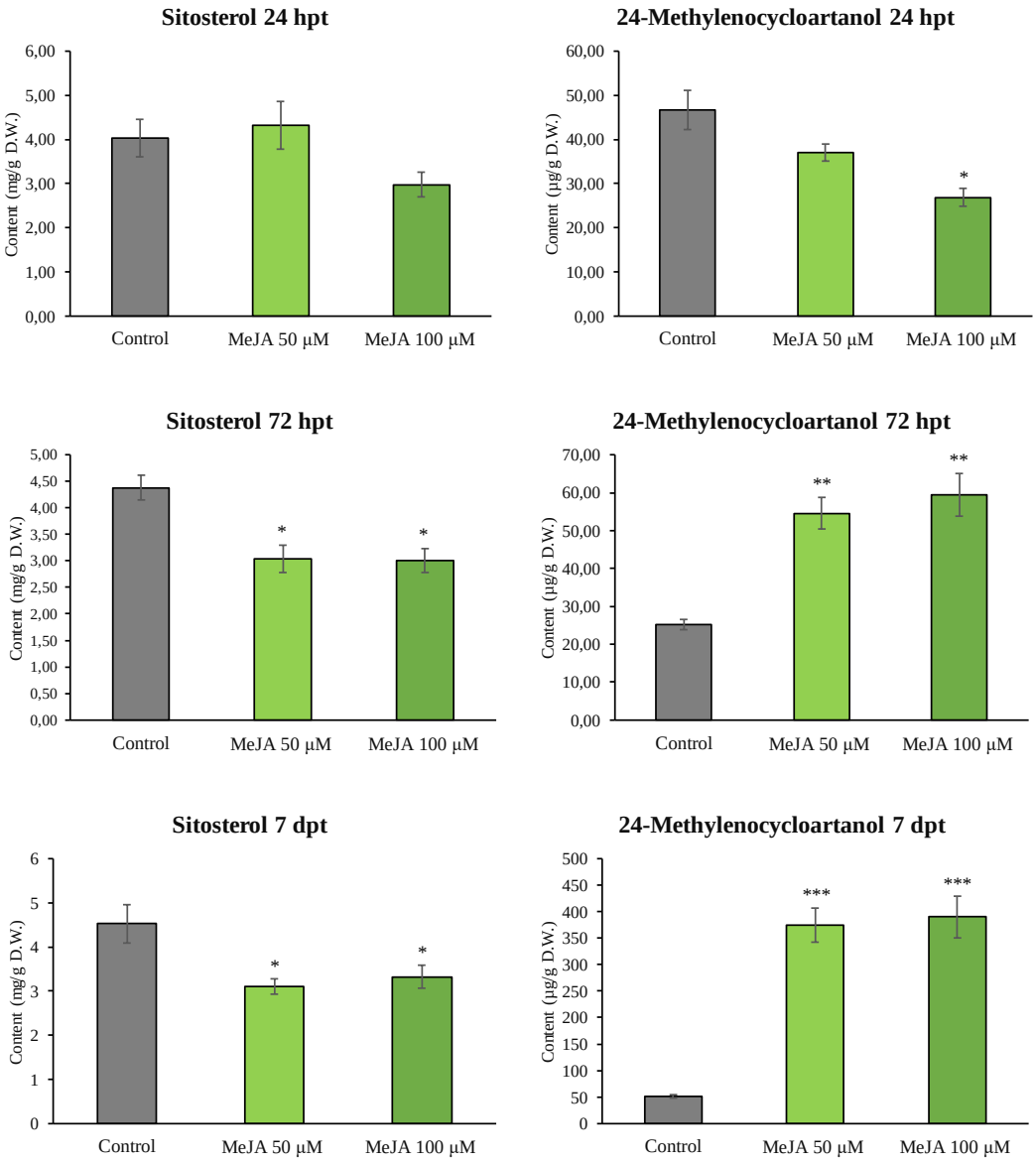

Figure 53. (to be continued) The profile of major triterpenoids (A, steroids; B, pentacyclic triterpenoids) in the cells from suspension cultures of *Vitis vinifera* cv. Gamay Fréaux var. Teinturier (GT_T strain) subjected to elicitation with 50 µM or 100 µM methyl jasmonate (MeJA), and collected after 24 h, 72 h and 7 days post-treatment. Results are referenced to cell dry weight and expressed in mg/g or µg/g D.W. as the means ± S.D. of three independent samples. Asterisks denote the significance levels at $p \leq 0.05$ with comparison to the control samples: 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*) as a result of one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's multiple comparison test.

B

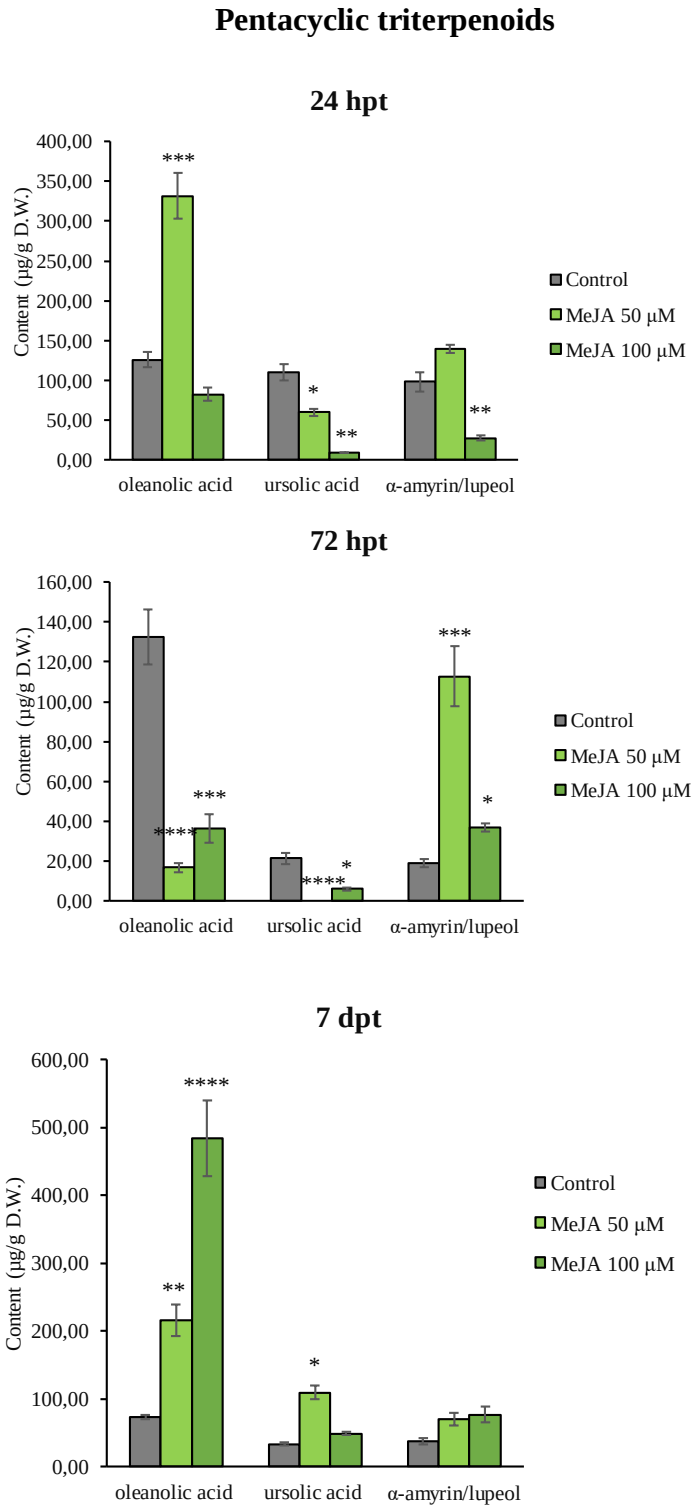

Figure 53. Continued. The profile of major triterpenoids (A, steroids; B, pentacyclic triterpenoids) in the cells from suspension cultures of *Vitis vinifera* cv. Gamay Fréaux var. Teinturier (GT_T strain) subjected to elicitation with 50 µM or 100 µM methyl jasmonate (MeJA), and collected after 24 h, 72 h and 7 days post-treatment. Results are referenced to cell dry weight and expressed in mg/g or µg/g D.W. as the means ± S.D. of three independent samples. Asterisks denote the significance levels at $p \leq 0.05$ with comparison to the control samples: 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*) as a result of one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's multiple comparison test.

2.1.2. Cultivar impact

Cell suspension cultures of Petit Verdot (PV), Cabernet Sauvignon (CS6) and Gamay Fréaux var. Teinturier (GT3) were subjected to elicitation with 50 µM MeJA and the cells were harvested after 48 h and 7 days. The similar triterpenoid profile was analyzed in this experiment as in the study with GT_T cultivar (subsec. 2.1.1.), i.e., 24-methylenocycloartanol, main phytosterols (campesterol, stigmasterol and sitosterol), oleanolic acid and α-amyrin/ lupeol. Betulin and betulinic acid were also studied, in turn, this time ursolic acid was not detected. The content of these triterpenoids is presented in Table 14. At 48 hpt the total contents of triterpenoids in the control samples were approximately 3.26, 3.43 and 6 mg/g D.W. in the cells of PV, CS6 and GT3, respectively. At 7 dpt, the control cells of PV, CS6 and GT3 contained respectively around 5.45, 1.17, and 9 mg/g D.W. of total triterpenoids. The amount of steroids in PV was approximately 794.58 µg/g and 1.72 mg/g D.W. at 48 hpt and 7 dpt, respectively. PV was the only cultivar in which the total content of pentacyclic triterpenoids exceeded that of steroids, it reached around 2.43 and 3.74 mg/g D.W. at 48 hpt and 7 dpt, respectively. The control cells of CS6 contained totally 3.2 mg/g and 876.75 µg/g D.W. of steroids at 48 hpt and 7 dpt, respectively. The total content of pentacyclic triterpenoids in CS6 did not differ in time and was on average 266 µg/g D.W. Moreover, oleanolic acid was not found in the cells of CS6. A particularly high content of total steroids was present in GT3, reaching 5.93 and 8.96 mg/g D.W. at 48 hpt and 7 dpt, respectively. In contrast, in this cultivar the only one pentacyclic triterpenoid was detected, oleanolic acid, in the quantity of 25.74 and 4.27 µg/g D.W. at 48 hpt and 7 dpt, respectively.

After elicitation with MeJA, the three phytosterols shared a similar pattern of changes in the cultivars studied. An example of sitosterol profile is shown in Figure 54A. At both time points the content of each phytosterols augmented in the cells of PV (around 2.4-fold). In GT3 a significant increase of the sitosterol amount was noted at 48 hpt (1.4-fold). At 7 dpt invariability of the quantity of sitosterol along with a decrease of campesterol (2.6-fold) was noted in GT3. In CS6 MeJA-treatment positively affected the level of phytosterols only at 7 dpt (around a 2.6-fold increase of campesterol and stigmasterol, and a 4.6-fold of sitosterol). The content of phytosterols precursor, 24-methylenocycloartanol, was noted to be enhanced in PV at 48 hpt (1.65-fold), and in the both time-points in GT3, with even a 3-fold change at 48 hpt, and being not detected in the control at 7 dpt.

Table 14. The profile of major triterpenoids in the cells from suspension cultures Petit Verdot, Cabernet Sauvignon (strain 6), Gamay Fréaux var. Teinturier (GT3 strain) subjected to elicitation with 50 µM methyl jasmonate (MeJA), and collected after 48 h and 7 days post-treatment. Results are referenced to cell dry weight and expressed in µg/g as the means ± S.D. of three independent samples. The significance levels at $p \leq 0.05$ was established with comparison to the respective control samples: 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*), by one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's multiple comparison test.

	Petit Verdot			
	48 hpt		7 dpt	
Steroids	Control	MeJA	Control	MeJA
campesterol	89.60 ± 6.68	197.71 ± 15.74 *	191.01 ± 15.25	343.4 ± 26.88 *
stigmasterol	28.1 ± 37.96	67.04 ± 6.0 **	70.99 ± 5.33	172.86 ± 14.74 **
sitosterol	494.57 ± 38.99	1307.91 ± 98.22 ***	1095.92 ± 82.72	2419.93 ± 190.47 **
24-methylenocycloartanol	182.29 ± 14.59	300.13 ± 23.87 *	358.72 ± 27.96	407.23 ± 30.85
Sum	**794.58 ± 68.25**	**1872.8 ± 141.28 ** **	**1716.66 ± 128.85**	**3343.44 ± 285.26 ***
Pentacyclic triterpenoids				
oleanolic acid	11.39 ± 0.98	9.28 ± 0.72	11.83 ± 0.98	14.54 ± 2.08
betulinic acid	28.98 ± 3.18	45.47 ± 3.58 *	43.37 ± 3.23	65.95 ± 4.99 *
α- amyrin/lupeol	2022.95 ± 160.59	1378.53 ± 103.88 *	2629.99 ± 151.22	2028.19 ± 153.69 *
betulin	398.8 ± 35.69	579.53 ± 44.21 *	1052.0 ± 145.6	1952.77 ± 146.78 *
Sum	**2462.13 ± 193.45**	**2012.83 ± 155.74**	**3737.2 ± 303.98**	**4061.47 ± 303.98**
Total	**3256.71 ± 243.83**	**3885.64 ± 299.72**	**5453.87 ± 553.89**	**7404.92 ± 555.11 ***

	Cabernet Sauvignon "6"			
	48 hpt		7 dpt	
Steroids	Control	MeJA	Control	MeJA
campesterol	182.18 ± 10.29	206.10 ± 17.54	85.68 ± 11.02	204.37 ± 10.88 **
stigmasterol	109.05 ± 11.61	151.26 ± 9.63	61.11 ± 5.11	172.89 ± 15.78 **
sitosterol	2863.51 ± 362.14	3350.61 ± 238.74	650.75 ± 41.14	3019.8 ± 406.40 ***
24-methylenocycloartanol	43.19 ± 4.17	39.87 ± 1.70	79.2 ± 7.72	79.3 ± 3.27
Sum	**3197.95 ± 267.16**	**3747.85 ± 341.93**	**876.75 ± 91.34**	**3476.37 ± 317.94 ***
Pentacyclic triterpenoids				
betulinic acid	40.34 ± 4.09	16.45 ± 1.24 **	52.66 ± 4.45	10.14 ± 0.87 ****
α- amyrin/lupeol	116.47 ± 13.57	99.9 ± 6.65	127.38 ± 9.75	149.89 ± 6.18
betulin	79.61 ± 7.68	37.75 ± 3.65 *	117.05 ± 11.9	26.88 ± 2.81 **
Sum	**236.42 ± 20.44**	**154.12 ± 12.53 ***	**297.1 ± 17.37**	**186.91 ± 10.82 ***
Total	**3434.37 ± 183.88**	**3901.97 ± 200.43**	**1173.86 ± 60.3**	**3663.29 ± 231.57 **

	Gamay Teinturier "3"			
	48 hpt		7 dpt	
Steroids	Control	MeJA	Control	MeJA
campesterol	669.74 ± 36.6	848.43 ± 46.97	1012.98 ± 56.36	826.48 ± 46.17
stigmasterol	149.15 ± 9.15	197.39 ± 10.88	273.86 ± 14.85	106.51 ± 6.74 **
sitosterol	5088.8 ± 299.4	7144.7 ± 399.45 *	7674.69 ± 410.04	7721.78 ± 435.19
24-methylenocycloartanol	21.59 ± 1.25	63.91 ± 3.88 ***	n.d.	392.69 ± 22.66 ***
Sum	**5929.29 ± 325.66**	**8254.44 ± 452.09 ***	**8961.54 ± 498.72**	**9047.48 ± 505.42**
Pentacyclic triterpenoids				
oleanolic acid	25.74 ± 1.42	5.0 ± 0.31 ****	4.27 ± 0.35	11.15 ± 0.98 ***
Total	**5955.03 ± 333.88**	**8259.45 ± 466.35 ***	**8965.81 ± 499.15**	**9058.63 ± 510.01**

Depending on the cultivar, MeJA-treatment specifically impacted the profile of pentacyclic triterpenoids (Fig. 54B). The content of oleanolic acid (being the only pentacyclic triterpenoid detected in GT3) decreased 5.1-fold at 48 hpt and increased 2.6-fold at 7 dpt, in regard to the control. The profile of betulinic acid, betulin and *α*-amyrin/lupeol was influenced in the same way in PV and CS6, at both time-points. The amount of betulinic acid and betulin increased in PV around 1.6-fold, and decreased in CS6 approximately 2.3- and 4.8-fold at 48 hpt and 7 dpt, respectively. MeJA-treatment led to a 1.4-fold decline of the content of *α*-amyrin/lupeol at both time-points.

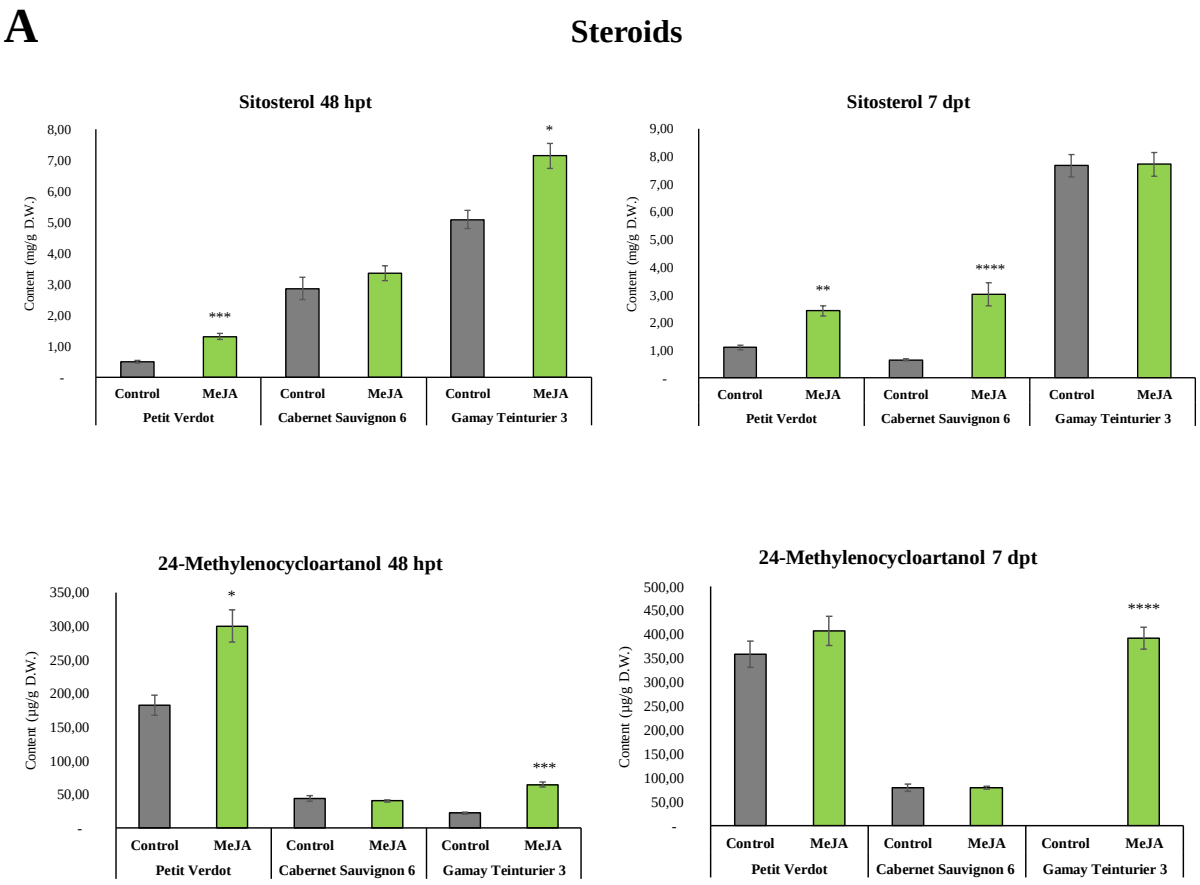

Figure 54. (to be continued) The profile of major triterpenoids (A, steroids; B, pentacyclic triterpenoids) in the cells from suspension cultures of *Vitis vinifera* cv. Petit Verdot, Cabernet Sauvignon (strain 6), Gamay Fréaux var. Teinturier (GT3 strain) subjected to elicitation with 50 µM methyl jasmonate (MeJA), and collected after 48 h and 7 days post-treatment. Results are referenced to cell dry weight and expressed in mg/g or µg/g D.W. as the means ± S.D. of three independent samples. Asterisks denote the significance levels at $p \leq 0.05$ with comparison to the respective control samples: 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*) as a result of one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's multiple comparison test.

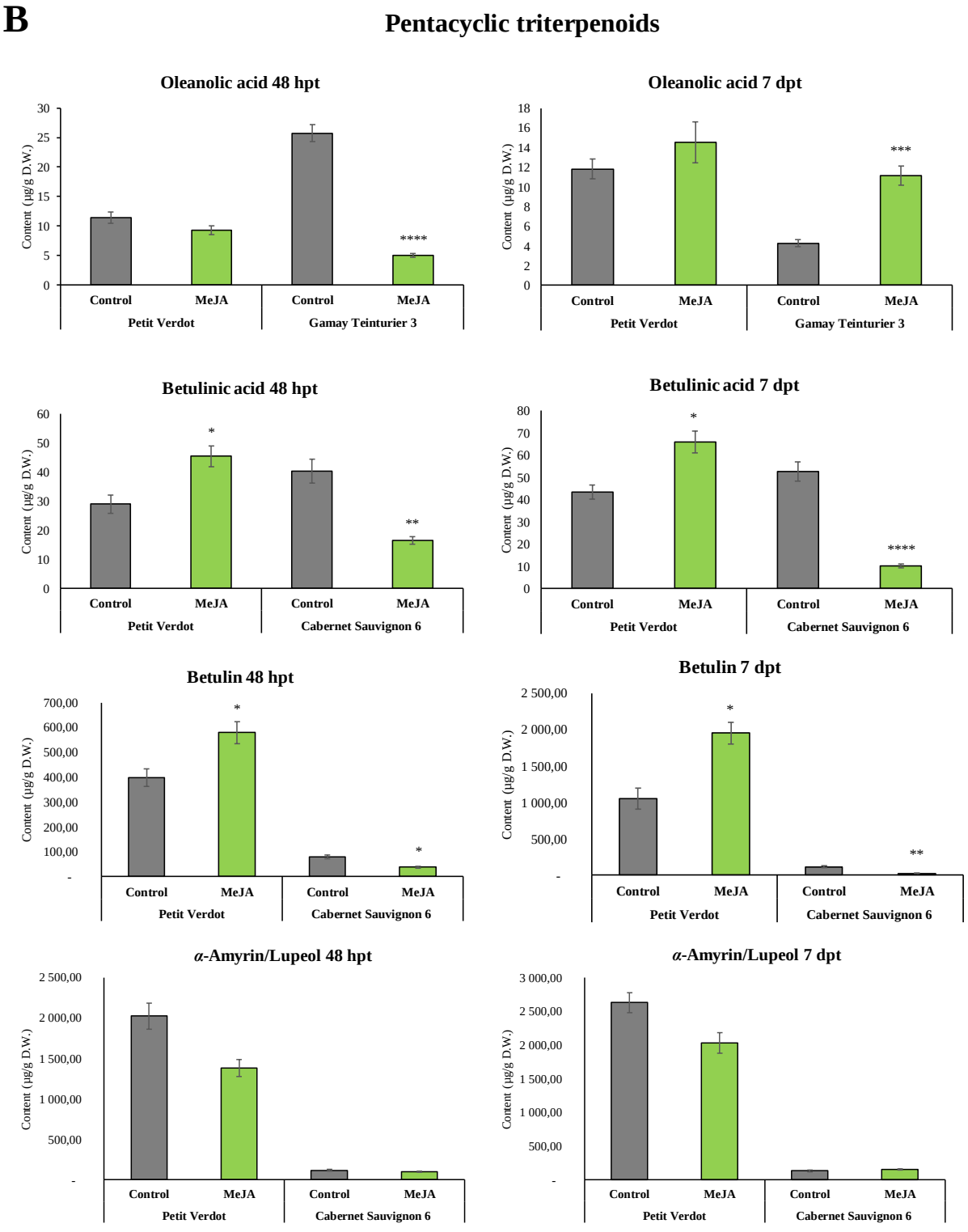

Figure 54. (continued) The profile of major triterpenoids (A, steroids; B, pentacyclic triterpenoids) in the cells from suspension cultures of *Vitis vinifera* cv. Petit Verdot, Cabernet Sauvignon (strain 6), Gamay Fréaux var. Teinturier (GT3 strain) subjected to elicitation with 50 µM methyl jasmonate (MeJA), and collected after 48 h and 7 days post-treatment. Results are referenced to cell dry weight and expressed in mg/g or µg/g D.W. as the means ± S.D. of three independent samples. Asterisks denote the significance levels at $p \leq 0.05$ with comparison to the respective control samples: 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*) as a result of one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's multiple comparison test.

2.2. *Vitis vinifera* leaves differently elicited

2.2.1. Triterpenoids in the whole leaf tissue

Foliar cuttings of *V. vinifera* cv. Cabernet Sauvignon cultivated in the greenhouse were sprayed with MeJA and BTH and the leaves were collected at 7 dpt and 14 dpt. Identified compounds were steroids (campesterol, stigmasterol, sitosterol, 24-methylenocycloartanol), and pentacyclic triterpenoids (taraxerol, α-amyrin/lupeol, oleanolic acid, olean-12-en-28-oic acid, 3-oxo-oleanolic acid, ursolic acid). The profiles of total sterols and pentacyclic triterpenoids are presented in Figure 55. At 7 dpt the total content of triterpenoids was approximately 2.17 mg/g D.W., with steroids and pentacyclic triterpenoids reaching 1.94 mg/g and 232.35 µg/g D.W (Table 15A). MeJA and BTH negatively impacted the total amount of steroids (1.2- and 1.3-fold respectively) at 7 dpt. This change was due to a 1.3-fold decrease of the content of campesterol in both modalities, and a 2.1-fold decline of the amount of 24-methylenocycloartanol by MeJA-treatment, in regard to the control. BTH was particularly effective in the enhancement of the accumulation of pentacyclic triterpenoids at 7 dpt. In this modality an increase of taraxerol (1.7-fold) and a spectacular one of oleanolic acid (11.6-fold) was noted. Moreover, in BTH-treated leaves olean-12-en-28-oic acid and 3-oxo-oleanolic acid were detected in the amounts of approximately 5.95 and 1.55 µg/g D.W., respectively. Olean-12-en-28-oic acid was also detected in the leaves sprayed with Triton in the quantity of 1.92 ± 0.15 µg/g D.W. At 7 dpt MeJA increased the amount of α-amyrin/lupeol and of oleanolic acid (2.4- and 1.9-fold, respectively), in comparison to the control. In the leaves sprayed with Triton, the content of oleanolic acid remained unchanged, but the one of its isomer, ursolic acid, slightly increased (1.3-fold).

At 14 dpt the total content of triterpenoids was approximately 1.54 mg/g D.W., with steroids and pentacyclic triterpenoids reaching 1.32 mg/g and 220.81 µg/g D.W. (Table 15B). Sterols were affected by MeJA and BTH in a positive way at this time point, in regard to the control. The content of campesterol increased respectively 1.8- and 2.6-fold in these modalities. In contrast, the amount of 24-methylenocycloartanol decreased 1.3-fold upon MeJA treatment. BTH also led to an accumulation of stigmasterol and sitosterol (1.8- and 1.1-fold). The total content of pentacyclic triterpenoids increased at 14 dpt after all the treatments, particularly in BTH- and MeJA-treated leaves (2.8- and 1.9-fold, respectively), as well as in Triton modality (1.7-fold). At this time point, all of pentacyclic triterpenoids were stimulated. Triton and BTH led to an accumulation of olean-2,12-dien-28-oic acid (1.29 and 2.21 µg/g D.W., respectively) and of 3-oxo-oleanolic acid (0.49 and 3.53 µg/g D.W., respectively). In

Triton-, MeJA-, and BTH-treated leaves the content of oleanolic acid increased 3.9-, 4.8- and 3-fold, respectively. Ursolic acid was not detected at 14 dpt in the control leaves, but was identified upon all the treatments in the quantity of approximately 3.4 µg/g D.W. on average.

Table 15. The profile of major triterpenoids in the leaves of *Vitis vinifera* cv. Cabernet Sauvignon from the greenhouse subjected to treatment with Triton (Triton X-100), methyl jasmonate (MeJA) or benzothiadiazole (BTH), and collected after 7 and 14 days post-treatment. Results are referenced to leaf dry weight and expressed in µg/g as the means ± S.D. of three independent samples. The significance levels at $p \leq 0.05$ was established with comparison to the respective control samples: 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*), by one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's multiple comparison test.

A

	7 dpt			
Steroids	Control	Triton	MeJA	BTH
campesterol	53.66 ± 3.55	57.88 ± 3.75	39.85 ± 2.58 *	40.37 ± 2.65 *
stigmasterol	207.85 ± 13.45	210.72 ± 14.61	119.06 ± 7.85	129.6 ± 8.37
sitosterol	1598.3 ± 102.15	1487.28 ± 97.05	1466.92 ± 95.78	1242.98 ± 80.25
24-methylenocycloartanol	76.86 ± 5.33	80.49 ± 5.25	36.79 ± 2.45 *	67.04 ± 4.45
Sum	1936.69 ± 125.04	1836.37 ± 115.55	1662.63 ± 108.25*	1480.01 ± 95.25 *
Pentacyclic triterpenoids				
taraxerol	187.37 ± 12.10	198.47 ± 12.85	138.17 ± 8.95	325.86 ± 21.06**
α- amyrin/lupeol	37.65 ± 2.55	52.63 ± 3.55	88.57 ± 5.65 **	52.55 ± 3.36
oleanolic acid	3.54 ± 0.25	5.27 ± 0.55	6.9 ± 0.46 *	41.17 ± 2.77 ****
olean-2,12-en-28-oic acid	n.d.	1.92 ± 0.15 ***	n.d.	5.95 ± 0.45 ****
3-oxo-oleanolic acid	n.d.	n.d.	n.d.	1.55 ± 0.15 ****
ursolic acid	3.78 ± 0.28	4.8 ± 0.33 *	2.99 ± 0.15	2.89 ± 0.15
Sum	232.35 ± 15.0	263.11 ± 15.99	236.64 ± 15.33	429.99 ± 28.45 **
Total	2169.04 ± 141.05	2099.49 ± 136.45	1899.28 ± 125.65 **	1910.0 ± 125.33 **

B

	14 dpt			
Steroids	Control	Triton	MeJA	BTH
campesterol	30.67 ± 1.75	29.98 ± 1.7	55.02 ± 3.15 *	80.74 ± 4.66 **
stigmasterol	110.34 ± 6.25	104.22 ± 5.89	126.55 ± 7.18	201.49 ± 12.01 **
sitosterol	1105.07 ± 65.48	1057.87 ± 59.99	1130.28 ± 65.74	1244.6 ± 71.38 **
24-methylenocycloartanol	70.48 ± 3.88	81.7 ± 4.55	56.33 ± 3.25 *	69.55 ± 3.95
Sum	1316.56 ± 74.55	1273.87 ± 72.04	1368.21 ± 78.65	1596.4 ± 90.27 *
Pentacyclic triterpenoids				
taraxerol	182.28 ± 10.31	299.05 ± 15.33	330.29 ± 18.99 *	518.54 ± 30.05 **
α-amyrin/lupeol	35.8 ± 2.02	54.67 ± 2.95 *	65.77 ± 3.75 *	82.99 ± 4.75 **
oleanolic acid	2.72 ± 0.15	10.61 ± 0.66 ****	12.98 ± 0.88 ****	8.14 ± 0.48 ****
olean-2,12-dien-28-oic acid	n.d.	1.29 ± 0.05 ***	n.d.	2.21 ± 0.15 ****
3-oxo-oleanolic acid	n.d.	0.49 ± 0.01 ***	n.d.	3.53 ± 0.25 ****
ursolic acid	n.d.	3.06 ± 0.19 ****	3.58 ± 0.25 ****	3.49 ± 0.19 ****
Sum	220.81 ± 12.55	369.2 ± 20.18 *	412.63 ± 23.33 **	618.93 ± 36.01 ***
Total	1537.38 ± 87.95	1643.07 ± 90.07	1780.85 ± 100.75	2215.33 ± 125.27 *

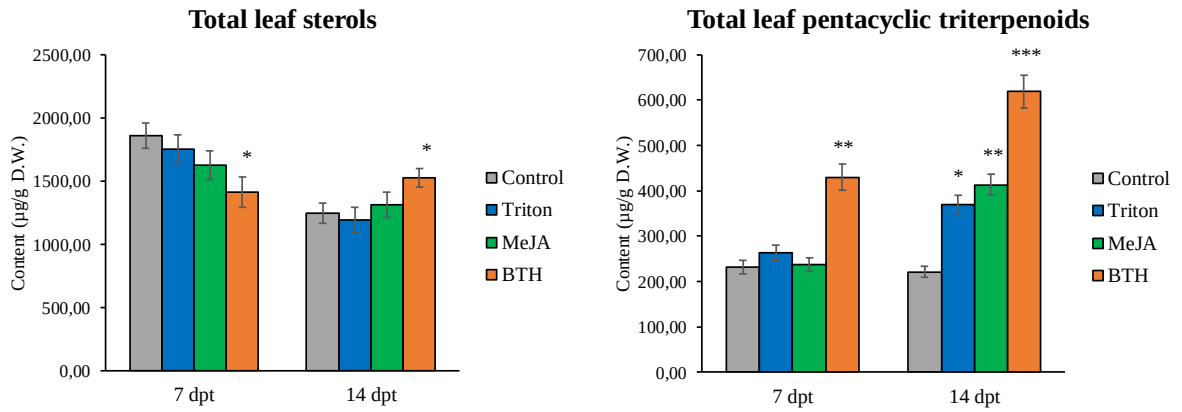

Figure 55. The profile of total sterols and pentacyclic triterpenoids in the leaves of *Vitis vinifera* cv. Cabernet Sauvignon from the greenhouse subjected to treatment with Triton (Triton X-100), methyl jasmonate (MeJA) and benzothiadiazole (BTH), and collected after 7 and 14 days post-treatment. Results are referenced to leaf dry weight and expressed in mg/g or µg/g D.W. as the means ± S.D. of three independent samples. Asterisks denote the significance levels at $p \leq 0.05$ with comparison to the respective control samples: 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*) as a result of one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's multiple comparison test.

2.2.2. Triterpenoids in leaf cuticular waxes

Strong modifications occurred in the triterpenoids profile of leaf cuticular waxes of *V. vinifera* cv. Cabernet Sauvignon upon treatment with Triton, MeJA and BTH at 14 dpt (Table 16). In the control leaves, the total content of identified compounds was approximately 193.72 µg/g F.W., with steroids and pentacyclic triterpenoids reaching 72.91 and 120.81 µg/g F.W. All the treatments led to a remarkable decrease of the amount of total steroids, 3.3-, 4.3-, and 2-fold in the leaves sprayed with Triton, MeJA and BTH, respectively (Fig. 56). Such effect was mainly the result of a decline of the amount of phytosterols in these modalities, on average of 27.8-, 2.2- and 2.7-fold respectively of campesterol, stigmasterol and sitosterol. The content of sterol precursors, cycloartenol and 24-methylenocycloartanol, augmented in BTH-treated leaves 2.1- and 1.2-fold, respectively. In the leaves sprayed with Triton, the amount of cycloartenol increased 2.2-fold and that of 24-methylenocycloartanol decreased 7.7-fold. The quantity of these two steroids was 6.8- and 3.1-fold lower in MeJA-treated leaves, in regard to the control. Some specificities could be observed for the two elicitors studied, as well as for Triton, in the stimulation of the biosynthesis of pentacyclic triterpenoids in the leaf cuticular waxes (Fig. 56). For example, taraxerol content decreased 2.6-fold in MeJA-treated leaves, and accumulated 1.3-fold in BTH-treated leaves, in regard to the control. In turn, MeJA led to a 2.3-fold increase of the amount of oleanolic acid, while BTH to its 2.5-fold decrease. Both MeJA and BTH augmented the quantity of *α*-

amyrin/lupeol (5.1- and 3.8-fold, respectively) and the quantity of oleanolic acid methyl ester (2.3- and 9.5-fold, respectively). BTH and Triton led to an accumulation of ursolic acid methyl ester, in the content of approximately 1.14 and 0.65 µg/g F.W., respectively. The leaves sprayed with MeJA and Triton shared some features of the profile of pentacyclic triterpenoids in cuticular waxes, such as a decrease of the amount of olean-2,12-dien-28-oic acid (about 2.4-fold) and an enhanced content of ursolic acid (about 4.4-fold). Moreover, betulinic acid was uniquely detected in Triton modality (0.49 ± 0.04 µg/g F.W.).

Table 16. The profile of major triterpenoids in the leaf cuticular waxes of *Vitis vinifera* cv. Cabernet Sauvignon from the greenhouse subjected to treatment with Triton (Triton X-100), methyl jasmonate (MeJA) or benzothiadiazole (BTH), and collected after 14 days post-treatment. Results are referenced to leaf fresh weight and expressed in µg/g as the means ± S.D. of three independent samples. The significance levels at $p \leq 0.05$ was established with comparison to the respective control samples: 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*), by one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's multiple comparison test.

Steroids	Control	Triton	MeJA	BTH
campesterol	34.53 ± 2.26	1.1 ± 0.08 ****	1.47 ± 0.1 ****	1.2 ± 0.08 ****
stigmasterol	5.04 ± 0.45	2.79 ± 0.19 **	3.04 ± 0.2 *	1.57 ± 0.1 ***
sitosterol	15.46 ± 1.0	3.85 ± 0.26 ****	7.84 ± 0.51 ***	7.32 ± 0.52 ***
cycloartenol	5.87 ± 0.45	12.76 ± 0.83 ***	0.86 ± 0.06 ***	12.0 ± 0.81 ***
24-methylenocycloartanol	11.99 ± 0.77	1.55 ± 0.1 ****	3.86 ± 0.27 ****	14.39 ± 0.94 *
Sum	72.91 ± 4.22	22.07 ± 1.45 ****	17.08 ± 1.11 ****	36.53 ± 2.37 ***
Pentacyclic triterpenoids				
taraxerol	116.56 ± 7.55	116.99 ± 7.76	44.16 ± 2.8 ***	153.83 ± 9.95 *
α- amyrin/lupeol	0.36 ± 0.01	0.66 ± 0.05	1.83 ± 0.13 ****	1.38 ± 0.09 ***
oleanolic acid	1.52 ± 0.1	2.01 ± 0.13	3.49 ± 0.25 ****	0.61 ± 0.06 **
betulinic acid	n.d.	0.49 ± 0.04 ****	n.d.	n.d.
olean-2,12-dien-28-oic acid	0.88 ± 0.06	0.3 ± 0.02 ***	0.47 ± 0.05 **	n.d.
3-oxo-oleanolic acid	n.d.	0.24 ± 0.03	0.28 ± 0.03	n.d.
ursolic acid	0.36 ± 0.02	1.86 ± 0.13 ****	1.31 ± 0.09 ***	n.d.
oleanolic acid methyl ester	1.1 ± 0.01	3.26 ± 0.22 ***	2.5 ± 0.04 ***	10.43 ± 0.74 ****
ursolic acid methyl ester	n.d.	0.65 ± 0.05 ***	n.d.	1.14 ± 0.08 ****
Sum	120.81 ± 7.75	126.51 ± 8.19	54.07 ± 3.37 **	167.4 ± 10.82 *
Total	193.72 ± 12.33	148.58 ± 9.63	71.15 ± 4.51 ***	203.94 ± 13.18

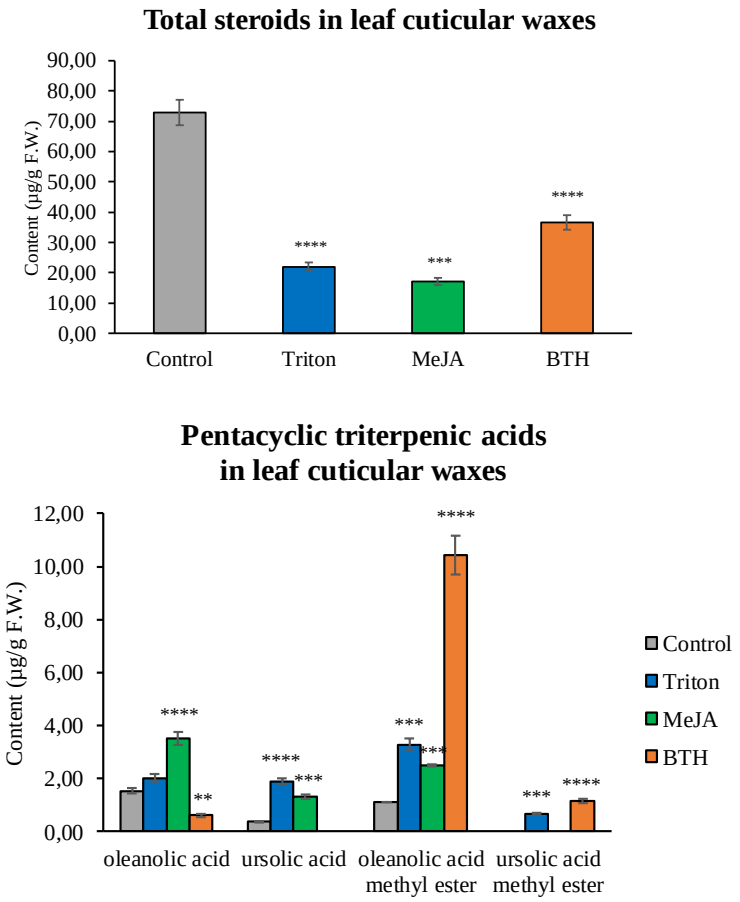

Figure 56. The profile of total steroids and pentacyclic triterpenic acids with their methyl ester derivatives, in the leaf cuticular waxes of *Vitis vinifera* cv. Cabernet Sauvignon from the greenhouse subjected to treatment with Triton (Triton X-100), methyl jasmonate (MeJA) and benzothiadiazole (BTH), and collected after 14 days post-treatment. Results are referenced to leaf dry weight and expressed in mg/g or µg/g D.W. as the means ± S.D. of three independent samples. Asterisks denote the significance levels at $p \leq 0.05$ with comparison to the respective control samples: 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*) as a result of one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's multiple comparison test.

2.3. Discussion

Steroids and pentacyclic triterpenoids are in general classified into one common class of triterpenoids, i.e., derivatives of the 30-carbon squalene (Croteau et al., 2007). However, the separation of these two groups of compounds has been proposed, based on their distinct biosynthetic pathways and different course of cyclization, as well as on a separate physiological role that they display in the plant (Phillips et al., 2006; Verpoorte, 2000). Indeed, the functions of steroids and pentacyclic triterpenoids can justify their classification into respectively primary and specialized metabolites (see part I chapter 4 and part IV chapter 1, subsec. 1.4). Consequently, biosynthetic pathways of steroids and pentacyclic triterpenoids pass through several branch points that can be regulated at various levels (Croteau et al., 2007). Thus, the competition between these two pathways may occur. Such effect was firstly observed in cell suspension cultures of *Tubernaemontana divaricata* L. treated with *Candida albicans* (Van der Heijden et al., 1989). In the frame of modular metabolic engineering, studies have been performed, in order to channelize precursor compounds for the production of pentacyclic triterpenoids, e.g., *β*-amyrin, by negative regulation of cycloartenol synthase, which catalyzes the biosynthesis of phytosterols precursor, cycloartenol (Kumar et al., 2013).

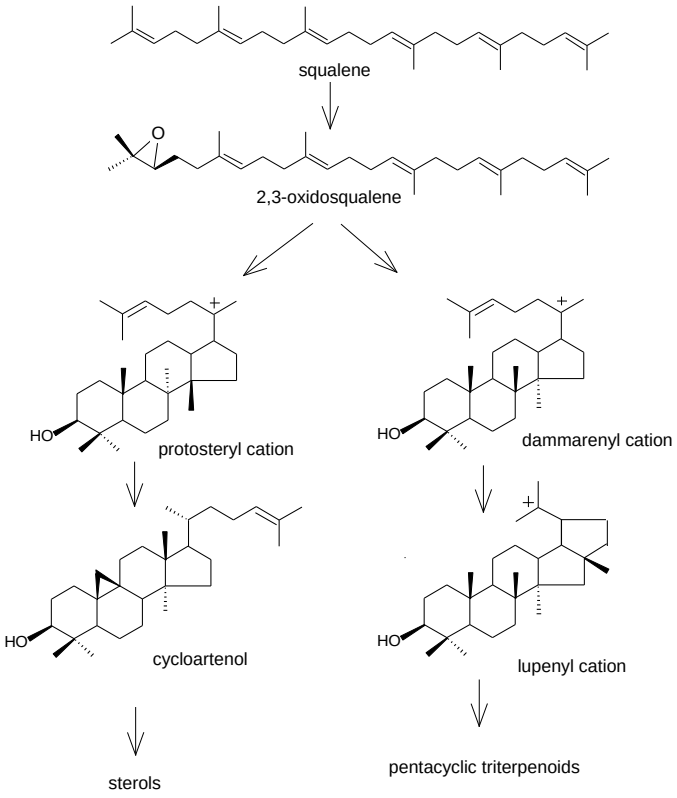

Figure 57. Pathways of biosynthesis of tetracyclic (steroids) and pentacyclic triterpenoids are competitive.

The follow-up of triterpenoids in grapevine subjected to elicitation seems to be important in the context of development of the use of PDS. It allows to simultaneously monitor the potential changes within primary metabolism and the induction of the biosynthesis of defensive molecules. In perspective, such observation could contribute to the evaluation of the balance between defense and fitness (growth and yield) in the plant. Indeed, the hypothesis according to which the defenses imply an energy cost to the plant was put forward by numerous authors (for review see Bolton, 2009). The pioneering work in this subject was performed by Smedegaard-Petersen and Stolen, (1981) who demonstrated a decrease in grain yield in barley (weight and protein content) due to infection with the causal agent of powdery mildew. Various studies have subsequently shown that the allocation of energy resources for defense reactions takes place to the detriment of their vigor (Heil et al., 2000; Huot et al., 2014; Zangerl et al., 1997). The latter, also called reproductive success or breeding value, is described in evolutionary biology as the ability of an individual of a certain genotype to reproduce (Heil, 2002). It can be measured, for example, by growth or fruit yield.

The research described in this chapter aimed to follow both steroids and triterpenoids, as primary (general) and secondary (specialized) compounds, in grapevine subjected to elicitation. First experiments were conducted on in vitro cell suspension cultures of three *V. vinifera* cultivars (PV, CS6 and GT3). MeJA was chosen as a recognized elicitor triggering grapevine defense mechanisms (e.g., stilbene accumulation) in several experimental models (Belhadj et al., 2006; Portu et al., 2015, Larronde et al. 2003; Krisa et al., 1999; Hatmi et al., 2014; Santamaria et al., 2011; Taurino et al., 2015). Among pentacyclic triterpenoids, α-amyrin/lupeol, betulin, betulinic, oleanolic, ursolic acids were analyzed. These compounds have potential role in plant defense (described in the part I, chapter 4, subsec. 4.3.2 and part IV, chapter 1, subsec. 1.4). The profile of typical phytosterols campesterol, stigmasterol and sitosterol, along with the one of their precursor, 24-methylenocycloartanol, was followed.

Impact of the elicitor concentration (50 or 100 µM) was evaluated on GT_T cultivar and the cells were collected at 24 hpt, 72 hpt and 7 dpt. At 24 hpt 50 µM MeJA led to an important increase in the content of oleanolic acid (with a decrease of its isomer, ursolic acid). No perturbation occurred in the amount of sitosterol and 24-methylenocycloartanol, the amount campesterol and stigmasterol only slightly increased. MeJA at concentration of 100 µM at this time-point was not effective as a stimulator, leading to a constant quantity (oleanolic acid) or a decrease of pentacyclic triterpenoids (ursolic acid and α-amyrin/lupeol), with no changes within phytosterols. Thus, at 24 hpt, either an upstream accumulation of phytosterols or no competing effect of sterols and pentacyclic biosynthetic pathways may be

suggested. Enhanced production of sterols upon elicitation has been already observed in the literature. For example, the content of sterols was positively influenced upon β-cyclodextrins treatment of cell suspension cultures of carrot (*Daucus carota*) (Sabater-Jara & Pendreño, 2013), in *Solanum malacoxylon* cell cultures treated with BTH (Burlini et al., 2011), or in vitroplants of *Lemna paucicostata* elicited with MeJA and silver nitrate (Suh et al., 2013). Such effect can be even desired for the production of sterols displaying positive biological activity on human health, such as cholesterol-lowering effect, anticancer, anti-atherogenic, and cardioprotective properties (MacKay and Jones, 2011). Miras-Moreno et al. (2016) give a complex review of the strategies enhancing the production of bioactive phytosterols in plant in vitro cultures.

At 72 hpt, MeJA at both concentrations negatively impacted oleanolic and ursolic acids, in turn, it increased the content of α-amyrin/lupeol. After the longer time (7 dpt), MeJA at both concentrations, particularly 100 µM enhanced the amount of oleanolic acid, and 50 µM MeJA also led to the accumulation of ursolic acid. Both at 72 hpt and 7 dpt, steroids showed the similar pattern of changes, with generally decreased amount of phytosterols along with the accumulation of their precursor, 24-methylenocycloartanol. Thus, the competition of biosynthetic pathways of sterols and pentacyclic triterpenoids could be suggested for these two time-points, however, with the accumulation of distinct defense molecules according to the time after treatment. The influence of duration of elicitor exposure, as well as of the concentration of elicitor, on the biosynthesis of different secondary metabolites in the same in vitro culture is well documented in the literature (for review see Namdeo, 2007).

Comparison of three *V. vinifera* cultivars was performed in term of their responsiveness to 100 µM MeJA at two time-points (48 hpt and 7 dpt). The profile of triterpenoids in GT3 changed in the similar way in comparison to GT_T, the other strain of the same cultivar (Gamay Fréaux var. Teinturier). The sterols and pentacyclic triterpenoids pathways competed in the cells of GT3 after the longer exposure to the elicitor (7 dpt), through an enhanced biosynthesis of oleanolic acid, the constant quantity or a decline of phytosterols, with the accumulation of the precursor of these latter, 24-methylenocycloartanol. The competition of the biosynthesis pathway of sterols and pentacyclic triterpenic acids, with the enhancement of the latter, was observed in the in vitro plantlets of *Centella asiatica*, *Ruscus aculeatus* and *Galphimia glauca* upon 100 µM MeJA treatment (Mangas et al., 2006).

In the cells of PV, at two time-points MeJA draw a metabolic flux away from α-amyrin/lupeol towards the further compounds biosynthesized in the lupane pathway, betulin and betulinic acid. However, no competition of the production of these pentacyclic

triterpenoids and the biosynthesis of phytosterols was observed (the content of the latter even increased). As it was mentioned above, simultaneous accumulation of both sterols and pentacyclic triterpenoids upon elicitation may occur. In contrast, MeJA applied to the cell suspensions of CS6 led to a decrease of the amount of pentacyclic triterpenoids detected in this cultivar (betulin and betulinic acid), or a constant quantity (α-amyrin/lupeol) at both time-points, with the accumulation of phytosterols after the longer exposure to the elicitor (7 dpt). The inverse effect of stimulation (sterols instead of pentacyclic triterpenoids) was observed, for example, in *C. officinalis* hairy root cultures treated with chitosan (Alsoufi et al., 2019).

The stimulation of pentacyclic triterpenoids biosynthesis (in particular saponins) in in vitro cultures with the use of jasmonates has been often reported in the literature and have a practical value for biotechnology (for reviews see Lambert et al., 2011; Ramirez-Estrada et al., 2016). The classical example is the production of ginsenosides in the cultures of *Panax ginseng*. An enhanced accumulation of the overall ginsenoside content was obtained in *P. ginseng* adventitious root culture (5-fold) or suspension cultures (10-fold) upon JA treatment (Yu et al., 2002; Hu et al., 2003). A 20-fold increase in the content of these compounds was achieved by the treatment of MeJA of *P. ginseng* suspension cultures (Lu et al., 2001). Recently, in the laboratory of the Department of Plant Biochemistry, 52-fold and 98-fold induction of respectively oleanolic acid and its glycosides was obtained in *C. officinalis* hairy roots treated with JA (Markowski, 2020). It is a much higher fold induction of the content of pentacyclic triterpenoids that there has been previously reported in the literature. MeJA applied as an elicitor to in vitro cultures stimulated also the accumulation of other pentacyclic triterpenoids, for example betulin and betulinic acid in the hairy root culture of *Betula pendula* Roth (Hajati et al., 2019). In the present study the highest fold of stimulation of pentacyclic triterpenoids was noted for oleanolic acid in the cultures of GT_T (a 7-fold-induction upon 100 µM MeJA treatment, at 7 dpt).

The impact of the elicitors studied as PDS in this thesis, MeJA and BTH, was evaluated on triterpenoids in both whole leaves and leaf cuticular waxes of *V. vinifera* cv. Cabernet Sauvignon from the greenhouse. In the literature there are only two studies reporting the effect of elicitation on the profile of triterpenoids in grapevine. Phytosterols content augmented in grape berries treated with BTH (Ruggiero et al., 2013), while an increased amount of ursolic, oleanolic, and betulinic acids was observed in grapes following the treatment of bunches with chitosan (Lucini et al., 2018). There is also a little information about the effect of elicitation on triterpenoids profile in other native plants. For example, MeJA treatment was reported to augment the content of betulin and oleanolic acid in different

tissues of white birch (Yin et al., 2013). Foliar application of osmotic stress agents, UV-C radiation or the treatment with JA and SA, was reported to increase bioactive saponins in *Quillaja brasiliensis* (de Costa et al., 2013). The current study provides a first insight into the effect of elicitation on a wider range of compounds belonging to both steroids and pentacyclic triterpenoids in grapevine leaves.

Different variants of the relation of the biosynthetic pathways of sterols and pentacyclic triterpenoids were observed in the native plant and depended on the treatment and the time of the exposure to the stimulus, as well as the matrix studied. In the whole leaves, the competition between the two pathways was clearly visible in the leaves treated with BTH at 7 dpt. The total content of sterols in this modality significantly decreased, and the constant amount of one of their precursor, 24-methylenocycloartanol was observed. BTH stimulated the biosynthesis of almost all of pentacyclic triterpenoids analyzed, particularly of oleanolic acid (11.6-fold induction). Oleanolic acid has been proposed as one of biomarkers of grapevine resistance towards *P. viticola* (Chitarrini et al., 2017a). The contribution of this molecule of the generally efficient protection conferred to grapevine by BTH against this pathogen may be suggested. Indeed, oleanolic and ursolic acids is known for its antifungal activities (Becker et al., 2005). At 14 dpt, all the pentacyclic triterpenoids (including taraxerol, α-amyrin/lupeol, ursolic acid and oleanolic acid along with its derivatives) were accumulated upon BTH-treatment, however, the content of sterols also slightly increased. The toxicity of α-amyrin, lupeol, and taraxerol towards insects was reported (Rodríguez et al., 1997). Thus, the elevated amount of these molecules in grapevine leaves due to elicitation could possibly contribute to its enhanced resistance to pests. However, to confirm such conclusion further investigation is needed.

In MeJA-treated leaves, at 7 dpt a decrease of the content of campesterol occurred with the accumulation of α-amyrin/lupeol and oleanolic acid. At 14 dpt, the profile of steroids in MeJA-treated leaves was similar to that observed at 7 dpt. After the longer-time of exposure to this elicitor, even more pentacyclic triterpenoids was positively affected (taraxerol, α-amyrin/lupeol, oleanolic and ursolic acids), and the content of sterols remained constant or also slightly increased. Triton also led to an accumulation of pentacyclic triterpenoids, particularly at 14 hpt. Such result may be associated with the surfactant activity of this substance, which can create a cuticle injury (Liu et al., 2016). Cutin monomers act as DAMPs that elicit plant immune responses through JA-pathway (Hou et al., 2019). An increased amount of oleanolic acid in both Triton- and MeJA-treated leaves should not be excluded as being a result of Triton surfactant activity, since this triterpenoid acid was

reported to be accumulated in grapevine leaves upon wounding (Chitarrini et al., 2017b). Moreover, BTH and Triton stimulated the accumulation of olean-2,12-dien-oic acid and 3-oxo-oleanolic acids in the whole leaf tissues. These oleanolic acid derivatives have been only detected in the leaf cuticular waxes in this research.

The biosynthetic pathways of sterols and pentacyclic triterpenoids competed in the leaf cuticular waxes upon all the treatments. Interestingly, in the leaf cuticular waxes the elicitors stimulated the accumulation of different pentacyclic triterpenoids than in the whole leaf tissue. For example, in the BTH-treated leaves the content of oleanolic acid decreased, olean-2,12-dien-oic acid and 3-oxo-oleanolic acids were not detected, but an important increase (9.5-fold) of the amount of oleanolic acid methyl ester occurred. In MeJA-treated leaves, the amount of taraxerol decreased in the leaf cuticular waxes, contrarily to the whole leaf tissue, while the amount of oleanolic acid increased in the both matrix (and in cuticular waxes its methyl ester accumulated as well). Some oleanolic acid derivatives were reported to act as feeding deterrent against potato beetle (*Leptinotarsa decemlineata*) (González-Coloma et al., 2011). However, there is no information about a role in plant defense of derivatives of pentacyclic triterpene acids detected in this study. In turn, some of them have been reported to display biological activities in human cell lines (Sultana and Ata, 2008; Ma et al., 2005).

To summarize, for the successful development of protection method based on PDS in the vineyard, the metabolic profiling of plants treated with such compounds is needed. Since essential biological functions other than defense (growth, differentiation) also require energy and primary metabolites, there could be direct or indirect competition between the latter and the activation of defense. The follow-up of steroids and triterpenoids upon elicitor treatment seems to be important since this group of molecules comprises the metabolites of both primary (sterols), and specialized (pentacyclic triterpenoids) functions. The results presented in the current chapter provides first insights on the effect of elicitation on the steroid and triterpenoid profile in grapevine cell suspension cultures and leaves. In some experimental models, the accumulation of bioactive pentacyclic triterpenoids occurred in the detriment of the biosynthesis of sterols. However, the time of exposure to the treatment, the matrix (whole leaves or cuticular waxes), as well as the cultivar and the concentration of the elicitor, played a crucial role in the modifications observed in the amounts of the studied compounds.

3. EFFECT OF ELICITATION ON GRAPEVINE DEFENSE STATUS

3.1. Protection conferred

The level of protection towards *P. viticola* conferred by the different treatments performed on the grapevine leaves and harvested at 6 dpt, varied according to the product applied (Fig. 58, 59). BTH and PHOS triggered the strongest protection to the plant, with an inhibitory effect on *P. viticola* growth, respectively at 98.5 ± 0.6 and 97.3 ± 1.1% in comparison to the control. MeJA also efficiently reduced the oomycete development but it was slightly less active than BTH and PHOS (85.8 ± 2.7% of growth inhibition). Triton, the co-formulant added in MeJA solution, induced a weak but significant protection against *P. viticola* (23.8 ± 5.8% of growth reduction). This efficiency was nevertheless considerably lower than the one provided by MeJA. No phytotoxicity effect was observed on the plants, regardless the treatment.

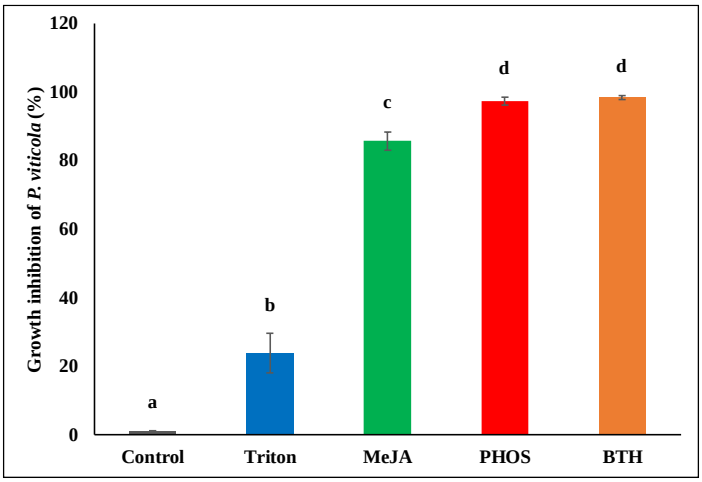

Figure 58. Growth inhibition of *Plasmopara viticola* on grapevine previously subjected to treatments with Triton (Triton X-100), MeJA (methyl jasmonate), PHOS (phosphonates) or BTH (benzothiadiazole). Data were expressed in percentage of inhibition relative to the control. Letters above columns show significant differences at $p \leq 0.05$ among modalities.

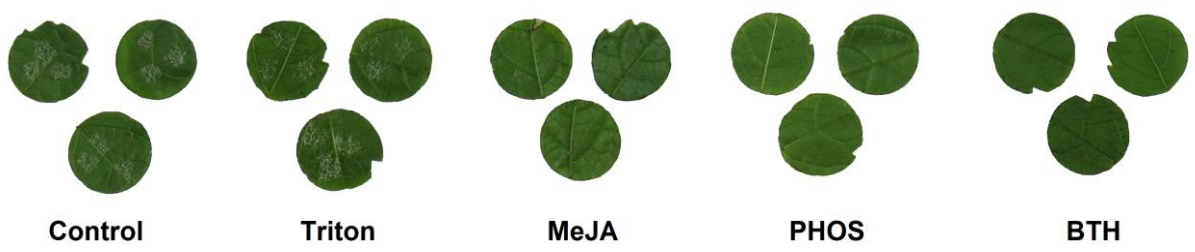

Figure 59. Visual evaluation of *Plasmopara viticola* sporulation symptoms on foliar discs generated from the grapevine leaves on which Triton X-100 (Triton), methyl jasmonate (MeJA), phosphonates (PHOS), or benzothiadiazole (BTH) was sprayed, and harvested after 6 days.

3.2. Expression of defense-related genes

Genes followed in this study are summarized in Figure 60.

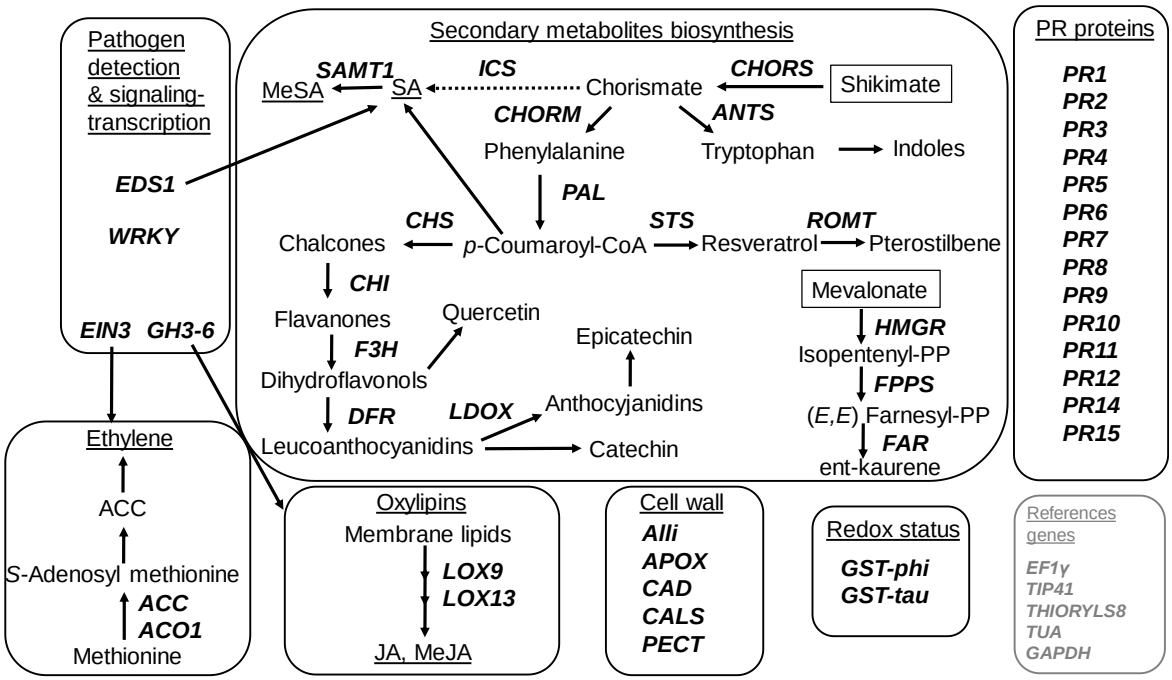

Figure 60. Genes studied and their implication in signaling or biosynthetic pathways related to defense. Based on Dufour et al. (2016). *ACC*, 1-aminocyclopropane-1-carboxylate synthase; *ACO1*, 1-aminocyclopropane-1-carboxylic acid oxidase; *Alli*, alliinase; *ANTS*, anthranilate synthase; *APOX*, ascorbate peroxidase; CAD, cinnamoyl-CoA-reductase; *CALS*, callose synthase; *CHI*, chalcone isomerase; *CHORM*, chorismate mutase; *CHORS*, chorismate synthase; *CHS*, chalcone synthase; *DFR*, dihydroflavonol 4-reductase; *EDS1*, lipase 3/enhanced disease susceptibility gene; *EF1γ*, elongation factor eEF1 gamma chain; *EIN3*, ethylene insensitive 3-Binding F Box Protein 1; *F3H*, flavanone-3-hydroxylase; *FAR*, ent-kaurene synthase; *FPPS*, farnesyl pyrophosphate synthase; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *GH3-6*, JA-Ile-synthase; *GST-phi*, glutathione *S*-transferase class-phi; *GST-tau*, glutathione *S*-transferase Tau class; *HMGR*, 3-hydroxy-3-methylglutaryl coenzyme A reductase; *ICS*, isochorismate synthase; *LDOX*, leucoanthocyanidin synthase; *LOX*, lipoxygenase; *PAL*, phenylalanine ammonia lyase; *PECT*, pectin methyl esterase; *PR1*, pathogen-related protein 1; *PR10*, ribonuclease; *PR11*, chitinase class V; *PR12*, defensin-like protein-oxalate oxidase; *PR14*, lipid transfer protein; *PR15*, germin-like protein-oxalate oxidase; *PR2*, beta-1,3-glucanase; *PR3*, endochitinase class; *PR4*, chitinase class IV; *PR5*, thaumatin-like protein; *PR6*, serine protease inhibitor; *PR7*, subtilisin-like endoprotease; *PR8*, acidic endochitinase-like; *PR9*, cationic peroxidase 1; *ROMT*, resveratrol *O*-methyltransferase; *SAMT*, salicylic acid methyl transferase; *STS*, stilbene synthase; *THIORYLS8*, catalytic thioredoxin-like protein 4A; *TIP41*, TIP41-like protein; *TUA*, tubulin alpha; *WRKY*, WRKY transcription factor.

3.2.1. Gene expression at 6 days post-elicitation

At 6 dpt (Fig. 61), gene overexpression occurred mainly after BTH and Triton treatments (22 and 15 induced genes, respectively), and was lower in the leaves treated with MeJA and PHOS (8 and 6 up-regulated genes, respectively). Conversely, BTH and Triton modalities significantly repressed few genes (2 and 1, respectively), while MeJA and PHOS treatments led to a significant repression of 50% or more of all modulated genes.

The expression of modulated genes at 6 dpt is presented on the heatmap (Fig. 62). All modalities triggered the overexpression of 2 PR proteins genes: *PR5* (thaumatin-like protein), and to a lesser extent *PR4* (chitinase). The leaves treated with Triton or BTH shared some additional up-regulated genes such as *PR2* (glucanase), *PR3* and *PR8* (chitinases class I and III, respectively). PHOS induced only 2 PR: *PR5* and *PR6* (serine protease inhibitor). The stimulation of the latter was also observed in the leaves sprayed with Triton. After MeJA treatment, 5 PR genes out of 14 were overexpressed (*PR4*, *PR5*, *PR9*, *PR10* and *PR14*), with more specifically the overexpression of *PR9* (lignin-forming peroxidase) and *PR14* (lipid transfer protein). *PR10* (ribonuclease) was induced both by MeJA and Triton treatments. Regarding the down-regulated genes, *PR7* (subtilisin-like endoprotease) and *PR11* (chitinase I) were repressed by both MeJA and PHOS. *PR6* was specifically repressed by MeJA, and *PR8* by PHOS.

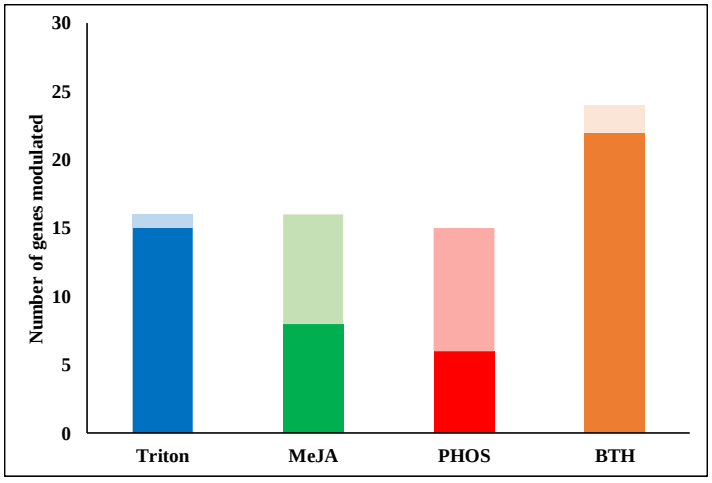

Figure 61. Numbers of defense-related genes significantly up- or down-regulated at 6 days after treatment. Over-expressed genes are represented in darker shade and down-regulated genes in lighter shade. Triton, Triton X-100; MeJA, methyl jasmonate; PHOS, phosphonates; BTH, benzothiadiazole.

Transcripts of phenylpropanoid pathway genes were significantly accumulated in Triton-treated leaves, and even more in BTH-treated leaves. Amongst these genes, we noted those involved in resveratrol biosynthesis (*PAL* and *STS*), and in the flavonoid pathway (chalcone synthase, *CHS*, and anthocyanidin synthase, *LDOX*). In PHOS and MeJA modalities, almost all genes remained unaffected.

The indole pathway was not or only slightly modulated by all treatments, except by PHOS with the down-regulation of anthranilate synthase (*ANTS*) and chorismate mutase (*CHORM*), and conversely, in the leaves treated with BTH, an up-regulation of *CHORM* and *ICS* (isochorismate synthase) was observed.

Responses of the 4 studied *GST* (glutathione *S*-transferase) genes also differed according to the treatment. They were similar for MeJA and PHOS with a down-regulation of *GST2* and *GST4*. *GST2* was also repressed by Triton, and *GST4* after BTH application. In BTH treated-leaves, the up-regulation of 3 *GST* genes (*GST2, GST3* and *GST5*) was noted.

Concerning the lipoxygenase genes, *LOX13* (lipoxygenase 13, involved in jasmonate biosynthesis) expression was stimulated in Triton and, more strongly, in MeJA modalities. PHOS had no effect, and BTH induced significantly lipoxygenase 9 (*LOX9*) expression.

In turn, no significant change was noted for the cell wall reinforcement genes in Triton- and BTH-treated leaves. MeJA triggered the up-regulation of *CAD* (cinnamyl alcohol dehydrogenase) and the down-regulation of *Alli* (alliinase). PHOS induced significantly the expression of 3 genes out of 4 (callose synthase (*CALS*), pectin methyl esterase (*PECT*), and cinnamyl alcohol dehydrogenase (*CAD*)).

Among the genes of hormone signaling class, only the transcription factor *WRKY6* was up-regulated after Triton and MeJA treatments. MeJA also induced a key gene of ethylene biosynthesis, *ACO1* (1-aminocyclopropane-1-carboxylic acid oxidase) but repressed a gene involved in SA pathway, *ICS* (isochorismate synthase). BTH led to a strong induction of genes of the SA pathway (SA methyl transferase (*SAMT*), and *ICS*), and also of *HT5* (hexose transporter). Conversely, PHOS down-regulated *GH3-6* (JA-Ile-synthase) and similarly to MeJA, *SAMT*.

Figure 62. Pattern of relative expression of defense-related genes in grapevine leaves 6 days after treatment with Triton (Triton X-100), MeJA (methyl jasmonate), PHOS (phosphonates) or BTH (benzothiadiazole). Expression data are given after log2 transformation. Gene expression of control leaves was used as reference to calculate the relative expression. Each column represents a treatment modality and each line corresponds to one gene represented by a single row of boxes. The color scale bars represent the ratio values corresponding to the mean of three independent experiments. Genes up-regulated appear in shades of red, with expression level higher than 5 in bright red, while those down-regulated appear in shades of blue, with intensity lower than -5 in dark blue. Numbers in boxes represent the significant changes in gene expression ($p \leq 0.05$) in treated-leaves compared to control. Significant differences are the result of one-way analysis of variance (ANOVA) followed by the post-hoc Dunnet's multiple comparison test.

3.2.2. Gene expression at 8 days post-elicitation and the effect of the pathogen

In the control leaves for the inoculated ones (8 dpt-2 dpi), referred as „8 dpt", modulation of gene expression was relatively low (from 2 to 12 genes) and depended on the treatment (Fig. 63). All modalities exhibited only over-expression of genes, except for PHOS which at contrary, inhibited gene expression (2 genes down-regulated). Five and 6 genes were up-regulated in Triton and BTH-treated leaves, respectively, and 12 genes in MeJA-treated leaves.

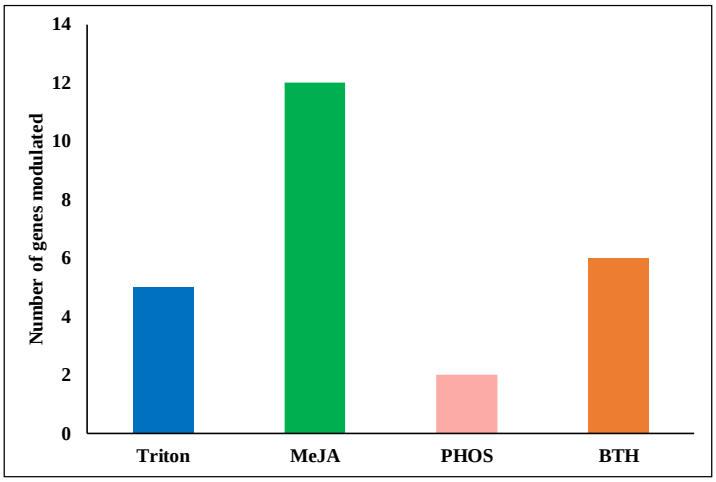

Figure 63. Numbers of defense-related genes significantly up- or down-regulated at 8 days after treatment. Over-expressed genes are represented in darker shade (Triton, MeJA, BTH) and down-regulated genes in lighter shade (concerns only PHOS which did not up-regulate any gene). Triton, Triton X-100; MeJA, methyl jasmonate; PHOS, phosphonates; BTH, benzothiadiazole.

In the control inoculated leaves (8 dpt-2 dpi) in comparison to un-inoculated leaves (8 dpt), all significant modulations of genes were down-regulations, except for *PR1* which was induced (Fig. 64 (first column)). Many PR proteins were significantly repressed such as *PR7*, *PR8*, *PR9* and *PR11*, as well as 8 genes out of 10 involved in phenylpropanoid biosynthesis, such as *PAL*, *STS*, *CHS*, *LDOX* and *F3H* (flavanone-3-hydroxylase). Numerous genes encoding other pathways also appeared to be repressed, as genes of the indole pathway, 2 glutathione *S*-transferases (*GST4* and *GST5*), *LOX9*, *WRKY6* and *GH3-6* (JA-Ile synthase).

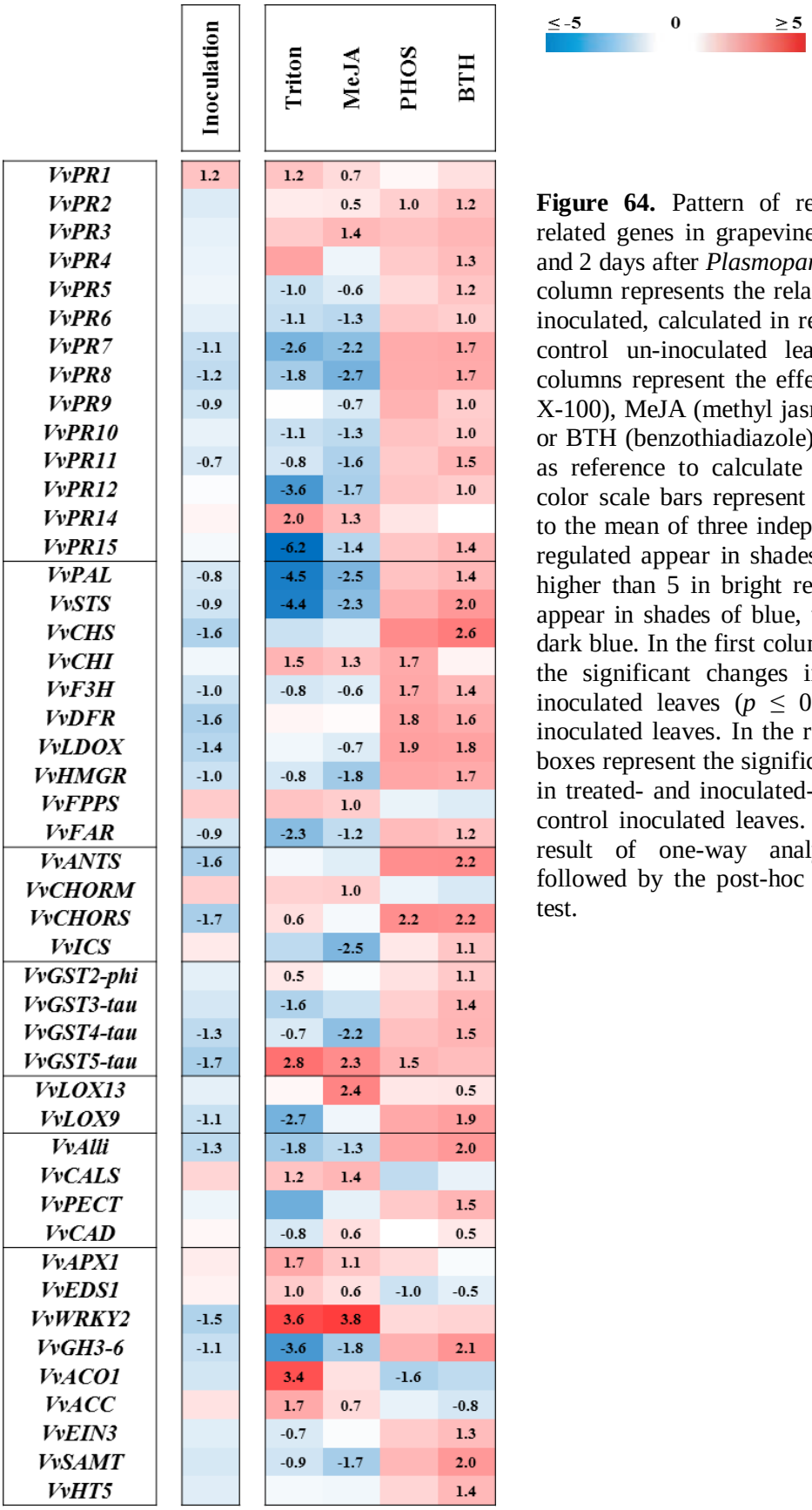

Figure 64. Pattern of relative expression of defense-related genes in grapevine leaves 8 days after treatment and 2 days after *Plasmopara viticola* inoculation. The first column represents the relative gene expression in control inoculated, calculated in regard to the gene expression in control un-inoculated leaves as reference. The other columns represent the effects of treatment Triton (Triton X-100), MeJA (methyl jasmonate), PHOS (phosphonates) or BTH (benzothiadiazole) with control inoculated leaves as reference to calculate relative gene expression. The color scale bars represent the ratio values corresponding to the mean of three independent experiments. Genes up-regulated appear in shades of red, with expression level higher than 5 in bright red, while those down-regulated appear in shades of blue, with intensity lower than -5 in dark blue. In the first column, numbers in boxes represent the significant changes in gene expression in control inoculated leaves ($p \leq 0.05$) compared to control un-inoculated leaves. In the remaining columns, numbers in boxes represent the significant changes in gene expression in treated- and inoculated-leaves ($p \leq 0.05$) compared to control inoculated leaves. Significant differences are the result of one-way analysis of variance (ANOVA) followed by the post-hoc Dunnet's multiple comparison test.

Major differences were revealed in the leaves after inoculation and depended on elicitor pretreatment (Fig. 64 (columns 2 to 5); Fig. 65). It is worth to note that the changes obtained in Triton, MeJA or BTH leaves at 8 dpt-2 dpi were more important (from 33 to 36 modulated genes) than those at 6 dpt (from 15 to 24), except for PHOS-treated leaves (8 modulated genes). Noticeably, for both Triton and MeJA, gene expression profiles were very similar with a majority of genes being significantly repressed (63.6 and 57.14%, respectively). Conversely, only 5.5% of the genes were down-regulated and 94.5% were overexpressed in BTH-treated and inoculated leaves. This profile tended to be similar for PHOS but with fewer genes significantly modulated (3-fold less than BTH).

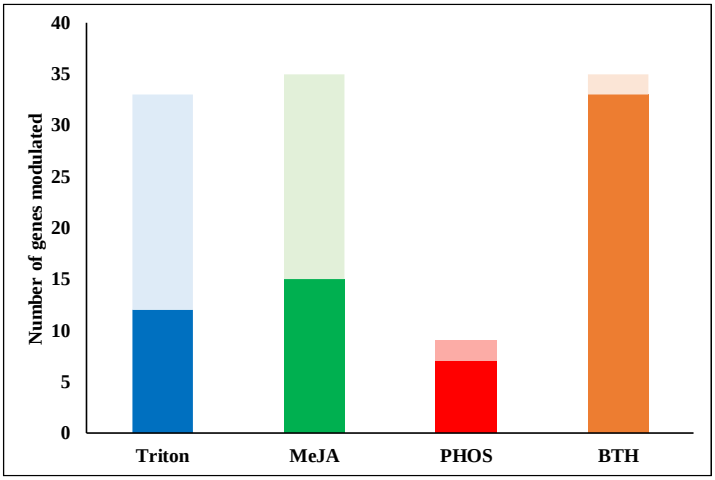

Figure 65. Numbers of defense-related genes significantly up- or down-regulated at 8 days after treatment. Over-expressed genes are represented in darker shade and down-regulated genes in lighter shade. Triton, Triton X-100; MeJA, methyl jasmonate; PHOS, phosphonates; BTH, benzothiadiazole.

More in detail (Fig. 64), Triton and MeJA triggered nearly the same gene expression changes. Their effects were characterized by a significant down-regulation of most of the PR-proteins genes studied (*PR5*, *PR6*, *PR7*, *PR8*, *PR10*, *PR11*, as well as defensin-like, *PR12* and germin-like protein-oxalate oxidase, *PR15*), of resveratrol biosynthesis genes (*PAL* and *STS*), and of some other genes involved in different pathways (*HMGR*, *F3H*, *GST4* and *Alli*), including genes related to JA (*GH3-6*) and SA pathways (*SAMT*). Among the genes commonly overexpressed, we identified two PR protein genes (*PR1* and *PR14*), a chalcone isomerase (*CHI*), *GST5*, genes involved in parietal reinforcement (*CALS* and ascorbate peroxidase (*APOX*)), and some genes involved in phytohormone signalization (*WRKY*, lipase 3/enhanced disease susceptibility gene, (*EDS1*) and *ACC*).

In PHOS-treated and inoculated leaves, only *EDS1* and *ACO1* were repressed. These two signaling genes were, on the contrary, overexpressed in Triton- and MeJA-treated and inoculated leaves. Some of the up-regulated genes were common to those identified in BTH treated and inoculated leaves such as *PR2*, *DFR* (dihydroflavonol 4-reductase), *LDOX*, *F3H* and *CHORS*. Compared to all other treatments, only one significant specific modulation by PHOS treatment could be noted, i.e., the repression of *ACO1* gene, which was induced by Triton and not affected by MeJA and BTH.

In contrast, in BTH-treated and inoculated leaves, 75% of studied genes (34 out of 48) were significantly up-regulated and only 2 genes were significantly repressed (*EDSI* and *ACC*) (Fig. 64). Among the 34 significantly overexpressed genes, the majority of them encoded PR proteins or belonged to the phenylpropanoid pathway. Genes of the indole pathway (*ANT*, *CHORS* and *ICS*), all the studied *GST* genes (*GST2*, *GST3*, *GST4* and *GST5*), lipoxygenase genes (*LOX9* and *LOX13*), genes involved in parietal reinforcement (*Alli*, *PECT* and *CAD*), and those involved in hormonal signaling (*GH3.6*, *EIN3* and *SAMT*) were also positively modulated.

3.3. Polyphenol analysis

The following polyphenols were quantified in this study: 12 stilbenes, including 4 monomers (*trans*-resveratrol, *trans*-pterostilbene, *cis*- and *trans*-piceids), 6 dimers (*trans*-δ-, *trans*-ε- and *trans*-ω-viniferins, pallidol, parthenocissin A, vitisinol C), and 2 tetramers (hopeaphenol, isohopeaphenol); 2 flavanols (catechin, epicatechin); 2 flavonols (quercetin-*O*-glucoside, quercetin-*O*-rutinoside). Besides, in the analyzed extracts other polyphenols were identified. Some of them were present in lower amounts (e.g., *cis*-resveratrol) or their profiles were not significantly altered, thus, they were not considered in this study. The analysis parameters are precised in the part III, chapter 5, subsec. 5.2. Fragment conditions and collision energies (CEs) are shown in Table A2. The chemical structures of the polyphenols studied are presented in Figure A2 (Appendix).

3.3.1. Polyphenol content at 6 days post-elicitation

At 6 dpt (Table 17) the total amount of polyphenols reached 14.65 ± 0.47 µg/mg D.W. in the un-treated leaves. The sum of stilbenes, flavonols, and flavanols content was respectively 7.28 ± 0.38, 7.13 ± 0.08 and 0.22 ± 0.01 µg/mg D.W. In Triton- and MeJA-treated leaves, the total amount of all the classes of polyphenols studied significantly

increased, respectively 2.4- to 2.9-fold in comparison to the control leaves. After PHOS or BTH treatments, stilbenes content decreased respectively 3.2- and 8.3-fold in comparison to the control leaves. The amount of flavanols and flavonols increased in BTH-treated leaves (approximately 3- and 2-fold, respectively), while in PHOS-treated leaves their content remained unchanged, in regard to the control.

The enhancement of total stilbenes content by the treatments with MeJA and Triton, in comparison to the control, was explained by the accumulation of *trans*-resveratrol, *cis*- and *trans*- piceids, *ε*-, *ω*-, and *δ*-viniferins, pallidol, hopeaphenol and isohopeaphenol, particularly in the case of MeJA. The content of pterostilbene was unchanged in all experimental conditions. Regarding flavanols, in MeJA-, Triton- and BTH-treated leaves, the content of catechin was respectively 5.1-, 3.1-, and 2.6-fold higher than in the control leaves, while the amount of epicatechin significantly increased in MeJA- and BTH-treated leaves (11.0- and 7.5-fold, respectively). The content of both flavonols, quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside) increased in the leaves on which Triton and BTH were sprayed (both compounds approximately 2 to 2.5-fold in these two modalities). The amount of quercetin-3-*O*-glucoside also significantly augmented after MeJA treatment (2.3-fold).

Table 17. Content of polyphenols in the grapevine leaves treated with Triton X-100 (Triton), methyl jasmonate (MeJA), phosphonates (PHOS), or benzothiadiazole (BTH), and harvested after 6 days. Results are referenced to leaf dry weight (µg/mg) as the means ± S.D. of three independent samples analysed in triplicate. Asterisks denote the significance levels at $p \leq 0.05$ with comparison to the control samples: 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*) as a result of one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's multiple comparison test.

	control	Triton	MeJA	PHOS	BTH
Stilbenes					
trans- resveratrol	0.27 ± 0.15	0.51 ± 0.1	0.83 ± 0.04 *	0.04 ± 0.01	0.07 ± 0.02
piceids (sum)	2.62 ± 1.56	5.5 ± 0.9	8.05 ± 0.524 *	0.29 ± 0.03	0.54 ± 0.16
pterostilbene	1.05 ± 0.52	1.5 ± 0.42	0.78 ± 0.05	0.81 ± 0.35	0.58 ± 0.15
ε- viniferin	1.63 ± 1.15	4.25 ± 0.88	9.64 ± 0.54 **	0.25 ± 0.1	0.12 ± 0.03
ω- viniferin	0.37 ± 0.19	0.2 ± 0.02	1.15 ± 0.08 **	0.17 ± 0.05	0.08 ± 0.01
δ- viniferin	0.2 ± 0.11	0.47 ± 0.06	1.1 ± 0.09 ***	0.01 ± 0	0.02 ± 0.01
pallidol	0.16 ± 0.05	2.23 ± 0.01 ***	0.87 ± 0.14 ***	0.10 ± 0.1	0.04 ± 0.01
parthenocissin A	0.35 ± 0.24	0.66 ± 0.05	1.89 ± 0.21 ***	0.16 ± 0.11	0.05 ± 0.02
vitisinol C	0.08 ± 0.01	0.06 ± 0.02	0.10 ± 0.01	0.02 ± 0 **	0.07 ± 0.02
hopeaphenol	0.11 ± 0.02	0.15 ± 0	0.04 ± 0.01 **	0.04 ± 0 **	0.11 ± 0.09
isohopeaphenol	0.41 ± 0	2.82 ± 0.33 ***	6.3 ± 0.29 ***	0.32 ± 0.06	0.47 ± 0.1
Sum	7.28 ± 0.38	18.41 ± 0.22 ***	30.81 ± 2.64 ***	2.26 ± 0.59 ***	2.20 ± 0.34 ***
Flavanols					
catechin	0.21 ± 0.01	0.65 ± 0.04 **	1.06 ± 0.11 ***	0.27 ± 0.05	0.53 ± 0.07 **
epicatechin	0.01 ± 0	0.09 ± 0.04	0.22 ± 0.01 *	0.03 ± 0.02	0.14 ± 0.06
Sum	0.22 ± 0.01	0.74 ± 0 **	1.28 ± 0.11 ***	0.31 ± 0.04	0.68 ± 0.09 **
Flavonols					
quercetin-3-*O*- glucoside	2.68 ± 0.1	7.09 ± 0.2 **	6.30 ± 0.54 *	3.56 ± 0.23	5.96 ± 0.91 *
quercetin-3-*O*- rutinoside	4.44 ± 0.15	9.05 ± 0.35 **	4.38 ± 0.68	5.39 ± 0.25	8.20 ± 0.61 **
Sum	7.13 ± 0.08	16.15 ± 0.22 **	10.68 ± 1.12 *	8.95 ± 0.01	14.17 ± 2.14 **
Total	14.65 ± 0.47	35.31 ± 0.45 ***	42.78 ± 2.42 ***	11.53 ± 0.65	17.06 ± 2.63

3.3.2. Polyphenol content at 8 days post-elicitation and 2 days post-inoculation

At 8 dpt (Table 18) in the control un-inoculated leaves, the total content of polyphenols was 50.55 ± 7.24 µg/mg D.W., with stilbenes, flavanols and flavonols reaching 15.94, 24.41, and 10.2 µg/mg D.W., respectively. After inoculation, the control inoculated leaves (8 dpt-2 dpi) exhibited no significant modification in the polyphenols content compared to the un-inoculated control ones (8 dpi). MeJA and BTH affected the total content of polyphenols in both un-inoculated and inoculated leaves, in comparison to their respective controls, i.e., control 8 dpt or 8 dpt-2 dpi. Such result was assigned by stilbenes, in the case of MeJA, and by flavanols for BTH. Triton triggered an increase of total polyphenols if 'Triton 8 dpt' was compared to 'control 8 dpt' (3.3-fold). The inoculation of MeJA-pretreated leaves triggered a significant enhancement of total stilbenes from 72.26 ± 6.27 µg/mg D.W. ('MeJA 8 dpt') to 170.90 ± 12.81 µg/mg D.W. ('MeJA 8 dpt-2 dpi'), i.e., 2.4-fold. The content of total stilbenes was 8.3-fold higher in 'MeJA 8 dpt-2 dpi' in comparison to 'control 8 dpt-2 dpi'. The content of the sum of flavonols augmented after inoculation in Triton-, MeJA-, and BTH-pretreated leaves in comparison to the control inoculated leaves (on average 1.4-fold). The flavanol content significantly increased in both un-inoculated and inoculated BTH-treated leaves and gained 9.6- and 7.4-fold higher level in regard to respective the controls. A slight increase of flavanols was also noted in Triton 8 dpt and MeJA 8 dpt-2 dpi (4.1- and 3.8-fold, respectively), in comparison to their respective controls, 8 dpt and 8 dpt-2 dpi.

The content of individual compounds within the classes of polyphenols studied elucidated their contribution in the main profiles. MeJA at 8 dpt showed an increased amount of the sums of piceids, *ε*-viniferin and isohopeaphenol, in regard to the control (8 dpt) (respectively 5.7-, 8.5-, and 40.9-fold). Inoculation of MeJA-pretreated leaves enhanced the amount of a half of stilbenic compounds studied. The content of piceids, *ε*- and *ω*-viniferins, parthenocissin, hopeaphenol and isohopeaphenol augmented in 'MeJA 8 dpt-2 dpi' respectively 7.3-, 14.9-, 43.2-, 42.7-, 6.6- and 50.4-fold, in regard to the 'control 8 dpt-2 dpi'. Inoculation of MeJA-treated leaves (MeJA 8 dpt-2 dpi), when compared to the control 'MeJA 8 dpt', led to an additional increase of the amount of stilbenes mentioned above 1.8-, 2.8-, 22.6-, 14.9-, 2,.4-, and 3.9-fold. In contrast, inoculation of MeJA-treated leaves caused a decrease of *δ*-viniferin (2.8-fold in regard to 'MeJA 8 dpt'). Concerning the remaining polyphenols for this modality, quercetin-3-*O*-glucoside augmented in 'MeJA 8 dpt' and 'MeJA 8 dpt-2 dpi' respectively 1.4- and 1.7-fold, in regard to their corresponding controls,

but not when compared to each other. In 'MeJA 8 dpt-2 dpi' leaves the amount of epicatechin was also significantly raised in regard to the 'control 8 dpt-2 dpi' (9.5-fold).

Piceids represented respectively from approximately 50 to 70% of total stilbenes in MeJA and Triton modalities. Like in MeJA modality, the content of these compounds was higher in Triton un-inoculated and inoculated leaves in comparison to the corresponding controls (respectively, 4- and 2.3-fold). However, their content was similar in 'Triton 8 dpt' and 'Triton 8 dpt-2 dpi'. Similarly, the content of ε-viniferin in Triton un-inoculated and inoculated leaves increased in comparison to their corresponding controls (respectively, 5.2- and 3.7-fold), but it remained unchanged when compared to each other. Other modifications in the content of stilbenes in Triton modality included a 4-fold increase of the content of pallidol in Triton un-inoculated leaves, and a 2.4-fold augmentation of the content of isohopeaphenol in the leaves pretreated with Triton and inoculated, in regard to the un-treated respective controls. Howeber, the fold induction of individual stilbenes by Triton, was always lower than that observed after MeJA treatment. Like in MeJA modality, the amount of quercetin-3-*O*-glucoside was noted to be higher approximately 1.4-fold in both 'Triton 8-dpt' and 'Triton 8 dpt-2 dpi', in comparison to the respective controls. In contrast, the content of quercetin-3-*O*-rutinoside increased in 'Triton 8 dpt-2 dpi' only if compared to 'Triton 8-dpt'.

In PHOS modalities, some important quantitative changes occurred within stilbenes and flavonols. The amount of pterostilbene augmented both in 'PHOS 8 dpt-2 dpi' in comparison to the control (2.4-fold). In PHOS un-inoculated leaves the quantity of δ-viniferin decreased 2.1-fold in regard to the control un-inoculated leaves. The content of both flavanols increased only after inoculation of PHOS-pretreated leaves (approximately 1.3-fold), in comparison to 'PHOS 8 dpt'.

In BTH-treated leaves the main modifications were noted in the content of both flavanols. The amount of catechin notably increased in 'BTH 8 dpt' (8.4-fold), as well as after inoculation (6.7-fold), in comparison to the controls. A spectacular change was noted for epicatechin in BTH-treated leaves where its content was 20.9- and 14.0-fold higher in, respectively, 'BTH 8 dpt' and 'BTH 8 dpt-2 dpi', in regard to their respective controls. However, inoculation did not significantly modify the quantity of flavanols, in BTH pretreated leaves. In contrast, the pterostilbene content increased only after inoculation in BTH-treated leaves (8 dpt-2 dpi) compared to 'BTH 8 dpt' (2.7-fold). The content of the two flavonols considered increased in BTH-treated and inoculated in regard to both the control at 8 dpt and BTH at 8 dpt, between 1.2- and 1.4-fold for each case.

Table 18. Content of polyphenols in the grapevine leaves treated with Triton X-100 (Triton), methyl jasmonate (MeJA), phosphonates (PHOS), or benzothiadiazole (BTH), harvested after 6 days and subjected to inoculation with *Plasmopara viticola* (8 dpt / 8 dpt-2 dpi). Results are referenced to leaf dry weight and expressed µg/mg D.W. as the means ± S.D. of three independent samples analysed in triplicate. Asterisks (in black) denote the significance levels at $p \leq 0.05$ with comparison to the respective control samples (8 dpt or 8 dpt-2 dpi): 0 to 0.001 (***), 0.001 to 0.01 (**), 0.01 to 0.05 (*) as a result of one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's multiple comparison test. Asterisks (in color) in parentheses indicate the significant differences between un- and inoculated leaves within a treatment. n.d. – not detected.

	control		Triton		MeJA		PHOS		BTH	
	8 dpt	8 dpt-2 dpi	8 dpt	8 dpt-2 dpi	8 dpt	8 dpt-2 dpi	8 dpt	8 dpt-2 dpi	8 dpt	8 dpt-2 dpi
Stilbenes										
trans- resveratrol	1.33 ± 0.09	1.69 ± 0.31	2.36 ± 0.26	2.69 ± 0.54	n.d *	n.d. ***	0.88 ± 0.29	1.00 ± 0.08	1.46 ± 0.09	0.89 ± 0.04
piceids (sum)	9.16 ± 0.28	12.91 ± 0.73	36.94 ± 0.75 ***	30.07 ± 0.22 ***	52.39 ± 1.79 *** (*)	94.14 ± 1.91 ***	13.44 ± 0.52	14.70 ± 0.32	13.24 ± 0.03	13.64 ± 0.32
pterostilbene	0.35 ± 0.13	0.52 ± 0.15	0.66 ± 0.01	0.80 ± 0.07	0.50 ± 0.15	0.26 ± 0.03	0.83 ± 0.07 * (*)	1.26 ± 0.10 **	0.28 ± 0.06 (*)	0.76 ± 0.08
ε- viniferin	0.80 ± 0.05	1.29 ± 0.14	4.16 ± 0.80 **	4.75 ± 0.46 ***	6.82 ± 0.13 *** (*)	19.3 ± 1.19 ***	0.73 ± 0.04	0.96 ± 0.07	0.86 ± 0.08	0.71 ± 0.04
ω- viniferin	0.92 ± 0.77	0.21 ± 0.05	0.57 ± 0.07	0.35 ± 0.02	0.4 ± 0.02 (*)	9.16 ± 3.51 **	0.1 ± 0.02	0.12 ± 0.02	0.59 ± 0.06	0.08 ± 0.01
δ- viniferin	1.34 ± 0.03	0.9 ± 0.26	1.79 ± 0.27	2.02 ± 0.22	1.24 ± 0.03 (*)	0.44 ± 0.02	0.64 ± 0.08 *	0.67 ± 0.04	0.33 ± 0.03 **	0.36 ± 0.02
pallidol	1.35 ± 0.47	1.92 ± 0.14	5.49 ± 0.36 **	4.92 ± 0.28 **	3.19 ± 2.57	1.12 ± 0.17	0.65 ± 0.3	1.1 ± 0.03	0.08 ± 0.01	0.28 ± 0.13
parthenocissin A	0.46 ± 0.03	0.52 ± 0.18	0.96 ± 0.1	1.06 ± 0.07	1.51 ± 0.05 (*)	22.55 ± 10.42 *	0.24 ± 0.03	0.4 ± 0.01	0.44 ± 0.25	0.1 ± 0.03
vitisinol C	0.02 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	0.03 ± 0.01	0.04 ± 0.02	0.08 ± 0.04	0.02 ± 0.01	0.01 ± 0	0.02 ± 0.02	0.01 ± 0.01
hopeaphenol	0.03 ± 0.02	0.06 ± 0.01	0.08 ± 0.03	0.1 ± 0.05	0.19 ± 0.02 (*)	0.45 ± 0.07 ***	0.07 ± 0.01	0.06 ± 0.02	0.03 ± 0	0.04 ± 0.02
isohopeaphenol	0.14 ± 0.03	0.46 ± 0.04	2.87 ± 0.08 * (*)	1.12 ± 0.05 *	5.95 ± 0.15 *** (*)	23.43 ± 1.08 ***	0.16 ± 0.05	0.26 ± 0.03	0.15 ± 0.07	0.31 ± 0.03
Sum	15.93 ± 1.17	20.54 ± 2.61	55.96 ± 3.49 ***	47.95 ± 1.92 ***	72.26 ± 6.27 *** (***)	170.98 ± 12.81 ***	17.79 ± 0.17	20.57 ± 0.7	17.52 ± 0.02	17.21 ± 0.44
Flavanols										
catechin	22.18 ± 2.98	22.97 ± 1.55	83.19 ± 19.48	56.82 ± 13.34	59.79 ± 0.22	72.79 ± 2.46	38.03 ± 5.98	58.58 ± 5.70	186.81 ± 42.81 ***	154.47 ± 13.30 ***
epicatechin	2.23 ± 0.31	2.52 ± 0.19	15.71 ± 4.54	10.22 ± 2.53	13.15 ± 0.59	23.99 ± 0.94 **	5.56 ± 1.01	6.71 ± 0.70	46.69 ± 8.83 ***	35.38 ± 4.65 ***
Sum	24.41 ± 3.29	25.49 ± 1.73	98.90 ± 24.02	67.04 ± 15.87	72.94 ± 0.81	96.78 ± 3.37	43.59 ± 6.99	65.29 ± 6.35	233.51 ± 51.64 ***	189.85 ± 17.90 ***
Flavonols										
quercetin-3-*O*- glucoside	5.05 ± 0.18	4.7 ± 0.36	6.88 ± 0.09 **	6.63 ± 0.26 **	6.93 ± 0.01 **	8.18 ± 0.29 ***	4.47 ± 0.04 (*)	5.81 ± 0.37	5.50 ± 0.03 (*)	6.83 ± 0.08 ***
quercetin-3-*O*- rutinoside	5.14 ± 0.15	4.68 ± 0.37	5.7 ± 0.07 (*)	7.6 ± 0.06	4.78 ± 0.21	4.54 ± 0.36	4.4 ± 0.06 (*)	5.67 ± 0.18	5.31 ± 0.03 (*)	6.77 ± 0.35 ***
Sum	10.2 ± 0.52	9.38 ± 1.25	12.58 ± 0.04	14.23 ± 0.45 ***	11.71 ± 0.31 (*)	12.73 ± 1.12 **	8.88 ± 0.14 (*)	11.48 ± 0.96	10.81 ± 0.09	13.61 ± 0.61 ***
Total	50.54 ± 7.24	55.42 ± 6.63	167.45 ± 37.5 **	129.23 ± 23.91	156.91 ± 5.44 **(**)	280.49 ± 9.25 ***	70.26 ± 12.13	97.34 ± 11.49	261.84 ± 73.15 ***	220.68 ± 30.56 ***

3.4. Combined analysis of grapevine responses to elicitor treatments, inoculation, and protection against downy mildew

Principal component analysis (PCA) was used to summarize and highlight the potential link between the protection conferred by the different treatments and the plant defense responses (biochemical and transcriptomic analyses) (Fig. 66). Most of the variance was contained in the first two principal components which captured 70.96% of the total variability. The axis 1, explaining 54.79% of the total variability, discriminated Triton and MeJA treatments against the control and of PHOS and BTH modalities. It mainly resulted from the modulation of some genes involved in SA signaling and in ET pathway (*WRKY, EDS1*, *ACC, ICS*, *EIN3*), as well as of *PR14*, combined with the content of quercetin-3-*O*-glucoside and stilbenes (piceids, parthenocissin, ε- and ω-viniferin, vitisinol C, isohopeaphenol). In the same way, PHOS and BTH treatments were well separated from the control along with Triton and MeJA treatments, essentially on the basis of the modulation of many genes, combined with pterostilbene and quercetin-3-*O*-rutinoside accumulation. The *P. viticola* growth was correlated to *trans*-resveratrol, pallidol and δ-viniferin content, and was negatively correlated to flavanols accumulation, as well as *PR3* and *CAD* genes regulation. The second axis explained only 16.17% of the variability, however, provided an explanation for the difference between MeJA- and Triton-treated leaves. The weak inhibition of downy mildew growth in Triton-treated leaves compared to the correct one obtained in MeJA-treated leaves could be supported by the low level of most of stilbenes, in particular complex ones, such as hopeaphenol, isohopeaphenol, and vitisinol C, post-Triton application. Five clusters corresponding to each treatment could be revealed, with PHOS and BTH treatments being very close but forming two distinct groups, as well as Triton and MeJA treatments, showing a similar pattern. All these 4 treatments were remarkably different from the control leaves.

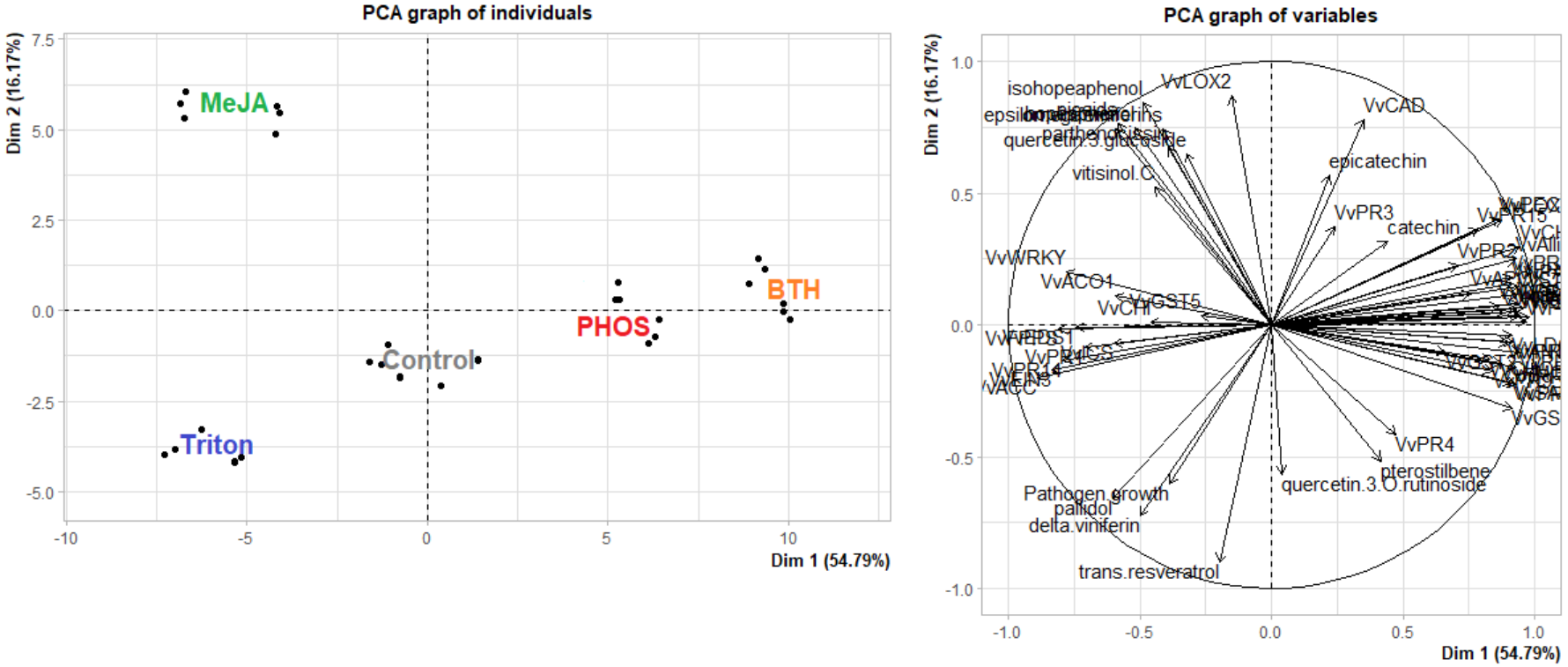

Figure 66. Distribution of grapevine responses and disease severity data on principal planes defined by two axes obtained through principal component analysis (PCA) of gene expression profiles, evaluation of *Plasmopara viticola* growth, and polyphenol analysis, using all treatment modalities data. BTH, benzothiadiazole; MeJA, methyl jasmonate; PHOS, phosphonates; Triton, Triton X-100.

3.5. Discussion

In the context of limiting pesticide inputs in vineyards by the use of PDS, some encouraging results have been obtained in enhancing grapevine innate immunity against downy and powdery mildews or gray mold (Delaunois et al., 2014; Belhadj et al., 2006; Perrazzoli et al., 2011; Dufour et al., 2016; Iriti et al., 2005; Wang et al., 2015; Bellée et al., 2018). Nevertheless, as it was described in the part I of this thesis (Introduction, chapter 6, sec. 6.2, subsec. 6.2.4.3), the effectiveness of different elicitor-like products varies and is difficult to obtain a high level of conferred protection in open field, since it depends on a number of factors, such as, formulation, plant variety, pathogen strain, or environmental conditions (Dagostin et al., 2011; Banani et al. 2014). Also, elicitors do not trigger identical reactions in the plant. It depends, obviously, to their origin and structure, so their capacity to interfere in a specific immune signaling pathway differs. However, a better comprehension of the mechanisms of action of PDS, and by providing reliable biomarkers of the protection that they confer to the plant, could offer a way to optimize the efficiency of PDS.

Within this framework, the study described in the current chapter aimed to assess the effect of treatments with different types of elicitors (MeJA, BTH and PHOS) applied on grapevine leaves, in term of their capacity to provide protection to the plant against the downy mildew causal agent, *P. viticola*. Immune responses triggered in the leaves, including the expression of defense-related genes (high-throughput microarray analysis) and polyphenol content modification (UHPLC-MS) were investigated. Such multidirectional approach has been developed by Corio-Costet et al. (2013) (the "BioMolChem" method). In this purpose, the leaves of greenhouse *V. vinifera* cv. Cabernet Sauvignon were subjected to the elicitations (or to pulverization with distilled water or Triton for the controls) and collected after 6 days. Subsequently, inoculation with *P. viticola* was performed on detached leaves and the responses of these latter were followed after 48 h post-inoculation (8 dpt-2 dpi), along with the non-inoculated leaves (8 dpt). A potential priming phenomenon was investigated. The content of polyphenols (stilbenes and flavonoids: flavanols and flavonols) and defense-related gene expression were assessed in all these modalities, as well as the efficacy of protection conferred by the treatments towards *P. viticola*.

As a result, a significant level of protection was achieved by all the treatments (inhibitory effect on *P. viticola* growth at 24% for Triton, and between 86 and 99% for the elicitors). Generally, numerous of the 48 genes studied and implicated in defense, were induced at 6 dpt and 8 dpt. The transcript accumulation was in accordance with the signaling

pathway specific to the elicitor, SA for BTH and JA for MeJA with some interconnections. PHOS tended to modulate the grapevine defenses as much as BTH. Moreover, by assessing the content of polyphenols, some biomarkers of conferred resistance to *P. viticola* could be proposed for each elicitor.

The accumulation of polyphenols, particularly stilbenes, is a characteristic response in grapevine subjected to biotic elicitation or pathogen infection (Bavaresco et al., 2009). However, the profile of these compounds may differ depending on the cultivar, organ, experimental conditions, or time of incubation (Pezet et al., 2004, Goufo et al., 2020, Taware et al., 2010). In this study, the vast majority of polyphenols followed was modified after elicitations and/or inoculation with *P. viticola*. The accumulation of more or less specific compounds was triggered according to the treatment and the time-point. Thus, a contribution of these molecules to the protection conferred can be suggested.

Treatment with MeJA led to an increased content of several stilbenes, i.e., monomers (*trans*-resveratrol, sum of piceids), dimers (δ-, ε- and ω-viniferins, pallidol, parthenocissin), and tetramer (isohopeaphenol) in the leaves. In MeJA-treated leaves upon *P. viticola* inoculation, the same compounds were altered, but the direction of changes differed due to the pathogen. In this case, a priming effect could be concluded for piceids, ε- and ω-viniferins, parthenocissin, isohopeaphenol, as well as an isomer of the latter, hopeaphenol. Stilbenes are known for their antifungal activities, and play an important role in the native or induced resistance of grapevines. In this regard, the abundance of oligomeric stilbenes in grapevine wood, canes and roots attract interest for the use of these by-products to obtain extracts displaying antifungal activities. These aspects were described in part I of this thesis (Introduction, chapter 5, subsec. 5.3.3.1). Stilbenes that were observed to be accumulated in this study in MeJA-treated leaves and/or after *P. viticola* inoculation were reported to be important biomarkers of grapevine resistance to this pathogen (Mattivi et al., 2011; Pezet et al., 2003, 2004; Langcake, 1981; Malacarne et al., 2011; Billet et al., 2020). The accumulation of ε-viniferin, a dimer of resveratrol, was reported as an effect of elicitation with MeJA in grapevine in vitro cell cultures and leaves (Belhadj et al., 2006; Hatmi et al., 2014; Santamaria et al., 2011; Taurino et al., 2015). In addition, we noted the formation of *trans*- and *cis*-piceids, the glycosylated forms of resveratrol, following both elicitation and inoculation in MeJA leaves. This enhanced accumulation of piceids in grapevine after MeJA treatment has been previously observed in in vitro cultures, leaves and grapes (Belhadj et al., 2006; Krisa et al., 1999; Portu et al., 2015). Thus, biosynthesis of piceids and viniferins as a result of biotic stresses seem to be confirmed as being mediated in part by jasmonates and its methyl ester. In

the literature, enhanced stilbene production after elicitation with MeJA was generally observed along with the accumulation of transcripts encoding enzymes implicated in the biosynthesis of these compounds (Belhadj et al., 2006; Portu et al., 2015, Larronde et al. 2003). In this study, despite an increased content of stilbenes mentioned above in MeJA-treated leaves, accumulation of *PAL* and *STS* transcripts was not observed, or even repressed at 8 dpt-2 dpi. This effect may be explained by the relatively late time-point of sample collection compared to previous studies, including the one conducted in the MIB laboratory (Belhadj et al., 2006). Indeed, it is possible that the time-point corresponding to the induction of these genes was missed. However, the co-formulant of MeJA, Triton, induced both the accumulation of some stilbenes (at 6 dpt and 8 dpt) and the induction of *PAL* and *STS* expression at 6 dpt. Triton treatment triggered a similar stilbene profile as MeJA, but with a significantly lower content of total stilbenes. Triton has a surfactant activity and thus, can create tissue injury which liberates cutin monomers acting as DAMPs that elicit plant immune responses (Heil et al., 2012). We can make the assumption that MeJA acts as a true plant defense compound by inducing strongly and rapidly defense responses whereas Triton, a surfactant, liberates few eliciting molecules thus triggering a slower plant response both in terms of intensity and timing. Moreover, similar gene transcription profiles were reported upon wounding and after JA elicitation (Heil et al., 2012). It may explain the similarity in the expression of defense genes between Triton and MeJA modalities in this study.

In contrast, BTH and PHOS treatments did not affect the content of stilbenes at 6 dpt (except for PHOS which decreased the content of hopeaphenol). In the literature, the inducing effect of these two elicitors on stilbenes content was reported to occur after relatively short periods of time (from 48 to 72 hpt) in comparison to the time of collection chosen in this study. On the other hand, in this study BTH significantly up-regulated *PAL* and *STS* gene expression. Perhaps, some post-transcriptional regulations could limit the production of stilbenes in this modality, or the newly synthesized monomeric and dimeric molecules were rapidly converted to, as instance, conjugated molecules not identified in this study. No change occurred in the gene expression of *STS* and *PAL* upon PHOS treatment. In turn, after inoculation with *P. viticola*, in the leaves pretreated with BTH and PHOS, an increase of the content of pterostilbene was observed. Pterostilbene, a methoxylated derivative of resveratrol, is also considered as biomarker of defense reaction in grapevine leaves and berries (Jeandet et al., 2002; Langcake, 1981; Pezet et al., 2004; Schmidlin et al., 2008; Vrhovsek et al., 2012). Despite its generally low amount even upon microbial challenge, pterostilbene was found to be one of the most toxic stilbenes to fungi and oomycetes, including *P. viticola* (Pezet et al.,

2004). Accumulation of pterostilbene as a result of priming effect was demonstrated in grapevine treated with *β*-aminobutyric acid and infected with downy mildew (Slaughter et al., 2008).

Besides, the profiles of the studied flavonoids were altered upon the treatments. The content of two flavonols, quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside, was enhanced in Triton- and BTH-treated leaves both at 6 dpt and 8 dpt-2 dpi, as well as in the leaves pretreated with PHOS and subjected to the inoculation, in regard to its control (PHOS 8 dpt). The level of quercetin-3-*O*-glucoside was also induced by MeJA at all the time points. Flavonols have a photoprotective role as well as an antioxidant function during plant responses to environmental stresses (Agati et al., 2013; Bouderias et al., 2020). In some populations of black currant, the level of quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside in leaves was suggested to play an important role in the resistance to foliar diseases (rust and septoria) (Vagiri et al., 2017). In grapevine, quercetin-3-*O*-glucoside was shown to be accumulated upon infection with *P. viticola* (Ali et al., 2012), phytoplasma (*Bois noir*) (Rusjan et al., 2012), as well as after a mechanical injury (Chitarrini et al., 2017b). In the work of Billet et al. (2020), the level of quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside was shown to be reduced in the leaves infected with downy mildew, but collected after much longer time after inoculation (6 or 15 days), in comparison to the current study (2 days post-inoculation). Also, all treatments, except PHOS, triggered an accumulation of flavanols in comparison to the controls. The positive impact of SA and JA and/or their analogs on the accumulation of grapevine flavanols has been already reported (Tassoni et al., 2012). Activation of flavanols biosynthesis by SA signaling as a defense reaction against poplar tree foliar rust has been noted (Ullah et al., 2019). Catechin and epicatechin are major flavanols in grapevines shoots, leaves, grapes, and seeds where they act as antioxidants themselves or they serve as monomers to produce more active polymeric proanthocyanidins (PAs) (Feucht et al., 1996; Teixeira et al., 2013; Yilmaz and Toledo, 2004). Elevated contents of catechin and epicatechin were detected in xylem sap and tissues of grapevine infected with *Xylella fastidiosa*, and in leaves affected with grapevine leafroll-associated viruses (GLRaV-3 and GVA) (Wallis and Chen, 2012; Guidoni et al. 1997). Protective activity of both flavanols studied herein was reported in the literature. For example, catechin was shown to inhibit infection in strawberry leaves by preventing the initiation of hyphae from appressoria of *Alternaria alternate*, while epicatechin present in avocado skin induced resistance to *Colletotrichum gloeosporioides* (Yamamoto et al., 2000). In grapevine, flavanols have been shown to have a beneficial effect against *P. viticola* (Andreu et al., 2018).

Simultaneously with the biochemical changes in the flavonoids profiles, at 6 dpt BTH induced expression of *CHS* and *F3H*, while Triton induced expression of *CHS* and *LDOX*, encoding enzymes that are implicated in the biosynthesis of flavanols. After inoculation with *P. viticola*, a range of genes related to the biosynthesis of flavonoids was up-regulated in the leaves pretreated with BTH (*CHS, F3H, DFR, and LDOX*) and with PHOS (*CHI, F3H, DFR, and LDOX*).

Many changes which occurred within genes related to other defense mechanisms were expected, according to the treatment applied. In grapevine, efficiency of BTH against *B. cinerea*, *P. viticola* and *E. necator* has been generally reported to be associated with the accumulation of total polyphenols in berry skins and leaves and PR-protein gene overexpression (Bellée et al., 2018; Dufour et al., 2013; Iriti et al., 2005; Wang et al., 2015). In this study, BTH treatment, along with the accumulation of certain polyphenols, up-regulated several PR-protein genes. At 6 dpt they included those encoding *β*-1,3-glucanases and chitinases (*PR2, PR3, PR4, PR5, PR8* and *PR11*). At 8 dpt-2 dpi almost all of the studied PR-proteins genes were induced in the leaves pretreated with BTH. Such effect of BTH has been already observed in different plants species, including grapevine (Dufour et al., 2013, 2016; Bellée et al., 2018; Harel et al., 2014; Banani et al., 2014; Thomas-Sharma et al., 2017, Landi et al., 2014). In contrast, few PR-protein genes were modulated in Triton- and MeJA-treated leaves, and also very few in PHOS-treated leaves. Previous works reported the ability of MeJA and potassium phosphite to induce PR-protein genes in grapevine and other plants, but at relatively early times of leaf collection after elicitation by the chemicals (Belhadj et al., 2006; Dufour et al., 2016; Liu et al., 2016; Massoud et al., 2012, Ramezani et al., 2018, Thomas-Sharma et al., 2017).

Moreover, at 6 dpt *GST* genes were nearly specifically overexpressed in BTH modality. At 8 dpt-2 dpi two TAU type *GST*- (*GST3* and *GST4*) and one PHI-type (*GST2*) genes were strongly overexpressed in the leaves pretreated with BTH. In a previous study, *GST4* was found repressed and *GST3* slightly induced after BTH treatment (Dufour et al., 2016). GSTs are a family of enzymes that detoxify cytotoxic compounds by conjugation of reduced glutathione to a wide range of substrates, and are involved in the transport of secondary metabolites (phytoalexins, anthocyanins) (Martínez-Márquez et al., 2017; Gullner et al., 2018). Thus, it could be suggested that phytoalexins synthesized in response to BTH are potentially conjugated and/or transported using the *GST* genes that were observed to be regulated in this modality. Moreover, GSTs play a key role in the detoxification and reduction of ROS (Gullner et al., 2018). The latter are well known to cumulate in cells if SA signaling is

activated, thus, also upon a stimulation of SA analogs, such as BTH (Clemente-Moreno et al., 2012). After Triton-, MeJA- and PHOS spraying, *GST* genes were repressed or remained unaltered, and after inoculation the up-regulated *GST5* gene seemed to be more specifically involved in the leaves pretreated with these elicitors. Thus, other mechanisms of defense are likely to be induced by MeJA and PHOS (and also Triton), distinct from those triggered by BTH.

Some cross-talks and specificities could be concluded within phytohormonal pathways, according to the treatment applied. The induction of expression of *LOX* genes, genes of the oxylipin pathway, is generally thought to occur under the JA signaling. Thus, the induction of the overexpression of *LOX13*, a protein involved in the biosynthesis of jasmonate (La Camera et al., 2004), was expected for MeJA modality and it indeed occurred at both time-points (and Triton at 6 dpt). In turn, BTH induced *LOX9*, and it was already reported in the literature (Dufour et al., 2013; Bellée et al., 2018). The induction of *LOX* both by MeJA and BTH in this study suggests cross-talk between the jasmonate and SA pathways.

The specificity of SA and JA signals was exhibited for the gene *SAMT*, encoding a salicylic acid carboxyl methyltransferase, which at 6 dpt was repressed in MeJA-sprayed leaves and up-regulated upon BTH treatment along with *ICS* transcripts. The same pattern was observed at 8 dpt-2 dpi suggesting the domination of SA pathway in BTH-treated leaves and not in MeJA-treated leaves, even after *P. viticola* inoculation. Similarly, genes of the chorismate pathway (*ICS*, *ANTS*, *CHORM* and *CHORS*) were induced by BTH, but poorly or even negatively regulated by Triton or MeJA treatment. Moreover, *G3H-6* was overexpressed in BTH-treated and repressed in MeJA-treated leaves, which is consistent with the function of these proteins consisting on the conjugation of amino acids to jasmonate or auxin, leading to their activation, inactivation or degradation (Westfall et al., 2010).

In contrast, *WRKY2* was strongly induced by both Triton and MeJA, but not by BTH. The WRKY gene family plays a key role in modulating genes expression upon biotic and abiotic stresses, thus their expression can be activated by pathogen infection or any kind of elicitation (Phukan et al., 2016). In grapevine, *WRKY2* was reported to be induced by wounding or after infection with *P. viticola* (Mzid et al. 2007). Moreover, a functional analysis of *WRKY2* in tobacco showed that the overexpression of this gene led to a reduced susceptibility of plants to various fungi (Mzid et al., 2007). The positive effect of Triton, the MeJA co-formulant, on *WRKY2* expression suggests the contribution of such compound in the grapevine stress responses that we noted in MeJA treated leaves. Indeed, the up-regulation of *WRKY2* observed in MeJA-treated leaves could potentially be the result of the surfactant

effect of Triton on the cell wall. This question concerns 77% of the genes commonly modulated by MeJA and Triton.

Genes involved in the ET pathway (*ACC* and *ACO*) were up-regulated at 8 dpt-2 dpi in the leaves pretreated with Triton and MeJA, and down-regulated in PHOS and BTH pretreated leaves. Nevertheless, a precocious induction of ET pathway upon BTH treatment could have occured, as it was reported in other studies (Bellée et al, 2018; Dufour et al., 2016; Hukkanen et al., 2008). This point may be supported by the fact that in BTH-pretreated and inoculated leaves occurred an overexpression of *EIN3*, a key positive factor of ET signaling, which affects many hormonal pathways, e.g., SA biosynthesis gene *SID2*, or cytokinine signaling (Dolgikh et al., 2019). The up-regulation of *EIN3* could be related either to a priming effect on the ET pathway, or to more complex cross-talk regulation between the different hormonal pathways.

Among the genes modulated after infection in this study, *APX1* was up-regulated in Triton- and MeJA-treated and inoculated leaves, but remained unaffected in PHOS- and BTH-treated leaves, which could be in agreement with the work of Durner and Klessig (1995), who described APX as an enzyme somewhat inhibited by SA induction. Ascorbate peroxidase (APX) regulates ROS levels in different subcellular compartments and prevents potential H_2O_2-derived cellular damage (Ozyigit et al., 2016).

After inoculation, *EDS1* was overexpressed in Triton- and MeJA-treated leaves, but was repressed by PHOS and BTH. This gene has been shown to be stimulated by SA treatment and inoculation with *P. viticola* in *Vitis* over short periods of time (Chong et al., 2008). The impact of time of sampling may explain the difference compared to the results obtained in this study. Furthermore, EDS1 and SA signaling were found to act redundantly on downstream resistance genes (Venugopal et al., 2009). Hypothetically, at 8 dpt-2 dpi, the actions of MeJA and SA on the *EDS1* gene would be reversed from what was observed 24 or 48 hours after treatment.

The number of genes affected by PHOS was generally lower in comparison to other treatments, but some specific modulations occurred for this elicitor. At 6 dpt the overall effect of PHOS treatment included a specific repression of the indole pathway and *GST* genes, accompanied by overexpression of genes involved in parietal reinforcement (*CALS*, *PECT* and *CAD*). The up-regulations of the latter genes would be in agreement with the prime callose deposition described in *Arabidopsis* (Eshragi et al., 2011).

To summarize, the PDS here studied conceivably activated the corresponding signaling pathways; i.e., BTH and MeJA mediated the responses via SA and JA signaling,

respectively, according to the literature (Lawton et al., 1996; Kazan and Manners, 2008). Moreover, the employment of the SA pathway by PHOS can be confirmed, since the PCA positioned this treatment close to BTH and opposite of MeJA. Indeed, the direct and indirect effect of phosphites on oomycetes (Smillie et al., 1989; Dufour and Corio-Costet, 2013) appears to be dose-dependent and would be implicated in the SA pathway at a concentration of 10 mM (Massoud et al., 2012). The up-regulation of some genes (*PR2* and genes implicated in flavonoids biosynthesis) can explain in part the indirect protection effect of PHOS treatment. In addition, the complexity of the interactions, the interdependence between different signaling pathways, and the establishment of effective defenses against the causal agent of downy mildew can be concluded through this study, as already mentioned by Guerreiro et al. (2016).

4. IMPACT OF DIFFERENT ELICITORS ON GRAPEVINE METABOLOME

4.1. NMR-based metabolic profiling of grapevine leaves

The obtained NMR spectra of the leaves extracts were characterized by the presence of three main chemical shift (ppm) regions where the signals of 29 metabolites could be identified (Fig. 67, Table 19). Most of amino acids (valine, threonine, alanine, GABA, proline, glutamine and glutamic acid) were recognized in the aliphatic region (δ 0.8-4.0 ppm). Several organic acids (acetic, pyruvic, succinic, malic and ascorbic acids) were identified in this area as well. The aliphatic region also revealed the signals of choline (δ 3.20 and 3.50), *myo*-inositol (δ 3.22, 3.46 and 3.60), syringic acid (δ 3.90) and shikimic acid (δ 3.93). Within the carbohydrate region (δ 4.0-5.5 ppm), the following signals could be recognized: anomeric protons of fructose (δ 4.07), sucrose (δ 4.16 and 5.39), β- and α-glucose (δ 4.57 and 5.18, respectively), as well as another signal, a third one, corresponding of malic acid (δ 4.38) and the one of tartaric acid (δ 4.41). The phenolic region (δ 5.5-8.5 ppm) showed the signals of (+)-catechin (δ 5.96 and 6.05), quercetin-3-*O*-glucoside (between δ 6.26 and 7.74), *trans*-feruloyl acid derivative (between δ 6.41 and 7.62), gallic acid (δ 7.10) and shikimic acid (δ 6.75). The signals of other compounds were also attributed in this region: tyrosine (δ 6.85 and 7.16), fumaric acid (δ 6.65), and two amines derivatives: adenine (δ 8.13) and trigonelline (δ 8.85 and 9.14).

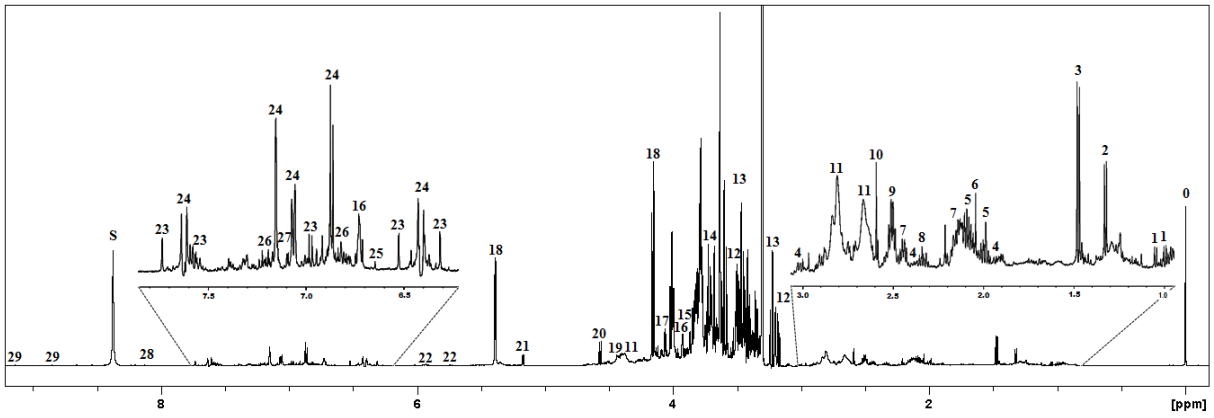

Figure 67. Representative proton nuclear magnetic resonance (1H-NMR) spectrum of a grapevine leaf extract (CD_3OD-KH_2PO_4 in D_2O, pH 6.0). 0, chemical shift reference standard (TMSP); S, standard for quantification (calcium formate); 1, valine; 2, threonine; 3, alanine; 4, γ-aminobutyric acid (GABA); 5, proline; 6, acetic acid; 7, glutamine; 8, pyruvic acid; 9, glutamic acid; 10, succinic acid; 11, malic acid; 12, choline; 13, *myo*-inositol; 14, ascorbic acid; 15, syringic acid; 16, shikimic acid; 17, fructose; 18, sucrose; 19, tartaric acid; 20, β-glucose; 21, α-glucose; 22, (+)-catechin; 23, quercetin-3-*O*-glucoside; 24, *trans*-feruloyl derivative; 25, fumaric acid; 26, tyrosine; 27, gallic acid; 28, adenine; 29, trigonelline.

Table 19. The proton nuclear magnetic resonance (1H-NMR) chemical shifts (δ), multiplicity of the signals (s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet), coupling constants (Hz), and the number of protons of the metabolites identified in grapevine leaves extracts (CD_3OD-KH_2PO_4 in D_2O, pH 6.0) by using one- and two-dimensional NMR (1D and 2D) spectra.

Compound	**Chemical shifts (δ), Multiplicity, Coupling constants (Hz) and Number of protons**
Valine	0.99 (d, *J* =7.0, 3H), 1.05 (d, *J* = 7.2, 3H)
Threonine	1.33 (d, *J* = 6.8, 3H)
Alanine	1.48 (d, *J* = 7.3, 3H)
γ-Aminobutyric acid (GABA)	1.92 (m, 2H), 2.42 (t, *J*= 7.4, 2H), 3.01 (t, *J* = 7.8, 2H)
Proline	2.0 (m, 3H), 2.08 (m, 1H)
Acetic acid	2.05 (s, 3H)
Glutamine	2.12 (m, 2H), 2.44 (m, 2H)
Pyruvic acid	2.34 (s, 3H)
Glutamic acid	2.50 (m, 2H)
Succinic acid	2.59 (s, 4H)
Malic acid	2.65 (dd, *J* = 7.8, 17.0, 1H), 2.81 (dd, *J* = 4.7, 17.0, 1H), 4.38 (dd, *J* = 4.6, 8.0, 1H)
Choline	3.20 (s, 9H), 3.50 (dd, *J* = 5.82, 4.16, 2H)
myo-Inositol	3.22 (t, *J* = 9.5, 1H), 3.46 (dd, *J* = 2.9, 10.0, 2H), 3.60 (t, *J* = 9.9, 2H)
Ascorbic acid	3.70 (d, *J* = 7.0, 1H)
Syringic acid	3.90 (s, 6H)
Shikimic acid	3.93 (m, 1H), 6.75 (m, 1H)
Fructose	4.07 (d, *J* = 3.8, 5H)
Sucrose	4.16 (d, *J* = 5.5, 1H), 5.39 (d, *J* = 4.0, 1H)
Tartaric acid	4.41 (s, 1H)
β-Glucose	4.57 (d, *J* = 7.9, 1H)
α-Glucose	5.18 (d, *J* = 3.8, 1H)
(+)-Catechin	5.96 (d, *J* = 2.3, 1H), 6.05 (d, *J* = 2.3, 1H)
Quercetin-3-*O*-glucoside	6.265 (d, *J* = 2.2, 1H), 6.53 (d, *J* = 2.2, 1H), 6.98 (d, *J* = 8.8, 1H), 7.57 (dd, *J* = 8.5, 2.0, 1H), 7.74 (d, *J* = 2.2, 1H)
trans-Feruloyl derivative	6.41 (d, *J* = 16.6, H), 6.87 (d, *J* = 8.2, H), 7.07 (dd, *J* = 2.0, 8.3, H), 7.16 (d, *J* = 1.8, H), 7.62 (d, *J* = 16.7, H)
Fumaric acid	6.65 (s, 2H)
Tyrosine	6.85 (d, *J* = 8.3, 2H), 7.16 (d, *J* = 9.0, 2H)
Gallic acid	7.10 (s, 2H)
Adenine	8.13 (s, 1H)
Trigonelline	8.85 (m, 2H), 9.14 (s, 1H)

4.2. Relative quantification of the metabolites identified

The relative abundance of the metabolites identified, and their contribution to the significant differences among the experimental conditions ($p \leq 0.05$), are presented in Figure 68A-E and Table A3 (Appendix). All treatments led to several distinct metabolomic alterations across the classes of the compounds studied, at 24 hpt and/or 6 dpt. Within carbohydrates (Fig. 68A) the modifications were particularly apparent after 24 h. In this time-point all the treatments negatively impacted each compound studied. Two characteristic patterns of sugars emerged. The first included α-glucose, β-glucose, and sucrose, of which the relative quantity decreased after each elicitor treatment, in comparison to the control (untreated plants). These compounds were perturbed the most by MeJA (approximately 2.5-fold). Fructose was negatively affected by MeJA, PHOS, and BTH to the same degree (approximately 1.5-fold). *Myo*-inositol followed the same pattern, i.e., its amount decreased at the same range as fructose after all elicitor treatments, but also after the treatment with Triton (1.2-fold), in regard to the control. At 6 dpt, definitely less changes occurred in carbohydrates metabolism. Only two compounds were affected: fructose (a 1.4-fold decrease in MeJA- and BTH-treated leaves), and sucrose (a 1.5-fold decline in MeJA sample).

The amino acids studied were either negatively, or positively affected by the treatments, in regard to the control (Fig. 68B). At 24 hpt, the relative content of some of these compounds remained unchanged, such as that of glutamic acid, threonine and tyrosine. All the elicitor treatments significantly reduced the amount of GABA (2.5-fold), in regard to the control. Alanine occurred in nearly equal amounts in all conditions except BTH, where it decreased (1.5-fold). The relative content of proline slightly increased in the leaves sprayed with Triton (1.3-fold). MeJA was the only elicitor which led to an enhancement of the level of valine and glutamine (respectively 1.6- and 1.5-fold). On the other hand, PHOS and BTH considerably inhibited the accumulation of glutamine (respectively 2.4- and 3.1-fold), while valine remained unaffected in these modalities. At 6 dpt, the vast majority of the amino acids was positively affected by the treatments, except for BTH sample where, like at 24 hpt, the content of alanine decreased (1.8-fold). In BTH-treated leaves, also a 12.7-, 1.7-, and 1.9-fold increase was observed in the relative quantity of respectively GABA, threonine, and tyrosine. PHOS led to an increase of both glutamic acid and glutamine, respectively 1.8- and 4.3-fold, in comparison to the control. MeJA significantly enhanced the amount of five amino acids: GABA (2.3-fold), proline (1.6-fold), threonine (3-fold), tyrosine (3-fold), and, like at 24 hpt,

of valine (1.8-fold). Triton positively affected threonine and tyrosine (a 1.8- and 2.4-fold increase, respectively, in regard to the control).

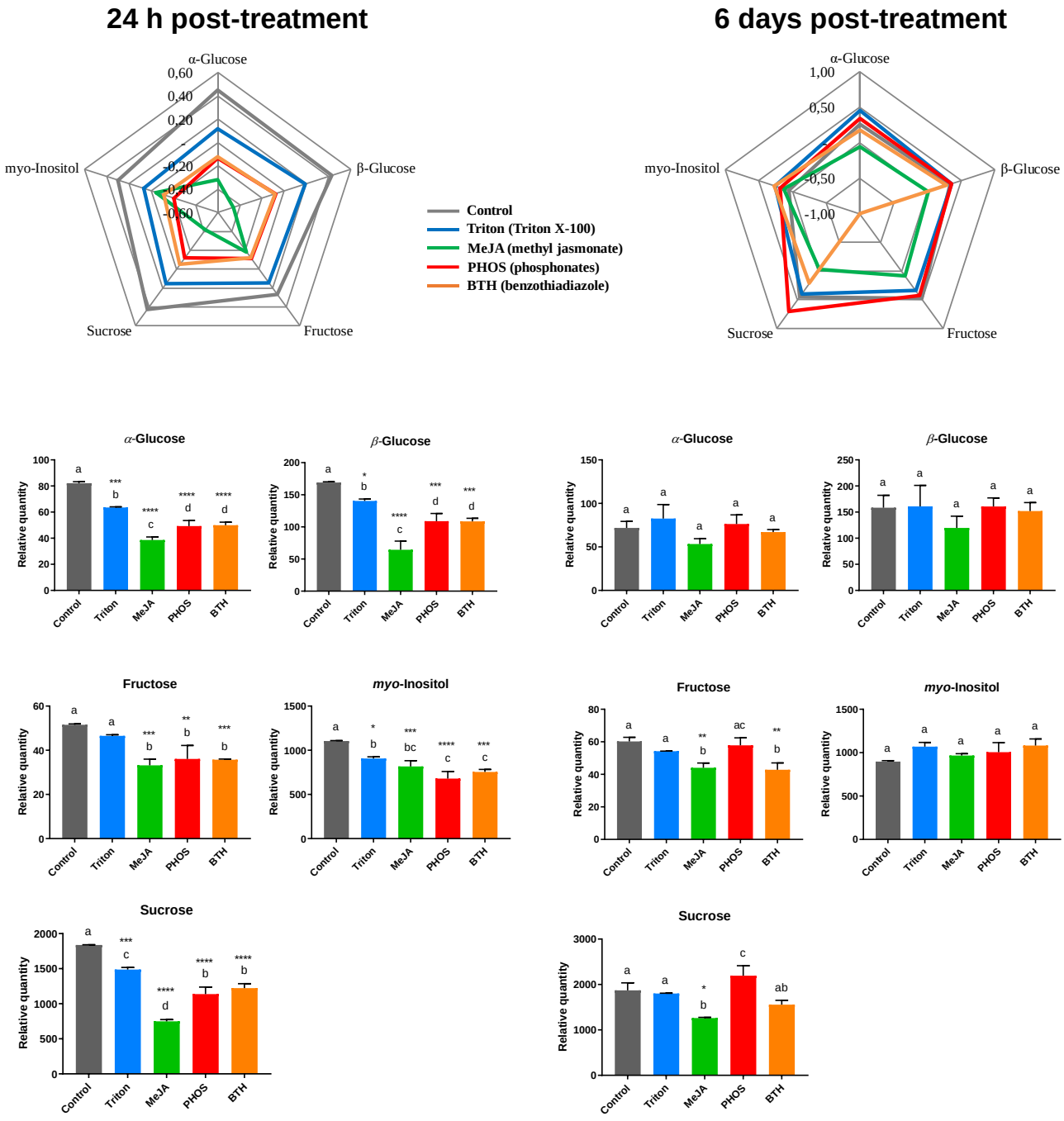

Figure 68A. Comparison of changes within carbohydrates in grapevine leaves differently treated and harvested at distinct time points (left: 24 h post-treatment, right: 6 days post-treatment). Histograms present relative abundance of the identified compounds by a proton nuclear magnetic resonance (1H-NMR) spectroscopy. Radar charts were effectuated on the means by normalizing the data. One-way analysis of variance (ANOVA) was applied ($p \leq 0.05$) followed by Tukey's range test. Asterisks denote the significance levels as compared to the control: $p \geq 0.05$ (ns), 0.01 to 0.05 (*), 0.001 to 0.01 (**), 0.0001 to 0.001 (***), <0.0001 (****). Letters indicate significant differences among all the samples.

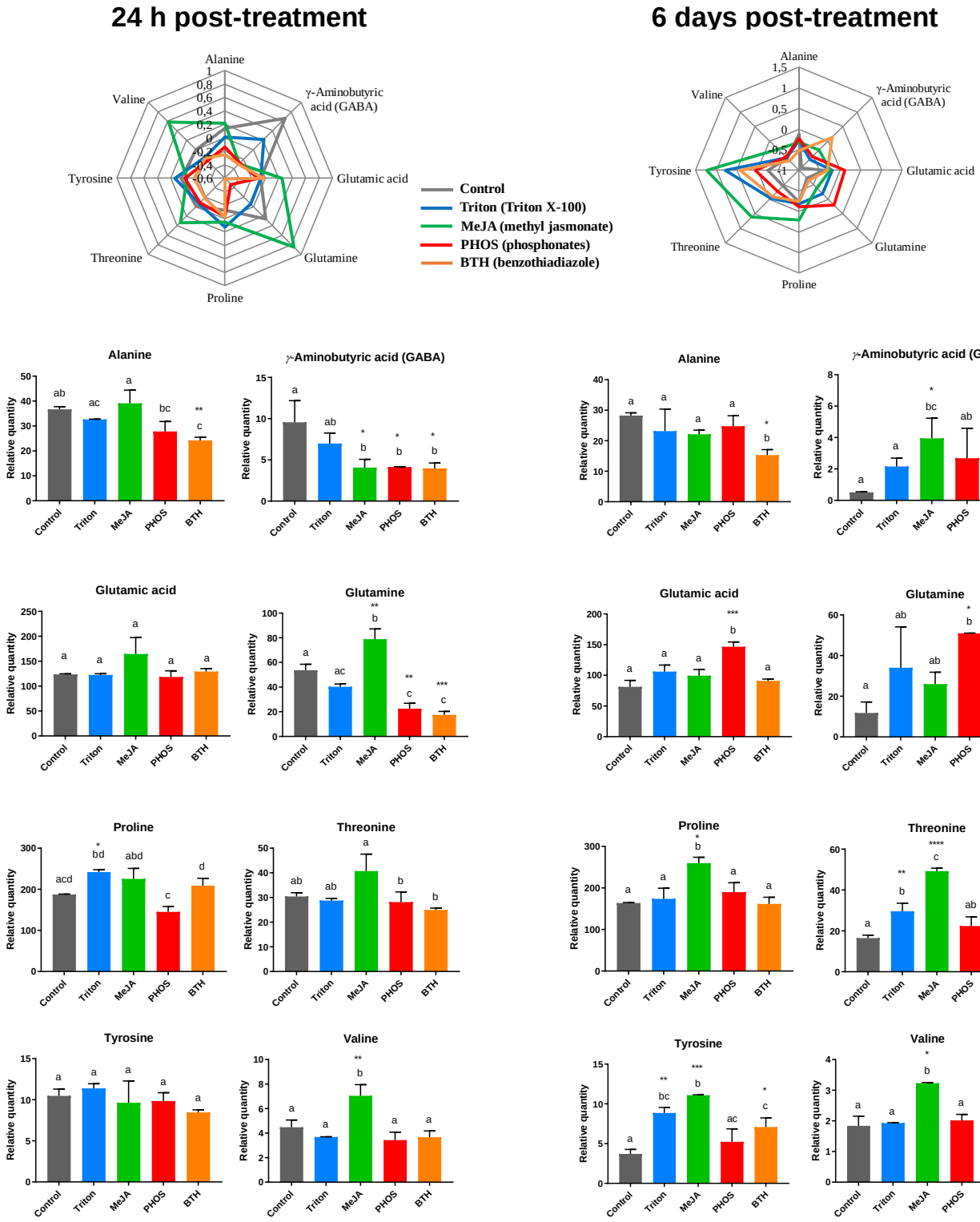

Figure 68B. Comparison of changes within amino acids in grapevine leaves differently treated and harvested at distinct time points (left: 24 h post-treatment, right: 6 days post-treatment). Histograms present relative abundance of the identified compounds by a proton nuclear magnetic resonance (1H-NMR) spectroscopy. Radar charts were effectuated on the means by normalizing the data. One-way analysis of variance (ANOVA) was applied ($p \leq 0.05$) followed by Tukey's range test. Asterisks denote the significance levels as compared to the control: $p \geq 0.05$ (ns), 0.01 to 0.05 (*), 0.001 to 0.01 (**), 0.0001 to 0.001 (***), <0.0001 (****). Letters indicate significant differences among all the samples.

All the organic acids identified were impacted by the treatments (Fig. 68C). At 24 hpt, each of the compound considered was negatively affected, except for acetic acid, of which the relative content slightly increased after MeJA treatment (1.3-fold in regard to the control). The amount of ascorbic and tartaric acids decreased in the leaves treated with MeJA (respectively 1.4- and 2.3-fold), PHOS, and BTH (approximately 1.7-fold for tartaric acid, while ascorbic acid was not detected in these samples). An average 3.3-fold decrease of malic acid was noted in the leaves treated by MeJA, PHOS, and BTH, and a 1.8-fold decline in the leaves sprayed with Triton. Pyruvic acid was mostly affected by PHOS and BTH (a decrease of around 2.4-fold), and less by Triton and MeJA (1.5-fold). The relative amount of fumaric acid decreased in Triton, MeJA and BTH modalities (approximately 2.5-fold), and of succinic acid in Triton and MeJA (1.2-fold the both), as well as PHOS (1.6-fold) samples, in regard to the control. At 6 dpt, the relative content of some of the amino acids studied was positively altered. The profile of fumaric, pyruvic and tartaric acids remained unchanged at this time point. The level of acetic acid increased in Triton, MeJA, and BTH samples, respectively 1.5-, 1.7- and 1.3-fold. Ascorbic acid was not detected in the control and BTH samples, and reached the relative content 173.49, 115.47, 198.83 mg/l respectively in Triton, MeJA and PHOS modalities. Quite similar pattern was observed for malic and succinic acids, both of which the level decreased around 0.7-, 0.5-, 0.3 and 3.2-fold respectively in Triton, MeJA, PHOS and BTH modalities.

Within phenolic compounds identified (Fig. 68D), two patterns of changes could be revealed according to the time-point after treatment considered. At 24 hpt, the relative content of several compounds decreased after treatments, whereas at 6 dpt, only positive alterations occurred, with the exception of gallic acid of which the amount declined in BTH-treated leaves at both time points (around 3.3-fold in regard to the control). In PHOS sample, the level of gallic acid decreased at 24 hpt and increased at 6 dpt (respectively 13.6- and 1.6-fold). At 24 hpt, the content of shikimic and syringic acids decreased at similar level in the leaves treated with MeJA, PHOS and BTH (on average 2- and 1.8-fold, respectively). The profile of these two molecules remained unaffected at 6 dpt. The relative content of quercetin-3-*O*-glucoside declined at 24 hpt in each treatment modality (around 1.5-fold), and increased at 6 dpt after Triton, MeJA and BTH applications (around 3-fold). At 24 hpt, the content of (+)-catechin and of a *trans*-feruloyl derivative was unaltered in every condition, while at 6 dpt significantly augmented in almost every modality. In the leaves treated with Triton, MeJA, and BTH, the quantity of (+)-catechin increased 2.2-, 3.1- and 1.6-fold, respectively, in regard

to the control. The level of a *trans*-feruloyl derivative showed a 2-fold accumulation in Triton and MeJA samples, and 1.6- and 1.3-fold in PHOS and BTH modalities, respectively.

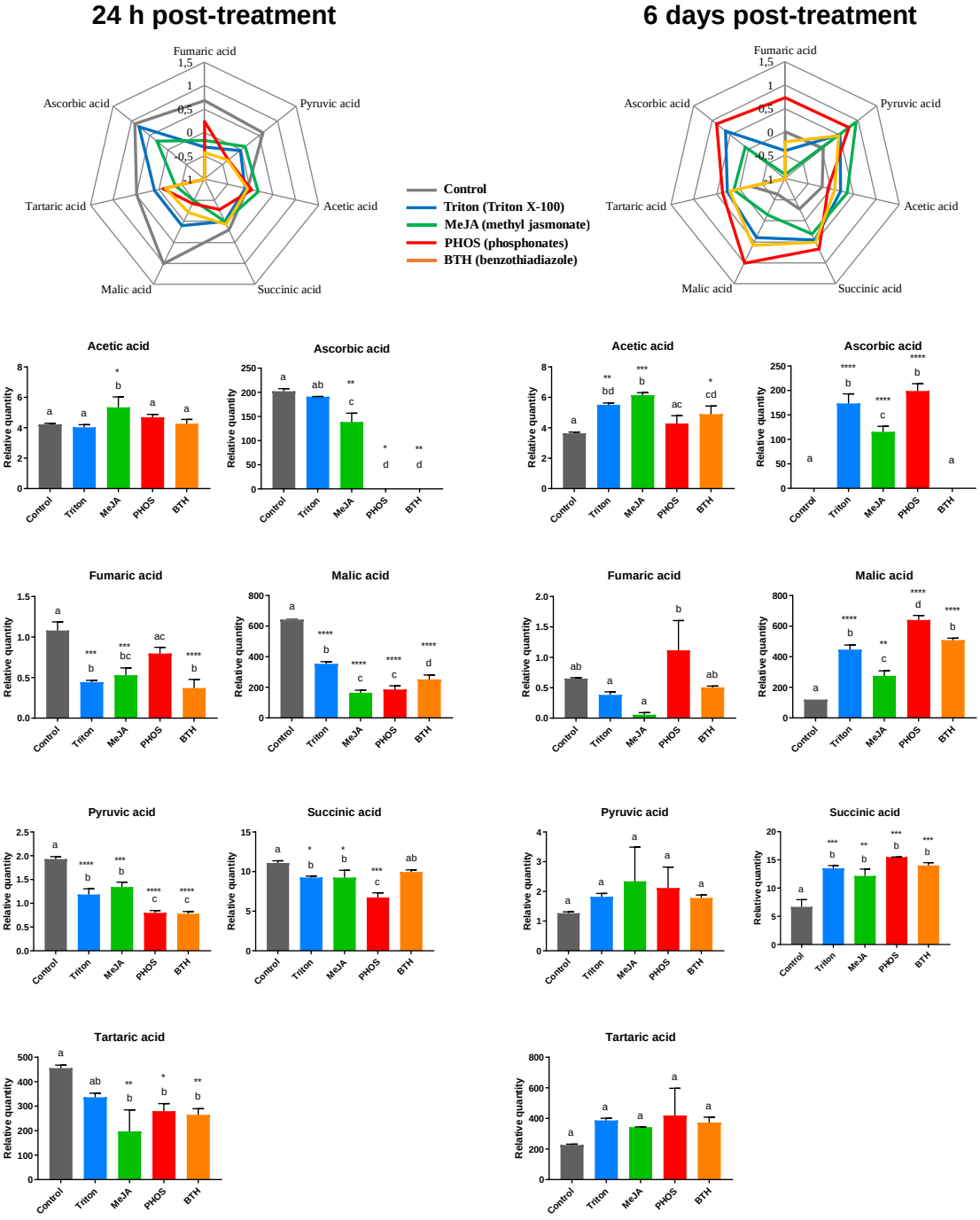

Figure 68C. Comparison of changes within organic acids in grapevine leaves differently treated and harvested at distinct time points (left: 24 h post-treatment, right: 6 days post-treatment). Histograms present relative abundance of the identified compounds by a proton nuclear magnetic resonance (1H-NMR) spectroscopy. Radar charts were effectuated on the means by normalizing the data. One-way analysis of variance (ANOVA) was applied ($p \leq 0.05$) followed by Tukey's range test. Asterisks denote the significance levels as compared to the control: $p \geq 0.05$ (ns), 0.01 to 0.05 (*), 0.001 to 0.01 (**), 0.0001 to 0.001 (***), <0.0001 (****). Letters indicate significant differences among all the samples.

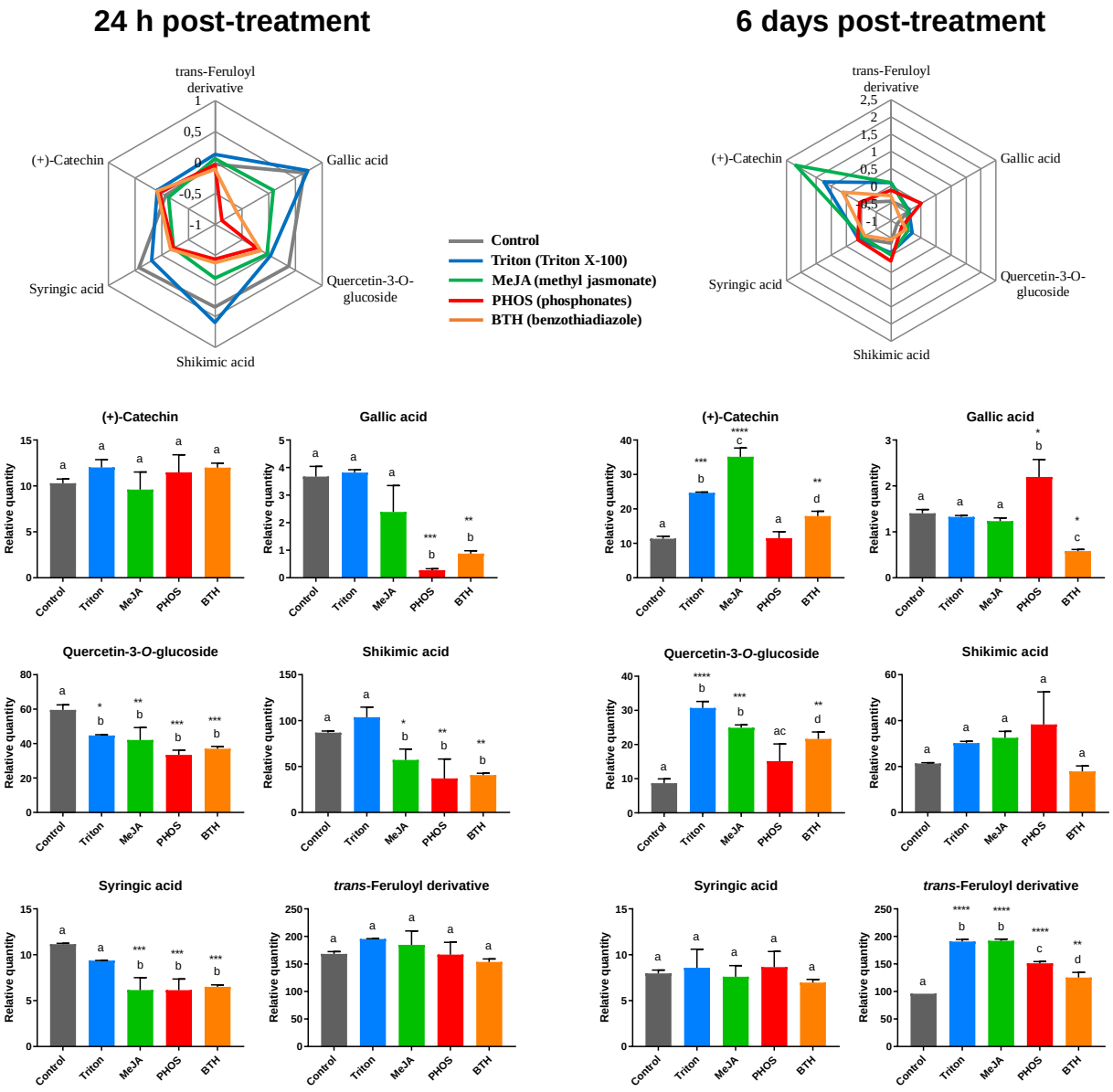

Figure 68D. Comparison of changes within phenolic compounds in grapevine leaves differently treated and harvested at distinct time points (left: 24 h post-treatment, right: 6 days post-treatment). Histograms present relative abundance of the identified compounds by a proton nuclear magnetic resonance (1H-NMR) spectroscopy. Radar charts were effectuated on the means by normalizing the data. One-way analysis of variance (ANOVA) was applied ($p \leq 0.05$) followed by Tukey's range test. Asterisks denote the significance levels as compared to the control: $p \geq 0.05$ (ns), 0.01 to 0.05 (*), 0.001 to 0.01 (**), 0.0001 to 0.001 (***), <0.0001 (****). Letters indicate significant differences among all the samples.

Among the three identifed amines (Fig. 68E), adenine remained unaffected both at 24 hpt and 6 dpt. The relative quantity of choline altered only at 24 hpt: its 1.5-fold increase, in regard to the control, was noted in PHOS- and BTH-treated leaves. Trigonelline was not detected in BTH samples at both time-points. Its level decreased after the treatment with Triton at 24 hpt (3.8-fold in regard to the control), and significantly increased at 6 dpt after elicitation with MeJA and PHOS (6.2- and 4.5-fold, respectively).

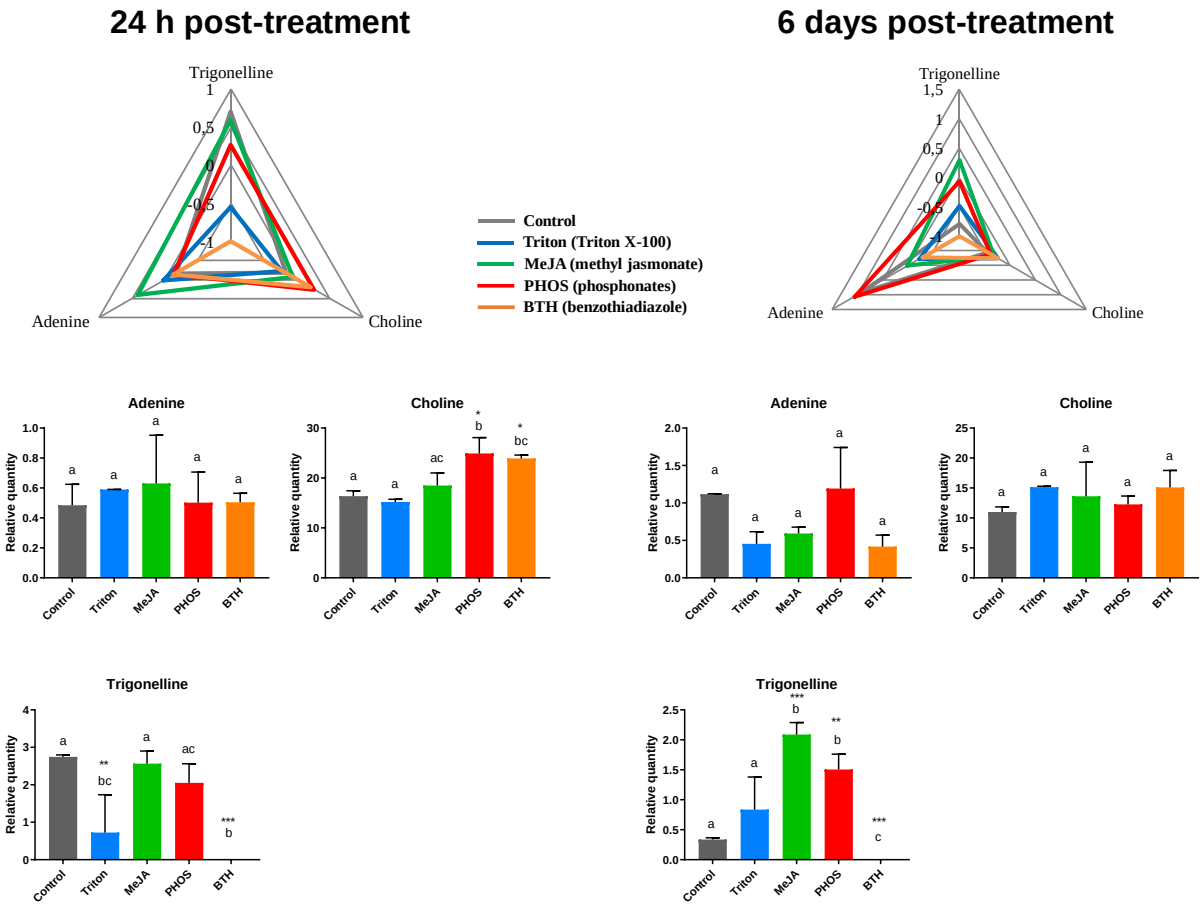

Figure 68E. Comparison of changes within amines in grapevine leaves differently treated and harvested at distinct time points (left: 24 h post-treatment, right: 6 days post-treatment). Histograms present relative abundance of the identified compounds by a proton nuclear magnetic resonance (1H-NMR) spectroscopy. Radar charts were effectuated on the means by normalizing the data. One-way analysis of variance (ANOVA) was applied ($p \leq 0.05$) followed by Tukey's range test. Asterisks denote the significance levels as compared to the control: $p \geq 0.05$ (ns), 0.01 to 0.05 (*), 0.001 to 0.01 (**), 0.0001 to 0.001 (***), <0.0001 (****). Letters indicate significant differences among all the samples.

4.3. Global view on the metabolic profile upon treatments

PCA was applied in order to gain more insight into the metabolic differences or similarities between the control and elicitor-treated leaves, as well as among all the modalities. At 24 hpt (Fig. 69A), a clear discrimination of the samples was enabled by the first two principal components (PC1 and PC2) which cumulatively accounted for 81.57% of the variation in all variables. PC1 explained 50.38% of the variation and separated the samples of elicitor-treated leaves (MeJA, PHOS, BTH) from the controls (the leaves un-treated and the ones on which Triton was sprayed), while PC2 which accounted for 31.19% distinguished MeJA modality from the controls and both elicitors, BTH and PHOS. In addition, BTH and PHOS were grouped not far from each other, as it similarly occurred for the two controls, whereas MeJA condition was apart. Based on the correlation coefficients of the factor loadings and the square of the cosine of the variables, the metabolites that contributed the most to the separation of the samples could be revealed. MeJA-treated leaves were distinguished by a relatively high abundance of threonine, valine, adenine, acetic acid, and glutamic acid. Both PHOS and BTH modalities were characterized by the presence of choline. The control and Triton were concentrated by the majority of the compounds, sugars (particularly fructose, α-glucose, β-glucose and sucrose), organic acids (mainly malic and tartaric acids), syringic acid, and GABA.

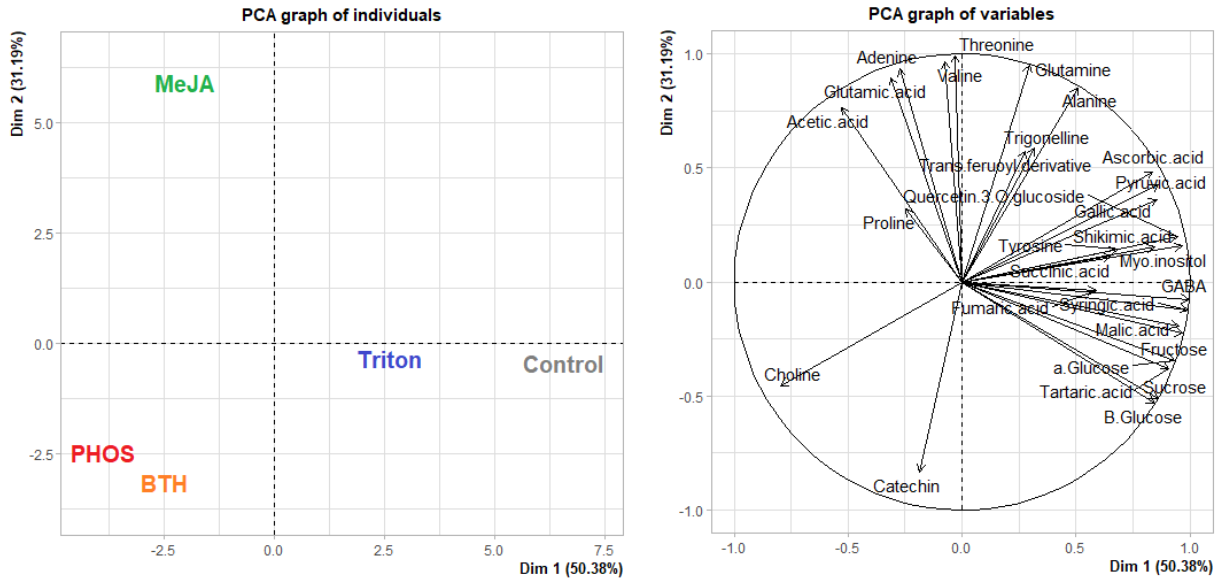

Figure 69A. Score (graph of individuals) and distribution of variables on loading plot (graph of variables) of principal component analysis (PCA) on the means of semi-quantified metabolites identified in the grapevine leaves at 24 h post-elicitation (Triton, Triton X-100; MeJA, methyl jasmonate; PHOS, phosphonates; BTH, benzothiadiazole).

At 6 dpt (Fig. 69B), the first two principal components captured 63.18% of the total variability. All the treatments and controls were well separated from each other. PC1 explained 43.13% of the variation and tended to put together Triton, MeJA and BTH samples, separating them from the control and PHOS modalities. PC2 which accounted for 29.05% distinguished Triton and PHOS from the control, BTH, and MeJA. Thus, contrarily to the result obtained at 24 hpt, BTH and MeJA acted similarly to each other, and together in a different way in regard to PHOS, on the set of the metabolites studied at this time-point. Such distribution of the samples resulted from a negative correlation of mainly pyruvic acid, a *trans*-feruoyl derivative and trigonelline in the control, and glutamine, ascorbic and tartaric acids in BTH-treated leaves. Conversely, the leaves of the condition Triton contained a higher abundance of these compounds. MeJA sample was characterized by the abundance of catechin, threonine and tyrosine, while PHOS was distinguished based on a high level of gallic and syringic acids.

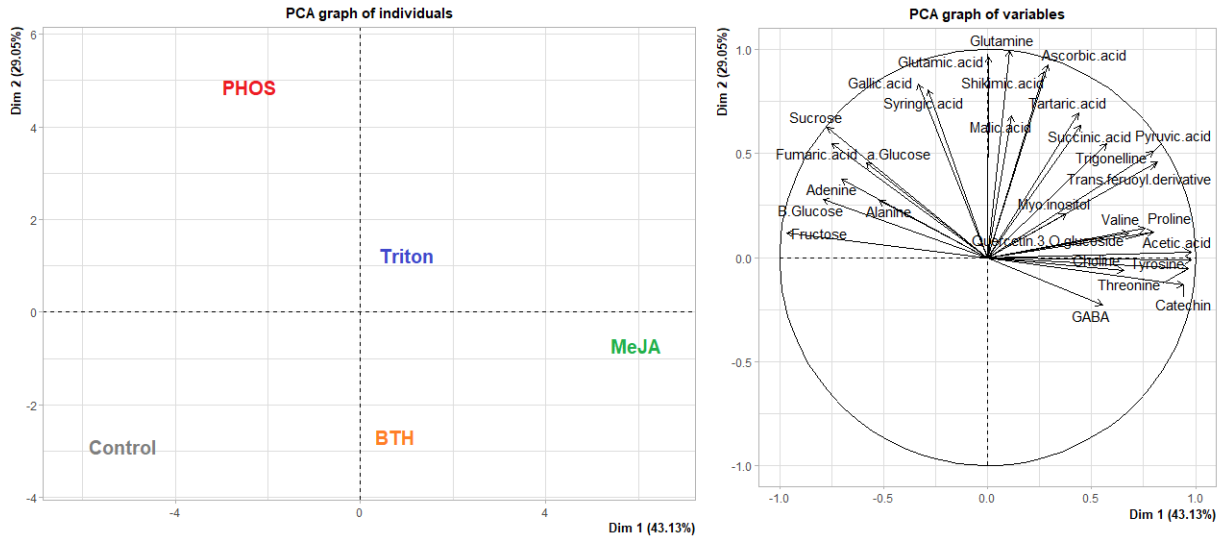

Figure 69B. Score (graph of individuals) and distribution of variables on loading plot (graph of variables) of principal component analysis (PCA) on the means of semi-quantified metabolites identified in the grapevine leaves at 6 days post-elicitation (Triton, Triton X-100; MeJA, methyl jasmonate; PHOS, phosphonates; BTH, benzothiadiazole.

4.4. Discussion

The impact of activating defense reactions by plants, either through a pathogen attack or upon PDS treatment, can conduct to metabolic perturbations at the level of both primary and specialized metabolisms. This is the so-called trade-off between defense and fitness (growth and yield). One of the mechanisms underlying this balance is the competition for the energy resource. Indeed, the activation of the defenses leads, among others, to the biosynthesis of phytoalexin-like secondary metabolites. The pathways for the production of the latter use primary metabolites as precursors and require chemical energy, e.g., in the form of ATP. For example, Vos et al. (2015b) measured that in modified yeast and observed that about 12 moles of ATP were required to produce 1 mole of resveratrol. Besides, many other catalytic reactions are ATP-dependent, such as the phosphorylation of shikimate to shikimate-3-phosphate. Similarly, the mobilization of amino acids used for the synthesis of PR proteins, makes them quite impossible to be invested in other proteins necessary, as instance, for cell division. For example, the genes encoding α- and β-tubulins (constituting microtubules cytoskeleton) have been reported to be repressed in grapevine cells cultures treated with β-cyclodextrin (Zamboni et al., 2009). Similar conclusions were found in the experiment where the production of *trans*-resveratrol in grapevine cells was synergistically induced by cyclodextrins and MeJA, in detriment of primary metabolism or cell division (Almagro et al., 2014). Photosynthesis-related genes and chlorophyll biosynthesis have been reported to be down-regulated following pathogen attack or elicitor treatment (Bolton, 2009), leading to a deterioration in plant fitness.

In this context, the study described in the current chapter aimed to evaluate the effect of different types of elicitors (SA- and/or JA-dependent) by measuring metabolomic responses in grapevine leaves after relatively early and long-term time after treatment (24 h and 6 days post-treatment). For this purpose, the leaves of *V. vinifera* cv. Cabernet Sauvignon greenhouse cuttings were treated with three different elicitors (MeJA, co-formulated with Triton X-100, BTH or a mix of potassium phosphonates (PHOS)), and the changes were compared in regard to non-treated (control) leaves, as well as to leaves which received a spray of Triton. In order to assess global modifications in metabolite pool under each condition, a 1H-NMR spectroscopy was chosen since it affords reproducibility, convenience for quantification, and straightforward metabolite identification (Kim et al., 2010). Only several studies report about alterations in grapevine leaf metabolism monitored by the use of NMR. These works investigated metabolomics of the leaves of different grapevine varieties, from

greenhouse and/or vineyard, upon disease attack: Esca disease (Lima et al., 2010), *P. viticola* (Ali et al., 2012), Flavescence dorée phytoplasma (Prezelj et al., 2016), impact of roots phylloxera on the foliar metabolism (Tucker et al. 2007). A thorough study of phytochemical composition of the leaves of *V. vinifera* cv. Falanghina, an ancient Italian variety, was conducted more recently with the employ of NMR (Tartaglione et al., 2018). Much more works using this technique have been performed on grapes and wines (for review see Fotakis et al., 2013) and MIB laboratory has acquired expertise in NMR techniques for wine analyses (Gougeon et al., 2019). No research has been carried out so far, in order to evaluate the impact of PDS in grapevine with the use of NMR.

In this study, a NMR-based method for the rapid determination and characterization of the influence of different treatments on grapevine leaves was proposed. This technique was found to be relevant as it covered a wide range of the metabolome. Twenty-nine leaf metabolites were identified and semi-quantified, including carbohydrates, amino acids, organic acids, phenolic compounds and amines. Multivariate statistical analyses in the form of PCA revealed differences between samples, of the significance statistically confirmed by one-way ANOVA followed by Tukey's post-hoc test. Depending on the time-point, all the treatments (MeJA, and even its co-formulant, Triton; PHOS and BTH) caused similar and/or specific changes within primary metabolism. Figure 70 shows the most likely metabolic interconnections of the compounds identified, and summarize their responsiveness to the treatments applied at two time-points studied.

As it was already underlined, one of the strategies that plant employs, if challenged by a stress factor, is diverting carbon skeletons from the primary to the specialized metabolism. In other words, the increase in defense associated biosynthetic pathways is often compensated by a reduction in other metabolic pathways. In the current work, this effect was reflected by a clear decrease in carbohydrates at 24 hpt in the leaves sprayed with all elicitors, and even with Triton. However, at 6 dpt the level of most of sugars stabilized. A similar effect was observed in the leaves of *Brassica rapa* treated with MeJA and collected at different time-points (Liang et al. 2006). *myo*-Inositol was affected as well at the early time point, which was expected since this molecule is involved in the biogenesis of the cell wall, the phospholipid signalling pathway and the regulation of the cytoskeletal structure. A clear explanation about the reduction of the content of *myo*-inositol as a result of a stress response has not been provided so far, but such effect was already reported in *Nicotiana tabacum* leaves infected with tobacco mosaic virus (Choi et al. 2006).

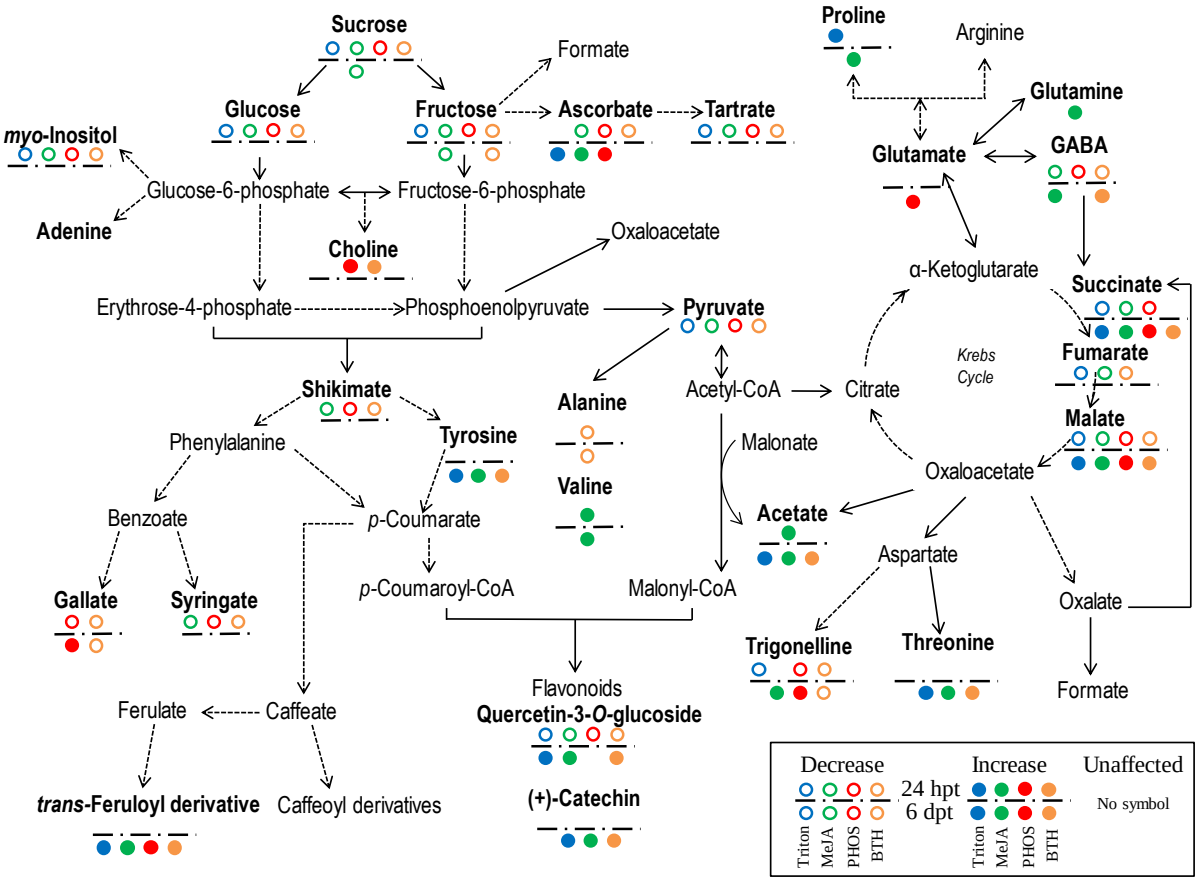

Figure 70. Simplified metabolic pathways influenced by different treatments in *Vitis vinifera* cv. Cabernet Sauvignon leaves at 24 h and 6 days post-treatment. Compounds identified in the experiment by a proton nuclear magnetic resonance (1H NMR) spectroscopy are shown in bold. Symbols associated with each metabolite show the significant changes with comparison to the control leaves assessed at $p \leq 0.05$ through one-way analysis of variance (ANOVA) test. Metabolites that are set with empty symbols are significantly decreased after treatment with respect to the control, whilst those set with filled symbols are significantly increased. Lack of symbol indicates metabolites unaffected by treatments. Blue, Triton X-100 (Triton); green, methyl jasmonate (MeJA); red, phosphonates (PHOS); orange, benzothiadiazole (BTH). KEGG database was used to establish metabolic networks.

Also, at 24 hpt the amount of several of the identified phenolics significantly decreased after treatments, or remained unaffected. A reduction in the level of phenolic metabolites was as instance observed in *A. thaliana* after BTH application (Hien Dao et al., 2009). The observed decrease of the content of shikimic acid, one of the crucial molecule in the phenylpropanoid pathway, may indicate an enhanced biosynthesis of the phenolic derivatives involved in defense responses. It could be also the case of the other phenolics studied, i.e., quercetin-3-*O*-glucoside, gallic and syringic acids, since they are precursors in the phenylpropanoid metabolism. In turn, at 6 dpt these phenolics accumulated in a common or specific way depending on the treatment applied. The content of a *trans*-feruloyl derivative

increased upon all treatments, that of quercetin-3-*O*-glucoside along with (+)-catechin raised in Triton, MeJA, and BTH modalities, while the amount of gallic acid specifically increased in PHOS-treated leaves. These results are consistent with those obtained by UHPLC-MS analyses, described and discussed in chapter 3.

Unlike carbohydrates and phenolics, amino acids were characterized by different patterns of changes. Amino acids, traditionally considered as precursors and constituents of proteins, have been recognized during last decades also as molecules implicated in regulatory and signaling processes linked to various stresses (Rai, 2002). In this study, some modifications within amino acids of molecules were linked to nitrogen signaling, i.e., GABA, glutamine and glutamic acid. Although the mode of action of GABA in plant defenses is not clear, its production might be activated by biotic and abiotic stresses via the induction of glutamate decarboxylase, an enzyme catalyzing its synthesis (Lima et al., 2010). The accumulation of glutamine is considered as evidence of activation of the PAL pathway (Figueiredo et al., 2008). An increase in glutamine was noted in a wheat-resistant cultivar upon infection (Hamzehzarghani et al., 2005). In this study, the direction of changes within these amino acids depended on both the treatment and the time-point. At 24 hpt, the glutamine content increased in MeJA-treated leaves, but decreased in PHOS and BTH modalities, glutamic acid remained unaffected, while the amount of GABA declined upon all the elicitor treatments. This pattern of changes was reported in *Agastache rugosa* Kuntze and grape berries, treated respectively with MeJA (Kim et al., 2013) and BTH (Iriti et al., 2005). The pool of amino acids was prominently changed in phosphite-treated Arabidopsis plants (Berkowitz et al., 2013). At 6 dpt, the quantity of glutamic acid and glutamine specifically increased in PHOS-treated leaves, and that of GABA raised in MeJA and BTH modalities. The role of these amino acids in stress was thus confirmed in the long-term time after treatment, and is consistent with elicitor-specific changes within phenolics discussed above. The remaining amino acids studied were also significantly affected, more after the longer time after treatment, and generally in the positive way. Triton, MeJA and BTH led to an increase of the amount of threonine and tyrosine at 6 dpt. Threonine was reported to be induced in *A. thaliana* treated with BTH (Hien Dao et al., 2009), and in *Agastache rugosa* Kuntze elicited with MeJA (Kim et al., 2013). Moreover, MeJA triggered an accumulation of valine at both time-points, as well as of proline at 6 dpt (the accumulation of this latter was also induced in Triton modality at 24 hpt). Valine, along with other amino acids (e.g., tyrosine), was reported to participate in plant-pathogen interactions (Rojas et al., 2014). Proline was proposed as one of defense biomarker against *P. viticola* of a resistant grapevine variety (Chitarrini et al.,

2017a). Consequently, this amino acid could be suggested as one of molecules involved in the protection conferred by MeJA (and also by Triton) against downy mildew in the results presented in chapter 3. Besides, the accumulation of all these amino acids was rather described as response to abiotic stresses, particularly to water stress, for example in barley leaves studied by Singh et al. (1973). In their work, alanine was conversely reported to decrease upon water stress. In this study, alanine specifically decreased at both time-points upon BTH treatment. The exact role of this latter amino acid has not been established.

Extensive alterations occurred in the metabolism of organic acids in the treated leaves. A remarkable decrease in pyruvic, fumaric and malic acids, and a moderate one in succinic acid was observed at 24 hpt. The simultaneous decrease in succinic, fumaric and malic acids may result either from the stronger demand for the Krebs cycle intermediates required for the biosynthesis of other metabolites, including those involved in defense, or from the increased consumption of pyruvic acid. The latter mechanism might be due to the allocation of pyruvic acid to the polyphenol biosynthetic pathway for the purpose of defense. A decrease in pyruvic acid and 2-oxoglutarate was also observed in *V. vinifera* cv. Gamay in vitro cultures elicited with oligogalacturonides (Krzyzaniak et al., 2018). Modifications of the concentrations of fumaric, malic, succinic and 2-oxoglutaric acids were monitored in opium poppy (*Papaver somniferum*) cell cultures treated with a fungal elicitor (Zulak et al., 2008). The levels of malate, succinate and fumarate were generally stable, reduced or even increased after treatment, depending on the moment of harvest (Zulak et al., 2008). In this study, an accumulation of malic and succinic acids occurred at 6 dpt upon all the treatments, suggesting a lesser demand for polyphenols, which at this time-point were already synthesized. At 24 hpt, the biosynthesis of tartaric acid was inhibited by all treatments, mainly elicitors, and was accompanied by a reduced level of its precursor, ascorbic acid, which plays a role in plant stress and physiology (growth and development). A possible stress generated by elicitation could contribute to its degradation. Such a phenomenon was already observed in salad leaves and broccoli as an effect of harvest and treatments with hormones like MeJA (Dewhirst et al., 2017). In contrast, at 6 dpt the level of tartaric acid remained stabilized, while ascorbic acid accumulated upon treatment with Triton, MeJA and PHOS. The involvement of this antioxidant in the enhanced protection conferred by these three treatments could be suggested. At 24 hpt, acetic acid was the only organic acid whose level did not change or even increased after application of MeJA. At 6 dpt this organic acid accumulated in Triton, MeJA and BTH modalities. Acetic acid is known to inhibit some plant pathogenic fungi (Sehirli and Saydam, 2016). Finally, the level of choline increased in PHOS and BTH conditions at 24 hpt. This

was probably a defense response since its role in plant stress has been demonstrated (Mou et al. 2002; Zhang et al. 2010). Overall, the elicitor treatments that were performed on grapevine plants significantly shifted their major metabolites, as it occurs in response to pathogenic infection (Parvaiz et al., 2018).

To summarize, differences and similitudes in the responses engendered were observed depending on the elicitor applied and therefore the defense signaling pathway used. As it was shown by PCA (Fig. 69A, B), distinct pattern of changes occurred according to the time-point considered. At 24 hpt, all the elicitors were grouped together (especially PHOS and BTH). It mainly resulted from the negative impact on carbohydrates and phenolics. Thus, the effect observed in this metabolic pool is more likely a consequence of fundamental metabolic repartitioning of carbon resources rather than a specific change. At 6 dpt, less pattern of changes was shared, and more specific modifications were observed according to the treatment. The variables involved in differentiating the samples involved the strongest accumulation of (+)-catechin, threonine and tyrosine in MeJA-treated leaves, the gallic acid content increase uniquely in the leaves elicited with PHOS, and the lowest level of metabolites in BTH modality, in comparison with remaining treatments, in particular a decrease of glutamine, ascorbic and tartaric acids. Moreover, even if Triton has not been recognized as elicitor, it importantly affected a number of metabolites followed in this study. It may result from the function of this molecule as surfactant creating an abiotic stress and facilitating cuticle penetration the soluble active ingredients in leaf (Liu et al., 2016). Besides, cutin monomers act as DAMPs eliciting plant immune responses (Hou et al., 2019) – they are released by wounding, a phenomenon triggering JA biosynthesis. Indeed, similar gene transcription profiles observed upon wounding were encountered after jasmonic acid elicitation (Peña-Cortès H., et al., 1995; Heil et al., 2012).

V. CONCLUSIONS AND PERSPECTIVES

Context

The consequences of climate change and the negative impact of conventional farming practices on eco-systems and human health are contemporary threats challenging the crop production. Thus, the need for the development of sustainable pest control methods in agriculture is of a high priority, especially for grapevine, the crop of a primary role in culture and global economics. Indeed, nowadays, in term of protection, vineyard agriculture is dominated by the use of copper-based formulations and pesticides. Attempts have been made for the withdrawal of some of these products from the market, at European and national levels. Recently, in the context of environmental preservation and to ensure healthy, sustainable food, the European Commission (EC) implemented the strategy „From Farm to Fork" which aims, among others, to reduce the use of synthetic pesticides and the risks associated with their application by 50% by 2030. The EU's agricultural land under organic farming is aimed to account 25% by 2030 (https://ec.europa.eu/food/farm2fork_en). In this purpose, the development of environmentally friendly practices will be supported. Additionally, similar objectives have been established in individual European countries in the frame of national action plans. In France, these are "Ecophyto" plans, and in Poland, the "National Action Plan to Reduce the Risk Associated with the Use of Plant Protection Products" (https://ec.europa.eu/food/plant/pesticides/sustainable_use_pesticides/nap_en). The progressive reduction of the quantity of harmful phytosanitary products applied concerns also copper-based fungicides, which in French viticulture are used as the main anti-mildew treatment. The EC approved the renewal of the copper compounds authorization for a period of 7 years from 2019, however, with the reduction in their quantities approved (passage of a maximum of 6 treatments on average at 4 kg/ha/year) (Commission Implementing Regulation 2018/1981). All these restrictions and the ones expected in the future imply the need to develop replacement means of diseases control of the crops.

During the past decade, one of the most promising alternative strategy of plant protection that has been proposed is based on the stimulation of defense responses (Walters et al., 2013). This strategy relies on the use of elicitors (in agriculture referred to Plant Defense Stimulators, PDS) which are biodegradable, non-toxic, non-polluting, and non-hazardous to eco-system substances. Elicitors stimulate endogenous plant defense mechanisms leading to the development of an enhanced resistance. Some of them can mobilize faster and more

robust reactions in response to a pathogen attack (priming) (Eder and Cosio, 1994; Conrath et al., 2006). Several PDS products have been marketed, including those adjusted for grapevine protection (Delaunois et al., 2014). The resistance conferred by PDS tends to be wide-spectrum and may be long-lasting, however, is rarely complete. Moreover, the efficiency of elicitors is likely to be influenced by a number of factors under field conditions, it also varies according to the plant species/cultivar and the specific pathogen (Héloir et al., 2019). Furthermore, the activation of defense reactions by such treatment may impose a cost on the plant fitness, leading to alterations in its primary metabolism which in long term can impact the plant vigor, and the yield and quality of berries (Heil, 2002). Thus, the successful application of PDS in viticulture requires a thorough understanding of the biological activity of such substances and relies on comprehensive knowledge of the plant immune reactions that are supposed to be stimulated, as well as on the consequences of such stimulation on the plant fitness.

The research presented in the frame of this thesis aimed, first of all, to provide an insight into the effect of foliar application of three recognized elicitors: MeJA (methyl jasmonate), BTH (benzothiadiazole) and phosphonates (PHOS) on grapevine leaves (*V. vinifera* cv. Cabernet Sauvignon from the greenhouse), in term of the protection that they confer to the plant against downy mildew, the activation of defense responses and the eventual impact on primary metabolism.

Grapevine leaf protection against Plasmopara viticola conferred by the three elicitors in relation to defense responses (gene expression and polyphenol analyses)

All of the three elicitors triggered a high protection to the grapevine leaves against *P. viticola*. An inhibitory effect on the oomycete growth was of approximately 85.8, 97.3 and 98.5% on the leaves treated with MeJA, PHOS and BTH, respectively, after 6 days post-elicitation. For analyses, the leaves were collected 6 days after elicitation plus or minus 2 days after inoculation. The induced resistance resulted from the up-regulation of defense-related genes and the enhanced biosynthesis of polyphenols. The transcript accumulation was consistent with the signaling pathway specific to the elicitor, salicylic acid for BTH (*SAMT* and *ICS* overexpression) and jasmonic acid for MeJA (*LOX13*), with some cross-responses (e.g., the up-regulation of *LOX9* by BTH). PHOS tended to modulate the defense responses like BTH, particularly in the up-regulation of genes encoding enzymes involved in the

biosynthesis of flavonoids (*CHS*, *F3H*, *DFR*, *and LDOX*) in the leaves pretreated and inoculated. As expected, BTH led to the up-regulation of genes encoding PR proteins (*PR2*, *PR4*, *PR5*, *PR8* and *PR11* both after elicitation and inoculation, and additionally *PR6*, *PR7*, *PR9*, *PR10*, *PR12*, *PR15* upon inoculation). A very complex cross-talk regulation between the different hormonal pathways and defense-related signaling molecules was generally noted. The responses observed were more or less consistent with the literature, however, the direction of changes sometimes differed. This could be attributed to the time of sampling which was crucial for such comparison. Thus, a kinetics of molecular and biochemical modifications occurring after elicitation plus or minus inoculation should be performed to better understand the responses that such treatments engender in the plant.

Different polyphenols were overproduced after treatment plus or minus inoculation. MeJA was particularly effective in the enhancement of the accumulation of stilbenes. Several stilbenes of different degrees of oligomerization were accumulated in MeJA-pretreated and inoculated leaves. These were monomers (sum of *trans*- and *cis*-piceids; 1.2-fold induction), dimers (ε- and ω-viniferins, parthenocissin; 2.8-, 22.9-, 14.9-fold induction, respectively), and tetramers (isohopeaphenol and hopeaphenol; 2.4- and 3.9-fold induction, respectively). Thus, the protection conferred by MeJA to grapevine leaves against *P. viticola* were likely to resulted from the accumulation of different stilbenes. The results on stilbenic monomers and dimers confirm the literature data of MeJA-mediated defense reactions in grapevine, but the enhancement of the content of the two tetramers by this elicitor is provided for the first time, for my knowledge, through the current work. Stilbenes of a high degree of oligomerization have been recognized as particularly effective against downy mildew by foliar discs assays previously performed in MIB laboratory (Gabaston et al., 2017).

The leaves pre-treated with PHOS and BTH overproduced a methoxylated derivative of resveratrol, pterostilbene (1.5- and 2.7-fold induction, respectively), which despite its generally low amount was previously reported as one of the most toxic stilbenes to fungi and oomycetes, including *P. viticola* (Pezet et al., 2004). Priming effect on pterostilbene accumulation was demonstrated in grapevine leaves pretreated with BTH (Dufour et al., 2013) and β-aminobutyric acid (Slaughter et al., 2008) and then inoculated by downy mildew. Also, two flavonols studied (quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside) were slightly accumulated in PHOS- and BTH-pretreated leaves (on average 1.3-fold induction). The content of two flavanols (catechin and epicatechin) significantly increased in the leaves pretreated with BTH (8.4- and 20.9-fold induction in comparison to non-elicited leaves, respectively), and then inoculated (7- and 15.9-fold induction, respectively), however, no

priming effect was observed for these two molecules. Flavonoids have been reported to be accumulated in grapevine upon stresses, including SA-mediated reactions and *P. viticola* infection (Fang and Huang, 2013; Andreu et al., 2018).

The effect of methyl jasmonate and benzothiadiazole on the biosynthesis of pentacyclic triterpenoids in grapevine whole leaf tissue and leaf cuticular waxes

The stimulating effect of the two elicitors studied, MeJA and BTH, on grapevine pentacyclic triterpenoids was investigated for the first time. Lucini et al. (2018) reported an elevated content of pentacyclic triterpenic acids, oleanolic, ursolic and betulinic, along with the accumulation of stilbenes, in grapes of *V. vinifera* bunches subjected to elicitation with chitosan. In this study, according to the elicitor, the time of exposure to it and the matrix considered (whole leaf tissue or leaf cuticular waxes), different bioactive pentacyclic triterpenoids were accumulated. In MeJA-treated leaves, the content of oleanolic acid increased both after 7 and 14 days post-treatment (1.9- and 4.8-fold induction, respectively). The amount of α-amyrin/lupeol also augmented at both time-points upon MeJA treatment (2.4- and 1.8-fold induction at 7 and 14 dpt, respectively). At 14 dpt MeJA-treated leaves accumulated taraxerol (1.8-fold induction), as well as ursolic acid (in the quantity of approximately 3.58 µg/g D.W.), which was not detected in the control leaves. BTH led to a more significant stimulation and the accumulation of a wider range of pentacyclic triterpenoids studied at both time-points. At 7 dpt, the content of taraxerol, α-amyrin/lupeol and oleanolic acid increased in BTH-treated leaves 1.7-, 1.4- and 11.6-fold. Moreover, BTH led to an accumulation of oleanolic acid derivatives, which were not detected in the control leaves, olean-2,12-dien-28-oic and 3-oxo-oleanolic acids, in the amount of approximately 5.95 and 1.55 µg/g D.W. At 14 dpt, the amount of taraxerol, α-amyrin/lupeol and oleanolic acid remained enhanced in BTH-treated leaves, in comparison to the control leaves (2.8-, 2.3- and 3-fold induction). The oleanolic acid derivaties as well as ursolic acid were also synthetized in BTH-treated leaves (in the quantity of approximately 2.21, 3.53 and 3.50 µg/g D.W., respectively), while they were not detected in the control leaves. The results obtained may suggest a potential contribution of the pentacyclic triterpenoids stimulated in grapevine defense. Indeed, oleanolic acid has been already proposed as a biomarker of grapevine resistance to *P. viticola* or wounding (Chitarrini et al., 2017a, 2017b). The accumulation of this compound upon elicitation with MeJA and BTH, as a response mediated by respectively JA and SA signaling pathways may partly confirm the previous results. Overall, further

investigation could be worth to associate an increased accumulation of pentacyclic triterpenoids in grapevine upon elicitation with an enhanced resistance to diseases.

Interestingly, in the leaf cuticular waxes different pattern of changes occurred within triterpenoids, studied at 14 dpt, in regard to those observed in the whole leaf tissue. Differences were particularly visible considering the effect of BTH, which in the leaf cuticular waxes did not stimulated neither the amount of oleanolic acid, nor the accumulation of its derivatives, olean-2,12-dien-28-oic and 3-oxo-oleanolic acids. However, BTH led to an increased biosynthesis of oleanolic acid methyl ester (9.5-fold induction) and the accumulation of ursolic methyl ester in the quantity of approximately 1.14 µg/g D.W. MeJA caused similar changes within the pentacyclic triterpenoids in the leaf cuticular waxes as in the whole leaf tissues, with an increased content of α-amyrin/lupeol, oleanolic acid, ursolic acid (5.1-, 2.3-, 3.6-fold induction, respectively). MeJA also led to the accumulation of oleanolic acid methyl ester (2.3-fold induction) uniquely in the leaf cuticular waxes. All these observations suggest different roles of pentacyclic triterpenoids accumulated in the cells of internal tissues and at the surface layers. Indeed, low-polar forms of triterpenoids (free and esters) secreted into the cuticular waxes, along with linear long-chain aliphatics, constitute the first line of plant defense against pathogens and herbivores (Zeisler-Diehl et al., 2018). It can be conceivable that according to the specific stress/pathogen, the plant responds by accumulating different types of triterpenoids with a particular localization to ensure its optimal defense. Modifications of the compounds occurring both in internal tissues and at surfaces, due to elicitation, needs to be taken into consideration while developing the protection method based on the use of PDS.

The impact of methyl jasmonate and benzothiadiazole on the profile of sterols

Phytosterols are essential compounds for the structure and stability of cell membranes. The profile of these compounds was importantly altered due to elicitation of grapevine leaves with MeJA and BTH. A strong reduction of total phytosterols was observed at 14 dpt particularly in the leaf cuticular waxes (a 4.3- and 2-fold decrease in respectively MeJA- and BTH-treated leaves, in comparison to the control). In whole leaf tissue, at 7 dpt both elicitors negatively impacted only campesterol (1.3-fold on average). In turn, at 14 dpt the level of the phytosterols studied increased. In MeJA-treated leaves, the content of campesterol augmented 1.8-fold, and in BTH-treated leaves a 2.6-, 1.8- and 1.1-fold induction was noted for campesterol, stigmasterol and sitosterol, respectively.

Regardless the effect of elicitation on sterols (negative or positive), the results obtained confirm the recognized role of these compounds in responses to stress in different plant species, including grapevine. Indeed, the fluctuations in the sterol composition, including changes in the ratio of campesterol to sitosterol or to sitosterol to stigmasterol, are suggested to be essential for some processes related to plant growth and development, as well as to stress compensation (Aboobucker and Suza, 2019; Schaeffer et al., 2001). For this reason, an expected competition of biosynthetic pathways of sterols and pentacyclic triterpenoids, of respectively primary and specialized functions in the plant, was often suggested; however, it was not always visible in this study. Similarly, it has been reported that if there are sufficient resources to ensure both the growth and defense, it is possible not to notice the negative impact of a stress condition, at least at the level of the physiology or the development of the plant (Walters and Heil, 2007). For example, in pine, the cost of resistance induction by MeJA could only be measured in a situation of phosphorus deficiency (Sampedro et al., 2011). The probability of the occurrence of a fitness-cost in the open field is strong, as the plant could be subjected simultaneously or not to various environmental fluctuations, with many of them being linked to climate change. To ensure a correct physiological state of the crop in an ever-changing environment, the use of biostimulants has been proposed. Biostimulants are substances or microorganisms applied to plants with the aim to improve the absorption of nutrients, tolerance to abiotic stress, and the quality traits of the crop. Thus, it could be relevant to associate the use of biostimulants with that of PDS. Such approach has been already tested on grapevine (Krzyzaniak, 2018), but for the development of this strategy and the validation of its efficiency more research is required.

The impact of methyl jasmonate, phosphonates and benzothiadiazole on grapevine leaf metabolome

In order to evaluate the potential fitness-cost linked to the activation of defense reactions in grapevine leaves, the effect of the elicitors was also studied on other primary metabolites, including carbohydrates, amino acids, organic acids, as well as some of precursors of phenolic compounds. Important alterations occurred and, like in the case of specialized metabolites, they depended on the time-point and the elicitor applied. Metabolic repartitioning of carbon resources was particularly visible as an early reaction (at 24 h post-treatment). In the un-treated leaves, the highest concentration of the majority of the identified metabolites was detected, mainly sugars (*myo*-inositol, fructose, sucrose, α- and β-glucose),

some organic acids (malic, pyruvic, tartaric, ascorbic and fumaric acids), and phenolics (quercetin-3-*O*-glucoside, syringic, gallic and shikimic acids). After the longer time, at 6 dpt, more specific changes occurred, including the strongest accumulation of (+)-catechin, threonine and tyrosine in MeJA-treated leaves, the gallic acid content increase uniquely in the leaves elicited with PHOS, and the lowest level of metabolites in BTH modality, in comparison with remaining treatments, in particular a decrease of glutamine, ascorbic and tartaric acids. In the literature, changes observed within all these metabolites have been reported to occur upon various stresses (Caretto et al., 2015).

From the results obtained, the question arises as how a treatment with PDS in vineyard could impact the quality of grape berries and wine. Several studies have been already performed in order to evaluate the composition of grapes and wines derived from grapevines subjected to elicitation (for review see Gutiérrez-Gamboa et al., 2018a). In all cases, important modifications of oenological parameters were noted in wine originated from grapevines of which bunches were directly treated with PDS, or even only after foliar application. In fact, plant natural defense stimulators have attracted an attention beyond their efficacy in crop protection, as agents improving grape and wine quality in term of volatile profile (Garde-Cerdán et al., 2014), amino acid (Garde-Cerdán et al., 2016), phenolic concentrations (Portu et al., 2017; Gil-Muñoz et al., 2017), and overall sensory attributes (Vitalini et al., 2014; D'Onofrio et al., 2018). Ruggiero et al. (2013) characterized phytosterols in berries and seed tissues of grapes at pre-veraison and veraison stages, following treatments with chitosan or BTH, and in the corresponding wines. In general, the elicitors applied augmented the amount of sitosterol, campesterol and stigmasterol both in grapes and the resulting microvinificates, in comparison to the control, as well as the modalities treated with conventional fungicides (Ruggiero et al., 2013). However, some studies demonstrated a negative impact of foliar elicitation on grape berry quality, but the degree of changes depended on the cultivar, the elicitor and the frequency of treatments (Gutiérrez-Gamboa et al., 2018b; Garde-Cerdán et al., 2017). Romanazzi et al. (2016) reported that chitosan was very effective against downy mildew in the vineyard, but it also perturbed the vigor of the vegetation, however, without negative impact on grapes yield and quality.

Overall, these observations underline the need for further research for the development of management tools for sustainable disease control. Broad categories of factors have to be taken into consideration, including the type of elicitor and the methods of its application (doses, frequency, and period of treatment). In addition, the specific environment in which the plant is localized, and in particular its nutrient intake, can play a role. Hence the importance of

eventual association with products acting in synergy, e.g., application of PDS combined with biostimulants products may respond to all the plant requirements. Also, elicitors that employ distinct signaling pathways, thus, induce diverse kind of defenses, could be associated in order to obtain a wide spectrum of responses. Finally, different sustainable strategies of diseases control could be developed as combined treatment to achieve synergized effects. The possible approaches include agronomic management of phytosanitary risks (prophylaxis, cultural practices, reasoned control) and alternative strategies, such as the improvement of resistance by genetic methods (production of hybrids or transgenesis), biological control, the use of antimicrobial plant products. A combination of these strategies could ensure the integrated pest management in the context of sustainable development.

Taken together the research presented in the current dissertation provide several supplementary information about grapevine responses to three elicitors (MeJA, PHOS and BTH), and may contribute to the development of application methods in vineyard of such products in the future. The results obtained in the experiments carried out underline that the thorough comprehension of biological dynamics characterizing the interaction between elicitor, plant molecular and metabolic responses and pathogen in a particular environmental context, is crucial for the advancement in the development of effective protection strategies based on the use of PDS for grapevine diseases control.

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APPENDIX

SUPPLEMENTARY DATA

Table A1. Characteristic ions of mass spectra of identified steroids and triterpenoids.

Compound	Mass spectrum *m/z* (relative intensity)
Steroids:	
Campesterol	400 (30), 107 (51), 105 (55), 95 (49), 83 (45), 81 (64), 71 (62), 57 (77), 55 (77), 43 (100), 41 (52)
Cholesterol	386 (26), 107 (50), 105 (48), 91 (57), 81 (54), 79 (46), 69 (47), 57 (87), 55 (73), 43 (100), 41 (55)
Clionasterol	414 (100), 396 (63), 329 (57), 213 (25), 107 (69), 105 (65), 95 (60), 81 (58), 55 (57), 43 (85)
Cycloartanol	428 (4), 205 (60), 109 (98), 95 (100), 93 (64), 81 (69), 69 (78), 57 (73), 55 (82), 43 (89), 41 (67)
Cycloartenol	426 (12), 121 (30), 109 (45), 105 (36), 95 (60), 93 (40), 81 (45), 69 (100), 55 (52), 41 (50).
Cycloart-23-ene-3,25-diol	442 (10), 203 (48), 121 (73), 109 (100), 107 (82), 95 (75), 81 (91), 69 (54), 55 (62), 43 (77)
Isofucosterol	412 (5), 314 (100), 105 (47), 95 (50), 91 (42), 83 (40), 81 (51), 69 (61), 55 (96), 43 (49)
24-methylenecycloartanol	440 (5), 121 (60), 119 (55), 109 (62), 107 (76), 105 (57), 95 (98), 93 (64), 81 (72), 69 (99), 55 (100)
Sitostenone	412 (37), 229 (34), 218 (31), 124 (100), 109 (31), 95 (41), 81 (27), 69 (32), 55 (37), 43 (44)
Sitosterol	414 (29), 145 (54), 107 (59), 105 (60), 95 (54), 91 (49), 81 (57), 57 (68), 55 (70), 43 (100), 41 (44)
Spinasterone	410 (31), 271 (98), 107 (66), 105 (67), 99 (80), 93 (65), 91 (60), 81 (61), 69 (45), 43 (100)
Sitostanol	416 (31), 215 (82), 109 (58), 107 (83), 95 (81), 93 (64), 81 (84), 69 (60), 57 (64), 55 (81), 43 (100)
Stigmasterol	412 (36), 145 (64), 107 (52), 95 (100), 83 (66), 81 (90), 78 (60), 69 (67), 67 (85), 55 (69)
Stigmastane-3,6-dione	428 (25), 135 (61), 107 (74), 98 (63), 95 (67), 79 (62), 69 (86), 57 (67), 55 (100), 43 (77), 41 (71)
Tremulone (stigmasta-3,5-dien-7-one)	410 (32), 187 (27), 174 (100), 161 (37), 159 (26), 91 (28), 57 (28), 55 (37), 43 (44), 41 (28)
Neutral triterpenoids:	
α-Amyrin	426 (4), 218 (100), 203 (20), 189 (36), 135 (35), 121 (32), 109 (32), 107 34), 95 (40), 81 (33), 55 (31)
α-Amyrenone	424 (12), 219 (19), 218 (100), 203 (24), 189 (16), 135 (19), 133 (18), 122 (18), 119 (17), 95 (16), 55 (18)
β-Amyrin	426 (27), 219 (18), 218 (100), 203 (49), 189 (17), 135 (11), 109 (13), 105 (12), 95 (15), 81 (18), 69 (14)

Betulin	442 (8), 203 (100), 189 (77), 133 (66), 121 (55), 107 (57), 105 (49), 95 (56), 93 (54), 81 (67)
Lupeol	426 (18), 207 (67), 189 (90), 135 (83), 121 (80) 109 (85), 121 (80), 95 (100), 93 (87), 81 (86)
Lupeol acetate	468 (8), 189 (100), 135 (63), 121 (76), 109 (71), 107 (78), 95 (77), 93 (80), 81 (68), 69 (53)
Taraxerol	426 (18), 411 (13), 302 (34), 287 (32), 269 (18), 218 (22), 204 (1000), 189 (39), 135 (63), 95 (41)
Uvaol	442 (1), 207 (13), 204 (17), 203 (100), 133 (33), 119 (13), 105 (11), 95 (12), 81 (10), 69 (10), 55 (11)
Triterpenoid acids:	
Betulinic acid methyl ester	470 (5), 207 (41), 203 (38), 189 (100), 175 (40), 119 (41), 107 (38), 105 (37), 95 (37), 93 (38)
Olean-2,12-dien-28-oic acid methyl ester	452(11), 425 (9), 263 (11), 262 (61), 221 (14), 203 (100), 190 (15), 189 (22), 133 (14), 119 (12)
Oleanolic acid methyl ester	470 (1), 262 (48), 207 (13), 204 (16), 203 (100), 202 (21), 189 (22), 133 (17), 119 (13), 105 (14)
3-Oxo-olean-12-en-28-oic acid methyl ester	468 (6), 262 (32), 204 (17), 203 (100), 202 (21), 189 (29),133 (17), 119 (14), 105 (12), 55 (12)
Ursolic acid methyl ester	470 (1), 263 (20), 262 (100), 207 (32), 203 (93), 189 (29), 133 (76), 119 (34), 105 (21), 95 (18)

Table A2. Analysis parameters for the polyphenols studied by ultra-performance liquid chromatography coupled to a triple quadrupole mass spectrometer (UHPLC-MS) in multiple reaction monitoring (MRM) mode.

Stilbenes	***Rt (min)***	***Fragmentor (V)***	***Precursor Ion***	***CE (eV)***	***Quantifier***	***CE (eV)***	***Qualifier***	***Ratio q/Q***	***Ion Polarity***
trans- resveratrol	12.96	100	**229**	12	**135**	20	**107**	87	Positive
trans- piceid	12.95	150	**389**	4	**227**	44	**143**	8	Negative
cis- piceid	14.58	150	**389**	4	**227**	44	**143**	9	Negative
pterostilbene	20.77	150	**257**	4	**135**	44	**181**	19	Negative
trans-ℰ- viniferin	18.00	154	**455**	20	**215**	36	**107**	99	Positive
ω- viniferin	18.80	154	**455**	20	**215**	36	**107**	96	Positive
δ- viniferin	19.30	154	**455**	12	**361**	12	**349**	72	Positive
pallidol	14.82	154	**455**	12	**361**	20	**215**	73	Positive
parthenocissin A	15.70	154	**455**	12	**361**	12	**349**	72	Positive
vitisinol C	17.99	125	**429**	40	**107**	24	**121**	59	Positive
hopeaphenol	16.94	218	**908**	36	**359**	13	**453**	55	Positive
isohopeaphenol	17.10	218	**908**	13	**453**	36	**359**	78	Positive
Flavanols									
catechin	9.50	100	**291**	12	**139**	12	**123**	43	Positive
epicatechin	10.60	100	**291**	12	**139**	12	**123**	45	Positive
Flavonols									
quercetin-3-*O*- glucoside	13.18	144	**463**	16	**301**	44	**271**	66	Negative
quercetin-3-*O*- rutinoside	12.79	216	**609**	28	**301**	60	**271**	57	Negative

Table A3. Identified and semi-quantified metabolites by a proton nuclear magnetic resonance (^{1}H NMR) spectroscopy in grapevine leaves elicited with Triton (Triton X-100), MeJA (methyl jasmonate), PHOS (phosphonates), or BTH (benzothiadiazole), and harvested 24 h and 6 days post-treatment (24 hpt and 6 dpt). Values are expressed as means of three replicates with standard deviations (SD). One-way analysis of variance (ANOVA) was applied ($p \leq 0.05$) in order to establish the significant differences between elicitor-treated and control leaves. Asterisks denote the significance levels as compared to control samples: $p \geq 0.05$ (ns), 0.01 to 0.05 (*), 0.001 to 0.01 (**), 0.0001 to 0.001 (***), <0.0001 (****).

Metabolite	Elicitor	24 hpt			6 dpt		
		Mean	SD	*p* -value	Mean	SD	*p* -value
Amino acids							
Alanine	Control	36,67	0,64		28,25	0,91	
	Triton	32,66	0,1		23,16	7,19	
	MeJA	39,1	3,06		22,11	1,35	
	PHOS	27,8	4,01		24,76	3,41	
	BTH	24,22	0,74	**	15,28	1,80	*
γ- aminobutyric acid (GABA)	Control	9,55	1,52		0,51	0,04	
	Triton	6,98	1,28		2,16	0,53	
	MeJA	4,06	0,58	*	3,95	1,28	*
	PHOS	4,14	0,03	*	2,68	1,91	
	BTH	3,97	0,38	*	6,52	0,33	*
Glutamic acid	Control	123,37	0,8		81,19	10,56	
	Triton	122,43	2,55		106,04	10,70	
	MeJA	164,44	19,08		99,37	10,05	
	PHOS	118,25	12,43		146,65	7,78	***
	BTH	129,24	3,43		91,00	2,99	
Glutamine	Control	53,71	2,81		11,77	5,38	
	Triton	40,28	2,29		34,05	20,09	
	MeJA	78,88	4,79	**	25,97	5,87	
	PHOS	22,5	4,36	**	50,90	0,16	*
	BTH	17,42	1,64	***	14,76	1,70	
Proline	Control	187,27	0,67		163,67	1,29	
	Triton	241,99	5,73	*	173,84	25,53	
	MeJA	225,47	14,63		259,64	14,35	*
	PHOS	145,2	12,84		189,84	22,74	
	BTH	208,87	10,38		161,55	16,05	
Threonine	Control	30,45	0,85		16,46	1,44	
	Triton	28,78	0,87		29,57	3,96	
	MeJA	40,71	3,98		49,24	1,43	****
	PHOS	28,13	4,13		22,36	4,53	
	BTH	24,99	0,44		27,43	3,18	*
Tyrosine	Control	5,23	0,24		3,71	0,56	
	Triton	5,69	0,28		8,85	0,69	**
	MeJA	4,82	0,76		11,09	0,06	***
	PHOS	4,92	0,5		5,23	1,63	
	BTH	4,23	0,09		7,08	1,14	*
Valine	Control	4,48	0,34		1,84	0,32	
	Triton	3,69	0,02		1,93	0,01	
	MeJA	7,04	0,53	**	3,23	0,02	*
	PHOS	3,43	0,65		2,01	0,20	
	BTH	3,66	0,3		1,44	0,09	

(to be continued)

Table A3. (continued)

Metabolite	Elicitor	24 hpt			6 dpt		
		Mean	**SD**	***p*-value**	**Mean**	**SD**	***p*-value**
Carbohydrates							
α-Glucose	Control	82,16	0,65		71,83	7,50	
	Triton	63,53	0,37	***	82,51	16,17	
	MeJA	38,62	1,36	****	53,31	6,08	
	PHOS	49,29	3,03	****	76,33	10,52	
	BTH	49,97	1,33	****	67,09	2,80	
β-Glucose	Control	169,05	0,67		158,34	23,86	
	Triton	140,44	2,1	*	160,86	40,36	
	MeJA	64,38	7,83	****	119,70	22,58	
	PHOS	108,87	8,55	***	160,79	16,31	
	BTH	108,53	2,79	***	151,95	16,36	
Fructose	Control	51,53	0,24		60,25	2,49	
	Triton	46,53	0,36		54,26	0,03	
	MeJA	33,16	1,62	***	44,05	2,89	**
	PHOS	36,09	4,3	**	57,83	4,61	
	BTH	35,74	0,17	***	42,86	4,14	**
myo-Inositol	Control	552,35	1,5		897,48	10,93	
	Triton	453,69	6,6	*	1069,25	48,93	
	MeJA	408,2	19,04	***	966,87	21,41	
	PHOS	339,5	28,58	****	1006,21	109,04	
	BTH	377,12	8,31	***	1081,48	77,11	
Sucrose	Control	1837,83	1,54		1870,68	164,47	
	Triton	1487,63	21,3	***	1800,25	8,95	
	MeJA	749,45	14,19	****	1260,06	16,87	*
	PHOS	339,5	28,58	****	2192,65	220,89	
	BTH	1222,85	36,09	****	1557,10	94,31	
Organic acids							
Acetic acid	Control	4,23	0,04		3,64	0,08	
	Triton	4,03	0,12		5,51	0,12	**
	MeJA	5,35	0,39	*	6,14	0,17	***
	PHOS	4,69	0,13		4,28	0,51	
	BTH	4,27	0,16		4,90	0,53	*
Ascorbic acid	Control	202,29	3,11		n.d.		
	Triton	190,94	0,37		173,49	19,55	****
	MeJA	138,68	10,48	**	115,47	11,40	****
	PHOS	n.d.		*	198,83	15,04	****
	BTH	n.d.		**	n.d.		
Fumaric acid	Control	1,08	0,06		0,65	0,02	
	Triton	0,44	0,01	***	0,38	0,05	
	MeJA	0,53	0,05	***	0,06	0,03	
	PHOS	0,8	0,05		1,12	0,49	
	BTH	0,37	0,06	****	0,51	0,02	
Malic acid	Control	643,1	2,26		119,80	0,01	
	Triton	353,82	9,69		446,15	30,08	****
	MeJA	164,5	9,69		274,86	33,05	****
	PHOS	185,54	17,19		640,58	29,31	****
	BTH	251,83	16,53		508,28	12,88	****

(to be continued)

Table A3. (continued)

Metabolite	Elicitor	24 hpt			6 dpt		
		Mean	**SD**	***p*-value**	**Mean**	**SD**	***p*-value**
Organic acids							
Pyruvic acid	Control	1,93	0,03		1,26	0,05	
	Triton	1,18	0,09	****	1,82	0,11	
	MeJA	1,34	0,06	***	2,34	1,15	
	PHOS	0,8	0,03	****	2,12	0,70	
	BTH	0,78	0,03	****	1,78	0,10	
Succinic acid	Control	11,09	0,16		6,67	1,29	
	Triton	9,25	0,13	*	13,50	0,49	***
	MeJA	9,24	0,53	*	12,15	1,22	**
	PHOS	6,73	0,41	***	15,49	0,03	***
	BTH	9,96	0,15		14,00	0,50	***
Tartaric acid	Control	455,77	6,96		226,21	5,45	
	Triton	336,38	11,87		387,17	13,52	
	MeJA	197,24	50,5		342,81	2,05	
	PHOS	279,83	21,74		418,82	178,17	
	BTH	264,69	14,53		373,14	35,10	
Phenolics							
(+)-Catechin	Control	10,29	0,27		11,35	0,70	
	Triton	12,02	0,6		24,69	0,21	***
	MeJA	9,61	1,1		35,15	2,54	****
	PHOS	11,48	1,35		11,48	1,91	
	BTH	11,99	0,28		17,91	1,41	**
Gallic acid	Control	3,67	0,21		1,40	0,08	
	Triton	3,82	0,07		1,33	0,03	
	MeJA	2,39	0,55		1,23	0,07	
	PHOS	0,27	0,04	***	2,20	0,38	*
Quercetin-3-*O*-glucoside	Control	59,55	1,69		8,68	1,28	
	Triton	44,67	0,35	*	30,71	1,90	****
	MeJA	42,11	4,16	**	24,91	0,84	***
	PHOS	33,44	1,91	***	15,15	5,04	
	BTH	37,04	0,77	***	21,65	2,04	**
Shikimic acid	Control	43,49	0,48		21,32	0,33	
	Triton	51,83	3,92		30,25	0,71	
	MeJA	28,57	3,43	*	32,53	2,79	
	PHOS	18,55	7,44	**	38,32	14,22	
	BTH	20,33	0,6	**	17,86	2,42	
Syringic acid	Control	11,15	0,05		7,96	0,37	
	Triton	9,37	0,01		8,58	2,03	
	MeJA	6,16	0,78	***	7,61	1,21	
	PHOS	6,15	0,85	***	8,66	1,70	
	BTH	6,5	0,13	***	6,96	0,36	
trans-Feruloyl derivative	Control	168,16	2,4		95,86	0,01	
	Triton	195,35	0,36		190,76	3,65	****
	MeJA	184,62	14,55		191,94	3,16	****
	PHOS	167,01	15,76		151,19	3,16	****
	BTH	153,65	3,11		125,24	9,55	**

(to be continued)

Table A3. (continued)

Metabolite	Elicitor	24 hpt			6 dpt		
		Mean	**SD**	***p*-value**	**Mean**	**SD**	***p*-value**
Amines							
Adenine	Control	0,48	0,08		1,12	0,01	
	Triton	0,59	0		0,45	0,16	
	MeJA	0,82	0,02		0,59	0,08	
	PHOS	0,5	0,14		1,19	0,55	
	BTH	0,5	0,03		0,42	0,15	
Choline	Control	16,37	0,61		10,98	0,87	
	Triton	15,19	0,38		15,14	0,14	
	MeJA	18,48	1,46		13,62	5,69	
	PHOS	24,91	2,23	*	12,26	1,39	
	BTH	23,92	0,47	*	15,10	2,82	
Trigonelline	Control	2,74	0,03		0,34	0,03	
	Triton	0,72	0,71	*	0,84	0,54	
	MeJA	2,56	0,19		2,09	0,20	***
	PHOS	0,05	0,36		1,51	0,26	**
	BTH	n.d.		***	n.d.		***

Figure A1. (to be continued) Chemical structures of triterpenoids identified in grapevine. A, steroids, B, pentacyclic triterpenoids.

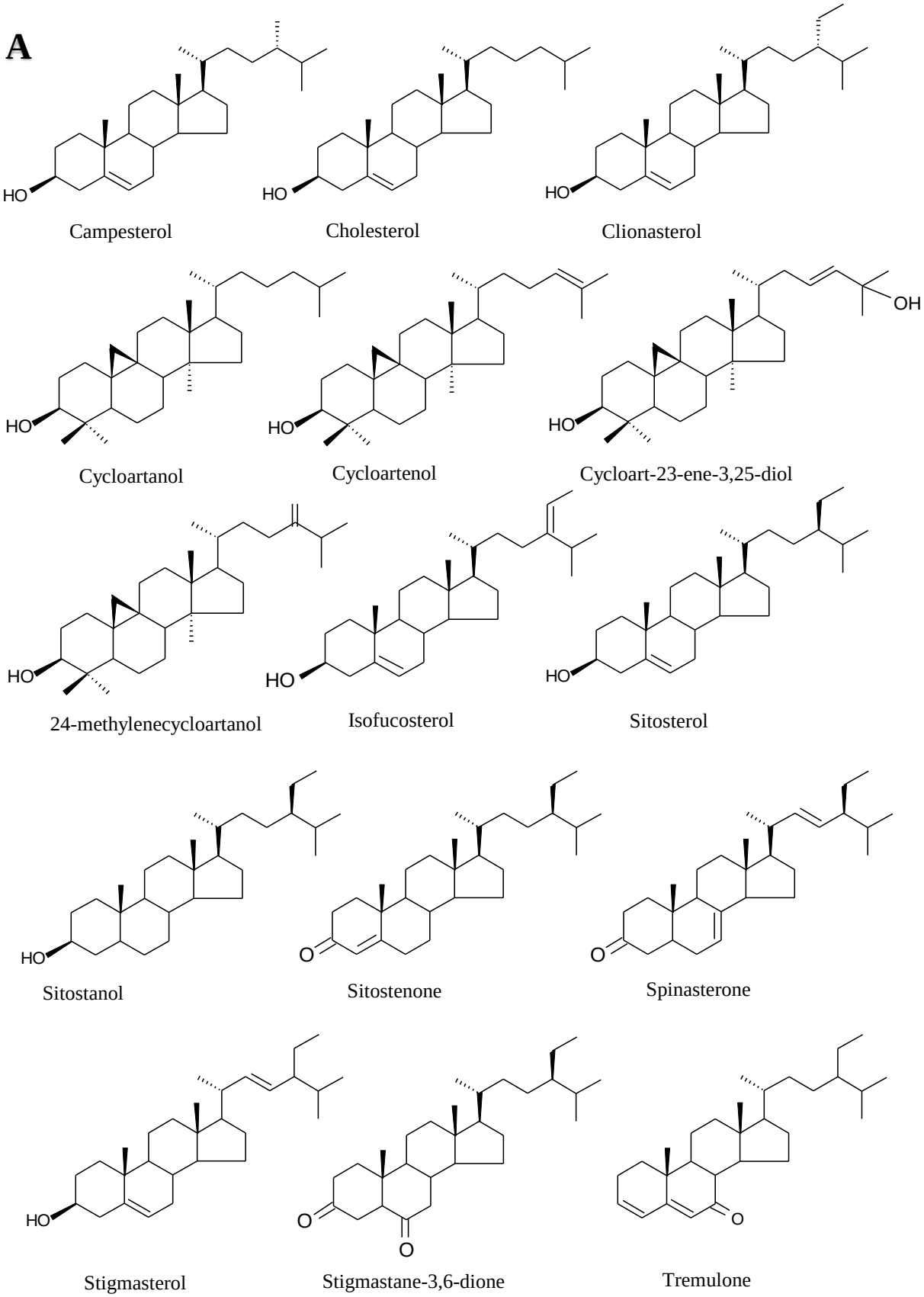


Figure A1. (continued) Chemical structures of triterpenoids identified in grapevine. A, steroids, B, pentacyclic triterpenoids.

B

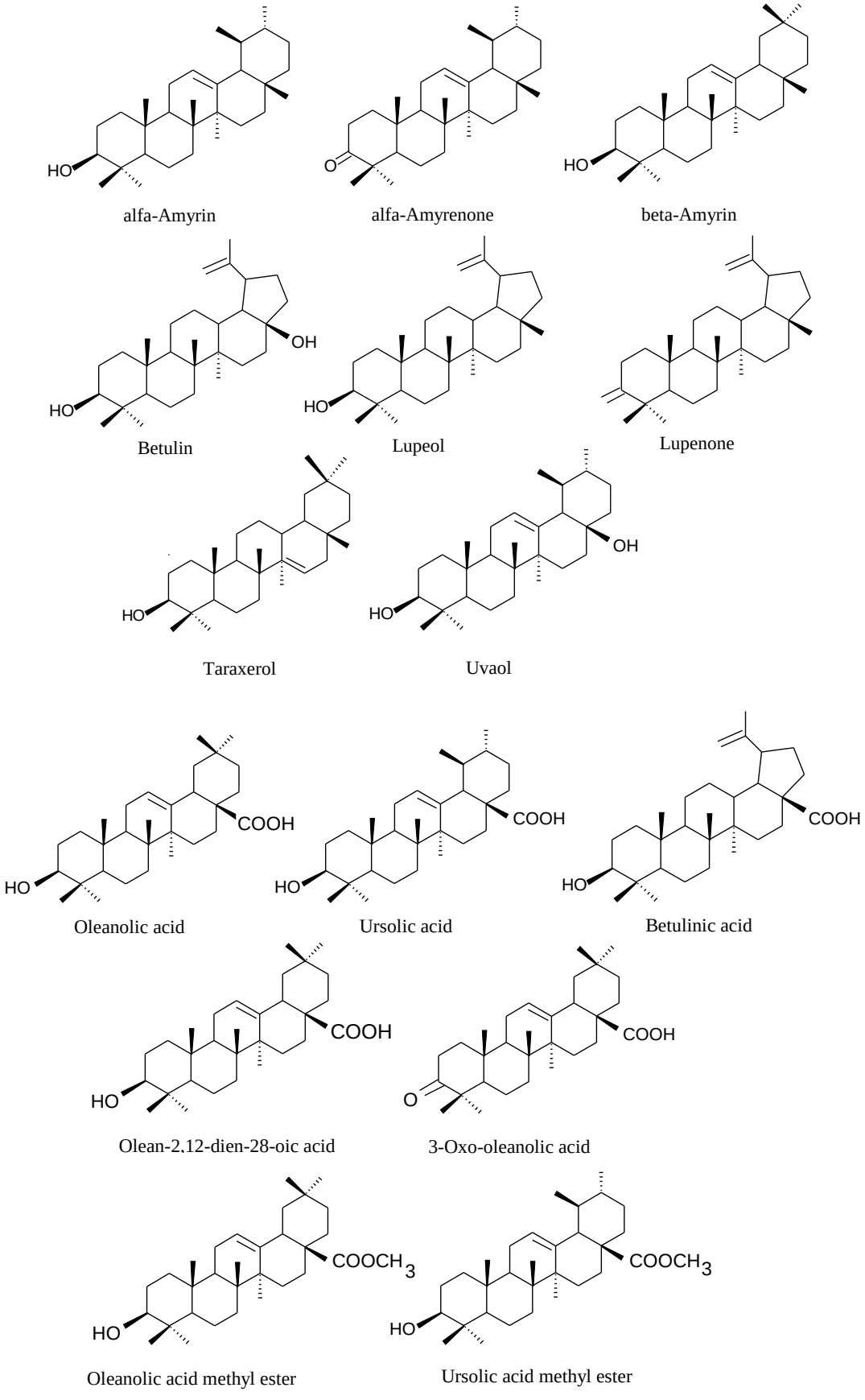
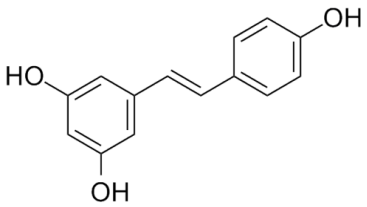
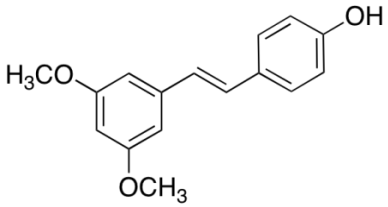


Figure A2. (to be continued) Chemical structures of the polyphenols studied. A, stilbenes; B, flavanols; C, flavonols (https://mib-polyphenol.eu/).

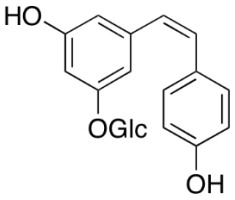
A



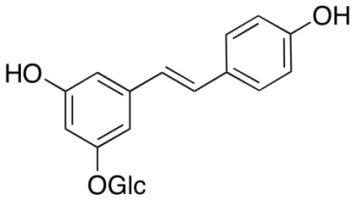
***trans*-resveratrol**



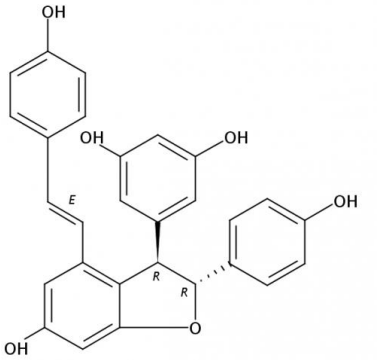
pterostilbene



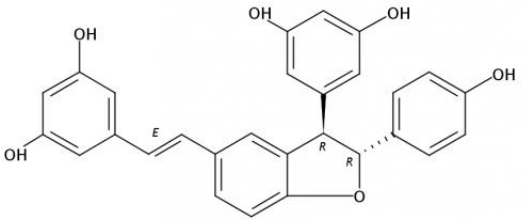
***cis*-piceid**



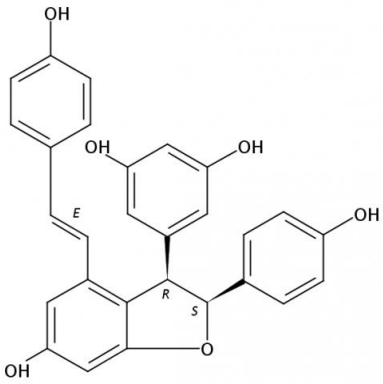
***trans*-piceid**



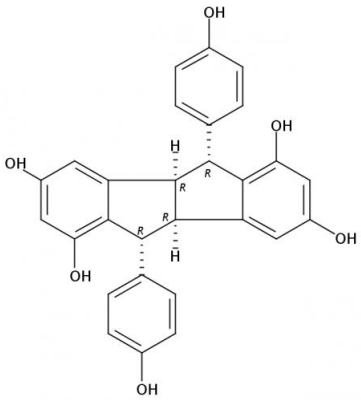
***trans*-ε-viniferin**



***δ*-viniferin**



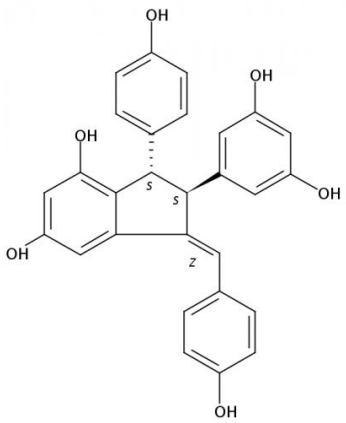
***ω*-viniferin**



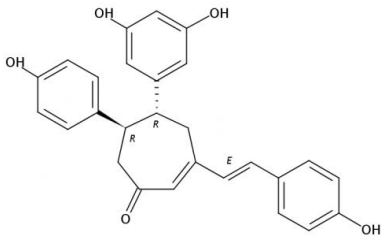
pallidol

Figure A2. (continued) Chemical structures of the polyphenols studied. A, stilbenes; B, flavanols; C, flavonols (https://mib-polyphenol.eu/).

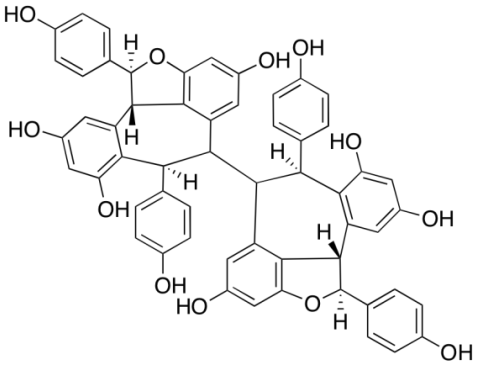
A



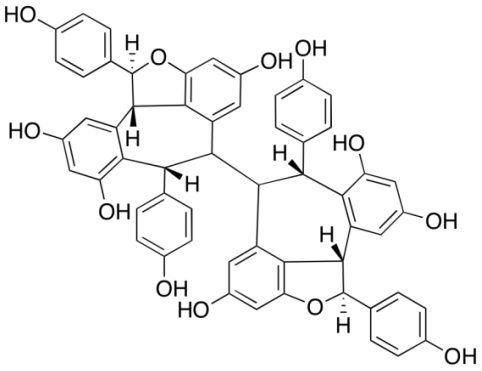
parthenocissin A



vitisinol C

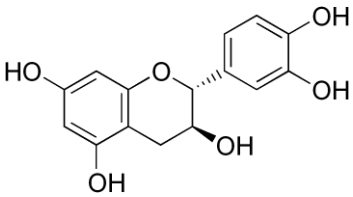


hopeaphenol

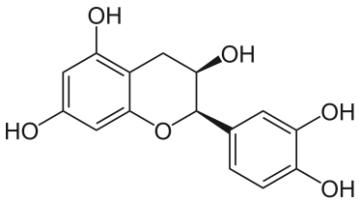


isohopeaphenol

B

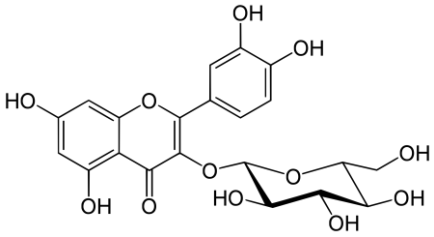


catechin

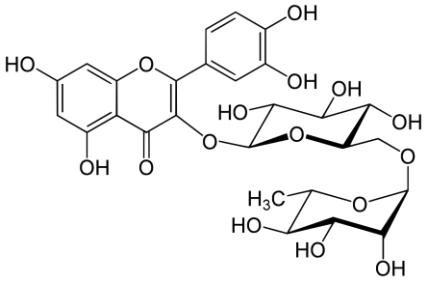


epicatechin

C



quercetin-3-*O*-glucoside



quercetin-3-*O*-rutinoside

LIST OF PUBLICATIONS

Published

Original papers:

Burdziej A. ✉**,** Pączkowski C., Destrac-Irvine A., Richard T., Cluzet S., Szakiel A. (2019). Triterpenoid profiles of the leaves of wild and domesticated grapevines. *Phytochemistry Letters, 30,* 302-308 (IF_{2019}=1.459).

Burdziej A., Da Costa G., Gougeon L., Le Mao I., Bellée A., Corio-Costet M.F., Mérillon J.M., Szakiel A., Richard T., Cluzet S. (2019) Impact of different elicitors on grapevine leaf metabolism monitored by 1H NMR spectroscopy. *Metabolomics, 15*(5), 67 (IF_{2019}=2.881).

Chapters in scientific monographs:

Burdziej A. ✉**,** Szakiel A. (2017) Vegetable raw materials in ethnopharmacology on the example of white sweet clover (*Melilotus alba*) and goat's-rue (*Galega officinalis*). *Postęp w badaniach nad roślinami – najnowsze doniesienia,* Wydawnictwo TYGIEL, ISBN 978-83-65598-64-6, pp. 91-100.

Rogowska A., **Burdziej A.,** Szakiel A. (2020) Competition of biosynthesis of sterols and triterpenoids in plants and *in vitro* cultures under biotic and abiotic stress conditions. *Rośliny w naukach medycznych i przyrodniczych – przegląd i badania,* Wydawnictwo TYGIEL, ISBN 978-83-66489-18-9, pp. 66-79.

Submitted to the journal

Burdziej A., Bellée A., Bodin E., Valls Fonayet J., Magnin N., Szakiel A., Richard T., Cluzet S., Corio-Costet M.F. Three types of elicitors induced grapevine resistance against *Plasmopara viticola*: common and specific immune responses. – submitted to *Journal of Agricultural and Food Chemistry* on September 2020 (Manuscript ID: jf-2020-061033).

SCIENTIFIC COMMUNICATIONS ON CONFERENCES

Oral presentations

Burdziej A., Pączkowski C., Cluzet S., Szakiel A. (2018) Comparison of triterpenoid profile of grapevine cv. Cabernet Sauvignon native plant and in vitro culture. *11th International Symposium on Chromatography of Natural Products,* Lublin, Poland, Book of Abstracts p. 61.

Burdziej A., Da Costa G., Gougeon L., Le Mao I., Bellée A., Corio-Costet M. F., Mérillon J. M., Szakiel A., Richard T., Cluzet S. (2019) Metabolomic alterations in elicitor-treated grapevine *Vitis vinifera* leaves monitored by 1H NMR. *Trends in Natural Product Research – PSE Young Scientists Meeting on Biochemistry, Molecular Aspects and Pharmacology of Bioactive Natural Products,* Budapest, Hungary, Book of Abstracts p. 50.

Burdziej A. (2019) Etude des réponses métaboliques de la vigne suite à différents stress environnementaux. *Journée des Doctorants de l'Institut des Sciences de la Vigne et du Vin (ISVV), 4ème Edition,* Villenave d'Ornon, France.

Poster presentations

Burdziej A., Szakiel A., Cluzet S. (2017) Wpływ elicytacji estrem metylowym kwasu jasmonowego na biosyntezę steroli i triterpenoidów pentacyklicznych w kulturach zawiesin komórkowych *in vitro* winorośli właściwej *Vitis vinifera* L. *IX Interdyscyplinarna Konferencja Naukowa Tygiel 2017 „Interdyscyplinarność kluczem do rozwoju",* Lublin, Book of Abstracts p. 408.

Burdziej A., Szakiel A., Cluzet S. (2017) Kultury *in vitro* zawiesin komórkowych winorośli właściwej *Vitis vinifera* jako model badawczy reakcji stresowych roślin. *III Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen,* Łódź, Poland, Book of Abstracts p. 37.

Burdziej A., Pączkowski C., Szakiel A., Cluzet S. (2017) Biosynteza triterpenoidów pentacyklicznych w elicytowanych kulturach zawiesin komórkowych winorośli właściwej *Vitis vinifera*. *I Konferencja Naukowa „Biotechnologia roślin – perspektywy i wyzwania",* Medical University of Warsaw, Warszawa, Poland, Book of Abstracts p. 24.

Burdziej A., Chojak M., Pączkowski C., Szakiel A., Cluzet S. (2017) Metabolism of triterpenoids during leaf development in grapevine (*Vitis vinifera*). *8th European Symposium on Plant Lipids,* Malmö, Sweden, Book of Abstracts p. 68.

Chojak M., **Burdziej A.,** Pączkowski C., Szakiel A., Cluzet S. (2017) Steroids and pentacyclic triterpenoids occurring in grapevine (*Vitis vinifera*) leaves. *5th International Conference and Workshop "Plant – the source of reseach material",* Lublin, Poland, Book of Abstracts p. 55.

Burdziej A., Pączkowski C., Szakiel A., Cluzet S. (2018) GC-MS analysis of isoprenoids in grapevine cv. Merlot and Gamay leaves after exposition to UV-B radiation. *11th International Symposium on Chromatography of Natural Products,* Lublin, Poland, Book of Abstracts p. 82.

Burdziej A., Chojak M., Pączkowski C., Cluzet S., Szakiel A. (2019) Metabolism of sterols and pentacyclic triterpenoids in grapevine *Vitis vinifera* leaves elicited with methyl jasmonate. *Trends in Natural Product Research – PSE Young Scientists Meeting on Biochemistry, Molecular Aspects and Pharmacology of Bioactive Natural Products,* Budapest, Hungary, Book of Abstracts p. 114.

Cluzet S., **Burdziej A.,** Pączkowski C., Destrac-Irvine A., Richard T., Szakiel A. (2019) Biochemical diversity of *Vitis* genus revealed from triterpenoids profiles of the leaves of wild and domesticated grapevines. *11th International Symposium of Enology of Bordeaux – ŒnoIVAS,* Bordeaux, France, Book of Abstracts p. 202.

Djemaa-Landri K., Vu M., Mandou C., Douillet A., **Burdziej A.,** Da Costa G., Franc C., Cluzet S., Valls Fonayet J. (2019) Phenolic compounds from grapevine leaves: methods of extraction and analysis. *2nd Indian - French Symposium Hyderabad, Plants with health benefits and biomolecules of interest. From plantlets to tablets,* Bordeaux, France, Book of Abstracts p. 18.

SCHOLARSHIPS

French Government Scholarship – International Dual Degree PhD (*Cotutelle*) (2016-2019, three stays of 5 months).

Motivational Scholarship – University's Integrated Development Programme (ZIP), co-financed by the European Social Fund within the framework of Operational Programme Knowledge Education Development (POWER), action 3.5.' (2018/2019 and 2019/2020).

Erasmus+ Scholarship for International Internship – The European Union's programme (research stay at University of Bordeaux, ISVV, UR Oenology, MIB axis, September-December, 2018)

Merit-based Scholarship – University of Warsaw (2019/2020)

GRANTS

The Ministry of Science and Higher Education through the Faculty of Biology, University of Warsaw intramural grant DSM for the projects:

501-D114-86-0115000-06 (2017) „Wpływ elicytacji jasmonianem metylu na metabolizm steroli i triterpenoidów pentacyklicznych w liściach winorośli właściwej (*Vitis vinifera*)" *("Effect of elicitation with methyl jasmonate on the metabolism of sterols and pentacyclic triterpenoids in the leaves of the grapevine (Vitis vinifera)")*.

501-D114-01-1140100 (2019) „Wpływ nalistnej elicytacji jasmonianem metylu i fosforanami potasu na akumulację stilbenów i triterpenoidów oraz ekspresję genów związanych z ich biosyntezą w liściach i korzeniach winorośli właściwej *Vitis vinifera*" ("*Effect of foliar elicitation with methyl jasmonate and potassium phosphates on the accumulation of stilbens and triterpenoids and the expression of genes related to their biosynthesis in the leaves and roots of the Vitis vinifera*")

AWARDS

Polish edition of "Ma thèse en 180 secondes" (My thesis in 180 seconds) competition – participation as the University of Warsaw doctoral candidate, June 2020 (online)

- **2nd Prize** (Prize of the Polish Academy of Sciences – PAN)
- **The Audience Prize** (Prize of the Polish National Agency for Academic Exchange – NAWA)