**Universidade do Minho**
Escola de Ciências

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UMinho | 2021

Financial support provided by:
FCT Fundação para a Ciência e a Tecnologia
REPÚBLICA PORTUGUESA
CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR
Co-financed by:
POCH
PORTUGAL 2020
UNIÃO EUROPEIA
Fundo Social Europeu
NORTE 2020

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# Physiology of photosynthetic grape berry tissues: the effects of canopy light microclimate and climate stress mitigation strategies

Doctoral Thesis
PhD Thesis in Biology

Work supervised by
**Professor Doctor Ana Cristina Gomes da Cunha**
**Doctor Artur Jorge da Silva Conde**
**Doctor Ric Cornelis Hendricus De Vos**

July 2021

# DECLARATION

## COPYRIGHT AND TERMS OF USE OF THE WORK BY THIRD PARTIES

## Acknowledgments

First and foremost, I want to deeply thank Professor Ana Cunha for giving me the opportunity to work together along the last few years. I thank the sharing of scientific and practical knowledge, as well as her support, dedication and effort on this project. It was a great privilege to work with her and I am very truly grateful for her guidance.

I also deeply thank Doctor Artur Conde for his supervision, dedication, concern, availability to help and kindness. I am very thankful for his useful scientific contribution and suggestions during the preparation of this work.

I would like to deeply thank the supervision of Doctor Ric De Vos from Business Unit Bioscience of Wageningen Plant Research Center at Wageningen University and Research (WUR). I thank for giving me the possibility to spend six months at his laboratory, which allow me to acquire new scientific and technical knowledge and many other interpersonal skills. It was a very rewarding experience, both professionally and personally. I deeply thank for his sharp and rigorous scientific analysis of the manuscripts. I am truly honored for his guidance.

The scientific collaboration of Professor João Serôdio, from the Center for Environmental and Marine Studies (CESAM) at the University of Aveiro, was also important for this work. I am thankful for his availability, patient and kindness.

I want to deeply thank the owners of Quinta Cova da Raposa, Manuel Taxa and his wife, for their availability and sympathy. I thank for kindly allowing me to implement and conduct the experimental design in their vineyards and for providing me the grape berry samples used in this work.

I thank the support given by the direction of this Doctoral Programme "Agricultural Production Chains – From Fork to Farm (AgriChains)", as well as given by the secretariat. I also deeply thank all the Professors involved on this PhD, especially Professor Teresa Lino Neto, for her tutoring and curricular guidance, and Professor Hernâni Gerós, for his sympathy and kindness.

I would like to thank the support given by all members of Business Unit Bioscience of Wageningen Plant Research Center (at WUR), in particular all from Plants Metabolomics group: Professor Doctor Robert Hall for his kindness and Roland Mumm, Bert Schipper, Henriette van Eekelen and Jeroen van Arkel for their help in the chemical analysis and data processing. A special thanks to Jasper Engel, for his help in the statistical analysis. I also want to thank the PhD colleagues, Giusi, Cristina and specially Sena for their companionship, help and for making me feel at home during my stay at Wageningen.

I also acknowledge the technical support given by the staff members of Biology Department of the School of Sciences from the University of Minho, namely Cristina Ribeiro, Luís Correia, Manuela Rodrigues, Amaro Rodrigues and Inês Pinheiro.

I thank the support and companionship of all members of Plant Biology laboratory: Henrique Noronha, Viviana Martins, António Teixeira, Richard Breia, Mariana Vale, Angélica Silva, Hélder Badim, António Freitas and Jorge Rodrigues. I also want to thank the Agrichains colleagues, namely Eunice Santos, Luis Giraldo for their sympathy and specially Ana Sofia Freitas for her support and friendship.

Finally, I would like to express my unmeasured gratitude to my parents and my brother for their unmatched support, patient and help.

## Financial support

Andreia Raquel Martins Garrido acknowledges the financial support provided by national funds through FCT - Portuguese Foundation for Science and Technology (PD/BD/128275/2017), under the Doctoral Programme "Agricultural Production Chains – from fork to farm" (PD/00122/2012) and from the European Social Funds and the Regional Operational Programme Norte 2020. This study was also supported by Centre for the Research and Technology of Agro-Environmental and Biological Sciences (UIDB/04033/2020) and by Centre of Molecular and Environmental Biology (UIDB/04050/2020).

The work was also supported by FCT and European Funds (FEDER/POCI/COMPETE2020) through the research project "MitiVineDrought—Combining "omics" with molecular, biochemical, and physiological analyses as an integrated effort to validate novel and easy-to-implement drought mitigation strategies in grapevine while reducing water use" with ref. PTDC/BIA-FBT/30341/2017 and ref. POCI-01-0145-FEDER-030341, respectively; and through the research project "BerryPlastid" with ref. POCI-01-0145-FEDER-028165 and ref. PTDC/BIA-FBT/28165/2017, respectively. The work was also support by project I&D&I "AgriFood XXI", ref. NORTE-01-0145-FEDER-000041, co-financed by the European Regional Development Fund (FEDER), through NORTE 2020 (Northern Regional Operational Program 2014/2020). This work also benefited from the networking activities within the European Union-funded COST Action CA17111"INTEGRAPE—Data Integration to maximize the power of omics for grapevine improvement".

# STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

# Fisiologia dos tecidos fotossintéticos do bago de uva: os efeitos do microclima de luz na copa e das estratégias de mitigação do stress climático

## Resumo

A cultura da videira tem uma elevada relevância económica e cultural. Atualmente, esta espécie enfrenta desafios difíceis pois, num contexto de alterações climáticas, a severidade dos stresses abióticos está a aumentar, causando impactos negativos na fisiologia da videira, em particular na atividade fotossintética e na regulação do estado hídrico. Desta forma, uma gestão adequada da luz/radiação na copa é essencial para garantir uma boa produção de uvas e vinho. Previamente, mostrámos que o exocarpo e os tegumentos das sementes de bagos de uva de uma casta branca (cv. Alvarinho) foram os tecidos mais fotossinteticamente ativos, que essa atividade variou ao longo das fases de desenvolvimento (verde, *véraison* e madura) e em resposta ao microclima de luz (LL - luz baixa; e HL - luz alta). No entanto, a função da fotossíntese da uva ainda é amplamente desconhecida. Neste trabalho pretendemos estudar os efeitos desses dois microclimas de luz e de duas estratégias de mitigação do stresse climático - aplicação foliar de caulino e irrigação - na atividade fotossintética, perfil de metabolitos e nos transcritos de genes-alvo desses tecidos do bago de uva, colhidos nessas três fases de desenvolvimento. Estudos por fluorometria de pulso de amplitude modulada (PAM) mostraram que HL aumentou a eficiência quântica máxima ($F_v/F_m$) e a atividade fotossintética ($rETR_{200}$) de ambos os tecidos da fase verde. Curiosamente, a aplicação foliar de caulino aumentou a atividade fotossintética dos exocarpos LL da fase verde em comparação com o controlo, enquanto que a irrigação diminuiu a atividade fotossintética das sementes HL nas fases *véraison* e madura, especialmente nas videiras de parcelas pulverizadas com caulino. Espectrometria de massa por cromatografia líquida (LCMS) revelou que apenas a "irrigação" e o "microclima de luz" levaram a diferenças significativas no perfil de metabolitos dos tecidos do bago. Análises transcricionais por reação em cadeia da polimerase (qPCR) mostraram que os níveis de transcrição de genes codificadores de elementos associados à fotossíntese, clorofila sintetase (*VvChlSyn*) e ribulose-1,5-bisfosfato carboxilase/oxigenase (*VvRuBisCO*), foram regulados positivamente pelo microclima HL. Paralelamente, o estudo de lipidómica mostrou que as sementes LL tiveram níveis mais altos de ácidos gordos livres, enquanto que HL levou à regulação positiva de ceramidas na fase verde e triglicerídeos e glicerofosfolípidos na fase madura. Globalmente, este trabalho fornece evidências sobre a contribuição da fotossíntese para a fisiologia do exocarpo e da semente do bago de uva, bem como novos conhecimentos para uma gestão adequada das práticas vitícolas.

**Palavras chave:** expressão de genes, fotossíntese, medidas de mitigação de curto-prazo - irrigação e caulino, microclima de luz, metabolismo.

# Physiology of photosynthetic grape berry tissues: the effects of canopy light microclimate and climate stress mitigation strategies

## Abstract

Grapevine is an agriculture crop with high economic and cultural relevance. Currently, this plant species faces a difficult challenge, as in the context of climate changes, the severity of abiotic stresses is increasing, causing negative impacts on grapevine physiology, namely on photosynthetic activity and water status regulation. Therefore, an appropriate management of the light/radiation intercepted by the canopy is essential to ensure a proper grape and wine production. Previously, we showed that grape berry exocarp or skins and seed integuments from a white variety (cv. Alvarinho) were photosynthetically active and that this activity varied along grape berry developmental stages (green, *véraison* and mature) and was responsive to the light microclimate that clusters experienced in the canopy (LL - low light; and HL - high light). However, the function of grape berry photosynthesis is still largely unknown. In this work we intended to study the effects of these two contrasting light microclimates under two short-term climate stress mitigation strategies - foliar kaolin application and irrigation - on the photosynthetic activity, metabolite profile and transcripts of target genes of the same two grape berry tissues, sampled at the same three developmental stages. Pulse amplitude modulation (PAM) fluorometry showed that HL increased the maximum quantum efficiency ($F_v/F_m$) and photosynthetic activity ($rETR_{200}$) of both tissues at the green stage. Interestingly, kaolin applied to leaves increased the photosynthetic activity of LL exocarps at green stage as compared with control, while the irrigation decreased the photosynthetic activity of HL seeds at *véraison* and mature stages, especially in those grapevine parcels sprayed with kaolin. Untargeted liquid chromatography mass spectrometry revealed that only "irrigation" and "light microclimate" led to significant differences in the metabolite composition of the berry tissues. Transcriptional analysis by real-time quantitative polymerase chain reaction showed that the transcript levels of genes encoding photosynthesis-related elements, chlorophyll synthase (*VvChlSyn*) and ribulose-1,5-bisphosphate carboxylase/oxygenase (*VvRuBisCO*), were up-regulated by HL microclimate. In parallel, lipidomics analysis showed that LL seeds had higher relative levels of free fatty acids, while HL led to up-regulation of ceramides at green stage and triacylglycerols and glycerophospholipids at mature stage. Overall, this work provides insights for the contribution of tissue-specific photosynthesis to grape berry's skin and seed physiology and metabolome, as well as new knowledge for a good management of viticultural practices.

**Keywords:** gene expression, irrigation and kaolin short-term measures, light microclimate, metabolism, photosynthesis.

# Index

## List of abbreviations and acronyms

| | |
|---|---|
| **2,4-D** | 2,4-dichlorophenoxyacetic acid |
| **3-PGA** | 3-phosphoglycerate |
| **AA** | Ascorbic acid |
| **ABA** | Abscisic acid |
| **ACCase** | Acetyl-CoA carboxylase |
| **ADH** | Alcohol dehydrogenase |
| **AL** | Actinic light |
| **ANOVA** | Analysis of Variance |
| **ANR** | Anthocyanidin reductase |
| **ASCA** | ANOVA simultaneous component analysis |
| **ATP** | Adenosine triphosphate |
| **B5** | Gamborg's B5 medium |
| **BAP** | 6-benzilaminopurina |
| **BHT** | Butylated hydroxytoluene |
| **C4H** | Cinnamate-4-hydroxylase |
| **CA** | Citric acid |
| **CAM** | Crassulacean acid metabolism |
| **cc-se** | Companion cells-sieve elements complex |
| **CHS** | Chalcone synthase |
| **cv** | Cultivar |
| **cwINV** | Cell wall invertases |
| **DFR** | Dihydroflavonol reductase |
| **ETC** | Electron transport chain |
| **$F_o$** | Minimal fluorescence from dark-adapted material |
| **FA** | Fatty acyls |
| **FAA** | Free fatty acids |
| **FAD** | Fatty acid desaturase |
| **FC** | Fold change |
| **FLS** | Flavonol synthase |
| **$F_m$** | Maximal fluorescence from dark-adapted material |
| **$F'_m$** | Maximal fluorescence from light-adapted material |

| | |
|---|---|
| **$F_s$** | Fluorescence emission from light-adapted material (steady state) |
| **$F_v/F_m$** | Maximum quantum efficiency of photosystem II |
| **G-3-P** | Glyceraldehyde-3-phosphate |
| **GCMS** | Gas Chromatography Mass Spectrometry |
| **GL** | Glycerolipids |
| **GP** | Glycerophospholipids |
| **HbA** | Hydroxybenzoic acids |
| **HEX** | Hexoses |
| **HL** | High light microclimate |
| **HPL** | Hydroperoxide lyases |
| **HPLC** | High performance liquid chromatography |
| **I** | Irrigation |
| **INVs** | Invertases |
| **IS** | Internal standard |
| **K** | kaolin |
| **KIN** | Kinetin |
| **LAR** | Leucoanthocyanidin reductase |
| **LCMS** | Liquid Chromatography Mass Spectrometry |
| **LDOX** | Leucoanthocyanidin dioxygenase |
| **LHC** | Light-harvesting complexes |
| **LL** | Low light microclimate |
| **LOX** | Lipoxygenase |
| **MS** | Murashige and Skoog medium |
| **NAA** | α-naphthaleneacetic acid |
| **NADPH** | Nicotinamide adenine dinucleotide phosphate |
| **NI** | Non-irrigation |
| **NK** | Non-kaolin |
| **NPQ** | Non-photochemical quenching |
| **OEC** | Oxygen evolving complex |
| **OIV** | International Organization of Vine and Wine |
| **PAL** | Phenylalanine ammonia lyase |
| **PAM** | Pulse amplitude modulated |

| | |
|---|---|
| **PAR** | Photosynthetically active |
| **PCA** | Principal component analysis |
| **PEP** | Phosphoenolpyruvate |
| **PEPC** | Phosphoenolpyruvate carboxylase |
| **PFD** | Photon flux density |
| **PFR** | Photosynthetic photon fluence rates |
| **PK** | Polyketides |
| **PSI** | Photosystem I |
| **PSII** | Photosystem II |
| **PUFA** | Polyunsaturated fatty acids |
| **PVP** | Polyvinylpyrrolidinone |
| **qPCR** | quantitative Polymerase Chain Reaction |
| **RC** | Reaction centers |
| **rETR** | Relative electron transport rate through PSII |
| **RuBisCO** | Ribulose-1,5-bisphosphate carboxylase/oxygenase |
| **RuBP** | Ribulose 1,5-bisphosphate |
| **SL** | Saccharolipids |
| **SP** | Saturation pulse |
| **SP** | Sphingolipids |
| **SPS** | Sucrose-phosphate synthase |
| **STS** | Stilbene synthase |
| **SUC** | Sucrose/$H^+$ symporters |
| **SuSy** | Sucrose synthase |
| **TAG** | Triacylglycerides or triacylglycerols |
| **TBA** | 2-thiobarbituric acid |
| **TBARS** | Thiobarbituric acid-reactive-substances |
| **TCA** | Tricarboxylic acid |
| **UDP-G** | Uridine diphosphate glucose |
| **WAA** | Weeks after anthesis |
| **ΦII** | Effective quantum yield of PSII |

# List of publications and communications

## Papers in the international peer-review journals

### Published:

Garrido, A., Engel, J., Mumm, R., Conde, A., Cunha, A.* & De Vos, R. C. H.* (2021). Metabolomics of Photosynthetically Active Tissues in White Grapes: Effects of Light Microclimate and Stress Mitigation Strategies. *Metabolites*, *11*(4), 205. https://doi.org/10.3390/metabo11040205 *Equal senior authorship.

Garrido, A., Serôdio, J., De Vos, R., Conde, A., & Cunha, A. (2019). Influence of foliar kaolin application and irrigation on photosynthetic activity of grape berries. *Agronomy*, *9*(11), 685. https://doi.org/10.3390/agronomy9110685

### Submitted:

Garrido, A., De Vos, R. C. H., Conde, A.* & Cunha, A.* (submitted). Light microclimate-driven changes at transcriptional level in photosynthetic grape berry tissues. *Plants*, *10*, x, https://doi.org/10.3390/xxxxx. (26th June 2021). *Equal senior authorship.

### Under final revision by the co-authors:

Garrido, A., Conde A., De Vos, R. C. H & Cunha, A. Fruit Photosynthesis: where, how and why? *(Provisional tittle)*.

Garrido, A., Conde A., De Vos, R. C. H & Cunha, A. The influence of light microclimate on the lipid profile and associated transcripts of photosynthetically active grape berry seeds. *(Provisional tittle)*.

Garrido, A., De Vos, R. C. H., Conde, A. and Cunha, A. Establishment of *callus* cultures from white grape berry tissues. *(Provisional tittle)*.

## Communications in international and national congresses

### Oral presentations:

Garrido, A., Conde, A., De Vos, R. C. H. & Cunha, A. (2021) Grapevine canopy light microclimate effects on the metabolism and gene expression of two photosynthetically active grape berry tissues. In II Plant Abiotic Stress Forum - An integrative lens over plant adaptation, 29th of May, Organized by iB2 Laboratory and PlantStress Laboratory PhD Students, University of Porto (online version), Portugal.

Garrido, A., Conde, A., Cunha, A. & De Vos, R. (2019). Phytochemicals and Photosynthesis of Grape Berry Exocarp and Seed (*Vitis vinifera*, cv. Alvarinho): Effects of Foliar Kaolin and Irrigation. In 21st International Conference on Grapevine Physiology and Biotechnology, 16th – 17th of December, Barcelona, Spain. (Note: This presentation allowed to receive the "Best Presentation Award").

Garrido, A., Serôdio, J., De Vos R., Conde, A. & Cunha, A. (2019). Photosynthesis of grape berry tissues under kaolin film application at canopy. In XXIII Meeting of the Spanish Society of Plant Physiology/ XVI Hispano-Portuguese Congress of Plant Physiology, 26th – 28th of June, Pamplona, Spain.

Garrido, A., Serôdio, J. & Cunha, A. (2018). Photosynthetic phenotype adaptation of grape berry (*Vitis vinifera*) to canopy light microclimate and acclimation to short-term light challenges: how to cope with Mediterranean summer stress? In National Congress on Climate Change, 19th – 21st of February, UTAD, Vila Real, Portugal. pp. 43. ISBN 978-989-704-259-1.

Garrido, A., Serôdio, J. & Cunha, A. (2017). Impact of the light microenvironment on photosynthetic activity of grape berry (*Vitis vinifera*): insights for light absorption mitigations measures. In 2nd World Symposium on Climate Change Adaptation, 6th – 8th of September, Coimbra, Portugal.

Garrido, A., Pimentel, D., Serôdio, J. & Cunha, A. (2017). Photosynthesis and phenylalanine ammonia lyase activity in grape berry (*Vitis vinifera*). In XV Spanish-Portuguese Congress of Plant Physiology, 26th – 29th of June, Barcelona, Spain.

### Poster presentation:

Garrido, A., Conde, A., De Vos, R. C. H. & Cunha, A. (2021). Metabolome and transcriptional changes in ripening grapes in relation to berry tissue-specific photosynthesis. In Plant Biology Europe 2021 Congress, 28th of June – 1st of July (online version). Torino. Italy.

# Chapter 1

## General Introduction

## 1.1. *Vitis vinifera*: a plant species with high social, cultural and economic relevance

Grapevine (*Vitis vinifera* L.) is a perennial woody species belonging to the Vitaceae family. Currently, more than 70 species grow in different geographical areas, being *Vitis vinifera* the most renowned one, with domestication of *Vitis vinifera sylvestris* beginning in Transcaucasia (today's Georgia, Armenia and Azerbaijan) over 8000 years ago, and subsequently spread to other countries (Estreicher, 2017).

According to the latest report of the International Organization of Vine and Wine (OIV) (OIV, 2019), world vineyards covered an area of approximately 7.449 million hectares (ha) in 2018, and 5 countries represented 50 % of this area, including, Spain (13 %) China (12 %), France (11 %), Italy (9 %), and Turkey (6 %). The same report indicated a record-breaking value of the grape production of 77.8 million tons, considering its all uses, namely, its production in the form of wine grapes (57 % of all grapes), table grapes (36 %), or dried grapes (7 %). Regarding to the production of wine grapes (in relation to the total), countries such as Germany (99.6 %), France (99.6%), Spain (96 %), Argentina (93.7 %), Romania (93.1 %), Australia (90.9 %), Italy (86.5 %) are the highest producers. Concerning the winemaking sector, the global wine production remained stable over the last two decades, with 292 million hectoliters (hl) in 2018, being the most important wine producers, Italy (54.8 million hl), France (48.6), Spain (44.4) and the USA (23.9). According to the same OIV 2018 report, Portugal was ranked as the 11$^{th}$ world and 5$^{th}$ European wine producer. The vineyard area in Portugal, in 2018, was 192,000 ha, which contributed for 6.1 million hl of wine production. The world trade, that is, the sum of exports from all countries, and considering the monetary value, was approximately EUR 30 billion in 2018.

Currently, grapevine is considered one of the most important agricultural crops cultivated in the world with high socioeconomic relevance due to the great diversity of modes of its fruit consumption and use: table grapes, raisins, juices, jellies, wine vinegar, food additives and most importantly, wine (Conde et al., 2007). Furthermore, other grape by-products (e.g., pomace, stems, leaves) have been used for pharmaceutical, nutraceutical and cosmetic purposes, due to their cardioprotective, antioxidant and anti-inflammatory properties, which are mainly associated with the high phenolic content (Teixeira et al., 2014). For instance, grape seed extracts have become popular in recent years, being used as nutritional supplement (Waterhouse et al., 2000). Nowadays, wine is considered an integral component of the culture of many countries and, when moderately consumed, has health benefits.

In addition to water, wine is composed by several compounds, as sugars, alcohol, phenolics, organic acids and mineral salts. The grapevine variety, the edaphoclimatic conditions and the enological practices are examples of factors that affect the wine chemical composition and its complexity. The main constituent of the wine is water, accounting for 75 to 90 % (v/v), followed by ethyl alcohol, which,

according to the type of wine, varies from 8 % to 13 % (v/v) and can achieves 15 % (v/v) due to climate changes (van Leeuwen et al., 2019). The content in sugar is important for the growth of fermentative yeasts, being directly responsible for the final alcoholic content of the wine. In addition, the higher content of phenolic compounds present in the wine, confers antioxidant features, which are beneficial for the human health, for instance, in preventing cardiovascular diseases (Cordova et al., 2005) and diabetes (Caimi et al., 2003).

## 1.2. Grape berry: histology, development and composition

Grape berry, a non-climacteric fruit, is comprised of different tissues and layers of cells (e.g., skin or exocarp, flesh or mesocarp and seeds) (Figure 1.1), with different anatomical characteristics and biochemical profiles, which can play distinct roles during the development and ripening of the fruit (Hardie et al., 1996). The growth and development of the grape berry presents a double sigmoid pattern (Coombe, 1992), that is, two successive growth phases separated by a phase where no increase in volume occurs (Figure 1.2).

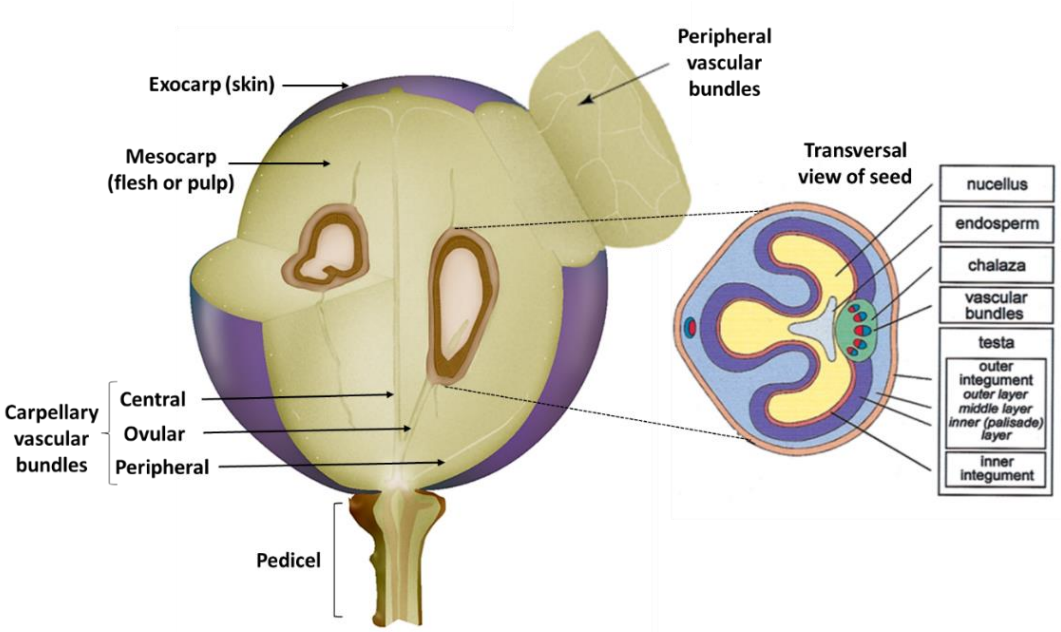


**Figure 1.1.** Anatomy and histology of a mature grape berry and close-up of a seed viewed in a transverse section. Adapted from Famiani et al., (2000), Kennedy (2002a). Illustration by Jordan Koutroumanidis.

The first stage (stage I), from flowering to approximately 60 days after, is characterized by rapid cell division and cell expansion, and in which the berry is formed and the seed embryos are produced (Kennedy, 2002a). Several organic compounds are accumulated in the berry during this first growth period, such as tartaric and malic acids. Tartaric acid accumulates at the beginning of this phase and its concentration is highest at the skin of the developing berry. By contrast, malic acid is accumulated in the

flesh at the end. Hydroxycinnamic acids (HcA) and tannins are also accumulated during this first growth stage. Hydroxycinnamic acids are accumulated in flesh and skin, while tannins accumulate in skin and seed tissues (Kennedy et al., 2000a; 2000b; 2001).

In addition, at this early stage of development, some grape berry tissues, namely the exocarp and seed outer integument, present photosynthetic activity, as we demonstrated before (Breia et al., 2013; Garrido et al., 2018), and as it will be exposed in more detail further ahead (in Chapter 2).

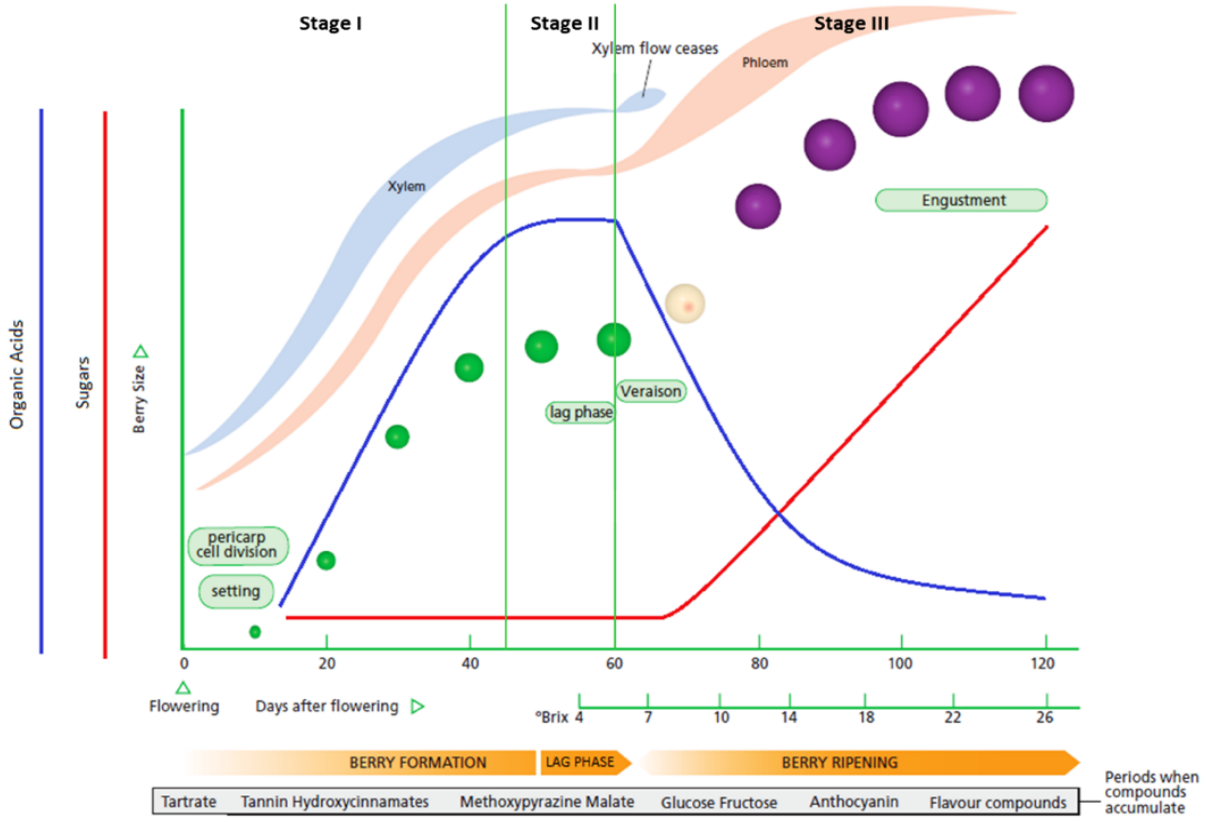


**Figure 1.2.** Structural, metabolic and physiological changes during the development and ripening of grape berry. Grape berry development occurs in three stages: stage I (green stage), stage II (lag phase) and stage III (ripening), which starts at *véraison*. Adapted from Kennedy, 2002a. Illustration by Jordan Koutroumanidis.

The second stage (stage II) is known as lag phase and it is characterized by an arrest in growth and grape berries begin to lose chlorophyll. After this lag phase, the beginning of second growth phase (stage III), which is known as *véraison*, coincides with the onset of ripening, and in red berries is when a change in the skin color is observed (in Portuguese this transition phase is called "pintor" (meaning painter) due to this changing in color). Besides the increase in volume, at this stage III berries become less acidic and sweeter due to the beginning of sugar accumulation. Sugars are transported to the grapes since the first stage, through the capillary vascular bundles, peripheral and central (Figure 1.1). A dorsal bundle network extends at the periphery of the fruit, and central vascular bundles connected to the seeds

irrigate the central flesh (Zhang et al., 2006). Some compounds produced and accumulated during the stage I subsist only in lower concentrations in the stage III. These compounds include malic acid, which is metabolized and used as a source of carbon and energy (Conde et al., 2007). The total tannin content also decreases during this stage in seed coat and in the exocarp (Kennedy et al., 2002a; 2000b). In the seed coat this decline accompanies the color changes that occur in seeds during ripening, suggesting that this seed browning represents oxidation of tannins during ripening (Kennedy et al., 2000a; 2000b). Despite of this decrease, in the stage III other metabolites determinants for the quality of the wine increase including, anthocyanins (in red grape varieties) and flavor compounds, which are important to the pleasant aroma of many varieties (Kennedy, 2002a).

### 1.3. Photosynthesis, photoassimilate distribution and plant growth

Photosynthesis is a physiological process performed by plants, algae and photosynthetic bacteria, crucial for all life on Earth. These photoautotrophic organisms are able to capture the energy of sunlight and use it to transform inorganic compounds (water and carbon dioxide) into highly energetic organic compounds (sugars). In eukaryotes, this process is divided into two distinct phases, spatially and functionally distinguished within the chloroplasts: the light-dependent photochemical reactions located in the thylakoid membranes and the carbon-reducing phase or Calvin-Benson cycle, which occurs in the stroma.

In the photochemical phase, bound pigment-protein complexes, the photosystems II and I work in series to intercept and convert sunlight energy into chemical energy stored in adenosine triphosphate (ATP) and reducing power in nicotinamide adenine dinucleotide phosphate (NADPH) (Keller, 2015) (Figure 1.3). The process begins with the energy of the photons being absorbed by the photosynthetic pigments (chlorophyll *a* and *b* and carotenoids) present in light-harvesting complexes (LHC) of photosystems II (PSII) and photosystem I (PSI), and channeled, by inductive resonance, to the respective reaction centers (RC). Here, the excitation energy (or exciton) is absorbed by the chlorophyll *a* molecules (*chl.a*$^*$), and the excited electron will eventually leave the molecule being transferred to an electron acceptor. In PSII, the strong oxidant produced when the excited chlorophyll *a* from the RC loses the electron (*chl.a*$^+$), promotes the separation of the water molecule into molecular oxygen, protons and electrons in the oxygen evolving complex (OEC). The high-energy electrons leaving both PS are transferred to other molecules creating a chain of redox reactions known as electron transport chain (ETC), leading to the formation of NADPH (Nelson and Ben-Shem, 2004). The transthylakoidal proton gradient ($\Delta$pH)

generated by the operation of the ETC in favor of a redox potential and the oxidation of water in the lumen, allows the formation of ATP in the stroma by the ATP synthase (Figure 1.3).

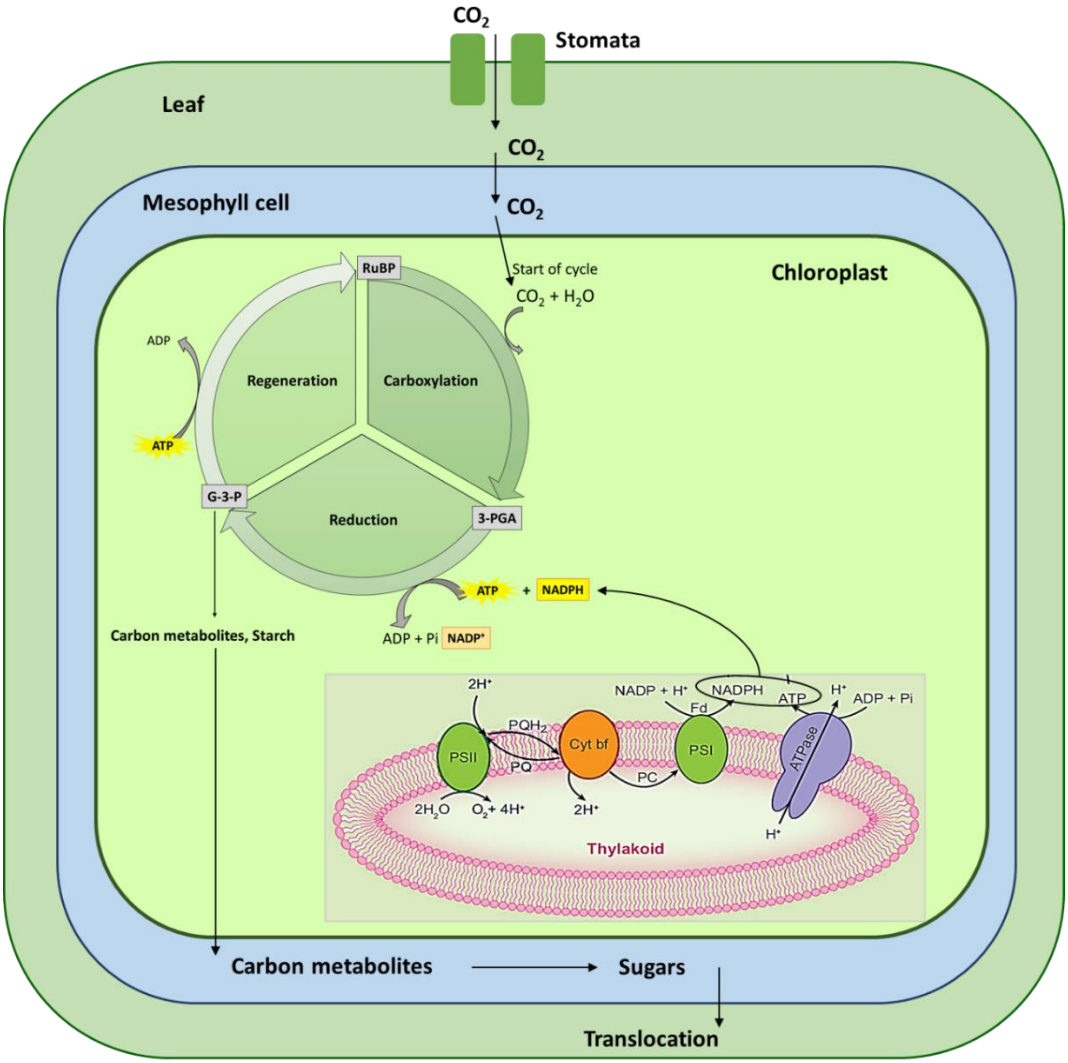


**Figure 1.3.** The main pathways of plant photosynthesis. Electron transport, driven by the excitation of photosystem I (PSI) and photosystem II (PSII), results in the reduction of $NADP^+$ to NADPH and the accumulation of protons in the thylakoid lumen, which are used to make ATP. The Calvin-Benson cycle proceeds in three stages: carboxylation, reduction and regeneration. Abbreviations: 3-PGA, 3-phosphoglycerate; Cyt bf, cytochrome b6f complex; Fd, ferredoxin; G-3-P, glyceraldehyde-3-phosphate; PC, plastocyanin; PQ, plastoquinone; PQH2, plastoquinol; RuBP, ribulose 1,5-Bisphosphate. Adapted from Baker (2008).

In the reactions of the Calvin-Benson cycle, the ATP and NADPH formed in the photochemical phase are used to assimilate $CO_2$, diffusing from the atmosphere through the stomata to the chloroplasts, into sugars. The Calvin-Benson cycle proceeds in three main phases: carboxylation, reduction and regeneration (Figure 1.3). In the first phase, the enzyme ribulose bisphosphate carboxylase/oxygenase (RuBisCO) combines the $CO_2$ with ribulose 1,5-Bisphosphate (RuBP) to produce two molecules of 3-phosphoglycerate (3-PGA). In the reduction phase, 3-PGA is transformed into glyceraldehyde-3-phosphate (G-3-P) using ATP and NADPH. Most of the G-3-P molecules (5/6) are used to regenerate RuBP allowing

to start the cycle again. The remaining G-3-P molecules can be used in the chloroplast or exported from the chloroplast, for instance to produce translocable sugars, like sucrose, and distributed among a significant number of pathways providing carbon compounds essential for plant growth and development (Smith and Stitt, 2007).

In plants, in addition to the leaves, other green-structures (e.g., fruits, stems, flower organs and roots) may present photosynthetic activity at some point of development, as reviewed by Aschan and Pfanz (2003) and Brazel and Ó'Maoileídigh (2019). Thus, they also might contribute partially for the carbon and energy budget, necessary for its own metabolism, growth and development (Cipollini and Levey, 1991; Cocaliadis et al., 2014; Ollat and Gaudillere, 2000).

## 1.4. Major compounds from primary metabolism in grape berries

Primary metabolism plays an essential role in grape berry development. During development, fruits act as strong sinks importing massive amounts of photoassimilates from the main photosynthesizing organs (Nath et al., 2014). These photoassimilates are translocated via phloem and used for growth and also as precursors of secondary metabolites (Nath et al., 2014). The products from primary metabolism are not only crucial for grape development and survival, but also endow grape berry specific characteristics decisive for its market value. Sugars, organic acids and the grape seed oils are among the most relevant primary metabolites in grape berries, as discussed below.

### 1.4.1. Sugars

Fruit sweetness is an essential characteristic of fruit quality and is determined by the total sugar content. In fact, for winemaking, the accumulation of sugars in mature grapes (65 to 91 % of mature grape berry dry weight is glucose and fructose) is important for the production of ethanol (Conde et al., 2007).

Sucrose is the main sugar transported at the plant level, being produced in leaf mesophyll cells, with a vital role of the sucrose-phosphate synthase (SPS). Sucrose is then transported to the companion cells-sieve elements (cc-se) complex of the phloem either by a symplastic (passive transport via plasmodesmata) or apoplastic pathway (reviewed by Boss and Davies, 2001) (Figure 1.4). The unloading of sucrose from cc-se to the fruit, can occur also by these two different pathways (Figure 1.4). It was demonstrated that during stages I and II of grape berry development, the phloem unloading was predominantly symplastic, but at the onset of ripening (with the beginning of stage III) the apoplastic pathway dominated (Zhang et al., 2006). This apoplastic mechanism is explained by the high sugar

concentrations found in the berry apoplast and by the availability of a sufficient plasma membrane surface area at the phloem/ storage parenchyma interface (Patrick, 1997).

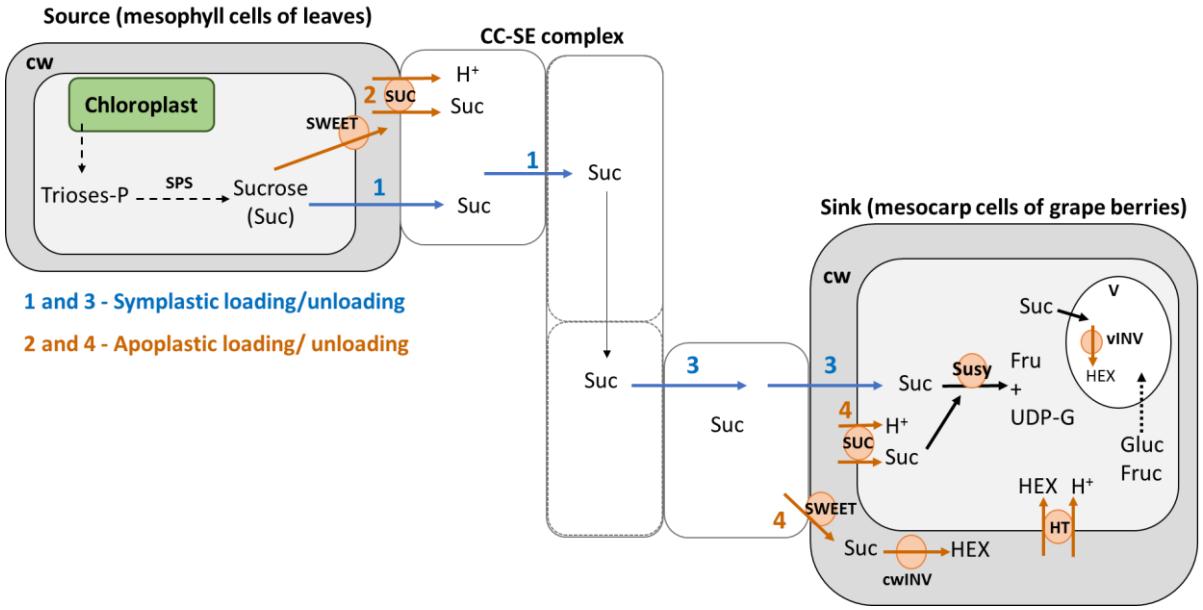


**Figure 1.4.** Model for sugar transport and accumulation in grapevine. Sucrose produced in autotrophic tissues is loaded into companion cell-sieve elements complex by symplastic (blue arrows) or apoplastic (orange arrows) pathways. At mesocarp cells in grape berry, the phloem unloading might occur through a symplastic route, via plasmodesmata, or through the apoplast. In the apoplastic pathway, sucrose is transported to the apoplast by sugar transporters, including SWEET transporters. At the apoplast sucrose might be cleaved into glucose and fructose by cwINV or transported to the cytoplasm by SUCs. The HEX might be imported to the cytoplasm by HTs. Sucrose imported to the cytoplasm might be cleaved by SuSys or transported to the vacuole and cleaved by vINVs. Abbreviations: cc-se, companion cells-sieve elements complex cw, cell wall; cwINV, cell wall invertase; Fru, fructose; Gluc, glucose; HEX, hexoses; HT, hexose transporters; SPS, sucrose-phosphate synthase; Suc, sucrose; SUC, sucrose/$H^+$ symporters; SuSy, sucrose synthase; SWEET, sugars will eventually be exported transporters; UDP-G, uridine diphosphate glucose; v, vacuole; vINV - vacuolar invertase. Adapted from Conde et al. (2007) and Stein and Granot (2019).

Both unloading mechanisms require the activity of invertases (INVs), which are localized in the cell wall (cwINV), vacuole and cytosol of mesocarp cells, and are responsible for the cleavage of sucrose into glucose and fructose. The expression and activity of cell wall invertases increase after *véraison* reaching a high level at the late stage (Zhang et al., 2006) and the expression of the two vacuolar invertases are high at the earlier stages of berry development and declines after *véraison* (Davies and Robinson, 1996; Deluc et al., 2007; Zhang et al., 2006).

After its unloading to the apoplast, sucrose enters the grape berry cells either by a disaccharide transporter (DST or SUC - sucrose/H+ symporter) or in the form of hexoses via monosaccharide transporters (MSTs, also known as hexose transporters - HTs), after invertases action (Figure 1.4). An example is VvHT1 transporter, a high affinity $H^+$-dependent symporter (Figure 1.4), with broad specificity

for various monosaccharides that is more expressed before *véraison*, that is, before hexoses accumulation in the mesocarp (Fillion et al., 1999; Vignault et al., 2005). In addition, Conde et al. (2006) demonstrated that VvHT1 is able to transport fructose but shows higher affinity for glucose. Other VvHTs have different affinities and are expressed at different berry developmental stages (Conde et al., 2006).

Sucrose imported to the cytoplasm of grape berries might be cleaved by SuSy (sucrose synthase), a cytosolic glycosyl transferase that is responsible for both synthesis and catabolism of sucrose, and thus in presence of uridine diphosphate, catalyzes the reversible conversion of sucrose into UDP-glucose and fructose (Zhu et al., 2017).

The presence of sugars (fructose and glucose) in mesocarp cells of the grape berry, from the onset of ripening onwards can lead to the initiation of other pathways of primary and secondary metabolism (Durán-Soria et al., 2020; Pott et al., 2019). In fact, hexoses can be directed to starch synthesis in the chloroplast or to glycolysis and subsequently the tricarboxylic acid (TCA) cycle, as well as the amino acid metabolism (Dai et al., 2013; Wang et al., 2017). In addition, some studies have shown a relationship between sugar and anthocyanin content (Dai et al., 2014; Larronde et al., 1998; Wang et al., 2017), which suggests that sugar is also important for the synthesis of secondary metabolites.

### 1.4.2. Organic acids

In grape berries, tartaric and malic acids are the most abundant organic acids (typically account for 90 % of total acids), but citric, succinic, lactic and acetic acid are also present (Conde et al., 2007). Besides influencing organoleptic characteristics, organic acids are important for the control of the pH of wine. In fact, the wine pH depends on two major factors, namely the ratio of malic acid to tartaric acid and the total amount of acids present (Conde et al., 2007).

Tartaric acid is accumulated in berry cell vacuoles from post anthesis until *véraison* and decrease during ripening, mostly by dilution due to water intake. Unlike to other organic acids that have their synthesis linked to the oxidative metabolism of sugars, tartaric acid is produced from L-ascorbic acid (vitamin C) in a five-step pathway (de Bolt et al., 2006). Although the intermediates of the metabolic pathway are known, the information about the enzymes involved in these reactions is still scarce. However, de Bolt et al. (2006) identified the gene and characterized the coded L-idonate dehydrogenase (L-IdnDH) enzyme, which catalyze the conversion of L-idonate to 5-keto D-gluconic acid.

Malic acid (or malate, in its deprotonated form) is accumulated in the berry during the first stage of development, reach its maximum value just prior to *véraison* and then decreases sharply (Sweetman et al., 2009). At the onset of ripening, there is a dramatic change from malate accumulation to its

breakdown. The pathways of malate synthesis and breakdown were characterized in several fruits, including in grape berry (Sweetman et al., 2009). The accumulation of malic acid in the vacuole in pre-*véraison* grapes is due to the metabolism of sugars that were translocated from the source leaves to the fruit (as reviewed by Sweetman et al. 2009). During the first stage of development, the sucrose transported from the leaves is broken down to glucose and fructose, which can enter into glycolysis, producing the phosphoenolpyruvate (PEP). The β-carboxylation of PEP in the cytosol catalyzed irreversibly by phosphoenolpyruvate carboxylase (PEPC, first carboxylative enzyme in the C4-type photosynthesis) leads to production of oxaloacetate. During the ripening process two putative PEPC isogenes decreased in expression (Deluc et al., 2007). The oxaloacetate is then reduced to malate by cytosolic $NAD^+$-dependent malate dehydrogenase (cytosolic MDH). After *véraison*, the cytosolic NADP-malic enzyme catalyzes the oxidative decarboxylation of malate to $CO_2$ and pyruvate, which is important to fulfill the carbon and energy needs of the berry, as the synthesis of sugars and secondary compounds (Sweetman et al., 2009). After *véraison*, malate liberated from the vacuole is catabolized in several pathways, including the tricarboxylic acid (TCA) cycle, respiration, gluconeogenesis and those associated with biosynthesis of secondary metabolites, among others (reviewed by Sweetman et al., 2009).

### 1.4.3. Grape seed oils

Nowadays, grape seed oil is produced as a specialty by-product of wine manufacture. In fact, the high-quality of virgin grape seed oil can be used as a source of edible vegetable oil, due to the low values of cholesterol and high content in vitamin E, both important for health benefits (Matthäus, 2008).

Grape seeds have about 10 to 20 % oil (w/w), mostly consist of storage lipids (i.e., triacylglycerides or triacylglycerols, TAG), which are rich in unsaturated fatty acids, such as oleic and linoleic acids (Baydar and Akkurt, 2001; Baydar et al., 2007; Ohnishi et al., 1990). Rubio et al. (2009) suggest that grapes collected from green harvesting near *véraison* could be suitable for seed oil extraction, with characteristics similar to those of the oil extracted from the seeds of mature grapes. The oil content differences among the cultivars are related to the maturity of the grape seeds (Ohnishi et al., 1990). It is estimated that about 90 % of grape seed oil is composed by unsaturated fatty acids (Matthäus, 2008). Linoleic acid is the most abundant fatty acid with a percentage ranging from 72.98 % in oils from green seeds to 67.61 % in oils obtained from the seeds of mature grapes (Baydar and Akkurt, 2001; Rubio et al., 2009). Other fatty acids can also be found in seeds of different grapevine cultivars, such as palmitic (6.5 to 9.7 %), stearic (3.5 to 7.3 %), oleic (17.8 to 26.5 %) and, in small quantities, linolenic (0.00 to 0.87 %) and eicosenoic acid (0.00 to 0.97 %) (Baydar and Akkurt, 2001; Ohnishi et al., 1990).

Other important constituents of grape seed oil are the tocopherols and tocotrienols, which are known generically as vitamin E (Kamal-Eldin and Appelqvist, 1996). Tocopherols appear to be universal constituents of all higher plants and are vital in preventing lipid peroxidation in seeds, ensuring seed longevity and healthy germination (Sattler et al., 2004). There are four forms of tocopherols: α (5,7,8-trimethyltocol), β (5,8-dimethyltocol), γ (7,8-dimethyltocol) and δ (8-methyltocol) (Baydar and Akkurt, 2001). Horvath et al. (2006) reported the biosynthesis, accumulation and physiological role of tocotrienols and tocopherols during grape seed development. Tocopherol is present in different tissues of grape, but in seeds the levels were high in the early stages of development, suffering a decrease in the short stationary phase and then a gradual decline after the onset of desiccation. The α-form is present throughout all development and in high concentrations, while the γ-form (50 %) and δ-form (10 %) are only found in high amounts in the first stage and afterwards suffer a decrease. Regarding to tocotrienols they are only present in the seeds (in endosperm and outer integument) and gradually increase at the beginning of the lag phase (stage II) and continues with the seed and endosperm maturation, suggesting the proposed function in protecting storage oil from oxidative damage (Cahoon et al., 2003).

## 1.5. Secondary compounds in grape berries

Secondary metabolites, like the phenolic compounds, play an important role in plant defense against biotic and abiotic factors. In grape berries, the presence of these compounds contribute to the color, taste, texture and astringency of wine (Garrido and Borges, 2013), as well as to their antioxidant properties and to potentially beneficial effects on health (Shrikhande, 2000; Weston, 2005). In general, these compounds are present mainly in the exocarp and seeds (Montealegre et al., 2006). The concentration in phenolic compounds depends on the vine variety, but it is also influenced by environmental factors and winemaking practices (Teixeira et al., 2013).

In terms of chemical structure, phenolic compounds exhibit of a phenyl ring backbone, having one or more hydroxyl groups or with other substituents (Garrido and Borges, 2013). Phenolic compounds may be divided into two main groups: nonflavonoids phenolics (with a simple C6 structure), comprising hydroxycinnamic acids, hydroxybenzoic acids, volatile phenols and stilbenes; and flavonoids, such as flavones, flavonols, flavanones, flavan-3-ols and anthocyanins (reviewed by Teixeira et al., 2013).

### 1.5.1. Nonflavonoid phenolics

Nonflavonoid phenolics are present in the berry and wine in lower concentrations, but the **hydroxycinnamic acids** (HcA) are the third most abundant class of soluble phenolics, after

proanthocyanidins (condensed tannins) and anthocyanins (Kennedy et al., 2006). HcA are present in all berry tissues but with greater incidence in the skin and pulp (Castellarin et al., 2012). The synthesis of these compounds occurs mainly before *véraison*, but during ripening their concentration decreases by a dilution effect due to berry size increase (Nagel et al., 1979). HcA are usually found in free-run juice and white wines, contributing to color browning oxidation (Adams, 2006; Kennedy et al., 2006). The most represented HcA are *p*-coumaric, caffeic and ferulic acids, which are present almost entirely as *trans* isomers but amounts of the *cis* isomers have also been identified. The esterification of these compounds with tartaric acid form, respectively, coutaric acid (*trans*-*p*-coumaroyl-tartaric acid), caftaric acid (*trans*-caffeoyl-tartaric acid), and fertaric acid (*trans*-feruloyl-tartaric acid) (Castellarin et al., 2012).

In addition to HcA, there are also the **hydroxybenzoic acids** (HbA) in wine, but in lower contents, being the gentisic, salicylic, gallic and *p*-hydroxybenzoic acids the most common in grape berry (Ali et al., 2010). Gallic acid is accumulated in higher levels comparing with the remaining and in seeds this acid can esterify the carbon 3 of flavan-3-ols (Adams, 2006).

**Stilbenes** are polyhydroxystilbenes (C6–C2–C6), that is, hydroxy and methoxy derivatives of stilbene (1,2-diphenylethylene), as well as their glycosides and polymers. Stilbenes are naturally-occurring compounds found in a large number of edible plants like *V. vinifera* L. In grape berry, the stilbenes are localized in the skin at mature stage (Fornara et al., 2008; Gatto et al., 2008), in accordance to their role as a barrier against pathogens (Bavaresco et al., 2009). The resveratrol is a stilbene with high importance for human health, due to its cardioprotective effects and for reducing the incidence of chronic diseases (Baur and Sinclair, 2006; Smoliga et al., 2011). Resveratrol can suffer glycosylations (e.g., piceids and astringin) or methylations (e.g., pterostilbene) (Chong et al., 2009). The α- β- γ- δ- ε-viniferins are a major group of resveratrol oligomers produced by oxidation of basic stilbenes (Castellarin et al., 2012).

### 1.5.2. Flavonoids

Flavonoids are a significant proportion of the phenolic compounds in grape berry and include several classes, as mentioned above. They are C6–C3–C6 polyphenolic compounds, consisting in two hydroxylated benzene rings (A and B), which are connected by an oxygen heterocycle (C) through a three carbons chain (Figure 1.5). The sub-division in different classes is due to the oxidation state of the heterocycle and position of the B-ring (Castellarin et al., 2012). In terms of their histological distribution, flavonoids are localized in the peripheral layers of berry skin and in some layers of the seed coat.

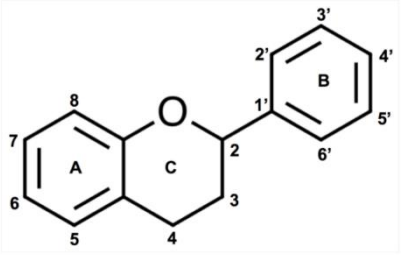


**Figure 1.5.** Flavonoid ring structure and numbering. Two hydroxylated benzene rings (A and B) connected by a three carbons chain that is part of a heterocyclic C ring.

**Flavonols** are widespread in fruits and are structurally diverse, differing by the B-ring substitution pattern, and in the nature and position of sugar substituents, which can also be acylated. In grape berry, flavonols such as kaempferol, quercetin, myricetin and the methylated forms isorhamnetin, laricitrin and syringetin were described (Mattivi et al., 2006). The synthesis of flavonol in the skin arises during in early stages of fruit and ends at *véraison* (Downey et al., 2003). However, in later stages of grape berry development, the accumulation of flavonols due to sunlight exposure can also occur (Matus et al., 2009). In fact, flavonol concentrations can be affected by environmental factors, notably sunlight, as shown in Pinot noir berries, where the berry skin on the sun-exposed side present higher levels of flavonols than skin on the shade side (Price et al., 1995).

**Anthocyanins** are pigments rather common in red, blue and purple fruits. In grape berries the anthocyanin content ranges from zero in white cultivars to 5 g $kg^{-1}$ in the *teinturier* cultivar Alicante Bouschet (Mattivi et al., 2006). In terms of structure the anthocyanins are glycosides and acylglycosides of anthocyanidins. The common six aglycones (anthocyanidins) found in grapes are malvidin, cyanidin, peonidin, delphinidin, pelargonidin and petunidin, which differ by the number of hydroxyl groups on their B-ring and their methylation pattern (He et al., 2010). In addition, anthocyanins can also be esterified (glycosylated or acylglycosylated) by acetic, coumaric or caffeic acids, in the 3-position.

**Flavan-3-ols** are the most abundant phenolics in grape berry (Singleton, 1992). These compounds comprise two monomers, catechins (2,3-*trans* configuration) and epicatechin (2,3-*cis* configuration), as well as polymers, called proanthocyanidins (syn. condensed tannins). Monomeric flavan-3-ols (catechins) are characterized for the presence of a hydroxyl group at the 3 position of the C ring and (+)catechin and its isomer (-)epicatechin, (+)gallocatechin, (-)epigallocatechin, and (-)epicatechin-3-*O*-gallate were found in grapevine (Su and Singleton, 1969).

**Tannins** are the polymeric flavan-3-ols and the class of soluble polyphenolics most abundant in grape berries. In wine, they are responsible for properties as astringency and bitterness (Kennedy, 2008) and were described as beneficial for many areas in health (Shi et al., 2003). They comprise a very diverse set of biomolecules, such as dimers, trimers and oligomers with more than 30 subunits (Adams, 2006;

Kennedy et al., 2006). In Figure 1.6 an hypothetical tetramer condensed tannin is composed by an extension of the subunits catechin, epicatechin, epigallocatechin and the terminal epicatechin gallate (Adams, 2006). These subunits are linked by C4-C6 and C4-C8 interflavan bonds. In addition, the structures of tannin polymers, which are composed by monomeric flavan-3-ols, suggests a precursor product relationship (Adams, 2006). In the grape berries, tannins are present in hypodermal layers of the skin and the soft parenchyma of the seed coat, inside the vacuole or bound to cell wall polysaccharides, being the degree of polymerization much larger in the skin than in seed (Adams, 2006; Hanlin et al., 2010; Kennedy et al., 2006). However, it is important to understand how tannins are transported across vacuole membranes and accumulated in these tissues. A study with mutants of *Arabidopsis* with a defective plasma membrane $H^+$-ATPase demonstrated that they were unable to accumulate tannins in vacuoles of the seed coat endothelial cells (Baxter et al., 2005), suggesting that tannin accumulation requires proper endomembrane trafficking and that at least one of the requisite $H^+$-ATPases plays a specific role in tannin biogenesis and vacuole accumulation.

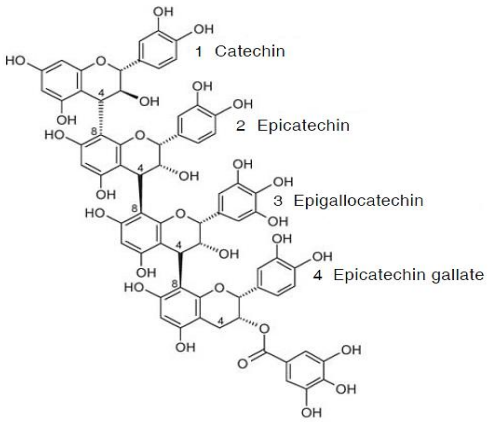


**Figure 1.6.** Hypothetical condensed tannin made up of four subunits: catechin, epicatechin, epigallocatechin, epicatechin gallate. The first subunit (catechin) is bound to the second (epicatechin) by an interflavan bond between carbon 4 of catechin and carbon 8 of epicatechin. Adapted from Adams (2006).

### 1.5.3. Biosynthesis pathways of phenolic compounds

Erythrose 4-phosphate and phosphoenolpyruvate, derivatives of the primary metabolism, are the initial precursors of the shikimate pathway. This biosynthetic pathway is responsible for the production of phenylalanine, as well as other aromatic amino acids, such as tyrosine and tryptophan. The biosynthetic pathway of soluble phenolic compounds, more specifically the phenylpropanoid pathway, begins with the aromatic amino acid phenylalanine (Figure 1.7) (Vogt, 2010). The first enzyme responsible for the synthesis of phenolic compounds is phenylalanine ammonia lyase (PAL), which converts phenylalanine to cinnamic acid. Cinnamic acid is then converted into *p*-coumaric acid by a hydroxylation at the 4-position by cinnamate-4-hydroxylase (C4H). The third step of the phenylpropanoid pathway is the esterification of *p*-coumaric acid with coenzyme A (CoA) by 4-coumaroyl:CoA-ligase (4CL) that produces 4-coumaroyl-CoA.

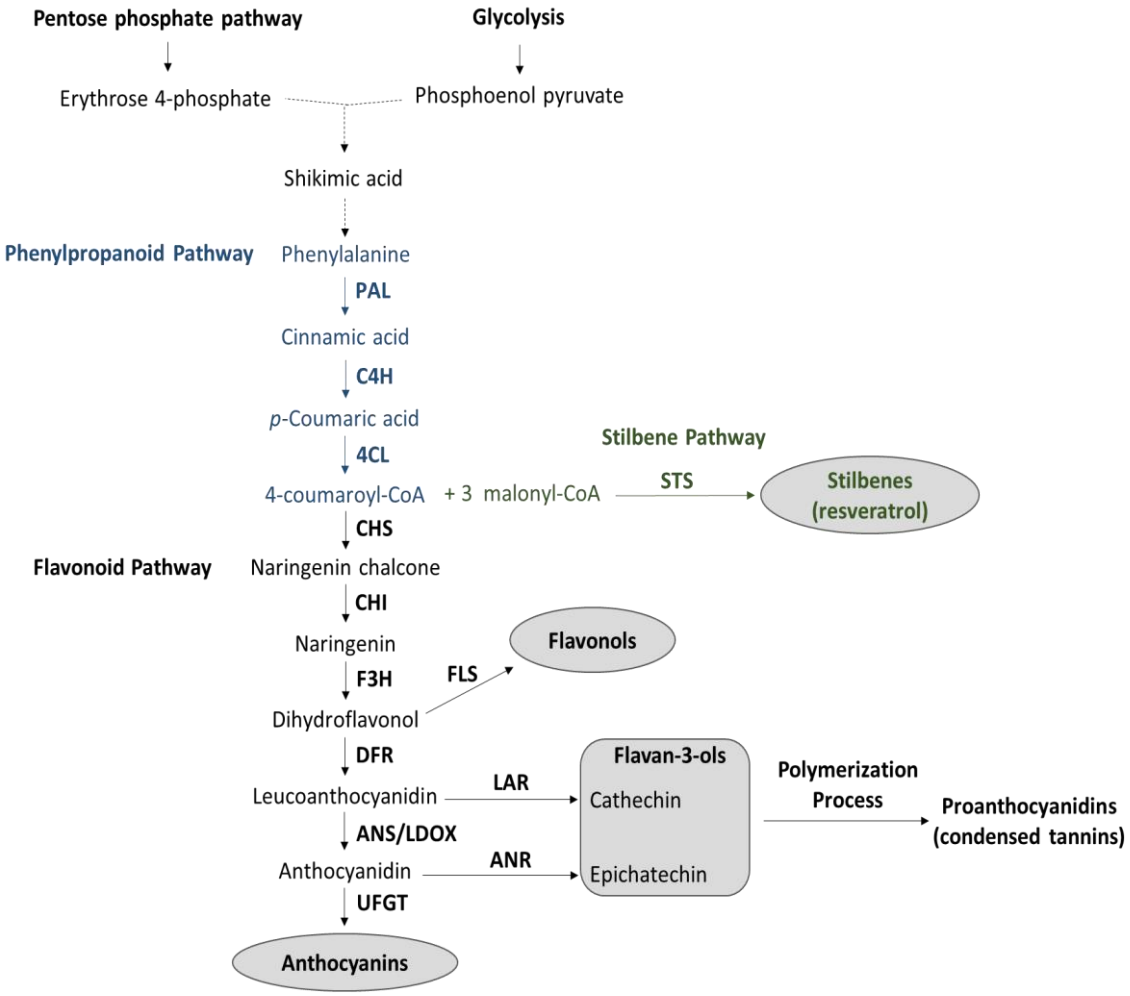


**Figure 1.7.** Biosynthetic pathways of phenolic compounds in grape berry. Abbreviations: PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl:CoA-ligase; CHS, chalcone synthase; STS, stilbene synthase; CHI, chalcone isomerase; F3H, flavonone 3-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; LDOX, leucoanthocyanidin dioxygenase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; UFGT, flavonoid glucosyltransferase. Full lines represent direct enzymatic conversion and dashed lines represent omitted intermediates. Adapted from Ageorges et al. (2014) and Teixeira et al. (2013).

The end-product of phenylpropanoid pathway, 4-coumaroyl-CoA, is used as substrate by stilbene synthase (STS) and chalcone synthase (CHS), beginning the stilbene and flavonoid pathways, respectively.

In the stilbene pathway, STS enzyme catalyzes three reactions of condensation of 4-coumaroyl-CoA with 3 molecules of malonyl-CoA producing resveratrol. In the STS reaction, the terminal carboxyl group is removed prior to closure of the A ring, so resveratrol has a different ring-folding compared to the CHS product naringenin chalcone.

#### 1.5.3.1. Flavonoid pathway

The flavonoid pathway leads to the synthesis of different classes of metabolites such as **flavonols**, **flavan-3-ols**, **proanthocyanidins**, and **anthocyanins** (Figure 1.7). In tissue-specific mRNA expression analysis it was verified that many of the genes encoding flavonoid biosynthetic enzymes were specifically expressed in the skin or seed (Grimplet et al., 2007). In grape berry, at least three genes encoding CHS exist, *Chs1* (AB015872), *Chs2* (AB066275), and *Chs3* (AB066274), which are transcribed under different controls (Goto-Yamamoto et al., 2002). After that, the naringenin results from the formation of the C ring by chalcone isomerase (CHI). The characterization of grape transcriptome allows to verified that a gene encoding a putative CHI is expressed strongly at the onset of *véraison* (da Silva et al., 2005). The next step of flavonoid pathway corresponds to hydroxylation of naringenin into different dihydroflavonols (He et al., 2010). When the hydroxylation occurs in the position 3, catalyzed by flavanone-3-hydroxylase (F3H), the dihydrokaempferol is formed. In addition, naringenin can also suffer hydroxylation at the 3' and 3',5' positions by the activity of flavonoid-3'-hydroxylase (F3'H) and flavonoid-3',5'-hydroxylase (F3'5'H), which catalyze the conversion into eriodictyol and pentahydroxyflavanone, respectively. These compounds are then converted by F3H into another dihydroflavonols, which are dihydromyricetin or dihydroquercetin.

The dihydroflavonols mentioned above can be oxidized into flavonols, like kaempferol, quercetin, and myricetin by flavonol synthase (FLS). In grapevine genome, five *FLS* genes were identified (Fujita et al., 2006). Three different isogenes encoding FLS were identified with each being expressed in different tissues, such as, skin (TC46143), pulp (TC46972) and seed (TC40373) (Grimplet et al., 2007). The putative flavonol regulator *VvMYBF1*, which activate *FLS* expression, was identified and functionally validated (Matus et al., 2008). Its expression in the berry was strongly reduced as a result of shading (Matus et al., 2009) and was induced by light (Czemmel et al., 2009).

On the other hand, the dihydroflavonols can also be reduced by dihydroflavonol reductase (DFR) to their corresponding leucoanthocyanidins (leucocyanidin, leucopelargnidin and leucodelphindin) (He et al., 2010). Recently, studies have been done in order to understand the structural and biochemical properties of the DFR, namely in grape berries, where the region of substrate binding and recognition was confirmed (Petit et al., 2007). It was also shown that *DFR* gene expression is induced by white light, calcium and sucrose (Gollop et al., 2002).

After this step, the leucoanthocyanidins are converted into the corresponding anthocyanidins (cyanidin, pelargonidin and delphinidin) by the action of leucoanthocyanidin dioxygenase (LDOX), also

called as anthocyanidin synthase (ANS). The promoter of the grape *Ans* gene (CAA53580) have several putative DNA binding motifs and can be induced by the same factors as *DFR* gene (Gollop et al., 2001).

The leucoanthocyanidins and anthocyanidins are considered as potential substrates for flavan-3-ols and consequently for proanthocyanidins biosynthesis. Leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) convert, respectively, leucoanthocyanidin and anthocyanidins into the flavan-3-ols catechin and epicatechin (Bogs et al., 2005). These flavan-3-ols monomers are required for the tannin polymerization process, which is not yet fully understood (Watrelot and Norton, 2020). In grape berry, two genes encoding LAR (*VvLAR1* and *VvLAR2*) and one encoding ANR (*VvANR*) were identified (Bogs et al., 2005). The genes encoding LAR were expressed in developing fruit, but had different patterns of expression in skin and seeds (Grimplet et al., 2007). *VvANR* was expressed only in seeds and detectable after *véraison* (Grimplet et al., 2007). In addition, those expressions were consistent with the accumulation of proanthocyanidins in the berry, suggesting that they are responsible for determining tannin composition of the fruit during ripening (Bogs et al., 2005). Furthermore, MYB transcription factors, *VvMYBPA1* and *VvMYBPA2*, were recognized in berry seeds and skin, respectively, and are expressed in parallel with proanthocyanidins accumulation during the early stages of grape berry development (Bogs et al., 2007; Terrier et al., 2008).

The last phase of the flavonoid pathway consists of the formation of anthocyanins. Anthocyanidins are inherently unstable under physiological conditions, and thus the glycosylation is an important modification for increasing their hydrophilicity and stability (He et al., 2010). The glycosylation catalyzed by flavonoid glucosyltransferase (UFGT) stabilizing cyanidin, pelargonidin and delphinidin, leads to formation of the anthocyanins cyanidin-3-*O*-glucoside, pelargnidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside, respectively (He et al., 2010). Normally, *UFGT* expression is only detected in red grape varieties skin after the onset of *véraison* (Boss et al., 1996).

## 1.6. Impact of environmental conditions on grape berry physiology, development and quality: the microclimate concept

Grapevine physiology and metabolism are affected by several environmental conditions (Blancquaert et al., 2019; Poni et al., 2018). Each wine-growing area presents a complex and interacting system, commonly called as *terroir*, which includes specific soil, topography, climate, landscape characteristics and biodiversity features, and its interaction with applied viticultural practices (Leeuwen and Seguin, 2006). In this way, the *terroir* determines grapevine development and physiology, and therefore grape berry composition and wine quality. On a finer scale at the grapevine level, grape berry

clusters and leaves have a **microclimate**, which is characterized by specific biotic and abiotic factors, including light and temperature. The concept microclimate was adopted by Smart et al. (1985) to define the environmental conditions in the vicinity of leaves and fruits. In this way, the structural organization of grapevines can influence the microclimate conditions that fruits and leaves experience, and consequently grape berry productivity and quality (Kraus et al., 2018).

**Water** availability is one of the most important factors for the growth and quality of the grapes. In general, water deficit, in particular associated with extreme temperatures, strongly affects key metabolic pathways of primary and secondary metabolism, like sugars and phenolics (Teixeira et al., 2013). In fact, in response to water stress, grape berries produce and accumulate osmotically active solutes, including sugars, amino acids, fatty acids, potassium ions, varying this response between grapevine varieties and stress intensity (Koundouras et al., 2006). For instance, the sugar content increased in Cabernet Sauvignon berries under water deficit, but no significant differences were observed in Chardonnay, Merlot and Shiraz (Castellarin et al., 2007a; Castellarin et al., 2007b; Ojeda et al., 2002). In addition, malate concentrations decreased under water stress conditions, while the anthocyanins and total phenols increased (Downey et al., 2006; Kennedy et al., 2002b; Matthews and Anderson, 1989). The increase in anthocyanin accumulation is due to up-regulation of genes, like *VvLDOX*, *VvDFR*, *VvUFGT1*, and transcription factors (e.g., *VvMYBA1*), which are responsible for encoding enzymes involved in this pathway (Castellarin et al., 2007a; Deluc et al., 2009). Water deficit also increased the flavonol content in a white grapevine variety Chardonnay, but this was not observed in a red variety (Cabernet Sauvignon), in which the contents were similar between irrigation treatments (Deluc et al., 2009). However, in another red variety (Aragonez), total flavonols content was higher in skins of berries from irrigated vines than from non-irrigated ones (Zarrouk et al., 2012).

**Light and temperature** are abiotic factors in close association, and both influence the overall grapevine physiology and berry composition (as reviewed by Palliotti and Poni (2016)). In vineyards, the increase of the number of days with high temperatures is particularly relevant. The production and quality of grape berries are sensitive to heat waves, especially at certain phenological stages, such as flowering and maturation. In fact, when the environment is warmer than the ideal for a given grapevine variety, it may result in a faster than desired phenological development (Gerós et al., 2016).

The effects of light microclimate conditions during growth on grape berry metabolism has been studied (Friedel et al., 2015; Koyama et al, 2012; Plessis et al., 2017; Reshef et al., 2017; Young et al., 2016). In general, grapes exposed to light have higher concentrations in sugars, anthocyanins and phenolic compounds and lower values of titratable acidity and malate, when compared to mature grapes

grown in the shade (Dokoozlian and Kliewer, 1995). This can be explained by a delay of grape berry maturation in the shade condition, as proposed by Zha et al. (2019) in a study testing different treatments of bagging. However, excessive higher temperatures can lead to a decline in sugar and anthocyanin content due to an increase in its degradation (Spayd et al., 2002). It was verified that light modulates the expression of flavonol synthase (*VvFLS*) and of *VvMYBF1*, a transcriptional regulator, being the flavonol content reduced in shaded berries (Azuma et al., 2012; Koyama et al, 2012). In addition, the shade can also induce lower anthocyanidin content in berries, due to down-regulation of genes/ transcription factors like *VvUFGT*, *VvMYBA1*, and *VvMYBA2* (Azuma et al., 2012; Koyama and Goto-Yamamoto, 2008; Matus et al., 2009).

### 1.7. Climate changes effects on viticulture and mitigation strategies

Climate changes effects are becoming a real concern for the agriculture sector in general, including viticulture, since its deeply dependent on weather and climate conditions (Santos et al., 2020). Indeed, the climate changes projections for the European viticulture, point to an intensification of summer-related environmental constraints, such as an increase in temperature and solar radiation, and a reduction in water availability (Fraga et al., 2013; Fraga et al., 2020). Projections for Portugal indicated that Douro, Alentejo and Minho regions will also be affected by these climate adversities (Fraga et al., 2014a; 2014b), thus imposing new challenges for these winemaking regions. In fact, several studies have already reported impacts of climate changes on grapevine phenology (Fraga et al., 2016) and physiology, as well as on grape berry composition and wine quality (Leeuwen and Darriet, 2016; Mira de Orduña, 2010). Moreover, it also enhances the severity of grapevine diseases caused by the biotic factors (Caffarra et al., 2012).

Stress mitigation strategies emerge as a solution to reduce the impacts caused by climate changes on grapevine physiology and can be classified as short- or long-term (Santos et al., 2020). Short-term mitigation strategies include, for instance: smart irrigation (Koech and Langat, 2018), training systems (i.e., grapevine canopy management) (Reynolds, 2010), application of leaf sunscreens (e.g., the mineral kaolin) (Brito et al., 2019), vine shadings (Caravia et al., 2016), and cover crops and soil tillage to maintain the water capacity in the soil (Parpinello et al., 2019). Long-term mitigation strategies, include relocations of vineyards to cooler sites, varietal selection and genetic breeding (Duchêne et al., 2012).

In particular, the white mineral **kaolin** ($Al_2Si_2O_5(OH)_4$), has become a cost-efficient mitigation strategy in vineyards, mainly due to its reflective properties, in alleviating the stress associated to excessive heat/radiation absorbed by leaves and grape berry clusters (Brito et al., 2019). In fact, recent investigations showed the positive effects of foliar kaolin application at the whole grapevine physiology,

as well as at the grape berry level (Conde et al., 2016; Conde et al., 2018; Dinis et al., 2016; Dinis et al., 2018; Frioni et al., 2019). Additionally, other studies also focused on the interaction between foliar kaolin application with irrigation treatments (Cooley et al., 2008; Glenn et al., 2010; Shellie and Glenn, 2008; Shellie and King, 2013). However, it is important to state that these strategies may have implications on the light received by leaves and fruits. On one hand, foliar kaolin will directly alter light reflection both outwards and into the canopy (Wünsche et al., 2004), while irrigation may indirectly lead to more shading due to an enhanced vegetative growth (Keller et al., 2016). Previously, we studied the response of grape berry photosynthetic tissues to long-term light microclimate conditions and to short-term light acclimation, giving insights for possible effects of mitigation measures on the light microclimate of grape berries (Garrido et al., 2018).

In summary, these mitigation measures aiming reducing radiation absorption by the vine canopy may also interfere with light spectrum and intensity for the grape berry photosynthesis, and thus, more investigations on that respect are required.

### 1.8. Research objectives

The main objective of this PhD dissertation was to study aspects of the grape berry physiology under different growing conditions, with a focus on the light microclimate experienced by the berry clusters during their development. In particular, we intended to investigate the effects of: i) two contrasting light microclimates within the canopy of grapevines, namely low light (LL) and high light (HL); and ii) two climate stress mitigation strategies, namely foliar kaolin application and grapevine irrigation, on various physiological and biochemical aspects of the two photosynthetically active tissues in developing grape berries (exocarp and seed) from the white grapevine variety Alvarinho. A set of complementary methodologies and approaches were used, including the assessment of the photosynthetic activity of the two grape berry tissues, sampled at three developmental stages in all treatments and microclimates, their respective metabolomic profiling, as well as the transcriptional analysis of target genes of key metabolic pathways. With this broad approach we also aim to unveil the potential functions of photosynthesis of these grape berry tissues on metabolic pathways/fingerprint metabolites and on the expression pattern of genes coding for respectively involved enzymes. Ultimately, we expect to contribute with knowledge that may help farmers on their decisions concerning viticultural practices, like sustainable adaptation strategies.

Firstly, in this Chapter 1 we intended to expose a general introduction. In the next chapter (Chapter 2) we will thoroughly review what is so far known about fruit photosynthesis. In that regard, histological

and physiological characteristics of fleshy fruits, with a particular emphasis on the grape berry will be addressed. Also, a special focus on photosynthesis in seeds will be given. Finally, the potential functions of photosynthesis in fruits and other organs, will be discussed.

The subsequent five chapters (Chapter 3 to 7) concern the research work carried out to meet the objectives of the thesis, as briefly described here:

In the Chapter 3 it is presented the influence of two mitigation strategies (foliar kaolin application and irrigation) on the photosynthetic activity of grape berry tissues (exocarp and seeds), grown in two light microclimates of the grapevine canopy. The photosynthetic activity (photochemical and non-photochemical parameters) of grape berry tissues was analysed using the Pulse amplitude modulated (PAM) chlorophyll fluorescence imaging technique (Baker, 2008). This analysis was performed in collaboration with Professor João Serôdio from the Center for Environmental and Marine Studies (CESAM) laboratory at the University of Aveiro. The characterization of canopy microclimates in terms of light intensity received by the clusters and associated grape berry temperatures, as well as results obtained from spectral analysis regarding transmittance and reflectance properties of leaves with and without kaolin are also presented here.

In the Chapter 4, metabolomic techniques were used to analyze the effects of tested factors - mitigation strategies and light microclimates - on the metabolic profile of photosynthetically berry tissues, aiming to evaluate the potential role of tissue-specific photosynthesis in their metabolomes. This part of the work was developed during a six-month internship at the Bioscience Department, Wageningen Plant Research, from Wageningen University & Research, The Netherlands, under the supervision of Doctor Ric C. H. De Vos. The untargeted metabolomics by Liquid Chromatography Mass Spectrometry (LCMS) and Gas Chromatography Mass Spectrometry (GCMS) allowed to find the global effects of all the treatments of this study, on the exocarp and seed metabolome. The observations were also complemented with targeted analysis, for instance, by High Performance Liquid Chromatography (HPLC) with PhotoDiode Array (PDA) and Fluorescence (FI) detectors, for the detection of chlorophylls, carotenoids and tocopherols.

Chapter 5 is devoted to study the expression patterns of selected genes coding for enzymes involved in primary and secondary metabolism that are directly or indirectly influenced by light exposure. This part of the work was carried out under supervision of Doctor Artur Conde from Biology Department at University of Minho. In this case, the photosynthetic grape berry tissues from the two light microclimates were considered for the gene expression analysis by real-time qPCR.

Chapter 6 is dedicated to study the effects of light microclimate on seed lipid profile, determined by LCMS analysis, since it is an important factor for the final quality of seeds, and to the best of our knowledge, there are no known studies on that respect.

In Chapter 7, it will be presented a sequence of different optimization protocols, which were tested in order to establish *in vitro* cultures from grape berry tissues. In particular, we tested: grape berry surface disinfection treatments, types of culture medium, hormonal combinations, special additives, among others. At the end, we obtained *callus* from exocarp. The main objective with this task was to establish cell lines with different photochemical competences allowing to relate photosynthetic activity, under control conditions, with metabolites and gene expression levels selected from previous results. Unfortunately, due to lack of time we were not able to perform it completely.

Finally, in the Chapter 8 we enclose a general integrative discussion of the present work, highlighting our main achievements and prospects of future work.

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# Chapter 2

## Fruit Photosynthesis: where, how and why?

The information presented in this Chapter 2 is being prepared for publication:

Garrido, A., Conde A., De Vos, R. C. H. and Cunha, A. Fruit Photosynthesis: where, how and why?

**Abstract**

Photosynthesis is a key physiological process for life on Earth. In addition to leaves, other plant organs, such as fruits and particularly their seeds can exhibit photosynthetic activity. There are several fruit-related aspects that influence fruit photosynthesis like anatomical and physiological characteristics, fruit developmental stage, but also the microenvironment where the fruits grow in the plant canopy - their microclimate. The main objective of this review was not only to compile the most recent information about these aspects, but also to address the challenging biological questions: why a sink organ has photosynthetic activity and what may be the main function(s) of fruit photosynthesis?

**Keywords:** fruit characteristics, photosynthetic activity, fruit tissues, roles and functions.

## 2.1. Introduction

Fruit is an important product derived from agriculture and its consumption is indispensable for the human diet. In addition to vitamins, inorganic minerals and fibers, the antioxidant capacity of several phytonutrients present in the fruits have beneficial effects in health, such as, to reduce the risk of a wide range of cancers, cardiovascular diseases and other diet-related diseases (Rodriguez-Casado, 2016). Due to the high economic value of fruits – a perishable staple – several studies have been carried out at the biochemical, physiological and molecular levels, namely to improve its nutritional quality and shelf life (Nath et al., 2014).

Evolutionary pressures have resulted in a diversity of fruits, ranging from small dry seed capsules that burst to allow seed dispersal, to relatively large complex fleshy fruits, that have evolved bright colors and complex aromas to attract seed-dispersing birds and animals (Lorts et al., 2008). That diversity can be organized under the following dichotomies: and fleshy or dry (without/with a soft succulent pericarp), and the dry fruits in dehiscent or indehiscent (open/not open to discharge seeds). For instance, capsules, siliques and legumes are dehiscent and dry; achenes, nuts and caryopsis of cereal grains are indehiscent and dry; drupes, pomes and berries are indehiscent and fleshy. Regarding to the type of fruit ripening, the classification in climacteric and non-climacteric is based on ethylene production and respiration rate (Kou and Wu, 2018). The onset of ripening in climacteric fruits is characterized by an increase in respiration with a simultaneous and a well-characterized peak of ethylene production (e.g., apple - Busatto et al., 2017; and tomato - Alexander and Grierson, 2002), while in non-climacteric fruits the ripening process occurs without sudden changes (e.g., strawberry - Symons et al., 2012; and grape - Chervin et al., 2004).

Photosynthesis depends essentially on the irradiance, on diffusive resistance to $CO_2$ - from the atmosphere to the sites of assimilation in the chloroplasts - and from the surface containing chlorophyll. In plants, it occurs predominantly in green leaves, which are the primary sources of photoassimilates to the whole plant. It was demonstrated, however, that throughout the life cycle of the higher plants, other vegetative and reproductive structures can be photosynthetically active, such as fruits, green stems, green flower organs and even roots (as reviewed by Aschan and Pfanz, 2003; Brazel and Ó'Maoileídigh, 2019) (Table 2.1). The photosynthetic activity of these organs and structures may be seasonal, expressed at specific developmental stages.

**Table 2.1**. Net photosynthetic ($P_N$) rates in different species and plant structures.

| Species | Structure | $P_N$ (μmol $CO_2$ $m^{-2}$ $s^{-1}$) | References |
|---|---|---|---|
| | **Fruit** | | |
| *Cucumis sativus* L. | Cucumber | 2.1 – 2.4 | Sui et al. (2017) |
| *Helleborus viridis* L. agg. | - | 0.1 | Aschan et al. (2005) |
| *Olea europaea* L. (cv. Leccino) | Olive | approx. 9[a] | Proietti et al. (1999) |
| *Ficus carica* L. | Figs | 18.2 (cv. Kalamon)<br>12.9 (cv. Fracasana and Mission | Vemmos et al., (2013) |
| *Fragaria* L. | Strawberry | 1-4 | Blanke (2002) |
| | **Floral Parts** | | |
| *Helleborus viridis* L. agg. | Sepals | 2.3 | Aschan et al. (2005) |
| *Lilium* hybrid L. (cv. Enchantment) | Anther<br>Tepals | 2.3<br>1.8 | Clément et al. (1997a, b) |
| *Spiranthes cernua* L. | Flower<br>Bud<br>Inflorescence | 2.5<br>3.7<br>0.2 | Antlfinger and Wendel (1997) |
| *Caesalpinia virgata* Torr. | **Stem** | 7.8 | Nilsen and Sharifi (1994) |
| *Senna armafa* L. | | 5.8 | |
| *Prunus persica* L. | | 0.4 - 1.0 | Alessio et al. (2005) |
| *Spartium junceum* L. | | approx. 8 | Nilsen et al. (1993) |
| *Vitis vinifera* L. | | approx. 0.8 ($F_v/F_m$ values) | Tikhonov et al. (2017) |
| | **Roots** | | |
| *Sonneratia alba* Sm. | Pneumatophores | 0.6 | Kitaya et al. (2002) |
| *Avicennia marina* (Forssk.) | | 0.2 | |
| *Tecticornia pergranulata* (J.M.Black) K.A.Sheph. & Paul G.Wilson | Aquatic adventitious | 0.5 | Rich et al. (2008) |

[a] Measured as gross photosynthetic rate.

Concerning to fruit photosynthesis, some early studies were reviewed by Blanke and Lenz (1989) in climacteric apple fruit, non-climacteric grape berry (both fleshy fruits), indehiscent fruits (e.g., cereal grain) and dehiscent fruits (e.g., pea pod). They reported that the photosynthetic profile of each type of fruit was dependent on some morphological and anatomical characteristics, as for instance, pea pods have much less resistance to $CO_2$ diffusion than fleshy fruits (Blanke and Lenz, 1989). There are still few works about this research topic, many using different experimental approaches. In addition, relevant lateral information is dispersed in the literature and should be recruited for a better understanding of the drivers and constraints for fruit photosynthesis to occur. Therefore, this review aims to compile and combine information focusing on characteristics of fruits, including, anatomical, physiological and biochemical, as well as raising discussion on the possible functions of photosynthesis on fruit metabolism and development.

## 2.2. Anatomical and physiological characteristics of fruits

### 2.2.1. Cuticular structure

Fleshy fruits are covered by an outer epidermis coated with a cuticle of variable thickness, which is composed by cutin and impregnated with waxy or greasy layers (Lara et al., 2015). During the development of fleshy fruits, the biosynthesis of cuticular wax is regulated by environmental factors like drought/humidity, light, temperature and pathogens (as reviewed by Trivedi et al., 2019). As in leaves, fruit cuticle provides a waterproof barrier between the epidermal cells and the relatively dry environment (Zarrouk et al., 2018). In addition, during fruit growth and development, the cuticle maintains its integrity with increasing volume and turgor pressure and also plays a central role in protecting the fruit against biotic stresses (e.g., insects and fungi) and abiotic stresses (e.g., UV radiation) (Lara et al., 2015).

Several studies have been carried out with the aim of understanding cuticle formation in fruits, including apple (Albert et al., 2013), grape (Becker and Knoche, 2012) and tomato (Segado et al., 2016). In general, with the growth of fruits, there is a rapid accumulation of wax in the cuticle, which makes it thicker and hinders the diffusion of gases (Blanke and Lenz, 1989). For instance, the cuticle of Riesling grape berries is present at early stages, with an increasing rate of cutin and wax deposition at pre-*véraison*, but after that, with the very rapid expansion of fruit surface area, the cuticular material flattens out (Casado and Heredia, 2001). In oranges from later developmental stages, the genes involved in the biosynthesis of wax, cutin and lignin were significantly induced, while genes involved in photosynthesis were repressed (Wang et al., 2016). In pepper fruit, it was verified that cuticle removal increased the gas permeability of

the surface (Banks and Nicholson, 2000), suggesting that this structure is crucial in determining compositional differences between the external and internal atmosphere.

The pea pod (*Pisum sativum* L.) have two distinct photosynthetic layers, the outer (exocarp) and the inner (endocarp) epidermis, containing a thick and thin cuticle, respectively, the first allowing a controlled $CO_2$ diffusion from the outside atmosphere by stomata and the second allowing the photoassimilation of $CO_2$ diffusing from the inner fruit cavity, and that is mainly released from seeds respiration process (Atkins et al., 1977).

### 2.2.2. Stomata frequency and functionality

Although stomata are present in the outer epidermal layers of fruits, its density is 10 to 100 times lower than in the abaxial epidermis of the respective leaves (Aschan and Pfanz, 2003). Despite this, in young fruits stomata are as sensitive as in leaves and regulate the rate of $CO_2$ exchange to a certain extent. However, with fruit growth the surface expands and the frequency of stomata decreases, after which lenticels (small, round or elliptical, pore-like structures that can be derived from nonfunctional stomata) dominate the diffusive resistance to $CO_2$ (Blanke and Lenz, 1989).

In grape berries, there are functional stomata till *véraison*, but after that the frequency decreases to less than one stoma per $mm^2$ (Blanke and Leyhe, 1987) and they become nonfunctional (lenticel covered by wax), causing a decrease in the transpiration rate and loss of water, but also higher $CO_2$ and lower $O_2$ concentrations in the berry core (Palliotti and Cartechini, 2001). Similarly, in *Citrus unshiu* about 300 stomata $mm^{-2}$ are present on fruit surface during early stages (~10-30 mm diameter fruit) collapsing steadily thereafter (Hiratsuka et al., 2015). In cucumber fruit, the stomatal frequency is only 1.58 % and 0.91 % of the upper and lower surfaces of leaves, respectively (Sui et al., 2017). Currant (*Ribes* species) varieties exhibited between 4 and 18 stomata per single fruit, corresponding to 0.2–0.3 stomata $mm^{-2}$ (Blanke, 1993). Stomata on a ripe apple are 30 times scarcer than on the abaxial surface of the respective leaf (Blanke and Lenz, 1989). In avocado fruit, the stomata are present in large number, that is, 20,000 to 30,000 per fruit, which corresponds between 50 to 75 stomata $mm^{-2}$, value that decrease with fruit expansion during ontogeny (Blanke, 1992). Equally, the number of stomata per strawberry decreased with growth and surface expansion from 6 stomata $mm^{-2}$ to 1-3 $mm^{-2}$ (Blanke, 2002). The high stomata density on the surface of young peach fruits leads to a high conductance, but at maturity stomata lose their function and differentiate into lenticels (de Oliveira Lino et al., 2016). Also, stomata are absent from the inner and outer epidermis of mature chili pepper fruits, and the gas exchange occurs only through

the cuticle (Blanke and Holthe, 1997). In mature olive fruit, scanning electron micrographs demonstrated that the stomata are covered by wax of a complex architecture (Proietti et al., 1999).

Stomata were also observed in the external surface of the pods, but again were fewer in number when comparing to leaves. For instance, the chickpea external pod surface had a stomatal density of 31±3 $mm^{-2}$ compared to 126±6 $mm^{-2}$ in leaves (Ma et al., 2001). The same study demonstrated that the higher rates of transpiration and the poorer water use efficiencies in old compared to young pods was due to the increased leakiness of stomata with age. The inner epidermis of endocarp of pea pods have thin cuticles and no stomata, while the outer epidermis, with thick cuticles, present stomata with a density approximately 25 % of the leaflet, suggesting its importance for the atmospheric $CO_2$ uptake (Atkins et al., 1977), and regulation of water losses. In soybean pods, the stomata were present at early stages of growth, being open and able for gas exchange (Andrews and Svec, 1975). Similarly, the stomata of *Brassica* pods were more functional at initial developmental stages with stomatal conductance reaching a maximum 30 days after anthesis and decreasing during the later phases (Singal et al., 1995).

The grain of cereals has a green pericarp where stomata are occasionally present in the external surface. In fact, there are contradictory data concerning this issue. Cochrane and Duffus (1979) reported very few stomata in wheat pericarp, and probably insufficient for gas exchange, while in another study, Tambussi et al. (2007) did not find stomata in the wheat pericarp. These latter authors suggested that the photosynthetic activity of the green pericarp is dependent of $CO_2$ internally generated by the respiration of endosperm cells.

### 2.2.3. Light diffusion inside the fruits

The morphology and anatomy of fleshy fruits (e.g., large volumetry) impose physical constraints to light penetration into the inner tissues, eventually reducing the photic zone to the outermost layers (Breia et al., 2013). Besides intensity, the quality of light reaching the inner regions is influenced by the cells of the outer pericarp. The presence of chlorophylls in green fruit can strongly influence the spectral composition of the light filtered through the fruit pericarp, as reviewed by Llorente et al. (2016).

The photon flux density (PFD) transmission through the skin ranges from 1 to 47 % of the incident PFD and, generally, only 2 % reach the internal regions (Aschan and Pfanz, 2003). For instance, the exocarp of grape berry transmitted 47.1 % of incident PFD and of apple Golden Delicious about 31.4 % (Aschan and Pfanz, 2003). In *Citrus unshiu*, photosynthesis was greater in fruits than in leaves under considerably low PFD (13.5 to 68 µmol $m^{-2}$ $s^{-1}$) (Hiratsuka et al., 2015). Green peel avocado fruit transmitted only 1.5 % of the incident light at 660 nm, but increase to 8.4 % at 730 nm (Blanke, 1992),

consistent with a selection of the spectrum by chlorophylls present in the peel. In inner tomato tissues, such as the locules, with low access to light, a high expression of genes associated with photosynthesis was observed (Lemaire-Chamley et al., 2005). Under a constant photosynthetically active radiation (PAR) of 1750 μmol $m^{-2}$ $s^{-1}$, the peel of apple fruit transmitted 1-3 % of incident PAR at 400 nm, increasing to 10-12 % at 850 nm (Blanke and Notton, 1992), and with fruit development this light transmission into the fruit core decreased by up to 80 %. Similarly, in olive fruits, PAR diffusion into internal tissue layers is very problematic, due to the increase in volumetry (Proietti et al., 1999). Chen and Cheng (2007) observed that sun-exposed peel of apple fruit had higher photosynthetic $O_2$ evolution capacities, as well as higher activities of enzymes from Calvin-Benson cycle. Additionally, in exocarp of cucumber fruit it was noted the expression of other key genes involved in the photochemical phase, for example, associated with light-harvesting complexes (Lhca) of photosystems I (PSI) and light-harvesting proteins (Lhcb) of photosystems II (PSII) (Sui et al., 2017). In the same fruit the decreased of PAR from 200 to 50 μmol $m^{-2}$ $s^{-1}$ led to a reduction of photosynthetic rate by 60-65 % (Marcelis and Hofman-Eijer, 1995).

In pea pods, up to 27 days after anthesis, under an incident PFD of 2200 μmol $m^{-2}$ $s^{-1}$, the exocarp and mesocarp (pericarp) absorbed around 67 %, the endocarp received 10 % and the remaining 23 % was transmitted to seeds in the pod cavity (Atkins et al., 1977). In later stages of pea pod development, a decline in chlorophyll content in outer layers allows an increase in PAR reaching the endocarp and seeds. In this way, there is a temporal separation in the loss of photosynthetic capacity between pods and seeds. Developing soybean embryos receive moderate levels of light (5–30 μmol $m^{-2}$ $s^{-1}$), but the amount of light transmitted by seed coat to the embryo is high (approx. 15 %), which influences seed photosynthesis (Allen et al., 2009). Similarly, in pod of chickpea, the seed coat light transmission increased with development leading to a greater light utilization by the embryo (Furbank et al., 2004).

The complex structure of the ear in C3 cereals, implies difficulties in transmission of light into the grains. In fact, the grain is surrounded by the lemma and palea, and it is shaded by the glume, which imposes low PFD levels reaching the green pericarp and endosperm (Tambussi et al., 2007).

### 2.2.4. Chloroplasts and photosynthetic pigments

Microscopic observations revealed the presence of chloroplasts in different fruits, as reviewed by Blanke and Lenz (1989). In fruit tissues, the density of chloroplasts is much lower as compared to the leaves, and thus the photosynthetic rate per unit of area (or per fresh or dry weight) is reduced (Aschan and Pfanz, 2003). For instance, strawberry contains 0.2-0.6 mg chlorophyll $g^{-1}$ fresh weight, i.e., 7-fold less chlorophyll than in the respective leaves (Blanke, 2002). But, differently from the leaves, where most

of the organ consist of chlorophyllin cells, generally in fruits only specific tissues or cells are chlorophyllin (e.g., Breia et al., 2013). In apple, for instance, chloroplasts can be only found at the hypodermal and inner perivascular tissue (Phan, 1973). Also, chloroplasts from different tissues may differ in structure, composition and function. Resorting to the apple example, in the hypodermal layers the chloroplasts are smaller than those from the inner tissue, exhibit grana throughout fruit development and contain starch granules, being more closely related with those found in leaves and perform photosynthesis-like C3 pathway (Phan, 1973). On the other hand, chloroplasts from perivascular tissue are larger and in a relatively small number compared with the respective leaf and with apparent absence of starch grains, being characterized by a C4-type photosynthesis (Phan, 1973). In the case of cucumber, the chloroplasts (with grana stacks 1.7-fold larger than in leaves) appear in inner walls of fleshy parenchyma cells, but its quantity per unit area is lower than in leaves (Sui et al., 2017). Avocado fruit contains sun-type chloroplasts that retain its structural integrity until the harvest. They are comprised by grana (with few thylakoids), with starch and lower chlorophyll content than the respective leaves on a per surface area basis (Blanke, 1992).

The pericarp of grape berries, at day zero after anthesis, have small plastids with their loosely arranged inter-granal lamellae and granal thylakoids, and with abundance of starch grains, and for that they can be regarded as amyloplasts (Hardie et al., 1996). During the post-anthesis period (i.e., between day 0 and approx. until day 42 after anthesis), it was observed an increase in chlorophyll content, as well as in plastid density within the pericarp tissues, as a result of the preceding period of cell division and enlargement of the fruit (Hardie et al., 1996). Thereafter, plastids acquire a larger pleomorphic form, being largely devoid of starch granules until the last stages of ripening, but contain large lipid-like globules (Hardie et al., 1996). In the same study, it was suggested that the plastids of the grape pericarp play a central role in the isoprenoid synthesis, such as monoterpenes, and thus in grape and wine flavor and aroma.

Chlorophyll fluorescence measurements showed that fruits have its photochemical machinery activated by the energy received from light – e.g., mango (Hetherington, 1997), lemon (Nedbal et al., 2000), tomato, (Carrara et al., 2001), papaya (Bron et al., 2004), eggplant (Calvo et al., 2017) and grape berry (Breia et al., 2013; Garrido et al., 2018; Garrido et al., 2019). In fact, the light growing conditions of fruits is a determinant factor affecting pigment concentration, the photosynthetic rate and the level of carbohydrates, as demonstrated for Nules Clementine mandarin fruit, that when growing inside the canopy had lower chlorophyll and carotenoid contents and lower carbohydrate levels (Cronje et al., 2013).

Similarly, in eggplant fruit, it was verified that different environmental light conditions influenced the fluorescence ratio of each photosystem (Calvo et al., 2017).

Throughout fruit development and maturation, the granal structure of the internal chloroplasts disintegrates and the chlorophyll content decreases as result of its substitution by other pigments, like carotenoids, or due to the activity of chlorophyllases (Hörtensteiner and Kräutler, 2011; reviewed by Seifert et al., 2014). For instance, in tomatoes there is fragmentation of the thylakoid membrane of the green chloroplasts and formation of colored chromoplasts with new carotenoid-bearing structures (Egea et al., 2011), as detected by confocal laser scanning microscopy (D'Andrea et al., 2014). The decrease in chlorophyll content during ripening, was observed in several fruits, such as papaya (Sanxter et al., 1992), olive (Proietti et al., 1999), tomato (Kozukue and Friedman, 2003), mandarin (Cronje et al., 2013), apple (Nagy et al., 2016) and grape berry (Garrido et al., 2018; Garrido et al., 2019). In grape berry tissues, while in seed integuments chlorophylls content decreased from 80 to 40 $\mu g\ g^{-1}$ fresh weight, in exocarps it decreased from 120 to 20 $\mu g\ g^{-1}$ fresh weight (Garrido et al., 2018). Our results of transcriptional analysis in exocarp and seed showed, however, that the *Chlorophyll Synthase* gene (*VvChlSyn*) keeps its relative expression of transcripts until later stages of development (Garrido et al., submitted).

The regulation of carotenoids in fruits can be rather complex due to the dramatic changes in content and composition during ripening, which are also dependent on the fruit tissue and the developmental stage. Recently, Lado et al. (2016) provided a comprehensive overview concerning the main carotenoid profiles in fleshy fruits and pattern of changes during ripening and of the different regulatory levels responsible for the diversity of carotenoid accumulation in fruit tissues. In grape berries, β-carotene and lutein are the predominant carotenoids, and there is a steady decline after *véraison*, which appears to be related to chloroplast disappearance and to the formation of carotenoid-derived norisoprenoid volatiles (β-ionone and β-damascenone) (Crupi et al., 2010; Young et al., 2012; Joubert et al., 2016), all important for wine aroma, because of their low olfactory perception threshold (Mendes-Pinto, 2009). In agreement with this, in our previous work the total carotenoids content (i.e., the sum values of α-, β- and lutein) decreased in grape berry exocarp and seed during development (Garrido et al., 2019).

Pea pod walls also have chloroplasts in different cells layers. The exocarp outer epidermis contains few chloroplasts, but they are abundant in the inner epidermis of the endocarp (with smaller starch grains), as well as in the parenchyma layers of mesocarp (Atkins et al., 1977). In the case of chickpea pod walls, low levels of chlorophyll were localized at the embryo and evenly distributed throughout the

cotyledons, but the seed coat contained a layer of chloroplast-rich cells directly below the epidermis (Furbank et al., 2004). The variation of chlorophyll concentration in pods is similar to that of fleshy fruits, with a decrease through development, as demonstrated for soybean pods (Andrews and Svec, 1975). These pods had 9.2-12.4 times less chlorophyll per gram fresh weight than leaves. However, the gross photosynthetic rates in pods were greater than in leaves on a per mg chlorophyll basis, which may be important for the rapid seed filling period of pods (Andrews and Svec, 1975).

The total chlorophyll content on a dry weight basis is lower on ear parts when compared with flag leaf (Lu and Lu, 2004). This differences in pigment composition can be explained by the distinct light environment between these two structures, being the ear localized at top of the canopy. In fact, the authors Lu and Lu (2004) verified that lumes, lemmas and awns of the wheat ears had a lower chlorophyll/carotenoid ratio, that reflect the required photoprotection. During the development of wheat grain, the maximum of chlorophyll content in the pericarp green layer was observed at 20 days after anthesis, when there was also a peak of photosynthetic activity (Caley et al., 1990).

### 2.2.5. Assimilation and refixation of internal $CO_2$

Fruit gas exchange with the external atmosphere takes place mainly through stomata and depends on a diversity of morphological and physiological aspects, namely: fruit type and size, fruit ontogeny stage, fruit temperature, shading and incident PFD, and chlorophyll content (Wahid et al., 2005). For instance, Aschan and Pfanz (2003) mentioned that fleshy fruits perfom basically internal $CO_2$ refixation, but dry fruits, during the younger stages, are able to assimilate atmospheric $CO_2$. Besides that, the incident light also influences the rate of $CO_2$ fixation, as demonstrated for mandarin fruit, in which the light-saturated net $CO_2$ assimilation rate ($A_{max}$) of fruit in the outer canopy was significantly higher than in the inner canopy (Cronje et al., 2013). Overall, fruits assimilate less atmospheric $CO_2$ via ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) when compared to the respective leaves (Blanke and Lenz, 1989), as observed in oranges (with 50 % to 75 % less) (Cronje et al., 2013).

As exposed above, at early stages, the cuticular and stomata characteristics enable gas exchange in fruits. Then, during fruit ontogeny, the great accumulation of wax in its surface leads to a reduction of $CO_2$ atmospheric exchange rate by 10-fold (Blanke and Lenz, 1989), resulting in an increase in internal $CO_2$ concentration and a simultaneous reduction of water loss through transpiration. Moreover, the increase in mitochondrial respiratory processes, fueled predominantly by imported photoassimilates from leaves to these sink organs at this later stages, results in a rise of internal $CO_2$ concentration (Blanke and

Lenz, 1989). This "excess" of internal $CO_2$ in the cytosol, can be transformed in carbonic anhydrase to bicarbonate ion ($HCO_3$), which is subsequently re-fixed by phosphoenolpyruvate carboxylase (PEPC).

In green and red-turning tomato fruit, PEPC enzyme activity is 2 times greater than RuBisCO activity (Carrara et al., 2001). Similar results were obtained for coffee green pericarp, in which ratio PEPC/RuBisCO activities was higher than in the respective leaves (Lopez et al., 2000). In mandarin fruit, the $CO_2$ refixation by PEPC was also registered contributing for a higher final sugar content, when compared with bagging fruit (Hiratsuka et al., 2012). More recently, the same authors using $^{14}CO_2$ suggested the presence of a C4/CAM (crassulacean acid metabolism) photosynthetic mechanism in mandarin fruits (Hiratsuka et al., 2015). In pre-climacteric avocado fruit mesocarp, occurs $CO_2$ recycling by PEPC, being the enzyme activity around 2.5 μmol $CO_2$ $s^{-1}$ $g^{-1}$ fresh weight (Blanke, 1992). In skin and mesocarp of grape berry, using $^{14}CO_2$ incorporation, it was verified that PEPC enzyme activity was about 17 times higher before *véraison* than after, leading to a higher concentration of malate, and therefore to acidification of the berries (Diakou et al., 2000). Also in apple fruit, PEPC activity was accompanied by malic acid synthesis, and a simultaneous regulation of cytoplasmic pH (Blanke, 1998), like in tomato fruit (Guillet et al., 2002).

More recently, a first quantitative data with *in vivo* respiratory $CO_2$ recapture in non-climacteric fleshy fruits, showed that cucumber can assimilate atmospheric $CO_2$ via RuBisCO and approximately 88 % of respiratory $CO_2$ was captured and re-fixed via PEPC, being this via quantitatively more important than the direct $CO_2$ fixation from the atmosphere (Sui et al., 2017).

In ears cereals, such as barley and bread/durum wheat, $CO_2$ refixation was considered a quantitatively relevant process (Tambussi et al., 2007). In fact, awns and external surface of glumes (where stomata are abundant) are the main structures responsible for external $CO_2$ assimilation, while the green pericarp and internal surfaces of lemmas (facing the grain), the recycling of respired $CO_2$ is the principal process (reviewed by Tambussi et al. (2007)). Indeed, it was observed that PEPC had a role in $CO_2$ respiratory refixation, both in barley ears (Bort et al., 1996) and in chloroplasts of pericarp cells and glumes of durum wheat (Araus et al., 1993).

In grain legumes, respiratory $CO_2$ released from the embryo is re-fixed by a layer of cells on the inner pod wall. In chickpea pods, experiments made with isotopically labelled $CO_2$ showed that more than 80 % of the $CO_2$ was fixed by the pod walls, rather than the seed tissues (Furbank et al., 2004). This internal recycling of $CO_2$ inside of the pod, may assist in maintaining seed filling in water-stressed chickpea (Ma et al., 2001).

## 2.3. Biochemical pathways proposed for fruit photosynthesis

More than 30 years ago Blanke and Lenz (1989) proposed a new biochemical process for the photosynthesis in fruits, called as malate-$CO_2$ shuttle (Figure 2.1). This mechanism was characterized as an intermediate status between the common types of photosynthesis, that is, a mechanism of $CO_2$-concentrating, analogous to C4/CAM-photosynthesis and also the presence of kinetics of C3 photosynthesis or the Calvin-Benson cycle.

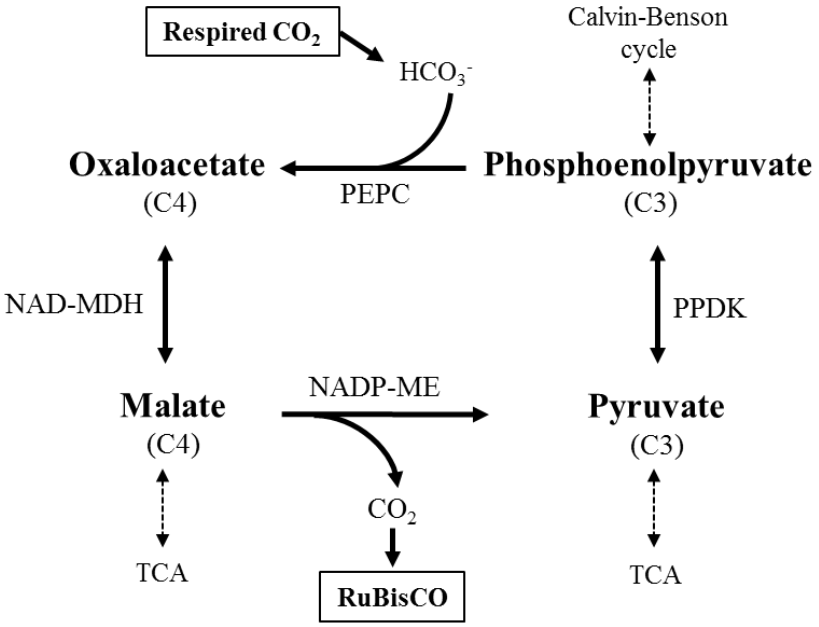


**Figure 2.1.** Malate-$CO_2$ shuttle proposed by Blanke and Lenz (1989). Abbreviations: $HCO_3^-$, bicarbonate ion; NAD-MDH, NAD-linked malate dehydrogenase; NADP-ME, NADP-linked malic enzyme; PEPC, phosphoenolpyruvate carboxylase; PPDK, pyruvate orthophosphate dikinase; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; TCA, tricarboxylic acid cycle. Adapted from Blanke and Lenz (1989).

With this mechanism, the excess of respiratory $CO_2$ is transformed by carbonic anhydrase to $HCO_3^-$ in the cytosol. Subsequently, occurs the β-carboxylation of phosphoenolpyruvate (PEP) with $HCO_3^-$, catalyzed irreversibly by PEPC, to synthesize oxaloacetate (OAA), which is then reduced to malate by cytosolic NAD-dependent malate dehydrogenase (NAD-MDH). Malate can diffuse to the chloroplast and suffer decarboxylation by NADP-malic enzyme, resulting in $CO_2$ and pyruvate, which can be regenerated into phosphoenolpyruvate, by pyruvate phosphate dikinase (PPDK). The released $CO_2$ can be fixated by RuBisCO in the Calvin-Benson cycle, while pyruvate, and also malate, can be directed to other pathways, like tricarboxylic acid (TCA) cycle and gluconeogenesis, or in the case of malate, accumulated in the vacuole till further use.

Despite the similarities between this mechanism and C4-type photosynthesis, it is argued that the fruits cannot be cataloged on this type of photosynthesis, since they do not present Kranz anatomy

(Blanke and Lenz, 1989). However, in literature it was also mentioned that in plants the C4 photosynthesis may be also achieved by compartmentalization (Edwards et al., 2001) within the cell without the presence of Kranz anatomy (Sage, 2002). Furthermore, a proxy to CAM mechanism does not imply also a spatial separation, or Kranz architecture, but a temporal separation of the two carboxylation reactions, eventually associated with fruit developmental stage.

Recently, Henry et al. (2020) reviewed the pathways of photosynthesis in non-leaf tissues, including stems, petioles, seeds and fruits. They verified that, in general, the C4 pathway has been reported in these non-leaf tissues in plants that employ C3 photosynthesis in the leaf. Moreover, the authors proposed a process of C4 photosynthesis for those structures, partitioned between the inner shaded tissues (e.g., cells of the endosperm in seeds of wheat), that are characterized by high levels of respiratory $CO_2$, and the outer green tissues (e.g., pericarp of wheat) more exposed to the light (Figure 2.2).

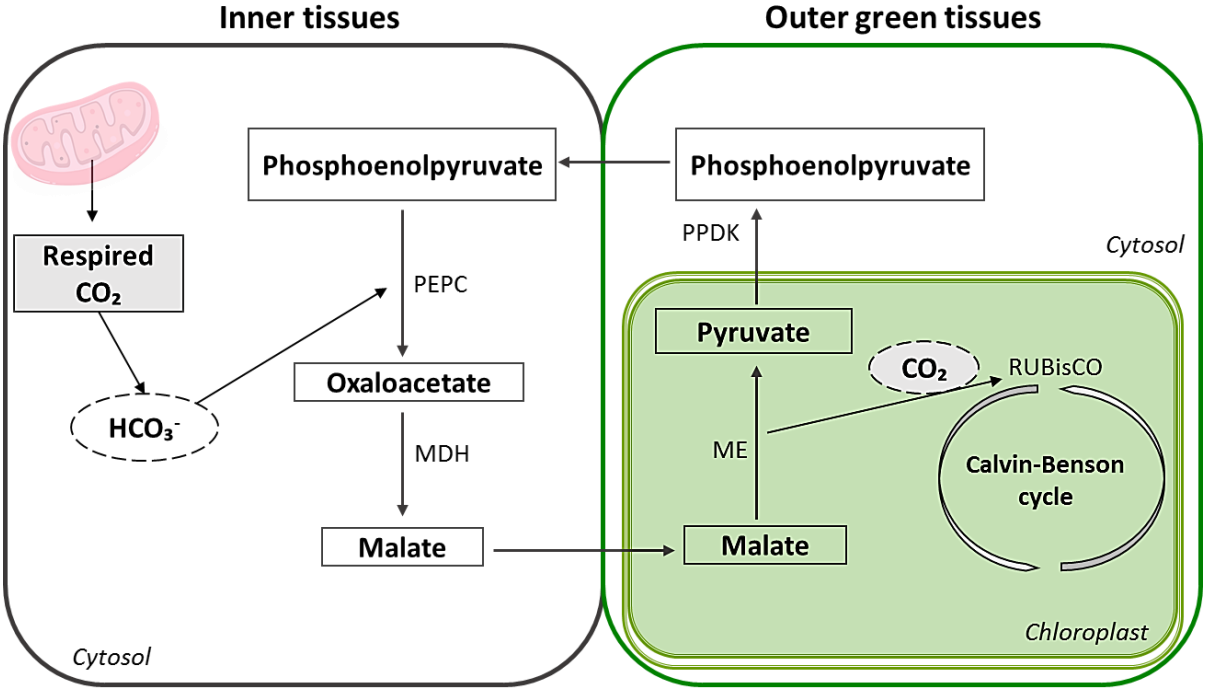


**Figure 2.2.** Photosynthesis in non-leaf tissues in outer and inner parts of the organ, proposed by Henry et al. (2020). Key reactions in inner tissues (without light) to capture respired carbon: PEPC, phosphoenolpyruvate carboxylase; MDH, malate dehydrogenase. Reactions in outer tissues (with light): ME, malic enzyme; RuBisCO, Ribulose-1,5-bisphosphate carboxylase/oxygenase; PPDK, Pyruvate orthophosphate dikinase. Adapted from Henry et al. (2020).

This internal recycling of respired $CO_2$ can be an evolutive adaptation of fruits and other non-foliar photosynthetic tissues to avoid carbon losses and thus improve locally the carbon balance (Aschan and Pfanz, 2003). In fact, Henry et al. (2020) refereed that the main contribution of the photosynthesis in

non-leaf tissues seemed to be associated with the need to re-capture carbon especially in storage organs that have high respiration rates.

For grape berries, Beriashvili and Beriashvili (1996) suggested the operation of two photosynthetic main routes, whereby $CO_2$ was assimilated primarily as malate in early stages of development (C4-type photosynthesis, involving PEPC), and primarily as sugars in berries at the initial stages of ripening (C3-type photosynthesis), what is consistent with the strong malic acid *vs* sugars pattern of C accumulation during development and ripening. The decline in expression of two putative PEPC isogenes and of PEPC activity at *véraison* (Deluc et al., 2007), supports this theory of a photosynthetic switch. In fact, Sweetman et al. (2009), in a review about the regulation of malate metabolism in grape berry, suggested that the accumulation of malic acid is in large part due to *de novo* synthesis in fruit, through the metabolism of photoassimilates translocated from leaf tissues, as well as resulted from the photosynthetic activity within the fruit itself. Corroborating this, proteomic studies revealed that skin of ripe berries still contained detectable amounts of proteins with functions related to photosynthesis and carbon assimilation (Grimplet et al., 2009). Indeed, the large subunit and the subunit binding-protein alpha of RuBisCO, were more abundant in the skin than in the pulp (mesocarp), in accordance with other previous studies (Famiani et al., 2000; Deytieux et al., 2007). Thus, the expression pattern of the carbon assimilation proteins indicates that the skin might retain a functional photosynthetic apparatus or its remnants undergoing degradation in mature berries (Grimplet et al., 2009). In accordance with this, results of our group using chlorophyll fluorescence analysis demonstrated that grape berry exocarp exhibit much higher photochemical efficiency than the mesocarp (Breia et al., 2013), and it keeps the photosynthetic activity till later stages of development (Garrido et al., 2018; Garrido et al., 2019). Moreover, our results of transcriptional analysis in exocarp showed that the relative expression of *VvRuBisCO* increased during berry development (Garrido et al., submitted).

In apple, a similar malate metabolism and simultaneous recycling of $CO_2$ respiratory was verified by Blanke (1998). In fact, PEPC is a very efficient isoform relative to the two substrates, $HCO_3^-$ and phosphoenolpyruvate, such as in CAM or $C_4$ photosynthesis. Here, PEPC gene expression was high only until the fruit set, decreasing thereafter during the maturation, and thus the fruit metabolism has to relay on its existing PEPC protein resources. The malate dehydrogenase was 80-fold in excess in comparison with PEPC, but both are concentrated in the vascular tissue linking the calyx, core and peduncle.

## 2.4. Possible roles of photosynthesis in fruits

Considering all the anatomical, physiological and biochemical aspects mentioned above, on the present section the possible roles of photosynthesis in fruits will be addressed, as well as the particular case of photosynthesis in seeds.

### 2.4.1. The relationship between photosynthesis and metabolism in fruits

The role of photosynthesis in fruit metabolism, and consequently on its quality, has been extensively discussed in tomato (Obiadalla-Ali et al., 2004; Carrari et al., 2006; Powell et al., 2012; Sagar et al., 2013; Cocaliadis et al., 2014). The main concern of those works was to obtain answers for the questions: What is the contribution of fruit photosynthesis to fruit metabolism before ripening? Does it have any effect on the final quality of the fruit?

In tomato fruit, genetic and molecular approaches have been applied to understand the relationship between photosynthesis and fruit metabolism, composition and nutritional value. For instance, experiences with transgenic tomato plants, where the expression of chloroplastidial isoform of fructose 1,6-bisphosphatase (*cp-FBPase*) was inhibited using the antisense technology, were performed (Obiadalla-Ali et al., 2004). The FBPase catalyzes the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate. In green plant tissues there are two isoforms, being the plastidial isoform an important enzyme for the control of Calvin-Benson cycle. The results showed that the transgenic lines had few changes in their carbohydrate metabolite levels, probably due to its ability to import sugars from leaves. This can be explained by considering that if there is indeed repression of photosynthesis in the fruits of the transgenic lines, an increased sucrose import becomes necessary as compensation. In addition, the same authors observed that the repression of cp-FBPase enzyme led to a reduction of average weights of fully ripe fruits. Interestingly, this decrease in weight was quantitatively similar to the estimated contribution of the fruit to the photoassimilates production, that is, around 15-20 %, although it represents a minor component when compared to that imported from the leaves (Cocaliadis et al., 2014).

In another study in tomato, a down-regulation of an auxin response factor (i.e., *ARF4*, which is from a class of transcription factors that regulate auxin-mediated gene expression) resulted in a higher transient starch accumulation at the early stages, together with an enhance of chlorophyll content and photochemical efficiency, which is consistent with the idea that the photosynthetic activity of fruit may be responsible, at least partially, for the production of photoassimilates and, consequently, for the high levels of starch (Sagar et al., 2013). In addition, it was verified that the overexpression of the transcription factor *SlGLK2* (important for chlorophyll accumulation and distribution in developing fruit), led to an enhance in

fruit photosynthesis gene expression and chloroplast development, and also to an increase in carbohydrates and carotenoids in ripe tomato fruit (Powell et al., 2012).

Overall, these studies pointed to the importance of fruit photosynthesis to carbon use efficiency, and its implications on fruit yield and quality, as recently reviewed by Simkin et al. (2020). Table 2.2 shows the contributions of fruit photosynthesis to overall C budget assessed in some fruits by: 1) calculations using $CO_2$ gas exchange parameters, dry weight and carbon content (e.g., Birkhold et al., 1992); 2) $^{14}CO_2$ feeding and gas exchange (Sui et al., 2017); or 3) by calculations using electron transport rate parameter, determined by pulse amplitude modulated (PAM) fluorometry, and surface area (Hetherington et al. 1998). But this is only about C, and photosynthesis can provide more than carbohydrates or even C-skeletons. Therefore, understanding the various functions of fruit photosynthesis is crucial, since it can provide a potential route for manipulating key photosynthetic genes to enhance the fruit development, composition, yield or nutritional quality, particularly under conditions of stress when leaf photosynthesis may be compromised.

**Table 2.2.** Fruit photosynthesis contribution to total carbon (%).

| Fruit | Fruit photosynthesis contribution to total carbon (%) | References |
|---|---|---|
| Grape berry | 10 | Ollat and Gaudillere (2000) |
| Tomato | 15 | Hetherington et al. (1998) |
| Blueberry | 15 | Birkhold et al. (1992) |
| Mango | 1 | Hetherington (1997) |
| Peach | 5-9 | Pavel and DeJong (1993) |
| Pea pod | 16-20 | Flinn et al. (1977) |
| Olive | 40 | Proietti et al. (1999) |
| Coffee | 20-30 | Lopez et al. (2000) |
| Cucumber | 9.4 | Sui et al. (2017) |

This theme is complex and still controversial. In fact, Lytovchenko et al. (2011) mentioned that tomato fruit photosynthesis is not important for photosynthate accumulation, including those metabolites impacting taste, and consequently it is not required for fruit metabolism and development. However, the same authors verified that tomato photosynthesis had a considerable role in seed development, impacting on seed set, composition and morphology during the early developmental stage.

With respect to grape berry, several approaches of transcriptomic (Degu et al., 2014; Deluc et al., 2007; Grimplet et al., 2007; Plessis et al., 2017), proteomic (Grimplet et al., 2009; Martínez-Esteso, et

al., 2011; Wang et al., 2017), metabolomic (Dai et al., 2013; Degu et al., 2014; Wang et al., 2017), and of these data integration in network analysis for identification of putative stage-specific biomarkers (Serrano et al., 2017; Zamboni et al., 2010), as well as data from co-expression gene networks (Zou et al., 2019), confirmed the presence of components directly related with photosynthetic activity, namely before *véraison* (e.g., light harvesting complexes, photosystem II oxygen evolving complex, Calvin-Benson cycle enzymes). In particular, the down-regulation of photosynthetic genes after *véraison* was first analysed by expressed sequence tag (EST) profiling (Terrier et al., 2001; Silva et al., 2005) and confirmed by microarray analysis in grape berries (Terrier et al., 2005; Deluc et al., 2007; Pilati et al., 2007; Zamboni et al., 2010), and specifically in berry skins (Waters et al., 2005), wherein photosynthesis-related transcripts are more abundant (Grimplet et al., 2007). All those investigations support our results obtained by chlorophyll fluorescence analysis by imaging-PAM fluorometry (Breia et al., 2013; Garrido et al., 2018; Garrido et al., 2019).

The Calvin-Benson cycle can supply precursors for pathways of primary and secondary metabolism (as reviewed for tomato by Cocaliadis et al., 2014). Secondary metabolites, like phenolic compounds, play important roles in plant defense against biotic and abiotic factors (Cheynier, 2012). Also, the diversity of compounds contributes for the nutritional quality of fruits and for its organoleptic properties, which are important to make them attractive and palatable. For instance, in grape berries, phenolics contribute to the color, taste, texture and astringency of the wine, as well as to its antioxidant properties and beneficial effects on health (Shrikhande, 2000; Weston, 2005). These compounds are mainly present in the exocarp and seeds of the grape berries (Garrido et al., 2021; Montealegre et al., 2006), both photosynthetically active (Breia et al., 2013; Garrido et al., 2018; Garrido et al., 2019).

### 2.4.2. The particular case of photosynthesis in seeds

In addition to fruit tissues, some seeds can perform photosynthesis at least during part of their development period. Investigations have been developed to understand the specific functions of photosynthesis in these organs (Ruuska et al., 2004; Rolletschek et al., 2005b; Tschiersch et al., 2011; Galili et al., 2014). In general, these investigations showed that seed photosynthesis may contribute in three distinct ways: 1) supply of oxygen to prevent and/or reduce hypoxia; 2) production of nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP) both fundamental for energetically demanding biosynthetic pathways in the chloroplast, such as fatty acid synthesis; 3) provision of C-intermediates for primary and secondary metabolism by the Calvin-Benson cycle; and 4) re-fixation of respiratory $CO_2$ by RuBisCO, what can improve the energy efficiency of seeds.

#### 2.4.2.1. Possible functions of $O_2$, ATP and NADPH from the photochemical phase

Most seeds have some peculiarities that hinders the absorption of oxygen, namely a thick coat and the accumulation of proteins and oils that become a glassy matrix during desiccation (Buitink and Leprince, 2008). Thus, during the maturation and desiccation process there is a decrease in oxygen diffusion into the dense inner seed tissues. This situation of hypoxia causes restrictions to the production of ATP by mitochondria (oxidative phosphorylation), which is pivotal for various metabolic pathways during seed development and embryo maturation (van Dongen et al., 2004; Vigeolas et al., 2011, 2003). Hypoxia can directly or indirectly affect several other processes in seeds, such as, nutrient uptake (e.g., wheat – van Dongen et al., 2004), storage activity and metabolite distribution (e.g., soybean – Rolletschek et al., 2005b), assimilate partitioning between endosperm and embryo (e.g., maize – Rolletschek et al., 2005a), and enzymatic activities associated with lipid metabolism (e.g., rapeseed – Vigeolas et al., 2003). It is impelling to relate seed photosynthesis – generally, a hidden structure deep in the fruit volume –, the production of $O_2$ in the photochemical phase and the benefits of avoiding hypoxia conditions inside of seeds.

Monocotyledonous barley caryopsis (*Hordeum vulgare* L.) has a green pericarp with chlorophyll (called chlorenchyma) (Figure 2.3A), where photosynthesis occurs in the mid-storage stage (Wobus et al., 2005). Chlorophyll fluorescence images of the effective quantum yield of PSII allowed to verify that the photosynthetic activity was restricted to the chlorenchymatic regions of the pericarp (Figure 2.3B) (Tschiersch et al., 2011). Besides that, the photosynthetic activity in these regions is responsible for the production of 3.5 µmol NADPH $h^{-1}$ and 2.3 µmol ATP $h^{-1}$, both important for storage (Tschiersch et al., 2011). The oxygen distribution ('oxygen maps') in barley allow to understand better the photosynthetic activity in its tissues (Figure 2.3C) (Rolletschek et al., 2004). In general, the pericarp exhibits high levels of oxygen, while at the central regions there is an oxygen deficiency. In the dark experiments, the oxygen levels decreased dramatically in the inner endosperm region and in the transfer cells (transport pathway of assimilates to endosperm) (Patrick and Offler, 2001). On the other hand, in the light assays, this reduction was not so great, suggesting that the oxygen released by photosynthesis may play an important role in nutrient transport to the endosperm. In addition, the expression of photosynthesis-related genes peaks before the assimilate storage phase (Sreenivasulu et al., 2004).

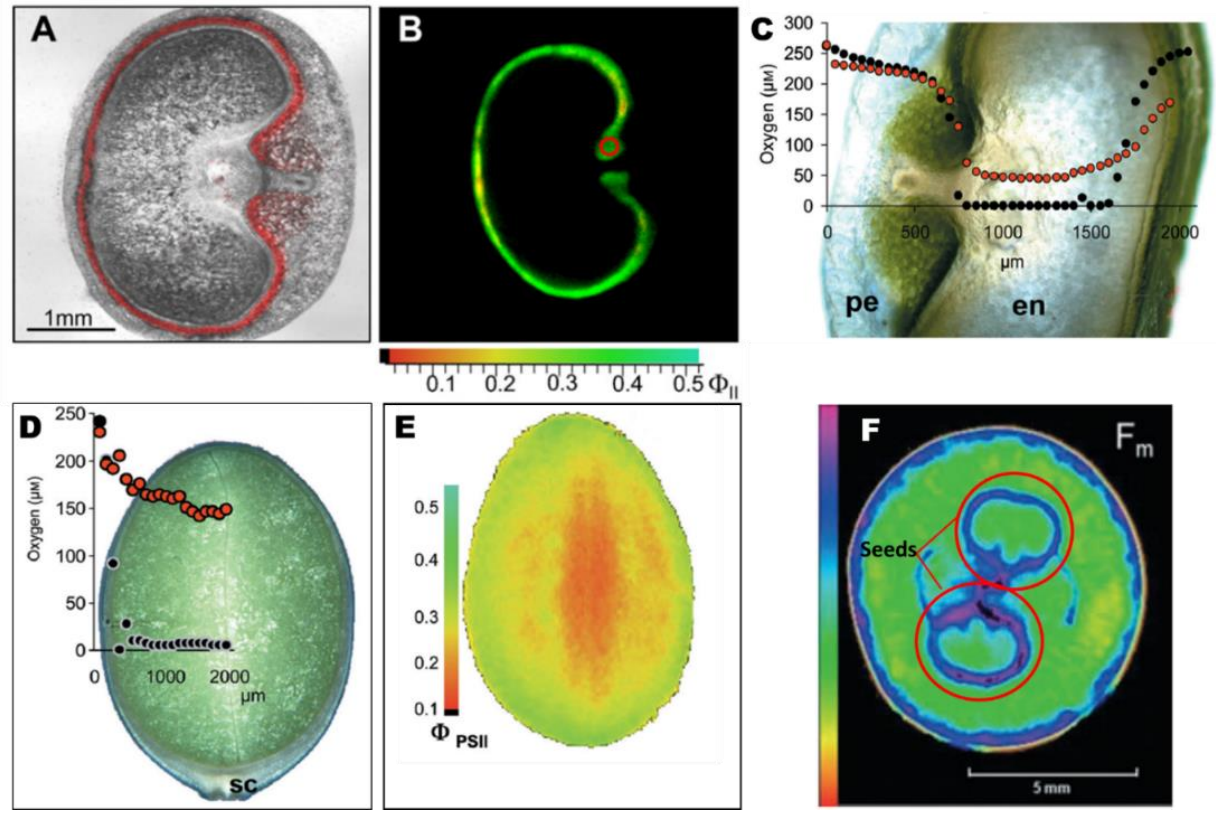


**Figure 2.3.** Representative oxygen maps and photosynthetic parameters of green seeds (**A** to **C** - barley; **D** and **E -** soybean; **F -** grape berry seeds). **(A)** Chlorophyll red auto-fluorescence in a barley seed cross-section performed by Confocal Laser Scanning Microscopy. **(B)** Image of effective quantum yield of PSII of a cross-section of barley caryopsis 12 days after pollination. Oxygen maps for barley seeds **(C)** and for soybean **(D)**, measured in either light (red circles) or dark (black circles), throughout pericarp (pe) and endosperm (en) and seed coat (sc). **(E)** Image of the effective quantum yield of PSII measured in soybean. **(F)** Image of maximum fluorescence of a grape berry cross-section**.** Adapted from Borisjuk and Rolletschek (2009), Breia et al. (2013) and Tschiersch et al. (2011).

Legume seeds, such as soybean [*Glycine max* (L.) Merril], present an embryo that turns green at the early stages of development. Approximately only 10 % of incident light is available to embryo surface (Rolletschek et al., 2005b). However, soybean embryos exhibit specialized chloroplasts with high grana stacking. The oxygen maps of soybean (Figure 2.3D) shows that the seed coat presents high concentrations of oxygen (at early storage stage), but these values decrease to minimum levels within the endospermal liquid. Furthermore, and when measured in the dark, the oxygen concentration within embryo is much lower (2 µM) comparatively to that under light conditions (220 µM) (Figure 2.3D) (Rolletschek et al., 2005b). At late-storage stage, soybean embryos have less oxygen concentration and a lower variation (in the same conditions), suggesting a decline in the capacity to balance oxygen consumption with its supply (Rolletschek et al., 2005b). Pulse amplitude modulated (PAM) fluorescence analysis allowed to confirm this situation. In fact, the effective quantum yield of PSII showed a homogeneous pattern for small embryos at the early storage stage, but at the mid-/late storage stage

(Figure 2.3E), there was a gradient declining towards the interior of the embryo, suggesting a gradual loss of photosynthetic ability (Borisjuk et al., 2005).

More recently, studies in grape berry using chlorophyll fluorescence imaging PAM demonstrate that, besides the exocarp, also the outer integument of the green seed had a very high fluorescence signal (Figure 2.3F) corresponding to high effective quantum efficiencies of PSII (Breia et al., 2013). Later on, it was shown that the seed integument had higher values for photochemical efficiency and capacity at green stages of development and then suffer a decrease in these parameters at later stages (Garrido et al., 2018; Garrido et al., 2019). Thus, and despite the increase in volume of the grape berry along developmental stages, seeds can receive diffuse transmitted light, as already reported by Aschan and Pfanz (2003), allowing photosynthetic activity at later stages even if at lower levels. This photosynthetic activity can provide the $O_2$ necessary to avoid the hypoxia that exist in grape berries (Xiao et al., 2018).

Additional research in developing seeds (soybean, rapeseed and oilseed rape) suggested that the photosynthetic activity can supply the energy (ATP) and reduction power (NADPH) necessary for lipid biosynthesis, storage metabolism and redox modulation of biosynthetic enzymes (Ruuska et al., 2004; Borisjuk et al., 2005; Goffman et al., 2005; Rolletschek et al., 2005b).

#### 2.4.2.2. Intermediates from photosynthesis used on seeds' metabolism and RuBisCO as a $CO_2$ rescue mechanism

Seeds have also high concentrations of internal $CO_2$ (Goffman et al., 2004). During embryogenesis, seeds receive photoassimilates from the phloem that are used for the synthesis of reserves, being this metabolic pathway characterized by the conversion of sucrose to pyruvate, through glycolysis, which is then transformed by pyruvate dehydrogenase (PDH) in acetyl-CoA. This is the main precursor of fatty acid biosynthesis, which are then used towards triacylglycerides or triacylglycerols synthesis (TAG, or storage lipids or oils) (Schwender et al., 2004). This conversion of sugars results in the loss of carbon, in the form of $CO_2$, for each acetyl-CoA unit produced. According to Ruuska et al. (2004), RuBisCO provides another route for fixation of $CO_2$ released by the PDH. Corroborating this view, from a study with embryos of *Brassica napus* L. (oilseed rape), Schwender et al. (2004) described a new metabolic pathway, in which RuBisCO acts without the Calvin-Benson cycle, in a mechanism previously unknown (Figure 2.4).

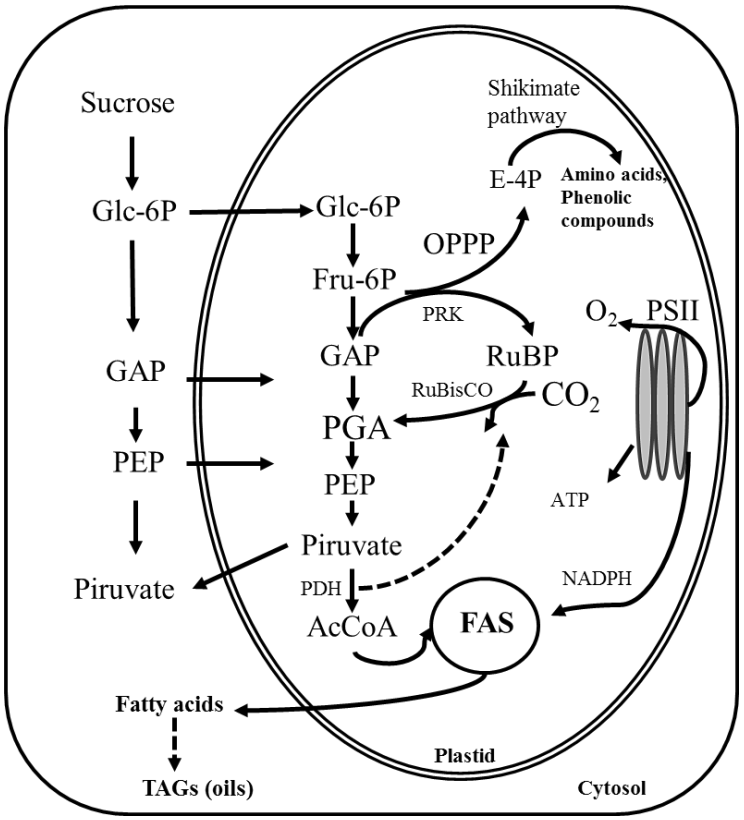


**Figure 2.4.** Metabolic pathway of transformation of sugars into fatty acids and highlighting the possible contributes of photosynthesis. Abbreviations: Glc-6-P, glucose-6-phosphate; GAP, glyceraldehydes-3-phosphate; PEP, phosphoenolpyruvate; Fru-6P, fructose-6-phosphate; PGA, 3-phosphoglyceric acid; PDH, pyruvate dehydrogenase; AcCoA, acetyl-CoA; FAS, fatty acid synthesis; OPPP, oxidative pentose phosphate pathway; PRK, phosphoribulokinase; E4P, erythrose-4-phosphate; RuBP, ribulose-1,5-bisphosphate. TAGs, triacylglycerides. Adapted from Schwender et al. (2004), Ruuska et al. (2004) and Allen et al. (2009).

This metabolic pathway involves three main steps. The first, is the conversion of hexose- and triose-phosphates to ribulose-1,5-bisphosphate by the non-oxidative reactions of the oxidative pentose phosphate pathway (OPPP) together with phosphoribulokinase (PRK); secondly, the conversion of RuBP and $CO_2$ (most of which is produced by pyruvate dehydrogenase - PDH) to 3-phosphoglyceric acid (PGA) by RuBisCO; and third, the metabolism of PGA to pyruvate and acetyl-CoA, and then to fatty acids. Therefore, this mechanism avoids loss of carbon by recycling internal $CO_2$, as well as provides several intermediates of the Calvin-Benson cycle for distinct pathways of metabolism.

Schwender et al. (2004) showed that this new pathway provides 20 % more acetyl-CoA for fatty acid synthesis and resulted in 40 % less loss of carbon as $CO_2$, comparatively to glycolysis. Similarly, Allen et al. (2009) verified that in soybean embryos, RuBisCO re-fixed 11 % of the $CO_2$ released by lipid synthesis and TCA cycle, and consequently the Calvin-Benson cycle contributed for the carbon economy. Moreover, in rapeseeds, at the early stage of oil accumulation, the fatty acids content was higher in seeds exposed to light when compared to those from dark conditions (Ruuska et al., 2004). Likewise, our recent results

showed that seeds from grape berries grown at two contrasting light microclimates at canopy, had distinct photosynthetic activity specially at green stage (Garrido et al., 2019), and also had different lipid profile (Garrido et al., manuscript in preparation). In addition, the relative expression of *VvRuBisCO* in those seeds was maintained in high levels during berry ripening, in values similar to than seen for exocarps (Garrido et al., submitted), suggesting that RuBisCO may have a function in re-assimilating the locally released $CO_2$ in these photosynthetic grape berry tissues, and thus contributing with intermediates for several metabolomic pathways, like storage lipids.

Additionally, intermediates of OPPP pathway, as erythrose-4-phosphate, together with PEP can be used for amino acid synthesis, which in turn can contribute as precursors for the shikimate pathway. This biosynthetic pathway is responsible for the production of phenylalanine, as well as other aromatic amino acids, such as tyrosine and tryptophan. Phenylalanine in the first substrate of a key secondary metabolic pathway, the phenylpropanoid pathway (Vogt, 2010).

### 2.4.3. Photosynthesis and the vascular system of fruits

Until later stages of development, sugars are transported from source organs to the fruits, through the phloem. The unloading of these sugars can occur by symplastic or apoplastic pathways, being the latter a mechanism dependent of energy (Lemoine et al., 2013). The photochemical phase of photosynthesis can provide the energy (ATP) necessary for the apoplastic unloading (Keller, 2015), a energetically demanding process.

Hibberd and Quick (2002) verified that the cells surrounding the peripheral vascular system of stems and petioles of tomato, have chlorophyll and photosynthetic activity (C4-type). Similar results were observed in young shoots and chlorenchyma of lignified shoots of grapevine (Tikhonov et al., 2017). Although it was refereed that, in grape berries, the unloading is predominantly symplastic in early stages of development, becoming the apoplastic pathway dominant with the onset of ripening (Zhang et al., 2006), our previous work in white grape berries tissues using the chlorophyll fluorescence imaging technique (Figure 2.3F), showed that there was a high concentration of chlorophyll/photochemical activity in perivascular cells (peripheral dorsal system) (Breia et al., 2013), consistent with a role of photosynthesis in the process.

Another interesting putative role for photosynthesis in the vascular bundles was observed in cucumber fruit, where the PEPC is present (Sui et al., 2017). The refixation of respiratory $CO_2$ by PEPC, followed by the synthesis of organic acids that can accumulate in the vacuole, can provide the turgor pressure necessary for cell expansion and fruit growth (Sui et al., 2017). The same was suggested for

tomato (Carrara et al., 2001; Guillet et al., 2002). In grape berry, immunohistochemical studies verified that PEPC is present in the vasculature, in the parenchyma cells of the pericarp and within the developing seeds, leading to the hypothesis that PEPC may play a role in the metabolism of the assimilates after their delivery to the fruit (Famiani et al., 2000), and also into the seed at the appropriate time during its development (Walker et al., 1999).

### 2.4.4. Ecological advantages of green fruits and seeds

The advantages or "services" of fruit photosynthesis can also be examined from an ecological point of view, and we can start this topic by saying that a green-colored fruit, located among green leaves, does not facilitate zoochoric seed dispersal. In fact, fruit color influences their ability to be dispersed by animals, namely birds (Cazetta et al., 2009). In this manner, the ripening process composes the mutualistic relationship between fleshy-fruit plants and seed-disperser animals (Duan et al., 2014). Besides the visibility conditions and the visual aptitude of the receiver, the visual signal detectability is determined by its contrast against the background, that is, the conspicuousness of the signal (Cazetta et al., 2009). Young fruits are usually green, but upon ripening they range from red, blue, yellow and orange to green and brown. Indeed, chlorophyll degradation is accompanied by a conversion of chloroplasts into chromoplasts that progressively accumulate high levels of carotenoids. However, some fruits are green when they are ripe ("green-ripe" or chlorophyllous), for example, cucumber, kiwi, pea, pepper and green apple varieties (Cipollini and Levey, 1991).

The dispersion of seeds of fleshy fruits is commonly done by animals (zoochory), that follow their visual and olfactory senses (Schaefer, 2011). For diurnal seed dispersers, such as birds, the visual stimuli are particularly important, whereas many of the nocturnal seed dispersers, such as bats and other mammals, rely to a large extent on olfactory stimuli. In case of the "green-ripe" fruits they are less conspicuous than fruits of other colors, being only dispersed by mammals, especially bats (Cipollini and Levey, 1991). In this way, the fruits that maintained green until maturation, tend to be dispersed by a limited variety of frugivores. Cipollini and Levey (1991) suggested that the ecological advantage of the "green-ripe" fruits consist in their ability to photosynthesize, reducing costs of production and enriching the pulp in nutrient rewards for frugivores. The same authors verified that at high light levels the green-ripe fruits have a positive carbon balance, but at low light levels the high rates of respiration often result in net $CO_2$ losses. These high respiration rates could be due to the maintenance of photosynthetic pigments and proteins, but also because these fruits are generally larger (Cipollini and Levey, 1991). The "green-ripe" fruits also present higher seed and pulp mass, fact that evidence an offer quantitatively larger

for dispersers. In addition, the presence of strong odors in these fruits, it is also indicative that some secondary metabolic pathways may be active, eventually fueled by photosynthesis.

The photosynthetic tissues of the grape berries, that is, the exocarp and seed outer integument (Breia et al., 2013), contain high levels of tannins, especially accumulated at green stage, decreasing afterwards (Garrido et al., 2021). Moreover, the two distinct light microclimate at canopy (LL and HL, low and high light, respectively) also led to a differences in the total flavan-3-ols in the exocarp at green and mature stage (Garrido et al., 2021). From a sensory standpoint, these compounds are correlated with astringency and bitterness of the wine (Ma et al., 2014), and in terms of ecophysiological functions, they confer protection against fungal and bacterial pathogens, insect pests and larger herbivores (as reviewed by Barbehenn and Constabel, 2011).

## 2.5. Concluding remarks

Fruits are vital organs in plant sexual reproduction and indispensable foods in our diet. Its quality depends on the physiological and biochemical mechanisms, that at the end contribute for the accumulation of several compounds. The main purpose of the present review was to compile and integrate information on anatomical, physiological and biochemical features and constraints of different types of fruits and of their seeds, in order to unveil potential functions of the photosynthesis performed by some of their green tissues. With this bibliographic research work, a diversity of photosynthetic mechanisms, or in some cases, the utilization of part of "old" photosynthetic routes in new solutions to meet tissue-specific demands or alleviate biochemical pressures were discussed. The relevance of photosynthesis in fruits is clearly supported by many findings.

Overall, the evidences point to some roles and functions, such as: firstly, the importance of energy (ATP) and reducing power (NADPH), both produced during the photochemical phase, and which can be important for energy-dependent biochemical processes, like the unloading of sugars from the vascular system or even the synthesis of fatty acids; secondly, the production of oxygen that can prevent and/or reduce the hypoxia inside of seeds; thirdly, the re-fixation of respiratory $CO_2$ by RuBisCO in the Calvin-Benson cycle or by PEPC (C4-type photosynthesis); and finally, the carbon skeletons, derived from Calvin-Benson cycle, that can fuel pathways of primary and secondary metabolism, and thus, contribute for the organoleptic properties of fruits.

This is a fascinating topic studied for over 40 years and from very different perspectives, from the molecular to the ecological one. However, due to fruit photosynthesis' complexity, in space (different tissues, compartments, fruit geometries), in time (variation associated with development) and in its

dependency on environmental factors, information is still lacking or conflicting. If using controlled simple systems is needed to better identify cause and effect mechanistic relationships, integrative global approaches like systems biology and omic networks are increasingly crucial. As an example of the first, *in vitro* cultures established from different tissues with distinct photosynthetic competences, would allow to study a more direct link between photosynthetic activity and specific changes at molecular and biochemical levels. Moreover, it is also important to understand the complexities of coordination between the environmental stresses, photosynthesis and stress responses, since it will represent key information for crops improvement in the context of the ongoing climatic changes.

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# Chapter 3

## Influence of foliar kaolin application and irrigation on photosynthetic activity of grape berries

The work presented in this chapter was published:

Garrido, A., Serôdio, J., De Vos, R., Conde, A. and Cunha, A. (2019). Influence of foliar kaolin application and irrigation on photosynthetic activity of grape berries. *Agronomy*, *9*(11), 685. https://doi.org/10.3390/agronomy9110685

Supplementary Materials are available online:
https://www.mdpi.com/2073-4395/9/11/685/s1

Author Contributions: Conceptualization, A.G., A.C. (Artur Conde), R.V., and A.C. (Ana Cunha) Methodology, A.G., J.S., and A.C. (Ana Cunha) Formal analysis, A.G. and A.C. (Ana Cunha). Investigation, A.G. and A.C. (Ana Cunha). Resources, J.S. and R.V. Writing—original draft preparation, A.G. Writing—review and editing, A.C. (Ana Cunha), J.S., R.V., and A.C. (Artur Conde). Supervision, A.C. (Artur Conde), R.V., and A.C. (Ana Cunha) Project administration, A.C. (Ana Cunha).

**Abstract**

Climate changes may cause severe impacts both on grapevine and berry development. Foliar application of kaolin has been suggested as a mitigation strategy to cope with stress caused by excessive heat/radiation absorbed by leaves and grape berry clusters. However, its effect on the light micro-environment inside the canopy and clusters, as well as on the acclimation status and physiological responses of the grape berries, is unclear. The main objective of this work was to evaluate the effect of foliar kaolin application on the photosynthetic activity of the exocarp and seeds, which are the main photosynthetically active berry tissues. For this purpose, berries from high light (HL) and low light (LL) microclimates in the canopy, from kaolin-treated and non-treated, irrigated and non-irrigated plants, were collected at three developmental stages. Photochemical and non-photochemical efficiencies of both tissues were obtained by a pulse amplitude modulated chlorophyll fluorescence imaging analysis. The maximum quantum efficiency ($F_v/F_m$) data for green HL-grown berries suggest that kaolin application can protect the berry exocarp from light stress. At the mature stage, exocarps of LL grapes from irrigated plants treated with kaolin presented higher $F_v/F_m$ and relative electron transport rates ($rETR_{200}$) than those without kaolin. However, for the seeds, a negative interaction between kaolin and irrigation were observed especially in HL grapes. These results highlight the impact of foliar kaolin application on the photosynthetic performance of grape berries growing under different light microclimates and irrigation regimes, throughout the season. This provides insights for a more case-oriented application of this mitigation strategy on grapevines.

**Keywords:** light microclimates, mitigation strategies, kaolin, irrigation, *Vitis vinifera* L., grape berry tissues, pulse amplitude modulated (PAM) fluorometry, photosynthesis, photosynthetic pigments.

## 3.1. Introduction

Viticulture is a historically important agronomic and socio-economic sector in Portugal. According to the last report from the International Organization of Vine and Wine (OIV), Portugal is the 11th world and 5th European wine producer (OIV, 2018). With 14 winemaking regions distributed throughout the country, the Vinhos Verdes or Minho region, as well as the Douro and Alentejo, are the major contributors for national exports and growth of this sector (Lavrador da Silva et al., 2018).

Grapevine is influenced by a complex and interacting system commonly called *terroir*, which, according to the OIV (OIV, 2010), includes specific soil, topography, climate, landscape characteristics and biodiversity features, and interaction with applied vitivini-cultural practices. This complex system

influences the canopy microclimate and grapevine physiology and development and, consequently, grape berry quality and the organoleptic properties of its wine, which is typical of each region.

Currently, climate change projections point to a particularly pronounced temperature variation, with an overall increase of up to 3.7 °C by the end of this century, compared to the 1985–2005 reference period (IPCC, 2019). These temperature changes will have great impacts in the Mediterranean wine regions (Ferrise et al., 2013). According to recent investigations using very high resolution bioclimatic zoning, both temperature and dryness are predicted to increase in several economically important Portuguese viticulture regions, including the Vinhos Verdes region (Fraga et al., 2014). Therefore, Portuguese vineyards will be subject to increased stress due to the interaction of the existing high radiation levels with the foreseen elevated air temperature and drought, which, all together, can have high impact on grapevine phenology, physiology, and productivity. Several of these climate impacts have already been reported, such as: earlier phenological timings and shortenings of the grapevine growing season (Fraga et al., 2016), sunburns in leaves and grape berries (Leeuwen and Darriet, 2016), reduction of stomatal conductance and decrease of photosynthetic rates, either by stomatal and non-stomatal limitations (Moutinho-Pereira et al., 2007), appearance and/or intensification of grapevine-related pests and diseases (Bois et al., 2017; Caffarra et al., 2012), increased grape sugar concentrations that lead to higher wine alcohol levels, lower acidities, and modification of varietal aroma compounds (Mira de Orduña, 2010), and higher inter-annual yield and wine production variability (Cunha and Richter, 2016).

In order to mitigate these adverse climate effects, new short-term measures have recently been implemented in Portuguese vineyards, such as smart irrigation (Costa et al., 2016; Fraga et al., 2018) and foliar application of kaolin (Brito et al., 2019). Vineyards are not traditionally irrigated and there are even restrictions on this practice in some regions, such as the Douro region (Costa et al., 2016). However, according to a recent projection model, a 10 % reduction in grapevine yield is expected in the Minho region if irrigation is not applied (Fraga et al., 2018). Kaolin is a white, chemically inert, and non-toxic clay material ($Al_2Si_2O_5(OH)_4$) that can reflect radiation, including photosynthetically active (PAR), ultraviolet (UV), and infrared radiation (IR) (Brito et al., 2019). Foliar application of this mineral has become a cost-efficient mitigation strategy to cope with water stress and excessive heat/radiation absorbed by leaves and grape berry clusters, which also proves effective in alleviating negative impacts on grapevines (Conde et al., 2016, 2018; Dinis et al., 2016a, 2016b, 2018). However, the amount and spectral quality of light intercepted by leaves and transmitted/ reflected into the canopy, crucial factors for leaf, and fruit physiology and development (Poni et al., 2018) are also important aspects to consider when mitigation practices are used.

Previous work done by our group, using pulse amplitude modulated (PAM) chlorophyll fluorescence imaging, has mapped grape berry photosynthesis at a histological level, and revealed both the exocarp and the seed outer integument as the main photosynthetically competent tissues (Breia et al., 2013). More recently, we have studied the photosynthetic performance of grape berry tissues from clusters growing at three distinct light microclimates in the canopy and observed microclimate-related differences in their photosynthetic capacity and acclimation status (Garrido et al., 2018). This led to the hypothesis that, if a specific viticulture practice changes the light reaching the clusters, and alters its light microclimate, it may impact the photosynthetic activity of berry tissues and associated tissue-specific biochemical processes. In fact, foliar kaolin application may have direct implications on light distribution at the whole canopy level, and irrigation is an indirect one, through increased vegetative growth. For instance, it has already been shown that kaolin application generally reduces the photosynthetic rates of individual leaves in other agricultural crops (e.g., apple, almond, and walnut canopies) (Le Grange et al., 2004; Wünsche et al., 2004), due to a 20–40 % increase in the reflection of PAR (Rosati et al., 2006). However, the photosynthesis of the whole canopy remained unaffected or even increased (9 %), because of the better light distribution within the canopy (Glenn and Puterka, 2007; Glenn, 2009; Rosati et al., 2007). In another study, decreased photosynthesis was observed in the inner leaves of irrigated grapevines due to higher vegetative growth (Escalona et al., 2003). While the function of photosynthesis in fruits is still poorly understood, it can be linked with primary and secondary metabolomic pathways (Cocaliadis et al., 2014; Obiadalla-Ali et al., 2004). Therefore, any effect on photosynthesis may impact grape berry development and composition.

Therefore, the main objective of the present work was to evaluate the effects of foliar kaolin application on the photosynthetic activity of grape berry tissues from clusters growing at two distinct microclimates, which include high light (HL) and low light (LL) microclimates, of irrigated and non-irrigated grapevines, during the season.

## 3.2. Materials and Methods

### 3.2.1. Site Description, Applied Treatments, and Sampling

Grape berry samples were collected in 2018 from field-grown 'Alvarinho' cultivar grapevines (*Vitis vinifera* L.) in the commercial vineyard *Quinta Cova da Raposa* in the Demarcated Region of Vinho Verde, Braga, Portugal (41°34'16.4" N, 8°23'42.0" W). The vineyard is managed by following standard cultural practices applied in organic farming, and is arranged in terraces along a granitic hillside with high drainage. The vine training system applied for this cultivar follows the settings of Sylvoz (Simple Ascending

and Recumbent Cord). The sector selected for the trial was located on a hill with NW-SE orientation and the vineyard rows with a NE-SW orientation. The treatments applied were: kaolin (K) and non-kaolin (NK) application on leaves, and irrigation (I) and non-irrigation (NI), in a complete factorial design (four treatment combinations) with two blocks, each with three to four vines per combination treatment (Figure 3.1B). A suspension of 5 % (w/v in water) kaolin (EPAGRO®, Sunprotect, Alverca do Ribatejo, Portugal) was applied twice on leaves on both sides of the rows. On July 6 and 27, corresponding to four weeks after anthesis (WAA) or BBCH-73 (BBCH-scale used for grapes by Lorenz et al. (1995) and seven WAA or BBCH-77, respectively. Irrigation of half of the plants, started on July 26 (seven WAA, BBCH-77), (Figure 3.1A,B). Water was applied by drip irrigation with one dripper per vine and a drip line placed approximately 80 cm above the soil. Irrigation occurred every three days, once a day either early in the morning or late in the afternoon, for 2 h, with an average dripper capacity of 5.5 ± 1.6 L $h^{-1}$ ($n$ = 12 randomly selected drippers, ± SD). Clusters with contrasting light exposure were also selected to harvest grape berries during their development. These were called low light (LL) and high light (HL) clusters. LL clusters grew in the shaded inner zones of the canopy, which were exposed only to diffuse, reflected, and transmitted light, while HL clusters were exposed to direct or reflected sunlight most of the day. Six independent sub-clusters (three per block), each containing 15–20 grape berries, were collected randomly from clusters growing at each of the experimental conditions (four treatments × 2 microclimates) from the southeast side of rows. Berries were harvested in the morning (9–10 a.m.) at three distinct developmental stages: Green (16 July, 6 WAA, BBCH-75), *Véraison* (29 August, 12 WAA, BBCH-83), and Mature (17 September, 15 WAA, BBCH-89). The material was transported in refrigerated boxes to the Center for Environmental and Marine Studies (CESAM) laboratory and used within 2–6 h for imaging fluorometry experiments. For other assays, berries were immediately frozen in liquid nitrogen and stored at −80 °C.

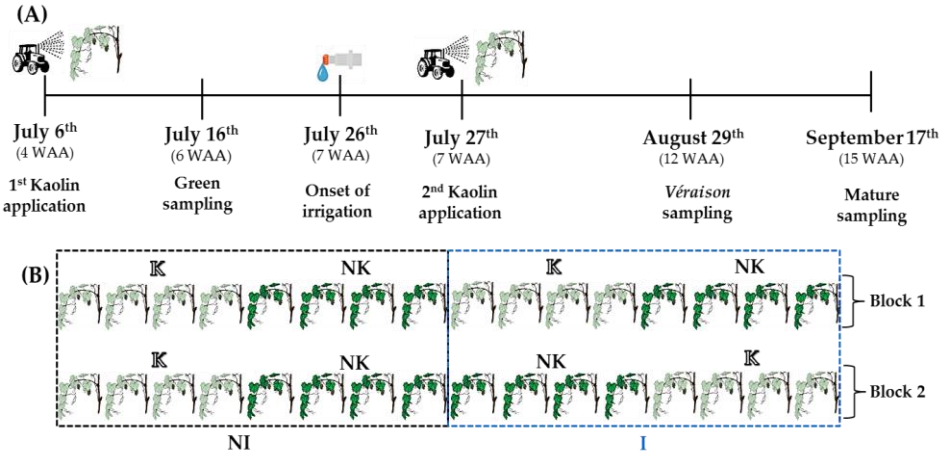


**Figure 3.1. (A)** Timeline of the grape growing season depicting the sampling times, foliar kaolin application dates, and the onset of irrigation. **(B)** Scheme of treatment combinations applied in the field: irrigation (I)/ non-irrigation (NI) x kaolin (K)/ non-kaolin (NK). (WAA - weeks after anthesis).

### 3.2.2. Light Intensity and Temperature Measurements for Microclimate Characterization

In order to characterize the microclimates in the vicinity of the growing clusters of all experimental conditions, light intensities and temperatures were registered on cloudless days (mean of 1500 ± 300 µmol photons $m^{-2}$ $s^{-1}$), between 15 h and 17 h, at green and mature stages, as described by Garrido et al. (2018). The light intensity (µmol photons $m^{-2}$ $s^{-1}$) was measured with a radiometer (LI-COR, LI-250 Light Meter, Lincoln, NE, USA) and the temperature (°C) was measured with an infrared digital thermometer (Infrared, DT8380, Beijing, China). Both parameters were determined in the frontal region of the clusters (LL and HL), at the southeast side of the row, and in full sun-exposed leaves. The devices were placed perpendicularly to the plant organ (cluster or leaf). The light sensor was placed on the organ surface facing outward, which registered the light intensity reaching at this point, and the thermometer was pointing to the organ at a distance of about 15 cm, which registered an average organ temperature. Sixteen replicate measurements per treatment were considered on randomly selected vines.

### 3.2.3. Kaolin Film Transmittance and Reflectance

Transmittance and reflectance spectra were obtained using a spectrometer (USB2000-VIS-NIR, grating #3, Ocean Optics, Duiven, The Netherlands), connected to a 400 mm-diameter fiber optic (QP400-2-VIS/NIR-BX; Ocean Optics), and recorded using the spectral acquisition software Spectra Suite (Ocean Optics, https://oceanoptics.com/). The transmittance spectrum was obtained by spreading a 5 % (w/v) kaolin suspension prepared in 70 % ethanol (allowing fast evaporation to prevent solvent interference), over a glass plate, which simulated particle distributions similar to those observed in the field. A halogen lamp was placed underneath to illuminate the spectrometer sensor positioned 3 cm above the glass plate. Different spectra were obtained from different areas randomly ($n$ = 3). The reflectance spectrum was obtained, according to Dinis et al. (2018), by pointing the fiber optics perpendicularly to the surface of collected leaves illuminated by the same halogen lamp. Three independent spectra were obtained from different leaf regions of both kaolin-treated and non-treated leaves. Transmittance and reflectance spectra were recorded for the 370–900 nm spectral range, with a spectral resolution of 0.33 nm. The transmittance spectra were expressed as a percentage of the controls (glass). Reflectance spectra were normalized to the spectrum reflected from a reference white panel (WS-1-SL Spectralon Reference Standard, Ocean Optics).

### 3.2.4. Chlorophyll Fluorescence Analysis

The photosynthetic activity of grape berry tissues was assessed as described by Garrido et al. (2018). For this, an imaging chlorophyll fluorometer was used (*Open FluorCAM 800 MF;* Photon Systems Instruments, Drásov, Czech Republic), which was comprised of four 13 × 13 cm LED panels emitting red light (emission peak at 621 nm, 40-nm bandwidth) and a 2/3 inch CCD camera (CCD381, Beijing, China) with a F1.2 (2.8–6 mm) objective. Two of the LED panels provided modulated measuring light (<0.1 μmol $m^{-2}$ $s^{-1}$) and the other two provided saturating pulses (>7500 μmol $m^{-2}$ $s^{-1}$, 0.8 s). Chlorophyll fluorescence images were captured and processed using FluorCam7 software (Photon Systems Instruments, Drásov, Czech Republic).

In a dark cabinet, exocarps and seeds were separated from dark-adapted berries and disposed in 8 × 8-well plates filled with water. Two independent plates were prepared for each microclimate (LL and HL), with each comprising all treatments and tissues. Exocarps and seeds were placed alternately in three rows each, using two columns per treatment, in a total of 12 biological replicates per condition and tissue ($n$ = 3 × 2 × 2 = 12). Each plate was subjected to the experiments described below.

The maximum quantum efficiency of photosystem II [$F_v/F_m = (F_m - F_0)/F_m$], which is a chlorophyll fluorescence parameter that reflects the probability of electrons being transferred from the PSII reaction center for the transport chain of electrons by quanta absorbed (Baker, 2008; Schreiber, 2004), was computed following a saturation pulse (SP). The isolated tissues were then acclimated to an actinic light (AL) of 200 μmol photons $m^{-2}$ $s^{-1}$ for 15 min, and, after a new SP, the effective quantum yield of PSII [$\Phi_{II} = (F'_m - F_s)/F'_m$] was computed. This parameter correlated with the quantum yield of $CO_2$ fixation in a wide range of physiological conditions (Genty et al., 1989). From $\Phi_{II}$ and PFR (photosynthetic photon fluence rates) (200 μmol photons $m^{-2}$ $s^{-1}$), the relative electron transport rate through PSII ($rETR_{200} = \Phi_{II} \times$ PFR) was calculated. Then, tissues were exposed to 1500 μmol photons $m^{-2}$ $s^{-1}$ for 15 min, with an SP being applied every 3 min. The last $F'_m$ values (at 15 min) were used to calculate the non-photochemical quenching [$NPQ = (F_m - F'_m)/F'_m$].

### 3.2.5. Analysis of Chlorophylls and Carotenoids by High Performance Liquid Chromatography Coupled to A Photodiode Array Detector (HPLC-PDA)

The extraction procedure was adapted from Fraser et al. (2000). Freeze-dried material (20 mg) of grape berry tissues, which includes exocarp and seed, was extracted in 1.8 mL of chloroform/methanol (1:1) (chloroform - Emsure®, Darmstadt, Germany, methanol - Biosolve®, Dieuze, France) with both 0.1 % (w/v) butylated hydroxytoluene (BHT, Sigma®, Zwijndrecht, The Netherlands) as an antioxidant and Sudan

1 (0.5 μg $mL^{-1}$) as the internal standard (IS). The samples were vortexed (10 s), kept on ice for 30 min (vortexed in between), and then sonicated for 15 min (Branson®, 3510 Ultrasonic Cleaner, Danbury, CT, USA). These steps were performed twice. After that, the samples were centrifuged at 16,100×*g* (Eppendorf®, Centrifuge 5415 R, Hamburg, Germany) and the supernatant (approx. 1200 μL) was transferred to a new Eppendorf tube with a perforated lid. The samples were dried for 1 h in a Speedvac (Savant®, SC100, Schiphol, The Netherlands) and then stored at −80 °C until the next steps. Prior to high performance liquid chromatography (HPLC) analysis, samples were dissolved in 200 μL ethylacetate solution containing 0.1 % (w/v) BHT, sonicated (10 min), and then centrifuged as above. Samples were protected from light and kept on ice during all of these procedures. The supernatant (180 μL) was transferred to amber-colored 2 mL HPLC vials with a glass insert and sealed.

The HPLC-PDA procedure was adapted from Mokochinski et al. (2018). The samples (20 μL) were analyzed using an HPLC (Waters Alliance e2695 Separations Module, Milford, MA, USA) coupled to a photodiode array detector (PDA) (Waters 2996) over the 240 to 700 nm UV/Vis range. Separation was performed on a reverse-phase $C_{30}$ column (250 × 4.6 mm i.d., S-5 μm - YMC Carotenoid, Komatsu, Japan) kept at 35 °C with a flow rate of 1.0 mL $min^{-1}$. The compounds were identified based on comparisons of retention times and absorption spectra (240 to 700 nm) with authentic standards.

### 3.2.6. Statistical Analysis

Results were statistically analyzed using Analysis of Variance tests (two-way ANOVA), followed by post hoc multiple comparisons using the Bonferroni test whenever the factors had significant effects (GraphPad Prism version 5.00 for Windows, GraphPad Software, La Jolla, CA, USA). Significant differences ($p \leq 0.05$) between sample groups are indicated with different letters. Notation with an asterisk means that only one factor (kaolin or irrigation) was significant.

## 3.3. Results and Discussion

### 3.3.1. Climatic Conditions and Microclimate Characteristics

In order to characterize the climate during the growing season at the study site (Braga), we used the official information available from the *Instituto Português do Mar e da Atmosfera* (IPMA) (IPMA, 2019), to determine the temperatures and total precipitation during 2018 (Figure S3.1). This growing season was atypical from a climatic point of view, with a relatively cold and extremely dry winter, which caused a delay of sprouting/flowering for two to three weeks (IVV, 2018), and a relatively cold spring with rainy periods during the vegetative growth of the grapevines.

To characterize the microclimates for the LL and HL berry clusters (two *a priori* selected distinct light microclimates within the canopy), measurements of light intensities and temperatures were performed at the cluster level, at two time points during the growing season, i.e., when the berries were still green (green stage) and, two months later, when the berries were at their mature stage of ripening (Figure 3.2 and Figure 3.3). Figure 3.2 depicts the average light intensities at LL and HL clusters growing under the different experimental conditions: i.e., irrigation/non irrigation (Figure 3.2a,c) and with kaolin/without kaolin (Figure 3.2b,d). The two microclimates were clearly distinct at both ripening stages, with HL clusters receiving about three-fold more light than LL clusters. At the green stage, i.e., before the onset of irrigation (Figure 3.1A), no significant differences were detected between the two sets of plots assigned to the subsequent irrigation experiment (four plots for irrigated (I) plants, i.e., 2 × NK-I and 2 × K-I) and four plots for non-irrigated (NI) control plants (2 × NK-NI and 2 × K-NI), see Figure 3.1B) (Figure 3.2a), which reveals that there were no plot-related effects on microclimate light intensity. At this early ripening stage, and with the adopted measurement procedure, no differences were detected with respect to light intensities reaching the berry clusters due to foliar kaolin application (Figure 3.2b). However, at a mature stage, both irrigation and kaolin had a small but significant effect on the light intensity reaching the HL clusters (Figure 3.2c,d). Irrigation slightly reduced the light intensity, likely due to the better vegetative growth of the plants, while foliar kaolin application increased it, likely due to an increased light reflection to both the interior and lower levels of the canopy. In the LL clusters, these effects of irrigation and kaolin on light intensity were not observed, at this time of day.

HL grapes consistently experienced higher temperatures than LL ones (Figure 3.3), and both I and K treatments exerted significant and contrasting effects on this microclimate parameter, mainly at the mature stage. Again, and consistent with what was observed and discussed above for light intensity, no effect was detected for I treatment on the grape berry temperature at the green stage (before the onset of irrigation) (Figure 3.3a).

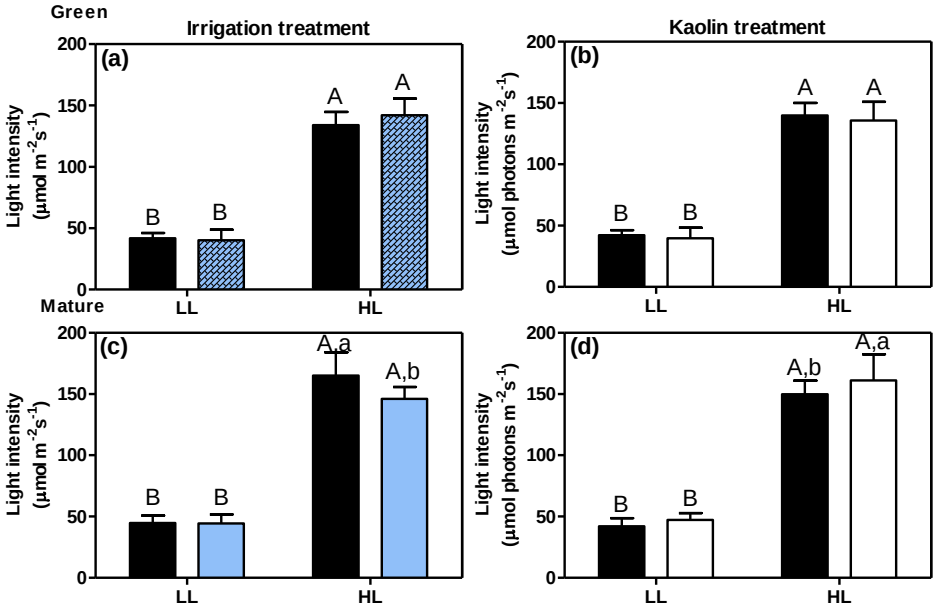


**Figure 3.2.** Light intensities received by LL and HL clusters at the green stage (**a**, **b**) and the mature stage (**c**, **d**), for plants with irrigation (blue columns, note: the textured blue columns at green stage i.e., before the onset of irrigation, represent the measurements in the plots that were later irrigated) and foliar kaolin application (white columns). Black columns correspond to the respective controls. Values represent means with a standard deviation ($n$ = 16 plants). Statistical notation: per ripening stage, different capital letters refer to significant differences (two-way ANOVA, $p \leq 0.05$) between the two light microclimates within the same plant treatment, and different lowercase letters for differences between treatments within each light microclimate. If the respective factor did not have a significant effect, the lowercase letters were omitted.

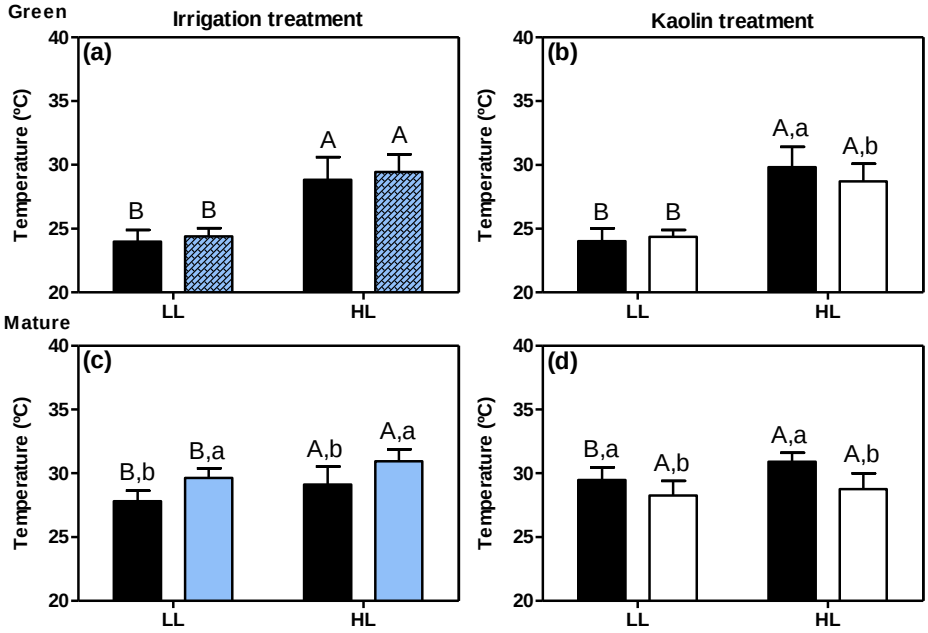


**Figure 3.3.** Temperatures of LL and HL clusters at the green stage (**a**, **b**) and the mature stage (**c**, **d**), for plants with irrigation (blue columns, note: the textured blue columns at the green stage i.e., before the onset of irrigation, represent the measurements in the plots that were later irrigated) and foliar kaolin application (white columns). Black columns correspond to the respective controls. Values represent means with standard deviation

($n$ = 16 plants). Statistical notation: per ripening stage, different capital letters refer to significant differences (two-way ANOVA, $p \leq 0.05$) between the two light microclimates within the same plant treatment, and different lowercase letters for differences between treatments within each light microclimate. If the respective factor did not have a significant effect, the lowercase letters were omitted.

The kaolin application led to a significant decrease in the temperature of the HL clusters at both green and mature stages (e.g., at this latter stage—from 31 °C to 28.7 °C), and of the LL clusters at the mature stage only (e.g., from 29.5 °C to 28 °C) (Figure 3.3b,d). The fact that LL clusters' temperature at the green stage were not affected by kaolin was likely related to the relatively low air temperature at this time of the growing season (early July, Figure S3.1). Thus, kaolin application on the leaves may increase the incident light by increasing the light reflection inside the canopy (Figure 3.2d), while maintaining a cooler microclimate for the growing berries, especially during the hot summer days, independently of the irrigation regime. This demonstrates one of the advantages of this mitigation strategy at the grape berry level. The kaolin solution sprayed on the leaves also resulted in leaf temperature reduction (Figure S3.2d). The present results are in agreement with previous studies in both grapevine leaves and berries (Oliveira, 2018; Shellie and King, 2013), as well as other crops (Rosati et al., 2006; Rosati et al., 2007). Therefore, it is likely that kaolin applied to leaves provides cooler temperatures throughout the grapevine by reducing the total amount of radiation transmitted into the canopy. This is also shown by thermal imaging in apple trees (Glenn, 2009). Furthermore, different training systems of the vineyard might influence the light intensities and temperatures inside the canopy (Kraus et al., 2018; Reynolds and Heuvel, 2009). For instance, the vine canopy was denser in our previous study in 2015 (Garrido et al., 2018), which resulted in an LL microclimate characterized by much lower light reaching the clusters compared to that in the present study, with major impacts on grape berry photosynthetic competence.

At the mature stage, plant irrigation resulted in a significant increase in the grape temperature of both LL and HL clusters (Figure 3.3c). This increase was unexpected, since a previous study reported lower berry temperature as a result of irrigation, rather than a higher temperature (dos Santos et al., 2007). This response is very interesting and clearly, additional studies, which are controlling/measuring the soil temperature in the rhizosphere, are required to determine the effect of irrigation and the irrigation procedure on the temperature of grape clusters.

### 3.3.2. Kaolin Film Transmittance and Reflectance Spectral Properties

To characterize the potential effects of kaolin applied to the leaves with regard to light intensity and quality, we performed transmittance and reflectance studies. For this purpose, the transmittance

spectrum of a film of kaolin solution (5 % w/v) on a glass plate was determined (Figure 3.4a). Although a high percentage of most photosynthetically active radiation (PAR) wavelengths is transmitted by the kaolin film, the blue light range is the least transmitted. The reflectance spectra obtained for leaves of grape plants sprayed with and without kaolin are represented in Figure 3.4b. Our results showed a relevant percentage of PAR is reflected by this white mineral, as compared to non-spayed (NK) vine leaves, rather than exclusively or mainly reflecting in the ultraviolet (UV) and infrared radiation (IR) ranges (Sharma et al., 2015). Additionally, kaolin was more efficient in reflecting UV light than IR light (in the measured ranges). These results are in accordance with previous studies using grapevine leaves (Dinis et al., 2018) and other crops (Glenn et al., 2002; Glenn, 2012; Rosati et al., 2006; Rosati et al., 2007). Thus, the beneficial effect of kaolin application is related to the reflection of excess radiation outwards, which reduces the risk of light stress-induced damage to leaves and fruit (Sharma et al., 2015), while transmitting a very significant proportion of PAR.

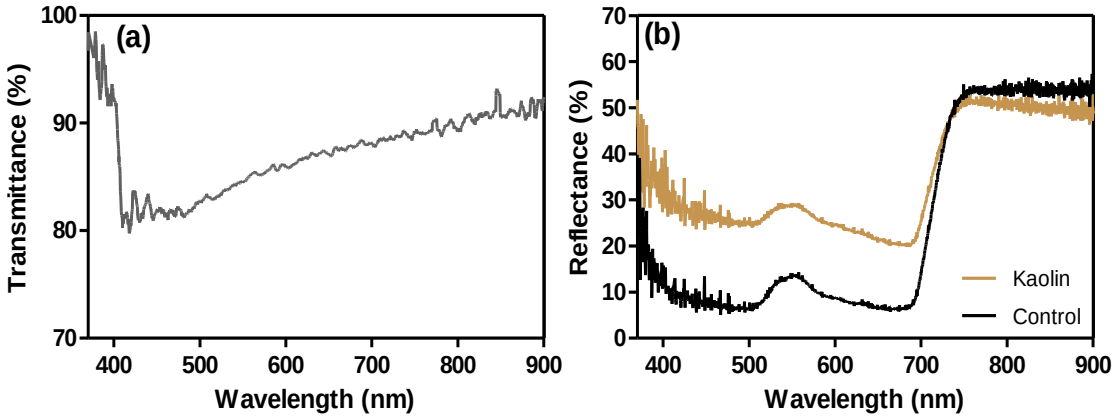


**Figure 3.4.** Transmittance (%) spectrum (**a**) of a kaolin suspension (5 % w/v) and reflectance (%) spectra (**b**) of leaves with and without kaolin (control) ($n$ = 3).

Together, these results call attention to the fact that foliar kaolin application may directly impact the photosynthesis of the sprayed leaves but also have an indirect effect on the non-sprayed leaves and grape berry clusters inside the canopy.

### 3.3.3. Effects on Berry Photosynthesis and Photosynthetic Pigments

#### 3.3.3.1. Maximum Quantum Efficiency of PSII

The maximum quantum efficiency of PSII ($F_v/F_m$) was determined *ex planta* under controlled conditions, using both exocarps and seeds from grape berries grown under the different treatments, microclimates, and three ripening stages (Figure 3.5). At the green stage, $F_v/F_m$ was similar in both berry tissues (Figure 3.5a,b). Upon further ripening, the exocarp kept its $F_v/F_m$ values (~0.7), while the seeds

showed a significant decrease in this parameter, which reached values around 0.4–0.5 at the mature stage, which was in accordance with what was reported by Garrido et al. (2018).

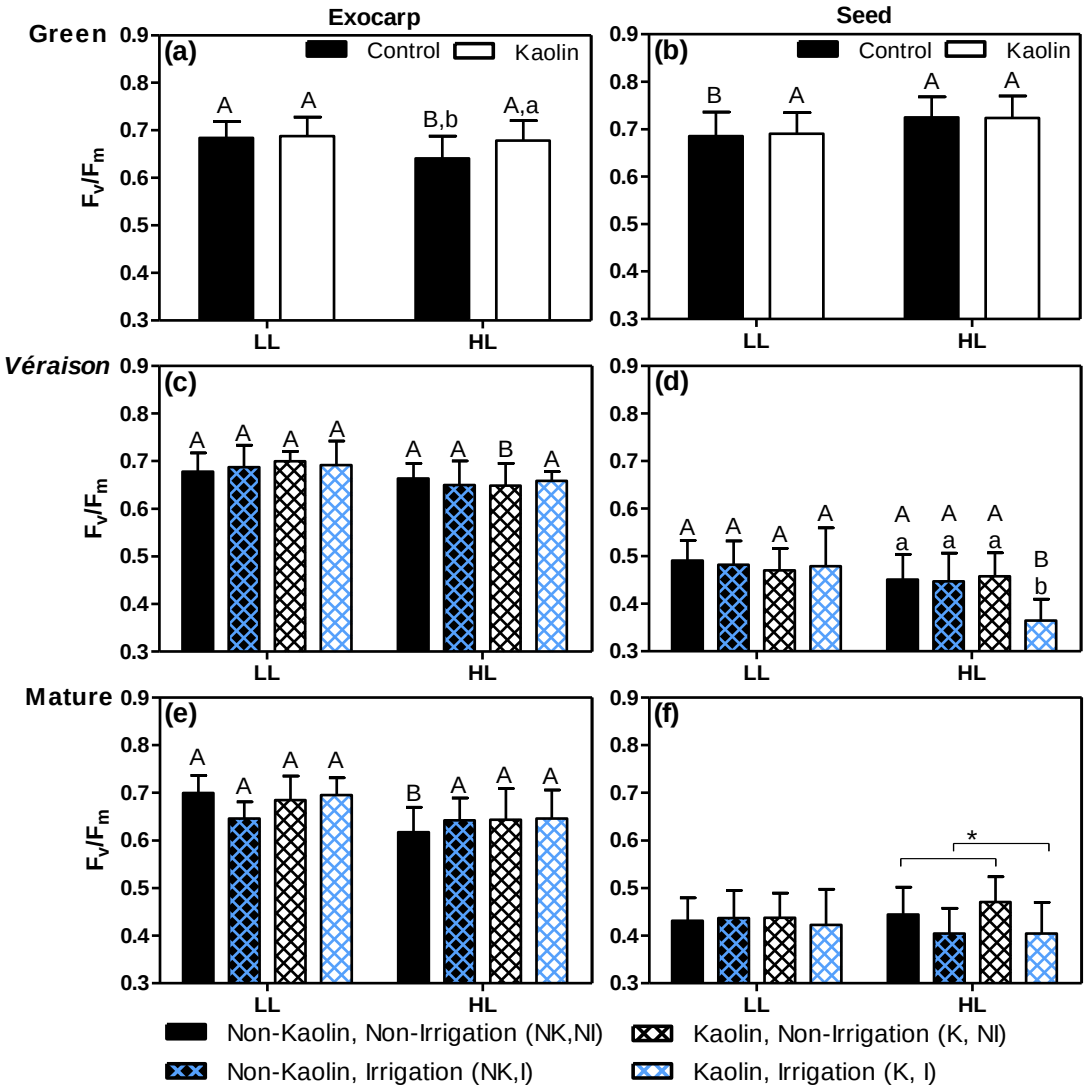


**Figure 3.5.** Maximum quantum efficiency of PSII ($F_v/F_m$) mean values ($n$ = 12–24 berries, +SD) of exocarps and seed integuments obtained from dark-adapted LL and HL grape berries grown under the four combinations of the two treatments applied: irrigation (I)/ non-irrigation (NI) × kaolin (K)/ non-kaolin (NK). Samples were collected at three development stages (green, *véraison*, and mature). Statistical notation: for each developmental stage, capital letters refer to differences between light microclimates within the same treatment combination, and lowercase letters refer to differences between treatment combinations within each light microclimate (mean values with a common letter were not significantly different). When capital and lowercase letters are omitted, the respective factor did not have a significant effect (two-way ANOVA, $p \leq 0.05$). Notation with an asterisk means that only one factor (kaolin or irrigation) was significant.

The cluster microclimate (LL vs. HL) had a significant effect on the $F_v/F_m$ values in both tissues and all developmental stages, with the exception of seeds at the mature stage, but exocarps and seed integuments responded differently to a light microclimate (Garrido et al., 2018). At the green stage, the

exocarps from berries under control conditions, showed lower $F_v/F_m$ values in HL clusters, while their seeds showed significantly higher values than those from LL berries (Figure 3.5a,b). This was likely due to their inner location where the light transmitted through the skin and flesh tissues reaches values as low as 2 % of the incident photon flux density (PFD) (Aschan and Pfanz, 2003), which eventually translates a light limitation effect in LL clusters. These microclimate effects were more or less maintained in exocarps upon subsequent ripening (see NK + NI Figure 3.5a,c,e, while not being significant in *véraison*), while, in seeds, the difference between LL and HL clusters disappeared (Figure 3.5b,d,f), likely related to the (large) intrinsic ripening-dependent decrease in photosynthetic competence of this tissue.

Both kaolin and irrigation treatments of plants differentially influenced the $F_v/F_m$ values of the two berry tissues, with the seeds globally more responsive than exocarps, particularly to the irrigation treatment, which induced a significant effect at *véraison* and mature stages (Figure 3.5d,f). On the other hand, at these latter stages, no effects from treatments were detected on the $F_v/F_m$ of exocarps. This tissue only responded to kaolin treatment at the green stage, where HL berries showed an increment (6 %) on $F_v/F_m$ by kaolin application (Figure 3.5a). In fact, the decrease in $F_v/F_m$ values of exocarps when comparing LL with HL berries at a green stage in control conditions (Figure 3.5a) had already been observed (Garrido et al., 2018), which revealed that microclimates with higher luminosity can decrease $F_v/F_m$ values of exocarps at this stage. Together, these results suggest that foliar kaolin may protect the berry exocarp from excess light at a stage when the grape berry photosynthetic phenotype is still developing (Garrido et al., 2018). In HL seeds, the most prominent effect was a decrease in $F_v/F_m$ in irrigated-treated plants (Figure 3.5d,f). However, at *véraison*, this effect was observed only in kaolin-treated plants (Figure 3.5d). This apparent paradoxical effect may be related with the increased temperatures observed in irrigated grape berries in the hottest months (Figure 3.3c and Figure S3.1). In fact, the inhibitory effect of higher temperatures on $F_v/F_m$ was already reported for grapevine leaves (Kadir et al., 2007).

#### 3.3.3.2. Relative Rate of Electron Transport Through PSII ($rETR_{200}$)

The relative electron transport rate through photosystem II (rETR) was determined after acclimation of the berry tissues to an AL intensity of 200 μmol photons $m^{-2}$ $s^{-1}$ ($rETR_{200}$) in order to simulate average field light conditions.

At the green stage, the $rETR_{200}$ was higher in HL grape berries than in LL berries (both in control and kaolin-treated plants), especially in seeds (Figure 3.6a,b). Interestingly, a positive influence of kaolin application was observed in LL exocarps at this stage (Figure 3.6a), which suggests that more PAR reflected by kaolin is reaching the inside of the canopy, which may improve the exocarp photosynthesis

of berries in shaded microclimates. At the *véraison* stage, this positive effect of kaolin was not detected, while it was evident at the mature stage (Figure 3.6e). In addition, a clear decrease in $rETR_{200}$ was observed in seeds, especially in HL clusters ($p < 0.0001$) during ripening (Figure 3.6d,d,f), which is similar to the results obtained for $F_v/F_m$. In addition, in seeds from HL-grown berries at the *véraison* stage of irrigated plants, the kaolin treatment led to a significant decrease in $rETR_{200}$ (Figure 3.6d: NK, I compared to K, I), which was observed for the parameter $F_v/F_m$ (Figure 3.5d). This pointed to a possible effect of berry temperature on seed photosynthesis. No reports were found for the effect of temperature on grape berry photosynthesis, but a continuous four-day exposure to high temperatures (38–40 °C) led to a decrease in photosynthetic activity of grapevine leaves (Greer and Weston, 2010; Luo et al., 2011; Xiao et al., 2017).

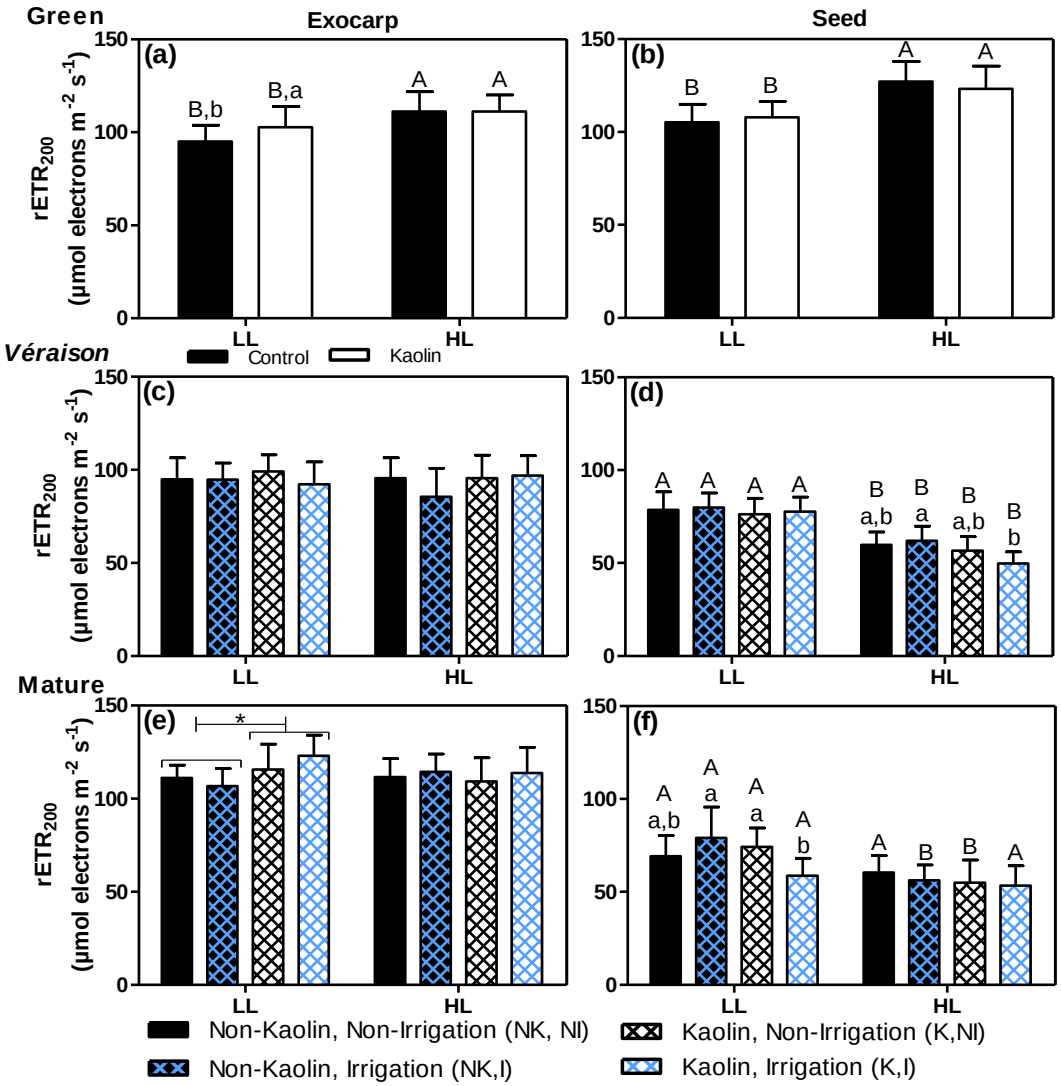


**Figure 3.6.** Mean values ($n$ = 12–24 berries, +SD) of relative rate of electron transport through PSII at 200 µmol photons $m^{-2}$ $s^{-1}$ ($rETR_{200}$). All the microclimate conditions, treatment combinations and statistical information are the same as in Figure 3.5.

### 3.3.3.3. Non-Photochemical Quenching

A major component of non-photochemical quenching (NPQ) is the primary protective mechanism against light-induced photoinhibition, which involves various processes dissipating excessive non-radiative energy (Krause and Jahns, 2007), including the xanthophylls cycle (e.g., as shown in grapevines, (Medrano et al., 2002)) and phosphorylation/dephosphorylation of light harvesting complexes (Szabó et al., 2005).

The NPQ results are represented in Figure 3.7. When comparing the berry tissues, it can be concluded that the exocarp tissue consistently exhibits roughly two-fold higher NPQ values than seeds. This result suggests that the exocarp exhibits more developed mechanisms of photoprotection, which is consistent with the fact that it is an external, more exposed tissue. During berry ripening, NPQ values decreased in both tissues, especially in seeds. Seeds attained very low NPQ values at later stages, which is in line with their $F_v/F_m$ (Figure 3.5b,d,f) and $rETR_{200}$ (Figure 3.6d,d,f) profiles, which likely reflect the normal ripening-related loss of photosynthetic functioning of seeds. For the exocarps, dissipation or quenching mechanisms other than NPQ may explain the result, since this tissue maintains high photosynthetic activity until the mature stage (Figure 3.6a,c,e). Accumulation of carotenoids in white berries ('Sauvignon Blanc') was also increased in response to increasing levels of solar light in the canopy, which shows that the berries utilize these photosynthesis-related pigments in photo-acclimation responses and/or as "sunscreens" (Joubert et al., 2016).

Regarding the effect of treatments, we found that the foliar kaolin application promoted lower NPQ in HL exocarps at the green stage, when compared with their NK controls (Figure 3.7a), which suggests that kaolin helps to protect these HL berries from excessive radiation absorption. This is similar to the $F_v/F_m$ results (Figure 3.5a).

At the *véraison* stage, both HL exocarps and HL seeds showed increased NPQ in irrigated-treated grape berries (Figure 3.7c,d). In LL exocarps, NPQ was also increased in K,I berries, when compared to the remaining treatment combinations (Figure 3.7c). This increase in NPQ values of grape berries in irrigated plants, was lost at the mature stage, even though it is important to note that NPQ values were already very low at this stage (Figure 3.7e,f). This irrigation-related feature had already been observed with other parameters and is discussed above. The increased temperatures registered at later developmental stages of grape berries (Figure 3.3c), can impose other limitations or impairments, and, thus, recruit more energy-dissipation by NPQ, and eventually by other dissipative mechanisms.

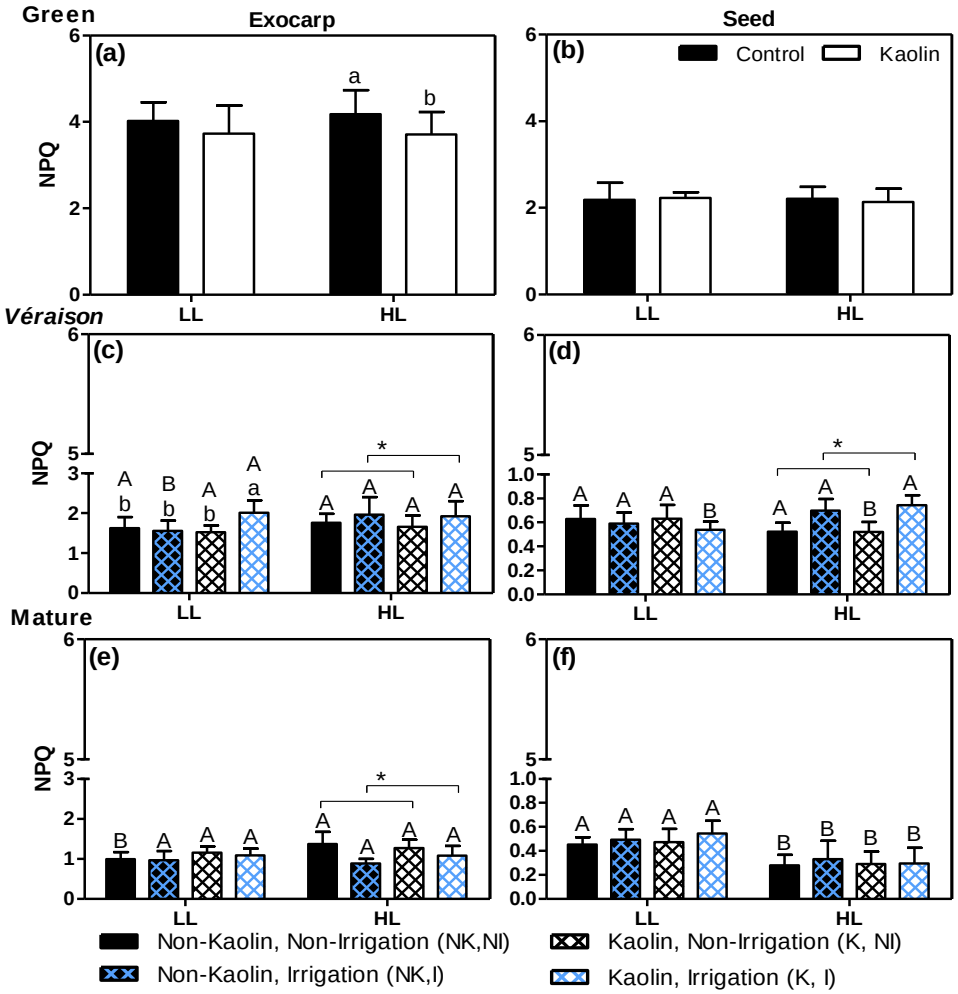


**Figure 3.7.** Non-photochemical quenching (NPQ) mean values ($n$ = 12–24 berries, +SD). All the microclimate conditions, treatment combinations, and statistical information are the same as in Figure 3.5.

### 3.3.3.4. Photosynthetic Pigments

To better evaluate the impact of foliar kaolin application on the light microclimate of grape berry clusters and its relationship with berry photosynthesis, and non-photochemical mechanisms, photosynthetic pigments were quantified in exocarps and seeds of both LL and HL-exposed grapes. Results obtained for the green stage are depicted in Figure 3.8 (for later stages, see Supplementary Materials). At control conditions, the HL berries had higher levels of both chlorophylls and carotenoids than LL berries, in both tissues. Additionally, and in line with the $rETR_{200}$ results (Figure 3.6a), kaolin application resulted in a marked increase by 26 % in chlorophylls and 82 % in carotenoids content in exocarps from LL berries (Figure 3.8a,c), which support the idea that more light reached the inner parts of the kaolin-sprayed canopy. This is fundamental to build the photosynthetic machinery (Tikkanen et al., 2012). During ripening, the photosynthetic pigments decrease in both tissues and especially in the seed integuments (Figure S3.3 and Figure S3.4) and no consistent and conspicuous effects by combined mitigation treatments were observed (Figure S3.3).

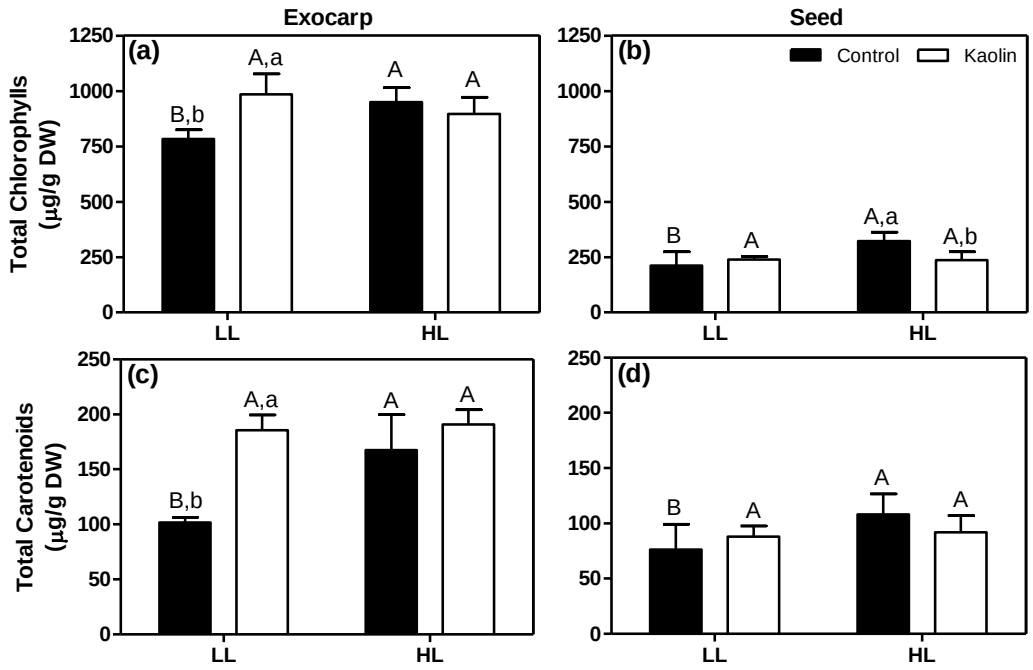


**Figure 3.8.** Chlorophylls (**a, b**) and carotenoids (**c, d**) concentration mean values ($n$ = 3, +SD) of exocarps and seeds obtained from LL and HL grape berries grown under non-kaolin (black columns) and kaolin (white columns) application, and collected at the green stage. Statistical notation: different capital letters refer to significant differences (two-way ANOVA, $p \leq 0.05$) between the two light microclimates within the same plant treatment, and different lowercase letters to differences between treatments within each light microclimate. If the respective factor did not have a significant effect, the letters were omitted.

In addition, and supporting the view discussed above, the higher grape berry temperature was registered in irrigated treatments at later developmental stages (Figure 3.3c), by imposing physiological impairments. The temperature recruits more energy-dissipation by NPQ (Figure 3.7), which is the fact that carotenoids contents (Figure S3.4), but not chlorophylls (Figure S3.3), were also increased by irrigation treatment, for both tissues at the *véraison* stage.

Overall, the results obtained by pulse amplitude modulated fluorometry showed that, for the external tissue, exocarp, and foliar kaolin application led to an increase of $F_v/F_m$ (Figure 3.5a, HL), $rETR_{200}$ (Figure 3.6a,e LL), and a reduction in non-photochemical quenching (Figure 3.7a, HL). To our best knowledge, this is the first work assessing the impact of foliar kaolin application on photochemical and non-photochemical functions in grape berries. Recently, it was verified that grapevine leaves with kaolin display the same response, i.e., an increase in $F_v/F_m$, $\Phi_{II}$, and ETR, and a decrease in NPQ (Dinis et al., 2018; Frioni et al., 2019). Similar results were also reported for olive leaves (Brito et al., 2019). In this way, and in terms of photochemical processes, those kaolin-treated leaves have lower photo-inhibitory damage (Dinis et al., 2016b; Maxwell and Johnson, 2000), and the open PSII reaction centers captured the light absorbed by PSII antenna more efficiently (Baker, 2008; Dinis et al., 2016b). This response was

likely due to a reduced loss of excitation energy by thermal dissipation, which could compete with its transference to PSII reaction centers, as shown by the lower NPQ values (Baker, 2008; Dinis et al., 2016b).

For exocarps of grape berries growing in inner parts of the canopy (LL microclimate), the photosynthetic results revealed that foliar kaolin application, may cause an extra "sunscreen" effect, and did not have a negative effect on those parameters, which we conjectured in our previous work (Garrido et al., 2018). The increased reflection provided by this mineral to inner parts of the canopy allowed good photochemical performance of LL exocarps, which is contrary to what we hypothesized in our previous work (Garrido et al., 2018). This contributes to higher carbon gains at the whole canopy level and also at the fruit level.

Regarding the results for the seed integument (internal organ), the positive effects of kaolin were observed mainly in non-irrigated plants such as an increase in $F_v/F_m$ (Figure 3.5f, HL) and a decrease in NPQ (Figure 3.7d, HL). In more temperate or Mediterranean regions, this seems like a positive effect, but these results also show the importance of the irrigation system. The interaction between kaolin application and irrigation treatments on grapevine leaves have been studied before (Cooley et al., 2008; Glenn et al., 2010; Shellie and Glenn, 2008; Shellie and King, 2013a; 2013b). However, based on our knowledge, no study has approached the impacts on photosynthetic activity at the grape berry level, using chlorophyll fluorescence analysis.

## 3.4. Conclusions

The purpose of the current study was to assess the effects of foliar application of kaolin and irrigation, as abiotic stress mitigation strategies, on the photosynthetic activity of exocarps (skins) and seeds of grape berries growing under different light microclimates in the canopy. One of the most relevant findings was that the kaolin applied to leaves increased the photosynthetic activity of both exocarps and seed integuments of berries growing under low light conditions in the canopy. This is likely due to higher reflection of PAR to the inner zones. We believe, though, that the beneficial effects will depend on the canopy structure and on the incident radiation, with denser canopies and higher radiations conferring higher overall photosynthetic gains. Somewhat puzzling was the observation that seeds of irrigated plants showed lower photosynthetic activities, in the *véraison* and mature stages, especially under kaolin treatment. Several causes may explain this unexpected phenomenon, so more detailed and ad-hoc design studies should be conducted to address this relevant finding.

This comprehensive study provides the first evidence of foliar kaolin application as a procedure allowing the modulation of photosynthesis in the grape berry, but also calls attention to the importance of the irrigation system. In this way, this knowledge can be used by farmers to support their decisions concerning sustainable adaptation strategies applied on vineyards. Research to unveil the function of berry tissues' photosynthesis on the metabolome of the grapes is already underway, which ultimately contributes to the final quality of the fruit and wine.

## 3.5. Supplementary Materials

The following are available online at https://www.mdpi.com/2073-4395/9/11/685/s1.

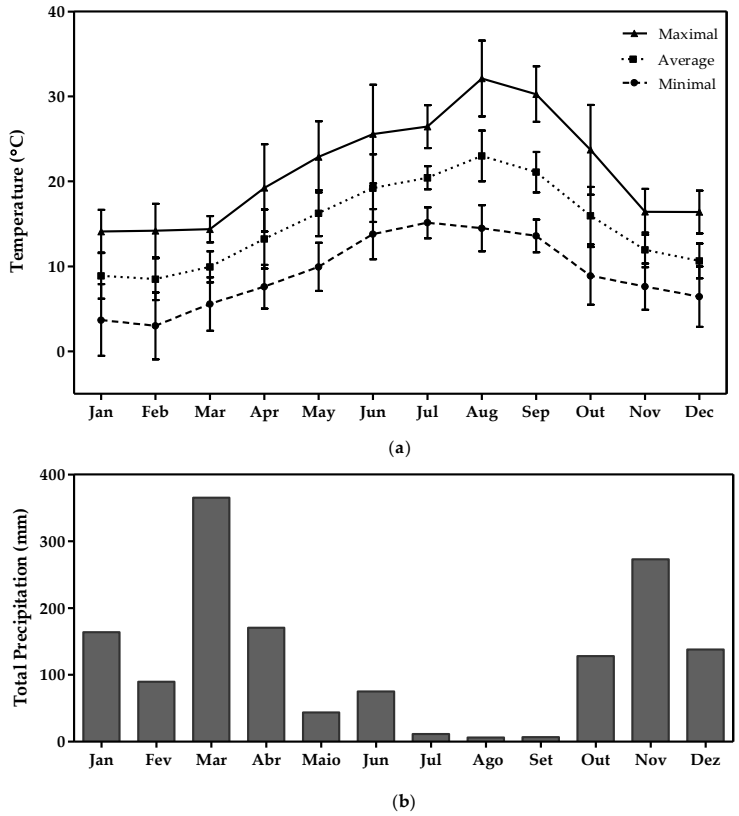


**Figure S3.1.** Meteorological elements from IPMA institute from Braga city. (**a**) Temperature (°C) maximal, average and minimal. (**b**) Total precipitation (mm).

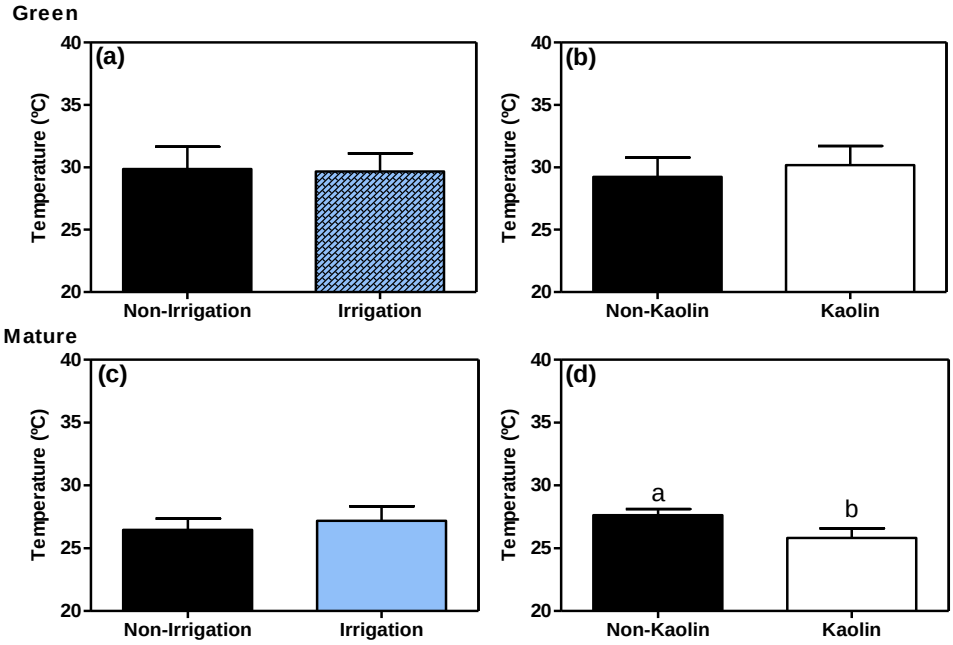


**Figure S3.2.** Temperatures of full exposed leaves at the green stage (**a**, **b**) and the mature stage (**c**, **d**), for plants with irrigation (blue columns; note: the textured blue columns at green stage i.e. before the onset of irrigation, represent the measurements in the plots that were later irrigated) and foliar kaolin application (white columns). Black columns correspond to the respective controls. Values represent means with standard deviation ($n$ = 16 plants). Statistical notation: per ripening stage, different lowercase letters refer to significant differences ($p \leq 0.05$) between treatments. Whenever letters are omitted it means that the respective factor did not have a significant effect.

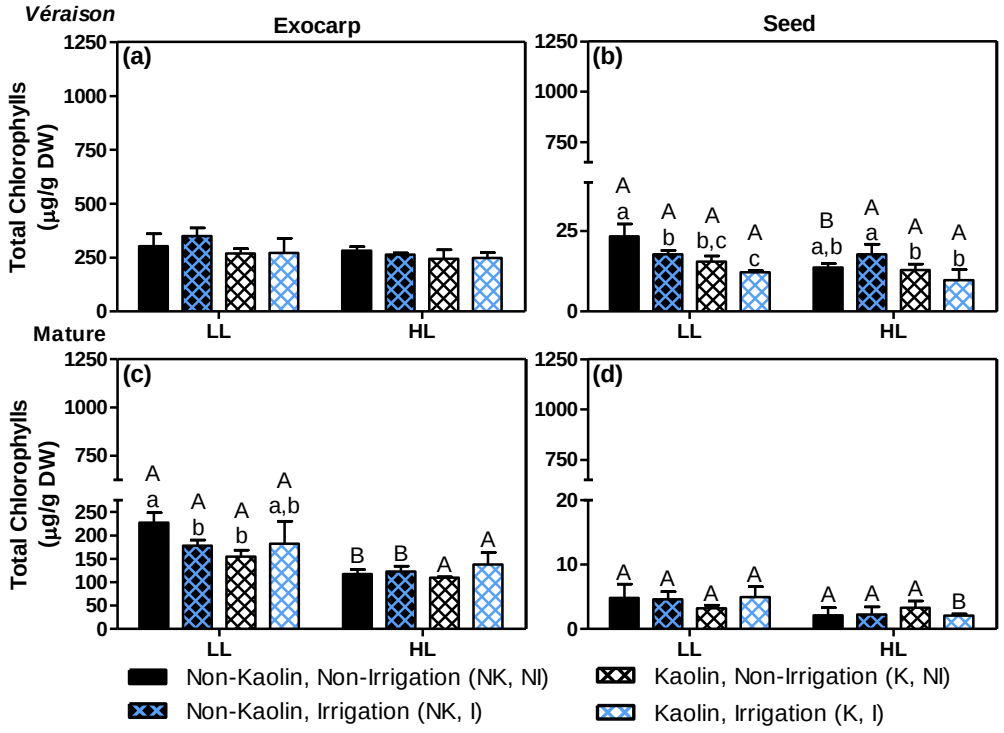


**Figure S3.3.** Chlorophylls concentration mean values ($n$ = 3, +SD) of exocarp and seed obtained from LL and HL grape berries grown under the four combinations of the two treatments applied: irrigation (I)/ non-irrigation

(NI) x kaolin (K)/ non-kaolin (NK). Samples were collected at three development stages (green, *véraison* and mature). Statistical notation: for each developmental stage, capital letters refer to differences between light microclimates within the same treatment combination, and lowercase letter refers to differences between treatment combinations within each light microclimate (mean values with a common letter were not significantly different). When capital and lowercase letters are omitted, the respective factor did not have a significant effect (two-way ANOVA, $p \leq 0.05$).

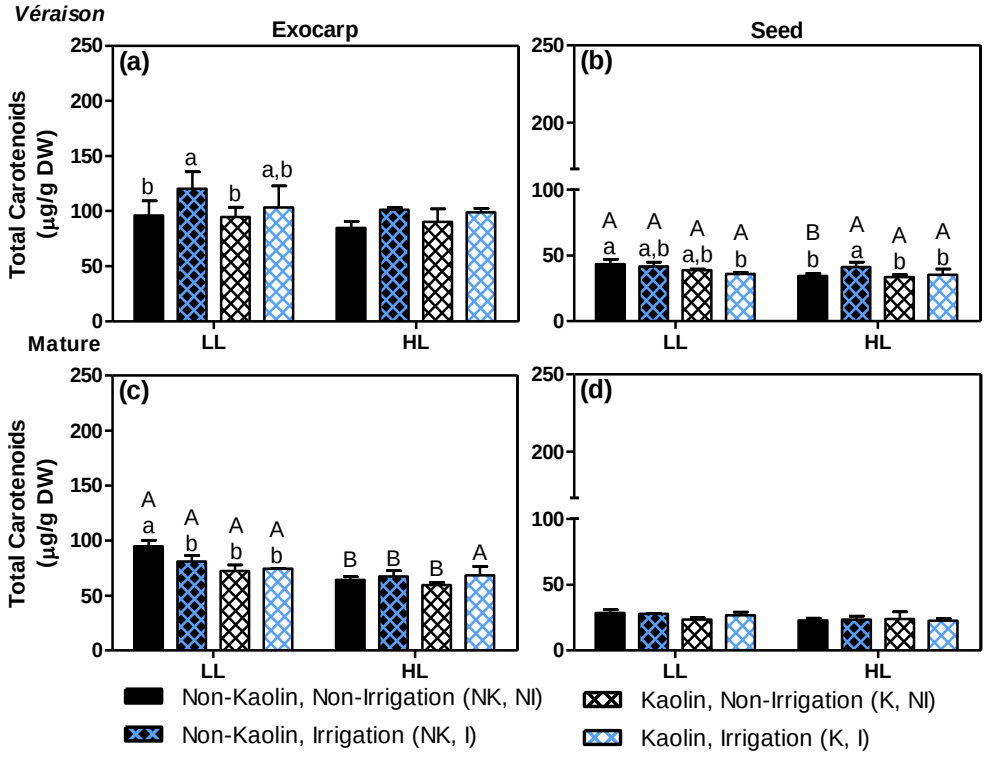


**Figure S3.4.** Carotenoids concentration mean values ($n$ = 3, +SD) of exocarp and seeds. All the microclimate conditions, treatment combinations, and statistical information are the same as in Figure S3.3.

## 3.6. Funding

The FCT-Portuguese Foundation for Science and Technology by the grant provided to Andreia Garrido (PD/BD/128275/2017), under the Doctoral Program "Agricultural Production Chains – from fork to farm" (PD/00122/2012), funded this research and APC.

## 3.7. Acknowledgments

The National Funds by FCT - Portuguese Foundation for Science and Technology, under the strategic programmes UID/AGR/04033/2019 and UID/BIA/04050/2019, and the project "INTERACT - VitalityWine - NORTE-01-0145-FEDER-000017 – funded by Norte2020 supported the work. The FCT and FEDER/COMPETE/POCI - Operational Competitiveness and Internationalization Program, under Project the projects MitiVineDrought – PTDC/BIA-FBT/30341/2017 (POCI-01-0145-FEDER-030341), and POCI-01-0145-FEDER-006958 also supported this work. Artur Conde was supported with a post-doctoral fellow

of the mentioned INTERACT/VitalityWine project with the Reference BPD/UTAD/INTERACT/VW/218/2016, and also supported by a post-doctoral researcher contract/position within the project "MitiVineDrought" (PTDC/BIA-FBT/30341/2017 and POCI-01-0145-FEDER-030341). This work also benefited from the networking activities within the European Union-funded COST Action CA17111 – "INTEGRAPE - Data Integration to maximize the power of omics for grapevine improvement". Authors acknowledge the owner from *Quinta Cova da Raposa*, Manuel Taxa, who provided the samples, Susana Chaves (from CBMA) for her English grammar revision, and also all support given by the Biology Department of the School of Sciences from the University of Minho.

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# Chapter 4

## Metabolomics of photosynthetically active tissues in white grapes: effects of light microclimate and stress mitigation strategies

The work presented in this chapter was published:

Garrido, A., Engel, J., Mumm, R., Conde, A., Cunha, A.* and De Vos, R. C. H.* (2021). Metabolomics of Photosynthetically Active Tissues in White Grapes: Effects of Light Microclimate and Stress Mitigation Strategies. *Metabolites*, *11*(4), 205. https://doi.org/10.3390/metabo11040205

*Equal senior authorship.

Supplementary Materials are available online:
https://www.mdpi.com/article/10.3390/metabo11040205/s1

Author Contributions: Conceptualization, A.G., A.C. (Artur Conde), A.C. (Ana Cunha), and R.C.H.D.V.; methodology, A.G., R.C.H.D.V., J.E., and R.M.; formal analysis, A.G., R.C.H.D.V., J.E., and R.M.; investigation, A.G. and A.C. (Ana Cunha); resources, R.C.H.D.V., R.M.; writing—original draft preparation, A.G.; writing—review and editing, A.C. (Ana Cunha), R.C.H.D.V., A.C. (Artur Conde), J.E., and R.M.; supervision, A.C. (Artur Conde), A.C. (Ana Cunha) and R.C.H.D.V.; project administration, A.C. (Ana Cunha).

**Abstract**

The effects of climate change are becoming a real concern for the viticulture sector, with impacts on both grapevine physiology and the quality of the fresh berries and wine. Short-term mitigation strategies, like foliar kaolin application and smart irrigation regimes, have been implemented to overcome these problems. We previously showed that these strategies also influence the photosynthetic activity of the berries themselves, specifically in the exocarp and seed. In the present work, we assessed the modulating effects of both canopy-light microclimate, kaolin and irrigation treatments on the metabolic profiles of the exocarp and seed, as well as the potential role of berry photosynthesis herein. Berries from the white variety Alvarinho were collected at two contrasting light microclimate positions within the vine canopy (HL—high light and LL—low light) from both irrigated and kaolin-treated plants, and their respective controls, at three fruit developmental stages (green, *véraison* and mature). Untargeted liquid chromatography mass spectrometry (LCMS) profiling of semi-polar extracts followed by multivariate statistical analysis indicate that both the light microclimate and irrigation influenced the level of a series of phenolic compounds, depending on the ripening stage of the berries. Moreover, untargeted gas chromatography mass spectrometry (GCMS) profiling of polar extracts show that amino acid and sugar levels were influenced mainly by the interaction of irrigation and kaolin treatments. The results reveal that both photosynthetically active berry tissues had a distinct metabolic profile in response to the local light microclimate, which suggests a specific role of photosynthesis in these tissues. A higher light intensity within the canopy mainly increased the supply of carbon precursors to the phenylpropanoid/flavonoid pathway, resulting in increased levels of phenolic compounds in the exocarp, while in seeds, light mostly influenced compounds related to carbon storage and seed development. In addition, our work provides new insights into the influence of abiotic stress mitigation strategies on the composition of exocarps and seeds, which are both important tissues for the quality of grape-derived products.

**Keywords:** grape berry tissues, light microclimate, irrigation, kaolin, metabolomics, photosynthesis.

## 4.1. Introduction

The grapevine (*Vitis vinifera* L.) is a perennial woody plant cultivated in many regions worldwide, spreading across temperate to semi-dry areas, but mainly in the latitudes spanning from 30 to 50 degrees (Estreicher, 2017). Viticulture represents an important agronomic activity with high socioeconomic relevance, due to the great diversity of grape derived-products consumption (e.g., table grapes, raisins and wine) (Ali et al., 2010), and its by-products (e.g., pomace, skins and seeds) (Teixeira et al., 2014). According to the International Organization of Vine and Wine (OIV), European viticulture accounts for 60 % of the world's wine production; Portugal is the 11$^{th}$ world and 5$^{th}$ European wine producer (OIV, 2018).

The *terroir*, unique to each wine-growing area, compiles a complex and interacting system of factors including specific soil, topography, climate, landscape characteristics and biodiversity features, in combination with applied viticultural practices (Leeuwen and Seguin, 2006). Together, these features influence the canopy microclimate, grapevine physiology, and, consequently, the grape berry composition and wine quality (Blancquaert et al., 2019). However, the intensification of abiotic stress due to a changing climate is already experienced in the Mediterranean regions, such as extended summer droughts and higher radiation and temperatures (Fraga et al., 2013; Fraga et al., 2014), imposing negative impacts on grapevine phenology, physiology, and productivity, and urges for measures to be taken (Fraga et al., 2019; Mira de Orduña, 2010).

Both short and long term strategies are being implemented in viticulture to maintain or even improve plant productivity and fruit quality under abiotic stress conditions (Bernardo et al., 2018). Long-term strategies encompass the relocating of vineyards to cooler sites or sites with lower solar exposure, and the selection of appropriate rootstocks and breeding for stress resistant varieties (Duchêne, 2016). Short-term measures include some existing viticultural practices, such as canopy management (Reynolds, 2010), vine shadings (Caravia, 2016), the introduction of cover cropping (Parpinello et al., 2019), smart irrigation (Fraga et al., 2018), and foliar kaolin applications (Conde et al., 2018). Kaolin, $Al_2Si_2O_5(OH)_4$, is a white inert clay mineral that reflects solar radiation. Not only does it reflect damaging ultraviolet (UV) and heat-generating infrared radiation (IR), but also photosynthetically active radiation (PAR) in a less significant manner (Garrido et al., 2019). Previous works demonstrated the beneficial effects of kaolin on both grapevine leaves, like a decrease in leaf temperature at midday with a parallel increase in both photosynthetic efficiency and photoassimilate synthesis (Conde et al., 2018; Dinis et al., 2016; Dinis et al., 2018), and the biochemical composition of the berries, including an increase in phenylpropanoids and flavonoids (Conde et al., 2016; Dinis et al., 2016).

Light is a key factor in the physiology of the plant, enabling photosynthesis (Garrido et al., 2018; Garrido et al., 2019) and related processes, including shoot development and biosynthesis of an array of primary and secondary metabolites (Reshef et al., 2017, 2018 and 2019). In fact, photosynthesis is not exclusive to leaves, but it can also occur in other green tissues including reproductive organs like fruits and seeds (Brazel and Ó'Maoileídigh, 2019). We previously characterized the photosynthetic activity of the two most photosynthetic competent grapevine fruit tissues, the exocarp (skin) and seed integuments (Breia et al., 2013), and of berries growing at contrasting light microclimates that naturally occur inside the plant canopy (Garrido et al., 2018; Garrido et al., 2019). The so-called Low Light (LL) grape berry clusters grow in the shaded inner zones of the canopy, where they are exposed to only diffuse, reflected and transmitted light and lower temperatures (approx. 50 μmol photons $m^{-2}$ $s^{-1}$ and a temperature of 26 °C), while the High Light (HL) clusters are exposed to both more direct or reflected sunlight and higher temperatures for the greatest part of the day (approx. 150 μmol photons $m^{-2}$ $s^{-1}$ and a temperature of 30 °C) (Garrido et al., 2019). Overall, our previous studies showed that the exocarp and seeds from HL berries exhibited a significantly higher photosynthetic capacity than the LL berries, especially at their green stage (Garrido et al., 2018; Garrido et al., 2019). Both tissues showed highest maximum quantum efficiency ($F_v/F_m$) and photosynthetic capacity ($ETR_m$) at the green stage, with the exocarp extending its activity up to the mature stage while seed photosynthetic activity was more restricted to the green and *véraison* stages (Garrido et al., 2018; Garrido et al., 2019).

Abiotic stress mitigation strategies like irrigation and foliar kaolin application may have implications on the amount and spectrum of light received by both leaves and fruits: foliar kaolin will directly alter the light reflection inside the canopy (Wünsche et al., 2004), while irrigation may indirectly lead to more shading due to an enhanced vegetative growth (Keller et al., 2016). Previously, we proved that such kaolin treatment also increased the photosynthetic activity of both exocarps and seed integuments of LL berries (Garrido et al., 2019). Irrigation lowered the photosynthetic activities in seeds of HL berries at both *véraison* and mature ripening stages, especially under kaolin treatment (Garrido et al., 2019).

Each grape berry tissue (exocarp, mesocarp and seed) contains numerous compounds (Conde et al., 2007) that are important for wine quality (Garrido and Borges, 2013), such as sugars, organic acids, amino acids, phenolic compounds and conjugated aroma compounds. The exocarp contains most of the flavonoids, which include flavonols such as quercetin glycosides, flavan-3-ols such as catechins, and in red cultivars also anthocyanins such as delphinidin-glycosides (Teixeira et al., 2013). The mesocarp is the primary site for the accumulation of sugars, hydroxycinnamic acids and organic acids (Teixeira et al., 2013). In the seed coats, high levels of tannins (polymers of flavan-3-ols) are observed (Kennedy et al.,

2000), while the endosperm contains mainly storage lipids (Hassanein and Abedel-Razek, 2009). Both the exocarp and seed contain photosynthetic pigments like chlorophylls and carotenoids (Garrido et al., 2018; Garrido et al., 2019).

The photosynthesis of fruits is generally linked to the biosynthesis of both primary and secondary metabolites (Cocaliadis et al., 2014). For instance, it has been reported for tomatoes that the photosynthesis of the fruit contributes 15–20 % of the carbon assimilates needed for fruit growth, although they represent only a minor part compared to the assimilates imported from the leaves (Obiadalla-Ali et al., 2004). However, Lytovchenko et al. (2011) mentioned that tomato fruit photosynthesis is not required for fruit energy metabolism nor for providing assimilates for growth, but is essential for properly timed seed development. In addition, in the grape berry, photosynthesis may contribute to the total carbon balance by supplying about 10 % of the carbon needed for fruit development and by recycling about 40 % of the carbon lost by mitochondrial respiration (Ollat and Gaudillere, 2000). Moreover, transcriptomics (Degu et al., 2014; Deluc et al., 2007; du Plessis et al., 2017), proteomics (Wang et al., 2017), metabolomics (Dai et al., 2013; Degu et al., 2014; Wang et al., 2017), and integration of omics data into biosynthetic networks (Serrano et al., 2017; Zamboni et al., 2010), confirmed the presence of components directly related to photosynthetic activity within the grape berry itself, in line with our results obtained by chlorophyll fluorescence analysis of the various berry tissues (Breia et al., 2013, Garrido et al., 2018; Garrido et al., 2019). However, the precise role of this photosynthesis of berry tissues on the physiology and product quality of the grapes is still not understood.

In this work, we compared the metabolite profiles of the two photosynthetically active tissues (exocarp and seed) of berries from three developmental stages (green, *véraison* and mature) from the white grape variety cv. Alvarinho grown under contrasting microclimate conditions inside the grapevine canopy with respect to perceived light (HL or LL). One purpose of the present study was to link the observed differential photosynthesis of HL- and LL-grown berries (Garrido et al., 2019), to possible differences in the tissue metabolite composition. In addition, we aimed to investigate the potential impact of two main short-term mitigation strategies applied in vineyards (i.e., foliar kaolin application and plant irrigation) on the metabolite profile of these grape berry tissues, in relation to their modulating effects on berry photosynthesis.

## 4.2. Results and Discussion

### 4.2.1. Global Effects on Grape Exocarp and Seed Metabolome

Untargeted metabolomics using liquid chromatography coupled with high resolution mass spectrometry (LCMS) was applied for comparing the composition of semi-polar compounds in the photosynthetically active berry tissues during their development, in both canopy light microclimates (LL and HL) and with both kaolin and irrigation as mitigation treatments. A large difference in the LCMS profiles was observed between exocarp and seed tissues (Figure S4.1 and Figure S4.2 in Supplementary Materials). Considering this large difference, the mass peak alignment and subsequent data processing steps were separately performed for each tissue. We obtained the relative peak intensity data for a total of 395 and 398 putative metabolites detected in the exocarp (Supplemental File S1, Table S1, available on this link) and seed (Supplemental File S2, Table S1, available on this link), respectively, across all 128 samples analyzed.

A principal component analysis (PCA), based on the relative intensities of the detected metabolites was performed for both tissues (Figure 4.1) in order to identify the main factors underlying the differentiation of the grape samples. Most of the total variability was explained by the first two PCs, i.e., 67.8 % and 80.4 % of the variation in the exocarp and in the seeds, respectively. For both tissues, PC1 was clearly related to the differences between the developmental stages, explaining 58.0 % of the variance in the exocarp and 73.4 % in the seed. In the exocarp (Figure 4.1a), the sample grouping was in line with their ripening order, i.e., green, *véraison* and mature stages, with the latter two stages being relatively similar compared to the green stage. In the case of the seed (Figure 4.1b), the *véraison* and mature stages were separated on PC2, explaining 7 % of the variability, rather than on PC1. These results indicate that in both berry tissues, the metabolites detected by LCMS, i.e., mainly secondary metabolites, are mostly affected during the development from the green to *véraison* stage.

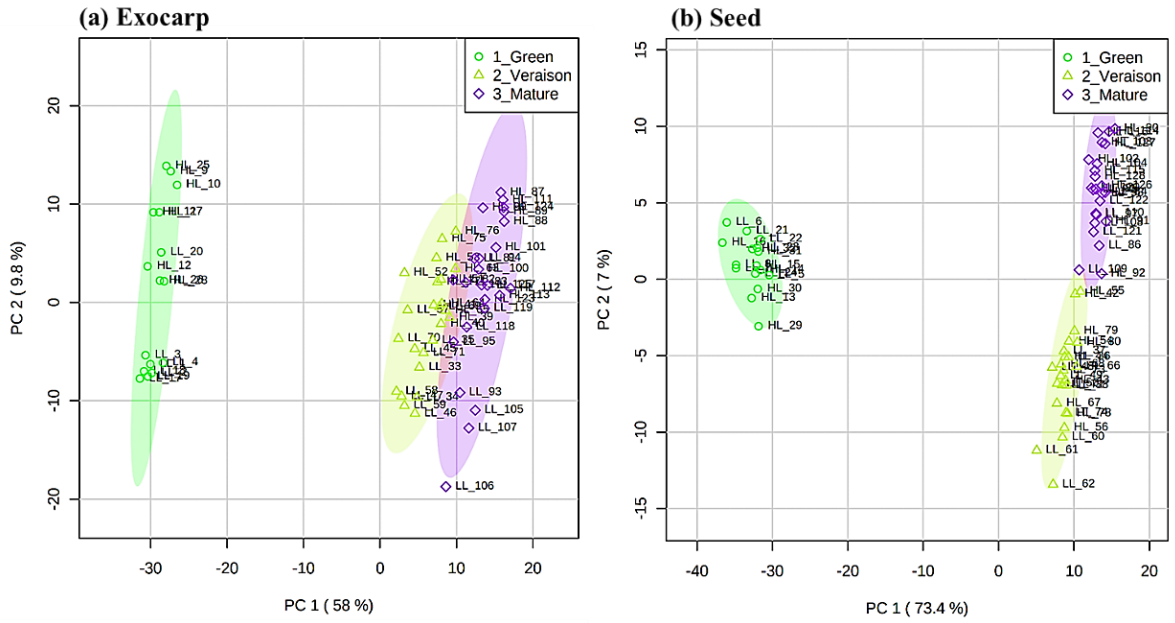


**Figure 4.1.** Principal component analysis (PCA) score plots of the liquid chromatography mass spectrometry (LCMS) metabolite data for the exocarp (**a**) and seed (**b**) in berries at three developmental stages (green, *véraison*, mature), including both microclimates (high light (HL) and low light (LL)) and both mitigation treatments. Colored ellipses represent 95 % confidence interval ($n$ = 4 for green stage and $n$ = 3 for *véraison* and mature stages).

Analysis of Variance (ANOVA) in combination with false discovery rate (FDR) correction indicated that 362 metabolites in the exocarp (i.e., 91.6 % of the metabolites detected by LCMS in this tissue) and 388 metabolites in seeds (i.e., 95.4 % of the detected seed metabolites) differed significantly between the three developmental stages. A heatmap plot was constructed based on the 25 top-ranking metabolites according to the ANOVA test (Figure S4.3). For both tissues, the heatmap shows two main blocks of metabolites, i.e., a group of metabolites with higher abundance in the green stage and another group of metabolites higher in the later stages.

The FDR corrected $p$-values and the fold change (FC) values between the averages of the green and mature groups for both the exocarp (Supplemental File S1, Table S2) and seed (Supplemental File S2, Table S2) of untreated control samples grown in a HL microclimate (as an example) were calculated to select those compounds that were most influenced by ripening (PC1). In fact, 17.2 % of the total of LCMS-compounds detected in the exocarp tissue appeared to be uniquely present (i.e., above detection threshold) in either green or mature grapes (Supplemental File S1, Table S2), while this was 19.8 % in seeds (Supplemental File S2, Table S2). In addition, among the metabolites present at both developmental stages, 39.4 % and 43.2 % were significantly different ($p < 0.05$) between these developmental stages in the exocarp and seed, respectively. In the exocarp, a range of procyanidins (also called flavan-3-ols) were higher in green than in mature grapes (Supplemental File S1, Table S2) including

procyanidin trimers (e.g., ID 308, 71-fold), dimers (e.g., ID 189, 4.5-fold and ID 140, epicatechin–gallocatechin, 17-fold) and the monomer catechin (ID 206, 4.2-fold), as well as stilbenes like resveratrol (ID 653, 58-fold) and piceid (ID 601, 2.8-fold). Their decrease upon ripening is in accordance with previous results with the exocarp of red grape berries (Jeandet et al., 1991; Jordão et al., 2001). Resveratrol in green berries has been suggested to play a role in preventing fungal infection and damage by UV irradiation (Hasan and Bae, 2017). On the other hand, a series of flavonol glycosides significantly increased upon ripening, such as quercetin 3-*O*-glucoside (ID 492, 5.4-fold), kaempferol-3-glucoside (ID 545, 46-fold) and an isorhamnetin-hexoside (ID 583, 49-fold), in accordance with previous results obtained with the skin of both white and red grapes (Downey et al., 2003). In the seed (Supplemental File S2, Table S2), there was a similar decrease in various procyanidins, such as gallocatechin (ID 229, 18-fold) and other polymeric compounds from the same class (e.g., ID 150, 28-fold; ID 377, 19-fold; ID 579, 12-fold), which is in accordance with previous results with seeds of red grape berries (Jordão et al., 2001). On the other hand, seed ripening coincided with an increase in resveratrol (ID 979, 7.2-fold) and several of its putatively-identified oligomers including a dimer (ID 926, 36-fold), a trimer-hexoside (ID 990, 33-fold) and a tetramer (ID 1074, 169-fold).

Subsequently, PCA was performed for each developmental stage and for each grape berry tissue separately (Figure 4.2 and Figure S4.4). For the PCA of the green stage, only the effects of microclimates and foliar kaolin application could be assessed, since at this developmental stage, no irrigation was yet applied to the vineyards. At the green stage, the PCA result plots indicated a separation of samples mainly according to the canopy microclimate (LL vs. HL), most specifically in exocarp samples (PC1 explaining 41.3 % and 29.3 % of total variance in exocarp and seed, respectively), while no clear sample grouping was observed for kaolin-treated versus untreated plants in either tissue, neither based on the first two PCs (Figure 4.2a,b) nor upon considering subsequent PCs (PC3 and PC4; data not shown). At the *véraison* stage (Figure S4.4), the berry exocarp metabolome (Figure S4.4a) was mainly influenced by microclimate as well (PC1, 24.9 %) and secondly also by irrigation (PC2, 18.9 %). In contrast, at this developmental stage, the seed metabolome (Figure S4.4b) was mainly affected by the irrigation treatment (PC1, 38.0 %). At the mature stage, the exocarp metabolome (Figure 4.2c) was primarily influenced by the canopy microclimate (PC1, 35.5 %) and secondly by the irrigation treatment (PC2, 11.5 %), while for mature seeds (Figure 4.2d) no clear grouping of the differently treated berries was detected.

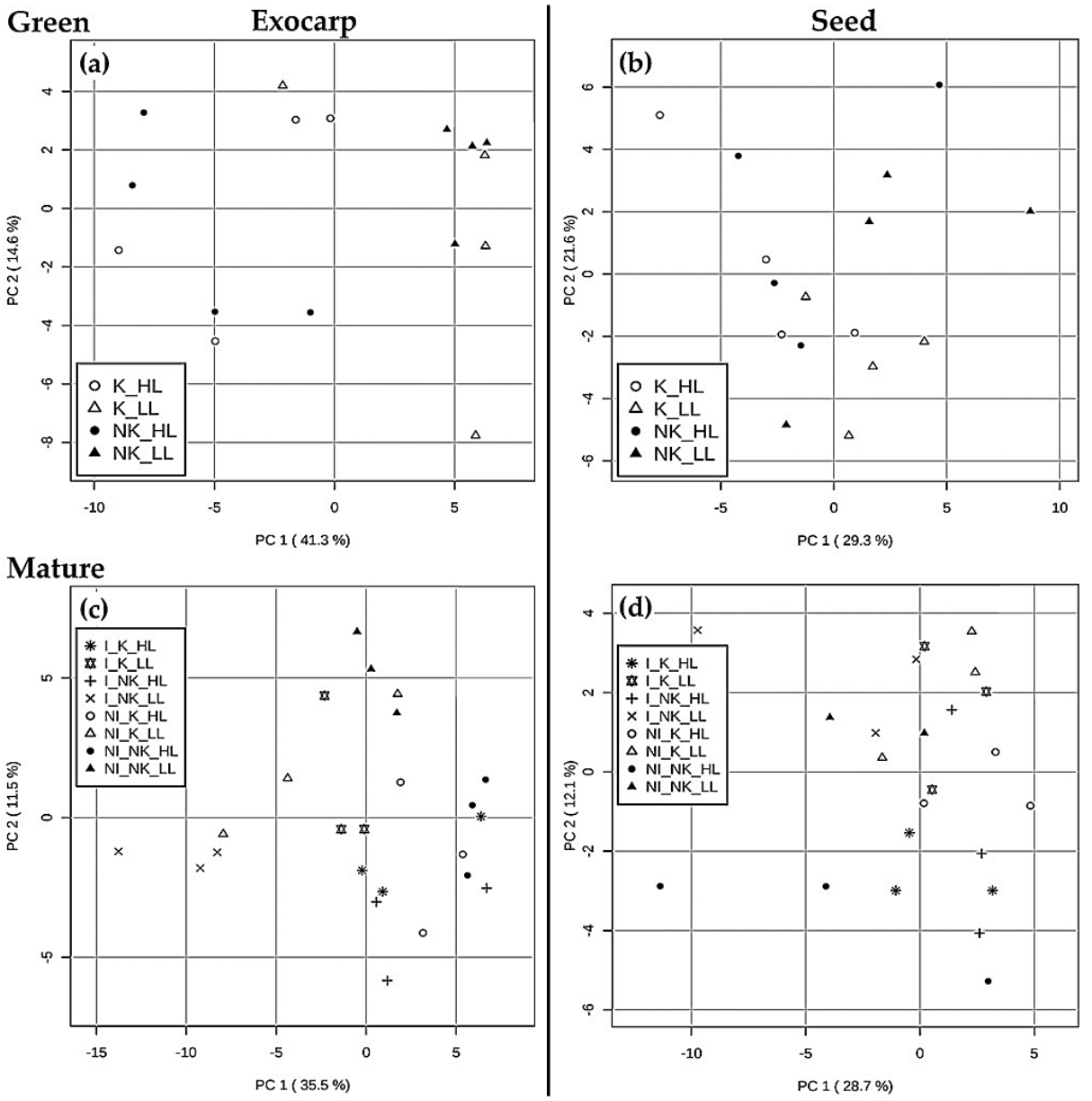


**Figure 4.2.** Principal component analysis (PCA) score plots based on the LCMS metabolite data for both exocarp (**a**, **c**) and seed (**b**, **d**) at the two most contrasting ripening stages (**a**, **b**—green and **c**, **d**—mature), including all microclimates and treatments ($n$ = 4 for green stage and $n$ = 3 for mature stage). The abbreviations in the legend represent: NI—Non-Irrigation; I—Irrigation; NK—Non-kaolin; K—Kaolin; LL—Low Light microclimate; HL—High Light microclimate.

To assess the composition of primary metabolites in exocarps, gas chromatography mass spectrometry (GCMS) analysis was performed. This was done for mature berries only, since this stage is most directly related to the quality of grapes and wine. The unbiased processing of the 24 exocarp samples resulted in the relative abundances of 99 metabolites, mainly sugars, amino acids and organic acids (Supplemental File S1, Table S3, Figure S4.5). In contrast to the PCA based on LCMS metabolites (Figure 4.2c), the PCA based on these GCMS compounds did not reveal clear effects of either microclimate, kaolin or irrigation on the metabolic composition of these mature exocarp samples (Figure

S4.6). The lack of irrigation effects suggests that the accumulation of primary compounds is unrelated to the effect of irrigation on the photosynthetic activity in these mature exocarps (Garrido et al., 2019).

ANOVA Simultaneous Component Analysis (ASCA) was subsequently used to determine which of the growth conditions, as well as their possible interactions, exerted a significant effect on the metabolome of the exocarp and seeds at each of the three berry developmental stages, based on either the LCMS and GCMS analysis (Table 4.1). In addition, we applied a N-way ANOVA to study the effect of the growth conditions on each metabolite in more detail (Table 4.1: numbers between brackets indicate numbers of significant metabolites). The significant compounds (all, or top 20) following from the ANOVA analysis were subsequently manually annotated (for exocarp—Supplemental File S1, Tables S4-S9; for seed—Supplemental File S2, Tables S3-S5; Files available on this link).

**Table 4.1.** Levels of significance (*p* values) obtained by ANOVA simultaneous component analysis (ASCA), for the effects of the various growth conditions and their interactions on the exocarp and seed metabolite composition, based on either the LCMS or GCMS analysis, during berry ripening (G—Green, V—*Véraison*, M—Mature). Significant effects ($p \leq 0.05$) are highlighted by the grey color; interactions that appear insignificant were omitted. The numbers between brackets indicate the total number of significant (FDR-adjusted $p \leq 0.05$) metabolites from N-way ANOVA.

| | **LCMS Data** | | | | | | **GCMS Data** |
|---|---|---|---|---|---|---|---|
| | **Exocarp** | | | **Seed** | | | **Exocarp** |
| **Growth Conditions** | **G** | **V** | **M** | **G** | **V** | **M** | **M** |
| Soil irrigation | - | 0.001 (78) | 0.002 (48) | - | 0.016 (30) | 0.264 | 0.147 |
| Kaolin | 0.472 | 0.112 | 0.165 | 0.262 | 0.197 | 0.145 | 0.036 (0) |
| Berry microclimate (HL/LL) | 0.001 (95) | 0.001 (88) | 0.001 (154) | 0.012 (26) | 0.115 | 0.006 (31) | 0.003 (3) |
| Irrigation × Kaolin | - | 0.044 (0) | 0.043 (0) | - | 0.084 | 0.194 | 0.001 (10) |

It is worth noting that the *p*-values of the univariate tests were adjusted for multiplicity by the Benjamini–Hochberg false discovery rate (FDR) procedure to control (in expectation) the proportion of false positive differential metabolites. Nevertheless, there is still a chance for false positive results, due to a relatively large number of variables of both the treatments tested and metabolites detected compared to the low number of biological replicates per group sample. Therefore, in the subsequent part we only focus on those significantly differing metabolites with the lowest *p*-values and for which the size of the effect (i.e., the fold change) was much larger than the overall technical variation for that specific compound (as determined from the quality control samples) (Supplemental Files S1 and S2, Table S1).

This ASCA approach identified the berry microclimate as the main growth condition influencing the LCMS-metabolites in both berry tissues at all three developmental stages, except for seeds at the *véraison* stage (Table 4.1), which is in accordance with the PCA results based on these LCMS-metabolites (Figure 4.2). In addition, the ASCA results for the GCMS-metabolites in the mature exocarp indicated that the irrigation treatment itself has no significant impact (cf. Figure S4.6); in contrast, here the berry microclimate has a significant impact, which was undetectable by the PCA model (Figure S4.6). Soil irrigation had a significant impact on the LCMS profiles: for exocarp at both *véraison* and mature stages and for seeds at the *véraison* stage only. In contrast, kaolin did not significantly influence the LCMS-profiles at either developmental stage or tissue, while it did significantly impact the GCMS profiles in the mature exocarp, but no significant differences could be shown for the individual metabolites. Previous studies with red grape varieties showed that kaolin application had a positive influence on both phenylpropanoids and flavonoids (Conde et al., 2016), while it only had a minor effect on both free and bound volatile organic compounds in the berries (Song et al., 2012).

Our ASCA models also showed a significant interaction effect between irrigation and kaolin on both LCMS and GCMS compounds in the berry exocarp, with the ANOVA model indicating a few significantly differing compounds in the GCMS-profiles only. The size of this interactive effect on individual GCMS compounds was rather small, i.e., less than 40 % change in abundance (Supplemental File S1, Table S4), while the direction of this effect differed between compounds: kaolin application reduced the irrigation-induced increase and decrease in L-alanine and quininic acid, respectively, while the (small but significant) increases in the abundance of several sugars induced by either kaolin or irrigation alone were counteracted when both treatments were applied together (Figure S4.7). Previous studies using whole red grape berries did not observe any significant interactive effect of kaolin and irrigation on free and bound volatile compounds (Song et al., 2012), or total soluble solids, total organic acids, anthocyanins and phenolics (Cooley et al., 2008). Altogether it seems likely that the interaction of these two mitigation treatments does not exert a large, if any, effect on the global metabolome of mature grapes in practice.

#### 4.2.1.1. Specific Effects of Microclimate

By comparing the fold change (FC) values of the average metabolite abundances in the HL and LL groups, i.e., the intensity ratio between the average of the HL and LL samples irrespective of mitigation treatment, we identified those metabolites that were most strongly affected by microclimate (for exocarps— Supplemental File S1, Tables S5-S7; for seeds— Supplemental File S2, Table S3 and S4; Files available on this link). Overall, the HL exocarps were characterized by a consistently higher level of several

flavonol conjugates (Figure 4.3), except for isorhamnetin hexoside at the green stage (Figure 4.3f). During berry development, the flavonol conjugates showed differential accumulation patterns (Figure 4.3). On the one hand, the relative intensities of quercetin-3-*O*-rutinoside (rutin) and quercetin 3-*O*-glucuronide decreased in HL exocarps during development, while in LL, exocarps kept their values constant (Figure 4.3a,c). The remaining flavonols showed an increase in intensity during development for both microclimates (Figure 4.3b,d–f). These results suggest that there was a development-specific flavonol composition and that this was significantly influenced by the light microclimate. If the relative abundance values of these six compounds are added up, notwithstanding their potentially differential ionization efficiencies in the MS source, the mean value of the total of these flavonols are significantly higher in HL-exocarps than in LL ones at all developmental stages (Figure S4.8a). These results suggest that HL berries had their maximum level of total flavonols already at the green stage and this high level was maintained upon subsequent ripening; in contrast, during ripening of LL berries, their flavonol content was continuously increasing to a level that at the mature stage was still lower than that of HL berries. By using a calibration curve of authentic standard, absolute quantities of the main flavonol quercetin 3-*O*-glucoside were obtained (Figure S4.8b), and these absolute values showed the same pattern as the relative peak values (Figure 4.3b).

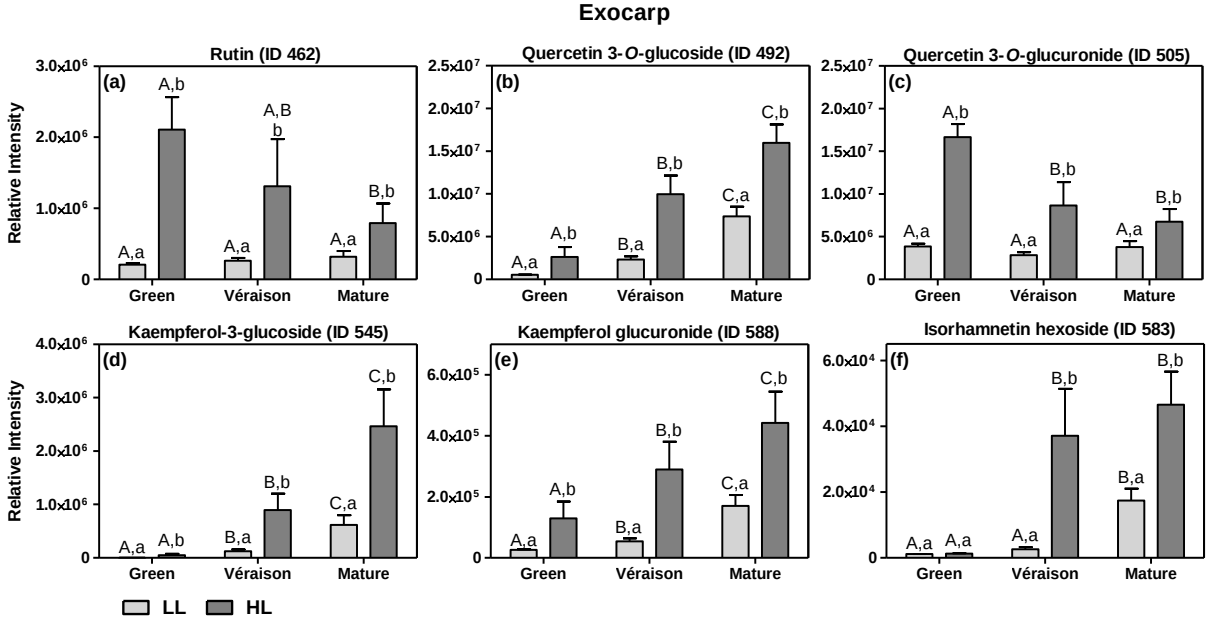


**Figure 4.3.** Relative intensities of the six main flavonol compounds as detected by LCMS (means and SD of $n$ = 8–12) for exocarp tissue at two canopy microclimates (LL and HL; independent of mitigation treatment) and at three developmental stages (green, *véraison* and mature). The flavonols considered are: (**a**) rutin (quercetin-3-*O*-rutinoside) (ID 462), (**b**) quercetin 3-*O*-glucoside (ID 492), (**c**) quercetin 3-*O*-glucuronide (ID 505), (**d**) kaempferol-3-glucoside (ID 545), (**e**) kaempferol glucuronide (ID 588) and (**f**) isorhamnetin hexoside (ID 583) (Supplemental File S1, Tables S5-S7). Statistical analysis (two-way ANOVA, $p \leq 0.05$) was applied after data $Log_2$ transformation. Statistical notation above the bars: the capital letters refer to differences between developmental stages for the

same microclimate, while the lowercase letters refer to differences between the two light microclimates for each stage.

The increase in flavonols by HL compared to LL is in accordance with previous reports on the microclimate effects on both white grape berries (du Plessis et al., 2017; Friedel et al., 2015; Joubert et al., 2016) and red varieties (Koyama et al., 2012; Reshef et al., 2018). This increase, especially relevant in the green stage when the total amount of flavonols peaks in exposed clusters, had a parallel with the increased photosynthetic activity of exocarps under HL conditions in the green stage (Garrido et al., 2019). Flavonols are generally considered to have antioxidant and/or "sunscreen" abilities, thereby protecting the photosynthetic apparatus as well as other macromolecules from excess solar radiation in situ (Agati and Tattini, 2010). Thus, the higher levels of flavonols in HL exocarps may represent an acclimation response to the higher intensity light microclimate, possibly to protect their photosynthetic system from radiation-mediated oxidative damage (Joubert et al., 2016), and therefore keeping its photosynthetic activity until the later stages of development (Garrido et al., 2019).

In HL exocarps, we also observed higher levels of some putatively annotated glycosylated aroma compounds, such as a vanillyl alcohol hexoside (ID 136) and geraniol-hexose-pentose (ID 685), as compared to LL berries (Supplemental File S1, Tables S6 and S7).

Another class of grape flavonoids, the flavan-3-ols or procyanidins, comprising both monomers and a range of oligomers/polymers of (epi)catechin and (epi)gallocatechins, are key to wine quality as they confer astringency and bitterness (Ma et al., 2014). In addition, they protect the plant and its fruits against pathogens, pest insects and herbivores (Barbehenn and Peter Constabel, 2011). Absolute quantities of six selected flavan-3-ols were obtained by using calibration curves of authentic standards; since their abundance patterns were more or less similar across samples (data not shown), their levels were added up to calculate total monomers and total procyanidins (Figure 4.4). At the green stage, HL berry exocarps had significantly higher contents of total flavan-3-ols, i.e., both (epi)catechin monomers (Figure 4.4a) and procyanidin dimers (Figure 4.4b), compared with LL ones (Figure 4.4a). During ripening, these contents decreased in both microclimates, but more so in HL than in LL. In addition, the HL microclimate led to an up-regulation of several other compounds including flavan-3-ols in the berry exocarp, when compared to LL; at the green stage, the HL exocarps contained more hydroxy-procyanidin trimers (e.g., ID 147 and ID 151) and a procyanidin conjugate (e.g., ID 310) (Supplemental File S1, Table S5). However, at the mature stage, the flavan-3-ols monomers, e.g., (+)-catechin (ID 206), dimers e.g., procyanidin B1 (ID 174) and trimers, (e.g., ID 215) were lower in HL exocarps (Supplemental File S1, Table S7). The analysis

by GCMS confirmed that mature exocarps accumulated less catechin monomers in HL than in LL conditions (ID 13276, FC HL/LL = 0.8).

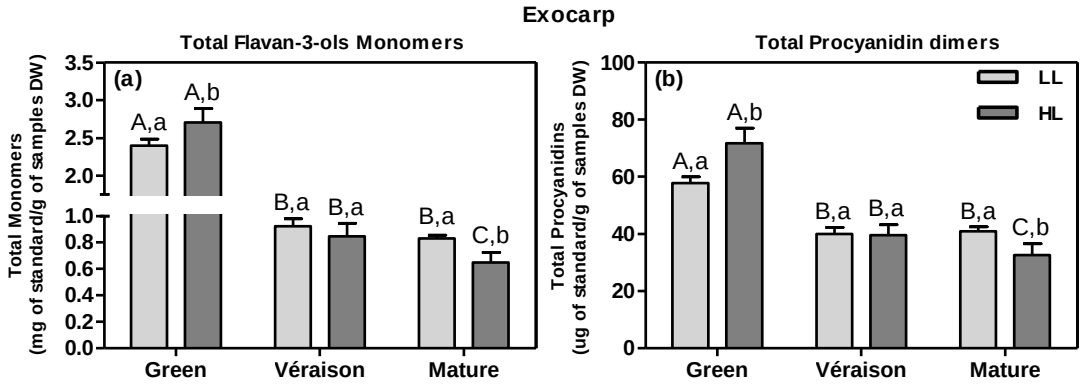


**Figure 4.4.** Total flavanols in exocarp tissue at two canopy microclimates (LL and HL; independent of mitigation treatment) and at three developmental stages (green, *véraison* and mature). (**a**) Total flavan-3-ols monomers levels (mg/g of dry weight (DW)): catechin, epicatechin, epicatechin-3-*O*-gallate. (**b**) Total procyanidin dimer levels (µg/g DW): procyanidin B1, B2 and B3. Statistical analysis with two-way ANOVA ($n$ = 8–12, +SD, $p \leq 0.05$). Statistical notation is the same as in Figure 4.3.

There are yet unexplained and conflicting results reported on the influence of light on the accumulation of flavan-3-ols in white grape berries. On the one hand, one study showed that the total flavan-3-ol content was affected neither by shading treatments nor by more incoming light due to leaf removal (Friedel et al., 2015). On the other hand, at the green stage, shaded white grapes contained more total flavan-3-ols monomers than exposed ones (du Plessis et al., 2017), while in another study with two different white varieties, the amount of various flavan-3-ols was greater in sun-exposed berries than in shaded ones (Rocchi, 2015), in line with our results at the green stage (Figure 4.4). However, in the skin of red mature grapes, shading resulted in a decrease in flavan-3-ols monomers and, subsequently, in a decreased level of condensed tannins (procyanidins) (Fujita et al., 2007); these results are in contrast to those previously obtained with white grapes and presented here for LL mature berry exocarps (du Plessis et al., 2017) (Figure 4.4).

The microclimate also affected the metabolic profile of seeds, at both green and mature stages (Supplemental File S2, Tables S3 and S4). At the green stage, seeds from HL showed an up-regulation of hydroxycinnamic acid compounds, i.e., upstream from the flavonoid pathway (Supplemental File S2, Table S3), including a coumaroyl conjugate (ID 285, FC HL/LL = 4.5) and the lignan-type hydroxycinnamic acid dimer isolariciresinol-β-4′-*O*-glucopyranoside (ID 613, FC = 2.2) (Figure 4.5). Hydroxycinnamic acids including lignans have been shown to possess antioxidant activity and are associated with the biosynthesis of lignins (Rodríguez-García et al., 2019), which are key in seed lignification (Lewis et al., 1998). In our experiments, the level of the lignan isolariciresinol increased from

the green to *véraison* stage and then its abundance was maintained up to the mature stage (Figure 4.5b); this pattern is in agreement with the degree of lignification of grape seeds (Cadot et al., 2006).

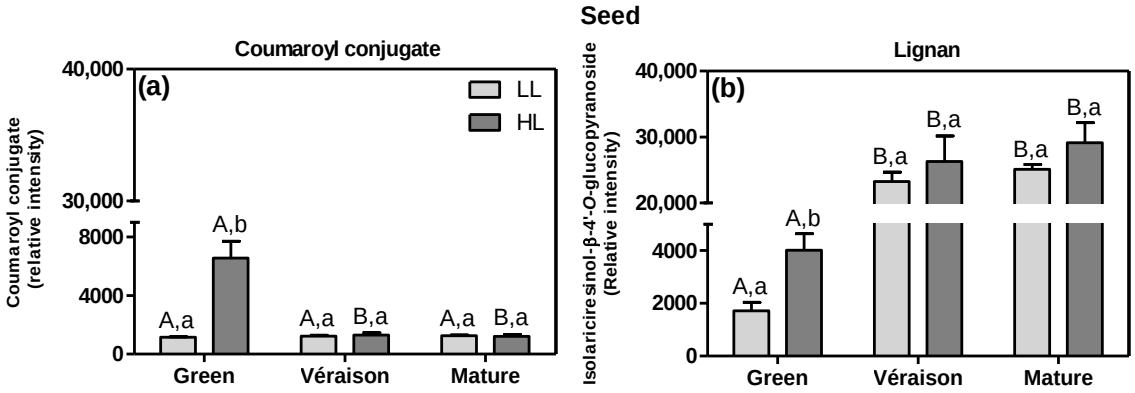


**Figure 4.5.** Examples of seed hydroxycinnamic acids, (**a**) a coumaroyl conjugate (ID 285) and (**b**) a lignan type—isolariciresinol-β-4′-*O*-glucopyranoside (ID 613) detected by LCMS (mean values + SD, $n = 8–12$) significantly differing between canopy microclimates (LL and HL; independent of mitigation treatment) and at three developmental stages (green, *véraison* and mature). Statistical analysis (two-way ANOVA, $p \leq 0.05$) was applied after data $Log_2$ transformation. Statistical notation is the same as in Figure 4.3.

The HL microclimate, as compared to LL, also led to a higher relative abundance of compounds from the flavonoid pathway itself, specifically flavan-3-ols in seeds at the green stage, including a procyanidin pentamer (ID 317, FC = 2.2), a (epi)gallocatechin-conjugate (ID 162, FC = 1.7), a pentahydroxyflavan dimer (ID 170, FC = 1.6) and the gallocatechin (ID 229, FC = 1.5) (Supplemental File S2, Table S3). In contrast, mature HL seeds accumulated less stilbene derivatives, such as (+)-alpha-viniferin-hexoside (ID 990, FC = 0.8) and viniferin 3″-glucoside (ID 1027, FC = 0.7) (Figure S4.9). Stilbenes are an effective response against pathogen infection and abiotic stress and contribute to the final nutraceutical quality of both seeds and wine (Ananga et al., 2017; Duarte et al., 2020; Hasan and Bae, 2017). To our knowledge, the effect of light conditions on stilbenes in grape seeds has not been reported before.

#### 4.2.1.2. Specific Effects of Irrigation

The irrigation of the soil resulted in an up-regulation of thonningianin B (ID 521, putatively identified), a tannin-type of compound, in the exocarp at both the *véraison* and mature stage (Figure 4.6a) (Supplemental File S1, Tables S8 and S9). In seeds, at the *véraison* stage, the irrigation led to an accumulation of several flavan-3-ols such as gallocatechin (ID 229, Figure 4.6b) and an (epi)gallocatechin-conjugate (ID 162) (Supplemental File S2, Table S5). Additionally, at the *véraison* stage, the irrigation resulted in a down-regulation of primary metabolites, including: D-fructose 1,6-bisphosphate (ID 79) and arginine (ID 77) in exocarps (Figure S4.10a,b, Supplemental File S1, Table S8) and both a hexose sugar

(ID 21) and the phenylpropanoid coutaric acid (ID 315) in seeds (Figure S4.10c,d, Supplemental File S2, Table S5). On the other hand, in exocarps at the mature stage, the irrigation treatment resulted in a lower accumulation of a hydroxy-procyanidin trimer (ID 151), a (epi)catechin-gallocatechin dimer (ID 122) and (+)-gallocatechin (ID 131) (Supplemental File S1, Tables S9).

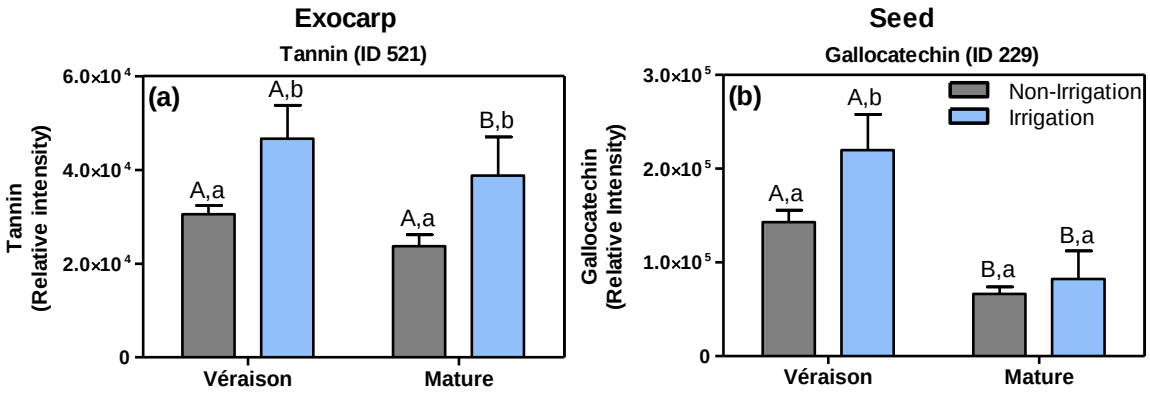


**Figure 4.6.** Relative abundance of (**a**) a tannin (ID 521) in the exocarp and (**b**) for gallocatechin (ID 229) in the seed, as obtained by LCMS analysis (means + SD, $n = 8–12$). Grape berries were grown under non-irrigation (grey bars) and irrigation conditions (blue bars) and collected at two developmental stages (*véraison* and mature). Data of HL and LL berries, combined. Statistical test: two-way ANOVA, $p \leq 0.05$. Statistical notation above the bars: the capital letters refer to differences between developmental stages for the same treatment condition, while the lowercase letters refer to differences between the control and irrigation treatment, within each developmental stage.

Genebra et al. (2014), likewise, showed that seeds from irrigated grapevines at full maturation had higher flavan-3-ols and tannins contents compared to non-irrigated ones, which was explained by a slower berry ripening upon irrigation. In fact, Castellarin et al., (2007) argued that water deficiency accelerates the ripening of grape berries. In another study, Koundouras et al. (2009) showed that the total amount of flavan-3-ols in the seed (per fresh weight) was higher in fully irrigated vines compared to non-irrigated ones, likely due to the effect of the more vigorous canopy growth on the berry microclimate. In addition, the skins and seeds of berries from fully irrigated plants of the red Syrah variety tasted more astringent than those from non-irrigated ones, which was attributed to the higher levels of various flavan-3-ol-type of polyphenols (Kyraleou et al., 2016). The present and previous studies thus indicate that irrigation can delay berry ripening and thereby result in the ripening-dependent decrease in flavan-3-ols (e.g., Figure 4.4 and Figure 4.6), which may have an effect on quality traits of both grape seed and wine.

### 4.2.2. Changes in Carbon Skeletons

Berries are strong sinks importing massive amounts of photoassimilates/carbon structures, mainly sucrose, from the leaves (Yamaki, 2010). Since exocarps and seeds are both photosynthetically active, especially in the green stage of berry development (Breia et al., 2013, Garrido et al., 2018; Garrido et al., 2019), they may locally contribute by supplying energy and carbon-skeletons needed for the biosynthesis of compounds accumulating in these berry tissues. The heatmap plot based on LCMS metabolites (Figure S4.3) showed that specific sugars and organic acids were relatively high at the green stage and decreased with ripening in either or both exocarps and seeds, including UDP-glucose (ID 97) and tartaric acid (ID 168) in the exocarp (Figure 4.7) and malic acid (ID 55) in the seed (not shown).

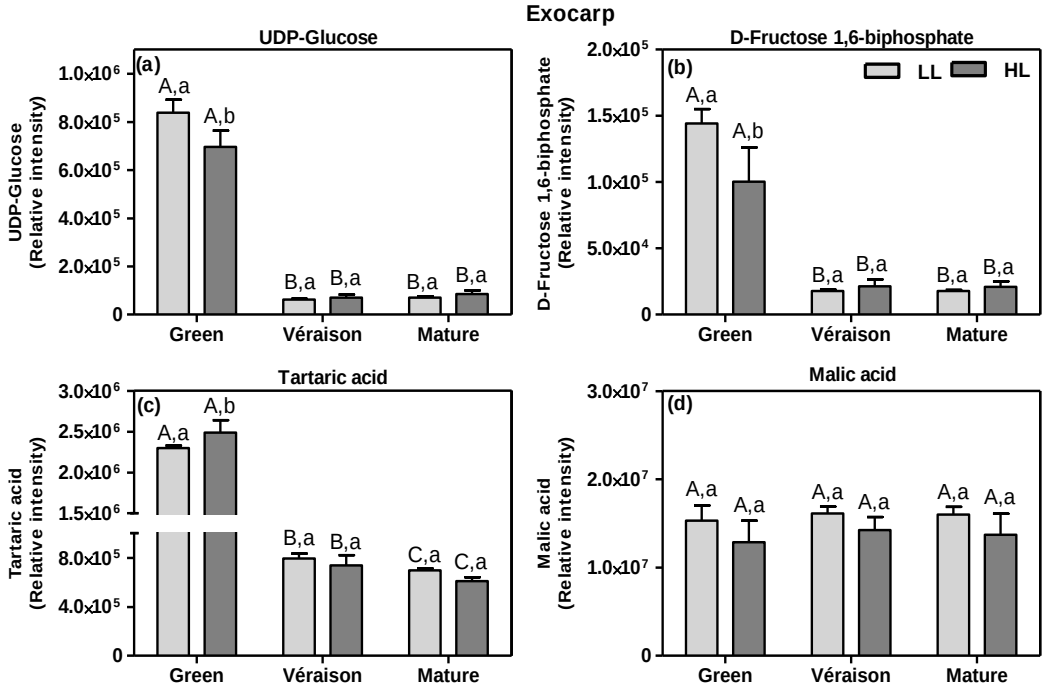


**Figure 4.7.** Relative intensity of some sugars (**a**, **b**) and organic acids (**c**, **d**) detected by LCMS (means + SD, *n* = 8–12) in exocarps of grapes grown in two light microclimates (LL and HL; independent of mitigation treatment) and harvested at three developmental stages (green, *véraison* and mature). (**a**) UDP-glucose (ID 97), (**b**) D-fructose 1,6-biphosphate (ID 79), (**c**) tartaric acid (ID 168) and (**d**) malic (ID 51) acid. See Figure 4.3 for statistical information.

UDP-glucose and D-fructose 1,6-biphosphate accumulate mainly at the green stage, especially in LL grapes, decreasing upon subsequent further grape berry development (Figure 4.7a,b). The result for UDP-glucose during development is in accordance with a previous study with whole red grape berries (Dai et al., 2013). Moreover, it was also shown that D-fructose 1,6-biphosphate was low at the green stage, peaked at the *véraison* stage, and afterwards it sharply decreased until the end of grape berry development (Dai et al., 2013).

The ripening-dependent decline in tartaric acid (Figure 4.7c) is consistent with previous reports (Deluc et al., 2007; Wang et al., 2017). Moreover, its level was significantly higher in green HL exocarps than in LL ones, which may be due to the higher photosynthetic activity of HL berries (Garrido et al., 2018; Garrido et al., 2019). The high amounts of organic acids endow green fruits with a sour taste for defense against herbivores (Vallarino and Osorio, 2018), while in mature berries, they are essential for both wine production, as they protect the fermentation process from bacterial contamination (Chidi et al., 2018), and wine taste. In contrast to tartaric acid, malic acid was more or less constant in exocarps throughout ripening (Figure 4.7d). The fact that malic acid did not decrease, as previously demonstrated for whole berries (Sweetman et al., 2009; Wang et al., 2017), suggests the tissue-specific effects of ripening on this organic acid. Here, we also show that malic acid did not significantly differ between the two light microclimates at any stage (Figure 4.7d), irrespective of their differential berry photosynthetic activity (Garrido et al., 2019), suggesting that malic acid accumulation in the exocarp occurs mainly through the metabolism of sugars translocated from leaves to the berries, rather than from fruit photosynthesis itself. Clearly, further studies are needed to determine the exact role of berry in situ photosynthesis in the biosynthesis of organic acids, since a previous study indicated a positive relation (Sweetman et al., 2009), but together, these results seem to point toward a role in the biosynthesis of specific organic acids. While the biosynthesis and catabolism of malic acid in grape berry is well established, being mainly produced from unloaded photoassimilates and accumulated in the vacuoles of mesocarp cells at the green phase (Sweetman et al., 2009), fewer studies are available for tartaric acid. However, it is known that the main pathway for the synthesis of its precursor, ascorbate (vitamin C), is fueled by carbon derived from photosynthesis (Cholet et al., 2016).

### 4.2.3. Lipid-Soluble Antioxidants and Lipid Oxidation in Photosynthetically-Active Grape Tissues

Tocopherols (vitamin E) are effective lipid-soluble antioxidants that can neutralize various reactive oxygen species, thereby protecting vulnerable membranes, such as chloroplast thylakoids and the photosynthetic apparatus, from photooxidative damage (Sattler et al., 2003). The main tocopherol species detected in both exocarps and seeds was α-tocopherol (Figure S4.11) followed by its γ-form (Figure S4.12), while δ-tocopherol was a minor compound (Figure S4.13) and β-tocopherol (not shown) was not detectable at all. As the effects of both microclimate and mitigation strategies were more or less similar on these three detected tocopherol species (i.e., α-, γ- and δ-), in each tissue, we added up their levels (Figure 4.8).

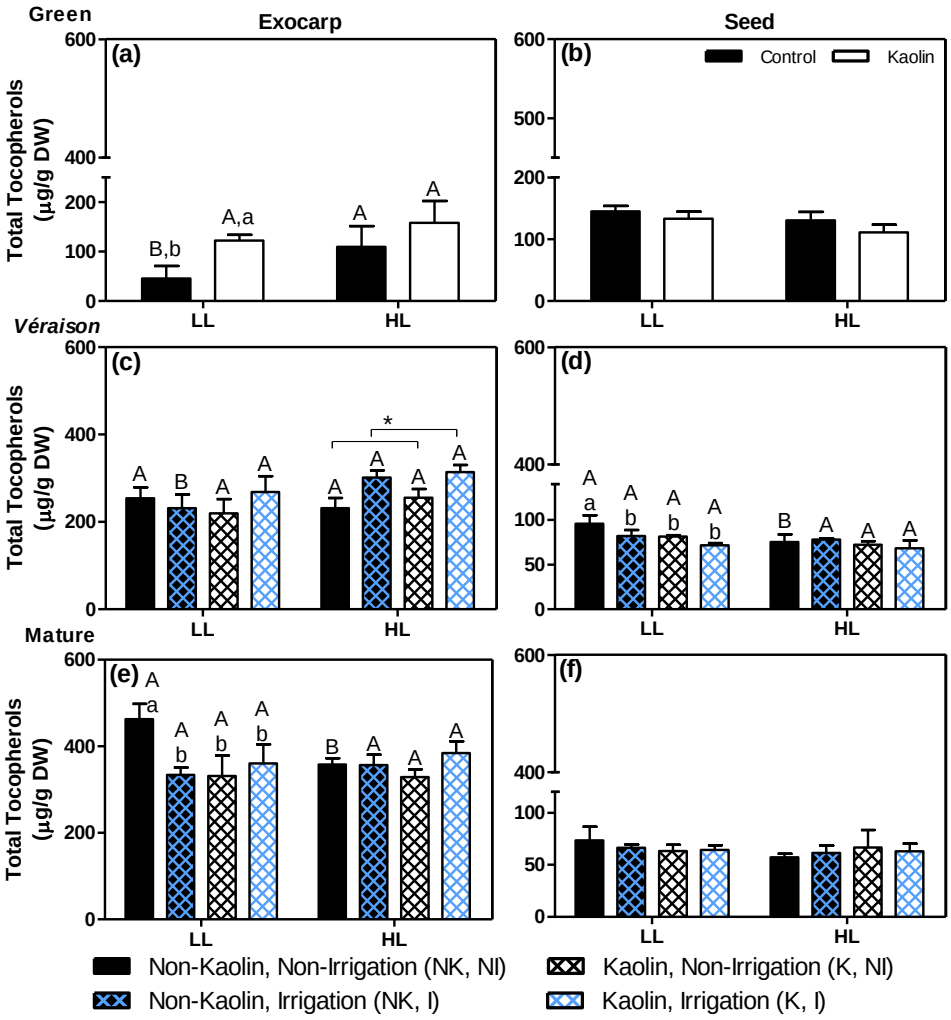


**Figure 4.8.** Total tocopherol contents (sum of the three main tocopherols detected, mean values + SD of *n* = 3 or 4) in exocarps (**a**, **c**, **e**) and seeds (**b**, **d**, **f**) of berries grown in either low light (LL) or high light (HL) microclimate conditions in the canopy. Kaolin (K) or no kaolin (NK) was applied to the plant leaves before fruit set; after green berries were developed, plants were either irrigated (I) or non-irrigated (NI). Samples were collected at three development stages: green (**a**, **b**), *véraison* (**c**, **d**) and mature (**e**, **f**). Statistical notations above the bars: at each developmental stage, the capital letters refer to differences between the two light microclimates within the same treatment, while the lowercase letters refer to differences between treatment combinations within the same light microclimate (bars with no or a common letter indicate no significant differences; two-way ANOVA, $p \leq 0.05$). Notation with an asterisk means that only one factor (in this case the irrigation) was significant.

In green berries (Figure 4.8a,b) the exocarp and seed contain about similar total tocopherol levels, with a significant effect of the microclimate on the exocarp of untreated plants (Figure 4.8a; HL/LL = 2.42), as well as a significant effect of kaolin on the LL-exocarps (K/NK = 2.71). During ripening, the tocopherol content of the LL-exocarp controls increased about 10-fold as compared to their green stage, while that of the HL-exocarp controls increased less, i.e., about 3.27-fold, and at the mature stage reached lower levels than LL ones (Figure 4.8a,c,e; black bars). In line with this elevated tocopherol level in green HL-exocarps, a previous study with Sauvignon Blanc grapes at the green stage indicated that elevated

light exposure induced the expression of tocopherol biosynthetic genes, which subsequently leads to the accumulation of lipophilic antioxidants, presumably to maintain the cellular redox balance and to protect the photosynthetic machinery from light stress (du Plessis et al., 2017; Pilati et al., 2014). In addition, the stimulating effect of kaolin on tocopherol content in green LL-exocarps is in line with previously reported elevated levels of both chlorophylls and carotenoids (Garrido et al., 2019), and is likely due to the fact that more light can reach the inner parts of the canopy by the increased reflection (Garrido et al., 2019).

In seeds, there were no relevant effects by either the light-microclimate or mitigation treatments, and in contrast to the marked increase in tocopherols in exocarps, a decrease of about 50 % was observed from the green to mature stage (Figure 4.8b,d,e). In green seeds, tocopherols may play an antioxidant role, preventing lipid peroxidation in both the thylakoid membranes, which are photosynthetically active (Garrido et al., 2019), and in the developing embryo and endosperm; in mature seeds, it is likely mainly present in the endosperm where it may play an important antioxidant role during seed storage and germination (Sattler et al., 2003).

The effects of the microclimate and kaolin on lipid peroxidation and lipid-breakdown products in the berry tissues were assessed using the thiobarbituric acid-reactive-substances (TBARS) assay. Due to the limited amount of available material, only exocarps from green berries and seeds from mature berries, both from non-irrigated plants, could be analyzed (Figure 4.9). In both tissues from non-kaolin treated control plants, there was a significant increase in TBARS in the HL berries as compared to the LL ones, suggesting enhanced lipid peroxidation due to photo-oxidative stress in the HL berries. This photo-oxidative stress effect may lead to photosynthetic impairment (Kadir et al., 2007), while in the mature seeds it may affect the lipid composition and thus the quality of the seed and its derived products (Bouhamidi et al., 1998). The kaolin treatment prevented the increase in the amount of TBARS in both tissues of HL-grown berries, suggesting a reduction in photo-oxidative damage, specifically in the direct-light exposed (HL) grapes. Indeed, previous studies likewise showed that kaolin-sprayed grapevines had lower TBARS levels compared to control ones, in both berries and leaves (Bernardo et al., 2017; Dinis et al., 2018).

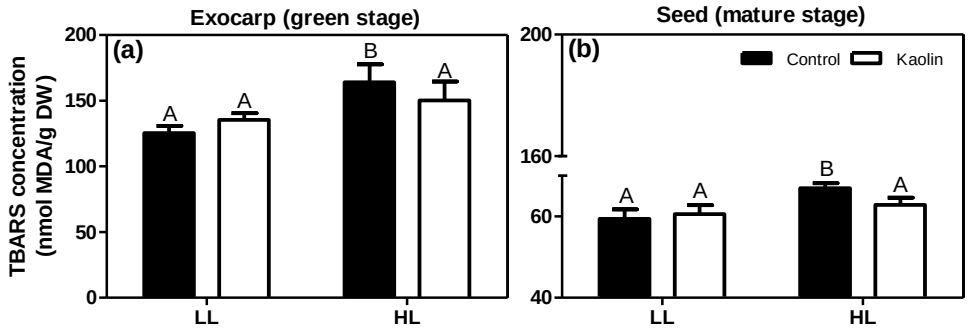


**Figure 4.9.** Levels of thiobarbituric acid-reactive-substances (TBARS; in nmol MDA/g DW) in green exocarps (**a**) and mature seeds (**b**). Grape berries were from LL and HL microclimates and from plants with or without (control) foliar kaolin application. Different capital letters above the bars refer to significant differences between microclimates within the same treatment ($p \leq 0.05$; $n = 3$ or 4).

Specific antioxidant mechanisms present in the exocarp may prevent or decrease this photo-oxidative stress in HL berries. Indeed, during berry development, the exocarp exhibited an increase in tocopherols (Figure 4.8a,c,e) and total flavonols (Figure S4.8a). In contrast, the total carotenoids content decreased during exocarp ripening (Garrido et al., 2019). Thus, carotenoids may be involved in the photoprotection of exocarp cells in the early (green) stages of berry development only, while both tocopherols and flavonols can act together upon subsequent ripening. Moreover, some of these ROS scavengers can support the non-photochemical quenching (NPQ) and may also compensate for the previously observed decrease in NPQ efficiency during berry ripening (Garrido et al., 2019; Pilati et al., 2014).

### 4.2.4. Effects on Grape Quality-Related Compounds: Total Phenolics and Sugars in Mature Fruit

In grape berries, the majority of phenolic compounds, a key parameter for wine quality, are located in the exocarp and seeds (Kennedy et al., 2000; Teixeira et al., 2013). As the untargeted LCMS analysis indicated significant effects on various phenolic compounds including flavonoids in both exocarps and seeds (e.g., Figure 4.3, Figure 4.4 and Figure 4.6; see also Supplemental Files S1 and S2, available on this link), we subsequently applied the Folin–Ciocalteu colorimetric method to determine the total amount of soluble phenolic compounds (Figure 4.10).

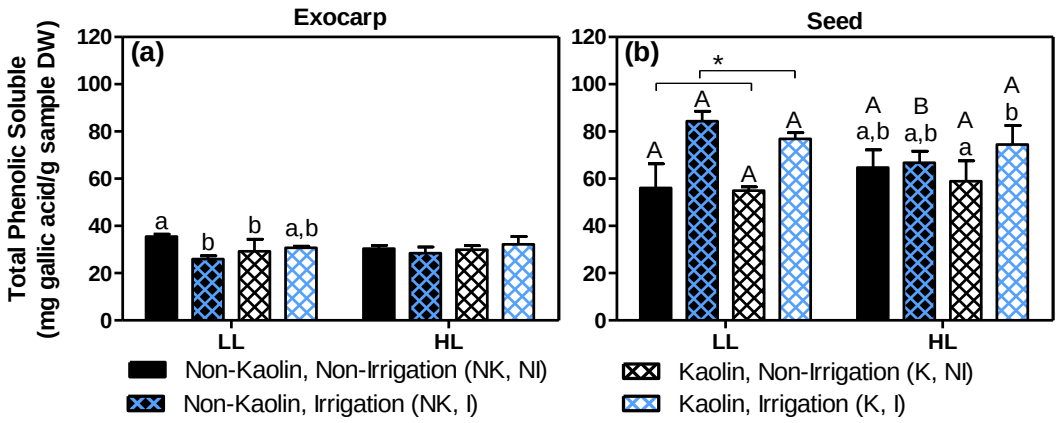


**Figure 4.10.** Total phenolic contents (mg gallic acid/g sample DW) in exocarps (**a**) and seeds (**b**) from mature stage berries. Growth conditions and treatments are the same as in Figure 4.8. Statistical notations above the bars: the capital letters refer to differences between the two light microclimates within the same treatment, while the lowercase letters refer to differences between treatment combinations within the same light microclimate (bars with no or a common letter indicate no significant differences; two-way ANOVA, $p \leq 0.05$). Notation with an asterisk means that only one factor was significant (here: irrigation in the case of LL seeds).

Overall, the total phenolic content was about two times higher in seeds than in exocarps. In exocarps (Figure 4.10a), neither a significant effect by microclimate nor a significant effect by either mitigation treatments in HL berries was observed, while in LL berries, both mitigation treatments and especially irrigation resulted in a decrease in total phenolics. This effect of irrigation is in accordance with previous studies showing an up-regulation of the grape flavonoid pathway by water deficiency (Castellarin et al., 2007; Deluc et al., 2009). In seeds (Figure 4.10b), this irrigation treatment induced a significant increase in total phenolics in LL-grapes, irrespective of kaolin treatment, while in HL grapes this increase by irrigation was only observed for kaolin-treated grapevines. It has been reported that water deficiency causes an up-regulation of the flavonoid pathway in both skins (=exocarp) and whole berries (Castellarin et al., 2007; Deluc et al., 2009). Our results (Figure 4.10) and previous ones (Kyraleou et al., 2016), showed that in seeds, the opposite occurs, i.e., the content of phenolic compounds increases by irrigation. Apparently, water deficiency exerts opposite effects on the (poly)phenolic pathways in seeds versus other berry tissues.

The major sugars present in ripe grapes are glucose and fructose, which both result from sucrose translocated from the leaves (Conde et al., 2006), while others are produced from the accumulated malate (Sweetman et al., 2009). Previous studies dealing with the influence of foliar kaolin on sugar biosynthesis in leaves showed that this application led to an increase in a vast array of leaf primary metabolites, including sucrose, glucose and fructose, as well as several organic acids (Conde et al., 2018). However, there is yet no information regarding sugar contents in relation to kaolin or other

mitigation effects in the grape exocarp, which is photosynthetically active, specifically. Figure 4.11 shows the levels of the three main individual sugars, as well as their summed values (i.e., here referred to as total sugar content) in the exocarp of the mature grapes.

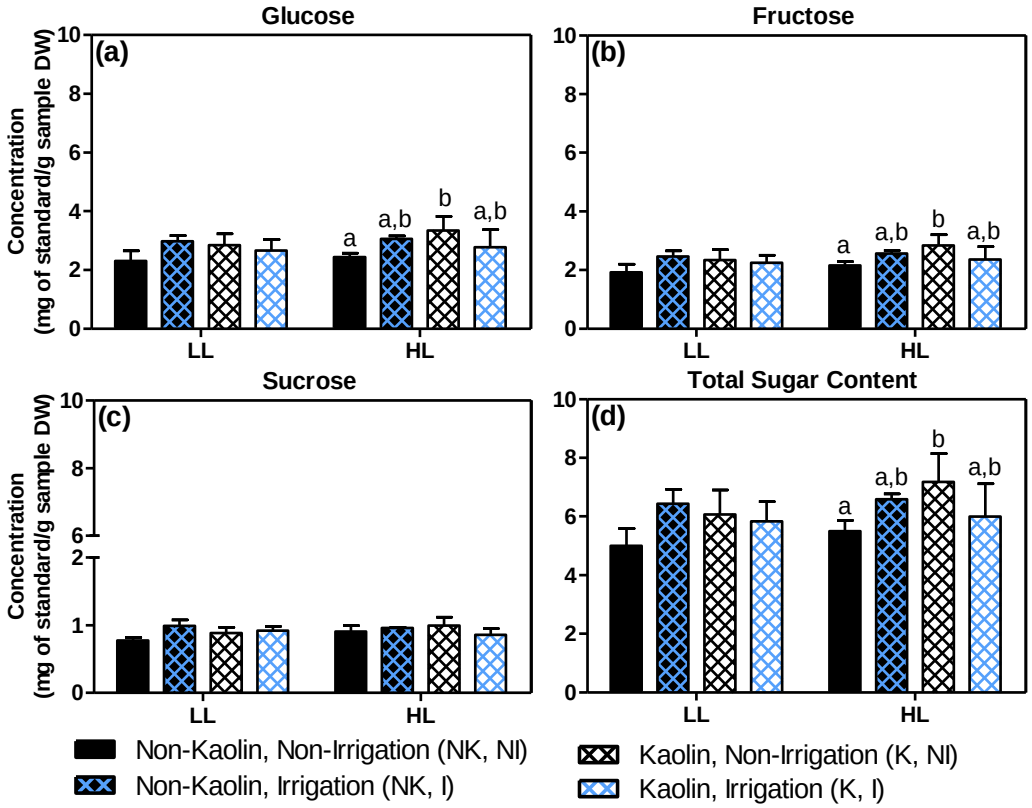


**Figure 4.11.** Levels of main sugars (in mg/g sample DW, mean values $n$ = 3–4, +SD) in the exocarp of mature berries grown in either low light (LL) or high light (HL) microclimate conditions in the canopy: (**a**) glucose, (**b**) fructose, (**c**) sucrose and (**d**) their summed values, in this paper referred to as total sugar content. Kaolin (K) or no kaolin (NK) was applied to the plant leaves before fruit set; plants were irrigated (I) or non-irrigated (NI) after the green stage. Statistical notations above the bars: the lowercase letters refer to differences between treatment combinations within the same light microclimate (values with a common letter or no letter at all, indicate no significant differences; two-way ANOVA, $p \leq 0.05$).

Under untreated control conditions (NK, NI), HL exocarps had sugar levels, similar to LL exocarps, while with kaolin only (K, NI), they contained significantly more glucose (Figure 4.11a; +37 %) and fructose (Figure 4.11b; +32 %), and consequently 30 % more total sugars (Figure 4.11d). Since the mature exocarps of HL and LL berries showed similar photosynthetic capacities (Garrido et al., 2019), we assume that this positive effect of kaolin on exocarp sugar content is related to the higher sugar biosynthesis in the grape leaves, leading to a higher import into the berries, including the exocarp, and/or to the higher biosynthesis from the accumulated malate that is used to produce sugars from the *véraison* stage, onwards.

## 4.3. Materials and Methods

### 4.3.1. Grapevine Field Conditions and Sampling

Grape berry samples were collected from Alvarinho cultivar grapevines (*Vitis vinifera* L.) grown in a field trial conducted in 2018 in the organic vineyard Quinta Cova da Raposa in the Demarcated Region of Vinho Verde, Braga, Portugal (41°34′16.4″ N 8°23′42.0″ W). Details concerning the vineyard and treatments are described in Garrido et al. (2019). Briefly, all treatments—kaolin (K) and non-kaolin (NK) application on leaves, and irrigation (I) and non-irrigation (NI) of grapevines—were applied in a complete factorial design in two blocks, each with three to four vines per treatment. The kaolin suspension (5 % *w/v* in water) was applied on both 6 July and 27 July 2018, corresponding to four and seven weeks after anthesis (WAA), respectively. Irrigation of half of the plants started on 26 July (seven WAA) by means of drip irrigation (one dripper per vine with an average capacity of 5.5 ± 1.6 L $h^{-1}$) for 2 h every three days. Grape clusters with contrasting light exposures in the canopy, called low light (LL) and high light (HL) microclimates, were collected randomly at each experimental condition and at three distinct developmental stages; green (16 July, 6 WAA), *véraison* (29 August, 12 WAA), and mature (17 September) berries were immediately frozen in liquid nitrogen and stored at −80 °C. The exocarp (=skin) and seeds were isolated from the whole frozen grape berries. Firstly, the berry was broken with a slight impact of a pestle in a mortar (both pre-cooled with liquid nitrogen), which allowed us to isolate the seeds. Secondly, the exocarps were dissected from the remaining frozen berry fragments in a petri dish placed in a box with ice, and then quickly transferred to a liquid nitrogen cooled falcon tube. Finally, both seeds and exocarp pieces were ground to a fine powder, using a mortar, a pestle and liquid nitrogen, and freeze-dried for 48 h before metabolomic analyses. At each ripening stage, we sampled berries from 3 (both *véraison* and mature) or 4 (green) biological replicates for each condition, in which 1 replicate resembled a mix of 5 to 10 berries from 3 to 5 clusters from 6 to 8 plants, resulting in a total of 128 samples. All dried samples, conditioned in boxes with silica to maintain the dehydration, were transported to Wageningen, the Netherlands, in order to analyze them by complementary targeted and untargeted metabolomics platforms.

### 4.3.2. Untargeted Metabolomics by Liquid Chromatography Mass Spectrometry (LCMS) and Gas Chromatography Mass Spectrometry (GCMS)

#### 4.3.2.1. LCMS Analysis

All 128 samples were used and extracted according to De Vos et al. (2007). Quality control (QC) samples ($n$ = 5, per each batch analysis) were also prepared with a mix of the grape berry tissues in

order to estimate the overall technical variation per compound. In short, 20 mg dry weight (DW) grape berry tissue was transferred to 2 mL plastic safe-lock Eppendorf tubes and extracted with 600 µL of 75 % (*v*/*v*) methanol/water + 0.1 % formic acid (FA). After vortexing (10 s) and sonication (15 min) (these steps were performed twice), samples were centrifuged (16,100× *g*) for 15 min and the supernatant was collected. Chromatographic separation (5 µL of in injection) was performed on an HPLC system (Waters Acquity, Milford, MA, USA) with a C18 column (Phenomenex Luna 150 × 2 mm i.d., 3 µm—Torrance, CA, USA) using ultra-pure water (eluent A) and acetonitrile (eluent B) both acidified with 0.1 % FA at a flow rate of 0.19 mL $min^{-1}$, starting with 5% B and increasing linearly to 35 % B in 45 min, followed by 15 min of re-equilibration at 5 % B. The column was kept at 40 °C and detection was done with both a PDA detector (Waters) at 210–600 nm and an LTQ-Orbitrap FTMS hybrid mass spectrometer (Thermo Scientific, Bremen, Germany) in negative ionization mode. A mass resolution of 60,000 FWHM was employed for data acquisition. Eluting compounds were detected in full-scan mode in the *m*/*z* range of 90–1350. Separate LCMS/MS runs were performed by re-injecting a random set of extracts, using data-dependent acquisition in discovery mode by selecting the 3 most intense ions per full scan for fragmentation up to MS3. Some selected phenolic compounds were identified or quantified using authentic standards: procyanidin B1, B2 (Extrasynthese®, Genay Cedex, France) and B3 (APIN Chemicals Ltd.®, Compton, United Kingdom), catechin (APIN Chemicals Ltd.®), epicatechin (Sigma®, Zwijndrecht, the Netherlands), epicatechin-3-*O*-gallate (Extrasynthese®), quercetin-3-glucoside (Fluka®, Munich, Germany), piceid (APIN Chemicals Ltd.®) and resveratrol (Sigma®).

#### 4.3.2.2. GCMS Analysis

For the analysis of polar (primary) compounds, we used an untargeted GCMS platform. In view of limited sample amounts, we only analyzed the 24 exocarp samples from the mature stage. Extraction was according to the protocol described by Carreno-Quintero et al. (2012). Briefly, 10 mg of dry weight powder was extracted with 1.4 mL of methanol/water 75 % (*v*/*v*) containing 8 µg $mL^{-1}$ of ribitol (Sigma®) as the internal standard. After sonication and centrifugation, 500 µL of the supernatant was mixed with 375 µL of chloroform (–20 °C) and 750 µL of distilled water (4 °C). After a new centrifugation, aliquots (50 µL) of the upper (polar) phase were transferred to an insert placed in a 2 mL vial. All samples were dried overnight (16 h) by vacuum centrifugation (Savant®, SPD121P, Thermo Scientific) at room temperature and the vials were closed under an argon atmosphere using magnetic crimp caps. Prior to analysis, dried samples were derivatized online using a TriPlusRSH autosampling/injection robot (Thermo Scientific) (Carreno-Quintero et al., 2012; Lisec et al., 2006). First, 12.5 µL of o-methylhydroxylamine

hydrochloride (20 mg $mL^{-1}$ pyridine) was added to the samples and incubated for 30 min at 40 °C with agitation. Then, the samples were derivatized with 17.5 µL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) for 60 min. An alkane mixture (C10-C32) was added to determine the retention indices of metabolites. The derivatized samples were analyzed by a GCMS system consisting of a Trace 1300 gas chromatograph (Thermo Scientific) with a PTV injector coupled to a TSQ8000 DUO-series triple quadrupole mass spectrometer (Thermo Scientific). One microliter of each sample was introduced to the injector at 70 °C using a split flow of 19 mL $min^{-1}$. Chromatographic separation was performed using a VF-5ms capillary column (Varian, Palo Alto, CA, USA; 30 m × 0.25 mm × 0.25 mm) including a 10 m guardian column with helium as the carrier gas at a column flow rate of 1 mL $min^{-1}$. The column effluent was ionized by electron impact at 70 eV. Mass spectra were acquired at a combined SRM and full scan mode with a $m/z$ range of 50 to 600 at an ion source temperature of 290 °C. A solvent delay of 420 s was set.

#### 4.3.2.3. Untargeted Data Processing and Multivariate Statistical Analysis

Unbiased mass peak picking and alignment of the raw data sets from LCMS and GCMS were carried out separately for each tissue using MetAlign software (Lommen, 2009). Irreproducible individual mass signals (present in <3 samples) were filtered out using an in-house script called MetAlign Output Transformer (METOT) (Houshyani et al., 2012). The remaining mass peaks, including molecular ions, in-source adducts (in case of LCMS), fragments and their natural isotopes, were subsequently clustered using MSClust software into so-called reconstructed metabolites (centrotypes) (Tikunov et al., 2012) according to their corresponding retention time and peak intensity pattern across samples. In the final LCMS dataset, the total number of non-detects, i.e., below the detection limit of 2500 ion counts per compound, was 12,154 and 14,308 for exocarp and seeds, respectively. These non-detects were subsequently filtered out when not present in all 3 or 4 biological replicates of at least one sample group. The values of the remaining non-detects (3394 and 2459 for exocarp and seeds, respectively) were randomized between 45 % and 55 % of the detection threshold, i.e., between 1125 and 1375. The resulting spreadsheets for exocarps (Supplemental File S1, Table S1, available on this link) and seeds (Supplemental File S2, Table S1, available on this link) with the relative intensity of each reconstructed metabolite in each sample were used for further statistical analyses.

The on-line tool MetaboAnalyst was employed to compare the three developmental stages for each tissue (Xia et al., 2015). The MSClust output was uploaded into this platform and was $Log_{10}$-transformed and scaled by the Pareto method (mean-centered and divided by the square root of standard deviation of

each variable). Principal component analysis (PCA) was used as an unsupervised approach. In addition, the heatmap plot was represented based on the 25 top-ranking metabolites according to Analysis of Variance (ANOVA) test. On this test, the *p*-values were adjusted using a false discovery rate (FDR) correction.

A multivariate statistical analysis was carried out using MATLAB software. An ANOVA simultaneous component analysis (ASCA), a common tool for analysis of metabolomics data (Smilde et al., 2005), was applied to the Log-transformed data. The model comprised the following factors: HL vs. LL, K vs. NK, I vs. NI and their interactions. The significance of each factor was assessed by a permutation test using 1000 permutations, and Wilks Lambda as a test statistic (Engel et al., 2015). Separate ASCA models were fitted to the data from each developmental stage to study the influence of the factors of interest on the overall metabolome of grape berry tissues. In addition to the ASCA analysis, the effect of the factors mentioned above on each metabolite was studied in more detail by N-way ANOVA. For each factor of interest, the *p*-values were adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR) approach. Adjusted *p*-values smaller than 0.05 were considered to be significant. For each factor, the significant metabolites showing the strongest effect (i.e., highest fold change (FC) values, estimated by the ANOVA) were considered for manual annotation.

The mass of the molecular ion was manually verified within the clustered mass signals of selected, reconstructed metabolites. Metabolites were then annotated using an in-house metabolite database based on comparisons of retention time, accurate mass and UV spectra, if available. On-line available metabolite databases (e.g., KNApSAcK) and literature on grape analyses were also employed for annotation.

In the case of GCMS metabolites, the mass spectrum of each ion cluster was compared with that in available EI-spectral libraries, such as the NIST2014 and the Golm spectral database (Hummel et al., 2010), as well as an in-house library of derivatized standards. In addition, the experimentally obtained RI was compared with reported RIs for verification of the automated spectra annotations.

The level of annotation of compounds was performed following the rules described by Sumner et al. (2007), being classified at four levels: identified metabolites by comparison with standards (level 1), putatively annotated compounds (level 2), putatively characterized compound classes (level 3), and unknown compounds (level 4).

### 4.3.3. Targeted Analysis

#### 4.3.3.1. Tocopherols

The extraction procedure for tocopherols was the same as recently described for chlorophylls and carotenoids (Garrido et al., 2019). Briefly, 20 mg DW of all 128 samples of the grape berry tissues (exocarp or seed) were extracted in 1.8 mL of chloroform/methanol (1:1) with both 0.1 % (*w/v*) butylated hydroxytoluene (BHT) as an antioxidant and Sudan 1 (0.5 μg $mL^{-1}$, Sigma®) as the internal standard (IS). The samples were vortexed, sonicated and centrifuged. The supernatant was dried for 1 h in a Speedvac and prior to analysis, the dried samples were dissolved in 200 μL ethylacetate containing 0.1 % (*w/v*) BHT, again sonicated and centrifuged, and the final supernatant (180 μL) was transferred to amber-colored 2 mL HPLC vials. Samples (20 μL for injection) were analyzed using an HPLC (Waters Alliance e2695 Separations Module, Milford, MA, USA) coupled to a fluorescence detector (Waters 2475) with excitation at 296 and emission at 340 nm. Separation was performed on a reverse-phase C30 column (250 × 4.6 mm i.d., S-5 μm—YMC Carotenoid, Komatsu, Japan) kept at 35 °C with a flow rate of 1 mL $min^{-1}$. The three tocopherol species detected (i.e., α-, γ- and δ-tocopherol) were identified based on comparisons of retention times with authentic standards. Waters Empower 3 software (Waters, Milford, MA, USA) was used for data processing. The total tocopherol content (μg per g of DW tissue) was obtained by adding up the levels of the three detected tocopherols.

#### 4.3.3.2. Sugars

All 24 exocarp samples from the mature stage (10 mg DW) were extracted with methanol/water 75 % (*v/v*). After sonification (10 min), followed by centrifugation (10 min) at maximal speed (16,100× *g*), the supernatant was transferred to new Eppendorf tubes and stored at −20 °C until use. For the sugar analysis, 20 μL was transferred to plastic vial and dried in a vacuum centrifugation without heating. The residue was resuspended in 0.2 mL MiliQ water and vortexed thoroughly. The residue dissolved in water was injected into a Dionex HPLC system (ICS 5000+DC) to analyze the sugar content, using a CarboPac PA 1, 4 × 250 mm column preceded by a guard column (CarboPac PA 1, 4 × 50 mm). Mono-, di-, and tri-saccharides were separated by elution in an increasing concentration of NaOH (20–350 mM) with a flow rate of 1 mL $min^{-1}$. Peaks were identified by co-elution of standards. The sugar amount was expressed in mg of sugar per g of dry material.

#### 4.3.3.3. Total Soluble Phenolics

The Folin–Ciocalteu colorimetric method was used for total phenolics quantification in all 24 exocarp and 24 seed samples from the mature stage (Waterhouse, 2003). Ten mg DW were extracted in 300 µL of 75 % (*v/v*) methanol/water with 0.1 % formic acid (FA), and after vortexing (10 s) and sonication (15 min), samples were centrifuged (16,100× *g*) for 15 min and the supernatant was collected. After that, 50 µL of extract was added to 300 µL of 10 % (*v/v*) Folin reagent and incubated for 5 min in the dark before adding 300 µL of 6 % (*w/v*) sodium carbonate. After 2 h of incubation in the dark, the absorbance was measured at 765 nm. The phenolic contents were determined using a gallic acid (Sigma®) calibration curve and expressed as mg of gallic acid equivalents [GAE]/g DW tissue.

#### 4.3.3.4. Lipid Peroxidation Products

The 16 green exocarp and 12 mature seed samples from the two distinct light microclimates were selected for the analysis of lipid peroxidation products. Ten mg DW were extracted in 800 µL of 0.5 % (*w/v*) 2-thiobarbituric acid (TBA) freshly dissolved in 20 % (*v/v*) trichloroacetic acid (TCA), and 800 µL of water was added. The mixture was vortexed, heated at 95 °C for 30 min in a water bath, cooled on ice and centrifuged at max speed for 10 min. The absorbance of the supernatant was measured at both 532 and 600 nm. Lipid peroxidation product levels were calculated as described by Hodges et al. (1999), and expressed in thiobarbituric acid-reactive-substances (TBARS) per g DW using malondialdehyde (MDA) as a standard.

#### 4.3.3.5. Statistical Analysis

Analysis of Variance tests (two-way ANOVA) were applied, followed by post hoc multiple comparisons using the Bonferroni test whenever the factors had significant effects (GraphPad Prism version 5.00 for Windows, GraphPad Software, La Jolla, CA, USA).

### 4.4. Conclusions

We previously showed that both the exocarp and seed of berries from the white grape variety Alvarinho are photosynthetically active and more so in berries exposed to full sunlight (HL microclimate) than in those of shaded locations in the vines (LL microclimate), especially at the green stage of their development (Garrido et al., 2019). Here, we used unbiased, comprehensive LCMS- and GCMS-based metabolomics approaches, as well as targeted analyses of selected key compounds for grape/wine quality traits, in order to get more insight into the effects of these contrasting canopy light microclimates, as well

as into the effects of soil irrigation and foliar kaolin spraying, on the metabolome composition of berry exocarps and seeds. Both strategies are regularly applied in viticulture as potential mitigation against abiotic stress. Our results indicate the significant influence of the microclimate in both photosynthetically active berry tissues, suggesting a potential role for in situ berry photosynthesis in contributing carbon-skeletons and energy for the biosynthesis of berry components during development and ripening. More experimental research, e.g., by specifically applying artificial shading and exposing grapes to additional light of specific wavelengths, is needed to get a better understanding of the exact role of berry photosynthesis in the final grape quality. In addition, the foliar kaolin application and especially the irrigation treatment appeared to exert their own or combined additional modulating effects on the metabolome of these two berry tissues, possibly due to their direct or indirect influence on photosynthesis in both the leaves and berries. Several compounds affected by these mitigation treatments are also relevant to viticulture, e.g., modulation of a series of phenolic compounds including mono- and polymers of flavan-3-ols, suggesting that good management of these treatments by farmers is necessary and may even further optimize their products by fine-tuning the berry metabolome. Moreover, it is also important to emphasize that this was an exploratory study which aimed to contribute new knowledge and generate hypotheses for future experiments. In addition, for more robust conclusions, more biological replicates and repeating campaigns are helpful in order to possibly link the grape berry metabolite composition to year-to-year variations in wine quality.

## 4.5. Supplementary Materials

The following are available online at:
https://www.mdpi.com/article/10.3390/metabo11040205/s1

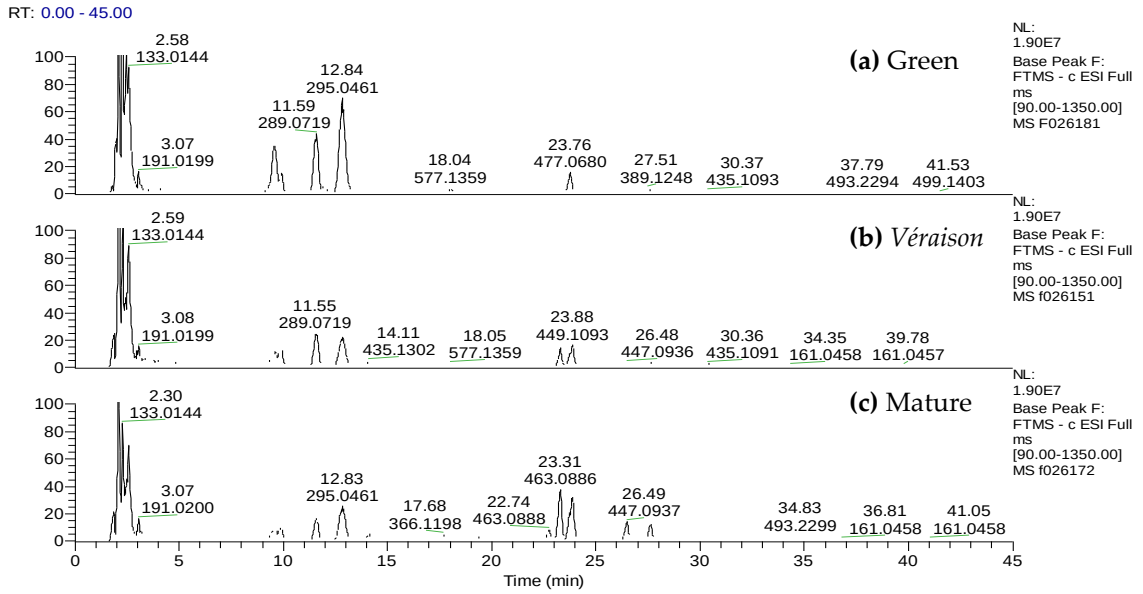


**Figure S4.1.** Liquid chromatography mass spectrometry (LCMS) chromatograms of exocarp at different berry developmental stages: green (**a**), *véraison* (**b**) and mature (**c**). Numbers above peaks represent, from top to bottom, the retention time and mass signals, respectively. The three chromatograms are in the same scale. Annotations of compounds, if known, are provided in (Supplemental File S1, Table S1, available on this link).

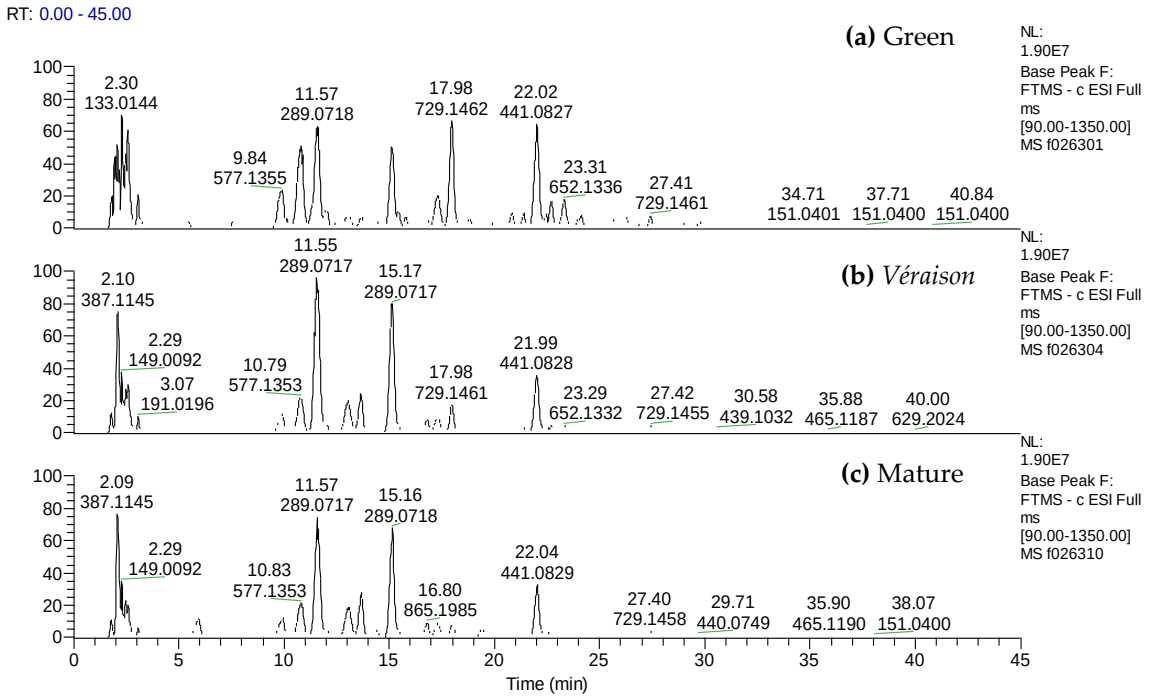


**Figure S4.2.** LCMS chromatograms of seeds at different berry developmental stages: green (**a**), *véraison* (**b**) and mature (**c**). Numbers above peaks represent, from top to bottom, the retention time and mass signals, respectively. The three chromatograms are in the same scale. Annotations of compounds, if known, are provided in Supplemental File S2, Table S1, available on this link.

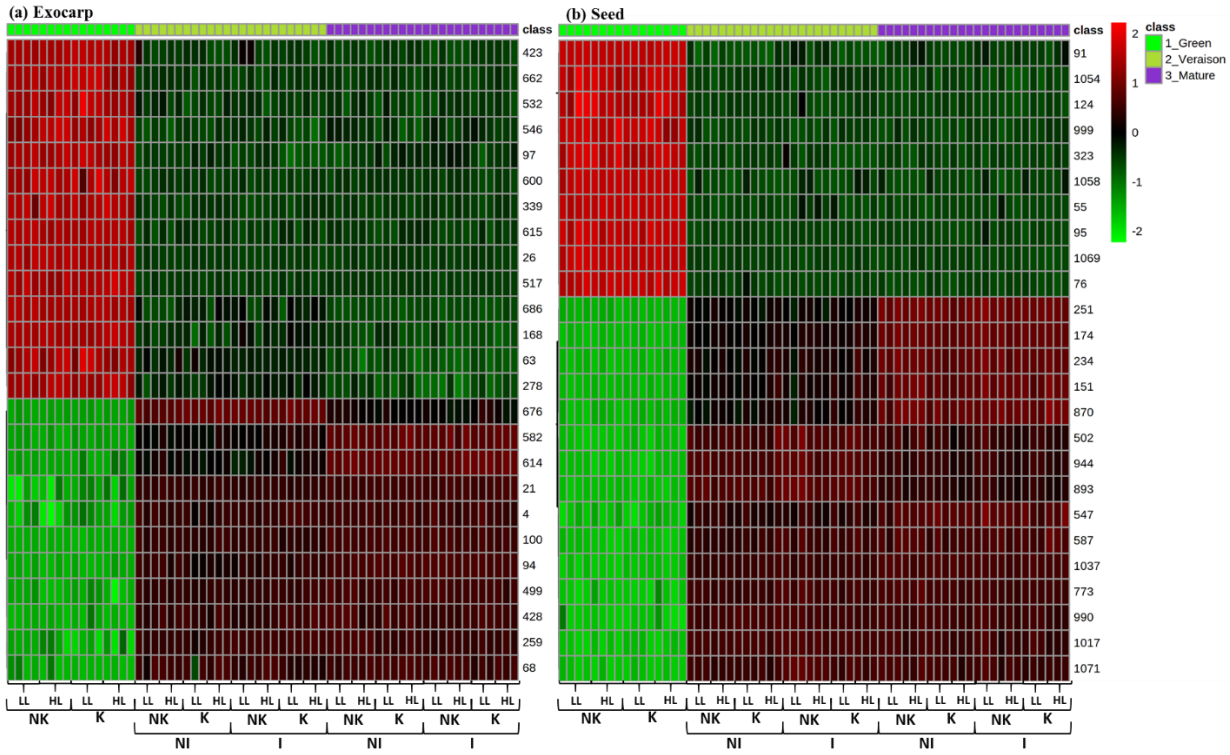


**Figure S4.3.** Heatmaps with the most significant LCMS-metabolites affected by developmental stages in exocarp (**a**) and seed (**b**). Heatmap plot with the 25 top-ranking metabolites from Analysis of Variance (ANOVA). Metabolites are represented as numbers on the right-hand side of the plot, using the metabolite number (metabolite ID) obtained from the data processing. Developmental stages are represented from left to right side of the plot by order, as well as, the replicates for low and high light (LL and HL, respectively) grape berries grown under the four combinations of the two treatments applied: irrigation (I)/ non-irrigation (NI) x kaolin (K)/non-kaolin (NK).

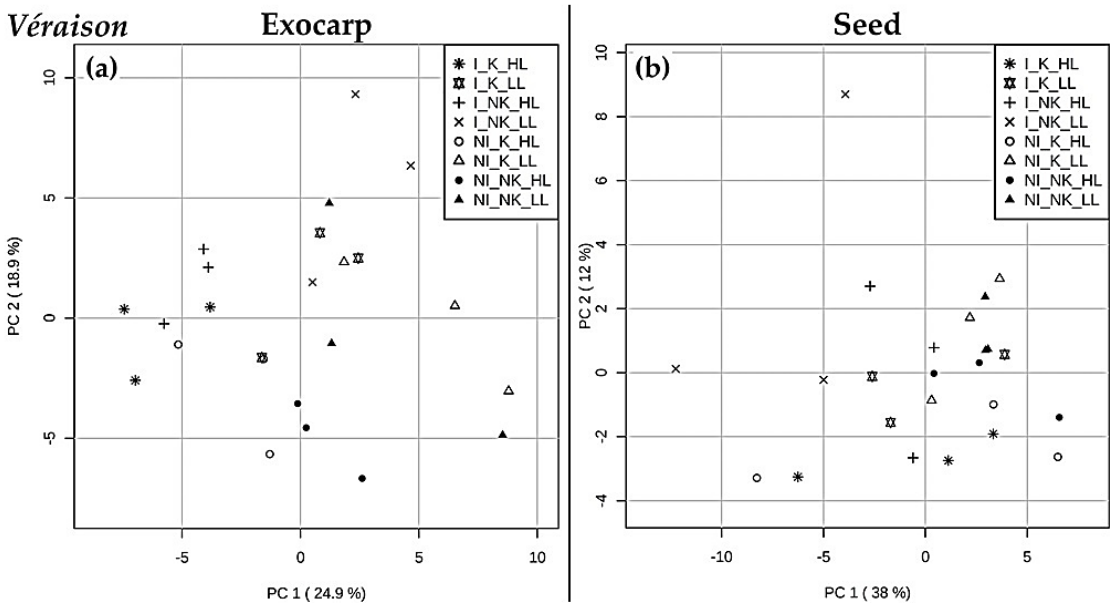


**Figure S4.4.** Principal component analysis (PCA) score plots of the liquid chromatography mass spectrometry (LCMS) metabolite data for exocarp and seed at *véraison* stage, including all microclimates and treatments ($n$ = 3).

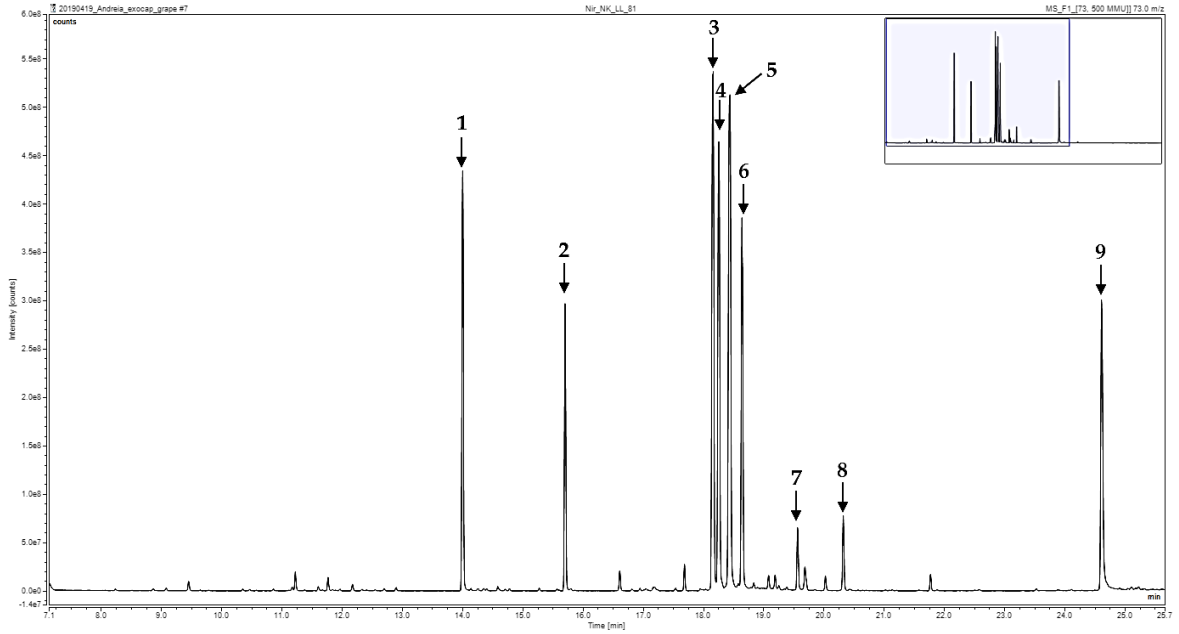


**Figure S4.5.** Gas chromatography mass spectrometry (GCMS) raw chromatogram of polar metabolites extracted from the exocarp of mature berries. **1 -** Malic acid (ID 1851); **2 -** Tartaric acid (ID 3244); **3 -** Fructose, isomer 1 (ID 5298); **4 -** Fructose, isomer 2 (ID 5745); **5 -** Glucose, isomer 1 (ID 6481); **6** - Galactose, isomer 2 (ID 7204); **7 -** D-Gluconic acid (ID 8359); **8 -** Myo-inositol (ID 9692); **9 -** Sucrose (ID 11573).

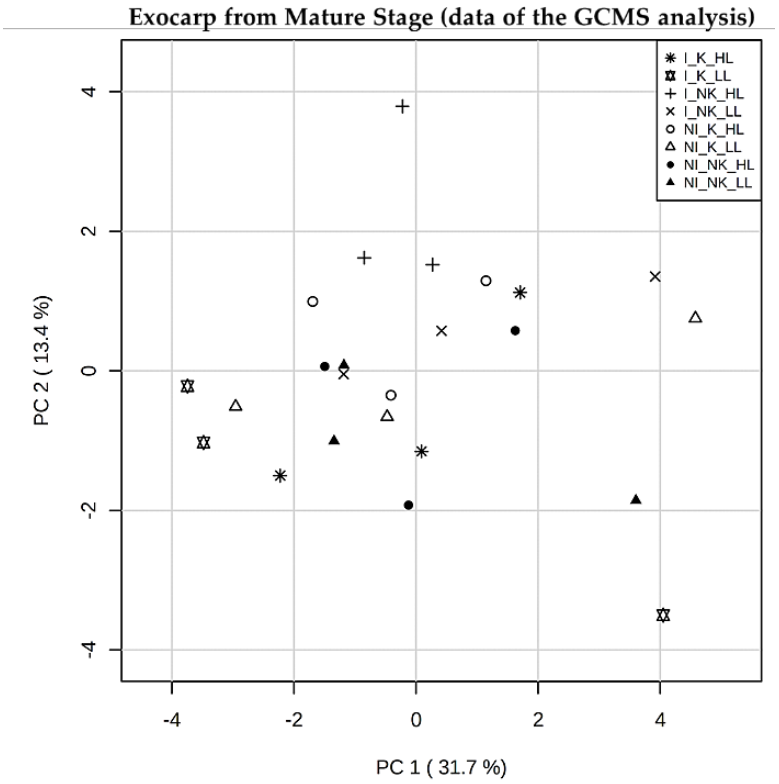


**Figure S4.6.** Principal component analysis (PCA) score plots of the gas chromatography mass spectrometry (GCMS) metabolite data for exocarp at mature stage, including all microclimates and treatments (*n* = 3). The abbreviations in the legend represent: NI - Non-Irrigation; I - Irrigation; NK - Non-kaolin; K - Kaolin; LL - Low Light microclimate; HL - Hight Light microclimate.

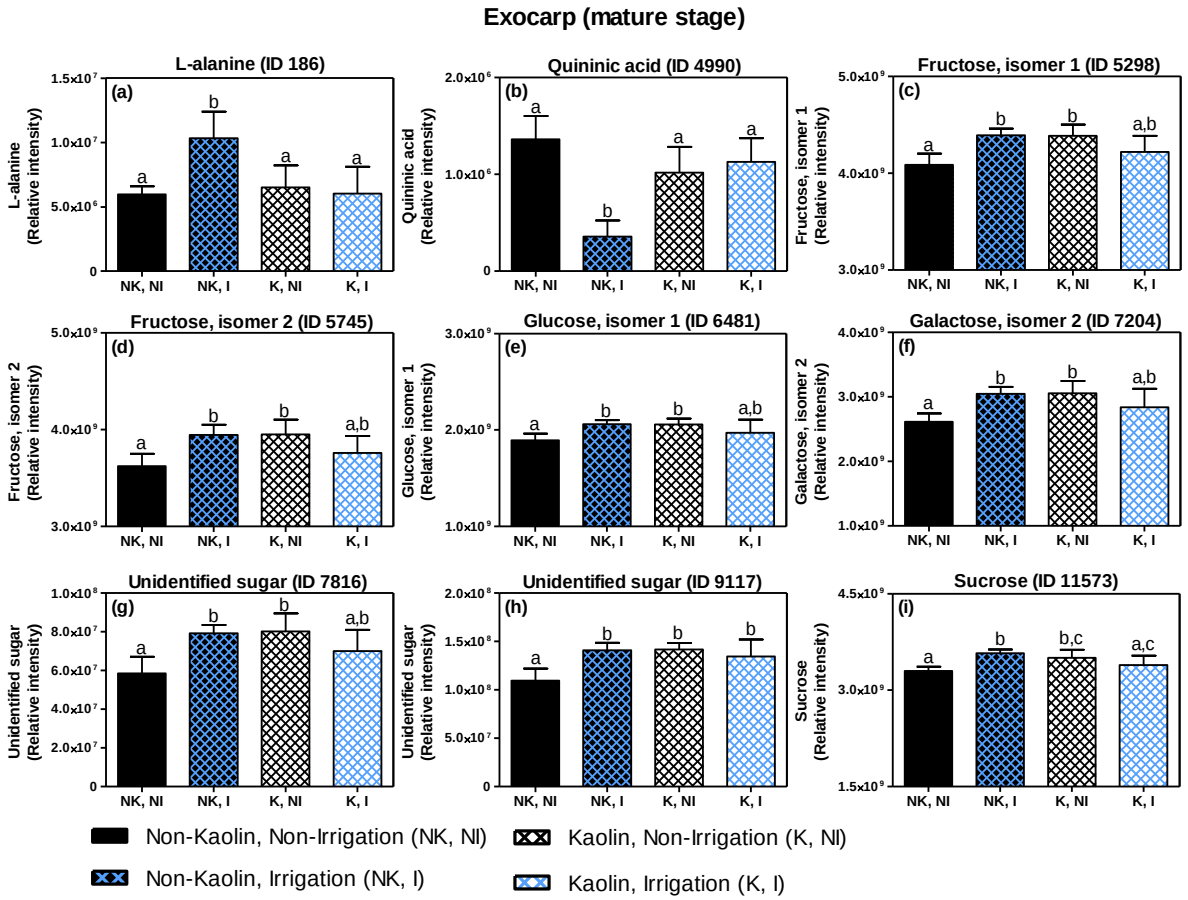


**Figure S4.7.** Relative abundance of some selected primary metabolites in the exocarp of mature berries as detected by GCMS (means and SD of $n = 6$, data of high light and low light microclimate combined): (**a**) L-Alanine (ID 186); (**b**) Quininic acid (ID 4990); (**c**) Fructose, isomer 1 (ID 5298); (**d**) Fructose, isomer 2 (ID 5745); (**e**) Glucose, isomer 1 (ID 6481); (**f**) Galactose, isomer 2 (ID 7204); (**g**, **h**) Unidentified sugar (ID 7816 and 9117); (**i**) Sucrose (ID 11573). Berries grown under the four combinations of the two treatments applied: irrigation (I)/ non-irrigation (NI) x kaolin (K)/ non-kaolin (NK). Statistical analysis (one-way ANOVA, $p \leq 0.05$) was applied after data $Log_2$ transformation. Statistical notation above the bars: letters refer to differences between treatment combinations.

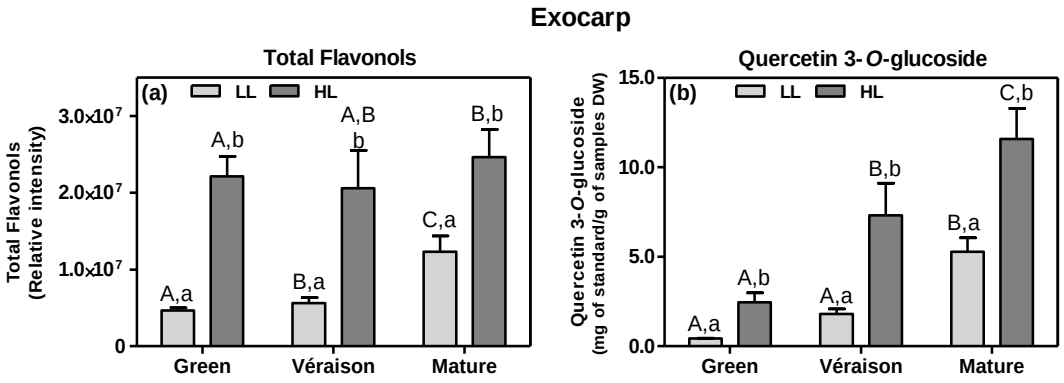


**Figure S4.8.** Flavonols in the exocarp. (**a**) Sum of relative intensities of the six main flavonol peaks as detected by LCMS (means and SD of $n = 8$-12) at two canopy microclimates (LL and HL; independent of mitigation treatment) and at three developmental stages (green, *véraison* and mature). The flavonols considered are: rutin (quercetin-3-*O*-rutinoside) (ID 462), quercetin 3-*O*-glucoside (ID 492), quercetin 3-*O*-glucuronide (ID 505),

kaempferol-3-glucoside (ID 545), kaempferol glucuronide (ID 588) and isorhamnetin hexoside (ID 583) (Supplemental File 1, Table S5-S7). Statistical analysis (two-way ANOVA, $p \leq 0.05$) was applied after data $Log_2$ transformation. (**b**) Quercetin 3-*O*-glucoside amount (mg/g of dry weight (DW)) in exocarp tissue grown at two canopy microclimates and from three developmental stages. Statistical notation above the bars: the capital letters refer to differences between developmental stages for the same microclimate, while the lowercase letters refer to differences between the two light microclimates for each stage.

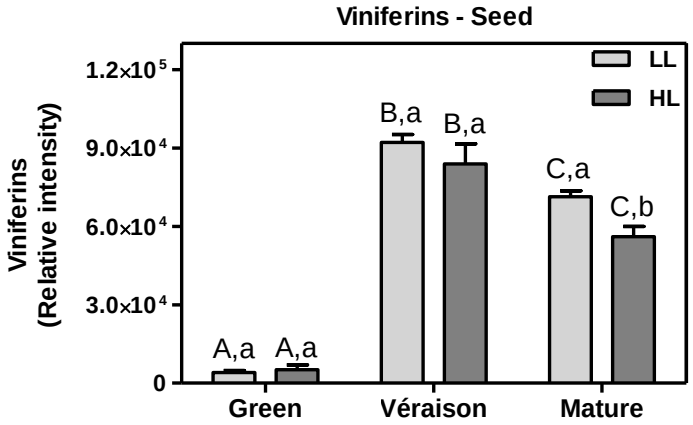


**Figure S4.9.** Sum of relative intensities of the two viniferins as detected by LCMS (means and SD of $n$ = 8-12) for seed tissue at two canopy microclimates (LL and HL; independent of mitigation treatment) and at three developmental stages (green, *véraison* and mature). The viniferins considered are: (+)-alpha-viniferin-hexoside (ID 990) and viniferin 3''-glucoside (ID 1027). Statistical analysis (two-way ANOVA, $p \leq 0.05$) was applied after data $Log_2$ transformation. Statistical information is the same as in Figure S4.7.

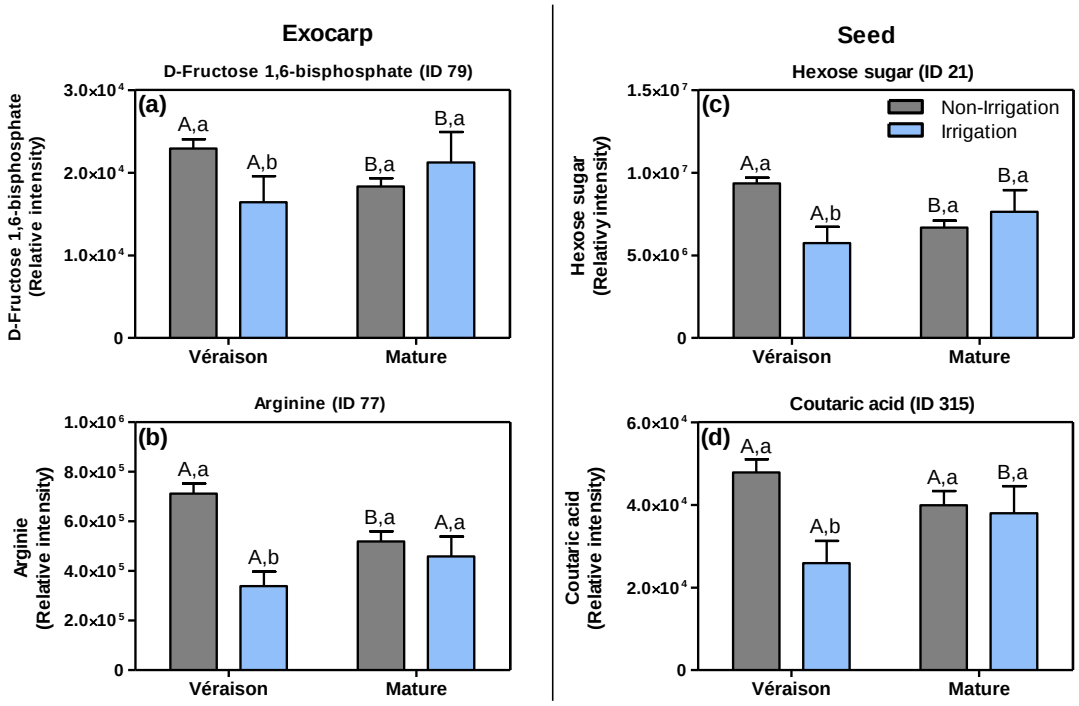


**Figure S4.10.** Relative abundance of some selected metabolites detected by LCMS ($n$ = 8-12, +SD) in exocarps and seeds under non-irrigation (grey bars) and irrigation conditions (blue bars) applied at two berry developmental stages (*véraison* and mature) (data of HL and LL berries combined). For exocarp: (**a**) D-Fructose 1,6-bisphosphate (ID 79), (**b**) arginine (ID 77). For seed: (**c**) hexose sugar (ID 21) and (**d**) coutaric acid (ID 315).

Statistical analysis (two-way ANOVA, $p \leq 0.05$). Statistical notation above the bars: the capital letters refer to differences between developmental stages for the same treatment condition, while the lowercase letters refer to differences between the control and irrigation treatment, within each developmental stage.

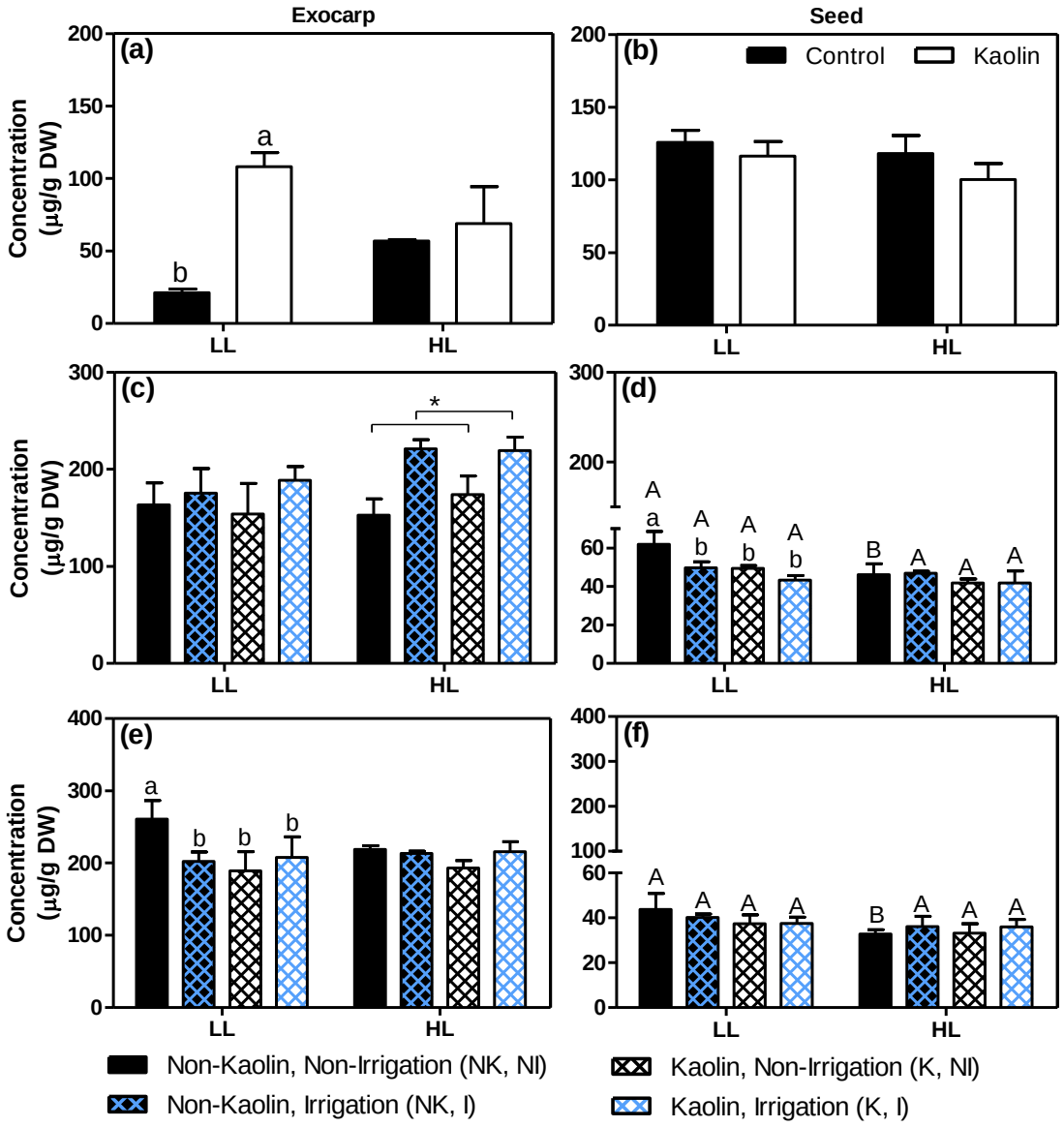


**Figure S4.11.** α-tocopherol contents (mean values of $n = 3$ or 4 biological replicates, +SD) in exocarps (**a**, **c** and **e**) and seeds (**b**, **d** and **f**) of berries from grape clusters, grown either low light (LL) or high light (HL) microclimate conditions in the canopy. Kaolin (K) or no kaolin (NK) was applied to the plant leaves before fruit set; after green berries were developed, plants were either irrigated (I) or non-irrigated (NI). Samples were collected at three development stages: green (**a** and **b**), *véraison* (**c** and **d**) and mature (**e** and **f**). Statistical notations above the bars: at each developmental stage, the capital letters refer to differences between the two light microclimates within the same treatment, while the lowercase letters refer to differences between treatment combinations within the same light microclimate (values with a common letter or no letter at all, indicate no significant differences; two-way ANOVA, $p \leq 0.05$). Notation with an asterisk means that only one factor (in this case the irrigation) was significant.

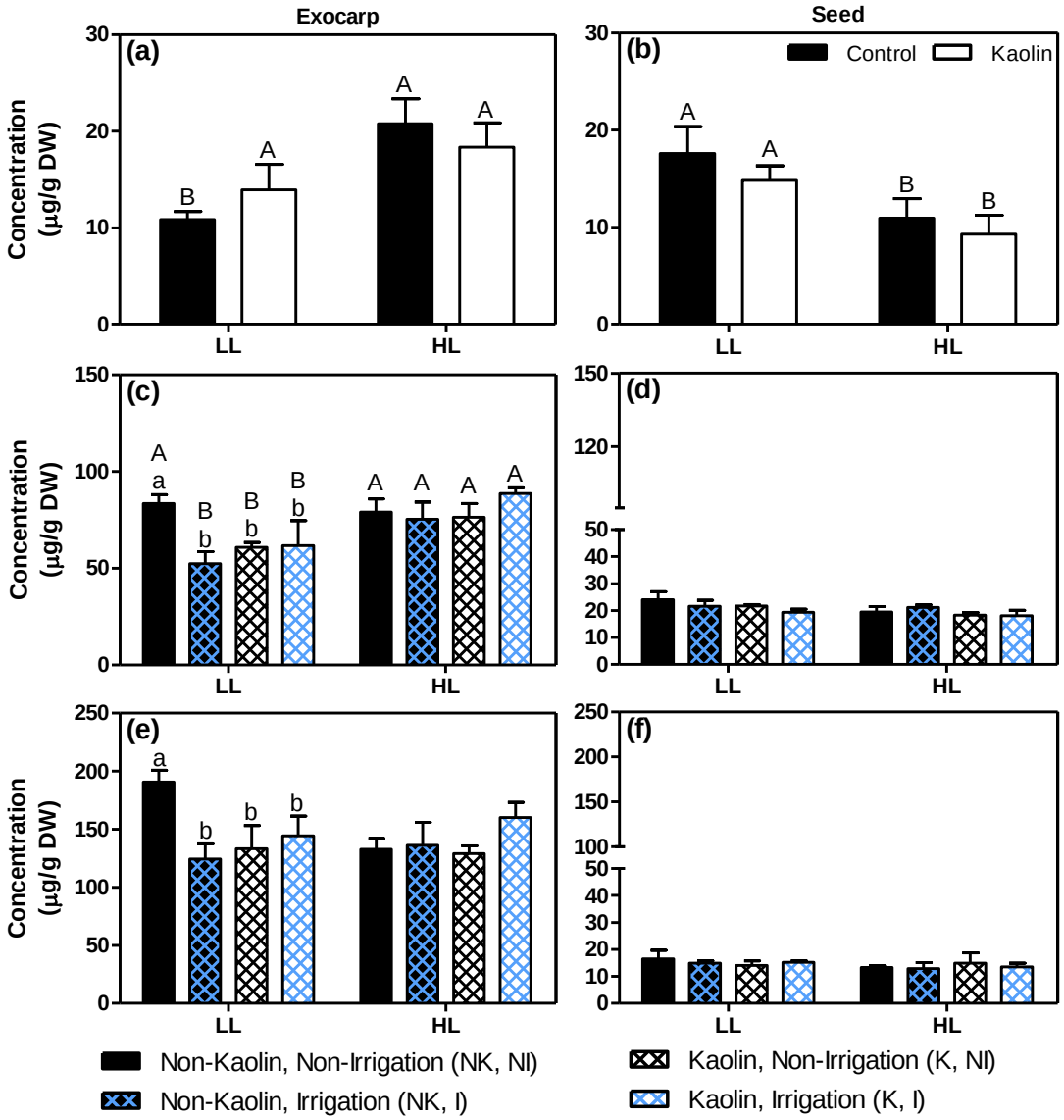


**Figure S4.12.** γ-tocopherol contents (mean values of *n* = 3-4 biological replicates, +SD) in exocarps (**a**, **c** and **e**) and seeds (**b**, **d** and **f**). All the microclimate conditions, treatment combinations, and statistical information are the same as in Figure S4.11.

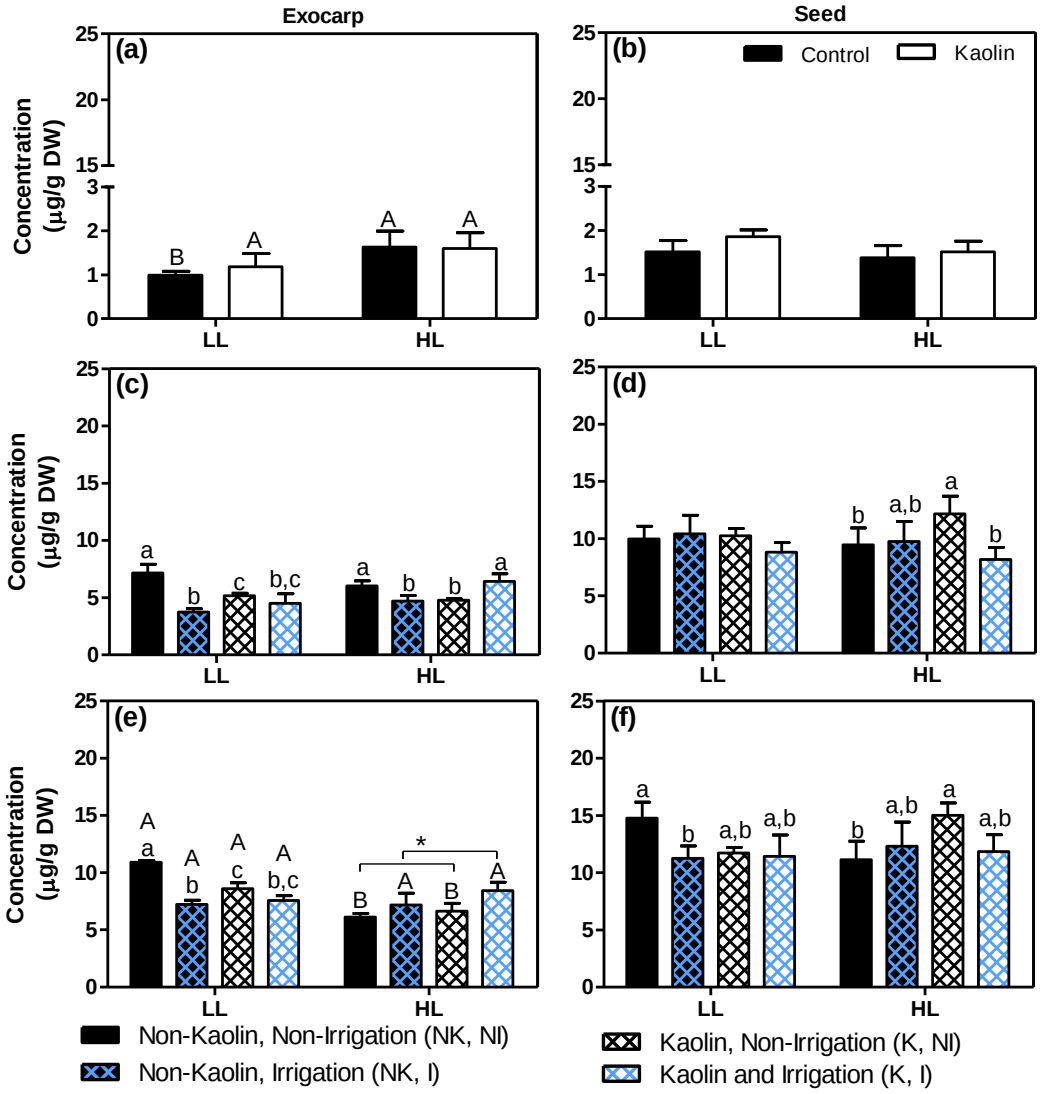


**Figure S4.13.** δ-tocopherol contents (mean values of *n* = 3-4 biological replicates, +SD) in exocarps (**a**, **c** and **e**) and seeds (**b**, **d** and **f**). All the microclimate conditions, treatment combinations, and statistical information are the same as in Figure S4.11.

## 4.6. Funding

This research and APC were funded by the FCT-Portuguese Foundation for Science and Technology, grant provided to Andreia Garrido (PD/BD/128275/2017), under the Doctoral Program "Agricultural Production Chains—from fork to farm" (PD/00122/2012).

## 4.7. Acknowledgments

The work was supported by National Funds by FCT—Portuguese Foundation for Science and Technology, under the project UIDB/04033/2020. The work was also supported by FCT and European Funds (FEDER/POCI/COMPETE2020) through the research project "MitiVineDrought—Combining "omics" with molecular, biochemical, and physiological analyses as an integrated effort to validate novel

and easy-to-implement drought mitigation strategies in grapevine while reducing water use" with ref. PTDC/BIA-FBT/30341/2017 and ref. POCI-01-0145-FEDER-030341, respectively; and through the research project "BerryPlastid—ref. POCI-01-0145-FEDER-028165 and ref. PTDC/BIA-FBT/28165/2017, respectively. Artur Conde was supported by a post-doctoral researcher contract/position within the project "MitiVineDrought" (PTDC/BIA-FBT/30341/2017 and POCI-01-0145-FEDER-030341). This work also benefited from the networking activities within the European Union-funded COST Action CA17111"INTEGRAPE—Data Integration to maximize the power of omics for grapevine improvement". Authors acknowledge the owner from Quinta Cova da Raposa, Manuel Taxa, who provided the samples, and also all support given by the Biology Department of the School of Sciences from the University of Minho. The authors would also like to acknowledge Bert Schipper, Henriëtte van Eekelen and Jeroen van Arkel (all Plant Metabolomics group of WUR-Bioscience) for their help in the chemical analyses and data processing.

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# Chapter 5

## Light microclimate-driven changes at transcriptional level in photosynthetic grape berry tissues

The work presented in this chapter was submitted (26th June 2021):

Garrido, A., De Vos, R. C. H., Conde, A.* and Cunha, A.* (submitted). Light microclimate-driven changes at transcriptional level in photosynthetic grape berry tissues. *Plants*, *10*, x, https://doi.org/10.3390/xxxxx.

*Equal senior authorship.

Author Contributions: Conceptualization, A.G., R.C.H.D.V, A.C. (Artur Conde) and A.C. (Ana Cunha); methodology, A.G. and A.C. (Artur Conde); formal analysis, A.G. A.C. (Artur Conde) and A.C. (Ana Cunha); investigation, A.G. and A.C. (Ana Cunha); resources, A.C. (Artur Conde) and A.C. (Ana Cunha); writing—original draft preparation, A.G.; writing—review and editing, A.G., R.C.H.D.V, A.C. (Artur Conde) and A.C. (Ana Cunha); supervision, R.C.H.D.V, A.C. (Artur Conde) and A.C. (Ana Cunha); project administration, A.C. (Ana Cunha).

**Abstract**

Viticulture practices that change the light distribution in grapevine canopy can interfere with several physiological mechanisms, like grape berry photosynthesis and other metabolic pathways, and consequently impact the berry biochemical composition, which is key to the final wine quality. We previously showed that the photosynthetic activity of exocarp and seed tissues was in fact responsive to the light microclimate in the canopy (low and high light, LL and HL, respectively), and that these different light microclimates also led to distinct metabolites profiles, suggesting a berry tissue-specific interlink between photosynthesis and metabolism. In the present work we analyzed the transcript levels of key genes in exocarps and seed integuments of berries collected from HL and LL microclimates at three developmental stages, using real-time qPCR. In exocarp the expression levels of genes involved in carbohydrate metabolism (*VvSuSy*), phenylpropanoid (*VvPAL1*), stilbenoid (*VvSTS1*) and flavan-3-ol synthesis (*VvDFR*, *VvLAR2* and *VvANR*) were highest at green stage. In seeds, the expression of several genes associated with both phenylpropanoid (*VvCHS1* and *VvCHS3*) and flavan-3-ols synthesis (*VvDFR* and *VvLAR2*) showed a peak at *véraison* stage, while that of RuBisCO was maintained up to mature stage. Overall, the HL microclimate, as compared to LL, resulted in a higher expression of genes encoding elements associated with both photosynthesis (*VvChlSyn* and *VvRuBisCO*), carbohydrate metabolism (*VvSPS1*) and photoprotection (carotenoid pathways genes) in both tissues. HL also induced the expression of the *VvFLS1* gene, which was translated into a higher activity of the FLS enzyme producing flavonol-type of flavonoids, while the expression of several other flavonoid pathway genes (e.g., *VvCHS3*, *VvSTS1*, *VvDFR* and *VvLDOX*) was reduced, suggesting a specific role of flavonols in photoprotection of berries growing in HL microclimate. This work suggests an interlink between berry photosynthesis and metabolism at the transcriptional level and provides relevant information for a smarter management of the light microenvironment at canopy level of the grapes.

**Keywords:** light microclimate, exocarp, seed, gene expression, enzyme activity, grape berry photosynthesis, metabolic pathways.

## 5.1. Introduction

Grapevine (*Vitis vinifera* L.) is commonly cultivated across temperate to semi-dry areas, including the Mediterranean region (Estreicher, 2017; Santillán et al., 2020). Currently, grape berry and wine production are being affected by the escalation of environmental constraints, due to the intensification of climate changes, thus, adaptation and/or stress mitigation strategies are being implemented for a better management of vineyards (as reviewed by Santos et al., 2020).

Grape berry is composed by different tissues and cell layers, including the exocarp (skin), mesocarp (pulp) and seeds, which present distinct anatomical characteristics and biochemical profiles during development (Cadot et al., 2006; Famiani et al., 2000; Hardie et al., 1996). Different tissues of the grape berry have different functions, mainly anatomical/structural, physiological and ecological, but they are also important in viniculture because their composition has a direct impact on the wine organoleptic properties (e.g., color, aroma, flavor and texture) (Garrido and Borges, 2013; Niimi et al., 2020). Indeed, the exocarp contributes to the integrity of the whole berry by protecting inner tissues from mechanical damage or pathogen attack (Hardie et al., 1996), allowing timely seed dissemination (Martin and Rose, 2014), and also confers protection against ultraviolet light exposure, especially due to its flavonols content (Martínez-Lüscher et al., 2019). The seed is rich in flavan-3-ol monomers and procyanidins (tannins), which confer protection against herbivory but are also responsible for the bitterness and astringency to the wine (Rousserie et al., 2019). Both primary and secondary metabolites of grape berry tissues are extremely important for fruit nutritional and organoleptic characteristics (Pott et al., 2019). Complex regulatory mechanisms are involved in their synthesis, such as many transcriptional, translational and biochemical, that can be also modulated by biotic and abiotic factors (as reviewed by Serrano et al., 2017).

It is well established that environmental conditions have a strong influence on metabolism of grape berry cells (Blancquaert et al., 2019). Light is an abiotic factor that influences the overall grapevine physiology but also grape berry composition (Poni et al., 2018). In this way, viticulture practices that involve canopy manipulations (e.g., leaf removal, shading covering, canopy conduction systems, and also irrigation) are directly related with levels of light reaching the leaves and grape berry clusters (Reynolds, 2010). Smart et al. (1985) introduced the concept of microclimate to define the specific environmental conditions in the vicinity of leaves and fruits. Several studies have addressed the effects of light conditions experienced by developing grape berries on their primary and secondary metabolites as well as on transcriptional changes related to key genes (Friedel et al., 2015; Koyama et al., 2012; Plessis et al., 2017; Reshef et al., 2017; Young et al., 2016). However, most of these studies focused on whole berries or just on the skin, and not specifically to other tissues/organs like seeds. To the best of our knowledge, no studies concerning the effects of light microclimate on grape seed metabolism and gene expression have been reported to date.

Like leaves, fruits may present photosynthetic activity, at least at their early stage of development (Aschan and Pfanz, 2003; Brazel and Ó'Maoileídigh, 2019). In grape berries, both the exocarp and the seed outer integument exhibit photosynthetic activity (Breia et al., 2013) and their photosynthetic

competence is responsive to the light microclimate experienced by the grapes throughout their development (Garrido et al., 2018). More recently, we characterized the photosynthetic profiles of these two berry tissues collected from clusters growing in two contrasting light microclimates in the canopy: LL (low light) i.e., shaded inner zones of the canopy; and HL (high light) i.e., grape berry clusters are exposed to direct sunlight part of the day, receiving 3-fold more light intensity than LL clusters (Garrido et al., 2019). Moreover, these photosynthetic profiles of these two berry tissues were also assessed in vineyards under short-term mitigation treatments against climate adversities, i.e., foliar kaolin application and soil irrigation (Garrido et al., 2019). A metabolomics study showed that both light microclimate and irrigation were the main environmental factors influencing the metabolite composition of exocarp and seed (Garrido et al., 2021).

Transcriptomics and genomics studies have disclosed the main elements involved in berry photosynthesis, especially in the skin (da Silva et al., 2005; Deluc et al., 2007; Grimplet et al., 2007; Terrier et al., 2005; Waters et al., 2005). In particular, Waters et al. (2005), using cDNA microarray analysis, verified that expressed sequence tag (ESTs) involved in photosynthesis and carbohydrate metabolism were co-regulated, suggesting that photosynthesis in the berry skin is a source of carbohydrate for the berry skin itself. However, much uncertainty still exists about the relation between grape berry photosynthesis and its primary and secondary metabolism, and whether this is regulated at the level of gene transcription or enzyme activity.

The main objective of the present work was to evaluate the transcriptional changes in key genes involved in photosynthesis, sucrose metabolism and secondary metabolite pathways (carotenoids, phenolics), in both exocarp and seed of grape berries exposed to either HL or LL microclimate, aiming to establish a potential link between transcripts, metabolites (Garrido et al., 2021) and photosynthetic activity (Garrido et al., 2019) in these berry tissues.

## 5.2. Results and Discussion

### 5.2.1. Transcriptional changes in photosynthetic machinery elements and primary metabolism

Chlorophylls are crucial elements from thylakoids membranes in the chloroplast, being responsible for light energy harvesting, together with the carotenoids, and for initiating the flow of electrons into the electron transport chain. We analyzed the transcription of the *Chlorophyll Synthase* gene (*VvChlSyn*) in exocarp and seed (Figure 5.1). *VvChlSyn* codes for chlorophyll synthase (Enzyme Commission Number - EC 2.5.1.62), the enzyme that catalyzes the last step of the biosynthetic pathway of chlorophylls *a* and *b*.

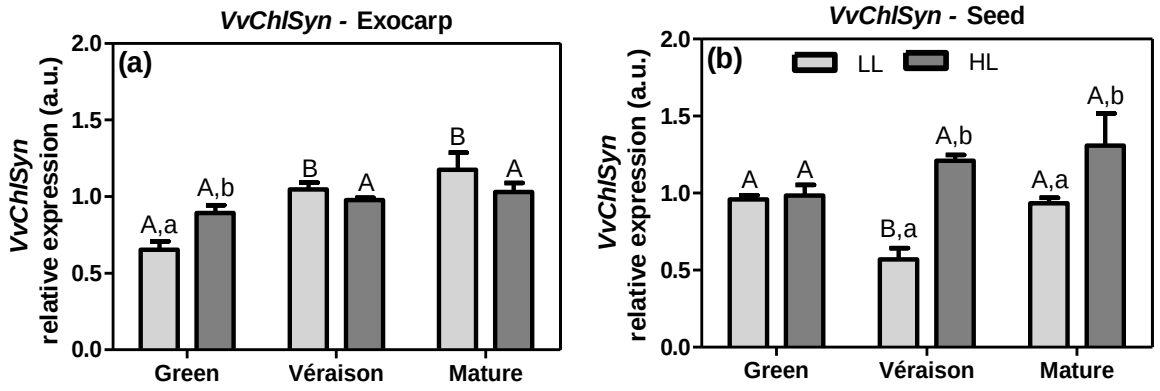


**Figure 5.1.** Relative expression of transcripts of chlorophyll synthase gene (*VvChlSyn*) in (**a**) exocarp and (**b**) seeds, from berries grown at two distinct light microclimates (LL and HL) and at three developmental stages (green, *véraison* and mature), as determined by real-time qPCR. Expression levels are normalized to the mean expression of reference genes *VvACT1* and *VvGAPDH*. Statistical analysis (two-way ANOVA, $p \leq 0.05$, $n = 3$) was applied after data Log(X+1) transformation. Statistical notation: capital letters refer to differences between developmental stages for the same microclimate, while lowercase letters refer to differences between microclimates for each stage. When the letters are omitted, it means that the respective factor did not have a significant effect.

Regarding to the microclimate effects on *VvChlSyn* expression in exocarp, statistical differences between HL and LL were observed already at green stage, in which HL led to a higher expression compared with LL (Figure 5.1a). During berry development, this *VvChlSyn* expression remained stable in HL exocarps, while it significantly increased (1.8-fold) in LL ones (Figure 5.1a). These results for *VvChlSyn* expression in exocarp are in agreement with the chlorophyll levels previously reported in this tissue (Garrido et al., 2019), and are also consistent with the observation that HL exocarps from green berries present a higher photosynthetic activity than LL ones (Garrido et al., 2019). In seeds, both light microclimates showed similar *VvChlSyn* expression at green stage, while at both later stages (*véraison* and mature) HL significantly increased *VvChlSyn* expression (2.1 and 1.4-fold at *véraison* and mature stage, respectively), as compared to LL (Figure 5.1b). The inner localization of seeds causes constrictions

to the level of light received, especially at the later stages of development, when the volume of the fruit increases (Aschan and Pfanz, 2003). Thus, this result of *VvChlSyn* expression in seeds may be partly explained by the dependence of these inner tissues for high light intensities and consistent with HL seeds being able to acclimate the photosynthetic capacity to higher light intensity challenges (Garrido et al., 2018). Overall, these results highlight the importance of HL microclimate to the *VvChlSyn* expression, which can also be reflected in the chlorophyll levels and in a functional photosynthetic system of both grape berry tissues.

Carotenoids not only contribute to the color and, through their cleavage into aromatic apocarotenoids like ionones, to the aroma of fruits, but are also important components of the photosynthetic apparatus, by playing a key role in light harvesting and transference of energy in the photosystems (Maoka, 2020). The carotenoid biosynthetic pathway comprises the synthesis of carotenes and their subsequent conversion into xanthophylls (Young et al., 2012). In land plants, the thermal dissipation of the excess light energy is performed by two xanthophyll cycles (Young et al., 2012): 1) the violaxanthin (Vx) cycle, i.e., the reversible enzymatic conversion of violaxanthin to zeaxanthin, via the intermediate antheraxanthin; and 2) the lutein-epoxide (Lx) cycle, i.e., the reversible enzymatic conversion of Lx to lutein.

In the present work we analyzed the expression of several genes coding for key enzymes of the carotenoid pathway (Figure S5.1), namely: *phytoene synthase 1* (*VvPSY1*) (Figure 5.2a,b), which encodes the first dedicated carotenoid biosynthetic enzyme (EC 2.5.1.32); *lycopene beta-cyclase 2* (*VvLBCY2*) (Figure 5.2c,d), a critical gene diverting to the branch of β-carotene and the violaxanthin cycle and thus competing with *lycopene epsilon cyclase 1* (*VvLECY1*) (Figure 5.2e,f). The latter is involved in the conversion of lycopene into δ-carotene, that is in the direction of α-carotene and lutein synthesis, diverting to the lutein-epoxide cycle; *carotene epsilon-monooxygenase* or *lutein-deficient 1* (*VvLUT1*) (Figure 5.2g,h), which is responsible for the synthesis of lutein and the lutein-epoxide cycle (Young et al., 2012); *violaxanthin de-epoxidase 1* (*VvVDE1*) and *zeaxanthin epoxidase 1* (*VvZEP1*) (Figure 5.2i-l), which are both involved in the violaxanthin cycle.

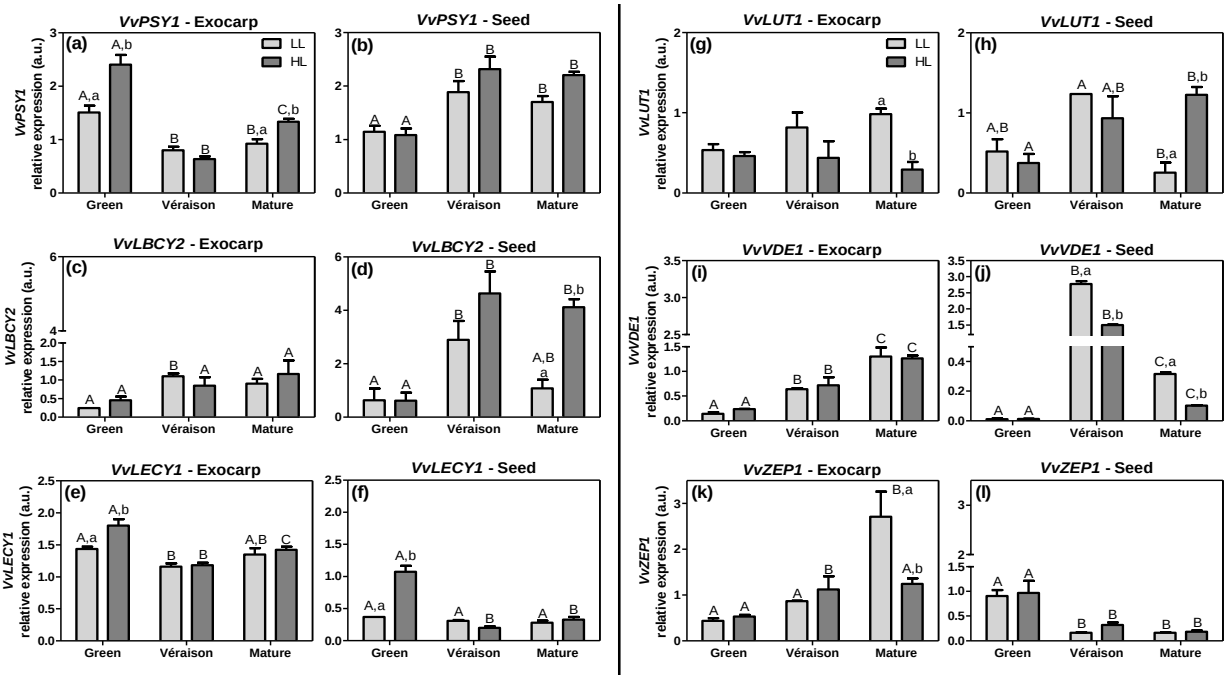


**Figure 5.2.** Relative expression of transcripts of phytoene synthase 1 (*VvPSY1*) (**a**, **b**), lycopene beta-cyclase 2 (*VvLBCY2*) (**c**, **d**), lycopene epsilon cyclase 1 (*VvLECY1*) (**e**, **f**), carotene epsilon-monooxygenase or lutein-deficient 1 (*VvLUT1*) (**g**, **h**), violaxanthin de-epoxidase 1 (*VvVDE1*) (**i**, **j**) and zeaxanthin epoxidase 1 (*VvZEP1*) gene (**k**, **l**) in exocarp and seeds, from berries grown at two distinct light microclimates (LL and HL), and at three developmental stages (green, *véraison* and mature), as determined by real-time qPCR. Expression levels are normalized to the mean expression of reference genes *VvACT1* and *VvGAPDH*. Statistical analysis (two-way ANOVA, $p \leq 0.05$, $n = 3$) was applied after data Log(X+1) transformation. Statistical notation: capital letters refer to differences between developmental stages for the same microclimate, while lowercase letters refer to differences between microclimates for each stage. When the letters are omitted, it means that the respective factor did not have a significant effect.

The expression pattern of all genes was altered during berry development (Figure 5.2), suggesting the importance of the ripening-dependent changes in carotenoid composition in both photosynthetic tissues. The results for both *VvPSY1*, *VvLBCY2* and *VvLECY1* (Figure 5.2a-f) show that at specific points of berry development, the HL berries exhibited a significantly increased expression in either exocarp or seed, in comparison to LL berries. This result suggests a higher carotenoid biosynthesis in fully exposed berries as compared to shaded berries. While *VvPSY1* expression peaked at green stage in the exocarp (Figure 5.2a), both *VvPSY1* and *VvLBCY2* expressions tend to increase during berry development in both tissues, most clearly in HL seeds (Figure 5.2a-d). In contrast, *VvLECY1* expression decreased from green to mature, again especially in HL seeds (Figure 5.2e,f), suggesting an up-regulation of the Vx route instead of Lx at later stages. This higher expression of *VvLECY1* in both tissues of green HL berries, together with relative low levels of *VvLBCY2* (Figure 5.2c,d), point to an up-regulation of the Lx route at green stage. In exocarps from green stage, the higher expression of genes *VvPSY1* and *VvLECY1* in HL (Figure 5.2a,e) is in accordance with the higher total carotenoid content (i.e., considering the summed values of α- and β-

carotene and lutein) in these HL berries compared to LL ones (Garrido et al., 2019). Moreover, the reduction of the expression of these two genes in exocarp throughout berry development (Figure 5.2a,e) is also in line with the previously reported decrease in total carotenoid levels (Garrido et al., 2019). These results suggest that, during their early development, the green berries growing in HL microclimate, i.e. with their exocarp exposed to direct sunlight, acclimate to the relative high light levels via an increased carotenoid/xanthophyll biosynthesis, supporting their higher photosynthetic activity (c.f. Garrido et al., 2019). Likewise, previous studies using berries from a different white grape variety (Sauvignon Blanc variety) report that at their green stage the exposed berries contained a higher pool of carotenoids compared to shaded ones, being also more acclimated to light stress (Joubert et al., 2016; Plessis et al., 2017).

The transcript levels of genes directly involved in the xanthophylls cycles (*VvLUT1*, *VvVDE1* and *VvZEP1* - Figure 5.2g-l) revealed that: in exocarp of mature grapes *VvLUT1* was significantly (>3-fold) less expressed in HL compared to LL microclimate (Figure 5.2g), while in seeds of the same stage it was the opposite (Figure 5.2h); the *VvVDE1* expression in exocarp increased throughout berry development and was similar between HL and LL conditions (Figure 5.2i), while in seeds its expression was lower in HL than in LL, except for green stage, clearly peaking at *véraison* stage (Figure 5.2j); *VvZEP1* expression in exocarp increased during development, reaching at mature stage significantly higher levels (>2-fold) in LL berries than in HL berries (Figure 5.2k); in contrast, its expression in seeds decreased and equally between HL and LL berries (Figure 5.2l). The increase in *VvVDE1* expression throughout exocarp development (Figure 5.2i) suggests that the Vx cycle is up-regulated in this external tissue, thereby protecting the photosynthetic apparatus from damage during berry development and ripening. Indeed, in the Sauvignon Blanc variety both *VvVDE1* expression and the pool of carotenoids (predominantly xanthophylls) increased in light-exposed berries, as compared to shaded ones, and were able to protect the photosynthetic machinery (Young et al., 2016).

In mature seed, the maintenance of the higher expressions in HL compared to LL of both *VvLUT1* (Figure 5.2h) and *VvLBCY2* (Figure 5.2d) together with the stronger decrease in *VvVDE1* expression (Figure 5.2j), are indicative of an up-regulation of both lutein and zeaxanthin synthesis by HL. In addition, in seeds the increase in *VvVDE1* expression and decrease in *VvZEP1* from green to *véraison* stages (Figure 5.2j,l) suggests the promotion of the zeaxanthin biosynthetic pathway in this tissue, upon berry development.

Globally, at green stage the higher expression of *VvLECY1* in both tissues (Figure 5.2e,f), together with the reduced levels of *VvLBCY2* (Figure 5.2c,d) and *VvLUT1* (Figure 5.2g,h), suggest that the

carotenoid pathway branch towards a-carotene, which seems to play a role in both tissues at this initial stage of development. At *véraison* and mature stage the exocarp relies on Vx, reducing the carbon flow to lutein synthesis (cf. Figure 5.2e,g,i,k), while in seeds both xanthophylls cycles seem to operate at these later stages of development (cf. Figure 5.2d,h,j).

Ribulose bisphosphate carboxylase/oxygenase (RuBisCO; EC 4.1.1.39) is a critical enzyme of the Calvin-Benson cycle of photosynthesis that catalyzes the condensation of $CO_2$ with ribulose 1,5-bisphosphate (RuBP) producing two molecules of 3-phosphoglycerate (3-PGA) as the stable products. In order to determine the effect of grape berry microclimate on this key enzyme in photosynthesis, the relative expression of *VvRuBisCO* gene in grape berry exocarps and seeds was analyzed in both microclimates (Figure 5.3).

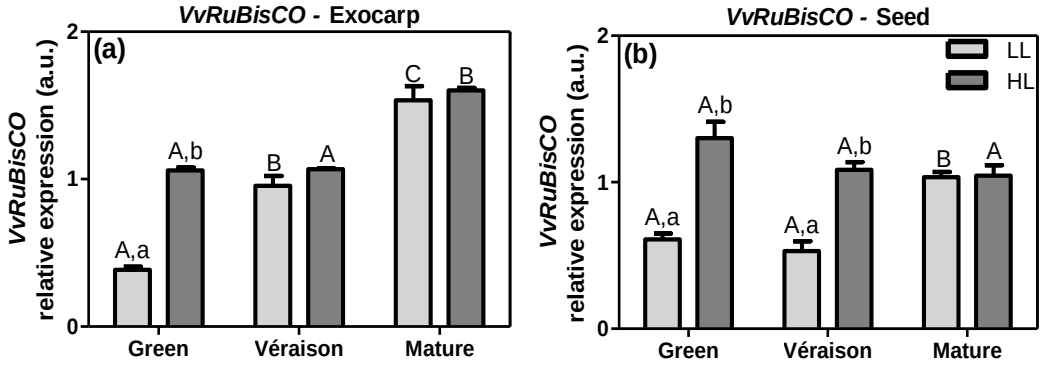


**Figure 5.3.** Relative expression of transcripts of ribulose bisphosphate carboxylase/oxygenase gene (*VvRuBisCO*) in (**a**) exocarp and (**b**) seeds, from berries grown at two distinct light microclimates (LL and HL), and at three developmental stages (green, *véraison* and mature), as determined by real-time qPCR. Expression levels are normalized to the mean expression of reference genes *VvACT1* and *VvGAPDH*. Statistical analysis (two-way ANOVA, $p \leq 0.05$, $n = 3$) was applied after data Log(X+1) transformation. Statistical notation: capital letters refer to differences between developmental stages for the same microclimate, while lowercase letters refer to differences between microclimates for each stage. When the letters are omitted, it means that the respective factor did not have a significant effect.

At green stage, both exocarps and seeds from HL berries showed a significant higher expression of *VvRuBisCO* (>2-fold), in comparison to LL ones (Figure 5.3a,b), in parallel with our previous results of their photosynthetic activities (Garrido et al., 2019). At *véraison* stage, HL microclimate led to a higher *VvRuBisCO* expression in seeds only (Figure 5.3b). No difference between HL and LL was observed at mature stage in either tissue. These results suggest that microclimate had an impact on carbon fixation capacity of both tissues, most specifically in immature fruits.

During berry development, the relative expression of *VvRuBisCO* in HL seeds was more or less constant while for exocarps it increased in both microclimates (Figure 5.3). These results are in accordance with the photosynthetic activity pattern of exocarps, which was kept high up to later ripening

stages, while in seeds this activity declined *post-véraison* (Garrido et al., 2018, 2019). During grape berry development, stomatal conductance decreases and gas exchanges with the atmosphere become virtually null after *véraison* due to blockage of the stomata with cuticular waxes (Palliotti and Cartechini, 2001), so the maintenance/increase in *VvRuBisCO* expression during ripening may be related to the fixation of internally produced $CO_2$. It is argued that refixation of respiratory $CO_2$ by RuBisCO after the onset of ripening (e.g., from malate catabolism) can provide carbon skeletons for other metabolic pathways, including seed storage lipids, as proposed for oilseed rape (Schwender et al., 2004).

Carbohydrate metabolism is a key point of interconnection between several metabolomic pathways, contributing for synthesis of intermediate compounds (Durán-Soria et al., 2020; Pott et al., 2019). In the present work we analyzed the expression of two genes *VvSPS1* and *VvSuSy* (Figure 5.4), both coding important enzymes in sugar metabolism. Sucrose-phosphate synthase (SPS; EC 2.4.1.14) is an enzyme involved in the sucrose biosynthesis, catalyzing the formation of sucrose-6-phosphate from uridine diphosphate glucose (UDP-glucose) and fructose-6-phosphate (Deluc et al., 2007). Sucrose synthase (SuSy; EC 2.4.1.13) is a glycosyl transferase, mainly present in the cytosol, that is responsible for both synthesis and catabolism of sucrose, and thus catalyzes the reversible conversion of sucrose and uridine diphosphate into UDP-glucose and fructose (Stein and Granot, 2019; Zhu et al., 2017). Wang et al. (2014), suggested that in grape berries the SuSy might cooperate with cell wall invertases cleaving the sucrose unloaded into the cytoplasm of sink cells. The products of sucrose cleavage by SuSy are available for many metabolic pathways, such as energy and primary-metabolites production and synthesis of complex carbohydrates (reviewed by Stein and Granot, 2019).

In both tissues the relative transcript levels of *VvSPS1* increased from green to later stages of berry development, being responsive to light microclimate (Figure 5.4a,b). The main difference between these tissues is that in the exocarp the HL microclimate, compared to LL, led to a significant up-regulation of *VvSPS1* expression at mature stage, while in seeds this effect was observed at the earlier stage *véraison*. These results reveal that sucrose biosynthesis was enhanced in exocarps and seeds during berry ripening. Previously we showed that the exocarp tissue is photosynthetically active up to mature stage of berry ripening, while the seeds show a slight decrease in photosynthesis especially after *véraison* stage (Garrido et al., 2019). The expression patterns of *VvSPS1* (Figure 5.4) and *VvRuBisCO* (Figure 5.3) were quite similar, suggesting an active photosynthetic/Calvin-Benson Cycle function until late in berry development in both grape berry tissues, most specifically in the exocarp. In this manner, from *véraison* stage onwards a flux of trioses phosphates from berry chloroplasts can fuel the biosynthesis of other intermediates and products, as the cytosolic synthesis of sucrose, the major translocatable product of photosynthesis.

Supporting this suggestion, Wu et al. (2011) showed that the enzyme activity of SPS in whole grape berries from several cultivars increased with development.

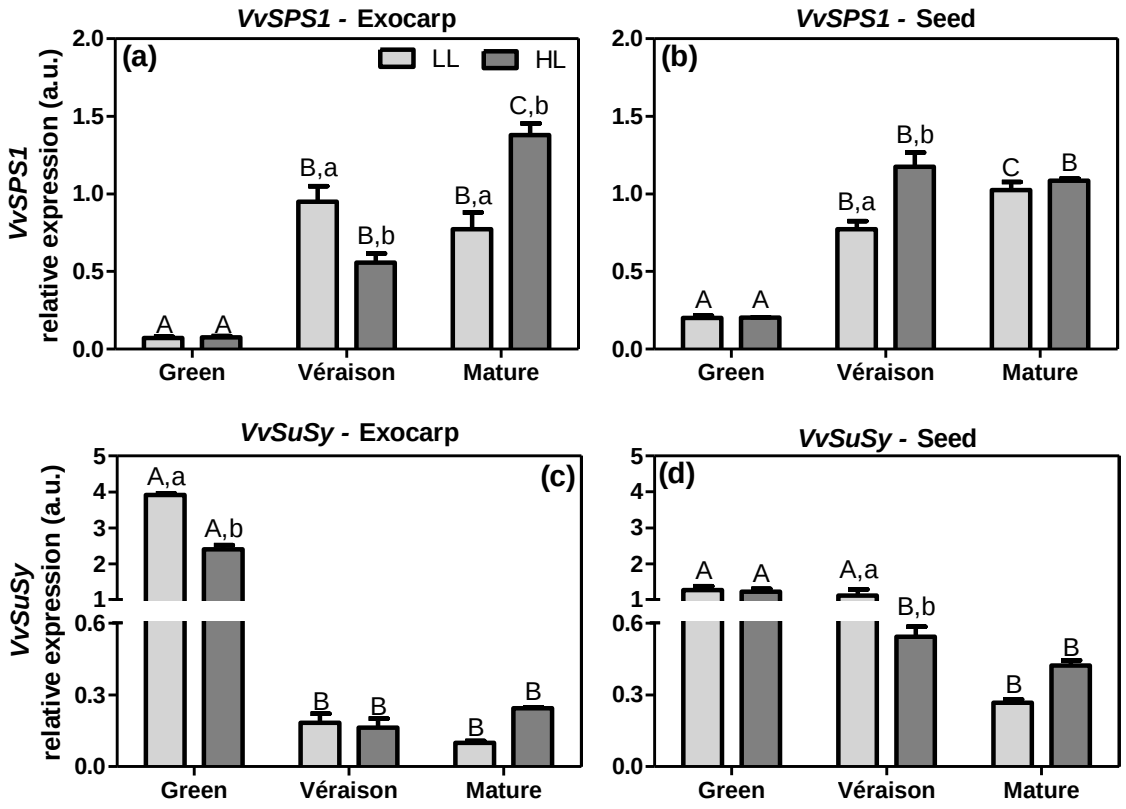


**Figure 5.4.** Relative expression of transcripts of sucrose-phosphate synthase 1 (*VvSPS1*) (**a**, **b**) and sucrose synthase (*VvSuSy*) gene (**c**, **d**) in exocarp and seeds, from berries grown at two distinct light microclimates (LL and HL), and at three developmental stages (green, *véraison* and mature), as determined by real-time qPCR. Expression levels are normalized to the mean expression of reference genes *VvACT1* and *VvGAPDH*. Statistical analysis (two-way ANOVA, $p \leq 0.05$, $n = 3$) was applied after data Log(X+1) transformation. Statistical notation: capital letters refer to differences between developmental stages for the same microclimate, while lowercase letters refer to differences between microclimates for each stage. When the letters are omitted, it means that the respective factor did not have a significant effect.

Both exocarp and seed had high levels of *VvSuSy* expression at green stage, that decreased at later ripening stages and particularly in exocarp (Figure 5.4c,d), thus in an opposite pattern compared to *VvSPS1*. This relative high expression level of *VvSuSy* and low expression of *VvSPS1* at the green stage in both tissues may suggest that sucrose imported from leaves, rather than sucrose produced locally in the berry, is crucial to cope with the high carbon and energy demands associated with the intense cell proliferation activity that occur during this phase of fruit growth (Dokoozlian, 2000).

### 5.2.2. Transcriptional and biochemical activity changes in the phenylpropanoid and flavonoid pathways

Both the expression patterns of *VvRuBisCO* and *VvSPS1*, and our previous metabolomics study (Garrido et al., 2021), suggest a role of berry photosynthesis in berry primary metabolism. Possibly, this berry photosynthesis can contribute with locally supplying carbohydrate resources to cope with the high demand of substrates needed for secondary metabolism, as also pointed by others (Dai et al., 2013; Degu et al., 2014; Martínez-Esteso et al., 2011; Wang et al., 2017; Zamboni et al., 2010). We therefore also intended to analyze the effects of the two contrasting canopy light microclimates on the expression of genes involved in the phenylpropanoid, flavonoid and stilbenoid pathways (Figure S5.2), which are responsible for the biosynthesis of key quality metabolites in both exocarp and seed of the grape berry (Ristic and Iland, 2005). According to our previous metabolomics study (Garrido et al., 2021), stilbenes were relatively abundant in both green exocarp and mature seed, while flavonols were mostly detectable in exocarp, especially at *véraison* and mature stages, and flavan-3-ols were most abundant at the initial stages of development (i.e., green and *véraison*) in both exocarp and seed.

The phenylpropanoid pathway starts with the conversion of phenylalanine into cinnamic acid by phenylalanine ammonia lyase enzyme (PAL; EC 4.3.1.24) (Singh et al., 2010). In grapevine, the analysis of the genome predicts 13 copies for *PAL* genes (Velasco et al., 2007), with *PAL1* the best characterized isoform (Boubakri et al., 2013). In both berry tissues, the transcript levels of *VvPAL1* decreased along development (Figure 5.5a,b). The canopy microclimate effect was more evident for the exocarp, in which HL led to a decrease in *VvPAL1* expression as compared to LL, at both green and *véraison* stages (Figure 5.5a). A previous study using berry skins of two red cultivars (Jingxiu and Jingyan) showed that *PAL* expression did not vary between exposed and shaded berries (Zheng et al., 2013). Another study with a red grape cultivar (Cabernet Sauvignon) showed that the expression levels of *PAL* in berry skin were up-regulated at both *véraison* and full mature stages upon increased sunlight exposure resulting from leaf removal (Sun et al., 2017) These apparently conflicting results may be explained by the fact that these latter studies were focused on the later stages of development and involved red varieties, in which the anthocyanin biosynthesis as well as the expression of genes up- and downstream in the pathway and their regulatory transcription factors (MYB family) are induced by light (Guan et al., 2016; Matus et al., 2009). On the other hand, the fact that *VvSuSy* expression (Figure 5.4c) was higher in LL than HL at the green berry stage may have resulted in a higher content in hexoses which are known to stimulate *PAL* expression (Dai et al., 2014; Roubelakis-Angelakis and Kliewer, 1986), and thus may have been responsible for the higher expression of *PAL* in these LL exocarps (Figure 5.5a).

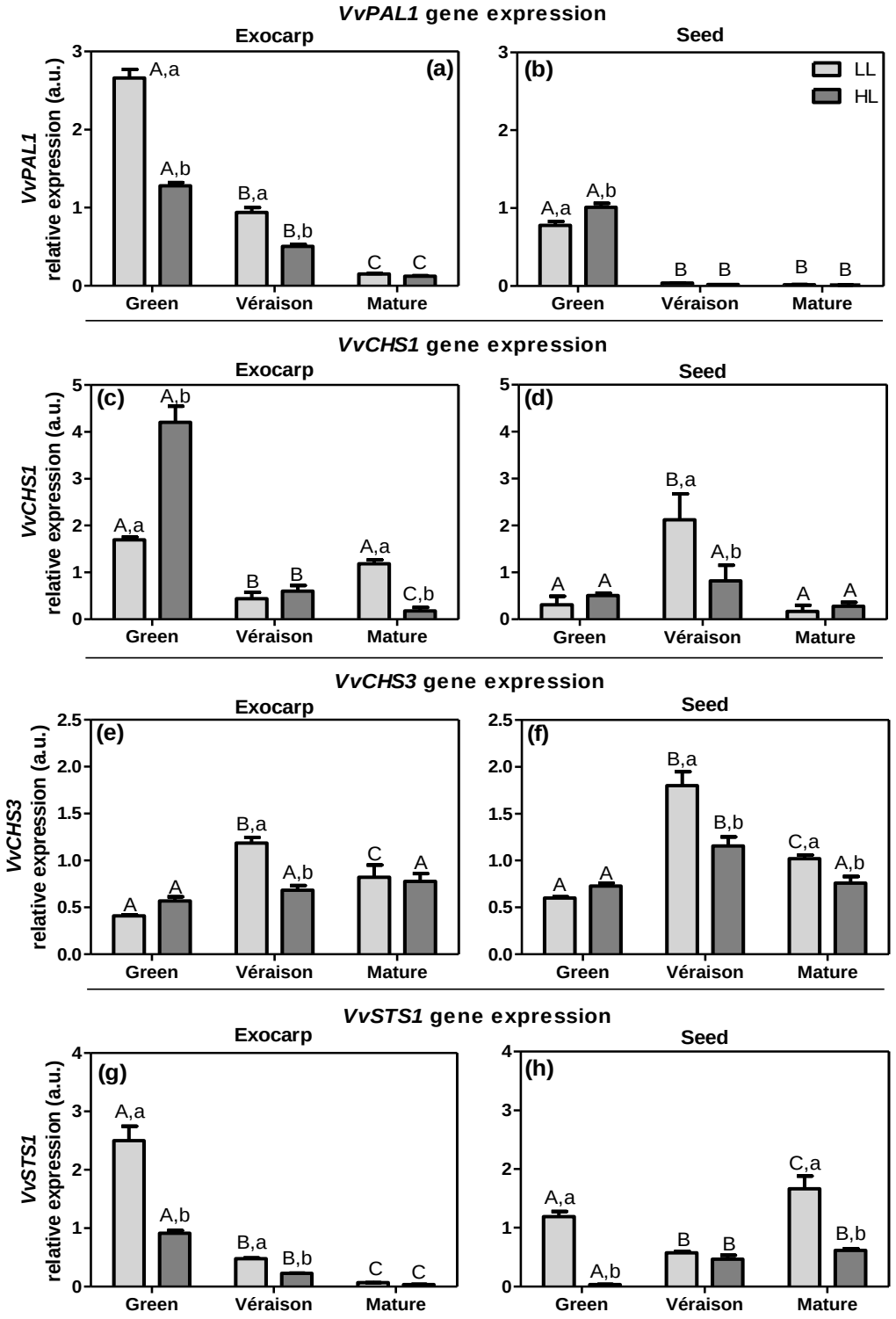


**Figure 5.5.** Relative expression of transcripts of phenylalanine ammonia lyase 1 (*VvPAL1*) (**a**, **b**), chalcone synthase 1 and 3 (*VvCHS1* and *VvCHS3*) (**c-f**) and stilbene synthase 1 (*VvSTS1*) gene (**g**, **h**), in exocarp and seeds, from berries grown at two distinct light microclimates (LL and HL), and at three developmental stages (green, *véraison* and mature), as determined by real-time qPCR. Expression levels are normalized to the mean expression of reference genes *VvACT1* and *VvGAPDH*. Statistical analysis (two-way ANOVA, $p \leq 0.05$, $n = 3$) was applied after data Log(X+1) transformation. Statistical notation: capital letters refer to differences between developmental stages for the same microclimate, while lowercase letters refer to differences between microclimates for each stage. When the letters are omitted, it means that the respective factor did not have a significant effect.

The flavonoid pathway is initiated by the chalcone synthase enzyme (CHS; EC 2.3.1.74) (Tian et al., 2006). We analyzed the gene expression of two isoforms (i.e., *VvCHS1* and *VvCHS3*; Figure 5.5c-f), because in white grapevine varieties these are known to be mostly expressed within the estimated total of four isoforms (Goto-Yamamoto et al., 2002). In exocarp tissue the expression of *VvCHS1* was highest at green stage, particularly in HL berries, and decreases during subsequent ripening (Figure 5.5c). In seeds *VvCHS1* was relatively low at green stage and showed a peak at *véraison* stage, particularly in LL berries (Figure 5.5d). Such peak at *véraison* stage with highest levels in LL berries was also observed for the *VvCHS3* gene in both tissues (Figure 5.5d,e,f). While *VvCHS3* exhibited a similar expression pattern in both tissues during development, *VvCHS1* expression pattern seems to be dependent on tissue and developmental stage and was differentially regulated by light microclimate. A previous study with grape berry from a red grapevine variety (Shiraz) showed that for skin, and in contrast to our results, the expression of *VvCHS1* and *VvCHS3* increased during development, while for seeds, and in agreement to our results, the expression of both genes peaked at *véraison* stage (Harris et al., 2013). The increase of *VvCHS* expression in skins of red varieties may be related to the ripening-induced biosynthesis of anthocyanins, which obviously does not take place in white cultivars. Moreover, cells suspension cultures of petiole *callus* from a white grapevine variety (Chardonnay) showed higher transcripts levels of *VvCHS1* when grown in the light as compared to cells grown in the dark, whereas *VvCHS3* was not induced by light (Harris et al., 2013), in line with our results for exocarp from HL vs LL at green stage (Figure 5.5c,e).

Stilbene synthase enzyme (STS; EC 2.3.1.95) is responsible for the condensation of 4-coumaroyl-CoA with 3 molecules of malonyl-CoA producing resveratrol. In grapevine, the *VvSTS1* is the best characterized isoform from a total of 48 *STS* genes (Parage et al., 2012). Its expression significantly decreased throughout exocarp development (Figure 5.5g) in a very similar pattern to that of *VvPAL1*. HL microclimate led to a decreased in *VvSTS1* expression in exocarp at green stage only, while in seeds this decrease was observed at both green and mature stages (Figure 5.5g,h). Considering that CHS and STS compete for the same substrate (Figure S5.2), the opposite responses of the respective genes to microclimate in the exocarp at green stage (Figure 5.5c,g) may be indicative of a down-regulation of the stilbene branch by HL and a channeling of substrates into the flavonoid pathway at this early stage of the berry development. In the seeds, the expression of *VvSTS1* was particularly evident at green and mature stages, in an inverse pattern compared to the *VvCHS1* and *VvCHS3* genes (Figure 5.5d,f,h).

Flavonol synthase (FLS; EC 1.14.20.6) is the key enzyme for the biosynthesis of flavonol-type of flavonoids, by catalyzing the oxidation of dihydroflavonols into flavonols. Among the five *FLS* genes in grape (Fujita et al., 2006), only the isoform *FLS1* showed a clear expression pattern corresponding to the accumulation of flavonols in berry skins (Downey et al., 2003), and thus this gene was selected for our study. In exocarp the expression of *VvFLS1* was relatively low: it was undetectable at green stage while increasing afterwards (Figure 5.6a), in agreement with previous studies with both red and white grape berry skins (Downey et al., 2003). In contrast, in seeds *VvFLS1* expression was detected at all stages, with a reduction along development (Figure 5.6b). In both tissues the HL microclimate, as compared to LL, significantly up-regulated *VvFLS1* expression (e.g., a 7-fold of increase for exocarp at mature stage). To check if these changes in expression of the *FLS1* gene isoform were translated into changes in enzyme activity, we prepared enzyme extracts and determined the potential FLS activity ($V_{max}$) using dihydroquercetin as a substrate. Indeed, in exocarp the activity of the FLS enzyme showed a similar profile as the *VvFLS1* gene expression, both with respect to development (802-fold of increase from green to mature) and microclimate (5-fold of increase by HL at mature stage) (Figure 5.6c). In seeds FLS enzyme activity was much less different between green and *véraison* stages (1.2-fold), while decreasing afterwards; at these first two stages it was 1.3-fold higher under LL than in HL (Figure 5.6d). Thus, in contrast to exocarp, in seed this FLS enzyme activity only partly corresponded to the expression pattern of *VvFLS1* across sample groups.

In our previous metabolomics study, we showed that the HL microclimate led to an increase of total flavonols in exocarp at all berry developmental stages (Garrido et al., 2021). Here we showed that this HL microclimate has also a clear positive influence on both *VvFLS1* expression and total FLS enzyme activity in this tissue. This difference between microclimates can be explained by a light acclimation mechanism in the HL exocarps (Plessis et al., 2017), thereby enhancing the biosynthesis of specific metabolites like flavonols that can protect the photosynthetic apparatus from light-induced damage, in order to maintain exocarp photosynthesis until the end of berry development. It has frequently been suggested that flavonols can limit photodamage through their ability to scavenge ROS and other free radicals generated by photooxidation, thereby contributing to the maintenance of oxidative homeostasis (Agati et al., 2013). Our results of the up-regulation of *VvFLS1* expression by HL microclimate as compared to LL are in accordance with previous studies performed with both white (Friedel et al., 2016; Plessis et al., 2017) and red (Koyama et al., 2012; Matus et al., 2009) grape berry varieties, as well as with the observation that cell cultures from white berries exposed to high light had an increase in *FLS* gene expression, as compared to the cells in the dark condition (Czemmel et al., 2009).

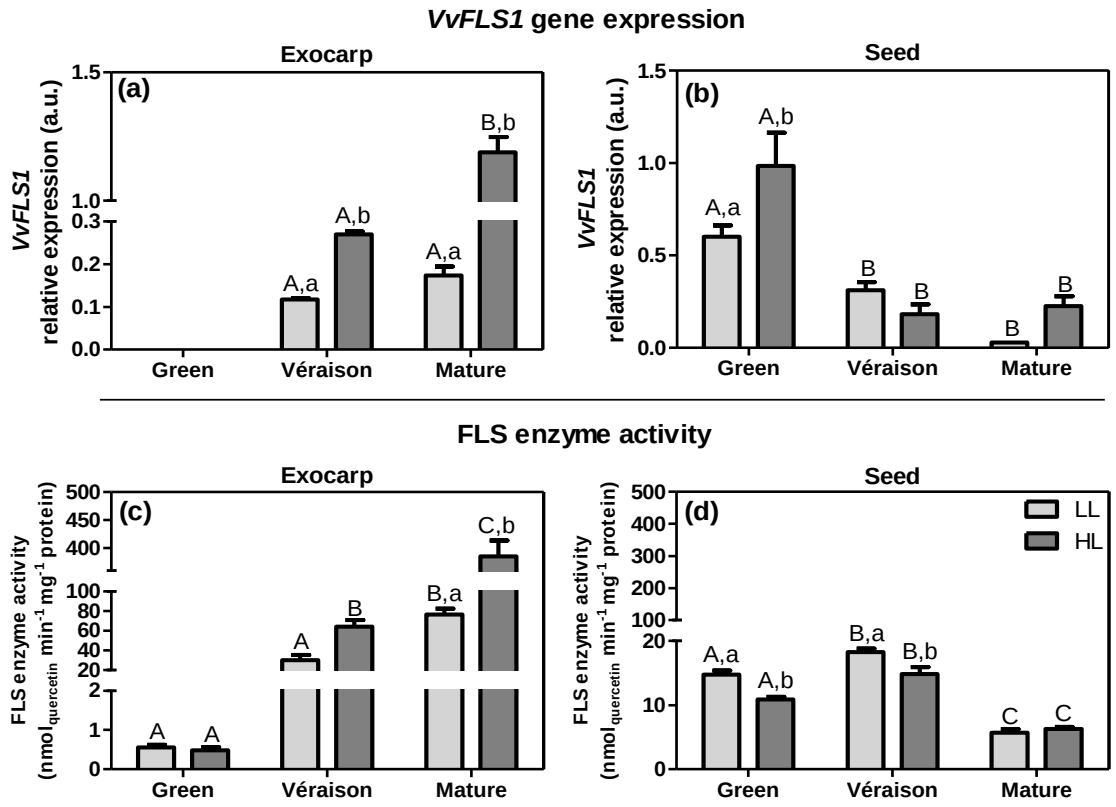


**Figure 5.6.** Relative expression of transcripts of flavanol synthase 1 gene (*VvFLS1*) in (**a**) exocarp and (**b**) seeds, from berries grown at two distinct light microclimates (LL and HL) and at three developmental stages (green, *véraison* and mature), as determined by real-time qPCR. Expression levels are normalized to the mean expression of reference genes *VvACT1* and *VvGAPDH*. Statistical analysis (two-way ANOVA, $p \leq 0.05$, $n = 3$) was applied after data Log(X+1) transformation. Flavonol synthase (FLS) biochemical activity [expressed in $V_{max}$ ($nmol_{quercetin}$ $min^{-1}$ $mg^{-1}$ protein)] in (**c**) exocarp and (**d**) seeds, from berries grown at two distinct light microclimates (LL and HL) and at three developmental stages (green, *véraison* and mature). Statistical analysis (two-way ANOVA, $p \leq 0.05$, $n = 5$) was applied. Statistical notation: capital letters refer to differences between developmental stages for the same microclimate, while lowercase letters refer to differences between microclimates for each stage. When the letters are omitted, it means that the respective factor did not have a significant effect.

The apparent discrepancy between *VvFLS1* gene expression and total FLS enzyme activity patterns observed in seeds may be due to other mechanisms associated with enzyme kinetics and/or post transcriptional regulation of this enzyme, and also due to the fact that FLS enzyme activity translates the sum of all 5 isoforms. Indeed, in seeds the abundance of flavonols was relatively low, as compared to exocarp, and not influenced by the berry microclimate (Garrido et al., 2021). In seeds from a red grape variety, the flavonols were even undetectable and *VvFLS1* gene expression was very low and only detected at *post-véraison* stages (Downey et al., 2003). Thus, in seeds the influence of microclimate on the flavonol branch of the flavonoid pathway is not as clear as it is in the exocarp. It is possible that along seed maturation other pathways like carotenoid biosynthesis are more important as photooxidative protection mechanism than the flavonol biosynthesis branch. In fact, at the green stage *VvFLS1* expression was

relatively high compared to that in both *véraison* and mature stages (Figure 5.6b), while the genes of the xanthophyll cycle were in general more expressed at the later stages of development (Figure 5.2).

Dihydroflavonols can also be converted into their corresponding leucoanthocyanidins, by the enzyme dihydroflavonol reductase (DFR; EC 1.1.1.219). These leucoanthocyanidins are subsequently converted into their corresponding anthocyanidins by the action of leucoanthocyanidin dioxygenase (LDOX; EC 1.14.20.4) (Figure S5.2). The leucoanthocyanidins and anthocyanidins are considered as potential substrates for the formation of flavan-3-ols (also named flavanols or condensed tannins). Leucoanthocyanidin reductase (LAR; EC 1.17.1.3) converts leucocyanidin into catechin, while anthocyanidin reductase (ANR; EC 1.3.1.77) converts cyanidin into epicatechin. The transcript levels of genes related with flavan-3-ols biosynthesis are represented in Figure 5.7.

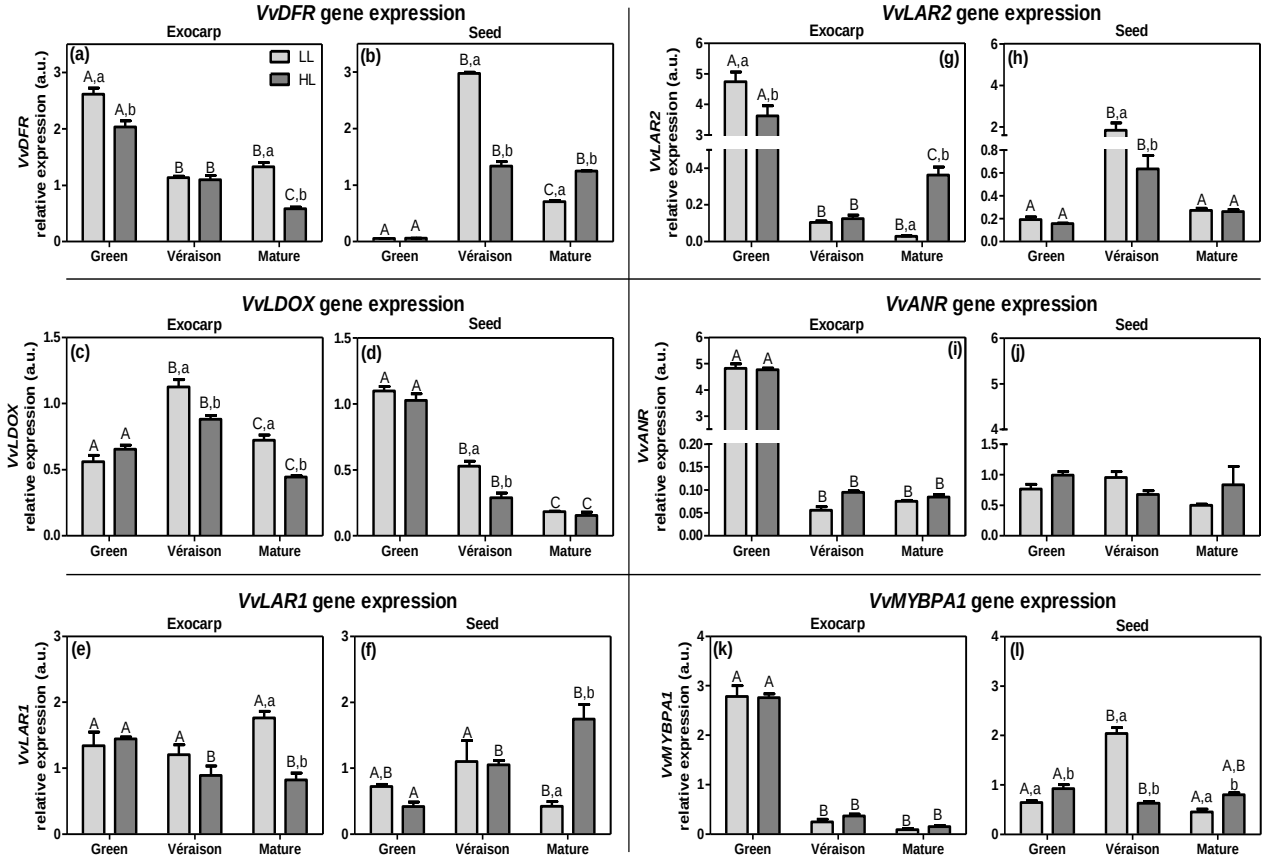


**Figure 5.7.** Relative expression of transcripts of dihydroflavonol reductase (*VvDFR*) (**a**, **b**), leucoanthocyanidin dioxygenase (*VvLDOX*) (**c**, **d**), leucoanthocyanidin reductase 1 and 2 (*VvLAR1* and *VvLAR2*) (**e-h**), anthocyanidin reductase (*VvANR*) gene (**i**, **j**) and of transcription factor MYBPA1 (*VvMYBPA1*) (**k**, **l**) in exocarp and seeds, from berries grown at two distinct light microclimates (LL and HL), and at three developmental stages (green, *véraison* and mature), as determined by real-time qPCR. Expression levels are normalized to the mean expression of reference genes *VvACT1* and *VvGAPDH*. Statistical analysis (two-way ANOVA, $p \leq 0.05$, $n = 3$) was applied after data Log(X+1) transformation. Statistical notation: capital letters refer to differences between developmental stages for the same microclimate, while lowercase letters refer to differences between microclimates for each stage. When the letters are omitted, it means that the respective factor did not have a significant effect.

In exocarp, *VvDFR* expression was highest in green stage and decreased along ripening (Figure 5.7a). In contrast, seeds exhibited a residual expression level at green stage, a high level at *véraison* which decreased again to mature stage (Figure 5.7b). The expression of *VvDFR* was generally significantly higher in LL compared to HL (Figure 5.7a,b). The expression of leucoanthocyanidin dioxygenase (*VvLDOX*) – route to the epicatechins – did hardly change in exocarp during berry development among the genes tested (Figure 5.7c), while in seeds it decreased along ripening to relatively low levels (Figure 5.7d).

For the leucoanthocyanidin reductase – route to the catechins – the expression of the genes *VvLAR1* and *VvLAR2*, coding for the only two isoforms identified in grapevine (Velasco et al., 2007), were analyzed (Figure 5.7e-h). *VvLAR1* expression was roughly constant along ripening in both exocarp and seed. There was a significant difference between tissues in their microclimate responses at mature stage: in exocarp the transcript levels were significantly lower (2-fold) in HL berries as compared to LL berries (Figure 5.7e), while in seeds they were significantly higher in HL (4.2-fold) (Figure 5.7f). For both tissues, the transcript levels of *VvLAR2* varied more between ripening stages than the transcripts of *VvLAR1*, with two clear peaks - at green stage for exocarp and at *véraison* stage for seed (Figure 5.7g,h), which is in accordance with previous work with skins and seeds from a red grape variety (Bogs et al., 2005). Together, the expression patterns of *VvLAR1* and *VvLAR2* seem to correspond to that of *VvDFR* (Figure 5.7a,b,e-h). Moreover, the Pearson correlation showed that both *VvDFR* and *VvLAR2* were significantly and positively correlated in both exocarp (Table S5.2; $r = 0.84$, $p < 0.0001$) and seed (Table S5.3; $r = 0.75$, $p < 0.001$).

The relative expression level of *VvANR*, coding for anthocyanidin reductase, in green exocarp was the highest of all the analyzed genes. However, this high expression in exocarp was only observed at the green stage, and its transcript levels at subsequent developmental stages were negligible and markedly lower than the levels observed in seeds at all stages (Figure 5.7i,j). In seeds, *VvANR* transcript levels were maintained constant along ripening, but at relatively low levels (Figure 5.7j).

The transcription factor *VvMYBPA1* in exocarp had a strong and significant positive correlation with genes related to flavan-3-ols biosynthesis (i.e., *VvDFR*, *VvLAR1*, *VvLAR2* and *VvANR*), while in seed this transcription factor had a significant correlation with only two of these genes (*VvDFR* and *VvLAR2*) (Figure 5.7, and Tables S5.2 and S5.3), conform its proposed role in regulating these structural flavonoid biosynthesis pathway genes (Bogs et al., 2007; Czemmel et al., 2012).

The activity of anthocyanidin reductase enzyme was also assessed, in order to investigate whether the observed changes in the *VvANR* gene expression were translated at the enzyme activity level (Figure 5.8). In the exocarp the ANR activity ($V_{max}$) was very consistent with the profile of its gene expression during

development (compare Figure 5.8a with 5.7i). For seeds, at the green and *véraison* stages the ANR activity was also similar to its gene expression, but it significantly increased at mature stage for both microclimates, especially in HL (Figure 5.8b). Although *VvANR* was the only gene whose expression was not significantly affected by light microclimate (Figure 5.7i,j), the ANR enzyme activity was stimulated by HL at *véraison* and mature stages, in exocarp and seeds, respectively (Figure 5.8). This difference between ANR transcripts levels and enzyme activity could be explained by other mechanisms associated with enzyme kinetics and/or post transcriptional regulation, possibly regulated by light (Matus et al., 2009).

It is worth noting that the significant differences registered in the enzyme specific activities (of both FLS and ANR) were not due to any constitutive difference in the soluble protein content between sample groups (data not shown).

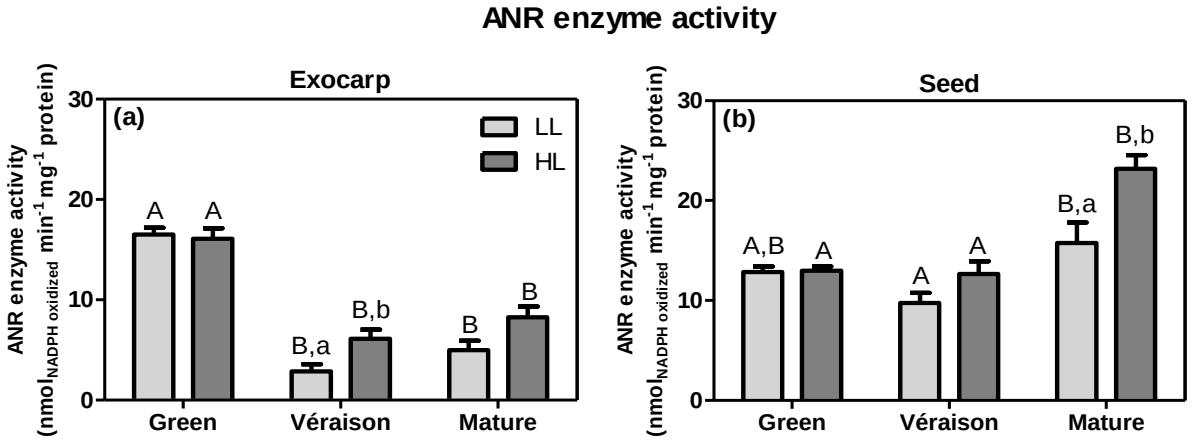


**Figure 5.8.** Anthocyanidin reductase (ANR; EC 1.3.1.77) biochemical activity, expressed in $V_{max}$ ($nmol_{NADPH\ oxidized}$ $min^{-1}$ $mg^{-1}$ protein) in exocarp (**a**) and seeds (**b**), from berries grown at two distinct light microclimates (LL and HL) and at three developmental stages (green, *véraison* and mature). Statistical analysis (two-way ANOVA, $p \leq 0.05$, $n = 4$-8) was applied. Statistical notation: capital letters refer to differences between developmental stages for the same microclimate, while lowercase letters refer to differences between microclimates for each stage. When the letters are omitted, it means that the respective factor did not have a significant effect.

Overall, along exocarp ripening there was a symmetry between the profiles of the gene expression of flavonols (Figure 5.6) and those of flavan-3-ols (Figure 5.7). In fact, the direction to flavan-3-ols synthesis was more evident at the green stage, considering the transcripts levels of *VvDFR*, *VvLAR2* and *VvANR* (Figure 5.7a,g,i), as well as the activity of the ANR enzyme (Figure 5.8a). In contrast, the *VvFLS1* expression in this external berry tissue was negligible in the green stage, but increased throughout ripening (Figure 5.6a) and in an opposite fashion to that seen for *VvDFR* (Figure 5.7a). The Pearson correlation matrix for the exocarp samples (Table S5.2) also showed that *VvFLS1* was negatively correlated with all the genes downstream in the flavonoid pathway, being significantly negatively correlated

with *VvDFR* ($r$ = - 0.77, $p$ < 0.001), which may be explained by the fact that both genes code for enzymes that compete for the same substrate, i.e., dihydroflavonols. This shift from green to mature stage is in accordance with the abundance profiles of the flavanols and flavonols detected in this exocarp tissue (Garrido et al., 2021). Moreover, *VvANR* and both isoforms of *VvLAR* were significantly positively correlated in exocarp (Table S5.2), suggesting that both pathways of flavan-3-ols monomers synthesis are active.

In the case of seeds, this shift between the expression of genes coding the enzymes of the flavonols and flavan-3-ols branches was not as evident as in the exocarp. In fact, *VvFLS1* expression was high at green stage (Figure 5.6b), decreasing afterwards, while *VvDFR* and *VvLAR2* expressions peaked at *véraison* stage (Figure 5.7b,h). Similar to the exocarp, in the seeds the *VvFLS1* expression was negatively correlated with some genes at downstream in the pathway, i.e., *VvDFR*, *VvLAR1* and *VvLAR2*, although not statistically different (Table S5.3). Furthermore, and in contrast to the exocarp, in the seeds the *VvFLS1* expression was significantly positively correlated with the expression of two genes, i.e., *VvLDOX* and *VvANR* (Table S5.3), although this correlation was only observed at the green stage (c.f. Figure 5.6b and Figure 5.7d). These results corroborate the profile of flavan-3-ols monomers along seed development.

## 5.3. Materials and Methods

### 5.3.1. Grapevine Field Conditions and Sampling

Grape berry samples were collected from a white cultivar (*Vitis vinifera* L. cv. Alvarinho) in the commercial vineyard Quinta Cova da Raposa in the Demarcated Region of Vinho Verde. Vineyard maintenance and sampling methods were as described in Garrido et al. (Garrido et al., 2019). Two distinct light microclimates in the grapevine canopy were considered for harvesting: low light (LL) clusters that grew in the shaded inner zones of the canopy (approx. 50 μmol photons $m^{-2}$ $s^{-1}$), and high light (HL) clusters that were exposed to direct sunlight most of the day (approx. 150 μmol photons $m^{-2}$ $s^{-1}$) (Garrido et al., 2019). Grape berries clusters were randomly collected, as 3 biological replicates, from both light microclimates and at three different developmental stages: Green [16$^{th}$ July, 6 weeks after anthesis (WAA) or BBCH-75 - BBCH-scale used for grapes by Lorenz et al., 1994], *Véraison* (29$^{th}$ August, 12 WAA, BBCH-83), and Mature (17$^{th}$ September, 15 WAA, BBCH-89). Each biological replicate corresponds to a mixture of 15 to 20 berries from 3 to 5 clusters from 6 to 8 plants growing in untreated vineyard plots (i.e., from the non-irrigated, non-kaolin controls plants, as described in Garrido et al., 2019). The whole berries were immediately frozen in liquid nitrogen and stored at −80 °C. Prior to the analysis, for each condition, one or more subsamples of grapes were prepared from each triplicate. The exocarps and seeds were

separated and grounded to a fine powder using a mortar and pestle under liquid nitrogen and stored till analysis or immediately used.

### 5.3.2. RNA extraction and cDNA synthesis

RNA was extracted from a total of 36 samples: 3 subsamples, one from each biological replicate x 2 tissues x 2 microclimates x 3 developmental stages. Total RNA was purified according to Reid et al. (Reid et al., 2006), with some adjustments. To 500 mg of frozen tissue, 3 mL of the extraction buffer containing 2 % of cetrimonium bromide (CTAB), 2 % of soluble polyvinylpyrrolidone (PVP) K-30, 300 mM TRIS-HCl (pH 8.0), 25 mM of ethylenediamine tetraacetic acid (EDTA), 2 M of sodium chloride (NaCl), and 40 mM of dithiothreitol (DTT, mixed just prior to use) were added. Samples were incubated at 60 °C for 30 minutes and shaken every couple of minutes. After this, the mixtures were extracted twice with 3 mL of chloroform:isoamyl alcohol (24:1) followed by a centrifugation step at 3,500 x *g* for 15 min at 4 °C were performed. The aqueous fraction (1.5 mL) was mixed with 0.1 vol of 3 M NaOAc (pH 5.2) and 0.6 vol of isopropanol, and maintained at -80 °C for 30 min, after which the samples were centrifuged at 3,500 x *g* for 30 min at 4 °C. The pellet was resuspended in 500 μL of plant RNA lysis solution from GeneJET Plant RNA Purification Mini Kit (Thermo Scientific®), following the manufacturer's instructions. RNA concentration was determined in a Nanodrop (Thermo Fisher Scientific Inc.) and its integrity was assessed in a 1 % agarose gel. Total RNA was further purified with DNase I Kit (Thermo Scientific®) to remove any contaminating DNA. First strand cDNA synthesis was synthesized from 1 μg of total RNA using the Xpert cDNA Synthesis Mastermix (Grisp®), following the manufacturer's instructions.

### 5.3.3. Transcriptional Analysis by Real-Time qPCR

Real-time qPCR was used for transcriptional analyses of target genes listed in the Table S5.1 (at the Supplementary Material section). The gene specific primer pairs used for each target or reference gene are also listed at Table S5.1. The analysis was performed with Xpert Fast SYBR (uni) Blue (Grisp®) using 1 μL cDNA (diluted 1:10 in ultra-pure distilled water) in a final reaction volume of 10 μL per well.

Experiments were performed in triplicate, as described above, in an CFX96 Real-Time Detection System (Bio-Rad) using the following cycler conditions: polymerase was activated with an initial step of 3 min at 95 °C, the double strand denaturation occurred at 95 °C for 10 s, the annealing temperature was 55 °C during 20 s and the extension temperature 72 °C during 20 s (amplification was performed using 40 cycles). Melting curve analysis was performed for specific gene amplification confirmation.

The reference genes, actin 1 (*VvACT1*) and glyceraldehyde-3-phosphate dehydrogenase (*VvGAPDH*) were selected, as these genes were proven to be very stable and ideal for qPCR normalization purposes in grapevine (Reid et al., 2006). Additionally, for each qPCR analysis, the target gene stability (i.e., the absence of significant variation of the expression of the reference genes in all samples), was always validated by the M-values and coefficient variance values calculated by CFX ManagerTM Software (Bio-Rad): for these parameters, the acceptable values for the stability, should be less than 1 and 0.5, respectively (Hellemans et al., 2007). Then, the expression values were normalized by the average of the expression of the reference genes, as described by Pfaffl (2001).

### 5.3.4. Enzyme assays

The activity of two enzymes (i.e., flavonol synthase – FLS, EC 1.14.20.6; and anthocyanidin reductase – ANR, EC 1.3.1.77), key in the biosynthesis of flavonols and flavan-3-ols, respectively, was also assessed as described below, in order to further analyze the observed changes in the gene expression at the enzyme activity level. For these assays, for each microclimate and developmental stage, grape berry tissues were prepared using 5 (for FLS) and 4 to 8 (for ANR) subsamples from the biological replicates, as described above.

#### 5.3.4.1. Enzyme extraction

Total protein extraction from grape berry powders was performed mainly as described by Stoop and Pharr (Stoop and Pharr, 1993). One gram of sample powder was mixed with 1 mL of buffer containing 50 mM bis-tris propane (pH 8.9, adjusted with HCl), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT) and 10 mg of polyvinylpolypyrrolidone (PVPP). The homogenates were thoroughly mixed and centrifuged at 18,000 x *g* for 20 min and the supernatants were maintained on ice and used for all enzyme assays. Total protein concentrations of the extracts were determined by the Bradford method (Bradford, 1976), reading at 595 nm and using bovine serum albumin (Sigma-Aldrich) as a standard.

#### 5.3.4.2. Flavonol synthase (FLS) activity

FLS activity was performed as described by Conde et al. (2016), with some modifications. The activity was determined spectrophotometrically (Shimadzu UV-1700) following quercetin production at 365 nm, during 30 minutes at 37 °C, in a total volume of 1 mL. The reaction medium (pH = 5.0) contained 85.77 mM of phosphate-buffered saline (PBS) buffer, 111 mM of sodium acetate, 83 µM of 2-oxoglutaric acid, 131 µM of taxifolin (dihydroquercetin), 100 µL of protein extract and started with 84 µM

of ferrous sulfate. FLS activity was calculated using the extinction coefficient of quercetin (ε = 13.2 $mM^{-1}$ $cm^{-1}$) and expressed in $nmol_{quercetin}$ $min^{-1}$ $mg^{-1}$ of protein, that is equivalent to 1 mU per mg of protein (the enzyme unit, U, corresponds to the conversion of one µmol of substrate per minute).

#### 5.3.4.3. Anthocyanidin reductase (ANR) activity

ANR activity was determined following NADPH consumption at 45 °C, in a total reaction volume of 1.5 mL with 89 mM of PBS buffer (pH = 6.5), 133 µM of nicotinamide adenine dinucleotide phosphate (NADPH), 66.7 µM of cyanidin chloride, 533 µM of ascorbic acid and 60 µL of protein extract. The enzyme activity was monitored spectrophotometrically (Shimadzu UV-1700) by the rate of NADPH oxidation at 340 nm, after adding its substrate cyanidin chloride. ANR activity was calculated using the extinction coefficient of NADPH (ε = 6.22 $mM^{-1}$ $cm^{-1}$) and expressed in $nmol_{NADPH}$ oxidized $min^{-1}$ $mg^{-1}$ protein, that is equivalent to 1 mU per mg of protein.

### 5.3.5. Statistical Analysis

Data from gene expression was firstly transformed [Log(X+1)] to meet homogeneity of variances. Then, a two-way ANOVA was applied followed by the *post hoc* Bonferroni test for multiple comparisons whenever the factors (microclimate or developmental stage) had a significant effect (GraphPad Prism version 5.00 for Windows, GraphPad Software, La Jolla, Calif., USA). Significant differences ($p \leq 0.05$) between sample groups are indicated with different letters: capital letters refer to differences between developmental stages for the same microclimate, while lowercase letters refer to differences between microclimates for each stage.

A correlation analysis between the relative expression levels of a selected set of genes (i.e., *VvFLS1*, *VvDFR*, *VvLDOX*, *VvLAR1*, *VvLAR2*, *VvANR* and *VvMYBPA1*) was made. The Pearson correlation coefficients ($r$) were calculated separately for both exocarp and seed, considering all samples regardless of light microclimate and developmental stage ($n$ = 18 per tissue). The calculations of $r$ and $p$-values were performed using Microsoft Excel® (version 2008: 13127.21624), and the correlation matrices for each tissue are presented at the Supplementary Material section (Tables S5.2 and S5.3).

## 5.4. Conclusions

In general, the results obtained in this study corroborate those of our previous studies (Garrido et al.,2021; Garrido et al., 2019), suggesting that tissue-specific photosynthesis coincides with the expression of photosynthesis and sucrose synthesis-related genes, as well as to the gene transcription and enzyme activities of key steps in secondary metabolism. Our results also point to a possible cross-talk between photosynthesis and sucrose unloading and breakdown/carbon usage in the berries. In the green stage the berries have a relative high demand for carbon and energy to sustain their high growth rate and the main part of the sucrose imported through the dorsal vascular system will be used to meet that demand: the relative high levels of transcripts of genes involved in sucrose catabolism (*VvSuSy*) combined with the low levels of *VvSPS1* in exocarp may corroborate this hypothesis. The high levels of expression of genes from the phenylpropanoid (*VvPAL1*) and stilbenoid (*VvSTS1*) pathways, as well as of those associated with flavan-3-ols biosynthesis (*VvDFR*, *VvLAR2* and *VvANR*) at this early stage of berry development may translate this cross-talk. At the later developmental stages, the flavonol branch of the flavonoid pathway (*VvFLS1*) in exocarp was more activated by HL as compared to LL microclimate, and the parallel pattern of the expression of *VvRuBisCO* suggest a role for photosynthesis/Calvin Cycle in the biosynthesis of these metabolites possibly associated with photoprotection. The results of seeds generally indicate that some genes peaked in their expression at either *véraison* or mature stage (i.e., the xanthophylls cycles - *VvVDE1* and *VvLUT1*; phenylpropanoids - *VvCHS3*; and flavan-3-ols pathway - *VvDFR* and *VvLAR2*). In this way, and despite of the reduction of photosynthetic activity along the season, it seems that at later stages of berry development additional mechanisms other than the assimilation of atmospheric $CO_2$ fixation may be involved to maintain the need for carbons for primary and secondary metabolism in the berries. Indeed, *VvRuBisCO* expression was maintained during ripening in both berry tissues, and especially at the latest stages of berry development this RuBisCO may have a function in re-assimilating the locally released $CO_2$, thus contributing with substrates required for the various metabolic pathways that are active in these berry tissues.

Clearly, further studies are needed to support the proposed link between actual photosynthesis in these grape berry tissues and the physiological/biochemical/transcriptional changes observed in these tissues (Garrido et al., 2021; Garrido et al., 2019). This fundamental science studies will also contribute with essential information for the viticulture practices that involve manipulations of canopy light microclimate.

## 5.5. Supplementary Materials

**Table S5.1.** Forward (F) and reverse (R) primers used for gene expression analysis by real-time PCR. Sequences accession numbers were obtained through Grape Genome Browser 12x or, for the carotenoid metabolism genes, from Plaza 1.0 (April, 2009; http://bioinformatics.psb.ugent.be/plaza_v1/), or Grape Genome Database (http://genomes.cribi.unipd.it/grape/) for *VvLAR2* and *VvRubisCO*.

| Gene | Accession number (Genoscope) | Primers |
|---|---|---|
| ***VvACT1 (reference gene)*** | GSVIVT01026580001 | F: 5′-GTGCCTGCCATGTATGTTGCC-3′ |
| | | R: 5′-GCAAGGTCAAGACGAAGGATA-3′ |
| ***VvGAPDH (reference gene)*** | GSVIVT00009717001 | F: 5′-CACGGTCAGTGGAAGCATCAT-3′ |
| | | R: 5′-CCTTGTCAGTGAACACACCAG-3′ |
| ***VvPAL1*** | GSVIVG01025703001 | F: 5′-CCGAACCGAATCAAGGACTG-3′ |
| | | R: 5′-GTTCCAGCCACTGAGACAAT-3′ |
| ***VvSTS1*** | GSVIVT01010590001 | F: 5′-CGAAGCAACTAGGCATGTGT-3′ |
| | | R: 5′-CTCCCCAATCCAATCCTTCA-3′ |
| ***VvCHS1*** | GSVIVT01032968001 | F: 5′-GTCCCAGGGTTGATTTCCAA-3′ |
| | | R: 5′-TCTCTTCCTTCAGACCCAGTT-3′ |
| ***VvCHS3*** | Lac14-SSH (CX126991) | F: 5′-TCGCATCACAAATAGCGAAC-3′ |
| | | R: 5′-CAGGGAAGCTGCCATGTATT-3′ |
| ***VvFLS1*** | GSVIVT01008913001 | F: 5′-CAGGGCTTGCAGGTTTTTAG-3′ |
| | | R: 5′-GGGTCTTCTCCTTGTTCACG-3′ |
| ***VvMYBPA1*** | GSVIVT01010590001 | F: 5′-AGATCAACTGGTTATGCTTGCT-3′ |
| | | R: 5′-AACACAAATGTACATCGCACAC-3′ |
| ***VvDFR*** | GSVIVT01009743001 | F: 5′-GGCTTTCTAGCGAGAGCGTA-3′ |
| | | R: 5′-ACTCTCATTTCCGGCACATT-3′ |
| ***VvANR*** | GSVIVG01035256001 | F: 5′-CAATACCAGTGTTCCTGAGC-3′ |
| | | R: 5′-AAACTGAACCCCTCTTTCAC-3′ |
| ***VvLAR1*** | GSVIVT01024419001 | F: 5′-CAGGAGGCTATGGAGAAGATAC-3′ |
| | | R: 5′-ACGCTTCTCTCTGTACATGTTG-3′ |
| ***VvLAR2*** | VIT_217s0000g04150 | F: 5′-CAAATCGCTTCATTTCCGACCTCCC-3′ |
| | | R: 5′-GTCATCTTCTTCCACGGTTACACGG-3′ |
| ***VvLDOX*** | GSVIVT01009743001 | F: 5′-ACCTTCATCCTCCACAACAT-3′ |
| | | R: 5′-AGTAGAGCCTCCTGGGTCTT-3′ |
| ***VvChlSyn*** | GSVIVT01011187001 | F: 5′-GCTGAGACTGATGCAAATGAAGCC-3′ |
| | | R: 5′-TGCCGAGAAGCTGATTGAAGTTCG-3′ |
| ***VvRubisCO*** | VIT_13s0019g02050 | F: 5′-TTCAAAGCTCTGCGCGCTCTAC3′ |
| | | R: 5′-TCTCTCAACTTGGATGCCATGAGG-3′ |
| ***VvSPS1*** | GSVIVT01012825001 | F: 5′-GGTTTGCCTATTGTTGCCACCAG-3′ |
| | | R: 5′-CAAGAAGGCCATTGTCAAGTACCC-3′ |
| ***VvSuSy*** | GSVIVT01015018001 | F: 5′-TGTTAAGGCTCCTGGATTTCAATTA-3′ |
| | | R: 5′-AGCCAAATCTTGGCAAGCA-3′ |
| ***VvPSY1*** | VV00G37410 | F: 5′-TCGATGTGGTGAAGTTTGTGCAG-3′ |
| | | R: 5′-TCAGGTGTCATCAGCATTGTTCCC-3′ |

| | | |
|---|---|---|
| ***VvLBCY2*** | VV08G15130 | F: 5′-AGGCTGGGTGGTTGAGAATTGG-3′ |
| | | R: 5′-TCCTCAACGACAAACCGAAGTGG-3′ |
| ***VvLECY1*** | VV11G01840 | F: 5′-TGGATGTGGCAGGGATTTCTTGG-3′ |
| | | R: 5′-TAGCATGGTTGCTCCAGTAGGG-3′ |
| ***VvLUT1*** | VV08G10880 | F: 5′-TGCTTGCTAGCAGAGAAGAGGTG-3′ |
| | | R: 5′-ACTGCCATTGAGTGCATCAGTTC-3′ |
| ***VvVDE1*** | VV00G14320 | F: 5′-GTTGTCTATGGTTGGTGGTTTGGC-3′ |
| | | R: 5′-ATGCCAGTGGGTCTCCATTTCC-3′ |
| ***VvZEP1*** | VV07G11310 | F: 5′-AGCGTGCTATTGGTGGAGAGTG-3′ |
| | | R: 5′-CCCGATTATGCAGGGTTTGTTCTC-3′ |

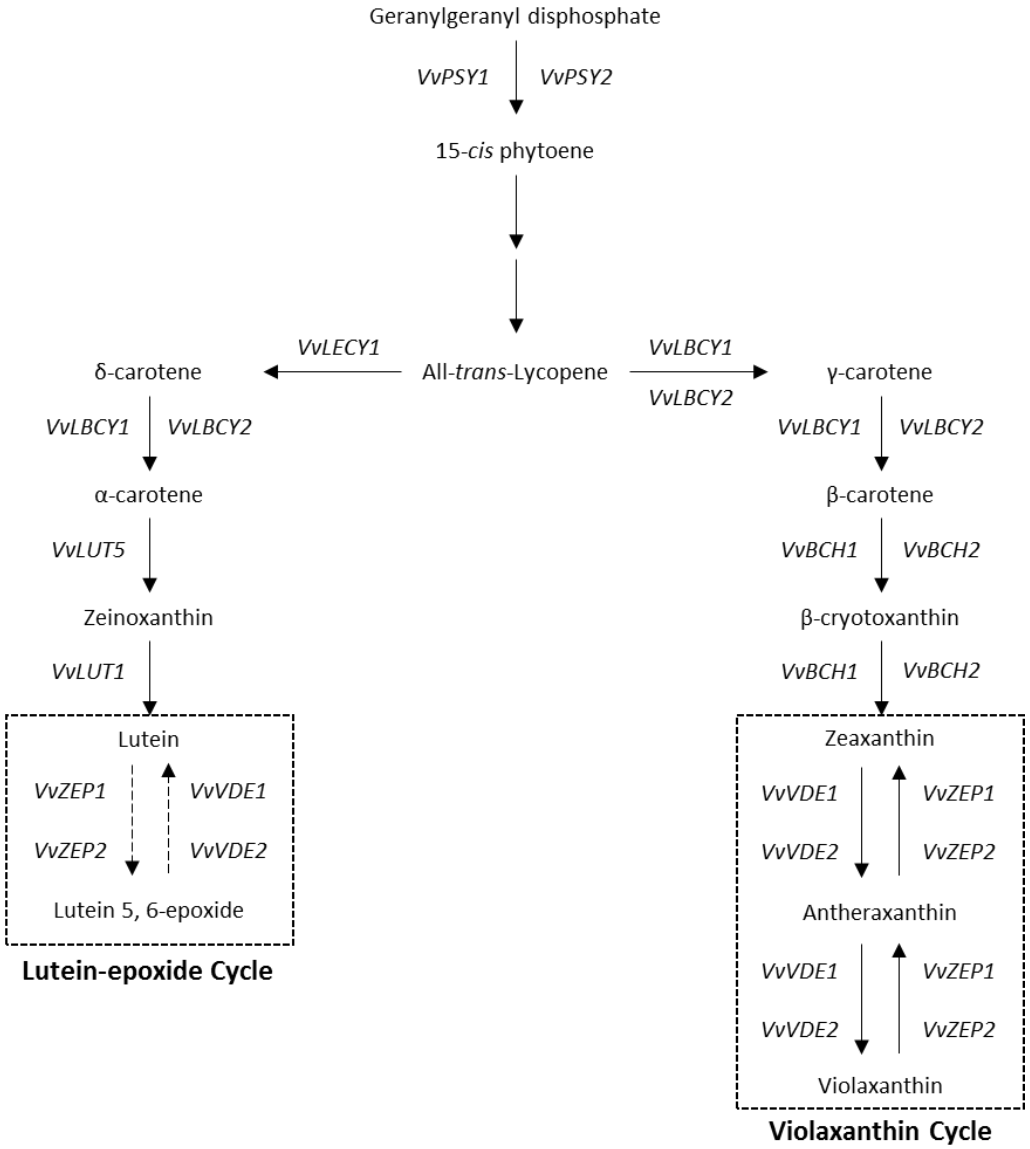


**Figure S5.1.** Simplified carotenoid metabolic pathway. *PSY1*/ *PSY2*, phytoene synthase; *LECY1*/ *LECY2*, lycopene epsilon cyclase; *LBCY1*/ *LBCY2*, lycopene β-cyclase; *LUT5*/ *LUT1*, lutein synthase; *BCH1*/ *BCH2*, β-carotene hydroxylase; *VDE1*/ *VDE2*, violaxanthin de-epoxidase; *ZEP1*/ *ZEP2*, zeaxanthin epoxidase. Adapted from Young et al. (2012).

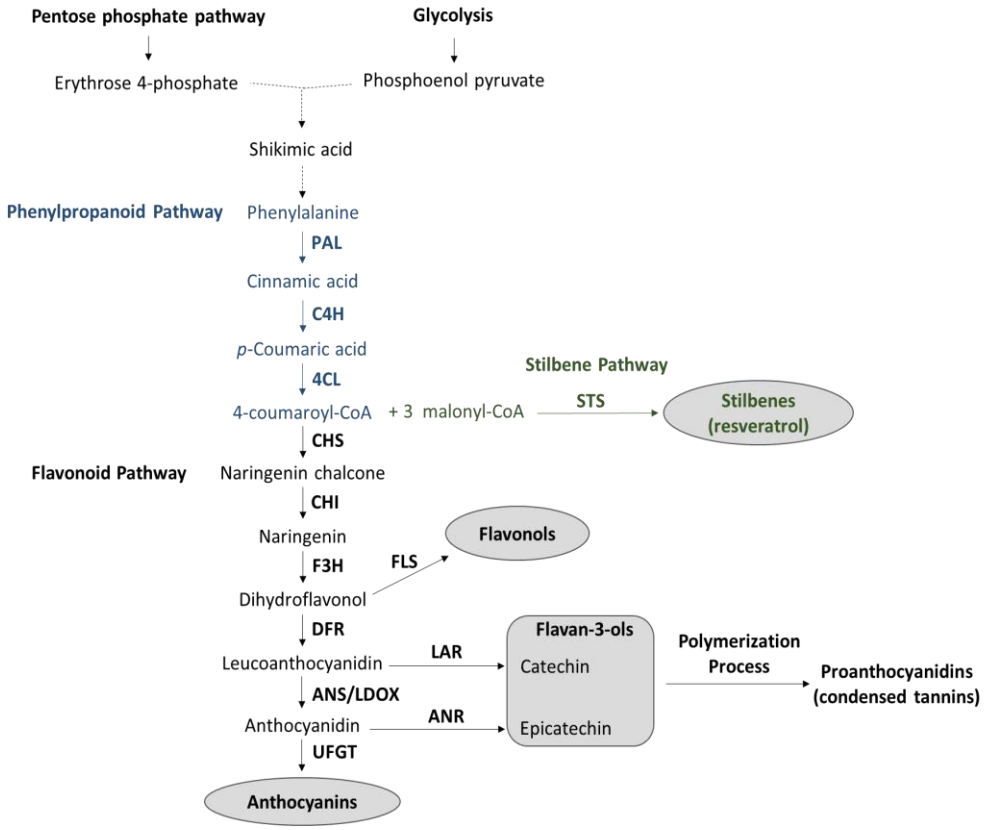


**Figure S5.2.** Biosynthetic pathways of phenolic compounds in grape berry. PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl:CoA-ligase; CHS, chalcone synthase; STS, stilbene synthase; CHI, chalcone isomerase; F3H, flavonone 3-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; LDOX, leucoanthocyanidin dioxygenase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; UFGT, flavonoid glucosyltransferase. Full lines represent direct enzymatic conversion and dashed lines represent omitted intermediates. Adapted from Ageorges et al. (2014) and Teixeira et al. (2013).

**Table S5.2.** Matrix with Pearson correlation coefficients ($r$), calculated for selected genes (*VvFLS1*, *VvDFR*, *VvLDOX*, *VvLAR1*, *VvLAR2*, *VvANR* and *VvMYBPA1*) in the exocarp, considering all samples collected in both light microclimate and developmental stages ($n$ = 18). The coefficient values in bold are statistically significant ($p \leq 0.05$).

| | *VvFLS1* | *VvDFR* | *VvLDOX* | *VvLAR1* | *VvLAR2* | *VvANR* | *VvMYBPA1* |
|---|---|---|---|---|---|---|---|
| *VvFLS1* | 1.000 | | | | | | |
| *VvDFR* | **-0.770** | 1.000 | | | | | |
| *VvLDOX* | -0.419 | -0.104 | 1.000 | | | | |
| *VvLAR1* | -0.439 | 0.456 | -0.021 | 1.000 | | | |
| *VvLAR2* | -0.452 | **0.842** | -0.414 | **0.567** | 1.000 | | |
| *VvANR* | **-0.504** | **0.845** | -0.381 | **0.567** | **0.982** | 1.000 | |
| *VvMYBPA1* | **-0.519** | **0.864** | -0.335 | **0.557** | **0.962** | **0.979** | 1.000 |

**Table S5.3.** Matrix with Pearson correlation coefficients ($r$), calculated for selected genes (*VvFLS1*, *VvDFR*, *VvLDOX*, *VvLAR1*, *VvLAR2*, *VvANR* and *VvMYBPA1*) in the seed, considering all samples collected in both light microclimate and developmental stages ($n$ = 18). The coefficient values in bold are statistically significant ($p \leq 0.05$).

| | *VvFLS1* | *VvDFR* | *VvLDOX* | *VvLAR1* | *VvLAR2* | *VvANR* | *VvMYBPA1* |
|---|---|---|---|---|---|---|---|
| *VvFLS1* | 1.000 | | | | | | |
| *VvDFR* | -0.379 | 1.000 | | | | | |
| *VvLDOX* | **0.877** | -0.399 | 1.000 | | | | |
| *VvLAR1* | -0.239 | 0.367 | -0.342 | 1.000 | | | |
| *VvLAR2* | -0.167 | **0.752** | -0.138 | 0.327 | 1.000 | | |
| *VvANR* | **0.556** | 0.098 | 0.357 | 0.420 | 0.212 | 1.000 | |
| *VvMYBPA1* | 0.125 | **0.698** | 0.167 | 0.215 | **0.782** | 0.404 | 1.000 |

## 5.6. Funding

This research and the APC was funded by FCT - Portuguese Foundation for Science and Technology, grant provided to Andreia Garrido (PD/BD/128275/2017), under the Doctoral Programme "Agricultural Production Chains – from fork to farm" (PD/00122/2012), and by the European Social Funds and the Regional Operational Programme Norte 2020.

## 5.7. Acknowledgments

Authors acknowledge the National Funds by FCT, via CITAB and CBMA research units under the projects UIDB/04033/2020 and UIDB/04050/2020, respectively. The work was also supported by FCT and European Funds (FEDER/POCI/COMPETE2020) through the research project "MitiVineDrought—Combining "omics" with molecular, biochemical, and physiological analyses as an integrated effort to validate novel and easy-to-implement drought mitigation strategies in grapevine while reducing water use" with ref. PTDC/BIA-FBT/30341/2017 and ref. POCI-01-0145-FEDER-030341, respectively; and through the research project "BerryPlastid" with ref. POCI-01-0145-FEDER-028165 and ref. PTDC/BIA-FBT/28165/2017, respectively. Artur Conde was supported by a post-doctoral researcher contract/position within the project "MitiVineDrought" (PTDC/BIA-FBT/30341/2017 and POCI-01-0145-FEDER-030341). The work was also support by project I&D&I "AgriFood XXI", ref. NORTE-01-0145-FEDER-000041, co-financed by the European Regional Development Fund (FEDER), through NORTE 2020 (Northern Regional Operational Program 2014/2020). This work also benefited from the networking activities within the European Union-funded COST Action CA17111"INTEGRAPE—Data Integration to maximize the power of omics for grapevine improvement". Ric C. H. De Vos was financed by business unit Bioscience of Wageningen University and Research Centre. Authors acknowledge the owner of Quinta

Cova da Raposa, Manuel Taxa, who provided the grape samples, and also all support given by the Biology Department of the School of Sciences from the University of Minho.

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# Chapter 6

## The influence of light microclimate on the lipid profile and associated transcripts of photosynthetically active grape berry seeds

The manuscript included in this chapter is prepared for the publication:

Garrido, A., Conde, A., De Vos, R. C. H. and Cunha, A. The influence of light microclimate on the lipid profile and associated transcripts of photosynthetically active grape berry seeds.

**Abstract**

Lipids and oils determine the quality and industrial value of grape seeds. Studies with legume seeds have demonstrated the influence of light on lipid metabolism and its association with seed photosynthesis. Likewise, grape berry seeds are photosynthetically active, at least until *véraison*, despite receiving only small amounts of transmitted sunlight. The main objective of the present work was to investigate the lipid profile of seeds from grape berries growing at two contrasting light microclimates in the canopy (low and high light, LL and HL respectively), previously reported to have distinct photosynthetic competences. Berries were collected at three developmental stages (green, *véraison* and mature) and from both LL and HL microclimates. Seeds were separated and analyzed for their lipid profiles using untargeted liquid chromatography high resolution mass spectrometry (LCMS; lipidomics). Overall, the seed lipid profiles differed greatly among both berry developmental stages and microclimates. The LL microclimate, coincided with a higher relative intensity levels of free fatty acids at all stages, while the HL microclimate led to an up-regulation of ceramides (i.e., sphingolipids present in plasma membrane and endomembrane system) at green stage and of triacylglycerols and glycerophospholipids at mature stage. The transcript levels of a gene from lipoxygenase family (i.e., *VvLOXO*) were down- and up-regulated by HL microclimate in seeds at green and *véraison* stages, respectively. These results suggest that seed photosynthesis may play distinct roles during seed growth and development, possibly by fueling different lipid pathways: at green stage, mainly towards the accumulation of membrane-bound lipid species that are essential for cell growth and maintenance of the photosynthetic machinery itself; and at mature stage mainly towards storage lipids (i.e., triacylglycerols) that contribute to the final quality of the grape seeds and consequently to the wine organoleptic properties.

**Keywords:** light microclimate, grape seeds, lipids, oils, photosynthesis roles.

## 6.1. Introduction

Grape seeds are an important co-product from winemaking process (Teixeira et al., 2014). Their biochemical composition, namely the oil content, sets their economic value for several industries (Lucarini et al., 2018), such as nutraceutical, pharmacologic and cosmetic (Ananga et al., 2017; Garavaglia et al., 2016).

Lipids are organic compounds formed by hydrocarbon chains of an hydrophobic or amphiphilic nature and have been divided into eight categories, following the classification proposed by LIPIDMAPS

(http://www.lipidmaps.org/): fatty acyls (FA), which encompasses the free fatty acids (FFA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), saccharolipids (SL), polyketides (PK) (derived from condensation of ketoacyl subunits); and sterols (ST) and prenols (PR) (derived from condensation of isoprene subunits) (Fahy et al., 2011). Furthermore, each of these categories includes distinct classes and sub-classes, increasing the complexity of this family of compounds.

The lipid biosynthetic pathway starts in the chloroplast, with the *de novo* synthesis FFA, and continues in the cytosol, endoplasmic reticulum and oil bodies (Bates et al., 2013; LaBrant et al., 2018). The biosynthesis of FFA is a light-dependent reaction using the reducing power and adenosine triphosphate (ATP) generated during the photochemical phase of photosynthesis (Rawsthorne, 2002; Ye et al., 2020). Part of the FFA synthesized in chloroplasts are directly assembled into polar lipids in the thylakoid membrane, while others are exported to produce polar lipids in the endoplasmic reticulum, in a mechanism also known as the Kennedy pathway (Wang and Benning, 2012). The chloroplast-specific glycerolipids, i.e., monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG), are particularly important to build a special hydrophobic matrix for pigment-protein complexes - the photosystems I and II - which are crucial for the photochemical phase of photosynthesis (Kobayashi, 2016; LaBrant et al., 2018). Therefore, the fatty acids that derive from the *de novo synthesis* in the chloroplast, like palmitic acid (C16:0) and oleic acid (C18:1), are important precursors not only for the synthesis of all the membrane lipids, as well as for the synthesis in the endoplasmic reticulum of polyunsaturated fatty acids (PUFA), like linoleic acid (C18:2) and linolenic acid (C18:3), and of storage lipids or oils (i.e., triacylglycerides or triacylglycerols, TAG) (Bates et al., 2013).

In grape berries, PUFA mainly consist of linoleic acid and are increasingly present in pulp (28 mg *per* 100 g of dry weight - DW), followed by skin (79 mg *per* 100 g DW) and seeds (7355 mg *per* 100 g DW) (Santos et al., 2011). Moreover, most of lipids present in grape seeds are TAG, which are in high concentrations in the endosperm (next to its interface with the inner layer of the seed coat), while they appear in lower amounts in skin and pulp (Pope et al., 1993). The oil content in grape seeds depends of grapevine cultivar and stage of seed maturity (Baydar and Akkurt, 2001; Rubio et al., 2009; Lachman et al., 2015). Seed development is divided in three distinct stages: stage I – morphogenesis (0 till 42 days after flowering, DAF); stage II – maturation or reserve accumulation (42 till 60 DAF); and stage III – desiccation (60 till 120 DAF) (Ristic and Iland, 2005; Angelovici et al., 2010). These three stages of seed

development coincide respectively with the stages of grape berry development, i.e., green, *véraison* and mature stages (Coombe, 1995). Seed oils are synthesized during the maturation stage, and then decrease, possibly due to partial breakdown along the seed desiccation process (Angelovici et al., 2010). In general, grape seed oil contents, depending on the cultivar, range between 10 to 20 % (v/w) and mostly consist of TAG rich in unsaturated fatty acids, like linoleic acid (C18:2, 60 to 70 % of total fatty acid acyl chains) and oleic acid (C18:1, 19 to 27 %) (Baydar et al., 2007; Baydar and Akkurt, 2001; Ohnishi, et al., 1990). For this, grape seed oil is gaining popularity for the production of edible vegetable oil, characterized as having beneficial effects for health (Yilmaz and Toledo, 2004; Matthäus, 2008). Rubio et al. (2009) verified that seeds collected from green to near *véraison* berries are most suitable for extraction to produce oil with characteristics similar to those found in seeds from mature grapes.

Despite their inner core localization in fleshy fruits (e.g., apple, tomato and grape) and even in dehiscent fruits (e.g., pea pods), seeds can receive diffuse transmitted light, as reviewed by Aschan and Pfanz (2003). Moreover, from the early developmental stages to maturation, seed coats are chlorophyllin and exhibit photosynthetic activity, as demonstrated in soybean seeds (Ruuska et al., 2004; Borisjuk et al., 2005; Rolletschek et al., 2005), and in grape berry seeds (Breia et al., 2013; Garrido et al., 2018; 2019). It has been suggested that seed photosynthesis in legumes has an important role in lipid metabolism in several ways: 1) the photochemical phase by producing nicotinamide adenine dinucleotide phosphate (NADPH) and ATP for energetically expensive FA synthesis (Borisjuk et al., 2005; Rolletschek et al., 2005) and 2) $O_2$ preventing anoxia inside seeds alleviating limitations to the mitochondrial respiratory process and thus allowing to generate more energy and reductant power (Borisjuk and Rolletschek, 2009); and 3) the Calvin-Benson cycle by re-fixing respiratory $CO_2$, providing intermediates for metabolism (Ruuska et al., 2004). In fact, studies with legume seeds point to an effect of light on lipid metabolism via photosynthesis (Goffman et al., 2005; Allen, et al., 2009). Ruuska et al. (2004) verified that seeds (*Brassica napus*) from siliques exposed to light *in planta* produce more FAs than seeds from shaded siliques.

To the best of our knowledge, there are no studies on the influence of light microenvironment at the canopy level on grape seed lipid metabolism and its potential relation with seed photosynthesis. In addition, viticulture management practices that can influence the light microclimate in the canopy, for instance, those applied in grapevines to alleviate the impacts of climate changes including spraying the plants with the white mineral kaolin and irrigation, can also interfere with the level of light that reach the

berries, and thus affect the photosynthetic activity of its tissues, including seeds (Garrido et al., 2019). Thus, the main objective of the present work was to evaluate the effects of two contrasting light microclimates at the canopy level (low light and high light, i.e., shaded and fully-exposed berry clusters) on the lipid profile of grape seeds, and to relate these effects to their differential photosynthetic activity (Garrido et al., 2019).

## 6.2. Material and Methods

### 6.2.1. Grapevine Field Conditions and Sampling

Grape berry samples were collected in 2018 from field-grown Alvarinho cultivar grapevines (*Vitis vinifera* L.) in the organic vineyard Quinta Cova da Raposa in the Demarcated Region of Vinhos Verdes, Braga, Portugal (41°34'16.4"N 8°23'42.0"W) (Garrido et al., 2019). Clusters with two contrasting light exposures were selected to harvest grape berries during their development: low light (LL) clusters that grew in the shaded inner zones of the canopy, and high light (HL) clusters that were exposed to direct sunlight most of the day (more details in Garrido et al., 2019). Grape berries were randomly collected in the morning (9–10 a.m.) from both light microclimates and at three distinct developmental stages: Green [6 weeks after anthesis (WAA) or BBCH-75 - BBCH-scale used for grapes by Lorenz et al. (1994)], *Véraison* (12 WAA, BBCH-83), and Mature (15 WAA, BBCH-89). At each developmental stage, grape berries were sampled as 3 (both *véraison* and mature) or 4 (green) biological replicates from each light microclimate condition, in which 1 replicate represented a mix of 15 to 20 berries, from 3 to 5 clusters from 6 to 8 plants growing in untreated vineyard plots (i.e., from the non-irrigated, non-kaolin controls plants, as described in Garrido et al., 2019). The whole berries were immediately frozen in liquid nitrogen and stored at −80 °C. Later, the berries were broken with a slight impact of a pestle in a mortar (both pre-cooled with liquid nitrogen), which allowed us to isolate the seeds. The seeds were then ground to a fine powder and the samples (a total of 20) were freeze-dried (48 h) for metabolomic analysis. For the transcriptional analysis, for each condition, 3 independent subsamples of grape seeds were prepared from the biological replicates, resulting in a total of 18, which were stored till analysis or immediately used.

### 6.2.2. Seeds' lipid extraction analysis by Liquid Chromatography Mass Spectrometry (LCMS) and data processing

The freeze-dried seed samples were extracted for lipidomics analysis, as described by Okazaki et al. (2013). Quality control (QC) samples ($n = 3$), prepared with a mix of lyophilized material of grape seed, were also prepared and extracted, in order to estimate the overall technical variation per compound. The samples with 10 mg of dry weight were extracted in 1.8 mL of chloroform/methanol (1:1, v/v), with 0.1 % (w/v) butylated hydroxytoluene (BHT) and 1 μM of internal standard 1,2-didecanoyl-sn-glycero-3-phosphocholine (*Sigma*® P7081). After two cycles of vortex and 20 min on ice, the samples were centrifuge at 16,100 x *g*, and the supernatant was transferred for new Eppendorf tubes (were stored at -80 °C, overnight). The organic solvent was evaporated during 1 h and 30 min. in a Speed vac (*Savant®, SC100*), in perforated tubes held closed in this step. Prior to LCMS analysis, samples were dissolved in 200 μL ethanol solution (96 %), vortexed and sonicated (5 min.) and then centrifuged 10 min. at 16,100 x *g*. The supernatants (150 μL) were transferred to amber-coloured 2-mL HPLC vials with glass insert and sealed. The analysis was performed at LCMS system, using a Waters ACQUITY UPLC® HSS T3 1.8 μm (1.0 x 100 mm) column, as described by Remmers et al. (2018).

Unbiased mass peak picking and alignment of the raw data sets from LCMS were carried out using MetAlign software (Lommen, 2009). Irreproducible individual mass signals (present in <3 samples) were filtered out using an in-house script called MetAlign Output Transformer (METOT) (Houshyani et al., 2012). The remaining mass peaks, including molecular ions, in-source adducts, fragments and their natural isotopes, were subsequently clustered using MSClust software into so-called reconstructed metabolites (centrotypes) (Tikunov et al., 2012), according to their corresponding retention time and peak intensity pattern across samples. In the final LCMS dataset, the total number of non-detected, i.e., below the detection limit of 5000 ion counts per compound, was 5127. These non-detected were subsequently filtered out when not present in all 3 or 4 biological replicates of at least one sample group. The relative intensities values were normalized by the internal standard. The values of the remaining non-detects were randomized between 45 % and 55 % of the detection threshold, i.e., between 2250 and 2750. The resulting spreadsheet with the relative intensity of each metabolite (a total of 376) in each sample was used for further statistical analyses.

### 6.2.3. RNA extraction and cDNA synthesis

RNA was extracted from the 18 samples: 3 replicates x 2 microclimates x 3 developmental stages. The total RNA was purified according to Reid et al. (2006), with some adjustments. To 500 mg of frozen tissue, 3 mL of the extraction buffer containing 2 % (w/v) of cetrimonium bromide (CTAB), 2 % (w/v) of soluble polyvinylpyrrolidone (PVP) K-30, 300 mM of TRIS-HCl (pH 8.0), 25 mM of ethylenediamine tetraacetic acid (EDTA), 2 M of sodium chloride (NaCl), and 40 mM of dithiothreitol (DTT, mixed just prior to use) were added. Samples were incubated at 60 °C for 30 minutes and shaken every couple of minutes. After this, the mixtures were extracted twice with 3 mL of chloroform:isoamyl alcohol (24:1) followed by a centrifugation step at 3500 x *g* for 15 min at 4 °C. The aqueous fraction (1.5 mL) was mixed with 0.1 vol of 3 M NaOAc (pH 5.2) and 0.6 vol of isopropanol, and maintained at -80 °C for 30 min, after which the samples were centrifuged at 3500 x *g* for 30 min at 4 °C. The pellet was resuspended in 500 μL of plant RNA Lysis Solution from GeneJET Plant RNA Purification Mini Kit (Thermo Scientific®), following the manufacturer's instructions. RNA concentration was determined in the Nanodrop (Thermo Fisher Scientific Inc.) and its integrity was assessed in a 1 % agarose gel. Total RNA was further purified with DNase I Kit (Thermo Scientific®) to remove any contaminating DNA. First strand cDNA synthesis was synthesized from 1 μg of total RNA using the Xpert cDNA Synthesis Mastermix (Grisp®), following the manufacturer's instructions.

### 6.2.4. Transcriptional Analysis by Real-Time qPCR

Real-time qPCR was used for transcriptional analyses of target genes (Table 6.1), in grape berries seeds from LL and HL microclimates of the three different developmental stages. The gene specific primer pairs used for each target or reference gene are also listed at Table 1. The analysis was performed with Xpert Fast SYBR (uni) Blue (Grisp®) using 1 μL cDNA (diluted 1:10 in ultra-pure distilled water) in a final reaction volume of 10 μL per well.

The transcriptional analyses were performed in an CFX96 Real-Time Detection System (Bio-Rad) using the following cycler conditions: polymerase was activated with an initial step of 3 min at 95 °C, the double strand denaturation occurred at 95 °C for 10 s, the annealing temperature was 55 °C during 20 s and the extension temperature was 72 °C during 20 s (amplification was performed using 40 cycles). Melting curve analysis was performed for specific gene amplification confirmation.

Actin 1 (*VvACT1*) and glyceraldehyde-3-phosphate dehydrogenase (*VvGAPDH*) were selected as reference genes, as the expression of these genes in berries is very stable and ideal for qPCR normalization purposes in grape plants (Reid et al., 2006). Additionally, for each qPCR analysis the actual stability of these target genes (i.e., no significant variation in their expression across sample groups) was validated by the M-values and coefficient of variance values calculated by CFX ManagerTM Software (Bio-Rad): values less than 1 and 0.5, respectively, were regarded as acceptable stabilities (Hellemans et al., 2007). Then, the expression values were normalized to the average of the expression of both reference genes, as described by Pfaffl (2001).

**Table 6.1.** Forward (F) and reverse (R) primers used for gene expression analysis by real-time PCR. Sequences accession numbers were obtained through Grape Genome Browser 12x.

| Gene | Primers | Reference |
|---|---|---|
| ***VvACT1 (reference gene)*** | F: 5′-GTGCCTGCCATGTATGTTGCC-3′ | Reid et al. (2006) |
| | R: 5′-GCAAGGTCAAGACGAAGGATA-3′ | |
| ***VvGAPDH (reference gene)*** | F: 5′-CACGGTCAGTGGAAGCATCAT-3′ | Gainza-Cortés et al. (2012) |
| | R: 5′-CCTTGTCAGTGAACACACCAG-3′ | |
| ***VvACCase1*** | F: 5′-TCCTTCAGGCAGGATCAACCATAC-3′ | Cramer et al. (2014) |
| | R: 5′-TGATCCGACTGTCCACAACAACC-3′ | |
| ***VvΔ9FAD*** | F: 5′-CTTGATGGGGTGAGAGATGAGA-3′ | Arita et al. (2017) |
| | R: 5′-ACCCAACCAGAAAGATAGAGATAGG-3′ | |
| ***VvFAD6*** | F: 5′-CAATTCGGCCTTGGATGTCT-3′ | |
| | R: 5′-TGCCAAACTTATCTTCACCCTCTT-3′ | |
| ***VvLOX0*** | F: 5'-TTCCACCCACTCGCCTGATG-3' | Podolyan et al. (2010) |
| | R: 5'-GCACCGCACCTGTTTCTTCG-3' | |

### 6.2.5. Statistical Analysis

The on-line tool MetaboAnalyst was employed to compare the three developmental stages (Xia et al., 2015). For this, the compound spreadsheet was uploaded into this platform and data was $Log_{10}$-transformed and scaled by the Pareto method (mean-centered and divided by the square root of standard deviation of each variable). Principal Component Analysis (PCA) was used as an unsupervised approach, to make a summary review of samples and to determine differences between developmental stages and between light microclimates. Analysis of Variance (ANOVA) test followed by post hoc multiple comparisons using the Tukey was employed to obtain the metabolites that contribute to the differences between

developmental stages. In addition, a heatmap plot was made based on the 23 top-ranking metabolites according to ANOVA test, after adjudgment of the *p*-values using the Benjamini–Hochberg false discovery rate (FDR) correction.

In addition, to select the compounds that were most influenced by light microclimate, in each developmental stage separately statistical analysis of the averages of the LL and HL groups was performed after $Log_2$ data transformation followed by Student's *t*-test using Analysis ToolPak from Microsoft Excel® (version: 16.0.1312721064). The list with significant metabolites ($p \leq 0.05$) and their respective fold change (FC) values were considered for manual putative identification, based on their accurate masses and the information available at LIPID MAPS® Lipidomics Gateway (http://www.lipidmaps.org/). It is worth noting that we only focus on those significantly differing metabolites with the lowest *p*-values and for which the FC-values (i.e., size of the effect) was higher than the overall technical variation for that specific compound (as determined from the quality control samples).

The gene expression data was Log(X+1) transformed to meet homogeneity of variances. Then, a two-way ANOVA was applied, followed by *post hoc* Bonferroni test whenever the factors (microclimate or developmental stage) had a significant effect (GraphPad Prism version 5.00 for Windows, GraphPad Software, La Jolla, Calif., USA). Significant differences ($p \leq 0.05$) between sample groups are indicated with different letters: capital letters refer to differences between developmental stages for each microclimate, while lowercase letters refer to differences between microclimates in each developmental stage.

## 6.3. Results and Discussion

### 6.3.1. Seed lipid patterns during grape berry development

Lipid profiles were assessed by high resolution Liquid Chromatography Mass Spectrometry (LCMS) of the apolar extracts of seeds from berries at three developmental stages (green, *véraison* and mature) and growing in two distinct light microclimates in the grapevine canopy (low light – LL, shaded, and high light – HL, fully exposed to sunlight). After data processing, a spreadsheet with the relative intensities of each detected compound was obtained, with a total of 376 putative lipids. The LCMS profiles of LL grape seeds (Figure 6.1) shows that seeds at green stage have lower relative abundance of lipids compared to both *véraison* and mature; these later stages showed rather similar profiles. The same trend was observed for HL grape seeds (data not shown). The highest peaks from retention time 18 till 25 minutes,

correspond to the main lipid class of triradylglycerols and sub-class triacylglycerols, i.e., seed storage lipids.

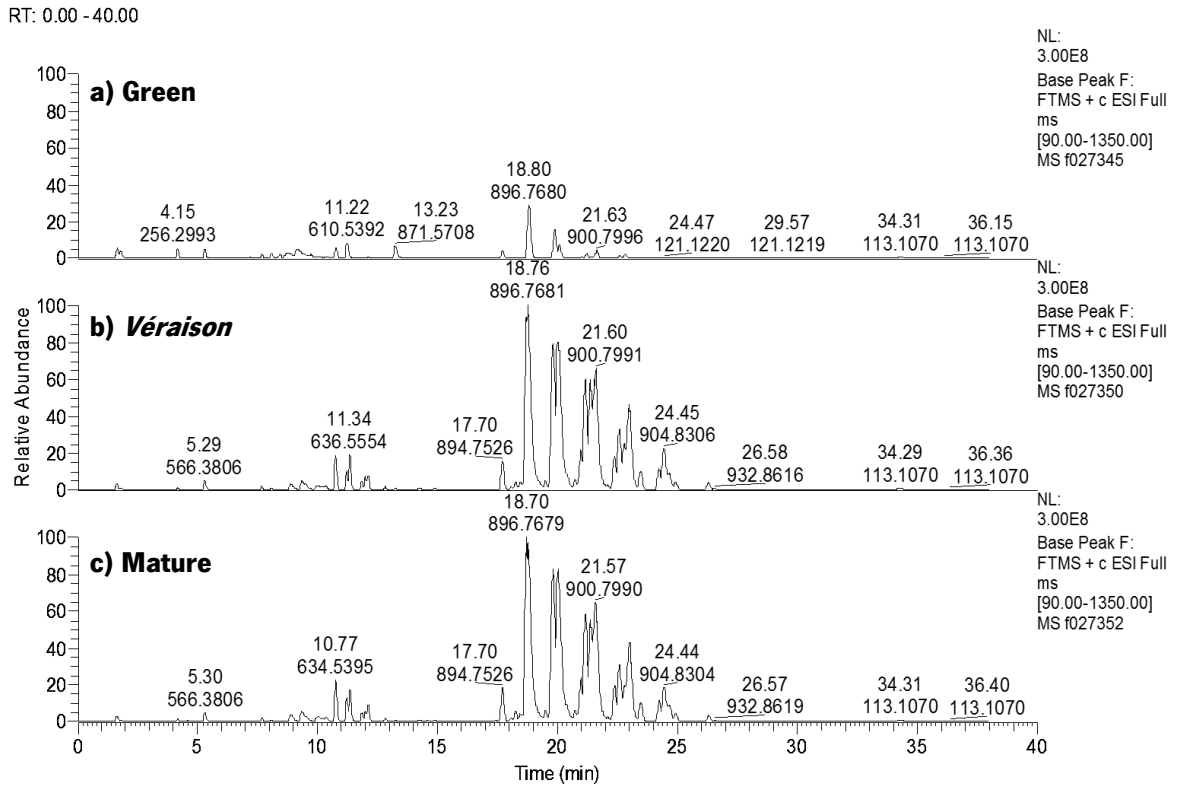


**Figure 6.1.** LCMS chromatograms of LL samples at different berry developmental stages: green (**a**), *véraison* (**b**) and mature (**c**). Numbers above peaks represent, from top to bottom, the retention time and accurate mass, respectively. The three chromatograms are in the same Y-scale.

Principal component analysis (PCA), an unsupervised multivariate analysis approach, was used to identify the main factors underlying the differentiation of grape seed samples (Figure 6.2). In this PCA plot, 18.4 % of total variance is explained by the first two principal components. PC1 clearly distinguished the green stage from *véraison* and mature, supporting the observations from the chromatographic (Figure 6.1). No conspicuous grouping was observed related to microclimate in this PC1-PC2 plot, nor in the PC2-PC3 plot (data not shown). The imposing effect of the developmental stage in explaining total sample variability can make it difficult to identify an eventual microclimate effect, so a more refined analysis must be taken.

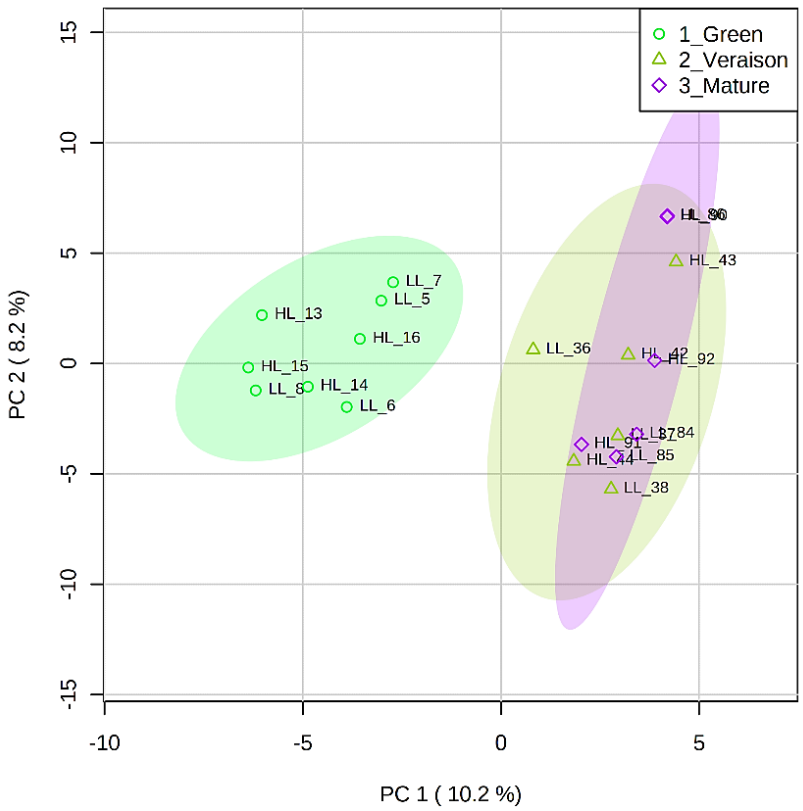


**Figure 6.2.** Principal component analysis (PCA) score plots of the liquid chromatography mass spectrometry (LCMS) lipid data for grape seeds at three developmental stages (green, *véraison*, mature) and light microclimates (LL - low light; HL - high light). Colored ellipses represent 95 % confidence interval ($n$ = 4 for green stage and $n$ = 3 for *véraison* and mature stages). The numbers next to the LL and HL indicate the sample identification.

The Analysis of Variance (ANOVA) test followed by *post hoc* multiple comparisons using the Tukey test allowed us to find 23 lipid compounds responsible for the differences between developmental stages (data not shown). Based on this result, a heatmap plot was constructed with 23 top-ranking metabolites according to ANOVA test between the developmental stages (Figure 6.3). Two main clusters (i.e., 1 and 2) highlighted the differences between the green, *véraison* and mature stages. The cluster 1 represents a group of lipids with high relative abundance at *véraison* and mature, when compared with green stage, including the following categories: ST, FA, GL and GP. The cluster 2 encompass lipids that had high relative abundance at green stage. In particular, the cluster 2 is divided in other two groups (2.1 and 2.2): the first group (i.e., 2.1) contains lipids mainly from GL category, which maintained high relative abundances till *véraison*; while the second group (2.2) is represented by ST, GL and GP categories, which had high relative abundances only in the green stage.

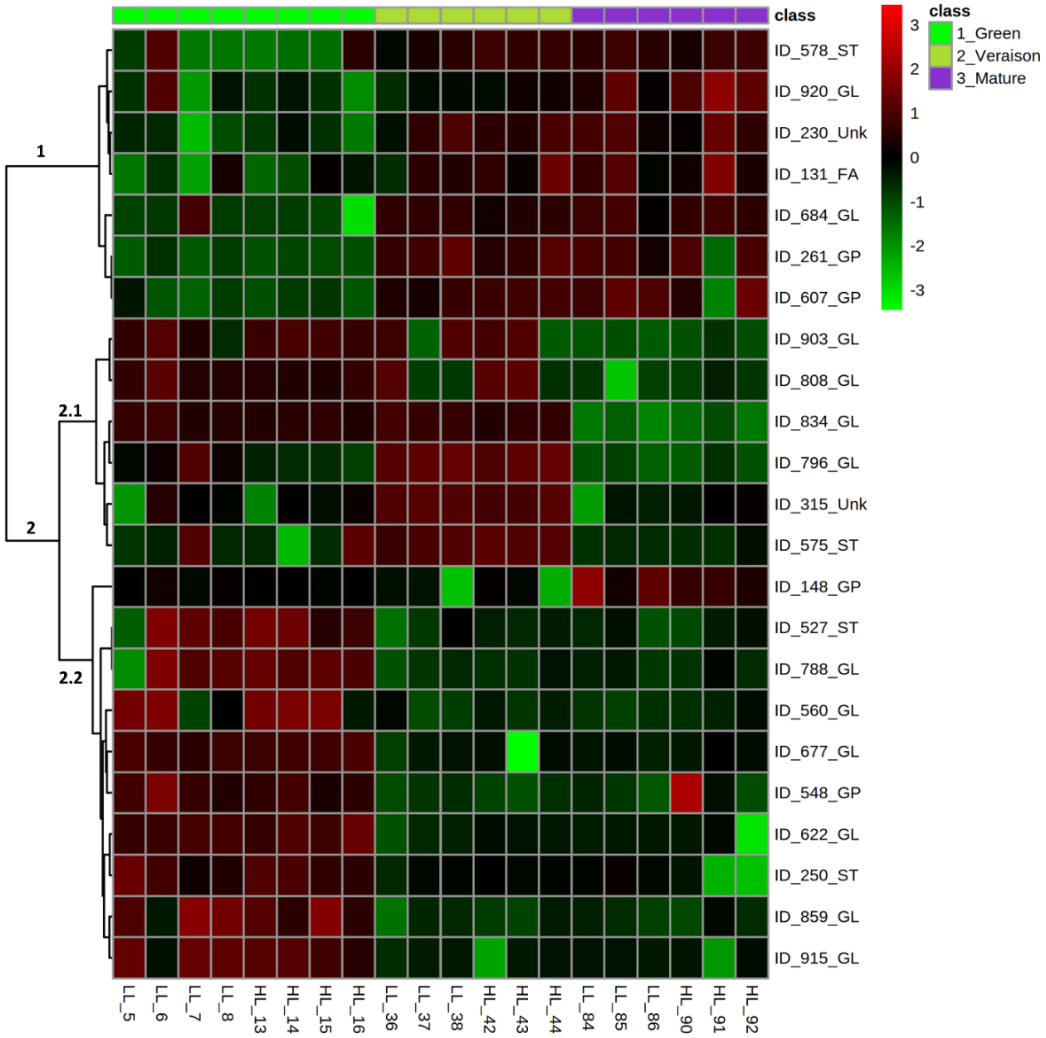


**Figure 6.3.** Heatmap with most significant lipids affected by developmental stages in grape seeds. Heatmap plot with the 23 top-ranking metabolites from Analysis of Variance (ANOVA). On the left-hand side, the numbers represent the main clusters of the heatmap. On the right-hand side of the heatmap, the metabolites are represented by their metabolite number (metabolite ID), followed by the abbreviation of the lipid category: ST – Sterols, GL – Glycerolipids (TAG, Triacylglycerols sub-class), FA – Fatty Acyls (or free fatty acids, FFA), GP – Glycerophospholipids, Unk – unknown. Developmental stages are indicated on top of the heatmap and ordered as green, *véraison* and mature (from left to right, while the biological replicates for low light (LL) and high light (HL) are indicated at the bottom. ($n$ = 4 for green stage and $n$ = 3 for *véraison* and mature stage each).

Both clusters 1 and 2 had high relative abundance of GL, which includes the TAG sub-class. Thus, the high levels of GL in seeds from *véraison* and mature stages (i.e., cluster 1), can be associated with the reserve accumulation that occurs at the stage II of seed development (Ristic and Iland, 2005); while the high relative levels of GL present in seeds from green and *véraison* stages (i.e., cluster 2), can have an important role in the photochemical phase of photosynthesis, since they are in the thylakoid

membranes of chloroplast (Kobayashi, 2016). Corroborating this, in our previous work we showed that grape seeds had high photosynthetic activity at green and *véraison* stages, decreasing slightly afterwards (Garrido et al., 2019). In addition, ST and GP are important components of the plasma membranes (Chen et al., 2009), and thus their high relative abundance at green and *véraison* stages can be related with high rate of cell growth (Ristic and Iland, 2005; Cadot et al., 2006).

From the analysis of this heatmap it is worth to note that there is a considerable variability between replicates, what can mask a potential effect of the microclimate in the PCA (Figure 6.2). A higher number of replicates should be considered in future field experiments.

### 6.3.2. Light microclimate effects on grape seed lipid metabolism: approach of the potential roles of seed photosynthesis

Grape berry seeds are located in the core of an ovoid fruit, shielded not only by a pigmented exocarp but also by a compact mesocarp, thus receiving low amounts of transmitted sunlight. Aschan and Pfanz (2003), using fruit halves reported a value of 2.3 % of the incident photon flux density. Therefore, and based on the light intensity previously measured at the grape berry surface level (Garrido et al., 2019), the grape seed samples used in this study received an estimated mean light intensity as low as 1.15 and 3.35 μmol photons $m^{-2}$ $s^{-1}$, at LL and HL microclimate, respectively, which led to a distinct photosynthetic activity in seeds (Garrido et al., 2019).

For each developmental stage, a Student's *t*-test was performed to select those lipids that mostly contributed for the differences between LL and HL microclimates ($p \leq 0.05$). The results show that 8, 10 and 20 metabolites were statistically different between LL and HL at green (Table 6.2), *véraison* (Table 6.3) and mature (Table 6.4) stage of development, respectively. The fold change (FC) values colored with the red-green patterns, indicate the differences between the two light microclimates.

At green stage, five lipids were influenced by the microclimate: one lipid was up-regulated (ID 412, FC HL/LL = 4.1), while four lipids were down-regulated by HL microclimate as compared with LL (ID 78, 128, 726, 680, all with FC < 0.5) (Table 6.2). Two of the four lipids less accumulated in HL, in relation to LL, were putatively annotated in the FA category (i.e., FFA). The compound up-regulated by HL belongs to the main class of ceramides. Ceramides belong to the SP category, which are components of plasma membrane and endomembrane system, and thus, they facilitate cell growth and also operate as signaling mediators during plant responses to abiotic and biotic stresses (Ali et al., 2018; Huby et al., 2020). SP

synthesis occurs in the endoplasmic reticulum, but it is directly linked to the lipid metabolic pathway in chloroplasts, where the synthesis of the initial precursor, i.e., the palmitoyl-CoA, takes place (Chen et al., 2009). Therefore, and assuming that this ceramide (ID 412) is representative for SP, its higher accumulation in seeds of HL green grapes compared to LL ones, might be related to the increased seed photosynthesis in HL (Garrido et al., 2018; 2019), and thus due to an increase in biosynthesis of palmitoyl-CoA precursor (Chen et al., 2009).

At *véraison* stage, one lipid from the main class of fatty acids (FA category) was up-regulated by HL, as compared to LL (ID 101, FC = 14) (Table 6.3). This high relative abundance of a hydroxy fatty acid in HL seeds, possibly is a result of lipid oxidation, caused by increased photooxidative stress in HL microclimate, in line with our results of thiobarbituric acid-reactive-substances (Garrido et al., 2021). In addition, at *véraison* stage the HL grape seeds had less relative abundance of three lipids (FC < 0.5), as compared with LL ones: ID 183 and ID 57, from GP and FA category, respectively, and ID 713, an unknown metabolite. The biosynthesis of fatty acids in plants is a light-dependent process (Ye et al., 2020) and requires reducing power and ATP (as reviewed by Rawsthorne, 2002). The free fatty acids synthesized in the chloroplast are need for the biosynthesis of other lipids, like phospholipids, glycolipids and oils (Bates et al., 2013).

**Table 6.2.** List of putatively annotated lipids with statistical differences between low light (LL) and high light (HL) microclimates at green stage (Student's *t*-test, $p \leq 0.05$). Metabolites are ordered by fold change values. ID – metabolite number identification. RT – retention time. SIM mass - putative molecular ion (highest signal in mass cluster). Lipid categories: SP – Sphingolipids, GP – Glycerophospholipids, GL – Glycerolipids, FA – Fatty Acyls.

| Cluster ID | Fold Change (HL/LL) | *p* value | RT (min) | SIM mass | Mass Calculated | Ionization mode | Elemental Formula | Main class | Lipid Category | Sub-class |
|---|---|---|---|---|---|---|---|---|---|---|
| **412** | 4.13 | 0.0085 | 11.64 | 784.658 | 784.6579 | $[M+H]^+$ | $C_{46}H_{90}NO_6P$ | Ceramides | SP | Ceramide 1-phosphates |
| **596** | 1.50 | 0.0189 | 16.15 | 789.593 | 789.5905 | $[M+NH_4]^+$ | $C_{46}H_{78}NO_6P$ | Glycerophosphocholines | GP | Dialkylglycerophosphocholines |
| **631** | 0.69 | 0.0352 | 17.28 | 644.596 | - | - | - | Unknown | - | - |
| **915** | 0.55 | 0.0315 | 23.88 | 890.815 | 890.8171 | $[M+NH_4]^+$ | $C_{56}H_{104}O_6$ | Triradylglycerols | GL | Triacylglycerols |
| **78** | 0.46 | 0.0375 | 4.17 | 256.299 | 256.2999 | $[M+NH_4]^+$ | $C_{17}H_{34}$ | Hydrocarbons | FA | - |
| **128** | 0.40 | 0.0293 | 5.59 | 695.390 | - | - | - | Unknown | - | - |
| **726** | 0.26 | 0.0150 | 19.53 | 698.643 | - | - | - | Unknown | - | - |
| **680** | 0.22 | 0.0149 | 18.66 | 656.596 | 656.5936 | $[M+NH_4]^+$ | $C_{38}H_{74}N_2O_5$ | Other Fatty Acyls | FA | - |

**Table 6.3.** List of putatively annotated lipids with statistical differences between low light (LL) and high light (HL) microclimates at *véraison* stage (Student's *t*-test, $p \leq 0.05$). Metabolites are ordered by fold change values. ID – metabolite number identification. RT – retention time. SIM mass - putative molecular ion (highest signal in mass cluster). Lipid categories: FA – Fatty Acyls, GL – Glycerolipids, GP – Glycerophospholipids. *-phosphoethanolamines.

| Cluster ID | Fold Change (HL/LL) | *p* value | RT (min) | SIM mass | Mass Calculated | Ionization mode | Elemental Formula | Main class | Lipid Category | Sub-class |
|---|---|---|---|---|---|---|---|---|---|---|
| **101** | 14.44 | 0.0077 | 4.90 | 283.226 | 283.2268 | $[M+H]^+$ | $C_{17}H_{30}O_3$ | Fatty Acids and Conjugates | FA | Hydroxy fatty acids |
| **480** | 1.65 | 0.0312 | 13.05 | 968.789 | - | - | - | Unknown | - | - |
| **601** | 1.44 | 0.0436 | 16.30 | 892.737 | 892.7389 | $[M+NH_4]^+$ | $C_{57}H_{94}O_6$ | Triradylglycerols | GL | Triacylglycerols |
| **626** | 1.42 | 0.0084 | 17.10 | 854.721 | 854.7232 | $[M+NH_4]^+$ | $C_{54}H_{92}O_6$ | Triradylglycerols | GL | Triacylglycerols |
| **952** | 1.29 | 0.0055 | 25.91 | 918.846 | 918.8484 | $[M+NH_4]^+$ | $C_{58}H_{108}O_6$ | Triradylglycerols | GL | Triacylglycerols |
| **148** | 1.27 | 0.0175 | 6.11 | 625.430 | 625.4251 | $[M+H]^+$ | $C_{31}H_{62}NO_8P$ | Glycero* | GP | Diacylglycero* |
| **780** | 1.20 | 0.0348 | 20.29 | 926.814 | 926.8171 | $[M+NH_4]^+$ | $C_{59}H_{104}O_6$ | Triradylglycerols | GL | Triacylglycerols |
| **183** | 0.40 | 0.0221 | 6.96 | 801.537 | 801.5389 | $[M+NH_4]^+$ | $C_{42}H_{74}NO_{10}P$ | Glycerophosphoserines | GP | Diacylglycerophosphoserines |
| **57** | 0.21 | 0.0005 | 3.17 | 223.096 | 223.0965 | $[M+H]^+$ | $C_{12}H_{14}O_4$ | Fatty Acids and Conjugates | FA | Dicarboxylic acids |
| **713** | 0.12 | 0.0031 | 19.06 | 228.977 | - | - | - | Unknown | - | - |

In our previous work, we verified that HL green seeds had more photosynthetic activity when compared with LL seeds from the same stage (Garrido et al., 2019). Moreover, during the ripening of grape berry and seeds, there was a decrease in photosynthetic activity of seed outer integument from grapes grown in both microclimates, but in particular for HL ones (Garrido et al., 2019). In this way, the higher levels of photosynthesis in HL green seeds can be pivotal to provide precursors, energy and reducing power for the synthesis of structural lipids, like ceramides. In fact, previous works in legume seeds (namely soybean) verified that photosynthesis plays an indirect role on lipid metabolism, by providing $O_2$ that is necessary for energy production (Borisjuk et al., 2005; Rolletschek et al., 2005; as reviewed by Borisjuk and Rolletschek, 2009; Tschiersch et al., 2011). In the case of grape seeds, the internal oxygen respiration peaked at around the beginning of ripening (i.e., around *véraison* stage) and then declined (Xiao et al., 2018). This way, this chemical energy can be generated to support the higher demands of lipid metabolomic pathways, including those of ceramides and fatty acids, mainly at early-mid stages of seed development (i.e., green and *véraison* stages). In addition, Calvin-Benson cycle intermediates can lead to the production of primary substrates, such as acetyl-CoA necessary to fuel lipid metabolism pathways. In particular, in soybean seeds studies with carbon isotopes allowed to find that high levels of light had influence on lipid metabolism, by increase in intermediates generated on Calvin-Benson cycle (Allen et al., 2009). Similarly, in rapeseed, the increase in light from 50 to 150 μmol $m^{-2}$ $s^{-1}$ also enhanced oil biosynthesis (Goffman et al., 2005).

At mature stage, HL seeds had high relative abundance of lipids from different categories, as compared with LL seeds (FC > 2), for instance: GL from TAG sub-class (ID 727, FC = 8.7; ID 714, FC = 5.4), ST (ID 215, FC = 6.1) and GP (ID 148, FC = 5.1) (Table 6.4). In fact, our results showed that HL mature grape seeds accumulated more TAG than LL ones. Several GP in mature grape seeds were up-regulated by HL microclimate as compared to LL (Table 6.4).

**Table 6.4.** List of putatively annotated lipids with statistical differences between low light (LL) and high light (HL) microclimates at mature stage (Student's *t*-test. $p \leq 0.05$). Metabolites are ordered by fold change values. ID – metabolite number identification. RT – retention time. SIM mass - putative molecular ion (highest signal in mass cluster). Lipid categories: GL – Glycerolipids, ST – Sterols, GP – Glycerophospholipids, PR – Prenols, SP – Sphingolipids, FA – Fatty Acyls. *-phosphoethanolamines.

| Cluster ID | Fold Change (HL/LL) | *p* value | RT (min) | SIM mass | Mass Calculated | Ionization mode | Elemental Formula | Main class | Lipid Category | Sub-class |
|---|---|---|---|---|---|---|---|---|---|---|
| **727** | 8.72 | 0.0003 | 19.60 | 925.803 | 925.8219 | $[M+H]^+$ | $C_{60}H_{108}O_6$ | Triradylglycerols | GL | Triacylglycerols |
| **215** | 6.16 | 0.0009 | 8.70 | 446.398 | 446.3993 | $[M+NH_4]^+$ | $C_{29}H_{48}O_2$ | Sterols/ Secosteroids | ST | Stigmasterols |
| **714** | 5.50 | 0.0331 | 19.20 | 822.753 | 822.7545 | $[M+NH_4]^+$ | $C_{51}H_{96}O_6$ | Triradylglycerols | GL | Triacylglycerols |
| **148** | 5.11 | 0.0182 | 6.11 | 625.430 | 625.4251 | $[M+H]^+$ | $C_{31}H_{62}NO_8P$ | Glycero* | GP | Diacylglycero* |
| **171** | 3.51 | 0.0273 | 6.71 | 796.547 | 796.5487 | $[M+NH_4]^+$ | $C_{44}H_{75}O_9P$ | Glycerophosphoglycerols | GP | 1-(1Z-alkenyl),2-acylglycerophosphoglycerols |
| **159** | 3.13 | 0.0074 | 6.29 | 631.417 | - | - | - | Unknown | - | - |
| **185** | 2.59 | 0.0386 | 7.20 | 772.547 | 772.5487 | $[M+NH_4]^+$ | $C_{42}H_{75}O_9P$ | Glycerophosphoglycerols | GP | eg. 1-alkyl,2-acylglycerophosphoglycerols |
| **36** | 2.40 | 0.0197 | 2.34 | 440.248 | 440.2443 | $[M+NH_4]^+$ | $C_{23}H_{31}FO_6$ | Steroids | ST | C21 steroids and derivatives |
| **192** | 2.32 | 0.0112 | 7.18 | 730.500 | 730.5041 | $[M+NH_4]^+$ | $C46H_{64}O6$ | Isoprenoids | PR | C40 isoprenoids (tetraterpenes) |
| **412** | 2.13 | 0.0191 | 11.64 | 784.658 | 784.6579 | $[M+H]^+$ | $C_{46}H_{90}NO_6P$ | Ceramides | SP | Ceramide 1-phosphates |
| **123** | 1.87 | 0.0341 | 5.46 | 678.470 | 678.4704 | $[M+H]^+$ | $C_{35}H_{68}NO_9P$ | Glycerophosphoserines | GP | 1-(1Z-alkenyl),2-acylglycerophosphoserines |
| **85** | 1.52 | 0.0031 | 4.40 | 316.284 | 316.2846 | $[M+NH_4]^+$ | $C_{18}H_{34}O_3$ | Octadecanoids | FA | Other Octadecanoids |
| **956** | 1.43 | 0.0386 | 26.33 | 880,831 | 880.8328 | $[M+NH_4]^+$ | $C_{55}H_{106}O_6$ | Triradylglycerols | GL | Triacylglycerols |
| **953** | 1.42 | 0.0414 | 26.10 | 830.794 | - | - | | Unknown | | - |
| **955** | 1.22 | 0.0076 | 26.22 | 944.862 | 944.8641 | $[M+NH_4]^+$ | $C_{60}H_{110}O_6$ | Triradylglycerols | GL | Triacylglycerols |
| **675** | 1.19 | 0.0474 | 18.48 | 880.716 | 880.7154 | $[M+H]^+$ | $C_{52}H_{98}NO_7P$ | Glycerophosphocholines | GP | e.g: 1-alkyl,2-cylglycerophosphocholines |
| **952** | 1.19 | 0.0169 | 25.91 | 918.846 | 918,8484 | $[M+NH_4]^+$ | $C_{58}H_{108}O_6$ | Triradylglycerols | GL | Triacylglycerols |
| **53** | 0.70 | 0.0065 | 3.02 | 228.195 | 228.1958 | $[M+NH_4]^+$ | $C_{13}H_{22}O_2$ | Fatty Acids and Conjugates | FA | Unsaturated fatty acids |
| **136** | 0.68 | 0.0042 | 5.81 | 354.336 | 354.3367 | $[M+NH_4]^+$ $[M+H]^+$ | $C_{22}H_{40}O_2$ | Fatty Acids and Conjugates Fatty amides | FA | Unsaturated fatty acids |
| **119** | 0.59 | 0.0095 | 5.34 | 352.320 | 352.3210 | $[M+NH_4]^+$ | $C_{22}H_{38}O_2$ | Fatty Acids and Conjugates | FA | Unsaturated fatty acids |
| **146** | 0.51 | 0.0419 | 6.07 | 282.279 | 282.2791 | $[M+NH_4]^+$ | $C_{18}H_{32}O$ | Fatty aldehydes | FA | - |
| **110** | 0.45 | 0.0032 | 5.20 | 415.389 | - | - | - | Unknown | - | - |
| **70** | 0.17 | 0.0003 | 3.66 | 256.227 | 256.2271 | $[M+NH_4]^+$ | $C_{15}H_{26}O_2$ | Fatty Acids and Conjugates | FA | Unsaturated fatty acids |
| **77** | 0.09 | 0.0000 | 4.11 | 437.373 | - | - | - | Unknown | - | - |

Light intensity is an environmental factor associated with temperature (Teixeira et al., 2013), and thus, the lipid profile of grape seeds from HL and LL microclimates can also be related with this abiotic factor. In a study performed with wheat by Djanaguiraman et al. (2018), it was shown that higher temperatures lead to a decrease in the photosynthetic rate, being this aspect related with thylakoid membrane lipid composition, among other factors (e.g., oxidative damage of cell organelle). In accordance with that, in our previous studies the photosynthetic activity/competence of grape seeds peaked at green phase and decreased onward especially in HL seeds (Garrido et al., 2018; 2019), whose grape berry clusters were hotter than LL ones. Similarly, in wheat the TAG levels were higher under high temperature stress, while polar lipid fatty acyl unsaturation was lower (Djanaguiraman et al., 2018). Indeed, HL seeds had a much lower relative abundance of unsaturated FA (e.g., ID 70 and 119) as compared to LL seeds (Table 6.4). This higher relative abundance of FA in LL mature seeds was also seen in LL seeds at the earlier stages, green (Table 6.2) and *véraison* (Table 6.3).

Overall, the results showed that light microclimate had influence on the lipid profile of grape seeds (Figure 6.4). The photosynthesis of grape berry seeds might have an important physiological role, especially at the early stages of seed development, on the biosynthesis of these lipids. In return, this diversity of lipids may be related with different biological functions but is also important for industrial applications. For instance, at harvest the lipids of grape seeds, are important for the fermentation process in winemaking industry (Tesnière, 2019), ultimately conferring sensorial characteristics to the wine.

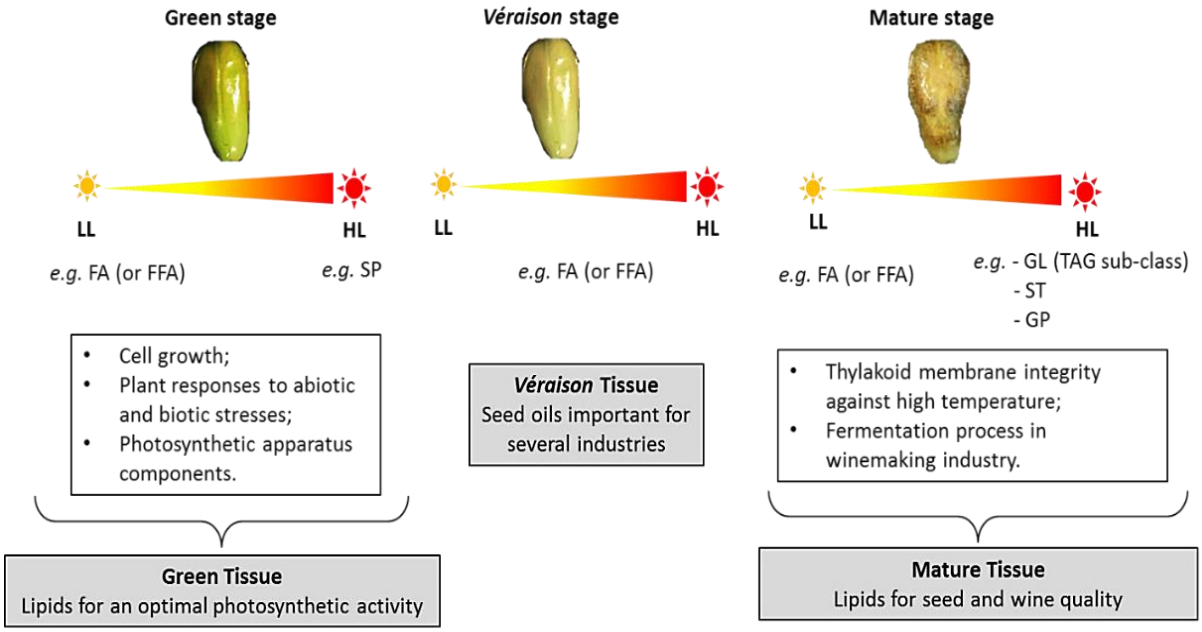


**Figure 6.4.** Overview of lipid profile in the seeds of grape berry clusters growing at two distinct light microclimates, low and high light (LL and HL, respectively) and from three developmental stage (green, *véraison* and mature). Possible biological functions of the lipids at each stage are represented in the boxes. Abbreviations of lipid categories: FA – Fatty Acyls (or free fatty acids, FFA), SP – Sphingolipids, GL – Glycerolipids (TAG, Triacylglycerols sub-class), ST – Sterols, GP – Glycerophospholipids.

### 6.3.3. Transcriptional pattern of key genes involved in fatty acid metabolism

The transcription of four key genes involved in fatty acid metabolism, i.e., acetyl-CoA carboxylase 1 (*VvACCase1*), stearoyl-[acyl-carrier-protein] *Δ*9-desaturase (*VvΔ9FAD*), fatty acid desaturase-6 (*VvFAD6*) and lipoxygenase (*VvLOXO*), was also evaluated. The enzymes encoded by these genes are not only involved in important steps of fatty acids biosynthesis in grape berry, but are also related to the production of flavor compounds (Cramer et al., 2014). In the chloroplast, the ACCase enzyme catalyzes the carboxylation of acetyl-CoA to malonyl-CoA in an ATP-dependent manner (Ye et al., 2020) (Figure 6.5). The C2 units derived from malonyl-CoA then build up C16:0, C18:0 fatty acids. Then, the desaturation of fatty acids occurs either in the chloroplast and in the endoplasmic reticulum (Figure 6.5). In the stroma of chloroplast, the Δ9 fatty acid desaturase (Δ9FAD) is a critical enzyme in the synthesis of unsaturated fatty acids, which has the capability to convert palmitic acid (C16:0) or stearic acid (C18:0) into palmitoleic acid (C16:1) or oleic acid (C18:1), respectively (Los and Murata, 1998). Fatty acid desaturase-6 (FAD6) and fatty acid desaturase-2 (FAD2) encode the desaturases that function in the plastid and in the endoplasmic reticulum, respectively, being responsible for converting oleic acid (C18:1) to linoleic acid (C18:2) (Mikkilineni and Rocheford, 2003; Dar et al., 2017). In the endoplasmic reticulum, other subsequently fatty acid desaturases (i.e., FAD3) play a key role in the synthesis of the

polyunsaturated fatty acids (PUFA), like linolenic acid (C18:3) (Lee et al., 2012; He et al., 2020). In the cytoplasm, lipoxygenases (LOX) catalyze the oxygenation of PUFA into oxylipins/hydroperoxides (Liavonchanka and Feussner, 2006; Mosblech et al., 2009), which are further converted by hydroperoxide lyases (HPL) to form smaller fragments of fatty acids including volatiles, like alcohols and esters (Figure 6.5).

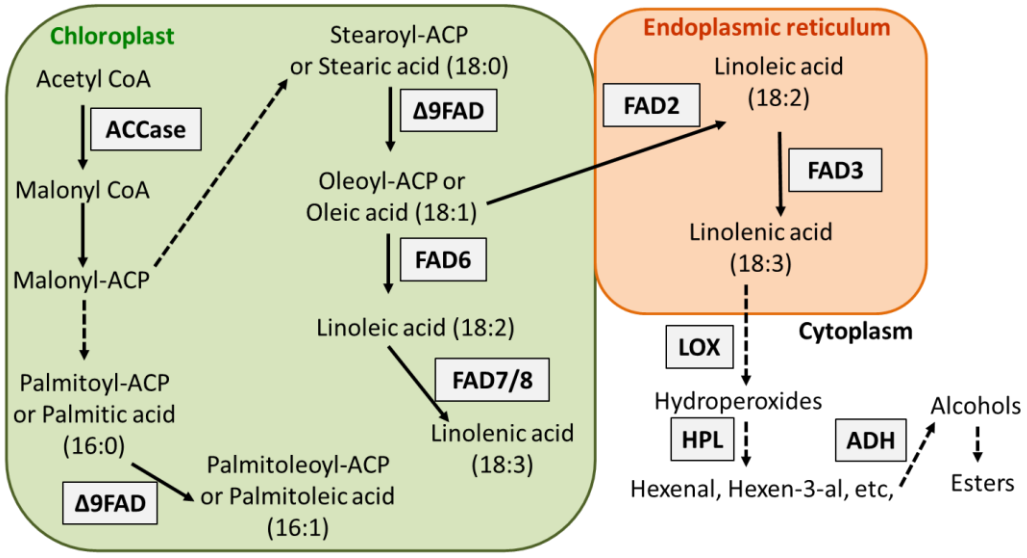


**Figure 6.5.** Simplified metabolomic pathway of free fatty acids synthesis and oxidation of polyunsaturated fatty acids into the production of flavor compounds like alcohols and esters. Abbreviations: Δ9FAD, stearoyl-[acyl-carrier-protein] Δ9-desaturase; ACCase, acetyl-CoA carboxylase; ADH, alcohol dehydrogenase, ACP, acyl carrier protein, FAD, fatty acid desaturase; HPL, hydroperoxide lyase; LOX, lipoxygenase. Adapted from Los and Murata (1998), Bates et al. (2013), Cramer et al. (2014), Dar et al. (2017) and He et al. (2020).

Overall, the statistical differences between samples were mainly observed in relation to developmental stages (Figure 6.6). The expression of *Δ9FAD* gene peaked at *véraison* stage and then decreased at mature stage (Figure 6.6b). This pattern along development is quite similar to the first gene of fatty acid biosynthetic pathway, i.e., *VvACCase1* (Figure 6.6a). These results are consistent with the fact that grape seeds at *véraison* stage accumulate high levels of TAG rich in unsaturated fatty acids (Rubio et al., 2009). Our results also showed that the expression of *VvFAD6* decreased along seed maturation, in both microclimates (Figure 6.6c). Information concerning the expression pattern of these genes along seed development is relatively scarce in the literature. However, in soybean seeds, the transcript levels of *FAD6* were relatively constant from young to mature stages (Heppard et al., 1996), contrasting our results for the same gene (Figure 6.6c). In addition, the relative expression of stearoyl-ACP desaturase (i.e., *Δ9FAD*) decreased along development of flax seeds (Fofana et al., 2006), while our results showed a peak at *véraison* stage (Figure 6.6b). Interestingly, a previous work with berry skin samples from the red grape variety Pinot Noir showed a decrease in *Δ9FAD* expression from post-*véraison*

to harvest stage (Arita et al., 2017), corroborating our results in seeds. Moreover, the same authors using berry skins from a white grape variety (Koshu) showed that the *VvFAD6* expression decreased along development, supporting our results in seeds also from a white grape variety (Arita et al., 2017).

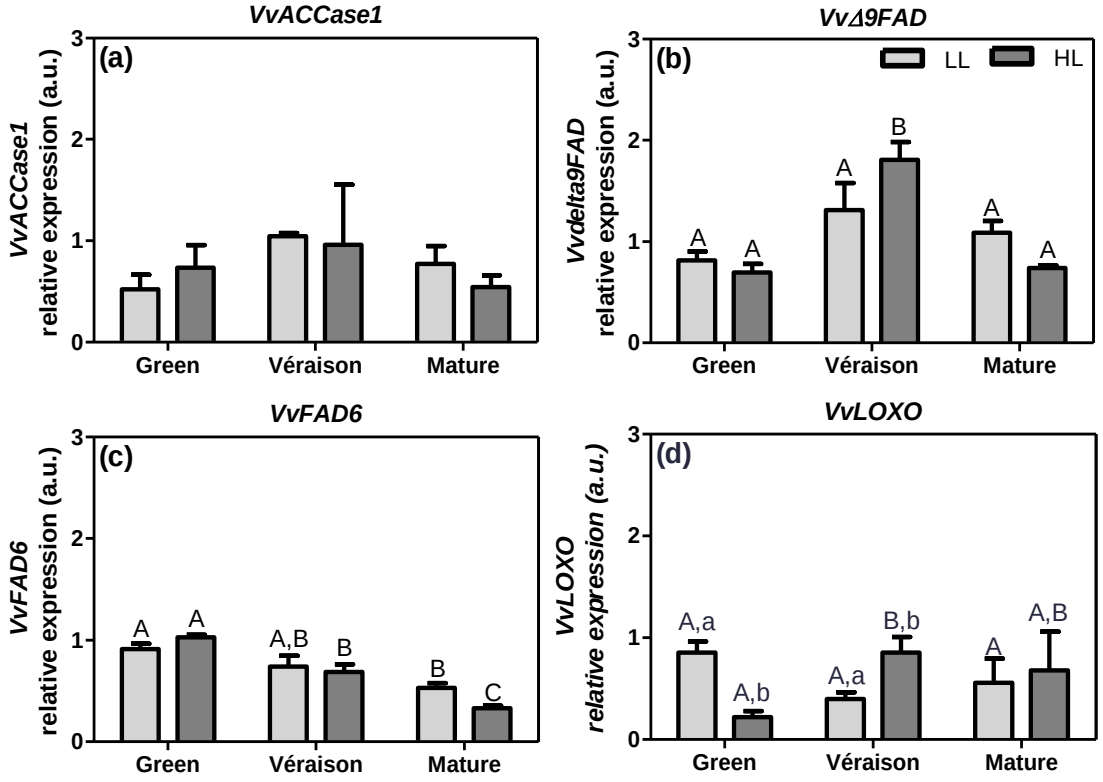


**Figure 6.6.** Relative expression of transcripts of: **(a)** acetyl-CoA carboxylase 1 (*VvACCase1*), **(b)** stearoyl-[acyl-carrier-protein] 9-desaturase (*VvΔ9FAD*), **(c)** fatty acid desaturase-6 (*VvFAD6*), and **(d)** lipoxygenase (*VvLOXO*). Gene expression analysis was performed by real-time qPCR in grape seeds from berries grown at two light microclimates (low and high light, LL and HL, respectively) and at three developmental stages (green, *véraison* and mature). Expression levels are normalized to the mean expression of reference genes *VvACT1* and *VvGAPDH*. Statistical analysis (two-way ANOVA, $p \leq 0.05$, $n = 3$) was applied after data Log(X+1) transformation. Statistical notation: capital letters refer to differences between developmental stages for the same microclimate, while lowercase letters refer to differences between microclimates for each stage. When the letters are omitted, it means that the respective factor did not have a significant effect.

Of the tested genes, the effects of the microclimate were only statistically significant for *VvLOXO*. The HL microclimate first led to a down-regulation of *VvLOXO* expression in seeds at green stage and then to an up-regulation at *véraison* stage (Figure 6.6d), as seen for *Δ9FAD* gene (Figure 6.6b).

Previously, Podolyan et al. (2010) characterized the enzymes and genes associated with the lipoxygenase pathway in a white grape variety. They verified that *VvLOXO* was mostly expressed in seeds (75 % of total expression) as compared to skin (21 %) and pulp (4 %). In plants LOXs can be localized in cytoplasm and plastids, but also in other cellular organelles, such as the vacuole, peroxysomes, lipid bodies, plasma membranes and microsomal membranes (as reviewed by Liavonchanka and Feussner,

2006). In grape berry the presence of putative targeting peptides and *in silico* analysis of *VvLOXO* sequences suggested that this enzyme may be localized in chloroplasts (Podolyan et al., 2010).

Alterations on LOX-HPL pathway may cause changes downstream, i.e., in alcohols and thiols and ultimately in the final quality of grapes and wine. In this way, recent investigations have focused on the effects of light on LOX enzymes (Xu et al., 2015; Joubert et al., 2016; Ju et al., 2016). For instance, Ju et al. (2016) showed that after removal of old leaves at *véraison* stage, the LOX activity in grape skins was significantly higher than in the controls. In addition, the same authors also tested bagging at *véraison* stage, which caused a significant decrease in LOX activity in grape skins. These results suggest that the activity of LOX in grapes is light dependent, and so viticulture practices that change grape berry exposure to light may have impact on lipid metabolism. In accordance with these studies, our experiments showed that *VvLOXO* expression was up-regulated by HL microclimate in seeds from grapes at *véraison* stage, but have also showed that this light regulation was dependent on the developmental stage (Figure 6.6d).

## 6.4. Conclusions

The aim of the present research was to analyze the lipid profile of grape seeds during the development of grape berries growing at two different light microclimates (shaded and exposed) in the canopy of grapevines. We also intended to relate potential microclimate-dependent differences in seed lipid metabolism to their previously reported differential photosynthetic activity (Garrido et al., 2019). Our results show that light microclimate influences seed lipid profiles in grapes at all developmental stages. At green stage, HL seeds had higher relative levels of ceramides, while at mature stage they contained higher relative levels of TAG and GP, as compared to LL seeds. At all stages, LL grape seeds had high relative abundance of the FA (i.e., FFA). Seed photosynthesis may play distinct roles and physiological functions at each developmental stage. At the green stage, seed photosynthesis can supply energy and oxygen to fuel *de novo* synthesis of FA and the pathway towards ceramides, which are important components for an operational photosynthetic apparatus. At the *véraison* stage, the HL microclimate led to a higher abundance of seed FA, probably associated with seed oil production. At the mature stage, although the photochemical activity of seeds was low (Garrido et al. 2019), the Calvin-Benson cycle of HL seeds may indirectly provide the acetyl-CoA needed to *de novo* synthesis of FA in the chloroplast and hence fueling the synthesis of TAG, ST and GP in these seeds, ultimately contributing to seed and wine quality. Very recent data revealed that *VvRuBisCO* had similar expression level in seeds during all developmental stages and was up-regulated by HL (Garrido et al., submitted).

To the best of our knowledge, this study provides the first comprehensive comparison of seed lipid profiles from grape berries growing in two light microclimates. In addition, it also highlights a possible role of berry photosynthesis on the lipidome in this inner tissue. This work also calls for attention for viticulture practices that change the light conditions at the grape berry level. Thus, this information might also be useful for growers in order to obtain grape seeds with optimal quality, not only as a by-product for diverse applications, but also for the winemaking industry and wine quality.

## 6.5. Acknowledgements

Andreia Garrido acknowledges the financial support provided by national funds through FCT - Portuguese Foundation for Science and Technology (PD/BD/128275/2017), under the Doctoral Programme "Agricultural Production Chains – from fork to farm" (PD/00122/2012) and from the European Social Funds and the Regional Operational Programme Norte 2020. Authors acknowledge the National Funds by FCT, via CITAB and CBMA research units under the projects UIDB/04033/2020 and UIDB/04050/2020, respectively. The work was also supported by FCT and European Funds (FEDER/POCI/COMPETE2020) through the research project "MitiVineDrought—Combining "omics" with molecular, biochemical, and physiological analyses as an integrated effort to validate novel and easy-to-implement drought mitigation strategies in grapevine while reducing water use" with ref. PTDC/BIA-FBT/30341/2017 and ref. POCI-01-0145-FEDER-030341, respectively; and through the research project "BerryPlastid with ref. POCI-01-0145-FEDER-028165 and ref. PTDC/BIA-FBT/28165/2017, respectively. Artur Conde was supported by a post-doctoral researcher contract/position within the project "MitiVineDrought" (PTDC/BIA-FBT/30341/2017 and POCI-01-0145-FEDER-030341). FEDER/COMPETE also supported this work through the project "AgriFoodXXI" (ref. NORTE-01-0145-FEDER-000041. This work also benefited from the networking activities within the European Union-funded COST Action CA17111"INTEGRAPE—Data Integration to maximize the power of omics for grapevine improvement". Authors acknowledge the owner from Quinta Cova da Raposa, Manuel Taxa, who provided the samples, and also all support given by the Biology Department of the School of Sciences from the University of Minho. The authors also acknowledge Bert Schipper and Henriëtte van Eekelen (Plant Metabolomics group of WUR-Bioscience) for their help in the chemical analyses and data processing.

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# Chapter 7

## Establishment of *callus* cultures from white grape berry tissues

The manuscript included in this chapter is being prepared for publication:

Garrido, A., De Vos, R. C. H., Conde, A. and Cunha, A. Establishment of *callus* cultures from white grape berry tissues.

**Abstract**

Plant cell cultures, offering an almost perfect controlled environment, are an alternative to study various physiological and biochemical aspects of whole plants or plant systems. Assays with cell suspensions, established from a cluster of less differentiated cells (i.e., *callus*), are quick and convenient, and offer the possibility to carried out relevant experiments throughout the year. However, *callus* induction from field material often becomes a hard work, namely in meeting an effective explant disinfection while avoiding explant death. On the present work we present a set of protocols used to optimize the induction of *callus* from a white grape berry cultivar. In particular, we intended to obtain *calli* from the external tissue exocarp (skin), a highly photosynthetic tissue when compared to the mesocarp, in order to assess possible relationships between fruit tissues photosynthesis and its primary and secondary metabolism. Several parameters were tested, such as: types of medium, disinfection treatments and antioxidant washing treatments of the explants. The disinfection treatments T1, T2, T5 and T7 avoided contamination of all replicates tested, but T7 [ethanol 70 % (2 min) + sodium hypochlorite 0.5 % (15 min)] promoted less browning of the explants and better *callus* growth, and thus it was chosen for further studies. Overall, good results were obtained with Nitsch medium supplemented with casein hydrolysate (mainly 1 g/L), 2,4-dichlorophenoxyacetic acid (2,4-D) (either with 1 or 2 mg/L) and 0.2 mg/L of 6-benzylaminopurine (BAP). This optimization allowed to obtain *calli* that can be used for the establishment of photomixotrophic suspensions and future experiments with cell lines of controlled photosynthetic activity.

**Keywords:** grape berry, disinfection, explant disposition, exocarp, *callus* induction.

## 7.1 Introduction

Grapevine (*Vitis vinifera* L.) is cultivated around the world to produce wine, fresh fruits and derived products (Alston and Sambucci, 2019). Grape berries experience a complex set of physical, physiological and biochemical changes during development (Ollat et al., 2002; Conde et al., 2007). In particular, grape berry tissue-specific photosynthesis has been studied (Garrido et al., 2018; Garrido et al., 2019) and its impact on metabolite profile was recently reported (Garrido et al., 2021), but all the potential functions of grape berry photosynthesis are still largely to unveil. In addition, environmental cues and viticulture practices influence grapevine physiology and grape berry metabolism (Mira de Orduña, 2010; Teixeira et al., 2013). In the field all these external factors occur in a combined and interactive manner, which makes it difficult to understand the specific regulatory mechanisms of stress in the grapevine and at the grape berry level (Mittler, 2006; Atkinson and Urwin, 2012).

Plant tissue culture is a useful tool to study, under controlled conditions, cell proliferation, tissue and organ differentiation and early plant development, as well as several cell physiological and biochemical mechanisms of whole plants or plant systems, either under optimal conditions or under individual and/or combined stresses. Since the first published works in grapevine tissue cultures (Mullins and Srinivasan, 1976), several studies have been made in manipulating grapevine explants, and with different proposes. For instance, in a previous study with a red grapevine cell suspension (established from skin-derived *callus*), it was demonstrated the influence of high light and temperature (separately and combined) in their metabolite profile and target gene transcripts (Ayenew et al., 2015). In addition, other study described the proteomic profiling of the cytosolic proteins, from synchronized cell suspension cultures that were established from *callus* lines originating along the development and ripening of a white grape berry cultivar (Sharathchandra et al., 2011). Moreover, grape cells suspensions have also been used to study the mechanisms of regulation (i.e., transcriptional and post-transcriptional) of monosaccharide transport by glucose (Conde et al., 2006) and the effects of abscisic acid (ABA) and sugar on the anthocyanin production (Hiratsuka et al., 2001). Thus, the grapevine cell suspension cultures are a useful tool to study individual berry-specific processes, as well as the regulation of metabolic pathways (Ferri et al., 2011). Besides that, they are also important for producing high-value metabolites (Calderón et al., 1993; D'Onofrio et al., 2009). More recently, Dai et al. (2014, 2015) have used intact grape berries, inserted by the pedicel into solid culture medium, which maintained their development/ ripening under controlled conditions.

The establishment of *in vitro* cultures from specialized tissues of grape berry, such as exocarp (or skin) and mesocarp, generally has a very low success rate (Torregrosa et al., 2001). Furthermore, the histological origin of *callus* is rarely known, which for us is crucial to obtain cell lines with distinct photosynthetic competences. In addition, establishing contamination-free cultures when the explants are obtained from material collected in the field can be a very time-consuming and difficult task to achieve. In this way, the main objective of the present work was to establish the protocols for grape berry disinfection and *callus* induction from grape berry tissues from a white grapevine variety (cv. Alvarinho). Several factors were tested, namely the disinfection and antioxidant treatments, topological disposition of explants on the plates, types of culture medium and hormonal combinations.

## 7.2. Material and Methods

### 7.2.1. Plant material

Grape berries were collected from a white grapevine cultivar (*Vitis vinifera* L., cv Alvarinho), grown in a farm under organic cultural practices, as described by Garrido et al. (2019). Grape berries clusters were collected early in the morning or at the end of the day, and they were kept refrigerated (4 °C) until inoculation (up to 24 hours maximum). Three growing seasons were considered (2018, 2019 and 2020) for the collection of the samples, and various factors were tested as described below.

### 7.2.2. Testing two types of medium culture: Gamborg's B5 and Murashige and Skoog

The Gamborg's B5 medium (B5) (Gamborg et al., 1968) and Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) were tested since they are often used in plant cell cultures. Grape berries collected at late green stage (1st August 2018) were surface-sterilized with ethanol 80 % (v/v) for 5 min, followed by sodium hypochlorite solution 0.8 % (v/v) for 10 min and then were rinsed three times in distilled sterile water. In the laminar flow chamber, exocarps were removed with a scalpel, cut in similar explants (approx. 1 $cm^2$) and transferred to flasks with solid culture medium (5 flasks/ replicates for each medium, each flask containing 4-5 explants). The two tested media (B5 and MS) (Duchefa Biochemie®) were buffered to pH 5.8, solidified with 0.8 % (w/v)[1] of agar and supplemented with 2 % sucrose and with 0.4 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/L 6-benzylaminopurine (BAP). Cultures were maintained in a growth room at 25 °C under a 16 h photoperiod and a light intensity of 50 µmol $m^{-2}$ $s^{-1}$.

### 7.2.3. Antioxidant washing treatments and MS medium supplemented with different antioxidants

As in a previous preliminary assay high levels of microbial contamination and browning of the explants occurred, we decided to test the efficiency of different antioxidant washing treatments, as well as antioxidant additives in the medium. For this, grape berries collected at mature stage (13th September 2018) were firstly washed with running water and a few drops of detergent (5 to 10 min), and then surface-sterilized with 0.8 % (v/v) sodium hypochlorite solution (5 min).

[1] Whenever the quantities, defined in percentage (%), are omitted, it is because they are defined on a weight/volume basis.

Subsequently, four antioxidant washing treatments were applied to the grape berries (15-20 berries for each treatment, during 5 min):

- **T1 (NA) -** without antioxidants;
- **T2 (PVP) -** polyvinylpyrrolidinone (5 g/L) (Bhatt and Dhar, 2000);
- **T3 (AA) -** ascorbic acid (100 mg/L);
- **T4 (AA+CA) -** ascorbic acid (100 mg/L) and citric acid (200 mg/L) (Patel et al., 2018).

Afterwards, in the laminar flow chamber, exocarp and mesocarp explants (3 explants per tissue; approx. 1 $cm^2$) were distributed in flasks with MS media with four antioxidant additives (4 flasks/replicates x 4 antioxidant washing treatment x 4 antioxidant additives, in a total of 64 flasks), as follows:

- **M1 (MS) -** MS medium without ascorbic acid;
- **M2 (MS+AA) -** MS medium with ascorbic acid (100 mg/L) (Ko et al., 2009; Çördük and Aki, 2011);
- **M3 (MS+VitB5+AA) -** MS basal inorganic medium with Gamborg's B5 vitamins (van Hengel et al., 1992) and with ascorbic acid (100 mg/L);
- **M4 (MS+VitB5) -** MS basal inorganic medium with Gamborg's B5 vitamins) and without ascorbic acid.

All the other medium supplements (sucrose, agar, hormones), as well as the culture maintenance conditions were the same as described above.

### 7.2.4. Testing a new disinfection treatment and different topological dispositions of exocarp explants

Since in the previous experiments none of the explants presented viability to continue with subcultures, another growing season was considered for new tests. Grape berries collected at the green stage (26$^{th}$ July 2019) (Figure 7.1a) were used to promote *callus* induction according to a protocol from Calderón et al. (1994). Briefly, berries without pedicel were washed with running water (5 min) and then surface-sterilized in 7 % aqueous solution of calcium hypochlorite ($CaClO_2$) for 15 min under agitation. At the laminar flow chamber, after four washes in sterile water, the explants (exocarp and mesocarp) were prepared in different sizes and geometries, and three topological dispositions of the explants on the culture medium were tested: A1 – from berry slices (2 mm), as performed by Sharathchandra et al. (2011), exocarp strips (ES) were separated from mesocarp (M) and inoculated vertically sideways and (5 explants per type) (Figure 7.1b); A2 - exocarp explants - small (0.5 $cm^2$) and large (1 $cm^2$) - were inoculated with hypodermal cells faced up (HCU), down (HCD) and vertically sideways (HCV) to the medium (2

explants per type) (Figure 7.1c). In this experiment Murashige and Skoog's medium was used supplemented with 250 mg/L of casein hydrolysate, 20 g/L of sucrose, 0.8 % of agar, 0.1 mg/L of α-naphthaleneacetic acid (NAA) and 0.2 mg/L of kinetin (KIN) and pH was adjusted to 5.8 (Calderón et al., 1994). A total of 24 plates were prepared, that is, 12 culture plates for the explants (ES and M) and disposition A1, and 12 plates for exocarp explants disposition and size A2. Half of each set (n = 12 plates) were maintained in the dark at 25 °C and the remaining were placed under light as described above.

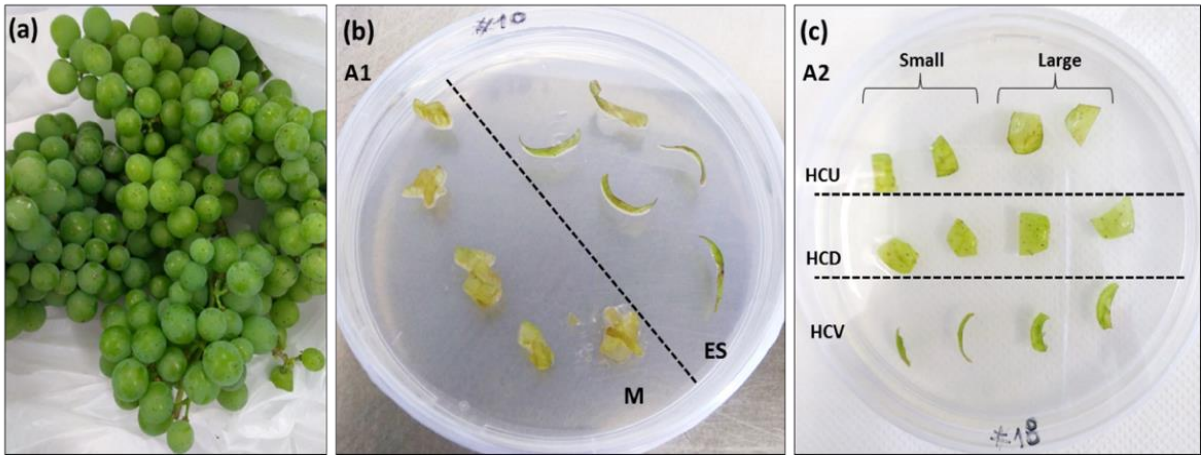


**Figure 7.1.** Grape berry cluster **(a)** from green stage (26th July 2019) used on this assay. Explant topological disposition: **(b) A1** - exocarp strips **(ES)** inoculated vertically and mesocarp **(M)** (5 explants per type); and **(c) A2** - exocarp explants (small - 0.5 $cm^2$; and large - 1 $cm^2$) with hypodermal cells inoculated faced up **(HCU)**, down **(HCD)** and vertically sideways **(HCV)** to the medium (2 explants per type).

### 7.2.5. Optimization of the disinfection procedure

Considering the main results from the previous assays, namely regarding the percentage of contamination, a new procedure for *callus* induction was performed at the same growing season (2019). For this, grape berries were collected at the mature stage (5th September). Firstly, berries without pedicel were washed with running water and some drops of detergent, after which ten disinfection treatments (T1 to T10) were tested, as listed at Table 7.1, according to the protocols from Calderón et al. (1994) and Dai et al. (2014) with some modifications.

**Table 7.1.** Ten disinfection treatments. From T1 until T9 was based on protocol from Dai et al. (2014), while T10 was performed according to Calderón et al. (1994).

| Treatment code | Disinfection process | | |
|---|---|---|---|
| | Ethanol 70 % | Sodium hypochlorite | |
| | Second (s) | Concentration (%, v/v) | Minutes (min) |
| **T1** | 30 | 0.5 | 15 |
| **T2** | 30 | 1 | 5 |
| **T3** | 30 | 2 | 1 |
| **T4** | 60 | 0.5 | 15 |
| **T5** | 60 | 1 | 5 |
| **T6** | 60 | 2 | 1 |
| **T7** | 120 | 0.5 | 15 |
| **T8** | 120 | 1 | 5 |
| **T9** | 120 | 2 | 1 |
| **T10** | Calcium hypochlorite 7 % (w/v) (15 min) | | |

In the laminar flow chamber, after four washing steps with sterile water, the explants were prepared. Per each culture plate, four mesocarp explants (M) and eight exocarp explants were inoculated (approx. 1 $cm^2$ of size), being four with hypodermal cells faced up (HCU) and four disposed vertically sideways (HCV). The medium used was the same of the previous assay: MS medium, 250 mg/L of casein hydrolysate, 20 g/L of sucrose, 0.8 % of agar, 0.1 mg/L of NAA and 0.2 mg/L of KIN, pH 5.8. Per each disinfection treatment, 3 replicates/culture plates were considered, with a total of 30 plates and of 120 explants per type of topological disposition. Per disinfection treatment 2 plates were maintained in the dark at 25 °C and the remaining were placed at light as described above.

### 7.2.6. Nitsch medium, two gelling agents, two concentrations of casein and two concentrations of dichlorophenoxyacetic acid (2,4-D)

#### Assay 1

Grape berry clusters collected at early green stage (1st July 2020) were used for *callus* induction (Figure 7.2a). Individual berries without pedicel were washed with running water with few drops of detergent (5 to 10 min), surface sterilized with ethanol 70 % (v/v) (2 min), followed by sodium hypochlorite 0.5 % (v/v) (15 min) (corresponding to the disinfection treatment T7 from the previous assay). At laminar flow chamber, and after four washes with sterile water, exocarp explants were prepared and inoculated using the topological dispositions HCU and HCV (Figure 7.2b).

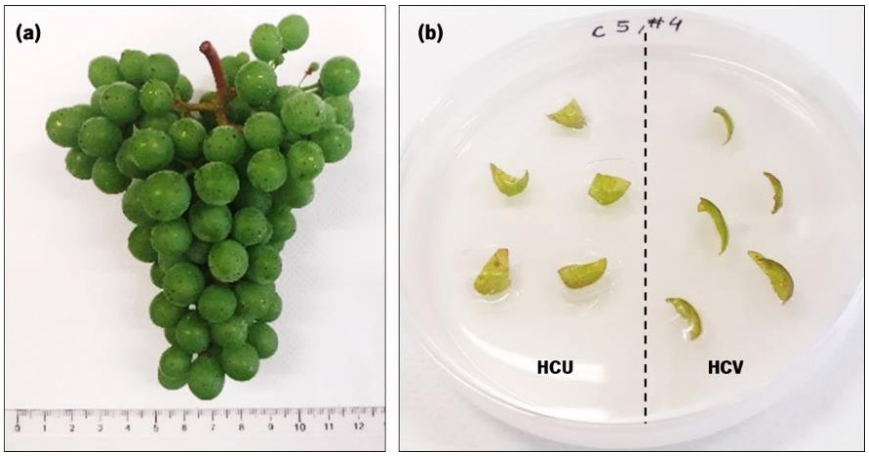


**Figure 7.2.** Grape berry cluster **(a)** from early green stage (1st July 2020) used on this assay. **(b)** Replicate example with two types of topological disposition of exocarp explants on a culture plate: hypodermal cells faced up **(HCU)** and disposed vertically **(HCV)** to the medium (5 replicates per type).

Ten explants (5 replicates per type) with similar size (approx. 1 $cm^2$) were placed on a culture plate with 15-20 ml of sterile autoclaved Nitsch medium (Nitsch and Nitsch, 1969) (Table S7.1), supplemented with: two concentrations of casein hydrolysate (0.5 and 1 g/L), sucrose (40 g/L), 0.8 % of two gelling agents (agar and phytagel), 0.2 mg/L of 6-benzylaminopurine (BAP) and two concentrations of 2,4-D (1 and 2 mg/L), and pH adjusted to 5.8, in a complete factorial design of $2^3$=8 combinations (Table 7.2). Per each medium combination 4 plates were prepared, with a total of 20 explants-replicates of each type of topological disposition per combination. Cultures were maintained at 25 °C under a 16 h photoperiod and with a light intensity of 50 µmol $m^{-2}$ $s^{-1}$.

**Table 7.2.** Eight combinations of Nitsch medium with different concentrations of casein hydrolysate, 2,4-D and gelling agent.

| Medium Combination code | Casein hydrolysate (g/L) | 2,4-D (mg/L) | Gelling agent |
|---|---|---|---|
| **C1** | 0.5 | 1 | Agar |
| **C2** | | | Phytagel |
| **C3** | | 2 | Agar |
| **C4** | | | Phytagel |
| **C5** | 1 | 1 | Agar |
| **C6** | | | Phytagel |
| **C7** | | 2 | Agar |
| **C8** | | | Phytagel |

### Assay 2

As these assays are very long, so as not to lose the green stage of development, a new inoculation was initiated based on an early visual inspection of the previous assay. For this, grape berry clusters from a later green stage (28th July 2020) were collected. The disinfection process, types of explants' disposition and medium conditions were the same as described on Assay 1. However, as in the previous assay, the extension of green color retained by the explants was higher in medium combinations supplemented with casein hydrolysate 1 g/L (i.e., from C5 until C8), only this concentration was used in this assay, and designated as C5', C6', C7' and C8'. Per each medium combination 5 plates were prepared, with a total of 25 explants-replicates of each type of topological disposition (i.e., HCU and HCV) per combination. Cultures were maintained in the same conditions, as described above.

## 7.3. Results

### 7.3.1. Effects of Gamborg's B5 and Murashige and Skoog media

In general, the explants were very brownish, and some flasks presented contaminations (2 replicates in case of MS medium and 3 in B5 medium, from a total of 5 replicates per each medium). At the end, only 1 flask of MS medium had explants (2) with a small *callus*. After 7 weeks, the *calli* were transferred for a new place inside the same flask; and after 2 more months, they were transferred for fresh medium, but no further growth occurred and the explants ended up dying.

### 7.3.2. Effects of antioxidant treatments

After 1 month in culture, from the initial total of 64 flasks, only 37 remained without contamination (57.81 %). Table 7.3 shows the percentage of flasks/replicates that remained in culture in each condition tested.

**Table 7.3 .**Percentage (%) of replicates after 1 month in culture in each type of MS media with four antioxidant additives (M1 - MS; M2 - MS+AA; M3 - MS+VitB5+AA; M4 - MS+VitB5) and antioxidant washing treatments (T1 - NA; T2 - PVP; T3 - AA; T4 - AA+CA). Abbreviations: AA, ascorbic acid; CA, citric acid; MS, Murashige and Skoog medium; NA, no antioxidants; PVP, polyvinylpyrrolidinone; VitB5, Gamborg's B5 vitamins.

| | **Antioxidant washing treatments** | | | | |
|---|---|---|---|---|---|
| **MS media with antioxidant additives** | **T1 (NA)** | **T2 (PVP)** | **T3 (AA)** | **T4 (AA+CA)** | **Total (%)** |
| **M1 (MS)** | 5.41 | 8.11 | 8.11 | 5.41 | **27.03** |
| **M2 (MS+AA)** | 2.70 | 5.41 | 8.11 | 5.41 | **21.62** |
| **M3 (MS+VitB5+AA)** | 5.41 | 8.11 | 5.41 | 8.11 | **27.03** |
| **M4 (MS+VitB5)** | 0.00 | 5.41 | 8.11 | 10.81 | **24.32** |
| **Total (%)** | **13.51** | **27.03** | **29.73** | **29.73** | |

Overall, the antioxidant washing treatment with ascorbic acid alone (T3) or combined with citric acid (T4) were the most efficient, with 29.73 % uncontaminated viable replicates. Concerning the different antioxidant additives tested, no clear effect was observed but M1 (MS) and M3 (MS+VitB5+AA) had higher percentages of uncontaminated replicates (27.03 %). The combination of M4 (MS+VitB5) medium with T4 (AA+CA) antioxidant washing treatment, had the highest total percentage of viable replicates (10.81 %), while the same medium (M4) combined with the T1 treatment (i.e., without pre-washing with antioxidant solution) revealed to be the worst combination, without any viable replicate in the end. Indeed, T1 treatment performed poorly, with about half of the percentage of replicates (13.51 %) when compared with the 3 pre-washing treatments, suggesting that washing grape berries with antioxidant solutions can be useful to prevent contaminations. Regarding to explants coloration, exocarps were still very brownish, more than the mesocarps (Figure 7.3), and no relevant differences were observed when comparing the four washing treatments and the four antioxidant additives. No explants presented viability to continue with subcultures.

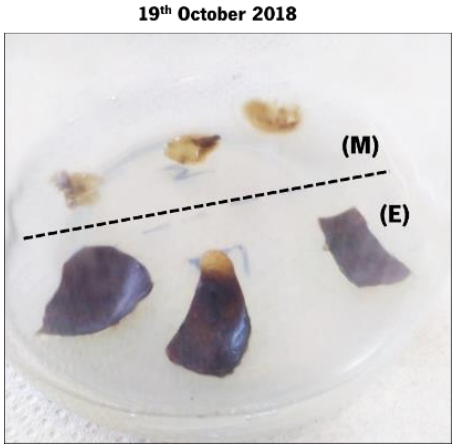


**Figure 7.3.** Examples of exocarp **(E)** and mesocarp **(M)** explants aspect, after 1 month in culture under the condition T4 (AA+CA) and M3 (MS+VitB5+AA).

### 7.3.3. Effects of different dispositions of exocarp explants in a MS medium supplemented with casein hydrolysate

Overall, and regarding to the topological disposition of exocarp explants on the plate, it seems that with the hypodermal cells face up (HCU) and vertically sideways (HCV) the explants had a greener aspect when compared with the remaining types of topological dispositions, exocarp strips (ES) and hypodermal cells faced down (HCD). Also, in HCV explants, it seems that the margin that is not in contact with medium was greener. No differences were observed between the two explant sizes tested. Additionally, the light and dark conditions did not have effect on the explants' color, since in both cases the tissues became brownish.

After 7 weeks in culture (3rd September 2019), only 29.16 % of the total number of replicates (i.e., 7 plates) kept free from contaminations. Only one replicate/plate (from the A2 type of disposition) presented some *callus* formation in exocarp explants with HCU and HCV (Figure 7.4). However, and after 4 subcultures performed every month, no further growth was observed and the *calli* were rejected (Figure 7.4).

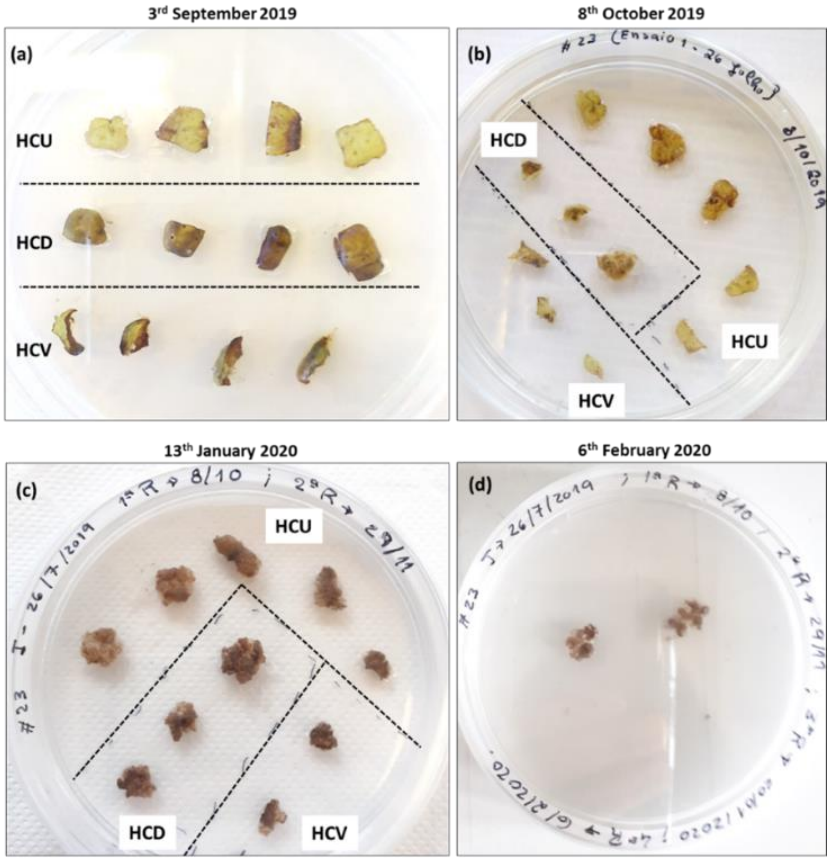


**Figure 7.4.** *Calli* aspect of one replicate, over the months in culture: **(a)** 3rd September 2019 (7 weeks after inoculation); **(b)** 8th October 2019; **(c)** 13th January 2020; and **(d)** 6th February 2020. Exocarp explants inoculated (on 26th July 2019) with hypodermal cells faced up **(HCU)**, down **(HCD)** and vertically **(HCV)** to the medium.

### 7.3.4. Effects of different disinfection treatments

After 1 month in culture, the efficiency of the disinfection treatments was calculated based on the numbers of plates-replicates per treatment and represented as the total percentage of contamination (Figure 7.5). The treatments T1, T2, T5 and T7 had no contamination in any plate-replicate, while T3, T4, T6, T8 and T9 had each one 14.29 % of contamination. The highest percentage of contamination was obtained in T10 (28.57 %), and so this treatment showed to be less efficient on grape berries disinfection.

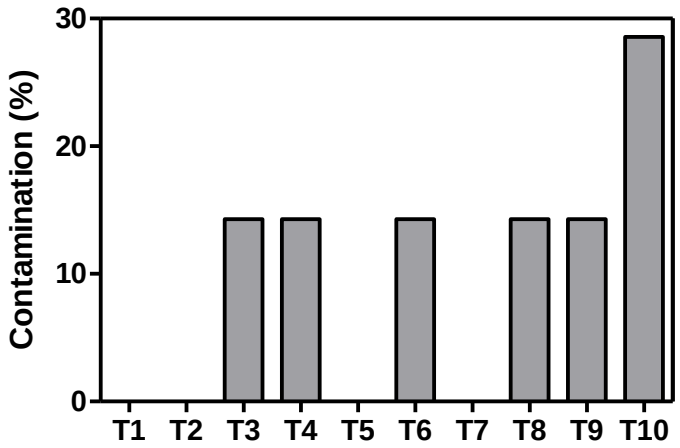


**Figure 7.5.** Total percentage of contamination for each disinfection treatment (T1 to T10), after 1 month in culture. The treatments are described above on Table 7.2.

One month after explant inoculation, relative *callus* growth (CG; relative to *callus* dimensions at the sub-culture) (Table 7.4) and explants browning (Table 7.5) were visually inspected for all disinfection treatments. The relative CG (relative to *callus* dimensions at the sub-culture) was registered according to an empirical semi-quantitative scale: 0 - without or with very little growth; 1 - little to medium growth; 2 - significant growth; and 3 - very significant growth. The visual evaluation of explant color was also based on an empirical semi-quantitative scale: 0 - heavy browning no browning; 1 - browning; 2 - slight browning; 3 - no browning.

In general, there was not much difference between light and dark conditions (Table 7.4 and Table 7.5). Overall, and regarding to *callus* growth, treatments T1, T2, T5, T6, T7 and T8 promoted higher cells proliferation (for example T7 #1 in Figure 7.6a). In addition, less browning of the explants was obtained for T6 and T7 treatments (Table 7.5).

**Table 7.4.** Visual evaluation of *callus* growth, after 1 month in culture, on all conditions tested: disinfection treatments (T1 to T10), light and dark, and exocarp explants with hypodermal cells inoculated faced up (HCU) and vertically (HCV) to the medium, as well as mesocarp (M) explants. Scale: **w.e. -** without explant; **0 -** without or with very little growth; **1 -** little to medium growth; **2 -** significant growth; **3 -** very significant growth.

| | T1 | | | T2 | | | T3 | | | T4 | | | T5 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** |
| **Light** | 3 | 1 | 0 | 3 | 2 | 0 | 0 | w.e. | 0 | w.e. | w.e. | w.e. | 3 | 3 | 0 |
| **Dark** | 1 | 3 | 0 | 2 | 2 | 0 | 2 | 2 | 2 | 3 | 3 | 0 | 2 | 1 | 0 |

| | T6 | | | T7 | | | T8 | | | T9 | | | T10 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** |
| **Light** | 3 | 3 | 0 | 3 | 2 | 0 | 3 | 3 | 0 | w.e. | w.e. | w.e. | w.e. | w.e. | w.e. |
| **Dark** | 3 | 1 | 0 | 3 | 3 | 0 | 1 | 1 | 0 | 3 | 3 | 0 | 2 | w.e. | 0 |

| | |
|---|---|
| **w.e.** | Without explant |
| **0** | Without or with very little growth |
| **1** | Little to medium growth |
| **2** | Significant growth |
| **3** | Very significant growth |

**Table 7.5.** Visual evaluation of explant color, after 1 month in culture, on all conditions tested: disinfection treatments (T1 to T10), light and dark, and exocarp explants with hypodermal cells inoculated faced up (HCU) and vertically (HCV) to the medium, as well as mesocarp (M) explants. Scale: **w.e. -** without explant; **0 -** heavy browning no browning; **1 -** browning; **2 -** slight browning; **3 -** no browning.

| | T1 | | | T2 | | | T3 | | | T4 | | | T5 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** |
| **Light** | 3 | 0 | 0 | 3 | 1 | 2 | w.e. | w.e. | w.e. | w.e. | w.e. | w.e. | 0 | 0 | 0 |
| **Dark** | 0 | 0 | 3 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 2 | w.e. | 3 | 0 | 3 |

| | T6 | | | T7 | | | T8 | | | T9 | | | T10 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** | **HCU** | **HCV** | **M** |
| **Light** | 3 | 3 | 0 | 3 | 0 | 0 | 3 | 3 | 1 | w.e. | w.e. | w.e. | w.e. | w.e. | w.e. |
| **Dark** | 2 | 0 | 3 | 2 | 3 | 3 | 0 | 0 | 1 | 2 | 1 | w.e. | 0 | w.e. | 0 |

| | |
|---|---|
| **w.e.** | Without explant |
| **0** | Heavy browning |
| **1** | Browning |
| **2** | Slight browning |
| **3** | No browning |

However, and after 4 subcultures performed every month, the small *calli* that had been formed in some replicates, did not exhibit further growth (Figure 7.6b).

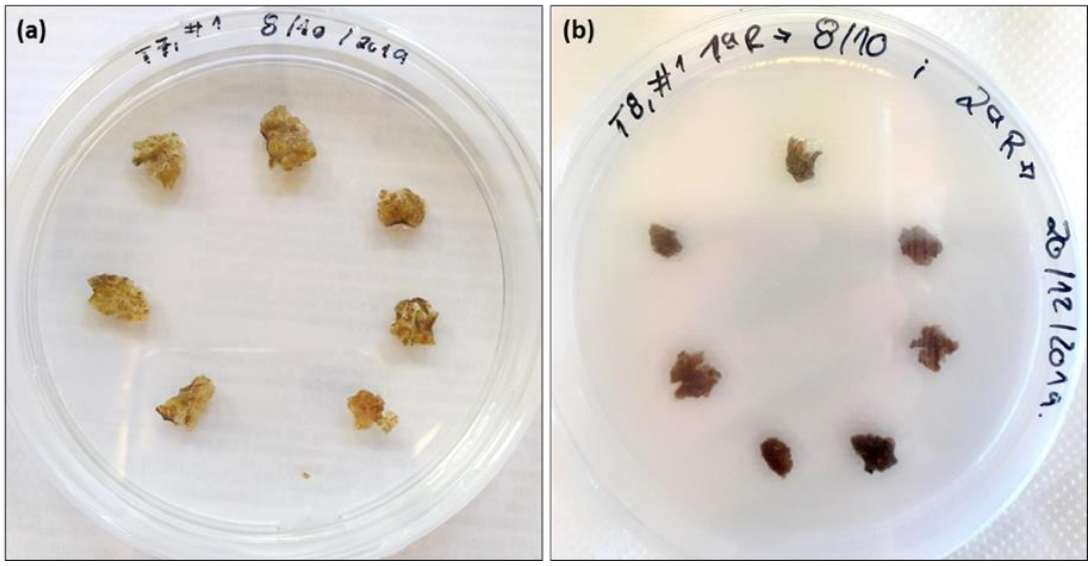


**Figure 7.6.** Aspect of a *callus* in one replicate (T7, #1): **(a)** after the first subculture (8th October 2019) and **(b)** at end of 4 subcultures (January 2020).

### 7.3.5. *Calli* induction in Nitsch medium

#### Assay 1

During the first month, the contaminated culture plates (i.e., 13 out of 32) were removed and the remaining kept in culture (in a total of 19 plates, at least one per each medium combination). After this, the viability of the explants was visually assessed according to an empirical scale of 4 levels of green area (GA) proportion of the explant surface (Figure 7.7): 0 - 0 % ≤ GA ≤ 2 %; 1 - 2 % < GA ≤ 40 %; 2 - 40 % < GA ≤ 80 %; 3 - GA ≥ 80 %.

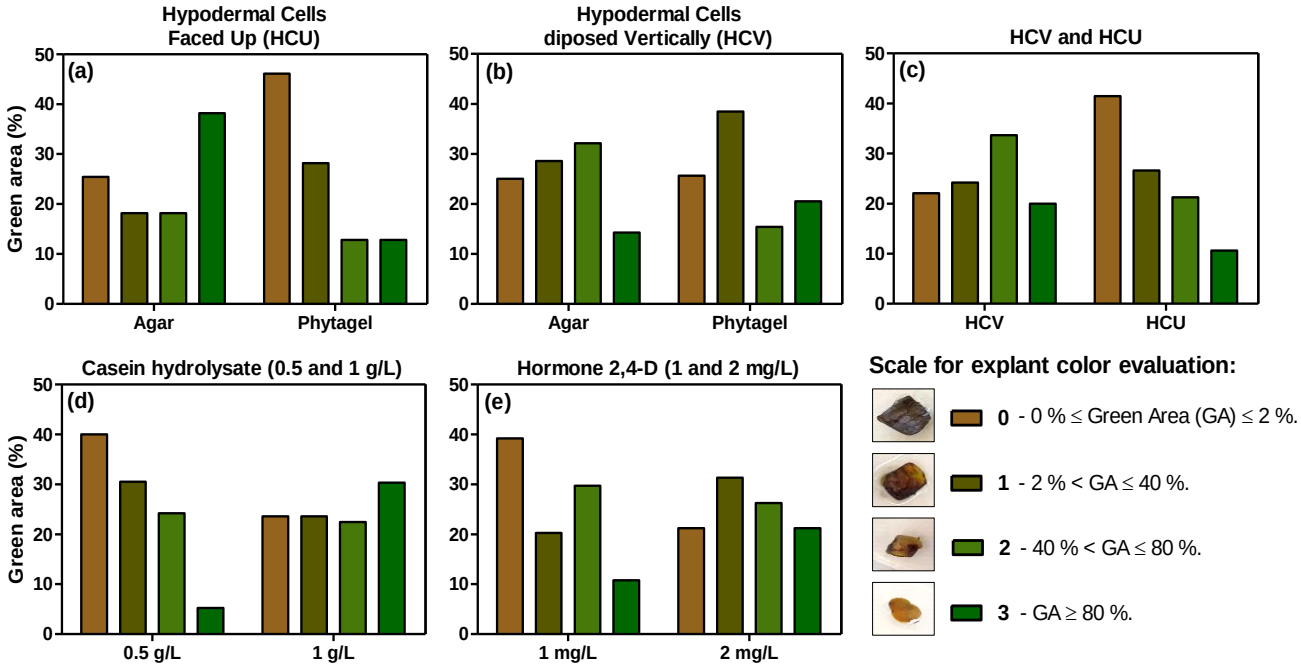


**Figure 7.7.** Explants evaluation according to a scale of 4 levels based on percentage of green area (GA): **0 -** 0 % ≤ GA ≤ 2 %; **1 -** 2 % < GA ≤ 40 %; **2 -** 40 % < GA ≤ 80 %; **3 -** GA ≥ 80 %. Factors analysed: **(a)** hypodermal cell faced up (HCU) and **(b)** vertically (HCV) in a medium with agar and phytagel; **(c)** HCV *versus* HCU; **(d)** two concentrations of casein hydrolysate (0.5 and 1 g/L) and **(e)** of 2,4-D (1 and 2 mg/L).

Explants with the hypodermal cells faced up (HCU) were greener in medium with agar than with phytagel (Figure 7.7a). That difference was not so evident with explants disposed vertically (HCV) (Figure 7.7b), but overall, the explants HCV had higher percentage of green area when compared with the explants HCU (Figure 7.7c). In terms of supplementation factors: explants inoculated onto media with higher concentration of casein hydrolysate (1 g/L) exhibited a higher percentage of green area, revealing that the addition of casein has a role in keeping the viability of the explants and that its concentration should be optimized (Figure 7.7d). A similar response was verified for the higher concentration of 2,4-D (2 mg/L) (Figure 7.7e).

After one month in culture, the explants were transferred to new plates with fresh medium (Table 7.2). During this process, the oxidized brownish explants were discarded and in case of those explants with some green area, the parts that were dried and brown were removed before transference. Despite the greenish-brownish aspect, some explants, mainly HCU, presented the formation of *callus* (Figure 7.8).

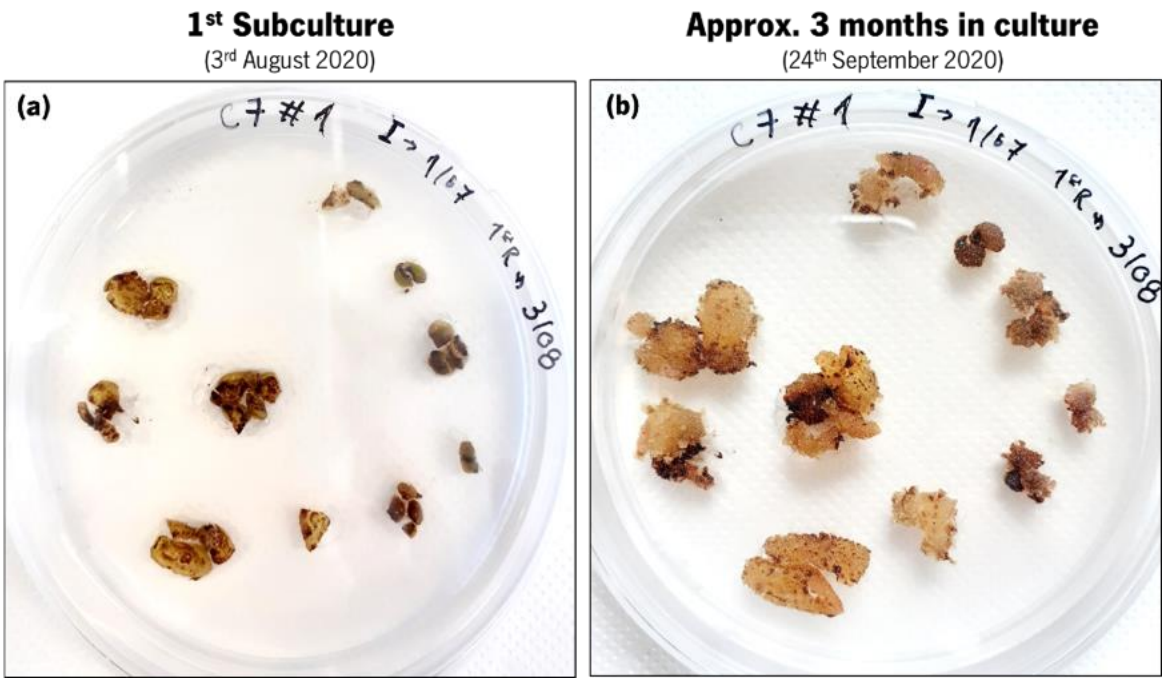


**Figure 7.8.** *Calli* aspect of one plate-replicate (C7 #1): **(a)** after the first subculture (1 month after inoculation, 3rd August 2020) and **(b)** after approximately 3 months in culture (24th September 2020).

After approximately 3 months in culture, explants were again visually inspected, and the relative *callus* growth (CG) was registered as before according to the same empirical semi-quantitative scale (0 to 3) (Figure 7.9). Overall, the results showed that the C7 and C8 medium combinations promoted higher *callus* growth (Figure 7.9) (mean value of 180.06 mg of fresh weight, n = 5), but in general the explants in C8 medium were more brownish. Similar to what was observed for the maintenance of explant viability (Figure 7.7d,e), this result also highlights the importance of casein hydrolysate and 2,4-D at the higher concentrations tested in promoting *callus* growth. Thus, after 3 months in culture, the explants/*callus* from all the eight combinations of medium, were transferred for new plates with C7 medium (Table 7.2).

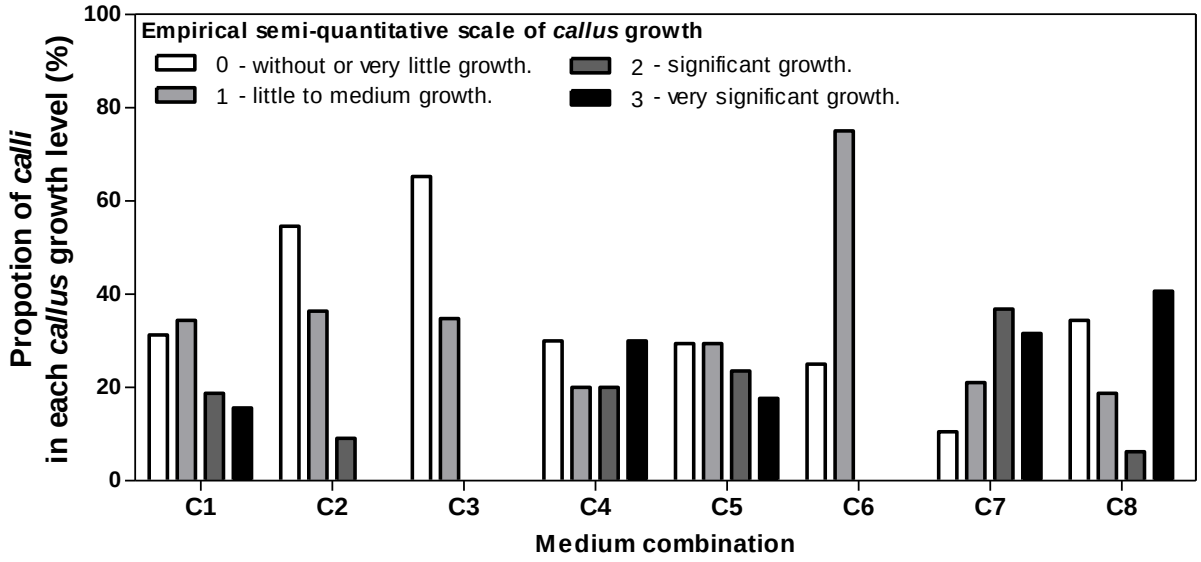


**Figure 7.9.** Explants evaluation according to a scale of 4 levels of relative *callus* growth (CG): **0 -** without or with very little growth; **1 -** little to medium growth; **2 -** significant growth; **3 -** very significant growth. The medium combinations are described in Table 7.2.

### Assay 2

After one month, all plates-replicates (i.e., 20 plates) remained in culture without any contamination. The viability of the explants was visually assessed and scored according to the empirical scale of 4 levels of green area (GA) (Figure 7.10), as already described above. Overall, the results showed that the exocarp explants placed on medium solidified with agar had a larger green area than those inoculated on medium with phytagel, independently of the disposition of the explants (Figure 7.10a,b,c). Although this gelling agent effect was much more prominent in exocarp explants disposed vertically (HCV) (Figure 7.10a,b), globally, no relevant differences were detected when comparing HCV and HCU (Figure 7.10d). Contrarily to what was observed in assay 1 (Figure 7.7e), no relevant differences were detected when comparing the two concentrations of 2,4-D (Figure 7.10e).

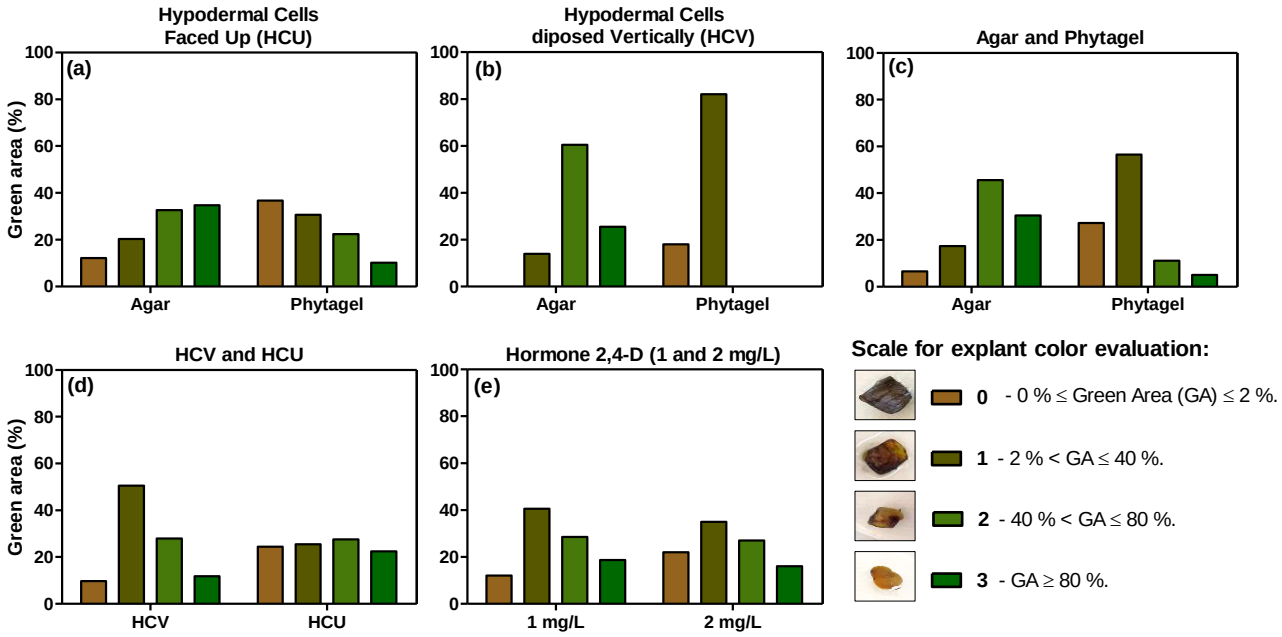


**Figure 7.10.** Explants evaluation according to a scale of 4 levels based on percentage of green area (GA): **0 -** 0 % ≤ GA ≤ 2 %; **1 -** 2 % < GA ≤ 40 %; **2 -** 40 % < GA ≤ 80 %; **3 -** GA ≥ 80 %. Factors analysed: **(a)** hypodermal cell faced up (HCU) and **(b)** vertically (HCV) in a medium with agar and phytagel; **(c)** gelling agent; **(d)** HCV *versus* HCU; and **(e)** two concentrations of 2,4-D (1 and 2 mg/L).

The explants were subcultured (1 month after inoculation) for new plates with the same medium conditions, as used initially (Table 7.2). After 3 months from this first subculture (i.e., in a total of 4 months in culture), the callosity level was visually inspected and the relative *callus* growth (CG) was registered as before according to the same empirical semi-quantitative scale (Figure 7.11). Both C5' and C7' medium combinations promoted more *callus* growth (levels 2 and 3), when compared with the other combinations tested (C6' and C8') (Figure 7.11). Thus, agar was the gelling agent more adequate to maintain explants viability, expressed in larger chlorophyllin (green) areas, as exposed above (Figure 7.10a,b,c), but also for cell proliferation and *callus* growth (Figure 7.11).

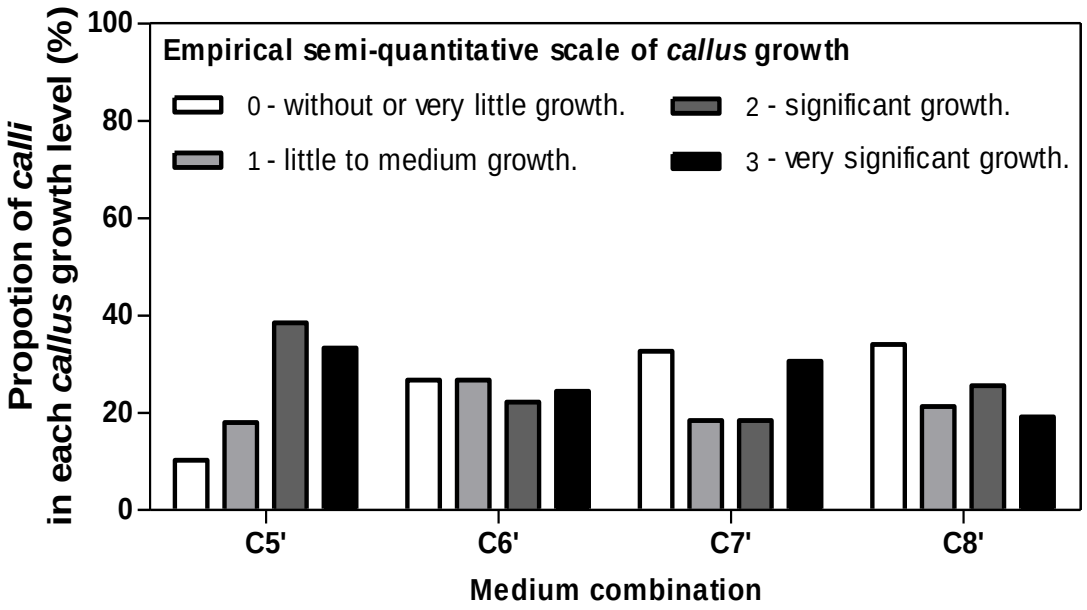


**Figure 7.11.** Explants evaluation according to a scale of 4 levels of relative *callus* growth (CG): **0 -** without or with very little growth; **1 -** little to medium growth; **2 -** significant growth; **3 -** very significant growth. The medium combinations are described in Table 7.2.

## 7.4. Discussion

Grapevine tissue culture is a useful biological tool for different applications, for instance: grape berry ripening studies, model systems to study the regulation of metabolomic and transcriptomic aspects, secondary metabolites production and fungal elicitor testing (Calderón et al., 1993; Ferri et al., 2011; Sharathchandra et al., 2011). However, grapevine is considered recalcitrant to *in vitro* manipulation (Martinelli and Gribaudo, 2009), which means low responsiveness of cells, tissues and organs to several stimuli used in plant tissue culture (nutrient, hormonal, other additives). In this way, further efforts and protocol optimizations are needed. The present work was designed to establish a protocol for *callus* induction using the exocarp of grape berry as the main explant. An additional objective was to subsequently establish cell suspension lines from this *callus*, but due to lack of time this was not possible.

The disinfection process is a crucial step to reduce contamination and to keep the viability of the plant material. The treatment T7 [ethanol 70 % (2 min) + sodium hypochlorite 0.5 % (15 min)], was one of the treatments that ensured an effective decontamination of the explants (Figure 7.5) and at the same time promoted the best *callus* growth and the least browning of the explants (Tables 7.4 and 7.5). However, in a previous study, that served as basis for this assay, 100 % of contamination occurred when using ethanol 70 % (10 s) and sodium hypochlorite 2 % (20 min) (Dai et al. 2014). This suggest that the

equilibrium between disinfectants concentration and treatment duration should be an important aspect to consider.

The topological disposition of exocarp explants on the medium was also an important factor for *callus* induction. In that regard, the results demonstrated that exocarps with the hypodermal cells inoculated faced up (HCU) or disposed vertically sideways (HCV) were the ones in which cell proliferation occurred (Figure 7.4), likely due to better air circulation around those cells (Smith, 2013), when compared with the others faced down (HCD).

Altogether, our results showed that *callus* induction occurred with Nitsch medium, using specific supplementations and gelling agents. The addition of casein hydrolysate to the medium, mainly at 1 g/L, revealed to be very effective in maintaining the explants green and viable (Figure 7.7d). Casein hydrolysate is an amino acid complex, which has been widely used as a supplement in the plant tissue culture media (Neumann et al., 2009). It can increase cell growth, mainly due to its high concentration of glutamine, which can contribute to balance the lack of synthesis of this amino acid (Bister-Miel et al., 1985). Moreover, casein hydrolysate also serves as a source of reduced nitrogen (Neumann et al., 2009), which can alleviate the problem of browning of the explants, as observed here in our results and in a previous study using petioles explants of eastern cottonwood (Thakur et al., 2012).

Auxins and cytokinins are equally necessary for *calli* production, since both hormones exert a strong influence on the cell growth and division, in particular in the different phases of the cell cycle: auxins affect DNA replication, whereas cytokinins seem to exert some control over the events leading to mitosis and cytokinesis (Gaspar et al., 1996). On the present work, we tested two concentration of the auxin 2,4-D (1 and 2 mg/L), but no relevant differences were found on *callus* growth. Besides that, two gelling agents were also tested, agar and phytagel (both at 8 g/L). Overall, the results for the visual evaluation of the *callus* growth and the percentage of green area showed that agar was the gelling agent more beneficial. Similarly to this result, a previous study showed that agar (7 g/L) was the gelling agent that ensured successful long-term maintenance of *Vitis* embryogenic cultures, when compared with phytagel (3 g/L) (Dhekney et al., 2009).

The present work opens the perspectives for future research by using this *callus* derived from exocarp tissue from a white grapevine variety (Alvarinho). In particular, the establishment of photomixotrophic cell suspensions will allow to manipulate their photosynthetic activity in controlled conditions and assess its impact on the metabolism, and thus, correlate this information with an *in vivo* study previously performed by our group (Garrido et al., 2019; Garrido et al., 2021).

## 7.5. Supplementary Information

**Table S7.1.** Nitsch medium composition: micro and macro elements and vitamins (Nitsch and Nitsch, 1969).

| Micro Elements | mg/L |
|---|---|
| FeNaEDTA | 36.70 |
| $MnSO_4.H_2O$ | 18.94 |
| $ZnSO_4.7H_2O$ | 10 |
| $H_3BO_3$ | 10 |
| $Na_2MoO4.2H_2O$ | 0.25 |
| $CuSO_4.5H_2O$ | 0.025 |

| Macro Elements | mg/L |
|---|---|
| $NO_3$ | 950 |
| $NH_4NO_3$ | 720 |
| $CaCl_2$ | 166 |
| $MgSO_4$ | 90.27 |
| $KH2PO_4$ | 68 |

| Vitamins | mg/L |
|---|---|
| myo-Inositol | 100 |
| Nicotinic acid | 5 |
| Glycine | 2 |
| Pyridoxine HCl | 0.50 |
| Thiamine HCl | 0.50 |
| Folic acid | 0.50 |
| Biotin | 0.05 |

## 7.6. Acknowledgements

Andreia Garrido acknowledges the financial support provided by national funds through FCT - Portuguese Foundation for Science and Technology (PD/BD/128275/2017), under the Doctoral Programme "Agricultural Production Chains – from fork to farm" (PD/00122/2012) and from the European Social Funds and the Regional Operational Programme Norte 2020. The work was supported by National Funds by FCT—Portuguese Foundation for Science and Technology, via CITAB and CBMA research units under the projects UIDB/04033/2020 and UIDB/04050/2020, respectively. The work was also supported by FCT and European Funds (FEDER/POCI/COMPETE2020) through the research project "MitiVineDrought—Combining "omics" with molecular, biochemical, and physiological analyses as an integrated effort to validate novel and easy-to-implement drought mitigation strategies in grapevine while reducing water use" with ref. PTDC/BIA-FBT/30341/2017 and ref. POCI-01-0145-FEDER-030341, respectively; and through the research project "BerryPlastid with ref. POCI-01-0145-FEDER-028165 and ref. PTDC/BIA-FBT/28165/2017, respectively. Artur Conde was supported by a post-doctoral researcher contract/position within the project "MitiVineDrought" (PTDC/BIA-FBT/30341/2017 and POCI-01-0145-FEDER-030341). The work was also support by project I&D&I "AgriFood" XXI, ref. NORTE-01-0145-FEDER-000041, co-financed by the European Regional Development Fund (FEDER), through NORTE 2020 (Northern Regional Operational Program 2014/2020). This work also benefited from the networking activities within the European Union-funded COST Action CA17111"INTEGRAPE—Data Integration to

maximize the power of omics for grapevine improvement". Authors acknowledge the owner from Quinta Cova da Raposa, Manuel Taxa, who provided the samples, and also all support given by the Biology Department of the School of Sciences from the University of Minho. A special thanks to Professor Ana Lúcia Pinto Sintra from Trás-os-Montes e Alto Douro University, for her tips to establish the grape berry tissues cultures presented here.

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# Chapter 8

# General Discussion, Conclusions and Future Perspectives

## 8.1. General Discussion

Light is a determinant abiotic factor for several physiological and developmental processes in plants. In viticulture, a good management of light interception by the canopy is essential to optimize the overall physiology of the grapevine, including photosynthesis, development and productivity, and ultimately to ensure the final organoleptic traits of the grape berries and wine. As previously mentioned, in addition to the leaves, other tissues/organs of the plants can perform photosynthesis, including fruits, seeds, green stems, green flower organs and even roots (Brazel and Ó'Maoileídigh, 2019). Previously, we showed that the exocarp and seed outer integument were the grape berry tissues with the highest photosynthetic activity (Breia et al., 2013), and that different light microclimates at the canopy of grapevine led to distinct photosynthetic activities and photosynthetic competences of these tissues (Garrido et al., 2018). However, there is still lack of information about the functions of this tissue-specific photosynthesis on the physiology and metabolism of the grape berry tissues and of the fruit as a whole.

Likewise, the effects of short-term mitigation strategies against climatic stresses, commonly applied in vineyards (such as foliar application of kaolin and plant irrigation), on grape berry photosynthesis and its impact on metabolism are also not fully understood. In this way, and as stated in Chapter 1, the main objective of this thesis was to understand the effects of canopy light microclimate and of the referred climate stress mitigation strategies on the physiology of photosynthetic grape berry tissues, in order to add new knowledge about the functions of photosynthesis in the grape berry, and ultimately to predict impacts on grape berry and wine quality.

Firstly, we studied the effects of two mitigation strategies - foliar kaolin application and irrigation - on the photosynthetic activity of exocarps and seeds of grape berries grown in two light microclimates (low and high light, LL and HL, respectively) and collected at three developmental stages (green, *véraison* and mature) (Chapter 3 – Garrido et al., 2019). Subsequently, a set of metabolomic approaches were used to evaluate the effects of the factors under study (mitigation strategies and light microclimate) on the metabolite profile of the photosynthetically grape berry tissues (Chapter 4 – Garrido et al., 2021). In addition, the transcriptional analysis of key genes from primary and secondary metabolomic pathways were also performed for exocarps and seeds grown at LL and HL microclimate (Chapter 5 – Garrido et al., 2021, submitted). As seeds are important co-products of the wine-making activity, with high value for several other industries, the lipid profile and the transcripts levels of genes involved on fatty acid synthesis and lipid metabolism, were assessed in seeds from LL and HL microclimate (Chapter 6 – Garrido et al., manuscript in preparation). With this global approach we intended to evaluate the interlink between the

tissue-specific gene expression levels, metabolite profile and the photosynthetic activity, aiming to unveil the potential functions of photosynthesis.

Overall, our results showed that the light microenvironment where grape berries grow induced significant effects on the photosynthetic, metabolite and transcripts profiles of both exocarps and seeds. The chlorophyll fluorescence technique showed positive effects of HL microclimate on photosynthetic parameters: at the green stage, the photosynthetic activity of both exocarp and seed was higher in the HL microclimate when compared to LL (Figures 3.5a,b and 3.6a,b). Likewise, the total concentration of chlorophylls and carotenoids (Figure 3.8), key elements in the photochemical reactions, the relative expression levels of genes coding for the photosynthesis-related enzymes, such as of chlorophyll synthase (*VvChlSyn*; Figure 5.1) and ribulose-1,5-bisphosphate carboxylase/oxygenase (*VvRuBisCO*; Figure 5.3), and even the expression of some genes coding for key enzymes of the carotenoid pathway (Figure 5.2), were also increased by HL microclimate. Thus, together, these results suggest that the HL microclimate had a positive impact either on the photochemical reactions, including photoprotection, and on the carbon fixation capacity of both grape berry tissues. Previous studies have also demonstrated that the light level was an important factor for the photosynthetic activity performed by the green tissues of fleshy fruits, like green peel of avocado (Blanke, 1992), peel of apple (Chen and Cheng, 2007), exocarp of cucumber (Sui et al., 2017), cherry tomato (Xiaoying et al., 2012) and tomato (Zhao et al., 2010), as well as in green oilseeds (Allen et al., 2009; Ruuska et al., 2004). Furthermore, it was also verified that in fruits and seeds, different light regimes, which affected photosynthesis, also influenced the oxygen ($O_2$) evolution (Rolletschek et al., 2005; Tschiersch et al., 2011), as well as the activity of Calvin-Benson cycle enzymes (e.g., in apple - Chen and Cheng, 2007).

The canopy light microclimate also had influence on the metabolomic profile (of hundreds of semi-polar primary and secondary metabolites), determined by liquid chromatography mass spectrometry (LCMS), of both exocarp and seed (Table 4.1) (Garrido et al., 2021). For instance, in the exocarp, the HL increased the relative abundance of total flavonols when compared to LL (Figure 4.3 and Figure S4.8), being this result in accordance with the relative expression of flavonol synthase gene (*VvFLS1*) and the higher activity of the respective enzyme (Figure 5.6a,c). In seeds, HL microclimate led to an up-regulation of compounds from upstream of the flavonoid pathway, i.e., the hydroxycinnamic acids (Figure 4.5). The flavan-3-ols monomers and procyanidins were also positively influenced by HL in both tissues at green stage (e.g., Figure 4.4). Overall, these results suggest that the photosynthesis of grape tissues, finely tuned and influenced by the light microclimate, can be decisive for their metabolite content and composition. At the end, these compounds contribute to the final quality of the wine (Garrido and Borges,

2013; Niimi et al., 2020) and they are also crucial in performing ecophysiological functions. For instance, flavan-3-ols/tannins confer protection against fungal and bacterial pathogens, insect pests and larger herbivores (Barbehenn and Constabel, 2011). Flavonoids, particularly flavonols, are generally considered to have antioxidant and/or "sunscreen" abilities, and thus they can limit photodamage through their ability to scavenge ROS and other free radicals generated by photooxidation, protecting the photosynthetic apparatus from light-induced damage (Agati et al., 2013).

Although it is now evident that photosynthesis occurs in fruits and in specific tissues of the fruit, its extent and importance is still unclear. Nevertheless, the products from the photochemical reactions of the photosynthesis, like the oxygen, ATP and NADPH, as well the carbon skeletons derived from the Calvin-Benson cycle, might be available to assist different metabolomic pathways and physiological mechanisms of grape berry tissues.

The relation between fruit photosynthesis and metabolism is well documented for tomato, as reviewed by Cocaliadis et al. (2014), and as exposed in more detail in the Chapter 2 of the present thesis. In general, those previous works suggested that fruit photosynthesis contributes with a percentage of carbon (by recycling internal respiratory $CO_2$), that at the end could be important for the nutritional quality. However, Lytovchenko et al. (2011) proposed that tomato fruit photosynthesis is dispensable for fruit size and metabolite content, but having a role in seed development during the early stage of development. In grape berries, Ollat and Gaudillere (2000) showed that photosynthesis contributes to the carbon balance by supplying about 10 % of the carbon needed for fruit development and by recycling about 40 % of the carbon lost by mitochondrial respiration. During ripening of grape berries the accumulation of cutin and wax in the cuticle (Becker and Knoche, 2012), difficult gas diffusion and gas exchange with the external atmosphere. Therefore, the levels of $CO_2$ released by mitochondrial respiration, increase in the grape berry core (Palliotti and Cartechini, 2001). This internal $CO_2$ can be re-fixed by phosphoenolpyruvate carboxylase (PEPC) (Figure 2.1) (Blanke and Lenz, 1989), but also by RuBisCO, if expressed and active as in leaves. Indeed, Schwender et al. (2004) proposed a new pathway of $CO_2$ re-assimilation by RuBisCO for green seeds of *Brassica napus* (oilseed rape). In our study, we observed that the expression of *VvRuBisCO* was maintained in seeds and increased in exocarps during grape berry development and ripening (Figure 5.3), suggesting an active Calvin-Benson cycle until later phases of development. In accordance with this, proteomic studies showed that skin of ripe berries contained proteins with functions related to photosynthesis and carbon assimilation, including the subunit binding-protein alpha of RuBisCO (Grimplet et al., 2009).

Still, considering variations in photosynthetic-related cues along development, our PAM-fluorometry results revealed that at the green stage both tissues showed highest maximum quantum efficiency ($F_v/F_m$) (Figure 3.5) and photosynthetic capacity ($rETR_{200}$) (Figure 3.6), with the exocarp extending its activity up to the mature stage, while seed photosynthetic activity was more restricted to the green and *véraison* stages. The physiological functions of photosynthesis may be different in the two grape berry tissues studied and along development, as discussed below.

The photochemical reactions in the exocarp at the early stages of development can supply the energy (in form of ATP) and oxygen required for the high demand for cell growth and accumulation of organic acids (malate and tartrate) (Dokoozlian, 2000; Sweetman et al., 2009). Moreover, the ATP can assist the unloading of sugars from the dorsal vasculature that are translocated from the leaves, namely from the onset of ripening onwards, when the energy-dependent apoplastic pathway dominates (Zhang et al., 2006). Still, in relation to sugar transport and metabolism, our results from transcriptional analysis showed that exocarp from green stage had low levels of expression of the sucrose-phosphate synthase 1 gene (*VvSPS1*), increasing afterwards (Figure 5.4a), in a similar pattern to that seen for *VvRuBisCO* (Figure 5.3). In an opposite manner, the expression of sucrose synthase gene (*VvSuSy*) was high at green stage but decreased with development of exocarp (Figure 5.4c). The *VvSuSy* codes the enzyme that might cooperate with cell wall invertases cleaving the sucrose unloaded from the leaves into the cytoplasm of sink cells (Wang et al. 2014). Therefore, these results may suggest that at the initial stage of development, the sucrose imported from leaves, rather than sucrose produced locally in the berry, is crucial to cope with the high carbon and energy demands.

Besides, in the exocarp, the photochemical products can also fuel primary and secondary metabolism with energy and reducing power. Likewise, the Calvin-Benson cycle, still active until later stages of development (c.f. *VvRuBisCO* expression) can contribute with carbon skeletons needed in those pathways. For instance, our metabolomics analysis showed that the exocarp from the green stage had high relative intensity levels of flavan-3-ols (Figure 4.4) and of stilbenes, like resveratrol, when compared with mature stage (Garrido et al., 2021). Similarly, the expression of genes from the phenylpropanoid (*VvPAL1*) and stilbenoid (*VvSTS1*) pathways (Figure 5.5a,g), as well as of those associated with flavan-3-ols biosynthesis (*VvDFR*, *VvLAR2* and *VvANR*) (Figure 5.7a,g,i), was also higher at the green stage than in subsequent stages. Moreover, the exocarp had an increase in the relative abundance of several flavonol glycosides along berry ripening (Figure 4.3; Figure S4.8b), which was consistent with the *VvFLS1* expression (Figure 5.6a,c). However, further studies are required to better understand this possible cross-

talk/relationship between photosynthesis/photochemistry/Calvin-Benson cycle and secondary metabolism in grape berry.

Photosynthesis in the seeds at the early stages of development, i.e., green and *véraison*, can provide $O_2$, which could be essential to avoid hypoxic conditions that may exist in the inner parts of the grape berries (Xiao et al., 2018). Moreover, the oxygen can fuel energy-generating biochemical pathways, including mitochondrial respiration, or others that require the consumption of $O_2$ like lignification (Lewis et al., 1998). In accordance to this, our results showed that from green to *véraison* stage, seeds had an increase in a lignan type (Figure 4.5b), which is associated with lignin synthesis (Lewis et al., 1998), in agreement with the degree of lignification of grape seeds along development (Cadot et al., 2006). Moreover, oxygen, NADPH and ATP are also essential for the storage metabolism and for the flux towards lipid biosynthesis, namely the *de novo* synthesis of free fatty acids in the chloroplast (Rawsthorne, 2002; Ye et al., 2020), as suggested previously for soybean, rapeseed and oilseed rape (Ruuska et al., 2004; Borisjuk et al., 2005; Goffman et al., 2005; Rolletschek et al., 2005). In that respect, our lipidomics results showed that the seeds from green and *véraison* stages had high relative intensity levels of membrane-bound lipid species, such as the glycerolipids (which encompass the triacylglycerols sub-class, TAG), sterols and glycerophospholipids (Figure 6.3). The first is an important element of the thylakoid membranes of chloroplast (Kobayashi, 2016), while the latter two can be related with high rate of cell growth that happens at these initial stages (Ristic and Iland, 2005; Cadot et al., 2006). At the mature stage, the biosynthetic pathway turns mainly to storage lipids (TAG), which are associated with the reserve accumulation that occurs at this stage of seed development (Ristic and Iland, 2005). In addition, we also showed that the seeds from HL microclimate had high relative intensity levels of ceramides (i.e., elements of plasma membranes and of the endomembrane system) at green stage and of TAG and glycerophospholipids at mature stage. Despite the decrease in the photosynthetic activity of the seeds at mature stage assessed by PAM fluorometry (Figure 3.6), the relative expression of *VvRuBisCO* was maintained in high levels along the three developmental stages of seeds, in values similar to than seen for the exocarps (Figure 5.3), which may suggest that an $CO_2$ rescue mechanism can be active, as proposed by Schwender et al. (2004) and Ruuska et al. (2004). Therefore, it may indirectly provide the acetyl-CoA needed to *de novo* synthesis of fatty acids in the chloroplast, or even providing other intermediates for primary and secondary metabolism. For instance, it can be important to support the high relative abundance of procyanidins at green stage and of viniferins at *véraison* and mature stages (Figure S4.9).

Regarding the effects of the studied mitigation strategies on grape berry photosynthesis, one relevant finding was that the kaolin applied to the leaves increased the photosynthetic activity ($rETR_{200}$) of exocarps from LL grape berries at green and mature stages (Figure 3.6a,e), as well as the total chlorophylls and carotenoids concentration at green stage (Figure 3.8a,c). This is in contrast to what we had hypothesized before, assuming that a white film of kaolin over the leaves could promote a shading effect in inner regions of the canopy (Garrido et al., 2018). However, the different angular orientations of the leaves in the canopy, can in fact increase the amount of light that is reflected to the inner regions (Figure 3.4b), stimulating the photosynthetic phenotype. Despite this, we did not register an increase in light intensity received by LL grape berry clusters from grapevines under kaolin treatment (Figure 3.2b), only in HL at mature stage (Figure 3.2d). However, these measurements were performed in a specific period of the day, and therefore to better understand the overall effect of kaolin application on the average light intensity of each microenvironment, a more detailed study with the registration of light intensities during the day and in different days along the season would be needed. Nevertheless, and in general, there were beneficial effects of foliar kaolin application on the photosynthetic activity of grape berry tissues, but dependent of each specific canopy structure.

Another interesting result, in this case regarding to the irrigation and the interactive effects between the two mitigation strategies, was observed for seeds from HL-grown berries at later stages of development. In fact, at the *véraison* stage, in the HL seeds from irrigated plants, the kaolin treatment led to a significant decrease in $F_v/F_m$ (Figure 3.5d) and $rETR_{200}$ (Figure 3.6d). In addition, the irrigation, regardless to the kaolin treatment, led to a decrease in $F_v/F_m$ (Figure 3.5f) and increase in non-photochemical quenching (NPQ) (Figure 3.7d), at mature and *véraison* stages, respectively. We hypothesized that the high air and soil temperatures registered during August and September (Figure S3.1) led to a significant increase in the temperature of the water used for irrigation, firstly inside the exposed black pipes and secondly when in contact with the top soil layers. The heated water absorbed by the root system and translocated by the vascular system to the whole plant, namely the fruits (grape berries), can cause a rise in their temperature and this way an effect on photosynthesis. Supporting a warming effect by the circulating xylem sap is the observation that the xylem of post-*véraison* grape berries retains structural integrity during berry growth (Chatelet et al., 2008). Choat et al. (2009) also suggested that the fruit is not hydraulically isolated from the parent plant by xylem occlusion but, rather, is "hydraulically buffered" by water delivered via the phloem. However, and contrarily to the leaves, grape berries, as compact spheroid structures, cools down with more difficulty by convection, especially inner regions, and cannot cool down by evapotranspiration due to the lack of functional stomata and high

cuticular resistances, especially at the later stages of ripening (Casado and Heredia, 2001; Rogiers et al., 2004), what may exacerbate the warming effect. An evidence that may support this idea is that even LL (shaded) grapes from irrigated plants exhibit higher temperatures when compared to those of non-irrigated plants (Figure 3.3c). The antagonistic interaction between irrigation and kaolin treatment may be explained by higher rates of leaf transpiration comparing to non-kaolin treated leaves (Dinis et al., 2018), and therefore higher water absorption and ascension rates in the xylem and higher fruit warming effect.

In terms of its agricultural relevance, the present research work showed that the irrigation was the mitigation treatment that influenced the metabolite profile of both berry tissues. For instance, the irrigation increased the relative levels of a tannin and a gallocatechin in the exocarp and seed, respectively, at later stages of development (Figure 4.6), and decreased primary metabolites like D-fructose 1,6-bisphosphate in exocarp and a hexose sugar in seed (Figure S4.10). Moreover, the total phenolic soluble content in the LL seeds at the mature stage, increased with irrigation treatment (Figure 4.10b). Therefore, an adequate irrigation system and management is crucial. On the other hand, the foliar kaolin application had no significant impact on grape berry tissues metabolome and exert beneficial effects on fruit photosynthesis and so it could be applied on the leaves to cope with extreme summer stresses, without apparent risks of reduced grape berry quality.

## 8.2. Conclusions and Future Perspectives

Overall, the results presented in this thesis showed that both the canopy light microclimate (LL and HL) and the mitigation strategies implemented in the vineyards to cope with climate stresses (foliar kaolin application and plant irrigation) influenced the photosynthetic activity of both grape berry tissues. In fact, the green HL-grown berries tissues had higher photosynthetic activity as compared with LL ones. The foliar kaolin application as compared with control, led to an increase of photosynthetic activity of LL exocarps at both green and mature stages, that was also observed in the total chlorophylls and carotenoids concentration at green stage only. The interactive negative effects of kaolin and irrigation in the photosynthesis were observed especially in HL seeds at *véraison* stage. Along the three developmental stages, the exocarp kept its photosynthetic activity, while seeds had a reduction but maintain RuBisCO expression. Moreover, the photosynthetic patterns responses to the light microclimate coincided with the expression of related genes, as well as those of sucrose synthesis. In fact, the HL microclimate, as compared to LL, resulted in a higher expression of genes encoding elements associated with both

photosynthesis (*VvChlSyn* and *VvRuBisCO*), carbohydrate metabolism (*VvSPS1*) and photoprotection (carotenoid pathways genes) in both tissues.

Furthermore, the metabolomics analysis indicated a significant influence of the microclimate in both photosynthetically active berry tissues, suggesting a potential role for *in situ* berry photosynthesis in contributing with carbon-skeletons and energy for the biosynthesis of berry components during development and ripening. For instance, HL exocarps, as compared with LL ones had high relative levels of flavan-3-ols at green stage. During the ripening of the exocarp, these metabolites decreased, which was also verified in the expression of genes related with this pathway (*VvDFR*, *VvLAR2* and *VvANR*). In addition, the HL exocarps had high relative abundance of flavonols when compared to LL ones, in line with results of the expression of flavonol synthase gene (*VvFLS1*) and the respective enzyme activity. In the case of seeds, the HL microclimate at green stage led to an up-regulation of hydroxycinnamic acid compounds (e.g., a coumaroyl conjugate and a lignan type). Moreover, the lipid profile of grape seeds was also influence by light microclimate: at green stage, the HL microclimate as compared with LL, led to an up-regulation of ceramides, which are membrane-bound lipid species essential for the cell growth; while at mature stage, HL seeds had higher levels of TAG and glycerophospholipids, as compared with LL ones.

The irrigation treatment influenced the metabolome of the two berry tissues studied, while the foliar kaolin application had no significant effect. In fact, irrigation led to an up-regulation of a series of phenolic compounds, including mono- and polymers of flavan-3-ols in both tissues. It is worth to note that the irrigation effects on the metabolome can be due to its direct or indirect influence on photosynthesis in both the leaves and berries. Nevertheless, to the best of our knowledge, this study was the first wide-ranging assessment of grape berry photosynthesis and metabolome under an experimental field setup employing a cost-efficient and environmentally-safe mitigations strategies, and thus, further studies are still required, specially under more controlled conditions.

Photosynthesis in fruits, in particular in grape berry tissues, is a fascinating research topic, with important practical applications, which deserves further investigation in the future. In fact, the increasing demand for food production together with the expected snowballing impacts of climate changes on agriculture, brings new challenges for research related with photosynthesis and the processes associated with it. Understanding the specificities of photosynthesis of non-foliar tissues, in particular those of grape berry tissues studied on the present thesis, will provide insights for viticulture management but also for genetic breeding programs in order to study the potential new target genes to enhance/maintain fruit development, yield and nutritional quality.

Despite the progress that has been made in tomato, there is little knowledge concerning this research topic in the grape berry. Thus, a set of emerging transgenic approaches can be employed to modify at transcriptional and post-transcriptional level the expression of a gene involved in photosynthesis, for instance using mRNA-inhibiting/degrading effectors or genome-editing technologies (Brazel and Ó'Maoileídigh, 2019). In this way, through specific modifications of steps in the pathways, either by knockout or overexpression of a gene, would allow the comprehension of molecular and biochemical mechanisms of photosynthesis in non-foliar tissues.

The carbon budget attained by the photoassimilates that come from the leaves, as well as those resulted from grape berry own photosynthesis, is crucial to its growth and development (Ollat and Gaudillere, 2000). The high levels of *VvRuBisCO* expression until later stages of development in both tissues, suggests that the enzyme can be active, refixing the "excess" of internal $CO_2$. However, the determination of biochemical activity of key enzymes of the Calvin-Benson cycle and carbohydrate metabolism (e.g., RuBisCO, SPS and SuSy), or even of phosphoenolpyruvate carboxylase (PEPC), would be determinant to understand the importance of photosynthesis in recycling the carbon by refixation of internal $CO_2$, either by RuBisCO or PEPC. In addition, measurements of gas exchange complemented with analysis of $^{14}CO_2$ labelling, as performed for cucumber fruit (Sui et al., 2017) and for soybean seeds (Allen et al., 2009), would also give a full picture of carbon flux for different metabolic pathways. A major challenge would be trying to perform those experiments in isolated skins and seeds. Furthermore, through the quantification of photosynthetic $O_2$, as performed in shaded versus sun-exposed peel of apple by Chen and Cheng (2007), the determination of ATP levels, as well as studying the mechanism involved on the sucrose unloading from the phloem, would be also relevant to better unveil the physiological functions of this derived products of the photosynthesis.

Moreover, the establishment of photomixotrophic suspensions from the exocarp *calli* (Chapter 7), would allow to perform photosynthesis-controlled experiments. For instance, by testing different light intensities and then measuring the causal effects in the cells at the metabolite and target gene-transcripts levels, as done by Ayenew et al. (2015), could elucidate better the contribution of photosynthesis in this berry tissue.

In summary, and despite its exploratory nature, this work highlights the importance of better understanding the physiological functions of photosynthesis of grape berry exocarp and seed, and the impact of different climate stress mitigations strategies on grape berry photosynthesis. As a whole, the results provide useful practical information to guide growers making rational-based decisions, and thus to promote sustainable and precision-based strategies applied in vineyards. Therefore, through the

precise adjustment of the agricultural practices, like canopy management and irrigation, or through stress mitigation strategies, it will allow to improve the grape berry and wine quality.

## 8.3. References


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