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**Study of the impact of atmospheric CO<sub>2</sub> level on grapevine leaf physiology, berry maturation and composition at harvest, using a FACE (Free Air Carbon dioxide Enrichment) system**

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## **Etude de l'impact d'une élévation du niveau de CO<sub>2</sub> atmosphérique sur la physiologie foliaire de la vigne, la maturation et la composition des baies à la récolte, à l'aide un système FACE (Free Air Carbon dioxide Enrichment).**

**Résumé :** Le changement climatique ne peut plus être ignoré à l'heure actuelle, et impacte tous les domaines de l'agriculture. L'élévation de la température moyenne globale, la réduction des précipitations dans certaines parties du globe ainsi que l'augmentation du taux de dioxyde de carbone sont autant de facteurs qui modifient l'environnement des espèces végétales. La vigne, connue pour son importance économique et culturelle, est très sensible aux modifications du climat. Une grande partie de la littérature scientifique existante concerne l'impact d'une hausse de température, et/ou d'un stress hydrique sur les plants de vignes, les effets de l'élévation du niveau de dioxyde de carbone atmosphérique restant assez peu étudiés, de par le challenge technologique que les dispositifs de plein champ représentent. Dans ce contexte, nous avons étudié l'effet de l'élévation de la concentration de dioxyde de carbone atmosphérique sur des vignes établies, étant traitées par fumigation depuis six ans au début de notre étude, et sur deux cépages (Cabernet Sauvignon et Riesling) en utilisant le dispositif VineyardFACE de l'Université de Geisenheim. L'impact du traitement CO<sub>2</sub> élevé sur la physiologie foliaire et sur la croissance végétative ayant déjà été démontré dans de récents travaux, notre but a été de déterminer les effets du traitement CO<sub>2</sub> sur les métabolites primaires (sucres, acides organiques, acides aminés) et secondaires (anthocyanes) de la baie de raisin, en étudiant des vignes déjà acclimatées au traitement CO<sub>2</sub>. De plus, les données agronomiques du suivi du développement des baies de raisin ainsi que leur dynamique de maturation ont permis de compléter les analyses métaboliques. L'étude des intermédiaires du métabolisme primaire (intervenant dans la glycolyse ou le cycle de Krebs) ont permis de vérifier l'impact du taux de CO<sub>2</sub> atmosphérique sur la plasticité métabolique de la baie de raisin en 2020 et 2021. Enfin, les composés aromatiques du moût de Riesling 2021 ont également fait l'objet d'analyses particulières. Les résultats montrent peu d'effets du traitement CO<sub>2</sub> sur les métabolites primaires ainsi que sur les dynamiques de maturation, pour les deux cépages (Riesling et Cabernet Sauvignon), bien que le taux de photosynthèse soit toujours stimulé par le traitement CO<sub>2</sub>. Cependant, il a été mis en évidence une tendance montrant la réduction des anthocyanes totales pour le Cabernet Sauvignon, principalement en 2020 et en 2021, tandis que la composition en anthocyanes restait similaire. La plasticité de la baie a également été étudiée, via l'investigation de composés impliqués dans le cycle de Krebs ainsi que d'intermédiaires du métabolisme des sucres. Ainsi des différences pour quelques composés ont été mises en évidence, l'effet année restant prédominant. Les composés aromatiques (terpènes libres et liés) ont également été analysés pour le moût du Riesling en 2021, leur composition étant peu impactée par le traitement CO<sub>2</sub>.

**Mots clés :** métabolisme des baies, changement climatique, niveau de CO<sub>2</sub>

## **Study of the impact of atmospheric CO<sub>2</sub> level on grapevine leaf physiology, berry maturation and composition at harvest, using a FACE (Free Air Carbon dioxide Enrichment) system.**

**Abstract:** Climate change cannot currently be ignored and impacts all fields of agriculture. IPCC reports forecast an increase of up to 700 ppm of atmospheric carbon dioxide concentration at the end of the 21st century (IPCC, 2021). Parameters such as elevated global temperature, reduced precipitations in certain areas of the world and increase of atmospheric carbon dioxide concentration are modifying plants' environment. Grapevine, which is a crop of economic and cultural importance, is very sensitive to climate modifications. The effects of temperature or water stress on grapevine have been widely investigated, and elevated carbon dioxide has been mainly studied in enclosed systems such as greenhouses. However, the impact of increased concentration of carbon dioxide on plants in open-field experiments remains scarcely studied because of the technical challenge that it represents. In this context, the aim of this PhD work was to investigate the impact of elevated carbon dioxide concentration using the set-up VineyardFACE located at Hochschule Geisenheim University. This open field set-up enables to apply a moderate and gradual increase (+20%) of ambient carbon dioxide concentration, for 6 years at the beginning of our study, on two grapevine cultivars, Riesling, and Cabernet Sauvignon. While knowing the impact of elevated carbon dioxide treatment on leaf physiology and vegetative growth from previous studies, as well as on berry composition during the early years of fumigation, the goal of the present work was to determine the effects of elevated carbon dioxide treatment on agronomical traits, primary metabolites (sugars, organic acids, amino acids) and secondary metabolites (anthocyanins) on vines undergoing six years of fumigation, as well as monitoring berry development and following berry ripening for successive seasons. Intermediates from central metabolism implied in glycolysis or TCA cycle were also studied for two years (2020 and 2021), and the aroma profile between ambient and elevated CO<sub>2</sub> was investigated by the analysis of aroma compounds on the must of Riesling 2021. Our results showed that although photosynthesis was still enhanced, little effects were found of elevated carbon dioxide treatment on primary metabolites as well as on berry ripening rates, for successive seasons for both Riesling and Cabernet Sauvignon. However, a trend of reduced total anthocyanins concentration in Cabernet Sauvignon was demonstrated by elevated carbon dioxide treatment mainly in 2020, while anthocyanins composition remained unchanged. The investigation of berry plasticity demonstrated slight differences for specific compounds, but the year effect was predominant. Terpenes (both bound and free) composition in Riesling in 2021 was not impacted by elevated carbon dioxide treatment.

**Keywords:** CO<sub>2</sub> levels, climate change, berry ripening

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# Summary

Climate change cannot currently be ignored and impacts all fields of agriculture. IPCC reports forecast an increase of up to 700 ppm of atmospheric carbon dioxide concentration at the end of the 21<sup>st</sup> century. Parameters such as elevated global temperature, reduced precipitations in certain areas of the world and increase of atmospheric carbon dioxide concentration are modifying plants' environment. Grapevine, which is a crop of economic and cultural importance, is very sensitive to climate modifications. The effects of temperature or water stress on grapevine has been widely investigated, and elevated carbon dioxide has been mainly studied in enclosed systems such as greenhouses. However, the impact of increased concentration of carbon dioxide on plants in open-field experiments remains scarcely studied because of the technical challenge that it represents. In this context, the aim of this PhD work was to investigate the impact of elevated carbon dioxide concentration using the set-up VineyardFACE located at Geisenheim University. This open field set-up enables to apply a moderate and gradual increase (+20%) of ambient carbon dioxide concentration on two grapevine cultivars, Riesling, and Cabernet Sauvignon. While knowing the impact of elevated carbon dioxide treatment on leaf physiology and vegetative growth from previous studies (Wohlfahrt et al., 2018), as well as on berry composition during the early years of fumigation, the goal of this study was to determine the effects of elevated carbon dioxide treatment on agronomical traits, primary metabolites (sugars, organic acids, amino acids) and secondary metabolites (anthocyanins, terpenes) on vines undergoing six years of fumigation, as well as monitoring berry development and following berry ripening for successive seasons. Intermediates from central metabolism were also studied for two years (2020 and 2021), and terpene analysis was realized on Riesling must 2021.

Our results showed that although photosynthesis was still enhanced, little effects were found of elevated carbon dioxide treatment on primary metabolites as well as on berry ripening rates, for successive seasons for both Riesling and Cabernet Sauvignon. However, a trend of reduced total anthocyanins concentration in Cabernet Sauvignon was demonstrated by elevated carbon dioxide treatment mainly in 2020, while anthocyanins composition remained fairly similar. Terpenes (both bound and free) composition in Riesling in 2021 was not impacted by elevated carbon dioxide treatment.

## Résumé

Le changement climatique ne peut plus être ignoré à l'heure actuelle, et impacte tous les domaines de l'agriculture. L'élévation de la température moyenne globale, la réduction des précipitations dans certaines parties du globe ainsi que l'augmentation du taux de dioxyde de carbone sont autant de facteurs qui modifient l'environnement des espèces végétales. La vigne, connue pour son importance économique et culturelle, est très sensible aux modifications du climat. Une grande partie de la littérature scientifique existante concerne l'impact d'une hausse de température, et/ou d'un stress hydrique sur les plants de vignes, les effets de l'élévation du niveau de dioxyde de carbone atmosphérique restant assez peu étudiés, de par le challenge technologique que cela représente. Dans ce contexte, nous avons étudié l'effet de l'élévation de la concentration de dioxyde de carbone atmosphérique sur des vignes établies, étant traitées par fumigation depuis six ans au début de notre étude, et sur deux cépages (Cabernet Sauvignon et Riesling) en utilisant le dispositif VineyardFACE de l'Université de Geisenheim. L'impact du traitement CO<sub>2</sub> élevé sur la physiologie foliaire et sur la croissance végétative ayant déjà été démontré dans de récents travaux (Wohlfahrt et al., 2018), notre but a été de déterminer les effets du traitement CO<sub>2</sub> sur les métabolites primaires (sucres, acides organiques, acides aminés) et secondaires (anthocyanes, terpènes) de la baie de raisin, en étudiant des vignes déjà acclimatées au traitement CO<sub>2</sub>. De plus, les données agronomiques du suivi du développement des baies de raisin ainsi que leur dynamique de maturation ont permis de compléter les analyses métaboliques. L'étude des intermédiaires du métabolisme primaire (intermédiaires intervenant dans la glycolyse ou le cycle de Krebs) ont permis de vérifier l'impact du taux de CO<sub>2</sub> atmosphérique sur le métabolisme de la baie de raisin.

Les résultats montrent peu d'effets du traitement CO<sub>2</sub> sur les métabolites primaires ainsi que sur les dynamiques de maturation, pour les deux cépages (Riesling et Cabernet Sauvignon). Cependant, il a été mis en évidence une tendance montrant la réduction des anthocyanes totales pour le Cabernet Sauvignon, principalement en 2020, tandis que la composition en anthocyanes restait similaire. Les composés aromatiques (terpènes libres et liés) ont également été analysés pour le moût du Riesling en 2021, leur composition étant peu impactée par le traitement CO<sub>2</sub>.

# Zusammenfassung

Die Klimaveränderung und Klimakrisen können heutzutage nicht geleugnet werden und machen sich im Anbau fast aller landwirtschaftlicher Kulturen bemerkbar. Die Erhöhung der globalen Temperatur, die Änderung in der Niederschlagverteilung und häufigkeit und die Steigerung des Kohlendioxides sind Faktoren, die die Umwelt und Interaktion der Kulturpflanzen modifizieren. Reben zählen zu der ökonomisch und kulturell bedeutsamen Kulturen, die sensibel auf Klimaänderungen reagieren. Die Wirkung von erhöhtem atmosphärischem Kohlendioxid ist bisher wenig untersucht, da diese Versuche große technische Herausforderung darstellen. In diesem Kontext wurde im Rahmen dieser Arbeit die Wirkung von atmosphärischem Kohlendioxid in einem Freilandversuch, dem VineyardFACE der Hochschule Geisenheim Universität untersucht. Die Wirkung einer "erhöhten CO<sub>2</sub>" Konzentration auf die Physiologie und das vegetative Wachstum wurde in vorausgegangen Arbeiten bereits beobachtet and beschrieben (Wohlfahrt et al., 2018). Das Ziel der eigenen Arbeit war es die Wirkung erhöhter CO<sub>2</sub> Konzentration auf primäre Metabolite (Zucker, Karbonsäuren, Aminosäure) und auch der sekundären Metabolite (Anthocyanine, Terpenes ) in Beeren zu untersuchen. Die Reben können sich von Versuchsbeginn diesen Bedingungen über einen Zeitraum von sieben Jahren akklimatisieren. Außerdem wurden im Rahmen der vorliegenden Arbeit agronomische Daten zur Phänologie sowie die Reifedynamik erfasst.

Die Auswertung der Ergebnisse zeigte keinen Einfluss der erhöhter CO<sub>2</sub> Behandlung bei den primären Metaboliten sowie auf die Reifedynamik, weder bei der Rebsorten Riesling noch bei Cabernet Sauvignon. Beim Gehalt der Gesamt-Anthocyanine konnte bei der Rebsorte Cabernet Sauvignon für das Jahr 2020 eine Abnahme festgestellt werden, die sich jedoch nicht im Verteilungsmuster der anderen Derivate der Anthocyane zeigte.

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Pour terminer, j'aimerais partager une citation d'une autrice qui me tient particulièrement à cœur.

*"I needed to put two critical ideas together: that I could both be mentally ill and lead a rich and satisfying life."*

Elyn R. Saks, *The center cannot hold*

# Résumé substantiel

## Changement climatique et viticulture

La crise climatique est plus que jamais d'actualité. Ainsi, l'environnement des espèces végétales est modifié, de par l'augmentation de la température à l'échelle du globe, le changement du régime de précipitations et l'augmentation du taux de dioxyde de carbone atmosphérique, qui devrait atteindre 700 ppm à la fin du siècle ; ces changements étant les conséquences directes des activités anthropiques. Les prévisions du GIEC mettent en avant, dans de nombreux rapports scientifiques, la fréquence accrue des événements climatiques extrêmes. Par conséquent, de nombreux climatologues alertent sur le dépassement de la limite d'augmentation de température, fixée à 1.5°C par les accords de Paris, qui pourrait générer des conséquences irréversibles, du fait de l'existence de points de bascule climatiques.

L'agriculture se trouve donc face à la nécessité de comprendre les impacts du changement climatique sur les cultures et de s'adapter à ceux-ci. De nombreuses études ont été réalisées sur les effets des facteurs liés au changement climatique sur différentes cultures, notamment sur la vigne (*Vitis vinifera* L.). La viticulture ayant une dimension économique et culturelle importante, l'impact de la combinaison d'une élévation de température, du stress hydrique et d'un niveau élevé de dioxyde de carbone sur la vigne et son métabolisme est relativement bien représenté par de nombreux articles de la littérature scientifique. Cependant, la majorité de ces études ont été effectuées en serres, ou dans des systèmes clos, donc dans des conditions contrôlées, qui présentent certains biais et dont les résultats ne peuvent pas nécessairement être appliqués au vignoble. De plus, ces études ont été réalisées sur des temps courts d'application de traitement et à des concentrations peu représentatives de l'augmentation graduelle et continue de dioxyde de carbone.

Par ailleurs, les composés métaboliques finaux de la baie de raisin sont représentés par les métabolites primaires (sucres, acides organiques, acides aminés) ainsi les métabolites secondaires (anthocyanes et flavonols). Ces composés ont un intérêt majeur car ils participent à la qualité de la baie de raisin et par extension celle du vin après le processus de vinification. De plus, de nombreuses études ont montré que le changement climatique modifie la composition de la baie lorsque le vignoble est vendangé, avec une augmentation du taux de

sucres et une diminution de l'acidité. En effet, van Leeuwen et al. (2019) a démontré que, depuis 35 ans, dans le Languedoc, le taux de sucres a augmenté de 11 à 14 % tandis que l'acidité totale a diminué de 6.0 à 4.5 g.L<sup>-1</sup>.

### **Intérêt de l'étude de l'impact du dioxyde de carbone sur le métabolisme de la vigne**

Les dispositifs FACE (qui est l'acronyme de Free Air Carbon dioxide Enrichment) implantés sur différentes cultures (pérennes ou annuelles) sont quasiment unanimes quant à l'effet du dioxyde de carbone sur la photosynthèse ainsi que sur la biomasse et le rendement. En effet, la photosynthèse est stimulée par l'augmentation de la concentration en dioxyde de carbone atmosphérique et cela se traduit par des augmentations des rendements. En outre, concernant la qualité de la baie de raisin, le premier dispositif FACE installé dans un vignoble a été étudié par Bindi et al. (2001), et les résultats obtenus démontrent que d'une part, la concentration en sucres et en acides organiques était modifiée par une forte concentration de traitement CO<sub>2</sub> (700 ppm) et que d'autre part, ces différences disparaissaient à maturité. Les travaux précédents en conditions contrôlées ont permis de renforcer l'idée selon laquelle la photosynthèse est stimulée par une augmentation du taux de dioxyde de carbone, bien que dans ces cas précis un phénomène d'adaptation a pu être mis rapidement en évidence (Kizildeniz et al. 2021).

Par ailleurs, il est également admis que les plants en conditions contrôlées comme les serres, ou même les Open Top Chambers, peuvent présenter des disparités au niveau de la morphologie et la physiologie de ces plants, impactant ainsi certains résultats d'analyse par rapport aux plants qui évoluent dans des dispositifs de plein champ (Poorter et al., 2016).

Ainsi, l'étude de l'effet du dioxyde de carbone atmosphérique, hors conditions contrôlées, représente un défi technique et économique important. En ce sens, le dispositif VineyardFACE est implanté à l'Université de Geisenheim, en Allemagne, et permet d'étudier un vignoble soumis à un traitement de dioxyde de carbone élevé. Un dispositif FACE comprend un système de fumigation de dioxyde de carbone, dans l'atmosphère proche des cultures sur lesquelles il est implanté, que ce soit sur des cultures annuelles ou des cultures pérennes comme la vigne dans notre cas. Il s'agit en effet du dispositif appelé VineyardFACE, composé de six anneaux, chaque anneau étant formé de 36 tours, au-dessus desquelles se trouvent un « souffleur » permettant soit la fumigation, c'est-à-dire 20% de dioxyde de carbone en plus que l'air ambiant (pour les anneaux « traitement »), correspondant aux prédictions du GIEC pour 2050, soit le brassage d'air ambiant (pour les anneaux « contrôle »). Le vignoble en question est composé de deux cépages plantés alternativement, à savoir un cépage blanc emblématique de la vallée de Rhin, le Riesling (clone 198-30 Gm), sur le porte-greffe SO4 (clone 47 Gm) et un cépage rouge, le Cabernet Sauvignon (clone 170), greffé sur le porte-greffe 161-49 Couderc.

L'impact de l'élévation du niveau de dioxyde de carbone atmosphérique sur la physiologie foliaire de la vigne a précédemment été étudiée par Wohlfahrt et al. (2018), démontrant une augmentation du taux d'assimilation, de la conductance stomatique, et du taux de transpiration (et de fait sur la capacité d'utilisation de l'eau) pour les deux cépages étudiés, Cabernet Sauvignon et Riesling. De plus, l'étude de l'impact à court terme, et sur les premières années de fumigation, du niveau de dioxyde de carbone élevé sur la composition de la baie et sur la qualité des vins issus de ce vignoble a pu mettre en évidence que le dioxyde de carbone n'avait

pas d'influence négative sur ces paramètres, même si certains composés étaient impactés à certains stades de développement, par exemple l'acide malique (Wohlfahrt et al., 2021). Ainsi, les présents travaux cherchent à étudier l'impact d'une élévation graduelle et modérée du dioxyde de carbone atmosphérique après six années de fumigation au début de notre étude, à la fois sur la physiologie foliaire de la vigne mais également sur la composition fine de la baie, en termes de produits finaux mais également d'intermédiaires du métabolisme carboné et de profil aromatique du moût.

## **Démarche**

Cette thèse a été effectuée en co-tutelle entre l'Université de Bordeaux et l'Université de Geisenheim, la saison estivale étant destinée aux expériences de plein champ sur le dispositif VineyardFACE, et les analyses métaboliques ont été effectuées à Bordeaux après l'envoi des baies récoltées sur le dispositif, sur les trois années du projet. Ainsi, des mesures physiologiques telles que les échanges gazeux, le rendement à la récolte et le poids des bois de taille ont été étudiés. Par ailleurs, la composition des baies récoltées sur le dispositif, en termes de métabolites primaires (sucres, acides organiques, acides aminés) et secondaires (anthocyanes) a également fait l'objet d'un intérêt particulier sur les trois années du projet, pour ainsi vérifier l'effet du traitement CO<sub>2</sub> sur les composés finaux de la baie de raisin. Ainsi, différentes approches analytiques ont été utilisées afin de quantifier ces composés, (dans des baies récoltées à différents stades de développement) tels que le dosage enzymatique pour les sucres, l'analyseur en flux continu pour les acides organiques et l'U-HPLC pour le dosage des acides aminés et des anthocyanes. L'étude des composés d'intérêt de la baie de raisin dans le contexte du changement climatique permet d'affiner la connaissance de l'impact de ces paramètres climatiques sur la qualité de la baie de raisin. Par ailleurs, une investigation fine du métabolisme primaire a été réalisée en collaboration avec l'Institut Max Planck à Potsdam Golm en Allemagne, permettant d'avoir à disposition un plus grand panel de concentration de métabolites, en condition CO<sub>2</sub> élevé ou CO<sub>2</sub> ambiant, pour deux années (2020 et 2021), deux cépages, et sur deux types de compartiments différents (pulpes et pellicules). Ces analyses ont été effectués dans un premier temps par une extraction chloroforme/méthanol sur les deux types de tissus puis la quantification des métabolites a été réalisée par LC-MS<sup>3</sup> à l'Institut Max Planck de Potsdam Golm. Enfin, l'étude de composés aromatiques tel que les monoterpènes et C<sub>13</sub>- norisoprenoids libres et liés, a été réalisée sur le moût du Riesling 2021, en collaboration avec l'Institut de Microbiologie et de Biochimie de l'Université de Geisenheim.

## **Résultats obtenus**

Dans le Chapitre 1, les mesures effectuées ont permis de mettre en évidence que le taux d'assimilation, ainsi que la conductance stomatique et le taux de transpiration, sont toujours impactés par l'élévation du niveau de dioxyde de carbone atmosphérique, pour les deux cépages, Cabernet Sauvignon et Riesling. La photosynthèse est donc également améliorée en conditions CO<sub>2</sub> élevé pendant la durée de notre étude. De plus, le rendement ainsi que le poids des baies étaient supérieurs pour le traitement CO<sub>2</sub> élevé, en particulier pour le Cabernet Sauvignon, tandis que des différences moindres ont été notées pour le Riesling. Par ailleurs, le

Chapitre 2 montre que la composition de la baie n'a pas semblé être modifiée de façon significative, que ce soit en termes de concentration de sucres, d'acides organiques ou d'acides aminés, l'effet de l'année étant prédominant. Cependant, la concentration en anthocyanes totales montre une tendance à la réduction sous traitement CO<sub>2</sub> élevé, notamment pour les années 2020 et 2021, surtout concernant les stades approchant la maturité et à maturité.

Par ailleurs, dans le Chapitre 3, étant donné que les composés finaux de la baie de raisin n'ont semblé être que peu affectés par le traitement CO<sub>2</sub> élevé, l'hypothèse selon laquelle les composés intermédiaires impliqués dans la régulation de ces composés finaux pourraient être modifiés par le traitement CO<sub>2</sub> a été envisagée et explorée. En effet, l'étude des composés impliqués dans le métabolisme des sucres (sucres phosphates ou intermédiaires de la glycolyse) ou le cycle de Krebs a permis de mettre en évidence quelques différences fines, impliquant les deux types d'échantillons (CO<sub>2</sub> élevé versus CO<sub>2</sub> ambiant). Cependant, l'effet année semblait prédominant en comparaison de l'effet du traitement. Enfin, dans le Chapitre 4, après étude quantitative des composés aromatiques tels que les terpènes libres et liés ainsi que les C<sub>13</sub>-norisoprenoids, le profil aromatique des moûts du Riesling en 2021 ne semble également pas être modifié négativement par le traitement CO<sub>2</sub> élevé, même si quelques différences fines ont pu être mise en évidence. En conclusion, les résultats obtenus ont permis d'affiner les connaissances de l'impact du dioxyde de carbone sur la physiologie, la maturité technologique ainsi que sur la composition fine de la baie de raisin.

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# Publications, presentations, posters

## Publications

Kahn, C., Tittmann, S., Hilbert, G., Renaud, C., Gomès, E., & Stoll, M. (2022). VineyardFACE: Investigation of a moderate (+20 %) increase of ambient CO<sub>2</sub> concentration on berry ripening dynamics and fruit composition of Cabernet-Sauvignon: This article is published in cooperation with Terclim 2022 (XIVth International Terroir Congress and 2nd ClimWine Symposium), 3-8 July 2022, Bordeaux, France. *OENO One*, 56(2), 193–204. <https://doi.org/10.20870/oeno-one.2022.56.2.5440>

## Oral presentations

TerClim Congress 2022 (XIVth International Terroir Congress and 2nd ClimWine Symposium), 3-8 July 2022, Bordeaux, France: VineyardFACE: Investigation of a moderate (+20%) increase of ambient CO<sub>2</sub> level on berry ripening dynamics and fruit composition  
Kahn, C., Tittmann, S., Hilbert, G., Renaud, C., Gomès, E., & Stoll, M.

Graduate school Geisenheim University: first presentation (7.01.2021) and final presentation (12.01.2023)

## Poster

Forschungsforum Geisenheim University: 30.09.2021

# **General introduction**

## General introduction

### 1.1 Climate change and viticulture

#### *Rising temperature and atmospheric carbon dioxide*

The first scientists considering the climate changes are respectively Svante Arrhenius, who published an article about the influence of CO<sub>2</sub> on planetary budget in 1896, and Guy Stewart Callendar, who calculated a global increase in land temperature of about +0.3°C between 1880 and the late 1930s (Anderson et al., 2016). Before the industrial era, atmospheric CO<sub>2</sub> concentration was stable for more than thousand years (Prentice et al., 2001). However, climate change crisis is occurring, and there is no doubt that anthropogenic influence is responsible for modification of atmospheric gas composition, especially for the major greenhouse gas (GHG) emissions, such as methane (CH<sub>4</sub>), nitrous oxide (N<sub>2</sub>O), and carbon dioxide (CO<sub>2</sub>). Indeed, atmospheric carbon dioxide concentration has increased by 40% in comparison to pre-industrial era, ranging from 278 ppm in 1750 to 390.5 ppm in 2011, which is firstly explained by the use of fossil fuels (IPCC, 2013). Moreover, 30% of anthropogenic emissions in carbon dioxide have been absorbed by the ocean, leading to their acidification, and consequently, seawater pH has decreased of 0.1 unit since industrial era (IPCC, 2013). Water cycle is then intensified by climate change, and these changes bring more intense rainfall in some regions of the world, whereas in others severe drought is expected. Consequently, sea level has also risen, according to the (IPCC, 2013) which could also provoke coastal flooding and coastal erosion.

Moreover, during the last three decades, the atmosphere and the ocean have been warmer than any time since 1850. In IPCC report (2013), it was assessed that global mean temperature is expected to increase at the end of the 21<sup>st</sup> century between 1.0°C and 3.7°C in average according to the different scenarios, from the most optimistic to the most pessimistic (RCP 2.6 to RCP8.5) (Table 1).

Table 1. Global Mean surface temperature change (°C) depending on the scenarios, relative on reference period of 1986-2005

		2046–2065		2081–2100	
	Scenario	Mean	Likely range <sup>c</sup>	Mean	Likely range <sup>c</sup>
Global Mean Surface Temperature Change (°C) <sup>a</sup>	RCP2.6	1.0	0.4 to 1.6	1.0	0.3 to 1.7
	RCP4.5	1.4	0.9 to 2.0	1.8	1.1 to 2.6
	RCP6.0	1.3	0.8 to 1.8	2.2	1.4 to 3.1
	RCP8.5	2.0	1.4 to 2.6	3.7	2.6 to 4.8
	Scenario	Mean	Likely range <sup>d</sup>	Mean	Likely range <sup>d</sup>
Global Mean Sea Level Rise (m) <sup>b</sup>	RCP2.6	0.24	0.17 to 0.32	0.40	0.26 to 0.55
	RCP4.5	0.26	0.19 to 0.33	0.47	0.32 to 0.63
	RCP6.0	0.25	0.18 to 0.32	0.48	0.33 to 0.63
	RCP8.5	0.30	0.22 to 0.38	0.63	0.45 to 0.82

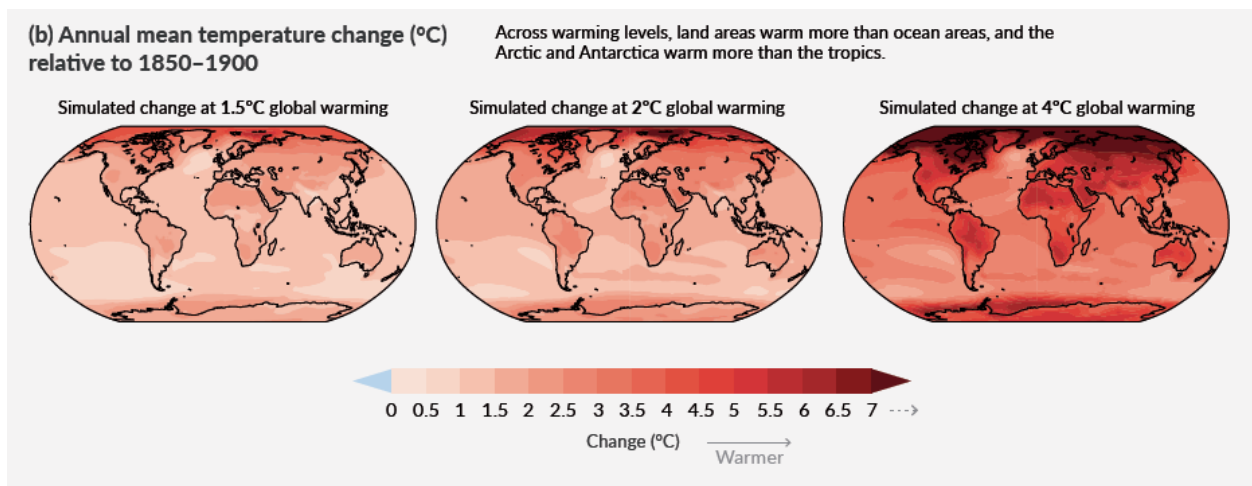


Figure 1. Annual mean temperature changes relative to 1850-1900 (IPCC 2021)

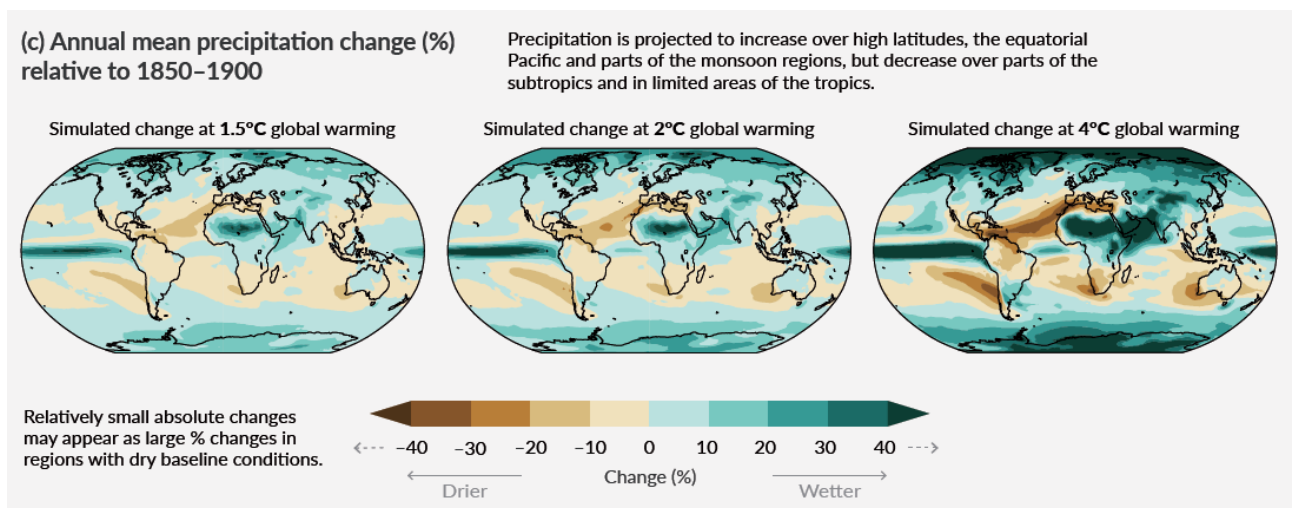


Figure 2. Annual mean precipitations change (%) relative to 1850-1900 (IPCC 2021)

In addition, in the 2021 IPCC report (IPCC, 2021), it was acknowledged that surface temperature will continue to increase until at least mid-century and that, under all emissions scenarios considered (Figure 1). It was pointed out that global warming of 1.5°C and 2°C will be exceeded during 21<sup>st</sup> century, except if emissions of carbon dioxide are drastically reduced in coming decades. Recent IPCC reports are acknowledging that global warming disrupts climate system, and this will lead to increases in the intensity and frequency of extremes hot events as well as heavy precipitation events, whereas some regions of the globe will experiment severe drought (Figure 2) (Masson-Delmotte et al., 2021) .

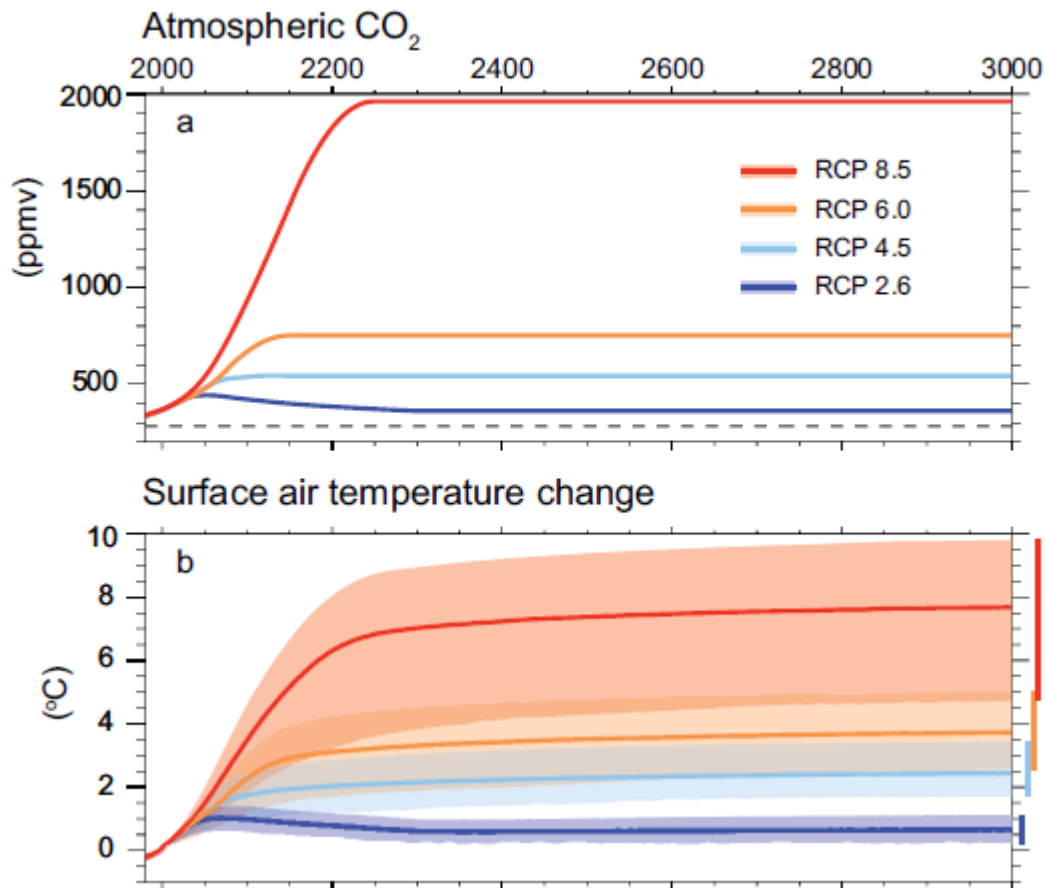


Figure 3. a) Provisions of atmospheric carbon dioxide concentration and b) surface air temperature change (IPCC, 2013)

Carbon dioxide atmospheric concentration is predicted to increase up to 700 ppm by the end of the century, which contributes to the increase of temperature during the same time (Figure 3). The contribution of carbon dioxide emissions from industrial processes is about 78% of total GHG emissions increase, in the period 1970-2010 and in the period 2000 to 2010 (IPCC, 2013).

Such climate changes have an irreversibility potential (Solomon et al., 2009) as the impact of rising atmospheric CO<sub>2</sub> can lead to irreversible rainfall reduction and a continuous sea level rise. In fact, even if CO<sub>2</sub> emissions cease, climate change is irreversible for at least 1000 years because of CO<sub>2</sub> concentration would still be above pre-industrial value (Solomon et al., 2009). Moreover, expected climate trajectories are more likely to be exceeding 1.5°C, which could have an impact on so-called climate tipping points, resulting in irreversible changes of the climate (Armstrong McKay et al., 2022).

Furthermore, these global changes affect the environment of plants, leading to various modifications depending on the different climate change-related parameters considered.



### Water status

Water scarcity is an issue which will persist and exacerbate under climate change conditions and is occurring due to higher evaporation and reduced rainfall. While water stress can enhance colour, flavour and aromas of grape berries and thus can improve wine quality, changes of weather conditions such as extreme and numerous drought periods (and severe water stress) are meant to affect plant metabolism. Grapevine permits the stomata closure to avoid hydraulic failure by reducing transpiration and conserving water. However, this mechanism limits photosynthesis (Flexas et al., 2004). In addition, water deficit during early growth phases reduces final berry size and consequently yield (Scholasch & Rienth, 2019). Severe drought affects both primary and secondary metabolites in grapevine leaves (Griesser et al., 2015). Moreover, markers of stress such as hydrogen peroxide, malondialdehyde (MDA) and proline were shown to increase in leaf samples under drought conditions (Ju et al., 2018). In grape berries, water deficit impacted amino-acid metabolic pathways by decreasing phosphoglycerate and shikimate derivatives (Arrizabalaga-Arriazu et al., 2021). Technological maturity parameters such as °Brix, titratable acidity and pH were also impacted by severe water stress in Pinot Noir (Zombardo et al., 2020). Sugar accumulation was more affected by water deficit when the conditions were imposed before *véraison* (Keller, 2006). Generally, water deficit is associated with an increase of total anthocyanins, although berry weight under water stress conditions is reduced (Castellarin et al., 2007).

### Temperature

Traditionally, viticultural regions worldwide are defined by isotherms of average growing season temperatures of 12-13°C and 22-24°C (from April to October in Northern Hemisphere and from October in April in Southern Hemisphere) (Schultz & Jones, 2010). It has become common knowledge that air temperature is an important driver of grapevine growth and development (Fraga et al., 2019).

### High temperature

As plant phenology is driven by temperature (van Leeuwen et al., 2008), climate change and in particular increased temperature, can affect the ripening of berries and thus can modify sugar content, acid balance and flavours (Coombe, 1987), which are the basis of wine quality (Jones et al., 2005). Malic acid content decreases with high temperature, whereas sugar accumulation increases (Coombe, 1987). Phenology is also impacted by elevated temperature, for example flowering in Bordeaux will be advanced by 15 days in 2020-2050 and by 30 days of the end of 21<sup>st</sup> century (van Leeuwen & Darriet, 2016). Moreover, fruit maturation and thus harvest occur earlier with increased temperature (Duchêne et al., 2010). Advanced phenology is also linked with higher risk of late frost occurring in spring after budburst (Sgubin et al., 2018).

Previous studies showed that elevated temperature hastened the onset of berry ripening (and at all important phenological stages) and decreased anthocyanins concentration, colour intensity

and titratable acidity, as summarized in the review of Kuhn et al. (2013). Berry weight was also reduced by elevated temperature (Greer & Weston, 2010).

In another trial, two temperature regimes (24°C/14°C or 28°C/18°C, day/night) were imposed on Tempranillo clones and results showed a decrease in anthocyanin concentration under the highest temperature regime (Arrizabalaga et al., 2018). Total leaf area was also reduced by elevated temperature at the onset of *véraison* and at maturity. These authors found that it did not affect grape yield whereas berry weight and diameter were significantly reduced (Arrizabalaga et al., 2018). Anthocyanins are indeed sensitive to temperature, as a negative correlation was demonstrated between high temperature and anthocyanins concentration (Mori et al., 2007; Movahed, 2016). More precisely, anthocyanins accumulation was reduced by high temperature (35°C) in Cabernet Sauvignon berries, starting one week before *véraison* until fruit maturity (Mori et al., 2007a).

### Heatwaves

Grapes are sensitive to heatwaves, especially at growth stages such as flowering and ripening. Cabernet Sauvignon grapes submitted to heat treatment (+8°C) at different developmental stages were studied by Lecourieux et al. (2017) and it was shown that di-hydroxylated anthocyanins content was significantly reduced, where no change was noticed for tri-hydroxylated anthocyanins. The impact of heatwaves on primary and secondary metabolites of grapevine cultivar Shiraz was investigated by Gouot et al. (2019). In consequence to these heatwaves, pulp metabolites such as amino acids and compounds from myo-inositol pathway were increased when high day temperature was applied, whereas under high night temperature the accumulation of malic acid decreased. Skin tannin composition changes were noticed when two consecutive heatwaves were applied, as well as modifications in seed physiology and seed tannins composition. However, no difference in total skin tannins was found (Gouot et al. 2019).

### Elevated CO<sub>2</sub>

As the plant assimilates CO<sub>2</sub> through the process of photosynthesis, the effects of elevated CO<sub>2</sub> on grapevine cannot be neglected. More precisely, elevated carbon dioxide concentration is expected to increase leaf photosynthesis rates (Leakey et al., 2009). Indeed, RuBisCO (ribulose 1,5 biphosphate carboxylase oxygenase), the key enzyme of photosynthesis, is limited by CO<sub>2</sub> substrate for C<sub>3</sub> plants such as grapevine (Ainsworth & Rogers, 2007). Yield and cluster weight were increased under elevated CO<sub>2</sub>, and the stimulation of photosynthesis was demonstrated by increased net photosynthesis rate, on grapevine cultivar Touriga Franca (Moutinho-Pereira et al., 2009).

The impact of carbon dioxide levels (elevated, 700 ppm; versus ambient, 400 ppm), temperature increase (ambient temperature, versus T<sub>a</sub> + 4°C) and irrigation levels (partial versus fully irrigated) on cultivar Tempranillo demonstrated that long term exposure to elevated carbon dioxide results in photosynthetic acclimation, suggested by reduced photosynthesis capacity (Salazar-Parra et al., 2015). This findings are also confirmed by

Kizildeniz et al. (2021). Moreover, what is also worth to mention is that in the same study, root dry weight increased, when elevated CO<sub>2</sub> was applied, in red but not in white Tempranillo (Kizildeniz et al., 2021), suggesting that the carbon excess could be allocated to the roots. In addition, as carbon metabolism is involved in the control of flowering occurrence, elevated CO<sub>2</sub> may induce some changes in flowering time (Springer & Ward, 2007). Besides, bud fertility was investigated under elevated CO<sub>2</sub> and this parameter seemed to be more cultivar dependent than affected by the treatment (Wohlfahrt et al., 2019).

It was also found that elevated CO<sub>2</sub>, irrespective of temperature, and under fully irrigated conditions, increased total anthocyanins concentration (Kizildeniz et al., 2015). More precisely, the effects of elevated CO<sub>2</sub> (700 ppm) and elevated temperature combined with UV-B doses were determined, and the authors found that UV-B radiation may alleviate the effects of elevated CO<sub>2</sub> and elevated temperature on anthocyanins concentration in grapes (Martínez-Lüscher et al., 2016).

Climate change, and especially elevated carbon dioxide, has also an impact on pest pressure (Reineke & Thiéry, 2016). In greenhouses, it was demonstrated that under elevated CO<sub>2</sub> conditions (800 ppm) the survival rate and the fertility of female vine mealybugs was increased, whereas in VineyardFACE facility (ambient +20% CO<sub>2</sub>), these parameters were not affected (Schulze-Sylvester & Reineke, 2019). However, in another study, grapevine demonstrated a CO<sub>2</sub> effect in response to European grapevine moth (*Lobesia botrana*), from the gene expression level (Reineke & Selim, 2019).

#### *Adaptation to climate change*

In the face of climate change, several strategies can be considered to mitigate the effects of elevated temperature and drought. As an example of short term adaptation, crop protection to high temperature through shading nets (Castellano et al., 2008; Shakak et al., 2008; Greer et al., 2011) or foliar sunscreens (Glenn et al., 2010) were proposed.

Indeed, as higher temperature is advancing grapevine phenology (Parker et al., 2011), the use of late-ripening varieties can be considered. Consequently, Duchêne et al. (2010) investigated the genetic variability of phenological parameters of 120 genotypes (F1 progeny from Riesling x Gewurztraminer cross). Moreover, the authors also created a virtual genotype, modelled to undergo *véraison* at a similar time than the latest ripening variety.

In this regard, modifying leaf to fruit ratio by removal of grape clusters or canopy pruning can delay *véraison* and thus sugar accumulation in grape berries, without affecting total acidity (Parker et al., 2014). Moreover, in the study of Parker et al. (2014), removal of grape clusters did not impact technological maturity of Sauvignon Blanc berries, which may indicate that carbohydrates translocation could occur at the whole vine level, rather than at single shoot units, and also that this technique could be considered to adapt to climate change. Leaf removal induces a greater exposition to UV-B, which were found to alleviate the effects of both CO<sub>2</sub> and increased temperature on anthocyanins concentration (Martínez-Lüscher et al., 2016). This

technique was also used by Wang et al. (2022), who investigated the relationship between sugar and anthocyanins accumulation, while applying carbon source limitation.

It can also be possible to use long-term adaptation strategies, for example to select cultivars and rootstocks which are more tolerant to temperature or water stress. Such adaptation could be the grafting of grapevine scions on drought resistant rootstocks in order to avoid quality and yield losses due to drought (van Leeuwen et al., 2019).

A recent study highlighted the capacity of ancient grapevine varieties from Spain to be more tolerant to climate change conditions (elevated CO<sub>2</sub> and elevated temperature). Indeed, under these conditions, must quality as well as must antioxidant properties remained stable for some of these genotypes (Antolín et al., 2020). Moreover, alternative cultivars suitability can be tested via Grapevine Flowering Véraison (GFV) and Grapevine Sugar Ripeness (GSR) models, which were found to be reliable (Parker et al., 2020).

On the other side, adaptation of viticulture to climate change could demand, as a last resort, the migration of viticulture regions to higher elevation or higher latitudes, which would in future be more suitable (Mosedale et al., 2016; Hannah et al., 2013), although the latter reference was challenged by van Leeuwen et al. (2013).

Elevated atmospheric CO<sub>2</sub> concentration is a parameter which is difficult, not to say impossible, to mitigate, and as it is expected to increase up to 700 ppm at the end of the 21<sup>st</sup> century, there is thus a need to further study the long-term effects of elevated CO<sub>2</sub> on grapevine, as suggested by the review of Clemens et al. (2022).

## **1.2 Effect of climate change on grapevine physiology and metabolism**

### *Grapevine physiology*

Grapevine is a fruit crop of economic importance, in Europe and worldwide. World vineyard area was estimated to be around 7.3 Mha in 2020, and wine production in 2020 was evaluated at 260 MhL (OIV, 2020). Grapevine is produced for table grapes, raisins, juice, but its main product is wine. In the timeframe 1993-2018, Europe was the greatest wine producer worldwide (66.7% of world production), with France, Italy, and Spain rating ahead respectively (FAO, 2021).

Grapevine is a woody perennial crop from the Vitaceae family, and the *Vitis* genus, which is essentially localized in temperate climates. The most cultivated grape species in Europe and worldwide is *Vitis vinifera*. Grapevine comprises numerous domesticated varieties, called cultivars (Cabernet Sauvignon, Pinot Noir, etc...). Today, around 10000 grape cultivars are grown commercially. Phylloxera crisis, due to the bug *Daktulosphaira vitifoliae* occurred at the end of the 19<sup>th</sup> century and caused severe losses in vineyards and vine diversity all around Europe. Grafting American rootstocks such as *V.rupestris*, *V.riparia* and *V.berlandieri*, which are resistant to phylloxera aphid, on *V.vinifera* scions was found to keep European vines

unspoiled (Galet & Smith, 1998). Currently, most grapes devoted to winemaking worldwide are grafted.

The cultivar Cabernet Sauvignon is a red variety emblematic from the Bordeaux region which represent 6% of the vineyard area in France (OIV, 2017). It comes from a cross between two other cultivars, Cabernet France (red) and Sauvignon Blanc (white), as demonstrated by their genetic parenthood (Bowers & Meredith, 1997). Riesling originated the Rheingau valley in Germany, has light, yellow-coloured berries and is a late-ripening variety compared to other German cultivars.

#### *Grapevine vegetative and reproductive cycle*

Grapevine development cycle is composed by vegetative and reproductive phases. In viticulture, phenology corresponds to the timing of specific stages of growth and development in the annual cycle (Keller, 2020). These developmental stages of grapevine growth are described according to the modified E-L (Eichhorn-Lorenz) system (Coombe, 1995) (Figure 4).

Vine activity begins with “bleeding” (drops) of xylem sap at pruning wounds in late winter or early spring, marking the transition from dormancy to active growth. This phase can last from few days to several weeks. This transition is related to the restoration of metabolic activity in roots. Cell division and auxin production in buds start one to three weeks before bud break. Buds are growing during bud breaks, marking the onset of vegetative growth in spring, and are going to form leaves and fruits.

Around 5-10 weeks after bud break, anthesis marks the beginning of bloom, which is also the period where growth only depends on plant reserves and thus makes the vine very vulnerable to stress (Lebon et al., 2005 ; Lebon et al., 2008a). The optimum temperature for flowers to open is between 20-25°C; the bloom period can last two or three weeks in a vineyard. Only 20 to 50 % of flowers will develop into berries.

After fecundation of the ovary (fruit set), grapevine bunches are being formed, firstly by a period of cell division. In the berry pericarp, the rate and duration of cell division is defined and controlled by embryos meaning that berries with more seeds will become larger than berries containing less seeds (Coombe, 1960). Mesocarp cells stops to divide after two to four weeks after anthesis but exocarp cells still divide (Pratt, 1971).

Harvest is organized when berries are at maturity. Usually, red grapevine berries are harvested based on soluble solids concentrations (°Brix) and titratable acidity. Otherwise, berries are overripened, characterized by withering and a high concentration of sugars.

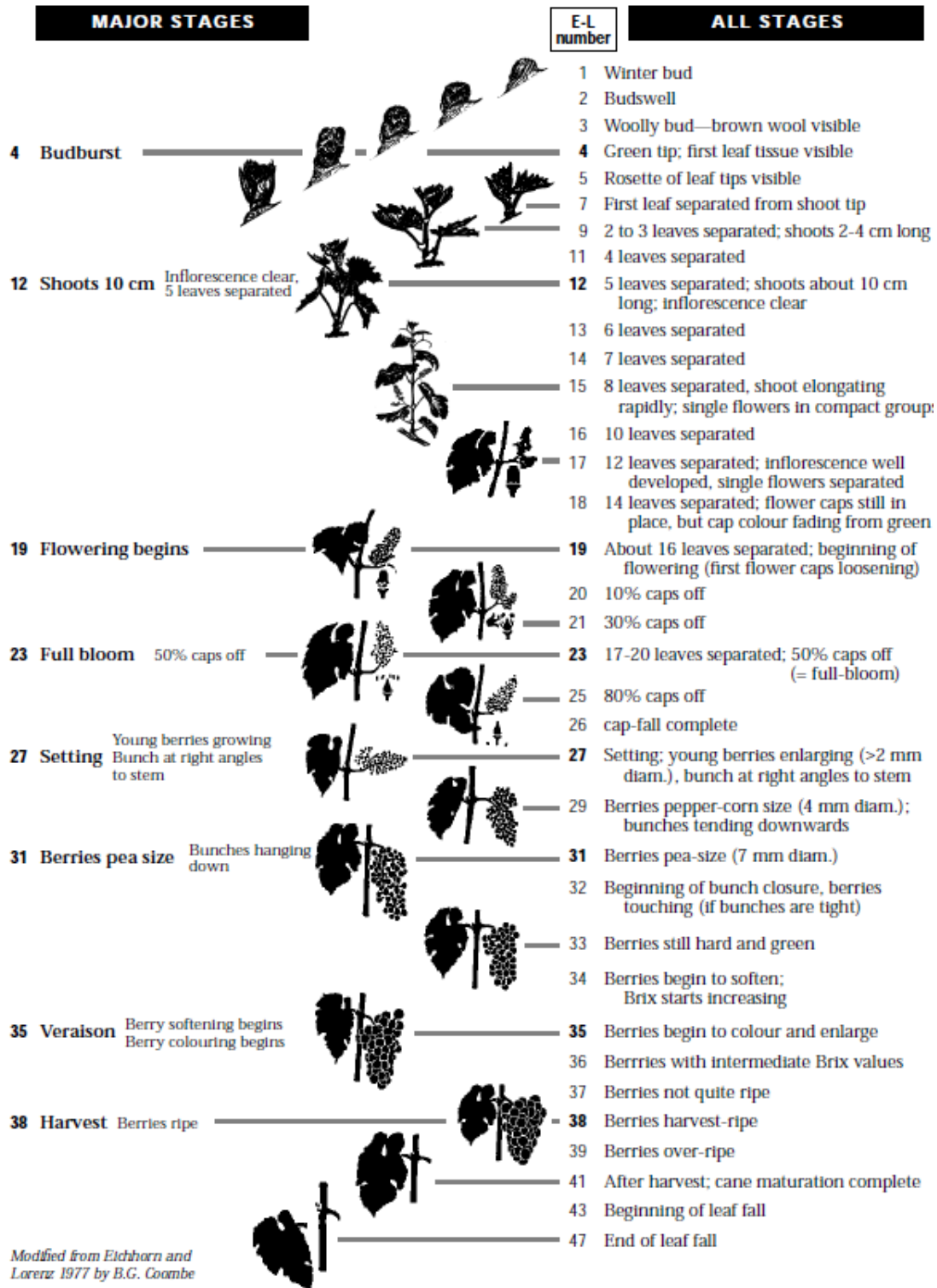


Figure 4. Grapevine growth stages according to modified EL system (Coombe, 1995)

Growth of grape berry consists in a double sigmoidal curve (Figure 5). Firstly, during the so-called herbaceous phase, after fruit set and before *véraison*, grape berry is hard and green, and accumulates malic and tartaric acid, but little sugar. Berry formation begins with setting,

pericarp cellular division resulting in intense berry growth. The first stage last six to nine weeks and ends when the skin cells are stopping to divide. This step is followed by a lag phase (Conde et al., 2007), when seeds are attaining their final size (Ristic & Iland, 2005). *Véraison* corresponds the onset of berry ripening period and lasts 5-10 weeks. It marks the changes in the acid to sugars balance, and it is described by a shift in berry colour in red varieties. During this stage, hexose sugars accumulate in flesh and skin. Berry starts softening, while malic acid and chlorophyll are degraded. Indeed, malic acid is consumed by respiration (Conde et al., 2007). Aroma compounds and polyphenolics such as anthocyanins (for red cultivars) are formed during ripening (maturation) and all these compounds are accumulated in the vacuoles (Coombe & McCarthy, 2000). In white cultivars, flavonols and terpenes are accumulating during maturation.

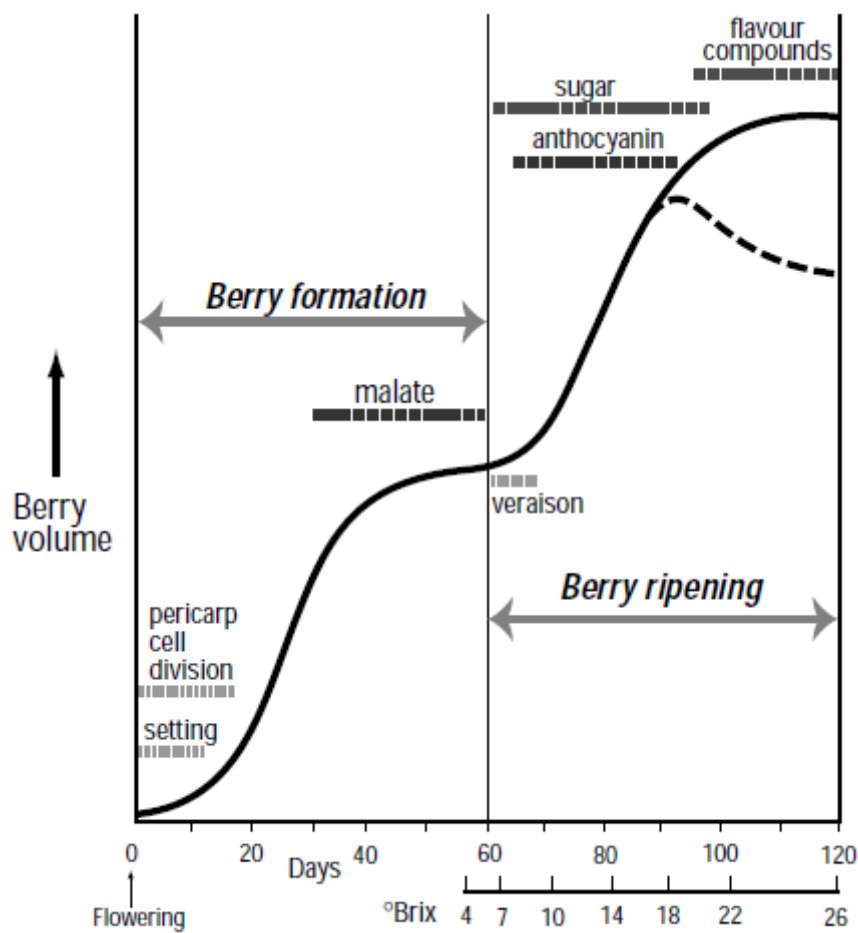


Figure 5. Grape berry development double sigmoidal curve and metabolites accumulation over time from flowering onwards (Coombe & McCarthy, 2000)

In the mesophyll cells of leaves, sugars produced by photosynthesis, after Calvin-Benson cycle, are used for respiration and to produce organic acids such as malic and tartaric acids as well as amino acids and polyphenols. Sucrose is then conducted through the stem to reach sink tissues, via the phloem (Van Bel, 2003). Water and mineral compounds are absorbed by roots and conducted via xylem sap to leaves. For grape berry, water flow from xylem slows and stops around *véraison* and the water transport continues via phloem sap (Coombe & McCarthy, 2000).

### 1.3 Primary metabolites in grape berry

Grapes are classified as a non-climacteric fruit. Major tissues composing grape berry are skin (exocarp), flesh (mesocarp) and seeds (Kennedy, 2002) (Figure 6). Skin is composed of two regions, the epidermis, and the hypodermis. Skin cells contain vacuoles which accumulate phenolic and aromatic compounds (Hardie et al., 1996) and in minor amounts sugars, organic acids, and amino acids. Flesh represents 75 to 85% of berry volume and its parenchymatic cells accumulate compounds such as organic acids, amino acids, and sugars. Seeds, which represent 3 to 6% of berry mass, accumulate tannins, and seeds number varies between one and four per berry. Moreover, skin represents 10 to 15% of total berry weight, and skin outer layer is recovered by wax, protecting the berry from physical, chemical and biological threats (Heredia, 2003).

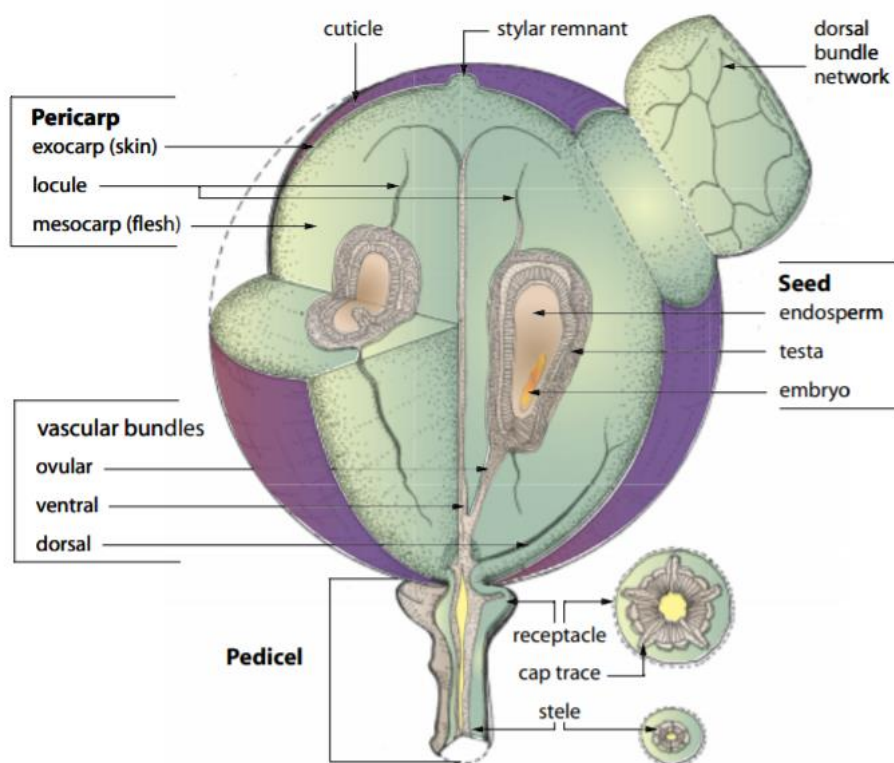


Figure 6. Grape berry structure, adapted from Kennedy (2002)



## *Sugars*

The organoleptic properties of grape berries are partly defined by their sugar concentration; the latter also determines the alcohol content in wines. Mature berries display the same amount of glucose and fructose, whereas at green stages glucose is predominant ( $G/F > 1$ ) (Varandas et al., 2004). When berry is over ripened, fructose is then predominant ( $G/F$  ratio  $< 1$ ). However, glucose to fructose ratio also depends on the different grape varieties (Kliewer, 1967). Assimilates from photosynthesis are transported by phloem in majority in the form of sucrose (Mullins et al., 1992) while soluble sugars in berries accumulated in vacuoles are mainly fructose and glucose.

Indeed, sucrose, produced through photosynthesis in leaves represent the main carbohydrate used for transport via the phloem to the berry (Hawker, 1969a). The sucrose loading is realized from mesophyll cells to the phloem either by a symplastic or by an apoplastic mechanism (Boss & Davies, 2009). Slightly before *véraison*, sucrose is unloaded from phloem by symplastic pathway, whereas after *véraison* sucrose is unloaded through apoplastic pathway, in response to grape sink development (Zhang et al., 2006). In the apoplastic pathway, sucrose is loaded and accumulated in the phloem, passing via the apoplast between phloem parenchyma cells and companion cells with transporters from the SWEETs family (Sugar Will Eventually be Exported Transporters) (Chen et al., 2012) and sucrose transporters (SUT1/SUC2) associated with proton pump (Gottwald et al., 2000). In the symplastic pathway, sucrose is loaded in the sieve tubes via plasmodesmata, between sieve elements and companion cell complex (Turgeon & Wolf, 2009).

Several key transporters have been identified and four of them (VvSUC11/VvSUT1, VvSUC12, VvSUC27 and VvSUT2), located in the plasma membrane, have been associated to sugar accumulation during berry ripening. Indeed, genes such as VvSuc11, VvSuc12 and VvSuc27 are involved in sucrose/ $H^+$  transport (Davies et al., 1999). While the expression of VvSuc27 declines during ripening, VvSuc21 and VvSuc12 are upregulated when hexoses accumulation starts (Davies et al., 1999 ; Lebon et al., 2008b).

## *Organic acids*

Organic acid composition in berries is a key point to wine quality. Acidity is an important parameter because it determines wine stability and contributes to its colour and flavour. The ratio of sugar to organic acids determines technological maturity, which helps to predict the harvest date. Organic acids start to accumulate in early stages of development and are, in grape berries, mainly malic and tartaric acids (Kliewer, 1966), which account for 69 to 92% of all organic acids in grape berries, however minor amounts of citric, succinic, lactic, and acetic acids can be found. Malic acid concentration reaches a peak just before *véraison* and decreases after, reaching concentration from 1 to 10  $g.L^{-1}$ . Tartaric acid concentration may be around 15

g.L<sup>-1</sup>, at the end of the vegetative phase (Ribéreau-Gayon et al., 2006). Citric acid, which plays an important role in the TCA cycle, has a concentration in must between 0.5 and 1 g.L<sup>-1</sup>.

Biosynthesis of malic acid originates with  $\beta$ -carboxylation of phosphoenolpyruvate (PEP) which forms oxaloacetate and then malate, the latter catalysed by cytosolic malate dehydrogenase (MDH) (Hawker, 1969b; Conde et al., 2007). During ripening, malate is liberated from the vacuole, becoming available for catabolism (TCA cycle and respiration, gluconeogenesis, amino-acids interconversions) (Ruffner & Kliewer, 1975). Malic acid degradation consists either in its decarboxylation into pyruvate and CO<sub>2</sub> via NADP-malic enzyme (Hawker, 1969b); or is diffusion through mitochondria. Mitochondrial MDH can catalyse its transformation into Oxaloacetate (OAA) or mitochondrial NAD-Malic enzyme would transform malate into pyruvate (Conde et al., 2007). In cool climates, malic acid concentration in grape berries is higher than in warmer ones (Conde et al., 2007), because malic acid degradation is sensible to temperature .

On the other hand, tartaric acid content on a berry basis stays rather constant, showing that its decrease in concentration is due to a dilution effect (Ruffner et al., 1983; Sweetman et al., 2009). Tartaric acid biosynthesis begins with L-ascorbic acid (Conde et al., 2007), and one key step in tartaric biosynthesis corresponds to the cleavage of a six carbon intermediates between either C2/C3 or C4/C5 (depending on the plant species) (DeBolt et al., 2006).

Moreover, malate concentration can affect wine's characteristics because of its involvement in carbonic maceration and malolactic fermentation (Kunkee, 1991). On the contrary, tartaric acid is not metabolized during fermentation, and is less sensitive to climatic conditions during ripening (Poni et al., 2018).

### *Amino-acids*

Amino-acids play an important role in plant metabolism and in the winemaking process, especially for yeast growth. Their biosynthetic pathways are presented in Figure 7. Major amino-acids found in grapes are arginine and proline (Stines et al., 2000). However, while ammonium ions and most amino acids are used by yeast, proline is not assimilated by them (Huang & Ough, 1991).

Amino acids are precursors of several quality-related secondary metabolites in grapes. Phenylalanine is a precursor of phenolic compounds such as anthocyanins and flavonols through the phenylalanine pathway (Tzin & Galili, 2010). Valine and leucine are precursors of aromas. GABA ( $\gamma$ -aminobutyric acid) is involved in TCA cycle as well as pH regulation, plant defence and development (Shelp, 1999). Amino-acids profile is relatively dependant on the variety, vintage, area and maturity level (Hernández-Orte et al., 2002).

For the cultivar Cabernet Sauvignon, proline concentration in mature grape berries is higher and arginine concentration is lower, respectively, when compared to the other cultivars studied (Grenache, Muscat Gordo, Pinot Noir, Riesling, and Sangiovese) (Stines et al., 2000). Moreover, when comparing both Riesling and Cabernet Sauvignon at harvest, it was found that

pulps from cultivar Cabernet Sauvignon have a double proline content compared to Riesling. In the same study, proline constituted 55% of total amino acids of pulp in Cabernet Sauvignon and 32% in Riesling, where arginine represented 12% of total amino acids for pulp in Cabernet Sauvignon, and 22% for Riesling (Stines et al., 2000). UV-B radiations did not impact total free amino acids content, but GABA displayed an increase whereas others amino acids decreased such as threonine, isoleucine, methionine, serine, and glycine (Martínez-Lüscher et al., 2014). The light exposure on Merlot berries was investigated on various metabolic compounds, and differences could be demonstrated for amino acids such as histidine, valine, GABA, alanine, and arginine between shaded and light-exposed berries (Pereira et al., 2006).

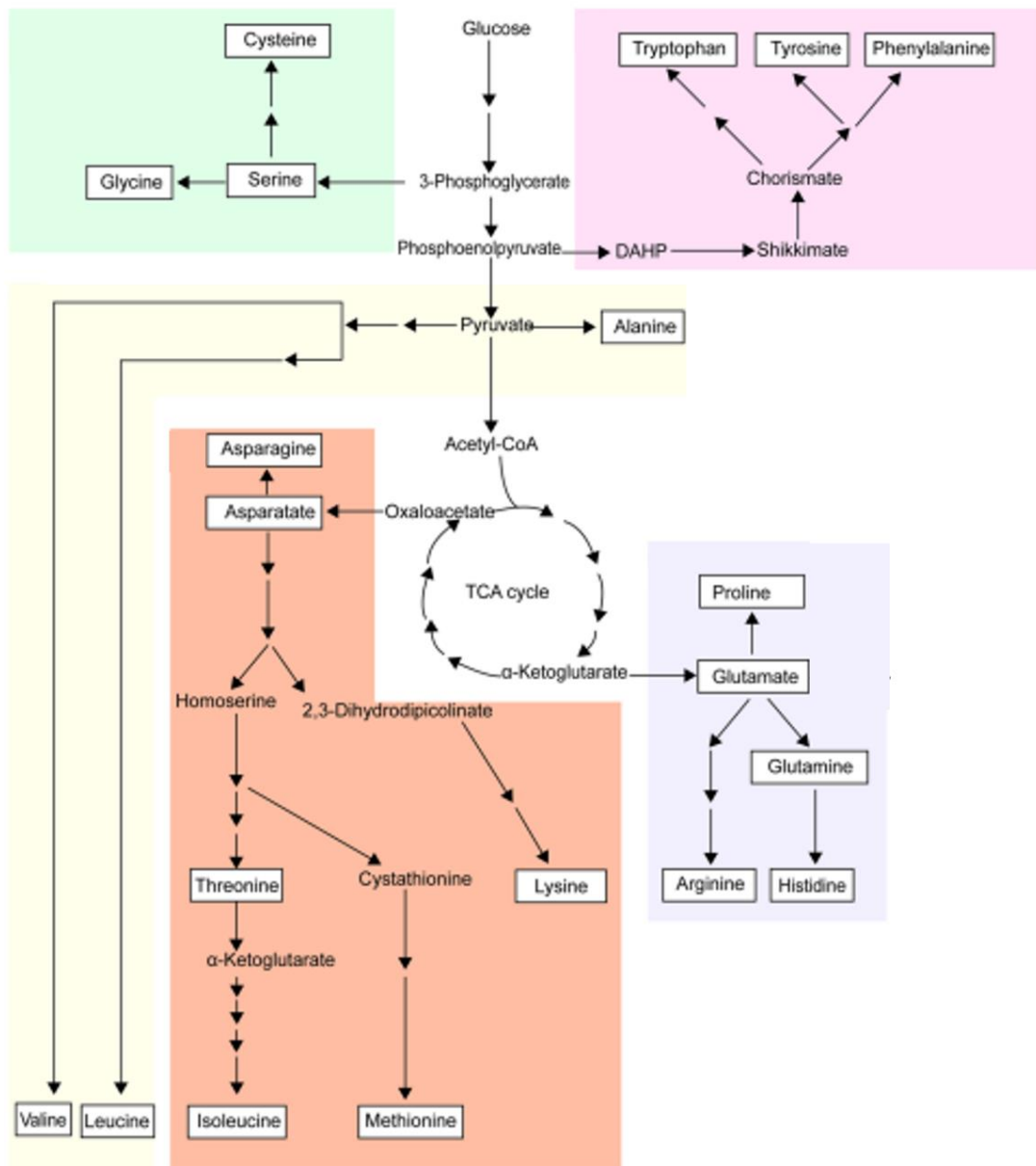


Figure 7. Biosynthesis pathways of amino acids, adapted from (Yang et al., 2020)

## *Minerals in grape*

Minerals also play a role in plant development and physiology, such as Potassium (K), Calcium (Ca), Phosphorus (P), Magnesium (Mg), and Sulphur (S). Potassium, which is the most abundant cation in grape berry, is absorbed by roots and transported towards all organs of the plant. At the beginning of berry development, most of the potassium is accumulated in the leaves (Blouin & Cruège, 2003). This macronutrient is involved in enzyme activation, plays a role in transmembrane potential difference of plasma membrane, and also on osmotic potential regulation (Conde et al., 2007). Calcium is involved in cell signalling and has also a structural role in cell walls and membranes (White, 2003). Phosphorus, as a component of key molecules, such as nucleic acids or ATP, has to be accumulated in sufficient amount (Gerós et al., 2012). Magnesium, which is the central atom of chlorophyll tetrapyrrolic moiety, is thus essential in the photosynthetic process (Gerós et al., 2012).

### **1.4. Secondary metabolites in grape berries**

#### *Phenolic compounds*

Phenolic compounds of grapevine can be divided in two groups: flavonoids and non-flavonoids. Flavonols, tannins and anthocyanins are among the first category, and non-flavonoids are represented by stilbenes and phenolic acids. They show beneficial properties for human health, promoting reduction of cardiovascular diseases for example (Khoo et al., 2017).

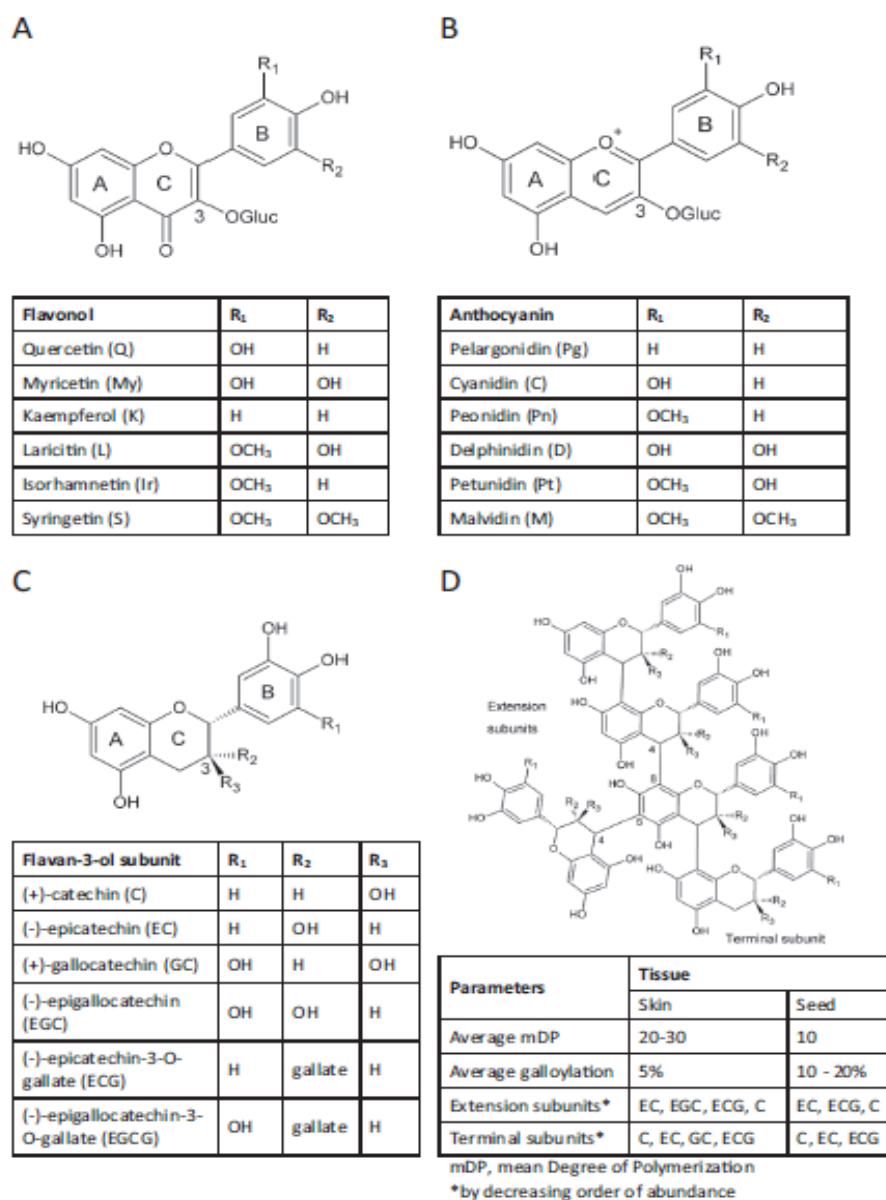
Anthocyanins are responsible for colour (from red to blue) in plants and thus used as food dye in industries to replace synthetic colorants. They are found in grape skin for most red cultivars; however, they can also be found in the pulp of Teinturier cultivars. Anthocyanins are key compounds for winemaking because of their contribution to wine astringency (Vidal et al., 2004). In grape berries, their accumulation occurs from *véraison* to maturity. Structurally, anthocyanins are glycosides and acyl-glycosides of anthocyanidins. Flavylium cation, which is the core of anthocyanidins, has a C6-C3-C6 flavonoid backbone, containing one heterocyclic benzopyran ring (C ring), one aromatic ring (A ring) and one phenyl constituent (B ring) (Mazza & Francis, 1995) (Figure 8). The C ring contains two conjugated double bonds, thus being charged positively (Boss & Davies, 2009).

In *Vitis vinifera*, five anthocyanidins have been quantified, known as malvidin (Mv), delphinidin (Dp), peonidin (Pn), cyanidin (Cy) and petunidin (Pt) (Kuhn et al., 2013), however in non-*Vinifera* grapes, pelargonidin (Pg) can also be found. Anthocyanins can be substituted with hydroxyl or methyl groups on the B ring, and thus can be differentiated as mono- (Pg), di- (C,Pn) and tri-hydroxylated (Dp, Pt, Mv) (Figure 8). They can also be esterified by acids (Conde et al., 2007). It is reported that in most plants, only O-glycosylation happens for anthocyanins, and the sugar linked is generally glucose. Whereas blueness is enhanced with the increase of free hydroxyl groups, redness intensifies with the raising of the methylation of the hydroxyl groups, so malvidin is the reddest individual anthocyanidins (Jackson, 2008). Consequently, kinetic of accumulation of red and blue anthocyanins determines the colour

variation in grape berries (Castellarin et al., 2006). Light exposure and temperature influence anthocyanins concentration, enhancing respectively their accumulation and their degradation, when temperature is above 35°C (Mori et al., 2007a).

Flavonols accumulate in berry skin and are odourless compounds in red cultivars, major flavonols are quercetin (Q, 44%) and myricetin (My, 37%). In white cultivars, kaempferol (K, 16,9%) and isorhamnetin (Ir, 1,7%) are found in majority (Mattivi et al., 2006a). Laricitin (L) and syringetin (S) are also found in grape berries. Depending on their number of hydroxy (-OH) or methoxy (-OCH<sub>3</sub>) groups on their B ring, flavonols can be mono-(K), di-(Q, Ir) or tri-hydroxylated (My, L and S) (Figure 8). Moreover, Isorhamnetin is a methylated form of Quercetin (Mattivi et al., 2006b). Like anthocyanins, only glycosylated forms of flavonols accumulate in grapes. Concerning their properties, quercetin is known to behave as UV-protectants (Doshi et al., 2006). White grape colour originates from carotenoids, xanthophylls and flavonols, which also exist in red varieties but are masked by anthocyanins (Jackson, 2020).

Among grape phenolic compounds, major flavan-3-ol monomers are (+)-catechin (C), (-)-epicatechin (EC) and (-)-epicatechin-3-O-gallate (Conde et al., 2007). Small quantities of these monomers can be found in berry skin, pulp and seeds or can also be polymerized as condensed tannins or proanthocyanidins (Pinasseau et al., 2016). Tannins, which are found in grape seeds and skin, confer astringency to wine. Their size is variable, ranging from dimers to oligomers. These compounds are responsible for major wine organoleptic properties and are as well involved in wine ageing process (Souquet et al., 1996). Proanthocyanidinins are indeed composed by flavan-3-ol subunits connected via C4-C8 and C4-C6 interflavan bonds. Last polymer subunit is called, by convention, the terminal subunit, whereas top and middle subunits are called extension (or upper) subunits (Gouot et al., 2019). Moreover, proanthocyanidins differ in composition and length, defined by mean Degree of Polymerization (mDP). Berry skin has a percentage of galloylation of ~5%, whereas this percentage in skin is around ~10-20% (Gouot et al., 2019). Moreover, seed colour changes (from green to brown) from *véraison* onwards, is probably due to proanthocyanidins oxidation or complexation with other compounds (Hanlin et al., 2010). Seed and skin tannins differ in the way that skin tannins are bigger than seed ones and contain epicatechin subunits. However seed tannins can have epicatechin gallate as subunit (Adams, 2006). Tannins are amphipathic molecules (having both hydrophobic aromatic rings and hydrophilic hydroxyl groups) hence can interact with proteins and polysaccharides from the cell wall (Hanlin et al., 2010).



**Fig. 1.** Flavonol (A), anthocyanin (B), flavan-3-ol (C), and proanthocyanidin (D) structures.

Figure 8. Structure of flavonols (A), anthocyanins (B), flavan-3-ols (C) and proanthocyanidin (D), from Gouot et al. (2019)

After tannins and anthocyanins, the third major phenolic compounds are hydroxycinnamates (Conde et al., 2007).

Stilbenes are associated with beneficial effects on health such as preventing from cancer and cardiovascular diseases (Landrault et al., 2002). They are produced via the phenylalanine/polymalonate pathway, with the action of stilbene synthase, which is the main

enzyme for resveratrol formation. Their chemical structure is constituted by a diphenylethylene group oriented cis or trans (Viret et al., 2018). In wine, the main stilbene is resveratrol in the cis and trans forms, and in grape only trans-resveratrol has been found (Bavaresco et al., 2002).

#### Biosynthesis of anthocyanins: phenylpropanoid & flavonoid pathways

Anthocyanins are synthesized at the cytosolic surface of endoplasmic reticulum via the flavonoid pathway (Boss et al., 1996) and are stored in vacuoles (Petrucci et al., 2013). Their synthesis was firstly described in grapevine by (Sparvoli et al., 1994).

Phenylpropanoid pathway starts with phenylalanine as precursor (He et al., 2010), converted by successive reactions in 4-coumaroyl-CoA (Figure 9). Firstly, phenylalanine is converted into cinnamic acid, catalysed by PAL (phenylalanine ammonia lyase). Afterwards, p-coumaric acid is produced by cinnamic acid catalysed by C4H (cinnamate-4-hydrolase). P-coumaric acid linked by Coenzyme A (CoA) forms p-coumaroyl CoA, by the enzyme 4CL (4-coumarate CoA ligase). The flavonoid pathway starts when one molecule of 4-coumaroyl-CoA and 3 molecules of malonyl-CoA produce, by the action of chalcone synthase (CHS), naringenin chalcone (Sparvoli et al., 1994). Goto-Yamamoto (2002) obtained three clones of the chalcone synthase gene, named Chs1, Chs2 and Chs3; moreover, they showed that Chs3 is expressed mostly in berry skin of red cultivars (Goto-Yamamoto et al., 2002).

CHS (chalcone synthase), CHI (Chalcone Isomerase), and F3H (flavanone-3-hydroxylase) are among the enzymes involved in early steps of flavonoid pathway (Figure 9).

Then flavonols and dihydroflavonols are formed from the chalcone intermediate, themselves leading to leucoanthocyanidins, precursors of anthocyanidins and flavan-3-ols (Gouot et al., 2019), according to the following steps. Indeed, naringenin can form dihydroxykaempferol through hydroxylation at the position 3 via F3H, at position 3', forming eriodictyol via F3'H, or at position 3'5' positions via F3'5'H catalysing pentahydroxyflavanone. The latter are converted in dihydroxyquercetin or dihydroxymyricetin respectively, via F3H. Dihydroxykaempferol can also be hydroxylated into these compounds, via F3'H or F3'5'H respectively. Key enzymes such as F3'H (flavonoid-3'-hydroxylase) and F3'5'H (flavonoid 3'-5'-hydroxylase) contribute respectively to the formation of di- and tri-hydroxylated flavonoids from mono-hydroxylated precursors (Bogs et al., 2006). DFR (Dihydroxyflavonol-4-reductase) and LDOX (leucoanthocyanidin dioxygenase, also known as ANS anthocyanidin synthase) reduce dihydroxyflavonols into their corresponding leucoanthocyanins (Sparvoli et al., 1994), then themselves into anthocyanidins. Indeed, colourless leucoanthocyanins are oxidized by catalysis of ANS into their corresponding-coloured anthocyanins (He et al., 2010).

Moreover, ANR (anthocyanidin reductase) and LAR (leucoanthocyanidin reductase) are main enzymes for flavan-3-ol production (Pfeiffer et al., 2006).

Under their initial form, anthocyanidins are unstable and cannot accumulate. Subsequently, stable anthocyanins are formed by glycosylation, acylation, and methylation. UFGT (Anthocyanidin 3-O-glucosyltransferase) catalyses the O-glycosylation of anthocyanidins (Ford et al., 1998), and OMT (O-methyl transferase) catalyses their methylation (Huguene et al., 2009).

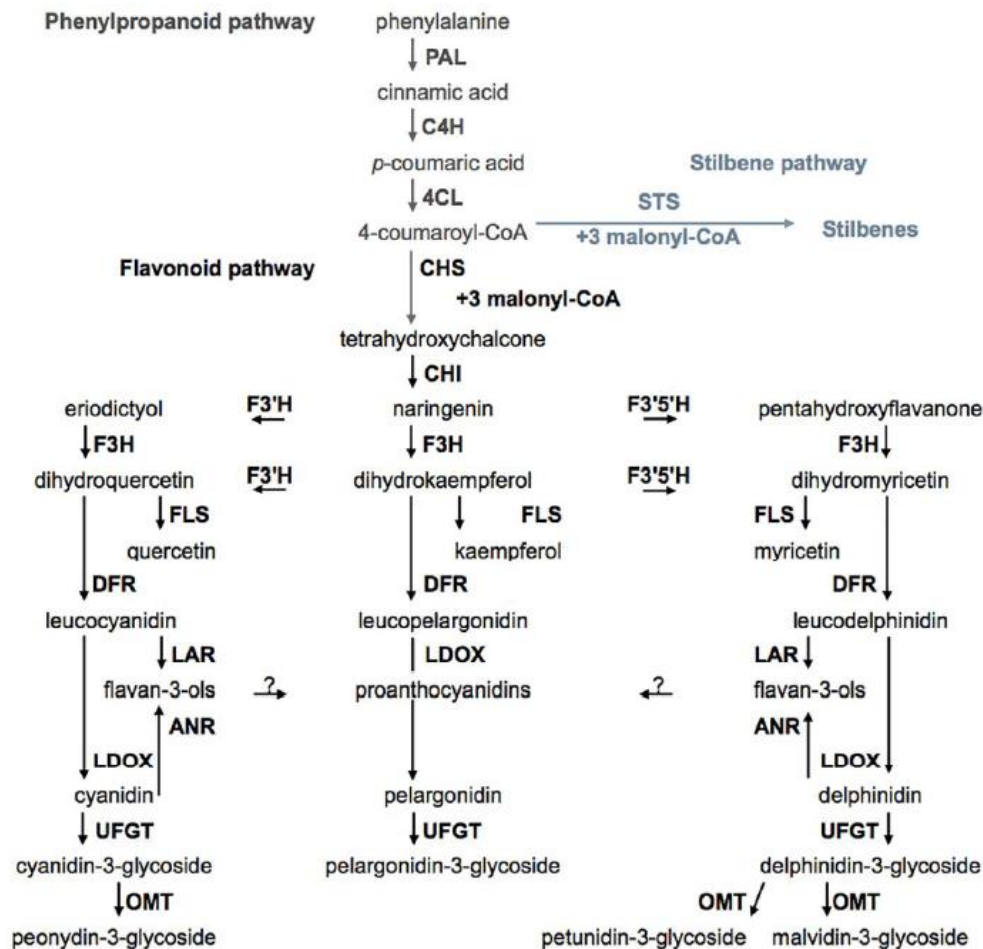


Figure 9. Biosynthesis pathways (phenylpropanoid and flavonoid pathways) of grape berry secondary metabolites (Teixeira et al., 2013)

Phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumaroylCoA-ligase (4CL), stilbene synthase (STS), Chalcone synthase (CHS), Chalcone isomerase (CHI), Flavonoid-3'-hydroxylase (F3'H) Flavonoid-3'5' hydroxylase (F3'5'H), flavonone-3-hydroxylase (F3H), flavonol synthase (FLS), dihydroxyflavonol reductase (DFR), leucoanthocyanidin dioxygenase (LDOX), dihydroxyflavonol 4-reductase (DFR), flavonoid glucosyltransferase (UFGT), O-methyltransferase (OMT)



## Impact of nitrogen availability on anthocyanins

As nitrogen supply modifies anthocyanins content, low nitrogen availability enhances anthocyanins production as in was shown in the study of Hilbert et al. (2003). In this condition, berries contained more Delphinidin-3-monoglucoside and Petunidin-3-monoglucoside. Soubeyrand et al. (2014) investigated the regulation of genes involved in anthocyanins biosynthesis and the stimulation of the phenyl-propanoid pathway was confirmed. A hypothesis was phrased, that flavonoids pathway plays the role of consuming ATP and NADPH overproduction. This assumption was verified in the study of Soubeyrand et al. (2018) which demonstrated that central metabolism was reduced in case of lower C/N ratio, whereas fluxes using energy and reducing power were increased (such as shikimate and flavonoid pathway) as suggested by Hernández & Van Breusegem (2010).

### *Aroma compounds*

Aroma compounds belong to chemical families such as monoterpenes, sesquiterpenes and C<sub>13</sub>-norisoprenoids, methoxyxypyrazines and sulfur (thiols) compounds (Kuhn et al., 2013). Among aroma compounds, terpenes have been the most extensively studied. They are located in berry skin and their biosynthesis begins, like other secondary metabolites, with Acetyl-coenzyme A (AcetylCoA) (Conde et al., 2007). Moreover, all terpenoids are synthesised from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), and most monoterpenes are synthesized from IPP and DMAPP derived from methylerythritol phosphate pathway (MEP pathway) (Bohlmann & Keeling, 2008). This family of compounds is responsible for fruity and floral aromas, such as linalool, geraniol, nerol,  $\alpha$ -terpineol and citronellol. However, some monoterpenes such as  $\alpha$ -terpinene, *p*-cimene, myrcene and famesol have resin-like odours (González-Barreiro et al., 2015). Terpene glycosides, which are odourless compounds, are also more common than free, volatile terpenes (Park et al., 1991).

C<sub>13</sub>-norisoprenoids derived from carotenoids degradation and adopt two main forms such as megastigmane and non-megastigmane. The concentration of both monoterpenes and norisoprenoids increases during ripening (Yang et al., 2011).

Methoxyxypyrazines are odorant compounds with vegetable-like notes. The major methoxyxypyrazine in amount is 2-methoxy-3 isobutylpyrazine (IBMP) followed by 2-methoxy-3-isopropylpyrazine (IPMP) and 2-methoxy 3 sec butylpyrazine (SBMP) (Ebeler & Thorngate, 2009). Their concentration increases during the first phase of berry development, then reaches a maximum before *véraison* and decreases hereafter (Guillaumie et al., 2013; Ryona et al., 2008). Vegetable-like fragrances associated with methoxyxypyrazines could be negatively perceived in red wines.

### 1.5. Experimental systems designed to study eCO<sub>2</sub>

Several set-ups were designed and used to study the effects of elevated CO<sub>2</sub> or more broadly climate change on grapevine, such as Temperature Gradient Greenhouses (Arrizabalaga-Arriazu et al., 2020; Kizildeniz et al., 2018; Salazar-Parra et al., 2015; Martínez-Lüscher et al., 2016), Open Top Chambers (OTC) (Gonçalves et al., 2009; Edwards et al., 2016) and FACE systems (Wohlfahrt et al., 2018 ; Bindi et al., 2001) as reported by the review of Clemens et al. (2022).

Open Top Chambers are enclosed systems where enriched CO<sub>2</sub> can be applied to potted plants. Using OTC, an application of 500 ppm of CO<sub>2</sub> resulted in increased net photosynthesis rate, intrinsic water use efficiency and C/N ratio whereas stomatal conductance and transpiration rate were not significantly affected (Gonçalves et al., 2009).

According to Long et al. (2004), a FACE system (which is an acronym for Free Air Carbon dioxide Enrichment) consists of a “circular set up surrounded by a ring of pipes releasing CO<sub>2</sub>, at vertical intervals from just above the ground to just above the top of the plant canopy”. Thus, FACE systems are open field setups used to create a near atmosphere enriched in CO<sub>2</sub> around crops.

Many FACE systems are installed on different crops such as pea (Bourgault et al., 2016), wheat, barley, maize (Erbs et al., 2015), and on trees such as poplars (Gielen & Ceulemans, 2001) or sour orange trees (Kimball et al., 2007). According to review articles (Ainsworth & Rogers, 2007 ; Kimball et al., 2002), the impact of elevated carbon dioxide on C<sub>3</sub> plants (including grapevine) consists in decreased stomatal conductance and increased light-saturated CO<sub>2</sub> uptake. Leaf area growth is also favoured thanks to increased CO<sub>2</sub> atmospheric concentration (Long et al., 2004). A review summarizing the crop response to elevated CO<sub>2</sub> in FACE experiments demonstrated that biomass and yield increased in all C<sub>3</sub> species studied, and that grape berry yield increased under elevated CO<sub>2</sub> for about 25% (Kimball, 2016).

It is worth to mention that between controlled environments such as greenhouses, and open field studies, there can be significant differences in experimental results (Poorter et al., 2016). Indeed, despite their cost and the technical challenge they represent, FACE systems are realistic settings to study the effects of elevated CO<sub>2</sub> on plants in *natura*.

Accordingly, Bindi et al. (2001) used a FACE system with three different carbon dioxide concentrations (ambient, 550 ppm, 700 ppm) on grapevine cultivar Sangiovese. Elevated CO<sub>2</sub> (700 ppm) increased significantly total weight and fruit dry weight. In addition, sugars and organic acid concentrations increased under elevated CO<sub>2</sub>, but at maturity, these effects tended to disappear.

Using the VineyardFACE facility in Geisenheim, Wohlfahrt et al. (2018) measured vegetative growth, leaf gas exchanges and yield parameters on the two cultivars Riesling and Cabernet Sauvignon under moderate (+20%) elevated and ambient CO<sub>2</sub>. Authors showed a significant increase of CO<sub>2</sub> assimilation rate, transpiration rate, stomatal conductance, and water use

efficiency. Usually results in literature report a decrease in stomatal conductance (Ainsworth & Rogers, 2007) although other authors stipulate that depending on weather conditions an increase in stomatal conductance can occur (Purcell et al., 2018). Moreover, according to Wohlfahrt et al. (2018), elevated CO<sub>2</sub> did not affect sugar concentration in must at harvest date. As well, further study of Wohlfahrt et al. (2020) showed that elevated CO<sub>2</sub> treatment did alter bunch parameters, but did not affect berry quality. However, elevated CO<sub>2</sub> treatment increased single berry weight and malic acid content at some stages. No negative impact of elevated CO<sub>2</sub> treatment was reported on must and wine composition for years 2014 to 2016, although there was some minor differences in galacturonic acid for Cabernet Sauvignon wines in 2015, and pH and volatile acidity in 2014 for Riesling (Wohlfahrt et al., 2021).

## 2. PhD thesis objectives

To understand the long-term effects of continuously increasing atmospheric carbon dioxide concentration (after 6 years of fumigation) on grapevine physiology and berry composition, the main aims of the thesis are structured around complementary axes. The duration of the study included three growing seasons (2019, 2020, and 2021) and focused on field experiments (at VineyardFACE facility in Geisenheim) and laboratory investigations (Bordeaux).

Firstly, we aimed to investigate if elevated atmospheric CO<sub>2</sub> has an impact on grapevine leaf physiology and as well, to determine the effects of elevated CO<sub>2</sub> on berry physiology, yield and ripening rates by phenological monitoring in open field set-up (Chapter 1).

Moreover, the second aim was to study the impact of elevated CO<sub>2</sub> on berry quality and composition for both primary metabolites (sugars, organic acids, and amino acids) and anthocyanins (Chapter 2).

Further investigations were conducted in both seasons 2020 and 2021 on intermediates from primary metabolism to understand the impact of elevated CO<sub>2</sub> on berry plasticity, thanks to a collaboration with the Max Planck Institut in Potsdam Golm (Chapter 3).

Finally, must composition in monoterpenes and C<sub>13</sub>-norisoprenoids permitted the investigation of the impact of elevated CO<sub>2</sub> on must aroma profile, for one year (Chapter 4).

### 3. General material & methods

#### *VineyardFACE system*

VineyardFACE (for Free Air Carbon dioxide Enrichment) is an open-field experimental set-up, located at Hochschule Geisenheim University (49° 59' N, 7° 57' E) in Rheingau, Germany, where one-year potted grapevines were planted in 2012, with a total area of 0.5 ha. Cabernet Sauvignon (clone 170, grafted on rootstock 161-49 Couderc) and Riesling (clone 198-30 Gm grafted on rootstock SO4, clone 47 Gm) are planted alternately. The vines are planted with rows oriented north-south. Six rings of 12 m diameter surround the field, three with ambient CO<sub>2</sub> (~400 ppm) and three with elevated CO<sub>2</sub> (aCO<sub>2</sub> + 20%), which is the concentration expected in 2050, according to IPCC. The fumigation started in 2014. These rings are built with 36 towers each, with a height of 2.5 m, whose head consists in one blower creating an airstream and one emitter releasing carbon dioxide (Wohlfahrt et al., 2018). Wind direction and velocity were measured to determine the release of CO<sub>2</sub>. In the center of each ring, CO<sub>2</sub> was recorded by carbon dioxide transmitters, and CO<sub>2</sub> was adjusted by a mass flow control valve maintaining target elevated CO<sub>2</sub> level (Wohlfahrt et al., 2018). Each parcel consists of seven rows, with the inner five rows used for sampling (23 vines for Riesling and 24 for Cabernet Sauvignon). Rings named A1, A2 and A3 are specified as ambient CO<sub>2</sub> rings and rings E1, E2 and E3 are specified as elevated CO<sub>2</sub> rings. Because blowers in eCO<sub>2</sub> rings were operated parallel to aCO<sub>2</sub> rings, A1-E1, A2-E2, A3-E3 are thus defined as experimental blocks (Figure 10).



Figure 10. Aerial view of VineyardFACE in Geisenheim (Wohlfahrt et al., 2018)

### Data collection

Samples were collected according to the following sample days.

Figure 11. Samples dates and collection for year 2019

<b>1</b> Green stages	<b>22.07.2019</b>	Riesling	<b>31.07.2019</b>	Cabernet Sauvignon
<b>2</b> 25% véraison	<b>13.08.2019</b>	Riesling	<b>28.08.2019</b>	Cabernet Sauvignon
<b>3</b> 50% véraison	<b>28.08.2019</b>	Riesling	<b>10.09.2019</b>	Cabernet Sauvignon
<b>4</b> 75% véraison	<b>10.09.2019</b>	Riesling	<b>24.09.2019</b>	Cabernet Sauvignon
<b>5</b> Maturity	<b>24.09.2019</b>	Riesling	<b>14.10.2019</b>	Cabernet Sauvignon

Figure 12. Samples dates and collection for year 2020

<b>1</b> Green stages	<b>27.07.2020</b>	Riesling	<b>04.08.2020</b>	Cabernet Sauvignon
<b>2</b> 25% véraison	<b>13.08.2020</b>	Riesling	<b>14.08.2020</b>	Cabernet Sauvignon
<b>3</b> 50% véraison	<b>28.08.2020</b>	Riesling	<b>28.08.2020</b>	Cabernet Sauvignon
<b>4</b> 75% véraison	/	Riesling	<b>25.09.2020</b>	Cabernet Sauvignon
<b>5</b> Maturity	<b>29.09.2020</b>	Riesling	<b>12.10.2020</b>	Cabernet Sauvignon

Figure 13. Samples dates and collection for year 2021

<b>1</b> Green stages	<b>10.08.2021</b>	Riesling	<b>10.08.2021</b>	Cabernet Sauvignon
<b>2</b> 25% véraison	<b>25.08.2021</b>	Riesling	<b>25.08.2021</b>	Cabernet Sauvignon
<b>3</b> 50% véraison	<b>09.09.2021</b>	Riesling	<b>09.09.202</b>	Cabernet Sauvignon
<b>4</b> 75% véraison	<b>21.09.2021</b>	Riesling	<b>21.09.202</b>	Cabernet Sauvignon
<b>5</b> Maturity	<b>06.10.2021</b>	Riesling	<b>22.10.2021</b>	Cabernet Sauvignon

# Results



**Chapter I: Influence of a moderate increase of atmospheric CO<sub>2</sub> (+20%) on agronomic traits of two grapevines cultivars, Cabernet Sauvignon and Riesling (*Vitis vinifera* L.) using VineyardFACE system**

# **Chapter I: Influence of a moderate increase of atmospheric CO<sub>2</sub> (+20%) on agronomic traits of two grapevines cultivars, Cabernet Sauvignon and Riesling (*Vitis vinifera* L.) using VineyardFACE system**

## **Introduction**

Climate change is modifying the environment of plants, and it becomes obvious that events such as extreme heatwaves are more likely to occur at increased intensity and frequency, according to IPCC (2021). Atmospheric carbon dioxide (CO<sub>2</sub>) concentration, resulting from anthropogenic activities, is expected to increase from 280 ppm before industrial revolution, to up to 700 ppm at the end of the 21<sup>st</sup> century, an increase which participates in the acceleration of global warming.

Viticulture is highly dependent to climate conditions. Moreover, agronomical traits such as yield at harvest, are affected by weather conditions, during the growing season and at different stages of grapevine development. (Petrie & Clingeleffer, 2005) demonstrated that the number of inflorescences is influenced by the temperature encountered during budburst. Bud fertility under elevated CO<sub>2</sub> treatment was also studied, in relation with bunch number and yield, but the response was mainly cultivar dependant (Wohlfahrt et al., 2019).

Moreover, many studies (using FACE systems, Open Top Chambers, greenhouses) were already conducted to understand the effects of elevated CO<sub>2</sub> at the plant scale, and have assessed the enhancement effect of elevated CO<sub>2</sub> on photosynthesis rate in C<sub>3</sub> plants (A. R. Reddy et al., 2010). In addition, the review of Ainsworth & Rogers (2007) summarized the effect of elevated CO<sub>2</sub> on different plant species, and it was unequivocal that assimilation rate increased under elevated CO<sub>2</sub> for C<sub>3</sub> crop, for about 10-15%. A decrease of stomatal conductance was mainly observed, in the range of 20 to 30% for C<sub>3</sub> crop (Ainsworth & Rogers, 2007). It has already been proved that elevated CO<sub>2</sub> has an impact on grapevine leaf physiology, increasing assimilation rate, stomatal conductance and transpiration rate and consequently water use efficiency, for the early years of the VineyardFACE study (Wohlfahrt et al., 2018).

In addition, berry diameter is an important parameter for wine making. It is usually assumed that wine produced with smaller berries would lead to wines of higher quality (Doligez et al., 2013). Berry diameter was recorded for various Tempranillo clones, under climate change conditions (elevated CO<sub>2</sub> and increased temperature, T+4°C), and it was demonstrated in the study of (Arrizabalaga-Arriazu, Gomès, et al., 2020) that elevated CO<sub>2</sub> treatment did not impact berry diameter, bunch weight and the number of berries.

However, studies investigating the effects of elevated CO<sub>2</sub> on grapevine (*Vitis vinifera* L.) were focusing mainly on shorts period of CO<sub>2</sub>-treatments (for example, from fruit set to maturity) (Arrizabalaga-Arriazu, Gomès, et al., 2020). Moreover, such studies were applying a high concentration of CO<sub>2</sub>, such as 700 ppm (Arrizabalaga et al., 2018) (Arrizabalaga-Arriazu, Gomès, et al., 2020) (Arrizabalaga-Arriazu et al., 2021) (Kizildeniz et al., 2015) (Edwards et al., 2016). Overall, although these numerous studies have investigated the short-term effects of elevated CO<sub>2</sub> on grapevine development, there is still a strong need for long-term adaptation studies, as suggested in the review of (Clemens et al., 2022).

The VineyardFACE facility was established in 2012 and the fumigation (+20% to ambient CO<sub>2</sub>), starting in 2014, was operated from sunrise to sunset during the entire year, until present. This facility offers a possibility to overcome technical challenges inherent to the difficulties of studying elevated CO<sub>2</sub> in open-field set-up.

The hypothesis that photosynthesis rate and field parameters were modified by elevated CO<sub>2</sub> conditions, after six years of fumigation, was thus investigated. The objectives of this study were to understand the effects of a moderate increase of CO<sub>2</sub>, i.e. +20% compared to ambient CO<sub>2</sub>, following 6 years of fumigation, on agronomic traits (assimilation rate, yield, berry weight and volume, and berry ripening dynamics, i.e., total soluble solids concentration and total acidity) of two grapevine cultivars (*Vitis vinifera* L.), Cabernet Sauvignon and Riesling, both planted in the VineyardFACE facility.

## Materials & methods

### *Assimilation rate*

In seasons 2019, 2020 and 2021, leaf gas exchange measurements were performed from full bloom to ripening (from June to September) using an open gas exchange measurement system (GFS-3000, Walz GmbH, Germany). One fully developed and sun-exposed leaf was measured from three plants per ring between 8.30 a.m. and latest until 2 p.m. to avoid shading conditions on the leaf surface, due to the row orientation in the VineyardFACE. A LED light source was used, to simulate the surrounding light conditions. Net assimilation rate (A), transpiration rate (E) and stomatal conductance ( $g_s$ ) were calculated in response to the predominant environmental conditions without extra cooling of the leaf chamber. A buffer tank was used to keep the surrounding CO<sub>2</sub> concentration of the measuring chamber stable.

### *Skin and seeds relative mass*

In 2020 and 2021, berries were counted and weighted. Then pulp, skin and seeds were separated manually under liquid nitrogen and the different compartments were weighted. Relative skin and seed mass, expressed in percentage, was calculated by dividing respectively skin frozen weight and seed frozen weight by berry frozen weight.

### *Total soluble solids (TSS) concentration and total acidity*

During the season 2020 and 2021, out of each ring forty berries from the three inner rows were taken, twenty from either side of the row. Berries were crushed and pressed (Longarone 85, QS System GmbH, Norderstedt, Germany), samples were centrifuged at 7830 rpm for 5 min (5430R, Eppendorf AG, Hamburg, Germany). For Oenofoss™ measurements, 1 mL of sample was added in Eppendorf tubes and then centrifuged. For N-OPA analysis, 500 µL of the sample was added to 500 µL of distilled water in Eppendorf tubes, according to a method described by (Dukes & Butzke, 1998) and (Wohlfahrt et al., 2020). The reagent o-phthaldialdehyde/N-

acetyl-l-cysteine (OPA/NAC) permits the derivation of  $\alpha$ - amino acids groups, and absorbance measurements were carried out at 335 nm.

#### *Berry volume calculation*

Equatorial and polar diameter were measured with a digital calliper on each frozen berry from samples 2020 and 2021, and volume of a spheroid, expressed in mm<sup>3</sup>, was calculated according to the following formula (Arrizabalaga-Arriazu et al., 2021):

$$\text{Equation 1: } V = \frac{4}{3} * \pi * r_1^2 * r_2$$

with  $r_1$  equatorial radius and  $r_2$  polar radius, both expressed in mm.

#### *Weather data*

Weather data were recorded in the weather station of VineyardFACE, mean temperature and daily precipitations amount were used to calculate Growing Degree Days (GDD) and precipitations amount during the growing seasons.

#### *Statistical analysis*

Two-ways ANOVA was performed with Rstudio (packages car and tidyverse were used to program the script) to verify the treatment and the year effect.

## **Results**

#### *Weather data*

The growing season 2020 was the warmest among the three years, and the lowest precipitations, in comparison to the growing season 2021, which was the coldest, and with highest precipitations from April to October. Concerning phenological events, flowering was around 12<sup>th</sup> of June 2020 for Riesling, and on 15<sup>th</sup> of June for Cabernet Sauvignon. In 2021, flowering was around 25<sup>th</sup> of June for both cultivars.

Table 2. Growing degree days (GDD) accumulation and precipitations (mm) from April 1<sup>st</sup> to October 31<sup>st</sup> for VineyardFACE weather station, for the growing seasons 2019-2021

<b>Growing season</b>	<b>GDD</b>	<b>Precipitations (mm)</b>
<b>2019</b>	1435.0	301.3
<b>2020</b>	1470.4	208.1
<b>2021</b>	1195.3	307.4

### Assimilation rate

Table 3. Average net assimilation rate ( $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ ) for 2019 (5 timepoints), 2020 (6 timepoints) and 2021, ambient  $\text{CO}_2$  and elevated  $\text{CO}_2$ , for Cabernet Sauvignon (data provided by Dr. Susanne Tittmann)

<b>Treatment / Average net assimilation rate A (<math>\mu\text{mol m}^{-2}\cdot\text{s}^{-1}</math>)</b>	<b>2019</b>	<b>2020</b>	<b>2021</b>
<b>aCO<sub>2</sub></b>	12.17 ± 3.08	12.77±1.25	16.91±2.67
<b>eCO<sub>2</sub></b>	14.19±4.38	16.72±2.13	20.33±2.81
<b>% of aCO<sub>2</sub></b>	+16.64	+30.91	+20.22

Table 4. Average net assimilation rate ( $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ ) for 2019, 2020 and 2021, ambient  $\text{CO}_2$  and elevated  $\text{CO}_2$ , for Riesling (data provided by Dr. Susanne Tittmann)

<b>Treatment\ Average net assimilation rate A (<math>\mu\text{mol m}^{-2}\cdot\text{s}^{-1}</math>)</b>	<b>2019</b>	<b>2020</b>	<b>2021</b>
<b>aCO<sub>2</sub></b>	13.17 ± 3.14	13.57 ± 2.79	16.14 ± 2.53
<b>eCO<sub>2</sub></b>	15.24 ± 3.41	16.51 ± 2.97	18.09 ± 2.79
<b>% of aCO<sub>2</sub></b>	+15.71	+21.67	+12.08

Net assimilation rate of Cabernet Sauvignon increased by 16.64%, 30.91% and 20.22 % under elevated  $\text{CO}_2$  compared to ambient  $\text{CO}_2$  treatment for years 2019, 2020, and 2021 respectively (Table 2). In 2021 the highest assimilation rate was measured for both treatments with 16.91 and 20.33  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for ambient and elevated  $\text{CO}_2$  treated plants respectively.

Net assimilation rate of Riesling increased by 15.71 %, 21.67% and 12.08 % in 2019, 2020, and 2021, respectively, under elevated  $\text{CO}_2$  compared to ambient  $\text{CO}_2$ . For both Cabernet Sauvignon and Riesling, there was an increase of photosynthetic rate during the recent years, determined in the VineyardFACE (Tables 2 & 3).

Table 5. Transpiration rate ( $\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for Cabernet Sauvignon and Riesling (data provided by Dr. Susanne Tittmann)

Average transpiration rate E $\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ Cabernet Sauvignon	2019	2020	2021
aCO <sub>2</sub>	2.12 ± 0.78	2.68 ± 0.88	2.99 ± 0.84
eCO <sub>2</sub>	2.34 ± 0.91	2.97 ± 1.08	3.14 ± 0.87
% of aCO <sub>2</sub>	10.38	10.82	5.01
<b>Riesling</b>			
aCO <sub>2</sub>	3.19 ± 1.06	2.92 ± 0.92	2.89 ± 0.89
eCO <sub>2</sub>	3.32 ± 0.94	3.13 ± 0.91	2.77 ± 0.70
% of aCO <sub>2</sub>	4.07	7.19	-4.15

Transpiration rate increased under elevated CO<sub>2</sub> treatment compared to ambient, except in 2021 for Riesling (Table 4). For Cabernet Sauvignon the percentage of change to ambient CO<sub>2</sub> were in 2019, 2020 and 2021 respectively of 10.38, 10.82 and 5.01 % for Cabernet Sauvignon and of 4.07, 7.19 and -4.15% for Riesling.

Table 6. Stomatal conductance  $g_s$  ( $\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for Cabernet Sauvignon and Riesling (data provided by Dr. Susanne Tittmann)

Average stomatal conductance $g_s$ $\text{mmol m}^{-2}\cdot\text{s}^{-1}$	2019	2020	2021
<b>Cabernet Sauvignon</b>			
aCO <sub>2</sub>	123.28 ± 38.55	165.64 ± 54.18	159.35 ± 29.50
eCO <sub>2</sub>	128.92 ± 45.00	196.05 ± 51.06	164.32 ± 30.69
% of aCO <sub>2</sub>	4.57	18.35	3.12
<b>Riesling</b>			
aCO <sub>2</sub>	132.8 ± 39.35	163.78 ± 47.82	147.85 ± 32.62
eCO <sub>2</sub>	135.28 ± 38.65	166.69 ± 53.92	138.27 ± 31.63
% of aCO <sub>2</sub>	1.86	1.77	-6.47

In addition, stomatal conductance also increased under elevated CO<sub>2</sub> conditions, except in 2021 for Riesling. Stomatal conductance increased by 4.57, 18.35 and 3.12% for Cabernet Sauvignon in 2019, 2020 and 2021 respectively. These changes were reduced for Riesling where increases of 1.86 and 1.77 % were noticed for 2019 and 2020 whereas in 2021 a decrease of 6.47% was noticed (Table 6).

### *Pruning weight*

Table 7. Pruning weight (kg per vine) of Cabernet Sauvignon and Riesling for 2019, 2020 and 2021, ambient CO<sub>2</sub> and elevated CO<sub>2</sub> (Data provided by Dr. Susanne Tittmann)

<b>Treatment/Year</b>	<b>2019</b>	<b>2020</b>	<b>2021</b>
<b>Cabernet Sauvignon</b>			
aCO <sub>2</sub>	0.67 ± 0.26	0.72 ± 0.29	0.54 ± 0.17
eCO <sub>2</sub>	0.66 ± 0.2	0.74 ± 0.19	0.59 ± 0.16
<b>Riesling</b>			
aCO <sub>2</sub>	0.65 ± 0.16	0.65 ± 0.17	0.55 ± 0.13
eCO <sub>2</sub>	0.64 ± 0.12	0.69 ± 0.15	0.56 ± 0.12

Pruning weight in 2019 did not demonstrate any significant difference between ambient (0.67 ± 0.26 kg) and elevated CO<sub>2</sub> treatment (0.66 ± 0.2 kg) in 2019 for Cabernet Sauvignon. Moreover, pruning weight for Riesling in 2019 was in average 0.65 ± 0.16 kg for ambient CO<sub>2</sub> treatment and 0.64 ± 0.12 kg for elevated CO<sub>2</sub> treatment, so no difference was found for this parameter between the two treatments (Table 6).

### *Crop yield at harvest*

Table 8. Crop yield at harvest (kg per vine) of Cabernet Sauvignon and Riesling, aCO<sub>2</sub> = ambient CO<sub>2</sub> treatment and eCO<sub>2</sub> = elevated CO<sub>2</sub> treatment (data provided by Dr. Susanne Tittmann)

<b>Treatment/Year</b>	<b>2019</b>	<b>2020</b>	<b>2021</b>
<b>Cabernet Sauvignon</b>			
aCO <sub>2</sub>	1.85 ± 0.65	2.14 ± 0.38	1.40 ± 0.61
eCO <sub>2</sub>	2.08 ± 0.65	2.30 ± 0.35	1.96 ± 0.64
<b>Riesling</b>			
aCO <sub>2</sub>	1.75 ± 0.63	2.70 ± 0.28	2.29 ± 0.65
eCO <sub>2</sub>	1.85 ± 0.75	2.77 ± 0.23	2.48 ± 0.77



Yield at harvest of Cabernet Sauvignon increased, between ambient CO<sub>2</sub> and elevated CO<sub>2</sub> treatment, of 12.4%, 7.5% and 40% in 2019, 2020 and 2021, respectively. Higher yield for elevated CO<sub>2</sub> was also determined for Riesling, which increased of 5.7%, 2.6% and 8.3%, respectively (Table 8). The highest yield increase between the two treatments was found in 2021, for both cultivars. However, the highest yield for both cultivars was reported in 2020, which was the year with the highest GDD (Table 1).

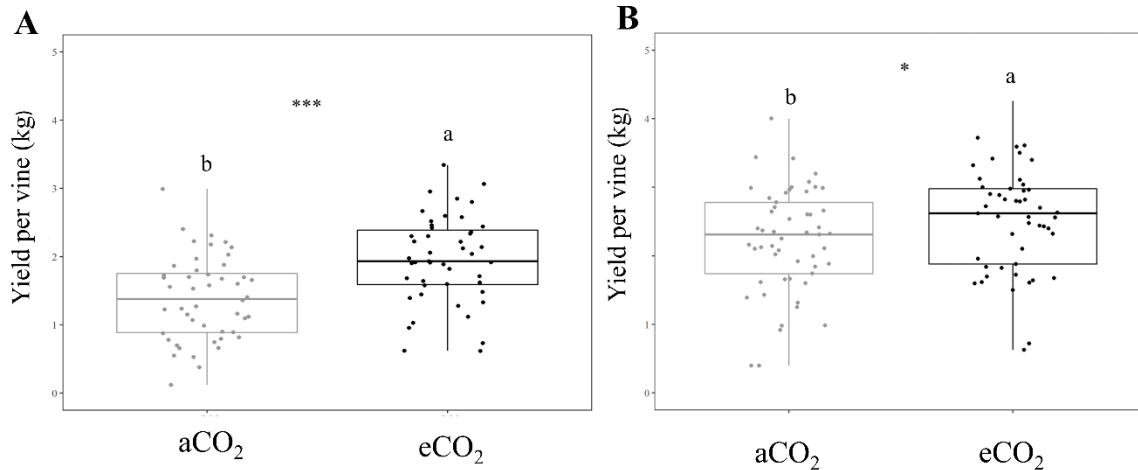


Figure 14. The crop yield per vine at harvest of A: Cabernet Sauvignon and B: Riesling, aCO<sub>2</sub> = ambient CO<sub>2</sub> treatment and eCO<sub>2</sub> = elevated CO<sub>2</sub> treatment, year 2021; \*\*\*, p<0.001, \*\*, p<0.01, \*, p<0.05 and n.s. not significant, means with letters in common within the same chart (A or B) are not statistically different

Cabernet Sauvignon yield of ambient treated plants was with  $1.40 \pm 0.61$  kg (mean  $\pm$  standard deviation) per vine in 2021, significantly lower than of elevated CO<sub>2</sub> treated grapevine with  $1.93 \pm 0.64$  kg per vine (high significance) (Figure 14A). Riesling yield in 2021 was as well higher for elevated CO<sub>2</sub> treatment with  $2.29 \pm 0.65$  kg per vine on average for ambient CO<sub>2</sub> treatment compared to elevated CO<sub>2</sub> treatment with  $2.48 \pm 0.77$  kg per vine (Figure 14B).

Yield at harvest in 2021 was higher for elevated CO<sub>2</sub> treatment in comparison to ambient treatment for both Cabernet Sauvignon and Riesling. After statistical analysis, it was found that yield at harvest was significantly higher for elevated CO<sub>2</sub> in 2021, and this was the case for the years 2019 and 2020 as well. Higher significance was found for Cabernet Sauvignon in 2021 (p<0.001) in comparison to Riesling (p<0.05). With the findings of assimilation rate, we can assume that reproductive growth as well as vegetative growth are still enhanced under elevated CO<sub>2</sub> treatment for the recent years of VineyardFACE use.

*Relative skin and seed mass*

Table 9. Relative skin and seed mass (%) of Cabernet Sauvignon, year 2020 and 2021, aCO<sub>2</sub>=ambient aCO<sub>2</sub> and eCO<sub>2</sub>=elevated CO<sub>2</sub>

<b>Year</b>	<b>Treatment/ DOY</b>	<b>217</b>	<b>227</b>	<b>241</b>	<b>269</b>	<b>286</b>
<b>2020</b>	<b>Relative skin mass</b>					
	aCO <sub>2</sub>	10.98 ± 1.14	9.50 ± 0.22	9.14±0.54	10.34±0.12	10.52±0.53
	eCO <sub>2</sub>	10.48±0.17	9.72± 0.3	9.46± 0.24	10.16± 0.18	11.02± 1.44
<b>2020</b>	<b>Relative seed mass</b>					
	aCO <sub>2</sub>	9.83 ± 0.70	9.41 ± 0.90	6.72 ± 0.61	4.25 ± 0.21	3.87 ± 0.33
	eCO <sub>2</sub>	9.24 ± 0.49	8.46 ± 0.68	6.60 ± 0.64	4.28 ± 0.12	3.95 ± 0.19
	<b>Treatment/ DOY</b>	<b>222</b>	<b>237</b>	<b>252</b>	<b>264</b>	<b>295</b>
<b>2021</b>	<b>Relative skin mass</b>					
	aCO <sub>2</sub>	11.48 ± 0.47	13.92 ± 2.65	12.47 ± 1.41	10.68 ± 0.66	20.30 ± 2.09
	eCO <sub>2</sub>	11.54 ± 0.26	13.13 ± 0.26	12.44 ± 1.60	12.49 ± 1.71	20.03 ± 1.51
	<b>Relative seed mass</b>					
	aCO <sub>2</sub>	10.11 ± 0.19	8.37± 0.19	5.98 ± 0.33	4.62 ± 0.08	5.12 ± 0.23
	eCO <sub>2</sub>	9.58 ± 0.88	8.18 ± 0.96	5.92 ± 0.11	4.92 ± 0.44	5.12 ± 0.78

Relative skin mass and seeds mass are the ratio between skin and seeds fresh weight and berry fresh weight. After applying one-way ANOVA test, neither relative skin mass nor relative seeds mass were significantly different between ambient and elevated CO<sub>2</sub> treatment in 2020. However, relative seed mass of Cabernet Sauvignon seemed to be lower for elevated CO<sub>2</sub> until

75% *véraison*, where relative seed mass was lower for ambient CO<sub>2</sub> treatment, even if the differences tended to disappear.

Seeds and skin mass were also not significantly different between the treatments in 2021. However, the same trend was observed in 2021, with relative seed mass being lower for elevated CO<sub>2</sub> treatment until 75% *véraison*, where the trend reversed, with minor differences (Table 9).

Table 10. Relative skin and seeds mass (%) of Riesling for year 2020, aCO<sub>2</sub>=ambient aCO<sub>2</sub>, and eCO<sub>2</sub>=elevated CO<sub>2</sub>

Treatment/ DOY	209	226	241	/	273
<b>Relative skin mass</b>					
aCO <sub>2</sub>	9.51 ± 0.88	8.12 ± 0.26	11.07 ± 4.86	/	8.29 ± 0.26
eCO <sub>2</sub>	9.98 ± 0.59	8.91 ± 0.23	8.99 ± 0.93		8.41 ± 0.44
<b>Relative seed mass</b>					
aCO <sub>2</sub>	9.52 ± 0.67	8.42 ± 0.69	5.3 ± 0.05	/	4.40 ± 0.28
eCO <sub>2</sub>	9.49 ± 0.49	8.32 ± 0.63	6.4 ± 0.85		4.41 ± 0.03

Table 11. Relative skin and seeds mass (%) of Riesling, for year 2021, ambient CO<sub>2</sub> and elevated CO<sub>2</sub>

Treatment/ DOY	222	237	252	264	279
<b>Relative skin mass</b>					
aCO <sub>2</sub>	8.68 ± 0.87	9.16 ± 0.88	10.52 ± 1.9	9.48 ± 1.21	12.43 ± 0.99
eCO <sub>2</sub>	8.60 ± 0.49	8.84 ± 0.84	10.22 ± 0.51	10.02 ± 0.56	12.98 ± 1.45
<b>Relative seed mass</b>					
aCO <sub>2</sub>	8.62 ± 1.23	7.00 ± 0.66	5.07 ± 1.02	4.01 ± 0.29	4.42 ± 0.3
eCO <sub>2</sub>	8.88 ± 0.74	7.41 ± 0.53	5.24 ± 0.26	4.14 ± 0.22	4.61 ± 0.5

For Riesling, in 2020, relative skin mass was higher for elevated CO<sub>2</sub> for the early *véraison* timepoints, and at 75% *véraison* the relative skin mass was lower for elevated CO<sub>2</sub> treatment, as the point 50% *véraison* was missing in 2020 the trend cannot be recognized (Table 10).

#### *Technological maturity*

Technological maturity is defined by Carbonneau et al. (1998) as the point where sugars reach a plateau (when 22 °Brix is attained for red cultivars) and that acidity is low. Measuring total

soluble solids and total acidity allows us to monitor berry ripening describing the technical maturity.

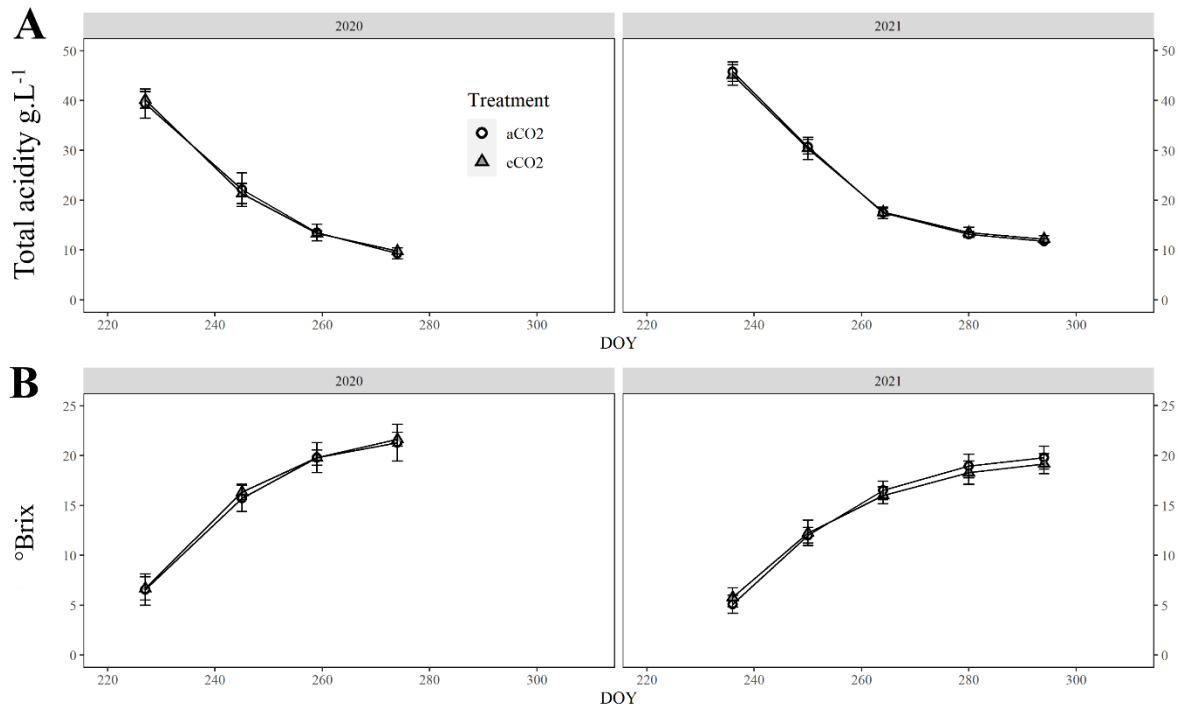


Figure 15. Technological maturity parameters (A: Total acidity in g.L<sup>-1</sup> and B: TSS in °Brix) of Cabernet Sauvignon in 2020 and 2021, aCO<sub>2</sub> = ambient CO<sub>2</sub> treatment and eCO<sub>2</sub>= elevated CO<sub>2</sub> treatment

In 2020, total acidity for Cabernet Sauvignon decreased from  $39.36 \pm 2.95$  g.L<sup>-1</sup> to  $9.33 \pm 1.14$  g.L<sup>-1</sup> for ambient CO<sub>2</sub> treatment and from  $40.15 \pm 1.65$  g.L<sup>-1</sup> for elevated CO<sub>2</sub> treatment, where four time points were analyzed. In 2021, five time points were realized and total acidity was ranging from  $45.76 \pm 1.95$  g.L<sup>-1</sup> to  $11.8 \pm 0.48$  g.L<sup>-1</sup> for ambient CO<sub>2</sub> treatment, and from  $45.13 \pm 2.07$  g.L<sup>-1</sup> to  $12.22 \pm 0.65$  g.L<sup>-1</sup> for elevated CO<sub>2</sub> treatment. TSS concentration did not reach 22°Brix in 2021 due to weather conditions (Table 1).

For Cabernet Sauvignon, in 2020 TSS concentration ranged from  $6.55 \pm 1.58$  °Brix to  $21.3 \pm 1.85$  °Brix for ambient CO<sub>2</sub> treatment, while for elevated CO<sub>2</sub> treatment TSS concentration was between  $6.69 \pm 1.17$  to  $21.65 \pm 0.69$  °Brix. In 2021, TSS concentration varied from  $5.09 \pm 0.9$  °Brix to  $19.8 \pm 1.15$  °Brix for ambient CO<sub>2</sub> treatment, and for elevated CO<sub>2</sub> treatment it varied from  $5.77 \pm 0.95$  to  $19.18 \pm 1.0$  °Brix. In both 2020 and 2021, 22 °Brix was not reached.

The vintage effect was predominant in comparison to the treatment effect. To sum up, technological maturity parameters such as TSS (expressed in °Brix) and total acidity (g.L<sup>-1</sup>), seemed to be not impacted by eCO<sub>2</sub> treatment in both 2020 and 2021, as previously published by Wohlfahrt et al. (2020) during the adaptation phase of the VineyardFACE.

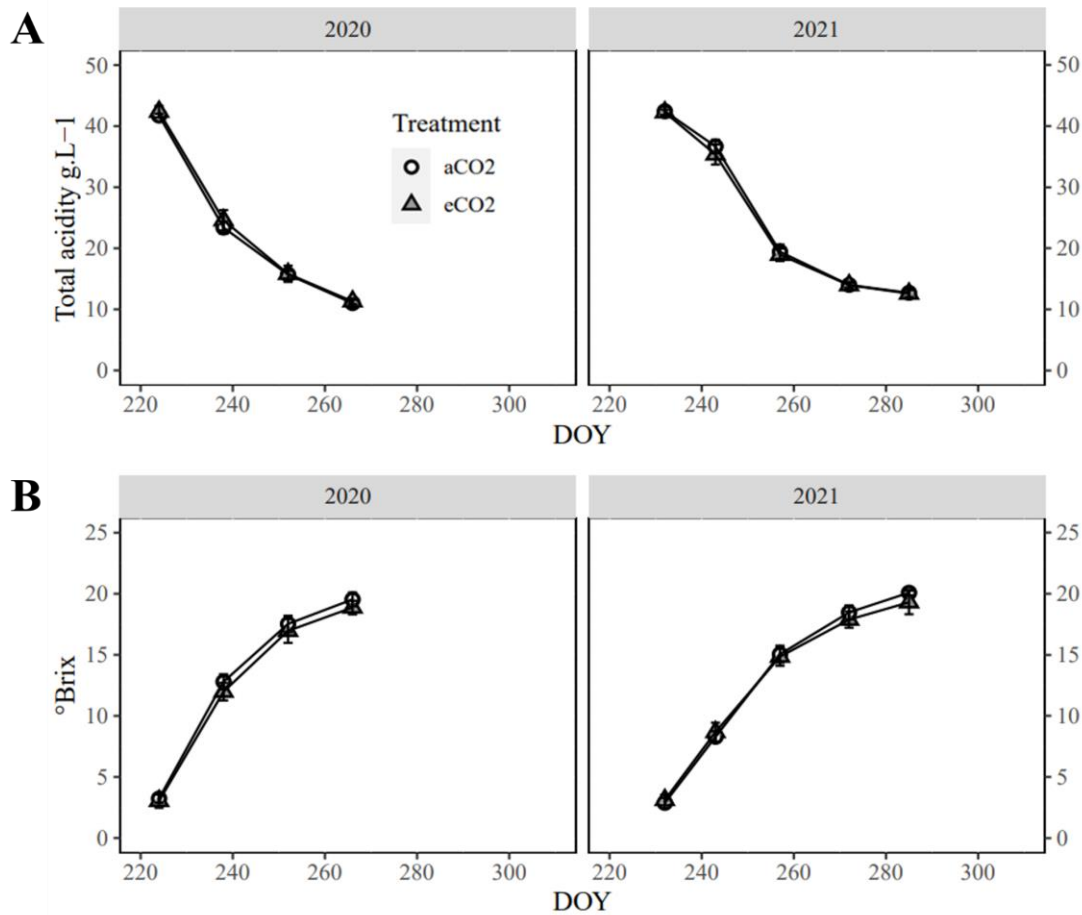


Figure 16. Technological maturity parameters of Riesling in 2020 and 2021 (A: Total acidity in g.L<sup>-1</sup> and B: TSS in °Brix), aCO<sub>2</sub> = ambient CO<sub>2</sub> treatment and eCO<sub>2</sub> = elevated CO<sub>2</sub> treatment

Concerning Riesling, total acidity ranged, in year 2020, from  $41.72 \pm 0.39$  g.L<sup>-1</sup> to  $11.01 \pm 0.53$  g.L<sup>-1</sup> for ambient CO<sub>2</sub> treatment and from  $42.36 \pm 0.96$  g.L<sup>-1</sup> to  $11.30 \pm 0.46$  g.L<sup>-1</sup> for elevated CO<sub>2</sub> treatment. In comparison, total acidity was in 2021, ranging from  $42.43 \pm 0.74$  g.L<sup>-1</sup> to  $12.7 \pm 0.73$  g.L<sup>-1</sup> for ambient CO<sub>2</sub> treatment and from  $42.25 \pm 0.42$  g.L<sup>-1</sup> to  $12.56 \pm 0.56$  g.L<sup>-1</sup> for elevated CO<sub>2</sub>.

TSS concentration ranged, for Riesling, in 2020 from  $3.23 \pm 0.33$  °Brix to  $19.53 \pm 0.56$  °Brix for ambient CO<sub>2</sub> treatment, and from  $3.04 \pm 0.56$  °Brix to  $18.87 \pm 0.56$  °Brix for elevated CO<sub>2</sub> treatment respectively. In 2021, TSS concentration started from  $2.87 \pm 0.41$  °Brix, attained  $20.08 \pm 0.22$  °Brix for ambient CO<sub>2</sub> treatment and ranged from  $3.11 \pm 0.43$  °Brix to  $19.29 \pm 0.97$  °Brix for elevated CO<sub>2</sub> treatment (Figure 16B).

Overall, no difference occurred for Riesling between the two treatments in 2020 and 2021 in terms of total acidity and TSS accumulation.

## Yeast assimilable nitrogen

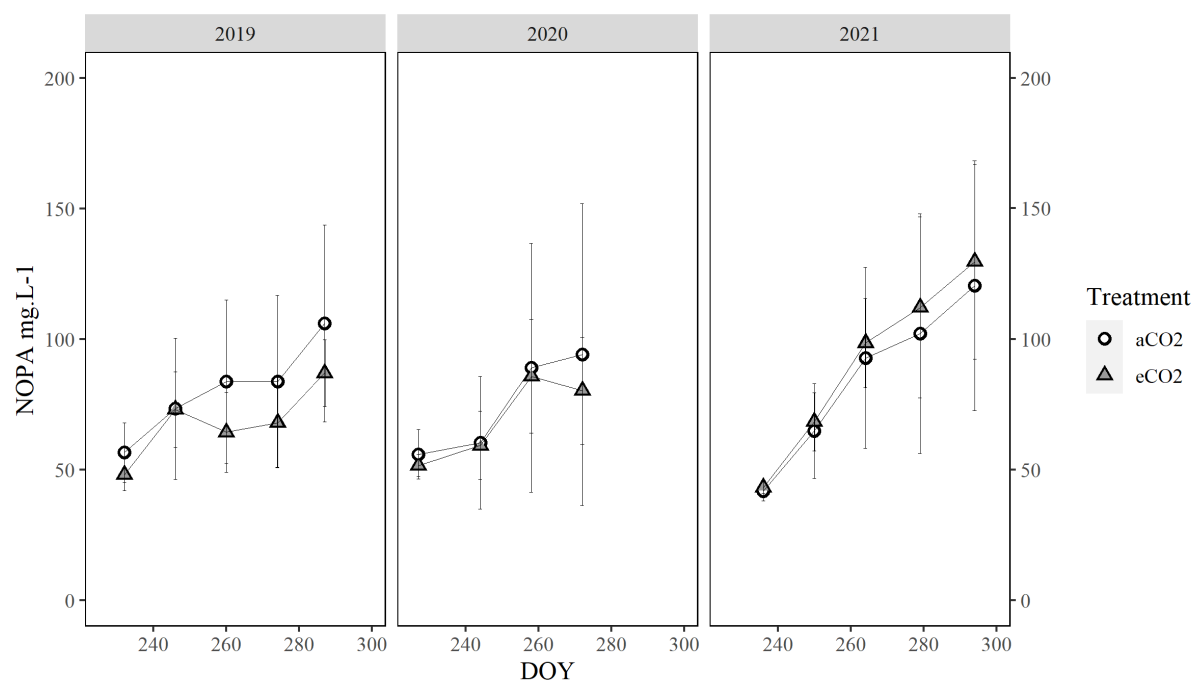


Figure 17. N-OPA values ( $\text{mg.L}^{-1}$ ) of Cabernet Sauvignon, in 2019, 2020 and 2021, aCO<sub>2</sub> = ambient CO<sub>2</sub> treatment and eCO<sub>2</sub>= elevated CO<sub>2</sub> treatment

N-OPA value corresponds to YAN (Yeast Assimilable Nitrogen), representing all amino acids, except proline. Amino acids values in must were very variable and impacted by the vintage. In 2019, N-OPA of Cabernet Sauvignon ranged from  $56.55 \pm 11.44 \text{ mg.L}^{-1}$  for E-L 33 to  $106 \pm 37.65 \text{ mg.L}^{-1}$  at harvest for ambient CO<sub>2</sub> treatment, whereas for elevated CO<sub>2</sub> treatment the values ranged from  $48 \pm 6.08 \text{ mg.L}^{-1}$  to  $87 \pm 12.84 \text{ mg.L}^{-1}$ . Moreover, at harvest in 2020, amino acids concentration reached  $94.11 \pm 57.88 \text{ mg.L}^{-1}$  for ambient CO<sub>2</sub> and attained  $80.22 \pm 20.47 \text{ mg.L}^{-1}$  for elevated CO<sub>2</sub>. The highest value were attained in 2021 (Figure 17), with  $120.56 \pm 47.89 \text{ mg.L}^{-1}$  for ambient CO<sub>2</sub>, and  $129.67 \pm 37.29 \text{ mg.L}^{-1}$  for elevated CO<sub>2</sub>. In 2019 and 2020, amino acids concentration of Cabernet Sauvignon was generally lower for elevated CO<sub>2</sub> treatment, whereas in 2021, amino acids concentration was higher under elevated CO<sub>2</sub> treatment, however not significantly.

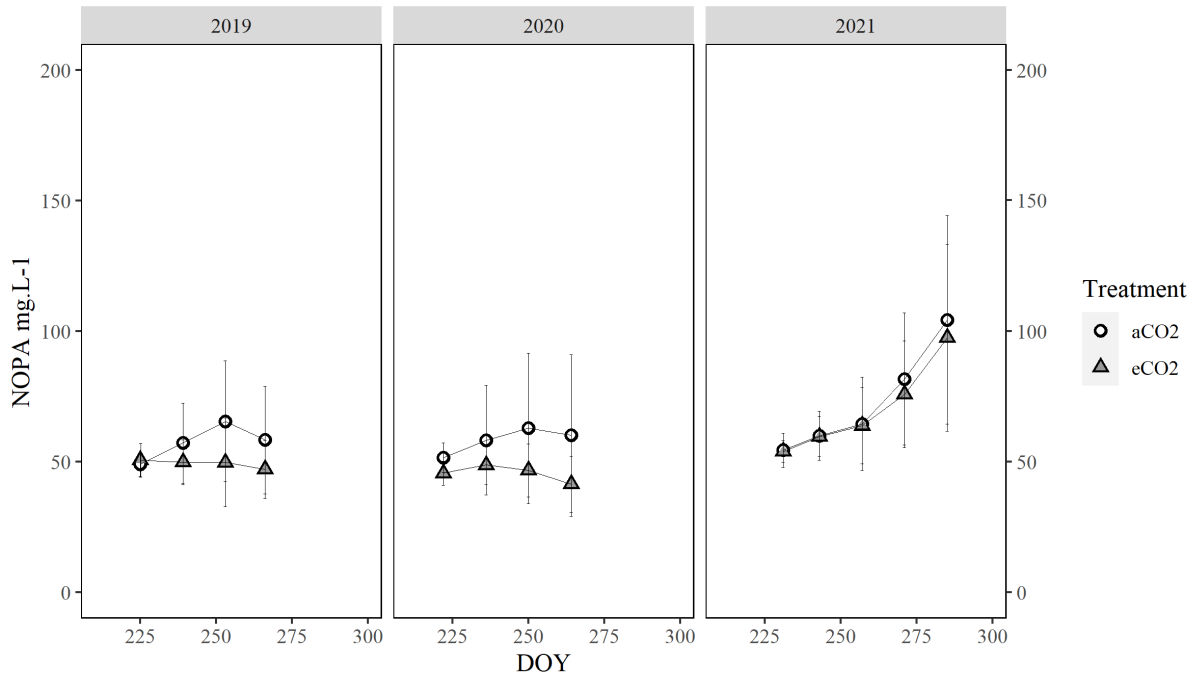


Figure 18. N-OPA values ( $\text{mg.L}^{-1}$ ) of Riesling in 2019, 2020 and 2021, aCO<sub>2</sub> = ambient CO<sub>2</sub> treatment and eCO<sub>2</sub>= elevated CO<sub>2</sub> treatment

N-OPA values of Riesling were lower in comparison to Cabernet Sauvignon. In 2019 and 2020, total amino acids concentration was lower for elevated CO<sub>2</sub> treatment in comparison to ambient CO<sub>2</sub> treatment, however not significantly. Moreover, the highest amino acids concentration of Riesling was reached at harvest in 2021 for both ambient and elevated CO<sub>2</sub> treatment. Indeed, amino acids concentration of must in 2019 reached  $58.25 \pm 20.68 \text{ mg.L}^{-1}$  for ambient CO<sub>2</sub> treatment, and for elevated CO<sub>2</sub> treatment  $47 \pm 11.05 \text{ mg.L}^{-1}$ , and in 2021 this specific value attained for ambient CO<sub>2</sub> treatment with  $104.33 \pm 39.9 \text{ mg.L}^{-1}$ , when elevated CO<sub>2</sub> treatment reached  $97.44 \pm 35.79 \text{ mg.L}^{-1}$  (Figure 18).

Berry weight and berry volume

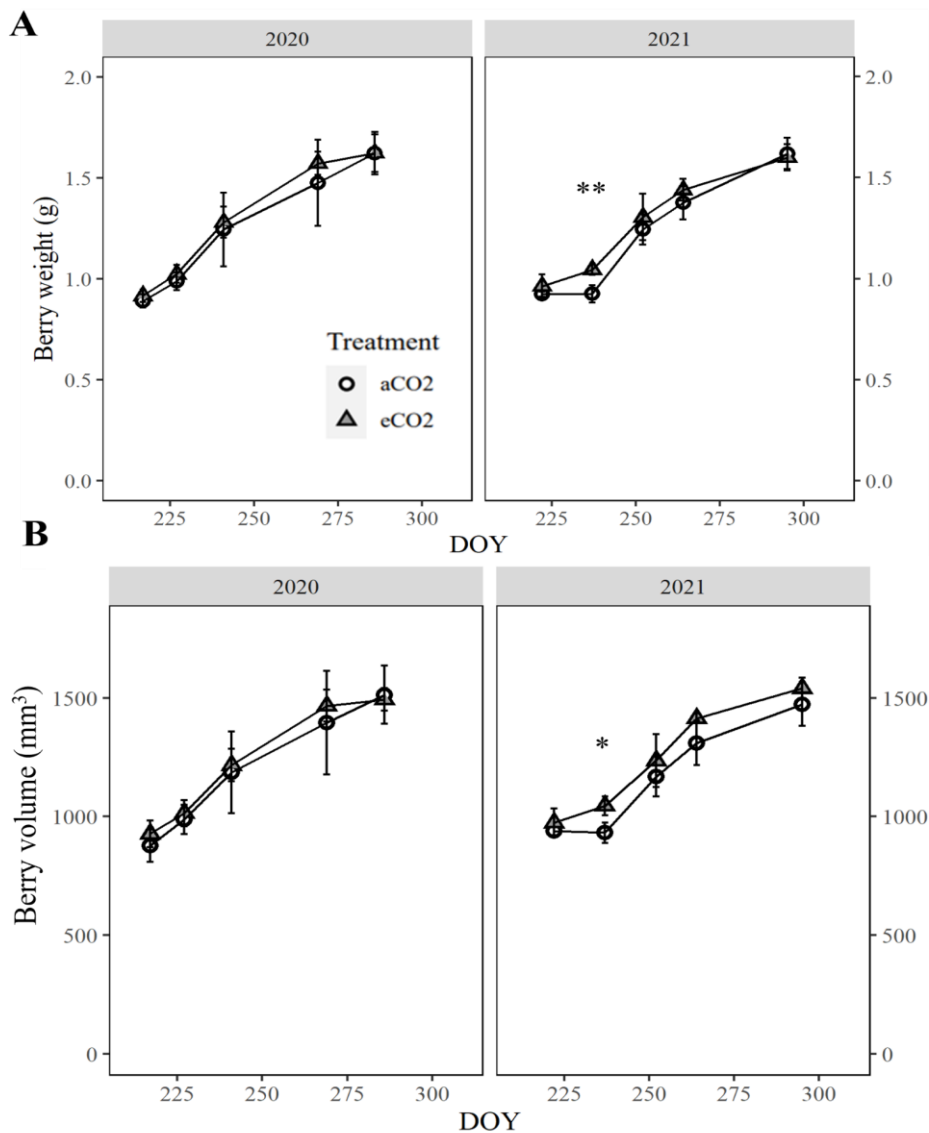


Figure 19. Berry weight (A) and berry volume (B) of Cabernet Sauvignon in 2020 and 2021, aCO<sub>2</sub> = ambient CO<sub>2</sub> treatment and eCO<sub>2</sub>= elevated CO<sub>2</sub> treatment

Berry weight and volume are also important to understand berry development and elevated CO<sub>2</sub> concentration could impact these parameters. For ambient CO<sub>2</sub> treatment, berry volume ranged from  $877.45 \pm 68.35 \text{ mm}^3$  in 2020 for the E-L stage 33 (green stage), to  $1513.62 \pm 123.10 \text{ mm}^3$  at harvest (Figure 19B). This corresponds to an increase of 73 % in berry volume in 2020. Concerning elevated CO<sub>2</sub> treatment, berry volume ranged for green stage from  $927.38 \pm 55.48 \text{ mm}^3$  to  $1493.36 \pm 47.60 \text{ mm}^3$  which corresponds to an increase of 61% in 2020. In the year 2021, for green stage and for ambient CO<sub>2</sub> treatment, berry volume varied from  $938.99 \pm 16.41 \text{ mm}^3$  to  $1473.53 \pm 92.10 \text{ mm}^3$  at harvest, which corresponds to an increase of 57%. For elevated CO<sub>2</sub> treatment, berry volume varied from  $974.41 \pm 59.65 \text{ mm}^3$  to  $1541.21 \pm 43.74 \text{ mm}^3$  (Figure 19B) corresponding to a very similar increase of 58%.



Berry weight showed a similar trend (Figure 19A), ranging from  $0.89 \pm 0.03$  g to  $1.62 \pm 0.1$  g at harvest in 2020 for the ambient CO<sub>2</sub> treatment. Moreover, for the elevated CO<sub>2</sub> treatment berry weight ranged from  $0.92 \pm 0.03$  g to  $1.62 \pm 0.09$  g, which corresponds to an increase of 82% and of 76%, respectively. However, during the ripening season, berry weight and berry volume demonstrated a trend of higher single berry weight as well as higher berry volume for Cabernet Sauvignon, but this trend was only significant at one developmental stage in one year (25% *véraison*) in 2021. An increase in berry weight was also noticed by (Wohlfahrt et al., 2020) where single berry weight of Cabernet Sauvignon increased for all dates during ripening in 2015 and 2016.

Moreover, at harvest, these differences tended to disappear, which was previously reported by (Bindi et al., 2001). The final berry weight and volume (at harvest) seemed to be not affected by elevated CO<sub>2</sub> treatment.

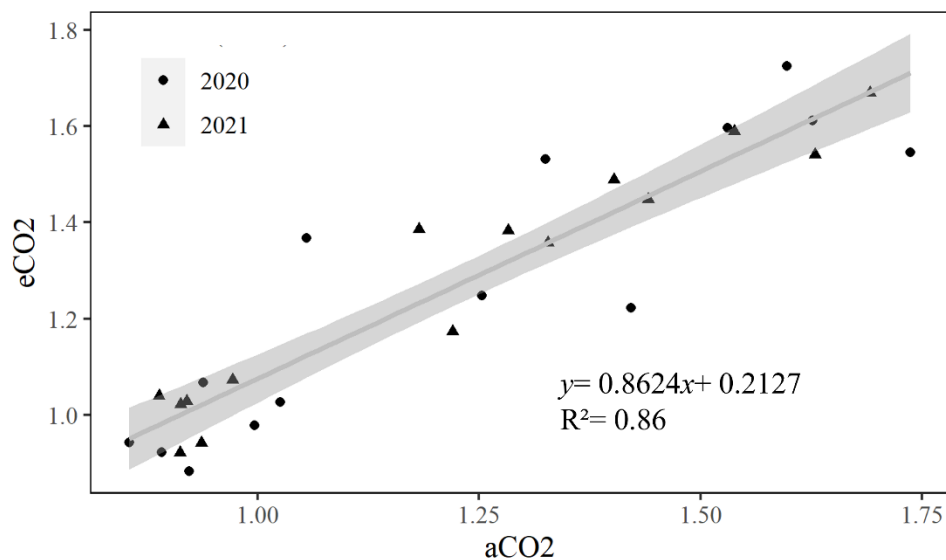


Figure 20. Relationship between aCO<sub>2</sub> and eCO<sub>2</sub> of single berry weight values for Cabernet Sauvignon in 2020 and 2021

The response curve of Cabernet Sauvignon was  $y = 0,8624x + 0,203$  ( $R^2 = 0,8051$ ) in 2020, and  $y = 0,8634x + 0,2196$  ( $R^2 = 0,9272$ ) in 2021, so the response was slightly higher for 2021 compared to 2020, however no general increase of single berry weight was drawn, for ambient CO<sub>2</sub> treatment compared to elevated CO<sub>2</sub>, when all sampling days are homogenized (Figure 20).

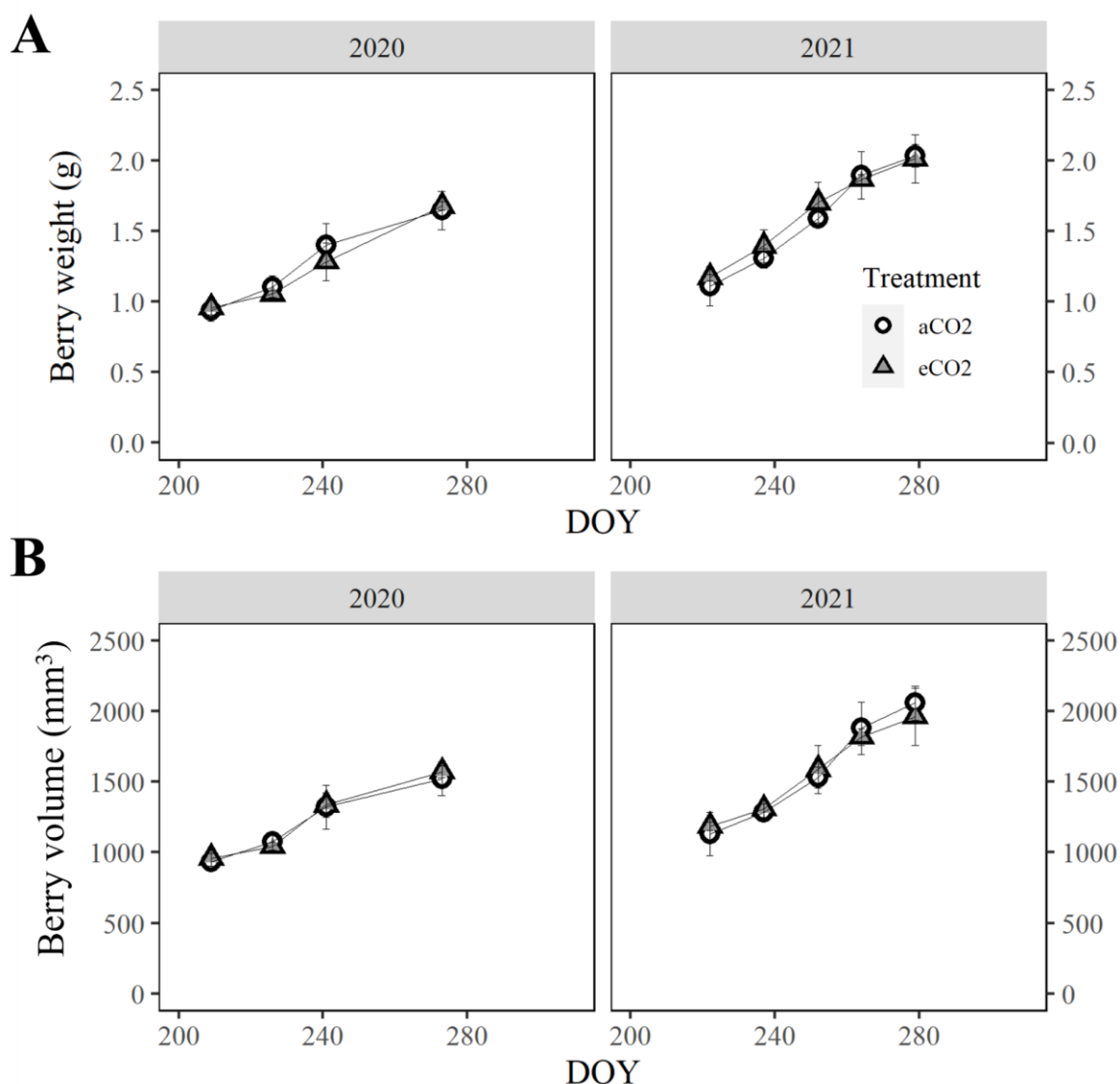


Figure 21. Berry weight (A) and berry volume (B) of Riesling in 2020 and 2021, aCO<sub>2</sub> = ambient CO<sub>2</sub> treatment and eCO<sub>2</sub>= elevated CO<sub>2</sub> treatment

For the ambient CO<sub>2</sub> treatment in 2020, berry weight for Riesling ranged from  $0.94 \pm 0.07$  g at green stage to  $1.65 \pm 0.14$  g at maturity, corresponding to an increase of 75,5% (Figure 21A). For elevated CO<sub>2</sub> treatment, this increase between green stage and harvest was of 75%, with berry weight being slightly higher for this treatment, however not significantly. In 2021, berry weight was higher compared to 2020, with  $1.11 \pm 0.14$  g ambient CO<sub>2</sub> treatment at green stage and  $2.04 \pm 0.08$  g at harvest. Although higher berry weight was found for elevated CO<sub>2</sub> treatment a green stage ( $1.17 \pm 0.022$  g), the differences tended to disappear at maturity ( $2.01 \pm 0.17$  g).

Berry volume for Riesling increased in 2020 for ambient CO<sub>2</sub> treatment, from  $930.65 \pm 61.85$  mm<sup>3</sup> at green stage, to  $1522.30 \pm 118.24$  mm<sup>3</sup>, corresponding to an increase of 63,6% (Figure 21B). For elevated CO<sub>2</sub> treatment, berries were slightly higher but not significantly. Moreover, at green stage, berry volume was  $959.98 \pm 56.27$  mm<sup>3</sup>, and at harvest it was  $1572.20 \pm 42.37$  mm<sup>3</sup>, corresponding to an increase of 63,7 %. In 2021, berry volume increased, for ambient

CO<sub>2</sub>, from  $1130.68 \pm 152.86 \text{ mm}^3$  to  $2058.48 \pm 122.36 \text{ mm}^3$  at harvest, and for elevated CO<sub>2</sub> from  $1185.22 \pm 31.73 \text{ mm}^3$  to  $1968.79 \pm 202.64 \text{ mm}^3$ . Generally, due to weather conditions and especially high precipitations (Table 1), Riesling berries had a bigger volume in 2021 compared to 2020.

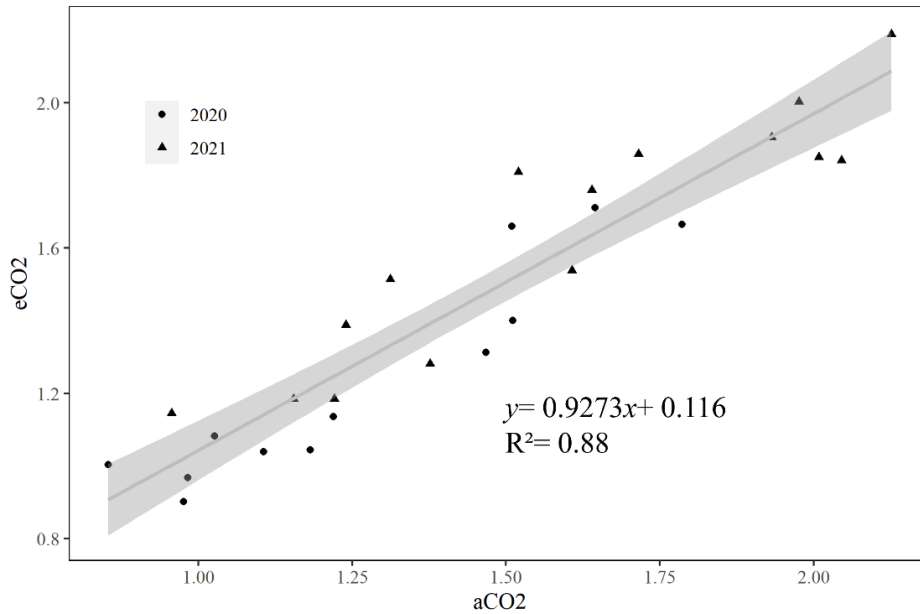


Figure 22. Relation between aCO<sub>2</sub> and eCO<sub>2</sub> of single berry weight values for Riesling in 2020 and 2021

The response curve of Riesling was in 2020:  $y = 0.9194x + 0.0744$  ( $R^2 = 0.8726$ ), and in 2021 was:  $y = 0.8297x + 0.3122$  ( $R^2 = 0.8558$ ), so surprisingly, the response was higher in 2020 than in 2021 (Figure 22). However, as for Cabernet Sauvignon, no conclusion can be drawn when all sampling days are homogenized.

## Discussion

The increase in assimilation rate in response to elevated CO<sub>2</sub> for C<sub>3</sub> plants is well documented in the literature (Ainsworth & Rogers, 2007) (Ainsworth & Long, 2005). This topic was studied for grapevine in various experimental conditions (Edwards et al., 2016). In temperature gradient greenhouses (TGG), (Arrizabalaga-Arriazu, Morales, et al., 2020) found higher photosynthesis activity, measured at *véraison*. Moreover, (Wohlfahrt et al., 2018) found an increase in assimilation rate (A), stomatal conductance (g<sub>s</sub>), transpiration rate (E) and water use efficiency (WUE<sub>i</sub>) under elevated CO<sub>2</sub> treatment in VineyardFACE, for both cultivars and for the three growing seasons. Assimilation rate of Cabernet Sauvignon increased by 12, 30 and 43 % respectively in 2014, 2015 and 2016 (Wohlfahrt et al., 2018) whereas in 2019, 2020 and 2021 the percentages of increase of 17%, 31% and 21% respectively were observed, in the present study.

However, as described by Salazar-Parra et al. (2015), photosynthetic rates increased under elevated CO<sub>2</sub> treatment (irrespective of water availability and temperature treatments, which were also applied) after 10 days of treatment (700 ppm of CO<sub>2</sub>). But after 20 days, no effect of CO<sub>2</sub> treatment on photosynthetic rate was noticed, suggesting an acclimation effect. Indeed, in the study of Kizildeniz et al. (2021) photosynthetic rates were also measured at 700 ppm of CO<sub>2</sub> in red and white Tempranillo. Both cultivars showed lower photosynthesis rates under elevated CO<sub>2</sub> treatment irrespective of temperature, water treatment and their combination.

These results suggest an acclimation of grapevine to elevated CO<sub>2</sub>, whereas in the VineyardFACE, net assimilation rate was still stimulated by elevated CO<sub>2</sub>, as demonstrated by an increase of up to 30% from 2019 to 2021. Indeed, the VineyardFACE system is designed to apply +20% of CO<sub>2</sub>, which means a gradual and continuous increase of CO<sub>2</sub>, compared to a transient application of 700 ppm of CO<sub>2</sub>, directly applied in greenhouse-controlled conditions by Kizildeniz et al. (2021). Some differences in methods (greenhouses versus open-field experiments, high doses of CO<sub>2</sub> versus moderate increase) could explain the differences in the obtained results. Arp (1991) showed that the downregulation of photosynthetic capacity under elevated CO<sub>2</sub> was occurring in potted plants, whereas plants grown in open field do not demonstrate this pattern. The increase of transpiration rate and stomatal conductance under elevated CO<sub>2</sub> in the present study were consistent with the previous work realized on VineyardFACE (Wohlfahrt et al., 2018) albeit this is not always the case in the literature. Indeed, C<sub>3</sub> crop were displaying a decrease in g<sub>s</sub> under elevated CO<sub>2</sub> conditions in the review of (Ainsworth & Rogers, 2007).

Yield enhancement under elevated CO<sub>2</sub> for C<sub>3</sub> plants is also widely reported in literature (Reddy et al., 2010). Indeed, a study of seventeen years on CO<sub>2</sub> enrichment on sour orange trees demonstrated that during this period, the biomass was overall enhanced by 70% (Kimball et al., 2007). Another study on FACE for rice crops demonstrated an increase of 30% of yield during the 2 years study (Zhu et al., 2015), which was previously reported by a meta-analysis on the effects of elevated CO<sub>2</sub> on rice (Ainsworth, 2008). According to Wohlfahrt et al. (2018), higher yield under elevated CO<sub>2</sub> was found for both cultivars for the initial three years of the considered period (2014 to 2016). The present study showed that yield was significantly higher from 2019 to 2021 for both Cabernet Sauvignon and Riesling. Moreover, no effect of elevated CO<sub>2</sub> on bunch number per vine during the period 2014-2016 was reported (Wohlfahrt et al.,

2018). Predawn leaf water potential (measured in 2015 and 2016) differed significantly between ambient and elevated CO<sub>2</sub> treatment with higher values for elevated CO<sub>2</sub>, however a significant effect of year was also noticed (Wohlfahrt et al., 2018).

Concerning our study, the following question can be phrased: where will the excess of assimilated carbon be allocated in the plant?

Berry weight and berry volume for Cabernet Sauvignon were higher under elevated CO<sub>2</sub> treatment, but not significantly, and for Riesling this trend was not apparent. Wohlfahrt et al. (2020) found that single berry weight of Cabernet Sauvignon increased for all dates under elevated CO<sub>2</sub> in 2015 and 2016, whereas for Riesling higher single berry weights were noticed for dates closer to harvest than *véraison*. Hence, it can be speculated whether the two cultivars replay differently to elevated CO<sub>2</sub> conditions in response to their adaptation in fruit size characteristics. The two cultivars seem to respond in a slightly different manner to elevated CO<sub>2</sub> treatment.

Relative skin mass under climate change conditions and water scarcity was studied by (Arrizabalaga-Arriazu et al., 2021), showing that for all clones considered, water deficit significantly increased the relative skin mass from mid-*véraison* onwards, with the exception of 2 weeks after mid-*véraison*, and climate change conditions (elevated temperature and elevated CO<sub>2</sub>) had the same effect 2 weeks after mid-*véraison* and at maturity .

Furthermore, no difference in technological maturity, such as total acidity and soluble solids, was found neither in 2020 nor in 2021 for both Cabernet Sauvignon and Riesling, as a consequence, this particular parameter seemed to be not impacted by elevated CO<sub>2</sub> treatment. Wohlfahrt et al. (2020) indicated a slight increase of sugar yield due to elevated CO<sub>2</sub> for both cultivars, but not significant. The present findings are in accordance with the results of Wohlfahrt et al. (2020), where no difference occurred in Cabernet Sauvignon for TSS and total acidity between the treatments and over three years. However, for Riesling, slight differences could be found in 2015 and 2016 for TSS, and in 2016 for total acidity. Moreover, amino acid concentration in must was not significantly different between ambient and elevated CO<sub>2</sub> neither in the timeframe 2014 to 2016 nor the timeframe 2019 to 2021, due to high variability within treatments (Wohlfahrt et al., 2020) .

If the berry composition is not modified, carbohydrates from photosynthesis enhancement could also be allocated to the root system, as some studies on grapevine demonstrated (Reddy et al., 2021). Indeed, carbohydrates excess induced to photosynthesis could be transported to the roots, where they could lead to greater root growth as it is suggested in the review of (Thompson et al., 2017) for C<sub>3</sub> crops. Not only was the root growth impacted by elevated CO<sub>2</sub> but also the root architecture of *Arabidopsis thaliana* was modified, in the study of Lee-Ho et al. (2007). Indeed, root length, number of roots and root diameter significantly increased under elevated CO<sub>2</sub>, of about 133%, 120% and 47%, respectively. Moreover, branching patterns were also modified, indicating a clear effect of elevated CO<sub>2</sub> on root system. In future studies, the impact of elevated CO<sub>2</sub> on roots could be investigated in depth regarding these parameters. However, it will need to be considered that root observations remain difficult and some of the quantifications will require destructive methods which will be counterproductive in long term experiments and may only be performed at the end of the experiment.

## **Conclusion**

In the present work, the impact of a moderate and gradual increase (+20%) of CO<sub>2</sub> was investigated on agronomic traits (i.e. assimilation rate, yield at harvest, pruning weight, berry weight and berry volume, skin and seed relative mass) as well as on ripening dynamics of Cabernet Sauvignon and Riesling, using VineyardFACE system in Geisenheim, for three years (2019, 2020, 2021). It was confirmed that, even though the vines received such kind of treatment for more than six years, photosynthesis (assimilation rate) and yield at harvest were still enhanced under elevated CO<sub>2</sub>, especially for Cabernet Sauvignon, as it was the case for the first years of VineyardFACE use. A trend was found for Cabernet Sauvignon berries having a higher volume and weight under elevated CO<sub>2</sub>, however not significant. For Riesling no major trend was found concerning this parameter.

## **Chapter II: The influence of elevated CO<sub>2</sub> on berry composition of Cabernet Sauvignon and Riesling using the VineyardFACE system**

## Chapter II : The influence of elevated CO<sub>2</sub> on berry composition of Cabernet Sauvignon and Riesling using the VineyardFACE system

### Introduction

Climate change predictions assess a mid-term (i.e. 2041-2060) increase of 1.6°C (most optimistic scenario) to 2.4°C (most pessimistic scenario) of global mean temperature (IPCC, 2021). Among crops, viticulture is meant to be greatly affected by climate change. Indeed, the increase of sugar concentration and thus alcohol content, as well as the reduction of total acidity, are already observed worldwide over the past three decades impacting one the profile of the wines. As a consequence, in Languedoc (France), alcohol content in wine increased from 11% to 14% and total acidity decreased from 6.0 to 4.5 g.L<sup>-1</sup> over the last 35 years (van Leeuwen et al., 2019).

The in-depth investigation of climate change related factors on berry composition at harvest, which is a critical point for wine quality, is thus highly requested (Jones et al., 2005). Organoleptic properties of grape berries are partly defined by their concentration in sugars (mainly glucose and fructose) and organic acids (i.e. mainly tartaric and malic acid). At the onset of ripening (called *véraison*) sugar start to accumulate in mesocarp cells and malic acid content starts to decline (Conde et al., 2007). Technological maturity, usually defined as sugars and organic acids ratios, is an indicator of berry ripeness and permits to determine the day of harvest.

On the other hand, amino acids, except proline, are an important nitrogen source for yeast during alcoholic fermentation. Moreover isoleucine, leucine, phenylalanine, and valine are precursors of esters and higher alcohols in wine (Garde-Cerdán & Ancín-Azpilicueta, 2008). The effects of some climate change related factors on berry composition have already been investigated, mainly regarding high temperature (Lecourieux et al., 2017; Rienth et al., 2014 ; Arrizabalaga et al., 2018) and water deficit (Deluc et al., 2009 ; Savoi et al., 2016). For example, in Western Australian regions, anthocyanins in berries of Cabernet Sauvignon are projected to decrease of about 18% by 2070 (Barnuud et al., 2014). Conversely, elevated CO<sub>2</sub> effect on grape berry composition has been less thoroughly studied and mostly using controlled or semi-controlled set-ups, in enclosed systems such as greenhouses or Open Top Chambers (OTCs) (Arrizabalaga-Arriazu et al., 2020 ; Kizildeniz et al., 2015). In temperature gradient greenhouses, water stress and elevated temperature plus elevated CO<sub>2</sub> concentration combined were applied, it was noticed that elevated CO<sub>2</sub> played a mitigation role in attenuating the effects of drought (Kizildeniz et al., 2015).

However, greenhouses or Open Top Chambers can introduce some biases inherent to enclosed systems, as demonstrated by (Poorter et al., 2016) where pot-grown plants displayed faster growth rates, different morphology, and higher nitrogen content. FACE systems are open field set-ups designed to study the effects of elevated CO<sub>2</sub> on various crops and showing less risks of such biases. Indeed, several FACE experiments are installed in annual or perennial crops worldwide, such as rice (Fukayama et al., 2011), cotton (Mauney et al., 1994), mustard (Ruhil et al., 2015), coffee (Ghini et al., 2015).



The first experiments on a FACE facility on grapevine (*Vitis vinifera* L.) studying berry composition were carried out by (Bindi et al., 2001), on already established field grown vines. These authors demonstrated that biomass was stimulated by elevated CO<sub>2</sub>, and that acids and sugars content were increased under elevated CO<sub>2</sub> during berry ripening. However, it was also noticed that the CO<sub>2</sub> effect disappeared at maturity, having consequently no significant effect on wine quality (Bindi et al., 2001).

Within the VineyardFACE plot, berry quality was already studied during the first years of vine's adaptation by Wohlfahrt et al. (2021), showing that must and the composition of young wines were not negatively affected by elevated CO<sub>2</sub>.

However, this chapter emphasizes to address the hypothesis whether an elevation in CO<sub>2</sub> concentration impacts berry quality in vines that have been grown under enriched carbon dioxide concentration for more than seven years. Thus, berry composition in primary metabolites (hexoses, organic acids, free amino acids) and secondary metabolites (anthocyanins) was determined to verify the effects of elevated CO<sub>2</sub> treatment over three growing seasons (2019, 2020, 2021), using VineyardFACE facility at Geisenheim.

## **Materials & methods**

### *Sugar quantification*

Sugar content in pulp samples is quantified by enzymatic reactions, by measuring at 340 nm the amount of NADPH formed after successive enzymatic reactions involving Hexokinase, Phosphoglucosomerase and Glucose-6- phosphate deshydrogenase. Samples were diluted with a Precision 2000 robot (Biotek Instruments Inc., Winooski, VT, USA) and analyzed with an EPOCH microplate reader (Biotek Instruments Inc., Winooski, VT, USA), and the software was Gen5, as described by (L. Wang et al., 2021).

### *Organic acids*

Tartaric acid and malic acid were analyzed in pulp samples with a continuous flux analyzer TRAACS800 (Bran & Luebbe, Plaisir, France) equipped with peristaltic pumps. Malic analysis is realized with an enzymatic quantitative method using L- malate dehydrogenase (L-MDH) which converts L-malate into oxaloacetate. Tartaric way uses a colorimetric quantitative analysis with reactant ammonium vanadate which forms a yellow-colored complex (metapervanadyl tartrate complex) quantified by spectrophotometry at 530 nm (Arrizabalaga-Arriazu et al., 2021). Software used for the report design is AACE (Bran & Luebbe, Plaisir, France).

### *Amino acids*

Free amino acids from pulp samples were derived with 6-aminoquinoly1-N-hydroxysuccinimidyl carbamate (AccQ-Tag) as derivatization reagent, and samples were analyzed on U-HPLC. Chromatograms were recorded with excitation at 250 nm and emission at 395 nm, with malvidin-3-glucoside as standard. The software Chroméléon 7.2.10 (ThermoScientific®) was used to calculate peak areas. Total amino acids concentration was

expressed as the sum of the 20 amino acids quantified (Alanine, Arginine, Aspartic acid, Asparagine, Cysteine, GABA, Glycine, Glutamic acid, Glutamine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tyrosine, Valine). Anthocyanins composition was calculated by regrouping amino acids according to their respective pathways:  $\alpha$ -ketoglutarate (proline, arginine, glutamic acid, glutamine, GABA, histidine), pyruvate (alanine, valine, leucine), aspartate (threonine, aspartic acid, asparagine, isoleucine, methionine, lysine), shikimate (tyrosine, phenylalanine), phosphoglycerate (serine, glycine).

### *Anthocyanins*

The analyses were carried out according to (Arrizabalaga-Arriazu et al., 2021). Freeze-dried skin (20-30 mg), initially frozen at  $100 \text{ mg} \pm 10 \%$  (Alph1-4, Christ, Osterode, Germany), were extracted using 500 or 750  $\mu\text{L}$  of methanol acidified with 0.1 % HCl (v/v). Samples were centrifuged (4000 rpm, 1 min) and filtered into U-HPLC vials through 0.2  $\mu\text{m}$  porosity filter (Millex-GS Syringe filter unit, Millipore) and 1 mL syringe (Normject®, Germany) The malvidin-3-glucoside standard was used to quantify anthocyanin concentration.

### *Statistical analysis*

One-way ANOVA was performed with Rstudio (packages rstatix and tidyverse were used to program the script) to verify the treatment effect on the various metabolites studied. Principal Component Analysis (PCA) were carried out with packages FactoMineR and factoextra.

## **Results**

### *Sugars*

Sugars started to accumulate in mesocarp cell vacuoles from *véraison* onwards when berries began to soften and changed color. In this work, total sugars concentration is assumed to be the sum of glucose and fructose concentrations. At harvest, for Cabernet Sauvignon, total sugars concentration showed the greatest differences in 2021 compared to 2019 and 2020, where total sugars concentration was  $170.87 \pm 3.56 \text{ mg.g}^{-1} \text{ FW}$  for ambient  $\text{CO}_2$  and  $158.89 \pm 9.95 \text{ mg.g}^{-1}$  for elevated  $\text{CO}_2$  (Figure 23), whereas in the previous years the differences tended to disappear at maturity. A trend, albeit not significant, was noticed for sugars (fructose, glucose, total sugars) to be lower under elevated  $\text{CO}_2$  throughout the ripening period in 2021, although in 2019 and 2020 it was the contrary at maturity. Overall, the vintage effect was obviously predominant compared to the  $\text{CO}_2$  effect.

Mostly, no major significant differences between the two  $\text{CO}_2$  treatments (ambient  $\text{CO}_2$  and elevated  $\text{CO}_2$ ) were observed in 2019, 2020 and 2021, when one-way ANOVA test was applied for Cabernet Sauvignon, and the observed trends tended to disappear at maturity (except in 2021). However, glucose concentration was significantly lower for elevated  $\text{CO}_2$  in 2019 at DOY=253 and total sugars concentration was lower at DOY=269.

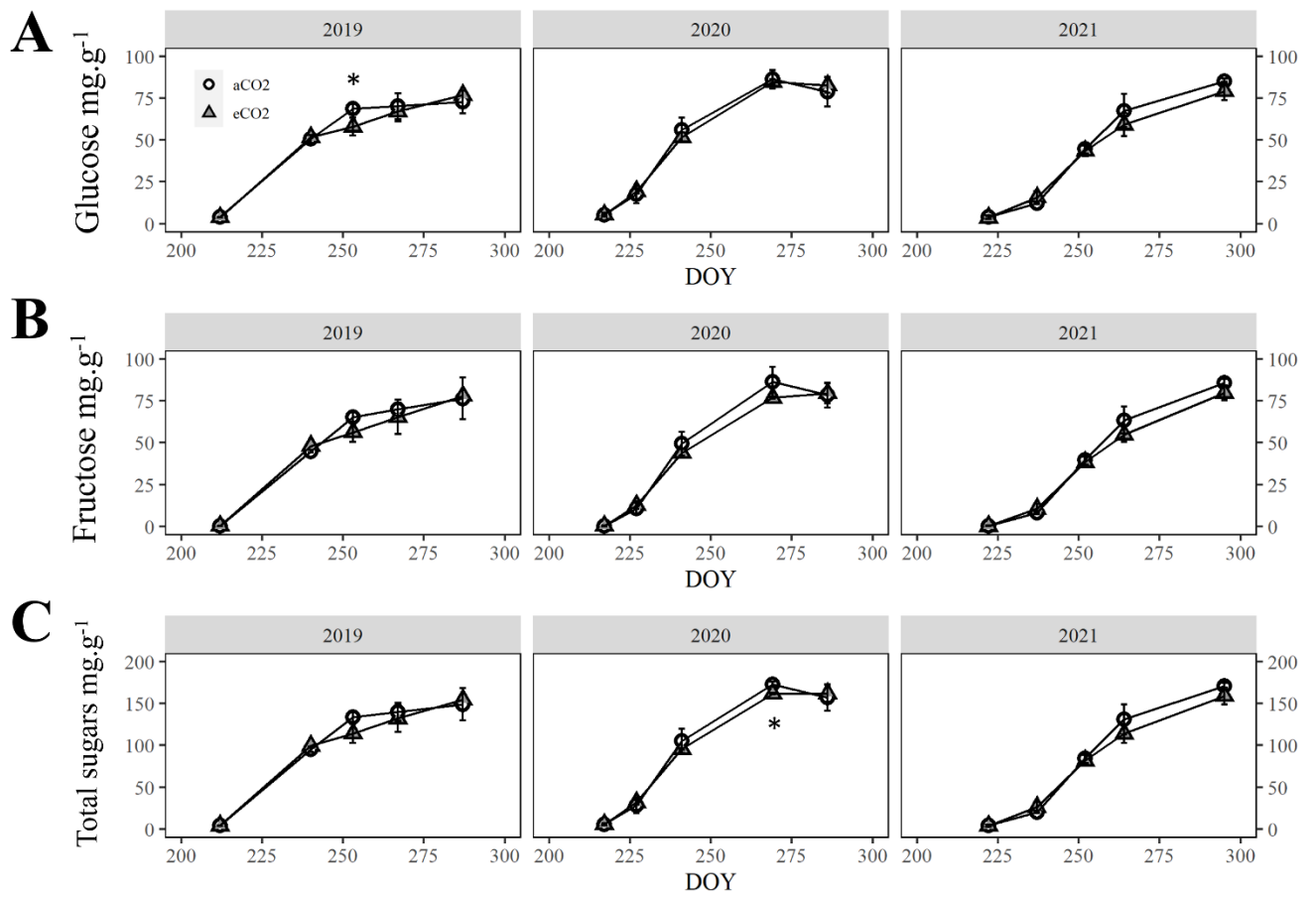


Figure 23. Sugars concentration (A: Glucose, B: Fructose, C: Total sugars) for 2019,2020 and 2021; cv. Cabernet Sauvignon, aCO<sub>2</sub> = ambient CO<sub>2</sub> and eCO<sub>2</sub>= elevated CO<sub>2</sub>; DOY Day Of Year

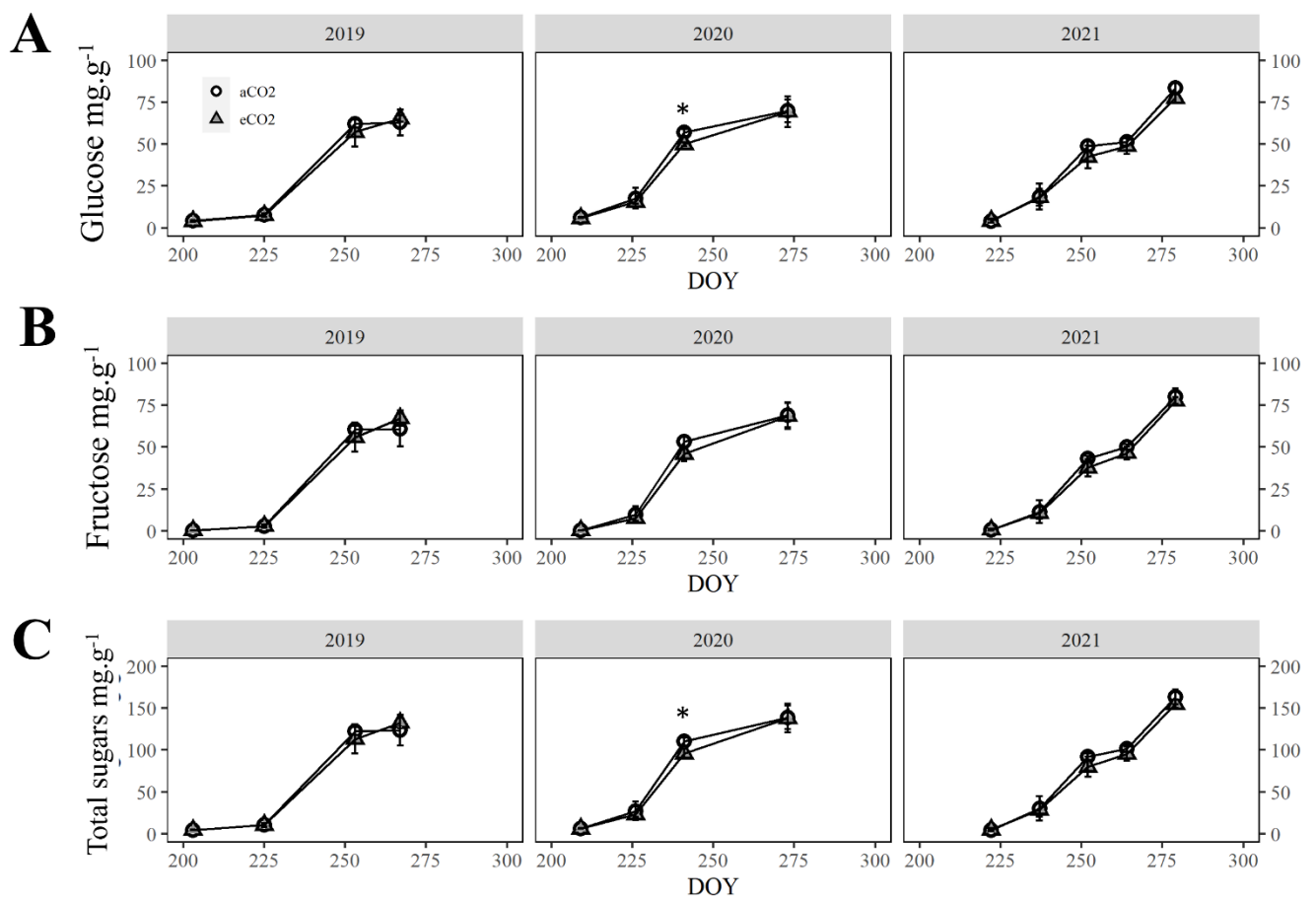


Figure 24. Sugars concentration (A: Glucose, B: Fructose, C: Total sugars) for 2019,2020 and 2021; cv. Riesling, aCO<sub>2</sub> = ambient CO<sub>2</sub> and eCO<sub>2</sub>= elevated CO<sub>2</sub>; DOY Day Of Year

Total sugars concentration in Riesling ranged from  $4.31 \pm 0.11 \text{ mg.g}^{-1}$  to  $123.62 \pm 18.1 \text{ mg.g}^{-1}$  for ambient CO<sub>2</sub> and from  $4.61 \pm 0.37 \text{ mg.g}^{-1}$  to  $132.46 \pm 6.2 \text{ mg.g}^{-1}$  for elevated CO<sub>2</sub> in 2019 (Figure 24). A trend of lower sugar concentration in Riesling under elevated CO<sub>2</sub> was found for all years. However, this trend is statistically not significant, total sugars and glucose concentration only showed a significant difference at DOY= 241 in 2020 when one-way ANOVA test was applied ( $p < 0.05$ ).

### Organic acids

Contrary to sugar accumulation, organic acids (mostly malic and tartaric acids) started to accumulate in the early stages of berry development (i.e. herbaceous developmental phase) and decrease after *véraison* (Figure 25). It is acknowledged that malic acid is degraded by respiration, whereas tartaric acid decrease is due to a dilution effect, jointly with increase in berry volume.

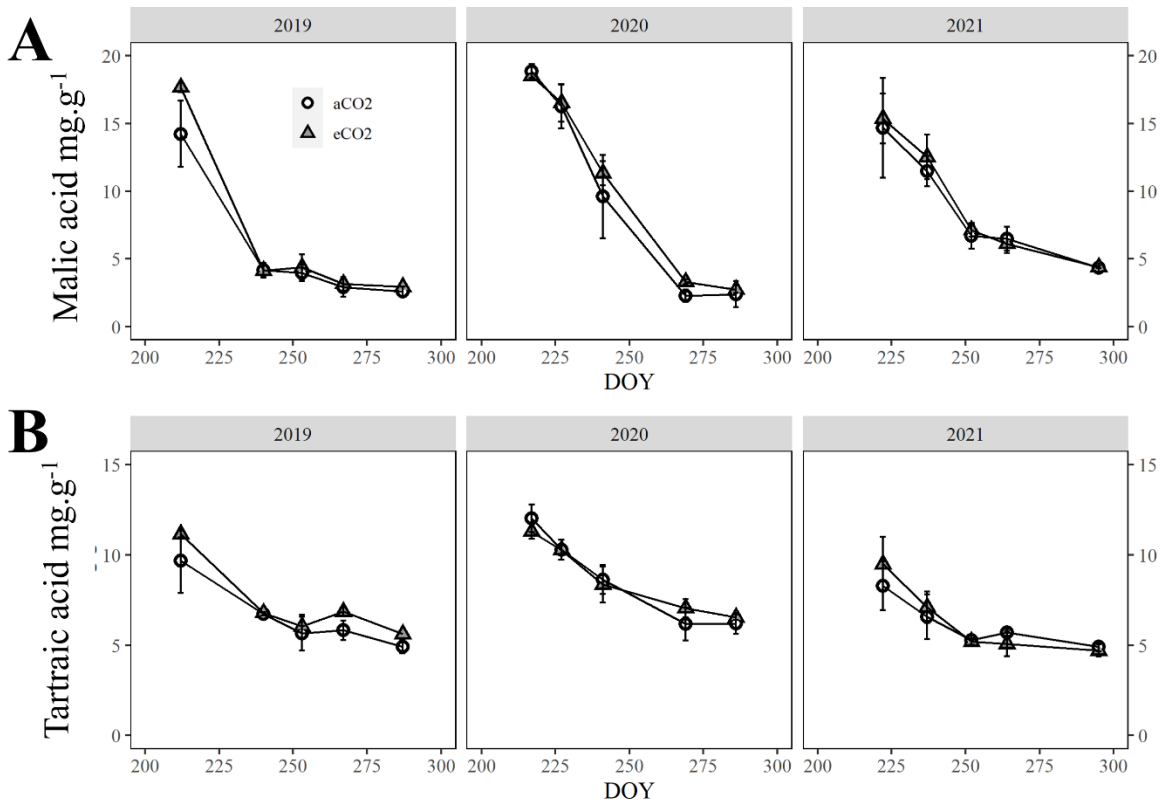


Figure 25. Organic acids (A: Malic acid, B: Tartaric acid) for the years 2019,2020 and 2021 (cv. Cabernet Sauvignon), aCO<sub>2</sub> = ambient CO<sub>2</sub> and eCO<sub>2</sub>= elevated CO<sub>2</sub>; DOY Day Of Year

From the first measurement to harvest, malic acid content in Cabernet Sauvignon decreased by 81,6 %, 87,3 % and 70,4 % in 2019, 2020 and 2021 respectively, for ambient CO<sub>2</sub> treatment and by 83,4 %, 85,1 % and 71,4 % for elevated CO<sub>2</sub> treatment. A trend can be assumed, with malic acid slightly higher for elevated CO<sub>2</sub> treatment, but firstly this trend was not statistically significant and secondly, at harvest all differences nullified (Figure 25A). Tartaric acid in Cabernet Sauvignon decreased by 49,2 %, 48,4% and 40,8% in 2019, 2020 and 2021 respectively, for ambient CO<sub>2</sub> treatment and by 49,6 %, 42% and 50,5% for elevated CO<sub>2</sub> treatment (Figure 25B).

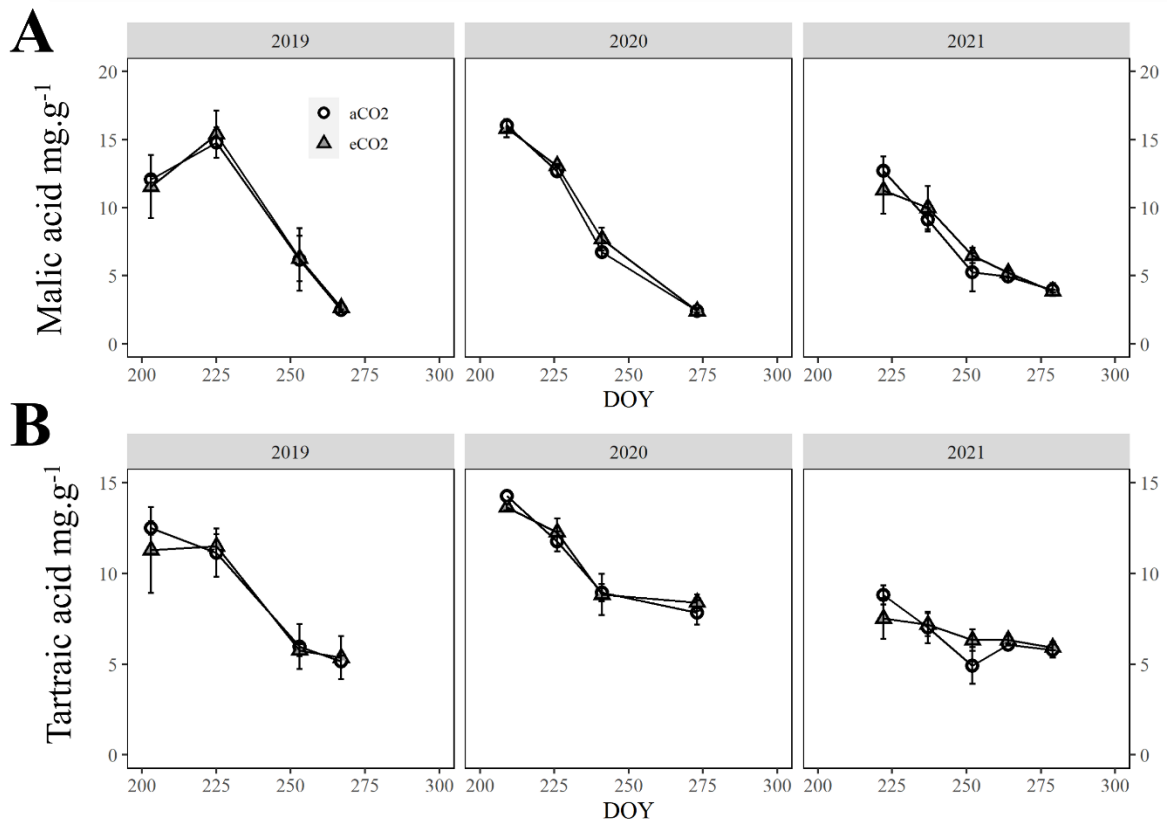


Figure 26. Organic acids (A: Malic acid, B: Tartaric acid) for the years 2019,2020 and 2021 (cv. Riesling) , aCO<sub>2</sub> = ambient CO<sub>2</sub> and eCO<sub>2</sub>= elevated CO<sub>2</sub>; DOY Day Of Year

Organic acids were also quantified in cultivar Riesling. Malic acid content decreased of 79,4 %, 85% and 68,8% in 2019, 2020 and 2021, respectively, for ambient CO<sub>2</sub> treatment and the decrease rates were of 77,1 %, 84,8 % and 65,8 % for elevated CO<sub>2</sub> treatment in 2019, 2020 and 2021, respectively. Malic acid content was also slightly higher under elevated CO<sub>2</sub> treatment; however, this trend was not significant (Figure 26A). Tartaric acid decrease rates were 58,8%, 44,9% and 34,2% in 2019, 2020 and 2021, respectively, for ambient CO<sub>2</sub> treatment. For the elevated CO<sub>2</sub> treatment, tartaric acid decreased of 52,7 %, 38,4 % and 21,2%, respectively (Figure 26B). After statistical analysis, organic acids in Riesling seemed to be more affected by the vintage effect than elevated CO<sub>2</sub> treatment.

Tartaric acid to malic acid ratio

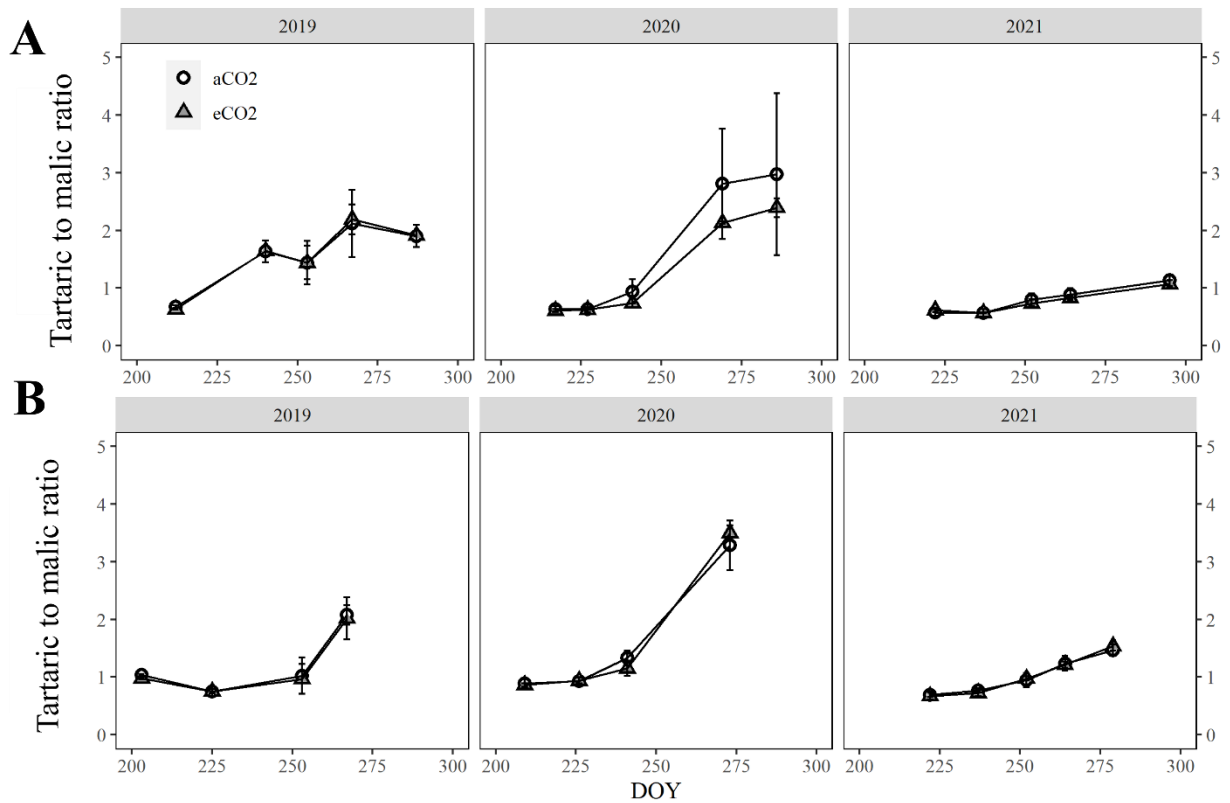


Figure 27. Tartaric acid to malic acid ratio (A: Cabernet Sauvignon, B: Riesling), for the years 2019,2020 and 2021, aCO<sub>2</sub> = ambient CO<sub>2</sub> and eCO<sub>2</sub>= elevated CO<sub>2</sub>; DOY Day Of Year

For Cabernet Sauvignon, tartaric to malic ratio seemed to be differ depending on the years, whereas for Riesling this ratio seemed to display the same curve but with different slopes. However, no difference was noticeable between the treatments for both cultivars (Figure 27A&B).

Amino-acids

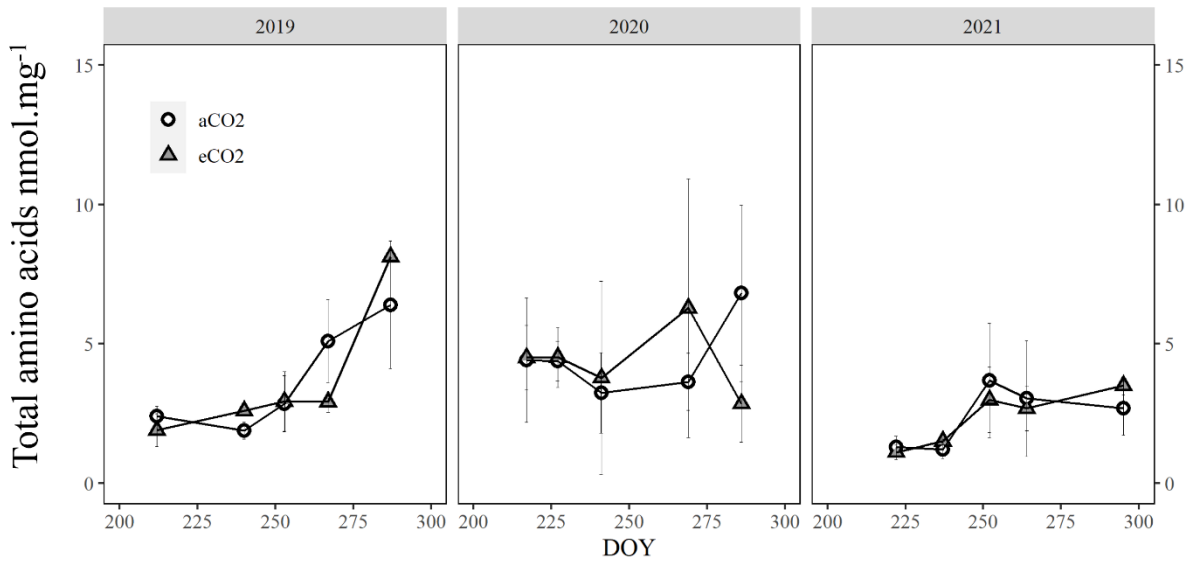


Figure 28. Total amino-acids (nmol.mg<sup>-1</sup>) in Cabernet Sauvignon, for the years 2019, 2020 and 2021, aCO<sub>2</sub> = ambient CO<sub>2</sub> and eCO<sub>2</sub>= elevated CO<sub>2</sub>; DOY Day Of Year

Total amino-acids content seemed to present large standard deviation especially in 2020 for Cabernet Sauvignon. In 2019, amino-acids content increased along berry development, and in 2020 amino acids concentration decreased sharply at harvest for elevated CO<sub>2</sub> treatment, whereas it increased for ambient CO<sub>2</sub> conditions (Figure 28). No trend was apparent for total amino acids of Cabernet Sauvignon between ambient and elevated CO<sub>2</sub> treatment within the three years of the study.

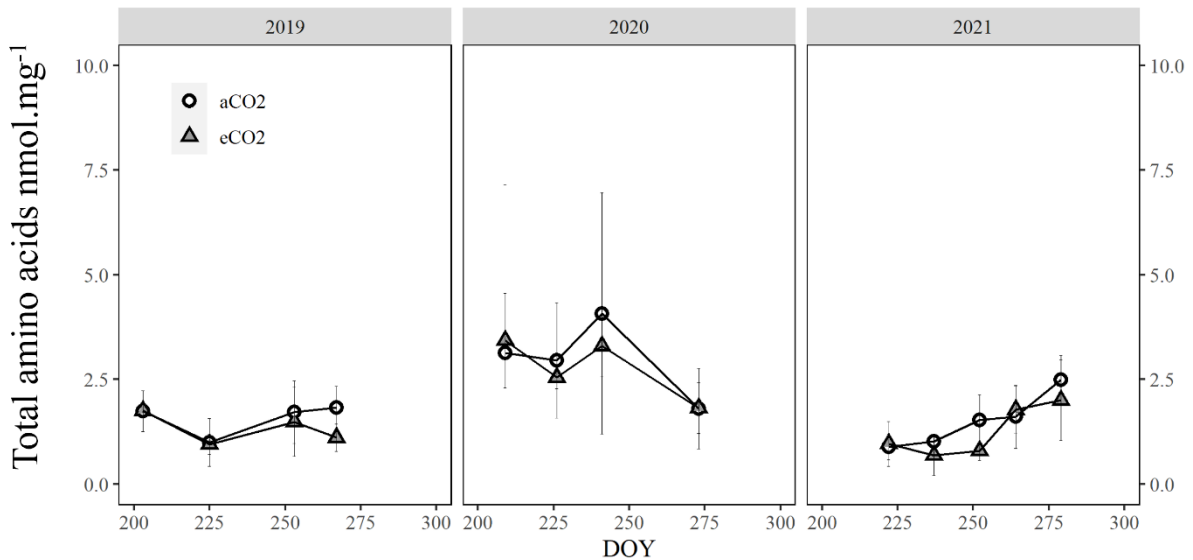


Figure 29. Total amino-acids expressed (nmol.mg<sup>-1</sup>) in Riesling, for the years 2019, 2020 and 2021, aCO<sub>2</sub> = ambient CO<sub>2</sub> and eCO<sub>2</sub>= elevated CO<sub>2</sub>; DOY Day Of Year

Total amino acids concentration in Riesling pulp was lower in comparison to Cabernet Sauvignon. Amino acids concentration in 2020 reached at harvest  $1.80 \pm 0.96$  nmol.mg<sup>-1</sup> for ambient CO<sub>2</sub> treatment and  $1.80 \pm 0.60$  nmol.mg<sup>-1</sup> for elevated CO<sub>2</sub> treatment, with the



differences noticed during ripening disappearing at maturity. In addition, total amino acids concentration reached at harvest  $2.48 \pm 0.58 \text{ nmol.mg}^{-1}$  for ambient  $\text{CO}_2$  treatment and  $2.00 \pm 0.97 \text{ nmol.mg}^{-1}$  for elevated  $\text{CO}_2$  treatment (Figure 29).

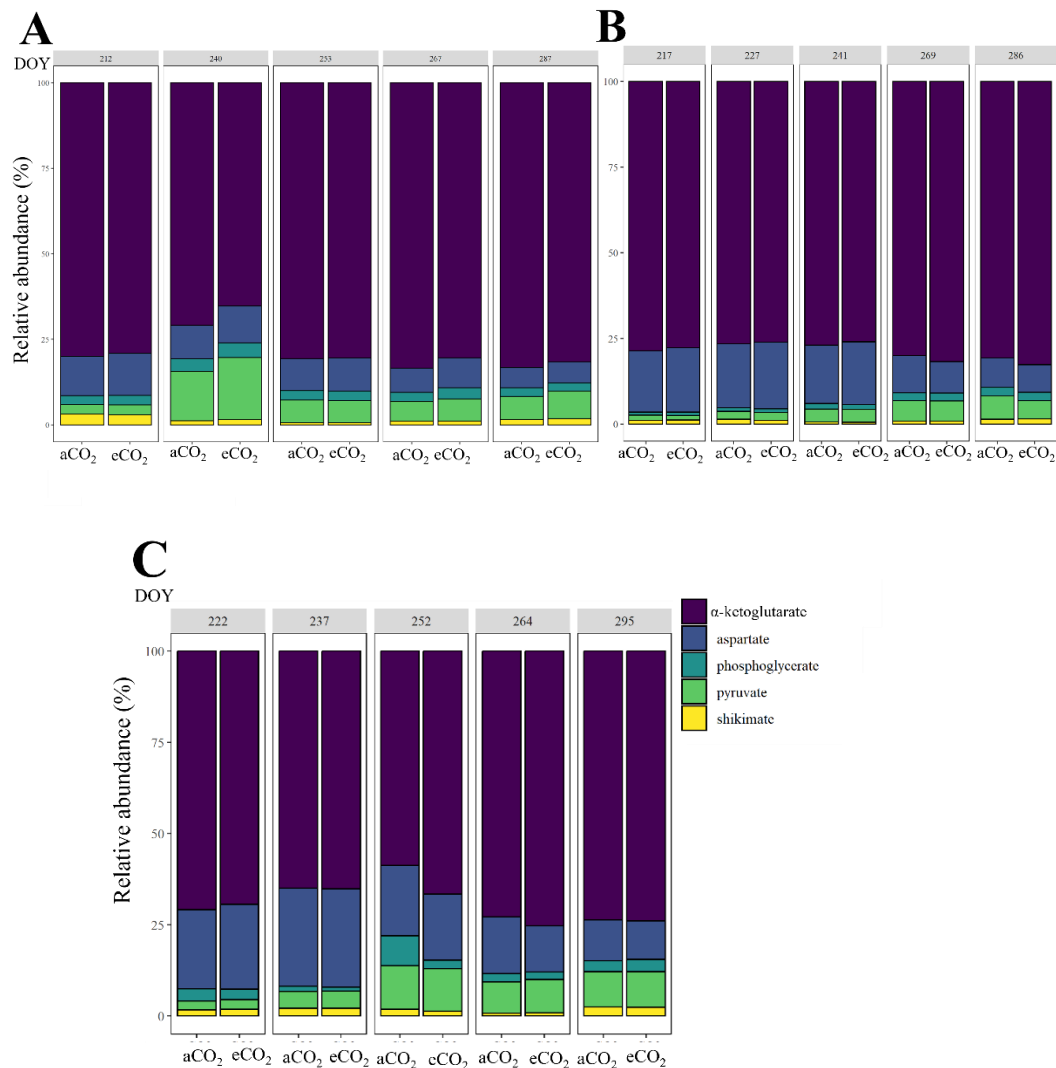


Figure 30. Relative abundance of amino-acids deriving from various biosynthetic pathways ( $\alpha$ -ketoglutarate, aspartate, phosphoglycerate, pyruvate, shikimate) for Cabernet Sauvignon in A: 2019 and B: 2020, C: 2021 a $\text{CO}_2$  = ambient  $\text{CO}_2$  and e $\text{CO}_2$ = elevated  $\text{CO}_2$ ; numbers above the bars are, Day Of Year (DOY)

In 2019, relative abundance of amino acids from different metabolic pathways varied during berry ripening, and in Cabernet Sauvignon  $\alpha$ -ketoglutarate derivatives which includes proline and arginine) where the most abundant ones (Figure 30A). In 2019, the contribution of  $\alpha$ -ketoglutarate derivatives to the total relative abundance of amino acids varied from 79.9 % at green stage for ambient  $\text{CO}_2$  treatment, to 83.3% at harvest. For elevated  $\text{CO}_2$  treatment,  $\alpha$ -ketoglutarate derivatives ranged from 79.1 % to 81.5 %. Amino-acids composition varied during berry development, and  $\alpha$ -ketoglutarate derivatives (Proline, Arginine, Glutamic acid, Glutamine, GABA, Histidine) were the most abundant amino acids. In Cabernet Sauvignon,  $\alpha$ -

ketoglutarate, aspartate, and pyruvate derivatives seemed to be more affected by elevated CO<sub>2</sub> than other metabolic pathways (shikimate, phosphoglycerate). Indeed, at harvest in 2020,  $\alpha$ -ketoglutarate derivatives showed an abundance of 80.7% for ambient CO<sub>2</sub> treatment, compared to 82.65% for elevated CO<sub>2</sub> treatment, and aspartate derivatives abundance was 8.52% for ambient CO<sub>2</sub> treatment compared to 8.08% for elevated CO<sub>2</sub> (Figure 30B). Pyruvate derivatives represented a proportion of 6.8% for ambient CO<sub>2</sub> compared to 5.32% for elevated CO<sub>2</sub>. Moreover, in 2021,  $\alpha$ -ketoglutarate derivatives were the greatest among all derivatives and varied from 70.8 % for green stages to 73.6% for maturity at ambient CO<sub>2</sub>, while under elevated CO<sub>2</sub> these derivatives ranged from 69.4% to 73.9%. Phosphoglycerate derivatives were decreased under elevated CO<sub>2</sub> at DOY=252, however this was not significant due to high variability between the samples (Figure 30C).

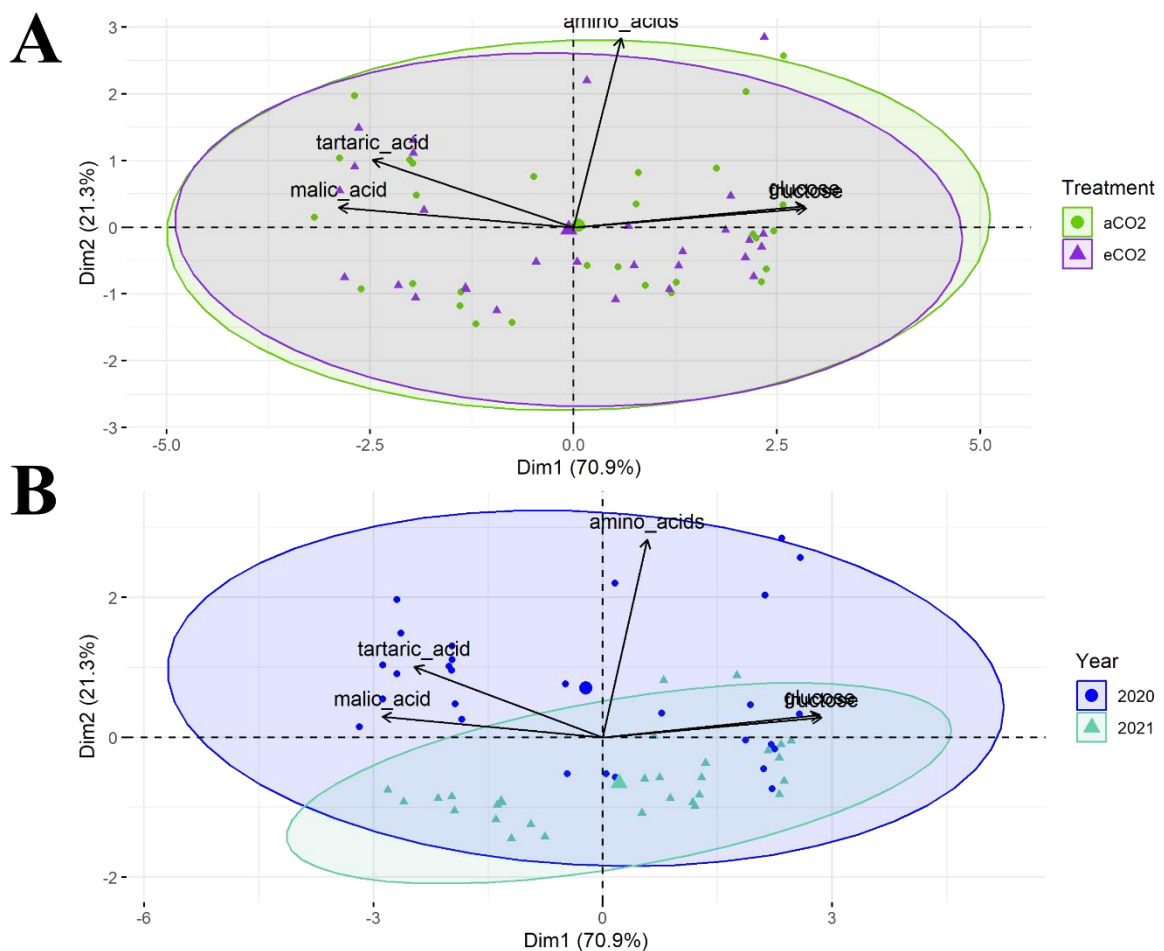


Figure 31. PCA biplot of Cabernet Sauvignon, organic acids (malic and tartaric acids), sugars (glucose and fructose) and total amino acids for years 2020 and 2021, aCO<sub>2</sub> = ambient CO<sub>2</sub> and eCO<sub>2</sub> = elevated CO<sub>2</sub>, differentiated by treatment (A) or by year (B)

Metabolic profiles of the samples were analysed in a PCA biplot (Figure 31). The first dimension explained 70.9% of the total variance and the second dimension explained 21.3% of the variance. Dimension 1 is positively correlated with glucose and fructose and negatively with tartaric and malic acids. Dimension 2 is correlated with total amino acids concentration. PCA plot allowed to clearly discriminate the year effect from the treatment effect, when

variables such as organic acids, sugars and total amino acids were studied. Indeed, the vintage effect was largely predominant in comparison to the treatment effect for Cabernet Sauvignon.

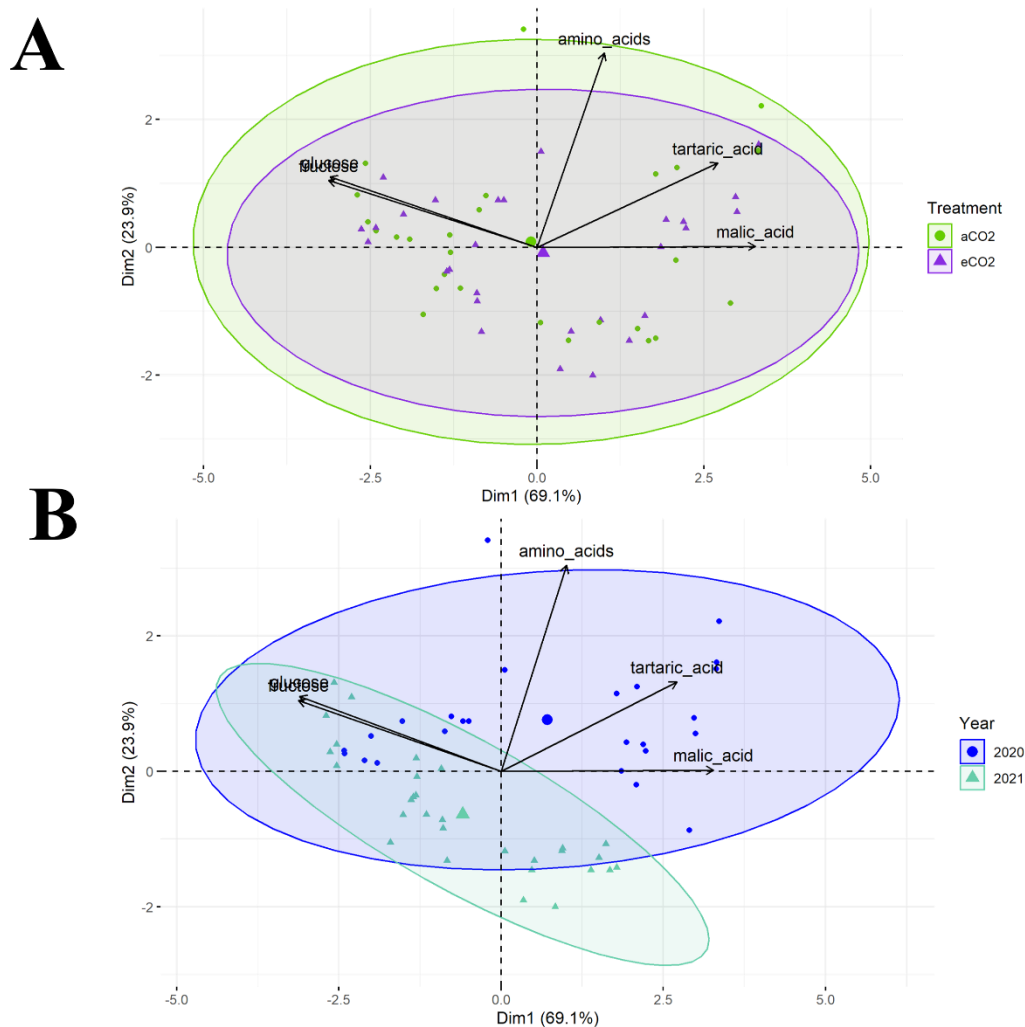


Figure 32. PCA biplot of Riesling, organic acids (malic and tartaric acids), sugars (glucose and fructose) and total amino acids for years 2020 and 2021, aCO<sub>2</sub> = ambient CO<sub>2</sub> and eCO<sub>2</sub> = elevated CO<sub>2</sub>, differentiated by treatment (A) or by year (B)

Concerning Riesling, the first dimension explained 69.1% of the variance and the second dimension explained 23% of the variance (Figure 32). PCA plot permitted to discriminate the year effect, but the treatment effect was not discernible. The vintage effect is undoubtedly predominant in comparison to the treatment effect for both Cabernet Sauvignon and Riesling.

### Total anthocyanins concentration

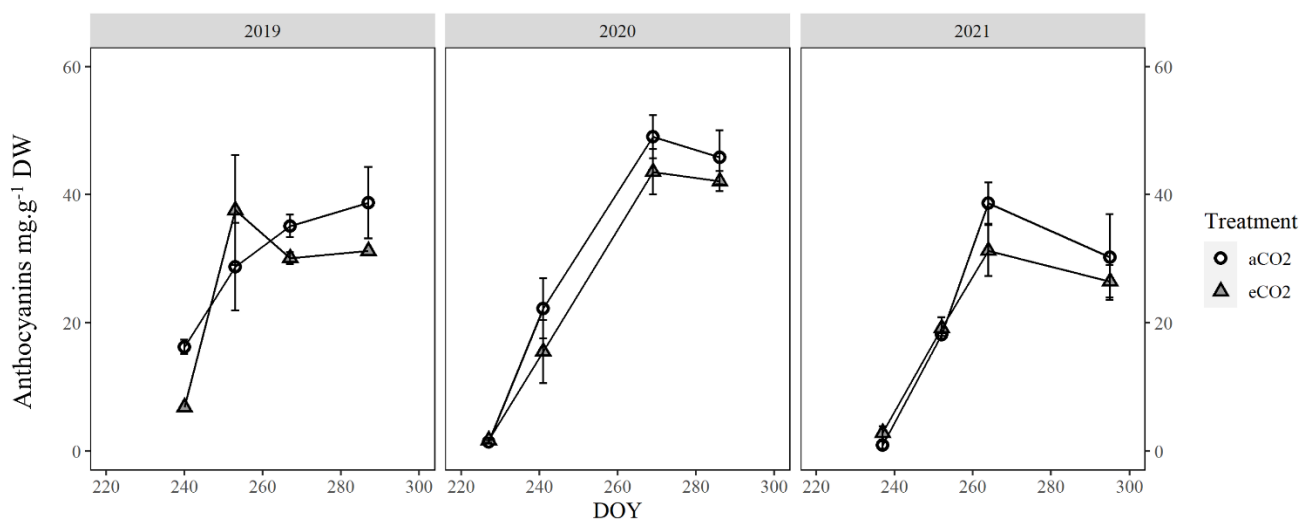


Figure 33. Anthocyanins content (mg.g<sup>-1</sup> DW) for Cabernet Sauvignon in 2019, 2020, and 2021, aCO<sub>2</sub> = ambient CO<sub>2</sub> treatment, and eCO<sub>2</sub>= elevated CO<sub>2</sub> treatment

The variation percentage of total anthocyanins content from 50% *véraison* to maturity in 2020 was 106.11% for ambient CO<sub>2</sub> treatment, whereas it was around 171.19% for elevated CO<sub>2</sub> treatment (Figure 33). At DOY=241 (50% *véraison*) in 2020, total anthocyanins content was  $22.24 \pm 4.68$  mg.g<sup>-1</sup> for ambient CO<sub>2</sub> treatment and is  $15.51 \pm 4.93$  mg.g<sup>-1</sup> for elevated CO<sub>2</sub> treatment, when at 75% *véraison* total anthocyanins content was  $49.02 \pm 3.35$  mg.g<sup>-1</sup> for ambient CO<sub>2</sub> and  $43.55 \pm 3.53$  mg.g<sup>-1</sup> for elevated CO<sub>2</sub>. At maturity, these differences were still discernible, with  $45.84 \pm 4.2$  mg.g<sup>-1</sup> for ambient CO<sub>2</sub> and  $42.09 \pm 1.58$  mg.g<sup>-1</sup> for elevated CO<sub>2</sub> respectively. Moreover, in 2021, a similar kinetic was observed, with a peak of total anthocyanins concentration for the timepoint 75% *véraison*, and a degradation of anthocyanins at harvest (Figure 33), which was delayed in 2021 due to the weather conditions. In 2021, at harvest, anthocyanins concentration was  $30.26 \pm 6.69$  mg.g<sup>-1</sup> for ambient CO<sub>2</sub> treatment, and  $26.47 \pm 2.54$  mg.g<sup>-1</sup> for elevated CO<sub>2</sub> treatment.

Consequently, a trend for anthocyanins content to be lower in skin under elevated CO<sub>2</sub>, which can be found also in the year 2019, except for the timepoint of 50% *véraison*, where the standard deviation made the result unclear. However, no significant difference can be drawn from the statistical analyses because the year effect was predominant.

## Anthocyanins composition

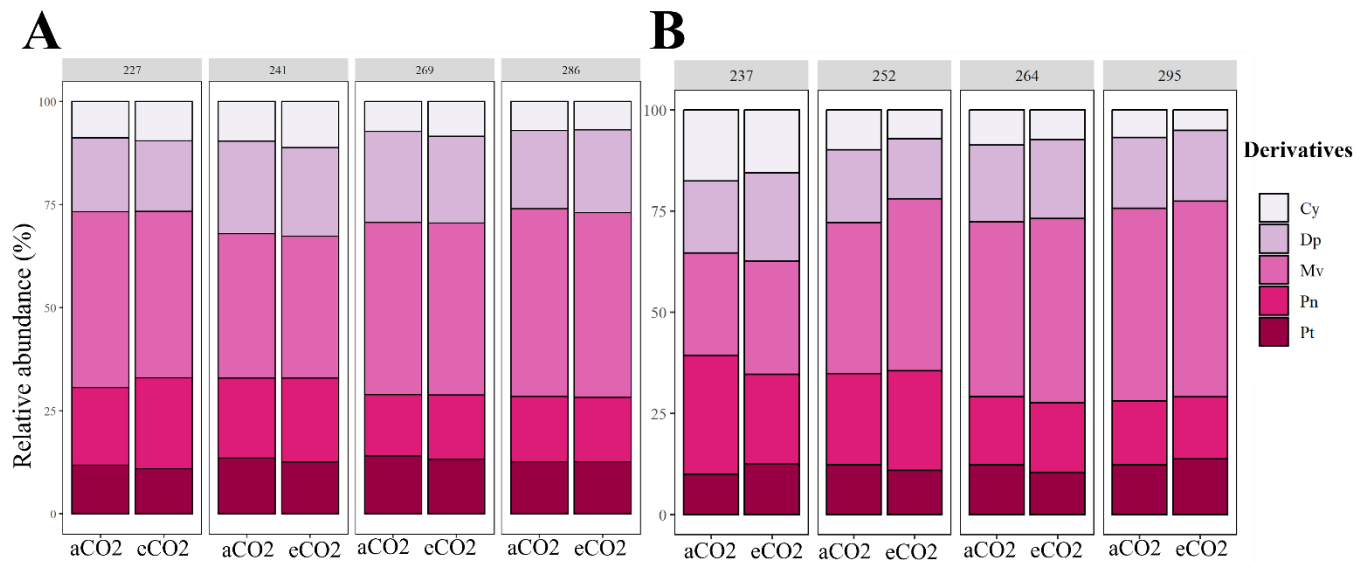


Figure 34. Anthocyanins composition for Cabernet Sauvignon in A: 2020 and B: 2021, aCO<sub>2</sub> = ambient CO<sub>2</sub> treatment and eCO<sub>2</sub>= elevated CO<sub>2</sub> treatment, derivatives as Cy=Cyanidin, Dp=Delphinidin, Mv=Malvidin, Pn=Peonidin, Pt= Petunidin, numbers above the bars are Day Of Year (DOY)

In 2020, the relative abundance of malvidin derivatives, the main anthocyanin in Cabernet Sauvignon grape skin, accounted for 42.7% for ambient CO<sub>2</sub> at DOY=227 (25% *véraison*), and for 40.4 % under elevated CO<sub>2</sub> (Figure 34). At maturity, malvidin represented 45.5 % for ambient CO<sub>2</sub> and 44.8 % for elevated CO<sub>2</sub> treatment. Peonidin derivatives accounted for 18.8% of total anthocyanins for ambient CO<sub>2</sub> treatment, whereas under elevated CO<sub>2</sub> treatment peonidin derivatives represented 22.04 % of total anthocyanins, at DOY= 227. At maturity, peonidin derivatives account for 15.8% for ambient CO<sub>2</sub> treatment, and for elevated CO<sub>2</sub> they represent 15.6% of total anthocyanins. Anthocyanins composition seemed to be slightly affected by elevated CO<sub>2</sub> treatment during ripening, however the final anthocyanin profile at harvest was not significantly affected.

*Di-hydroxylated to tri-hydroxylated ratio of anthocyanins*

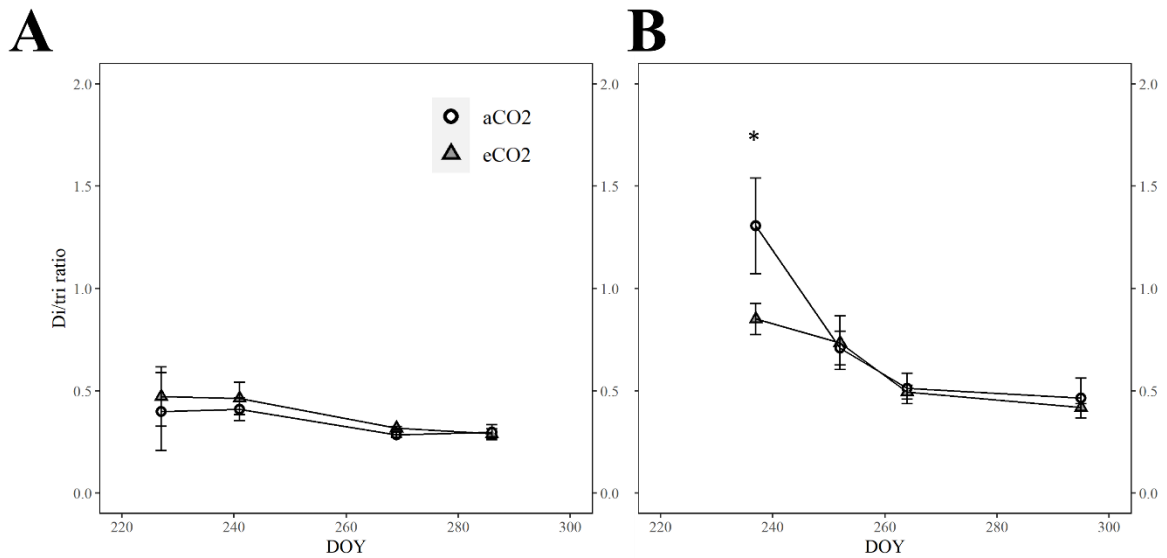


Figure 35. Di-hydroxylated (Cyanidin, Peonidin) to tri-hydroxylated (Delphinidin, Malvidin, Petunidin) ratio of Cabernet Sauvignon skins in A: 2020 and B: 2021, aCO<sub>2</sub>= ambient aCO<sub>2</sub> and e CO<sub>2</sub>= elevated CO<sub>2</sub>

Interestingly, di-OH to tri-OH anthocyanins ratio was higher for ambient CO<sub>2</sub> ( $1.31 \pm 0.24$ ) compared to elevated CO<sub>2</sub> ( $0.85 \pm 0.08$ ) for the timepoint 25% *véraison* in 2021. Cyanidin and Peonidin derivatives seemed to be more abundant in ambient CO<sub>2</sub> samples at this timepoint compared to Delphinidin, Malvidin and Petunidin (Figure 24B). In 2020, di-OH to tri-OH ratio did not differ when ambient CO<sub>2</sub> and elevated CO<sub>2</sub> were compared and was in the range of 0.4 during berry ripening (Figure 35A).

## Discussion

Even though the vines were grown under the VineyardFACE conditions for more than seven years, the present study showed that primary metabolites were not affected by elevated CO<sub>2</sub> treatment, for both Riesling and Cabernet Sauvignon. However, studies previously conducted in temperature gradient greenhouses, in which three levels of temperature (ambient, +2°C, +4°C) and two CO<sub>2</sub> levels (400 and 700 ppm) were applied on various Tempranillo clones, noticed an increase of malic acid at the onset of *véraison*, with differences disappearing at maturity (Arrizabalaga-Arriazu et al., 2020). In greenhouses, malic acid concentration was higher under climate change conditions (elevated temperature and 700 ppm of CO<sub>2</sub>) at mid-*véraison*, but its degradation rate was also enhanced during the ripening phase (Arrizabalaga-Arriazu et al., 2021). Titratable acidity and malic acid content were affected by elevated CO<sub>2</sub> (700 ppm) and elevated temperature (+3°C) applied in combination in the study of Martínez-Lüscher et al. (2016), performed in fully controlled conditions. Accumulation of malic acid in the green berries comes from photosynthesis, for around 50% (Conde et al., 2007) or inside mitochondria via enzymes such as fumarase or mMDH (mitochondrial malate dehydrogenase) (Sweetman et al., 2014). As malic acid biosynthesis begins with β-carboxylation of phosphoenolpyruvate (PEP) (Conde et al., 2007), elevated CO<sub>2</sub> concentration might have an impact on malic acid concentration. Tartaric acid, on the contrary, is less sensitive to environmental conditions during ripening (Poni et al., 2018). Moreover, in the early FACE experiments, organic acids and sugars were affected by elevated CO<sub>2</sub> concentration, still differences disappearing at maturity (Bindi et al., 2001). In the previous years of the VineyardFACE experiments, the vintage effect was predominant in comparison to elevated CO<sub>2</sub> effect for primary metabolites such as sugars and organic acids (Wohlfahrt et al., 2021). Concerning amino acids, (Arrizabalaga-Arriazu et al., 2020) showed that, considering all the clones studied, elevated CO<sub>2</sub> significantly decreased the proportion of α-ketoglutarate derivatives, and thus increased the abundance of derivatives synthesized from pyruvate and aspartate precursors at maturity, which is not the case in our results. In addition, a decrease in total amino-acids concentration occurred as well at the onset of *véraison* (Arrizabalaga-Arriazu et al., 2020). Finally, phenylalanine, the precursor of anthocyanins, was strongly enhanced in all clones at the onset of *véraison*, under elevated CO<sub>2</sub> (Arrizabalaga-Arriazu et al., 2020). In our study, total anthocyanins were consistently lower under elevated CO<sub>2</sub>, compared to ambient conditions mainly in 2020, and in 2021 around maturity albeit not significantly. This change is not related to an increase of skin to pulp ratio under elevated CO<sub>2</sub>, which seemed to decrease. Usually, total anthocyanins concentration expressed in mg/berry increased with berry size, and when this concentration was expressed in mg/g skin it was correlated with the number of berries (Barbagallo et al., 2016). Generally, anthocyanins composition was not affected by elevated CO<sub>2</sub> in our study. Anthocyanins are more affected by climate conditions (water supply, temperature, and total intercepted solar radiations) and yield, than sugar for example, as it was assessed by Sadras et al. (2007).

Anthocyanins concentration is expected to increase during berry development because of the accumulation of anthocyanins in the hypodermal layers of berry skin which occurs from *véraison* onwards (He et al., 2010). It was demonstrated that a trend of lower total anthocyanins in young wines under elevated CO<sub>2</sub> treatment can be predicted for years following the preliminary studies on the VineyardFACE (Wohlfahrt et al., 2021). Moreover, conducting an OTC experiment, Gonçalves et al. (2009) showed that total anthocyanins from Touriga Franca grapes were decreased by elevated CO<sub>2</sub> treatment in red wine, but were not affected in berries. In another study, (Arrizabalaga-Arriazu et al., 2020) investigated the effects of climate change parameters (700 ppm, +4°C) and demonstrated that, irrespective of temperature, anthocyanins concentration was higher under elevated CO<sub>2</sub> during at the onset of *véraison* onwards, whereas 2 weeks after mid-*véraison*, anthocyanins concentration was lower under this treatment. However, in our study, differences nullified at maturity. Upregulation of anthocyanins biosynthesis under elevated CO<sub>2</sub> and elevated temperature was also noticed, although anthocyanins concentration increased two weeks after *véraison* and decreased at harvest (Martínez-Lüscher et al., 2016). However, under elevated temperature (T = 35°C), grape berry anthocyanins decreased significantly and their composition was also affected with the exception of malvidin derivatives (Mori et al., 2007a). On the other hand, water deficit may increase anthocyanins content by the decrease in berry size (Ojeda et al., 2002), potentially partially alleviating the effect of elevated temperature. This illustrates the importance of studying the interaction of the various climate change-related factors.

Another report on table grapes (*Vitis vinifera* L. cv. Cardinal) showed that even a very short-term treatment (3 days) of elevated CO<sub>2</sub> (+20%) can induce a decrease in total anthocyanins concentration, affecting the genes coding for PAL (Phenylalanine ammonia lyase), STS (Stilbene synthase) as well as CHS (Chalcone synthase) (Sanchez-Ballesta et al., 2007). Another study on soft fruit such as strawberries demonstrated the impact of elevated temperature and elevated CO<sub>2</sub> on various compounds from secondary metabolism, showing the increase of polyphenols such as flavonoids, antioxidants, and anthocyanins (Balasooriya et al., 2019). Indeed, pelargonidin-3-glucoside content was gradually increased by elevated CO<sub>2</sub> (Balasooriya et al., 2019). Concerning another fleshy fruit or vegetable crop such as tomato, although elevated CO<sub>2</sub> did increase fruit yield, no major changes in fruit composition was noticeable (total phenols, glucose, fructose, sucrose), whereas higher temperature had an effect (Pimenta et al., 2022).

## **Conclusion**

The impact of a long-term elevated CO<sub>2</sub> treatment (+20%) on grape composition was studied on well-established vines, of Riesling and Cabernet Sauvignon. Primary metabolites such as sugars, organic acids, amino acids, did not show statistically different results when ambient and elevated CO<sub>2</sub> conditions were compared. Overall, the vintage effect was by far of larger importance compared to the impact of the treatment. However, total anthocyanins showed a trend of being lower under elevated CO<sub>2</sub> treatment compared to ambient conditions in 2020 during berry development and in 2021 after 50% *véraison*. Anthocyanin content could be further studied in young wines of Cabernet Sauvignon, to determine if the trend noticed in berries is also remarkable in wines.



## **Chapter III: Influence of a moderate increase of CO<sub>2</sub> on central carbon metabolites in Cabernet Sauvignon and Riesling berries**

## Chapter III: Influence of a moderate increase of CO<sub>2</sub> on central carbon metabolites in Cabernet Sauvignon and Riesling berries

### Introduction

The final composition of a grape berry is determined partly by metabolic pathways such as sucrose synthesis and degradation, glycolysis, tricarboxylic acid (TCA) cycle and the shikimate pathway. The TCA cycle is of importance in plant cells because it provides energy (ATP-Adenosine triphosphate), reducing power (NADH; Nicotinamide Adenine Dinucleotide Hydrogen), and carbon skeletons, which allows it to be a central hub in metabolism and a key element in the coordination of primary and secondary metabolism pathways (Araújo et al., 2014 ; Soubeyrand et al., 2018). Compounds produced from sugar metabolism are usually incorporated in glycolysis, which also generates energy (ATP), reducing equivalents (NADH), and intermediates for amino acids synthesis, lipids and secondary metabolites (Wang et al., 2017).

A previous study investigating in depth metabolic plasticity of central carbon metabolism either from berries of fruiting-cuttings or of open-field plots alongside fruit developmental and ripening curves. The results showed that metabolic intermediates profiles exhibited strong coordination within their respective pathways, clustering in sugar-phosphate metabolism, glycolysis, TCA cycle, pentose-phosphate and shikimate pathways (Dai et al., 2013). Moreover, it was also shown that despite differences in metabolic profile trajectories during berry development and ripening, fruit composition at ripeness was not significantly different in fruit from fruiting cuttings compared to vineyard sampled berries, demonstrating the plasticity of berry metabolism. Other authors studied the response of rice to low CO<sub>2</sub> of various metabolites from Calvin-Benson cycle (CBC), and found that CBC cycle intermediates maintained a high level of concentration, indicating that end-product synthesis could have been inhibited (Borghi et al., 2019). Similar results were found in *Arabidopsis thaliana* rosettes, where different CO<sub>2</sub> concentration were applied, and most of intermediates from CBC cycle were not affected by low CO<sub>2</sub> concentration (i.e. at the compensation point for CO<sub>2</sub>) whereas end-products synthesis (sucrose, starch, amino acids) was inhibited (Arrivault et al., 2009).

In the context of climate change, berry metabolism plasticity can be affected by numerous environmental changes including higher temperature, increased solar irradiance, water stress, and increased atmospheric CO<sub>2</sub> concentration. For example, in the study of Lecourieux et al. (2020), Cabernet Sauvignon berries were exposed to heat stress (+8°C), and intermediates from sugar metabolism, glycolysis and TCA cycle were quantified, showing the impairment of high temperature on carbohydrate and energy metabolism, depending on developmental stages and heat stress treatment duration. Malic acid and shikimate were particularly affected, their levels in the ripening berries being reduced, with deleterious effects on the fruit composition at ripeness (Lecourieux et al., 2020). Solar irradiance was found to be correlated with lower malate and aspartate content, and higher levels of valine, leucine, serine, and stress markers such as proline and GABA (Reshef et al., 2017). Root restriction, which can induce water stress, was shown to increase flavonoids in skin tissues from pre-*véraison* onwards, having thus a marked effect on secondary metabolism in ripening berries (Duan et al., 2019).

A limited number of metabolites is generally analyzed (Dai et al., 2013) and research focuses mainly on quality-related end-products such as glucose, fructose, amino acids, and organic acids. However, in our study, end-products of grapevine metabolism seemed to be mostly unaffected by elevated CO<sub>2</sub> treatment (please see Chapter 2) However, if these end-products (sugars, organic acids, amino-acids, and anthocyanins) display a similar concentration between ambient and elevated CO<sub>2</sub>, it could be conceivable that the regulation of the metabolic fluxes in the pathways that lead to these compounds could be modified by elevated CO<sub>2</sub>, by virtue of metabolism plasticity. To test this hypothesis, we have then chosen to investigate the effects of elevated CO<sub>2</sub> on intermediates from primary metabolism for samples of the years 2020 and 2021, for both Cabernet Sauvignon and Riesling and in the compartments of skins and pulps, respectively.

## Materials & methods

A collaboration has been initiated several years ago with Pr. John Lunn (Max Planck Institute of Molecular Plant Physiology, Potsdam Golm) and was put to good use to realize the LC-MS<sup>3</sup> analysis berry metabolome, of berry skin and pulp of Cabernet Sauvignon and Riesling.

### *Extraction and LC-MS<sup>3</sup> analysis*

Frozen skin and pulp powder of samples 2020 were weighted (15-20 mg) and sent to Max Planck Institut in Potsdam Golm to be analyzed by LC-MS<sup>3</sup>, according to the following protocol, without modifications (Lunn et al., 2006).

For samples of 2021, an aliquot of 15-20 mg of frozen tissue (skin or pulp) was weighed in Safe Lock tubes and 175 µL of ice-cold mixture of chloroform/methanol, CHCl<sub>3</sub>/CH<sub>3</sub>OH (3:7, v/v) was added to frozen samples. Right after, the tubes were vortexed. In addition, 175 µL of ice cold CHCl<sub>3</sub>/CH<sub>3</sub>OH (3:7, v/v), was added afterwards while the tubes were sitting in ice. Then, tubes were placed in the -20 °C freezer and left for 2 h with occasional vortex mixing (2-3 times). After this time, 350 µL of ice-cold water was added, and tubes were vortexed, for at least 5s to ensure the two phases are dispersed, then allowed to be warmed up to 4°C with repeated shaking. Tubes were then centrifuged at 13000 g, 4°C, for 10 min. The supernatant was transferred to screw-capped tubes and kept at 4°C, sitting in ice. The extraction steps were repeated using 300 µL of ice-cold water, and the supernatants were combined. Then, solvents were evaporated to dryness using a centrifugal vacuum dryer at 35° C. Metabolites were separated by anion-exchange high-performance liquid chromatography (LC) using an ICS 3000 chromatograph (Dionex Corporation, Sunnyvale, USA) coupled to a QTrap 5500 triple quadrupole mass spectrometer (MS, AB Sciex, Foster City, USA), as described by (Dai et al., 2013).

Quantified metabolites are the following : Trehalose-6-P (T6P), Sucrose-6-P (Suc6P), ADP-Glucose (ADPG), Galactose-1-P (Gal1P); Fructose-1-P (F1P), Glucose 1,6 BP (GlcBP), UDP-Galactose (UPDGal), Glucose 1P (Glu1P), Glycerol 3P (G3P), Phosphoenolpyruvate (PEP), Fructose-6-P (Fru6P), Mannose-6-P (M6P), Fructose1,6BP (F1,6BP), UDP-Glucose (UDPG), 3-phosphoglyceric acid (3PGA), shikimate, 2-oxoglutarate (2-OG), aconitate, citrate, iso-

citrate, malate, pyruvate, succinate, Glucose-6-P (G6P), 3-deoxy-manno-octulosonate (3-dmO), 4-O-methyl-glucuronic acid (4-O-MGA), galacturonic acid, glucuronic acid, ascorbate.

### *Statistical analysis*

Two-way ANOVA was performed on combined datasets of metabolites content, for two years studied in this chapter (2020 and 2021), to verify the year and the treatment effect (packages tidyverse and car were used to program scripts). Moreover, one-way ANOVA was also performed to verify the treatment effect for each year. Heatmaps (using the mean of the three biological replicates per treatment) were also realized with the package corrplot. The transformation of data ( $\log_2$ ) for heatmaps was performed according to the publication of Savoi et al. (2016).

## Results

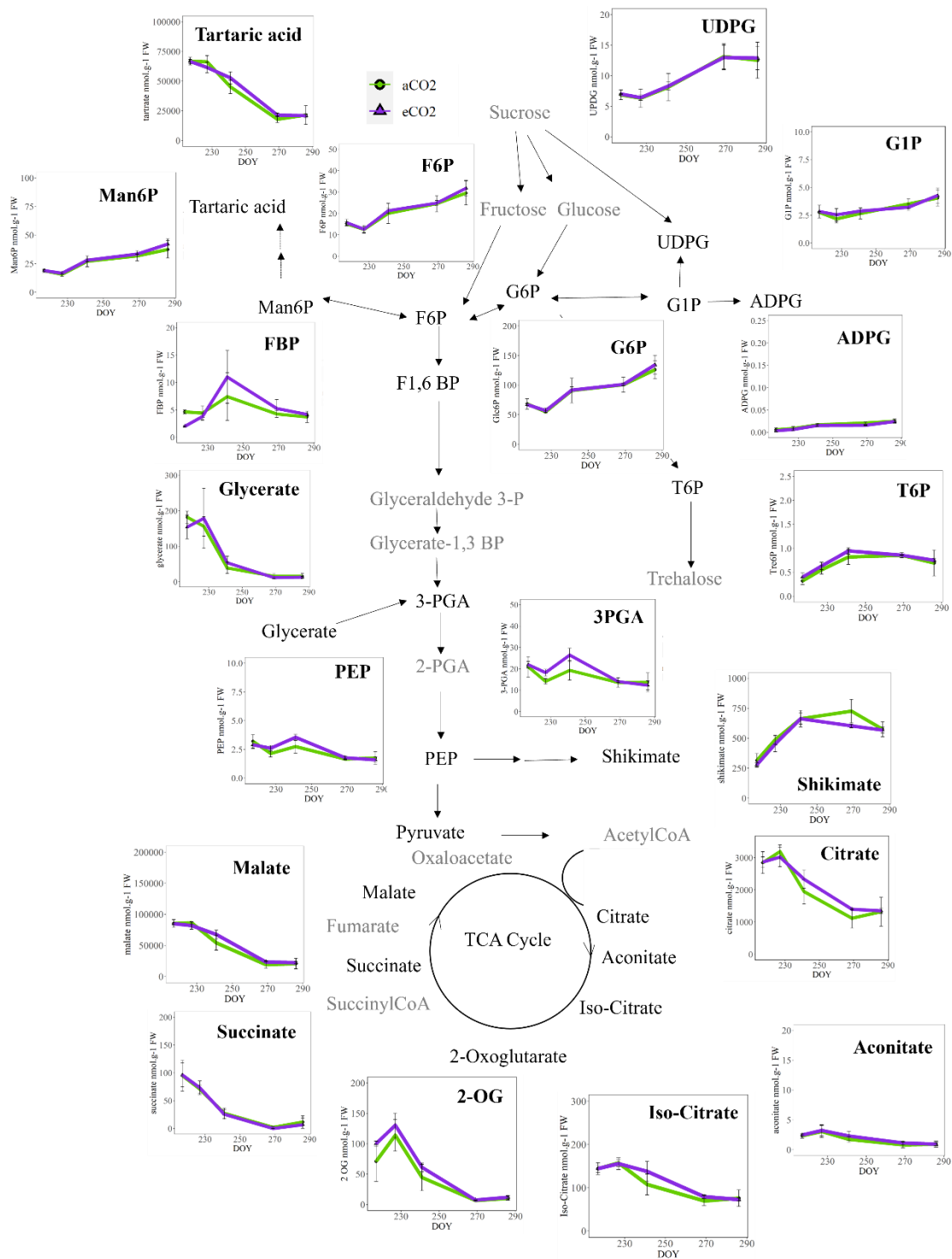


Figure 36. Concentration of compounds (nmol.g<sup>-1</sup>) involved in metabolic pathways including TCA cycle and sugar metabolism in year 2020 of Cabernet Sauvignon pulps, under ambient CO<sub>2</sub> (green circles) and elevated CO<sub>2</sub> (violet triangles)

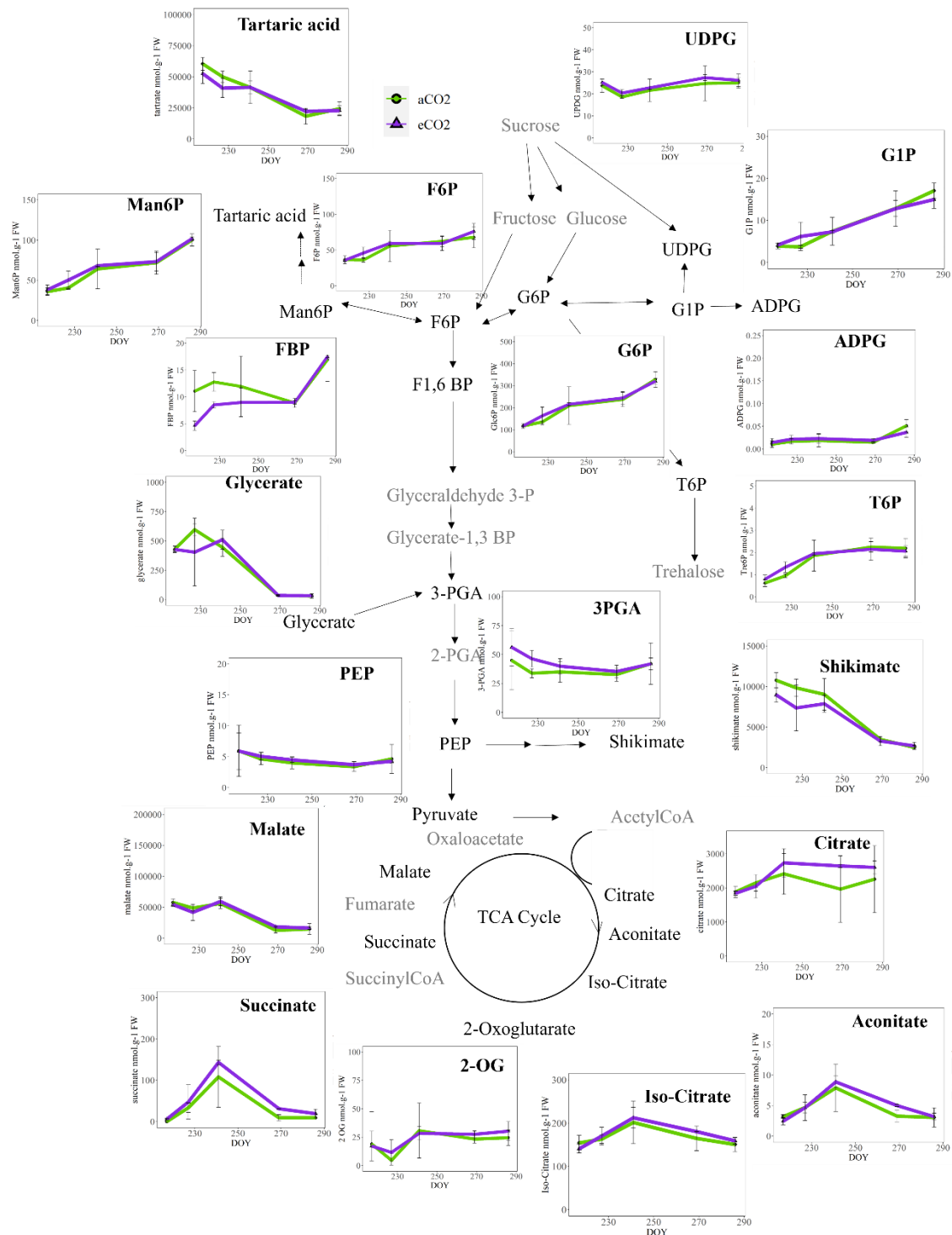


Figure 37. Concentration of compounds (nmol.g<sup>-1</sup>) involved in metabolic pathways including TCA cycle and sugar metabolism in year 2020 of Cabernet Sauvignon skins, under ambient CO<sub>2</sub> (green circles) and elevated CO<sub>2</sub> (violet triangles)

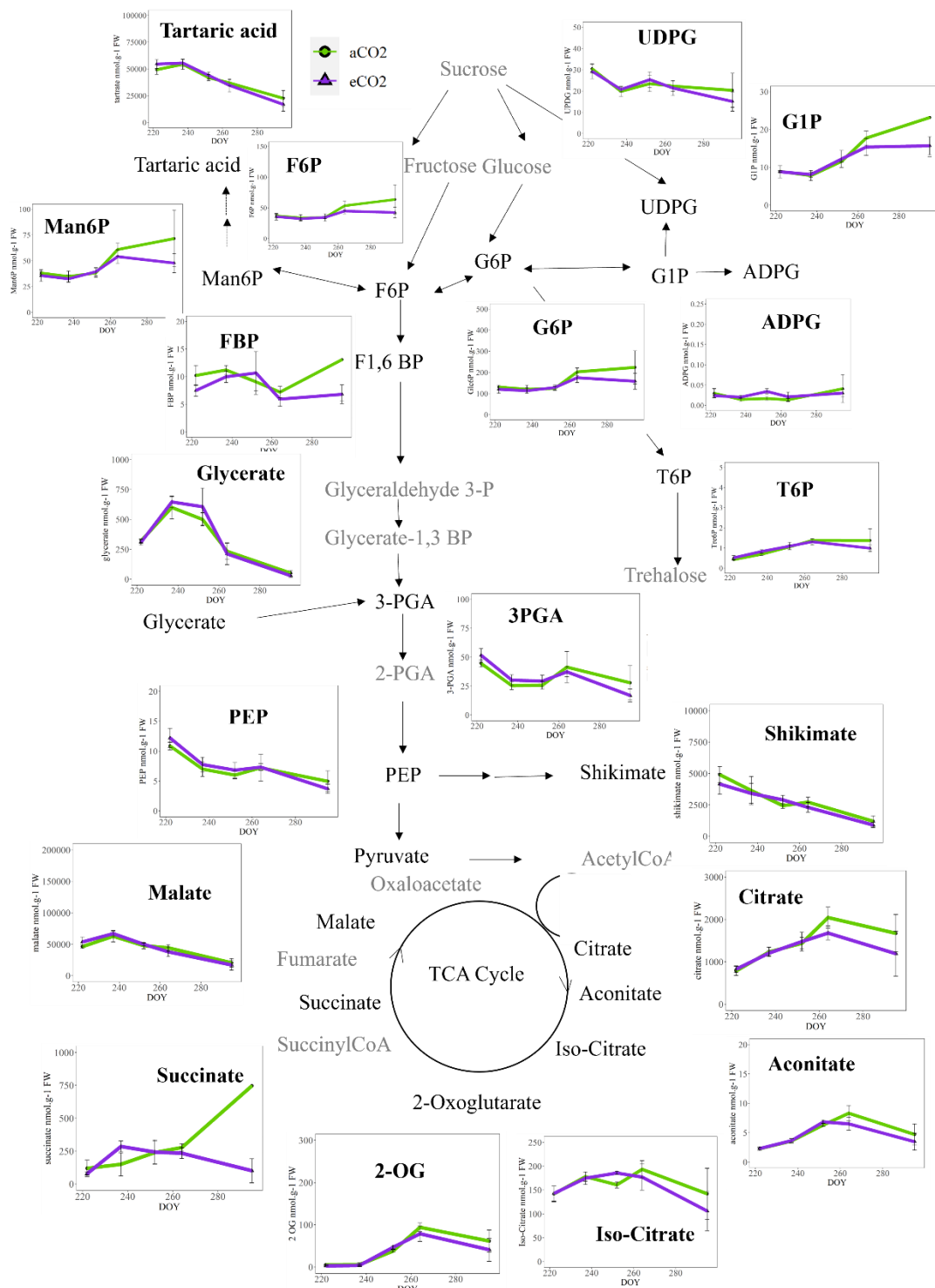


Figure 38. Concentration of compounds (nmol.g<sup>-1</sup>) involved in metabolic pathways including TCA cycle and sugar metabolism in year 2021 of Cabernet Sauvignon skins, under ambient CO<sub>2</sub> (green circles) and elevated CO<sub>2</sub> (violet triangles)

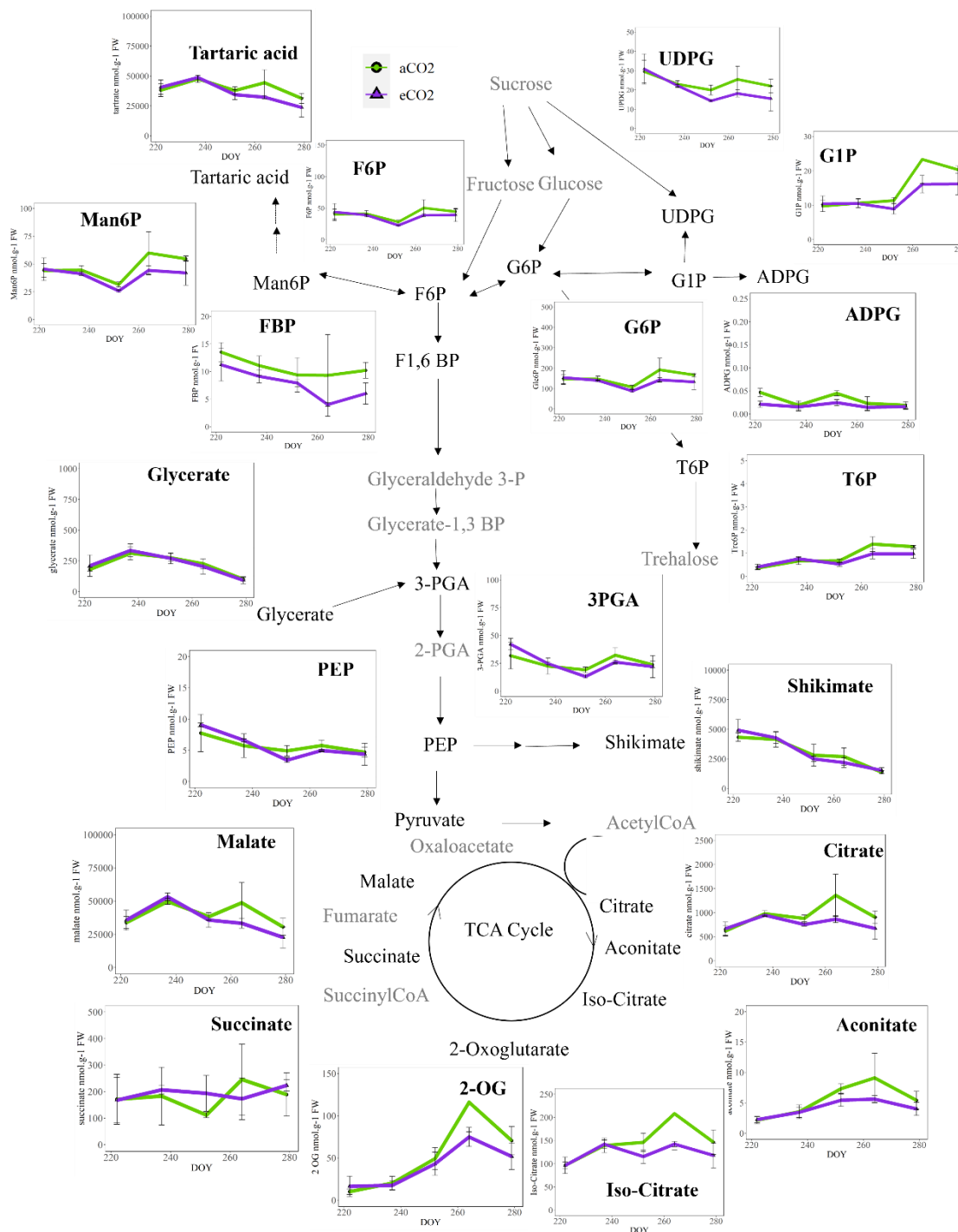


Figure 39. Concentration of compounds (nmol.g<sup>-1</sup>) involved in metabolic pathways including TCA cycle and sugar metabolism in year 2021 of Riesling skins, under ambient CO<sub>2</sub> (green circles) and elevated CO<sub>2</sub> (violet triangles)



The kinetic of TCA cycle intermediates seemed to be different in terms of peak occurrence and rates of degradation in 2021 and in 2020 (Figure 37 & 38). Sugar intermediates displayed an increase during berry ripening, at different rates depending on the metabolite considered, and TCA compounds demonstrated a peak and afterwards a decrease, as well at different rates (Figure 37 & 38). Moreover, at harvest in 2021, all compounds quantified in Cabernet Sauvignon skins seemed to be lower under elevated CO<sub>2</sub> in comparison to ambient CO<sub>2</sub>, although it was mainly not significantly different in the ANOVA analysis (Figure 38).

After statistical test (two-way ANOVA considering year and treatment effects), the year effect was significant for many of the metabolites, considering the different DOY and the corresponding developmental stages. Regarding the treatment effect, only a few metabolites of Cabernet Sauvignon skins demonstrated an effect such as Fru1,6BP at 25 % *véraison*, with a decrease in Fru1,6BP under elevated CO<sub>2</sub>, or a treatment and a year effect, such as Glycerol-3P and shikimate, at EL-33 and Tre6P, succinate and 3-PGA at 25% *véraison*. The main differences were noticed during the early stages of ripening, whereas at maturity, near the harvest date no major differences seemed to occur, at least from the statistical point of view. When the sum of skin metabolites analyzed, elevated CO<sub>2</sub> samples displayed higher content of such metabolites near maturity, whereas it was the opposite during early developmental stages. Moreover, in Riesling skins, some compounds such as Fru1,6BP, tartrate and aconitate had a treatment effect at some developmental stages. Fru1,6BP displayed a treatment effect at EL-33, and a treatment and a year effect at maturity. Significant year effect was shown for treatment, for tartrate skin at 25% *véraison*, and for aconitate at 50 % *véraison*.

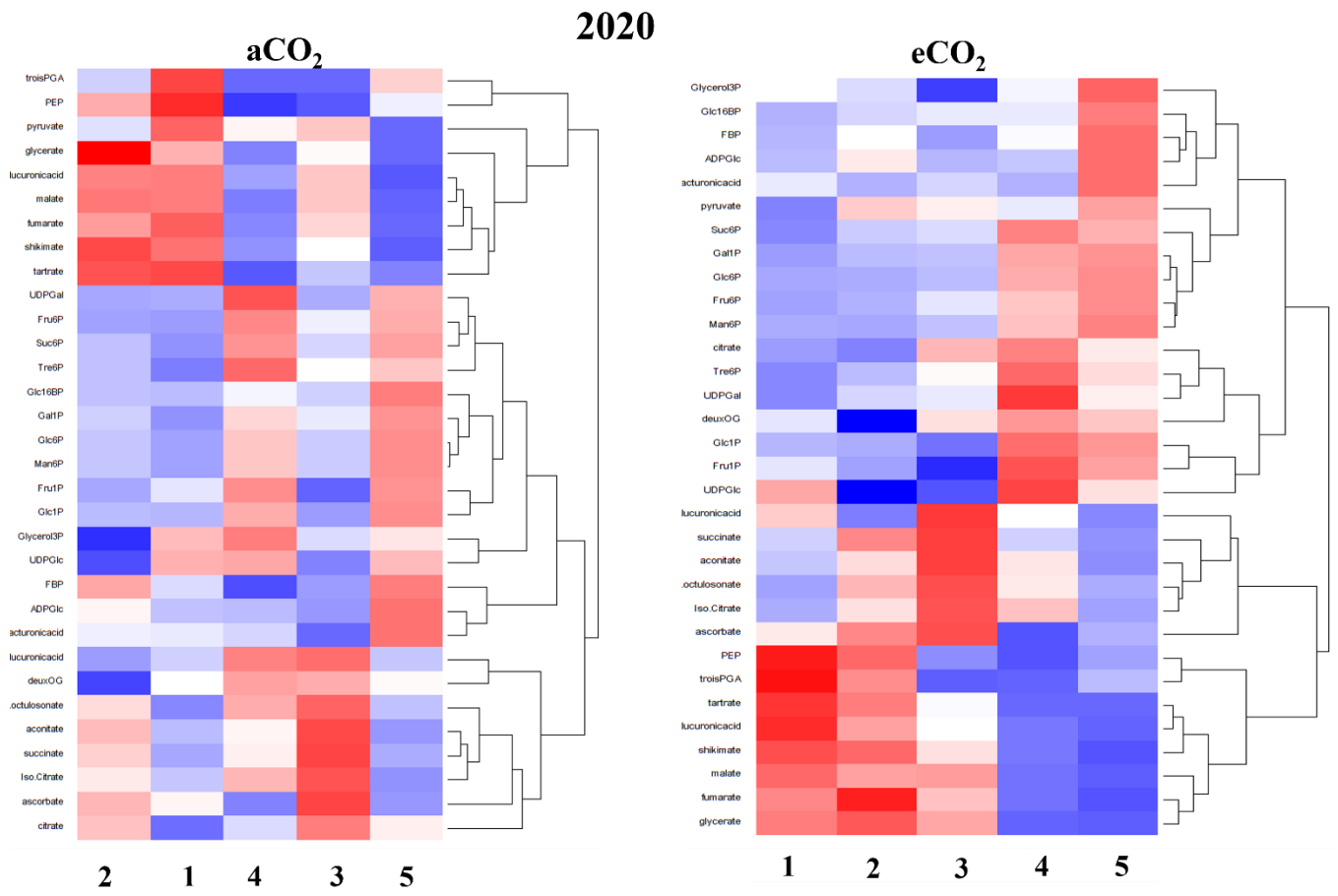


Figure 40. Heatmap and clustering of metabolite changes in berry skins for ambient CO<sub>2</sub> and elevated CO<sub>2</sub> treatment of Cabernet Sauvignon skins in 2020, each column represents a developmental stage and each line represent a metabolite

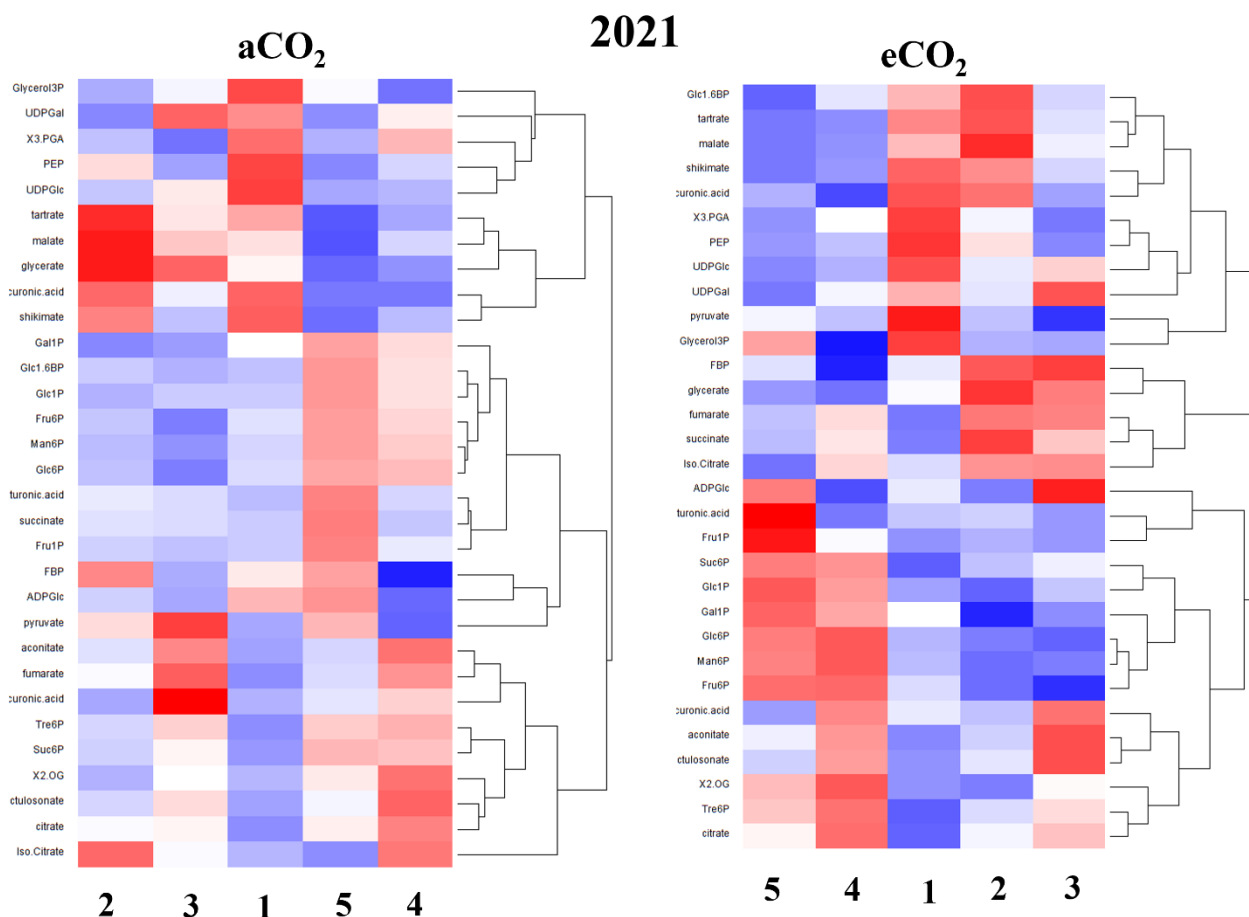


Figure 41. Heatmap and clustering of metabolite changes in berry skins for ambient CO<sub>2</sub> and elevated CO<sub>2</sub> treatment of Cabernet Sauvignon skins in 2021, each column represents a developmental stage and each line represent a metabolite

Heatmaps were drawn for years 2020 and 2021 respectively, to observe the correlations of the metabolites depending on the developmental stages and clustered together. Three groups were drawn from the cluster analysis, such as TCA cycle intermediates, glycolysis intermediates and sugar phosphates. Indeed, early developmental stages are more correlated with organic acids, and intermediates from TCA cycle, whereas late ripening stages are more correlated with sugars phosphate and intermediates of glycolysis, which is coherent with grape general metabolism.

According to Figure 40 and Figure 41, Fru1,6BP (FBP) demonstrated a different correlation within the developmental stages, when ambient and elevated CO<sub>2</sub> conditions were compared. The differences between ambient CO<sub>2</sub> and elevated CO<sub>2</sub> seemed to be relatively scarce in comparison to the year effect.

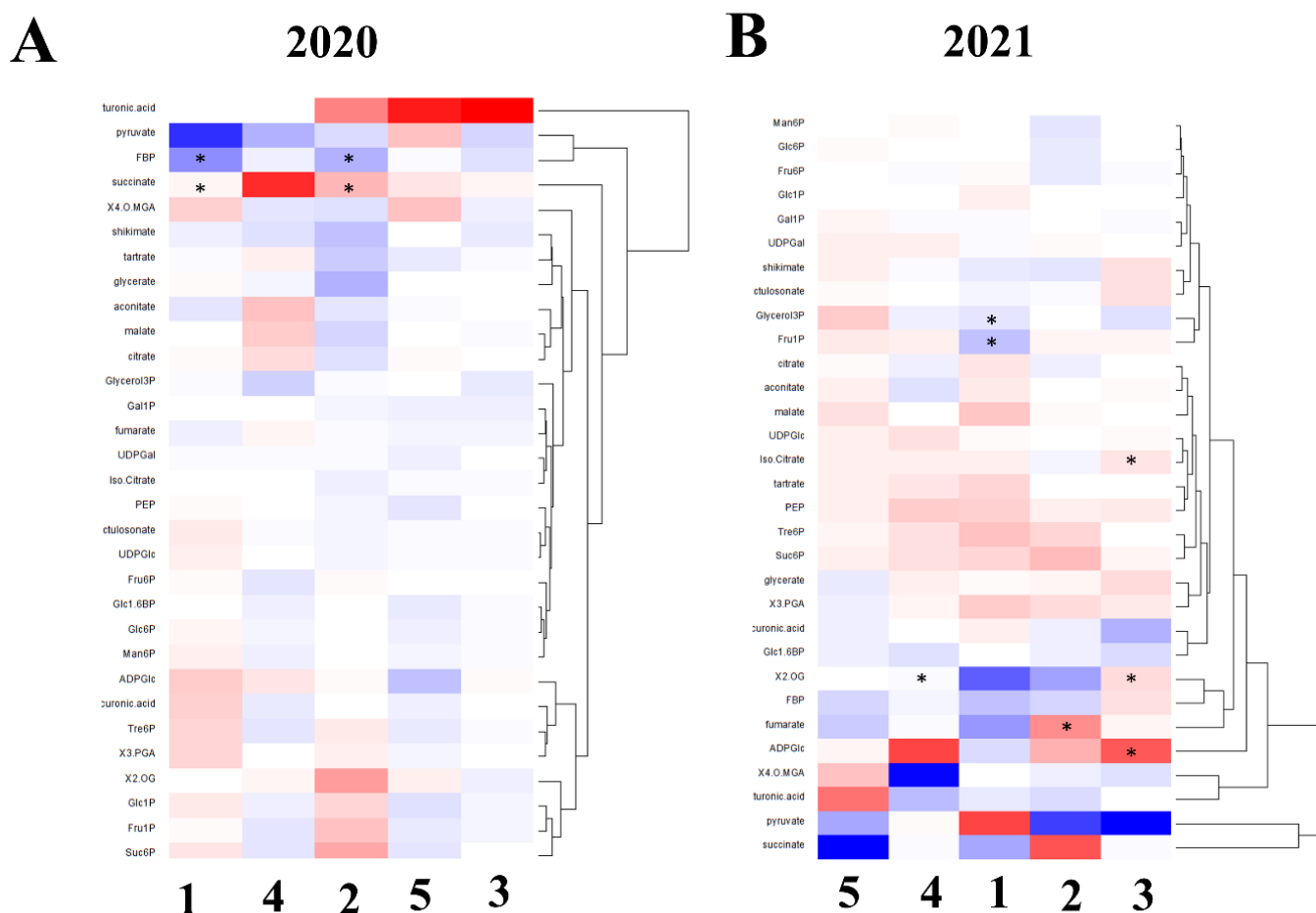


Figure 42. Heatmap representing the  $\log_2(e\text{CO}_2/a\text{CO}_2)$  of intermediates of primary metabolites in Cabernet Sauvignon skins in A: 2020 and B: 2021, for different developmental stages (without automatic scaling), blue and red boxes indicate a lower and higher concentration in elevated CO<sub>2</sub>, respectively, asterisk indicate significant differences between treatments ( $p < 0.05$ ), according to one-way ANOVA

In 2020, only two compounds were significantly affected by elevated CO<sub>2</sub> in Cabernet Sauvignon skins. It was indeed the case of F1,6 BP and succinate, which were affected both at green stage and 25% *véraison*, showing a decrease under elevated CO<sub>2</sub> for F1,6 BP and an increase under elevated CO<sub>2</sub> for succinate (Figure 42A). However, elevated CO<sub>2</sub> affected significantly in 2021 the concentration of seven out of 31 metabolites at one developmental stage each, for Cabernet Sauvignon skins. These metabolite concentrations were increasing, decreasing, or not affected by elevated CO<sub>2</sub> conditions. Globally, in the skin compartment in 2021, compounds such as succinate, pyruvate, galacturonic acid and 4-O-methylglucuronic acid were affected by elevated CO<sub>2</sub> at different developmental stages, albeit not significantly. Significant differences were found for Fructose-1-P, Glycerol 3-P, fumarate, iso-citrate, 2-Oxoglutarate and Glucose-1,6 BP, for stages before maturity (Figure 42B).

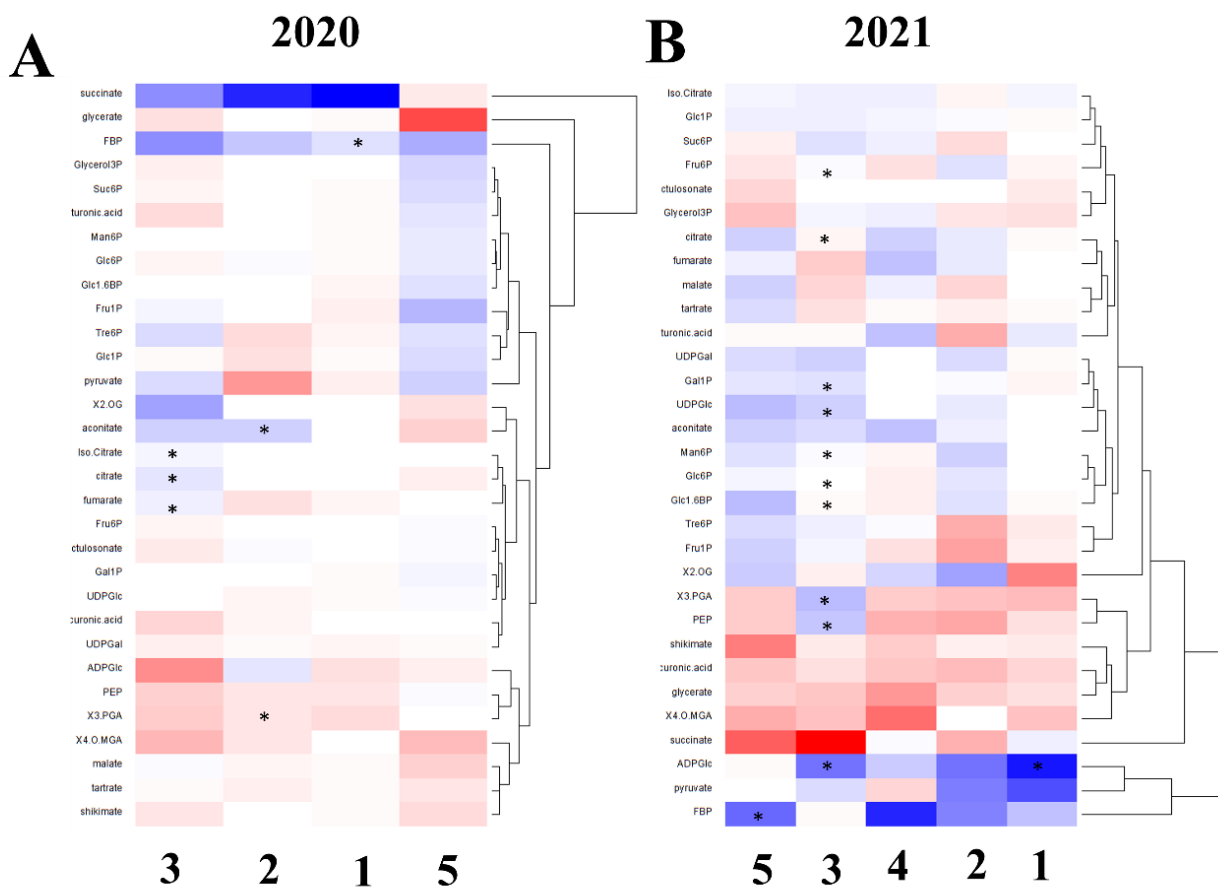


Figure 43. Heatmap representing the log<sub>2</sub>(eCO<sub>2</sub>/aCO<sub>2</sub>) of intermediates of primary metabolites in Riesling skins in A: 2020 and B: 2021, for different developmental stages (without automatic scaling), blue and red boxes indicate a lower and higher concentration in elevated CO<sub>2</sub>, respectively, asterisk indicate significant differences between treatments (p < 0.05), according to one-way ANOVA

In 2020, six compounds were significantly affected by elevated CO<sub>2</sub> in Riesling skins, at one developmental stage each. F1,6 BP was also significantly decreased under elevated CO<sub>2</sub> at green stage, whereas TCA cycle compounds such as aconitate, citrate, iso-citrate, fumarate were also decreased under elevated CO<sub>2</sub>, but at a later stage. Moreover, in 2021, one stage (50% *véraison*) displayed the major significant changes, for compounds such as Fru6P, Glc6P, PEP, 3-PGA, F1,6 bisphosphate, and citrate. Galactose-1P, UDP-Glucose, ADP-Glucose were also affected at this stage (Figure 43B).

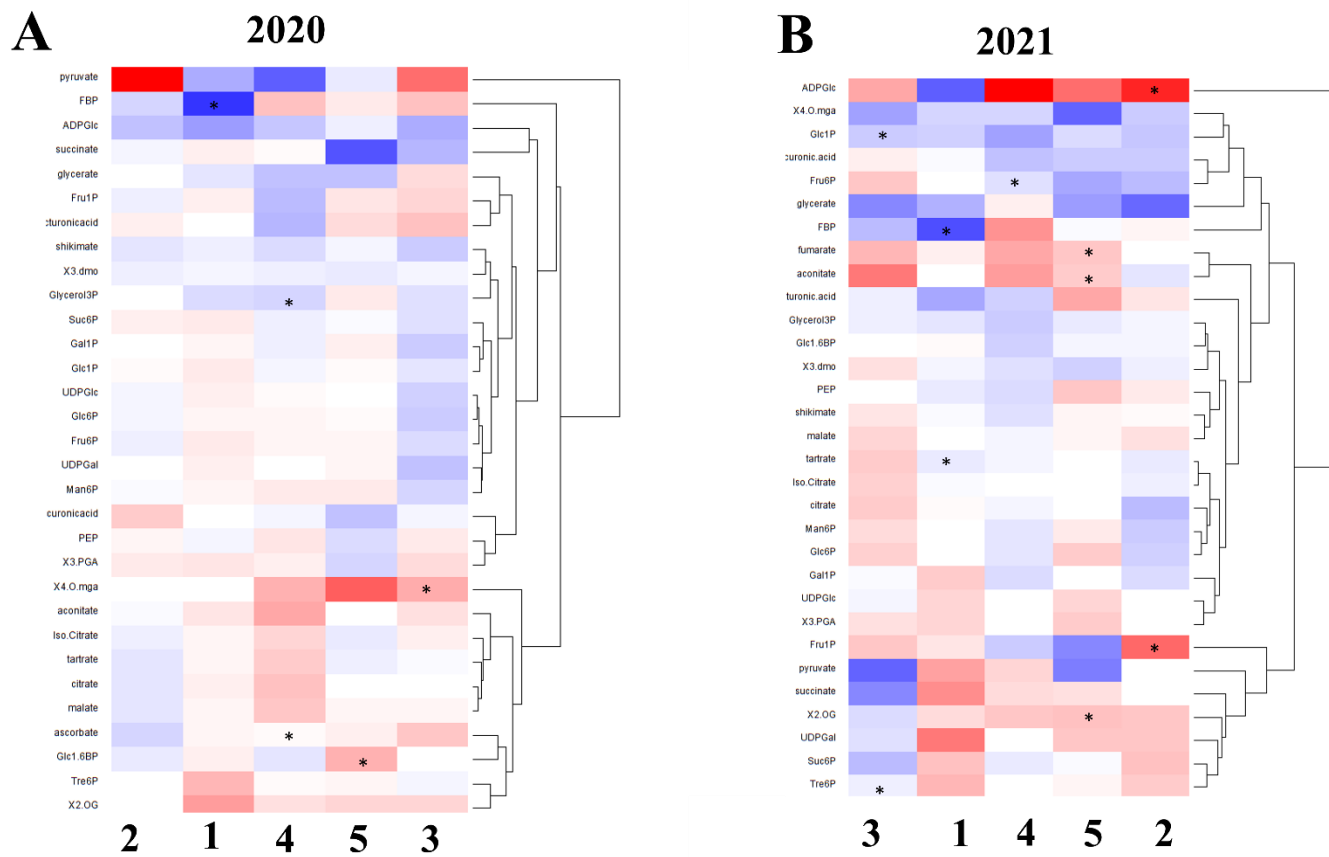


Figure 44. Heatmap representing the log<sub>2</sub>(eCO<sub>2</sub>/aCO<sub>2</sub>) of intermediates of primary metabolites in Cabernet Sauvignon pulps in A: 2020 and B: 2021, for different developmental stages (without automatic scaling), blue and red boxes indicate a lower and higher concentration in elevated CO<sub>2</sub>, respectively, asterisk indicate significant differences between treatments (p<0.05), according to one-way ANOVA

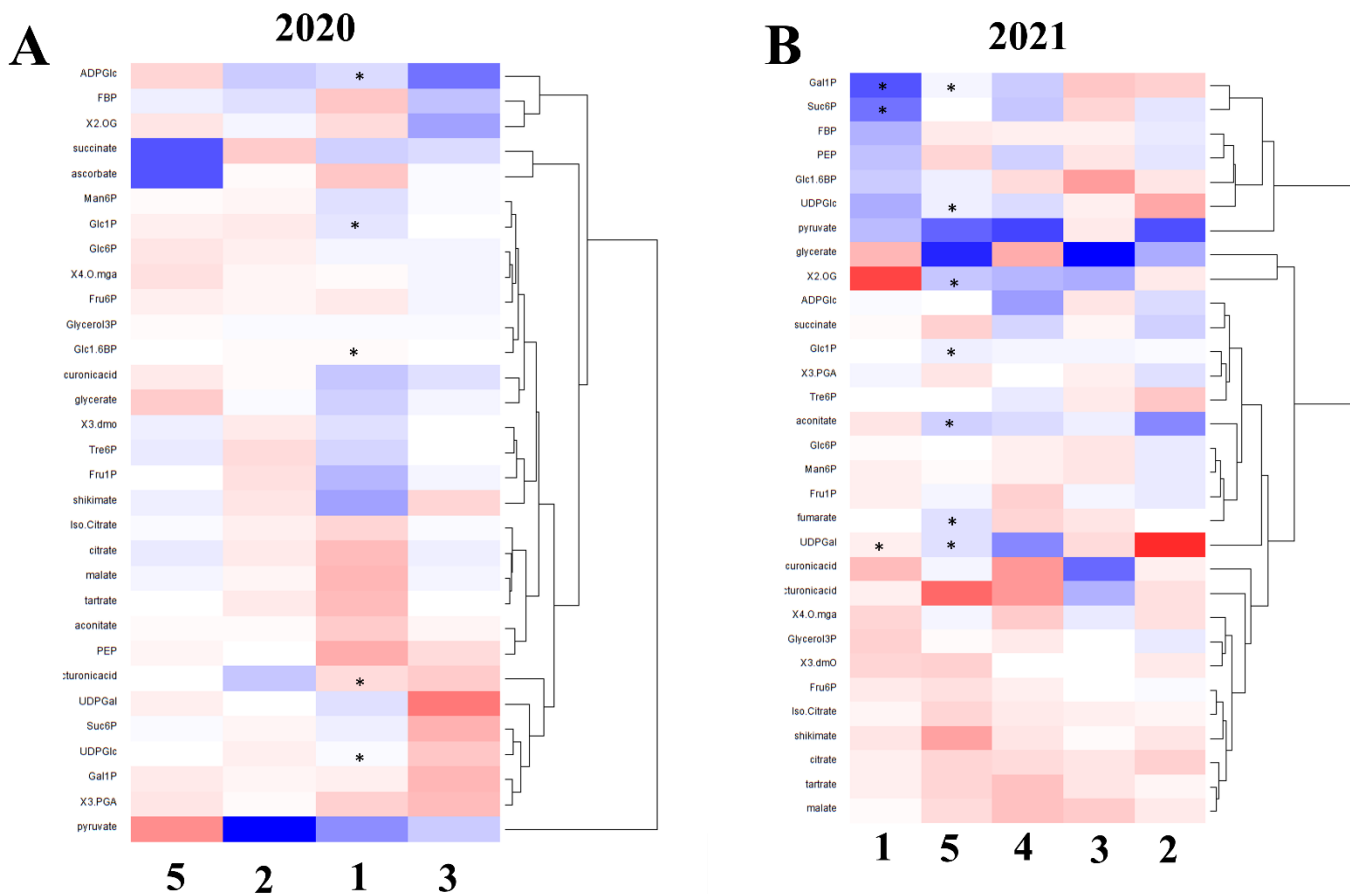


Figure 45A&B. Heatmap representing the  $\log_2(e\text{CO}_2/a\text{CO}_2)$  of intermediates of primary metabolites in Riesling pulps in A: 2020 and B: 2021, for different developmental stages (without automatic scaling), blue and red boxes indicate a lower and higher concentration in elevated CO<sub>2</sub>, respectively, asterisk indicate significant differences between treatments ( $p < 0.05$ ), according to one-way ANOVA

Concerning pulps, the compound which stood out was F1,6 BP, which decreased significantly according to one-way ANOVA, at green stage in 2020 for Cabernet Sauvignon. Moreover, Glycerol 3-P and 4-O-methylglucuronic acid, as well as ascorbate and Glucose 1,6-BP were also impacted by elevated CO<sub>2</sub>, but at stages approaching maturity and at maturity. In 2021, 10 compounds were impacted at one specific developmental stage, such as F 1,6-BP at early stage, fumarate, aconitate and 2-OG at maturity (Figure 44A).

According to Figure 45 A & B, 10 compounds were significantly different in 2021, compared to 5 in 2020, for Riesling pulps. In 2021, fumarate and aconitate were as well lowered under elevated CO<sub>2</sub> at maturity.

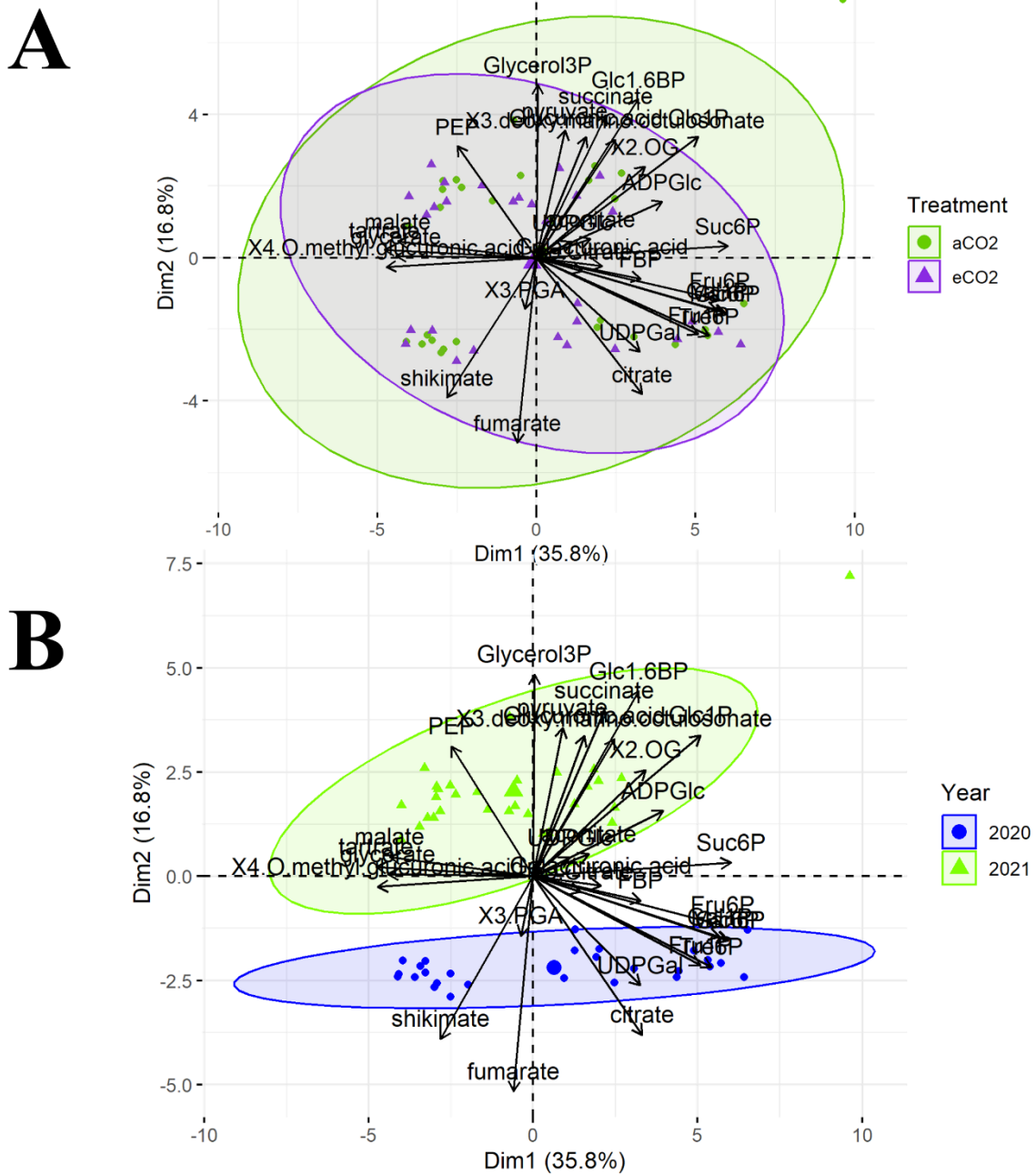


Figure 46. PCA biplots of Cabernet Sauvignon skins in 2020 and 2021, differentiated by years: A and by treatment: B; aCO<sub>2</sub> = ambient CO<sub>2</sub> treatment and eCO<sub>2</sub> = elevated CO<sub>2</sub> treatment

To try to clearly evaluate the year and treatment respective effect, PCA biplot were realized on skins samples, regrouping 2020 and 2021. The PCA biplots showed that the year is more discriminant than the treatment (Figure 46A and B), where only the 20 first variables contributing to the axes were shown. Indeed, there was a clear distinction between the samples from year 2020 and 2021, whereas no clear distinction could be assumed between ambient CO<sub>2</sub> and elevated CO<sub>2</sub>. PC1 was associated with Suc6P and malate/tartrate, whereas PC2 was associated with Glycerol 3P and fumarate. The distinction between the two years can be



attributed along PC2, with samples from 2021 which were associated to increased Glycerol-3-P and decreased fumarate (Figure 31A and B).

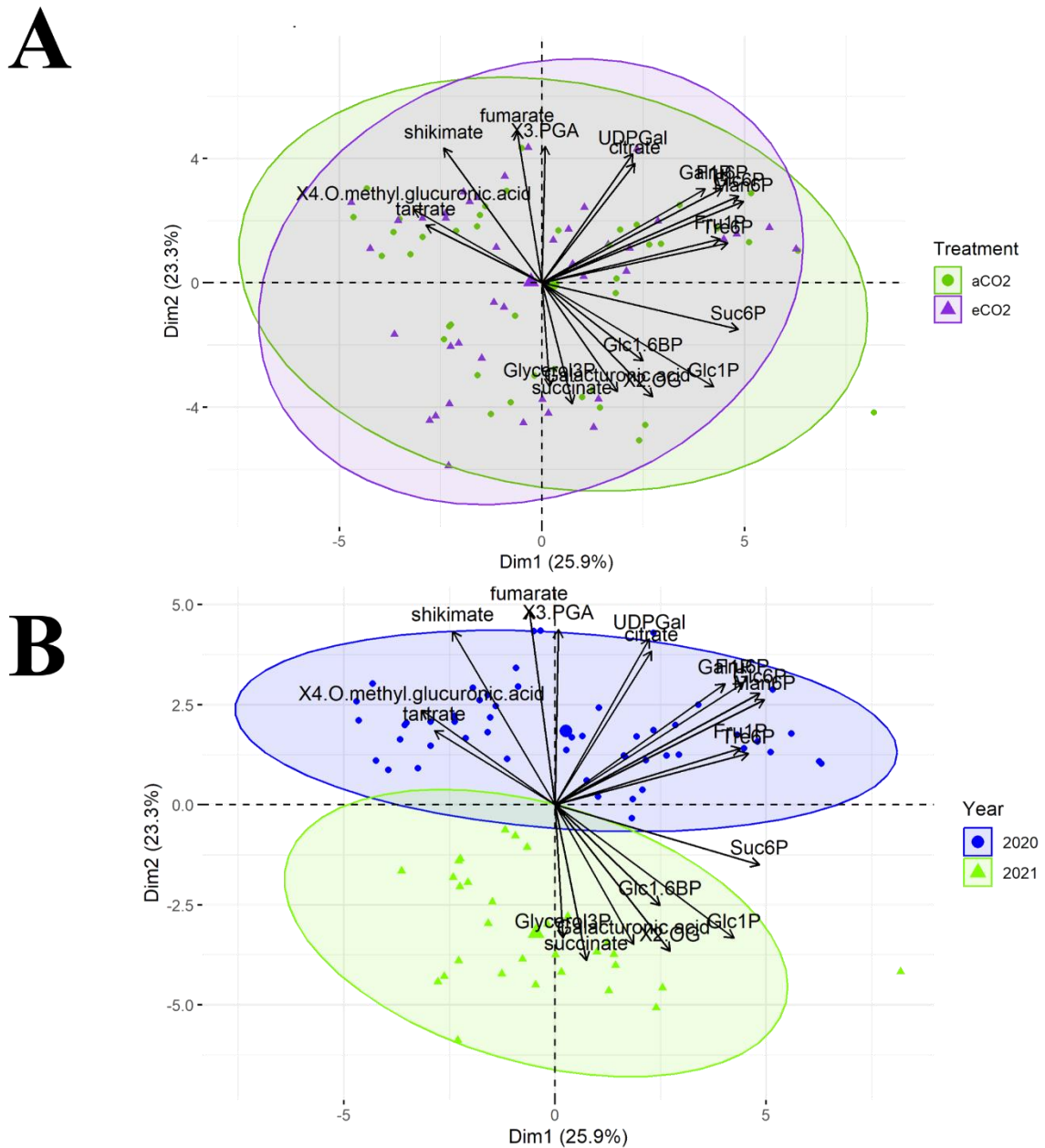


Figure 47. PCA biplots of Riesling skins in 2020 and 2021, differentiated by years: A and by treatment: B; aCO<sub>2</sub> = ambient CO<sub>2</sub> treatment and eCO<sub>2</sub>= elevated CO<sub>2</sub> treatment

As for Cabernet Sauvignon, Riesling demonstrated a clear difference for the years, and not for the treatment. Dimension 1 was associated with Suc6P and Fruc1P, and tartrate, whereas dimension 2 was associated with fumarate and Glycerol3P.

## Discussion

Intermediates from central metabolism were quantified in berry compartments such as skin and pulp samples, in years 2020 and 2021, for both cultivars, Cabernet Sauvignon and Riesling. Some statistical differences at certain developmental stages were noticeable for compounds such as Fru1,6BP, shikimate, Tre6P, 3-PGA and Glycerol-3-P for Cabernet Sauvignon skins, and Fru1,6BP, tartrate and aconitate for Riesling skins, when the year and the treatment effect were taken in account. When the treatment only was taken in account, statistical differences were mostly visible for Fru1,6 BP, for both cultivars.

Dai et al. (2013) investigated similar metabolites and showed that sugars and organic acids had a similar temporal profile between the two conditions considered by the authors (berries from fruiting-cuttings and vineyards), however some differences were shown in profiles of phosphorylated intermediates. An overview of metabolic changes during ripening for tomato fruit indicated general trend such as an increase in glucose, fructose, and mannose; and a decrease in shikimate and TCA cycle intermediates, when anthocyanins also increased (Quinet et al., 2019). The impact of abiotic factors was also reviewed, and it was noticed that elevated CO<sub>2</sub> generated an increase in total soluble sugars, as well as a decrease in malic acid and flavonoids content (Quinet et al., 2019). However, the authors mentioned performed their experiments on higher concentrations of CO<sub>2</sub> such as 550 and 700 ppm (Mamatha et al., 2014) or 800 ppm (Wei et al., 2018).

Within our study, TCA cycle intermediates were affected by elevated CO<sub>2</sub> for Riesling skins in 2020. Moreover, Riesling skins also displayed changes in glycolysis intermediates in 2021. In addition, Cabernet Sauvignon skins were also affected but in a more moderate manner. The regulation of TCA cycle under high temperatures was investigated by Sweetman et al. (2014) on grapevine cv. Shiraz, and these authors found out that the activity of NAD-ME (NAD-dependent malic enzyme) increased under heating stress. This enzyme catalyses the conversion of mitochondrial malate to pyruvate. Moreover, an increase in numerous amino acids derived from pyruvate, oxaloacetate and  $\alpha$ -ketoglutarate (for example proline) did demonstrate a change in TCA regulation, possibly linked to the increased activity of NAD-ME (Sweetman et al., 2014). Concerning intermediates from TCA cycle (succinate, citrate, fumarate and malate), it was demonstrated that their content gradually increased from early stages of development onwards, up to a peak at EL-31 (fumarate, succinate), EL-33 (malate) and around *véraison* (citrate); and decreased hereafter (Wang et al., 2017). It was also assessed that 2-oxoglutarate, a compound included in TCA cycle, could play a role in signalling in plants (Araújo et al., 2012). In another study, heat stress applied on Cabernet Sauvignon berries decreased the content of several metabolites such as intermediates of glycolysis (F6P, 3PGA, pyruvate), sugar phosphates (T6P, S6P and M6P) and shikimate, glycerate and G3P. Lower malate content was also noticed (Lecourieux et al., 2020).

Tre6P, which is the phosphorylated intermediate of trehalose biosynthesis, plays a central role in signaling in plants. It was established that T6P is implied in the regulation of stomatal

conductance (Figueroa & Lunn, 2016), which therefore is linked to atmospheric CO<sub>2</sub> concentration. T6P is also involved in partitioning the sugar assimilates between sucrose, organic acids, amino acids, via post translational regulation of PEPC (phosphoenolpyruvate carboxylase), in source leaves. Moreover, in sink organs, T6P regulates the sucrose consumption (probably by inhibition of SnrK1) (Figueroa & Lunn, 2016). However, in our study, Tre6P seemed to be not affected by elevated CO<sub>2</sub>, both in 2020 and 2021.

The shikimate pathway permits the flow of carbon from carbohydrates metabolism to aromatic compounds biosynthesis. Chorismate, an intermediate from the shikimate pathway, is a precursor of three aromatic amino acids such as phenylalanine, tyrosine and tryptophane (Zhang et al., 2012), and phenylalanine is also a precursor of secondary metabolites such as anthocyanins (Tzin & Galili, 2010). Shikimate usually displays a decreasing curve during berry ripening. The trend of decreased anthocyanins under elevated CO<sub>2</sub> demonstrated in 2020 and 2021 in Chapter 2 during ripening could possibly be linked to reduced shikimate, as statistically, for Cabernet Sauvignon skins, a year effect but also a treatment effect was noticed. Phenylalanine is also of importance as it is a precursor of phenolic compound through phenylpropanoid pathway. In Cabernet Sauvignon berries, phenylalanine content increased under elevated temperature applied at *véraison*, and this increase was significant when heat treatment was applied at ripening stage (Lecourieux et al., 2017).

In another study investigating the effects of water stress on skin and pulps of Cabernet Sauvignon grape berries, some metabolites differed between pulps of well-watered plants and water deficit treated plants, among them shikimate (Grimplet et al., 2009). In addition, grapes of Cabernet Sauvignon and Shiraz were submitted to water deficit, and the resulted skins were displaying a decrease in amino acids and intermediates of TCA cycle, from *véraison* onwards. Nevertheless, proline, alanine and ascorbate contents increased, especially in Shiraz (Hochberg et al., 2015).

However, only slight differences could be assumed between ambient and elevated CO<sub>2</sub> for such metabolites, which was confirmed by the PCA biplots where the year effect was more discriminant than the treatment, as previously suggested by Chapter 2.

## **Conclusion**

As end products were not significantly different under elevated CO<sub>2</sub> compared to ambient CO<sub>2</sub>, metabolite profiling was of interest to investigate berry plasticity in terms of intermediates from central carbon metabolism. Although the year effect was predominant, fine differences have been observed, mainly regarding Fructose 1,6BP, and the investigation of enzymes activities and genes regulation of these respective pathways under ambient and elevated CO<sub>2</sub> could be a next step in future analyses to sharpen the comprehension around the effects of elevated CO<sub>2</sub> on metabolism.

## **Chapter IV: Effects of elevated CO<sub>2</sub> on the content of must terpenes in Riesling**

## Chapter IV: Effects of elevated CO<sub>2</sub> on the content of must terpenes in Riesling

### Introduction

Terpenoid is a group of molecules which includes monoterpenes and C<sub>13</sub>-norisoprenoids. Monoterpenes are C<sub>10</sub> compounds, which play a role in the resistance of plants towards diseases caused by fungi or bacteria (Gershenson & Dudareva, 2007), influence the sensory properties of grape berries, and, after the wine-making process, of wines. For example, in white wines, they contribute to the aroma profile, more precisely fruity and floral aromas. Monoterpenes in grapes are mainly present in their glycosylated form (Park et al., 1991), frequently conjugated to sugars such as arabinose, glucose, apiose, and rhamnose (Voirin et al., 1992), and are odorless compounds. Free forms can also be found, and are mainly represented by linalool, geraniol, as well as nerol, with pyran and furan forms of linalool oxides. Terpenes such as linalool, nerol,  $\alpha$ -terpineol and geraniol have a great olfactory impact and a low perception threshold (Alem et al., 2019). Depending on the process, some other terpenes can also be found such as citronellol, hotrienol, nerol oxide, and myrcenol among others (Conde et al., 2007). Monoterpene content also permits the classification of grape varieties according to Muscat and non-Muscat varieties, among the latter, Riesling (Mateo & Jiménez, 2000). Terpene biosynthesis begins with the production of IPP (isopentenyl diphosphate) and DMAPP (dimethylallyl pyrophosphate) (Oldfield & Lin, 2012). C<sub>13</sub>-norisoprenoids are formed by carotenoids breakdown, and  $\beta$ -damascenone as well as  $\beta$ -ionone are of particular importance, because they contribute to flowery and fruity notes in young wines (Mendes-Pinto, 2009) whilst  $\beta$ -damascenone has a low odor threshold of 2 ng/L in water (Mendes-Pinto, 2009). Another norisoprenoid, TDN (1,1,6-trimethyl-1,2-dihydronaphthalene), is responsible for the typical kerosene-like aroma in Riesling wines (Tarasov et al., 2020). Although this odor is appreciated in aged Riesling wines, it is undesired in younger wines, and likely to be rejected by most consumers. Higher TDN concentrations were observed for Riesling from areas with warm to high temperatures compared to colder climates (Winterhalter & Gök, 2013).

Climate change questions the impact of changing environmental conditions such as increased global mean temperature, water stress/drought and increased atmospheric carbon dioxide on the aromatic potential of grapes and furthermore wine quality. Generally, in plants, an increase in aroma compound production, particularly for terpenes, can be caused by high temperature, around 40°C (Guenther et al., 1993; Rienth et al., 2021). However, elevated temperature was shown to reduce the aromatic potential of grapevine (Rienth et al., 2014). On the contrary, a study about drought effect on grape berry secondary metabolism using metabolic profiling showed that phenylpropanoids and monoterpenes production was stimulated under water deficit, thus having a potential effect on grape and wine sensory qualities (Savoi et al., 2016). Moreover, increased light exposure had an impact on volatile terpenoids in Sauvignon Blanc by increasing their content (Young et al., 2016).

In another study, whereas the final concentration of free monoterpenes was not dependent on sun exposure either before or after *véraison*, higher temperature did influence the monoterpenes

concentration before *véraison*, but not after (Brandt, 2020). It was also found that  $\beta$ -damascenone increased under high temperature whereas it decreased under higher sun exposure. In addition, higher bound TDN concentrations were displayed under warmer temperature (Brandt, 2020). Furthermore, light exposure influenced positively the monoterpenes content, by the upregulation of monoterpene synthases (Friedel et al., 2016). The effects of elevated CO<sub>2</sub> on must aromatic profile of white varieties have been scarcely studied, because research focused mostly on drought or temperature effect. Indeed, one study concerning the red cultivar Touriga Franca, whose vines have been grown under elevated CO<sub>2</sub> conditions (500 ppm), did not demonstrate significant differences when compared to ambient CO<sub>2</sub> level for citronellol and  $\beta$ -damascenone concentrations (Gonçalves et al., 2009). Thus, in our study, the aim was to test the hypothesis that elevated CO<sub>2</sub> could have an impact on grapevine aroma compounds and aroma profile in must, thus free as well as bound monoterpenes and C<sub>13</sub>-norisoprenoids were quantified in this matrix.

## Material & methods

Must was collected by the doctoral student with support according to Chapter 1 material and methods, and terpene analyses were conducted by Stefanie Fritsch at the Institute for Microbiology and Biochemistry at Geisenheim University. For free terpenes, 1.7 g of NaCl was added to the vial, and then 5 mL of sample was added (30% NaCl in 5 mL sample). Ten  $\mu$ L of standard mix was also added, and samples were analyzed by GC-MS. For bound terpenes, SPE cartridges (SDB-L, Phenomenex, Torrance, CA, USA) were conditioned with 3 mL of pentane/dichloromethane (2:1, v/v) followed by 4 mL of methanol, 4 mL of methanol/H<sub>2</sub>O (1:1, v/v) and 4 mL of H<sub>2</sub>O. Then, 5 mL samples and 5 mL of H<sub>2</sub>O were loaded on SPE cartridges. When the cartridges were empty, 4 mL H<sub>2</sub>O was added to wash the cartridges, they were then allowed to dry for 30 min under vacuum and nitrogen flow. After this step, 4 mL of pentane/dichloromethane (2:1, v/v) permitted the removal of free terpenes. Then, elution of bound terpenes was realized with 4 mL ethyl acetate. This fraction was thus evaporated to dryness at 40°C with a low nitrogen flow. Residues were resolved with 5 mL citric acid buffer (0.2 M, pH 2.5) and placed in an ultrasonic bath. Vials were then placed into beakers, closed with aluminum foil, and heated for 1h at 100°C. Then samples were cooled for 5 min on ice and for 5 min at room temperature. Afterwards, as for free terpenes, 10  $\mu$ L of internal standard mix and 1.7 g NaCl were added. Agilent 6890 GC coupled to a quadrupole mass spectrometer Agilent 5973 N (Agilent Technologies, Palo Alto, CA, USA) was used for GC-MS analysis, equipped with 30 m DB-WAS column, as described by (Brandt, 2020). For both bound and free compounds, the following monoterpenes and C<sub>13</sub>-norisoprenoids were quantified:  $\beta$ -myrcene, limonene, 1,8-cineol, rose oxide 1, rose oxide 2, linalool oxide 1nerol oxide, linalool oxide 2, vitispirane, linalool, hotrienol,  $\alpha$ -terpineol, TDN, citronellol, myrtenol, nerol,  $\beta$ -damascenone, geraniol and  $\beta$ -ionone.

## Statistical analysis

One-way ANOVA was performed with Rstudio version 2022.07. 2+576, to verify the treatment effect. PCA were plotted, using the packages FactoMineR and factoextra.

## Results

Table 12. Free monoterpenes and C13-norisprenoids concentration in Riesling must during ripening, year 2021, ambient CO<sub>2</sub> = aCO<sub>2</sub> and elevated CO<sub>2</sub> = eCO<sub>2</sub>; DOY: Day Of Year

Day of sampling/free terpenes and norisoprenoids (µg.L <sup>-1</sup> )	19/08/2021 (DOY 231)		31/08/2021 (DOY 243)		14/09/2021 (DOY 257)		28/09/2021 (DOY 271)		12/10/2021 (DOY 285)	
	aCO <sub>2</sub>	eCO <sub>2</sub>	aCO <sub>2</sub>	eCO <sub>2</sub>	aCO <sub>2</sub>	eCO <sub>2</sub>	aCO <sub>2</sub>	eCO <sub>2</sub>	aCO <sub>2</sub>	eCO <sub>2</sub>
β -myrcene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
limonene	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
1,8-cineol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Rose oxide 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Rose oxide 2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Linalool oxide 1	15.23 ±2.33	17.67 ±.33	10.27 ±1.17	8.73 ±0.74	4.97 ±1.02	6.40 ±1.47	7.73 ±1.96	7.37 ±1.23	4.43 ±1.27	5.67 ±0.91
Nerol oxide	1.03 ±0.15	1.13 ±0.15	1.03 ±0.11	0.80 ±0.0	0.67 ±0.15	0.83 ±0.11	1.07 ±0.21	1.07 ±0.53	0.90 ±0.17	1.10 ±0.17
Linalool oxide 2	5.90 ±1.04	7.43 ±1.27	3.4 ±0.5	3.07 ±0.32	1.10 ±0.44	1.87 ±0.68	1.07 ±0.47	0.93 ±0.50	0.43 ±0.40	0.73 ±0.45
Vitispirane linalool	n.q. ±3.46	n.q. ±3.06	n.q. ±1.47	n.q. ±1.80	n.q. ±4.2 ±0.79	n.q. ±6.83	n.q. ±6.67	n.q. ±6.80	n.q. ±10.50	n.q. ±14.23 ±4.67
Hotrienol	1.58 ±78.70	0.35 ±74.90	0.15 ±68.00	0.10 ±57.73	1.88 ±38.13	2.14 ±44.23	0.80 ±57.53	4.25 ±54.20	81.87 ±81.87	106.00 ±106.00
α -terpineol	18.88 ±6.20	4.61 ±5.93	9.50 ±5.23	5.26 ±5.03	11.35 ±5.20	5.49 ±6.03	10.49 ±6.63	10.34 ±6.57	20.56 ±8.47	16.15 ±10.00
TDN	±0.1	±0.49	±0.06	±0.32	±1.36	±0.67	±1.11	±0.51	±2.00	±2.10
citronellol	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
myrtenol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
nerol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
β -damascenone	1.23 ±0.31	1.40 ±0.17	1.67 ±0.15	1.70 ±0.10	3.07 ±0.40	3.90 ±0.10	3.40 ±0.36	2.63 ±0.58	1.67 ±0.15	1.97 ±0.06
Geraniol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
β -ionone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table 13. Bound monoterpenes and C13-norisprenoids concentration in Riesling must during ripening, year 2021, ambient CO<sub>2</sub> = aCO<sub>2</sub> and elevated CO<sub>2</sub> = eCO<sub>2</sub>; DOY: Day Of Year

Day of sampling/bound terpenes and norisoprenoids (µg.L <sup>-1</sup> )	19/08/2021 (DOY 231)		31/08/2021 (DOY 243)		14/09/2021 (DOY 257)		28/09/2021 (DOY 271)		12/10/2021 (DOY 285)		
	Treatment	aCO <sub>2</sub>	eCO <sub>2</sub>	aCO <sub>2</sub>	eCO <sub>2</sub>	aCO <sub>2</sub>	eCO <sub>2</sub>	aCO <sub>2</sub>	eCO <sub>2</sub>	aCO <sub>2</sub>	eCO <sub>2</sub>
β -myrcene	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .
limonene	2.06 ± 0.15	2.23 ± 0.32	2.2 ± 0.0	1.93 ± 0.11	1.77 ± 0.06	1.7 ± 0.0	1.83 ± 0.06	1.87 ± 0.06	1.97 ± 0.06	2.07 ± 0.11	
1,8-cineol	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	
Rose oxide 1	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	
Rose oxide 2	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	
Linalool oxide 1	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	
Nerol oxide	6.3 ± 3.8	7.3 ± 1.05	5.30 ± 2.2	3.93 ± 0.87	6.87 ± 1.95	7.37 ± 0.35	19.76 ± 0.23	23.73 ± 1.39	28.97 ± 3.7	33.87 ± 0.67	
Linalool oxide 2	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	
Vitispirane	9.73 ± 3.67	12.67 ± 0.74	18.17 ± 7.08	18.63 ± 1.18	31.9 ± 4.48	31.67 ± 0.4	41.00 ± 10.59	44.17 ± 5.92	41.67 ± 5.81	47.07 ± 3.60	
linalool	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	
Hotrienol	10.87 ± 6.87	15.03 ± 1.63	14.17 ± 5.83	10.83 ± 1.85	14.07 ± 3.86	17.73 ± 1.48	37.67 ± 3.53	46.87 ± 5.2	62.07 ± 12.55	71.47 ± 13.51	
α -terpineol	27.87 ± 5.10	31.63 ± 7.65	31.97 ± 4.30	29.67 ± 7.56	20.20 ± 3.44	20.60 ± 2.98	22.60 ± 2.62	24.07 ± 4.05	28.07 ± 4.86	30.63 ± 6.52	
TDN	2.50 ± 0.76	3.07 ± 0.76	4.33 ± 1.76	5.43 ± 0.74	17.10 ± 3.97	14.40 ± 0.95	25.50 ± 7.78	29.93 ± 5.18	28.97 ± 4.38	31.73 ± 2.41	
citronellol	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	
myrtenol	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	
nerol	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	
β -damascenone	0.73 ± 0.57	1.40 ± 0.46	2.63 ± 0.38	2.63 ± 0.57	3.73 ± 0.46	3.63 ± 0.40	4.63 ± 0.21	5.33 ± 0.71	5.07 ± 0.15	5.4 ± 0.52	
Geraniol	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	
β -ionone	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	n.d .	



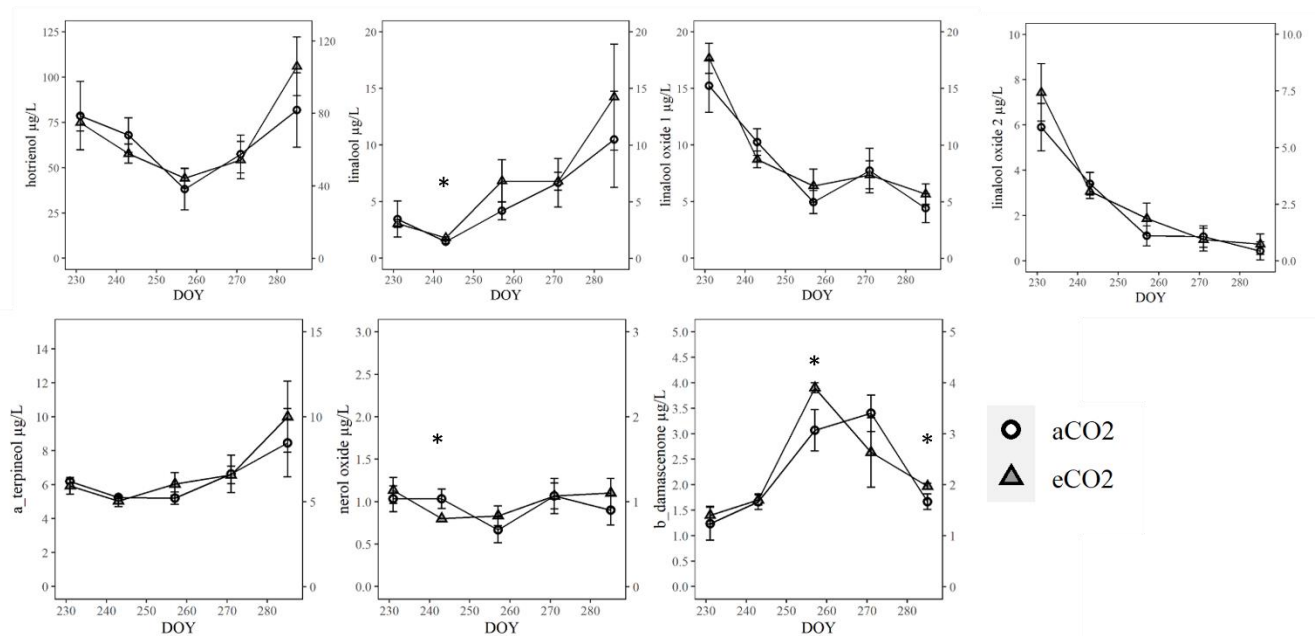


Figure 48. Free monoterpenes and C<sub>13</sub>-norisoprenoids concentration in Riesling must during ripening, year 2021, ambient CO<sub>2</sub> = aCO<sub>2</sub> and elevated CO<sub>2</sub> = eCO<sub>2</sub>; DOY: Day Of Year

The various free monoterpenes and C<sub>13</sub>-norisoprenoids studied had different accumulation kinetics in the must during berry ripening. Compounds such as α-terpineol and linalool increased during ripening, whereas linalool oxide 1 and linalool oxide 2 decreased. For other compounds, there were changes in concentration during berry development (hotrienol and β-damascenone) or they remained quite stable (e.g. nerol oxide). Whereas at maturity hotrienol had a concentration of  $78.7 \pm 18.89 \mu\text{g}\cdot\text{L}^{-1}$  for ambient CO<sub>2</sub> treatment and  $74.9 \pm 4.61 \mu\text{g}\cdot\text{L}^{-1}$  for elevated CO<sub>2</sub> treatment, the concentration of linalool oxide 2 decreased from  $5.9 \pm 1.04 \mu\text{g}\cdot\text{L}^{-1}$  for ambient CO<sub>2</sub> treatment to  $0.43 \pm 0.4 \mu\text{g}\cdot\text{L}^{-1}$  and from  $7.43 \pm 1.27 \mu\text{g}\cdot\text{L}^{-1}$  to  $0.73 \pm 0.45 \mu\text{g}\cdot\text{L}^{-1}$  for elevated CO<sub>2</sub> treatment. In addition, free β-damascenone accumulation seemed to be hastened for elevated CO<sub>2</sub> samples in comparison to ambient conditions (Figure 49). At maturity, β-damascenone was higher significantly higher for elevated CO<sub>2</sub> conditions. When one-way ANOVA was applied, significant differences (p < 0,05) was noticed for free linalool at DOY= 243 (sampling day 31.08.2021), where its content was higher for elevated CO<sub>2</sub> treatment, and for nerol oxide, where its content was lower under elevated CO<sub>2</sub> conditions. However, there was mainly no difference between ambient and elevated CO<sub>2</sub> treatment for both free and bound terpenes in must from 2021, in terms of concentration.

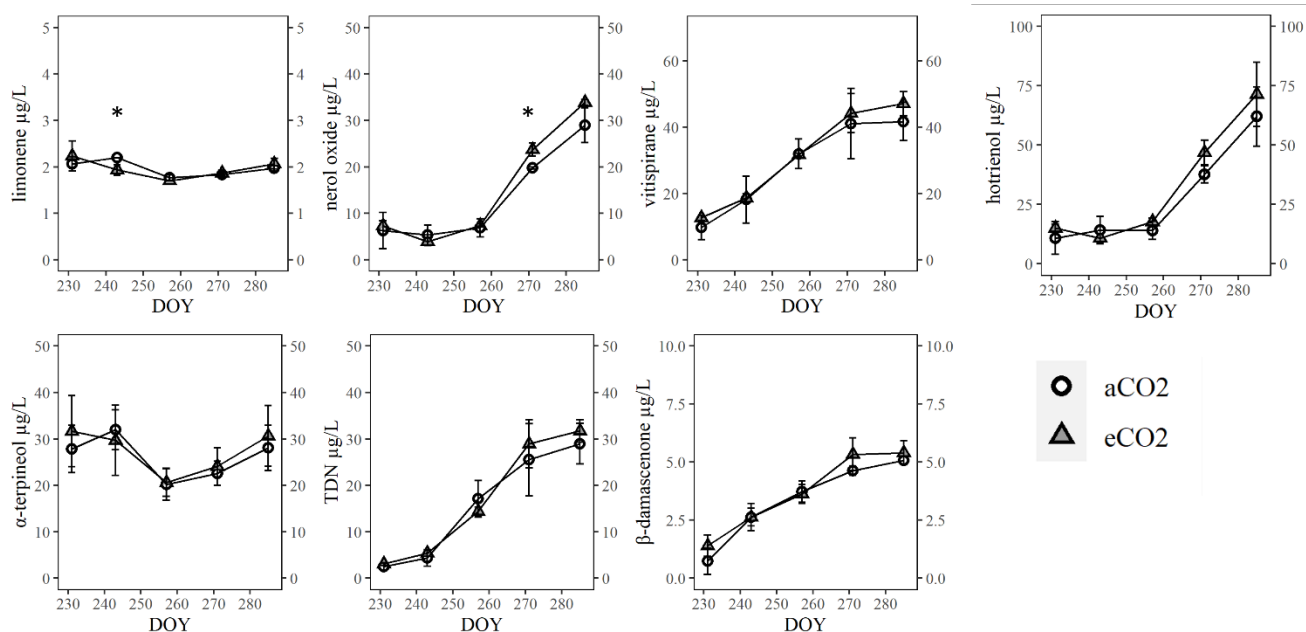


Figure 49. Bound monoterpenes and C<sub>13</sub>-norisoprenoids concentration in Riesling must during ripening, year 2021, ambient CO<sub>2</sub> = aCO<sub>2</sub> and elevated CO<sub>2</sub> = eCO<sub>2</sub>; DOY: Day Of Year

Bound terpenes and C<sub>13</sub>-norisoprenoids displayed mostly an increase during ripening (nerol oxide, vitispirane, hotrienol, TDN and β-damascenone), except for α-terpineol which showed a decrease at DOY=257 where its concentration was  $20.2 \pm 3.44 \mu\text{g.L}^{-1}$  for ambient CO<sub>2</sub> conditions and  $22.6 \pm 2.62 \mu\text{g.L}^{-1}$  for elevated CO<sub>2</sub>, and increased hereafter (Figure 36). Hotrienol had the highest concentration at maturity, increasing from  $10.87 \pm 6.87 \mu\text{g.L}^{-1}$  to  $62.07 \pm 12.55 \mu\text{g.L}^{-1}$  under ambient CO<sub>2</sub> and from  $15.03 \pm 1.63 \mu\text{g.L}^{-1}$  to  $71.47 \pm 13.51 \mu\text{g.L}^{-1}$  under elevated CO<sub>2</sub> (Figure 50). When ANOVA test was applied to verify the effect of the treatment, one difference (\*,  $p < 0.05$ ) was noticed for nerol oxide at DOY = 271, with a concentration of  $19.77 \pm 0.23 \mu\text{g.L}^{-1}$  for ambient CO<sub>2</sub> and  $23.73 \pm 1.39 \mu\text{g.L}^{-1}$  under elevated CO<sub>2</sub> conditions, and for limonene at DOY=243.

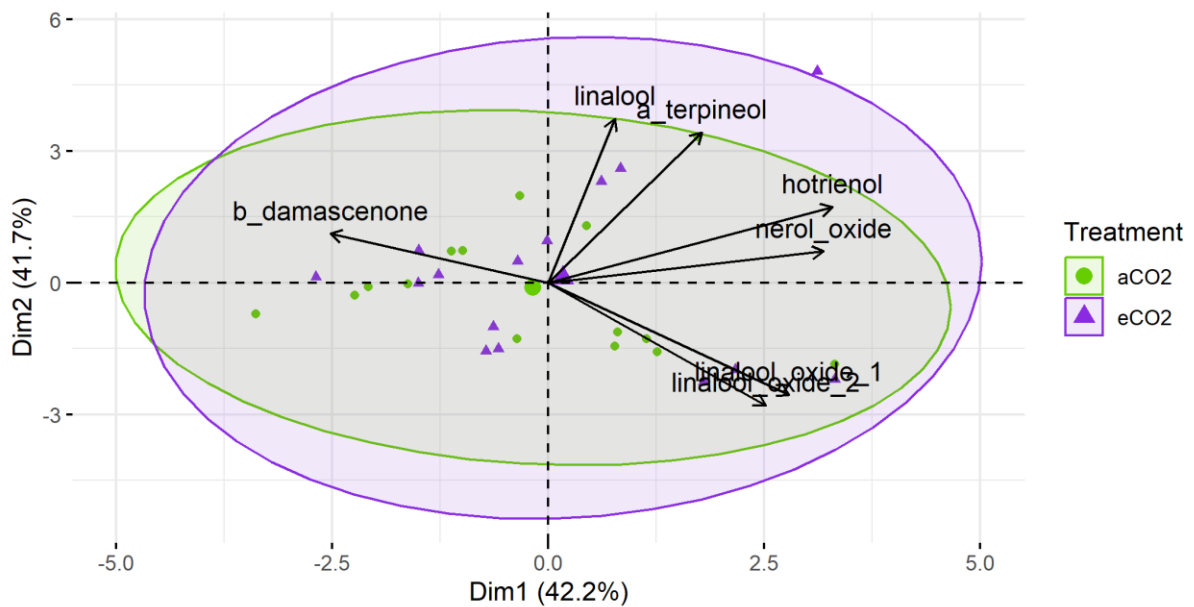


Figure 50. PCA biplot of free terpenes on Riesling must 2021, aCO<sub>2</sub> = ambient CO<sub>2</sub> and eCO<sub>2</sub>= elevated CO<sub>2</sub>

For free terpenes, in the PCA biplot the first dimension explains 42.2% of the variance, and the second dimension explains 41.7% of the variance (Figure 51), together the two principal components were explaining 83.9% of data variability. The PCA did not show a clear separation for free terpenes between the data points of berries sampled from elevated CO<sub>2</sub> treatment and from ambient CO<sub>2</sub> treatment. Dimension 1 was mostly explained by hotrienol and nerol oxide, whereas dimension 2 was explained by linalool. Indeed, terpenes at the beginning of ripening were highly represented by linalool oxide 1 and linalool oxide 2, and at maturity samples were more represented by linalool or by  $\alpha$ -terpineol. Dimension 2 corresponds more to the stability of the compound during ripening, with  $\beta$ -damascenone being less stable and nerol oxide remaining stable (Figure 51).

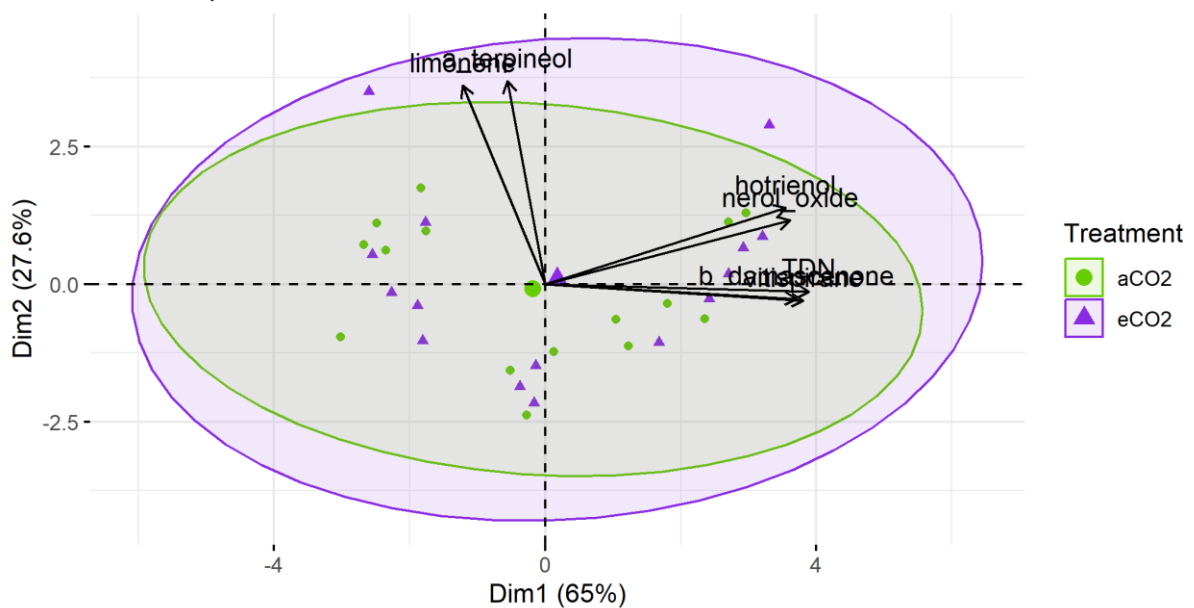


Figure 51. PCA biplot of bound terpenes on Riesling must 2021, aCO<sub>2</sub> = ambient CO<sub>2</sub> and eCO<sub>2</sub> = elevated CO<sub>2</sub>

For bound terpenes, the first dimension of the PCA biplot explained 65% of the variance, and the second dimension explained 27.6% of the variance. The first dimension was mainly explained by TDN and  $\beta$ -damascenone, whose concentrations were increasing during ripening, and the second dimension was explained by  $\alpha$ -terpineol and limonene (Figure 52). As for free terpenes and norisoprenoids, no distinct difference between the two treatment groups could be displayed for bound compounds.

## Discussion

Terpenes and C<sub>13</sub>-norisoprenoids are usually not studied in must during berry development, but mostly in young wines.

It was reported that the onset of bound C<sub>13</sub>-norisoprenoids accumulation in Riesling began 1-2 weeks earlier than monoterpenes glycosides accumulation (Ryona & Sacks, 2013).

Within our study, slight differences could be found for free aroma compounds between ambient and elevated CO<sub>2</sub> conditions, with linalool displaying a treatment effect, as well as for bound terpenes with nerol oxide, but these differences were only significant for one developmental stage during ripening. In another study, total free monoterpenes increased from EL-34 to EL-38 by 46.56% and total bound monoterpenes increased by 72%, where linalool, limonene,  $\beta$ -myrcene and  $\alpha$ -terpineol were related to post *véraison* stage in Riesling and Muscat Hamburg grapes (Yue et al., 2020). Free terpenes in Riesling decreased from EL-31 to EL-33, and increased hereafter until maturity; monoterpenes and C<sub>13</sub>-norisoprenoids studied also had different accumulation kinetics (Luo et al., 2019). Another study investigating the effects of a double cropping system on aroma compounds showed a cultivar dependent response for aroma composition, but berries from winter season showed higher concentration of terpenes and norisoprenoids for different cultivars, including Riesling (Lu et al., 2022).

The effect of regulated deficit irrigation at harvest was investigated on terpenes composition in Gewürztraminer, and differences in free terpenes were found ( $\alpha$ -terpineol, citronellol, farnesene-a, farnesene-b, geraniol, methyl geranate), whereas no difference seemed to occur for bound terpenes (Kovalenko et al., 2021). Also, the authors reported a strong year effect. Water deficit in white grapes allowed the accumulation of free monoterpenes, including hotrienol, linalool, nerol and  $\alpha$ -terpineol (Savoi et al., 2016). The plant response to water deficit is usually linked to stomatal closure, which restricts water loss but also carbon assimilation (Chaves et al., 2010). Elevated CO<sub>2</sub> could have opposite physiological effects compared to drought, especially in our study where stomatal conductance and transpiration rate were increased under elevated CO<sub>2</sub>, as Chapter 1 suggested, whereas under drought these parameters usually decreased. Indeed, free hotrienol, linalool and  $\alpha$ -terpineol increased during berry ripening within our study, for both CO<sub>2</sub> conditions.

Moreover, within our study, aroma profile in Riesling seemed to be not negatively affected by elevated CO<sub>2</sub> concentration.

## **Conclusion**

Free and bound aroma compounds were investigated under two CO<sub>2</sub> conditions, and the aroma profile of Riesling must in 2021 seemed to be overall not affected by the elevated CO<sub>2</sub> treatment. In complement to these analyses, it would be of further interest to investigate the aroma profile of young Riesling wines of vines grown under elevated and ambient CO<sub>2</sub> conditions at VineyardFACE. Moreover, aroma profile as well as the ageing parameters of wines from previous vintages grown under elevated and ambient CO<sub>2</sub> could also be considered for future studies.

# **General discussion**

## General discussion

Climate change is affecting grapevine physiology and berry composition, mostly through a temperature and drought effect. Elevated CO<sub>2</sub>, as a climate change-related factor was demonstrated to have an impact on grapevine physiology, by increasing assimilation rate, stomatal conductance, and transpiration rate (Wohlfahrt et al., 2018). However, the need of long-term studies on the effect of elevated CO<sub>2</sub> on grapevine was stated in this regard in the review of Clemens et al. (2022). VineyardFACE is designed in the purpose of studying the long-term effects of a moderate and gradual increase of CO<sub>2</sub>. Thus, within the present study, the impact of a gradual increase of CO<sub>2</sub> was explored, after more than six years of fumigation in an open-field set-up, on grapevine physiology, berry composition and metabolism, as well as aroma profile.

### VineyardFACE, a long-term study

The present study is part of the VineyardFACE experiment, which was established in 2013. As previously demonstrated by Wohlfahrt et al. (2018) over the timeframe 2014 to 2016, photosynthesis rate was enhanced during the years of the present study, for both Cabernet Sauvignon and Riesling. These results are in accordance with other previous outcomes, as assimilation rate is generally stimulated by elevated CO<sub>2</sub>, to an extent which depends also on species and experimental conditions (Leakey et al., 2009). Although berry yield was higher under elevated CO<sub>2</sub>, the excess in carbohydrates noticed from previous seasons onwards could be allocated to the woody tissues rather than the reproductive organs, as stated by Wohlfahrt (2021) where pruning wood of Riesling was richer in glucose under elevated CO<sub>2</sub> from 2014 to 2016. Furthermore, it was also suggested by Salazar-Parra et al. (2015) and stated by Kizildeniz et al. (2021), that the exposure to elevated CO<sub>2</sub> (700 ppm) could induce photosynthetic acclimation in grapevine (i.e. decrease in photosynthesis performance). However, this is not the case in our study where photosynthesis still displayed an enhancement in response to elevated CO<sub>2</sub> conditions but was a lower CO<sub>2</sub> level compared to other greenhouse studies. Besides, leaf anatomy changes under elevated CO<sub>2</sub> were studied in a recent communication of Wohlfahrt et al. (2022), changes in leaf morphology mainly through differences in palisade parenchyma and epidermis thickness were reported under elevated CO<sub>2</sub> in Cabernet Sauvignon, whereas no difference occurred in Riesling. A study on tomato fruit, another C<sub>3</sub> plant, demonstrated broader and thicker leaves under elevated CO<sub>2</sub> concentrations, when CO<sub>2</sub> was enriched to 550 and 700 ppm (Rangaswamy et al., 2021). Elevated CO<sub>2</sub> seemed to impact mostly the leaves, by increasing assimilation rate, but also stomatal conductance and transpiration rate within the present study. However, most FACE studies have demonstrated a decrease in stomatal conductance, by 20 to 30% for C<sub>3</sub> crops (Ainsworth & Rogers, 2007). This assumption can be mitigated, as Purcell et al. (2018) demonstrated that, in some cases, elevated CO<sub>2</sub> could lead to increased stomatal conductance.

On the other hand, technological maturity is not impacted by elevated CO<sub>2</sub> conditions, as previously demonstrated by Wohlfahrt et al. (2020), which is also the case in the timeframe of

our study. In addition, berry composition in terms of primary metabolites for both Riesling and Cabernet Sauvignon seemed to remain mostly unaffected by elevated CO<sub>2</sub> treatment. These results were different from the findings of the initial FACE experiment in Italy, where both sugars and organic acids concentration were stimulated by elevated CO<sub>2</sub> treatment (Bindi et al., 2001). The duration (two years), and the timeframe of the experiment from the onset of budbreak towards harvest and the concentration of CO<sub>2</sub> treatment (550 and 700 ppm) were also different from the present study using the VineyardFACE, where a moderate and gradual increase of CO<sub>2</sub> was applied since 2014 during the entire time of the experiment and from sunrise to sunset. The short duration of most of the previous experiments and their mainly large increase of CO<sub>2</sub> treatment has a direct effect on plant metabolism. Indeed, in tomato fruit, an elevation of CO<sub>2</sub> (850 ppm) stimulated both photosynthesis and carbohydrates content (Islam et al., 2006). Health-promoting compounds of tomato (lycopene,  $\beta$ -carotene, ascorbic acid), as well as sugars, titratable acidity, and sugar to acid ratio were greatly increased under elevated CO<sub>2</sub> (800-900 ppm). Within our study, the year effect was also predominant in comparison to the treatment effect, which might be due to the disparity between weather conditions among the years studied, with 2020 being a warmer and drier year, compared to 2021.

In the literature, some studies seemed to contrast with the expected results. A high increase of CO<sub>2</sub> concentration would theoretically lead to increased carbohydrates available in leaves (Rogers & Ainsworth, 2006). On the contrary, in goji berries, elevated CO<sub>2</sub> (570 and 760 ppm) decreased glucose and fructose content after 90 and 120 days of applied treatment, while photosynthesis was promoted (Ma et al., 2021).

### **The context of climate change involves various abiotic parameters**

Climate change results in a combination of factors, as many studies were conducted, studying the impact of these parameters (elevated temperature, drought, elevated CO<sub>2</sub>) and their interactions. Indeed, an increase of carbon dioxide could minimize the deleterious effects of other climate change-related factors. Indeed, elevated CO<sub>2</sub> was demonstrated to mitigate the adverse effects of drought, by stimulating biomass and yield in C<sub>3</sub> crops even under dry conditions (van der Kooi et al., 2016). In a FACE experiment involving a C<sub>4</sub> crop such as sorghum, elevated CO<sub>2</sub> increased the crop biomass despite drought conditions, meaning that elevated CO<sub>2</sub> could have a mitigation effect when drought is also involved (Ottman et al., 2001). A TGG experiment (400 versus 700 ppm, T<sub>a</sub> versus T<sub>a</sub>+4°C and well irrigated versus drought) on peach demonstrated that elevated CO<sub>2</sub> alleviated the effects of drought; but this response was also depending on the rootstock used (Jiménez et al., 2020). The effects of combined factors, such as elevated temperature and elevated CO<sub>2</sub>, were mitigated by UV-B treatment on anthocyanins concentration (Martínez-Lüscher et al., 2016). Moreover, in the study of Arrizabalaga-Arriazu et al. (2020) a significant interaction was found, between elevated temperature and elevated CO<sub>2</sub>, concerning anthocyanins to sugars ratio, which could suggest that elevated CO<sub>2</sub> could mitigate the effects of elevated temperature, for this parameter.



## Berry quality under elevated CO<sub>2</sub>

Anthocyanins content is greatly decreased by temperature, as demonstrated by (Mori et al., 2007a). In Cabernet Sauvignon, heating treatment (+8°C) decreased anthocyanin content at maturity (Lecourieux et al., 2017). Although within the present study, total anthocyanins content displayed a decrease under elevated CO<sub>2</sub>, especially in 2020 and 2021, this trend was not significantly different between the treatments. (Wohlfahrt et al., 2021) assumed that Cabernet Sauvignon would display lower total anthocyanins concentration under elevated CO<sub>2</sub>. However, these results contrasted with the study of Arrizabalaga-Arriazu (2020), where elevated CO<sub>2</sub> treatment result in increased anthocyanins concentration at the onset of ripening and decreased anthocyanins concentration from two weeks after mid-*véraison* onwards. Moreover, Arrizabalaga-Arriazu (2019) demonstrated a modification of the anthocyanins profile towards more stable forms, such as tri-hydroxylated, di-methylated and acylated forms, under elevated CO<sub>2</sub> conditions. Within the present study, in 2021, higher di-hydroxylated to tri-hydroxylated ratio was found for ambient CO<sub>2</sub> conditions, meaning that either cyanidin or peonidin derivatives were decreased under elevated CO<sub>2</sub> or delphinidin, malvidin and petunidin derivatives increased under elevated CO<sub>2</sub>, but this was noticeable only for one developmental stage. Another study on Tempranillo demonstrated that UV-B affected flavonols more than anthocyanins, with less abundant tri-substituted forms while UV-B doses increased (Martínez-Lüscher et al., 2014).

Intermediates of primary metabolism, such as compounds from TCA cycle and glycolysis intermediates displayed some differences between the treatments, but these differences seemed to be dependent on the cultivar and the year, rather than the treatment. After statistical analyses and data visualization, F1,6 biphosphate was found to be affected both in Cabernet Sauvignon and Riesling, being significantly lower under elevated CO<sub>2</sub> at early stages in Cabernet Sauvignon in 2020.

In another FACE experiment using ambient CO<sub>2</sub> (375 ppm) and elevated CO<sub>2</sub> (550 ppm) concentrations in *Arabidopsis thaliana* rosettes, metabolite and transcript profiles revealed changes such as increased sugars, as well as increased isocitrate, malic acid and succinate. Besides, the transcript profile revealed a down-regulation of transcript related to amino-acids biosynthesis (Li et al., 2008). However, the short life span of *Arabidopsis thaliana* (48 days in average) rather reflected short-term response to elevated CO<sub>2</sub> (a snapshot, as the authors mentioned in their publication) than long-term response.

However, compounds involved in cell wall of berry skins, such as 4-O-methylglucuronic acid (Urbanowicz et al., 2012), seemed to be affected by elevated CO<sub>2</sub> albeit not significantly, but these changes could be correlated with increased berry weight and berry volume, especially in Cabernet Sauvignon. Effects of elevated CO<sub>2</sub> on cell wall has already been investigated in other species. Cell wall plasticity and elasticity in *Plantago* media leaves were increased under elevated CO<sub>2</sub>, whereas in *Anthyllis vulneraria* no effect was demonstrated, showing a specie-dependent response (Taylor et al., 1994). Moreover, a review has been dedicated to understanding the effects of elevated CO<sub>2</sub> on mechanistic basis of growth processes at the cellular level (Pritchard et al., 1999).

To the best of our knowledge, aroma profile of grape must under elevated CO<sub>2</sub> has not been yet investigated in white grapevine cultivars. Gonçalves et al. (2009) showed no negative effect of elevated CO<sub>2</sub> on aroma profile of Touriga Franca young wines, despite lower methionol, 1-octanol, 4-ethylguacaol concentrations, higher linalool, and ethyl lactate concentrations for one particular year under elevated CO<sub>2</sub> conditions. Water deficit increased the monoterpene concentration in a white cultivar, together with phenylpropanoids and tocopherols; these changes were also visible at the transcriptomic level (Savoi et al., 2016). In Cabernet Sauvignon, aroma potential could be decreased by elevated temperature by the deregulation of aroma and aroma precursor related genes (Lecourieux et al., 2017). Within our study, β-damascenone seemed to be hastened under elevated CO<sub>2</sub>, However, within the present study, only few differences were noticed between elevated and ambient CO<sub>2</sub> conditions for monoterpenes and C<sub>13</sub>-norisoprenoids (Chapter 4).

### **Root system possibly affected under elevated CO<sub>2</sub> ?**

Roots parameters could not have been studied in the VineyardFACE, because it would require the destruction of experimental the set-up. However, the effects of elevated CO<sub>2</sub> in root systems of soybeans have already been studied, demonstrating a direct influence of CO<sub>2</sub> on root system architecture, impacting its micromorphology and physiology, with the greatest impact being on root dry weight (Rogers et al., 1992). Reddy et al. (2021) demonstrated that elevated CO<sub>2</sub> mitigated the effect of elevated temperature for all cultivars studied on grapevine roots parameters. Moreover, depending on the cultivar, different root parameters (such as root volume, total root length, root length density) were increased by elevated CO<sub>2</sub> (Reddy et al., 2021). The effects of elevated CO<sub>2</sub> on the rhizosphere microbiome were also studied on wheat and soybean by Cheng et al. (2011), demonstrating that elevated CO<sub>2</sub> affected soil microbial community, microbial biomass, and activities. In addition, another study on nonmycorrhizal versus mycorrhizal plant species grown under elevated atmospheric CO<sub>2</sub> for three years demonstrated changes in rhizosphere carbon dynamics (Drigo et al., 2013). In tomato plants, elevated CO<sub>2</sub> significantly impacted the microbial ecosystem, with a greater impact on fungal community compared to the effects on bacteria (H. Wang et al., 2020). A meta-analysis indicated that root morphology, rooting depth and fungal colonization as well as biomass allocation responded positively to increased atmospheric CO<sub>2</sub> concentration (Nie et al., 2013). Elevated CO<sub>2</sub> also influences pest pressure, by directly or indirectly influencing pathogenesis- and herbivory-related traits in both pest and pathogens populations (Kazan, 2018).

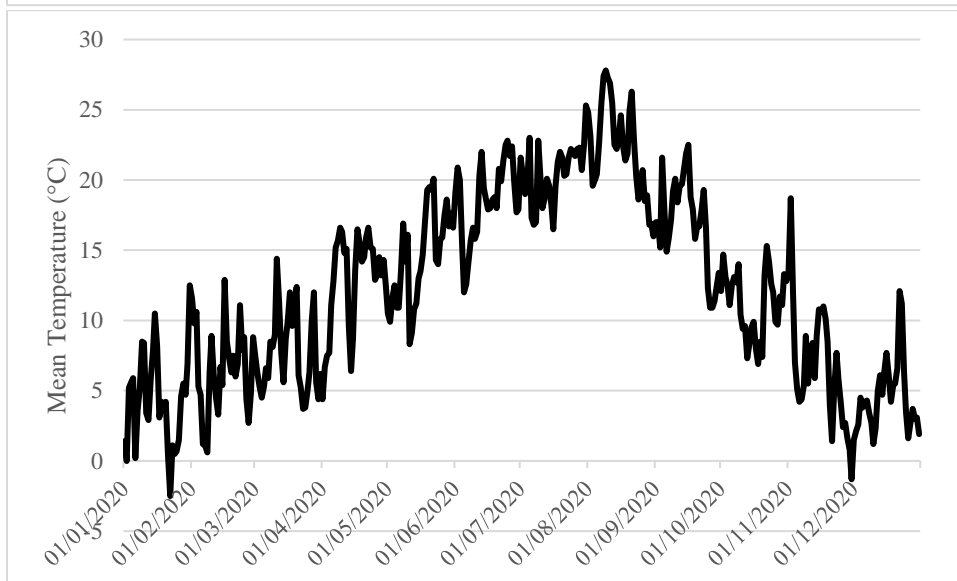
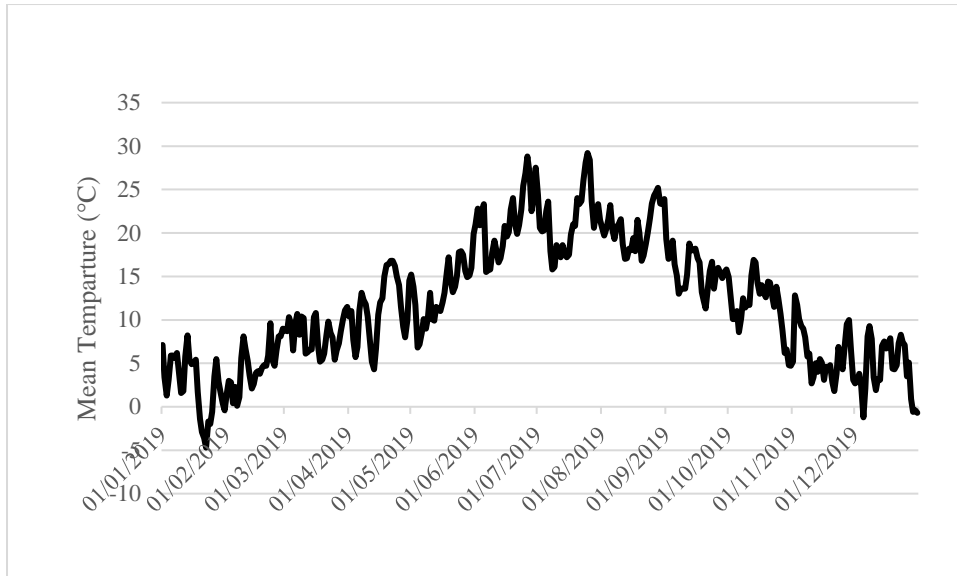
## Conclusion & perspectives

First general conclusion drawn from the experiments at VineyardFACE from the timeframe 2019 to 2021 could be that, although vegetative growth seemed to be impacted by elevated CO<sub>2</sub> treatment according to Chapter 1, berry composition, both for final compounds (Chapter 2) and intermediates from central metabolism (Chapter 3) were not negatively impacted by elevated CO<sub>2</sub>, at least for the timeframe of this study. Moreover, in Chapter 4, monoterpenes and C<sub>13</sub>-norisoprenoids composition, and to extend aroma profile seemed to be similar between the two treatments for the must of Riesling in 2021. However, atmospheric carbon dioxide concentration is still increasing, according to IPCC. Indeed, the predictions are expecting 700 ppm of CO<sub>2</sub> at the end of the 21<sup>st</sup> century, which strongly contributes to climate change through greenhouse effect. Consequently, a question arising could be phrased: how far are we from the tipping point, concerning the effects of elevated CO<sub>2</sub> on berry composition and berry plasticity? In other words, how long could the grapevine stay unaffected by elevated CO<sub>2</sub>?

Moreover, climate change encompasses a combination of factors, known as higher temperature, water stress and elevated CO<sub>2</sub>, as a simplified approach. As our investigation dealt only with one factor, a question remaining opened could be the following: what could be the interaction between elevated CO<sub>2</sub> and other factors of climate change such as elevated temperature or drought and how much they interact with each other? Moreover, as increased atmospheric CO<sub>2</sub> concentration contributes to greenhouse effect, and thus to increased global mean temperature, this parameter would likely have an indirect impact on climate change consequences.

Root system can be studied in VineyardFACE, but with difficulty, and may require destructive methods, so an important part of the question remains opened: what are the effects of long-term acclimation to elevated CO<sub>2</sub> on root system and rhizosphere microbiome at VineyardFACE?

## Appendix



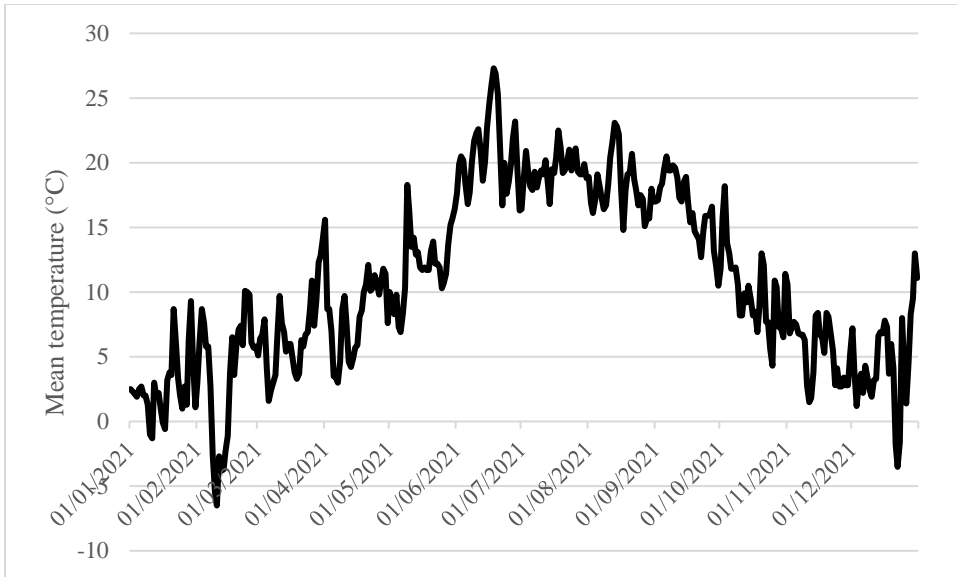
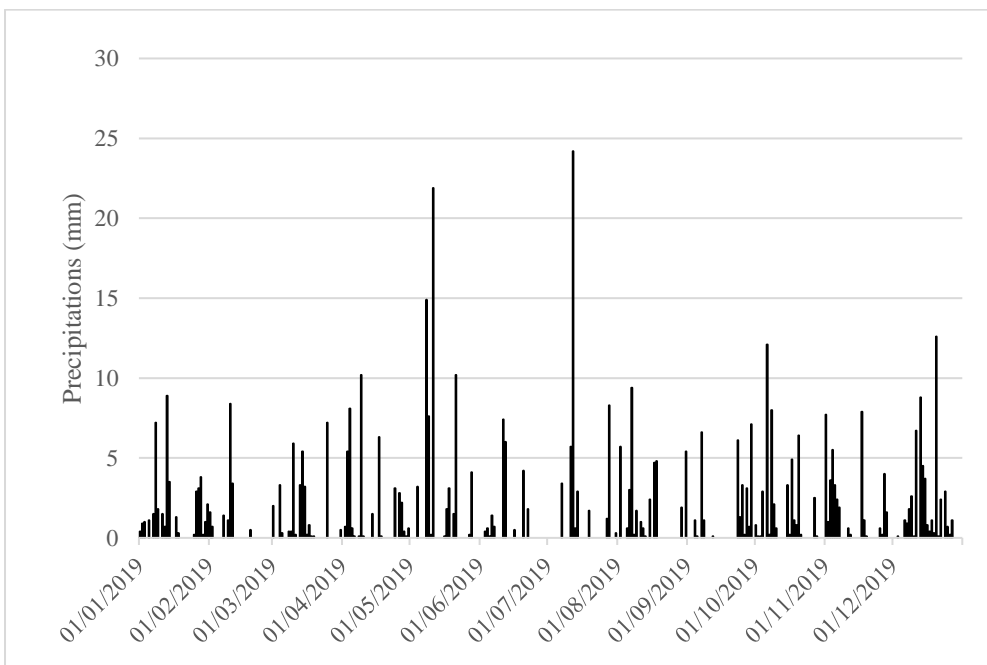


Figure 52. Mean temperature (°C) during the years 2019, 2020 and 2021 for VineyardFACE weather station



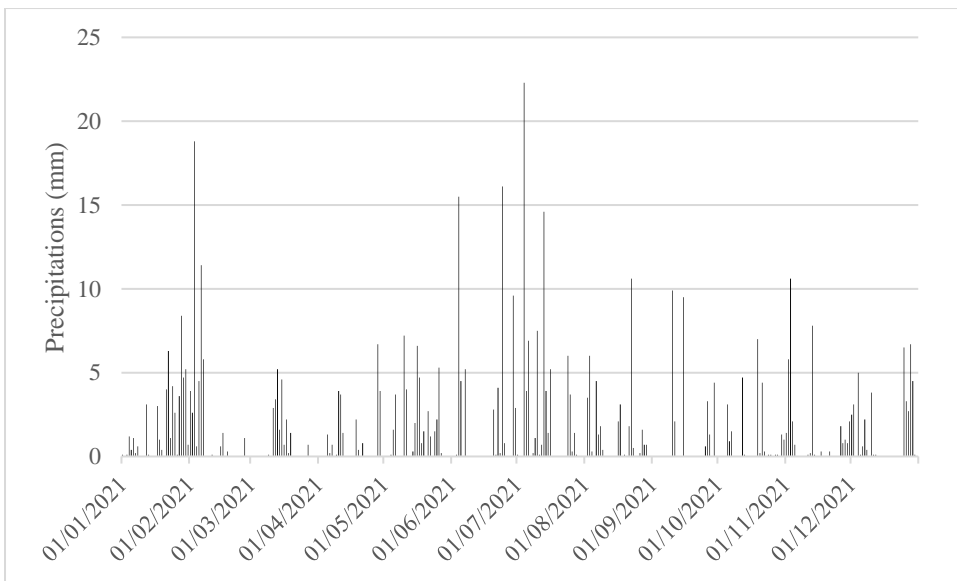
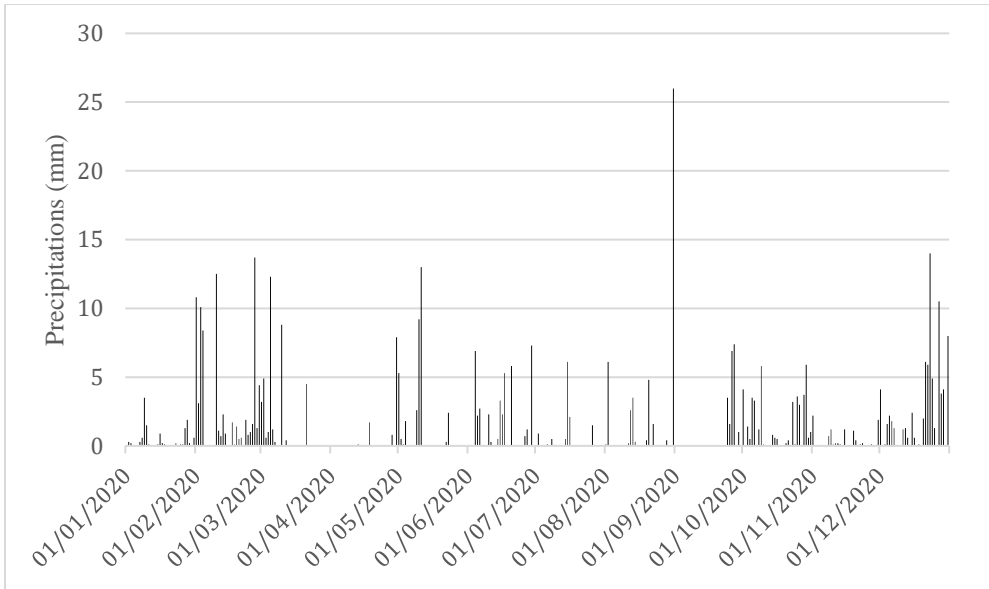


Figure 53. Precipitations amount (mm) during the years 2019, 2020 and 2021 for VineyardFACE weather station

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ORIGINAL RESEARCH ARTICLE

# VineyardFACE: Investigation of a moderate (+20 %) increase of ambient CO<sub>2</sub> concentration on berry ripening dynamics and fruit composition of Cabernet-Sauvignon

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## ABSTRACT

Climate change and rising atmospheric carbon dioxide concentration are a concern for agriculture, including viticulture. Studies on elevated carbon dioxide have already been conducted on grapevines, mainly taking place in greenhouses using potted plants or using field-grown vines under instant and higher CO<sub>2</sub> enrichment, i.e., > 650 ppm. The VineyardFACE, located at Hochschule Geisenheim University, is an open field Free Air CO<sub>2</sub> Enrichment (FACE) experimental set-up designed to study the effects of elevated carbon dioxide using adapted, field-grown vines (*Vitis vinifera* L. cv. Cabernet-Sauvignon). As the carbon dioxide fumigation started in 2014, the long-term effects of elevated carbon dioxide treatment can be investigated on berry ripening parameters and fruit metabolic composition.

The present study investigates the effect on fruit composition under a moderate increase (+20 %; eCO<sub>2</sub>) of carbon dioxide concentration, as predicted for 2050 on Cabernet-Sauvignon. Berry growth, ripening dynamics and composition were determined and primary (sugars, organic acids, amino acids) and secondary metabolites (anthocyanins) were analysed. Compared to previous results of the early adaptive phase of the vines (Wohlfahrt *et al.*, 2020), our results show little effects of eCO<sub>2</sub> treatment on primary metabolites composition in berries. However, total anthocyanins concentration in berry skin was lower for eCO<sub>2</sub> treatment in the hot and dry season of 2020, although the ratio between anthocyanins derivatives did not differ.

**KEYWORDS:** Free Air CO<sub>2</sub> Enrichment, carbon dioxide, berry ripening, berry composition

## INTRODUCTION

Ongoing changes in global mean temperature, precipitations and continuously increasing carbon dioxide (CO<sub>2</sub>) concentration are reshuffling conditions in which plants are growing. Indeed, atmospheric carbon dioxide concentration increases continuously due to anthropogenic emissions and currently reaches 410 ppm, while global surface temperature is already 1.09 °C higher in 2011–2020 compared to the 1850–1900 era (IPCC, 2021). Global surface temperature at the end of the century is predicted to increase by 1.0 °C to 1.8 °C (low greenhouse gas GHG emissions scenario), 2.1 °C to 3.5 °C (intermediate GHG emissions scenario) and by 3.3 °C to 5.7 °C (very high GHG emissions scenario; IPCC, 2021). Furthermore, according to the most pessimistic scenarios, rainfall variability should be amplified in the near future, and climate change is predicted to intensify the severity of wet and dry events (IPCC, 2021).

Crops are sensitive to environmental conditions, and grapevine is no exception to the rule. Berry oenological potential (i.e., its composition at harvest) is a complex trait that mainly results from genotypes (scions and rootstocks) and environmental interactions. Thus, climate change's impact on grapevine fruit composition at harvest needs to be thoroughly studied to help the wine industry adapt to future climate conditions (Duchêne *et al.*, 2010; Schultz, 2000). Numerous studies have already characterised how grapevine and wine characteristics are impacted by elevated temperature (Luchaire *et al.*, 2017; Sadras *et al.*, 2013), heat stress (Lecourieux *et al.*, 2017), water deficit (Deluc *et al.*, 2009) or UV-B (Martínez-Lüscher *et al.*, 2013).

Studies concerning the impact of atmospheric carbon dioxide concentration increase are scarcer and it should be noticed that studying carbon dioxide concentration effects is technically more challenging. Thus, prior studies on the impact of carbon dioxide on *Vitis vinifera* L. were mostly conducted in greenhouses or enclosed (tunnel or chamber) systems, using potted plants (fruiting cuttings) (Arrizabalaga-Arriazu *et al.*, 2020; Arrizabalaga-Arriazu *et al.*, 2021; Martínez-Lüscher *et al.*, 2016). However, the translation of the results obtained from greenhouses to vineyard-based studies can be biased, as reported by Poorter *et al.* (2016). FACE (Free Air Carbon dioxide Enrichment) systems are open field setups designed to create a CO<sub>2</sub> enriched atmosphere around crops and represent a more realistic experimental system. Several FACE systems have been installed already, whether on various herbaceous crops such as pea (Bourgault *et al.*, 2016), wheat and rice (Cai *et al.*, 2016), barley and maize (Erbs *et al.*, 2015) or trees such as poplars (Gielen and Ceulemans, 2001). According to literature reports on FACE based experiments, elevated CO<sub>2</sub> caused decreased stomatal conductance (Ainsworth and Rogers, 2007; Leakey *et al.*, 2009) and increased light-saturated CO<sub>2</sub> uptake/net assimilation rate (Reddy *et al.*, 2010) in C<sub>3</sub> plants. Consequently, the carboxylation efficiency of RuBisCo, compared to the oxygenation efficiency, was increased under elevated

CO<sub>2</sub>. An increase in leaf area was also observed in response to an increase in CO<sub>2</sub> atmospheric concentration (Ainsworth and Long, 2005).

The impact of CO<sub>2</sub> atmospheric concentration on grapevine vegetative growth and berry composition has also been investigated by Bindi *et al.* (2001) using a FACE system, with different carbon dioxide concentrations (550 ppm, 700 ppm compared to ambient), on cv. Sangiovese. Vegetative growth, as well as fruit fresh and dry mass, were significantly increased by elevated CO<sub>2</sub> concentrations. Sugars and organic acid concentrations increased during berry development and ripening, but at maturity, these effects tended to disappear (Bindi *et al.*, 2001). However, in these experiments, the treatment was applied instantaneously as a “shock” of CO<sub>2</sub>, i.e. by suddenly rising local CO<sub>2</sub> to the desired level, which will not mimic realistic climate change scenarios, in which CO<sub>2</sub> increases gradually by approximately 2 ppm per year (IPCC, 2021). Therefore, such results must be discussed with caution since vines did not face a long-term acclimation towards gradual changes in carbon dioxide.

Using the VineyardFACE system described by Wohlfahrt *et al.* (2018), vegetative growth, leaf gas exchanges and yield parameters of cvs. Riesling and Cabernet-Sauvignon were compared under elevated and ambient CO<sub>2</sub> conditions. A significant increase in net assimilation rate, transpiration rate, stomatal conductance and water use efficiency was reported during the early years of grapevine adaptation, i.e., up to six years after planting (Wohlfahrt *et al.*, 2018). Conversely, other results in the literature rather reported a decrease in stomatal conductance (Ainsworth and Rogers, 2007), although some authors agree with the fact that depending on weather conditions, an increase in stomatal conductance can occur under enriched CO<sub>2</sub> conditions (Purcell *et al.*, 2018). Further studies showed that elevated CO<sub>2</sub> altered bunch parameters, increased single berry weight as well as malic acid content (Wohlfahrt *et al.*, 2020). Moreover, minor differences in galacturonic acid for Cabernet-Sauvignon wines, pH or volatile acidity for Riesling were observed (Wohlfahrt *et al.*, 2021). However, fruit quality at ripeness was globally not affected by elevated CO<sub>2</sub>. Indeed, sugar concentration did not differ at harvest (Wohlfahrt *et al.*, 2018). No negative impact of elevated CO<sub>2</sub> treatment was reported on must and wine composition for the years 2014 to 2016, and no difference occurred in total anthocyanin concentration in young wines of Cabernet-Sauvignon (Wohlfahrt *et al.*, 2021).

In previous studies of the VineyardFACE, young grapevines adapted to the fumigation from an early stage of their development, and vegetative growth, as well as berry composition, was extensively studied. However, the long-term effect of elevated CO<sub>2</sub> has hardly been studied yet, as recently highlighted by Clemens *et al.* (2022). In VineyardFACE, vines have been well established since 2014, and the duration of the fumigation allows us to study grape berry parameters and composition of well-adapted vines using Cabernet-Sauvignon under near future (i.e. mid-century, 2050) forecasted scenarios.



# MATERIALS AND METHODS

## 1. Experimental set-up

VineyardFACE is an experimental setup located at Hochschule Geisenheim University (49° 59' N, 7° 57' E; Rheingau, Germany) with a total area of 0.5 ha planted in 2012 with Cabernet-Sauvignon (clone 170, grafted on rootstock 161-49 Couderc). Six Free Air Carbon dioxide Enrichment (FACE) rings surround the vineyard, three with ambient CO<sub>2</sub> levels (~410 ppm, aCO<sub>2</sub>, “A” rings) and three with elevated CO<sub>2</sub> levels (aCO<sub>2</sub> plus 20 %: eCO<sub>2</sub>, “E” rings). These rings are built with 36 towers, with a built-in blower creating an airstream and emitters releasing carbon dioxide (Wohlfahrt *et al.*, 2018). Each ring consists of seven rows, with the inner five rows used for sampling and rings A1-E1, A2-E2, A3-E3 are defined as experimental blocks.

## 2. Berry sampling and processing

Berries were collected in seasons 2019, 2020 and 2021 and sampled from E/L 33-34 onwards (Coombe, 1995). *Véraison*'s progress was assessed by the percentage of individual berries starting to change colour. Between 14 and 18 berries were picked per sampling day (Supplementary Table 1) and selected randomly from the inner row of each ring (half of the berries from the eastern side of the canopy and half from the west), put in pre-chilled Falcon tubes, immediately frozen in liquid nitrogen and stored in freezers at –80 °C until processing. Berries were counted and weighted. Pulp, skin and seeds were separated, and all compartments were weighted. Pulp and skins were reduced into a fine powder using an MM400 grinder (Retsch, Haan, Germany) under liquid nitrogen. Relative skin and seed mass, expressed in percentage, was calculated by dividing respectively skin fresh weight and seed fresh weight by berry fresh weight. Seed and ground powders were then stored in a –80 °C freezer until further analysis.

## 3. Berry volume calculation

Equatorial and polar diameters were measured on each frozen berry from samples 2020 and 2021 with a digital calliper. A formula for the volume of a spheroid was applied to calculate berry volume, according to Arrizabalaga-Arriazu *et al.* (2021).

$$V = \frac{4}{3} * \pi * r_1^2 * r_2$$

r<sub>1</sub> equatorial radius, r<sub>2</sub> polar radius.

## 4. Primary metabolites analysis

Primary metabolites in berries (sugars, organic acids and amino acids) were extracted and their contents were determined as previously described by Arrizabalaga-Arriazu *et al.* (2021). Briefly, pulp frozen powder (250 mg ± 10 %) was successively hot extracted with ethanol 80 % (v/v), ethanol 50 % (v/v) and Milli-Q water (80 °C for 15 min). Supernatants of all three extractions were combined, dried (Speed Vac System ISS110, Savant) then resuspended in Milli-Q water. Extracts were filtered through

a 0.45 µm filter (Millipore) and stored in a –20 °C freezer for further analysis.

Sugar analysis was performed using a Pipette Robot (Robot Precision 2000) for the dilutions and the adding of enzyme steps. Sugar quantification was done by a plate reader (Epoch) using software Gen5, as previously described in Arrizabalaga-Arriazu *et al.* (2021).

Tartaric acid and malic acid were analysed with a continuous flux analyser TRAACS800 (Bran and Luebbe, Plaisir, France). Malic acid was quantified using L-malate dehydrogenase, which converts L-malate into oxaloacetate. Tartaric acid was determined by colorimetric quantitative analysis with reactant ammonium vanadate which forms a yellow-coloured complex quantified by spectrophotometry at 530 nm (Arrizabalaga-Arriazu *et al.*, 2021).

Individual amino acid quantification was determined after filtered extracts derivatisation with 6-aminoquinolyl-N-hydroxy-succinimidyl-carbamate AQC (AccQ-Tag derivatisation reagent, Waters, Milford, MA, USA) using U-HPLC Ultimate 3000 (Thermo Electron SAS, Whaltman, MA, USA) according to Arrizabalaga-Arriazu *et al.*, 2021. The software used to acquire the data and integrate the peaks is Chroméléon version 7.1 (ThermoScientific).

## 5. Anthocyanins profiling

Freeze-dried skin powder (20–30 mg), frozen initially at 100 mg ± 10 % (Alpha-4, CHRIST, Osterode, Germany), were extracted using 500 or 750 µL methanol acidified with 0.1 % HCl (v/v). Extracts were filtered into U-HPLC vials through a 0.2 µm porosity filter (Millex-GS Syringe filter unit, Millipore) and then analysed according to Arrizabalaga-Arriazu *et al.* (2021). The malvidin-3-glucoside standard was used to quantify anthocyanin concentration.

## 6. Berry must analyses

During the season 2020 and 2021, forty berries from the three inner rows were taken, twenty from each side of the row, for each ring. Berries were crushed and pressed (Longarone 85, QS System GmbH, Norderstedt, Germany), then samples were centrifuged at 7830 rpm for 5 min (5430R, Eppendorf AG, Hamburg, Germany). For N-OPA analysis, 500 µL of the sample was added to 500 µL of Milli-Q water in Eppendorf tubes, according to a method described by Wohlfahrt *et al.* (2020). For Oenofoss™ measurements, 1 mL of sample was added in Eppendorf tubes and then centrifuged.

## 7. Gas exchange measurements

In both seasons (2019 and 2020), the gas exchange measurements were performed from June to October using an open gas exchange measurement system (GFS-3000, Walz GmbH, Germany). Three grapevines (*Vitis vinifera* L., cv. Cabernet-Sauvignon) per ring and one fully developed and sun-exposed leaf per plant were measured (in total nine per treatment) between 8.30 a.m. and latest until 2 p.m. to avoid shading conditions on the leaf surface due to the row orientation in the VineyardFACE. To simulate

the surrounding light conditions, a LED light source was used. Net assimilation rate  $A$ , transpiration rate  $E$  and stomatal conductance  $g_s$  were calculated in response to the predominant environmental conditions without extra cooling of the leaf chamber. A buffer tank was used to keep the surrounding  $CO_2$  concentration stable.

## 8. Statistical analyses

Statistical analyses were performed with RStudio (version 4.1.2.). The packages *cowplot*, *tidyverse* and *ggplot2* were used in RScripts. Datasets were subjected to two-way ANOVA to verify the effects of time (DOY), treatment and their interaction. When parameters were measured for two years, two-way ANOVA was performed on combined datasets for each developmental stage to verify treatment and vintage effect.

## RESULTS

### 1. Net assimilation rate

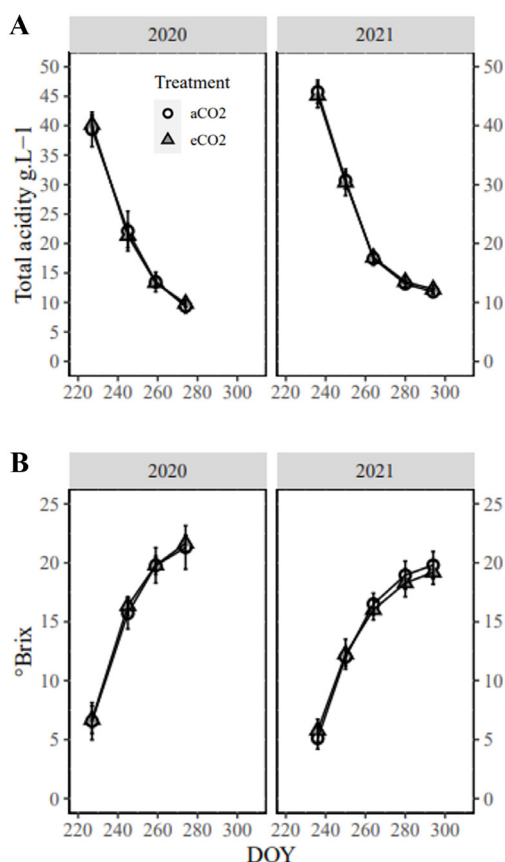
The net assimilation rate  $A$ , averaged over the season, was 16.6 % higher under elevated  $CO_2$  ( $eCO_2$ ) concentration in 2019 with  $14.19 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  compared to  $12.17 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  under ambient  $CO_2$  ( $aCO_2$ ; Table.1). This effect was even more pronounced in 2020, with an increase of approximately 31 % compared to ambient conditions.

### 2. Total acidity and total soluble solids

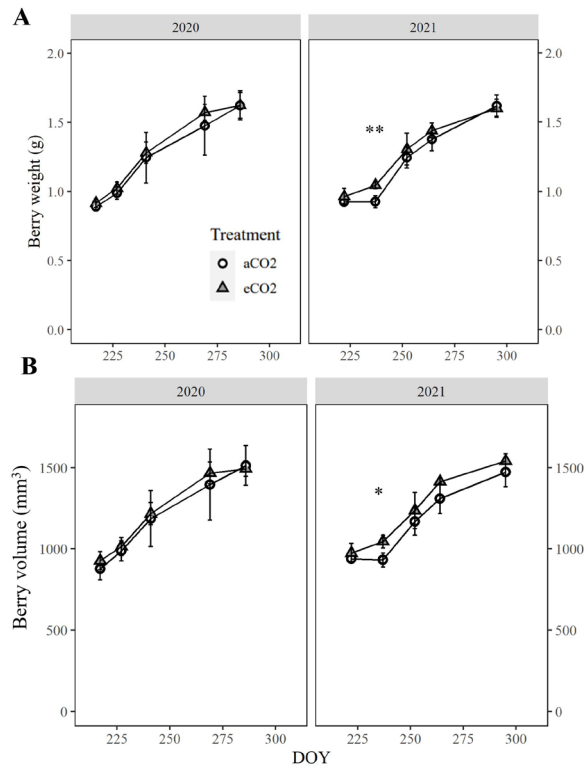
Total acidity in must decreased during berry development, ranging from  $39.37 \pm 2.95 \text{ g}\cdot\text{L}^{-1}$  to  $9.33 \pm 1.14 \text{ g}\cdot\text{L}^{-1}$  for  $aCO_2$  at the green stage and from  $40.16 \pm 1.65 \text{ g}\cdot\text{L}^{-1}$  to  $9.81 \pm 0.6 \text{ g}\cdot\text{L}^{-1}$  for  $eCO_2$  at maturity in 2020 (Figure 1A).

**TABLE 1.** Cabernet-Sauvignon vine net assimilation rate for 2019 and 2020.

treatment\ Net assimilation rate A ( $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ )	2019	2020
$aCO_2$	12.17	12.77
$eCO_2$	14.19	16.72
% to $aCO_2$	116.64	130.91



**FIGURE 1.** Total acidity ( $\text{g}\cdot\text{L}^{-1}$ ), Cabernet-Sauvignon, year 2020 A) year 2021 B) and TSS ( $^{\circ}\text{Brix}$ ), year 2020 C) year 2021 D) under ambient carbon dioxide treatment/ $aCO_2$  (open circles) and elevated carbon dioxide treatment/ $eCO_2$  (grey triangles) treatment.



**FIGURE 2.** Berry weight (A) and berry volume (B) for Cabernet-Sauvignon under ambient carbon dioxide treatment/aCO<sub>2</sub> (open circles) and elevated carbon dioxide treatment/eCO<sub>2</sub> treatment (grey triangles), \*\* p < 0.001, \* p < 0.01, n.s. not significant.

No significant difference in total acidity was observed between the two CO<sub>2</sub> treatments (Supplementary Table 2). Interaction between treatment and day of year was not significant. Statistical analyses performed on both years, for each sampling date, demonstrated a clear vintage effect but no treatment nor year vs. treatment interaction effects.

Total soluble solids (TSS) showed no difference between the two treatments throughout each season (Figure 1B). Due to particular weather conditions in 2021, Cabernet-Sauvignon did not reach 22 °Brix but nevertheless was harvested at the end of October. For each developmental stage, the vintage effect was significant for TSS (Supplementary Table 3).

### 3. Berry volume and berry weight evolution

Berry volume increased in 2020 from  $877.4 \pm 68.3$  to  $1513.6 \pm 123.1$  mm<sup>3</sup> for aCO<sub>2</sub> and from  $927.4 \pm 55.5$  to  $1493 \pm 47.6$  mm<sup>3</sup> for eCO<sub>2</sub> (72.5 % and 60.9 %, respectively, Figure 2B). In 2021, berry volume ranged from  $939.9 \pm 16.4$  to  $1473.5 \pm 92.1$  for aCO<sub>2</sub> and from  $974.4 \pm 59.6$  to  $1541.2 \pm 43.7$  for eCO<sub>2</sub> (56.7 % and 58.2 %, respectively). Indeed, there was a trend of higher berry volume for both years. Although berry volume was significantly impacted by sampling day as expected, neither treatment nor interaction between day of year and treatment did demonstrate significant differences (Supplementary Table 4). However, by combining datasets both years and for each stage of development, there was no vintage effect but a treatment effect at 25 % *véraison*.

Single berry weight increased throughout berry development, with berries in 2020 weighing  $0.89 \pm 0.03$  g for aCO<sub>2</sub> and  $0.91 \pm 0.03$  g for eCO<sub>2</sub> at green stages and  $1.62 \pm 0.11$  g for aCO<sub>2</sub> and  $1.62 \pm 0.09$  g for eCO<sub>2</sub> at maturity (Figure 2A, Supplementary Table 5). Berry weight under eCO<sub>2</sub> seemed to be slightly higher than for berries under aCO<sub>2</sub>. However, this trend seemed to be alleviated around harvest time for both seasons 2020 and 2021.

### 4. Skin and seeds mass

Relative skin mass did not differ between the treatments for 2020 (Table 2). Seed mass reported to berry weight seemed to be decreased under elevated CO<sub>2</sub> treatment compared to ambient, and during ripening, the difference was alleviated. The difference between the two treatments was, however, not significant.

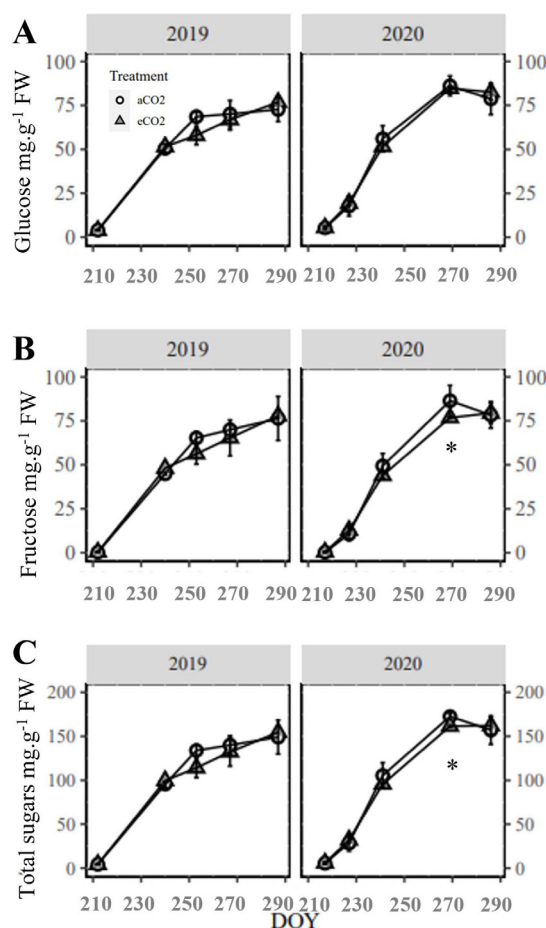
### 5. Primary metabolites

#### 5.1. Sugars

Sugar concentration was expressed as glucose, fructose and total sugars (sum of glucose and fructose). In 2019, total sugar concentration increased from green stages to maturity from  $4.2 \pm 0.004$  mg.g<sup>-1</sup> to  $149.05 \pm 19.3$  mg.g<sup>-1</sup> for aCO<sub>2</sub> and from  $4.17 \pm 0.19$  mg.g<sup>-1</sup> to  $154.63$  mg.g<sup>-1</sup> for eCO<sub>2</sub>, compared to 2020 where it increased from  $5.48 \pm 1.23$  mg.g<sup>-1</sup> to  $157 \pm 16$  mg.g<sup>-1</sup> for aCO<sub>2</sub> and from  $5.69 \pm 0.68$  mg.g<sup>-1</sup> to  $162.03 \pm 10.35$  mg.g<sup>-1</sup> for eCO<sub>2</sub> (Figure 3). Sugar concentration seemed to be more affected by the vintage effect than by the treatment effect when combining both years 2019 and 2020.

**TABLE 2.** Relative skin and seed mass (%) for Cabernet-Sauvignon, year 2020, ambient aCO<sub>2</sub> or elevated CO<sub>2</sub>.

Treatment\DOY		217	227	241	269	286
Relative skin mass	aCO <sub>2</sub>	10.98 ± 1.14	9.50 ± 0.22	9.14 ± 0.54	10.34 ± 0.12	10.52 ± 0.53
	eCO <sub>2</sub>	10.48 ± 0.17	9.72 ± 0.3	9.46 ± 0.24	10.16 ± 0.18	11.02 ± 1.44
Relative seed mass	aCO <sub>2</sub>	9.83 ± 0.70	9.41 ± 0.90	6.72 ± 0.61	4.25 ± 0.21	3.87 ± 0.33
	eCO <sub>2</sub>	9.24 ± 0.49	8.46 ± 0.68	6.60 ± 0.64	4.28 ± 0.12	3.95 ± 0.19

**FIGURE 3.** Sugars for year 2019 and 2020 expressed as Glucose (A), Fructose (B) and total sugars (C) for Cabernet-Sauvignon under ambient carbon dioxide treatment/aCO<sub>2</sub> (open circles) and elevated carbon dioxide treatment/eCO<sub>2</sub> treatment (grey triangles) \*\* p < 0.001, \* p < 0.01, n.s. not significant.

However, at 75 % *véraison*, a significant treatment effect was noticeable for fructose, visible for both years (Supplementary Table 6).

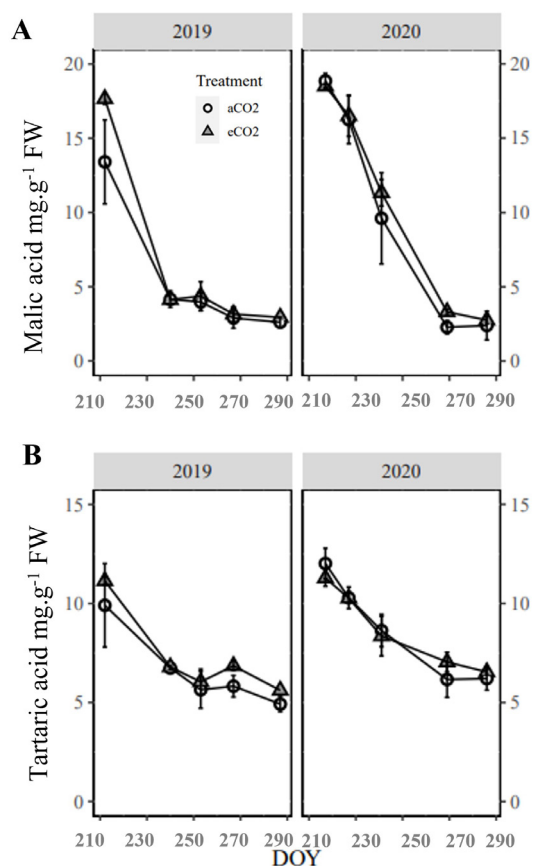
## 5.2. Organic acids

Malic and tartaric acid concentrations decreased during ripening as maturation progressed. Indeed, malic acid decreased by 80.61 % for aCO<sub>2</sub> and 83.4 % for eCO<sub>2</sub> in 2019, while in 2020, it decreased by 87.3 % for aCO<sub>2</sub> and by 85.2 % for eCO<sub>2</sub> (Figure 4A). Tartaric acid in 2019 decreased by 50.3 % for aCO<sub>2</sub> and by 49.6 % for eCO<sub>2</sub> compared to 2020, when it decreased by 48.4 % for aCO<sub>2</sub> and 42.02 % for eCO<sub>2</sub> (Figure 4B). As for total acidity, no significant difference was demonstrated between the two treatments

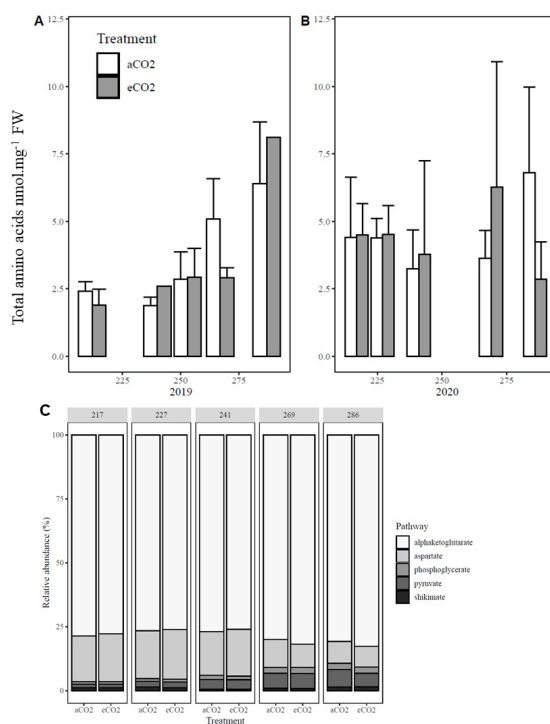
for both malic and tartaric acids (Supplementary Table 7). The vintage effect was indeed more predominant at some stages, namely at 25 % *véraison* and 50 % *véraison*.

## 5.3. Amino acids content and composition

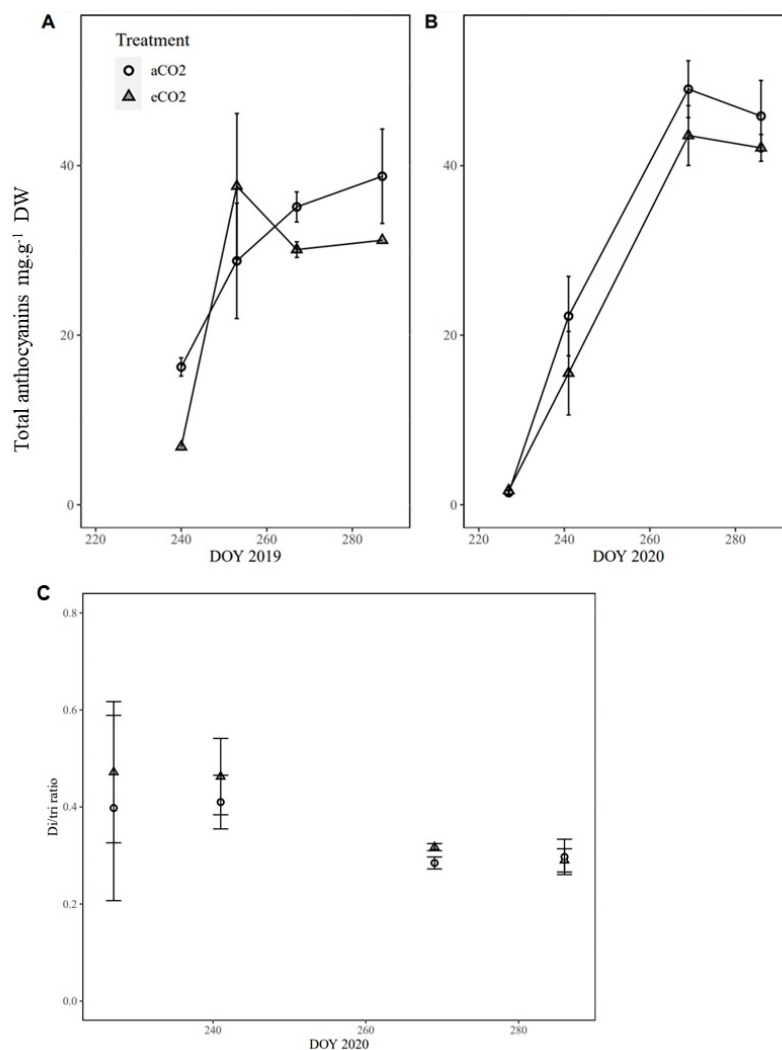
In 2019, amino acid content did increase along berry development, from 2.4 ± 0.35 nmol.mg<sup>-1</sup> to 6.4 ± 2.29 nmol.mg<sup>-1</sup> for aCO<sub>2</sub> and from 1.9 ± 0.58 nmol.mg<sup>-1</sup> to 8.11 nmol.mg<sup>-1</sup> for eCO<sub>2</sub> (Figure 5A). In comparison amino acids content was steadier or even decreased in 2020, ranging from 4.41 ± 2.23 nmol.mg<sup>-1</sup> to 6.81 ± 3.16 nmol.mg<sup>-1</sup> for aCO<sub>2</sub> and from 4.50 ± 1.16 nmol.mg<sup>-1</sup> to 2.89 ± 1.39 nmol.mg<sup>-1</sup> for eCO<sub>2</sub> (Figure 5B). Combining both years datasets, total amino acid content demonstrated a vintage effect at early



**FIGURE 4.** Organic acids 2020; malic (A) and tartaric acid (B) for Cabernet-Sauvignon, year 2019 and 2020, ambient carbon dioxide treatment/aCO<sub>2</sub> (open circles) and elevated carbon dioxide treatment/eCO<sub>2</sub> (grey triangles) treatment, \*\* p < 0.001, \* p < 0.01, n.s. not significant.



**FIGURE 5.** Total amino acids expressed in nmol.mg<sup>-1</sup>, for Cabernet-Sauvignon, year 2019 (A) and 2020 (B), ambient carbon dioxide treatment/aCO<sub>2</sub> (white bars) or elevated carbon dioxide treatment/eCO<sub>2</sub> (grey bars) treatment and (C) amino acids composition (Cabernet-Sauvignon) in 2020 for each DOY.



**FIGURE 6.** Total anthocyanins for Cabernet-Sauvignon skins, (A) year 2019, (B) year 2020 and (C) ratio between di- and tri-hydroxylated anthocyanin forms at ambient carbon dioxide treatment/aCO<sub>2</sub> (open circles) and elevated carbon dioxide treatment/eCO<sub>2</sub> (grey triangles).

stages (Supplementary Table 8). However, during ripening, neither vintage nor treatment effects were significant. In 2020, the alpha-ketoglutarate amino-acid derivatives (Pro, Arg, Gln, Glu, GABA, His) were the most abundant, followed by the aspartate pathway derivatives (Thr, Asp, Asn, Ile, Met, Lys) for the first three sampling stages (Figure 5C). Pyruvate derivatives (Ala, Val, Leu) abundance increased throughout berry development, ranging from 2.17 % to 6.81 % for aCO<sub>2</sub> and from 1.36 % to 5.32 % for eCO<sub>2</sub>.

#### 5.4. Anthocyanins

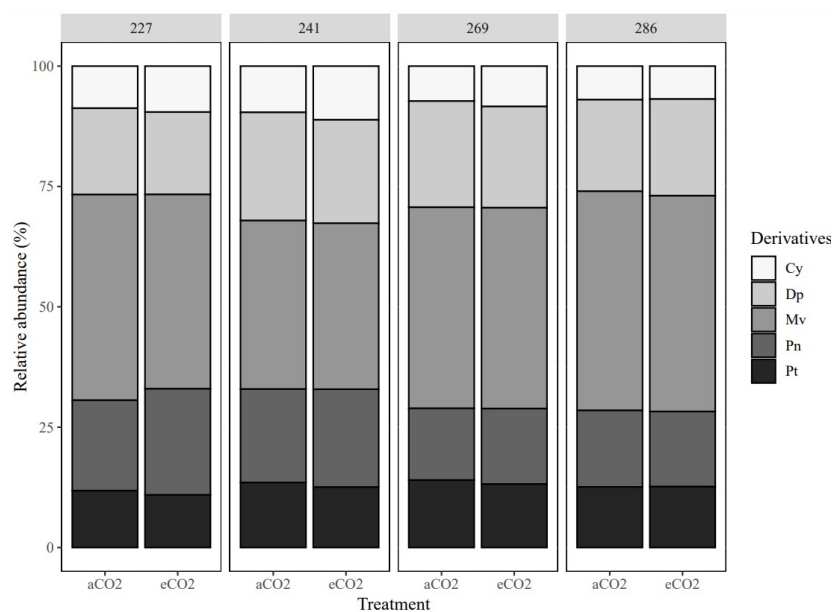
The concentration of anthocyanins in skin samples in 2019 did not significantly differ among treatments according to two-way ANOVA (Figure 6A). For 2019 and 2020, respectively, the sampling date effect was significant, but the treatment was not (supplementary table 9). However, there is a consistent trend in decreased anthocyanins concentration in berries under eCO<sub>2</sub> treatment (mostly in 2020), but not statistically significant. Neither anthocyanin composition nor

di- to tri-hydroxylated forms ratio were modified in 2020 for the two treatments (Figure 6C).

However, when combining both year datasets for statistical analysis, a treatment effect was noticeable at 25 % *véraison* and 75 % *véraison*, although the vintage effect was still predominant.

According to Figure 6, a trend of higher di-hydroxylated anthocyanins (cyanidin and peonidin derivatives) compared to tri-hydroxylated anthocyanins (malvidin, petunidin and delphinidin) was noticeable in 2020 in samples under elevated CO<sub>2</sub> treatment but the difference tended to decline at maturity. Around *véraison*, the standard deviation was important because of berry heterogeneity in colours.

Malvidin derivatives were the most abundant derivatives in the samples at all developmental stages, with a percentage of malvidin derivatives being respectively 45.5 % for aCO<sub>2</sub> and 44.8 % for eCO<sub>2</sub> at maturity. Even if slight differences



**FIGURE 7.** Anthocyanins derivatives composition (Cabernet-Sauvignon) of year 2020 under ambient carbon dioxide treatment/aCO<sub>2</sub> and elevated carbon dioxide treatment/eCO<sub>2</sub> treatment. Cy: cyanidin, Dp: delphinidin, Mv: malvidin, Pn: peonidin and Pt: petunidin

could occur during berry development between elevated and ambient CO<sub>2</sub> treatment, no major change in anthocyanins composition is noticeable in 2020 (Figure 7).

## DISCUSSION

This study is unique since it has been performed in a vineyard located FACE setup (VineyardFACE) where vines have been treated by elevated CO<sub>2</sub> concentration for almost one decade from shortly after planting to mature vines. Hence, the results of the investigations can be interpreted as long-term acclimation responses of the vines. The net assimilation rate was higher under elevated CO<sub>2</sub> treatment for both the 2019 and 2020 seasons (Table 1). Similar physiological responses were reported in previous seasons (Wohlfahrt *et al.*, 2018), resulting in enhanced vegetative growth.

Regarding reproductive plant biomass, berry volume did not differ significantly between ambient and elevated CO<sub>2</sub> treatment. However, when combining the years 2020 and 2021, there was a significant increase in berry volume at 25 % *véraison* and a trend of higher berry volume under elevated CO<sub>2</sub>. Moreover, single berry weight demonstrated a clear trend of an increase under elevated CO<sub>2</sub> treatment for both seasons 2020 and 2021, a trend that was alleviated at maturity. Biomass increase in yield under eCO<sub>2</sub> for red cultivars such as Sangiovese was reported by Bindi *et al.* (2005), assuming a higher berry weight under eCO<sub>2</sub>. For Cabernet-Sauvignon, single berry weight increased under elevated CO<sub>2</sub> on the same VineyardFACE experimental setup (Wohlfahrt *et al.*, 2020) when the vines were younger. Berry size is reported to be related to berry and wine quality, particularly at *véraison*, which coincides with a simultaneous sugar accumulation and organic acid degradation (Chen *et al.*, 2018). Sugars accumulated in berries are

mostly glucose and fructose (Kliewer, 1966). Mature berries display the same amount of glucose and fructose, whereas, at the early stages, glucose is predominant. In this study, no difference was noticeable between the two treatments, neither for TSS in must nor in total sugars from frozen pulp powder, as it was shown for TSS at the beginning of VineyardFACE experiments (Wohlfahrt *et al.*, 2020).

Organic acids start to accumulate in the early stages of grape berry development and are mainly represented by malic and tartaric acid (Kliewer, 1966). During ripening, malic acid is degraded by increased respiration rate (Conde *et al.*, 2007), whereas tartaric acid concentrations decrease by dilution effect due to berry volume increase. Our results suggest no significant effect of elevated CO<sub>2</sub> on both malic and tartaric acids. The effect of temperature on organic acid and especially malic acid degradation has already been reviewed (Etienne *et al.*, 2013). Using potted plants in greenhouses, Arrizabalaga-Arriazu *et al.* (2020) found that malic acid in berry decreased from mid-*véraison* onwards under elevated temperature, whereas under elevated CO<sub>2</sub> (700 ppm), malic acid increased at *véraison* but was significantly reduced at maturity. In VineyardFACE, malic acid degradation was slowed down under elevated CO<sub>2</sub> concentration for Cabernet-Sauvignon (Wohlfahrt *et al.*, 2020). Our results suggest that this effect is alleviated by long-term acclimation of the vines.

Concerning berry composition, it was firstly reported that vines in FACE systems under elevated CO<sub>2</sub> were displaying a change in sugar and organic acid concentration, but this CO<sub>2</sub> effect did disappear at maturity (Bindi *et al.*, 2005). In temperature gradient greenhouses, a mitigating role of elevated CO<sub>2</sub> was demonstrated on grapevine vegetative

growth and yield when combined with elevated temperature and drought (Kizildeniz *et al.*, 2015).

No effect of elevated CO<sub>2</sub> was found at maturity on total amino acid concentration, although CO<sub>2</sub> treatment reduced alpha-ketoglutarate derivatives in later stages. Moreover, phenylalanine content was significantly increased at *véraison*, and it was found that elevated CO<sub>2</sub> treatment decreased amino acid concentration at *véraison* and two weeks after mid-*véraison* (Arrizabalaga-Arriazu *et al.*, 2020).

Anthocyanins are considered important metabolites in fruit, which are usually altered during wine ageing to form polymeric pigments. Elevated temperature decreased anthocyanins concentration (Spayd *et al.*, 2002) and accumulation. Furthermore, genes for anthocyanins biosynthesis were down-regulated under high-temperature conditions (Mori *et al.*, 2007). Indeed, elevated temperature conditions decreased anthocyanins concentration (Arrizabalaga-Arriazu *et al.*, 2021). However, the effect of elevated CO<sub>2</sub> on anthocyanins remains less evident in the literature. Berry characteristics were described to be unaffected by elevated CO<sub>2</sub> in Open Top Chambers on cv. Touriga Franca, however, total anthocyanins and polyphenol concentrations in red wine were reported to decrease under elevated CO<sub>2</sub> treatment (Gonçalves *et al.*, 2009). When elevated temperature and elevated CO<sub>2</sub> were combined, it was reported that total anthocyanins and malic acid declined (Salazar Parra *et al.*, 2010). Studies applying different UV-B doses and two temperature/CO<sub>2</sub> regimes to grapevine fruit cuttings cv. Tempranillo demonstrated that anthocyanins concentration differed during berry development between the treatments. Indeed, their concentration was higher under elevated CO<sub>2</sub> and elevated temperature combined two weeks after *véraison*, but at maturity, the trend reversed (Martínez-Lüscher *et al.*, 2016). When studying the two parameters independently, Arrizabalaga-Arriazu *et al.* (2020) found that elevated CO<sub>2</sub> treatment did increase anthocyanin concentrations at the onset of *véraison* and mid-*véraison*, whereas after mid-*véraison*, their concentration decreased, in a clone genotype-dependent manner. In the case of the current study, the decreased anthocyanin concentration could be due to increased single berry weight. Indeed, an increase in berry size could lead to a lower skin surface to berry volume ratio (Ojeda *et al.*, 2002). Moreover, it was found that under carbon limitation induced by variation in source-to-sink ratio using Cabernet-Sauvignon the proportion of di-hydroxylated anthocyanins decreased (Bobeica *et al.*, 2015; Wang *et al.*, 2021). In our study, the di- to tri-hydroxylated anthocyanins ratio seemed to be higher, although not significantly, under elevated CO<sub>2</sub> treatment. To gain more knowledge, in the future experiment, the degradation rate of monomeric anthocyanins under eCO<sub>2</sub> conditions forming polymeric pigments will need to be investigated to better follow the ageing potential of the red wines.

In a recent study, the parameters of young wine, such as total anthocyanins and organic acids, were more affected by the vintage effect than the CO<sub>2</sub> treatment (Wohlfahrt *et al.*, 2021). These results confirm that, even if photosynthesis is still

enhanced by elevated CO<sub>2</sub> treatment, primary and secondary metabolites content of berries may not differ under scenarios of near-future atmospheric CO<sub>2</sub> conditions.

## CONCLUSION

This study aims to evaluate the effects of 2050 atmospheric carbon dioxide conditions on the berry composition of cv. Cabernet-Sauvignon that has been grown under elevated CO<sub>2</sub> concentration for almost one decade. No major differences in primary metabolites were found under elevated carbon dioxide treatment in two recent seasons, although it was shown that photosynthesis and the net assimilation rate are still enhanced by elevated carbon dioxide. However, from the berry quality point of view, elevated carbon dioxide has little effect on berry ripening dynamics and fruit composition. Concerning at least atmospheric carbon dioxide concentration, the tipping point for grapevine seems to be not already crossed. However, climate change results in a combination of factors such as elevated temperature, drought in certain regions, and of course, elevated CO<sub>2</sub>. Further investigations focusing on combined environmental factors on primary metabolism intermediates, as well as aroma compounds, are part of ongoing studies within the VineyardFACE.

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