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Resilience of grapevine to climate change: From plant physiology to adaptation strategies, volume II

Edited by
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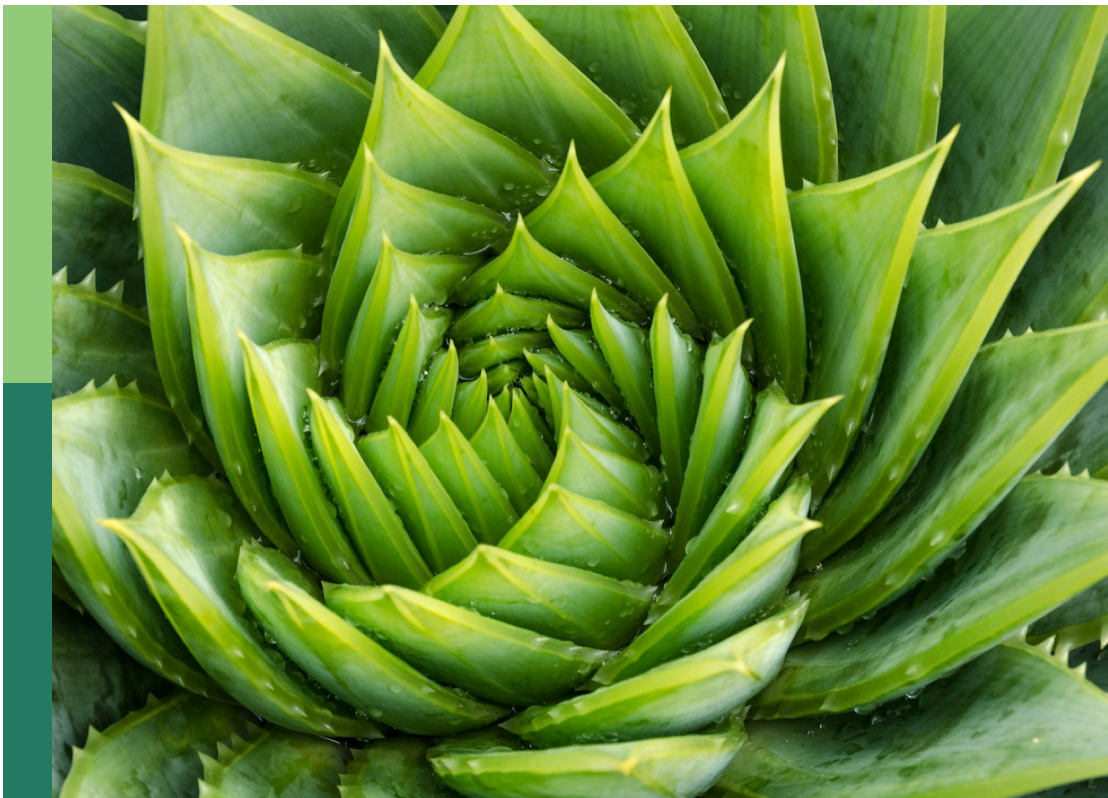
Published in
Frontiers in Plant Science



ISSN 1664-8714
ISBN 978-2-8325-3347-5
DOI 10.3389/978-2-8325-3347-5

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Resilience of grapevine to climate change: From plant physiology to adaptation strategies, volume II

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Citation

Pastore, C., Diago, M. P., Frioni, T., eds. (2023). *Resilience of grapevine to climate change: From plant physiology to adaptation strategies, volume II*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-3347-5

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frontiers | Frontiers in Plant Science

TYPE Editorial
PUBLISHED 11 August 2023
DOI 10.3389/fpls.2023.1268158

OPEN ACCESS

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RECEIVED 27 July 2023
ACCEPTED 31 July 2023
PUBLISHED 11 August 2023

CITATION
Frioni T, Pastore C and Diago MP (2023) Editorial: Resilience of grapevine to climate change: from plant physiology to adaptation strategies, volume II. *Front. Plant Sci.* 14:1268158. doi: 10.3389/fpls.2023.1268158

Editorial: Resilience of grapevine to climate change: from plant physiology to adaptation strategies, volume II

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KEYWORDS

temperature, berry composition, climate change, *Vitis vinifera* L., soil management, canopy management, breeding

Editorial on the Research Topic
Resilience of grapevine to climate change: from plant physiology to adaptation strategies, volume II

Introduction

Under a dramatic and progressive increase of vineyard stress incidence, frequency, and unpredictability, new solutions to defend the world wine industry from climate change-related problems are urgently needed. Following Volume I (Pastore et al., 2022), in this Research Topic, 12 studies from 7 different countries provide significant knowledge on the effects of key environmental stressors on specific grapevine physiologic/metabolic traits or propose groundbreaking strategies to improve vineyard resilience to climate change (Table 1).

How climate change factors are affecting grapevine morpho-physiology and berry traits

The wine sector faces important challenges related to sustainability issues and the impact of climate change. Extreme climate events, together with the increase of carbon dioxide (CO_2), have become a matter of concern for the wine sector worldwide. In this context, yield and quality losses due to increasing temperatures, altered solar radiation, and scarce water availability are challenging grape growers, especially if associated with the appearance of sunburn symptoms that can cause sunburn necrosis in berries. Knowledge of the temperature conditions that can induce sunburn on berry surface can be useful to predict the appearance of sunburn damage and to adopt specific mitigation strategies. The probability of a berry developing symptoms of sunburn necrosis depends on both the intensity and duration of the heat that it receives. Müller et al. reported that with longer

TABLE 1 An overview of the topics and of the adaptation strategies proposed by the 12 papers included in the Research Topic.

Authors	Location	Limiting factors studied						Type of adaptation proposed				
		Water stress	Temperature, humidity and VPD	Light	Salinity	CO_2	Biotic stresses	Genetic material	Canopy management	Soil management	Irrigation	Foliar applications
Arguedas et al.	CAN		X	X					X			
Oliver et al.	SPA		X						X			
Yu et al.	USA	X	X						X		X	
Ramos et al.	SPA						X	X				X
Daldoul et al.	TUN				X			X				
Dami and Zhang	USA		X					X				X
Wohlfart et al.	GER					X		X				
Costa et al.	POR	X	X							X	X	
Müller et al.	GER	X	X	X					X		X	
Buesa et al.	SPA	X	X	X					X	X		
Fichtl et al.	GER	X						X				
Poni et al.	ITA	X	X	X					X			

10.3389/fpls.2023.1268158

durations of heat exposure, lower berry surface temperature is required for berries to show symptoms.

Volatile compounds (VCs) play an important role in wine quality and can be considered one of the most important secondary metabolites in the grapevine berry. Volatile compounds biosynthesis in grape berries is affected by environmental conditions and, in particular, by UV-exposure and temperatures of the bunches. Moderate temperature increases and reduced ultraviolet (UV) exposure in different stages of berry ripening (pre-veraison, post-veraison, and ripening) can affect the composition of VCs in *Vitis* sp. 'L' Acadie blanc', in Nova Scotia (Canada), suggesting that climate change events can affect VCs biosynthesis at harvest, even if they occur at early berry developmental stages (Campos-Arguedas et al.). Increasing temperatures can impact the vines directly, as previously described, or through an indirect effect linked to the raising of soil temperature. Soil conditions (topography, properties, and management) influence the vine morpho-physiology, as reviewed by Costa et al. The highest biomass production and shoot growth rates are linked to an increase of soil temperature conditions (24°C compared to 13°C), while the exposure of grapevine roots to high temperatures often decreases primary root length and lateral root density.

The increase of CO_2 concentration induces increased photosynthesis in most plant species, including *Vitis vinifera* grapevines, but the mechanisms behind this improvement are not completely understood. Elevated CO_2 can also bring a modification of leaf physiology and morphological leaf characteristics without altering leaf pigments. Wohlfahrt et al. observed that depending on the cultivar, palisade parenchyma could increase and the epidermal tissue could decrease in thickness, suggesting the existence of seasonal adaptation strategies of grapevines under elevated CO_2 concentrations.

Plant material to improve climate change resilience

In Volume I, the importance of grapevine genetic diversity in the adaptation to climate change was illustrated. In this second volume, physiological mechanisms and genetic patterns underlying the differential response of varieties and rootstocks to various biotic and abiotic stresses were studied. With regard to better adaptation to increasing drought conditions, the role of rootstocks is essential. Fichtl et al. focused on modeling root architecture traits (e.g., rooting depth, root length density, and specific root length, among others) by integrating interactions with physiological processes, such as different water availability scenarios. In this work, a range of phenotyping methods and the integration of these phenotyping data into different models—to advance the understanding of rootstock x environment x management interactions and to predict rootstock genotype performance in a changing climate—were investigated, and the lessons learnt towards optimizing rootstock breeding were presented. Strongly related to drought increase is the phenomenon of salinity stress. Daldoul et al. revisited the physiological and biochemical response of wild grapevines (*V. Sylvestris*), in particular, that of the genotype "Tababa" versus a well-known rootstock (Paulsen 103), against salinity stress conditions. From this study, the dynamic salt mechanism tolerance of wild grapevines was elucidated, and specific candidate genes that could be valuable for appropriate breeding strategies to increase salt tolerance were identified.

As mentioned, the increase of CO_2 concentration is another effect of climate change. While elevated CO_2 induces increased photosynthesis in most plant species, including *Vitis vinifera* grapevines, the mechanisms behind this improvement are not completely understood. Wohlfahrt et al. presented their observations on the leaf physiology and morphological characteristics of cvs. Riesling and Cabernet Sauvignon subjected to elevated CO_2 conditions using a unique free-air carbon dioxide enrichment set-up (FACE) and provided first insights to seasonal adaptation strategies of grapevines under future elevated CO_2 concentrations.

New frontiers on vineyard soil and canopy management

While selecting proper plant material at vineyard establishments will be the foundation of the adaptation of viticulture to climate change, a no lesser role will be taken by technical aspects of seasonal vineyard management, starting from the ground. Indeed, as this Research Topic also confirms, soil management is one of the trending Research Topics. In their extensive review, Costa et al. analyzed vine response at varying soil temperature levels, pointing out that different floor management techniques could affect vine yield and fruit composition by directly modulating water and nutrients availability or weeds competition and also, indirectly, changing soil temperature and organic matter mineralization time evolution. In this framework, the authors acknowledge the relevance of irrigation in order to improve vineyard soil quality and resilience to climate change.

Irrigation was one of the patterns also followed by Müller et al. and Yu et al. In the first article, the authors pointed out that early water deficit could improve grape sunburn tolerance later in the season. Yu et al. showed that different irrigation volumes were interacting with trellis systems at determining vine yield and fruit composition traits, suggesting the revision of an applied water × training system combination in arid environments.

This was also one of the points raised by the review of canopy management by Poni et al. In their article, the authors pointed out the substantial change has occurred in the last twenty years in respect to the directions of such techniques, which were once aimed at boosting ripening and today have the main goal of sheltering clusters from direct exposure or controlling sugars excesses. In this framework, Müller et al. showed that early exposure of grapes to sunlight elicits a priming effect, making grapes more tolerant to sunburn later in the season. Oliver-Manera et al. verified the effects of the vine forcing technique applied after fruit set and at the

beginning of bunch closure on yield, vegetative growth, and non-structural carbohydrate reserves over two consecutive years, suggesting that this technique, if allowing the forced vines to restore carbon reserves at the whole-canopy level, could be promising in order to delay harvest. However, in the vineyard climate change adaptation "game", soil and canopy management cannot be conceived as unrelated topics. Buesa et al. compared canopy shading, bud forcing, and mulching + vine shading *vs* an untreated control, looking for the best strategy to improve cv. Macabeo wines in eastern Spain. The authors found that bud forcing was the most suitable technique for the production of premium sparkling wine, while the additive effects of shading nets and mulching could be the best strategy to improve grapevine physiological performances and grape composition for the production of other styles of wine.

Foliar applications

With the aim of improving the physiological and biochemical performance of grapevines towards biotic and abiotic stressors, new biological and hormonal products are released to be applied in the leaves. Alternatively, the mechanisms behind the specific response of treated grapevines with one of these products to a particular stressor are sometimes unknown. Two articles covering this approach are included in Volume II. Likewise, the genetic and phenotypic responses of three Mediterranean cultivars to *Dittrichia viscosa* extract and *Bacillus velezensis* strain were investigated under greenhouse conditions (Ramos et al.), showing that markers specific to some differentially expressed genes presented a stable overexpression after being treated (foliar application) with these two biocontrol products, regardless of the grapevine variety. While the global warming of most viticultural areas is predicted as a consequence of climate change, cold damage still remains a strong devastating weather event to grape production in some viticultural regions worldwide. To mitigate freezing stress, grapevines undergo cold acclimation (transition from a cold-sensitive to a cold-hardy state) through a series of physiological changes derived from either the up- or downregulation of more than 3000 genes. As a key player in freezing tolerance, abscisic acid (ABA) and its application at different doses was studied under greenhouse conditions in *Vitis* spp cv. Chamboucin and *Vitis vinifera* cv. Cabernet Franc cultivars (Dami and Zhang). It was revealed that fructose, glucose, and sucrose are the main soluble sugars that correlate with the freezing tolerance of grape buds and that the synthesis of these sugars is enhanced by ABA treatment after raffinose decay in mid-winter.

Reference

Pastore, C., Frioni, T., and Diago, M. P. (2022). Resilience of grapevine to climate change: From plant physiology to adaptation strategies. *Front. Plant Sci.* 13, 994267.

Author contributions

TF: Writing – original draft, Writing – review & editing. CP: Writing – original draft, Writing – review & editing. MD: Writing – original draft, Writing – review & editing.

Acknowledgments

Topic Editors wish to express their gratitude to all the authors and reviewers who contributed to the Research Topic. Special thanks to the Frontiers in Plant Science Editorial Board who gave us the opportunity to build up the Research Topic, and to the Editorial Office for the prompt assistance.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

frontiers | Frontiers in Plant Science

ORIGINAL RESEARCH
published: 30 June 2022
doi: 10.3389/fpls.2022.862259

Different Temperature and UV Patterns Modulate Berry Maturation and Volatile Compounds Accumulation in *Vitis* sp.

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OPEN ACCESS

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Specialty section:
This article was submitted to Plant Abiotic Stress, a section of the journal Frontiers in Plant Science

Received: *25 January 2022*
Accepted: *24 May 2022*
Published: *30 June 2022*

Citation:
Campos-Arguedas F, Sarrailhé G, Nicolle P, Dorais M, Brereton NJB, Pitre FE and Pedneault K (2022) Different Temperature and UV Patterns Modulate Berry Maturation and Volatile Compounds Accumulation in Vitis sp. Front. Plant Sci. 13:862259. doi: 10.3389/fpls.2022.862259

Volatile compounds (VCs) in grapevine berries play an important role in wine quality; however, such compounds and vine development can be sensitive to environmental conditions. Due to this sensitivity, changes in temperature patterns due to global warming are likely to further impact grape production and berry composition. The aim of this study was to determine the possible effects of different growing-degree day accumulation patterns on berry ripening and composition at harvest. An experimental field was conducted using *Vitis* sp. L'Acadie blanc, in Nova Scotia, Canada. Using on-the-row mini-greenhouses, moderate temperature increase and reduced ultraviolet (UV) exposure were triggered in grapevines during pre-veraison (inflorescence to the beginning of berry softening), post-veraison (berry softening to full maturity), and whole season (inflorescence to full maturity), while controls were left without treatment. Free and bound VCs were extracted from berries sampled at three different phenological stages between veraison and maturity before analysis by gas chromatography–mass spectrometry (GC-MS). Berries from grapevines exposed to higher temperatures during early berry development (pre-veraison and whole) accumulated significantly higher concentrations of benzene derivatives 2-phenylethanol and benzyl alcohol at harvest, but lower concentrations of hydroxy-methoxy-substituted volatile phenols, terpenes, and C_{13}-norisoprenoids than the control berries. These results illustrate the importance of different environmental interactions in berry composition and suggest that temperature could potentially modulate phenylpropanoid and mevalonate metabolism in developing berries. This study provides insights into the relationships between abiotic conditions and secondary metabolism in grapevine and highlights the significance of early developmental stages on berry quality at harvest.

Keywords: fruit aroma, secondary metabolites, gas chromatography-mass spectrometry, climate change, berry ripening, abiotic stress

INTRODUCTION

Understanding the impact of environmental change (e.g., temperature, altered solar radiation, and water availability) on plants and developing adaptation strategies to climate change are essential to maintaining the productivity of cultivated lands in forestry and agriculture. According to projections from the Intergovernmental Panel on Climate Change (IPCC), global increases in temperature will reach 1.4–4.4°C by 2100 depending on greenhouse gas emissions (Masson-Delmotte et al., 2021). In Eastern Canada, mean temperatures are predicted to increase from 2 to 4°C in the summer and 1.5 to 6°C during the winter (Vasseur and Catto, 2008), leading to a northward expansion of growing degree day (GDD) $\geq$ 1,200 by 400–600 km by 2099 (King et al., 2018).

Temperature is considered one of the main factors influencing the phenology of grapevine (*Vitis* sp.) (Keller, 2015), and variations in temperature during the developmental and ripening phases have an impact on the biosynthesis of primary and secondary metabolites, specifically during berry growth (Mira de Orduña, 2010; González-Barreiro et al., 2015). Temperature rises due to global warming have been reshaping the dynamics of grapevine harvest and wine production worldwide by globally increasing seasonal heat accumulation (Santos et al., 2020; Venios et al., 2020), a variable often assessed through the sum of GDDs. Increases in GDD accumulation have been shown to accelerate plant development and advance grapevine phenology (leading to earlier harvest dates), shorten the time between bud break and flowering, increase the concentration of sugars, and decrease the acidity content of berries (Mira de Orduña, 2010; van Leeuwen and Darriet, 2016; Cameron et al., 2021). However, in addition to temperature rises, larger temperature ranges throughout the growing season could also have a potential impact on plant growth, phenology, and metabolism (Barnuud et al., 2014). Less is known about the impact of these new seasonal temperature patterns on grapevine development and fruit biochemistry.

Grapevine berry production follows a double sigmoidal growth pattern involving a developmental phase (I; berry formation) that goes from flowering to fruit softening, and a ripening phase (II) that goes from berry softening to fully mature fruit, separated by a short lag phase (Keller, 2015). The first phase (hard, green berries) is characterized by cell division and elongation along with the accumulation of proanthocyanidins (tannins) and organic acids (malic and tartaric acids). During the second phase, called veraison, berries soften and organic acids break down, while sugars and many secondary metabolites, such as terpenes, C_{13}-norisoprenoids, and other volatile compounds (VCs), accumulate (Jackson, 2008; Keller, 2015). Variation in abiotic conditions, including temperature, can influence the biosynthesis of these metabolites (Mira de Orduña, 2010; González-Barreiro et al., 2015).

Volatile compounds include different chemical families, such as aliphatic alcohols, aliphatic aldehydes, monoterpenes, benzene derivatives, and C_{13}-norisoprenoids, among others (Lund and Bohlmann, 2006; Dunlevy et al., 2009; Roubelakis-Angelakis, 2009). In the fruit, most VCs are bound to a sugar moiety (glycosylated) and a fraction is present as free (aglycon). VCs are involved in plant development and both biotic and abiotic stress defense signaling (Dudareva et al., 2013; Vivaldo et al., 2017). As such, the biosynthesis of VCs can be influenced by environmental changes such as seasonal conditions and agricultural practices, and these factors can, therefore, influence the grape and wine aroma profile of the same cultivars within the same area (Lund and Bohlmann, 2006; Dunlevy et al., 2009; Moreno and Peinado, 2012).

The biosynthesis of VCs as a response of plants exposed to high temperatures varies with the intensity and time of exposure. Previous studies on the effect of temperature on VC accumulation have found that compound production and emission increase at high temperatures (Guenther et al., 1993; Copolovici and Niinemets, 2016; Rienth et al., 2021); however, in grapevines, this effect is not consistent (Selmar and Kleinwächter, 2013; Rienth et al., 2016; Lecourieux et al., 2017; Pastore et al., 2017) as many other variables, such as radiation, cultivar, and terroir, can play an important role (Mira de Orduña, 2010; Rienth et al., 2021). In other model plants, such as *Camellia sinensis*, compounds, such as 2-phenylethanol (a product of the shikimate pathway), have been favored at high temperatures (Huang et al., 2001; Shu et al., 2021) and in *Polygonum minus*, the biosynthesis of certain terpenes and aldehydes was related to elevated temperatures (Goh et al., 2016).

Variations in temperature due to climate change and the evolution of VCs during ripening can affect berry quality (Bonada et al., 2015). Several studies have assessed the effects of temperature in controlled conditions such as greenhouses or growth chambers (Salazar Parra et al., 2010; Luchaire et al., 2017; Kizildeniz et al., 2018) and approaches that are valuable in providing important information on the fundamental responses to abiotic stressors and grapevine's physiology and development. However, interactions with other factors, such as rainfall, radiation, humidity, and soil management, can be better explored under field conditions.

Several studies have used experimental field trials where vines have been exposed to different environmental conditions, and plant physiology, berry quality, and berry yield responses were assessed (Sadras and Soar, 2009; Soar et al., 2009; Sadras and Moran, 2012; Sadras et al., 2012a,b, 2013; Bonada et al., 2015). A similar field trial was used here to test whether changes in temperature patterns at different stages of berry development (pre-veraison, veraison, and post-veraison) or a global rise in temperature (whole season) could lead to modifications of the profile of VCs in mature berries. On-the-row mini-greenhouses were used at different berry developmental stages of *Vitis* sp. cv L'Acadie blanc to alter GDD accumulation patterns along the growing season. Berries were sampled at three phenological stages around full maturity, and the composition of free VC (FVC) and glycosylated VC (GVC) was analyzed.

MATERIALS AND METHODS

Treatments and Experimental Design

The experiment was carried out in a commercial vineyard located in the Gaspereau Valley, Wolfville, NS, Canada (45°4'19, 56"N,

64°17'44.7108"W) during the 2020 growing season. The 11-year-old, self-rooted "L'Acadie blanc" (Cascade X Seyve-Villard 14-287) was used. The vines were pruned to 12 buds per vine and trained in vertical shoot position. Row spacing and vine spacing were 6.0 and 1.2 m, respectively. A complete randomized block design, including five blocks and four treatments, was used. Each block consisted of four plots with four consecutive vines each (**Figure 1A**). To avoid edge effects, border rows were included between blocks (**Figure 1**).

Treatments consisted of temperature increases triggered by polycarbonate mini-greenhouses (**Figure 1A**) installed on-the-row at specific times and locations. Each structure (4.9 m length × 0.6 m width × 2.4 m height) was covered with polycarbonate panels (Tuftex, Onduline, VA, USA), transmitting 89% of the incident light within the visible spectrum and blocking ultraviolet (UV) radiation below the 380 nm. Due to this UV blocking effect, significant differences in VC accumulation should be considered a result of mini-greenhouse treatment, which includes both altered GDD and UV exposure. Two sides of the structure include covering by a clear polyethylene film cover (A&A Grower Supply) at the bottom, with a 20-cm gap left at ground level, and leaving two sides of the mini-greenhouse open to allow the circulation of air (**Figure 1**). Holes (∼5 mm diameter) were made at the top of the structure to avoid rain accumulation. The modified Eichhorn-Lorenz (EL) system was used to describe phenological stages as described by Coombe (1995). The vines were exposed to four different treatments (**Figure 1A**), namely, 1. whole season (W): mini-greenhouse installation from the shoot and inflorescence developmental stage at EL-15 (3 June) until harvest at EL-38 (9 October), 2. pre-veraison (Pre): mini-greenhouse installation from EL-15 (3 June) until veraison at EL stage 35 (11 September), 3. post-veraison (PT): mini-greenhouse installation from EL-35 (11 September) until harvest at EL-38 (9 October), and 4. control (CT): without mini-greenhouse. To achieve a target of two clusters per shoot, cluster and shoot thinning were performed prior to veraison to level the number of clusters and the number of shoots in each plant (clusters per plant = 1.70 × shoot number + 1.70, with $r^2 = 0.89$).

Climatic Measurements

Temperature and humidity were monitored in each plot. Relative humidity at canopy level was measured hourly by TemLog 20H data logger from Elitech Technology Inc. (Milpitas, CA, USA). Temperature and light (lux) were measured hourly by Hobo Pendant Temperature/Light Data Logger (UA-002-64) from ONSET (Bourne, MA, USA) above the canopy. The following equation is used to calculate the accumulation of degree days (GDD):

$$GDD = \sum_{i=1}^{n} T_i - T_b$$

where a base temperature (T_b) of 10°C is used, n is the total number of days in each period, and T_i is the mean daily temperature (max+min/2).

Sampling

Berries were sampled at the following stages: EL-36 ("*berries with intermediate brix values*"), EL-37 ("*berries not quite ripe*"), and EL-38 ("*harvest-ripe*") (Coombe, 1995). The samples were immediately frozen with liquid nitrogen, transported in dry ice, and stored at −80°C until analyzed. Each sample consisted of 6–8 clusters randomly picked on all four vines of each treatment.

Physicochemical Analysis of Grapes

For each experimental unit, 200 fresh berries were weighted, hand-crushed, and filtered through a sieve. The concentration of total soluble solids (TSS; °Brix) of the must was measured with a PAL-1 pocket refractometer (Atago Co., Ltd., Tokyo, Japan). Titratable acidity (TA; as g L^{-1} of tartaric acid equivalent) was quantified with an HI 84502 titratable total acidity mini-titrator (Hanna Instruments. Woonsocket, RI, USA), as follows: 2 ml of juice were added and diluted to a final volume of 50 ml (specifications for high range TA; 4.0–25.0 g L^{-1} of tartaric acid) and titrated with the HI 84502-50 titrant solution (NaOH) to a final pH of 8.2. pH was measured using an MP 200 pH meter (Mettler Toledo, Columbus, OH, USA).

Analysis of Volatile Aroma Compounds

Chemicals

Ethanol (97%), methanol (HPLC grade), dichloromethane (HPLC grade) n-Hexane (99%), and insoluble polyvinylpolypyrrolidone (PVPP) were purchased from Millipore Corporation (Billerica, MA, USA). n-Pentane (HPLC grade) and citric acid (anhydrous) were purchased from Anachemia (Mississauga, ON, CA). Sodium sulfate anhydrous was purchased from VWR (Solon, OH, CA) and sodium phosphate dibasic was purchased from Spectrum Chemicals (Gardena, CA, USA). Rapdidase Revelation Aroma enzyme (a mixture of pectinases and glycosidases) was purchased from ScottLabs Canada (Niagara-on-the-lake, ON, CA). Internal standards (±)-2-octanol (≥ 99.5%) and nonyl-ß-∂-glucopyranoside (≥ 97%) were purchased from Sigma Aldrich (Oakville, ON, CA). All the other authentic standards used to determine the retention time are listed in **Supplementary Table 1**.

Sample Extraction

Free and glycosylated volatile compounds were extracted from 200 g of grape berries. The berries were thawed at 4°C overnight. The juice was extracted, filtered through cheesecloth, and centrifuged at 4°C at 4,234 g for 20 min. The juice was again filtered on cotton and 1 g of PVPP was added per 100 g of juice and stirred for 20 min. The juice was vacuum filtered with Whatman paper (grades 3, 4, and 5). Fractions of 100 ml of juice were taken and 100 ml of distilled water was added, as well as 100 µl of 2-octanol (230 mg L^{-1} in ethanol) and 0.45 ml of nonyl-ß-∂-glucopyranoside (1,000 mg L^{-1} in ethanol/water, 50:50) were used as internal standards (Paolini et al., 2018).

Volatile compounds were extracted by adsorption on Isolute ENV + solid-phase extraction (SPE) cartridges (500 mg, 6 ml) purchased from Biotage (Charlotte, NS, USA). The cartridges were conditioned with 20 ml of methanol and 20 ml of

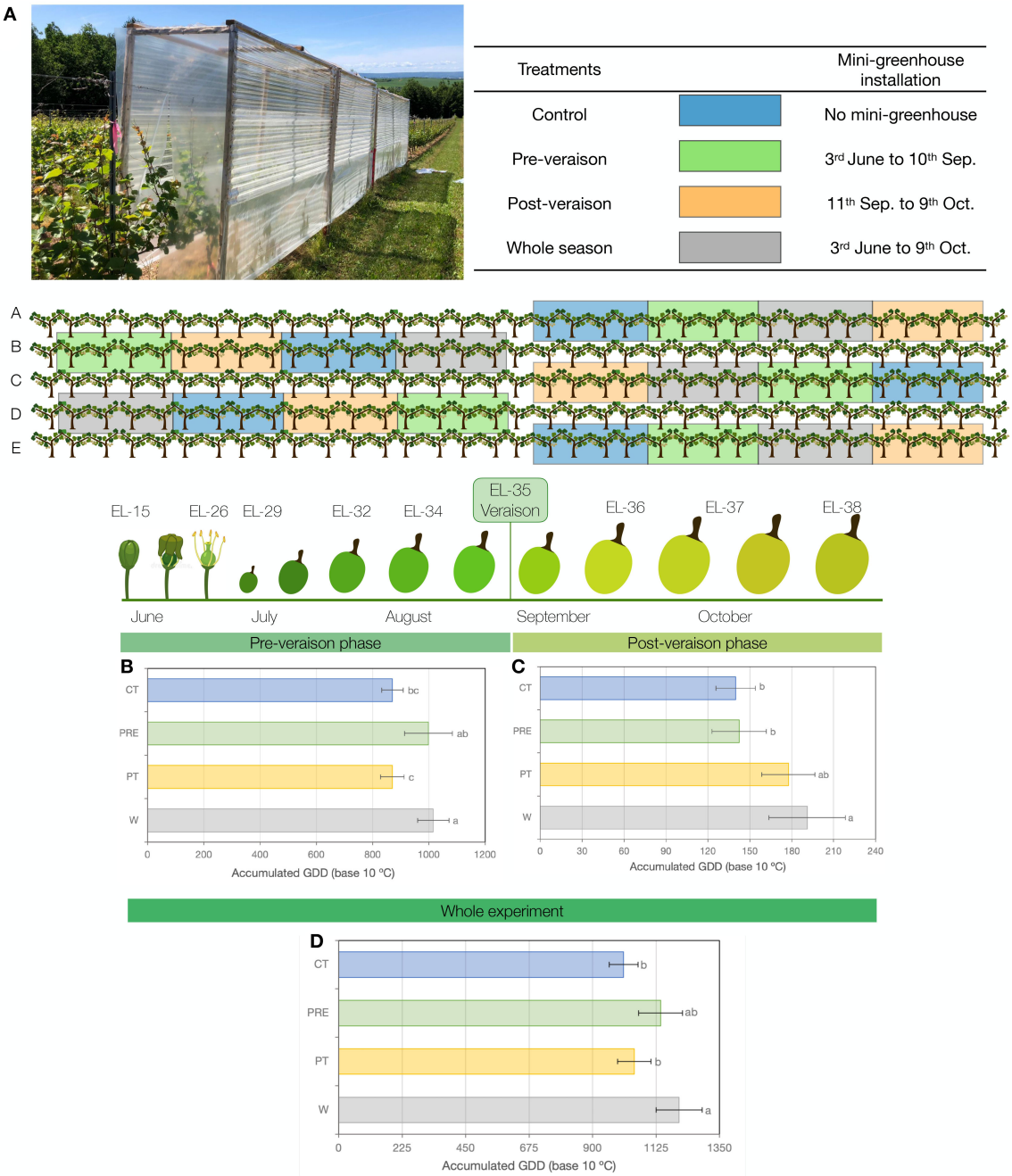

FIGURE 1 | (A) Mini-greenhouse, temperature treatments over the growing season, and experimental design. **(B)** Accumulation of growing degree days (GDD; based on 10°C) during the experiment. Pre-veraison phase (from 3 June 2020 to 10 September 2020). **(C)** Post-veraison phase (from 11 September 2020 to 9 October 2020). **(D)** Whole experiment (from 3 June 2020 to 9 October 2020). Values are means ± SD ($n = 5$ for Whole, Pre, and PT; $n = 3$ for CT). For each graph, values with different letters indicate significant differences between treatments (Tukey's test at $p \leq 0.05$).

distilled water at a flow rate of 1 ml min^{-1}. The sample was loaded onto the cartridge and then washed with 50 ml of distilled water. The FVCs were then eluted with 25 ml of dichloromethane (Schneider et al., 2004). The extract was filtered through anhydrous sodium sulfate and 50 ml of pentane was added (to a ratio of dichloromethane: pentane, 1:2), and the solution was concentrated to 200 µl using a Kuderna-Danish at 35–40°C (Schneider et al., 2004). The extract was stored at −80°C until gas chromatography–mass spectrometry (GC-MS) analysis.

The GVC fraction was eluted with 25 ml of methanol and evaporated to dryness under nitrogen at 45°C. All traces of FVCs were removed with dichloromethane: pentane (1:2). The remaining residue was then dissolved in 500 μl of phosphate/citrate buffer at pH 5 (Lanaridis et al., 2016). A volume of 200 μl of enzyme solution from Rapdidase Revelation Aroma (70 mg ml^{-1} in phosphate/citrate buffer, pH 5) was added (Schneider et al., 2004; Crespo et al., 2017), and the solution was kept at 40°C for 24 h. Once the enzymatic reaction was completed, internal standard 2-octanol (25 μl; 230 mg L^{-1} in ethanol) was added and the volatile fraction was extracted with dichloromethane: pentane (1:2) solution; the sample was concentrated at 200 μl using a Kuderna-Danish at 35°C−40°C. The extract was stored at −80°C until GC-MS analysis.

GC-MS Analysis

Compounds were separated on an HP-5MS Ultra Inert chromatographic column (30 m × 250 μm × 0.25 μm). Injection volumes were 1 μl and it was carried out in splitless mode. Helium was used as the carrier gas. The oven temperature was 40°C for 2 min, increased to 240°C at a rate of 3.5°C min^{-1}, maintained at 240°C for 2 min, increased to 250°C at a rate of 20°C min^{-1}, and held isothermal for 5 min at 250°C. Compounds were analyzed using an Agilent 7890A gas chromatography instrument coupled to an Agilent 5975 mass selective detector and a flame ionization detector using a splitter (Agilent Technologies, Santa Clara, CA, USA) and equipped with the ChemStation software for data acquisition and analysis. Compound identification was performed by mass spectra comparison, by NIST MS Search 2.0 library, and by retention indexes (Slegers et al., 2015), and for certain compounds, identification was possible using authentic standards (**Supplementary Table 1**). As commonly used in the analysis of aroma compounds (Azzolini et al., 2012; Ghaste et al., 2015), the response of the internal standard 2-octanol was used to normalize the peak area and to make a relative estimation of the concentration of the identified compounds, considering a response factor equal to 1.00.

Statistical Analysis

All statistical analyses were performed using the R software (RCoreTeam, 2022). Analysis of variance (ANOVA) was carried out for the climatic measurements, VCs, TSS, pH, berry weight (g), and TA. Means were compared using Tukey's *post hoc* honestly significant difference (HSD) at $p \leq 0.05$. Repeated measure ANOVA was also performed, and means were also compared using the Tukey HSD test to determine the significance of the VCs in response to the study factors (temperature and phenological stage). Principal component analysis (PCA) was carried out for compounds that were significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ in terms of treatment and phenological stage. Pearson's correlations between environmental variables (GDD) and berry maturity variables (TSS, TA, and pH) were estimated and considered significant at $p \leq 0.001$.

RESULTS

Evolution of the Growing Degree-Days Pattern and the Climatic Conditions

The mini-greenhouses used in this experiment efficiently modulated mean temperatures (**Supplementary Figure 1**) and GDD accumulation during the pre-veraison phase (Pre treatment), post-veraison phase (PT treatment), whole experiment (W treatment), and/or CT (treatment without mini-greenhouses) (**Figure 1**). GDD accumulation was higher in the Pre and W treatments during the pre-veraison phase from 3 June 2020 to 10 September 2020, accumulating 999 and 1,016 GDD, respectively, compared to the 870 GDD and 871 GDD accumulated in PT and CT treatments, respectively (**Figure 1B**). During the post-veraison phase from 11 September 2020 to 9 October 2020, the W treatment accumulated more GDD than the Pre and CT treatments (**Figure 1C**). During the whole experiment (3 June 2020 to 9 October 2020), the GDD was significantly higher in the W treatment with 1,207 GDD compared to 1,048 GDD and 1,010 GDD in the PT and CT treatments, respectively, while no significant difference was observed between the control and Pre or PT treatments (**Figure 1D**). Canopy temperature for W and PRE shows higher mean values at the beginning of the season, in line with the establishment of the mini-greenhouses (**Supplementary Figure 1**). Lately, during the season, specifically in September, the patterns of mean temperature switched from Pre to PT, being the W and PT the treatments showing higher mean temperatures during the last 2 months of the experiment (refer to **Supplementary Figure 1**).

Berry Growth and Juice Basic Metrics

Berries were sampled at three different phenological stages around full ripeness (EL-36, EL-37, and EL-38). Juice metrics and berry weight showed significant differences between phenological stages (**Table 1**). The highest value of TSS was found at full ripeness, 22.4 °Brix at EL 38, when compared to EL-37 (21.4 °Brix) and EL-36 (19.5 °Brix), which were also significantly different from each other. TA ($g{\cdot}L^{-1}$ tartaric acid eq) showed a similar trend in all treatments, as the values decreased with berry maturity, with the lowest value of 8.55 g L^{-1} tartaric acid eq reached at EL 38. Berry weight increased during ripening until EL-38 was reached, with a mean value of 1.2 g $berry^{-1}$.

The mini-greenhouse treatments had less impact on berry weight and juice metrics (**Table 2**). Productivity varied from 1.86 to 2.44 kg $vine^{-1}$ between different treatments but was not significantly different (**Supplementary Table 2**).

Berries from the W treatment had a value of 21.8 °Brix, a higher TSS compared to the control (CT, 20.4 °Brix; **Table 2**) although pH, TA, and berry weight were similar. CT and Post treatments had visibly increased red coloration compared to Whole and Pre treatments (**Supplementary Figure 3**).

Berry pH increased as berries matured and was negatively correlated with TA (**Supplementary Figure 2**). Similarly, TA was also negatively correlated with TSS (**Supplementary Figure 2**), and TSS was positively correlated with pH (**Supplementary Figure 2**). Only TSS was

TABLE 1 | Effects of the four mini-greenhouse treatments (CT, control; Pre, pre-veraison; PT, post-veraison; W, whole season) on berry weight (g), total soluble solids (°Brix), pH, and titratable acidity (g L^{-1} of tartaric acid eq) of L'Acadie blanc berries, vintage 2020.

Variables[a]	CT	Pre	PT	W	*p*-value
Berry weight	1.14 ± 0.08	1.13 ± 0.11	1.16 ± 0.07	1.13 ± 0.09	0.774
Total soluble solids	20.39 ± 1.64a	21.42 ± 1.23ab	20.89 ± 1.43ab	21.79 ± 1.47b	0.0543
pH	3.01 ± 0.09	2.96 ± 0.08	3.02 ± 0.10	2.95 ± 0.14	0.179
Titratable acidity	9.89 ± 1.50	10.14 ± 1.27	9.69 ± 1.16	10.22 ± 1.46	0.696

[a]*Data are means ± standard deviation of n = 15. For each variable, values with different letters indicate significant differences between treatments according to Tukey's test at p < 0.1.*

TABLE 2 | Effects of the phenological stage (EL-36, EL-37, and EL-38) on berry weight (g), total soluble solids (°Brix), pH, and titratable acidity (g L^{-1} of tartaric acid eq) of *Vitis* sp. cv. L'Acadie blanc berries, vintage 2020.

Variables[a]	EL-36	EL-37	EL-38	*p*-value
Berry weight	1.10 ± 0.09a	1.15 ± 0.06ab	1.17 ± 0.09b	0.0209
Total soluble solids	19.50 ± 1.17a	21.45 ± 0.72b	22.43 ± 0.71c	<0.001
pH	2.88 ± 0.10a	3.02 ± 0.06b	3.05 ± 0.05b	<0.001
Titratable acidity	11.37 ± 0.77c	10.05 ± 0.70b	8.55 ± 0.53a	<0.001

[a]*Data are means ± standard deviation of n = 20. For each variable values with different letters indicate significant differences between treatments according to Tukey's test at p < 0.05.*

significantly correlated with GDD ($r^2 = 0.49$, $p < 0.001$; **Supplementary Table 3**).

Influence of Ripening and Mini-Greenhouse Treatments on Volatile Compounds

A total of 46 and 53 compounds were detected in the FVC and GVC fractions, respectively, and were classified into nine classes (**Tables 3**, **4**), namely, (1) aliphatic alcohols, (2) aliphatic aldehydes, (3) aliphatic acids, (4) terpenes, (5) C_{13}-norisoprenoids, (6) aliphatic esters, (7) volatile phenols, (8) benzene derivatives, and (9) other volatiles. Among these, aliphatic esters were only detected as FVC and terpenes, and C_{13}-norisoprenoids were only detected as GVC. Aliphatic aldehydes represented the largest proportion of FVC, with a mean concentration of 11.74 μg g^{-1} FW. The GVC fraction was mainly composed of benzene derivatives (2,254 ng g^{-1} FW, on average) followed by C_{13}-norisoprenoids (973 ng g^{-1} FW, on average) and terpenes (895 ng g^{-1} FW, on average).

The total concentration of FVC did not change from EL-36 to EL-38 (**Supplementary Table 4**), but 12 variable compounds showed significant differences from one stage to another, mostly among aliphatic alcohols and aliphatic acids. Noticeably, methyl salicylate significantly increased from EL-36 to EL-37/EL-38, whereas benzyl alcohol significantly decreased from stage EL-36/EL-37 to stage EL-38. In contrast to the total FVC fraction, the total concentration of GVC significantly increased with ripening and was significantly higher at EL-38 with a concentration of 5.6 μg g^{-1} FW, compared to EL-36 and EL-37 with 4.4 μg·g^{-1} FW and 4.8 μg g^{-1} FW, respectively (**Supplementary Table 4**). The rise of total GVC is related to a significant increase in the concentration of 23 GVC, mostly aliphatic alcohols and terpenes, which totalized 181 and 1,327 ng g^{-1} FW, respectively, at EL-38. Of interest, the concentration of the monoterpenes linalool and nerol increased by 2.5 times, and the concentration of the sesquiterpene nerolidol increased by 1.3 times from stage EL-37 to EL-38.

The VC profiles also varied depending on mini-greenhouse treatment applied during the pre-veraison (Pre) or the post-veraison (PT), or during the whole season (W) (**Tables 3**, **4**). The CT also showed different VC profiles than other treatments, especially from the Pre and W treatments. CT berries showed the highest concentration of total FVC, with 14.06 μg g^{-1} FW, compared to the W and Pre treatments, with 12.61 and 12.46 μg g^{-1} FW, respectively (**Table 3**). The total GVC did not vary from one treatment to another (**Table 4**); however, the mini-greenhouse treatments affected specific classes of compounds, and most of these changes were observed in the GVC fraction.

Pre and W treatments resulted in a higher accumulation of benzene derivatives when compared to no greenhouse (CT) and mini-greenhouse used during post-veraison (PT). Indeed, the average concentrations in the Pre and W treatments of benzyl alcohol with 1,349 ng g^{-1} FW and 2-phenylethanol with 1,138 ng g^{-1} FW were significantly higher compared to the average concentrations of benzyl alcohol of 1,049 ng g^{-1} FW and 2-phenylethanol of 763 ng g^{-1} FW found in the CT and PT (**Table 4**). Conversely, CT berries had higher concentrations of bound volatile phenols at 554 ng g^{-1} FW when compared to Pre and W treatments at 371 and 354 ng g^{-1} FW, respectively, comprising isoeugenol, isovanillyl alcohol, acetovanillone, methyl-3-hydroxybenzoate, (*E*)-coniferyl alcohol, and sinapyl alcohol. CT berries also contained significantly higher concentrations of terpenes at 1,322 ng g^{-1} FW and C_{13}-norisoprenoids at 1,126 ng g^{-1} FW when compared to Pre and W treatments at 714 and 904 ng g^{-1} FW for terpenes and C_{13}-norisoprenoids, respectively, comprising (*E*)-linalool oxide, (*E*)-8-hydroxylinalool, nerolidol, β-ionol, and 3-oxo-7,8-dihydro-α-ionol.

The use of mini-greenhouses during the post-veraison (PT) phase had a limited impact on the FVC and GVC profiles of berries but, interestingly, berries from the PT treatment showed VC profiles somewhat intermediate between the Pre and W treatments, and the CT. Besides the similar accumulation of benzene derivatives (as mentioned earlier), PT and CT berries also had a similar concentration of the terpenes nerol and nerolidol. On the other side, CT berries had significantly higher

TABLE 3 | Impact of the temperature treatments (Control; Pre, pre-veraison; Post, post-veraison; Whole, whole season) on the profile of free volatile compounds (ng·g^{-1} FW) of berries from *Vitis* sp. cv. L'Acadie blanc in 2020.

Compounds[a]	CT	Pre	PT	W	*p*-value
Aliphatic alcohols					
(Z)-2-Penten-1-ol	41.7 ± 10.8	34.2 ± 13.1	34.5 ± 13	30.9 ± 9.2	0.0941
1-Hexanol	170 ± 240	139 ± 139	142 ± 165	107 ± 113	0.7940
2-Hexanol	3.0 ± 1.7	2.1 ± 0.9	3.3 ± 2.0	3.2 ± 2.6	0.6600
(E)-2-hexen-1-ol	408 ± 110b	336 ± 172ab	376 ± 81.4ab	272 ± 134 a	0.0330
2,4-Dimethyl-1-heptanol	382 ± 235b	220 ± 160ab	290 ± 233ab	174 ± 139 a	0.0443
2,6-Dimethyl-2-octanol	11.7 ± 13.0	12.8 ± 7.2	15.8 ± 10.8	14.0 ± 9.6	0.8020
3,7,11-Trimethyl-1-dodecanol	27.3 ± 39.2	10.7 ± 9.0	16.0 ± 13.9	14.9 ± 14.7	0.3120
Sum	988 ± 234c	732 ± 244ab	837 ± 194bc	595 ± 200 a	<0.0001
Alpihatic aldehydes					
Hexanal	2 608 ± 325	2 289 ± 367	2 445 ± 591	2 392 ± 234	0.1890
2-Hexenal	86.1 ± 21.5	83.2 ± 13	80.4 ± 16	80 ± 23	0.7950
(E)-2-Hexenal	9,876 ± 1 019	8,918 ± 986	8,924 ± 1,524	9 082 ± 784	0.0660
2,3,4-Trimethyl-hex-3-enal	2.9 ± 0.7	41.9 ± 67.1	2.9 ± nd	3.8 ± nd	0.7500
(E,E)-2,4-Hexadienal	3.2 ± 0.8	2.7 ± 1.2	2.8 ± 1.2	3.1 ± 0.7	0.6510
Nonanal	9.4 ± 7.8	10.7 ± 9.2	8.6 ± 3.8	10.9 ± 6.8	0.8060
(E,E)-2,6-Nonadienal	10.9 ± 2.9	13.2 ± 4.9	12.5 ± 4.2	10.4 ± 3.8	0.1960
(E)-4-Undecenal	1.4 ± 0.4	2.0 ± 1.5	1.6 ± 0.8	1.6 ± 0.7	0.4690
Sum	12 595 ± 1,308	11 325 ± 1,333	11,474 ± 2,062	11,579 ± 946	0.0856
Aliphatic acids					
2-Propenoic acid pentyl ester	98.6 ± 29b	66.2 ± 35.9a	80.6 ± 32.8ab	70.9 ± 32.8ab	0.0446
Butyric acid-5-hexenyl ester	11.0 ± 5.0b	7.4 ± 2.1a	8.1 ± 2.5ab	8.5 ± 3.0ab	0.0398
Butyric acid octyl ester	12.6 ± 5.6	14.9 ± 7.4	14.8 ± 7.7	16.7 ± 9.6	0.5450
Hexanoic acid	32.0 ± 18.9	27.6 ± 10.9	29.4 ± 17.3	26.1 ± 8.2	0.7040
2-Ethyl-hexanoic acid	3.1 ± 2.2	2.5 ± 1.2	3.0 ± 1.3	2.5 ± 1.1	0.8240
(E)-2-Hexenoic acid	34.7 ± 16.9	31.1 ± 19.5	43.7 ± 22.7	24.8 ± 19.5	0.3680
Heptanoic acid	4.4 ± 2.1	3.6 ± 0.9	4.2 ± 2.8	4.5 ± 1.8	0.6540
Octanoic acid	12.2 ± 3.7	11.4 ± 6.2	12.8 ± 6.3	13.0 ± 6.1	0.8790
7-Oxooctanoic acid	10.5 ± 6.6	11.1 ± 4.3	10.7 ± 5.5	11.2 ± 3.2	0.9810
Sum	197 ± 52.5	156 ± 53.5	179 ± 57.7	164 ± 56.3	0.1910
Aliphatic esters					
2-Butoxy-ethanol	4.2 ± 1.4	4.2 ± 1.3	4.4 ± 1.5	4.7 ± 1.1	0.6940
2,2-Butoxyethoxy-ethanol	14.4 ± 7.2	16.8 ± 8.6	14.4 ± 6.3	19.9 ± 8.6	0.1750
Sum	18.3 ± 7.8	20.5 ± 9.1	18.8 ± 7.5	24.7 ± 9.2	0.1690
Volatile phenols					
Methyl salicylate	7.3 ± 4.1a	14.9 ± 7.8b	10.1 ± 6.4ab	8.9 ± 5.8ab	0.0105
Vanillin	27.5 ± 16.1	32.9 ± 28.8	23.9 ± 13.1	30 ± 12.1	0.6030
	17.0 ± 12.7	15.2 ± 15.1	14.7 ± 9.7	12.1 ± 7.5	0.7180
4-Hydroxy-3,5-dimethoxy-benzaldehyde					
Sum	51.8 ± 24.5	63.0 ± 43.9	48.7 ± 16.2	51.1 ± 15.0	0.4970
Benzene derivatives					
Benzyl alcohol	35.0 ± 9.4	33.1 ± 13.5	27.6 ± 14.7	29.4 ± 12.8	0.3800
Phenylethanal	24.6 ± 21.8	12.1 ± 7.2	26.4 ± 24.8	15.3 ± 11.8	0.0908
p-Tolualdehyde	10.8 ± 13.3	9.6 ± 11	10.3 ± 11.3	13.7 ± 14.7	0.8610
Benzophenone	11.4 ± 1	11.8 ± 1	11.8 ± 2.1	12.0 ± 1.5	0.7190
2-Phenoxy-ethanol	4.7 ± 0.8	6.3 ± 3.8	5.6 ± 2.2	5.6 ± 2.4	0.4470
Sum	85.1 ± 23.2	67.9 ± 23.2	79.2 ± 37.6	73.8 ± 27.0	0.3980
Other volatiles					
3,4,4-Trimethyl-2-hexene	7.0 ± 0.2	6.3 ± 0.1	6.9 ± 1.2	6.5 ± 0.2	0.6760

(Continued)

TABLE 3 | Continued

Compounds[a]	CT	Pre	PT	W	*p*-value
Heptane	50.8 ± 21.9	48 ± 20.6	49.2 ± 24.4	57.1 ± 17.4	0.6500
4-Methyl-heptane	12.6 ± 9.7	8.1 ± 2.8	16.4 ± 14.7	11.8 ± 10.2	0.3840
2,4-Dimethyl-heptane	6.3 ± 6.4	3.8 ± 2.0	6.8 ± 4.8	4.8 ± 3.5	0.4750
2,4-Dimethyl-1-heptene	55.4 ± 28.4	34.8 ± 19.7	44.4 ± 14.1	23.3 ± 15.3	0.0582
4-Methyl-nonane	5.5 ± 3.0	4.8 ± 1.6	5.0 ± 2.6	5.4 ± 1.4	0.8140
4-Propyl-3-heptene	2.4 ± 0.2	2.1 ± 0.7	2.7 ± 1.0	2.4 ± 0.3	0.6050
2,2-Dimethyl-3-octene	5.9 ± 2.6	4.8 ± 2.0	5.8 ± 2.9	5.4 ± 2.0	0.6090
2,3,3-Trimethyl-1,7-octadiene	4.2 ± 3.8	2.4 ± 1.1	4.1 ± 4.5	4.4 ± 3.0	0.8940
Decane	28.6 ± 13.3b	17.5 ± 10.1ab	25.4 ± 11.8ab	16.7 ± 9.7a	0.0433
4-Ethyl-decane	3.1 ± 1.2	3.7 ± 1.5	5.3 ± 3.5	3.3 ± 2.7	0.2660
y-Undecalactone	3.6 ± 3.1	4.5 ± 5.6	3.7 ± 2.6	3.3 ± 1.6	0.8370
Total	14,055 ± 1,362b	12,455 ± 1,359a	12,750 ± 1 971ab	12,607 ± 954a	0.0147

*Variables showing a significant interaction between treatments and phenological stages are in bold (refer to **Supplementary Table 4** for repeated measure ANOVA and Tuckey's comparison test). [a]All compounds were quantified as 2-octanol equivalents. Values are means ± standard deviation of 15 biological replicates. For each treatment, values with different letters indicate significant differences according to Tukey's test at $p < 0.05$. ns, not significant; nd, not determined. Repeated measure ANOVA was also carried out to detect possible interactions between temperature; treatments and phenological stages (**Supplementary Table 6**).*

(*E*)-8-hydroxylinalool concentrations of 101 ng g^{-1} FW and β-ionol concentrations of 236 ng g^{-1} FW when compared to PT berries at 77 and 174 ng g^{-1} FW, respectively.

Certain compounds showed a significant interaction between phenological stages and the mini-greenhouse treatments, meaning that treatment impact varied according to the phenological stage (refer to **Supplementary Tables 6**, **7** for detailed statistics of interactions). Among FVC, the 1-hexanol concentration of 471 ng g^{-1} FW was significantly higher in CT berries when compared to 253 ng g^{-1} FW of W berries at stage EL-36, but no differences were observed between treatments at other stages (**Supplementary Table 6**). Conversely, bound 1-hexanol was more concentrated in PT berries at 53.6 ng g^{-1} FW when compared to W berries at 20.4 ng g^{-1} FW (**Supplementary Table 7**). The accumulation of bound (*Z*)-linalool oxide, hotrienol, (*Z*)-8-hydroxylinalool, and lilac alcohol C was significantly modulated by grapevine phenology and treatments. Globally, these compounds accumulated at a faster rate in CT berries and were significantly more concentrated in CT berries at stages EL-37 and/or EL-38 when compared to other treatments. The volatile phenol methyl vanillate showed a similar accumulation pattern and was significantly more concentrated in CT berries at EL-38 when compared to other treatments and phenological stages.

Principal Component Analysis of Volatile Compounds

Principal component analyses were performed to show the relationships between VCs and the mapping of phenological stages (**Figure 2**). The matrix used for this analysis consisted of FVC and GVC that varied significantly from one ripening stage to another (EL-36, EL-37, and EL-38). PC1 and PC2 of the compounds from the FVC fraction explained 45.1% of the total variability (**Figure 2A**). Based on this PCA, the aliphatic alcohol 1-hexanol was the main contributor to the FVC (PC 1) and was mainly associated with the ripening stage EL-36, while 2,4-dimethyl-1-heptanol, butanoic acid-5-hexenyl ester, decane, and methyl salicylate were associated with the ripening stages EL-37 and EL-38, which were less discriminated from each other. In the PCA of the GVC (**Figure 2B**), PC1 and PC2 explained 65.3% of the total variability between samples (PC 1, 50.4%; PC 2, 14.9%). The compounds used in this PCA were mostly associated with the phenological stage EL-38 (quadrants II and III, **Figure 2B**), but the terpenes (*E*)- and (*Z*)-8-hydroxylinalool, hotrienol, (*Z*)-linalool oxide, and nerolidol and lilac alcohol C were the most significant contributors to the mapping, mostly over PC 1. Groups (EL-36, EL-37, and EL-38) were better discriminated with regard to GVC when compared to FVC.

The PCAs of FVC and GVC and temperature treatments were also performed only using significant variables (**Figure 3**). The PCA of FVC (**Figure 3A**) explained 53.5% of the variability between treatments. The aliphatic alcohols *(E)*-2-hexen-1-ol and 2,4-dimethyl-1-heptanol were the main contributors to PC 1 and mainly associated with the CT, whereas the volatile phenol methyl salicylate, the main contributor to PC 2, was associated with the treatment Pre. The PCA of GVC showed that the variability between samples was explained at 45.4% by PC 1 and at 14.0% by PC 2 (**Figure 3B**). The mapping of compounds and samples showed two different patterns. The first pattern showed that the aliphatic alcohol 5-(2-tetrahydrofurfuryl)-heptan-2-ol, the aliphatic aldehyde (*E*)-2-hexenal, and the monoterpenes (*Z*)-linalool oxide, hotrienol, nerolidol, and pyranoid linalool oxide were mainly associated with the CT. The second pattern showed that benzyl alcohol and 2-phenylethanol were strongly associated with the treatments W and Pre (quadrant A-I, **Figure 3B**). Overall, the mapping showed that treatments Pre and W were quite similar in terms of GVC profile, whereas PT and CT showed some similarities, while not being totally alike.

TABLE 4 | Impact of the temperature treatments (Control; Pre, pre-veraison; Post, post-veraison; Whole, whole season) on the profile of glycosylated volatile compounds ($ng \cdot g^{-1}$ FW) of berries from *Vitis* sp. cv. L'Acadie blanc in 2020.

Compounds[a]	CT	Pre	PT	W	*p*-value
Aliphatic alcohols					
3-Methyl-1-butanol	26.8 ± 5.6	25.5 ± 7.3	22.7 ± 9.6	24.4 ± 5.5	0.4786
2-Methyl-1-butanol	24.4 ± 8.5	24.2 ± 10.3	19.0 ± 8.5	19.5 ± 7.6	0.1838
2-Methyl-2-buten-1-ol	13.4 ± 5.2	13.4 ± 4.0	13.1 ± 5.5	12.3 ± 4.2	0.9034
3-Methyl-3-buten-1-ol	48.5 ± 10.2	46.5 ± 10.1	41.0 ± 11.4	46.1 ± 10.0	0.2540
1-Pentanol	9.0 ± 3.8	7.8 ± 2.3	7.8 ± 4.2	6.4 ± 2.2	0.1790
1-Hexanol	25.3 ± 15.7	20.3 ± 8.9	28.9 ± 23.9	17.7 ± 7.4	0.1991
3-Hexen-1-ol	15.2 ± 5.3c	8.3 ± 3.4a	13.8 ± 5.3bc	9.8 ± 5.4 ab	0.0008
Sum	163 ± 41.9	146 ± 35.0	146 ± 60.5	136 ± 30.8	0.4250
Aliphatic aldehydes					
Hexanal	11.8 ± 6.3	11.1 ± 4.6	12.7 ± 6.3	8.6 ± 2.4	0.1738
(*E*)-2-Hexenal	32.1 ± 16.9ab	23.1 ± 9.8ab	36.6 ± 27.8b	19.5 ± 11.1a	0.0422
Sum	43.9 ± 21.1ab	34.2 ± 12.3ab	49.3 ± 32.1b	28.1 ± 11.6a	0.0349
Aliphatic acids					
(*Z*)-9-Octadecenoic acid	28.8 ± 13.2	28.3 ± 10.5	32.8 ± 18.2	31.7 ± 13.5	0.7834
Tetradecanoic acid	49.5 ± 20.1	41.4 ± 20.1	45.0 ± 23.3	43.8 ± 15.0	0.7253
Sum	78.3 ± 27.7	69.6 ± 23.3	77.7 ± 36.5	75.5 ± 18.6	0.8153
Mono- and sesquiterpenes	63.8 ± 26.3b	38.3 ± 11.7a	45.6 ± 14.5a	36.1 ± 12.8a	0.0002
(*Z*)-Linalool oxide					
(*E*)-Linalool oxide	37.1 ± 9.7b	29 ± 4.1a	33.2 ± 5.3ab	30.6 ± 5.5a	0.0073
Linalool oxide pyranoid	56.5 ± 22.4b	32.9 ± 11.7a	43.6 ± 12.1 ab	32.2 ± 12.3a	0.0001
Linalool	11.2 ± 13.5	5.7 ± 4.7	9.0 ± 11.1	5.3 ± 4.8	0.2699
Hotrienol	110 ± 81.8b	53.4 ± 27.4a	58.3 ± 31.9 a	45.9 ± 35.7a	0.0027
Nerol	20.2 ± 12.1b	10.0 ± 7ab	20.4 ± 19.4b	9.2 ± 8.0a	0.0190
Lavandulol	17.1 ± 3.9	15.6 ± 2.9	18.7 ± 8.0	16.7 ± 1.6	0.3489
(*E*)-8-Hydroxylinalool	101 ± 34.8b	58.9 ± 21.4a	77 ± 22a	59.9 ± 17.7a	<0.0001
(*Z*)-8-Hydroxylinalool	839 ± 472b	414 ± 225a	632 ± 235 ab	439 ± 202a	0.0009
Linalyl isobutyrate	32.3 ± 15.4	24.8 ± 7.7	26.0 ± 6.9	26.0 ± 8.5	0.1844
2,6-Dimethyl-2,6-Octadiene-1,8-diol	8.8 ± 10.1	7.0 ± 6.2	8.8 ± 8.5	6.4 ± 5.5	0.7737
Nerolidol	14.4 ± 4.5b	9.4 ± 2.5a	11.9 ± 3.5 ab	8.9 ± 2.7a	<0.0001
Lilac alcohol C	10.7 ± 6.9	6.6 ± 3.7	9.5 ± 4.6	6.8 ± 4.4	0.0817
Sum	1,322 ± 656b	705 ± 317a	994 ± 353ab	723 ± 294a	0.0005
C_{13}-norisoprenoids					
3-Hydroxy-β-damascone	172 ± 34.0	148 ± 26.5	152 ± 27.7	142 ± 45.0	0.0940
3-Hydroxy-7,8-dihydro-β-ionol	87.1 ± 37.7	66.7 ± 15	70.2 ± 18.6	72.6 ± 14.7	0.0998
3-Oxo-α-ionol	349 ± 67.3	302 ± 55.2	315 ± 64.3	290 ± 85.4	0.1160
β-Ionol	236 ± 102b	155 ± 37.1a	174 ± 38.9a	164 ± 34.9a	0.0021
3-Hydroxy-5,6-epoxy-β-ionone	24.5 ± 5.4	20.3 ± 6.5	23.8 ± 5.0	20.9 ± 4.2	0.0954
3-Oxo-7,8-dihydro-α-ionol	236 ± 39.8b	191.7 ± 31a	206.1 ± 34.6ab	201.1 ± 22.9a	0.0033
Dihydro-3-oxo-β-ionol	21.5 ± 7.8b	15.4 ± 4.2a	18.4 ± 5.0ab	18.4 ± 4ab	0.0332
Sum	1,126 ± 269b	899 ± 160a	959 ± 181ab	909 ± 144a	0.0076
Volatile phenols					
p-Vinylguaiacol	21.3 ± 6.2	17.1 ± 9.1	19.9 ± 10.7	16.4 ± 6.8	0.3397
Eugenol	36.7 ± 16.3	30.9 ± 10.8	36.8 ± 18.2	44.5 ± 14.6	0.1120
Methoxyeugenol	10.8 ± 2.4	10.9 ± 2.6	10.7 ± 3.6	9.8 ± 2.0	0.6660
2-Hydroxy-benzeneethanol	18.8 ± 16.1	11.5 ± 7.2	14.1 ± 7.7	9.9 ± 4.8	0.0867
Isoeugenol	22.4 ± 13.3b	10.3 ± 3.6a	16.6 ± 4.6ab	10.8 ± 2.4a	<.0001
Isovanillyl alcohol	33.6 ± 17.3b	18.8 ± 6.6a	28.4 ± 11.8ab	19.9 ± 8.6a	0.0024
Acetovanillone	30.8 ± 6.0b	25.3 ± 5.0a	29.2 ± 6.1ab	24.3 ± 4.8a	0.0053

(Continued)

TABLE 4 | Continued

Compounds[a]	CT	Pre	PT	W	*p*-value
Methyl vanillate	129 ± 134b	67.4 ± 68.3ab	68.5 ± 21.2ab	53.8 ± 19.6a	0.0445
Methyl 3-hydroxybenzoate	32.0 ± 10.9b	22.4 ± 8.4a	28.2 ± 7.9ab	20.6 ± 8.8a	0.0034
(*E*)-Coniferyl alcohol	56.4 ± 31.9b	29.7 ± 20.2a	42.6 ± 30.3ab	24.2 ± 13.3a	0.0042
Sinapyl alcohol	34.3 ± 27.1b	19.3 ± 9.9ab	22.6 ± 12.0ab	17.0 ± 9.8a	0.0266
Salicyl alcohol	21.0 ± 8.1	18.8 ± 7.5	16.2 ± 6.1	14.8 ± 4.7	0.0693
5-(3-Hydroxypropyl)-2,3-dimethoxyphenol	13.9 ± 9.7	9.5 ± 4.9	10.9 ± 6.1	7.5 ± 2.4	0.0552
2-Hydroxy-4,5-dimethylacetophenone	40.0 ± 12.5	32.2 ± 10.3	31.5 ± 10.0	31.6 ± 8.8	0.0846
4-tert-Butyl-2-methylphenol	53.5 ± 13.8	46.6 ± 10.1	45.4 ± 12.2	48.9 ± 9.6	0.2434
Sum	554 ± 250b	371 ± 129a	422 ± 113ab	354 ± 69.0a	0.0034
Benzene derivatives					
Benzyl alcohol	1 094 ± 96.6a	1 390 ± 298b	1 004 ± 157a	1 308 ± 182b	<0.0001
2-Phenylethanol	770 ± 85.1a	1 152 ± 143b	756 ± 132a	1 123 ± 163b	<0.0001
3-Tridecyl ester-m-toluic acid	86.1 ± 17.8	71.9 ± 19.4	78.9 ± 18.7	91.8 ± 67.2	0.4968
4-Benzyloxy-3-methoxybenzyl alcohol	22.8 ± 9.6ab	28.5 ± 8.5b	17.0 ± 3.0a	21.9 ± 8.3ab	0.0022
Sum	1,972 ± 151a	2,643 ± 432b	1,856 ± 251a	2,544 ± 302b	<0.0001
Other volatiles					
2-Butyltetrahydro-furan	9.1 ± 1.2	8.0 ± 2.0	8.3 ± 2.1	8.1 ± 1.9	0.3807
5-(2-Tetrahydrofurfuryl)-heptan-2-ol	48 ± 30.3b	24.4 ± 12.7a	33.4 ± 14.3ab	23.3 ± 12.8ab	0.0024
6-Ethenyl-2,2,6-trimethyloxan-3-ol	36.3 ± 6.2c	29 ± 3.8a	34.9 ± 5.1bc	31.3 ± 4.3ab	0.0005
Total	5,354 ± 1,106	4,929 ± 950	4,580 ± 803	4,833 ± 619	0.1278

*Variables showing a significant interaction between treatments and phenological stages are in bold (refer to **Supplementary Table 5** for repeated measure ANOVA and Tuckey's comparison test). [a]All compounds were quantified as 2-octanol equivalents. Values are means ± standard deviation of 15 biological replicates. For each treatment, values with different letters indicate significant differences according to Tukey's test at $p < 0.05$. ns: not significant; nd: not determined. Repeated measure ANOVA was also carried out to detect possible interactions between temperature; treatments and phenological stages (**Supplementary Table 7**).*

DISCUSSION

Climate change will likely modify viticulture practices and further impact wine properties in the near future (van Leeuwen and Darriet, 2016). In northern areas such as Eastern Canada, temperatures have been predicted to increase noticeably (Vasseur and Catto, 2008) but, as longer growing seasons happen more often, larger variations in temperature patterns during the season could make it even more challenging to properly ripen berries year after year. In this study, the impact of different temperature patterns on berry composition was explored at three harvest stages using on-the-row mini-greenhouses at different times during the season. The mini-greenhouses efficiently modulated temperature during the season by creating warmer conditions during the first phase of berry development (Pre treatment) or during veraison (PT treatment) while keeping a similar amount of total GDD during the season, between the treatments Pre and PT and the CT (**Figure 2**). The treatment lasting throughout the season (Whole) increased GDD accumulation, mimicking a temperature rise that could be expected from global warming. Both the phenological ripening stage and the mini-greenhouse treatment affected the berry biochemistry and the accumulation of VCs in *Vitis* sp. L'Acadie blanc berries.

Berry Basic Composition

Grapevines under the mini-greenhouses throughout the whole growing season (W treatment) resulted in berries with higher TSS than the control and there was a strong positive correlation of GDD with TSS but not TA of pH (**Supplementary Table 3** and **Supplementary Figure 2**). Elevated temperatures can impact berry biochemical composition by altering grapevine physiology and metabolism (Keller, 2015). Several studies that have previously evaluated the effects of thermal stress on berries indicate the concentration of primary metabolites and physicochemical properties vary due to the duration, intensity, and moment of exposure during development (Soar et al., 2009; Greer and Weston, 2010; Greer and Weedon, 2013; Crespo et al., 2017; Pastore et al., 2017). A positive impact of temperature on TSS was reported in Muscat à petits grain blancs and Sangiovese (Crespo et al., 2017; Pastore et al., 2017). These authors also observed a significant impact on TA and pH, whereas others (Sadras and Soar, 2009) observed no alteration of TA nor pH at a higher temperature, suggesting that other factors could be involved in organic acid metabolism in response to temperature. Several studies (Sadras and Soar, 2009; Soar et al., 2009; Greer and Weedon, 2013; Sadras et al., 2013; Pastore et al., 2017) suggested that differences in parameters, such as berry growth, TSS, pH, and TA, can respond differently depending on the duration and intensity of temperature increases and interactions with other factors (solar radiation, UV, water, and nutrient supply) and, more importantly, that the response is cultivar-dependent.

Volatile Compounds

A total of 99 VCs were quantified in the free and glycosylated fractions of L'Acadie blanc berries. Approximately 90% of the

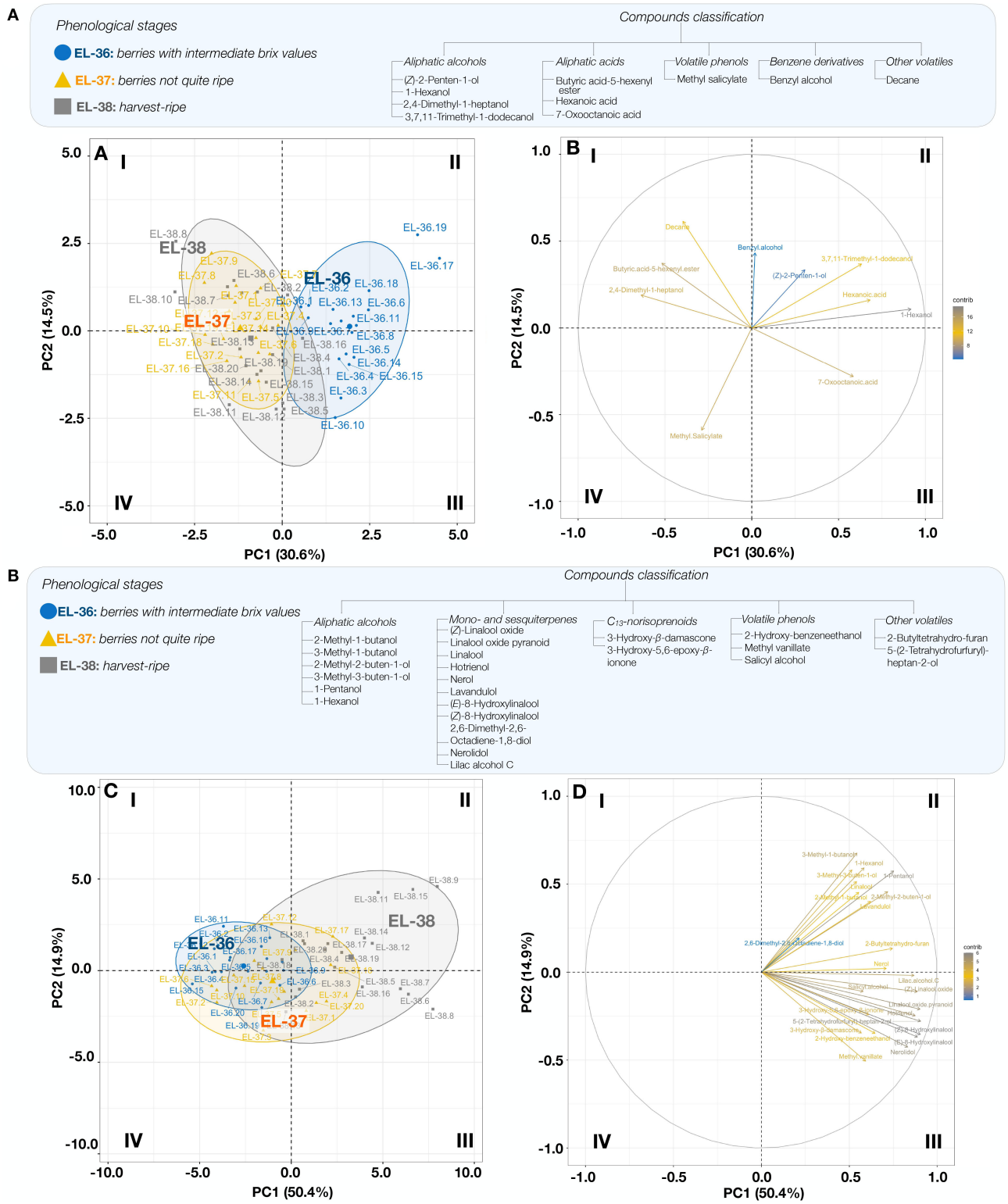

FIGURE 2 | Principal component analysis (PCA) of free volatile compounds (FVCs) **(A)** and glycosylated volatile compounds (GVCs) **(B)** (compounds; right plot) at phenological stages (EL-36, EL-37, and EL-38; samples; left plot). Biological replicates are labeled 1 to 20. FVCs include the following variables: (*Z*)-2-penten-1-ol, 1-hexanol, 2,4-dimethyl-1-heptanol, 3,7,11-trimethyl-1-dodecanol, butyric acid-5-hexenyl ester, hexanoic acid, 7-oxooctanoic acid, methyl salicylate, benzyl alcohol, and decane. GVCs include the following variables : 2-methyl-1-butanol, 3-methyl-1-butanol, 2-methyl-2-buten-1-ol, 3-methyl-3-buten-1-ol, 1-pentanol, 1-hexanol, (*Z*)-linalool oxide, linalool oxide pyranoid, linalool, hotrienol, nerol, lavandulol, (*E*)-8-hydroxylinalool, (*Z*)-8-hydroxylinalool, 2,6-dimethyl-2,6-octadiene-1,8-diol, nerolidol, lilac alcohol, 3-hydroxy-*β*-damascone, 3-hydroxy-5,6-epoxy-*β*-ionone, 2-hydroxy-benzeneethanol, methyl vanillate, salicyl alcohol, 2-butyltetrahydro-furan, and 5-(2-tetrahydrofurfuryl)-heptan-2-ol. Quadrants are identified I-IV (clockwise).

total FVC was composed of two compounds biosynthesized from the lipoxygenase pathway, namely, ∼70% of the FVC being (*E*)-2-hexenal (derived from α-linolenic acid; C18:3Δ9,12,15) and ∼19% of the FVC being hexanal (derived from linoleic acid; C18:2Δ9,12) (Moreno and Peinado, 2012; Lin et al., 2019). The higher concentrations of (*E*)-2-hexenal over hexanal agree with previous studies on *Vitis vinifera* and interspecific hybrids that suggested that the C18:3 route dominates over the C18:2 route, as α-linolenic acid is the preferred substrate for the lipoxygenase enzyme (Kalua and Boss, 2009; Pedneault et al., 2013).

The distribution of the GVCs was spread across eight different chemical families, but benzene derivatives and monoterpenes represented ∼37 and ∼25% of the total GVC fraction, respectively. 2-Phenylethanol and benzyl alcohol represented the largest proportion of benzene derivatives (∼95%). High levels of 2-phenylethanol are consistent with results from other interspecific *Vitis* sp. varieties, as 2-phenylacetaldehyde and 2-phenylethanol from the juice of "St. Croix" and "Sabrevois" studied by Slegers et al. (2015) represented 51.2% and 37.7% of the total free aroma profile, respectively. Similarly, Ghaste et al. (2015) showed that varieties, such as Isabella (*V. vinifera* × *Vitis labrusca*), *Vitis arizonica texas*, and *Vitis cinerea*, also have high levels of compounds, such as 2-phenylethanol and benzyl alcohol in the GVC fraction with concentrations reaching up to of 9,420 and 3,710 $\mu g\ kg^{-1}$, respectively.

Influence of the Phenological Ripening Stage on Volatile Compounds

The phenological ripening stage significantly affected the accumulation of VCs in L'Acadie blanc berries, as the concentration of most compounds increased as berries reached maturity.

Aliphatic acids from the FVC fraction, aliphatic alcohols, and monoterpenes from the GVC fraction represented the main chemical families significantly impacted by the ripening stage, the majority of which increased in the later ripening stages (**Supplementary Tables 4, 5**). These results agree with the literature, as many of these compounds are known to be synthesized during the last stages of berry development (Moreno and Peinado, 2012; Crespo et al., 2017). However, although the presence of VC increased with ripening, their accumulation is not necessarily uniform (Pedneault et al., 2013). For example, linalool was found at phenological ripening stages EL-37 and EL-38, but not at stage EL-36 (**Supplementary Table 5**). In contrast, the concentration of *(Z)*-8-hydroxylinalool, which constituted a large proportion of the GVC profile (∼67% of the total monoterpenes), increased as maturity was reached (**Figure 2B**).

Globally, when comparing the FVC with GVC, FVC represented a larger proportion of VCs in L'Acadie blanc berries. GVC is highly soluble in aqueous media (polar compounds), which facilitates plant transport through the phloem (Moreno and Peinado, 2012). In wine, GVC can represent the potential aroma that is revealed by acid or enzymatic hydrolysis during winemaking (Dunlevy et al., 2009). Interestingly, monoterpenes were only found as glycosides in L'Acadie blanc berries. Among these, (*E*)-8-hydroxylinalool (a highly oxidized monoterpene) can undergo several oxidation and cyclization processes during the winemaking process and, ultimately, result in the production of wine lactone, an important contributor to wine aroma (Lin et al., 2019); thus, the contribution of glycosides to the wine aroma goes beyond a restricted definition of varietal aroma compounds.

Grapevine Plasticity in Response to Temperature Increase

Besides genotype specificities, the accumulation of VCs can be influenced by seasonal and regional climates and vine-growing practices and factors that can influence the characteristics of a given variety grown in different areas (Dunlevy et al., 2009; Moreno and Peinado, 2012). In this study, 99 VCs were quantified in the free and glycosylated fractions (FVC and GVC, respectively), and their concentration was influenced by the type of treatment they were exposed to (**Tables 3**, **4**). Besides a limited number of compounds showing significant differences [e.g., (*E*)-2-hexen-1-ol, 2,4-dimethyl-1-heptanol, 2-propenoic acid pentyl ester, and decane], the FVC profile of the berries was relatively stable across treatments. Conversely, large differences were observed between the GVC profiles of berries across different classes of compounds, including benzyl and phenolic derivatives, terpenes, and C_{13}-norisprenoids.

Benzyl alcohol (∼22%) and 2-phenylethanol (∼18%) were the main GVC found in berries for all treatments, but their accumulation patterns differed between temperature treatments. Berries from the treatments exposed to elevated temperatures during the early developmental stages (pre-veraison phase; W and Pre treatments) had the highest concentrations of benzyl alcohol and 2-phenylethanol when compared to those exposed to higher temperature only during veraison (PT treatment) or not exposed (CT treatment; **Figure 3B** and **Table 4**). In contrast, the hydroxy-methoxy-substituted volatile phenols, such as isoeugenol, isovanillyl alcohol, acetovanillone, methyl-3-hydroxy benzoate, and (*E*)-coniferyl alcohol, had reduced accumulation in the Pre and W treatments when compared to the CT treatment. These findings suggest that mini-greenhouse treatments selectively modulated the metabolic pathways related to the biosynthesis of benzyl and phenyl derivatives at early developmental stages. 2-Phenylethanol is biosynthesized through the L-phenylalanine pathway *via* phenylpyruvic acid and phenylacetaldehyde, whereas both benzyl alcohol and hydroxy-methoxy-substituted volatile phenols derive from the phenylpropanoid pathway. Benzyl alcohol synthesis occurs during the early steps of the phenylpropanoid pathway, through the shortening by two carbons of the side chain of *trans*-cinnamic acid, while hydroxy-methoxy-substituted volatile phenols are produced later downstream, from caffeic, ferulic, and sinapic acids (Huang et al., 2001; Widhalm and Dudareva, 2015; Shu et al., 2021).

The bioconversion of L-phenylalanine into 2-phenylethanol has been found to be favored at high temperatures (Huang et al., 2001; Shu et al., 2021), but different species of plants tend to have different accumulation patterns of the compound.

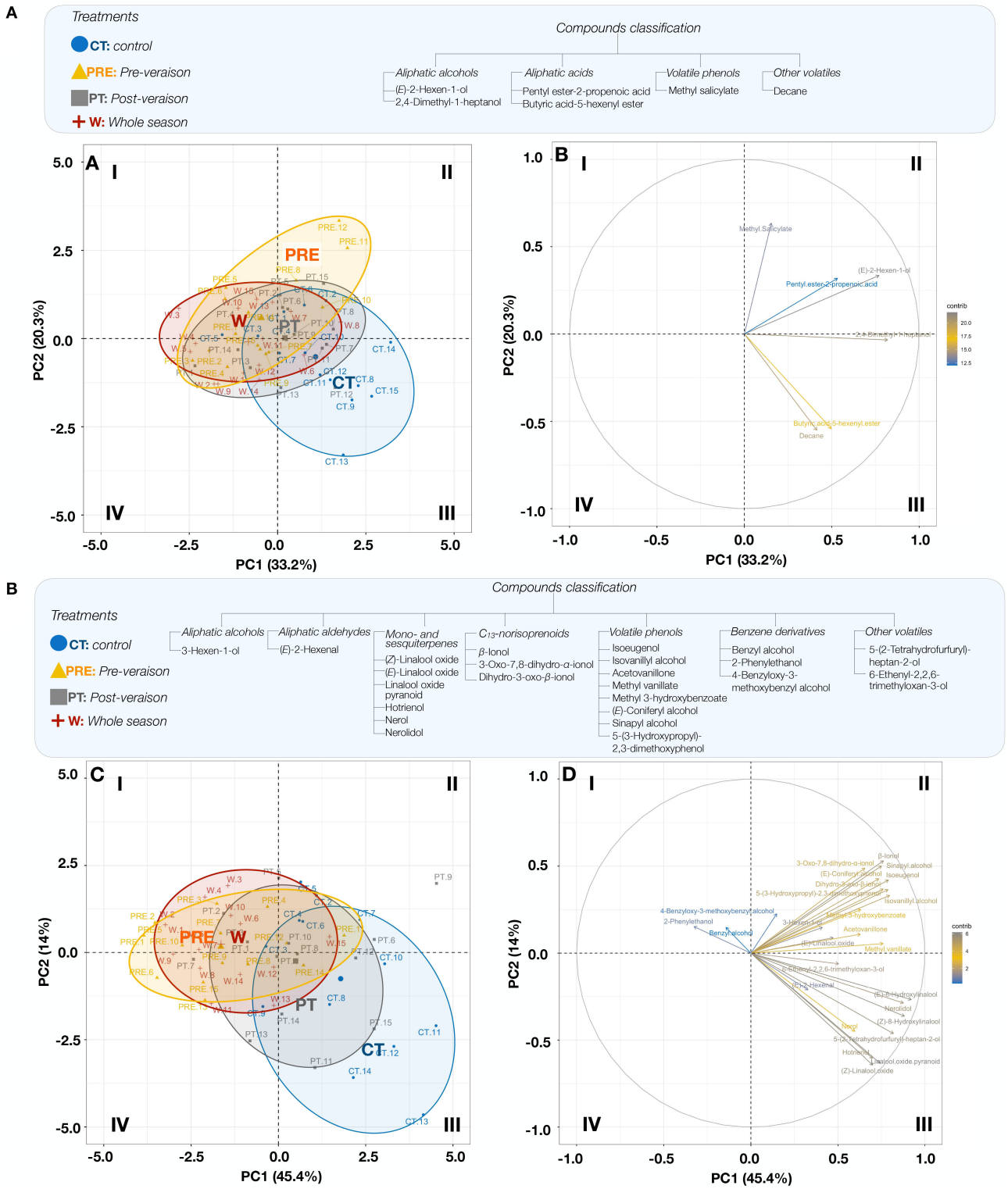

FIGURE 3 | PCA of FVCs **(A)** and GVCs **(B)** (variables; right plot), plotted according to the treatments (CT, control; PRE, pre-veraison; PT, post-veraison; W, whole season; samples; left plot). Biological replicates are labeled 1 to 15. FVCs include the following variables: (*E*)-2-hexen-1-ol, 2,4-dimethyl-1-heptanol, pentyl ester-2-propenoic acid, butyric acid-5-hexenyl ester, methyl salicylate and decane. GVCs include the following variables 3-hexen-1-ol, (E)-2-hexenal, (*Z*)-linalool oxide, (*E*)-linalool oxide, linalool oxide pyranoid, hotrienol, nerol, nerolidol, *β*-ionol, 3-Oxo-7,8-dihydro-*α*-ionol, dihydro-3-oxo-*β*-ionol, isoeugenol, isovanillyl alcohol, acetovanillone, methyl vanillate, methyl 3-hydroxybenzoate, (*E*)-coniferyl alcohol, sinapyl alcohol, 5-(3-hydroxypropyl)-2,3-dimethoxyphenol, benzyl alcohol, 2-phenylethanol, 4-benzyloxy-3-methoxybenzyl alcohol, 5-(2-tetrahydrofurfuryl)-heptan-2-ol, and 6-ethenyl-2,2,6-trimethyloxan-3-ol. Quadrants are identified I-IV (clockwise).

Zeng et al. (2019, 2020) showed an increase in the 2-phenylethanol concentration of tea (*Camellia sinensis*) leaves and cut roses (*Rosa hybrida*), following a temperature increase. In petunias (*Petunia* × hybrida cv. Mitchel Diplod), high temperatures reduced the levels of 2-phenylethanol, whereas no change was observed in tomato (*Solanum lycopersicum*) (Zeng et al., 2019). Phenylpropanoid derivatives, such as benzyl alcohol and hydroxy-methoxy-substituted volatile phenols, are also known to have different patterns of accumulation in plants, in part because they are thought to be produced on-demand in response to abiotic and biotic stress (Widhalm and Dudareva, 2015). For instance, benzyl alcohol has been shown to increase in cut roses following temperature treatment but was found in lower concentrations in Albarino grapes grown on a warm site when compared to a cooler site (Vilanova et al., 2007; Zeng et al., 2020). However, the biosynthesis of complex phenylpropanoid derivatives, such as flavonoids, produced further down the phenylpropanoid pathway, can be reduced at high temperatures, resulting in the accumulation of the benzyl alcohol precursor cinnamic acid (Austen et al., 2019). Thus, the differential accumulation of phenylpropanoid derivatives in the CT treatment (more hydroxy-methoxy-substituted volatile phenols) when compared to the Pre and W treatments (more benzyl alcohol) could be attributable to the treatment-specific differences in abiotic conditions, with the observed temperature increases potentially resulting in increased production of benzyl alcohol at the expense of other compounds in the phenylpropanoid pathway. Additional targeted analyses of phenolic compounds within the pathway are necessary to test this hypothesis; however, the red coloration (likely attributable to anthocyanins) observed on the CT berries when compared to the greener berries of W and Pre treatments (reduced anthocyanins) supports this potential impact of treatments upon the phenylpropanoid pathway (**Supplementary Figure 3**). From a wine perspective, such variation in phenol profiles may affect wine sensory properties and suggests that climate change could have important potential impacts on wine quality.

Terpenes contributed significantly to the glycosidic fraction (∼23%) and were significantly affected by the treatments. In contrast to volatile phenols, the terpenes, such as (*Z*)-linalool oxide, (*E*)-linalool oxide, hotrienol, (*Z*)-8-hydroxylinalool, and (*E*)-8-hydroxylinalool, predominantly accumulated in the CT (**Figure 3B** and **Table 4**). As previous research (Goh et al., 2016) has shown a close positive relationship between temperature and terpene production in plants, which is dependent on the geographic origin and genotype of plant populations, this finding was unexpected. Indeed, the W treatment accumulated more GDD due to an overall higher temperature than all other treatments but had the lowest levels of monoterpenes. One explanation for decreased terpenes could be the polycarbonate panels used in on-the-row mini-greenhouses, which block UV rays below 380 nm in addition to raising the canopy temperature. Polycarbonate panels with high visible light transmission and high UV absorbance have traditionally been used in order to support plant growth while protecting equipment from long-term UV damage, but research has highlighted the interactions of UV light with plant physiology. One such interaction is thought to be with terpene biosynthesis, which is typically associated with veraison in grapevine ripening and involves, among others, a family of proteins called terpene synthases (Matarese et al., 2013). The terpene synthase family includes several genes in grapevine (up to 25 for the terpene synthase-b subfamily), and some of these genes are thought to be UV responsive and differentially expressed at different developmental stages (Carbonell-Bejerano et al., 2014; Wen et al., 2015). Differential UV exposure has been directly shown to affect the accumulation of compounds, such as C_{13}-norisoprenoids and monoterpenes (Joubert et al., 2016; Young et al., 2016), generating changes in the VC profile by activating the various genes responsible for their synthesis (Lin et al., 2019). Exposure is thought to increase the production of several compounds (e.g., monoterpenes, aldehydes, alcohols, norisoprenoids, and flavonols) in order to protect the berries against UV damage (Gil et al., 2013; Joubert et al., 2016). Specifically, in grapevine, increases in monoterpenes, such as limonene and geraniol in *V. vinifera* cv Malbec (Gil et al., 2013); hotrienol, limonene, and linalool in Sauvignon blanc (Joubert et al., 2016); and geraniol, citronellol, and nerol from Pinot noir (Song et al., 2015) have been associated with UV exposure. Similar to terpenes, C_{13}-norisoprenoids, including 3-oxo-α-ionol, β-ionol, and 3-oxo-7,8-dihydro-α-ionol, also accumulated in the CT treatment. Previous findings demonstrated that norisoprenoid precursors, such as β-carotene, may increase in berries exposed to UV radiation, which could in turn lead to higher C_{13}-norisoprenoid concentrations (Joubert et al., 2016).

CONCLUSION

This study explored the impact of mini-greenhouse treatments, installed in the vineyard at different times during the growing season, on the FVC and GVC profiles during the ripening of L'Acadie blanc berries cultivated in Nova Scotia, Canada. Mini-greenhouse treatments, even when applied only during early berry development, affected berry composition at harvest. Of interest, berries from the treatments with an increased accumulation of GDD during the first developmental stages (W and Pre) had the highest concentrations of benzyl alcohol and 2-phenylethanol, suggesting that early exposure to higher temperatures could potentially impact berry VC composition. In contrast, terpenes, C_{13}-norisoprenoids, and hydroxy-methoxy-substituted volatile phenols predominantly accumulated in the CTs, possibly as an effect of reduced UV radiation due to the polycarbonate panels used in mini-greenhouses. Although plants were exposed to natural light during veraison, grapevines under the mini-greenhouses during early berry development (Pre treatment) had reduced levels of terpenes and C_{13}-norisoprenoids compared to CTs, suggesting UV exposure might be more important during berry development as well as temperature.

Despite the study only being conducted over a single growing season, these findings provide insight into the biochemical plasticity of *Vitis* sp. and the possibility of modifying the accumulation of VCs in berries. 2-Phenylethanol and benzyl alcohol were the main varietal aroma compounds found in

L'Acadie blanc berries and were increased in vines with higher GDD, suggesting that increased temperatures attributable to climate change could potentially affect the quality of L'Acadie blanc wines in the future. Future research would need to establish the consistency of this pattern in field trials without differential UV exposure to confirm this possibility.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KP conceived, planned this study, and supervised FC-A and GS. KP, NB, and FP contributed to the planning of the experiment and the realization in the field. FC-A and GS implemented and maintained the viticultural treatments and carried out berry sampling. FC-A did the processing and analysis of climatic data and berry samples. PN developed the method for FVC and GVCs extraction. FC-A and KP drafted the original manuscript and finalized the manuscript. MD, NB, and FP reviewed and edited the manuscript. All authors approved the manuscript for publication.

FUNDING

This research was made possible by the financial support of Agriculture and Agri-Food Canada (AAFC), the Nova Scotia Department of Agriculture, the Canadian Grapevine Certification Network (CGCN), the Grape Growers' Association of Nova Scotia, through Canadian Agricultural Partnership's AgriScience Clusters (Activity 9B), and the Natural Sciences and Engineering Research Council of Canada (NSERC Discovery grant).

ACKNOWLEDGMENTS

The authors would like to thank Mayank Pathak and Kumuduni Lakmali for their contribution to the establishment of the treatments. They also deeply thank Marcel Kolb and Luckett Vineyards for their assistance and for kindly providing the experimental site and berries used in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.862259/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 03 October 2022
DOI 10.3389/fpls.2022.1015574

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

RECEIVED 09 August 2022
ACCEPTED 09 September 2022
PUBLISHED 03 October 2022

CITATION
Yu R, Torres N, Tanner JD, Kacur SM, Marigliano LE, Zumkeller M, Gilmer JC, Gambetta GA and Kurtural SK (2022) Adapting wine grape production to climate change through canopy architecture manipulation and irrigation in warm climates. *Front. Plant Sci.* 13:1015574. doi: 10.3389/fpls.2022.1015574

Adapting wine grape production to climate change through canopy architecture manipulation and irrigation in warm climates

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Grape growing regions are facing constant warming of the growing season temperature as well as limitations on ground water pumping used for irrigating to overcome water deficits. Trellis systems are utilized to optimize grapevine production, physiology, and berry chemistry. This study aimed to compare 6 trellis systems with 3 levels of applied water amounts based on different replacements of crop evapotranspiration (ET_c) in two consecutive seasons. The treatments included a vertical shoot position (VSP), two modified VSPs (VSP60 and VSP80), a single high wire (SH), a high quadrilateral (HQ), and a Guyot pruned VSP (GY) combined with 25%, 50%, and 100% ET_c water replacement. The SH had greater yields, whereas HQ was slower to reach full production potential. At harvest in both years, the accumulation of anthocyanin derivatives was enhanced in SH, whereas VSPs decreased them. As crown porosity increased (mostly VSPs), berry flavonol concentration and likewise molar % of quercetin in berries increased. Conversely, as leaf area increased, total flavonol concentration and molar % of quercetin decreased, indicating a preferential arrangement of leaf area along the canopy for overexposure of grape berry with VSP types. The irrigation treatments revealed linear trends for components of yield, where greater applied water resulted in larger berry size and likewise greater yield. 25% ET_c was able to increase berry anthocyanin and flavonol concentrations. Overall, this study evidenced the efficiency of trellis systems for optimizing production and berry composition in Californian climate, also, the feasibility of using flavonols as the indicator of canopy architecture.

KEYWORDS

anthocyanins, climate change, irrigation, trellis systems, viticulture

Introduction

Grapes are profitable fruit crop that are widely grown in the state of California, with an increasing need to accomplish cultural tasks mechanically (California Department of Food and Agriculture (CDFA), 2020; Kurtural and Fidelibus, 2021). However, there are many factors that are currently challenging the productivity, quality, and sustainability in wine grape vineyards, one being the increasingly significant global warming trend affecting California and the whole world (Venios et al., 2020; Rienth et al., 2021), where more frequent heat waves (Torres et al., 2021a) and continued warming of air temperature imposes great threats to vineyard yield, berry and wine composition (Gambetta and Kurtural, 2021).

Grape berry and wine quality are determined by the composition and concentration of secondary metabolites accumulated in berries. Flavonoids are the most abundant secondary metabolites and contribute to many quality-determining traits, including color, mouthfeel, and aging potential of wine (Poni et al., 2018). There are generally three classes of flavonoids in wine grapes, including anthocyanins, flavonols, and proanthocyanidins. Anthocyanins are responsible for grape berry and wine color, and they are sensitive to external environmental conditions when clusters are exposed to solar radiation and heat, with overexposure resulting in anthocyanin degradation (Torres et al., 2020; Torres et al., 2021a). On the other hand, flavonols tend to be positively related to solar radiation (Martínez-Lüscher et al., 2019a). Solar radiation, especially UV-B, can often up-regulate flavonols' biosynthesis, resulting in more flavonols accumulated in berry skins. However, excessive solar radiation received and heat accumulated in California would accelerate the degradation of not only anthocyanins, but also flavonols, which will cause a decline in the antioxidant capacity of resultant wine and a possible reduction in wine aging potential (Torres et al., 2021a).

In viticulture, trellis system selection is a critical aspect grower needs to consider when establishing a vineyard. An ideal trellis can promote grapevines' photosynthetic capacity through optimizing light interception by the grapevine canopy. Most importantly, a suitable trellis can optimize canopy microclimate by providing sufficient solar penetration into canopies since solar radiation is necessary to enhance the berry composition (Bavougian et al., 2012; Sanchez-Rodriguez and Spósito, 2020) without excessive exposure of clusters to direct sunlight to avoid flavonoid degradation (Martínez-Lüscher et al., 2020; Torres et al., 2021a). There is evidence that grape clusters over-exposed to solar radiation are prone to occur with some of the widely used trellis systems. For example, vertical shoot position (VSP), a traditional and commonly used trellis system in viticulture production, has been found to produce canopies with high porosity which increases vulnerability of clusters to over-exposure (Dry, 2009), causing overly enhanced maturity and considerable degradation in berry anthocyanins (Torres et al., 2021a). However, there is a lack of evaluations of the performance among various trellis systems in relation to the warming climate trends, and how their specific architectures contribute to variations in berry chemical profiles.

In warm climates such as California, viticulture relies on irrigation for maintaining production, and previous work in the area showed that the application of different amounts of crop evapotranspiration (ET_c) can significantly modify polyphenolic and aromatic profiles in wine (Torres et al., 2022). Due to the increasingly frequent drought condition in many wine grape growing regions, recent studies have been focusing on the grapevine physiological and berry chemical responses towards specific levels of water deficits imposed by different ET_c replacements, where water deficits are affective in manipulating grapevine water status, leaf gas exchange, components of yield, and berry composition: often, more water deficits applied to the grapevines would diminish photosynthetic capacities, but promote berry maturity (*i.e.* sugar and flavonoid accumulation) (Torres et al., 2021d; Torres et al., 2021c; Torres et al., 2022). In some extremely drought conditions, however, severe water deficit might lower flavonoid concentration due to encouraged chemical degradation (Yu et al., 2020). Moreover, these effects resulted from different irrigation regimes can be modified by the canopy architecture as functions of trellis system since trellis systems can directly determine canopy sizes, hence resulting in different water demands from grapevines accordingly (Williams, 2000). On the other hand, over extraction of ground water to irrigate permanent crops have recently been questioned and legislation has been enacted in the state of California called the 'Sustainable Groundwater Management Act' (Kiparsky, 2016). As a result, in some regions such as Napa Valley of California, grape growers will only be allowed to irrigate 120 mm per year. However, there is a lack of information on how the existing vineyards will cope with this water limitation in terms of irrigation scheduling.

Therefore, the objectives of this study were to evaluate and compare 6 different trellis systems in combination with 3 irrigation strategies to understand the impact of trellis system and applied water amount on canopy architecture, grapevine physiology and berry composition. We hypothesized that traditional VSP systems would not be as efficient as the other trellis systems in terms of yield production and flavonoid accumulation, leading to greater berry flavonoid degradation and overall lower flavonoid concentrations.

Materials and methods

Vineyard site, plant materials, and weather conditions

The experiment was conducted in 2020 and 2021 on *Vitis vinifera* `Cabernet sauvignon´ (Clone 8) grapevines grafted on

3309C rootstock (*V. riparia* × *V. rupestris*). The vineyard for this study was located at the University of California Oakville Experimental Station in Oakville, Napa County, CA, USA and planted in 2016. Grapevines were spaced at 1.52 m × 2.13 m (vine × row). The rows had NE-SW orientation.

Weather data at this vineyard was obtained from the California Irrigation Management Information System (CIMIS) (station #77, Oakville, CA). The weather station was located approximately 100 m from the experimental vineyard block. Growing Degree Days (GDD) were used to assess the accumulated heat units at the experimental site, and calculated with the following equation (McMaster and Wilhelm, 1997):

$$GDD = \sum_{Apri\,1}^{Harvest}\left(\frac{\text{Daily maximum temperature} + \text{Daily minimum temperature}}{2} - 10\right) \quad 1)$$

where negative values were not included in the accumulated GDD value, and the time period recorded for the calculation was from 1 April until harvest in each year.

Experimental design

The study was conducted in a split-plot factorial design that utilized 2 separate sets of factors. The main factors of the experiment were 6 trellis systems randomly combined with 3 different water amounts applied at random to each row with 4 replications in each treatment, which consisted of seven vines. There were 72 treatment-replicates in total. The main plot factor (trellis systems) was applied to every row, and the sub-plot (applied water amounts) was applied randomly to 7 consecutive vines within each row so that 3 separate irrigation sub-plot factors were contained in every row within the vineyard block. The 5 middle vines in each treatment-replicate were used for on-site measurements as well as berry sampling.

Trellis systems and applied water amounts

Trellis systems

6 trellising systems were used for the measurements in this experiment (Figure 1). The 6 trellis systems included a vertical shoot position (VSP, Figure 1A), 2 additional VSP designs that were modified with more opened canopies (with ~60°C and ~80°C shoot orientation: VSP60 and VSP80, Figures 1B, C, respectively), a VSP trellis cane-pruned with a Guyot method (GY, Figure 1F), a high quadrilateral trellis (HQ, Figure 1D), and a single high wire trellis (SH, Figure 1E). The cordon height (h) for 1A, 1B, 1C and 1F were 0.96 m above vineyard floor. The cordon height for 1D was 1.54 and for 1E it was 1.70 m above vineyard floor respectively.

The canopy management was conducted based on the common local practices for these trellis designs for the traditional VSP, VSP60, and VSP80. The grapevines were spur-pruned to two buds per spur retaining approximately 30 spurs per plant. After bud break, the shoot numbers were corrected to approximately 25 shoots per vine for VSP types. The HQ grapevines were spur pruned to retain 60 spurs per plant and then shoot thinned to 50 shoots per vine. The SH vines were box-pruned mechanically to a spur height of approximately 10 cm, and 45% of the shoots were mechanically thinned at 40 cm shoot length as per common local practice to mimic manual shoot thinning operations (Terry and Kurtural, 2011; Kurtural et al., 2019). The GY vines were cane-pruned by hand to 2, 12-node canes with 2 renewal spurs at the head of each vine. There was no leaf removal or cluster removal conducted in either year.

Applied water amounts

The irrigation treatments applied to the grapevines were based on calculated ET_c by using the following equation:

$$ET_c = ET_o \times K_c \quad 2)$$

where ET_o was the reference evapotranspiration and K_c is the crop coefficient. ET_o was measured from the CIMIS station weekly throughout both seasons, and K_c was assessed by using the shade cast method previously described by Williams and Ayars (2005). Briefly, three neighboring VSP trellised rows were irrigated to 100% of ET_o to create unstressed grapevines. Shade cast on to the berm and row middles were measured weekly then to calculate the K_c. Irrigation was initiated when the general grapevine stem water potential for the field fell below – 1.0 MPa (28 May 2020 and 10 June 2021). The applied water amounts used in this study were to replace 100% crop evapotranspiration (ET_c), 50% ET_c and 25% ET_c. These treatments were applied by varying the emitter numbers per vine with irrigation duration determined based on 100% ET_c treatment. NETAFIM™ pressure compensating on-line button drippers were installed to apply different rates of irrigation: 2 drippers with a rate of 4 L/h at each vine to simulate 100% ET_c replacement, 2 drippers with a flow rate of 2 L/h at each vine to simulate 50% ET_c replacement, and 2 drippers with a flow rate of 1 L/h to simulate 25% ET_c. In total, 100% ET_c treated grapevines received 308 mm and 246 mm of water in 2020 and 2021, respectively.

Leaf gas exchange, leaf area index, and yield component assessment

Leaf gas exchange

At mid-day (between 12:00 – 14:00 h), leaf gas exchange measurements were taken bi-weekly in both seasons to assess leaf photosynthetic activities as well as plant water status by using a portable infrared gas analyzer CIRAS-3 (PP Systems, Amesbury, MA, USA). Each time, three different fully sun-

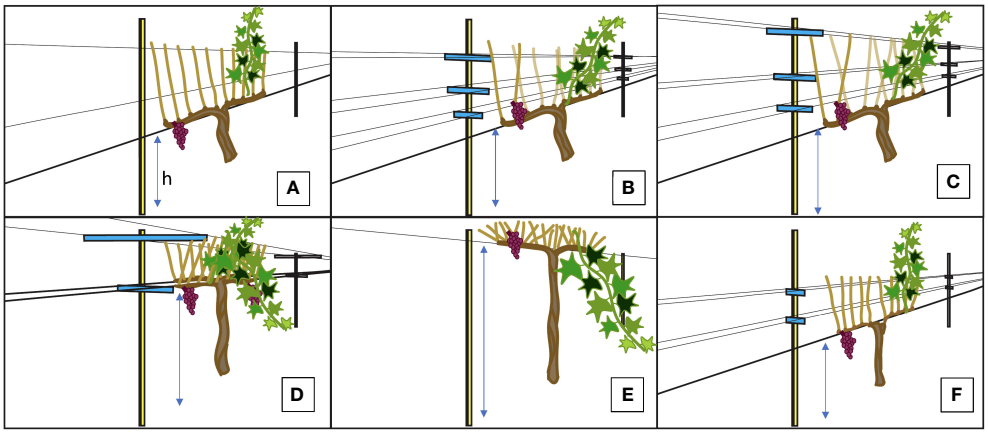

FIGURE 1
Illustrations for the Trellis Systems Established at the Oakville Experimental Vineyard: **(A)** Traditional Vertical Shoot Position (VSP); **(B)** Vertical Shoot Position 60° (VSP60); **(C)** Vertical Shoot Position 80° (VSP80); **(D)** High-Quadrilateral (HQ); **(E)** Single High Wire (SH); **(F)** Guyot-pruned Vertical Shoot Position (GY). "h" stands for the cordon height from the vineyard ground and the h for each trellis system was described in Materials and Methods.

exposed leaves were selected from the main shoot axis on the middle three grapevines in each treatment-replicate. In both years, the measurements were taken when sunlight condition were at photosynthesis saturation levels, where the average photosynthetic active radiation (PAR) was approximately at 1708.43 ± 282.81 µmol mol^{-1} (mean ± one standard deviation from the mean) in 2020 and 1764.85 ± 287.84 µmol mol^{-1} (mean ± one standard deviation from the mean) in 2021. CIRAS-3 was set to a relative humidity at 40% and a reference CO_2 concentration at 400 µmol mol^{-1}. From the measurement, leaf net carbon assimilation (A_{net}) and stomatal conductance (g_s) were assessed directly. Intrinsic water use efficiency (WUE_i) was calculated as the ratio between g_s to A_{net}.

Canopy microclimate and leaf area

Canopy microclimate was assessed using digital photography as previously reported (Martínez-Lüscher et al., 2019a). Crown porosity (% of gaps in the canopy) and leaf area index (LAI) was assessed with a smartphone application VitiCanopy on an iOS operating system (Apple Inc., Cupertino CA, USA) (De Bei et al., 2016). The settings used for this vineyard site were described previously (Yu et al., 2021b). Total leaf areas were calculated based on the LAI multiplied by the unit ground area for each vine (3.24 m^2).

Yield components

Clusters were harvested by hand at approximately 23 - 25 ° CBrix, and all clusters in each treatment-replicate were harvested, counted, and weighed on a single harvest day each season (14 September 2020, 6 September 2021). Yield components were assessed or calculated for cluster number per vine, cluster weight, berry fresh weight, leaf area to fruit ratio, and yield per vine.

Berry sampling and berry quality assessment

10 berries were randomly sampled from each of the five central vines for a total of 50 berries. Berry samplings took place at harvest in both seasons. The 50 berries were divided into two subsets of 30 berries and 20 berries. The 30-berry set was used for berry weight and berry composition analysis. Berry must total soluble solids (TSS) was recorded in the unit of °CBrix with a digital refractometer (Atago PR-32, Bellevue, WA, USA). Measurements of the berry must pH and titratable acidity (TA) were determined with an autotitrator (862 Compact TitroSampler, Metrohm, Switzerland) and were recorded as g L^{-1} of tartaric acid at the titration end point of pH 8.2 (Ough and Amerine, 1988).

Sample preparation for determination of skin flavonoids

The second subset of 20 berries was used for the determination of skin flavonoids from each individual treatment-replicate. Skins were manually removed from the subset of 20 berries and subsequently lyophilized (Centrivap Benchtop Centrifugal Vacuum Concentrator 7810014 equipped with Centrivap -105°C Cold Trap 7385020, Labconco, Kansas City, MO, USA). After lyophilization, dry skin weights were recorded and then, the dried skins were ground into fine

powder with a mixing mill (MM400, Retsch, Mammelzen, Germany). 50 mg of the freeze-dried berry skin powder were collected, and the skin flavonoids were extracted with 1 mL of methanol:water:7M hydrochloric acid (70:29:1, V:V:V) to simultaneously determine flavonol and anthocyanin concentration and profile as previously described by Martínez-Lüscher et al. (2019a). The extracts were stored overnight in a refrigerator at 4°C. In the next day, the extracts were centrifuged at 30,000 *g* for 15 minutes, and the supernatants were separated from the solids and transferred into HPLC vials after being filtered by PTFE membrane filters (diameter: 13 mm, pore size: 0.45 µm, VWR, Seattle, WA, USA). Then, the samples were injected into HPLC for chromatographic analysis.

Determination of berry skin flavonoids

Anthocyanin and flavonol concentrations (expressed in the unit of mg per g of berry fresh weight) in berry skin tissues were analyzed with a reversed-phase HPLC (Model 1260, Agilent, Santa Clara, CA, USA) with the use of two mobile phases: (A) 5.5% formic acid in water and (B) 5.5% formic acid in acetonitrile. The specific method used for this study required a C18 reversed-phase HPLC column for the analysis (LiChrosphere 100 RP-18, 4 × 520 mm^2, 5 mm particle size, Agilent Technologies, Santa Clara, CA, United States). The flow rate of the mobile phase was 0.5 mL min^{-1} and the flow gradient started with 91.5% A with 8.5% B, 87% A with 13% B at 25 min, 82% A with 18% B at 35 min, 62% A with 38% B at 70 min, 50% A with 50% B at 70.01 min, 30% A with 70% B at 75 min, 91.5% A with 8.5% B from 75.01 min to 90 min. The column temperature was maintained at 25°C on both left and right sides of the column. All chromatographic solvents were of high-performance liquid chromatography (HPLC) grade, including acetonitrile, methanol, hydrochloric acid, formic acid. These solvents were purchased from Thermo-Fisher Scientific (Santa Clara, CA, USA). Detection of flavonols and anthocyanins was recorded by the diode array detector (DAD) at 365 and 520 nm, respectively. Investigated anthocyanin derivatives included di-hydroxylated forms: cyanidin and peonidin, and tri-hydroxylated forms: delphinidin, petunidin, and malvidin; investigated flavonols included a mono-hydroxylated form: kaempferol, di-hydroxylated forms: quercetin and isorhamnetin, and tri-hydroxylated forms: myricetin, laricitin, and syrigintin.

Post-run chromatographic analysis was conducted with Agilent OpenLAB software (Chemstation edition, version A.02.10) and identification of individual anthocyanins and flavonols was made by comparison of the commercial standard retention times found in the literature (Martínez-Lüscher et al., 2019a). Malvidin 3-*O*-glucoside used for anthocyanin identification was purchased from Extrasynthese (Genay, France). Myricetin-3-*O*-glucuronide, myricetin 3-*O*-glucoside, quercetin 3-*O*-glucuronide, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside, isorhamnetin 3-*O*-glucoside, and syringetin 3-*O*-glucoside used for flavonol identification were purchased from Sigma-Aldrich (St. Louis, MO, United States). Flavonol molar abundant (molar %) was calculated as the percentage of specific flavonol derivatives' concentration over total flavonols' concentration.

Statistical analysis

The statistical analysis for the experiment was performed using MIXED procedure of SAS (v 9.4. SAS Institute, Cary, NC, USA). All the datasets were first checked for normal distribution using a Shapiro-Wilkinson test before running the two-way MIXED procedure. A Tukey's HSD *post-hoc* test was performed to analyze the degree of significance among the various measurements. The levels of significance ≤ 0.10 were the results that were considered for the Tukey's *post hoc* tests. Season-long measurements of leaf gas exchange variables were analyzed for each year *via* three-way Analysis of Variance using the MIXED procedure of SAS using REPEATED option for measurement dates. A regression analyses was performed between variables of interest and, *p* values were acquired to present the significances of the linear fittings, as well as the regression coefficient (as R^2).

Results

Weather at the experimental site

Both seasons were considerably arid as the experimental site only received 233.9 mm and 276.9 mm of precipitation from the previous dormant season until harvest in 2020 and 2021, respectively (Table 1). During the growing seasons, from April to September, the site received 23.2% of the total precipitation in 2020 (54 mm) and only 2.1% in 2021 (5.9 mm). In addition, there was minimal precipitation during data collection of this study from June to September, where only 2 mm and 1.2 mm of precipitation were received in 2020 and 2021. As for the air temperature during the growing seasons, the average maximum air temperature was slightly higher in July, August, and September in 2020 compared to 2021, but lower in March and April. The average minimum air temperature was constantly higher in 2020 compared to 2021 from March until harvest in September, except July. Similarly, the average air temperature was generally higher in 2020 than 2021 except both Julys which had the same average air temperature. As for GDD accumulation (as calculated until harvest), the two seasons were slightly different. In 2020, there was 1525.4°C GDD accumulated when the berries reached 23.9°Brix on average; in 2021, there was 1292.3°C GDD accumulated when the berries reached 22.6°Brix on average. Thus, 2020 was a slightly drier and hotter season than 2021.

TABLE 1 Weather information at the experimental site as obtained from california irrigation management information system (cimis) station located in oakville (#77, Oakville, Napa County)[a].

Month-Year	Precipitation (mm)	Average Maximum Air Temperature (°C)	Average Minimum Air Temperature (°C)	Average Air Temperature (°C)	Growing Degree Days (°C)
Oct-19	0.2	26.6	4.9	15.4	–
Nov-19	24.4	20.8	3.3	11	–
Dec-19	66	14.3	5.7	9.5	–
Jan-20	58.5	15.4	3.5	8.8	–
Feb-20	1	20.6	3.7	11.4	–
Mar-20	29.8	17.6	4.4	10.7	–
Apr-20	25.9	23	7.1	14.6	154.2
May-20	26.1	26.2	8.8	17.4	385.05
Jun-20	0.2	29.5	10.4	19.7	683.8
Jul-20	0.2	30.2	10.1	19.2	997.35
Aug-20	1.6	31.8	12.3	21.1	1359.15
Sep-20	0	31.4	11.1	20	1525.35
Oct-20	0.3	29.7	8.1	17.4	–
Nov-20	31.9	19.8	2.2	10.2	–
Dec-20	46.4	17.1	2	8.5	–
Jan-21	97.5	15.7	3.6	9	–
Feb-21	35.3	18	3.3	10.5	–
Mar-21	59.6	18.5	2.6	10.3	–
Apr-21	4.3	23.5	4.3	13.1	116.55
May-21	0.4	27.7	7.2	17.3	347.6
Jun-21	0.3	28.7	9.3	18.8	618.1
Jul-21	0.2	29.5	10.8	19.2	932.6
Aug-21	0.2	29.7	10.1	19	1239.5
Sep-21	0.5	30	8.7	18.6	1292.25

[a]Growing degree days were calculated from 1 April to harvest in each year.

Canopy microclimate

LAI and crown porosity were assessed in both seasons, and leaf areas were calculated based on the unit ground area and LAI (Figure 2). In 2020, VSP80 had the most leaf area among the six trellis systems, VSP60 and GY had similar leaf areas, followed by VSP (Figure 2Aa-1). SH and HQ had the lowest leaf areas as the canopies in these two trellises still had gaps. This was also confirmed with the fact that SH and HQ had the highest crown porosities among the six trellis systems (Figure 2Aa-2). The other trellis systems had similar lower crown porosities than SH and HQ. There was no difference in canopy architecture among the three irrigation regimes in the first season (Figures 2Ab-1, Ab-2).

In 2021, all the trellis systems had similar leaf areas (Figure 2Ba-1). HQ had higher crown porosity than VSP60, but the other trellis systems had similar crown porosities to either HQ or VSP60 (Figure 2Ba-2). These effects were not modified by the irrigation treatments and no significant interactions between factors were found. For applied water amounts, 50% ET_c had higher leaf area than 25% ET_c, but there was no difference between 100% ET_c with either 25% or 50% (Figure 2Bb-1). However, 50% ET_c still had the highest crown porosity compared to 100% ET_c, and 25% ET_c did not show any difference with the other two irrigation treatments (Figure 2Bb-2).

Grapevine leaf gas exchange

Grapevine leaf gas exchange was monitored throughout both seasons, and their integrals were calculated to represent the season-long plant response of grapevines for net carbon assimilation rate (A_n), stomatal conductance (g_s), and intrinsic water use efficiency (WUE_i) (Figure 3). In 2020, there were no differences in g_s and A_n among the six trellis systems (Figures 3Aa-1, Aa-2). However, HQ had the highest WUE_i, whereas VSP, VSP60, and SH had lower WUE_i (Figure 3Aa-3). Regarding the irrigation treatments, there was no difference in g_s integrals (Figure 3Ab-1). However, a linear response to water amounts were observed for A_n and WUE_i, with 100% ET_c having the highest values of both gas exchange variable monitored (Figures 3Ab-2, Ab-3).

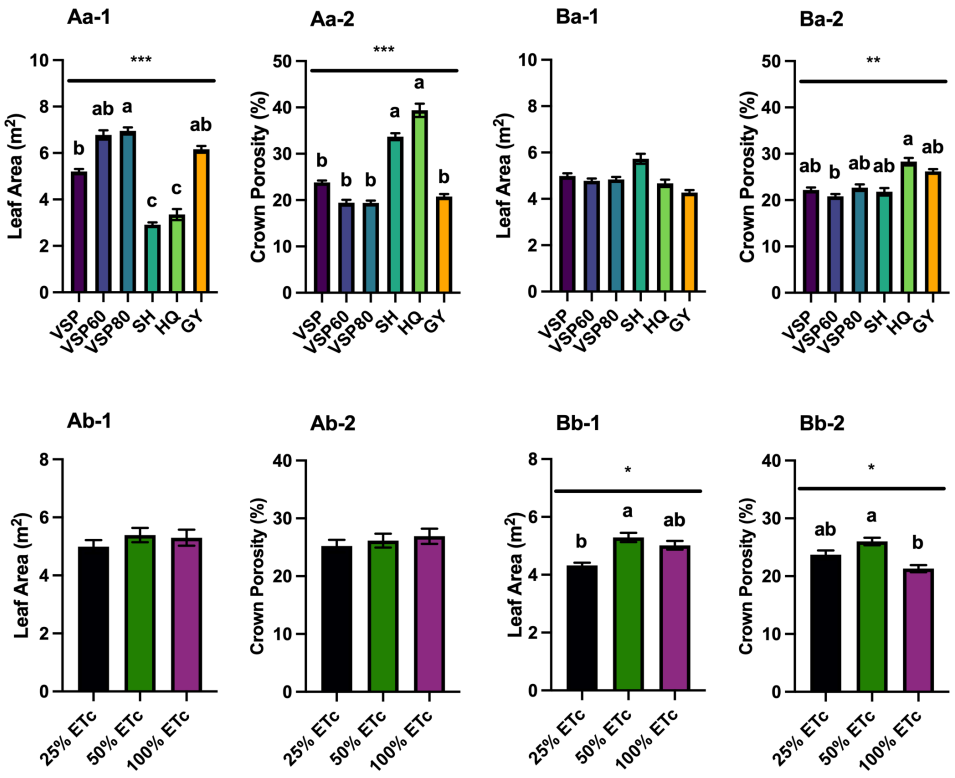

FIGURE 2
Canopy Architecture as Affected by Trellis Systems and Applied Water Amounts of 'Cabernet Sauvignon' in Oakville, CA, USA in **(A)** 2020 and **(B)** 2021; **(a)** the main effect of trellis systems, **(b)** the main effect of applied water amounts; (1) leaf area, (2) crown porosity. Error bars represent one standard deviation from the mean, letters represent ranking after Tukey's *post hoc* analyses. Asterisks represents significant levels *p*, '***'$p<$ 0.001, '**'$p < 0.01$, '*'$p < 0.05$. VSP, vertical shoot position; VSP60, vertical shoot position 60°; VSP80, vertical shoot position 80°; SH, single hire wire; HQ, High-Quadrilateral; GY, guyot-pruned vertical shoot position; ET_c, crop evapotranspiration.

In 2021, there were no differences in g_s, A_n, and WUE_i among the six trellis systems (Figures 3Ba-1, Ba-2, Ba-3). Nevertheless, a linear response to water amounts was recorded, with 100% ET_c showing the highest A_n and g_s, followed by 50% ET_c, and 25% ET_c (Figure 3Bb-1, Bb-2) which accounted for a higher WUE_i in 25% ET_c with 50% treatments compared with 100% ET_c (Figure 3Bb-3).

The analysis of the gas exchange recorded at each measurement day indicated that in 2020, despite starting with the highest g_s, SH had lower g_s over the season (Figure 4Aa-1). Contrarily, HQ trellis system showed higher g_s in July and August which was connected with higher A_n over the season (Figure 4Aa-2). On the other hand, GY and VSP80 systems enhanced A_n during some periods over the season. Regarding WUE_i, VSP60 and HQ had the highest values while SH decreased it in the early season and increased it in early August (Figure 4Aa-3). However, all these differences tended to diminish at the end of the season. For irrigation treatments, a constant effect of water amount was observed with 100% ET_c increasing g_s and A_n and decreasing WUE_i (Figures 4Ab-1, Ab-2, Ab-3).

In 2021, GY and VSP60 showed higher g_s and A_n values in general (Figures 4Ba-1, Ba-2). HQ showed lower g_s values and VSP had lower A_n values compared to the other trellis systems throughout the season. HQ increased WUE_i throughout the whole season (Figure 4Ba-3). Although GY had higher WUE_i in the early season, it showed constantly lower WUE_i values after 23 June 2021. Besides GY, VSP showed lower WUE_i in July and August. A similar effect of irrigation treatments was observed over the second season, with a linear response for increased g_s and A_n and decreased WUE_i when the irrigation water amount was increased (Figures 4Bb-1, Bb-2, Bb-3).

Yield components and berry quality parameters

Yield components and berry quality parameters were assessed at harvest in both seasons (Table 2). SH and HQ had the smallest berries among the six trellis systems in the two seasons. In 2020, SH and VSP increased the cluster number, while VSP80 and GY decreased it whereas, in 2021, SH and HQ

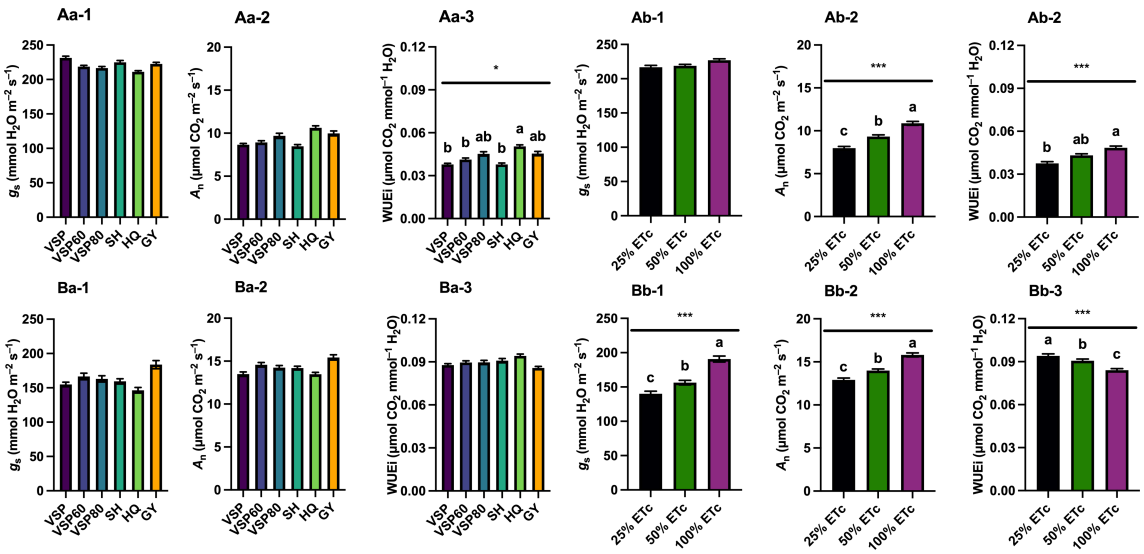

FIGURE 3
Season-long Leaf Gas Exchange Integrals as Affected by Trellis Systems and Applied Water Amounts of 'Cabernet Sauvignon' in Oakville, CA, USA in **(A)** 2020 and **(B)** 2021; **(a)** the main effect of trellis systems, **(b)** the main effect of applied water amounts; (1) stomatal conductance (g_s), (2) net carbon assimilation rate (A_n), (3) intrinsic water use efficiency (WUE_i). Error bars represent one standard deviation from the mean, letters represent ranking after Tukey's *post hoc* analyses. Asterisks represents significant levels *p*, '***'$p < 0.001$, '*'$p < 0.05$. VSP, vertical shoot position; VSP60, vertical shoot position 60°; VSP80, vertical shoot position 80°; SH, single hire wire; HQ, High-Quadrilateral; GY, guyot-pruned vertical shoot position; ET_c, crop evapotranspiration.

accounted for increased the cluster number. VSP, VSP60, VSP80, and GY increased the cluster weight compared to SH in 2020. In 2021, SH showed the lowest cluster weight and skin weight. Regarding yield, differences were only significant in 2020 where SH enhanced vine yield compared to the other trellis systems. On the other hand, 100% ET_c enhanced berry weight, cluster weight, and yield over the two seasons with no difference on leaf area to fruit ratio. Regarding berry quality parameters, SH had the highest TSS among and the lowest pH in 2020, whereas in 2021, VSPs and GY enhanced the TSS and the pH. Results also showed that irrigation treatments had little effect on the berry quality parameters over the two seasons with only TSS being increased in the 25% ET_c treatment in the harvest of 2020.

Berry skin anthocyanins and flavonols

Berry skin anthocyanins were assessed in both seasons at harvest (Table 3). Different trellis systems affected not only the total anthocyanin concentration but also modified the anthocyanin composition, leading to modifications in the profile stability. In both seasons, SH had the highest concentrations in all the anthocyanin derivatives besides di- and tri-hydroxylated anthocyanins among the six trellis systems. In 2021, HQ also notably increased most of the anthocyanin derivatives, tri-hydroxylated, di-hydroxylated, and total anthocyanins compared to the VSP trellis systems. On the other hand, VSP trellis systems tended to decrease the anthocyanin concentrations. Regarding the irrigation treatments, 25% ET_c generally showed the higher concentrations in petunidins, di- and tri-hydroxylated anthocyanins, and total anthocyanins in 2020 compared to 100% ET_c. In 2021, 25% ET_c increase most of the anthocyanin concentration in berries. 50% ET_c performed similarly in 2021 and showed higher concentrations in malvidins, tri-hydroxylated anthocyanins, and total anthocyanins.

In parallel with anthocyanin assessments, berry skin flavonols were measured at harvest in both seasons (Table 4). In 2020, SH showed the highest concentration in myricetins. SH and HQ showed the highest concentrations in quercetins, isorhamnetins and kaempferols in both seasons. SH and HQ also showed the highest concentration in tri- and di-hydroxylated as well as total flavonols in both seasons. In 2020, there were no differences among the six trellis systems in laricetins and syringetins. While in 2021, VSPs enhanced syringetin concentration. Regarding applied water amounts, little effects of irrigation treatments were shown in 2020. However, in 2021, 25% ET_c increased most of the flavonol derivatives except laricetins and syringetins compared to the other two treatments.

Flavonols and their correlations with canopy crown porosity and leaf area

The relationships between berry skin flavonol concentrations and canopy architecture were investigated in both seasons

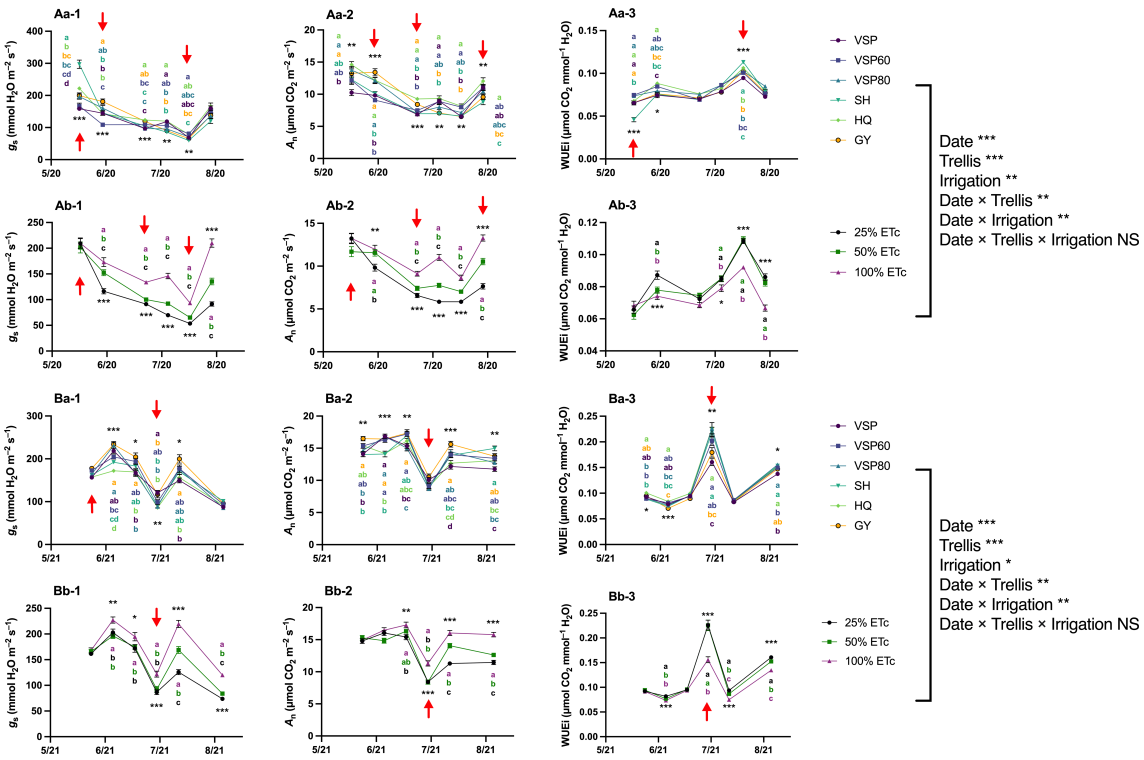

FIGURE 4
Progression of Leaf Gas Exchange as Affected by Trellis Systems and Applied Water Amounts of 'Cabernet Sauvignon' in Oakville, CA, USA in **(A)** 2020 and **(B)** 2021; **(a)** the main effect of trellis systems, **(b)** the main effect of applied water amounts; (1) stomatal conductance (g_s), (2) net carbon assimilation rate (A_n), (3) intrinsic water use efficiency (WUE_i). Error bars represent one standard deviation from the mean, letters represent ranking after Tukey's *post hoc* analyses. Asterisks represents significant levels *p*, '***'*p*< 0.001, '**'*p*< 0.01, '*'*p*< 0.05. Arrows above individual dates indicate statistical difference between starting date and the indicated date. VSP, vertical shoot position; VSP60, vertical shoot position 60°; VSP80, vertical shoot position 80°; SH, single hire wire; HQ, High-Quadrilateral; GY, guyot-pruned vertical shoot position; ET_c, crop evapotranspiration. ns, not signigficant.

(Figure 5). In 2020, crown porosity had positive and significant correlations with quercetin (molar %, R^2 = 0.383, p< 0.0001, Figure 5Aa-1), total flavonol concentration (mg per g of berry fresh weight (FW), R^2 = 0.248, p< 0.0001, Figure 5Aa-2), and total flavonol concentration (mg per berry, R^2 = 0.118, p = 0.003, Figure 5Aa-3). Leaf area was also correlated with these variables, but the correlations were negative with quercetin (R^2 = 0.356, p< 0.0001, Figure 5Ab-1), total flavonol concentration (R^2 = 0.312, p< 0.0001, Figure 5Ab-2, and total flavonol concentration (R^2 = 0.115, p = 0.004, Figure 5Ab-3). In 2021, the correlations were similar but not as significant as 2020. Crown porosity still had significant and positive relationships with quercetin (R^2 = 0.173, p = 0.0003, Figure 5Ba-1) and total flavonols concentration (R^2 = 0.170, p = 0.0003, Figure 5Ba-2). However, the relationship between crown porosity and total flavonol concentration (R^2 = 0.043, p = 0.081, Figure 5Ba-3) did not persist, as was observed in 2020. The relationships between leaf area and quercetin (molar %) and total flavonol concentration were significant, although not as strong (R^2 = 0.090, p = 0.010 and R^2 = 0.067, p = 0.030, respectively). Leaf areas were negatively correlated with these two variables (Figure 5Bb-1 and Bb-2). The significant correlation between leaf area and total flavonol concentration did not hold in 2020 as compared to 2020 (R^2 = 3.86E-04, p = 0.870, Figure 5Bb-3). It was evident that when crown porosity was greater, there was greater flavonol accumulation as well greater molar percentage of quercetin.

Discussion

Grapevine physiology was affected by canopy architecture and grapevine water status

A trellis system selected in grapevine vineyard is usually aimed at optimizing canopy architecture to further maximize canopy photosynthetic activity and improve canopy microclimate, which can yield desirable production and berry composition (Kliewer and Dokoozlian, 2005; Wessner and Kurtural, 2013; Sanchez-Rodriguez and Spósito, 2020). In historically cooler regions according to Winkler's Index, VSP

TABLE 2 Effects of trellis systems and applied water amounts on yield components and berry composition of 'Cabernet Sauvignon' in Oakville, CA in 2020 and 2021[a,b].

		Trellis							*Irrigation*				Trellis×Irrigation
		VSP	**VSP60**	**VSP80**	**SH**	**HQ**	**GY**	***p* value**	**25% ET_c**	**50% ET_c**	**100% ET_c**	***p* value**	
2020	**Berry Weight (g)**	0.97 **a**	1.00 **a**	1.01 **a**	0.86 **b**	0.87 **b**	0.97 **a**	**	0.83 **c**	0.96 **b**	1.05 **a**	***	ns
	Cluster No.	62.97 **a**	32.39 **bc**	30.36 **c**	62.97 **a**	38.11 **b**	27.67 **c**	***	35.96	38.04	38.07	ns	ns
	Cluster Weight (g)	132.76 **a**	133.40 **a**	120.48 **a**	74.17 **c**	97.24 **b**	138.97 **a**	***	100.00 **b**	118.39 **a**	130.12 **a**	***	ns
	Skin Weight (mg)	30.09 **b**	30.87 **b**	34.52 **b**	44.90 **a**	32.73 **b**	32.27 **b**	***	3491	35.97	31.81	ns	ns
	Yield (kg vine^{-1})	4.27 **ab**	4.34 **ab**	3.56 **b**	4.71 **a**	3.56 **b**	3.87 **b**	*	3.39 **b**	4.19 **a**	4.58 **a**	**	ns
	Leaf Area: Fruit (m^2 kg^{-1})	1.09	1.03	1.07	1.45	1.10	1.12	ns	1.19	1.13	1.12	ns	ns
	TSS (°Brix)	23.5 **b**	23.7 **b**	23.7 **b**	24.6 **a**	24.1 **ab**	24.2 **ab**	*	24.8 **a**	24.1 **b**	22.9 **c**	***	ns
	pH	3.47 **a**	3.49 **a**	3.46 **ab**	3.40 **c**	3.42 **bc**	3.48 **a**	**	3.47	3.45	3.44	ns	ns
	TA (g L^{-1})	7.74	7.36	7.69	7.53	7.78	7.71	ns	7.50	7.70	7.80	ns	ns
2021	**Berry Weight (g)**	1.00 **ab**	1.03 **a**	1.03 **a**	0.88 **ab**	0.83 **b**	1.03 **a**	**	0.88 **b**	0.94 **b**	1.07 **a**	***	ns
	Cluster No.	45.51 **b**	48.86 **b**	44.50 **b**	88.28 **a**	82.61 **a**	39.86 **b**	***	55.94	56.97	62.04	ns	ns
	Cluster Weight (g)	148.72 **ab**	153.96 **a**	149.24 **ab**	96.97 **b**	126.54 **ab**	168.59 **a**	**	121.78 **b**	156.19 **a**	144.04 **ab**	.	ns
	Skin Weight (mg)	64.82 **abc**	71.66 **ab**	66.84 **abc**	53.55 **c**	57.00 **bc**	73.86 **a**	**	60.40	64.11	69.36	ns	ns
	Yield (kg vine^{-1})	6.83	7.47	6.56	8.20	10.47	6.71	ns	6.13 **b**	8.68 **a**	8.21 **a**	.	ns
	Leaf Area: Fruit (m^2 kg^{-1})	0.74	0.66	0.76	0.70	0.56	0.65	ns	0.72	0.70	0.62	ns	ns
	TSS (°Brix)	23.1 **a**	23.2 **a**	23.3 **a**	21.7 **b**	21.9 **b**	22.7 **ab**	*	22.6	22.5	22.9	ns	ns
	pH	3.62 **a**	3.59 **ab**	3.57 **ab**	3.55 **b**	3.53 **b**	3.58 **ab**	.	3.59	3.56	3.57	ns	ns
	TA (g L^{-1})	5.98	5.96	5.89	5.63	5.71	8.43	ns	6.81	5.82	6.18	ns	ns

[a]Analysis of variance (p value indicated) Letters within columns indicate significant mean separation according to Tukey's test at where ".": p value< 0.1; where "*": p value< 0.05; "**": p value< 0.001, "***": p value< 0.0001.
[b]VSP, vertical shoot positioned; VSP 60, vertical shoot positioned 60°; VSP 80, vertical shoot positioned 80°; SH, single high wire; HQ, high quadrilateral; GY, guyot; TSS, total soluble solids; TA, titratable acidity; ET_c, crop evapotranspiration; ns, not significant.

trellis system is widely used as it offers relatively higher compatibility with mechanization and is suitable for the regional production goals (Tardaguila et al., 2008). However, with the warming trend in air temperature getting more pronounced, VSPs have been showing greater chances of getting cluster overexposure, resulting in sunburnt berries with yield loss and color degradation (Martínez-Lüscher et al., 2017b; Torres et al., 2020a). Under our experimental conditions, HQ trellis system showed less leaf area and greater crown porosity than the other trellis systems in 2020 in accordance with previous studies, where split trellis designs might allow more solar radiation to penetrate the canopy interior (Wessner and Kurtural, 2013; Martínez-Lüscher et al., 2019a). Conversely, SH had similar leaf area but lower crown porosity in 2020. However, the differences in leaf areas and crown porosities were not as noticeable in 2021. This could be attributed to the fact that the HQ and SH might have still been filling up spaces with new growth compared to the VSPs, which might have already had relatively more established canopy architectures. In addition, the differences in leaf areas and crown porosities could be minimized by arid growing season in 2021, despite the supplemental irrigation applied to them, accounting for diminished leaf areas (mostly in VSPs) as shown in 2021 than 2020. Furthermore, precipitation received at the vineyard prior to bud break (Martínez-Lüscher et al., 2017a), as well as precipitation received immediately prior to flowering (Yu et al., 2021a) in semi-arid regions were deemed key determinants of canopy response for latter parts of the growing season.

TABLE 3 Effects of trellis systems and applied water amounts on berry skin anthocyanins of 'cabernet sauvignon' in Oakville, CA, USA in 2020 and 2021[a, b, c].

		Trellis							*Irrigation*				Trellis×Irrigation
		VSP	**VSP60**	**VSP80**	**SH**	**HQ**	**GY**	***p value***	**25%ET$_c$**	**50%ET$_c$**	**100%ET$_c$**	***p value***	
2020	**Cya**	0.02 **c**	0.03 **abc**	0.02 **bc**	0.04 **ab**	0.04 **a**	0.03 **abc**	*	0.03	0.03	0.03	ns	ns
	Peo	0.10 **b**	0.10 **b**	0.11 **b**	0.14 **a**	0.14 **a**	0.11 **b**	**	0.11	0.12	0.11	ns	ns
	Di-OH	0.12 **b**	0.13 **b**	0.13 **b**	0.18 **a**	0.17 **a**	0.13 **b**	*	0.14	0.15	0.14	ns	ns
	Del	0.15 **c**	0.18 **bc**	0.18 **bc**	0.31 **a**	0.22 **b**	0.17 **bc**	***	0.21	0.21	0.18	ns	ns
	Pet	0.12 **c**	0.14 **bc**	0.14 **bc**	0.23 **a**	0.17 **b**	0.13 **bc**	***	0.17 **a**	0.16 **ab**	0.14 **b**	**	ns
	Mal	0.96 **b**	0.95 **b**	1.01 **b**	1.36 **a**	1.05 **b**	0.94 **b**	***	1.20 **a**	1.03 **b**	0.92 **b**	***	ns
	Tri-OH	1.23 **b**	1.28 **b**	1.33 **b**	1.89 **a**	1.44 **b**	1.25 **b**	***	1.58 **a**	1.40 **ab**	1.23 **b**	***	ns
	Total	1.35 **b**	1.41 **b**	1.46 **b**	2.06 **a**	1.61 **b**	1.39 **b**	***	1.72 **a**	1.55 **ab**	1.38 **b**	**	ns
2021	**Cya**	0.02 **c**	0.02 **bc**	0.02 **c**	0.04 **a**	0.03 **ab**	0.02 **c**	***	0.03	0.03	0.02	ns	ns
	Peo	0.11 **b**	0.11 **b**	0.11 **b**	0.17 **a**	0.15 **a**	0.10 **b**	***	0.14 **a**	0.13 **ab**	1.74 **b**	ns	ns
	Di-OH	0.13 **b**	0.14 **b**	0.13 **b**	0.21 **a**	0.19 **a**	0.12 **b**	***	0.16 **a**	0.16 **ab**	0.13 **b**	*	ns
	Del	0.20 **b**	0.23 **b**	0.22 **b**	0.45 **a**	0.39 **a**	0.19 **b**	***	4.69	4.46	3.58	ns	ns
	Pet	0.16 **b**	0.19 **b**	0.19 **b**	0.34 **a**	0.31 **a**	0.16 **b**	***	0.26 **a**	0.24 **ab**	0.19 **b**	***	ns
	Mal	1.58 **b**	1.67 **b**	1.69 **b**	2.10 **a**	2.13 **a**	1.55 **b**	***	1.93 **a**	1.84 **a**	1.58 **b**	***	ns
	Tri-OH	1.95 **b**	2.09 **b**	2.11 **b**	2.89 **a**	2.83 **a**	1.90 **b**	***	2.51 **a**	2.38 **a**	1.99 **b**	***	ns
	Total	2.08 **b**	2.22 **b**	2.24 **b**	3.09 **a**	3.01 **a**	2.02 **b**	***	2.68 **a**	2.54 **a**	2.12 **b**	***	ns

[a]Analysis of variance to compare data (p value indicated); Letters within columns indicate significant mean separation according to Tukey's HSD test at p value< 0.1, where ".": p value< 0.05, where "*": p value< 0.05; "**": p value< 0.001, "***": p value< 0.0001.
[b]VSP, vertical shoot positioned; VSP 60, vertical shoot positioned 60°; VSP 80, vertical shoot positioned 80°; SH, single high wire; HQ, high quadrilateral; Del, delphinidins; Cya, cyanidins; Pet, petunidins; Peo, peonidins; Mal, malvidins; Tri-OH, tri-hydroxylated anthocyanins; Di-OH, di-hydroxylated anthocyanins; ET$_c$, crop evapotranspiration; ns, not significant.
[c]All compounds were expressed in the unit of mg per g of berry fresh weight.

TABLE 4 Effects of trellis systems and applied water amounts on berry skin flavonols of 'cabernet sauvignon' in Oakville, CA, USA in 2020 and 2021[a, b, c].

		Trellis							*Irrigation*				Trellis×Irrigation
		VSP	**VSP60**	**VSP80**	**SH**	**HQ**	**GY**	***p value***	**25%ET$_c$**	**50%ET$_c$**	**100%ET$_c$**	***p value***	
	Kae	0.46 **bc**	0.36 **c**	0.42 **bc**	0.53 **ab**	0.66 **a**	0.55 **ab**	**	0.55	0.49	0.45	ns	ns
2020	**Que**	4.27 **b**	3.94 **b**	4.29 **b**	5.76 **a**	6.47 **a**	4.55 **b**	***	5.18	4.82	4.64	ns	ns
	Iso	0.47 **b**	0.42 **b**	0.48 **b**	0.52 **ab**	0.66 **a**	0.51 **ab**	**	0.53	0.51	0.48	ns	ns
	Di-OH	4.74 **b**	4.36 **b**	4.77 **b**	6.29 **a**	7.12 **a**	5.06 **b**	***	5.71	5.33	5.13	ns	ns
	Myr	2.40 **b**	2.40 **b**	2.57 **b**	3.46 **a**	3.17 **b**	2.59 **b**	**	2.84	2.81	2.65	ns	ns
	Lar	0.41	0.41	0.46	0.45	0.47	0.45	ns	0.46	0.45	0.41	ns	ns
	Syr	0.69	0.64	0.73	0.79	0.71	0.74	ns	0.79	0.72	0.63	ns	ns
	Tri-OH	3.49 **c**	3.46 **c**	3.76 **bc**	4.70 **a**	4.35 **ab**	3.78 **bc**	*	4.10	3.98	3.69	ns	ns
	Total	8.69 **b**	8.18 **b**	8.95 **b**	11.52 **a**	12.13 **a**	9.38 **b**	***	10.36	9.80	9.27	ns	ns
2021	**Kae**	0.34 **b**	0.36 **b**	0.40 **b**	0.65 **a**	0.67 **a**	0.34 **b**	***	0.57 **a**	0.42 **b**	0.39 **b**	**	ns
	Que	2.68 **b**	2.92 **b**	2.98 **b**	5.16 **a**	5.37 **a**	2.67 **b**	***	4.40 **a**	3.22 **b**	3.26 **b**	**	ns
	Iso	0.42 **b**	0.43 **b**	0.45 **b**	0.77 **a**	0.70 **a**	0.40 **b**	***	0.62 **a**	0.51 **ab**	0.46 **b**	**	ns
	Di-OH	3.09 **b**	3.34 **b**	3.44 **b**	5.93 **a**	6.07 **a**	3.07 **b**	***	5.02 **a**	3.74 **b**	3.71 **b**	**	ns
	Myr	0.24 **b**	2.66 **b**	2.86 **b**	4.00 **a**	4.10 **a**	2.49 **b**	***	3.53 **a**	3.04 **ab**	2.69 **b**	***	ns
	Lar	0.36	0.33	0.38	0.32	0.36	0.38	ns	0.40	0.34	0.32	ns	ns
	Syr	0.50 **ab**	0.49 **ab**	0.52 **a**	0.40 **c**	0.42 **bc**	0.51 **a**	**	0.49	0.47	0.46	ns	ns
	Tri-OH	3.27 **b**	3.48 **b**	3.76 **b**	4.72 **a**	4.89 **a**	3.39 **b**	***	4.42 **a**	3.84 **b**	3.50 **b**	***	ns
	Total	6.71 **b**	7.18 **b**	7.60 **b**	11.30 **a**	11.63 **a**	6.70 **b**	***	10.00 **a**	7.99 **b**	7.60**b**	**	ns

[a]Analysis of variance to compare data (p value indicated); Letters within columns indicate significant mean separation according to Tukey's HSD test at p value< 0.05, where "*": p value< 0.05; "**": p value< 0.001, "***": p value< 0.0001.
[b]VSP, vertical shoot positioned; VSP 60, vertical shoot positioned 60°; VSP 80, vertical shoot positioned 80°; SH, single high wire; HQ, high quadrilateral; Myr, myricetins; Que, quercetins; Kae, kaempferols; Lar, laricetins; Iso, isorhamnetin; Syr, syringetins; Tri-OH, tri-hydroxylated flavonols; Di-OH, di-hydroxylated flavonols; ET$_c$, crop evapotranspiration; ns, not significant.
[c]All compounds were expressed in the unit of 10^{-2} mg per g of berry fresh weight.

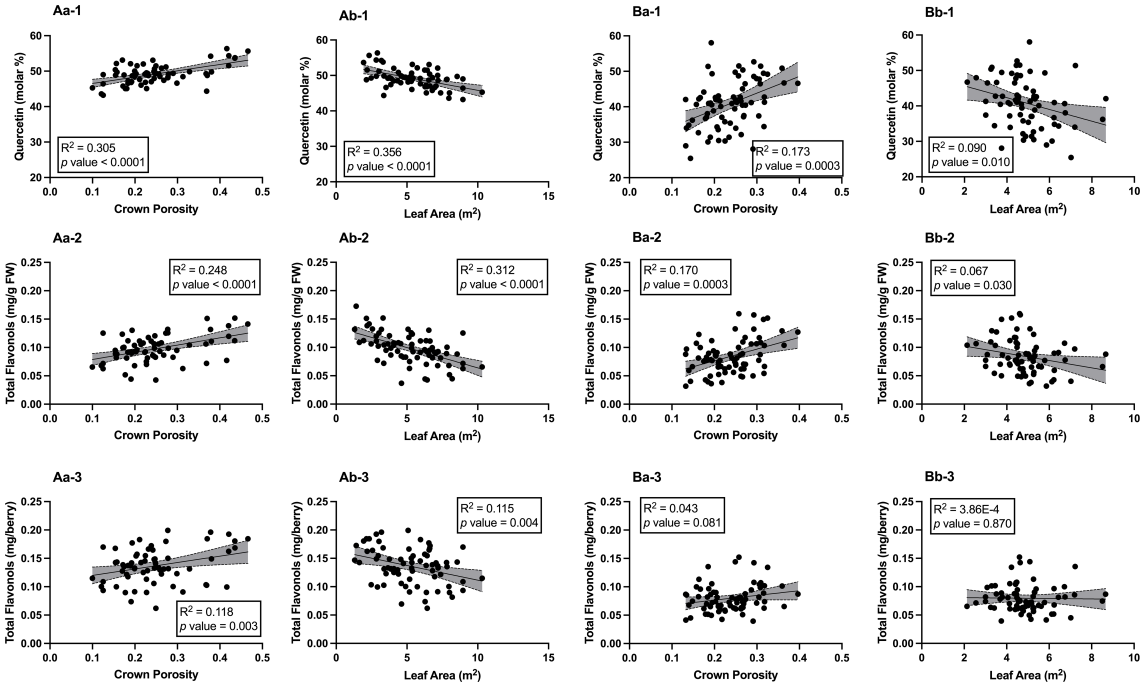

FIGURE 5
Relationships between Canopy Architecture and % molar Quercetin, Total Flavonol Concentration, and Total Flavonol Content in Berry Skins of 'Cabernet Sauvignon' in Oakville, CA, USA in 2020 **(A)** 2020 and **(B)** 2021; **(a)** correlations with crown porosity, **(b)** correlations with leaf areas; (1) quercetin (molar %), (2) total flavonols (mg/g FW), (3) total flavonols (mg/berry).Grey shade areas indicate 95% confidence intervals, and correlation values were expressed in R^2 and *p* values. FW, berry fresh weight.

In this study, yield per vine was not constantly determined by the trellis systems in both years, although similar bud densities at pruning were achieved. Furthermore, more leaf area did not account for more yield at harvest, despite it was well established that a sufficient leaf area would support fruit development (Martínez-Lüscher and Kurtural, 2021), and in contrast with some previous studies (Kliewer and Dokoozlian, 2005; Herrera et al., 2015). This could be attributed to not only the total amount leaf area but also how the leaves were distributed within the canopy. Commonly, HQ would have more open space to distribute more exposed and photosynthetically active leaves to the sunlight to optimize production (Bettiga et al., 2003; Brillante et al., 2018).

Previous studies have shown that greater leaf area can also contribute to higher TSS accumulation (Parker et al., 2015; Martínez-Lüscher and Kurtural, 2021), which was not observed in this study. On the contrary, more leaf area resulted in less TSS accumulation. This might be explained by the fact that the leaf area to fruit ratio, which represented the source-sink balance within the grapevine, might have a greater influence on the berry TSS accumulation. In this study, even though no statistical differences were observed in leaf area to fruit ratio, SH in 2020 showed relatively higher values (not statistically significant) with higher TSS accumulated at harvest. A similar situation was observed during the second season, where VSP80 showed relatively higher leaf area to fruit ratio (not statistically significant) and subsequently higher TSS at harvest. When crown porosity was considered, higher porosity resulted in greater TSS accumulation in berries, which could be attributed to the higher potential of berry exposure to the hot environment, causing the berries to experience greater dehydration (Torres et al., 2017). This relationship was not observed in 2021, and it might be derived from the relatively higher crop load and lower leaf area to fruit ratio in 2021 compared to 2020, especially by SH and HQ (not statistically significant). SH and HQ did not have a similar source-sink balance as 2020, which lowered their capacity to translocate photosynthates into the berries, this might have been the reason why they had a more reduced TSS at harvest compared to the other trellis systems.

Regarding the applied water amounts, the results were clear and consistent, with increased water status in grapevines irrigated with higher water amounts, and consequently, greater berry weight, cluster weight, and yield. These results agreed with previous studies on the relationships between grapevine water status and yield components (Torres et al., 2021b; Torres et al., 2021c). However, leaf area and crown porosity were not affected by applied water amount treatments in 2020. This might have

been resulted from the remarkably high air temperature at the experimental site, diminishing the grapevine vegetative growth despite the water compensation from irrigation (Greer and Weedon, 2016). Consequently, berry quality parameters were slightly affected by irrigation treatments, with only TSS being higher with greater water stress in the harvest of the first season due to berry dehydration (Torres et al., 2017) and potential promotion in sugar accumulation (Zarrouk et al., 2016).

Influences of trellis systems and grapevine water status on berry anthocyanins and flavonols

There were two flavonoid classes monitored in this study, anthocyanins and flavonols. They are highly sensitive towards environmental conditions (Martínez-Lüscher et al., 2014; de Rosas et al., 2017; Arrizabalaga-Arriazu et al., 2020; Yu et al., 2020). This study evidenced that SH increased berry skin anthocyanin and flavonol concentrations compared to the other trellis systems over the two seasons. SH might have had more advancement in berry development due to more efficient leaf area to fruit ratio achieved in this trellis system (Torres et al., 2017; Gambetta and Kurtural, 2021). Furthermore, the crown porosity of SH was ranging from 0.20 to 0.30, a window of inferred solar radiation exposure identified in previous works (Torres et al., 2020) for `Cabernet Sauvignon´. As for VSPs, anthocyanin degradation was unlikely to be the reason why VSPs had lower anthocyanin concentration since the greater leaf area could have provided berries some degree of protection from receiving excessive solar radiation (Torres et al., 2020). This can be confirmed by the fact that TSS and berry skin anthocyanin concentration were still synchronized in 2020. However, there was a decoupling of TSS and berry skin anthocyanin concentration in 2021, where the VSPs had higher TSS but lower skin anthocyanin content. Unlike the first season, the leaf area and canopy crown porosity showed no difference among the trellises, but the effective leaf area that can provide protection against excessive solar radiation might differ between SH and HQ from the other trellis systems. Hence, even with similar leaf areas, the VSPs still exposed clusters to the environmental stresses, which promoted TSS accumulation due to dehydration but greater anthocyanin degradation, similar to what was observed in previous studies (Martínez-Lüscher et al., 2019b; Yu et al., 2020). Although the TSS levels in this study were not at the level for reaching the tipping point of anthocyanin degradation as previously reported (approximately 24-25°Brix), compared to the SH and HQ with greater height from the vineyard floor, the VSPs might have been more easily affected by the solar radiation and heat reflected from soil surface, causing hotter and drier canopy microclimate and inevitably lead to greater anthocyanin degradation (Martínez-Lüscher et al., 2019a; reviewed by van Leeuwen et al., 2019). Additionally, some previous studies have shown negative relationships between yield and berry composition (Uriarte et al., 2016). Similar observations in this study might be due to source organs (leaves) of the VSPs were not distributed widely enough to be as efficient as those of the SH and HQ, resulting in lower photosynthetic capacity in their canopies, which further reduced the translocation of photosynthates flowing into berries to promote TSS accumulation and flavonoid biosynthesis.

As for flavonols, previous studies have shown that flavonols are very sensitive to solar radiation, especially UV radiation, where more light will often increase flavonol concentration in berry skins (Martínez-Lüscher et al., 2014; Torres et al., 2022). The results from this work corroborated previous observations that less leaf area with more crown porosity would increase solar radiation inside the canopy, and further increase flavonol concentrations in berry skins (Martínez-Lüscher et al., 2019a; Torres et al., 2021a). Additionally, SH and HQ showed greater concentrations in di-hydroxylated flavonols (quercetins and isorhamnetins) as well as some tri-hydroxylated flavonol (myricetins) derivatives.

Water deficits, achieved by manipulating applied water amounts through irrigation, can significantly improve flavonoid concentrations in grape berries (Torres et al., 2021d; Torres et al., 2021c). Similar results were observed in our findings as well, where 25% ET_c was able to increase anthocyanin and flavonol concentrations in grape berries. One previous study at the same experimental site showed that 25% ET_c could potentially increase the possibility for flavonoid degradation and decrease the wine antioxidant capacity (Torres et al., 2022). However, we did not see such effects in this study. This might be because berry sugar accumulation was not affected among the three applied water amounts, and the overall TSS levels did not exceed the tipping point (~25°Brix). It was repeatedly been noticed that, beyond this TSS level, skin anthocyanins and even flavonols would start to significantly degrade in a hot climate (Yu et al., 2020; Gambetta and Kurtural, 2021). Hence, in our study, all the treatments might have ended up having similar advancements in berry flavonoid accumulation because of the similar levels of TSS without any promoted accumulation or degradation among the three irrigation strategies (Ferri et al., 2011), but 25% ET_c was able to decrease berry weights, which resulted in higher concentrations in anthocyanins and flavonols.

Flavonols as an indicator of canopy architecture determined by solar radiation

Positive relationships between flavonols and solar radiation, especially UV-B, have been consistently observed in previous research, clearly indicating that more solar radiation penetrating

into the canopy interior promotes flavonol concentration in berry skins (Koyama et al., 2012; Martínez-Lüscher et al., 2014). Further, flavonol content and derivative proportions exhibited strong relationships with solar radiation (Martínez-Lüscher et al., 2019a), which was confirmed in this study, where quercetin proportion and both total flavonol concentration correlated strongly with leaf area and crown porosity especially with VSP types.

When the high air temperature or drought conditions became extreme, flavonoids in berry skins started to degrade (Martínez-Lüscher et al., 2017b). For all six trellis systems in 2020, the relationships between flavonols and canopy architecture were strong. These relationships between leaf area/crown porosity and flavonols can provide a feasible way of assessing canopy architecture in terms of the canopy's contribution towards berry composition and *vice versa*. This approach is not limited only to red cultivars and can also be applied to white cultivars since flavonols are still synthesized in their skin tissues (Pérez-Navarro et al., 2021). Also, for quercetin specifically, it is the most abundant flavonol derivative in grape berry skins. Hence, the compound would be unchallenging to isolate and extract, offering an easy assessment of the effects of solar radiation on berry flavonol profiles. Interestingly, in this study, the VSPs did not result in higher quercetin or total flavonol concentrations, indicating that these trellis systems might not be suitable for accumulating or maintaining flavonoids in berry skins in a hot climate regardless of TSS levels compared to other trellis systems. Although the relationships between canopy architecture and flavonols were strong in this study and align with previous reports, the influence of canopy structure imposed by trellis system on berry chemical development needs more investigation to understand the contributions of trellis systems to canopy architecture and canopy microclimate.

Conclusion

As growing season temperatures continue to rise in viticultural regions, grape growers are looking for ways to adapt to maintain consistent production volume and quality. However, legislative pressure on grape growers harnessing their ability to extract ground water for irrigation purposes will limit this adaptation. Overall, this study provided evidence of how different trellis systems combined with irrigation strategies affected grapevine physiological development and berry chemical profiles. Our results indicated that SH and HQ trellis systems could enhance the efficiency of grapevine canopy in promoting TSS accumulation and yield as well as higher capacity for flavonol and anthocyanin accumulation in berry skins with less chemical degradation compared to the traditional VSPs. Additionally, we purposely aimed to study the relationships between flavonols and canopy architecture. We observed strong correlations between molar % quercetin, and total flavonol concentration and content with leaf area and canopy porosity, indicating that berry skin flavonols can be feasible indicators for canopy architecture to register berry development in response to solar radiation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SK conceptualized and designed the trial. RY, NT, JG, LM, MZ, JT, and SK executed the trial, curated the data. RY and NT wrote the first version of the manuscript. GG and SK revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

UC Davis Library has provided partial funding to defray publication costs.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 22 November 2022
DOI 10.3389/fpls.2022.998910

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

RECEIVED 20 July 2022
ACCEPTED 17 October 2022
PUBLISHED 22 November 2022

CITATION
Oliver-Manera J, Anić M, García-Tejera O and Girona J (2022) Evaluation of carbon balance and carbohydrate reserves from forced (*Vitis vinifera L.*) cv. Tempranillo vines. *Front. Plant Sci.* 13:998910. doi: 10.3389/fpls.2022.998910

Evaluation of carbon balance and carbohydrate reserves from forced (*Vitis vinifera L.*) cv. Tempranillo vines

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Elevated temperatures during berry ripening have been shown to affect grape quality. The crop forcing technique (summer pruning that 'force' the vine to start a new cycle) has been shown to improve berry quality by delaying the harvest date. However, yield is typically reduced on forced vines, which is attributed to vine low carbon availability soon after forcing and likely incomplete inflorescence formation. The present study aims to estimate the carbon balance of forced vines and evaluate vine responses to changes in carbon patterns due to forcing. Three treatments were studied on Tempranillo cultivar: non-forced vines (Control), vines forced shortly after fruit set (CF_{early}) and vines forced one month later at the beginning of bunch closure (CF_{late}). Whole canopy net carbon exchange was modelled and validated using two whole canopy gas exchange chambers. In addition, non-structural carbohydrate reserves at budburst, forcing date and harvest, were analysed. Yield, yield components and vegetative growth were also evaluated. Harvest date was delayed by one and two months in the CF_{early} and CF_{late}, respectively, which increased must acidity. However, yield was lower in the forced treatments compared to the Control (49% lower for CF_{early} and 82% for CF_{late}). In the second year, at the time when CF_{early} and CF_{late} dormant buds were unlocked (forced budburst), forced vines had significantly lower non-structural carbohydrates than Control vines at budburst. Although the time elapsed from budburst to reach maximum net carbon exchange was longer for the Control treatment (80 days) than for the forced treatments (about 40 days), average daily net carbon exchange until harvest was comparable between Control (60.9 g CO_2/vine/day) and CF_{early} (55.9 g CO_2/vine/day), but not for CF_{late} (38.7 g CO_2/vine/day). In addition, the time elapsed from budburst to harvest was shorter in forced treatments (about 124 days) than for the Control (172 days). As a result, the cumulative net carbon exchange

until harvest was reduced by 35% (CF_{early}) and 55% (CF_{late}) in the forced treatments. However, no differences in carbon reserves at harvest were observed between treatments partly helped by the higher source:sink ratio observed in forced than Control vines.

KEYWORDS

delayed ripening, forcing regrowth, photosynthesis, climate change, net carbon exchange, source:sink

1 Introduction

Temperatures in the Mediterranean region are expected to increase in the coming decades due to global warming (Allen et al., 2018). In the Ebro Valley (north-east of Spain), warming projections predict an advance of harvest dates of up to 18 days for the Chardonnay variety (Prats-Llinàs et al., 2017). Other studies predict an advance of 35 days for Tempranillo in the Duero Valley (Ramos et al., 2018). The effects of global warming are not limited to plant development. Berry and wine quality will be severely affected (Gutiérrez-Gamboa et al., 2021). At high temperatures, there is a decoupling between the accumulation of sugar and phenolic compounds in berries (Sadras and Moran, 2012), alcohol content may be higher and aroma, flavor, and acidity content lower in wine (Keller, 2010; Mira de Orduña, 2010).

To minimize the effects of global warming, it has been proposed to move the ripening period and harvest date to more suitable (cooler) conditions (Palliotti et al., 2014). Many different management techniques and strategies have been investigated including irrigation strategies, chemical treatments, late winter pruning, leaf removal, fruit thinning and severe trimming (Palliotti et al., 2014; Van Leeuwen and Destrac-Irvine, 2017; Santos et al., 2020; Gutiérrez-Gamboa et al., 2021). However, for most of these techniques, the delay in ripening would be limited to only a few (one or two) weeks with different effects on grape quality depending on the variety and the local climatic conditions. For instance, in a trimming study with Tempranillo cultivar conducted in a temperate Spanish region, a 5-day delay of veraison resulted in a decrease of the grape sugar-acid ratio (Santesteban et al., 2017). On the other hand, in a study carried out in a warmer region of Spain also with Tempranillo, the removal of all mature apical leaves caused a 10-day delay in the harvest date, but without any effect on sugar content and even reducing grape acidity at harvest in defoliated vines (Buesa et al., 2019). Forced regrowth (Gu et al., 2012) -better known as the crop forcing technique- can delay the berry ripening phase by up to two months (Martinez De Toda et al., 2019) resulting in a decrease of the sugar-acid ratio (Gu et al., 2012; Lavado et al., 2019; Martinez De Toda et al., 2019; Martínez-Moreno et al., 2019). The crop forcing technique involves heavy pruning from mid-spring to early summer, removing all leaves and clusters. A total of about 2-6 buds are left on the remaining shoots. The result is a break in dormancy in the remaining buds, forcing a new vegetative and reproductive cycle (Gu et al., 2012).

Promising results for improving berry quality at harvest, such as higher acidity and phenolic compound concentrations, have been obtained in Cabernet Sauvignon in California using the forcing technique (Gu et al., 2012). Some recent studies in Spain have reported similar improvements in fruit and wine quality in the Tempranillo cultivar. However, a significant reduction in yield and vine vigor has also been observed (Martinez De Toda et al., 2019; Martínez-Moreno et al., 2019; Lavado et al., 2019; Pou et al., 2019). This decrease in yield could be related to the phenological stage at which forced pruning is performed (Martinez De Toda et al., 2019). As a general rule, lower number of bunches per vine are observed the earlier the treatment, (Martinez De Toda et al., 2019; Martínez-Moreno et al., 2019) which suggests an incomplete inflorescence primordia formation at the time when vines are forced. It has also been suggested that the low carbohydrate availability at the time of forcing together with the environmental conditions of the forced vines, could explain the reduction in both yield (the reduced number of bunches per vine and berries per bunch) and vine vigour (Martínez-Moreno et al., 2019). Therefore, the two main limitations of the crop forcing technique are i) an incomplete inflorescence primordia formation before dormant bud unlocking and ii) the low carbohydrates availability caused by removing all leaves which can lead to a source limitation affecting yield for the current and the next year (Poni et al., 2020). An alternative crop forcing technique (named double cropping) in which neither the primary crop nor the leaves bellow the trimming point (node six) were removed, was validated on potted Pinot Noir vines by Poni et al. (2021). The dormant buds were successfully unlocked and, as a result, a forced crop was added to the primary crop in forced vines. In addition, the presence of new functional leaf area improved the whole vine carbon balance in forced vines compared to non-forced vines. The same technique was used in Tempranillo

(Martínez De Toda, 2021), but the forced crop did not reach the total sugar concentration of the non-forced vines.

The use of whole-plant carbon balance simulation models is a powerful tool for analyzing the effects of canopy management on carbon dynamics, especially if the model is properly site validated. Poni et al. (2006) successfully adapted and validated the Charles-Edwards canopy photosynthesis model (Charles-Edwards, 1982) together with the Arrhenius equations for respiration to estimate the carbon balance of grapevines. This model is characterized by its simplicity and robustness when the vines are not under water stress. The net carbon exchange models, however, requires information about carbohydrate reserves to better interpret carbon dynamics (Holzapfel et al., 2010).

The aim of this study is to analyze the effect on carbon balance and carbon reserve dynamics of vines under the crop forcing technique to better interpret the effects on vine performance.

2 Materials and methods

2.1 Experimental site, plant material and experimental design

The experiment was conducted in 2018 and 2019 in a commercial vineyard in Lleida (41.65°N, 0.52°E; 320 m.a.s.l.) on Tempranillo vines grafted on R110 rootstock and planted in 2013. The rows were north-south oriented (31.6°N-E) with 1.65 m between vines and 2.5 m between rows. Vines were trained with double cordons and had a vertically-positioned canopy. The criterion for winter pruning was to leave about 12 spurs on each vine and two buds per spur. Vines were drip irrigated with 2.3 L/h emitters spaced at 0.6 m intervals. Irrigation scheduling was calculated using the water balance approach (Allen et al., 1998) and based on a stem water potential threshold of -0.8 MPa, as proposed by Marsal et al. (2008) for non-stressed vines.

Weather data were collected from a weather station located 6.8 km from the plot. The weather station forms part of the regional weather service of Catalonia. In 2019, phenology was assessed weekly according to the modified E-L system (Coombe, 1995). Growing degree days (GDD) were calculated with 10°C as the baseline temperature.

Three treatments were studied: unforced vines grown conventionally according to winery criteria (Control), early forcing (CF_{early}) and late forcing (CF_{late}). In 2019, in Control vines, a standard removal of excessive/basal lateral shoots was undertaken on July 2, together to the removal of the bunch zone leaves (bellow nodes 3-6) to allow exposure of the bunch to the sun, while a mechanical hedging and lateral trimming was executed on July 10, and a fruit thinning to retain about 20 bunches per vine was operated on 19 July. Forcing treatments were applied shortly after fruit set (E-L 27) for CF_{early} and at the beginning of bunch closure (E-L 32) for CF_{late}. The exact dates were June 14 and June 3 for CF_{early} and July 13 and July 1 for CF_{late}, for 2018 and 2019, respectively. Crop forcing pruning was performed mechanically with a pre-pruner (Pellenc DISCO, Pellenc SAS, Pertuis, France) attached to a tractor, retaining 6-8 buds per shoot, and manually removing the remaining leaves and bunches. It should be taken into account that the phenological shift due to the crop forcing technique required adaptation of pest and disease control, as well as irrigation system and irrigation scheduling to each treatment. Therefore, the experimental design was designed to allow viticulture personnel to manage each treatment differently. Based on a high resolution NDVI map performed in 2016 of the same field and described in Bellvert et al. (2020), in an area as much homogeneous as possible, three parallel and adjacent plots were established with four rows and 12 vines per row (Supplementary Figure 1 in Supplementary Material). Each plot was randomly assigned a treatment. The most likely gradient of vigor was observed following the direction of the vine rows. Then, the central 16 vines of each plot were grouped into four replicates of four vines each, according to the orientation of the plot. The maximum distance between two vines of each replicate of different treatments was 22.5 m (between the Control and the CF_{early}). Before any treatment was applied, trunk diameter perpendicular to the row at a 0.5 height was measured in each vine of each plot and statistical analysis were performed (Supplementary Table 1 in the Supplementary Material). Trunk diameters were 38.0 cm (Control), 41.2 cm (CF_{early}) and 40.9 cm (CF_{late}). No statistical differences were observed between treatments ($P = 0.28$) but between replicates ($P = 0.01$). The edge vines of each plot were used as buffers to avoid the influence of the neighboring treatment. All the vine measurements are described in the next sections. However, due to the large amount of data from the experiment, all measured, and some estimated parameters are summarized in Supplementary Table 2.

2.2 Yield, yield components, grape quality, and carry-over effects

The optimal harvest date was set at a total soluble solids content (TSS) between 22.5 and 23.5 °Brix for all treatments. Thus, from one month after veraison until harvest, a sample of berries was collected approximately every three days to extract the juice and measure TSS with a refractometer (Pallette, PR-32α, ATAGO Co., LTD., Tokyo, Japan) to ensure the optimal harvest date. At harvest, yield, number of bunches per vine and bunch weight were determined. A sub-sample of 50 berries per replication was weighed (berry weight). The number of berries per bunch and per vine was then estimated. All samples were weighed shortly after collection and dried at 65°C to constant

weight to determine the dry weight. Berry juice was extracted from one sample of each replication and TSS was determined. The same juice was used to measure pH using a pHmeter (Crison PLG-22, HACH LANGE, SLU, Barcelona, Spain) and titratable acidity (TA). To measure TA (g/L tartaric acid) of the must, 10 mL of filtered juice was diluted with 10 mL of distilled water and titrated with a 0.1 N NaOH solution to a final pH of 8.2. In 2019, bunch compactness was estimated from the ratio between bunch and rachis weight of a subsample of 10 bunches per treatment at harvest. Before performing the forcing in 2019, the number of bunches per shoot on one vine per replicate was counted to evaluate carry-over effects from the 2018 season.

2.3 Vegetative growth, light interception, and biomass

In 2018, the winter pruning of 10 vines per treatment was collected and weighed, and the number of shoots of one vine per replicate was counted.

In 2019, one vine per replicate was selected (four vines per treatment) to measure biomass (leaves, shoots, and fruit), leaf area (LA), trunk cross-sectional area (TCSA) and the fraction of intercepted photosynthetically active radiation (FIPAR). On forcing dates (June 3 for CF_{early} and July 1 for CF_{late}) total biomass removed by forcing pruning was collected and weighed. Shoots and bunches were also counted. On each forcing date, four vines located outside the experiment managed as the Control treatment were forced. Biomass was then measured, and shoots and bunches were counted. In addition, total biomass removed by the vineyard management actions described in section 2.1 for the Control treatment was recorded. In the forced vines, no management actions were performed other than forcing except for light defoliation of CF_{early} vines in September which was also weighed. At the end of the season, the four selected vines per treatment were bagged and their leaves and shoots collected and counted. The collected biomass was dried at 65°C until constant weight and then weighed.

Every three weeks, LA was determined on four representative fruiting shoots per vine using the Lopes and Pinto procedure (Lopes and Pinto, 2005). In this procedure, total LA is estimated by multiplying the leaf area of individual shoots by the number of shoots per vine. The leaf area of the individual shoot was calculated as the mean of the leaf with the largest and smallest area on the shoot multiplied by the number of leaves per shoot. Individual leaf area was then estimated from leaf central vein (CV) measurements using a linear regression between the two parameters ($LA = 21.531CV - 93.98$; $R^2 = 0.89$). To obtain the regression, 150 leaves per treatment were sampled from July to October. For each leaf, the central vein was measured with a tape-measure and individual leaf area was measured with the Li3000 (Li-3000, Li-Cor, Inc., Lincoln, NE, USA). Only leaves in which the central vein was longer than 4.5 cm were considered.

Trunk diameter was calculated as the average of two diameter measurements per vine taken with a digital caliper (Absolute Digimatic Caliper, Mitutoyo Corp., Aurora, IL, USA) parallel and perpendicular to the row at a height of 50 cm above the ground. Then, the increase in trunk cross-sectional area (ΔTCSA) was calculated as the difference in TCSA between the beginning and end of the season.

The fraction of photosynthetically active radiation intercepted by the vines (FIPAR) was measured every three weeks from May to the end of September. This was carried out between 11:00 and 12:00 (GMT) using an Accupar LP-80 ceptometer (Meter group, Inc. USA). For each vine, one measure was taken above the canopy (I_{above}) and 12 measurements were taken below the canopy (I_{below}). Measurements below the canopy were taken parallel to the row and 0.5m apart to cover the entire ground allocated per vine. The FIPAR was calculated as follows (Equation 1):

$$\text{FIPAR} = 1 - \frac{\sum I_{below}/12}{I_{above}} \quad (1)$$

These punctual FIPAR measurements were used as input for estimating daily FIPAR using a model based on the specific site and plant characteristics (canopy height and width) measured on the same dates (Oyarzun et al., 2007).

The winter pruning weight of 10 vines per treatment was also measured.

2.4 Plant water status, leaf photosynthesis, and quantum yield

Physiological measurements were made during the 2019 season from May to October. Stem water potential (Ψ_s) was measured every 15 days from May to October following the Shackel et al. (1997) methodology. On four vines in each treatment (one per replicate), a shaded leaf located near the trunk was bagged in an aluminum bag 30 min before the measurement. Measurements were carried out between 11:30 and 12:30 (GMT), using a pressure chamber (Model 3005, Soil moisture, Corp. Sta. Barbara, CA, USA).

Stomatal conductance (g_s) and leaf net photosynthesis (Pn) were determined using an LCA-4 portable open gas exchange system (ADC, Hoddesdon, UK). Measurements were performed on two fully developed, sunlit leaves (PAR > 1200 μmol/s/m^2) per vine, located at mid-height of the canopy. Measurements were made monthly at between 11:00 and 12:30 (GMT) on the same four vines per treatment in which biometric measurements were realized. Therefore, a total of 8 measurements per treatment were performed on each measurement date.

Quantum yield (α) was measured, approximately once a month, on one fully expanded and sunlit leaf from three vines per treatment. A Li-6400 infrared gas analyser system (Li-6400, Li-Cor, Inc., Lincoln, NE, USA), equipped with a 6400-40 leaf chamber fluorometer (10% blue light and 90% red light), was

used to establish the photosynthetic response to photon flux density (Pn/I curves) within a photon flux density range of 50, 100 and 200 μmol/s/m^2. Leaf temperature was set at 25 ± 2°C, except in October, when a leaf temperature of 20 ± 2 °C was considered more appropriate. The reference CO_2 concentration was set at 400 ppm. Measurements were performed from 7:00 to 11:00 (GTM) to avoid a relative humidity lower than 40% as proposed by Escalona et al. (1999).

2.5 Canopy photosynthesis model

A simple variant of the Charles-Edwards (1982) model was used to estimate daily canopy photosynthesis during the 2019 growing season (Equation 2) (Poni et al., 2006). Data obtained from the same four vines per treatment where biomass, leaf area and FIPAR were monitored were used to run the model. This model is based on the Big Leaf approach and requires light and plant characteristics as inputs:

$$Pn_{canopy} = \frac{\propto S\ h\ dailyFIPAR\ Pn}{\propto k\ S + h\ Pn} G \quad (2)$$

where Pn_{canopy} is the net photosynthesis of all the canopy leaves (g CO_2/vine/day); α is the quantum yield (μg CO_2/J); S is the total daily integral of PAR radiation on the horizontal plane (MJ/m^2/day); h is daylength (s); Pn is leaf photosynthesis (mg CO_2/m^2/s); k is extinction coefficient (dimensionless); and G is vine spacing (m^2/vine). We assumed that 48% of the total incident radiation was in the PAR range (Tsubo and Walker (2005)).

k is based on Russell et al. (1989) (Equation 3):

$$k = \frac{\ln\left(I_{above}/I_{below}\right)}{LAI} \quad (3)$$

where I_{above} is the incident radiation above the canopy and I_{below} is the radiation measured bellow the canopy described in section 2.3, and LAI is the leaf area index, calculated as leaf area per ground area (m^2/m^2).

Parameters that fell between two different measured parameters were estimated linearly to allow the model to run continuously.

2.6 Net carbon exchange model

Using the same four vines per treatment for which Pn_{canopy} was calculated, daily net carbon exchange was estimated as follows (Equation 4):

$$NCE_m = \sum_{sunrise}^{sunset} Pn_{canopy} - \sum_{sunset}^{sunrise} R_{leaf} - \sum_{0}^{24} R_{shoot} - \sum_{0}^{24} R_{fruit} - \sum_{0}^{24} R_{trunk} \quad (4)$$

where NCE_m is the modelled daily net carbon exchange (g CO_2/day), Pn_{canopy} is calculated canopy net photosynthesis (g CO_2/day) from sunrise to sunset; and R is respiration of aerial organs (g CO_2/day). Because leaf respiration during the day was already included in Pn_{canopy}, only nocturnal leaf respiration was estimated. Respiration was calculated according to Equation 5:

$$R = q_m\ X\ Q_{10}^{\left(\frac{T}{10}\right)} \quad . (5)$$

where q_m is the maintenance coefficient, X is the organ size parameter (g DW or m^2), Q_{10} is the temperature coefficient and T is the air temperature (°C). Both q_m and Q_{10} were taken from the literature (Palliotti et al., 2005; Poni et al., 2006; Escalona et al., 2012 and Hernández-Montes et al., 2020) (see Supplementary Table 3 in the Supplementary Material). It was assumed that respiration coefficients did not change because of the treatment. Respiration coefficients that fell between two different phenological stages were estimated linearly. The organ size parameters (X) were LA, trunk area, fruit dry weight (DW), and shoot DW. Trunk area was calculated as the product of the perimeter of the trunk by the trunk height. From pea size of berry development (E-L31) to harvest, 2 berries were collected from ten vines per treatment at approximately 15-day intervals, dried at 65°C, and weighed. Fruit DW was then estimated by multiplying the dry weight of each berry by the final number of berries at harvest. Shoot DW was estimated from shoot length of the same shoots on which LA was measured multiplied by the number of shoots per vine. The conversion from shoot length to shoot dry weight was calculated using a linear regression between shoot density (SD) (g DW/cm) and GDD (SD = 0.00005 GDD + 0.0394; R^2 = 0.95). To obtain the regression, 16 shoots per treatment were measured, dried at 65°C, and weighed on each forcing pruning date (June 3 and July 1) and at the end of the season (November 18). The organ size parameters that fell between measurement dates were estimated linearly using GDD as the physiological time scale.

The modelled net carbon exchange was normalised relative to the total LA of each vine (NCE/LA) and expressed as g CO_2/m^2 leaf area.

2.7 Canopy net carbon exchange 7model validation

To validate the model, two open-top gas exchange chambers were constructed which were similar to those described by Corelli-Grappadelli and Magnanini (1993) (see Supplementary Figure 2). The chamber volume was approximately 5 m^3 and they were made of Mylar^R plastic. Air was introduced into the chamber from a height of 3.5 m *via* a 20 cm diameter aluminum tube. A 186.5 W centrifugal fan (Casals Ventilación Industrial IND, S.L., Girona, Spain) was installed in each chamber to blow the air into the chamber through a 19 cm diameter PVC pipe.

Once the air was in the chamber, it was distributed through a perforated aluminum tube around the base of the canopy. Air mixing inside the chamber was enhanced by two 12V cpu fans (F12 PWM PST, Arctic GmbH), positioned directly above the canopy. The air velocity (m/s) at the center of the inlet tube was measured using an air velocity transmitter (Dwyer Series 641, USA) and the flow rate (F_v), in m^3/s, was calculated as follows (Equation 6):

$$Fv = air\ velocity \cdot pipe\ area\,. \quad (6)$$

Air flow was adjusted to approximately 12 m^3/min using a REG-5 fan speed controller (Casals Ventilación Industrial IND, S.L., Girona, Spain). Temperature and relative humidity inside and outside the chamber were monitored using a Vaisala HMP110 sensor (Vaisala Corporation, Helsinki, Finland). Global solar radiation was measured inside and outside the chamber using pyranometers (Apogee SP-110, Apogee Instruments, Inc., North Logan, USA). CO_2 concentrations at the inlet ($CO_{2\ ref}$) and outlet ($CO_{2\ an}$) were measured using an infrared gas analyzer (Li-820, Li-Cor, Inc., Lincoln, NE, USA). A handmade system of valves, micropumps, and tubes controlled by an SDM-CD16AC relay controller (Campbell Scientific, Inc., N Logan, USA) was used to pump the air from the inlet and outlet of the chamber to the gas analyzer device. The delay between each reference and corresponding analysis measurement was 40 s. Net carbon exchange in the chamber was calculated as follows (Equation 7):

$$NCE_{ch} = F_m(CO_{2\,ref} - CO2_{2\,an}) \quad (7)$$

where NCE_{ch} is the net carbon exchange in the chamber (µmol CO_2/s/vine), and F_m is the air flow (mol/s) which was calculated as follows (Equation 8):

$$F_m = F_v \frac{1000}{22.41} \frac{273.15}{(T + 273.15)} \quad (8)$$

where T is the air temperature in Celsius.

The recording frequency was 20s using a CR1000 datalogger equipped with two AM16-32B multiplexers (Campbell Scientific, Inc., N Logan, USA).

The model was validated by comparing the sum of NCE_m with the sum of NCE_{ch} (g CO_2/vine). Measurements were taken from May to the end of September on 8 different days from approximately 6:00 to 17:00 (GMT). On each day, a Control and a CF vine were measured simultaneously. The coefficient of determination (R^2), root mean square error (RMSE) and the Nash-Sutcliffe Efficiency (NSE) coefficient were used to validate the model.

2.8 Carbohydrate reserves

Carbohydrate reserves were evaluated at the beginning of the 2019 season shortly after budbreak (April 2), at forcing pruning dates (June 3 for CF_{early} and July 1 for CF_{late}) and at harvest (September 16 for Control, October 16 for CF_{early} and November 11 for CF_{late}). On each sampling date, one trunk and one root sample (with a diameter between 5-10 mm) were collected from three vines per treatment. Because the sampling procedures were destructive, samples were collected from vines at the edges to avoid affecting vines used for other measurements. Trunk samples were collected with a corer inserted 10 mm from mid trunk heigh for each vine after removing the bark. Two holes per vine were made to ensure that there were sufficient sample for analysis. All samples were stored in a portable refrigerator and quickly transported to the laboratory. At the laboratory, samples were microwaved at 600W for 90 s to inactivate enzymes, as described by Landhäusser et al. (2018), and shortly thereafter oven dried at 65°C to constant weight. To determine soluble sugars concentration, 50 mg of the ground sample was used following a modification of the phenol-sulfuric acid colorimetric method (Buysse and Merckx, 1993). The solid residue of the process was used to determine the starch concentration by using amyloglucosidase to hydrolyze the starch. The starch concentration was determined by spectrophotometry at 490 nm as glucose. The total concentration of non-structural carbohydrates (%DW) in the trunk (TNSC) and root (RNSC) was calculated as the sum of soluble sugars and starch.

2.9 Statistical analysis

A univariate analysis of variance (ANOVA) was performed to reveal differences between treatments ($P< 0.05$). The normal distribution of experimental errors was assessed with Shapiro-Wilk test. Homogeneity of error variances was assessed with Levene's test ($P< 0.05$). Differences between means were determined using the Tukey test. All statistical analyses were performed with JMP14 software (SAS Institute Inc., Cary, NC, 1989-2021).

3 Results

3.1 Weather and phenological data

In 2018, vines were harvested on September 21 (Control), October 26 (CF_{early}) and November 27 (CF_{late}). In 2019, budburst (E-L 4) was observed on March 26 (Control), on June 14 (CF_{early}), 11 days after forcing, and on July 11 (CF_{late}), 10 days after forcing (Figures 1A, B). Veraison (E-L 35) of 50% of berries was observed on July 26 (Control), September 2 (CF_{early}), and October 2 (CF_{late}) while harvest dates were on September 16 (Control), October 16 (CF_{early}), and November 11 (CF_{late}). The period from budburst to veraison lasted 127 days (1112 GDD) for Control, 81 days (1179 GDD) for CF_{early} and 84 days (1082 GDD) for CF_{late} treatments. Average temperatures from budburst to veraison were 18.3, 24.3 and 22.5°C for the

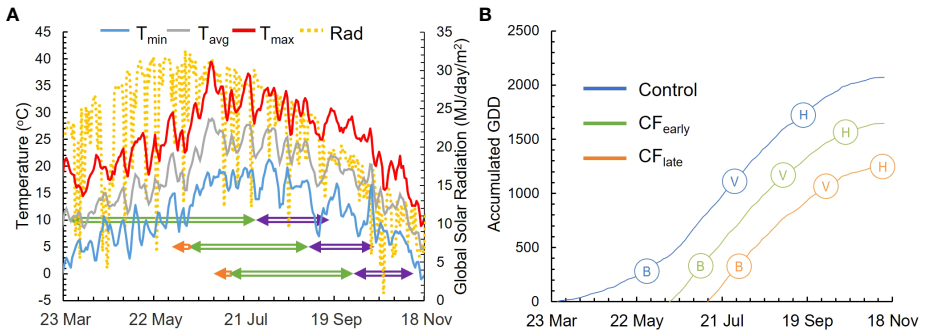

FIGURE 1
Weather and phenological data for season 2019. Daily maximum (red line), mean (grey line), and minimum (blue line) temperatures and daily global solar radiation (dotted yellow line) are represented. Phenological periods represented are from budburst to veraison (green arrowed line) and from veraison to harvest (purple arrowed line). Period from forcing treatment to budburst is also represented (orange arrowed line) **(A)**. The seasonal cumulative GDD for 2019 season for Control (blue line), CF_{early} (green line) and CF_{late} (orange line) **(B)**. Full bloom (B), veraison (V) and harvest (H) are also represented.

Control, CF_{early} and CF_{late} vines, respectively (Figure 1B). Average ripening temperatures were lower in the forcing treatments (22.8, 19 and 14°C for the Control, CF_{early} and CF_{late} vines, respectively). Mean daily global solar radiation was similar between treatments before veraison but was reduced by 18% and 48% for the CF_{early} and the CF_{late} treatments respectively, during the ripening period compared to Control (Figure 1A).

3.2 Vine performance and grape quality

In 2018, vines were severely affected by downy mildew in May and early June which affected some inflorescences and fruits. Therefore, yield was negatively affected in the Control treatment although individual bunch weight at harvest was still higher than in the forced treatments (Table 1). Because downy mildew attack occurred before forcing, yields of the forced treatments were not affected. Forced vines reduced pruning weight by about 40% (Table 1). As a result, the highest Ravaz index was observed in CF_{early} vines because yield was the same as the Control but with lower vigour.

In 2019, both forced treatments reduced yield and yield components compared to the Control, except for the number of bunches per shoot, which was reduced in CF_{early} but not in CF_{late} (Table 1). The CF_{early} exhibited a higher bunch weight due to a higher number of berries per bunch and higher berry weight compared to CF_{late}. Bunch compactness was not affected by the forcing (Table 2). Lower pruning weight was observed only in the CF_{late} treatment (Table 1). Control vines had a higher Ravaz index (Table 1) and a lower LA/fruit ratio than forced vines (Table 2). We did not observe any differences in trunk growth between treatments (Table 2).

Yield was reduced by 39% in CF_{early} but increased 10-fold in CF_{late} in 2019 when compared to 2018. In contrast, pruning weight increased 12.5% in CF_{early} but decreased by 50% in CF_{late} in 2019 when compared to 2018.

Regarding the berry quality traits, differences between treatments were observed in 2018 in TSS because sugar accumulation stopped in November 15 in CF_{late} treatment (data not shown), but not in 2019 (Table 1). An increase in TA was observed in the forced treatments. The later the harvest date, the greater the TA. Except in 2019 when the same pH was observed for the Control and the CF_{early} treatments, the must pH was generally lower in forced treatments.

3.3 Leaf area and fraction of intercepted radiation

Before forcing in 2019, differences in LA were observed between the Control and CF_{early} in the measurement taken on May 20, but not after (Figure 2A). On the other hand, differences in FIPAR were observed between the Control and both forced treatments until the measurement taken on June 3 (Figure 2B). After forcing in 2019, CF_{early} vines reached LA and FIPAR values comparable to Control within approximately one and a half months after budburst (Figures 2A, B). CF_{late} vines failed to reach maximum levels of LA and FIPAR comparable to the other treatments (Figures 2A, B). However, compared to Control, the reduction of LA was higher than the reduction of FIPAR (36% and 26% respectively), which may be related to a sparser canopy (visual observation) in CF_{late} vines.

3.4 Biomass partitioning

Although dry matter partitioned to fruit and shoots differed between 2018 and 2019, the sum of both was maintained in the forced treatments (Table 3). In 2019, biomass dry matter was higher

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TABLE 1 Effects of crop forcing on yield components, grape quality, and vine vigour in 2018 and 2019.

Season	Treatment	TSS ° Brix	TA (g/L)	pH	Bunches per vine (No)	Shoots per vine (No)	Bunches per shoot (No)	Bunch weight (g)	Berryweight (g)	Berriesper bunch (No)	Berriesper vine (No)	Yield (kg/ vine)	Pruning weight (kg/ vine)	Ravaz Index (kg/ kg)
2018	Control	22.4 ± 0.1 b	5.8 ± 0.1 c	3.6 a	17 ± 2 b	22 ± 1 b	0.8 ± 0.1 b	200 ± 9 a	2.39 ± 0.09 a	83 ± 4 a	1455 ± 176b	3.5 ± 0.4a	1.46 ± 0.10a	2.4 ± 0.3b
	CF_{early}	23.8 ± 0.1 a	8.4 ± 0.3 b	3.3 b	33 ± 4 a	28 ± 2 a	1.3 ± 0.1 a	119 ± 6 b	1.62 ± 0.10 c	74 ± 4 b	2435 ± 316a	3.9 ± 0.5a	0.83 ± 0.07b	4.4 ± 0.6a
	CF_{late}	21.6 ± 0.0 c	12.5 ± 0.5 a	3.1 c	5 ± 0 c	22 ± 1 b	0.2 ± 0.0 c	34 ± 10 c	1.23 ± 0.03 b	30 ± 8 c	128 ± 21c	0.2 ± 0.1b	0.89 ± 0.06b	0.2 ± 0.0c
	Significance	*	*	*	*	*	*	*	*	*	*	*	*	*
2019	Control	23.4 ± 0.1	4.7 ± 0.0 c	3.3 a	23 ± 1 b	27 ± 1 b	0.9 ± 0.0 a	387 ± 31 a	2.56 ± 0.05 a	141 ± 14 a	3364 ± 199a	8.7 ± 0.5a	0.99 ± 0.14a	8.8 ± 0.5a
	CF_{early}	23.4 ± 0.1	6.9 ± 0.2 b	3.3 a	19 ± 2 b	38 ± 4 ab	0.5 ± 0.0 b	118 ± 6 b	2.16 ± 0.05 b	55 ± 3 b	1095 ± 154b	2.4 ± 0.3b	0.99 ± 0.21a	2.4 ± 0.4c
	CF_{late}	23.7 ± 0.2	10.2 ± 0.3 a	3.1 b	36 ± 3 a	42 ± 6 a	0.9 ± 0.1 a	58 ± 4 b	1.51 ± 0.02 c	38 ± 3 b	1307 ± 101b	2.0 ± 0.2b	0.42 ± 0.10b	4.8 ± 0.4b
	Significance	ns	*	*	*	*	*	*(*)	*	*(*)	*	*	*	*

Treatment effects were analysed using ANOVA and the means were separated with the Tukey test. Means followed by different letters are different at P< 0.05. Significance levels in brackets correspond to differences only analysing forced treatments through t-test. ns, not significant. *=significant. The grape quality parameter data are the mean of 4 samples (one per each replication) per treatment ± standard error. Yield, bunches per vine, bunch weight, berries per bunch and berries per vine at harvest data are the means of 16 vines per treatment ± standard error. Berry weight is the mean of 50 berries from each of four replications per treatment. Shoots per vine are the means of four vines (one per each replication) per treatment ± standard error. Pruning weight data are the means of 10 vines per treatment ± standard error.

in the Control than for the forced treatments (Table 4). Forcing reduced biomass by 20% and 54% in the CF_{early} and CF_{late} treatments, respectively. After forcing, total biomass was 41% lower in CF_{late} than in CF_{early}. The relative proportion of biomass partitioned among vegetative organs (leaves + shoots) after forcing was 62.2% in CF_{early} and 48% in CF_{late}, compared to 31.5% in Control vines. The CF_{late} vines invested a lower proportion of DM in the shoots, but the same in the leaves than the CF_{early} vines.

3.5 Carry-over effects

Carry-over effects from the 2018 season, such as a lower number of bunches per shoot before forcing, were detected in CF_{early} but not in CF_{late} (Table 2). In addition, LA before forcing was lower in CF_{early} than in the Control vines (Figure 2A) suggesting lower vigour in CF_{early} vines.

3.6 Plant water status, leaf stomatal conductance, leaf net photosynthesis and quantum yield

The Ψ_s (Figure 3A) was always above -0.8 MPa. The only exception was on October 10, after an irrigation failure. Measured g_s (Figure 3B) was above or close to 0.3 mol/m^2/s throughout the growing season. The CF_{late} treatment tended to keep g_s nearly constant throughout the season; the same pattern was observed with Ψ_s.

While the Control and CF_{early} treatments reached their maximum Pn rate (16 µmol/m^2/s) in late July and then Pn began to decline, Pn was constant throughout the season for the CF_{late}, at about 14 µmol/m^2/s (Figure 3C). However, quantum yield followed similar patterns in the three treatments, although lower values were observed for CF_{early} on August 25 and higher values were observed for the forced vines than for the Control at the end of the season, probably because the Control leaves began to senesce (Figure 3D).

3.7 Validation of the canopy net carbon exchange model

As mentioned earlier, whole-canopy gas exchange chambers were used to validate the model. The temperature inside the chamber exceeded the ambient temperature by 7°C in exceptional cases, but rarely exceeded 35°C. The maximum vapour pressure deficit recorded in the chamber was 4.6 kPa but remained below 3.5 kPa most of the time (see Supplementary Table 4 for more information on chamber conditions). Canopy NCE_{ch} values were between 4 and 12 µmol/m^2/s (see more information about chamber results in Supplementary Table 5).

TABLE 2 Effects of crop forcing on number of bunches per shoot before forcing (before fruit thinning and shoot removal for the Control treatment), bunch compactness at harvest, LA/fruit ratio and TSCA in 2019.

Parameter	Control	CF_{early}	CF_{late}	Significance level
Number of bunches per shoot before forcing (No)	1.7 ± 0.2a	0.8 ± 0.0b	1.5 ± 0.1a	*
Bunch compactness (g/g)	26.8 ± 0.7	29.6 ± 1.3	30.7 ± 1.0	ns
LA/fruit (cm^2/g)	7.4 ± 0.7c	35.3 ± 2.8a	22.7 ± 2.5b	*
ΔTCSA (cm^2/vine)	3.3 ± 0.7	3.1 ± 0.7	2.7 ± 0.2	ns

Treatment effects were analysed using ANOVA and the means were separated with the Tukey test. Means followed by different letters are different at P< 0.05. ns, not significant. *=significant. Bunch compactness is the mean of 10 bunches ± standard error. LA/fruit ratio and ΔTCSA data are the means of four vines (one per replication) per treatment ± standard error.

From May 29 to September 26, 14 different whole-canopy NCE_{ch} measurements were obtained and used to validate the model. Not all measurements included the entire day. The inputs used to run the model the same days on which the chambers were operated are summarized in Supplementary Table 6. The NCE_m correlated well with NCE_{ch} measurements (Supplementary Figure 3) regardless of the treatment. NCE_{ch} was well explained by the model (R^2 = 0.95), and adequately adjusted to the 1:1 line.

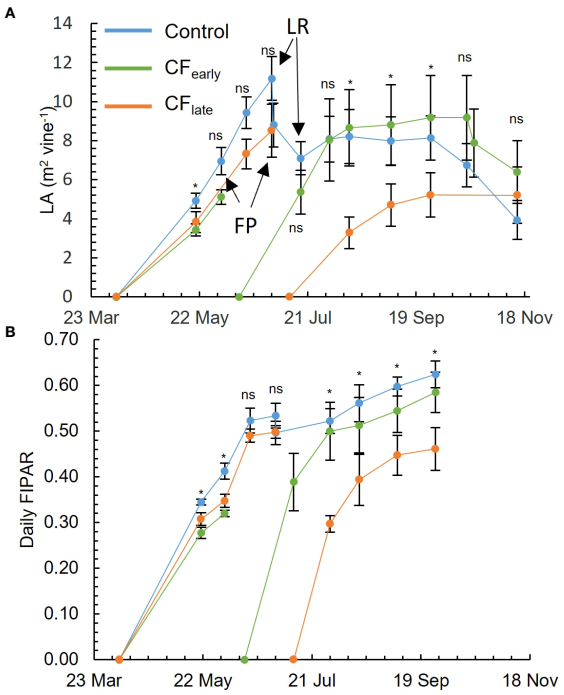

FIGURE 2
Seasonal evolution for 2019 of leaf area (LA). FP, forcing pruning; LR, leaf and shoot removal, only affecting control treatment **(A)**. Daily fraction of intercepted radiation (FIPAR) evolution for each treatment modelled according to the Oyarzun et al. (2007) model **(B)**. Blue, green, and orange lines correspond to Control, CF_{early} and CF_{late} treatments, respectively. Each plot is the average of four vines for each treatment. Bars mean standard deviation. Treatment effects were analysed using ANOVA at P< 0.05. ns, not significant; *=significant.

The NSE was 0.95 indicating a good model performance, and the error was low (RMSE = 5.8 g CO_2/vine/day). Typical and representative days of the regional summer weather and high levels of variability in canopy size and crop phenology were covered. On average, the estimated cumulative Pn_{canopy} was 15348, 10924 and 10201 and cumulative respiration was 3159, 2335 and 1926 g CO_2/vine for Control, $CF_{early,}$ and CF_{late} respectively (Figures 4A, B).

3.8 Daily and seasonal carbon balance

Before forcing, Pn_{canopy} and respiration (R) did not differ between treatments (Figures 4A, B). However, after forcing, carbon loss by respiration was 21.8% and 16.8% for CF_{early} and CF_{late} (P< 0.05), respectively. NCE_m was 6 g CO_2/day higher in Control than in CF_{early} when comparing the time before forcing between the Control and CF treatments. However, there were no significant differences in CF_{late}, compared with Control. Note that the period before forcing in CF_{early} ranged from March 26 to June 3, whereas in CF_{late} it ranged from March 26 to July 1, 28 days longer. Forcing reduced NCE_m to zero or below zero for 17 (CF_{early}) and 13 (CF_{late}) days. CF_{early} vines reached the maximum NCE_m 55 days post-forcing and CF_{late} 41 days. In Control vines, NCE_m averaged 60.9 g CO_2/day from budburst (March 26) to harvest (September 16). Similar NCE_m was observed in CF_{early} vines from forced budburst (June 14) to harvest (October 16), with an average of 55.9 g CO_2/day (Figure 4C). However, from budburst to harvest (from July 11 to November 11), average NCE_m was lower in CF_{late} (38.7 g CO_2/vine/day) than the other two treatments. When NCE_m were normalized by leaf area, higher NCE/LA was observed only for 10 days in CF_{early} vines. In contrast, CF_{late} had the highest NCE/LA values for 46 days after forcing treatments (Figure 4D). In the CF_{late} treatment, maximum NCE/LA was 24.5 g CO_2/m^2/day compared with 19.7 g CO_2/m^2/day in CF_{early} and 15.5 g CO_2/m^2/day in Control.

Pn_{canopy} and NCE_m began to decline after August 11 in CF_{early} (Figure 4A), which was due to a sharp decline in leaf photosynthesis and quantum yield (Figures 3C, D). This decline was milder in Control vines and delayed until September 8 in

TABLE 3 Effects of crop forcing on dry matter partitioned to fruit and shoots between years 2018 and 2019.

	Year	Control	CF_{early}	CF_{late}
Fruit DM (g)	2018	808 ± 65	1008 ± 16	50 ±7
	2019	2541 ± 249	686 ± 170	551 ± 72
Significance		*	ns	*
Shoot DM (g)	2018	786 ± 56	450 ± 16	486 ± 19
	2019	532 ± 75	536 ± 114	224 ± 53
Significance		*	ns	*
Fruit + Shoot DM (g)	2018	1593 ± 107	1458 ± 25	536 ± 24
	2019	3073 ± 296	1222 ± 282	776 ± 115
Significance		*	ns	ns

Treatment effects were analysed using t-test at P< 0.05. ns, not significant. *=significant. Data are the means of four replicates ± standard error.

CF_{late}. During berry ripening, average NCE_m levels differed among the three treatments ($P< 0.05$). Higher NCE_m levels were observed in Control (85.1 g CO_2/day) than in CF_{early} (45.8 g CO_2/day) and CF_{late} (19.7 g CO_2/day) (Figure 4C).

Throughout the season, $CumNCE_m$ was higher in Control vines than in the two forced treatments, which accumulated a similar amount of carbon between them (Figure 4C). Prior to forcing, CF_{late} vines accumulated 37% more carbon than CF_{early} vines. However, after forcing, lower $CumNCE_m$ was found in CF_{late} vines than CF_{early} (33%). From budburst (after forcing in the forced treatments) to harvest, $CumNCE_m$ differed greatly between treatments, with values of 10672 g CO_2/vine in Control, 6883 g CO_2/vine in CF_{early} and 4763 g CO_2/vine in CF_{late} (Figure 5). Forced vines reduced CumNCE by 60-70% from budburst to full bloom. From veraison to harvest, CF_{late} vines accumulated 60% less carbon than CF_{early} and 81% less than Control vines (Figure 5).

3.9 Carbohydrate reserves

At budburst, $TNSC_{initial}$ was lower in CF_{early} vines (Table 5). At forcing dates, both forced treatments reduced TNSC (0.9 %DW CF_{early} and 8.2 %DW CF_{late}) and RNSC (2.2 %DW CF_{early} and 1 %DW CF_{late}) compared to pre-forcing budburst level (March 26). Moreover, at forcing dates, forced vines had lower TNSC than the Control vines at budburst, and TNSC was lower in CF_{late} than in CF_{early}. At harvest, no differences in RNSC were observed between treatments, which may be attributable to the high variability of the Control treatment. However, in CF_{early} RNSC was 20% higher than in Control and 18% higher than in CF_{late}. From forcing to harvest, CF_{early} increased both TNSC (6.2 %DW) and RNSC (5 %DW) whereas CF_{late} increased TNSC (7.7 %DW) but slightly decreased RNSC (0.8 %DW).

TABLE 4 Biomass removed and distribution on forcing dates and at the end of the season in year 2019.

Date	Treatment	B_t (g DW/vine)	%fruit	%leaves	%shoots
3 June	Control	714 ± 79	7.2 ± 0.9	69.6 ± 2.6	23.2 ± 1.7
	CF_{early}	505 ± 46	2.4 ± 0.3	70.3 ± 3.2	27.3 ± 3.5
Significance level		*	*	ns	ns
1 July	Control	1737 ± 168	33.2 ± 3.3	47.2 ± 1.7	19.6 ± 1.8
	CF_{late}	1318 ± 255	34.5 ± 1.0	49.0 ± 2.0	16.5 ± 1.1
Significance level		ns	ns	ns	ns
Whole season	Control	4284 ± 478 a	68.5 ± 1.8 a	17.9 ± 0.9 b	13.6 ± 1.0b
	CF_{early}	2453 ± 548 b	29.9 ± 3.1 b	42.0 ± 2.1 a	28.1 ± 1.3a
	CF_{late}	2844 ± 623 b	42.3 ± 3.0 b	39.6 ± 2.7 a	18.1 ± 1.0b
Significance level		*	*	*	*
Forced season	CF_{early}	1947 ± 507 b	37.8 ± 2.6 c	33.1 ± 1.6 a	29.1 ± 2.6a
	CF_{late}	1156 ± 293 c	52.0 ± 5.1 b	29.8 ± 3.7 a	18.2 ± 1.8b
Significance level		*	ns	ns	*

Treatment effects were analysed using ANOVA and the means were separated with the Tukey test. Means followed by different letters are different at P< 0.05. ns, not significant. *=significant. Data are the means of four replicates ± standard error. "Whole season" includes from March 26 to November 12 for all treatments. "Forced season" involves the second cycle of the forced treatments (from forced budburst to November 12). The "Forced season" Tukey test was carried out comparing to "Whole season" Control. Significance level under "Forced season" only refers to differences between both forced treatments using ANOVA.

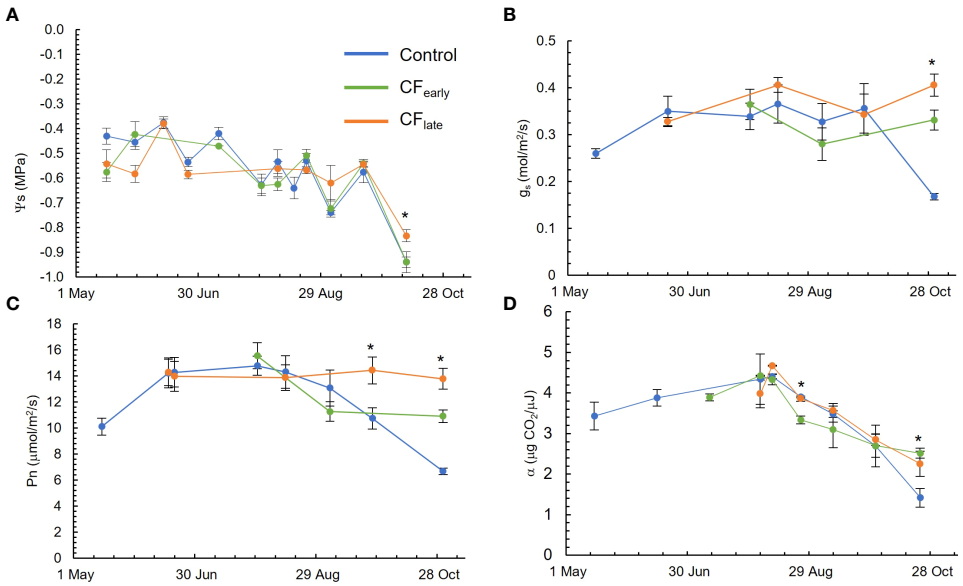

FIGURE 3
Seasonal evolution of stem water potential (Ψ_s) **(A)**, leaf stomatal conductance at midday (g_s) **(B)**, sunlit leaf net photosynthesis at midday (Pn) **(C)** and quantum yield (α) **(D)** in the 2019 season. Blue, green, and orange lines correspond to the Control, CF_{early} and CF_{late} treatments, respectively. Bars indicate standard deviation of eight measurements excepting for quantum yield in which three measurements were carried out. * indicates statistical differences using ANOVA ($P < 0.05$).

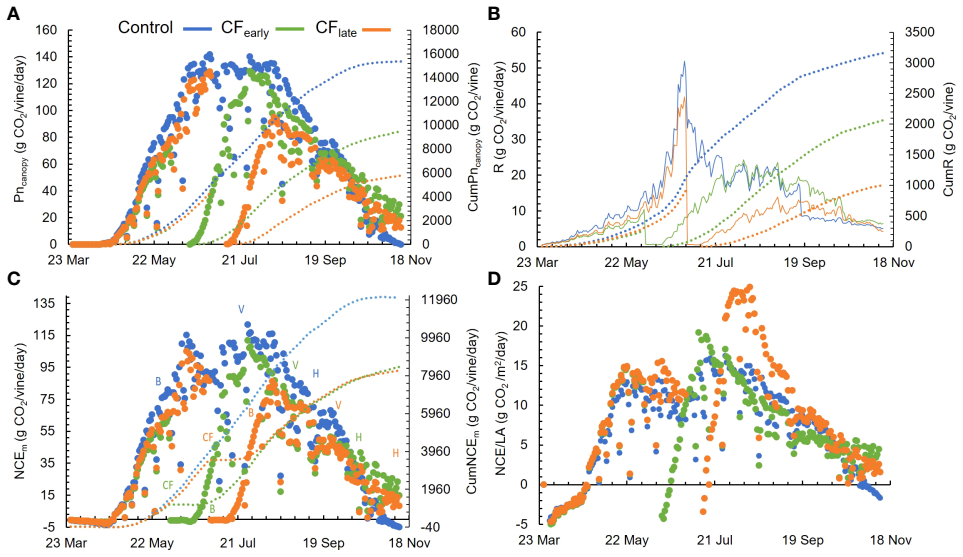

FIGURE 4
Seasonal patterns for daily modelled whole-canopy net photosynthesis (Pn_{canopy}) and cumulative Pn_{canopy} ($CumPn_{canopy}$) **(A)**, daily (R) and cumulative (CumR) above ground modelled respiration **(B)**, daily (NCE_m) and cumulative ($CumNCE_m$) net carbon exchange **(C)** and daily modelled NCE_m per unit of leaf area (NCE/LA) **(D)**. B, bloom; V, veraison; H, harvest. Dotted lines represent the cumulated parameters. Blue, green, and orange colours correspond to the Control, CF_{early} and CF_{late} treatments. Each plot is the average of modelling four vines for each treatment in 2019.

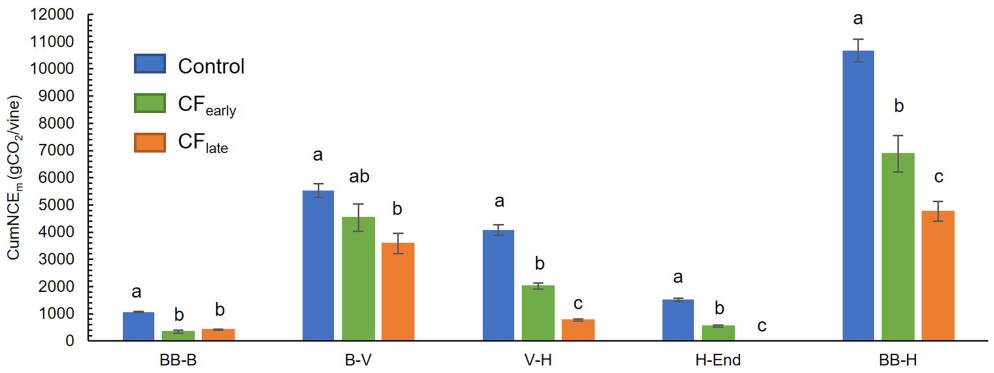

FIGURE 5
Cumulative net carbon exchange (CumNCE$_m$) between two different phenological stages in 2019. Forced treatments included only post-forcing data. BB, bud burst; B, bloom; FP, forcing pruning; V, Veraison; H, harvest; End, end of season. Blue, green, and orange correspond to Control, CF$_{early}$ and CF$_{late}$ treatments, respectively. Each column indicates the mean of four vines. Bars indicate standard deviation. Treatment effects were analysed using ANOVA and the means were separated with the Tukey test. Means followed by different letters are significantly different at $P < 0.05$.

4 Discussion

4.1 Leaf area and light interception

In contrast to the CF$_{late}$ vines, after forcing, the CF$_{early}$ vines recovered leaf area and daily fraction of intercepted radiation that were comparable to those of the Control (Figures 2A, B). However, leaf and shoot removal, lateral trimming and hedging were performed on the Control vines but not on the forced treatments, limiting potential vegetative growth on the Control vines. These observations are consistent with other studies reporting reduced vegetative growth in forced vines (Gu et al., 2012; Martinez De Toda et al., 2019; Martínez-Moreno et al., 2019), and the later the forcing occurred, the lower the leaf area (Martinez De Toda et al., 2019). Reduced growth performance in forced treatments has been attributed to lower carbon reserve status in forced vines (Martínez-Moreno et al., 2019) as early growth stages are dependent on carbon reserves (Smith and Holzapfel, 2009; Holzapfel et al., 2010). Our observation of lower TNSC at forcing date (Table 5) and lower vigour (Figure 2A) in CF$_{late}$ than in CF$_{early}$, supports this hypothesis.

4.2 Vine water status and leaf physiological parameters

We did not observe a significant difference in water status between treatments. Stem water potential was above the threshold of -0.8 MPa that we established to avoid limiting photosynthesis (Marsal et al., 2008). In addition, leaf stomatal conductance was greater than 0.3 mol/m^2/s, which did not limit leaf net photosynthesis (Escalona et al., 1999). Therefore, we excluded water stress as a cause of photosynthesis limitation. Because younger leaves (less than 100 days) have higher photosynthetic rates than older leaves (Poni et al., 1994), we expected higher leaf net photosynthesis rates in both forced treatments. In CF$_{late}$, leaf net photosynthetic rates were constant until October (Figure 3C), indicating that leaves remained more active. In contrast, this effect was not observed in CF$_{early}$ leaves (Figure 3C). In vines with a high LA/fruit ratio, leaf net photosynthesis tends to decrease (Iacono et al., 1995), which may be attributed to feedback inhibition in response to low sink activity (Paul and Foyer, 2001). Therefore, feedback inhibition may explain the lower leaf net photosynthesis observed in CF$_{early}$ leaves because the LA/fruit ratio was extremely high. The quantum yield levels we measured (Figure 3D) were in the range (0 to 4 µg CO_2/J) of those previously reported for grapevine (Poni et al., 2006; Mirás-Avalos et al., 2018). However, more significant differences in the seasonal quantum yield patterns due to the treatments were expected.

4.3 Net carbon exchange NCE model (NCE$_m$) and carbohydrate reserves

4.3.1 Validation of the model

The model showed high correlation with the chamber measurements and had an acceptable error (Supplementary Figure 3). Modelled and measured NCE values were within the range of values reported by other studies on Cabernet Sauvignon (Poni et al., 2006) and Tempranillo (Pagay, 2016). Total above ground respiration losses were 23.2%, 20.7% and 18.4% of the Pn$_{canopy}$ for the Control, CF$_{early}$, and CF$_{late}$, respectively (Figures 4A, B). These values are close to the 25% reported for Cabernet Sauvignon (Poni et al., 2006) and the 19.3% for Tempranillo cultivar (Medrano et al., 2015). Therefore, the

TABLE 5 Total non-structural carbohydrate concentration in trunk (TNSC) and roots (RNSC) at budburst (initial), forcing date (forcing) and at harvest (harvest) for Control and forced treatments in 2019.

Parameter	Control	CF_{early}	CF_{late}	Significance level
$TNSC_{initial}$ (%DW)	16.0 ± 0.3 a	12.3 ± 0.1 b	16.3 ± 1.1 a	*
$TNSC_{forcing}$ (%DW)	16.0 ± 0.3 a	11.2 ± 0.9 b	8.1 ± 0.6 c	*
$TNSC_{harvest}$ (%DW)	20.2 ± 0.2	17.4 ± 3.1	15.8 ± 2.3	ns
$RNSC_{initial}$ (%DW)	37.2 ± 4.6	34.6 ± 1.3	32.5 ± 3.3	ns
$RNSC_{forcing}$ (%DW)	37.2 ± 4.6	32.4 ± 1.1	31.5 ± 2.3	ns
$RNSC_{harvest}$ (%DW)	29.7 ± 6.5	42.7 ± 0.3	30.7 ± 3.6	ns(*)

Treatment effects were analysed using ANOVA and the means were separated with the Tukey test. Means followed by different letters are different at $P< 0.05$. Significance levels in brackets correspond to differences only analysing forced treatments. ns, not significant. *=significant. $TNSC_{initial}$ and $RNSC_{initial}$ was sampled on April 2 for all treatments. For the Control treatment, $TNSC_{forcing}$= $TNSC_{initial}$ and $RNSC_{forcing}$ = $RNSC_{initial}$. TNSC data are means of three vines ± standard.

model can be considered a realistic tool for carbon balance analysis.

4.3.2 Whole canopy net carbon exchange dynamics

As we suspected, the crop forcing technique affected daily and seasonal net carbon exchange patterns. The capacity to accumulate carbon in a forced season (from forced budburst to November 12) is reduced (Figure 4C) mainly because forced seasons are shorter than non-forced. In addition, photosynthesis ceased in forced vines due to the forcing, and a recovery period of approximately 45 days was required to reach maximum canopy photosynthesis and net carbon exchange (NCE_m) rates (Figure 4C). Since whole-canopy net carbon exchange is proportional to the radiation intercepted by the canopy (Poni et al., 2003; Petrie et al., 2009) maximum daily NCE_m was recovered in CF_{early} but not in CF_{late} (Figure 4D), which also negatively affected the cumulative NCE_m for the forced season (Figure 5).

The initial FIPAR and LA in 2019 were slightly greater in Control compared to the forced treatments (Figures 2A, B). This might have biased the result presented in Figure 5. A larger LA at the begging of the season should result in a larger NCE_m before the forcing. However, we found non-significant differences in LA and FIPAR (Figures 2A, B) from June to CF_{late} forcing date. Besides, the trunk diameter of all vines was measured right before the begging of the experiment in 2017. Trunk diameters were 38.0 cm (Control), 41.2 cm (CF_{early}) and 40.9 cm (CF_{late}). No statistical differences were observed between treatments (P = 0.28) (Supplementary Table 1). The treatments were severe enough (removal of all leaves and bunches) to overcome the experimental error in LA and FIPAR. All in all, confirms that plants presented the same vigour at the beginning of the experiment. Therefore, the differences observed in Figure 5 were attributed to the treatments imposed than to an experimental error.

In a fruit removal experiment, net carbon exchange at a canopy level was reduced in vines without fruits compared to vines with fruit load (Petrie et al., 2000). Because leaf net photosynthesis is an input to the whole canopy net carbon exchange model, the decline in leaf net photosynthesis observed in CF_{early}, which we attributed to the extremely high LA/fruit ratio, was also observed by the whole-canopy model (Figure 4C). In a forced double cropping experiment, NCE/LA was reported to be higher in forced vines than in unforced vines due to the younger canopy (Poni et al., 2021). However, these vines had a LA/fruit ratio of 2 m^2/kg because primary crop was not removed at forcing, which is close to the LA/fruit ratio we observed in CF_{late} but significantly lower than that observed in CF_{early} (Table 2). Therefore, after vegetative growth slowed down in CF_{early} (in August), the sink activity of the fruit was not sufficient to maintain a high NCE_m rate and, NCE/LA was similar to the Control despite the younger canopy (Figures 4C, D). On the other hand, respiration (R) is a function of biomass (Amthor, 2000). By partitioning a lower proportion of biomass to shoots and maintaining the proportion of leaves (Table 4), CF_{late} vines improved the daily carbon balance and increased the photosynthesis/respiration ratio. Thus, higher NCE/LA for CF_{late} (Figure 4D), at least until mid-October when CF_{early} vines lost some of LA (Figure 2A) increasing NCE/LA for this treatment, could therefore be explained by a higher proportion of sunlit leaves due to a sparser canopy, improved daily carbon balance and to a higher leaf photosynthetic activity. In a late-pruning experiment, vines in which budburst was delayed by 31 days, the same delay we observed between CF_{early} and CF_{late}, were able to compensate for the accumulated carbon budget of traditionally managed vines by increasing the NCE/LA ratio (Gatti et al., 2016). In our experiment, higher canopy efficiency in CF_{late} (Figure 4D) could not compensate for the carbon accumulation (Figure 5). This was partly because canopy photosynthesis was limited by the reduced leaf area of the vines but also because solar radiation and daylength decreased rapidly from September onward (Figure 1A). Delaying harvest until mid-November therefore drastically reduces the capacity to gain carbon from veraison to harvest (Figure 5), although CF_{late} vines could reach TSS comparable to the other treatments (Table 1) likely using previously accumulated carbohydrate reserves.

4.3.3 Carbohydrate reserves: trunk and root non-structural carbohydrates

Regarding the carbohydrate reserve analysis, the concentrations observed in trunk and roots were close to other values reported for fully irrigated vines in hot climates (maximum TNSC of 20 %DW and RNSC of 40 %DW) (Smith and Holzapfel, 2009; Holzapfel et al., 2010). The lower carbohydrate reserves observed at forcing dates compared to the time of budburst (March 26) (Table 5) indicated that the NCE accumulated prior to forcing was insufficient to replenish carbohydrates reserves and, therefore, the capacity to refill carbohydrate storage at the whole-vine level depends mainly on the photosynthetic capacity from forcing to the end of season. These results are consistent with previous reports in which the minimum of carbohydrate reserves was observed between full bloom and one month later (Zapata et al., 2004; Bennett et al., 2005; Holzapfel et al., 2010; Zufferey et al., 2012) and are supported by the fact that, although cumulative net carbon exchange was the same for both forced treatments for the whole season (Figure 4C), the RNSC was lower at harvest for CF_{late} because the capacity to accumulate carbon is reduced in the CF_{late} forced season (Figure 5). However, LA/fruit indicates a highly favourable source:sink relationship for forced vines, especially for CF_{early} (Table 2), which can be confirmed by calculating NCE_m/yield (3.6 g CO_2/g yield for Control, 9.14 g CO_2/g yield for CF_{early}, and 7.84 g CO_2/g yield CF_{late}). As a result, at harvest, forced treatments increased carbohydrate reserves (CF_{early}) or maintained them (CF_{late}) compared to pre-forcing budburst (sampled on April 2) (Table 5), compensating for the lower seasonal cumulative NCE_m (Figure 4C). The large increase in carbohydrate reserves from forced budburst to harvest in the CF_{early} treatment supports the hypothesis of feedback inhibition of photosynthesis acting carbohydrate storage organs as major carbon sinks, despite being among the lowest priority sink (Minchin and Lacointe, 2005). On the other hand, the Control treatment reduced RNSC by 8% compared to budburst (Table 5), which is in accordance with other studies which revealed that carbon reserves in roots, are very sensitive to source:sink relationships and fruit yield (Smith and Holzapfel, 2009; Zufferey et al., 2012).

4.4 Agronomic implications of the crop forcing technique related to carbon availability

In 2018, the CF_{late} treatment was applied too late and did not reach the target TSS (Table 1). However, in our experiment, the main objective of the crop forcing technique which was delaying berry ripening in a cooler environment (Figure 1A) and harvesting at the desired TSS but higher acidity, was achieved, although yield was reduced (Table 1). Under different environmental conditions, with different cultivars and irrigation strategies, a reduced bunch weight, due to a lower number of berries per bunch and smaller berry size, is a characteristic of forced vines (Gu et al., 2012; Lavado et al., 2019; Martinez De Toda et al., 2019; Martínez-Moreno et al., 2019; Pou et al., 2019) which is consistent with our results (Table 1). Low carbon availability at the beginning of the berry development is crucial for the final berry weight, since an imbalance at that time cannot be compensated later (Candolfi-Vasconcelos and Koblet, 1990). Besides, the number of berries per bunch is highly sensitive to carbon stress (Bennett et al., 2005), as carbohydrates are required for proper flower formation (Lebon et al., 2008) although elevated temperatures around budburst may also have the same effect (Petrie and Clingeleffer, 2005; Pagay and Collins, 2017). In addition to the observed decrease in trunk carbon reserves at forcing (Table 5), the period from budburst to full bloom was reduced from 65 days in the Control to 23 days in the forced treatments (Figure 1B), resulting in a reduced capacity to provide carbon (Figure 5) for flower formation and the first stages of the berry development. However, our results do not allow to distinguish between carbon or temperature stress or, the most likely hypothesis, a combination of both stress factors as the cause of the large decrease in bunch weight. Since bunch compactness was not reduced in the forced treatments (Table 2), we ruled out a reduction in fruit set percentage.

In the crop forcing technique, vines that are forced early in the season (e.g. around bloom and fruit set) are generally more sensitive to a reduction in the number of bunches per shoot suggesting an incomplete formation of the inflorescence primordia (Gu et al., 2012; Martinez De Toda et al., 2019; Martínez-Moreno et al., 2019). This is consistent with our observations of a reduced number of bunches per shoot in CF_{early} but not in CF_{late} in 2019 (Tables 1 and 2). However, we did not observe the same effect in the season 2018 probably because the treatment was applied 11 days later than in 2019. Therefore, the timing of forcing is a relevant aspect in forcing performance as reported in previous experiments (Martinez De Toda et al., 2019; Martínez-Moreno et al., 2019). In addition, in forcing treatments, inflorescence formation, which is highly sensitive to carbon availability (Keller and Koblet, 1995; Sánchez and Dokoozlian, 2005; Lebon et al., 2008), occurred partly before forcing and partly after forcing, shortly after carbon assimilation ceased. Vines forced earlier have a lower capacity to accumulate carbon before forcing (Figure 4C), and after a high yield season, as was the case with CF_{early} in 2018 (Table 1), the level of carbohydrate reserves may be lower at non-forced budburst (Table 5). Note that CF_{early} reduced the number of bunches per shoot even before the forcing treatment (Table 2). Therefore, the state of carbon reserves at the time of non-forced budburst, which depends on the photosynthetic capacity and source-sink relationship in the previous year (Lebon et al., 2008) and at forcing, is a determining factor for the number of forced bunches per shoot.

Since biomass in forced vines was quite conservative from year to year (Table 3), an increase in yield implies a decrease in pruning weight (Table 1), which is closely related to vine photosynthetic capacity (Kliewer and Dokoozlian, 2005). Therefore, high yields in forced vines must lead to a reduction in carbon reserves for the following season because both, reduced carbon assimilation and a stronger fruit sink activity. However, in a climate change scenario, increased temperatures extend the growing season. Together with the expected increase in ambient CO_2 concentration, higher photosynthetic capacity of vines as well as higher yields and vine vigour are expected (Kizildeniz et al., 2015). The yield reduction associated to the crop forcing technique could be milder and wine producers could consider crop forcing as a less risky tool to produce high quality wines. On the other hand, the crop forcing technique is already a valuable tool for research. Recently, this technique has been successfully used to study and validate the robustness of vine development models (Prats-Llinàs et al., 2020).

As far as we know, there is only one other technique that can delay harvest by two months on grapevines. This technique, double cropping, is a version of forced regrowth in which the primary crop and the leaves of the six basal nodes are retained at the time of forcing. It was validated in Pinot Noir in two consecutive years, (Poni et al., 2021) and in Tempranillo in only one season (Martínez De Toda, 2021) demonstrating that dormant buds are capable to break dormancy without removing all the leaves. Double cropping overcame the yield reduction observed with the crop forcing technique (Lavado et al., 2019; Martinez De Toda et al., 2019; Martínez-Moreno et al., 2019; Pou et al., 2019), as the sum of primary and forced yields was even higher than in the non-forced vines (Martínez De Toda, 2021; Poni et al., 2021). Moreover, the carbon balance of the vine was even improved in forced vines as the leaf area was substantially restored with younger leaves, which ended up in a more functional canopy than the canopy of the unforced vines (Poni et al., 2021). In addition, unlike our experiment in which NCE_m was reduced to zero for about two weeks after forcing (Figure 4C), in Poni et al. (2021) the reduction of NCE soon after forcing was only about 55% of the pre-forcing NCE. Therefore, the retained basal leaves continued to provide sugars to the entire vine during the interval between forcing and the emergence of new functional leaf area which, probably, helped to drive regrowth of the forced shoots and minimize the depletion of carbohydrate reserves of the permanent organs of the vine. It is relevant that, unlike our experiment for CF_{early} treatment, no carry-over effects were observed in vines in which double cropping was applied, which can be related to a better vine carbohydrate status soon after forcing. However, it should be noted that the experiment of Poni et al. (2021) was conducted with an early cultivar following the quality criteria for sparkling wine, on potted vines and with site-specific conditions different from those of our experiment. Therefore, further research is necessary to explore the promising benefits of retaining leaves and fruits on forced vines in medium to late ripening cultivars and in field conditions.

5 Conclusions

Our study confirms that the forcing technique has a negative impact on the seasonal carbon balance under our experimental conditions. The shorter season, smaller vines, and the environmental conditions at the end of the season limit the seasonal carbon balance. However, the capacity to restore carbohydrate reserves after forcing was demonstrated in early and late forcing dates. Therefore, the yield reduction seems to be a necessary strategy to increase the source:sink ratio, allowing the forced vines to restore carbon reserves at the whole-canopy level. Because the state of carbon reserves before and at the time of forcing plays an important role in forced yield, techniques that can modulate carbon reserve dynamics applied before forcing, such as mild water stress or sink organs removal (e.g. fruit removal) would be of interest for improving the carbon availability in the forced season. However, after forcing, any viticultural practises that restrict carbon assimilation and vegetative growth must be used with caution, as they may affect the carbon reserves for the next season. Of particular interest is the possibility of not removing all the leaves when vines are forced to provide carbon for shoot regrowth, as well as not removing all the bunches to counteract the drastic reduction in yield of the forced vines to make this technique more acceptable to winegrowers.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

JG and OG-T conceived, planned, and supervised this study. JO-M contributed to the planning of the experiment. JO-M and MA developed the net carbon exchange chambers and realized most of the field measurements and tasks. JO-M did the processing and analysis of all the data and drafted and finalized the manuscript. JG, OG-T and MA reviewed the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by funds from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) research project RTA2015-00089-C02-02 and from the H2020 programme project Vineyards´ Integrated Smart Climate. Application (VISCA) 730253. The participation of Jori Oliver-Manera was founded by the Ministerio de Ciencia e Innovación in an Instituto Nacional de Investigación fellowship BES-2017-082089.

Acknowledgments

The authors would like to thank Mercè Mata, Jesús del Campo, Pinelopi Mavropouli, Konstantina Moschou, Arnau Roig, Ana Pelechá and Christian Jorfré for their hard work in the field and Dr Jaume Casadesús and Dr Joaquim Bellvert for their scientific advice. Special thanks to Raïmat Wineries for their support throughout the experiment.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.998910/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 09 December 2022
DOI 10.3389/fpls.2022.1085878

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

RECEIVED 31 October 2022
ACCEPTED 23 November 2022
PUBLISHED 09 December 2022

CITATION
Wohlfahrt Y, Krüger K, Papsdorf D, Tittmann S and Stoll M (2022) Grapevine leaf physiology and morphological characteristics to elevated CO_2 in the VineyardFACE (Free air Carbon dioxide Enrichment) experiment. *Front. Plant Sci.* 13:1085878. doi: 10.3389/fpls.2022.1085878

Grapevine leaf physiology and morphological characteristics to elevated CO_2 in the VineyardFACE (Free air Carbon dioxide Enrichment) experiment

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Atmospheric carbon dioxide (CO_2) concentration has continuously increased since pre-industrial times and has currently reached an average growth rate of 2.3 ppm per year. For the majority of plant species elevated CO_2 (eCO_2) improves photosynthesis and thus plant biomass production. To investigate the effects of eCO_2 on leaf physiology and morphological leaf characteristics two *Vitis vinifera* L. cultivars, Riesling and Cabernet Sauvignon, grown in the VineyardFACE (Free Air Carbon dioxide Enrichment) system were used. The VineyardFACE is located at Geisenheim, Rheingau comparing future atmospheric CO_2-concentrations (eCO_2, predicted for the mid-21st century) with current ambient CO_2-conditions (aCO_2). Experiments were operated under rain-fed conditions for two consecutive years (2015 and 2016). For both varieties and CO_2 treatments, leaf gas exchange measurements were performed as well as measures of epidermal flavonoid (Flav) and leaf chlorophyll (Chl) indices by using a portable leaf clip. Furthermore, leaves were sampled for spectrophotometric analysis of the leaf pigments chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid (Car). Additionally, leaf cross-sections were produced as permanent preparations to investigate morphological characteristics of the leaf structure. Both cultivars did not differ in leaf chlorophyll meter readings or leaf pigments between the two CO_2 treatments while net assimilation was highly stimulated under elevated CO_2 for both seasons. Differences found in leaf cross-sections were detected in palisade parenchyma and epidermal thickness of Cabernet Sauvignon under eCO_2, whereas Riesling net assimilation increased by 40% under a 20% CO_2 enrichment while remaining unaffected in different leaf layer thickness. The observed results within grapevine leaf tissues provide insights to seasonal adaptation strategies of grapevines under elevated CO_2 concentrations predicted in future.

KEYWORDS

leaf morphology, chlorophyll, *Vitis vinifera*, carbon dioxide, leaf physiology, histology, FACE (Free Air CO_2 Enrichment)

Introduction

Atmospheric carbon dioxide, one of the most relevant greenhouse gases has been increasing continuously since pre-industrial times from 280 ppm in 1750, and is predicted to exceed 700 ppm by the end of 21st century (IPCC, 2021). This accumulation of CO_2 - among other air pollutants in the atmosphere - leads to a changed re-radiative effect and thus to an increase in global mean surface temperature - widely known as global warming. Besides that, high-pressure "blocking" weather systems (Davini and D'Andrea, 2020), an altered wind frequency and a shifting precipitation pattern are also consequences of a worldwide changing climate with an increasing intensity of extreme weather events (Manning and Tiedemann, 1995).

Plant and ecosystem performance is influenced by increasing CO_2 levels leading to a modified plant physiology and thus to altered plant growth as well as developmental changes. For most of C_3 plant species, elevated CO_2 improves the photosynthetic apparatus resulting in an increased plant biomass production (Reddy et al., 2010) – in both – vegetative and reproductive performance. Besides agricultural crops, various CO_2 enrichment experiments have been conducted worldwide for various plant types with CO_2 effects on plant growth and ecosystems *via* a multitude of mechanisms (Ainsworth and Long, 2005). The up-regulation of photosynthesis under elevated CO_2 as one main outcome is reported for most plant types. Likewise, water use efficiency, which is referred to net assimilation related to either transpiration or stomatal conductance, is shown to be improved under eCO_2 conditions. Carbon metabolism in C_3 plants is promoted under eCO_2 due to higher carboxylation rates by RuBisCO and together with higher net assimilation rates are accountable for an enhanced biomass production.

Field studies on grapevines under elevated CO_2 conditions that have been conducted are rare, showing higher yield and vegetative growth due to enhanced net assimilation rates (Bindi et al., 2001; Moutinho-Pereira et al., 2009; Edwards et al., 2017). Furthermore, in a previous study emerged from the VineyardFACE, Riesling and Cabernet Sauvignon resulted in higher lateral leaf area and leaf biomass, as well as increased bunch and berry weight under elevated CO_2 concentrations (Wohlfahrt et al., 2018). Crop yield of Riesling showed a 10.4% (2015) and 17.8% (2016) increase under eCO_2 and Cabernet Sauvignon gained 17.3% (2015) and 10.1% (2016) higher yield under eCO_2. Effects on grapevine leaf transpiration and stomatal conductance are distinct, but most of the times the water demand decreased under eCO_2 conditions when vines were mature at an age of 9 up to 20 years (Bindi et al., 2005; Tognetti et al., 2005; Moutinho-Pereira et al., 2009; Edwards et al., 2016; Edwards et al., 2017). Younger vines, at an age of 4 to 6 years showed a higher water consumption under eCO_2 and therefore an increased leaf transpiration and stomatal conductance (Wohlfahrt et al., 2018). Nevertheless, independent of vine age, all previous studies observed an eCO_2 effect on vine water use efficiency, which was shown to improve and has been supported by higher photosynthetic capacity under eCO_2. As leaf photosynthesis occurs in chloroplasts of the mesophyll (palisade and spongy parenchyma) it is likely that an increased photosynthesis rate leads to an adaptation in morphological characteristics of leaves. Furthermore, spongy parenchyma has larger intercellular space for gas transportation, while palisade parenchyma is higher in chloroplast number and thus more beneficial to increase leaf photosynthesis.

Morphological alteration of leaves under eCO_2 has been reported for several tree and agricultural C_3 species, e.g. increase in leaf thickness and layers, extension of leaf cells and chloroplast development (Thomas and Harvey, 1983; Robertson and Leech, 1995; Saxe et al., 1998). The increase in leaf thickness of the grapevine cultivar Touriga Franca was derived from an extended spongy parenchyma and only partially due to an increase in palisade parenchyma under eCO_2 conditions (Moutinho-Pereira et al., 2009).

The aim of this study was to investigate the effects of eCO_2 on leaf physiology and morphological characteristics of the two *Vitis vinifera* L. cultivars Riesling and Cabernet Sauvignon grown in the VineyardFACE system and under temperate oceanic climate conditions.

Material and methods

Field site

The study was conducted at the VineyardFACE experimental site (49°59'N, 7°57'E) of Hochschule Geisenheim University, located in the Rheingau Valley, Germany, and was established as a ring system with six rings and a total area of 0.5 hectares. The vineyard used for the study was planted in 2012 using one-year-old pot-grown vines which were trained into a vertical shoot positioning system (VSP) and cane pruned to five nodes per square meter. Rows were north–south-orientated, while vine spacing was 0.9 m within rows and 1.8 m between rows. Two cultivars were used, *Vitis vinifera* L. cv. Riesling (clone 198–30 Gm) grafted on rootstock SO4 (clone 47 Gm) and cv. Cabernet Sauvignon (clone 170) grafted on rootstock 161–49 Couderc. Both rootstocks used are not considered to show a high tolerance against drought stress and were selected according to scion growth characteristics. Cultivars were bearing fruit for the first time in 2013, at an age of three years.

The soil at the field site is characterized as low-carbonate loamy sand to sandy loam with an average pH of 7.0 (0-30 cm, 30-60 cm, 60-90 cm). The available water capacity is 300 mm according to BFD5W (HLNUG, 2008). Management of vines was in accordance with the code of good practice (Bundesministerium für Ernährung Landwirtschaft und Verbraucherschutz - BMELV, 2010) and

considered an Integrated Pest Management (IPM). Mineral fertilizer was amended with 50 kg N ha^{-1} a^{-1} before bloom (May). Cover crop consisted of Freudenberger WB 130 mulch mixture III, permanent vineyard greening I (Feldsaaten Freudenberger, Krefeld, Germany) in every second row, while every other row was ploughed. The cover crop mixture consisted of 10% perennial ryegrass, 20% Chewing's fescue, 30% creeping red fescue and 40% Kentucky bluegrass and was mowed several times during vegetation. Shoot trimming was performed twice during vegetation, besides that no other canopy manipulation was conducted. Experiments were conducted under rain-fed conditions for two years, 2015 and 2016.

VineyardFACE system and carbon dioxide treatments

For the simulation of an elevated atmospheric CO_2 concentration, the VineyardFACE as a ring-shaped system started operating with a testing phase in 2013 comparing future atmospheric CO_2-concentrations (eCO_2) with current ambient CO_2-conditions (aCO_2). It is part of a special crop FACE system for permanent and annual crops implemented at Geisenheim University (Supplementary Figure 1A). Full operation of the still ongoing experiment started in 2014, including three ambient rings (aCO_2) and three elevated rings (eCO_2) with a targeted 20% CO_2 increase in the eCO_2 rings, which was the predicted concentration for 2050 (IPCC, 2014). Examples of an aCO_2 and eCO_2 ring during vegetation and the VineyardFACE experimental set-up are shown in Supplementary Figure 1. The VineyardFACE was described by Wohlfahrt et al. (2018) earlier. However, in brief each ring of the VineyardFACE system consisted of 36 jets, distributed in 10° steps, along a vertical double tubing system mounted at a height of 2.5 m, equipped with fans (MP25/4 T; CasaFan GmbH, Hasselroth, Germany) to create a high velocity downward air stream when activated and to allow a force-free pre-dilution of CO_2. Real time measurements of wind direction and wind speed were used to determine the release of CO_2 *via* transmitters (Thies Clima GmbH, Goettingen, Germany) installed in 3 m height. Depending on wind direction and wind speed fans operated in the upwind direction and only solenoid valve emitters on upwind-orientated side released CO_2, unless wind speed was less than 0.1 m s^{-1} by Azimuth regulation (upwind control). The released CO_2 was distributed throughout the ring by wind movement. Depending on the wind direction, the fans were switched on or off, with nine fans continuously on, covering a sector of 90° (Supplementary Figure 2). The CO_2 release varied as a function of wind speed by adjusting the pulse-pause ratio of the CO_2 releasing valves, the on time (pulse time) was fixed to 200 ms. According to the wind direction, five emitting valves were activated as shown in Supplementary Figure 2. No CO_2 enrichment was carried out at wind speed < 0.1 m s^{-1} or air temperatures < 7°C. Fans in aCO_2 rings were operated parallel to fans in eCO_2 rings (E1-A1, E2-A2 and E3-A3) and where therefore defined as blocks. The data was recorded by a datalogger (CR800, Campbell Scientific, Logan, Utah, USA). Fumigation of CO_2 was maintained during the entire year and from sunrise to sunset - mathematically calculated for the location of Geisenheim, Germany. To validate CO_2 distribution within FACE rings, CO_2 concentrations were recorded during an intensive period of monitoring in July 2015 using an infrared gas analyser (Li-Cor LI-8100CO_2/H_2O Analyzer and LI-8150 Multiplexer, Li-Cor Biosciences, Lincoln, NE, USA) at two different heights (0.8 and 1.7 m). Monitoring of the period from 14th to 22nd of July in 2015 is shown in Supplementary Figure 3. In 0.8 m height eCO_2 concentration was 476 ppm, whereas aCO_2 concentration remained at 397 ppm. At 1.7 m, CO_2 concentration measured was 395 ppm for aCO_2 and 458 ppm for eCO_2. Whereas CO_2 enrichment at 0.8 m was at the target of 20%, the CO_2 enrichment concentration in 1.7 m was at 16%.

Weather conditions

The climatic conditions are characterized by a temperate oceanic climate (Köppen-Geiger climate classification: Cfb (C-mild temperate, f-fully humid, b-warm summer); Chen and Chen, 2013) with mild winters and warm summers represented by an average annual temperature of 11.0°C (long-term average from 1991 to 2020) and mean annual rainfall of 527 mm. Mean daily temperature and precipitation data were collected from a weather station within the VineyardFACE. Precipitation and air temperature for the seasons 2015 and 2016 are shown in Supplementary Figure 4. Average growing season (1 April to 31 October) temperature was 15.9°C in both years, accumulated precipitation during the same time was 227 mm and 371 mm, in 2015 and 2016, respectively.

Leaf gas exchange measurements

Leaf gas exchange measurements were conducted by using a portable open gas exchange system (GFS-3000, Walz, Effeltrich, Germany) to detect net assimilation rate (A). Measurements were performed on fully developed and physiological active, sun-exposed leaves on high solar irradiation days between 9 am to 1 pm at five or six time points per season. On each date three leaves of three vines per FACE-ring were measured. An external LED light source (1200 µmolm^{-2} s^{-1}) was used which represented the mean light intensity of the measuring period. A 10-Liter buffer container was used for each of the two CO_2 treatments to sample air within the rings by air intake of the gas analyser and to buffer short-term CO_2 fluctuations. The carbon dioxide concentrations ($CO_{2\ abs}$) of the gas analyser was set to ambient to enable realistic CO_2 conditions present in the field.

Optical measurements

In both growing seasons, six mature primary leaves of six different vines per FACE-ring were measured on the adaxial and abaxial side with a Dualex Scientific portable optical leaf clip meter (Force A, Orsay, France) to determine epidermal flavonols (Flav) and leaf chlorophyll (Chl) indices according to Cerovic et al. (2012). Additionally, a nitrogen balance index (NBI) was calculated as the ratio of Chl and Flav. After execution of field measurement (02/09/2015 and 30/08/2016) same leaves were sampled to analyse leaf pigments.

Leaf pigment analyses

Following optical measurements leaf samples were collected in black tubes and immediately frozen in liquid nitrogen in the field. Until further processing samples were stored at -80° C. Subsequently, leaves were grinded with pestle and mortar using liquid nitrogen under dark conditions to avoid damaging of pigments. Then samples were freeze-dried through the application of lyophilisation. For further analysis, 30 mg of freeze-dried sample were weighed in a 2 ml reaction tube with a spatula tip of sodium bicarbonate. The samples were extracted with 700 ml 100% aceton on ice for half an hour, mixed using a vortex (Reac control, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) and centrifuged at 4° C at 13.800 rpm (MiniSpin® plus, Eppendorf SE, Hamburg, Germany). This washing step was repeated seven times. The supernatant was filtered using a syringe filter (0.45 µm) and 1 ml (10fold dilution) was transferred in a quartz cuvette (1 mm) for photometric analysis. The absorption at 400 to 780 nm was measured using a UV/Vis spectrophotometer (Specord 50, Analytik Jena GmbH, Jena, Germany). Chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid (Car) content were determined according to Lichtenthaler (1987).

Leaf histological analyses

For morphological traits six leaves per repetition of each CO_2 treatment were sampled on the same dates in 2015 (02/09) and 2016 (30/08). Cut leaves were rolled and immediately fixed in tubes containing a FAA solution (70% ethanol, 20% H_2O, 5% formaldehyde and 5% glacial acetic acid). After 24 h leaf samples were transferred and stored in tubes with an 70% ethanol solution until further processing. Later, rolled leaves were cut in slices following dehydration by using an increasing ethanol/isopropanol series, infiltration and embedding in paraffin under low air pressure conditions. By using a rotary microtome (Leica, RM 2155, Nussloch, Germany) sections of 5 µm were prepared and fixed on microscopic slides. Then, the sections were triple stained after the W3A method according to Wacker (2006) by using acridine red CI45000, acriflavin CI46000 and astral blue CI48048 in combination with ethanol, dest. water and glacial acetic acid following washing and differentiation with isopropanol. Pictures of the leaf cross-sections were taken using a fluorescence microscope (Keyence, Biozero BZ-8000K, Neu-Isenburg, Germany). Measurements of pictures were conducted with ImageJ, an image analysis software (National Institutes of Health, Bethesda, MD, USA). Then, thickness of the upper and lower epidermis, the palisade and sponchy parenchyma were recorded (Figure 1). Pictures published in this work were taken with an additional microscope (Mikroskop BX53 Olympus Deutschland GmbH).

Statistical analyses

Statistical analyses were performed with the statistical software R, version 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria). Data for all parameters were tested using multi-factor (treatment, block, year and

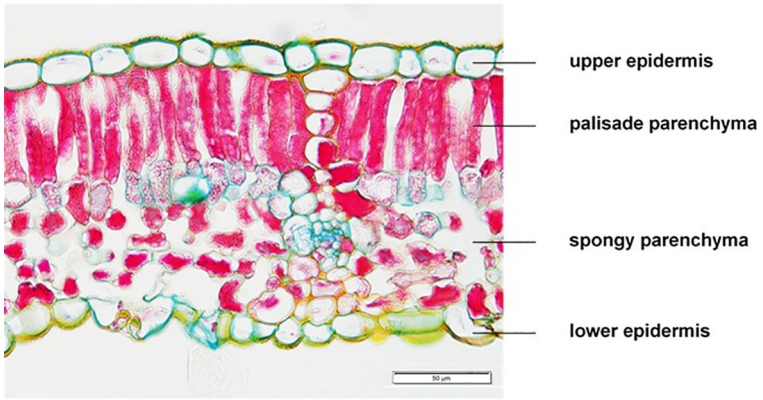

FIGURE 1
Histological tissue section of a *Vitis vinifera* cv. Riesling leaf as basis for analysis of epidermal and parenchymatic shares.

interaction treatment x year as well as treatment x date) analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test for significant differences ($P \leq 0.05$ level). For all parameters, means per ring were calculated and used for statistical analyses.

Results

The net assimilation rates were significantly stimulated under eCO_2 for both cultivars and seasons, which are presented in Figure 2 and have previously been described for stomatal conductance, water use efficiency, pre-dawn leaf water potential as well as for pruning weight or leaf area (Wohlfahrt et al., 2018). Additionally, results of the statistical output are shown in Table 1. Cabernet Sauvignon net assimilation rate increased from 18% up to 41% in 2015 under eCO_2 conditions, and showed +31% on a seasonal average. In 2016, the increase was 25% up to 63% with an average of +42% under eCO_2. Net assimilation of Riesling was 19% to 62% higher under eCO_2 in 2015 showing a seasonal average of a 41% increase. The gain in 2016 ranged between 31% to 46% with a seasonal average of +40%. Overall, Riesling was stimulated higher in net assimilation under eCO_2 in 2015, whereas in 2016 cultivars did not differ in their rate of increase (approx. 40%). It was obvious that in both cultivars the year as well as the measuring date have to be considered as independent factors. For Cabernet Sauvignon an interaction between the treatment and year and date occurred (Table 1).

Optical leaf clip meter indices did not differ between treatments or years for both cultivars (Table 2). Only for Riesling a trend to higher Chl index under CO_2 enrichment over the two years (*P=0.0629*) was observed. Whereas NBI index was higher, Flav index was lower in leaves of Cabernet Sauvignon compared to Riesling in both years. As shown in Table 2, leaf pigments (Chl a, Chl b, total Chl and Car) were affected by the year and not by eCO_2.

Total leaf thickness and width of spongy parenchyma of Cabernet Sauvignon (Figure 3A) and Riesling (Figure 3B) remained less affected under eCO_2 conditions (Table 3). However, significant differences were found in histological analyses of the leaf cross-sections between the two CO_2

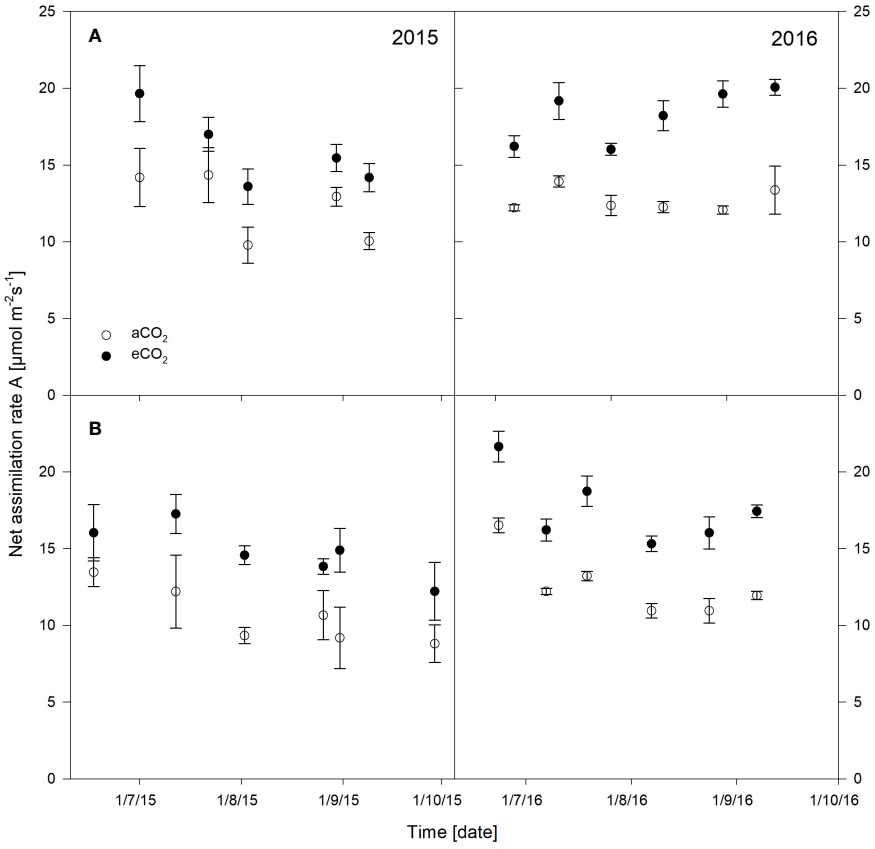

FIGURE 2
Net assimilation rate of *Vitis vinifera* cvs. Cabernet Sauvignon **(A)** and Riesling **(B)** measured over the seasons 2015 and 2016 under aCO_2 and eCO_2 conditions. Data represent mean ± SD of the three rings and nine leaves per treatment.

TABLE 1 Results of the multi-factor analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test for net assimilation of the two cultivars Riesling (R); Cabernet Sauvignon (CS) over the two seasons and measuring dates. Significant differences appear at $P \leq 0.05$ level and are displayed in bold type.

	P value	
	R	CS
treatment	***2.2-e16***	***2.2-e16***
block	*0.3130*	*0.8637*
year	***1.365e-11***	***5.522e-06***
date	***1.041e-12***	***4.374e-11***
treatment x year	*0.1804*	***0.0012***
treatment x date	*0.3289*	***0.0156***

treatments in upper and lower epidermis and the palisade parenchyma of Cabernet Sauvignon (Figure 3A). Whereas under eCO_2 the palisade parenchyma increased, the epidermal tissue decreased in thickness. Also, palisade parenchyma in Riesling showed a trend in increase under eCO_2 (Table 3), no significance difference was detected. However, the ratio between palisade and spongy parenchyma hardly differed between the CO_2 treatments in Riesling whilst in Cabernet Sauvignon the treatment effect was significantly pronounced (*P=0.017*) with an increasing ratio under eCO_2. Leaf layer thickness of both cultivars was affected by the year, like the epidermis and palisade parenchyma, the latter appeared to have higher values in 2015 (Table 3). Additionally, total leaf thickness and ratio between palisade and spongy parenchyma showed an effect by the year in Cabernet Sauvignon. Block effects occurred for both cultivars in total thickness of the leaf and the spongy parenchyma thickness.

Discussion

Responses of two different grapevine cultivars grown in the VineyardFACE-system indicate that an increase in atmospheric CO_2 predicted for the mid-century affects leaf gas exchange, and especially enhances net assimilation. This is in accordance with results obtained from previous studies on field-grown grapevines (Bindi et al., 2001; Moutinho-Pereira et al., 2009; Edwards et al., 2017) and a multitude of other C_3 crop species under elevated CO_2 concentrations. In a previous VineyardFACE trial both, Riesling and Cabernet Sauvignon had frequently higher photosynthetic rates in their early years of adaptation and increased in leaf as well as fruit biomass production. Hence, an impact on single berry weight, cluster weight and bunch architecture has been shown (Wohlfahrt et al., 2018 and Wohlfahrt et al., 2020). Even though net assimilation was highly stimulated for both cultivars under a relative low CO_2 increase (+39% net assimilation vs. +20% CO_2 increase), no impact was found in chlorophyll content nor lead to changes in other leaf pigments or leaf nitrogen status.

That the NBI index in leaves differs within different grapevine cultivars and that Chl index is used as indicator for leaf nitrogen content was reported by Cerovic et al. (2015), and could further provide information about the nutrition status of berries. Interestingly, the differences found between the leaves of the two cultivars for Chl index and NBI were also detected earlier during berry ripening in 2015 and 2016 by higher amino acid concentration in berries of Cabernet Sauvignon in comparison to Riesling (Wohlfahrt et al., 2020). These cultivar dependent differences, probably influenced by the choice of rootstock and the scion-rootstock combination as well, were found for various plant growth parameters, e.g. lateral leaf area or perennial wood growth (Wohlfahrt et al., 2018). Differences in leaf nutrition status by using optical leaf clip meter indices or leaf pigment content have not been found between the two CO_2 treatments and for neither of the two cultivars, which corroborates the results of Moutinho-Pereira et al. (2009) when using a SPAD meter. Leaf nitrogen status relates to the photosynthetic capacity and that is why leaves form the highest growth demand for nitrogen (Evans, 1989), while under elevated CO_2 leaf nitrogen content generally decreases by a N-dilution effect caused by the increase in carbohydrate accumulation through enhanced net assimilation (Feng et al., 2015). Thus, it remains unclear if the two cultivars within the VineyardFACE will decrease in leaf nitrogen under eCO_2 in future as variations in nitrogen content are also depending on the initial nitrogen limitation status of the single plant (Stitt and Krapp, 1999; Ainsworth and Long, 2005).

Leaf pigments (Chl a, Chl b, total Chl and Car) were not altered under eCO_2 which is in accordance with results of total chlorophyll and carotenoid content in beech leaves, where eCO_2 revealed no effects (Polle et al., 1997). Only a varying nutrient supply caused significant differences in leaf pigments of beech. The seasonal differences in leaf pigments shown for both cultivars were expected due to their dependence on environmental factors such as water availability (Fanizza et al., 1991), which differed in rainfall 2015 (230 mm) and 2016 (369 mm) during growing season. Leaf chlorophyll pigments (Chl a, Chl b, Chl total) were reduced about 50% and carotenoids by 30% in 2015, when precipitation was shortened in comparison to 2016.

Histological analyses of the grapevine leaf cross-sections revealed no increase in total leaf thickness under elevated CO_2. Other C_3 species, particularly soybean, loblolly pine and sweet gum showed an increase in leaf thickness under different CO_2 enrichment scenarios (Thomas and Harvey, 1983), and in different poplar clones in the early phase of growth (Radoglou and Jarvis, 1990). Furthermore, leaves of crop species were reported to exhibit greater increases in leaf thickness compared to wild species (Pritchard et al., 1999), but in this review only experiments conducted in chambers (growth chamber and open top chamber), glass houses and phytotrons

TABLE 2 Results of optical leaf clip meter readings of leaf chlorophyll (Chl), flavonols (Flav) and nitrogen balance index (NBI) as well as leaf pigment content (in dry matter, DM) for chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Chl total) and carotenoid (Car) of the two cultivars Riesling (R) and Cabernet Sauvignon (CS) under aCO_2 and eCO_2 conditions.

	Dualex indices			mg g^{-1} DM			
	Chl	**Flav**	**NBI**	**Chl a**	**Chl b**	**Chl total**	**Car**
2015							
R aCO_2	26.09 ± 2.26	2.90 ± 0.04	9.02 ± 0.68	2.16 ± 0.28	1.04 ± 0.16	2.67 ± 0.36	0.64 ± 0.07
R eCO_2	29.11 ± 2.63	2.92 ± 0.04	10.04 ± 0.76	2.16 ± 0.29	1.07 ± 0.14	2.68 ± 0.35	0.69 ± 0.09
2016							
R aCO_2	27.02 ± 3.04	2.80 ± 0.07	9.75 ± 1.31	3.95 ± 0.30	3.58 ± 0.27	5.70 ± 0.39	0.94 ± 0.13
R eCO_2	30.31 ± 1.06	2.86 ± 0.11	10.65 ± 0.57	3.81 ± 0.31	3.43 ± 0.18	5.48 ± 0.40	0.93 ± 0.10
P value							
treatment	*0.0629*	*0.3883*	*0.1152*	*0.8264*	*0.7360*	*0.7966*	*0.6282*
block	*0.5896*	*0.8216*	*0.6142*	*0.3345*	*0.1627*	*0.2655*	*0.5203*
year	*0.4799*	*0.1030*	*0.2499*	***1.897e-05***	***5.658e-08***	***2.306e-06***	***0.0023***
treatment x year	*0.9256*	*0.7516*	*0.9143*	*0.8375*	*0.4907*	*0.7397*	*0.7439*
2015							
CS aCO_2	29.66 ± 3.52	2.64 ± 0.09	11.32 ± 1.58	2.09 ± 0.31	0.98 ± 0.15	2.58 ± 0.38	0.60 ± 0.10
CS eCO_2	29.54 ± 2.81	2.62 ± 0.21	11.50 ± 1.99	2.41 ± 0.28	1.17 ± 0.15	2.98 ± 0.35	0.70 ± 0.08
2016							
CS aCO_2	29.03 ± 3.07	2.53 ± 0.11	11.63 ± 1.68	4.22 ± 0.23	3.73 ± 0.11	6.04 ± 0.28	0.96 ± 0.11
CS eCO_2	31.41 ± 1.63	2.58 ± 0.07	12.28 ± 0.99	4.54 ± 0.48	3.71 ± 0.08	6.36 ± 0.48	1.11 ± 0.21
P value							
treatment	*0.4835*	*0.8502*	*0.6456*	*0.1576*	*0.3410*	*0.1603*	*0.1748*
block	*0.1780*	*0.3764*	*0.1891*	*0.2723*	*0.5223*	*0.2817*	*0.2614*
year	*0.6995*	*0.3339*	*0.5484*	***1.055e-05***	***3.584e-09***	***9.703e-07***	***0.0016***
treatment x year	*0.4388*	*0.6339*	*0.7930*	*0.9500*	*0.1701*	*0.7615*	*0.8164*

Data represent mean ± SD of the three rings and six leaves per treatment. Tukey's honestly significant difference (HSD) test for significant differences appear at $P \leq 0.05$ level and are displayed in bold type.

have been considered. However, effects of elevated CO_2 on leaf anatomy were summarized to depend on leaf development stage, soil fertility, and again, season of the year (Pritchard et al., 1999). The latter is in accordance with the total thickness of epidermis and palisade parenchyma of Riesling and Cabernet Sauvignon, which were enlarged in 2015 compared to 2016 and thus affected by the season. The differences in leaf thickness could be attributed to extreme temperatures in the growing season 2015 (29 heat days (≥30°C) compared to 17 heat days in 2016) since under high temperature conditions an increase in thickness of grapevine leaves was reported (Salem-Fnayou et al., 2011). Still, both types of ground tissue, palisade and spongy parenchyma contain chloroplasts. Even though the palisade parenchyma contains a high number of chloroplasts compared to the spongy parenchyma, the latter is very prominent in terms of the intercellular air space in the lower mesophyll. Chlorenchyma and aerenchyma are both of utmost importance for the photosynthetic rate which in parts may help to explain that under eCO_2 the photosynthetic activity will be further stimulated, since a higher internal leaf surface enhances the ability to absorb CO_2 to a larger extent. In a previous study on grapevines (cv. Touriga Franca) under open top chamber conditions authors assumed that an increased leaf and therefore parenchyma thickness under eCO_2 was due to an enlargement of cells rather than increased cell division (Moutinho-Pereira et al., 2009), which was previously suggested by Pritchard et al. (1999). This could be explained by the same amount of parenchyma layers in both CO_2 treatments (Figure 3, data not shown). Nevertheless, thickness of palisade parenchyma increased, at least for Cabernet Sauvignon under eCO_2. These morphological alterations of leaf layers and extension of cells under eCO_2 were found in other agricultural C_3 species (Thomas and Harvey, 1983; Robertson and Leech, 1995). Surprisingly, instead of an expansion in leaf thickness Cabernet Sauvignon epidermal thickness decreased under higher CO_2 concentration. That an increase in leaf tissues within the mesophyll happens at the expense of epidermis (Garnier et al., 1999), and could therefore lead to increasing foliage photosynthetic potentials was proposed by Niinemets (1999) and approved in this study. Contrary to the leaf morphology of the red cultivar Touriga Franca, which resulted in thicker spongy parenchyma and thus lower or unchanged palisade to spongy parenchyma ratio (Moutinho-Pereira et al., 2009), the palisade to spongy

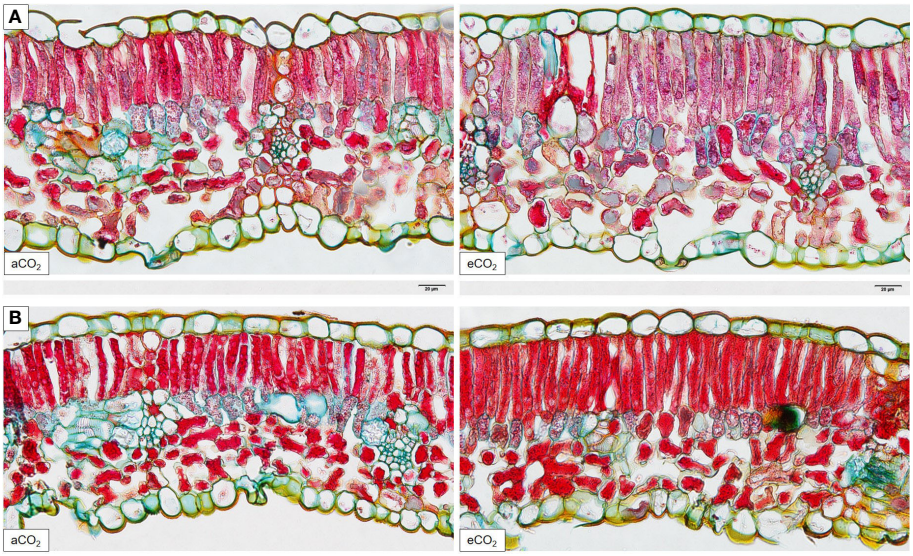

FIGURE 3
Histological analysis of aCO_2 and eCO_2 leaf cross-sections of *Vitis vinifera* cvs. Cabernet Sauvignon **(A)** and Riesling **(B)** stained with W3A (20 µm, 400x).

TABLE 3 Thickness of total leaf tissue, palisade parenchyma, spongy parenchyma and ratio of palisade to spongy parenchyma of the two cultivars Riesling (R) and Cabernet Sauvignon (CS) under aCO_2 and eCO_2 conditions.

	Thickness [µm]				
	total thickness	**upper/lower epidermis**	**palisade parenchyma**	**spongy parenchyma**	**palisade/spongy parenchyma ratio**
2015					
R aCO_2	171.98 ± 5.71	34.82 ± 3.09	51.65 ± 4.08	86.64 ± 1.61	0.61 ± 0.04
R eCO_2	177.35 ± 4.93	39.75 ± 1.28	55.60 ± 4.74	83.15 ± 6.16	0.69 ± 0.11
2016					
R aCO_2	166.89 ± 14.30	34.40 ± 2.71	45.79 ± 1.97	83.48 ± 9.15	0.56 ± 0.06
R eCO_2	176.22 ± 14.62	33.98 ± 1.90	50.37 ± 4.09	87.35 ± 11.98	0.58 ± 0.04
P value					
treatment	*0.1528*	*0.0954*	*0.0937*	*0.9553*	*0.1987*
block	***0.0250***	*0.0901*	*0.3021*	***0.0169***	*0.2110*
year	*0.5189*	***0.0329***	***0.0396***	*0.8780*	*0.0687*
treatment x year	*0.6794*	*0.0555*	*0.8892*	*0.2980*	*0.4998*
2015					
CS aCO_2	191.99 ± 7.58	40.05 ± 5.99	59.40 ± 2.85	89.52 ± 3.18	0.68 ± 0.03
CS eCO_2	195.97 ± 9.87	34.78 ± 1.35	66.66 ± 3.97	93.91 ± 4.62	0.72 ± 0.00
2016					
CS aCO_2	172.37 ± 10.80	34.78 ± 0.40	48.88 ± 4.30	87.19 ± 7.97	0.56 ± 0.01
CS eCO_2	175.00 ± 11.42	31.20 ± 3.17	56.00 ± 0.38	85.42 ± 10.07	0.67 ± 0.08
P value					
treatment	*0.3301*	***0.0405***	***0.0039***	*0.6341*	***0.0170***
block	***0.0030***	*0.1136*	*0.1404*	***0.0107***	*0.0960*
year	***0.0004***	***0.0403***	***0.0004***	*0.0786*	***0.0080***
treatment x year	*0.8367*	*0.6470*	*0.9689*	*0.2801*	*0.1764*

Data represent mean ± SD of the three rings and six leaves per treatment. Tukey's honestly significant difference (HSD) test for significant differences appear at $P \leq 0.05$ level and are displayed in bold type.

parenchyma ratio increased under eCO_2 within Cabernet Sauvignon under open field conditions. This leads to the assumption that chamber experiments are not fundamentally comparable with studies conducted under field conditions on the one hand, and cultivar specific leaf characteristics and responses on the other hand (Boso et al., 2010). Also, different 'climatic' effects are possibly responsible for the differences in parenchyma responses. In addition, Riesling (cool to intermediate) and Cabernet Sauvignon (warm) belong to different climate maturity groupings based on average growing season temperatures (Jones et al., 2005). Under these requirements, different plant reaction of the two cultivars are expected with the accessory climatic changes apparent from season to season which were recently shown (Wohlfahrt et al., 2018). In a study based on climate and developmental plasticity with regards to the seasonal variability in grapevine leaf morphology, results demonstrated that besides environmental, genetic and developmental effects influence the leaf shape in a way largely independent of each other (Chitwood et al., 2016).

Eventually, free air CO_2 enrichment studies are essential to understand plant responses to a changing climate, especially for permanent plant crops and obtained results are likely to improve the current understanding of physiological and structural responses of plants to future environmental conditions, e.g. elevated CO_2 levels.

Conclusion

Results observed on leaf physiology and morphological characteristics of cvs. Riesling and Cabernet Sauvignon can provide first insights to seasonal adaptation strategies of grapevines under a changing climate and in particular to future elevated CO_2 concentrations. However, regardless of the CO_2 treatment the effect of the season and in particularly high temperature and low precipitation can modify the plant response to eCO_2. Thus, the plant water as well as nutrition status may have a large impact on leaf morphology too. For these reasons, field studies on the effect of elevated CO_2, especially by using non-herbaceous perennial plants, are complex and difficult to execute and thus need a long-term investigation over at least two decades. Therefore, studies like the present one are welcome to improve our knowledge about the response of plants to future environmental conditions under realistic conditions.

Furthermore, as the plant nutrient status is suggested to be linked to the antioxidative enzyme response under elevated CO_2 concentrations (Schwanz et al., 1996) the nutritional status of the leaves and the whole plant needs to be intensified in further VineyardFACE studies. In addition, investigations should be carried out in the direction of carbon sink and in regards to the C/N ratio in the soil if it is assumed that a higher surface litter input due to more leaf biomass under eCO_2 could also stimulate the rate of mineralization.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

Author contributions

Conceptualization: YW. Methodology: YW, KK, DP, and ST. Formal analysis: YW. Investigation: YW and ST. Resources: MS. Data curation: YW and ST. Writing—original draft preparation: YW, KK, DP, ST, and MS. Writing—review and editing: YW and MS. Visualization: YW, KK, and DP. Supervision: MS. Project administration: YW, ST, and MS. Correspondence with the journal's editor: YW. All authors contributed to the article and approved the submitted version.

Funding

The authors would like to thank the LOEWE excellence cluster FACE2FACE of the Hessian State Ministry of Higher Education, Research and the Arts for funding this work. For funding the research exchange with the Leibniz Institute of Vegetable and Ornamental Crops (IGZ), Erfurt, Germany, the authors are grateful to Hochschule Geisenheim University. The cooperation and financial support through the Hessian Agency for Nature Conservation, Environment and Geology (HLNUG) is also acknowledged. The authors are thankful for financial support by the Open Access Publishing Fund of Geisenheim University.

Acknowledgments

We acknowledge the technical staff of the Department of General and Organic Viticulture for operating vineyard management of the VineyardFACE. Thanks are due to the laboratory team of the Department for helping with sampling, preparation, and analyzation of leaf pigments. We thank Anna-Sophie Düker and Nikolina Škobić for conducting histological analyses of the leaf cross-sections during their Bachelor and Master thesis, respectively.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1085878/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 09 December 2022
DOI 10.3389/fpls.2022.1077710

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

RECEIVED 23 October 2022
ACCEPTED 16 November 2022
PUBLISHED 09 December 2022

CITATION
Daldoul S, Hanzouli F, Hamdi Z, Chenenaoui S, Wetzel T, Nick P, Mliki A and Gargouri M (2022) The root transcriptome dynamics reveals new valuable insights in the salt-resilience mechanism of wild grapevine (*Vitis vinifera* subsp. *sylvestris*). *Front. Plant Sci.* 13:1077710. doi: 10.3389/fpls.2022.1077710

The root transcriptome dynamics reveals new valuable insights in the salt-resilience mechanism of wild grapevine (*Vitis vinifera* subsp. *sylvestris*)

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Introduction: Most of elite cultivated grapevine varieties (*Vitis vinifera* L.), conventionally grafted on rootstocks, are becoming more and more affected by climate changes, such as increase of salinity. Therefore, we revisited the valuable genetic resources of wild grapevines (*V. sylvestris*) to elaborate strategies for a sustainable viticulture.

Methods: Here, we compared physiological and biochemical responses of two salt-tolerant species: a wild grapevine genotype "Tebaba" from our previous studies and the conventional rootstock "1103 Paulsen". Interestingly, our physio-biochemical results showed that under 150mM NaCl, "Tebaba" maintains higher leaf osmotic potential, lower Na+/K+ ratio and a significant peaked increase of polyphenol content at the first 8h of salinity stress. This behavior allowed to hypothesis a drastic repatterning of metabolism in "Tebaba's" roots following a biphasic response. In order to deepen our understanding on the "Tebaba" salt tolerance mechanism, we investigated a time-dependent transcriptomic analysis covering three sampling times, 8h, 24h and 48h.

Results: The dynamic analysis indicated that "Tebaba" root cells detect and respond on a large scale within 8h to an accumulation of ROS by enhancing a translational reprogramming process and inducing the transcripts of glycolytic metabolism and flavonoids biosynthesis as a predominate non-enzymatic scavenging process. Afterwards, there is a transition to a largely gluconeogenic stage followed by a combined response mechanism based

on cell wall remodeling and lignin biosynthesis with an efficient osmoregulation between 24 and 48 h.

Discussion: This investigation explored for the first time in depth the established cross-talk between the physiological, biochemical and transcriptional regulators contributing to propose a hypothetical model of the dynamic salt mechanism tolerance of wild grapevines. In summary, these findings allowed further understanding of the genetic regulation mechanism of salt-tolerance in V. sylvestris and identified specific candidate genes valuable for appropriate breeding strategies.

KEYWORDS

cell wall remodeling, metabolic repatterning, root resilience, ROS scavenging, salt tolerance, transcriptomic analysis, *Vitis sylvestris*, wild grapevine

Introduction

Viticulture is one of the major horticultural industries of the world (Creasy and Creasy, 2018) with an area of cultivation exceeding 7.5 million ha (Berhe and Belew, 2022). Grapevines are well adapted to semi-arid climates such as the Mediterranean region and are considered relatively tolerant to water deficit, but are susceptible to significant damage from long-term salinity (Vincent et al., 2007). Rising temperature and reduced rainfall will accentuate soil salinity in irrigated areas caused by the climate change in the Mediterranean area (Santos et al., 2020). Resilience of *Vitis* to these abiotic factors is mainly linked with root, because rootstocks developed from breeding American wild grapevine species have been used for more than a century to control infection by Phylloxera and to confer tolerance to the grafted grapevines (Serra et al., 2014). However, these rootstocks are becoming more and more affected by climate changes such as increase of salinity and drought, newly emerging diseases, or heat. Thus, it is critical to improve our understanding of the molecular mechanisms deployed by tolerant species to adapt to these environmental stresses in order to elaborate strategies of a sustainable viticulture (new rootstocks with better ability to adapt to climate changes and their consequences).

In this respect, the Wild Grapevine [*Vitis vinifera* subsp. *sylvestris*, (*i.e. hereafter referred to as Vitis sylvestris*)] is of particular interest, due to their naturally occurring tolerance to abiotic stress reviewed in Daldoul et al. (2020). Previously, an identification of *Vitis sylvestris* genotypes was determined based on morphological descriptors established by the International Organization of Vine and Wine (OIV) and prospected in different countries [e.g. from Portugal (Cunha et al., 2007), Spain (Ocete et al., 2011), Romania (Popescu et al., 2013), Tunisia (Zoghlami et al., 2013) and Italy (Schneider et al., 2015)]. Furthermore, our molecular investigations demonstrated that there is a clear differentiation between cultivated and Tunisian wild genotypes as well as within *Vitis sylvestris* populations, using a set of molecular markers such as: the nuclear microsatellites (SSR; Zoghlami et al., 2013) and the single nucleotide polymorphisms (SNP; Riahi et al., 2013). Thus, these studies revealed a significant pattern of isolation by distance which implies that each wild population would constitute a distinct pool of genetic variation excluding any possibility of hybrids generation among the *Vitis sylvestris* populations (Riahi et al., 2012). Twenty *Vitis sylvestris* populations were characterized and each one was named according to its region of origin (Zoghlami et al., 2013). One of them, "Tebaba" population was selected in this study.

Vitis sylvestris is considered as the ancestor of the cultivated form *Vitis vinifera* subsp. *vinifera*, and do harbor resistance factors against several pests, such as Phylloxera (Campus et al., 2013), Downy Mildew (Duan et al., 2015), wood decaying fungi (Guan et al., 2016), Powdery Mildew and Black Rot (Schröder et al., 2015), as well as against some abiotic factors, such as: calcareous soil (Cambrollé et al., 2014), salinity (Askri et al., 2012) and drought (Azri et al., 2020). So far, Tunisian farmers have been using *Vitis sylvestris* as rootstock in their traditional vineyards. However, no *Vitis sylvestris* genotype has been certified as rootstock. Recently, our comparative physiological studies demonstrated that among the Tunisian wild *Vitis sylvestris* genotypes, "Tebaba" was selected as the most salt-tolerant genotype towards a high NaCl concentration (Askri et al., 2018). This salt tolerance phenotype was also reported in other *Vitis sylvestris* genotypes (Baneh et al., 2015; Carrasco et al., 2022). Indeed under severe stress, "Tebaba" was able to maintain well hydrated leaves through efficient osmotic adjustment and sufficient potassium flux and selectivity of K^+ versus Na^+ in the root part (Askri et al., 2018). Furthermore, our comparative proteomic investigations focusing on leaf responses

to drought stress of "Tebaba" versus a salt-sensitive wild grapevine genotype revealed that several ROS scavenging proteins were up-regulated only in "Tebaba" (Azri et al., 2020).

All previous studies have examined the response of wild grapevine genotypes to abiotic and biotic stresses by comparing different genotypes within the wild grape pool but only one recent attempt has been done to compare the wild grapevine genotype to the conventional rootstock response. Recently, a comparative transcriptomic study compared the new M-rootstocks to the conventional salt-tolerant 1103-Paulsen rootstock and showed a lower transcriptomic changes and lower accumulation of Na^+ and Cl^- ions in the leaves of the grafted scion on 1103P which is in favor to maintain their physiological response in the longer term (Buesa et al., 2022). Interestingly, the only study that has been conducted in parallel to our investigation and recently reported the root transcriptome patterns of the coastline wild grapevine AS1B (Spain ecotype) compared to the commercial rootstock 110R cultivated under different salt concentrations and different timings (Carrasco et al., 2022). However, choices of salt concentrations and time sampling points were unsupported by physiological and/or biochemical analysis and thus generated a descriptive transcriptomic results without any predictive mechanism of salt tolerance in wild grapevine. An early comparative transcriptomics based study of wild grapevine is a crucial first step toward gaining a molecular understanding of tolerance mechanism and all cellular changes triggered by salinity stress. In fact, targeting the genetic components of the early responses to salt stress could improve salinity tolerance in many plant species. In this context, the early response of *Populus euphratica* to salt stress revealed to be crucial to elucidate the tolerance mechanism through an integrated regulatory network and revealed the key roles of calcium-related genes in the tolerance trait (Chen et al., 2017). In wild grapevines AS1B ecotype, a changes in gene expression was observed at early stage of salt stress while it remain constitutive in the 110R rootstock (Carrasco et al., 2022). In Quinoa, the early physiological root response to salinity was critical in shaping how plants control the salt load and their overall response in the long-term stress (Kiani-Pouya et al., 2020). These results were further supported by the transcriptomic analysis of the early-stage response to salt stress suggesting that the restricted changes in gene expression in tolerant genotype Q68 was the key of the tolerance mechanism (Vita et al., 2021).

In preliminary efforts that set the stage for this work, we found that the most salt-tolerant wild grapevine "Tebaba" displayed a high physiological salt-tolerance in its roots than the most known salt-tolerant conventional rootstock «1103 Paulsen» and showed time-dependent biochemical responses. Therefore, a transcriptomic effort was initiated to characterize in time-resolved detail the response of "Tebaba" genotype to salt stress. We propose that deep metabolic remodeling in wild grapevine follows a biphasic modality, where a limited number of transcription factors induce a gene set involved in the process of non-enzymatic Reactive Oxygen Species (ROS) scavenging. Our hypothesis support that early-response regulatory genes establish a short-term acclimatization that could be a rapidly reversed as early as 8h after salt stress (150mM NaCl) application. When the salinity stress is prolonged, deeper metabolic changes induce novel transcription family members, which appear directly involved in cell-wall remodeling between 24h and 48h. To our knowledge, this is the first in-depth report involving interacting physiological traits, biochemical pathways and molecular mechanisms of salt-stressed roots from wild *Vitis sylvestris* genotype. This is not only crucial to understand the adaptation and survival of these species under abiotic stresses, but also to promote novel rootstocks *via* molecular breeding that can better cope challenging climate changes than conventional rootstocks and reassure about the viability of viticulture under such threats.

Materials and methods

Grapevine material and growth conditions

A Wild grapevines [*Vitis sylvestris*, (Arnold et al., 2010)] "Tebaba" genotype was identified in the northwest of Tunisia (interval of latitude-longitude: 36°53'54N/009°06'48E). It was found in high humid area along the continuous water streams and channels in the down side of "Tebaba" forest at 100 m of altitude, hence its name is referring to its main region of origin. Tebaba genotype is usually found in association with *Crategus azarolus* and *Rubus ulmifolius* as host species. According to Zoghlami et al. (2013), "Tebaba" genotype is distinguished mainly by a pentagonal blade shape, three combined lobes with toothed margins and high density of young twings. The indigenous wild grapevine "Tebaba" was compared to the conventional rootstock 1103 Paulsen (1103P) originating from a cross between the North American wild species *Vitis berlandieri* cv. Rességuier number 2 and *Vitis rupestris* cv. Lot. by Federico Paulsen, 1896. 1103P morphological characteristics were linked mainly to the low hair density of the shoots, a slightly bronzed young leaves and an open petiole sinus in the adult leaves (Rahemi et al., 2022). Tebaba's woody cuttings of diameter (0.5 to 0.8 cm) were harvested from a well-localized individual plant grown in Tebaba forest, while the 1103P rootstock cuttings with the same diameter were collected from our germplasm collection. Plants were cultivated in sandy soil for two months under controlled greenhouse. When the grapevine shoots had reached 12-14 nodes, 18 grapevines for each genotypes were transferred into 7 l pots of inert sandy soil (pH 7.4 and electrical conductivity (EC) 0.46 dS/m) and grown for two additional months (16 h light period, PAR of 300 $\mu mol.m^{-2}.s^{-1}$, 30/22°C ± 3°C day/night temperatures, and an

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average humidity of 70%). "Tebaba" and 1103P plants were randomized into two groups: Salt stressed and control, trained vertically by one wire and watered with 5X diluted of commercial nutrition solution Villmorin Universal. Once salt treatment began (Figure 1A): Five months old grapevine plants from the stressed group received a step-wise increase of NaCl concentration by 25 mM NaCl every two irrigations until reaching 150 mM (EC 18 dS/m at 25°C). This concentration of 150mM NaCl was previously defined as discriminating for the Tunisian wild grapevine genotypes (Askri et al., 2012), while control plants did not receive any supplemental NaCl. After reaching 150 mM, roots were harvested at three different time points (8h, 24h and 48h) for biochemical and transcriptomic analyses. All measurements were conducted on three replicates

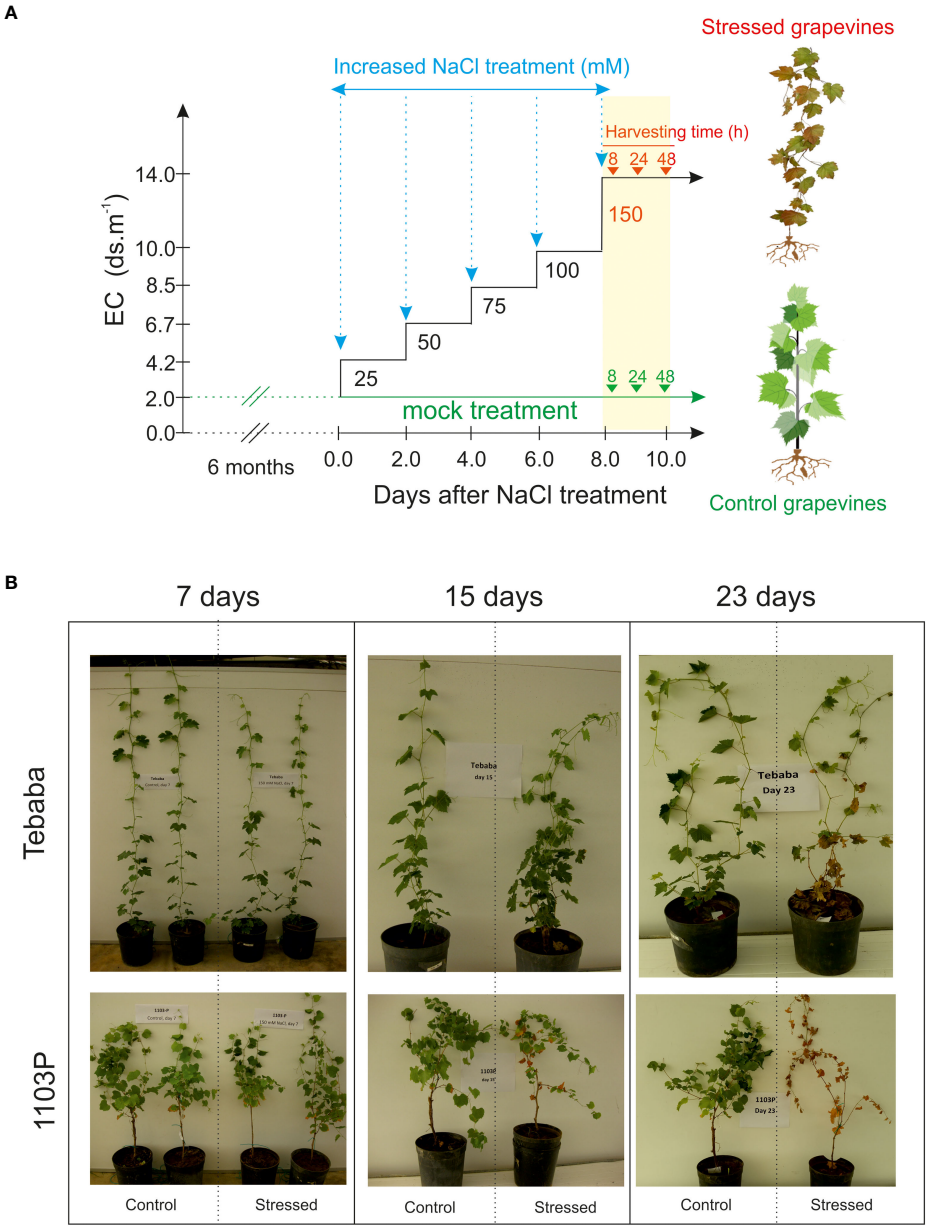

FIGURE 1
Salt stress application. **(A)** Gradual application of NaCl levels in soil medium and time course harvesting. During the experiment, salinity levels of soil media was increased gradually by 25mM NaCl until a final salt concentration is reached (150mM) leading to a gradual enhancement of the electric conductivity (EC). EC of solution was 2, 4.2, 5.1, 6.7, 8.5, 10 and 14 dS m-1 respectively. **(B)** Visual inspection of salt treated grapevines after 7, 15 and 23 days of salt exposure.

for each treatment; each separate replicate was composed of three vines.

Leaf osmotic potential

Leaf osmotic potential (Ψs) was determined using an osmometer (Herman Roebling, Type 13/13 DR, Berlin, Germany). After freezing the leaf blades with N_2 liquid cell sap was pressed on by a syringe (Moutinho Pereira et al., 2001). After centrifugation (12,0009g, 3 min, 4°C), 100 µl of the cell sap were used for measurements in the osmometer (mOsmol/Kg H20). The (Ψs) was calculated according to Van't Hoff equation, Ψs: -nRT, where n=m Osmol (g. H_2O)$^{-1}$, R = 8.314 ×10^{-6} MPa mol^{-1} K $^{-1}$ and T = 298.2 K.

Determination of sodium, potassium and chloride content

For the ion concentration determinations, dried root samples at 70°C were extracted in 0.1 N of nitric acid. The sodium (Na^+) and potassium (K^+) concentrations were measured using flame photometry (Tandon, 1993), Cl^- ion was measured by an automatic chloridometer (Buchler–Cotlove chloridometer) which was analyzed by colorimetric amperometric titration with silver ions (Munns et al., 2010).

Activity measurements of antioxidant enzymes

To estimate the activities of catalase (CAT), superoxide dismutase (SOD) and the steady-state levels of hydrogen peroxide (H_2O_2), fresh root samples (~100 mg. fw) were ground by mortar and pestle in 1 mL of ice-cold potassium phosphate buffer (0.1 M, pH 7.5) according to (Venisse et al., 2001). The liquid extracts were centrifuged at 12000×g for 10 min, at 4°C (Hermel Z 383 K), the supernatants were collected and evaporated to dryness. An aliquot of 100 µl was used to determine protein content according to (Bradford, 1976). The powder was redissolved in 70% HPLC grade methanol to a concentration of 10 mg/ml. The activity of SOD was quantified from the scavenging of light induced superoxide radicals generated in the riboflavin-nitroblue tetrazolium (NBT) according to Beauchamp and Fridovich (1971). The reaction mixture contained 3 ml of 50 mM phosphate buffer (pH 7.6), to which 20 g riboflavin, 12 mM EDTA, and 0.1 mg of NBT were added in sequence, along with 5 µL of extract. Reaction was started by illuminating the tubes for 2 minutes at room temperature. Immediately after illumination, the absorbance was measured at 590 nm with a negative control to determine the quantity of formazan produced in the absence of the extract. The percentage of superoxide anion scavenged was calculated from the ratio between A_{590} of the sample over A_{590} of the negative control. After 2 min of incubation at 25°C, the color was read with a Hitachi U-2000 spectrophotometer at 590 nm against blank samples. The percentage of scavenging activities (%) was calculated as follows: Scavenging activities % (capacity to scavenge the superoxide radical) = [1 - (absorbance of sample at 590 nm)/(absorbance of control at 590 nm)]. From this, the enzyme activity based on an extinction coefficient of 12.8 mM^{-1}cm^{-1}. In analogy, scavenging of hydrogen peroxide was used as proxy for catalase activity, following the method by (Ruch et al., 1989). Here, different amounts (60-420 µg) of the dried extract were dissolved in 3.4 ml of 0.1 M phosphate buffer (pH 7.4) and mixed with 600 µl of H_2O_2 (43 mM), recording A_{230} nm against Butyl-Hydroxy-Toluol (BHT) as positive control yielding 100% scavenging. The concentration of hydrogen peroxide was estimated based on an extinction coefficient of 40 mM^{-1} cm^{-1}. All data on redox parameters are means and standard errors from three biological replicates.

Polyphenol content

The total phenolic compounds were extracted from fresh root sample (1g) using methanolic solvent (methanol/water 80/20, v/v; 10 ml). Then the mixture was sonicated in an ultrasonic bath for 30 min at 37°C. The suspension was centrifuged at 5000 g for 10 min at room temperature and the supernatant was collected. Phenolic compounds were determined using Folin–Ciocalteu reagent method (Singleton et al., 1965) with minor modifications. For the assay, 50 µl of the diluted methanolic extract were added to 250 µl of the Folin–Ciocalteu reagent. Before adding 1 ml of sodium carbonate (7.5%), the reaction mixture was shaken thoroughly and allowed to stand for 2 min at room temperature. The absorbance of the samples was measured at 760 nm after an incubation for 30 min in the dark, at room temperature. Gallic acid was used as a standard and the results were expressed as milligrams of gallic acid equivalent (GAE)/g of fresh weight.

RNA isolation

Root samples from three time points 8h, 24h and 48h of TC and TSS treatments were used for transcriptomic analysis, as these time points showed distinct physiological differences between treatments. Total RNA was extracted from 100 mg of multiple root tips (5 cm) for each replicate using the Spectrum Plant Total RNA Kit from Sigma Aldrich according to manufacturer protocol. DNA was removed using an RNase-Free DNase kit (On-Column DNase I Digestion Set, Sigma Aldrich). RNA quality and quantity of root tissue were verified using an Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit).

Library construction, RNA sequencing and analysis

The library construction was carried out according to the bench manual of TruSeq RNA Sample Prep Kit v2 (Illumina) at BGI Tech solutions (Honkong). Bar coded libraries were prepared for two separate biological replicates of each time point and treatment (3 time points of control roots, 3 time points of stressed roots, 2 replicates =12 separate libraries) and they were sequenced using an Illumina® HiSeq™ 4000 Sequencing System (Illumina, Inc., San Diego, CA, USA). Illumina sequences from each TC and TSS treatment was generated as 100 bp pair-end reads in FASTQ format. The cleaning procedure included, trimming low quality reads from the ends to a Phred quality score > 20 and filtering reads with a length less than 10 bp and with adaptors and reads with unknown bases (N bases more than 5%). Samples after cleaning had high quality reads (20 to100 bp). Raw sequencing reads were filtered to get clean reads by using SOAPnuke (v1.5.2, parameters -l 15, -q 0.2, -n 0.05) (https://github.com/BGI-flexlab/SOAPnuke). HISAT pipeline was applied to align reads against the grapevine reference genome assembly (PN40024 12X, V1), a nearly homozygous inbred of the *V. vinifera* Pinot Noir cultivar (Kim et al., 2015). StringTie was then used for transcript reconstruction (Pertea et al., 2015). Subsequently, Cuffcompare (Cufflinks tools) was utilized to compare reconstructed transcripts and the grapevine reference annotation (Trapnell et al., 2012). Coding potential of novel transcripts were predicted by CPC (Kong et al., 2007). SNP and INDEL calling was carried out by using GATK (v 3.4-0, https://www.broadinstitute.org/gatk) with parameters (call): allow Potentially Misen coded Quals, stand call conf 20.0, stand emit conf 20.0 and parameters (filter): -window 35, -cluster 3, -filterName FS, -filter "FS > 30.0", -filterName QD, -filter "QD < 2.0" (Mckenna et al, 2010). In addition, the mapped clean reads to reference genes using Bowtie2 was used to quantify transcript abundance in terms of Fragment Per Kilobase per Million mapped reads (FPKM) (Langmead and Salzberg, 2012). The total uniquely mapped read ratios for TC and TSS samples were ranging from 71% to 74% (Table S1). The identification of DEGs was based on the negative binomial distribution of DEseq2 package (Love et al., 2014). The cutoff of DEGs was Fold Change ≥ 2 and adjusted by a false discovery rate (FDR) and *P*-value (q-value) < 0.05. The terms up- or down-regulated will be used to refer to the expression values of the TSS root relative to TC root expression values. The datasets generated for this study can be found in the NCBI sequence read archive under accession SRA Bioproject PRJNA507974. For Gene Ontology (GO) enrichment and pathway analysis, all DEGs that were identified in all pairwise comparisons were mapped to GO terms using an R function *phyper* and Kyoto Encyclopedia of Genes and Genomes (KEGG) through R package clusterProfiler (Yu et al., 2012), and significantly enriched terms were identified in comparison with the genome background. Heat maps were drawn using R packages of pheatmap (Kolde R. Pheatmap: Pretty Heatmaps. R Package Version 1.0.12).

Real-time PCR validation of RNA-seq data

The quantitative RT-PCR analysis was carried out using SYBR green master mix (2X Brilliant III Ultra-Fast QRT-PCR master mix; (Agilent Technologies, Santa Clara, CA, USA), on AriaMx Agilent system (AriaMx; Agilent Technologies, Santa Clara, CA, USA) with the following reaction conditions: reverse transcription step at 50°C for 10min; initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 30 s, 60°C for 60 s and a melt-curve program (65–95°C with a temperature increase of 0.5°C after every 5 s). The melting curve was generated to determine the amplicon specificity. The qRT-PCR experiments were performed using three biological and three technical replicates. A reaction with no template control and a reverse transcription negative control were performed to check the potential reagents and genomic DNA contamination. The expression of *VvEF1γ* and *VvActin* genes were found to be stable in our transcriptome database and hence were used as the normalization control in real time PCR. Primers were designed for selected transcripts from transcriptome database using QuantPrime QPCR. Details of the primers are represented in supplementary table. Relative expression of the transcripts was calculated using 2 -ΔΔCT method (Livak and Schmittgen, 2001).

Results

Salinity affected shoot growth, osmotic potential and mineral composition in wild and rootstock grapevines

At shoot level, 15 days after adding 150mM NaCl, salt stress symptoms were visible only in salt treated rootstock 1103P plants. After a longer exposure of 23 days salt stress symptoms on 1103P plants have become more intense in both leaves and shoots (leaf burn, defoliation and shoot necrosis). However, under these same experimental conditions, the Tunisian wild grapevine genotype "Tebaba" displayed mild symptoms and greater plant viability (Figure 1B). Its leaf osmotic potential showed a pronounced increase (-1,210 MPa and -0,9632 MPa) compared to 1103P (-0, 7390 MPa, -0,746 MPa), under control and stress conditions respectively (Figure 2A).

At the root zone, increasing salinity in the irrigation solution significantly raised both Na^+ and Cl^- content in roots of both species (1103P and "Tebaba"). Accumulation ratios of sodium ions compared to control were similar in both "Tebaba" and 1103P at 8h. The highest accumulations were observed at 24h with ratios of 8,61 and 9,32 compared to control in "Tebaba" and 1103P respectively (Figures 2B, C). A similar trend was observed for Cl^-

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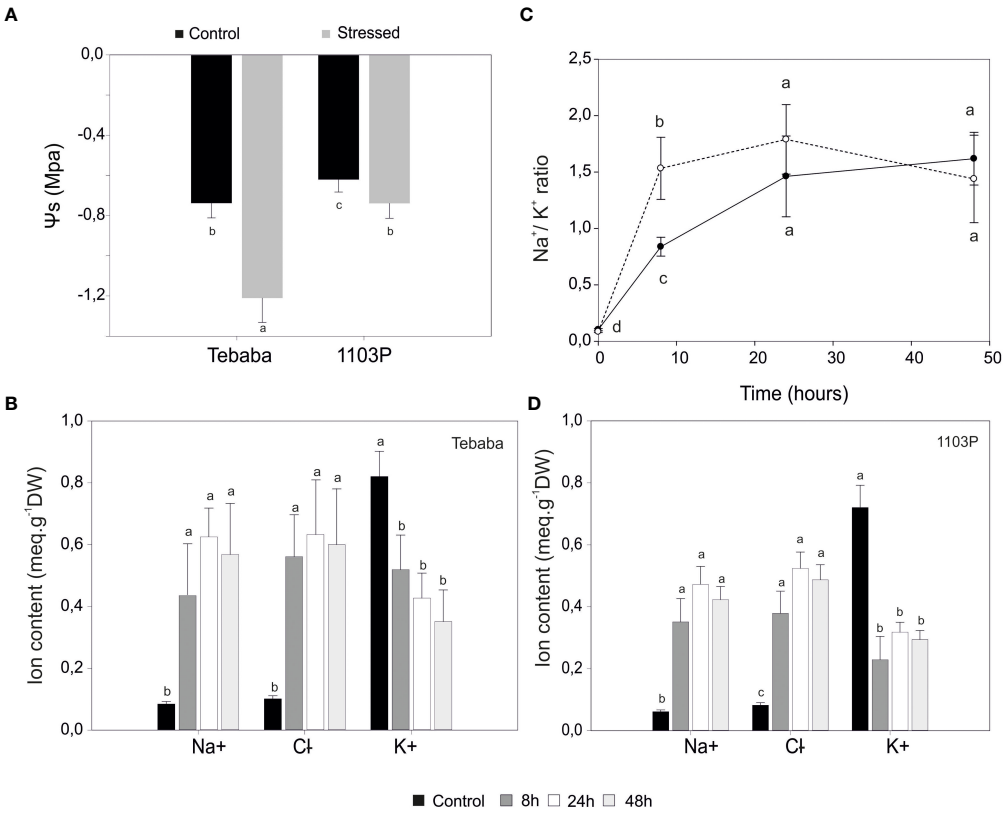

FIGURE 2
Physiological characterization of salt response in grapevine plants. **(A)**, Leaf water potential of control and salt stressed plants exposed to 48h of NaCl stress. Changes in Na^+, Cl^-, K^+ contents in roots of "Tebaba" **(B)**, and 1103P **(C)** at 8h, 24h and 48h of control and NaCl stress exposure. **(D)**, Time dependent changes in Na^+/K^+ selectivity ratio normalized to control in "Tebaba" and 1103P. Values are means (± SD) of at least 3 replications. Data labeled with different letters are significantly at P<0,05.

accumulations. At the same time, a significant decrease in potassium content was observed in both grapevines. The 1103P showed a pronounced reduced content in K^+ compared to "Tebaba", especially after 8h of salt stress exposure with a reductions ratio of 1,57 in "Tebaba" against 3,14 in 1103P. Consequently, Na^+/K^+ ratio tended to increase progressively from the early phase (8h) to the late phase (24-48h) of the stress. However, it remains significantly lower in "Tebaba" compared to 1103P especially at 8h with a Na^+/K^+ ratios of 8,18; 16,55 and 15,79 for 8h,24h and 48h, respectively (stressed samples versus control), compared to 18,09; 21,11 and 20,07 for 1103P (Figure 2D).

Enzymatic and non-enzymatic defense against Reactive Oxygen Species (ROS) in response to salt stress

The induced activity of the SOD increased at late time points 24h-48h of salt exposure in roots of "Tebaba" concomitantly with a decrease in the amount of the anion superoxide O_2^-, suggesting that SOD might be efficient in O_2^- detoxification (Figures 3A, B). For 1103P, the O_2^- scavenging activity decreased along with a decrease in the SOD activity suggesting a less efficient ROS scavenging ability. "Tebaba" genotype showed an increase in H_2O_2 content at early time points 8h-24h of salt stress exposure before stabilizing under longer stress exposure 48h indicating a potential quick adaptive response to oxidative stress. In this respect, 1103P showed a different behavior with a high H_2O_2 content at 8h, before decreasing progressively along with exposure time duration to NaCl (Figure 3C). Regarding the detoxifying CAT enzyme activity, it showed opposite trends (gradual increase) compared to H_2O_2 content. Thus, in "Tebaba", an efficient inhibition of H_2O_2 occurred at 24h of salt stress due to an increase of CAT enzyme activity. However, at the early stage (8h), the level of CAT enzyme in "Tebaba" was low and was not able to ensure an efficient scavenging of the H_2O_2 production (Figure 3D). In our study the polyphenol content increased only at 8h and just in "Tebaba" (no differences were noticed in 1103P, Figure 3E). This phenolic compound is among the non-enzymatic antioxidants and would contribute as scavenging free radicals in "Tebaba" to maintain redox homeostasis under salt stress. The scavenging of the H_2O_2 is supported by mechanisms other than

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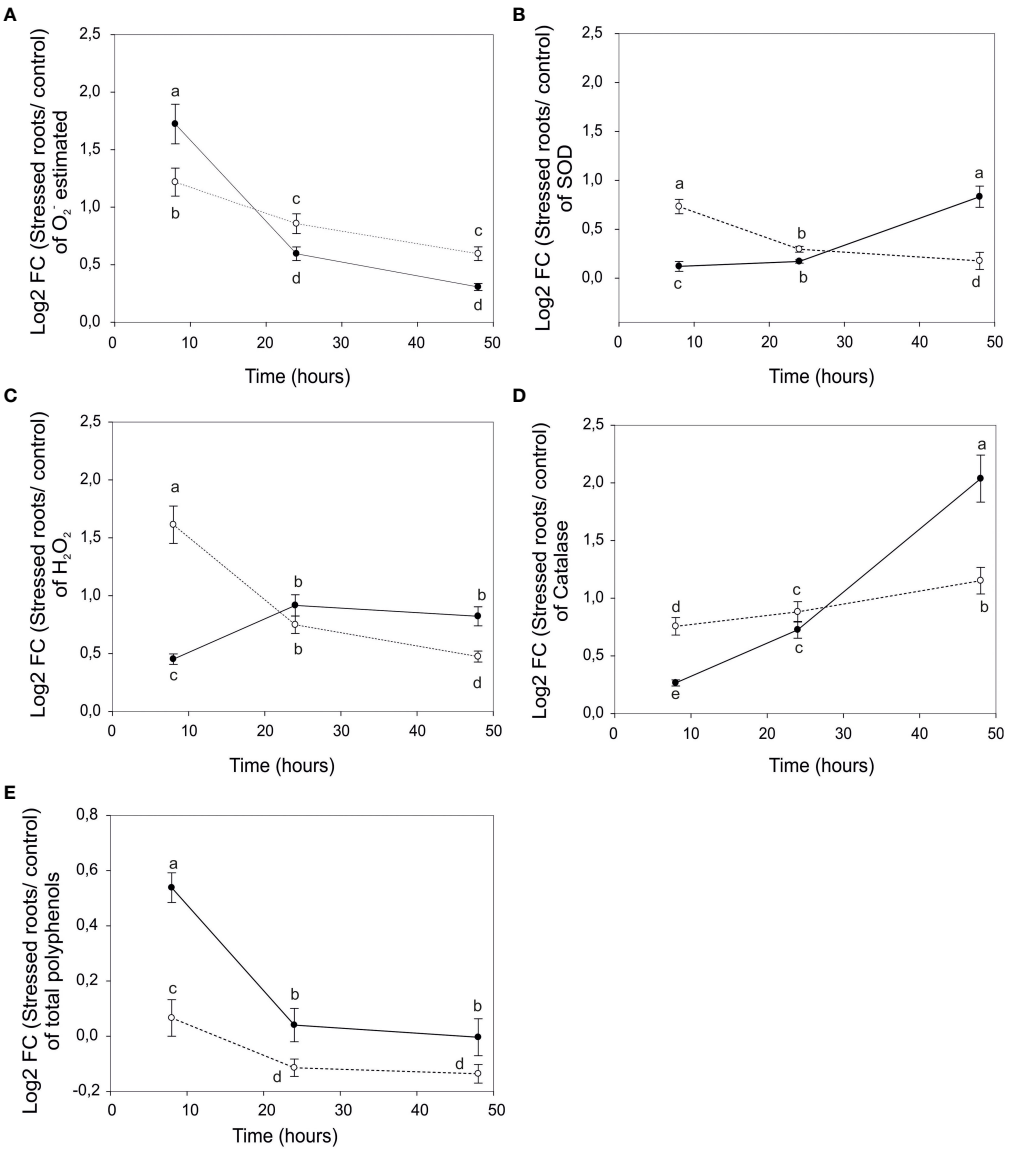

FIGURE 3
The effect of salt stress on the antioxidant enzymes activities in the rootstock 1103P and wild grapevine "Tebaba". Log2 fold changes of anion superoxide O_2^- levels **(A)**, Superoxide Dismutase SOD **(B)**, levels of hydrogen peroxide H_2O_2 **(C)**, levels of catalase CAT **(D)** and Polyphenols **(E)** in "Tebaba" (continuous trait) and 1103P (pointy trait) at 8h, 24h and 48h of NaCl stress.Concentration of H level of lipid peroxidation (MDA), and activities of the enzymes SOD, CA Concentration of Hzymes SOD, Values represent the mean of at least three independent experiments ± SE. Significant differences amongst different treatments are indicated by different letters, according to Tukey's Honestly Significant Difference (HSD) test (P<0.05).

CAT and most probably by non-enzymatic mechanisms mediated by polyphenol compounds.

Overview of RNA-seq data from "Tebaba" roots subjected to salt stress

To gain comprehensive insights into the wild grapevine (*Vitis sylvestris*) transcriptomic response to salinity stress, "Tebaba" and 1103P plants were treated with 150 mM NaCl solution for 8h, 24h and 48h and the root samples were used for RNA-sequence analysis along with their TC samples. We used principal-component analysis (PCA) to examine the similarity between samples according to the components that explain most of the variance in the data as shown in (Figure S1). A high correlation between biological replicates was observed (R2 > 0.96) for all the treatments, which indicate that the biological replicates were reliable in this study. In addition, the reliability of

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our transcriptome profiling dataset was validated by examining the expression of selected genes by using RT q-PCR and by comparing them to the normalized data obtained in the RNA-Seq analysis. We found highly significant and positive correlations between RT q-PCR and RNA-Seq results in all time points (8h, 24h and 48h) which means that the results of RNA-seq were reliable (Figure S2).

A total of 5093 DEGs were detected in the roots of "Tebaba" following salt treatment (Figure 4). The number of DEG at each point of the time course, increased from 2002 (8h) and, 2373 (24h) and to 2782 (48h). Large proportion of salt responsive genes were induced at 48h of stress due to greater accumulation of salt in roots following longer exposure to salt stress. This might reflect a sequential response of the plant as adaptive strategy to overcome salinity stress. The DEGs whose expression was modified at every time point were compared using Venn diagrams to identify the genes which were specifically induced or repressed after salt treatment (Figure 4). Lower numbers of genes are specifically differentially up or down-regulated at 8h comparisons (910), followed by 24h (1041) and 48h comparisons (1399). On other hand, the high number of common genes, differentially up- or down- regulated, between 24h and 48h (651) compared to those between 8h and 24h (360). 321 DEGs were commonly identified during the time course of 150mM NaCl stress. The expression of these genes showed either a continuous up-regulation pattern or a continuous down-regulation pattern.

A greater number of molecular pathways were significantly enriched following salinity time course

GO term mainly involved cellular components, molecular function and biological process (Figure S3). Under the biological process, the predominant transcripts were found in metabolic process (72.7% and 71,4%) at 8h and 48h respectively, and in cellular process (47%) at 48h. In the molecular function, the predominant transcripts found to be in catalytic activity (74,6%, 76,6% and 76,5%), while in the cellular process the highest prevalence of transcripts were recorded in cell and cell part with (79,5%, 69% and 57,9%) at 8h, 24h and 48h respectively. Across all time point, biological process was the most enriched GO category followed by the molecular function category. Metabolic process, single-organism process, cellular process, response to stimulus, localization and biological regulation were the most abundant groups in the biological process category. In the molecular function category, catalytic activity and binding were the most abundant groups. In the cellular component category, cell and cell part, membrane, membrane part and organelle were the most abundant groups (Figure S3).

A total of 119, 247 and 358 pathways were identified after 8, 24 and 48h respectively. The study revealed that the highest levels of transcripts were found to be involved in the metabolic pathway at 8h, 24h and 48h (31.09%, 32.39% and 32.68%) followed by biosynthesis of secondary metabolites (21.85%, 22.27% and 21.79%), then in phenylpropanoid biosynthesis (10.08%,6.88% and 7.54%).

To understand the regulatory networks of DEGs genes, we carried out a pathway mapping against a pathway database KEGG using the KOBAS tool (version 3.0). The results revealed that all genes were mapped to 103 different pathways. Among those, 41pathways were common for all time points indicating a continuous gene regulation in these pathways. Four, eight and nineteen, additional pathways were specific to 8h, 24h and 48h, respectively suggesting that longer salt stress treatment affected more pathways. The most enriched DEGs pathways at all of the time points were metabolic pathway, biosynthesis of secondary metabolites and phenylpropanoid biosynthesis. However, MAPK signaling pathway was only enriched only at 8h, nitrogen metabolism pathway was preferentially enriched at 24h and Galactose metabolism only at 48h (Figure S4).

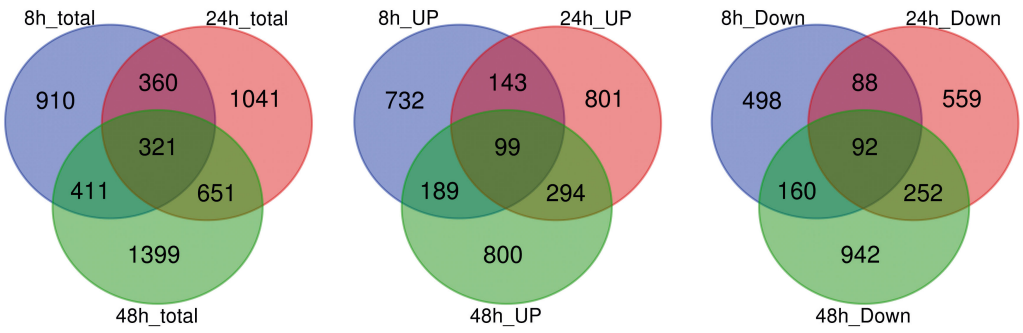

FIGURE 4
Analysis of global differentially expressed genes. Venn diagram illustrating the number of differentially expressed genes (DEGs) in repose to 150mM NaCl at 8h, 24h and 48h of treatment and their overlaps.

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Osmotic stress signaling and osmoregulation pathways in salt treated "Tebaba" roots

DEGs encoding osmotic sensing (Figure 5A) and osmo-adaptation (Figure 5B) were modulated during the time course of salinity stress. At 8h of NaCl stress, three DEGs (VIT_03s0038g02980, VIT_00s0399g00030, and VIT_10s0523g00050) encode ion channels and seven DEGs (VIT_14s0060g01590, VIT_05s0049g00560, VIT_06s0004g06430, BGI_novel_G000074, VIT_08s0007g01230, VIT_09s0002g02790, and VIT_07s0005g04390) encode receptor-like kinases (RLKs) were up-regulated. At 24 to 48h of salt stress, DEGs encoding vesicular trafficking (VIT_17s0000g01080, VIT_13s0019g05190, VIT_05s0124g00030), Hyperosmotically inducible periplasm protein (VIT_18s0001g04800) were upregulated. Particularly, at 48h of salt stress, we observed the up-regulation of the phosphoinositide signaling pathway related DEGs (VIT_07s0031g00920, VIT_02s0012g00550, VIT_18s0157g00210) and the calcium signaling pathway genes (VIT_18s0001g11830, VIT_18s0122g00180, VIT_01s0026g00880, VIT_05s0020g03980). However, DEG encoding for the *Cl^- loading to xylm*/SLAH, (VIT_18s0001g13440) was down-regulated.

DEGs encoding four protein kinases (PTKs: VIT_10s0003g01990, VIT_05s0020g01690, VIT_08s0040g01580, VIT_08s0007g06570) and four protein phosphatases 2C (PP2C) (VIT_06s0004g05460, VIT_11s0016g03170, VIT_10s0523g00020, VIT_16s0050g02680) were gradual increased over time and correlated to the upregulation of Abscisic acid (ABA) biosynthesis/transport genes: *NCED* (9-cis-epoxy-carotenoid dioxygenase VIT_10s0003g03750, VIT_19s0093g00550, VIT_02s0087g00910) and (VIT_05s0049g02240). However, DEGs encoding ABA receptors (VIT_13s0067g01940, VIT_02s0012g01270) were dramatically down-regulated. Since signaling is necessary for re-establishing osmotic equilibrium in "Tebaba" plants, several osmoprotective genes such as *dehydrin* (VIT_04s0023g02480), *LEA* (VIT_16s0115g00170, VIT_06s0004g03010, VIT_08s0007g05580), *Osmotin* (VIT_02s0025g04300, VIT_18s0001g04800) and *Osmy* (VIT_18s0001g04800) DEGs were continuously upregulated over time.

DEGs involved in secondary metabolites: Flavonoid/isoflavonoid pathways under salt stress

ROS scavenging was performed *via* the activation of enzymatic and/or non-enzymatic antioxidant defense pathways, in "Tebaba" roots following a time course pattern (Figure 6). Genes in this pathway can be classified into three sub-pathways (i) stilbenoids, (ii) isoflavonoids, (iii) flavonoids and anthocyanidins. DEGs coding for stilbenoids pathway (VIT_16s0100g00840, VIT_16s0100g00940, VIT_16s0100g00920, VIT_12s0028g02890, VIT_16s0100g00850) were repressed in all time point. However, enzymes encoding for *CHS* (VIT_05s0136g00260), *CHI* (VIT_13s0067g02870: *chalcone isomerase*) were upregulated. Transcripts coding for *Isoflavone methyltransferase/OrcinolO-methyltransferase* (*IOMT*: VIT_15s0045g01490; VIT_12s0028g02930) were mostly down-regulated at 48h with a fold change of -2,5 except for the DEG (*OMT*: VIT_10s0003g00470) which was upregulated at all of the time points (Figure S5A). Flavanone encoding DEGs (*F3H*: VIT_18s0001g14310, VIT_03s0063g01210, VIT_06s0009g02840) were particular upregulated at 24h with fold changes ranging from 1,1 to 1,5. Three glycoside flavonol *UDP-glucose glucosyltransferase* DEGs (VIT_12s0055g00160, VIT_12s0055g00200 and VIT_03s0017g01990) were upregulated at 48h except for VIT_03s0017g01990 whose upregulation was over time course. Leucoanthocyanidin dioxygenase (*LDOX*) is the key enzyme leading to the synthesis of anthocyanins. Two transcripts annotated *LDOX* VIT_08s0105g00380 and VIT_13s0067g01020) were found to be repressed at all-time points. DEG of *Anthocyanin biosynthesis* (VIT_18s0041g00900) was repressed under salt stress. "Tebaba" roots does not activate the flavonoid pathway under prolonged salt stress.

DEGs involved in phenylpropanoid metabolism under salt stress

The time-course analysis revealed 15 differentially expressed genes related to lignin, among which two were homologous of a caffeic acid O-methyltransferase homolog (*COMT*: VIT_11s0016g02600, VIT_10s0003g04160), a key enzyme in the lignin pathway, downregulated and decreased overtime reaching -2,35 at 48h. The Cinnamyl alcohol dehydrogenase encoding DEG (*CAD*: VIT_00s0371g00050) was upregulated at 24h by a FC of 1,51. DEGs encoding lignin biosynthesis (VIT_00s1677g00010, VIT_00s0510g00030, VIT_08s0058g00980, VIT_00s0226g00030), VIT_12s0055g01010, VIT_04s0023g01240, VIT_00s0481g00020, VIT_00s0731g00010, VIT_18s0117g00600, VIT_18s0075g00960) were mostly up-regulated at 24h (Figure S5B). The present study supports the hypothesis that "Tebaba" might be diverting substrates from the isoflavonoids and anthocyanins pathway to increase production of lignin, especially after 24h of salt stress. These results suggest that "Tebaba" root cells may respond to salt stress by thickening cell walls, due to an increased expression of many lignin biosynthesis genes under saline conditions. Physical reinforcement of the root cell wall could be an important component of the long-term salt stress adaptation in plants.

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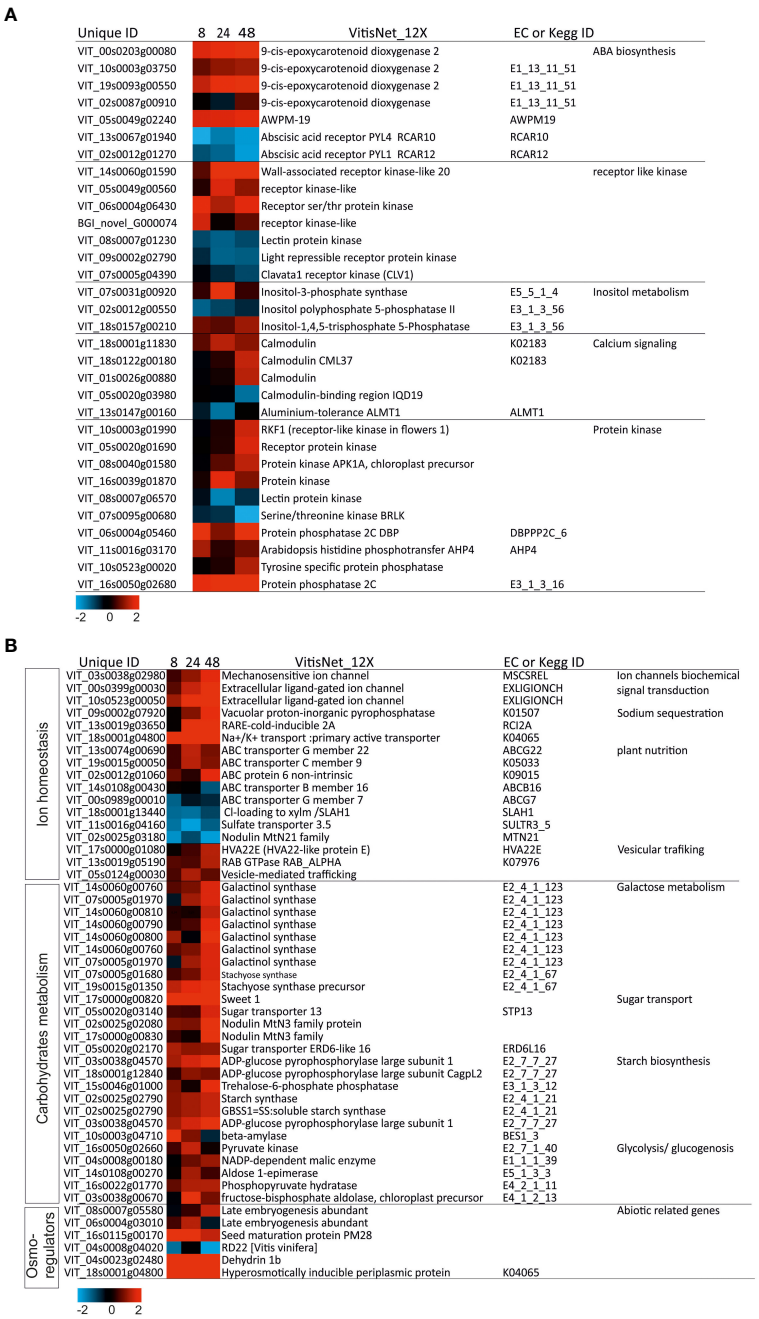

FIGURE 5
Heatmap of DEGs involved in osmotic sensing and osmo-adaptation in wild grapevine subjected to salt stress treatments. HeatMap Analysis of expression of transcripts related to osmotic sensing **(A)**, and osmoadaptation **(B)** at different time points. The Data were ln-transformed. Red and blue colors indicate up- and down- regulated transcripts, respectively, from both control and salt treated roots. False Discovery Rate (FDR) ≤ 0.001 and the maximum value of log2 (ratio of stress/control) ≥ 1 was used as the cut-off to evaluate significant differences in expression.

DEGs involved in cell wall pathways under salt stress

Sixty-three DEGs associated with cell wall metabolism were identified (Figure S6). The metabolic processes of the cell wall were particularly activated at 24 and 48h of salt stress (Figure 7). It mostly included the synthesis of enzymes and proteins involved in cell wall modifications. Our results showed that under salinity stress, the expression of some cell-wall extensibility related genes exhibited a general

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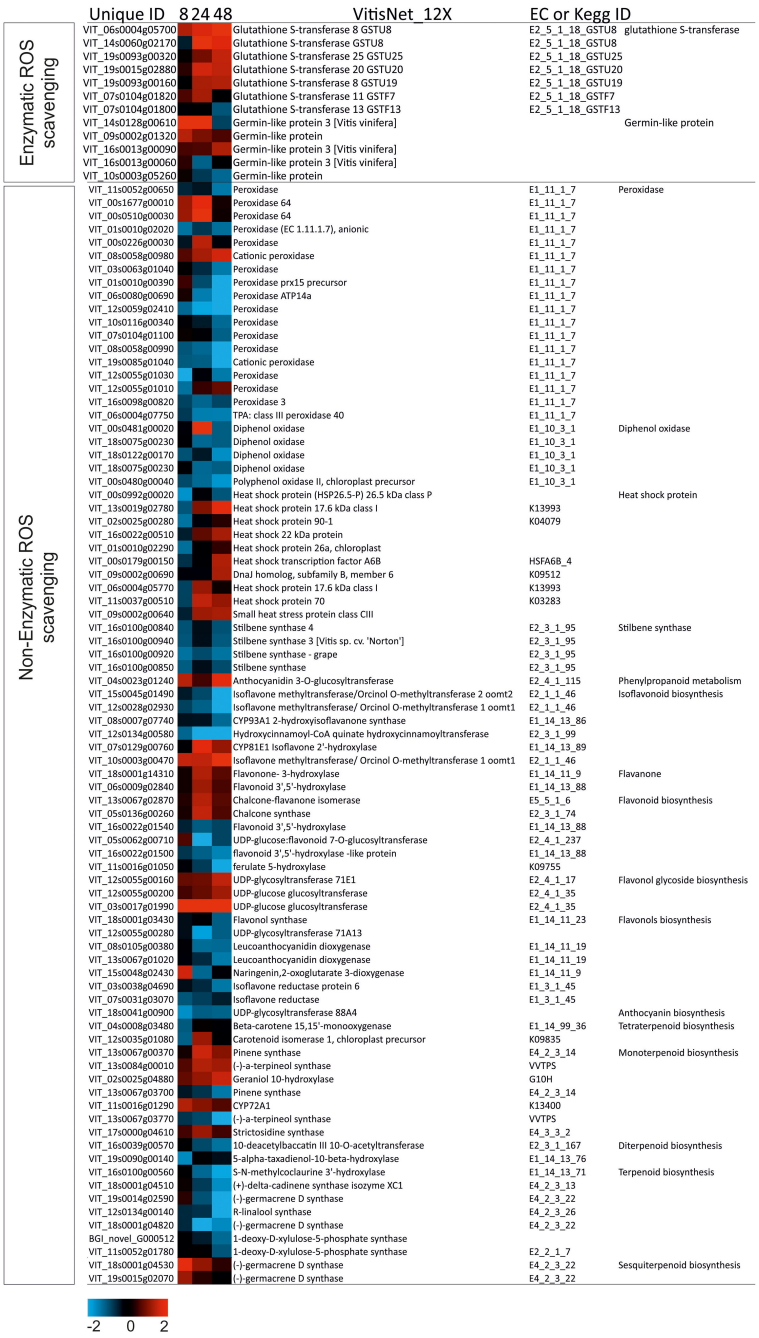

FIGURE 6
Heatmap of DEGs involved in Reactive Oxygen Species (ROS) scavenging in wild grapevine subjected to salt stress treatments. HeatMap Analysis of expression of transcripts related to ROS scavenging at different time points. The Data were ln-transformed. Red and blue colors indicate up- and down- regulated transcripts, respectively, from both control and salt treated roots. False Discovery Rate (FDR) ≤ 0.001 and the maximum value of log2 (ratio of stress/control) ≥ 1 was used as the cut-off to evaluate significant differences in expression.

trend favoring cell wall-loosening. In fact, most genes involved in cell wall loosening were up-regulated at 24h-48h, such as genes encoding *Expansin* (12 DEGs) and *Xyloglucan* (14 DEGs). A down regulation of various cell wall degradation related enzymes were noticed (e.g., cellulases: 5 DEGs) while genes involved in cross-linking of cell-wall polymers or cell wall stiffening, such as *Pectinesterase*, were down-regulated (3 DEGs).

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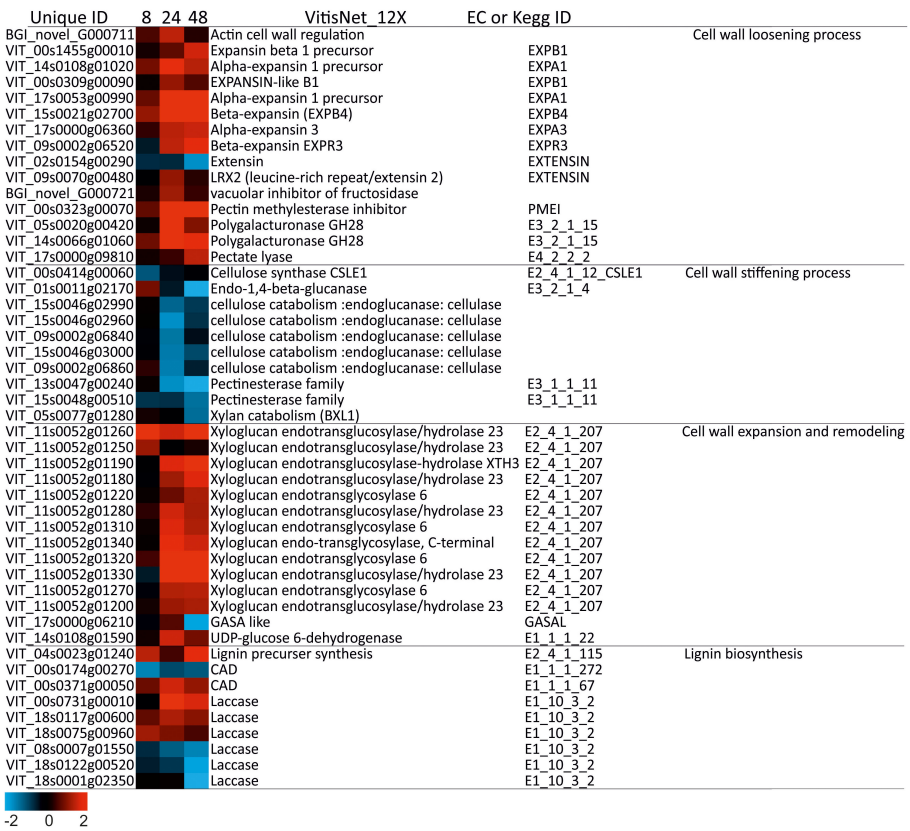

FIGURE 7
Heatmap of DEGs involved in cell wall metabolism in wild grapevine subjected to salt stress treatments. HeatMap Analysis of expression of transcripts related to cell wall at different time points. The Data were ln-transformed. Red and blue colors indicate up- and down- regulated transcripts, respectively, from both control and salt treated roots. False Discovery Rate (FDR) ≤ 0.001 and the maximum value of log2 (ratio of stress/control) ≥ 1 was used as the cut-off to evaluate significant differences in expression.

DEGs involved in starch, glycolysis and Galactose metabolisms under salt stress

Regarding starch metabolism, the upregulation of the DEG *β-amylase* (VIT_10s0003g04710) at early salt stress phase (8h) was followed by the induction of the glycolytic pathway genes such as those encoding: *Aldolase-1-epimerase* (VIT_14s0108g00270), *Fructose-1,6-bisphosphate aldolase* (*FBA*, VIT_03s0038g00670), *Enolase* (VIT_16s0022g01770, VIT_16s0022g01770), and *Pyruvate kinase* (*PK*, VIT_16s0050g02660, VIT_16s0050g02660). When extending the salinity exposure, DEGs related to starch synthesis (*AGPase*) were continuously up-regulated in a time dependent-(VIT_03s0038g04570, VIT_02s0025g02790, VIT_18s0001g12840). DEGs encoding carbohydrate catabolism, such as *beta-galactosidase* (VIT_11s0016g02200, VIT_11s0016g02200, VIT_18s0001g02230), *beta-glucosidase* (VIT_13s0064g01720, VIT_19s0014g04750) and Sucrose catabolism (VIT_02s0154g00090, VIT_04s0008g01140), were down-regulated under salt stress.

DEGs of *Galactinol synthase* (VIT_14s0060g00760, VIT_07s0005g01970, VIT_14s0060g00760, VIT_14s0060g00790, VIT_14s0060g00810, VIT_14s0060g00800, VIT_14s0060g00810, VIT_07s0005g01970, VIT_14s0060g00800, VIT_07s0005g01680) and *Stachyose synthase* (VIT_07s0005g01680, VIT_03s0038g04570, VIT_02s0025g02790, VIT_03s0038g04570, VIT_02s0025g02790, VIT_15s0046g01000) as well as the *sugar transport* (VIT_17s0000g00820, VIT_05s0020g03140, VIT_02s0025g02080, VIT_17s0000g00830) mostly increased at 24h (Figure S7).

Heat shock protein (HSP) and sesquiterpenoids biosynthesis in response to salt stress

Heat shock proteins (*HSP*) are stress responsive proteins known as molecular chaperone, protecting plants from the stress damage. In this pathway, 10 DEGs related to *HSP* were identified. The high molecular weight *HSP* including

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VIT_02s0025g00280 and VIT_11s0037g00510 were down-regulated at 8h, but upregulated at 48h. *HSP* (VIT_13s0019g02780, VIT_16s0022g00510, VIT_06s0004g05770 and VIT_09s0002g00640) were down-regulated at 8h but overexpressed at 24h and 48h. The *HSP* VIT_00s0992g00020 was down-regulated under salt stress. Heat-stress transcription factors (VIT_00s0179g00150, VIT_09s0002g00690) showed reduced expression levels at 8h and 24h. Besides *HSP*, DEGS encoding *(-)-germacrene D synthase* (VIT_19s0014g02590, VIT_18s0001g04530 and VIT_19s0015g02070) from the sesquiterpenoids pathway were up-regulated at 8h of salt stress (Figure 6).

Different transcription factors profiles were associated with salinity in "Tebaba" roots

Transcription Factors (TF) are important modules in regulating gene expression. In our research, members of various TF families involved in salt tolerance were identified. Thirty-one DEGs encoding transcription factor were differentially regulated in "Tebaba" after 8, 24 and 48h of salt stress treatment (Figure 8). Two of them were specifically expressed at 8h, nine at 24h, thirteen at 48h and seven were commonly repressed at all experimental time points. Most of these TFs belong to *MYB* (8), AP2/ERF (8), *WRKY* (3), *bHLH* (4), *BES* (1), LOB (1), *RW4* (1), *TRAF* (1), *NAC* (1) and *HSF* (1) families. The most represented families correspond to MYB TF and AP2/ERF. The majority of TF encoding MYBs were overexpressed at all-time points; especially, *MYB 305* (VIT_05s0049g02260) and *MYB 120-4* (VIT_00s0203g00070) which showed the highest fold induction overtime course. However, the *AP2/ERF* DEGs were either overexpressed at 24h, such as *AP2.124* (VIT_11s0016g03350), *AP2.76* (VIT_02s0025g04460) and *AP2.140* (VIT_04s0008g06000) or at 48h, such as *ERF9-1* (VIT_12s0028g03270), *AP2.137* (VIT_16s0013g01070 48) and *AP2.10* (VIT_13s0067g01960). Besides the TFs, 5 transcripts corresponding to translational regulators, increased markedly at 8h of salt stress then decreased over time (Figure 8).

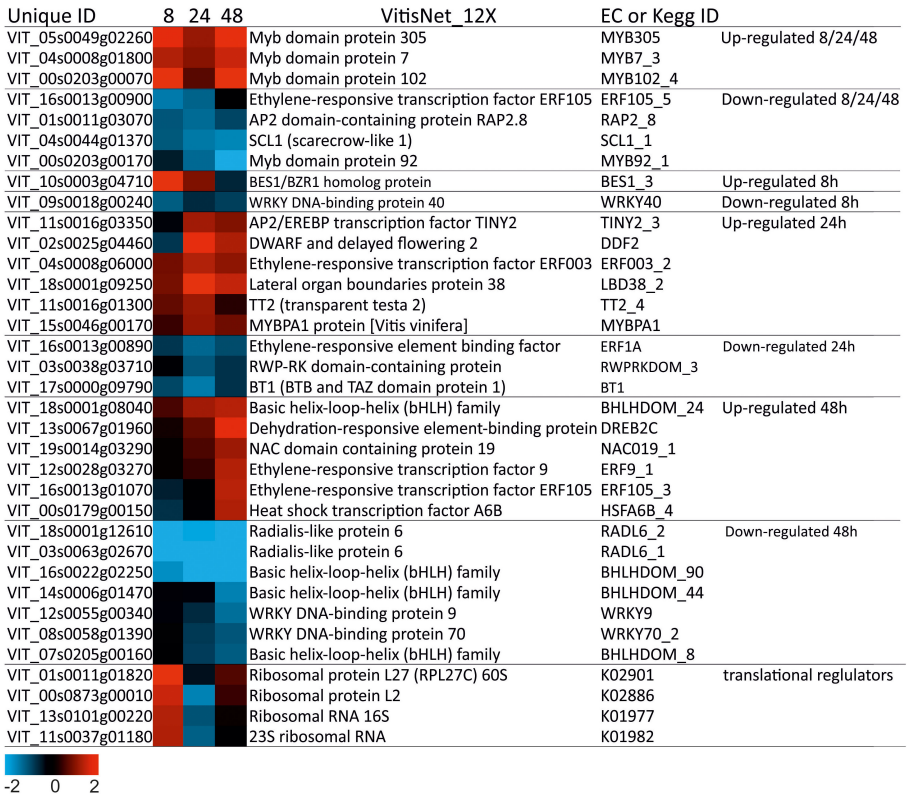

FIGURE 8
Heatmap of Transcription factors and translational regulators in wild grapevine subjected to salt stress treatments. HeatMap Analysis of expression of transcripts related to Transcription factors at different time points. The Data were ln-transformed. Red and blue colors indicate up- and down- regulated transcripts, respectively, from both control and salt treated roots. False Discovery Rate (FDR) ≤ 0.001 and the maximum value of log2 (ratio of stress/control) ≥ 1 was used as the cut-off to evaluate significant differences in expression.

Discussion

Given that genetic variation is the basis for crop improvement, characterization of genetic control of salt tolerance traits using locally adapted plants such as crop wild relatives and landraces would provide a great potential to dissect contributing traits and mechanisms (Bohra et al., 2022) Populations of wild grapevine distributed in different geographical environments showed a wide range of salt tolerance. For example, the North African wild grapevine genotype "Tebaba" (from Tunisia) was able to tolerate 150mM NaCl for 15 days without any symptoms (Askri et al., 2018) and salt injury were visible only after 23 days (Figure 1B). By contrast, the wild grapevine AS1B ecotype from the coastline of Asturias, showed a complete mortality rate at 14 days of 120mM NaCl (Carrasco et al., 2022).

Here, we have evaluated the physiological and biochemical responses of "Tebaba" wild grapevine genotype (*Vitis Sylvestris*) and compared it to the well-known salinity-tolerant and widely used rootstock 1103P under controlled salinity conditions. "Tebaba" showed the lowest osmotic potential values and less salt damage compared to 1103P rootstock suggesting that "Tebaba" responses to salt stress by maintaining required water relation parameters for positive root cell turgor (Haider et al., 2019). Furthermore, rootstocks with lower osmotic adjustment capacity are those with greater capacity to restrict the leaf accumulation of Na^+ and Cl^-, thus, preventing their possible phytotoxic effects (Stevens and Walker, 2002; Zhang et al., 2002). At the ionic level, "Tebaba" maintained a lower Na^+/K^+ ratio than 1103P, particularly at very early time (after 8h of stress application), suggesting that minimizing Na^+ accumulation in the root cells under salt stress could be a tolerance trait in the "Tebaba's" roots compared to 1103P (Zhang et al., 2018). This is on line with our previous results that showed that the tolerance of "Tebaba" genotype was due to an effective excluding of Na^+ from roots and lamina and an adaptation to osmotic adjustment *via* a better overall selectivity of potassium versus sodium (Askri et al., 2018). At the biochemical level, "Tebaba" showed also at very early time point lower levels of CAT and SOD activation concomitant with a lower H_2O_2 production which was probably due to a low Na^+/K^+ ratio as compared to 1103P. A possible explanation could be the lower translocation of sodium ions into "Tebaba" roots implying low capacity of inducibility of the antioxidative enzymes. Thus, the H_2O_2 did not reach a critical level that trigger CAT activation, since this enzyme has a low affinity to H_2O_2 (Ransy et al., 2020). Unexpectedly, "Tebaba" genotype showed efficient O_2^- scavenging and an increased accumulation of polyphenols only at early time point 8h underlining that the adverse effects of oxyradicals are prevented by the non-enzymatic antioxidant system at very early time followed, later on, by an enzymatic system. This outcome suggested that "Tebaba" roots have efficient sophisticated enzymatic and non-enzymatic antioxidant defense systems that work in concert to control cascades of uncontrolled oxidation and protect plant cells from oxidative damage at very early time. Oppositely, 1103P roots relied mostly on enzymatic antioxidant defense system. This last observation is not surprising since the synthesis of phenolic compounds is affected either positively or negatively among grape rootstocks in response to abiotic stresses, where the rootstock 1103P was reported to have reduced phenolic content under increased salinity stress condition (Jogaiah et al., 2014). Thus, accumulation of polyphenols at early time in resistant specimens can be one of the indicators for screening novel generation of grape rootstocks more resilient to abiotic stresses. Taken together, these outcomes showed that to resist the salinity conditions, "Tebaba" and 1103P mediated distinct physiological and biochemical responses following a biphasic mode. To deep our understanding on the tolerance mechanism of "Tebaba", we conducted a high throughput transcriptomic analyses in order to characterize the potential gene involved in regulating important metabolic processes in "Tebaba" and that are associated with the transition from early (8h) to late response (24-48h) under salinity condition. Each general area of metabolic pathway will be discussed in turn.

Enzymatic and non-enzymatic antioxidant pathways

Four DEGs encoding *Germin like-proteins* (*GPLs*) were highly upregulated after 8h of salinity application. This family of cell wall glycoproteins was reported to be associated with SOD activity (Gucciardo et al., 2007) and to play a structural role as targets for protein cross-linking to reinforce the cell wall during abiotic stresses (Zimmermann et al., 2006). This suggests that "Tebaba" *GLPs* are strongly implicated in cell wall strengthening and resistance to salinity stress even in the presence of very low hydrogen peroxide contents at 8h.

Following the time course, seven DEGs encoding plant *GSTs* were upregulated after 24h and 48h of salt stress. Among of these, 5 belong to Tau class (GSTU) and the two others belong to Phi class (GSTF, (Mohsenzadeh et al., 2011). Phi and Tau class *GSTs* are plant-specific and predominantly present and they have been well documented to regulate oxidative stress metabolism generated by drought and salt stresses as reviewed by (Kumar and Trivedi, 2018). It has also been suggested that *GSTF* class can recycle glutathione adducts of oxidized flavonols back to the parent flavonols maintaining consequently the antioxidant pools. Thus, we suggest that *GSTFs* play pivotal role in combining enzymatic and non-enzymatic antioxidant mechanisms in "Tebaba" roots (Dixon et al., 2011).

Beside the enzymatic antioxidant response, an increase of the non-enzymatic antioxidants, reflected by the increase of the total polyphenol content and mediated by several DEGs

encoding enzymes involved in the biosynthesis of different phenolic compound classes, was occurred during the biphasic response. Flavonol Glycoside DEGs were simultaneously upregulated with three sesquiterpenoids DEGs after 8h of salt treatment. The glycoside forms of flavonols were suggested to act as H_2O_2 scavengers during water stress in the Mediterranean species *Fraxinus ornus* (Fini et al., 2012). Furthermore, they conferred salt stress tolerance in transgenic rice since they act as ROS scavengers to reduce oxidative damage (Zhan et al., 2019). Recently, Dong et al. (2020) reported that the upregulation of *UDP-glucose glucosyltransferase* was required for the redirection of metabolic flux from lignin biosynthesis to flavonoid glycoside biosynthesis under abiotic stress. The biosynthesis of flavonols, could be an early defensive mechanism developed by "Tebaba" roots in order to mitigate oxidative stress as an effective ROS scavenging pathway (Šamec et al., 2021). Recently, flavonols were proposed to be a crucial process allowing Mediterranean plant species to adapt to climate change, especially in the Mediterranean area considered as one of the most sensitive regions to climate change over the globe (Laoué et al., 2022). In addition to the flavonol glycoside, ROS scavenging mechanism was mediated by the sesquiterpenoids *via* their efficient removal of singlet oxygen generated under oxidative stress (Velikova et al., 2004). DEGs encoding monoterpenoids, isoflavoinoids, flavanons and HSP pathway biosynthesis were downregulated after 8h of salt stress and were induced only after 24 to 48 h suggesting a minor metabolic adjustment. Several studies showed that the 24 h might be a turning point at which the salt response strategy might begin to change in the roots of soybean (Liu et al., 2019) and common Bermuda grass (Shao et al., 2021). For example, the *HSP* induction after prolonged salt stress would play a role of protector of their target proteins from denaturation (Guo et al., 2020). At 48h, our results showed the upregulation of the lignin biosynthesis DEGs (*CAD*, *SAD*) was concomitant with a downregulation of DEGs related to anthocyanin metabolism (*LDOX*), suggesting a time-depend switch from the accumulation of anthocyanin compounds at 8h to the accumulation of lignin compounds at 48h (Zúñiga et al., 2019) hypothesized that the upregulation of the lignin biosynthetic pathway negatively affected the anthocyanin production by lowering the levels of the common precursor p-coumaric acid. This was already evidenced by Van Der Rest et al (2006) in tomato transgenic plants. Lignin is most likely to constitute the phenolic compounds pool of "Tebaba" rather than antocyanins.

Osmotic stress-related signaling and sodium-sequestration/exclusion

Sensing salt signals is a prerequisite for initiating the reestablishment of cellular ionic homeostasis. Starting after 8h of salinity stress, the upregulation of DEGs encoding ion channels and *RLKs*, suggests that these transporters establish, as early response, signaling circuits to transduce information from outer plant cell under salinity conditions. At the same time (8h post stress application), DEG encoding vacuolar *H^+-ATPase* was also up-regulated suggesting an active transport of Na^+ from the cytoplasm to the vacuole, generating thus a vacuolar Na^+ sequestration process (Graus et al., 2018; Yang and Guo, 2018). Such a signaling system is important to maintain ionic homeostasis through an efficient Na^+ compartmentalization mechanism as a trait of wild grapevine adaptation to salt stress. DEGs encoding vesicular trafficking were upregulated by 24 to 48h of salt stress. This up-regulation was concomitant with the transcript accumulation of the *hyperosmotically inducible periplasm protein* and the *HVA22E*, which are part of the regulatory mechanism for vesicle movement between the plasma membrane and vacuole. They are also reported to involved in maintaining a lower Na^+/K^+ ratio and in conferring salt tolerance in transgenic Arabidopsis plants (Liu et al., 2012). Vesicles trafficking encoding genes are known to be correlated with Na^+ exclusion and are important for the salinity stress response (Sweetman et al., 2020). Taken together, this suggests that the vesicle trafficking mechanism could be the main process for Na^+ relocation/exclusion and thus favoring the K^+ accumulation in the roots of wild grapevine under salinity condition.

In addition, restriction of Cl^- transport into the roots could be achieved by the *Cl^- loading to xylm/SLAH1* DEG which its down-regulation is known to reduce Cl^- efflux and maintain anion homeostasis. Such mechanism was already reported in *Vitis* species with enhanced salt stress tolerance (Henderson et al., 2014). DEGs encoding *PTKs* and *PP2C* were gradually increased over time and correlated to the upregulation of ABA biosynthesis and transport genes and most likely regulated by *WRKY52* (VIT_09s0018g00240), known to be involved in the ABA signaling pathway (Geilen and Böhmer, 2015). However, DEGs encoding ABA receptors were dramatically down-regulated, suggesting that the transduction of osmotic signal in the "Tebaba" roots could rely mainly on the ABA independent pathway.

Osmoregulation and osmoprotection

To strength the osmotic adjustment, particularly during in the late phase, cell roots most likely induce DEGs encoding enzymes related to the raffinose oligosacharide family, suggesting that the galactionl was essentially accumulated in order to prevent cell dehydration and loss of turgor after longer exposure to NaCl. This is on line with what was reported for the stress tolerance mechanism in wild *Vitis Amurensis* (Chai et al., 2019).

Starch turnover for energy saving in wild grapevine under salinity stress

Plants remobilize their starch reserve to release energy, sugars and derived metabolites to help mitigate the stress (Zeeman et al., 2010). At early salt stress phase, the upregulation of the DEG encoding *β-amylase* followed by the induction of the glycolytic pathway genes such as: (*Aldolase-1-epimerase*), *Fructose-1,6-bisphosphate aldolase* (*FBA*), *Enolase*, and *pyruvate kinase* (*PK*), suggest that the increased flow of carbon through the Calvin cycle leads to an increased sucrose and amino acid production. This might result in osmoprotectant and compatible solutes that are involved to support plant growth and/or to maintain the osmotic balance and scavenging hydrogen peroxide under salinity conditions (Du et al., 2019). When extending the salinity exposure, DEGs of starch synthesis were highly upregulated with maximum increase up to 48h. This upregulation was concomitant with a down-regulation of DEGs starch catabolism, suggesting that the turnover of starch metabolism under salinity stress could be an alternative source of energy and carbon for the biosynthesis of compatible solutes, allowing thus "Tebaba" roots to mitigate the effects of salt stress (Thalmann and Santelia, 2017).

Lignin biosynthesis and cell wall remodeling

Starting at 24h of salt stress, several DEGs of lignin biosynthesis pathway (e.g. *Laccase*) were up-regulated, suggesting that enhancing lignin biosynthesis pathway would enhance rigidity of "Tebaba" roots and provide mechanical support for water and osmolytes transport through the xylem vessel (Hu et al., 2009). This is on line with previous studies reporting that strong expression of lignin biosynthetic genes is a crucial factor in plant adaptation and tolerance to salt stress (Chun et al., 2019). The transcriptional induction of lignin biosynthesis is maybe controlled by both TFs: *MYBPA1*, (Koyama et al., 2014) and *NAC019-1* (Tu et al., 2020). In addition, our transcriptomic analysis revealed the upregulation of several DEGs related to the cell wall-loosening pathway such as *expansins* and *pectinases* as well as DEGs encoding cell wall expansion and remodeling (e.g. *xyloglucan*). This occurred concomitantly with a downregulation of the cell wall stiffening DEGs (*cellulases* and *pectin esterases*), supporting one of the explanations that cell wall extensibility may play a crucial role in adaptation to salinity by maintaining normal turgor pressure under a longer exposure to salt stress (Chen et al., 2019). In the same line, transcriptomic reprogramming revealed that cell wall remodeling mainly occurred in the wild *Vitis sylvestris* AS1B ecotype when the salinity increased (Carrasco et al., 2022). Here, it is important to stress out that "Tebaba" genotype establish a combined response mechanism based on cell wall remodeling with an efficient osmoregulation. At transcriptional level, the cell expansion most likely regulated *via* both transcription factors: the *VviERF045* [VIT_04s0008g06000, (Leida et al., 2016)] and *Lateral organ boundaries protein 38* [VIT_18s0001g09250, (Chavigneau et al., 2012)]. Taken together, these outcomes suggest that modulation of lignin and cell wall-related pathway could be a good indicator of an acclimation mechanism used by "Tebaba" roots to mitigate salt stress. This cell wall modification could be regulated by *AP2/EREBP* TF (Saelim et al., 2018).

Time-independent transcription factors in wild grapevine

Interestingly, the *ERF9-1* from the ERF family was induced at late time point 48h in "Tebaba" roots which is online with our previous comparative study showing the upregulation of this TF exclusively in the tolerant cultivated grapevine versus sensitive cultivars under salt stress (Daldoul et al., 2010). We thus suggest that this TF could be a salt tolerance biomarker in *vitis*. The *MYB102-4* from the MYB family, was upregulated over time in this study under salinity condition but it is also known to contribute to the water deficit induced suberization of grapevine roots (Zhang et al., 2020) suggesting that probably this TF is involved in "Tebaba" root suberization. The *MYB305* was reported to be up-regulated in shade-treated inflorescence in *Vitis vinifera* (Domingos et al., 2016) and was found to be upregulated in this study under salinity stress.

Besides TFs, gene expression regulation is influenced by translational regulators. Their related DEGs were markedly upregulated only at very early time point 8h in "Tebaba" roots. Translational reprogramming process used by "Tebaba" genotype could be a faster and more dynamic strategy to quickly adjust the translation process to the environmental changes to alleviate salt stress (Dias-Fields and Adamala, 2022).

A hypothetical mechanism for salt stress response in wild grapevine roots

The integration of physiological, biochemical and transcriptomic data shed new light on response of wild grapevine to salt stress over a time course. The combined results increase our understanding of the chronological changes that occur during two distinct phases: after 8h (very early response phase) and after 24 to 48h as late response phase, and allow us to propose a schematic model of the transcriptional cascade during salinity stress in *Vitis sylvestris* (Figure 9). According to this model, very early response TF and signaling genes sense and respond to salinity stress by activating ion channel receptors, receptor-like kinases, the H^+-*ATPase* for ion

10.3389/fpls.2022.1077710

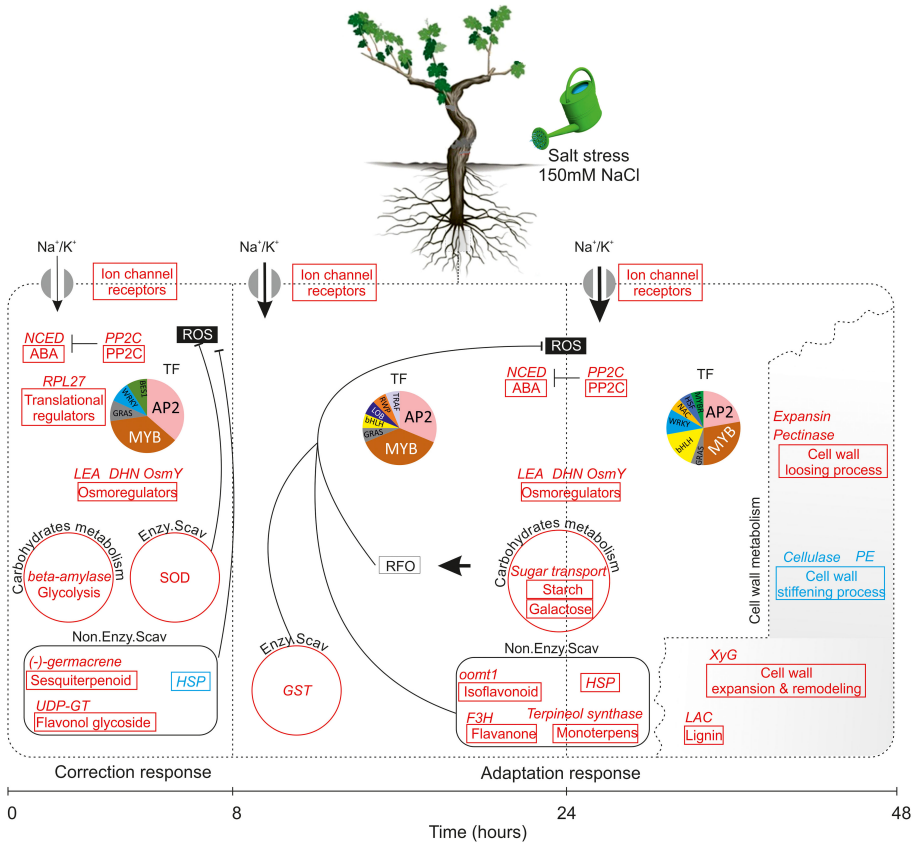

FIGURE 9
Model of how *vitis sylvestris* response to salinity stress during the early phase (8h) and the late phase (24-48h). Gene pathways significantly upregulated highlighted in red and those in blue are significantly downregulated; each pathway was represented by a box and the key DEGs related to each pathway were indicated in the top of the box; the size of arrow indicated the amount of ions transported. Abbreviations: NCED, 9-cis-epoxy-carotenoid dioxygenase; ABA, Abscisic Acid; PP2C, Protein Phosphatases 2C; LEA, Late Embryogenesis Abundant; DHN, Dehydrin; Osmy, Hyperosmotically inducible periplasmic protein; SOD, Superoxide dismutase; HSP, Heat shock protein; GST: Glutathion S-transferases; XyG, Xyloglucan; LAC, Laccase; PE, Pectine Esterase; TF, Transcription Factor; ROS, Reactive Oxygen Species; RFO, Raffinose Family of Oligosaccharides. The association of putative transcription factors (TF) with each state is depicted in pie charts. Sizes are proportional to the number of each TF family detected. TFs that were significantly enriched under those conditions are listed in Figure 8.

transport, preferentially K^+, and increasing expression of genes of sesquiterpenoids biosynthesis, flavonol glycoside as non-enzymatic antioxidant system against ROS and glycolysis to compensate low carbon assimilation. These early responding changes are reinforcing the hypothesis that the early response establish a short term acclimatization to what could be a quickly reversed stress. When salt stress is prolonged, it seems that metabolic changes induce some novel transcription factor family members (e.g. *NAC*) which appear to execute more specific function related to the induction of a gene directly involved in lignin biosynthesis, isoflavonoids, monoterpenes, *HSP*, galactose and starch anabolism to generate osmoprotectants against RFO in parallel to the induction of the enzymatic antioxidant system. These changes also include, the activation of genes related to cell wall-loosening pathway concomitant with a down-regulation of the cell wall stiffening offering a cell wall flexibility and metabolic reprogramming under salt stress. Our findings allow further understanding of the genetic regulation mechanism of salinity tolerance in *Vitis sylvestris*. Specific validation and functional characterization of the key biomarkers would help in selecting suitable traits for the design of appropriate breeding strategies and the development of new generation of rootstocks (of Mediterranean origin) with highly enhanced abiotic stress tolerance.

Data availability statement

The RNA-seq datasets by using Illumina-Hiseq platform are available from the NCBI Sequence Read Archive database (SRA; http://www.ncbi.nlm.nih.gov/sra) under project number accession PRJNA507974. The cDNA libraries were obtained from the

controls and their respective salt-stress of 150mM NaCl for 8h, 24 h and 48h with two biological replicates, respectively.

Author contributions

SD, MG, and AM conceived and designed the study. FH and ZH performed the physiological and biochemical analyses. SD performed the molecular analyses. MG performed the bioinformatic and statistical analyses. SD and MG wrote the original draft of the manuscript. All authors read and contribute to the review and editing of the final manuscript.

Funding

This research was carried out as part of the Tunisian National Research Program (CP2019-2022) of the laboratory of Plant Molecular Physiology at the Centre of Biotechnology of Borj-Cedria, funded by the Tunisian Ministry of Higher Education and Scientific Research.

Acknowledgments

The authors would like to thank the technical staff of the laboratory as well as the editors and reviewers of the special issue of Frontiers in Plant Science «Resilience of Grapevine to Climate Change: From Plant Physiology to Adaptation Strategies» Volume II.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1077710/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 16 December 2022
DOI 10.3389/fpls.2022.1075231

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

RECEIVED 24 October 2022
ACCEPTED 28 November 2022
PUBLISHED 16 December 2022

CITATION
Ramos M, Daranas N, Llugany M, Tolrà R, Montesinos E and Badosa E (2022) Grapevine response to a *Dittrichia viscosa* extract and a *Bacillus velezensis* strain.
Front. Plant Sci. 13:1075231.
doi: 10.3389/fpls.2022.1075231

Grapevine response to a *Dittrichia viscosa* extract and a *Bacillus velezensis* strain

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The present study aims to evaluate the response of the three Mediterranean local grapevines 'Garnacha Blanca', 'Garnacha Tinta', and 'Macabeo' to treatments with biocontrol products, namely a botanical extract (Akivi, *Dittrichia viscosa* extract) and a beneficial microorganism (*Bacillus* UdG, *Bacillus velezensis*). A combination of transcriptomics and metabolomics approaches were chosen in order to study grapevine gene expression and to identify gene marker candidates, as well as, to determine differentially concentrated grapevine metabolites in response to biocontrol product treatments. Grapevine plants were cultivated in greenhouse under controlled conditions and submitted to the treatments. Thereafter, leaves were sampled 24h after treatment to carry out the gene expression study by RT-qPCR for the three cultivars and by RNA-sequencing for 'Garnacha Blanca'. Differentially expressed genes (DEGs) were investigated for both treatments and highly influenced DEGs were selected to be tested in the three cultivars as treatment gene markers. In addition, the extraction of leaf components was performed to quantify metabolites, such as phytohormones, organic acids, and phenols. Considering the upregulated and downregulated genes and the enhanced metabolites concentrations, the treatments had an effect on jasmonic acid, ethylene, and phenylpropanoids defense pathways. In addition, several DEG markers were identified presenting a stable overexpression after the treatments in the three grapevine cultivars. These gene markers could be used to monitor the activity of the products in field treatments. Further research will be necessary to confirm these primary results under field conditions.

KEYWORDS

biocontrol products, transcriptomics, RT-qPCR, gene markers, metabolites, grapevine

1 Introduction

The European Union is the main world producer, consumer, and exporter of grapevine for wine-making (*Vitis vinifera*), and the production is mainly concentrated in three countries: Italy (29.7%), Spain (27.1%), and France (24.2%) (European Commission, 2021). Vineyards are threatened by several diseases, including powdery mildew and gray mold caused by the fungal pathogens *Erysiphe necator* and *Botrytis cinerea*, respectively, and downy mildew caused by the oomycete *Plasmopara viticola* (Avenard et al., 2003; Gessler et al., 2011; Reynier, 2011; Beris et al., 2021). These causal agents are able to infect several grapevine tissues starting from flowers and leaves (*E. necator*), from leaves (*P. viticola*), and from berries (*B. cinerea*). If the first infections are not controlled, the diseases spread quickly in the vineyard and mildews can infect berries as well. These diseases can cause severe crop losses depending on the season and the cultivation area, reducing the harvest quality and yield, plant vigor and photosynthesis (Calonnec et al., 2006; Leroy et al., 2013; Kunova et al., 2021).

The main grapevine cultivars are susceptible to these diseases and vineyard protection requires intensive treatments with plant protection products (PPPs), such as chemical fungicides from bud burst until ripening (Boubakri et al., 2013). The frequency average of the applied fungicide treatments is around ten treatments per year, which can rise up to 20 treatments under the most critical conditions (Butault et al., 2010; Leroy et al., 2013; Pertot et al., 2017). This intensive use of PPPs can affect the treated crops, the environment, and the consumer health as well (Alavanja et al., 2004; Boubakri et al., 2012; Boubakri et al., 2013; Krzyzaniak et al., 2018; Zambito Marsala et al., 2020). To prevent the negative impact of the intensive use of synthetic PPPs, more environmentally friendly compounds, such as biocontrol products are promoted by European governments (European Parliament, Council of the European Union, 2009a; European Parliament, Council of the European Union, 2009b). Among the different types of biocontrol products, there are natural substances derived from plant, animal, or mineral extracts and beneficial microorganisms able to protect the plant from pests and diseases.

Natural substances as well as beneficial microorganisms used as biocontrol products present modes of action mainly relying on (i) direct action against the pathogen (Bonaterra et al., 2012; Persaud et al., 2019) or (ii) indirect action by stimulating plant defense (Perazzolli et al., 2011; Perazzolli et al., 2012; Pieterse et al., 2014; Rienth et al., 2019; Nishad et al., 2020; Burdziej et al., 2021). It has been reported that a plant extract from *Vitis* can present direct activity in grapevine against downy mildew (Schnee et al., 2013). Some beneficial microorganisms are able to compete against pathogens for space and nutrient supplies (Bonaterra et al., 2012) or to show antagonism activity against pathogens through antimicrobial or lytic enzyme production (Ongena and Jacques, 2008; Wang et al., 2013; Mora et al., 2015; Vilà et al., 2016). Moreover, laminarin (algae extract) and chito-oligosaccharides associated with oligogalacturonides (COS-OGA) are already used in vineyards as plant defense stimulators, protecting grapevine against downy mildew and powdery mildew (Van Aubel et al., 2014; Bodin et al., 2020). Some beneficial microorganisms are already authorized and used in vineyards (Otoguro and Suzuki, 2018). It is reported *Bacillus subtilis* strains that show antagonism activity against gray mold (Maachia et al., 2015) and a *Saccharomyces cerevisiae* cell wall derivatives-based product that induce resistance against downy mildew, gray mold and powdery mildew (De Miccolis Angelini et al., 2019). Biocontrol products with a combination between the two types of mechanisms are described as well (Krzyzaniak et al., 2018; Esmaeel et al., 2020; Zhou et al., 2020).

Plant defense response to biotic stresses relies on different levels of recognition. After pathogen infection, molecular patterns or effectors of the pathogen are recognized leading to (pathogen-associated molecular patterns) PAMP-triggered immunity (PTI) or effector-triggered immunity (ETI). Both PTI and ETI stimulate plant systemic acquired resistance (SAR) (Abdul Malik et al., 2020). Beneficial microorganisms recognition can also trigger plant defense response called induced systemic resistance (ISR). SAR and ISR responses involve phytohormones, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), being SA more specific to SAR and JA/ET pathway to ISR (Pieterse et al., 2014). It is reported that SA is involved in the defense against biotrophic pathogens, including *P. viticola* and *E. necator*, whereas JA/ET pathway against necrotrophic pathogens, such as *B. cinerea*. However, the two pathways can be activated simultaneously (Burdziej et al., 2021). Direct application of phytohormones or analogues are able to trigger defense response in grapevine against downy mildew (Bodin et al., 2020; Burdziej et al., 2021). Despite biocontrol products modes of action are not always well-understood in several plant species and cultivars, it is important to assess that they have no impact on the treated plants or the environment.

This study aims to evaluate the response of three appreciated and autochthonous grapevine cultivars of the Mediterranean zone, concretely of Catalonia (Spain): Garnacha Blanca, Garnacha Tinta, and Macabeo to biocontrol product treatments. Two biocontrol products from different origins were investigated: a botanical extract (Akivi, *Dittrichia viscosa* extract) and a beneficial bacterial strain (*Bacillus* UdG, *Bacillus velezensis* living bacteria). Both products are still in development and the combination of a whole genome transcriptomics approach and a targeted metabolomics approach was chosen to elucidate the grapevine response to the treatments. The objectives of this work are: (i) to study grapevine gene expression response after biocontrol product treatment using transcriptomics; (ii) to identify robust gene marker candidates

presenting stable differential expression after treatment within the three grapevine cultivars; and (iii) to determine grapevine metabolites variations after biocontrol product treatment using targeted metabolomics.

2 Materials and methods

2.1 *Bacillus* UdG production and plant extract

Bacillus velezensis UdG strain was isolated from a wild plant collected during a sample screening as reported by Mora et al. (2011). *B. velezensis* UdG was routinely cultivated on a Luria-Bertani agar and incubated at 28°C for 24h. For the assays, two different products consisting of lyophilized and fresh cells were prepared.

For lyophilized *Bacillus* (BL), a fermentation process was done in a pilot-scale bioreactor (Biostat® C, Sartorius, Germany) with a working volume of 30 L of production medium for 48 h at 28°C, pH7 and agitation ramp from 50 to 500 rpm. The production medium consisted of a modification of the original recipe of Walker and Abraham (1970). Specifically, the following modifications were considered: 7 g L^{-1} instead of 1 g L^{-1} of KH_2PO_4, 1 g L^{-1} instead of 4 g L^{-1} of L-monosodium glutamate, 5 g L^{-1} of molasses and 1 g L^{-1} of soy flour instead of 342 g L^{-1} of saccharose, 1 mL L^{-1} instead of 5 mL L^{-1} of ferric citrate solution, and 1 mL L^{-1} of oligoelement solution at 0.1 mg mL^{-1} instead of at 0.1 mg L^{-1}. After fermentation, the cells were harvested by centrifugation (SA-1-02-175, GEA Westfalia, Granollers, Spain) at 10,000 rpm and the concentrated cell suspension was mixed with skimmed milk (15% final concentration). The bacterial suspension was frozen at −70°C and lyophilized in a laboratory scale freeze-dryer (Unitop HL, VirTis, Gardiner, NY). Dried samples were stored in vacuum sealed plastic-coated aluminum bags.

For fresh *Bacillus* (BF), a fermentation process was carried out in a 2-L Erlenmeyer flask for 48 h at 28°C and shaking at 150 rpm with 500 mL of the original recipe of production medium (modification: oligoelement solution was used at 0.1 mg mL^{-1} instead of at 0.1 mg L^{-1}). After fermentation, the cells were harvested by centrifugation at 13,200 g for 10 min (Centrifuge 5810R, Eppendorf) and concentrated 10X with the corresponding volume of supernatant.

The plant extract Akivi was provided by S.A.S. AkiNaO (France). It is a formulated botanical extract prototype from *Dittrichia viscosa* composed of a high content of polyphenols and terpenes (Tamm et al., 2017).

2.2 Plant material, treatments, and experimental design

Three grapevine cultivars (*Vitis vinifera* L.), namely Garnacha Blanca, Garnacha Tinta and Macabeo, grafted on rootstock 110R, were obtained from commercial nurseries (Agromillora Iberica and Viveros Villanueva Vides, Spain). One-year-old bench-grafted grapevine rootlings were planted in a 2 L pot with 80% of the growing media (Prodeasa BV35, Burés Profesional, Spain), 20% of perlite (A-13, Agroteibe, Spain), and 4 g of the fertilizer (Osmocote® Exact Mini 3-4M, ICL Specialty Fertilizers, France). Bench-grafted grapevines were grown in a greenhouse at 25 ± 2°C, 60 ± 10% relative humidity and a 16:8 h light:dark photoperiod. Young stocks with at least about 4 to 6 expanded leaves were used for the experiments.

The treatments consisted of Akivi at 0.521 g L^{-1} (Aki), and *Bacillus* UdG at 10^8 CFU mL^{-1} lyophilized (BL) and fresh (BF). Water was used as the solvent to prepare each treatment. The BF treatment was only used in the experiment with cv. Garnacha Blanca. A non-treated control (NTC) using water was included in all the experiments. The products were sprayed on adaxial and abaxial leaf surfaces using an airbrush until near run-off.

The experimental design for cv. Garnacha Blanca stocks included 4 randomized blocks corresponding to the different treatment modalities (Aki, BL, BF, and NTC), while for cvs. Garnacha Tinta and Macabeo included 3 blocks (Aki, BL, and NTC). Each block was composed of 4 biological replicates of 5 plants.

2.3 Sampling plant material and RNA isolation

Sampling was carried out 24 h after spraying plants with the products. At that time transcriptomics response, as well as, phytormone signalization and metabolomics responses can be evaluated. Four biological replicates were sampled for each treatment for RNA-sequencing analysis (RNA-seq), and three biological replicates for reverse transcription quantitative PCR (RT-qPCR) analysis. Two leaves from each plant (5 plants per biological replicate) were harvested, grounded, and soaked in liquid nitrogen. Each ground leaf sample was added to 2 mL tubes containing two borosilicate glass beads in order to obtain a fine powder using Tissuelyzer II system (Qiagen, USA) for 1 min at 30 Hz.

For total RNA isolation from grapevine leaves, the commercial kit Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) was used (Supplementary Table 1) following manufacturer's instructions. Residual DNA was removed using Invitrogen™ TURBO DNA-free™ Kit (Applied Biosystems, USA).

The concentration and purity of RNA was assessed by spectrophotometric measurements using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). RNA quality was evaluated using electrophoresis on 1.0% agarose gels.

Prior to RNA-seq analysis, a R.I.N. measurement was carried out using an Agilent 2100 Bioanalyzer (Agilent technologies, USA) to check RNA integrity from cv. Garnacha Blanca samples

and RNA extracted in each sample was quantified by using the Qubit 2.0 Fluorometer (Invitrogen, USA).

2.4 RNA-sequencing and reads mapping

The plant response to treatments using transcriptomics was studied on cv. Garnacha Blanca grapevine leaves after spray application with Aki, BL, BF or water (NTC). A total of 16 samples were used for the library construction.

The RNA-seq transcriptome library was prepared using the TruSeq Stranded mRNA Sample Prep kit (Illumina, USA) following the manufacturer's instructions using 1-2 μg of good quality RNA (R.I.N. > 7) as input. The RNA was fragmented by 3 minutes at 94°C and each purification step was performed by using 0.81X Agencourt AMPure XP beads. Final libraries were quantified by using the Qubit 2.0 Fluorometer (Invitrogen, USA) and quality tested by Agilent 2100 Bioanalyzer RNA Nano assay (Agilent technologies, USA). Libraries were then processed with Illumina cBot for cluster generation on the flowcell, following the manufacturer's instructions and sequenced on paired-end (2x150 bp, 30M reads per sample) at the multiplexing level requested on NovaSeq6000 (Illumina). The CASAVA 1.8.2 version of the Illumina pipeline was used to process raw data for both format conversion and de-multiplexing.

Raw sequence files were first subjected to quality control analysis by using FastQC v0.10.1 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) before trimming and removal of adapters with BBDuk (https://jgi.doe.gov/data-and-tools/bbtools/) setting a minimum base quality of 25 and a minimum read length of 35 bp. Reads were then mapped against the *V. vinifera* L. genome (*V. vinifera* cv. Pinot noir var. PN40024) (version 12X Ensembl) with STAR v2.6 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3530905/). FeatureCounts v1.6.1 (https://academic.oup.com/bioinformatics/article/30/7/923/232889) was then used to obtain raw expression counts for each annotated gene using only uniquely mapping reads (MAPQ>=30). The differential gene expression (DGE) analysis was conducted with the R package edgeR (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2796818/) using the Trimmed mean of M-values (TMM) normalization method and considering as significant the genes with a False Discovery Rate (FDR) ≤ 0.05. Fragments Per Kilobase Million (FPKM) were obtained with edgeR. Gene Ontology (GO) Enrichment Analysis was performed using in-house scripts based on the AgriGO publication (https://academic.oup.com/nar/article/45/W1/W122/3796337). GO enrichment analysis was carried out using a threshold value (p-Value) < 0.05. The main biological functions were selected considering the Gene Ontology (GO) terms that showed at least 4 affected DEGs. Then the selected GO terms were analyzed using REVIGO web platform (http://revigo.irb.hr/) in order to summarize GO terms by removing redundancies. For each biological function category, different GO terms clusters (representative groups) that presented semantic similarity were obtained. The affected DEGs corresponding to all the GO terms of each cluster were added. In addition, GO terms that presented a background number over 1000 genes (BG-Item) were discarded since they are general GO terms. Clusters that showed less than 10 DEGs were joined under the term "other" considering the total number of genes. In addition, metabolic pathways influenced by the treatments were defined using Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation (Kanehisa et al., 2016). KEGG pathways with a corrected p-Value < 0.05 were considered significantly influenced by the treatments.

2.5 Screening of differentially expressed genes

Screening of DEGs was carried out for each treatment modality (Aki, BL and BF) in comparison with the NTC. The two *Bacillus* modalities were studied together in order to identify common genes exclusively due to the bacterial activity, eliminating the effect of the freeze-drying.

Gene expression levels were assessed on the basis of unique mapped genes and were calculated using the FPKM method. FPKM values were used to analyze the differences in gene expression between treatments (Aki, BL, and BF) and NTC, by calculating a Fold-Change (FC) value.

Due to the high biological variability, the DEGs screening was conducted on the three biological replicates that presented less variability between each other, in order to avoid hiding a part of the treatment impact on the plant. DEGs exclusively altered by each treatment were targeted. The criteria of selection during the screening were based on DEGs presenting high differential expression value, specifically $Log_2(FC) > |1.4|$ and good repeatability among the three biological replicates.

2.6 Validation of DEGs by RT-qPCR

To confirm the transcriptome data obtained by RNA-seq analysis, 27 DEGs were selected ($Log_2(FC) > |1.4|$ and good repeatability among the three biological replicates) and their expression level was validated by RT-qPCR (Supplementary Table 2). The UBQ gene, coding for the Ubiquitin-conjugating enzyme, was used in this study as the endogenous gene for data normalization. This endogenous gene was previously selected according to the method described by Silver et al. (2006) (Supplementary Figure 1).

Standard curves for DEGs and the endogenous gene were obtained using decimal dilutions of extracted recombinant plasmid DNA (target sequences were cloned into a vector pSpark® in *Escherichia coli* DH5α cells) corresponding to

copy numbers ranging between 10^2 and 10^7. Ct values in each dilution were measured in triplicate and a negative non-template control was included in each run. Real-time PCR reactions included 10 µL SYBR® Green PCR Master Mix (Applied Biosystems), 6 µL RNase-free water, 1 µL of each forward and reverse primer (Supplementary Table 2) at the corresponding concentration, and 2 µL DNA in a final volume of 20 µL. The optimal primer concentration (100, 300 or 600 nM) was previously defined. The thermal cycling conditions were as follows: 10 min at 95˚C for initial denaturation; 40 cycles of 15 s at 95˚C, and 1 min at 60˚C; and a final melting curve program of 60 to 95˚C with a heating rate of 0.5˚C s^{-1}. Ct values were plotted against the logarithm of their initial template copy numbers and each standard curve was generated by a linear regression of the plotted points. The efficiency of each standard curve was calculated using the formula $E = (10^{(-1/a)} -1) \times 100$, where "a" is the slope of the curve.

For RT-qPCR, total RNA was extracted from leaf samples of treated plants using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) as explained above. First-strand of cDNA was synthetized from RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The absence of chromosomal DNA contamination was confirmed by minus-reverse transcriptase control in qPCR. Quantitative PCR was carried out in a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems) to assess the transcriptional level of 27 DEGs. All the information of the selected genes and primers designed by Primer-BLAST tool from the Nacional Centre for Biotechnology Information (NCBI) are shown in Supplementary Table 2. Optimized qPCR reactions and the thermal cycling conditions were described above. Each qPCR assay included duplicates of each cDNA sample, no-template and RNA controls to check for contamination. Ct values from three biological replicates were averaged, and UBQ gene was used for data normalization.

The comparative critical threshold (ΔΔCt) method was used to assess the relative quantification of gene expression. Similar amplification efficiencies of all gene primer pairs were checked (Supplementary Table 3) making the ΔΔCt method appropriate to calculate the Fold-Change (FC). The ΔCt of the NTC leaf samples was used as the calibrating condition to calculate the FC. Genes were considered to be up- or downregulated if their FC were at least two-fold ($FC = 2^1$ or 2^{-1}) higher or less than the calibrator condition (Livak and Schmittgen, 2001) and showed statistically significant differences with the NTC.

2.7 Metabolite analysis

Metabolite extractions were carried out from powdered samples of grapevine leaves of cvs. Garnacha Blanca, Garnacha Tinta, and Macabeo obtained 24 h after spraying them with Aki, BL, or water (NTC) as explained above.

For phytohormone extraction, 250 mg of fresh grapevine leaves were grounded in an ice-cold mortar with 750 µL of extraction solution (methanol:isopropanol:acetic acid; 20:79:1 by vol.) (Llugany et al., 2013). Then, the supernatant was collected after centrifugation at 1000 g for 5 min at 4°C. These steps were repeated two more times and pooled supernatants were lyophilized. Finally, samples were dissolved in 250 µL pure methanol and filtered with a Spin-X centrifuge tube filter of 0.22 µm cellulose acetate (Costar, Corning Incorporated, USA). Phytohormone quantification was done using a standard addition calibration curve spiking control plant samples with the standard solutions of gibberellin A1 (GA1), gibberellin A4 (GA4), methyl jasmonate (MeJA), salicylic acid (SA), (±)-jasmonic acid (JA), (+)-cis, trans-abscisic acid (ABA) and 1-aminocyclopropane-1-carboxylic acid (ACC) ranging from 5 to 250 ppb and extracting as described above. Deuterated hormones jasmonic acid-d5 (JA-d5) and salicylic acid-d6 (SA-d6) at 30 ppb and 300 ppb, respectively, were used as internal standards in all the samples and standards measurements. Standards were purchased from Merk (Germany).

Plant hormones were analyzed by LC-ESI-MS/MS system in multiple reaction monitoring mode (MRM) according to Segarra et al. (2006). Phytohormones were separated using HPLC Acquity (Waters, USA) on a Luna Omega C18 column 1.6 µm 100 Å 50 x 2.1 mm (Phenomenex, USA) at 50°C at a constant flow rate of 0.8 mL min^{-1} and 10 µl injected volume. The elution gradient was carried out with a binary solvent system consisting of 0,1% of formic acid in methanol (solvent A) and 0,1% formic acid in milli-Q H_2O (solvent B) with the following proportions (v/v) of solvent A (t (min), %A): (0, 2) (0.2, 2), (1.6, 100), (2, 100), (2.1, 2) and (3, 2). MS/MS experiments were performed on an ABI 4000 Qtrap mass spectrometer (Sciex). All the analyses were performed using the Turbo Ionspray source in negative ion mode except for MeJA and ACC.

Quantification was made by injection of extracted and spiked samples in multiple reaction monitoring (MRM) mode. Identification of phytohormones was based on retention time and presence of peak in the MRM trace compared with those of the standards.

Organic acids (OA) were extracted with a classical extraction protocol. Briefly, 250 mg of fresh grapevine leave powder was grounded in an ice-cold mortar with 2 mL of hydrochloric acid (0.025N). Then, the supernatant was collected after centrifugation at 1000 g for 15 min at 4°C. Meanwhile, Sep-Pack C18 cartridges (Waters, USA) were activated with (i) 1.4 mL of methanol, (ii) 0.7 mL of milli-Q water, and (iii) 1.4 mL of hydrochloric acid (0,025M). Supernatant (1.4 mL) were passed through the cartridge to recover 0.7 mL of clean extract. Finally, samples were filtered at 0.22 µm just prior to injection into an HPLC system.

Organic acids were analyzed by HPLC-UV system (Shimazu, Japan) in the following conditions (Tolrà et al., 2005): YMC-Pack ODS-A HPLC column 5µm 120Å 250 x 4.6 mm (YMC,

Germany) at a constant flow rate of 0.8 mL min^{-1} and 10 µl injected volume. The injection method ran 15 min with an isocratic flow of 50 nM Potassium dihydrogen phosphate (KH_2PO_4) adjusted at a pH of 2.8 using Phosphoric acid (H_3PO_4).

The following standards were used for OA measurements: acetic acid, cis-aconitic acid, trans-aconitic acid, ascorbic acid, citric acid, isocitric acid, formic acid, fumaric acid, galacturonic acid, gluconic acid, glucuronic acid, glutamic acid, glycine, glycolic acid, glyoxylic acid, lactic acid, maleic acid, malic acid, malonic acid, oxalic acid, oxoglutaric acid, pyruvic acid, quinic acid, succinic acid, tannic acid, and tartaric acid.

Four peaks corresponding to OA were detected on samples HPLC-UV chromatograms. Then, these peaks' retention times were compared with the retention times of 26 standards injected in the same conditions, and the identification was confirmed by standard enrichment injection within the grapevine samples. The four OA were identified (oxalic acid, tartaric acid, malic acid, and oxoglutaric acid) and quantified thanks to calibration curves. Calibration curves: malic acid ($y=1.2967x+7.0154$, $R^2 = 0.9967$), oxalic acid ($y=0.2891x+4.7116$, $R^2 = 0.9993$), oxoglutaric acid ($y=1.4261x+17.324$, $R^2 = 0.9972$), tartaric acid ($y=2.6801x+2.4512$, $R^2 = 0.9998$).

Putative identification was carried out by comparing the retention time of the standards with the peaks obtained in the grapevine leaf samples. The standard addition to the samples was done to check that the standard matches the targeted peak in leaf matrix conditions. Calibration curves were done at an appropriate range for each putatively identified organic acid and R^2 must be above >0.99 to allow quantification. Quantification was made within the samples using the calibration curves.

Phenolic compounds were extracted according to Solecka et al. (1999) with modifications (Kidd et al., 2001). Briefly, leaves were extracted with 70% methanol and after centrifugation (10 min, 5000 x g) the supernatant was re-extracted three times with ethyl ether to eliminate ether soluble lipids. The remaining water phase was treated with 2 M HCl for acid hydrolysis of soluble conjugated phenolic compounds. After extraction with ethyl acetate and drying, the residue was re-dissolved in 50% methanol. Total phenolic compounds levels were determined by spectrophotometry (Shimadzu UV-2450, Duisburg, Germany) following the method of Folin-Ciocalteau (Singleton et al., 1999), using gallic acid (Sigma, Steinheim, Germany) as the standard with detection at 765 nm. The results were expressed in Gallic Acid Equivalents (GAE).

2.8 Statistical analysis

Principal Component Analysis (PCA) was applied to the RNA-seq data comparing the biological replicates of each treatment modality with the NTC. The statistical analysis of the RT-qPCR data was done using REST2009 Software (Pfaffl et al., 2002). DEGs standard curves for gene expression quantification were made by linear regression on Excel. Validation of the DEGs was performed by a correlation study between the gene expression measured by RNA-seq and RT-qPCR techniques. Pearson correlation analysis was applied to the data for each treatment modality using R software (R version 3.5.2).

For metabolite measurements, to identify significant differences between treated (Aki and BL) and NTC leaves, several statistical tests were performed. All tests were performed on R software (R version 3.5.2) with a significant level of p-Value < 0.05. First, each of the metabolite datasets were tested (Shapiro-Wilk and Bartlett tests) to determine the suitability of parametric or non-parametric tests. For parametric tests, one-way analysis of variance (ANOVA) was carried out followed by a Tukey's multiple comparisons test. For non-parametric tests, Kruskal-Wallis test followed by Dunn test were carried out.

3 Results

3.1 Quality assessment of RNA-seq data and gene expression estimation

The 16 sequencing samples produced around 39 million of total sequencing raw reads for NTC and Aki treatments, while around 36 million for BL and BF treatments (Supplementary Table 4). Following the filtering and trimming process, around 33 (NTC and Aki) and 31 (BL and BF) millions of cleaned reads were obtained (85 and 86%, respectively, of the total sequencing reads).

When the reads were paired and aligned to the reference *V. vinifera* L. PN40024 genome, around 14.7 (BL and BF) and 15.8 (NTC and Aki) million reads from each treatment could be mapped, (94.9 and 94.6%, respectively, of the input paired reads) (Supplementary Table 5). Moreover, between 85.6 (NTC and Aki) and 86.7% (BL and BF) of the input paired reads were assigned to genes.

The overall quality of the experiment was evaluated considering the consistency between the biological replicates using the normalized gene expression values (normalization of the FPKM) from each treatment. The PCA analysis revealed that one out of four biological replicates of each treatment (Aki_R1, BL_R1, BF_R1, NTC_R3) did not cluster as expected from the experimental design (Supplementary Figure 2). This variability among replicates could hide some of the treatment effect on gene expression, thus, this replicate was not included in further analysis.

PCA on normalized gene expression using the three retained biological replicates showed that the two first principal

components explained 83.57% (Aki), 84.21% (BL) and 85.43% (BF) of the variance. In addition, the PC1 explained 63.33% (Aki), 60.5% (BL) and 49.38% (BF) of the variability in gene expression between each treatment and the NTC.

The RNA-seq raw transcriptomic data were submitted to the GEO repository of the National Center for Biotechnology Information (NCBI) (GSE211268).

3.2 Analysis of the differential expression of genes after the treatments

Gene transcription in cv. Garnacha Blanca grapevine leaves was triggered by Aki, BL and BF treatments to varying degrees. The volcano plots show the degree of variation of the Differential Expression of Genes (DEGs) based on red and green dots (Supplementary Figure 3). The relationship between the fold-change (Log_2(FC)) and the statistical significance of the differential expression test (-Log_{10}(FDR)) is displayed.

Plot similarities within *Bacillus* treatments (BL and BF) were observed since the most of genes were distributed between Log_2 (FC) values of -4 and 4 and with significance values (-Log_{10} (FDR)) up to 75 (downregulated genes) and 50 (upregulated genes). However, Akivi plot differed from *Bacillus* ones since the main of genes were distributed between Log_2(FC) values of -3 and 3 and with lower significance values of 20 (downregulated genes) and 60 (upregulated genes).

Additionally, heatmaps of these DEGs for each treatment effect are shown in Supplementary Figure 4. The expression patterns of DEGs were consistent within the three biological replicates but differed between treatments in comparison with the NTC. After Aki and BL treatments, the number of genes that were over-expressed (red) and down-expressed (green) in comparison with the NTC were equivalent. However, after BF treatment a higher number of genes were over-expressed (red) in comparison with the NTC.

As shown in Venn diagrams (Figure 1), 793 genes were upregulated and 652 genes were downregulated (log2(FC)>|1.4|) within the different treatments (Aki, BL and BF) in grapevine leaves after the treatments. *Bacillus* treatments (BL and BF) altered the expression level of a higher number of genes than the botanical extract Akivi treatment (Aki). BL and BF treatments showed 438 and 396 upregulated DEGs, respectively, and 481 and 313 downregulated DEGs, respectively, whereas Aki treatment showed a total of 278 upregulated and 225 downregulated DEGs. In addition, the plant response towards *Bacillus* (both BL and BF) and Akivi (Aki) treatments was fairly specific since only 31 upregulated and 68 downregulated genes were common to all three treatments.

However, the *Bacillus* treatments, both lyophilized and fresh, shared a high number of up- (43.1%) and downregulated (41.3%) genes. These genes were altered by the *Bacillus* treatments, independently of being the product lyophilized or not. Therefore, these shared genes were used for the following validation of RNA-seq results by RT-qPCR. From the 583 upregulated genes after either BL or BF treatments, 251 genes were shared. From the rest of genes, 187 and 145 were only upregulated after BL and BF treatments, respectively. Whereas from the 562 downregulated genes after BL or BF treatments, 232 genes were shared. From the remaining genes, 249 and 81 were only upregulated after BL and BF, respectively.

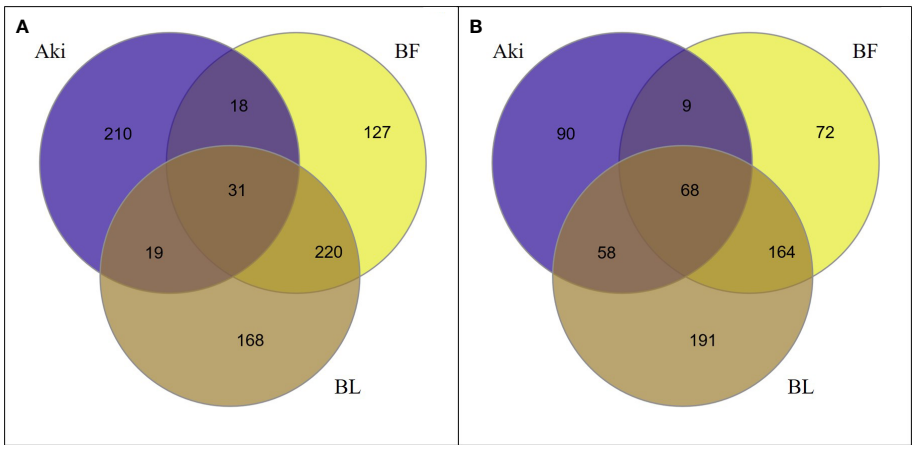

FIGURE 1
Venn diagrams showing the relationship between upregulated **(A)** and downregulated **(B)** differentially expressed genes (DEGs) identified in leaves of cv. Garnacha Blanca grapevine. Data correspond to 24h after treatments with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG, compared to the non-treated control (NTC).

3.3 Functional analysis of DEGs in grapevine after treatments

3.3.1 GO analysis of DEGs

GO enrichment analysis was carried out to evaluate the major biological functions of DEGs influenced by the Aki, BL, and BF treatments. The biological functions are classified into three categories: biological process (BP), cellular component (CC), and molecular function (MF). Upregulated GO terms were identified in 34.4, 25.8 and 25.0% of DEGs after the Aki, BL and BF treatments, respectively, whereas 35.3, 32.9 and 41.4% were downregulated (Supplementary Table 6).

Figures 2, 3 show the upregulated and downregulated GO term clusters obtained by REVIGO analysis. Three biological processes associated with upregulated genes, namely "transmembrane transport", "stress response", and "regulation of defense response" were shared by the three treatments. The GO term clusters "Phosphorylation", "biosynthetic process", "cell differentiation", and "recognition of pollen" were exclusively enriched by Aki treatment, whereas "protein catabolic process", "organelle organization", "protein folding", and "developmental process", "RNA modification", and "protein refolding" were related to by BL and/or BF treatments.

Four biological processes associated with downregulated genes, namely "metabolic process", "microtubule-based movement", "stress response" and "transmembrane transport" were shared by the three treatments. "Catabolic process", "aerial part development", and "cell wall biogenesis" were exclusively reduced by Aki treatment. Whereas "carbohydrate metabolic process" and "mitotic cell cycle" were reduced by both Aki and BF treatments, "organelle organization", "photosynthesis", and "biosynthetic process" were related to BF and/or BL treatments.

Some of the upregulated GO terms from BP category that were arranged in two well-defined clusters are related to plant defense response, namely "stress response" and "regulation of defense response" (Figure 4). These two clusters include 30 GO terms (Table 1). In general, only 6 out of 30 GO terms were shared by Aki and *Bacillus* (BF and/or BL) treatments. Five GO terms were shared by the two *Bacillus* treatments (BF and BL), while eight, three, and eight GO terms were unique for Aki, BF and BL, respectively.

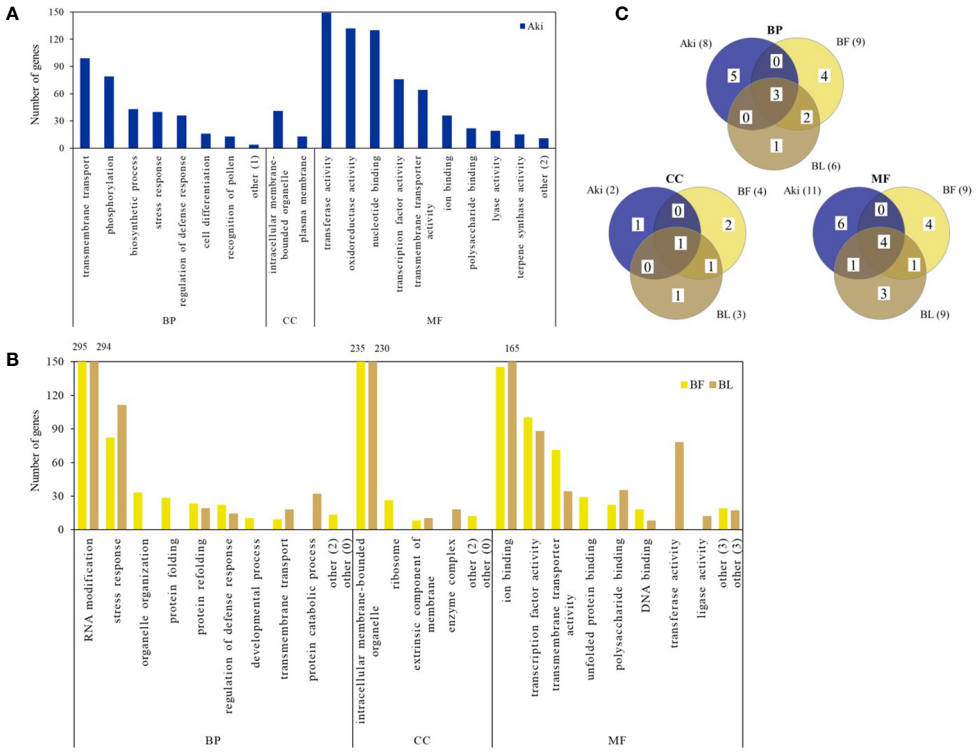

FIGURE 2
Upregulated genes according to Gene Ontology (GO) enrichment and REVIGO analysis in cv. Garnacha Blanca grapevine after treatment with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG, compared to the non-treated control (NTC). **(A, B)** Bar graphs show the number of upregulated DEGs in each GO term cluster. Clusters that showed less than 10 DEGs were included under the term "other", indicating in parenthesis the number of clusters that represent. **(C)** Venn diagrams show the total upregulated GO term clusters. Categories of processes: biological (BP), cellular component (CC), and molecular function (MF).

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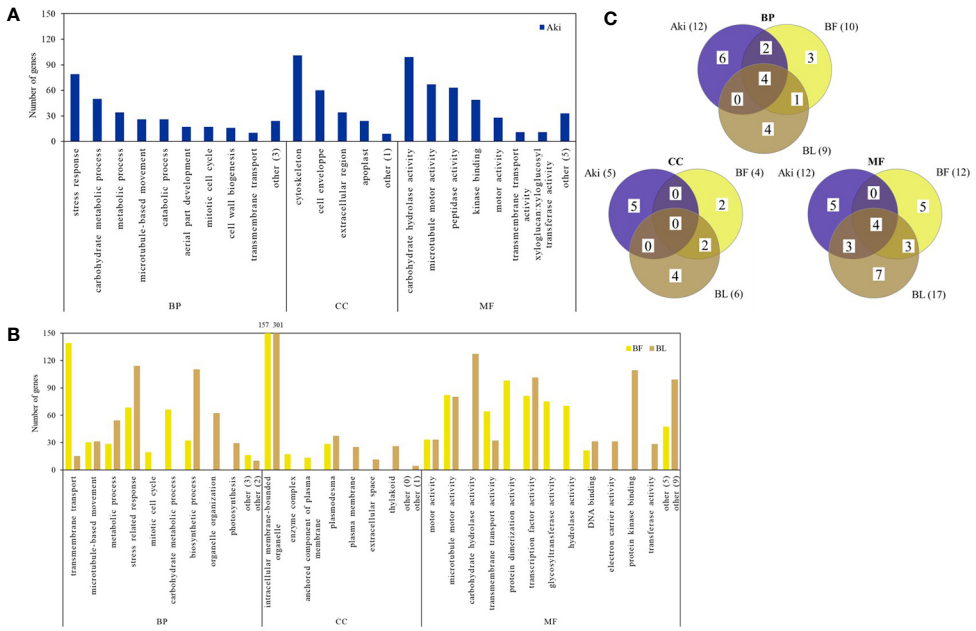

FIGURE 3
Downregulated genes according to Gene Ontology (GO) enrichment and REVIGO analysis in cv. Garnacha Blanca grapevine after treatment with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG, compared to the non-treated control (NTC). **(A, B)** Bar graphs show the number of downregulated genes in each GO term cluster. Clusters that showed less than 10 DEGs (BP and CC for Aki, BF and BL and MF for Aki) or 20 DEGs (MF for BF and BL) were included under the term "other", indicating in parenthesis the number of clusters that represent. **(C)** Venn diagrams show the total downregulated GO term clusters. Categories of processes: biological (BP), cellular component (CC), and molecular function (MF).

From the cluster named "regulation of defense response", the GO term regulation of jasmonic acid mediated signalling pathway was shared by all treatments. Whereas the GO term regulation of defense response was shared by Aki and BF, regulation of systemic acquired resistance was unique for Aki. From the cluster named "stress response", the GO terms response to osmotic stress, response to karrikin, and response to hydrogen peroxide were unique for *Bacillus* (BF and BL), while the GO terms immune response and plant-type hypersensitive response, and response to wounding, biotic stimulus and ethylene were unique for Aki.

The upregulated genes ($Log_2(FC) > 1.4$) related to the GO terms included in "regulation of defense response" and "stress response" clusters are shown in Table 2 (Supplementary Tables 7, 8). Interestingly, some upregulated DEGs were also unique for each treatment (Aki and *Bacillus*). After *Bacillus* treatment, one gene related to the regulation of jasmonic acid, and several genes related to transcription factors, chaperones, enzymes as catalase, PR protein with antimicrobial activity, abscisic acid receptor, and cold induced protein were upregulated. However, after Akivi treatment, two genes related to the regulation of SAR, one defense response related gene, three genes related to the response to chitin, and one gene related to PR protein with antimicrobial activity were upregulated.

Some of the downregulated GO terms from BP category were arranged in a cluster related to plant defense response, namely "stress-related response" (Figure 5). This cluster include 18 GO terms related to regulation of cellular cycle and cell population proliferation, plant development, metabolic processes and their regulation, stress response, defense and response to stimuli and signal transduction (Table 3). The GO terms related to cellular cycle, stress and stimuli response, metabolic processes regulation and signal transduction were shared by the three treatments. However, GO terms related to plant development, defense response and metabolic processes were unique for *Bacillus* treatments.

The downregulated genes ($Log_2(FC) < 1.4$) related to the GO terms included in "stress-related response" cluster are shown in Table 4 (Supplementary Table 9). Interestingly, after both Aki and *Bacillus* treatments, some DEGs related to cellular cycle were downregulated. In particular, six cyclin proteins and one annexin protein were downregulated for Aki treatment. While four cyclin proteins were downregulated for *Bacillus* treatments.

Moreover, genes related to plant growth and development, such as transcriptional factors and zinc finger proteins, DNA replication, and two lipid transfer protein (LTP) that intervene in systemic acquired resistance SAR were downregulated after *Bacillus* treatments.

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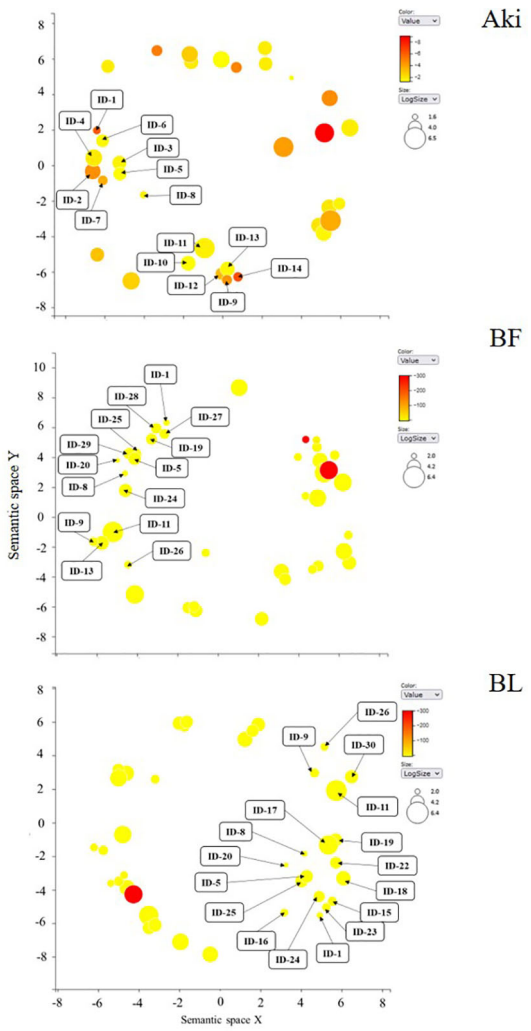

FIGURE 4
REVIGO graphs of upregulated GO term clusters (regulation of defense response and stress response) in leaves of cv. Garnacha Blanca grapevine included in biological process category after treatments with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG. ID: identification of GO terms associated with Table 1.

Considering different stress responses, after Aki treatment two genes connected with receptor like kinases that intervene in plant innate immunity were downregulated, while transcription factors to several stresses and abiotic stresses were downregulated after *Bacillus* treatments.

3.3.2 KEGG pathway analysis of DEGs

KEGG pathway analysis was performed to evaluate the biological mechanisms influenced by the Aki, BL, and BF treatments. Few pathways were associated with DEGs affected by the treatments and none was shared between Akivi and *Bacillus* treatments (Supplementary Table 10).

The following pathways "Glutathione metabolism", "Linoleic acid metabolism", "alpha-Linolenic acid metabolism", "Terpenoid backbone biosynthesis", "beta-Alanine metabolism", and "Fatty acid degradation" were triggered after Aki treatment. Whereas "Starch and sucrose metabolism" was triggered by both BL and BF treatments, "Ribosome biogenesis in eukaryotes" was exclusively triggered by BF treatment.

The pathway "Porphyrin and chlorophyll metabolism" was reduced after Aki treatment, while "DNA replication" was reduced after both BL and BF treatments. "Cysteine and methionine metabolism" was reduced after BL treatment; while "Fructose and mannose metabolism", "Phenylpropanoid biosynthesis", and "Starch and sucrose metabolism" were reduced after BF treatment.

3.4 Gene marker candidates on grapevine

3.4.1 Selection of DEGs

A total of 27 DEGs were selected since their expression level was modified due to Aki, BL and BF treatments according to the results of RNA-seq analysis (Table 5).

From the 12 DEGs highly triggered by Aki treatment, eight genes are related to defense response (A1, A3, A4, A5, A6, A7, A11, and A12). Specifically, two genes are involved in detoxification of reactive oxidative species (A5 and A6); two genes are related to hormone signalling pathway (A3, and A4), one gene is involved in biosynthesis of secondary metabolites (A7), and one gene is a marker of SAR response (A11). From the 15 DEGs highly triggered by BL and BF treatments, four genes are involved in defense response (B1, B6, B8, and B14).

3.4.2 Validation of selected DEGs by RT-qPCR

Standard curves of the 27 DEGs showed R-squared values above 0.99 and, in general, amplification efficiencies above 90%, except for three DEGs (A1, A3 and A4) that showed slightly lower efficiencies above 80% (Supplementary Table 3). The expression levels of the 27 DEGs within the NTC samples on the 'Garnacha Blanca' experiment were stable showing FC values close to 1 (Table 6). The selected DEGs were upregulated after Aki (12) and BL treatments (14) with significant differences in comparison with the NTC, with the exception of B5 gene that was downregulated.

Moreover, the relative expression levels of the 27 DEGs on cv. Garnacha Blanca obtained by RT-qPCR and RNA-seq analysis were highly consistent for both Aki and BL treatments (Figure 6). That was confirmed by Pearson correlation test that showed high correlation coefficient values, 0.729 and 0.938 for Aki and BL, respectively, and statistical significances with p-values<0.05 (Supplementary Figure 5). Therefore, the 27 DEGs

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TABLE 1 Representative groups (clusters) of upregulated GO terms of biological processes obtained with REVIGO and associated to plant defense responses, after treatments of cv. Garnacha Blanca grapevine leaves with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG.

Representative group	ID	GO ID: GO Term	Uniqueness*		
			Aki	BF	BL
Regulation of defense response	9	GO:2000022: regulation of jasmonic acid mediated signalling pathway			
	13	GO:0031347: regulation of defense response			
	26	GO:0051096: positive regulation of helicase activity			
	14	GO:0010112: regulation of systemic acquired resistance			
	10	GO:0045454: cell redox homeostasis			
	12	GO:0010469: regulation of signalling receptor activity			
	30	GO:0006879: cellular iron ion homeostasis			
Stress response	1	GO:0010200: response to chitin			
	8	GO:0061408: positive regulation of transcription from RNA polymerase II promoter in response to heat stress			
	5	GO:0034605: cellular response to heat			
	11	GO:0006355: regulation of transcription, DNA-templated			
	19	GO:0000165: MAPK cascade			
	25	GO:0006970: response to osmotic stress			
	20	GO:0080167: response to karrikin			
	24	GO:0042542: response to hydrogen peroxide			
	2	GO:0006955: immune response			
	3	GO:0009611: response to wounding			
	4	GO:0009607: response to biotic stimulus			
	6	GO:0009723: response to ethylene			
	7	GO:0009626: plant-type hypersensitive response			
	27	GO:0046686: response to cadmium ion			
	28	GO:0009739: response to gibberellin			
	29	GO:0009651: response to salt stress			
	15	GO:0010039: response to iron ion			
	16	GO:0070413: trehalose metabolism in response to stress			
	17	GO:0035556: intracellular signal transduction			
	18	GO:0009617: response to bacterium			
	21	GO:0006073: cellular glucan metabolic process			
	22	GO:0009738: abscisic acid-activated signalling pathway			
	23	GO:0010167: response to nitrate			

Akivi (Aki), lyophilized (BL) and fresh (BF) Bacillus UdG treatments
ID: GO term assigned identifier
White space means not GO term
*Smaller values denote higher uniqueness. Red (0.7-0.8), orange (0.8-0.9), yellow (0.9-1.0)

that were previously selected by RNA-seq analysis were validated by RT-qPCR on grapevine cv. Garnacha Blanca.

3.4.3 Expression of validated DEGs in the three grapevine cultivars

The expression levels of the 27 DEGs were subjected to RT-qPCR using samples from experiments performed with two other grapevine cultivars, namely Garnacha Tinta and Macabeo. Within the NTC samples of the 'Garnacha Tinta' and 'Macabeo' experiments, the expression levels of the 27 DEGs were stable showing fold change values close to 1 (Table 6).

Concerning the 12 selected DEGs by Akivi treatment, nine (A2, A3, A4, A5, A7, A8, A9, A10, and A12) and six genes (A1, A2, A3, A4, A9, and A12) in 'Garnacha Tinta' and 'Macabeo', respectively, showed differential expression levels with statistical significance compared to the NTC (regardless of the FC value) (Table 6). After Akivi treatment, the A1, A4, and A12 genes were upregulated on the three grapevine cultivars (Figure 7). However, the A1 gene showed a FC value of 4.86 without significant differences with the NTC on 'Garnacha Tinta'. Whereas the A9 gene was upregulated on 'Garnacha Blanca' and 'Garnacha Tinta', this gene was downregulated on 'Macabeo'. In the case of A8 gene, despite it was upregulated on 'Garnacha Blanca' and 'Garnacha Tinta', its gene expression was not affected on 'Macabeo'. Seven genes, namely, A2, A3, A5, A6, A7, A10 and A11, were only upregulated on 'Garnacha

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TABLE 2 Upregulated genes included in the GO terms that belong to regulation and stress response groups after treatments of cv. Garnacha Blanca grapevine leaves with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG.

Description	Gene ID	GO ID	Aki	BF	BL
Enzymes					
Leucoanthocyanidin dioxygenase	VIT_13s0067g01020	9	■	■	■
Trehalose 6-phosphate synthase	VIT_17s0000g08010; VIT_01s0026g00280	16			■
Trehalose-phosphatase	VIT_12s0028g01670	16			■
Xyloglucan endotransglucosylase/hydrolase	VIT_11s0052g01280; VIT_05s0062g00250; VIT_01s0026g00200	21			■
Catalase	VIT_00s0698g00010	27		■	■
Proteins that mediate the attachment of integral membrane proteins to the cytoskeleton					
Ankyrin repeat	VIT_14s0081g00370	13	■	■	■
Ankyrin repeat	VIT_05s0165g00010; VIT_14s0081g00360	13		■	■
Transcriptional regulators/Transcriptional factors					
Jasmonate ZIM domain-containing protein 8	VIT_10s0003g03790	9, 13		■	■
Cold induced protein	VIT_17s0000g08010	27		■	■
Zinc finger (C2H2 type) family	VIT_13s0019g00480	1	■		
Myb domain protein 14	VIT_05s0049g01020	1, 27, 29		■	■
Salt tolerance homolog2	VIT_03s0038g00340	1, 20	■	■	■
WRKY DNA-binding protein 33	VIT_08s0058g00690	1, 5, 25, 29		■	■
Heat shock transcription factor C1	VIT_11s0016g03940	5, 8		■	■
Modulators and regulators of related defense responses and cell death program					
NIM1	VIT_07s0005g02070; VIT_01s0011g03430	14	■		
NSL1 (necrotic spotted lesions 1)	VIT_01s0011g05950	2, 7	■		
Abscisic acid receptor PYL1 RCAR12	VIT_13s0067g01940	22			■
Plant peptide growth factors.					
Phytosulfokines PSK1	VIT_08s0007g03870	12	■		
DNA replication					
DNA mismatch repair protein MSH3	VIT_00s0388g00030	26		■	■
Iron storage and transport proteins					
Ferritin	VIT_08s0058g00440, VIT_08s0058g00430, VIT_08s0058g00410	15, 18, 24, 30			■
Metal-nicotianamine transporter YSL1	VIT_02s0025g02510	15			■
Chaperones (HSP)					
Heat shock protein 18.2 kDa class II	VIT_12s0035g01910	24, 29		■	■
Heat shock protein 17.6 kDa class I	VIT_13s0019g03160	24, 29		■	■
HSP (HSP26.5-P) 26.5 kDa class P	VIT_00s0992g00020	24, 29		■	■
Pathogenesis related proteins					
Pathogenesis protein 10	VIT_05s0077g01570	22			■
Pathogenesis protein 10	VIT_05s0077g01600	4	■		
Unknown					
unknown	VIT_09s0002g03340	27		■	■

Go ID: GO term assigned identifier (Table 1)

Blanca', while the expression pattern of these genes was not affected on 'Garnacha Tinta' and 'Macabeo'. Therefore, the expression pattern after Akivi treatment was quite similar in the 'Garnacha Blanca' and 'Garnacha Tinta' (5 out of 12 genes were upregulated). However, the expression pattern obtained on 'Macabeo' differed from 'Garnacha Blanca' and 'Garnacha Tinta' since only 3 out of 12 (A1, A4, A12) genes were upregulated on all cultivars tested. In particular, three genes (A9, A10 and A11) showed FC below 1 only on 'Macabeo', being A9 downregulated.

In relation to the 15 selected DEGs by BL treatment, twelve genes showed differential expression levels with statistical significance compared to the NTC within 'Garnacha Tinta' (B1, B2, B3, B5, B6, B7, B9, B10, B12, B13, B14, and B15) and 'Macabeo' (B1, B2, B3, B5, B6, B7, B8, B9, B11, B12, B13, and B14) (regardless of the FC value) (Table 6). After BL treatment, B1, B2, B3, B9, B12, B13, and B14 genes were upregulated on the three grapevine cultivars (Figure 7). The only gene that was downregulated on 'Garnacha Blanca' was B5, which was

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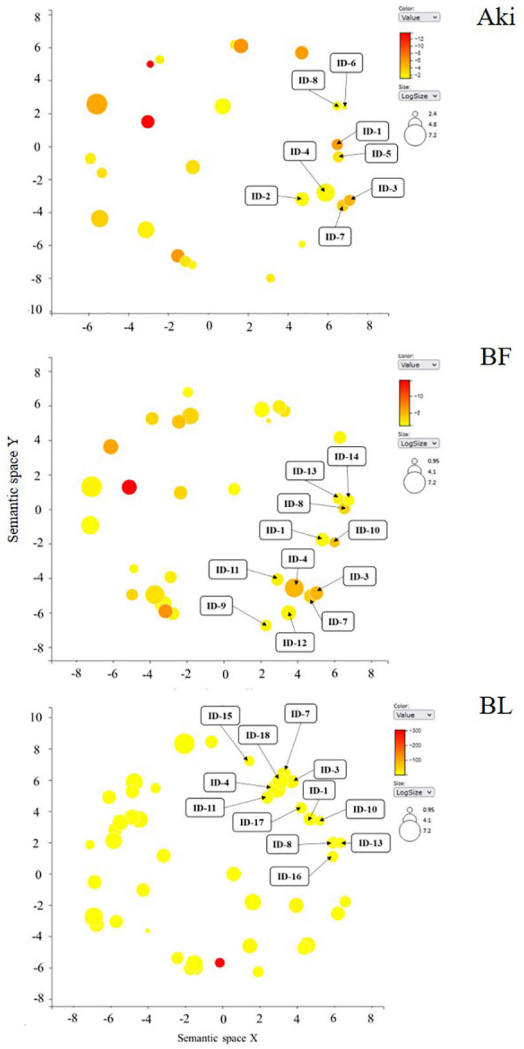

FIGURE 5
REVIGO graphs of downregulated GO term clusters (regulation of defense response and stress response) in leaves of cv. Garnacha Blanca grapevine included in biological process category after treatments with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG. ID: identification of GO terms associated with Table 3.

unaltered and upregulated on 'Garnacha Tinta' and 'Macabeo', respectively. Three genes, namely B4, B6, and B7, were only upregulated on 'Garnacha Blanca', while they were unaltered (FC between 0.5-2) on 'Garnacha Tinta' and 'Macabeo'. In the case of B8 and B11 genes, their expression levels were upregulated on both 'Garnacha Blanca' and 'Macabeo', while their expression levels were not affected on 'Garnacha Tinta'. Two genes, namely B10 and B15, were upregulated on both 'Garnacha Blanca' and 'Garnacha Tinta', while their expressions were not affected on 'Macabeo'. Therefore, the expression pattern after BL treatment was quite similar in the 'Garnacha Blanca' and 'Garnacha Tinta' (9 out of 15 genes were upregulated). Similar results were also observed comparing expression patterns in 'Garnacha Blanca' and 'Macabeo' (9 out of 15 genes were upregulated) despite B5 gene was clearly upregulated on 'Macabeo' and downregulated on 'Garnacha Blanca'. However, the expression pattern obtained on 'Macabeo' differed from 'Garnacha Tinta' since a smaller number of genes (7 out of 15) shared the same upregulation transcriptional pattern.

3.5 Metabolite concentrations

Foliar Aki and BL treatments slightly influenced some of the mineral nutrient concentrations in the grapevine leaves, but not enough to affect plant development in any of the three cultivars (Supplementary Table 11).

Phytohormones, organic acids (OA) and total phenolic compounds concentrations were compared for each treatment (Aki and BL) with the NTC (Table 7).

Regarding Aki treatment, no phytohormones were significantly influenced in the same way among the three grapevine cultivars. The SA tended to present higher levels after Aki treatment in 'Garnacha Blanca' and 'Macabeo' and was significantly enhanced in 'Garnacha Tinta'. The GAs showed an opposite pattern being their levels significantly increased in 'Garnacha Blanca', but reduced in 'Macabeo'. The MeJA in 'Garnacha Blanca' was significantly enhanced after Aki treatment. Although all the studied phytohormones, with the exception of JA, tended to be enhanced after Aki treatment in 'Garnacha Blanca'; JA, ACC, and ABA were not significantly influenced by Aki treatment in any of the three cultivars.

BL treatment significantly enhanced JA in 'Garnacha Tinta' and MeJA in 'Garnacha Blanca'. Oppositely, GAs were significantly reduced after BL treatment in 'Macabeo'. However, neither SA, ACC, nor ABA were significantly influenced by BL treatment in any of the three cultivars.

After BL or Aki treatment, phytohormone content changed without a clear pattern and the establishment of a defense signalling triggering mechanism was not possible to infer from our data.

It is worth to mention that ABA global values detected in 'Macabeo' are higher than the values detected in the two 'Garnacha' varieties.

Four OA were identified in the leaves of the three grapevine cultivars: oxalic, tartaric, malic, and oxoglutaric (Table 7). Aki and BL treatments caused a significant reduction in oxoglutaric acid in 'Macabeo', but BL significantly increased the amount of this organic acid in 'Garnacha Blanca'. BL treatment also

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TABLE 3 Representative groups (clusters) of downregulated GO terms of biological processes obtained with REVIGO and associated to plant defense responses, after treatments of cv. Garnacha Blanca grapevine leaves with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG.

Representative group	ID	GO ID: GO Term	Uniqueness* Aki	BF	BL
Stress-related response	3	GO:0045787: positive regulation of cell cycle			
	4	GO:0006355: regulation of transcription			
	8	GO:0009414: response to water deprivation			
	7	GO:0008284: positive regulation of cell population proliferation			
	1	GO:0009734: auxin-activated signaling pathway			
	10	GO:0010017: red or far-red light signaling pathway			
	13	GO:0009744: response to sucrose			
	11	GO:0045910: negative regulation of DNA recombination			
	2	GO:0043086: negative regulation of catalytic activity			
	5	GO:0007178: transmembrane receptor protein serine/threonine kinase signaling pathway			
	6	GO:0071249: cellular response to nitrate			
	9	GO:0009909: regulation of flower development			
	12	GO:0043085: positive regulation of catalytic activity			
	14	GO:0046686: response to cadmium ion			
	15	GO:0010112: regulation of systemic acquired resistance			
	16	GO:0009627: systemic acquired resistance			
	17	GO:0000076: DNA replication checkpoint signaling			
	18	GO:0045893: positive regulation of transcription			

Akivi (Aki), lyophilized (BL) and fresh (BF) Bacillus UdG treatments
ID: GO term assigned identifier
White space means not GO term
* Smaller values denote higher uniqueness. Purple (0.7-0.8), blue (0.8-0.9), clear blue (0.9-1.0)

reduced the level of tartaric acid in 'Garnacha Tinta'. The rest of organic acids were not altered.

Total phenolic compounds concentration was significantly enhanced after BL treatment in 'Macabeo' and in 'Garnacha Blanca'. Aki treatment only tend to increase the level of total phenolic compounds.

4 Discussion

The response of grapevines to foliar treatments with the botanical extract Akivi (Aki) and the beneficial microorganism *Bacillus* UdG (fresh, BF or lyophilized, BL) was investigated. Among the environmentally friendly compounds considered as PPPs by the European Union legislation, beneficial microorganisms and natural substances (i.e. plant extracts) are included. *Bacillus* UdG and Akivi were used in this study representing each modality. Previous studies have reported the features and potential of both biocontrol products (Mora et al., 2011; Mora et al., 2015; Ramos et al., 2022). Figure 8 shows a scheme as a summary of the genes related to the main plant defense response pathways (Jasmonic Acid, JA; Salicylic Acid, SA; Ethylene, ET; Abscisic Acid, ABA; phenylpropanoids pathway; and mitogen activated protein kinases and Ca^{2+} signalling induction, MAPKs) whose expression levels were influenced by the treatments. Interestingly, the two *Bacillus* UdG treatments, both BF and BL triggered the same pathways. However, BF and BL did not always trigger the same genes of the above-mentioned pathways. These results underline the importance of the product formulation since it may determine its efficacy and mode of action. Only Aki and BL treatments will be discussed hereafter.

Gene transcription related to JA biosynthesis was slightly influenced by either Aki and BL treatments since lipoxygenases (LOX) related genes did not show overexpression with Log_2(FC) values higher than 1.4. However, phytohormone concentrations related to JA defense pathway were affected in cvs. Garnacha Blanca and Tinta. Specifically, MeJA concentration was significantly higher after Aki and BL treatments in 'Garnacha Blanca' and JA concentration was doubled after BL treatment in 'Garnacha Tinta'. In agreement with metabolite contents, several genes regulated by JA pathway were upregulated by the treatments. Namely, the expression level of genes related to non-inducible immunity 1 (NIM1) were upregulated by Aki treatment and the expression level of a gene related to enhanced disease susceptibility (EDS1) and to nonexpressor of pathogenesis-related genes 1 (NPR1) was slightly upregulated by both treatments. Moreover, Aki and BL treatments upregulated the expression of several genes involved in different transcription factors with WRKYs domain (WRKYs

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TABLE 4 Downregulated genes included in the GO terms that belong to stress-related response groups after treatments of cv. Garnacha Blanca grapevine leaves with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG.

Gene description	Gene ID	GO ID	Aki	BF	BL
Transcription factor related to auxin signalling pathway					
IAA31	VIT_05s0020g01070	1			
Proteins that control the cell cycle by activating cyclin-dependent kinase (CDK)/Cycle regulators					
Cyclin delta-3 (CYCD3_1)	VIT_18s0001g09920				
Cyclin D3_2	VIT_03s0180g00040	2, 3, 6, 7			
Cyclin CYCB1_2	VIT_06s0009g02090	2, 3, 6, 7			
Cyclin B-type	VIT_08s0040g00930	2, 3, 6, 7			
Cyclin 1b (CYC1b)	VIT_13s0067g01420	2, 3, 6, 7			
Cyclin-dependent protein kinase regulator CYCB2_4	VIT_18s0001g14170	2, 3, 6, 7			
Cyclin B2;4	VIT_03s0038g02800	3, 7			
Cyclin delta-2	VIT_03s0091g01060	3, 7			
Cyclin A1	VIT_18s0001g02060	3, 7			
Cyclin-dependent protein kinase CYCB3	VIT_19s0085g00690	3, 7			
Annexin ANN4	VIT_00s0131g00080	3,8			
Protein kinase WEE1	VIT_07s0104g01740	17			
Proteins that join DNA to form nucleosomes					
Histone H4	VIT_06s0004g04370; VIT_13s0019g00780; VIT_13s0019g00800	8			
Histone H1	VIT_07s0005g01060; VIT_07s0141g00730; VIT_14s0081g00500	11			
Receptors like-Kinases (RLK)					
Proline extensin-like receptor kinase 1 (PERK1)	VIT_01s0127g00670	3			
Receptor protein kinase	VIT_05s0020g01690	3			
DNA replication and repair					
ATP-dependent DNA helicase RecQ	VIT_01s0010g02590	3, 7			
Origin recognition complex subunit 5	VIT_01s0011g04400	13			
Origin recognition complex subunit 4	VIT_17s0000g01960	13			
DNA mismatch repair protein	VIT_01s0011g03440	15			
B ZipDNA binding proteins/Transcription factors/Zinc finger proteins					
BZIP protein HY5 (HY5)	VIT_04s0008g05210	10			
BZIP protein HY5 (HY5)	VIT_05s0020g01090	10			
BZIP transcription factor BZIP6	VIT_00s0541g00020	18			
AP2/ERF domain containing protein	VIT_08s0007g08150	18			
NAC Secondary wall thickening promoting factor1	VIT_02s0025g02710	18			
Late meristem identity1 HB51/LMI1	VIT_08s0007g04200	18			
Homeodomain leucine zipper protein HB-1	VIT_01s0026g01550	18			
Homeobox-leucine zipper protein HB-7	VIT_15s0048g02870	18			
Constans 2 (COL2)	VIT_14s0083g00640	9			
Zinc knucle	VIT_01s0010g01670	17			
Lipid Transfer Proteins (LTP)					
DIR1 (defective IN induced resistance 1)	VIT_00s0333g00050	16			
Protease inhibitor/seed storage/lipid transfer protein (LTP)	VIT_08s0007g01370	16			
Unknown					
unknown	VIT_04s0008g04200	5			
unknown	VIT_04s0023g03760	5			
unknown	VIT_07s0129g00200	3, 7			
unknown	VIT_13s0067g02560	18			

Go ID: GO term assigned identifier (Table 3)

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TABLE 5 Selected Differentially Expressed Genes (DEGs) on cv. Garnacha Blanca grapevine leaves after treatment with Akivi (Aki) and lyophilized *Bacillus* UdG (BL).

Code	Gene ID	Log_2 (FC)	FC	FDR	vCOST Description
Akivi					
A1	VIT_12s0059g02600	4.96	31.18	5.01E-15	Receptor protein kinase RK20-1
A2	VIT_06s0004g03350	3.46	11.03	9.18E-24	Lateral organ boundaries protein 1
A3	VIT_05s0077g00520	3.17	8.99	7.76E-11	Gibberellin 2-oxidase
A4	VIT_17s0000g00200	3.23	9.40	1.58E-17	Ethylene-responsive transcription factor ERF114
A5	VIT_08s0058g00970	2.39	5.23	1.67E-17	Cationic peroxidase
A6	VIT_12s0055g01010	3.04	8.23	1.38E-28	Peroxidase
A7	VIT_00s0372g00040	2.74	6.68	1.68E-08	1,8-cineole synthase, chloroplast
A8	VIT_04s0023g02240	2.83	7.11	7.56E-56	S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase
A9	VIT_12s0034g01140	2.07	4.21	5.28E-21	Plastocyanin domain-containing protein
A10	VIT_19s0090g00660	2.01	4.03	1.03E-32	Lipase GDSL
A11	VIT_03s0088g00810	1.88	3.67	3.94E-16	Pathogenesis-related protein 1 precursor (PRP 1)
A12	VIT_07s0005g06090	1.67	3.19	9.06E-19	Pore-forming toxin-like protein Hfr-2
Bacillus					
B1	VIT_16s0022g00860	5.23	37.41	1.25E-28	Invertase/pectin methylesterase inhibitor
B2	VIT_06s0004g07210	5.45	43.58	4.78E-65	CCT motif constans-like
B3	VIT_16s0100g00740	4.26	19.16	2.49E-15	unknown
B4	VIT_14s0068g01160	2.91	7.53	5.22E-12	Cytokinin-repressed protein CR9
B5	VIT_00s1490g00010	-2.46	0.18	9.93E-38	5'-adenylylsulfate reductase (APR1)
B6	VIT_13s0064g01370	3.08	8.43	4.30E-07	Polygalacturonase inhibiting protein 1 PGIP1
B7	VIT_09s0002g04280	3.14	8.81	2.33E-47	Dynein light chain LC6, flagellar outer arm
B8	VIT_03s0091g00310	2.96	7.80	6.23E-16	Indole-3-acetic acid-amido synthetase GH3.8
B9	VIT_01s0011g01980	2.47	5.52	3.91E-22	fasciclin arabinogalactan-protein (FLA21)
B10	VIT_01s0026g02740	2.64	6.25	9.54E-28	unknown
B11	VIT_08s0058g00430	1.82	3.52	1.32E-02	ferritin
B12	VIT_10s0116g00530	1.96	3.89	1.07E-30	Thiazole biosynthetic enzyme, chloroplast (ARA6)
B13	VIT_00s0480g00060	1.49	2.81	7.91E-20	Polyphenol oxidase [Vitis vinifera]
B14	VIT_07s0031g02610	2.98	7.92	3.48E-14	NAC domain containing protein 2
B15	VIT_13s0067g02130	2.50	5.64	6.67E-10	Dehydration-induced protein (ERD15)

FC, fold change
FDR, false discovery rate

TFs) and pathogenesis related proteins (PR). Interestingly, one of the PR related genes (VIT_03s0088g00810) was highly influenced by Aki treatment. Therefore, JA defensive pathway seemed to be triggered by both treatments. This is in agreement with the essential role of JA as a phytohormone involved in the regulation of defense gene expression (Rienth et al., 2019), such as EDS1, NPR1, or NIM1 related genes (Ochsenbein et al., 2006; Pajerowska-Mukhtar et al., 2013; Backer et al., 2019). These results enlightened the link between PR upregulated genes and WRKYs TFs but they did not underline the bond with transcriptional regulators jasmonate-zim domain (JAZ) intermediate related genes as previously described (Kazan and Manners, 2012; Guerreiro et al., 2016).

The expression level of genes related to SA pathway were not clearly affected by neither Aki nor BL treatments. However, the measured concentration of SA phytohormone in grapevine leaves treated with Aki tended to be higher than in leaves treated with NTC or BL on all three cultivars, especially in 'Garnacha Tinta' in which significant differences were observed. It could be explained by the upregulation of the expression of some genes related to EDS1, NIM1, and NPR1 already commented above in JA paragraph. Actually the mentioned genes are described as modulators that intervene in SA accumulation and they are produced in crosstalk between SA and JA pathways (Rustérucci et al., 2001; Zhu et al., 2011; Chen et al., 2021). Interestingly, the expression level of the glutathione-S-transferase (GST) related gene was also upregulated by Aki treatment. It is reported that SA is able to regulate several genes from GST family that are upregulated through SA pathway in treated plants with beneficial microorganisms resulting in ISR priming (Gullner et al., 2018). GST family enzymes are involved in detoxifying cytotoxic compounds and the process implies transmembrane transport (Burdziej et al., 2021), which is in agreement with these results

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TABLE 6 Expression levels of the selected DEGs influenced by treatments of cvs. Garnacha Blanca, Garnacha Tinta, and Macabeo with Akivi (Aki) and lyophilized *Bacillus* UdG (BL).

	'Garnacha Blanca'				'Garnacha Tinta'				'Macabeo'			
DEGs	NTC	Aki			NTC	Aki			NTC	Aki		
A1	1.29	14.37	± 3.96	*	1.29	4.86	± 4.91		1.11	4.46	± 1.73	*
A2	1.08	10.12	± 0.79	*	1.00	1.88	± 0.71	*	1.02	1.58	± 0.50	*
A3	1.05	5.64	± 0.57	*	1.03	1.43	± 0.25	*	1.04	1.74	± 0.81	*
A4	1.12	3.27	± 0.38	*	1.02	2.22	± 0.72	*	1.02	3.20	± 1.19	*
A5	1.05	8.01	± 1.07	*	1.02	1.55	± 0.32	*	0.99	1.68	± 0.30	
A6	1.05	9.11	± 0.44	*	1.09	1.39	± 0.49		1.05	1.16	± 0.80	
A7	1.06	3.39	± 0.61	*	1.06	1.65	± 0.59	*	1.10	1.10	± 0.60	
A8	1.08	4.71	± 1.71	*	1.05	2.94	± 1.90	*	1.16	1.78	± 1.27	
A9	1.06	4.56	± 1.14	*	1.01	2.79	± 2.32	*	1.01	0.44	± 0.21	*
A10	1.01	3.15	± 0.72	*	1.00	1.69	± 0.53	*	1.01	0.92	± 0.29	
A11	1.01	3.24	± 0.96	*	1.12	1.44	± 0.38		1.02	0.69	± 0.40	
A12	1.10	3.54	± 0.40	*	1.01	9.96	± 3.41	*	1.01	2.23	± 0.68	*
DEGs	NTC	BL			NTC	BL			NTC	BL		
B1	1.16	58.79	± 23.12	*	1.06	14.51	± 3.64	*	1.01	4.07	± 1.64	*
B2	1.05	22.43	± 3.57	*	1.03	3.19	± 0.34	*	1.02	4.05	± 1.17	*
B3	1.06	7.98	± 2.92	*	1.00	2.21	± 0.17	*	1.00	2.02	± 0.19	*
B4	1.15	5.49	± 1.84	*	1.09	0.77	± 0.20		0.90	1.63	± 0.13	
B5	1.04	0.22	± 0.07	*	1.02	0.50	± 0.18	*	1.09	3.03	± 0.58	*
B6	1.01	5.17	± 1.71	*	1.01	1.48	± 0.15	*	1.04	0.54	± 0.08	*
B7	1.29	6.86	± 1.57	*	1.04	1.39	± 0.25	*	1.07	0.71	± 0.12	*
B8	1.03	4.10	± 0.67	*	1.01	1.38	± 0.24		1.08	2.43	± 0.21	*
B9	1.09	8.30	± 3.71	*	1.01	5.07	± 1.22	*	1.06	2.76	± 0.59	*
B10	1.07	7.66	± 1.43	*	1.01	3.17	± 0.73	*	1.01	1.26	± 0.38	
B11	1.15	4.71	± 1.45	*	1.02	1.16	± 0.44		1.07	7.18	± 1.72	*
B12	1.01	4.68	± 0.90	*	1.01	3.76	± 0.54	*	1.03	8.59	± 1.48	*
B13	1.00	4.32	± 0.62	*	1.02	2.84	± 0.28	*	1.02	2.07	± 0.51	*
B14	1.12	5.96	± 1.32	*	1.04	3.15	± 0.76	*	1.22	2.50	± 0.22	*
B15	1.03	6.26	± 1.58	*	1.04	2.66	± 0.58	*	1.03	0.92	± 0.27	

DEG functions are indicated in Supplementary Table 2. Data correspond to RT-qPCR. The relative expression level of each gene was calculated by the comparative critical threshold (ΔΔCt) method using the non-treated control samples (NTC) as the calibrator and UQB gene as internal control for data normalization. Data mean the fold change ($2^{-\Delta\Delta Ct}$) ± confidence interval and significant differences according to REST2009 Software are represented by *.

since "transmembrane transport" GO term was influenced by Aki and BL treatments.

The biosynthesis of ET seemed to be triggered by both treatments through the upregulation of the expression level of 1-aminocyclopropane-1-carboxylate oxidase (ACO) related genes. Moreover, the concentration of the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) tended to present higher levels in 'Garnacha Blanca' leaves after Aki and BL treatments. These results were in agreement with the upregulation of expression of genes related to response factors regulated by ET (ERF TF, (AP2)/ERF TF, AP2 TF) by both treatments. In addition, the expression level of one of the genes related to ERF TF was highly influenced by Aki treatment (VIT_17s0000g00200). Therefore, ET defensive pathway seemed to be triggered by both treatments. ET response factors are key regulators of JA, ET, and ABA pathways in response to biotic and abiotic stresses, activating PR genes, such as osmotins (PR-5), chitinases (PR-3) and β-1,3-glucanases (PR-2) (Mizoi et al., 2012; Bahieldin et al., 2016; Rienth et al., 2019), which were indeed upregulated after both Aki and BL treatments.

The ABA biosynthesis was triggered by BL treatment through the upregulation of 9-cis-epoxycarotenoid dioxygenase (NCED) related gene expression. In fact, ABA biosynthesis starts with carotenoids and involves NCED enzyme that is strongly upregulated by stress (Xiong and Zhu, 2003). ABA is involved in the response to water stress and particularly intervenes in stomatal closure (Catacchio et al., 2019; Postiglione and Muday, 2020). It is expected variability in water stress response between grapevine cultivars because the two 'Garnacha' are more resistant to drought than 'Macabeo' (Mirás-Avalos and Araujo, 2021). These results are consistent

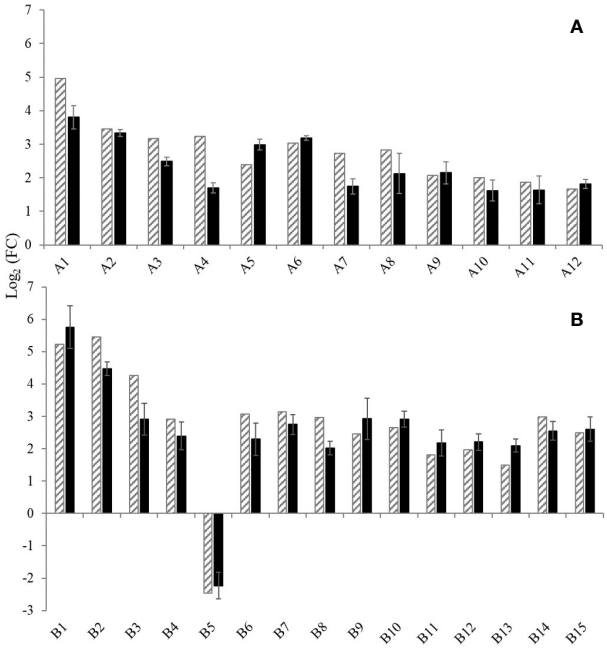

FIGURE 6
Expression levels of twenty-seven genes selected for validation of the RNA-Seq data by RT-qPCR. The gene expression was analysed after treatments with Akivi **(A)** and lyophilized *Bacillus* UdG **(B)**. RNA-seq (stripped bars) and RT-qPCR (black bars) analysis. Gene functions are indicated in Supplementary Table 2. RT-qPCR data are shown as the mean of $Log_2(FC)$of three biological replicates, where FC is the fold-change value and was calculated as $2^{-\Delta\Delta Ct}$ using non- treated control (NTC) samples as the calibrator and UBQ gene for data normalization. Error bars mean confidence interval of three biological replicates.

with ABA measured concentrations that were twice or three times higher in 'Macabeo' than in the two 'Garnacha'. Actually, 'Macabeo', which is less resistant to drought, is more likely to trigger water stress response involving ABA signaling. ABA is also involved in pathogen response signaling pathway and linked with SA, JA, and ET related genes regulation (Nishad et al., 2020). For instance, ABA biosynthesis induction by laminarin treatment triggered JA production in grapevine (Balestrini et al., 2020). However, ABA relation with JA-dependent related genes are closely linked with MYCs TF (Pieterse et al., 2014) but were not influenced by any of the treatments in the present study.

This study also underlined that the expression of a Phenylalanine Ammonia Lyase (PAL) and a Chalcone Synthase (CHS) related genes were upregulated after BL treatment, whereas the expression of one Leucoanthocyanidin dioxygenase (LAR) related gene was upregulated after both Aki and BL treatments. PAL, CHS, LAR, and flavonol synthase (FS) are key enzymes for biosynthesis of several secondary metabolites, such as phenylpropanoids, or phytoalexins isoflavonoids (Campos et al., 2003; Yonekura-Sakakibara et al., 2019). These enzymes are related to SA biosynthesis sharing PAL enzyme as showed in the results. Stilbene biosynthesis was also triggered by both treatments through the upregulation of Stilbene Synthase (STS) and Myb TF related gene expression. The transcriptomic results were in accordance with the total phenolic concentration in leaves, which tended to be higher after Aki and BL treatments in 'Garnacha Blanca' and statistically higher in 'Macabeo' after BL treatment. It is worth to mention that the phytohormones JA, MeJA, SA, ET, and ABA positively regulate stilbene biosynthesis (Dubrovina and Kiselev, 2017). In agreement with these results, JA and ET strongly trigger phenylpropanoids pathway, notably stilbene biosynthesis (Belhadj et al., 2008; Rienth et al., 2019).

The expression level of several genes related to Mitogen-Activated Protein Kinases (MAPKs) and Calcium ion (Ca^{2+}) signaling pathways were slightly upregulated after Aki treatment and some of them after BL treatment as well, such as Ca^{2+} Dependent Kinases (CDPKs), Calmodulin (CaM), Respiratory Burst Oxydase Protein (RBOHF) and Heat Shock Transcription Factors (HS TFs) with an upregulation lower than $Log_2(FC) > 1.4$. In addition, one CaM and several peroxidases (PO) related genes were clearly upregulated after Aki treatment, and two of them were highly upregulated by Aki treatment (VIT_12s0055g01010; VIT_08s0058g00970) and involved in hypersensitive response

10.3389/fpls.2022.1075231

TABLE 7 Phytohormone, organic acids, and total phenolic contents in leaves of grapevine cvs. Garnacha Blanca, Garnacha Tinta, and Macabeo treated with Akivi (Aki) and lyophilized *Bacillus* UdG (BL) and water (NTC).

	'Garnacha Blanca'						'Garnacha Tinta'						'Macabeo'					
	NTC		Aki		BL		NTC		Aki		BL		NTC		Aki		BL	
Phytohormone																		
JA	4.39	± 1.65	4.83	± 1.21	6.15	± 1.24	4.18	± 1.74	5.54	± 1.48	10.68	± 0.72*	5.75	± 0.73	6.95	± 1.70	5.85	± 2.25
MeJA	3.71	± 0.26	4.95	± 0.24*	5.12	± 0.55*	4.98	± 0.23	4.76	± 0.16	4.31	± 0.13	4.41	± 0.34	4.13	± 0.22	4.00	± 0.33
SA	375	± 78	553	± 75	321	± 68	215	± 52	654*	± 147	375	± 67	131	± 17	218	± 26	159	± 34
ACC	8.86	± 0.28	10.44	± 0.48	10.65	± 0.65	11.06	± 0.65	10.23	± 0.38	11.36	± 0.33	12.69	± 0.67	11.54	± 1.13	12.13	± 0.55
ABA[1]	1.51	± 0.20	0.94		1.54		6.15	± 0.99	7.37	± 0.39	5.71	± 0.60	21.25	± 3.48	21.55	± 1.05	17.96	± 0.71
GA1&4	8.39	± 1.07	31.05	± 5.96*	18.29	± 2,77	9.49	± 2.47	6.02	± 4.34	6.70	± 0.29	27.34	± 5.57	4.13*	± 1.60	7.85*	± 5.43
Organic acid																		
Oxalic	4.47	± 0.50	3.75	± 0.95	3.28	± 0.66	3.38	± 0.32	3.54	± 0.90	5.35	± 1.59	2.33	± 0.05	1.76	± 0.42	2.80	± 0.38
Tartaric	15.51	± 0.65	15.23	± 0.61	16.30	± 0.52	19.45	± 0.40	17.48	± 0.20	17.20*	± 1.20	17.13	± 1.20	16.43	± 0.30	15.02	± 0.55
Malic	1.48	± 0.24	1.87	± 0.38	1.78	± 0.13	1.34	± 0.20	2.02	± 0.24	1.39	± 0.14	0.92	± 0.16	0.88	± 0.18	1.18	± 0.29
Oxoglutaric	346	± 63	413	± 96	631*	± 21	667	± 21	674	± 30	671	± 21	155	± 33	88*	± 4	78*	± 11
Total phenolic	200	± 25	359	± 18	395*	± 61	876	± 35	912	± 51	808	± 19	656	± 52	679	± 79	984*	± 46

JA: jasmonic acid; MeJA: methyl jasmonate; SA: salicylic acid; ACC: ethylene precursor; ABA: abscisic acid; and GA1&4: Gibberellins A1 and A4.
[1] ABA concentration values in 'Garnacha Blanca' was at the limit of detection and only one value was detected for the treatment modalities (Aki and BL), thus, they were not included in statistical analysis. The following concentrations correspond to phytohormone (ng/g FW), organic acid (mg/g FW, except for oxoglutaric acid in µg/g FW), and total phenolic (µg gallic acid equivalent/g PF). Results are means ± standard deviation (n=3 biological replicates). Significant differences according to the Tukey test (parametric tests) or the Dunn test (non-parametric tests) between treatment (Aki or BL) and NTC are represented by asterisks (*).

(HR). As no phytotoxicity was observed after the treatments, Aki treatment may prime HR to be faster in case of pathogen infection. A crosstalk is described between MAPKs, JA, SA, and ET pathways (Rasmussen et al., 2012; Jagodzik et al., 2018; Nishad et al., 2020) and it was confirmed in this study since all these pathways were upregulated by the treatments.

In addition, Aki and BL treatments had an effect on other metabolites, including the Oxoglutaric acid (2-OG) that showed higher concentration in 'Garnacha Blanca' leaves after BL treatment and lower concentration in 'Macabeo' leaves after Aki and BL treatments. The 2-OG is involved in gibberellin (GA), alkaloid and flavonoid biosynthesis (Puhl et al., 2008;

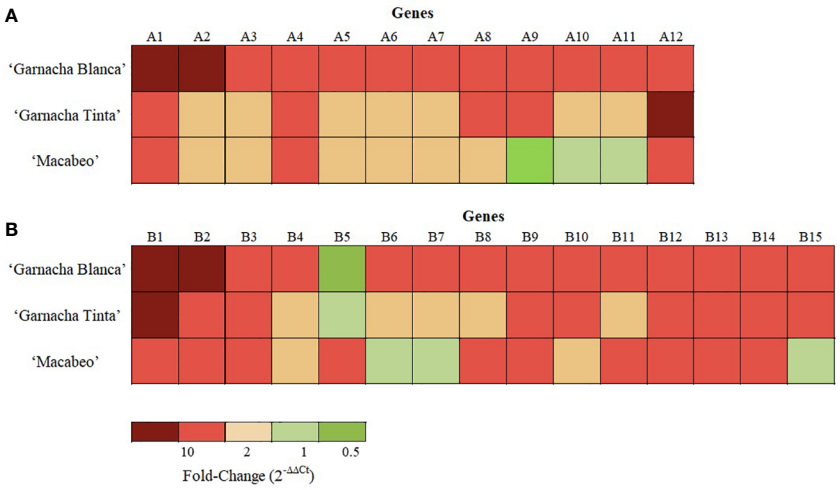

FIGURE 7
Transcriptional pattern of DEGs after treatments of grapevine cvs. Garnacha Blanca, Garnacha Tinta, and Macabeo with Akivi **(A)** or lyophilized *Bacillus* UdG **(B)**. The fold change was assessed by the ΔΔCt method. The UBQ gene was used as the internal control for data normalization. The ΔCt of the non-treated control (NTC) samples was defined as the calibrator. Three independent biological replicated were performed. Gene functions are indicated in Supplementary Table 2.

Araújo et al., 2012). Indeed, it was reported that treating grapevine with a structural mimic of 2-OG (prohexadione-Ca) inhibit the enzyme and alter flavonoid biosynthesis (high amount of unusual flavonoids) (Puhl et al., 2008). This is in agreement with the obtained results since phenylpropanoids pathway was triggered by both Aki and BL treatments. Moreover, the GA content in leaves was also affected in the present study, being GA1 and GA4 concentrations higher after the treatments (Aki or BL) in 'Garnacha Blanca' and lower in 'Macabeo'. However, the link between 2-OG and GA concentrations' variations was not clear, that reinforce the hypothesis linking the 2-OG with flavonoid biosynthesis.

The concentration of Tartaric acid in grapevine leaves was also affected since lower concentrations were detected in 'Garnacha Tinta' leaves treated with BL. Grapevine presents a high concentration on tartaric acid and its biosynthesis occurs in leaves and berries. Tartaric acid was shown to be involved in various processes and abrupt changes in its biosynthesis were linked with oxidative burst as well as ascorbate/glutathione redox state in berries (Burbidge et al., 2021). More insight on this matter could give interesting results like a kinetics study of tartaric acid after BL treatment.

Grapevine response to Aki and BL treatments at transcripts and metabolic level indicate the ability of these products to trigger plant defense response. In fact, many transcripts related to defense responses were detected by the RNA-seq analysis of leaves. Some of the transcripts did not present differential expression, but others were highly affected by Aki treatment (VIT_17s0000g00200, VIT_08s0058g00970, VIT_12s0055g01010, and VIT_03s0088g00810) and were selected as DEGs markers candidates (A4, A5, A6, and A11, respectively). Considering all the upregulated transcripts in JA, ET, SA, and ABA pathways (Figure 8) and the higher concentrations of some phytohormones; the application of Aki and BL treatments to grapevine can stimulate several processes related to plant defense immune system like SAR. Particularly, the treatments upregulated JA, ET, and phenylpropanoid pathways. Moreover, Aki treatment seemed to trigger several genes involved with HR. Further investigations are necessary to identify the mode of action of the two biocontrol product candidates (Aki and BL).

These results also indicate that the treatments with Aki and the BL might prime a defense response through ISR. However, the study was designed to investigate the interaction between the biocontrol products and grapevine without pathogen infection. If the mode of action is priming ISR, the effect could be seen only with the presence of the pathogen attack (Van Wees et al., 2008; Pieterse et al., 2014; Esmaeel et al., 2020). Actually, a complex effect acting in two steps was observed on various biocontrol products, such as the *Rheum palmatum* plant extract (Godard et al., 2009), *Trichoderma harzianum* T39 (Perazzolli et al., 2011), and sulphated laminarin (Trouvelot et al., 2008), being this last one already used in vineyards against downy mildew. These products show plant defense stimulation activity through the induction of some genes immediately after the treatment and the reinforcement of the modulation of defense response through other genes after pathogen inoculation. The pathogen infection may trigger biocontrol product activity and different grapevine response as it was observed using transcriptomics in watermelon (*Citrullus lanatus*) roots treated with the beneficial microorganism candidate *B. velezensis* against *Fusarium oxysporum* fungal pathogen (Jiang et al., 2019). More insights on Aki and BL possible modes of action could be revealed through another investigation introducing pathogen inoculation in the study, such as *P. viticola*, *E. necator*, or *B. cinerea* and analyzing grapevine response to the treatment after pathogen infection. This new research could be more accurate by doing a sampling kinetics to study the plant response to both treatments and pathogen inoculation along time by transcriptomic and metabolomics approaches (Jiang et al., 2019).

From the twelve DEGs selected for Aki treatment eight are related to plant defense (A1, A3, A4, A5, A6, A7, A11, and A12). Some of them (A4, A5, A6, and A11) are involved in the main pathways related to plant defense response (Figure 8) and were discussed above. From the fifteen DEGs selected for BL treatment, four of them are related to plant defense (B1, B6, B8, and B14). In addition, another gene could be related to defense response (B12-VIT_10s0116g00530) as it is involved in thiazole biosynthesis. Thiazole is a precursor of thiamine that has been showed to be able to stimulate defense response (Boubakri et al., 2012; Boubakri et al., 2013). It is reported that thiamine is able to induce resistance to downy mildew defense response elicitation in leaves of 'Chardonnay' cultivated in greenhouse-controlled conditions. The elicited defense response included accumulation of hydrogen peroxide (H_2O_2), callose deposition in stomata cells, phenylpropanoid compounds accumulation (stilbenes, phenolic compounds, flavonoids and lignin) and hypersensitive response. Thiamine triggered several genes involved in defense response like PR genes (glucanase, chitinases, serine protease inhibitor, glutathione-S-transferase) and lipoxygenases pathway involved in JA biosynthesis (Boubakri et al., 2012; Boubakri et al., 2013). The high rate of DEGs highly impacted by the treatments and related to defense response is consistent with the transcripts analysis previously mentioned, and with the hypothesis that Aki and BL treatments could be able to induce resistance on grapevine.

Several DEGs markers presented stable overexpression after Aki (A1, A4 and A12 genes) and BL (B1, B2, B3, B9, B12, B13, and B14 genes) treatments in the three grapevine cultivars. Therefore, they could be considered as appropriate markers of Akivi and *Bacillus* treatments and could be used to test different doses and formulations of the biocontrol products in greenhouse-controlled conditions. Actually, defining treatment dose and formulation are crucial steps in product development that highly impact its efficacy. As observed in the present study, grapevine response is variable according to the studied cultivar as described other studies (Bota et al., 2016; Catacchio et al.,

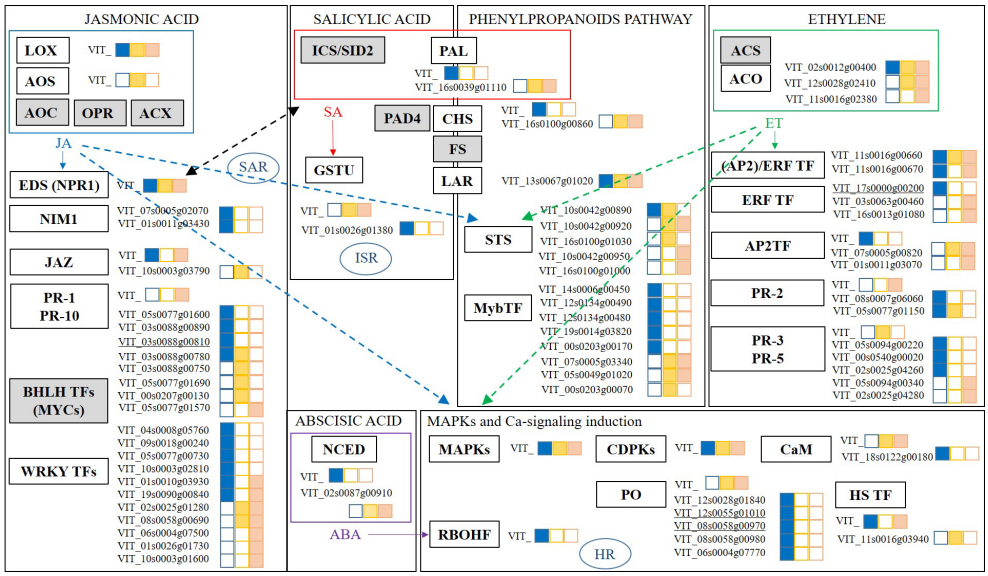

FIGURE 8
Scheme of main pathways related to plant defense response: Jasmonic Acid (JA); Salicylic Acid (SA); Ethylene (ET); Abscisic Acid (ABA); phenylpropanoids pathway; and mitogen activated protein kinases, Ca^{2+} signalling induction (MAPKs). DEGs results are presented from RNA-seq analysis of grapevine leaves treated with the botanical extract (Aki, blue) and the microbial product (BF, yellow; or BL, orange). Complete DEGs transcript codes are written when the differential expression is above Log (FC) > 1.4; only VIT_ is written otherwise. DEGs highly impacted by one of the treatments are underlined. Gene groups from the different pathways are indicated, the box is white coloured when transcripts related to the genes' groups were found, the box is grey coloured otherwise. JA and ET interactions with other pathways are represented with arrows. Black arrow represents JA and SA crosstalk. LOX, LipOXygenase; AOS, Allene Oxide Synthases; AOC, Allene Oxide Cyclase; OPR, OPDA Reductase; ACX, Acetyl-CoA oXidase; EDS1/NPR1, Enhanced Disease Susceptibility/Non-expressor of Pathogen Related genes 1; NIM1, Non-Inducible Immunity 1; SAR, Systemic Acquired Resistance; JAZ, JAsmonate-Zim domain; PR, Pathogenesis Related proteins; BHLH TFs, Helix Loop Helix TFs, WRKY TFs, Transcription Factors with domain WRKY. ICS/SID2, IsoChorismate Synthase; PAL, Phenylalanine Ammonia Lyase; PAD4, PhytoAlexin Deficient 4; FS, Flavonoid Synthase; LAR, LeucoAnthocyanicin Dioxygenase; GSTs, Glutatione-S-Tranferase; ISR, Induced Systemic Resistance; STS, STilbene Synthase; MyB TF, MyB Transcription Factors. ACS; 1-Aminocyclopropane-1-Carboxylate Synthase; ACO, 1-Aminocyclopropane-1-Carboxylate Oxidase; ERF TF, (AP2)/ERF TF/AP2TF; Ethylene Response Factors Transcription Factors; PR, Pathogenesis Related proteins. NCED, 9-Cis-Epoxycarotenoid Dioxygenase. MAPKs, Mitogen Activated Protein Kinases; CDPKs, Ca^{2+} DePendent Kinases; CaM, CalModulin; RBOHF, Respiratory Burst Oxidase Homologue protein F; PO, PerOxidases; HS TF, Heat Shock Transcription Factors.

2019; Fasoli et al., 2019; Balestrini et al., 2020). Therefore, the identified markers are only robust for the three tested cultivars and should be tested on other cultivars to extend their use. The markers could also be tested in field conditions, but it could be difficult to detect an impact on transcriptome in field conditions due to vineyard biological variability (Balestrini et al., 2020).

5 Conclusion

Grapevine response to the Aki and BL treatments at transcripts and metabolites levels gave insights on modes of action of these biocontrol products that are under development. RNA sequencing analysis showed different gene expression patterns after foliar treatments with the biocontrol products compared with the NTC in 'Garnacha Blanca'. Furthermore, RT-qPCR enabled the quantification of several selected genes (DEG) in three different cultivars. This information was complemented with metabolic analysis (phytohormones, phenols, and organic acids). Considering all the upregulated transcripts and enhanced metabolites concentrations related to JA, ET, and phenylpropanoids pathways, strong indication was found of grapevine defense induction by the treatments. However, further studies are necessary to confirm these first results and a kinetics study could be interesting. In addition, several DEGs markers were identified presenting a stable overexpression after the treatments (Aki or BL) in the three grapevine cultivars. They could be used as markers of activity of the products for further investigations.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE211268.

Author contributions

ML, EM, and EB obtained the financial support. MR, ND, ML, EM, and EB conceived and designed the research. MR, ND, ML, RT and EB conducted and performed the experiments. MR, ML, and EB analyzed the data. MR and ND wrote the first draft of the manuscript. ML, EM, and EB revised and edited the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by PALVIP (EFA182/16) project that has been 65% co-financed by the European Regional Development Fund (ERDF) through the Interreg V-A Spain France Andorra programme (POCTEFA 2014-2020). POCTEFA aims to reinforce the economic and social integration of the French-Spanish-Andorran borders. Its support is focused on developing economic, social and environmental cross-border activities through joint strategies favouring sustainable territorial development.

Acknowledgments

The authors would like to thank Gemma Roselló, Héctor Naún Saravia, and Josep Pereda for the technical support in the greenhouse experiments and for growing up the grapevine stocks, and Rosa Padilla for her help in the laboratory. We are grateful to Anna Bonaterra for her help and expertise in cultivating *Bacillus* UdG strain and to S.A.S. AkiNaO for providing Akivi biocontrol product. We are also thankful to Anna Bonaterra and Luis Moll for their help during the grapevine leaves samplings.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1075231/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 17 February 2023
DOI 10.3389/fpls.2023.1084590

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

RECEIVED 30 October 2022
ACCEPTED 30 January 2023
PUBLISHED 17 February 2023

CITATION
Dami I and Zhang Y (2023) Variations of freezing tolerance and sugar concentrations of grape buds in response to foliar application of abscisic acid. *Front. Plant Sci.* 14:1084590. doi: 10.3389/fpls.2023.1084590

Variations of freezing tolerance and sugar concentrations of grape buds in response to foliar application of abscisic acid

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The purpose of this study was to explore the mechanism of ABA-induced freezing tolerance increase in grapevines. The specific objectives were to evaluate the impact of ABA treatment on soluble sugar concentration in grape buds and determine the correlations between freezing tolerance and ABA-affected soluble sugar concentration. *Vitis spp* 'Chambourcin' and *Vitis vinifera* 'Cabernet franc' were treated with 400 and 600 mg/L ABA in the greenhouse and field. The freezing tolerance and soluble sugar concentration of grape buds were measured monthly during the dormant season in the field and at 2wk, 4wk, and 6wk after ABA application in the greenhouse. It was observed that fructose, glucose, and sucrose are the main soluble sugars that correlate with freezing tolerance of grape buds and the synthesis of these sugars can be enhanced by ABA treatment. This study also found that ABA application can promote raffinose accumulation, however, this sugar may play a more important role in the early acclimation stage. The preliminary results suggest that raffinose accumulated first in buds, then its decrease in mid-winter corresponded with the increase of smaller sugars, such as sucrose, fructose, and glucose, which in turn, corresponded with reaching maximum freezing tolerance. It is concluded that ABA is a cultural practice tool that can be used to enhance freezing tolerance of grapevines.

KEYWORDS

cold hardiness, Chambourcin, Cabernet franc, raffinose, sugars, ABA

Introduction

Cold damage is by far the most devastating weather event to grape production (Snyder and de Melo-Abreu, 2005). Grapes and the established wine reputation of certain regions are sensitive to climate extremes, and there are concerns about how changing climate patterns will impact these industries. Although average winter temperatures have been trending upwards over the last 20 years, so has the variability in winter temperatures (https://mrcc.purdue.edu). The rise of average temperature has a two-fold impact on grapevines: it retards cold acclimation in the fall and reduces winter freezing tolerance (FT), resulting in a faster

deacclimation and earlier budburst, which renders grapevines more vulnerable to spring frost (Wolf and Cook, 1992; Kovaleski et al., 2018).

To mitigate freezing stress, grapevines adapt to cold climates by undergoing physiological changes that result in a transition from a cold-sensitive to a cold-hardy state, a process known as *cold acclimation*. During this process, nearly 800 genes are upregulated and 2300 genes are downregulated (Londo et al., 2018). During cold acclimation, plants increase their desiccation and freezing tolerances by involving various physiological and biochemical changes, such as sugar accumulation. A decrease in photoperiod (*i.e.* daylength) has been reported to induce the synthesis of the phytohormone ABA, which is then translocated to the various plant tissues, resulting in shifts in gene expression and a cascade of cellular signaling responses (Chao et al., 2007). ABA has been suggested to play a role during these changes by promoting soluble sugar accumulation (Xue-Xuan et al., 2010). Soluble sugars, including sucrose, glucose, fructose, and raffinose family oligosaccharide (RFO) such as raffinose and stachyose accumulate when plants develop freezing tolerance in winter and decrease during deacclimation in spring (Wanner and Junttila, 1999). Among these soluble sugars, sucrose and raffinose have been suggested to play an important role in cold acclimation of woody plants. A sudden increase of sucrose and raffinose concentrations at the start of cold acclimation was observed in poplar wood (*Populus x canadensis Moench* ''robusta'') and remained high during the winter season (Sauter et al., 1996). Furthermore, cold tolerant grapevines were found to accumulate more soluble sugars and have differential expression of sugar metabolism genes compared to cold-sensitive plants (Chai et al., 2019). Genes involved in sugar metabolism, such as galactinol synthase (GolS), raffinose synthase (RafS), β-amylase (BAMY), and phosphoglycerate kinase (PGK), were differentially expressed under freezing temperatures (Londo et al., 2018; Chai et al., 2019; Wang et al., 2021).

The functional role of soluble sugars during cold acclimation has been proposed as osmotic regulator and cryoprotectant. It has been reported that soluble sugars can be used to adjust osmotic pressure in leaf and root cells under water stress (Ogawa and Yamauchi, 2006). It has also been suggested that some soluble sugars, such as sucrose, can interact with the lipid bilayer of cell membranes to prevent damage caused by dehydration (Anchordoguy et al., 1987). Furthermore, the hydrogen bond between glucose and protein can stabilize protein structure and prevent dehydration-induced protein unfolding (Allison et al., 1999). As cryoprotectants, sugars can prevent ice crystallization by inhibiting the nucleation of ice crystals. In this case, the water in cells can be solidified as an amorphous glass (Sacha and Nail, 2009).

Endogenous ABA has been suggested to play an important role in promoting the production of soluble sugars during cold acclimation of many plant species, such as Lily (*Lilium rubellum* L.) and moss (*Physcomitrella patens* L.) (Xu et al., 2006; Bhyan et al., 2012). It has also been reported that exogenous ABA application (40 and 100 mg/L) induced the accumulation of fructose and sucrose in wheat (*Triticum aestivum* L.) along with increased freezing tolerance (Kerepesi et al., 2004). In grape (*Vitis vinifera*), sucrose and raffinose have been found to correlate with the variation of the ABA content in buds and ambient temperature during dormant season (Koussa et al., 1998). In gentian (*Gentiana scabra* L.), it has also been found that the concentrations of sucrose and raffinose in buds are sensitive to ABA application since incubating buds with ABA solution increases the sucrose and raffinose concentration with increased desiccation tolerance (Suzuki et al., 2006). Moreover, applying ABA inhibitor (fluridone) decreased the sugar concentration and desiccation tolerance (Suzuki et al., 2006).

Additionally, the ABA-inducible raffinose production in seed embryos in relation to desiccation tolerance is well documented. The seedlings grown from exogenous ABA-incubated cucumber (*Cucumis sativus* L.) seeds showed higher raffinose concentration in tissues with a higher desiccation tolerance than control groups (Wang et al., 2012). There is evidence showing that the ABA-activated galactinol synthase is mainly responsible for the accumulation of raffinose in seeds (Blochl et al., 2005).

Previous greenhouse and field studies demonstrated that exogenous ABA application advanced cold acclimation and increased freezing tolerance of grapevines (Zhang et al., 2011; Zhang and Dami, 2012a). In this study, the relationship between ABA and sugar metabolism was investigated. The specific objectives were to: 1) evaluate the effect of exogenous ABA on soluble sugar concentration in grape buds of 'Chambourcin' and 'Cabernet franc' cultivars; and 2) determine the correlations between freezing tolerance and ABA-affected soluble sugar concentration.

Materials and methods

Plant materials, experimental design, and treatments

This study consisted of two field experiments conducted during the dormant season and one greenhouse experiment.

Greenhouse experiment

One-year-old dormant *Vitis vinifera* 'Cabernet franc' grafted on *Vitis riparia* × *Vitis rupestris* 'Couderc 3309' were planted in 7.6 L pots and placed on benches in the greenhouse. In the second year, one-year-old own-rooted *Vitis spp* 'Chambourcin' grapevines were also planted under the same conditions. The greenhouse conditions were consistent with the settings described in Zhang et al. (2011). Briefly, the greenhouse settings were as follows: 22/19°C and 50/50% relative humidity (day/night). The light intensity was maintained at 300 umol.m^2. s^{-1} using metal halide 1000-W high-pressure sodium lights (Sunlight Supply, Woodland, AZ). All grapevines were pruned back and kept in 4°C cooler to satisfy their chilling requirements then placed in the greenhouse to promote budburst.

The grapevines were pruned to the twelfth basal nodes when the leaf age was approximately 50 days. ABA was applied with the concentration of 0 (control) and 400 mg/L. All the 'Chambourcin' and 'Cabernet franc' grapevines were sprayed when the leaf age was approximately 80 days. Prior to applying ABA, the average leaf number and shoot length per vine were 18 and 140 cm, respectively. Leaf and bud samples were collected at 2, 4, and 6wk after ABA application, corresponding to leaf age of 94, 108, and 122 days, respectively. At each sample collection time, four vines from control and ABA treated groups were randomly selected. The

experimental design was a completely randomized design. Buds on node positions 1 to 5 were used for the freezing tests. Leaves and buds on the same position on the adjacent shoot of the same vine were used for sugar analysis.

Leaf age was determined based on Eichorn-Lorenz (EL) stages of shoot development (Eichorn and Lorenz, 1977) with one-day leaf age corresponding to the first unfolded leaf (EL stage 7) originating from the third basal node.

The same ABA sample and surfactant from the field experiments were used in the greenhouse experiment. Whole vines were sprayed with ABA solutions to run off with a 7.6 L hand-held sprayer (Gilmour Gardening Innovation, Peoria, IL) averaging a spray volume of 0.2 L/vine.

Field experiment

During 2010-2011 dormant season, two field experiments were conducted at the Research Vineyard in Wooster and the Ashtabula Agricultural Research Station, Kingsville, OH. In Wooster, OH, grafted 'Chambourcin' (Seyve-Villard 12417 × Seibel 7053) grapevines on *Vitis riparia* × *Vitis rupestris* rootstock 'Couderc 3309' and planted in 1996 at the Research Vineyard, were used for this study. Vines were spaced 1.25 × 3 m (vine × row), trained to high-cordon system (height = 1.83 m), and spur-pruned to 2 buds per spur and 16 buds per meter of cordon, followed by shoot and cluster thinning to 13 and 20 per meter of cordon, respectively prior to ABA treatment. Seven ABA treatments were assigned to vines on a randomized complete block consisting of four blocks with 5 vines per plot unit as follows: control (deionized water), 400 and 600 mg/L ABA sprayed at 50% véraison stage (4V and 6V, respectively), 400 and 600 mg/L ABA sprayed at 20 days after véraison stage (4V20 and 6V20, respectively), 400 and 600 mg/L ABA sprayed at 40 days after véraison stage (4V40 and 6V40, respectively). In Kingsville, OH, 'Cabernet franc' (Clone 1) grafted on *Vitis riparia* × *Vitis rupestris* 101-14 Millardet et de Grasset rootstock were planted in 2005. Vines were spaced 1.8 × 2.4 m (vine × row), trained to a bi-lateral cordon system with vertically-shoot positioned, and spur-pruned to 16 buds/m of cordon. The vineyard block was divided into four blocks. Each block consisted of 20 grapevines, which were divided into five panels (four vines per panel). Each panel was randomly assigned to one of five treatments: control (same as above), 600 mg/L ABA sprayed at V, V20, V40, and V55 (20, 40, and 55 days after 50% véraison stage). In each panel, the first three vines were used as a replicate for one treatment and the fourth was an untreated buffer vine. Canopy management practices consisted of leaf removal of the basal three leaves on both sides of the canopy in late July and shoot hedging performed in early August. A spring frost event (-1.2°C) occurred on 10 May 2010 which caused injury of shoots and inflorescences and resulted in uneven number of clusters per vine. In order to avoid the potential confounding effect of crop level, the shoot number per vine was adjusted and all clusters were removed from all treated vines. Daily temperatures from weather stations at the vineyard sites were recorded during the field experiments (Supplemental Figures 1, 2).

The ABA sample (VBC-30051) was provided by Valent Bioscience (Libertyville, IL). The a.i. was 20.0% (w/w) S-ABA. The ABA sample was dissolved in deionized water with 0.05% Tween-20 (Acros Organic, Hampton, NH). Whole vine canopies (leaves and clusters) were sprayed with ABA solutions to runoff with a 15-L back sprayer (SP System LLC. Model SP0, Santa Monica, CA) averaging a spray volume of 0.5 L/vine.

Determination of freezing tolerance

In the field experiments, one representative one-year-old cane with a minimum of 12 to 15 lignified internodes was collected from each replication and buds on node positions 3 to 7 were used. There were five buds used from each replication and 20 buds per treatment in both 'Chambourcin' and 'Cabernet franc'. Buds were excised and mounted on thermoelectric modules (MELCOR, Trenton, N.J.), which were placed in a Tenney environmental chamber (Thermal Products Solutions, New Columbia, PA). The chamber temperature was lowered from -2 to -50°C at 4°C/hr. Freezing tolerance of buds was determined using differential thermal analysis and was expressed as the average lethal temperature exotherm that kills 50% of the bud population or LT50 (Wolf and Pool, 1987). For Chambourcin, LT50s were determined five times from October 2010 to February 2011. For Cabernet franc, LT50s were determined six times from October 2010 to January 2011. In the greenhouse experiment, the buds were excised and tested following the same procedures applied in the field experiments.

Sugar analysis

In the field experiments, the cane selection followed the same protocol applied in freezing tolerance determination. The buds were excised and frozen immediately in a box with dry ice and then stored at -80°C freezer. In the greenhouse experiment, the buds and leaves were immediately plunged in liquid nitrogen and then stored at -80°C freezer. The sugar extraction, derivatization, and quantification followed a modified protocol based on Grant et al. (2009).

Extraction

Five frozen buds were ground by mortar and pestle in liquid nitrogen and then freeze dried. The freeze-dried buds were weighed and transferred to a 2-ml microcentrifuge tube before extraction. Leaf samples were freeze dried first and then ground to powder. For each replicate/treatment, approximate 5-6 mg leaf samples were weighed and transferred to a 2-ml microcentrifuge tube before extraction. One mL of 75% ethanol (Fisher Scientific, Pittsburgh, PA) was added to bud and leaf samples and the samples left at room temperature for 3h and shaken every 30 min. The samples were then centrifuged at 6708 x g for 10 min and the supernatants were transferred to a glass vial for collection. The extraction procedure was repeated twice and the glass vials ended up with approximate 2-3 mL supernatant collection. The glass vials were placed on the Reacti-Therm Heating Modules (Thermo Fisher Scientific, Waltham, MA) at 45°C and dried under an air stream overnight.

Derivatization

For bud samples, 250 µL pyridine (Sigma-Aldrich, St. Louis, MO) and 250 µL STOX solution (For 750 µg/vial internal standard: 100 mL pyridine, 2.5 g hydroxylamine hydrochloride, and 0.6 g phenyl-β-D-

glucopyranoside; For 100 µg/vial internal standard: 100 mL pyridine, 2.5 g hydroxylamine hydrochloride, and 80 mg phenyl-β-D-glucopyranoside) were added to each dried glass vial. The vials were shaken for 10 sec and placed on the Reacti-Therm Heating Modules at 70°C for 40 min. Vials were then removed from the heating block and cooled under room temperature. Four hundred µL hexamethyldisilazane (HMDS, Sigma-Aldrich, St. Louis, MO) and 40 µL trifluoroacetic acid (TFA, Sigma-Aldrich, St. Louis, MO) were added to each glass vial and the vials were shaken for 10 sec. Finally, the vials were placed at 4°C refrigerator overnight for precipitation. The supernatants were transferred to 1.5 mL vials and ready for sugar analysis the next day. Leaf samples were derivatized following the same procedure except the volumes for pyridine, STOX solution, HMDS, and TFA were 125, 125, 200, and 20 µL respectively.

Gas chromatography/Flame ionized detector

GC/FID was used to analyze samples from the field and 2011 greenhouse experiments. The derivatives were injected to a gas chromatograph (Hewlett Packard 5890 Series II, Hewlett Packard, Boulder, CO) with a 30-m capillary column (HP5-MS, 250 µm inner diameter and 0.25 µm thickness). Injection temperature was 280°C and oven ramp was: 180°C for 2 min, 6°C·min^{-1} ramp to 215°C, held for 1 min, and then 40°C·min^{-1} to 320°C, held for 22 min. The flow rate of the carrier gas, Helium, was 1.0 mL·min^{-1}. Soluble sugars were identified and quantified (Chemstation Quantiation Process Program, Agilent Technologies, Santa Clara, CA) by comparison with standard sugars and the internal standard, phehyl β-D glucopyranoside (Sigma-Aldrich, St. Louis, MO).

Statistical analysis

The data were subjected to one-way analysis of variance using Minitab statistical software (Minitab Inc., State College, PA). The model tested for main effects of different treatments. When appropriate, means were separated using LSD (α=0.05). The correlation between bud freezing tolerance and sugar concentration was determined using Pearson Correlation Analysis.

Results

Effect of ABA on freezing tolerance of greenhouse-grown and field-grown grapevines

In the greenhouse, between 2wk and 6wk after ABA application, the LT50s of 'Chambourcin' and 'Cabernet franc' grape buds consistently decreased by 6 and 2.5°C on average, respectively. In 'Chambourcin' grape buds, ABA treatment started to affect freezing tolerance 4wk after ABA application and decreased the LT50s by 3.8°C on average (Figure 1A). In 'Cabernet franc' grape buds, ABA treatment started to affect freezing tolerance 2wk after ABA application and decreased the LT50s by 2.5°C on average (Figure 1B). In sum, the LT50s between 2wk and 6wk decreased and ABA-increased freezing tolerance was consistent between 2wk and 6wk. The data of the freezing tolerance in the field experiments of

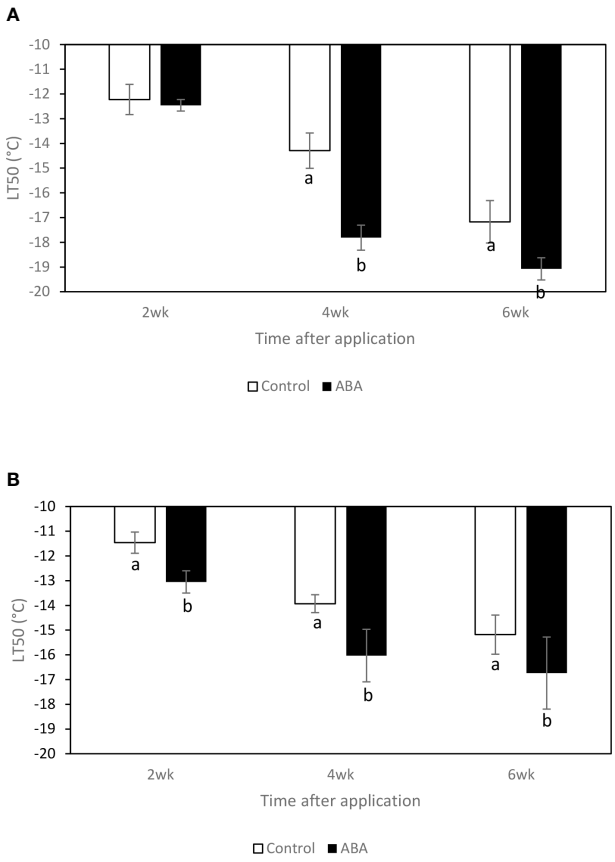

FIGURE 1
Effect of ABA on bud freezing tolerance (LT50) of greenhouse-grown grapevines, **(A)** 'Chambourcin' and **(B)** 'Cabernet franc'. Bars with different letters are significantly different at $p \leq 0.05$. (n = 4). Standard errors are presented.

'Cabernet franc' and 'Chambourcin' has been reported by Zhang and Dami (2012a) and Zhang and Dami (2012b), respectively. The lowest temperatures recorded in 2011 were -23.3°C on 24 January in Kingsville and -21.3°C on January 13 in Wooster (Supplemental Figures 1, 2). Overall, ABA at veraison and post-veraison increased freezing tolerance in 'Cabernet franc' (Zhang and Dami, 2012a) and in 'Chambourcin (Zhang and Dami, 2012b) in midwinter.

Effect of ABA on the seasonal changes of soluble sugar concentrations in the field-grown grapevines

In 'Chambourcin' grape buds, the concentrations of fructose, glucose, sucrose, galactinol consistently increased from late fall to mid-winter and did not reach their maximum values until February (Table 1). The concentrations of raffinose and stachyose reached peak values in December, and then started to decrease in January and February (Table 1). In 'Cabernet franc' grape buds, the concentrations of soluble sugars followed a similar pattern as in 'Chambourcin' by reaching maximum concentrations in January (Table 2). Raffinose and stachyose reached peak values in November, and then started to decrease in December and January (Table 2).

TABLE 1 Effect of ABA on the seasonal changes of the sugar concentrations (mg/g dry wt) in 'Chambourcin' buds.

Sugar	Treatment*	29 Oct.	22 Nov.	15 Dec.	15 Jan.	27 Feb.
Fructose	Control	18.3 ± 1.6	29.6 ± 2.2 c	31.1 ± 1.3 c	34.0 ± 1.6 b	37.7 ± 2.1 b
	4V	18.7 ± 0.6	33.5 ± 1.0ab	37.8 ± 2.0 b	46.2 ± 2.6 a	44.9 ± 1.9 a
	4V20	18.5 ± 0.8	34.6 ± 1.7 a	49.0 ± 0.2 a	41.9 ± 2.5 a	44.5 ± 2.7 a
	4V40	17.5 ± 1.3	35.2 ± 1.6 a	31.7 ± 1.0 bc	26.5 ± 0.6 b	34.6 ± 2.4 b
	6V	16.9 ± 1.0	35.0 ± 1.9 a	44.9 ± 1.5 a	40.0 ± 1.3 a	40.3 ± 2.3 b
	6V20	19.0 ± 1.8	31.5 ± 1.7 bc	34.4 ± 1.5 bc	47.0 ± 2.0 a	38.1 ± 2.3 b
	6V40	15.8 ± 1.2	29.6 ± 0.2 c	28.0 ± 1.7 c	42.9 ± 1.6 a	40.0 ± 2.3 b
	p-value	ns	0.001	<0.001	0.048	0.001
Glucose	Control	13.8 ± 0.8 b	22.6 ± 0.8 c	23.9 ± 1.1 b	27.9 ± 1.2 b	26.9 ± 1.4 c
	4V	14.2 ± 0.7 b	24.3 ± 1.6 b	25.4± 1.9ab	28.4 ± 1.3 b	27.9 ± 1.2 c
	4V20	14.3 ± 0.8 b	25.0 ± 0.8 ab	26.5 ± 1.2 ab	28.7 ± 1.7 b	30.0 ± 1.7 b
	4V40	13.1 ± 0.8 b	25.2 ± 0.8ab	28.3 ± 1.2 ab	28.6 ± 1.4 b	31.9± 1.6 ab
	6V	17.3 ± 1.7 a	26.5 ± 2.0 a	29.6 ± 1.8 a	28.2 ± 1.3 b	32.2 ± 1.1 ab
	6V20	17.3 ± 0.6 a	25.9 ± 1.4 a	29.1 ± 1.2 a	29.9 ± 1.3 a	33.4 ± 1.7 a
	6V40	13.6 ± 0.6 b	25.2 ± 1.2 ab	29.7 ± 1.5 a	32.1 ± 1.7 a	32.8 ± 1.8 ab
	p-value	<0.001	<0.001	0.024	0.001	0.011
Sucrose	Control	17.5 ± 0.9	16.5 ± 1.1 c	26.3 ± 1.8 d	23.6 ± 1.6 c	32.4 ± 1.4 c
	4V	19.0 ± 1.2	23.8 ± 1.9 b	33.0 ± 2.1 ab	41.6 ± 1.9 a	43.2 ± 1.6 a
	4V20	18.3 ± 1.5	23.8 ± 1.3 b	33.0 ± 2.1 ab	41.0 ± 1.0 a	41.0 ± 1.1 a
	4V40	18.5 ± 1.1	23.3 ± 1.3 b	28.5 ± 1.3 c	33.1 ± 1.3 b	36.6 ± 1.1 ab
	6V	19.0 ± 1.0	27.0 ± 1.6 a	34.5 ± 1.1 a	42.3 ± 1.3 a	35.2 ± 1.1 b
	6V20	20.0 ± 1.4	27.0 ± 0.7 a	35.5± 2.1 a	34.9 ± 1.0 b	38.2 ± 1.7 ab
	6V40	17.8 ± 1.1	22.0 ± 1.2 b	31.0 ± 2.1 b	36.0 ± 1.1 b	31.0 ± 1.8 c
	p-value	ns	<0.001	0.015	0.001	<0.001
Myo-inositol	Control	13.1 ± 0.5	21.2 ± 1.7	13.6 ± 0.7	8.6 ± 0.5	11.4 ± 0.8
	4V	14.4 ± 0.4	22.8 ± 1.0	14.4 ± 0.4	8.5 ± 0.8	11.9 ± 1.0
	4V20	13.2 ± 1.1	21.2 ± 0.5	13.3 ± 0.8	8.7 ± 0.8	10.2 ± 1.2
	4V40	14.4 ± 0.3	20.9 ± 1.1	14.0 ± 0.7	7.5 ± 1.8	13.4 ± 2.1
	6V	13.7 ± 0.5	20.2 ± 1.8	13.9 ± 0.2	9.0 ± 1.0	12.7 ± 1.2
	6V20	14.1 ± 0.4	20.1 ± 1.0	14.5 ± 0.9	10.3 ± 1.5	13.4 ± 2.0
	6V40	14.3 ± 0.6	22.5 ± 0.2	15.6 ± 1.0	12.5 ± 1.4	13.2 ± 1.4
	p-value	ns	ns	ns	ns	ns
Galactinol	Control	0.30 ± 0.04	0.42 ± 0.05 ab	0.54 ± 0.06	0.46 ± 0.03	0.83 ± 0.15 ab
	4V	0.27 ± 0.02	0.47 ± 0.03 a	0.53 ± 0.07	0.54 ± 0.04	0.83 ± 0.13 ab
	4V20	0.20 ± 0.02	0.44 ± 0.05 ab	0.51 ± 0.05	0.47 ± 0.05	0.86 ± 0.15 a
	4V40	0.30 ± 0.02	0.35 ± 0.05 b	0.45 ± 0.09	0.52 ± 0.07	0.63 ± 0.15 ab
	6V	0.31 ± 0.03	0.43 ± 0.07 ab	0.49 ± 0.03	0.54 ± 0.04	0.68 ± 0.06 ab
	6V20	0.30 ± 0.04	0.41 ± 0.07 ab	0.43 ± 0.02	0.54 ± 0.02	0.48 ± 0.03 b
	6V40	0.29 ± 0.02	0.51 ± 0.02 a	0.48 ± 0.07	0.62 ± 0.05	0.49 ± 0.02 b
	p-value	ns	0.046	Ns	ns	0.019

(Continued)

TABLE 1 Continued

Sugar	Treatment*	29 Oct.	22 Nov.	15 Dec.	15 Jan.	27 Feb.
Raffinose	Control	2.03 ± 0.19 c	2.73 ± 0.16 c	2.94 ± 0.08 c	1.37 ± 0.17 ab	0.48 ± 0.03 ab
	4V	2.36 ± 0.07 ab	3.29± 0.14a	3.31 ± 0.11 bc	1.60 ± 0.15 a	0.44 ± 0.02 b
	4V20	2.62 ± 0.17 a	3.16 ± 0.20 ab	3.59 ± 0.19 bc	1.39 ± 0.11 ab	0.65 ± 0.02 a
	4V40	2.13 ± 0.08 bc	2.80 ± 0.11 c	3.22 ± 0.10 bc	1.54 ± 0.06 a	0.54 ± 0.03 ab
	6V	2.54 ± 0.15 a	3.18 ± 0.125 ab	4.58 ± 0.14 a	1.40 ± 0.07 ab	0.45 ± 0.02 b
	6V20	2.37 ± 0.21 ab	2.88± 0.07 bc	4.00 ± 0.15 b	1.64 ± 0.10 a	0.49 ± 0.03 ab
	6V40	2.23 ± 0.11 bc	3.19 ± 0.12 ab	3.26 ± 0.10 bc	1.60 ± 0.15 a	0.41 ± 0.02 b
	p-value	0.003	0.013	<0.001	0.002	<0.001
Stachyose	Control	1.30 ± 0.13 b	1.75 ± 0.11 b	2.21 ± 0.16 bc	2.02 ± 0.18 c	1.63 ± 0.07 ab
	4V	1.42 ± 0.09 ab	2.50 ± 0.11 a	3.14 ± 0.17 a	2.42 ± 0.09 ab	1.63 ± 0.11 ab
	4V20	1.59 ± 0.15 a	2.44 ± 0.17 ab	2.57 ± 0.07 b	2.31 ± 0.18 b	1.64 ± 0.14 ab
	4V40	1.32 ± 0.08 b	1.98 ± 0.17 b	2.43 ± 0.08 bc	2.23 ± 0.13 b	1.51 ± 0.17 b
	6V	1.40 ± 0.06 ab	2.43 ± 0.15 ab	2.65 ± 0.14 b	2.61 ± 0.04 ab	1.72 ± 0.14 a
	6V20	1.38 ± 0.09 ab	2.20 ± 0.13 ab	2.71 ± 0.17 b	2.69 ± 0.15 a	1.46 ± 0.09 b
	6V40	1.30 ± 0.11 b	1.91 ± 0.14 b	2.06 ± 0.04 c	2.30 ± 0.12 b	1.54 ± 0.04 b
	p-value	0.033	<0.001	<0.001	0.007	0.05

*4V, 4V20, 4V40 correspond to ABA application of 400 mg/L at 50% véraison stage and 20 and 40 days after 50% véraison stage, respectively. 6V, 6V20, and 6V40 correspond to ABA application of 600 at 50% véraison stage and 20 and 40 days after 50% véraison stage, respectively. (n=4). ns, not significant. Letters indicate significant differences among means.

TABLE 2 Effect of ABA on the seasonal changes of the sugar concentrations (mg/g dry wt) in 'Cabernet franc'.

Sugar	Treatment*	11 Oct.	25 Oct.	8 Nov.	29 Nov.	21 Dec.	18 Jan.
Fructose	Control	10.2 ± 0.2 b	16.3 ± 1.6 c	22.0 ± 1.2 b	32.9 ± 1.6 b	33.8 ± 2.2 b	35.4 ± 0.4 b
	Véraison	13.7 ± 0.6 a	17.0 ± 1.0 b	27.6 ± 1.6 a	37.4 ± 1.9 a	35.8 ± 2.3 a	37.6 ± 1.4 a
	V20	14.6 ± 1.0 a	22.3 ± 0.9 a	26.1 ± 1.3 ab	38.1 ± 1.5 a	38.0 ± 1.8 a	39.5 ± 1.5 a
	V40	12.7 ± 0.8 b	21.7 ± 1.0 a	24.0 ± 0.9 ab	33.8 ± 1.6 b	35.1 ± 1.8 b	36.7 ± 1.5 ab
	V55	N/A	16.8 ± 0.7 b	23.3± 0.8 ab	34.1 ± 1.9 b	34.7 ± 1.4 b	35.6 ± 1.3 b
	p-value	0.001	<0.001	0.046	<0.001	0.011	0.038
Glucose	Control	18.9 ± 0.4 b	19.9 ± 1.6 c	22.7 ± 1.6 c	32.1 ± 1.9 c	38.6 ± 1.5 b	36.0 ± 2.0 b
	Véraison	19.9 ± 0.6 b	24.2 ± 2.0 a	26.5 ± 1.9 a	38.1 ± 1.5 a	37.5 ± 1.2 b	39.8 ± 1.8 a
	V20	20.9 ± 0.7 a	23.7 ± 1.8 a	24.3 ± 1.3 b	38.4 ± 1.5 a	43.6 ± 1.9 a	42.9 ± 0.6 a
	V40	20.0 ± 1.4 a	22.5 ± 1.6 b	24.1 ± 1.2 b	36.3 ± 1.9 b	38.7 ± 1.4 b	40.2 ± 1.2 a
	V55	N/A	22.2 ± 1.1 b	25.3 ± 0.9 a	33.3 ± 2.0 c	36.5 ± 1.2 c	37.5 ± 1.7 b
	p-value	0.049	0.046	0.022	0.01	0.038	0.035
Sucrose	Control	9.6 ± 0.5 b	15.2 ± 0.8 d	24.3 ± 0.8 b	22.9 ± 1.8 b	28.5 ± 1.7 bc	30.4 ± 0.8 b
	Veraison	13.4 ± 0.8 a	28.0 ± 0.6 b	29.5 ± 1.0 a	27.7 ± 2.2 a	31.8 ± 1.6 a	32.5 ± 1.4 a
	V20	13.0 ± 0.3 a	31.2 ± 1.8 a	32.0 ± 1.6 a	28.1 ± 1.4 a	25.9 ± 1.4 c	32.6 ± 1.2 a
	V40	9.3 ± 0.6 b	19.1 ± 1.0 c	23.2 ± 1.1 bc	21.0 ± 1.1 b	29.2 ± 1.6 ab	31.6 ± 1.5 ab
	V55	N/A	16.6 ± 1.5 cd	20.9 ± 0.8 c	22.1 ± 1.1 b	26.2 ± 1.2 bc	32.1 ± 2.2 a
	p-value	<0.001	<0.001	<0.001	<0.001	0.006	0.041
Myo-inositol	Control	3.9 ± 0.5	6.5 ± 0.3	3.3 ± 0.8	3.8 ± 0.3	2.9 ± 0.3	3.1 ± 0.4

(Continued)

TABLE 2 Continued

Sugar	Treatment*	11 Oct.	25 Oct.	8 Nov.	29 Nov.	21 Dec.	18 Jan.
	Veraison	4.1 ± 0.7	5.6 ± 0.1	3.6 ± 0.1	3.3 ± 0.5	2.9 ± 0.1	2.8 ± 0.3
	V20	4.8 ± 1.3	5.7 ± 0.9	3.2 ± 0.3	3.6 ± 0.5	2.9 ± 0.9	3.4 ± 0.5
	V40	4.1 ± 1.3	6.1 ± 0.4	3.8 ± 0.1	3.5 ± 0.4	3.7 ± 0.7	3.5 ± 0.4
	V55	N/A	4.4 ± 0.3	3.2 ± 0.5	2.1 ± 0.5	2.7 ± 0.8	3.4 ± 0.4
	p-value	ns	ns	ns	ns	ns	ns
Galactinol	Control	0.23 ± 0.02 b	0.39 ± 0.05 a	0.22 ± 0.07	0.26 ± 0.01	0.20 ± 0.02	0.19 ± 0.02 b
	Veraison	0.41 ± 0.12 a	0.27 ± 0.01 b	0.24 ± 0.02	0.23 ± 0.03	0.22 ± 0.01	0.19 ± 0.01 b
	V20	0.33 ± 0.12 ab	0.25 ± 0.04 b	0.20 ± 0.02	0.26 ± 0.03	0.20 ± 0.04	0.22 ± 0.02 a
	V40	0.23 ± 0.03 b	0.37 ± 0.08 a	0.25 ± 0.02	0.24 ± 0.02	0.25 ± 0.04	0.23 ± 0.01 a
	V55	N/A	0.26 ± 0.02 b	0.21 ± 0.03	0.20 ± 0.03	0.29 ± 0.02	0.24 ± 0.02 a
	p-value	0.046	0.005	ns	ns	ns	0.008
Raffinose	Control	0.25 ± 0.02	0.55 ± 0.06 bc	0.82 ± 0.08 c	0.67 ± 0.05 c	0.67 ± 0.04 c	0.26 ± 0.04 c
	Veraison	0.37 ± 0.03	0.60 ± 0.02 b	0.99 ± 0.09 bc	0.85 ± 0.05 ab	1.01 ± 0.09 a	0.42 ± 0.04 a
	V20	0.34 ± 0.02	0.72 ± 0.06 a	1.34 ± 0.04 a	0.89 ± 0.04 a	1.04 ± 0.05 a	0.38 ± 0.02 a
	V40	0.33 ± 0.02	0.78 ± 0.09 a	1.38 ± 0.02 a	0.68 ± 0.05 bc	0.82 ± 0.08 b	0.32 ± 0.01 b
	V55	N/A	0.47 ± 0.08 c	1.09 ± 0.07 b	0.47 ± 0.12 ab	0.87 ± 0.14 bc	0.30 ± 0.02 bc
	p-value	ns	<0.001	<0.001	<0.001	0.008	<0.001
Stachyose	Control	0.66 ± 0.08 c	0.31 ± 0.03 c	0.30 ± 0.08 b	0.37 ± 0.02 b	0.28 ± 0.04 c	0.25 ± 0.03 b
	Veraison	1.00 ± 0.11 a	0.43 ± 0.01 bc	0.36 ± 0.05 a	0.40 ± 0.06 a	0.37 ± 0.02 a	0.29 ± 0.02 a
	V20	0.95 ± 0.04 ab	0.55 ± 0.12 ab	0.39 ± 0.04 a	0.38 ± 0.11 a	0.36 ± 0.02 a	0.30 ± 0.03 a
	V40	0.83 ± 0.10 b	0.67 ± 0.15 a	0.36 ± 0.02 a	0.34 ± 0.04 b	0.34 ± 0.03 ab	0.30 ± 0.01 a
	V55	N/A	0.45 ± 0.08 bc	0.31 ± 0.10 b	0.27 ± 0.03 b	0.29 ± 0.06 bc	0.32 ± 0.02 a
	p-value	0.002	0.026	0.05	0.042	0.033	0.027

Veraison, V20, V40 and V55, correspond to ABA application of 600 mg/L at 50% véraison stage and 20 days after 50% véraison stage, respectively. 6V and 6V20 correspond to ABA application of 600 at 50% véraison stage and 20 days after 50% véraison stage, respectively. ns, not significant; N/A, not available. Letters indicate significant differences among means.

Compared to control, some ABA treatments increased the concentrations of fructose, glucose, sucrose, galactinol, raffinose, and stachyose during the dormant season in both cultivars. In 'Chambourcin' buds, ABA increased the concentrations of the above sugars, relative to the control, on average by 12% (galactinol) to 58% (fructose) (Table 1). In 'Cabernet franc' buds, ABA increased the concentrations of the above sugars on average by 6% (glucose) to 100% (galactinol) (Table 2).

Effect of ABA on the sugar concentrations of greenhouse-grown grape leaves and buds

Between 2wk and 6wk after ABA application, there were no trends of soluble sugar concentrations in either 'Chambourcin' or 'Cabernet franc' grape leaves (data not shown). In 'Chambourcin' buds, the concentrations of fructose, glucose, and sucrose increased on average by 47%, 35%, and 56%, respectively (Figures 2A, 3A, 4A). In 'Cabernet franc' buds, the concentrations of fructose, glucose, and sucrose increased on average by 42%, 37%, and 82%, respectively (Figures 2B, 3B, 4B). The ABA treatment significantly increased the concentrations of the above three soluble sugars. In 'Chambourcin' buds, ABA treatment increased the concentrations of fructose, glucose, and sucrose on average by 8%, 13%, and 17%, respectively (Figures 2A, 3A, 4A). In 'Cabernet franc' buds, ABA treatment increased the concentrations of fructose, glucose, and sucrose on average by 26%, 22%, and 27%, respectively (Figures 2B, 3B, 4B). There were variations of galactinol (data not shown) and raffinose (Figure 5) concentrations between 2wk and 6wk after ABA application. However, neither the trend of variation nor ABA effect was significant. The only significant difference was at 2wk in 'Chambourcin' with higher raffinose in ABA-treated than in control (Figure 5).

Association between freezing tolerance and soluble sugar concentrations of grape buds

The concentrations of fructose, glucose, and sucrose consistently correlated with freezing tolerance of grape buds in the greenhouse and field (Table 3). The concentrations of these sugars increased while the LT50 decreased (freezing tolerance increased). The correlation

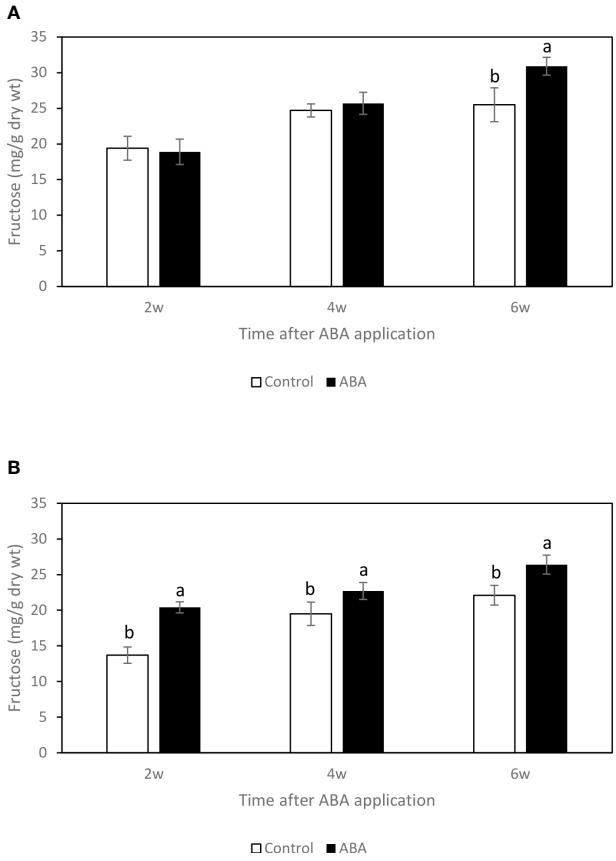

FIGURE 2
Effect of ABA on the bud concentrations of fructose in greenhouse-grown grapevines **(A)**'Chambourcin' and **(B)** 'Cabernet franc'. Bars with different letters are significantly different at $p \leq 0.05$. (n = 4). Standard errors are presented.

between galactinol and freezing tolerance varied between greenhouse and field study and between 'Chambourcin' and 'Cabernet franc'. In the field study, the correlation between raffinose and freezing tolerance was not significant for either cultivar. The raffinose concentration did not correlate with bud freezing tolerance because it increased in late fall and reached the peak in early winter. However, during the early acclimation stage (October – December for 'Chambourcin' grapevines and October – November for 'Cabernet franc' grapevines), the raffinose concentrations negatively correlated with freezing tolerance expressed as LT50s in 'Chambourcin' (R=-0.702, p<0.001, Figure 6A) and 'Cabernet franc' (R=-0.696, p<0.001, Figure 6B). There was also correlation between the other sugars and freezing tolerance during the early acclimation stage (Supplemental Table 1). In the greenhouse study, the correlation was significant for 'Chambouricn' only (Table 3).

Discussion

Effect of ABA on freezing tolerance of grape bud under greenhouse conditions

ABA treatment increased freezing tolerance of both 'Chambourcin' and 'Cabernet franc' grapevines under greenhouse conditions. ABA treatment started to increase freezing tolerance 2wk after application. In the greenhouse experiment, the ABA-treated grapevines had higher freezing tolerance than untreated ones. In 'Chambourcin' buds, the effect was seen 4wk after application with a rapid decrease of LT50 between 2wk and 4wk after ABA application. The greenhouse study confirmed other findings that ABA treatment increased freezing tolerance of greenhouse-grown 'Cabernet franc' (Wang et al., 2020), and field-grown 'Cabernet franc' (Zhang and Dami, 2012a), 'Pinot gris' (Li and Dami, 2016), and 'Chambourcin' grapevines (Zhang and Dami, 2012b). It is also suggested that it takes time (2wk to 4wk) for grapevines to show the effect of ABA on freezing tolerance.

Seasonal changes of soluble sugar concentrations and their correlations with freezing tolerance of grape buds

Soluble sugars have been suggested to play an important role in protecting cells from cold damage. In the greenhouse and field studies, three soluble sugars, fructose, glucose, and sucrose, consistently increased during the winter season and reached their maximum values when the grape buds were at their maximum

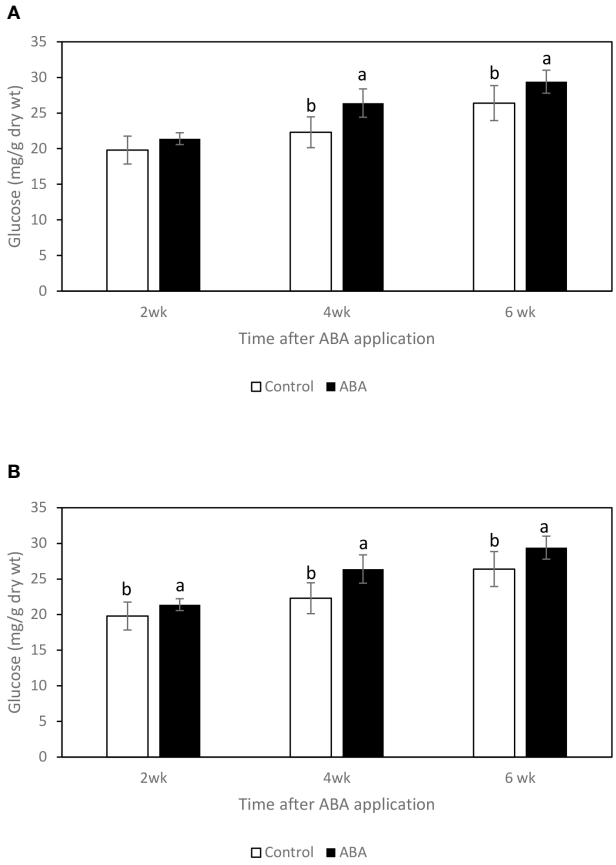

FIGURE 3
Effect of ABA on the bud concentrations of glucose in greenhouse-grown grapevines **(A)** 'Chambourcin' and **(B)** 'Cabernet franc'. Bars with different letters are significantly different at $p \leq 0.05$. (n = 4). Standard errors are presented.

freezing tolerance. This result is consistent with previous studies on the seasonal carbohydrate changes in grape buds (Hamman et al., 1996; Jones et al., 1999). Soluble sugar concentrations increase in the fall in response to low temperatures, reach a maximum during the coldest months in mid-winter, and decrease in the spring (Sakai and Larcher, 1987). The raffinose family oligosaccharides (RFO) appear to be the most important in mediating FT, as they change exclusively with cold acclimation. Even though raffinose is a very minor carbohydrate in grape tissues there is reason to believe that it is very important in freezing tolerance of *Vitis* species. In fact, raffinose has been shown to play a cryoprotective role by protecting cell membranes, stabilizing proteins, and retaining enzyme activities during a freeze-thaw event (Anchordoguy et al., 1987; Hincha et al., 1993; Stushnoff et al., 1997). However, the relationship between raffinose and LT50 was not consistent in grapes. For example, some reports demonstrated that the maximum level of freezing tolerance of grape buds was not always associated with the highest level of raffinose concentration in those tissues (Koussa et al., 1998; Jones et al., 1999). In Arabidopsis, it has been suggested that raffinose is not essential for freezing tolerance development. The raffinose-deficient mutant could still develop cold acclimation without raffinose accumulation within the tissues (Zuther et al., 2004). In this study, we found a similar discrepancy where raffinose concentration in buds peaked in late fall to early winter (October-December) and did not correspond to the maximum level of freezing tolerance which was reached in January. These results also corroborate a French report that showed that raffinose peaked in November on Chardonnay vines grown in the Burgundy region (Koussa et al., 1998). The early findings coupled with the studies described above may explain that while the correlation between LT50 and raffinose exists, it does not support its critical role to maximize freezing tolerance in mid-winter like in other plant species. For these reasons, a different mechanism to explain the role of raffinose in grapes is proposed as follows. First, raffinose accumulation has been demonstrated as an early response triggered by low but non-freezing temperatures that prepare buds for dormancy and cold acclimation (Grant et al., 2009). Second, in the field study, bud tissue dehydration began early in the fall (Zhang and Dami, 2012a; Zhang and Dami, 2012b) coinciding with raffinose accumulation which peaked before the occurrence of sub-freezing temperatures. In the greenhouse study, raffinose concentration also peaked before the occurrence of increased freezing tolerance. Therefore, it is suggested that raffinose might play a more important role in desiccation rather than freezing tolerance in grapevines. However, this needs further investigation.

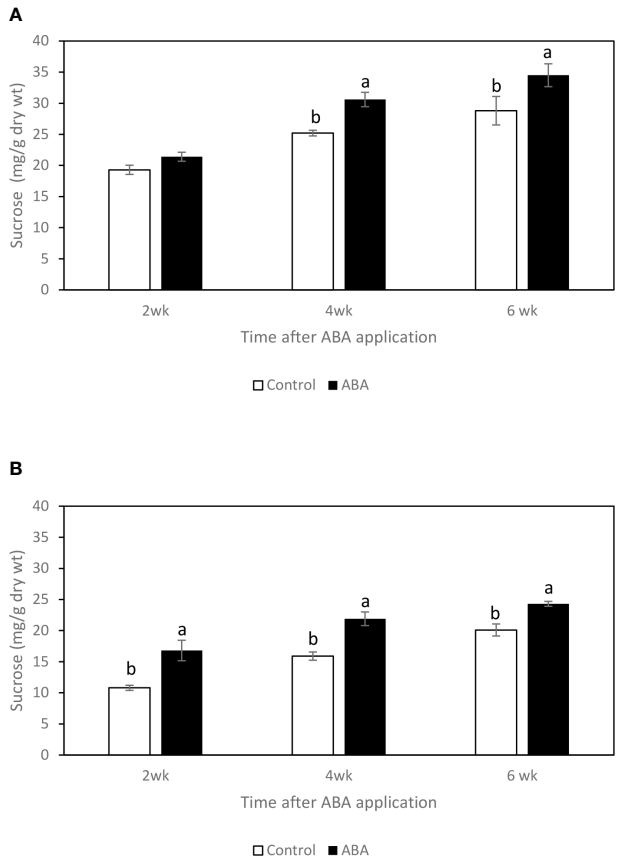

FIGURE 4
Effect of ABA on the bud concentrations of sucrose in greenhouse-grown grapevines **(A)** 'Chambourcin' and **(B)** 'Cabernet franc'. Bars with different letters are significantly different at $p \leq 0.05$. (n = 4). Standard errors are presented.

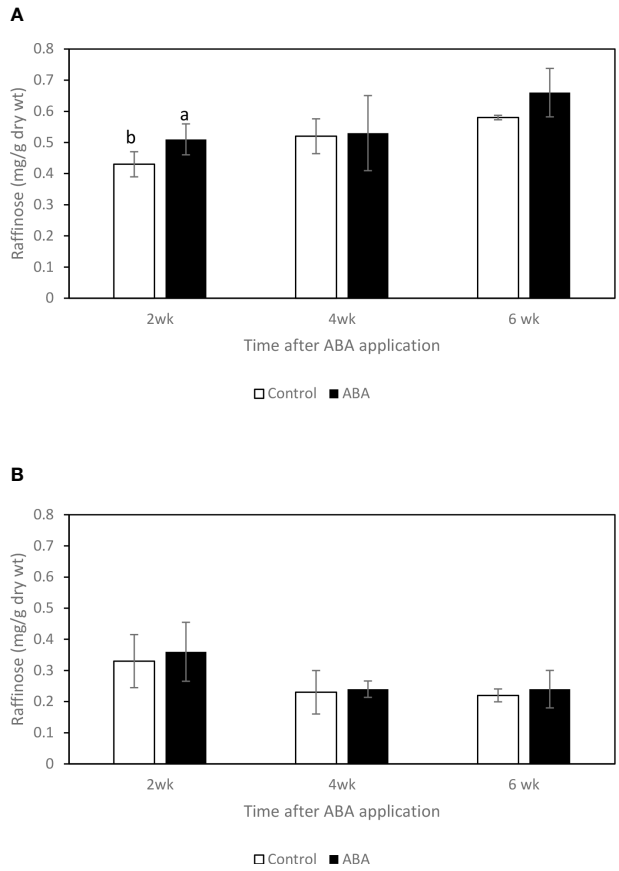

FIGURE 5
Effect of ABA on the bud concentrations of raffinose in greenhouse-grown grapevines **(A)** 'Chambourcin' and **(B)** 'Cabernet franc'. Bars with different letters are significantly different at $p \leq 0.05$. (n = 4). Standard errors are presented.

Raffinose has been demonstrated to stabilize membrane during desiccation by forming hydrogen bonds and substituting for water during desiccation (Crowe et al., 1989; Hoekstra et al., 2001). Furthermore, raffinose has been suggested as an osmoprotectant like proline (Gilmour et al., 2000; Taji et al., 2002). In the greenhouse study, at 2wk after ABA application, it was observed that the sucrose concentration significantly increased, the LT50s started to decrease, and the raffinose decreased. It is suggested that when grapevines start to increase freezing tolerance by accumulating soluble sugars, raffinose is one source for that. The field study showed that raffinose concentration was at the lowest level in mid-winter, which is likely metabolized into small sugars such as fructose and glucose which are important for freeze protection in mid-winter. Actually, this has been verified in previous studies which reported that glucose and fructose, but not raffinose are the predominant sugars during maximum hardiness in the 'Riesling' and 'Chardonnay' (*Vitis vinifera*) (Hamman et al., 1996; Koussa et al., 1998; Jones et al., 1999). These small sugars protect against freezing by depression of the nucleating temperature to promote supercooling (Sakai and Larcher, 1987; Crowe et al.,

TABLE 3 Correlations between soluble sugar concentrations and freezing tolerance (LT50) of grape buds.

Sugars	Field		Greenhouse	
	Chambourcin	Cabernet franc	Chambourcin	Cabernet franc
Fructose	-0.593***[z]	-0.861***	-0.822***	-0.756***
Glucose	-0.708***	-0.790***	-0.833***	-0.666***
Sucrose	-0.719***	-0.769***	-0.928***	-0.763***
Galactinol	-0.411*	0.314**	0.442*	-0.130 ns
Raffinose	0.117 ns	0.049 ns	0.559**	0.284 ns

[z] ns, *, **, and *** No significant, significant at p ≤ 0.05, 0.01, and 0.001, respectively.

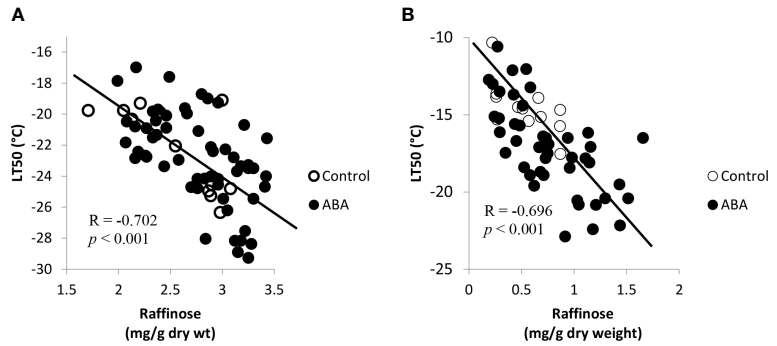

FIGURE 6
Correlations between freezing tolerance (LT50) and the bud raffinose concentrations in field grown grapevines during the acclimation stage, October – December for **(A)** 'Chambourcin' and October – November for **(B)** 'Cabernet franc'. (n = 4).

1989; Takata et al., 2007). This hypothesis, however, needs further investigation.

Effect of ABA on the soluble sugar accumulation

Both in the greenhouse- and field-grown grape buds, it has been observed that fructose, glucose, and sucrose increased in ABA-treated grape buds. Additionally, ABA promoted the accumulation of raffinose in buds from field and greenhouse-grown 'Cabernet franc' grapevines (2wk after application). The effect of ABA on the productions of these sugars has also been found in other plants. It has been reported that ABA treatment significantly promoted the accumulation of fructose, glucose, sucrose, and raffinose in the seedling of cucumber (*Cucumis sativus* L.) (Meng et al., 2008). In winter rapeseed (*Brassica napus* L.) shoots, exogenous ABA treatment significantly promoted the accumulation of soluble sugars with the increased freezing tolerance (Burbulis et al., 2010). In barley (Hordeum vulgare *L.*), exogenous ABA application increased freezing tolerance of plant tissues by promoting the production of sucrose (Bravo et al., 1998). Raffinose is closely related to the ABA-inducible desiccation tolerance, especially in seeds. For instance, it has been reported that exogenous ABA treatment can significantly increase the raffinose concentration in alfalfa (*Medicago sativa* L.) seeds by increasing the galactinol synthase activity (Blochl et al., 2005).

Conclusion

In summary, this study has demonstrated that fructose, glucose, and sucrose are the main soluble sugars that correlate with freezing tolerance of grape buds. Exogenous ABA application increased freezing tolerance of grape buds by promoting the accumulation of these soluble sugars. This study also suggested that ABA application can promote raffinose accumulation, but this sugar may play an important role in the early acclimation stage for increasing the desiccation tolerance. The preliminary result suggested that chronologically raffinose accumulates first in the buds before cold treatment. Then, a decrease of raffinose concentration coincided with the increase of smaller sugars, sucrose, fructose, and glucose. The accumulations of the latter sugars correspond to the maximum increase of freezing tolerance.

Cold damaging events are predicted to be exacerbated with the warming trend of climate (Schultze and Sabbatini, 2019). In this era of climate change and increasingly variable weather, there is a great need to advance the science of freezing tolerance in plants by developing more resilient grapevines to cold damage. This study demonstrates the importance of soluble sugars, including RFO in gaining freezing tolerance and that ABA can be used as a cultural practice to enhance freezing tolerance in grapevines and reduce economic losses associated with cold damage.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

ID designed experiments and developed treatments and data collection and co-wrote manuscript. YZ conducted experiments, collected data, analyzed results and co-wrote manuscript. All authors contributed to the article and approved the submitted version.

Funding

Authors are thankful for the financial support provided by the Department of Horticulture and Crop Science, OARDC/OSU and the Lonz Foundation.

Acknowledgments

The ABA sample was graciously provided by Valent Bioscience Co. (Libertyville IL). We also thank Trudi Grant for her technical assistance with sugar analysis.

Conflict of interest

Author YZ was employed by the company Grapery.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1084590/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Perspective
PUBLISHED 14 March 2023
DOI 10.3389/fpls.2023.1162506

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

RECEIVED 09 February 2023
ACCEPTED 27 February 2023
PUBLISHED 14 March 2023

CITATION
Fichtl L, Hofmann M, Kahlen K, Voss-Fels KP, Cast CS, Ollat N, Vivin P, Loose S, Nsibi M, Schmid J, Strack T, Schultz HR, Smith J and Friedel M (2023) Towards grapevine root architectural models to adapt viticulture to drought. *Front. Plant Sci.* 14:1162506. doi: 10.3389/fpls.2023.1162506

Towards grapevine root architectural models to adapt viticulture to drought

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To sustainably adapt viticultural production to drought, the planting of rootstock genotypes adapted to a changing climate is a promising means. Rootstocks contribute to the regulation of scion vigor and water consumption, modulate scion phenological development and determine resource availability by root system architecture development. There is, however, a lack of knowledge on spatio-temporal root system development of rootstock genotypes and its interactions with environment and management that prevents efficient knowledge transfer into practice. Hence, winegrowers take only limited advantage of the large variability of existing rootstock genotypes. Models of vineyard water balance combined with root architectural models, using both static and dynamic representations of the root system, seem promising tools to match rootstock genotypes to frequently occurring future drought stress scenarios and address scientific knowledge gaps. In this perspective, we discuss how current developments in vineyard water balance modeling may provide the background for a better understanding of the interplay of rootstock genotypes, environment and management. We argue that root architecture traits are key drivers of this interplay, but our knowledge on rootstock architectures in the field remains limited both qualitatively and quantitatively. We propose phenotyping methods to help close current knowledge gaps and discuss approaches to integrate phenotyping data into different models to advance our understanding of rootstock x environment x management interactions and predict rootstock genotype performance in a changing climate. This could also provide a valuable basis for optimizing breeding efforts to develop new grapevine rootstock cultivars with optimal trait configurations for future growing conditions.

KEYWORDS

vineyard, sustainability, root phenotypes, water, plant architecture, stress

1 Introduction

Grapevine (*Vitis vinifera* L.) is recognized as being well adapted to challenging environments (Ollat et al., 2019). With current climate change projections, however, abiotic stress in viticulture is likely to increase to levels that potentially jeopardize grape production, quality and wine typicity (Fraga et al., 2016; Schultz, 2016; Ollat et al., 2019). Temperatures and evaporative demand have risen and are expected to continue rising in many viticultural areas (Schultz, 2017). In addition, precipitation patterns are likely to be affected, with rainfall events becoming more erratic, resulting in increased frequency, severity and duration of drought periods and making water availability arguably one of the most crucial environmental factors limiting future growth and productivity of crops in general and viticulture in particular (Costa et al., 2016; Delrot et al., 2020; Gambetta et al., 2020; IPCC, 2021).

Winegrowers have high awareness of the effects of climate change. The vast majority of wineries in Europe (Loose and Pabst, 2019) and worldwide (Neethling et al., 2020) state that they have noticed climate change effects in the recent past, with a majority referring specifically to drought stress and water scarcity. Climate change effects are perceived as particularly detrimental in already hot and dry regions such as Spain and southern France (Neethling et al., 2020), and even in parts of the world with extensive irrigation infrastructure recent experience shows that water security is not guaranteed under extended drought (Van Dijk et al., 2013; Lund et al., 2018). In the case of Germany, growers perception of climate change effects on yield and quality has changed from positive (Battaglini et al., 2009) to overwhelmingly negative in the past decade (Loose and Kiefer, 2020), with drought risk in steep slope viticulture (Strub and Loose, 2021) and young vineyards perceived as particularly critical scenarios (Friedel et al., 2022).

There is a wide diversity of adaptation levers to improve the management of viticulture under future climatic conditions (Naulleau et al., 2021). Among them, the choice of existing or breeding of novel rootstocks suitable for site-specific environmental characteristics represents an elegant way ensuring adaptation to a range of abiotic and biotic stresses (Delrot et al., 2020), while maintaining traditional scion varieties that are familiar to the market (Ollat et al., 2016; Zhang et al., 2016). There is wide consensus that rootstocks will be central to adapting to the challenges of climate change, notably the rising risk of drought stress, with both choice of the right plant material and breeding of new rootstock genotypes presenting key strategies (Marguerit et al., 2012; Corso and Bonghi, 2014; Berdeja et al., 2015; Grossi et al., 2016; Ollat et al., 2016; Delrot et al., 2020; Gambetta et al., 2020). The great diversity of existing rootstocks, however, still remains widely underexploited in viticultural practice (Ollat et al., 2016), possibly due to a lack of decision support for growers regarding the choice of rootstocks (e.g. for Germany: Friedel et al., 2022). The choice of adequate rootstock varieties to reduce drought stress is a challenging task, as plant performance under drought is subject to strong genotype x environment (G x E) interactions (Tardieu, 2012). Grapevine drought tolerance is a particularly complex integrative trait with multiple underlying physiological mechanisms subject to rootstock x scion x environment x management (G_1 x G_2 x E x M) interactions. Such complexity and lack of mechanistic understanding of many drought responses can also prevent accurate prediction of drought stress risk under future climatic scenarios (Gambetta et al., 2020).

There is a general consensus in the plant research community that, among plant traits that play a role in drought stress physiology, root system architecture stands out as being of utmost relevance (Wasson et al., 2012; Comas et al., 2013; White et al., 2013; Lynch, 2018; Shoaib et al., 2022). The importance of root system architecture (i.e. the spatial distribution and shape of different root types within a volume of soil) and its temporal development lies in the fact that water is heterogeneously distributed in the soil in space and time. The spatio-temporal deployment of roots will therefore substantially determine the ability of plants to take up water (De Dorlodot et al., 2007; Rogers and Benfey, 2015; Tron et al., 2015). The challenge is that root architecture traits are complicated to assess in a meaningful spatial and temporal resolution, particularly in perennials grown under field conditions (Dumont et al., 2016).

To better match rootstocks to target growing areas, it is necessary to combine detailed knowledge of current and future drought stress scenarios with an understanding of root architecture traits that may contribute to drought tolerance (White et al., 2013). In that sense, modeling can assist with explaining observed data, testing hypotheses and integrating drought conditions and plant performance on the scale of individual plants up to crop stands and deepen our understanding of the complex high-dimensional space of G x E x M interactions (Soualiou et al., 2021). Root architecture models can assist in our understanding how roots access and extract soil resources. They enable researchers to plan and interpret the results of root sampling strategies and help to explore how single or sets of root architecture traits contribute to drought adaptation within various growing scenarios, without the necessity of executing numerous experiments that would be required to display the vast array of soil and drought conditions found in agricultural regions (Dupuy et al., 2010; Schnepf et al., 2018a; Schnepf et al., 2018b). This can also provide a basis to formulate breeding targets for the development of improved rootstock cultivars with desired trait configurations (Cooper et al., 2021).

In this perspective, we review state-of-the-art knowledge from different disciplines and propose an approach to bridge knowledge gaps in the role of grapevine root system architecture under drought, with an emphasis on dynamic root development of young vines. We investigate how a highly interdisciplinary collaboration with a strong focus on modeling might enhance our understanding of spatio-temporal interplays of both soil water availability and root architecture, and thereby identify modeling strategies that may advance our understanding of grapevine rootstock traits. Such an integrative approach would facilitate knowledge transfer into viticultural practice and broaden the possibilities of decision support for winegrowers. It could also help to improve rootstock genetic improvement programs that target specific future environmental scenarios.

10.3389/fpls.2023.1162506

2 Simulating realistic environments for rootstocks

Environmental effects and G x E interactions have been shown to be larger sources of variance for target traits than genotype effects alone, particularly under stress conditions (Chenu, 2015). Hence, detailed knowledge on the spectrum of drought stress scenarios representative for the majority of future vineyard situations is necessary to advise growers on their choice of rootstock and inform breeders about the requirements rootstocks will have to meet in the course of climate change. Such drought stress scenarios can be adequately characterized for different environments by modeling the vineyard water balance (Hofmann et al., 2022).

The capacity to model the vineyard water balance based on observed or simulated weather data (e.g. Lebon et al., 2003; Pieri et al., 2012; Gaudin et al., 2014; Hofmann et al., 2014) is also of high importance for the identification of traits with high agronomic relevance, and to define trait combinations in new rootstocks with improved performance in future vineyards. For example, Hofmann et al. (2022) modeled future drought stress risk on a vineyard plot scale for two winegrowing regions using an ensemble of climate change projections. In their model, topographical, geological, meteorological and vineyard management factors (e.g. slope and aspect, cover crop use, row spacing) were integrated to obtain precise predictions of the vineyard water balance. While such model approaches fulfill the requirements for the description of future drought stress scenarios for established vineyards, they do not specifically use root parameters such as rooting depth or root length density (RLD), and assume that roots can extract water from the complete soil reservoir defined by a default effective root zone. Further, soil water content is modeled only by the fraction of the available water capacity without considering the vertical distribution of soil water. Hence, their use for specific predictions such as young vine survival might be limited, as only a fraction of soil water may be available for young vines due to limited rooting depth and radius. Under the assumption that water extraction of young vines can be represented by limiting the effective root zone, the model was applied to predict the water balance of young vineyards, but such predictions remain to be validated by targeted experiments (Hofmann et al., 2022). As genetic variability for root parameters has been shown to exist (e.g. Tandonnet et al., 2018), extended models that are capable of capturing a range of root trait configurations would provide the opportunity to consider root genetic diversity in the modelling process.

Due to the high variability of topologic and geologic parameters typically associated with many traditional winegrowing regions, and a high variability of monetary and cultural value of vineyards often located in close spatial proximity, a plot-scale resolution as chosen by Hofmann et al. (2022) seems adequate for water balance simulations in viticulture. Water balance modeling has also shown the importance of E x M interactions for the water balance of target growing environments and demonstrated the particular importance of cover cropping (Celette et al., 2010; Gaudin et al., 2014; Hofmann et al., 2014). Figure 1 illustrates simulated annual

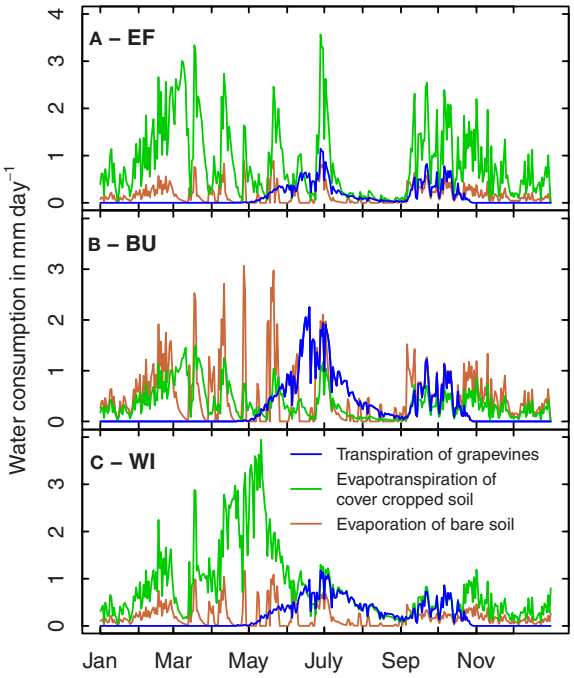

FIGURE 1
Example simulation of grapevine transpiration, evapotranspiration of cover cropped soil and evaporation of bare soil for three vineyards in the Rheingau region in 2022 **(A–C)**. **(A)** Ehrenfels vineyard, row spacing 2.50 m, 85 mm available water capacity (AWC), fully cover cropped with exception of the undervine area (width 0.4 m). **(B)** Burgweg vineyard, row spacing 1.60 m, 115 mm AWC, every other row cover cropped. **(C)** Wilgert vineyard, row spacing 1.60 m, 320 mm AWC, fully cover cropped with exception of the undervine area. Development of cover crops is divided into several growth stages.

courses of water consumption by grapevine transpiration, evapotranspiration of cover cropped soil and evaporation of bare soil of three different vineyards located in close spatial proximity. In the extreme example illustrated in Figure 1A (steep slope, southern inclination, shallow soil, wide, cover-cropped rows), a large fraction of the available water is transpired by the cover crop before the grapevines start to consume water. This could lead to early drought stress during shoot development and flowering in dry springs. As cover cropping practice differs substantially among dry farmed winegrowing regions (e.g. Celette et al., 2008; Abad et al., 2021), the inclusion of a cover crop component in modeling drought stress scenarios is fundamental to apply water balance models on a large scale and may justify further model refinements such as the type of cover crop used.

In perspective, relevant drought stress scenarios might be extracted from a cluster analysis (e.g. Mishra and Singh, 2011; Crespo-Herrera et al., 2021) performed on the results of a large scale application of water balance models combined with ensembles of climate change projections (Chenu, 2015). Input data needed to run water balance models on large scales with high (plot-scale) spatial resolution have become increasingly available in the past decades by use of digital elevation models and the increased availability of high-resolution soil maps for many European regions (Panagos et al.,

2022). Data on soil water availability may also become available in higher spatial (i.e. horizontal and vertical) resolution using novel techniques such as cosmic ray neutron sensing (Baroni et al., 2018).

3 Root architecture is a key determinant of grapevine performance under drought

Grapevines can cope with water deficits through a range of mechanisms that help to delay the onset of severe water stress, and through mechanisms that help the plant tolerate more negative water potentials without significant tissue damage (Chaves et al., 2010; Tsegay et al., 2014; Lovisolo et al., 2016; Simonneau et al., 2017; Gambetta et al., 2020). In this regard, various single traits have been discussed to play a pivotal role in grapevine drought adaptation, including an array of morphological, anatomical and physiological characteristics of both aerial and underground organs (Simonneau et al., 2017; Gambetta et al., 2020). Although difficult to rank according to their importance for rootstock drought tolerance with the current state of knowledge, a large body of evidence suggests that root architectural traits, the temporal pattern of their deployment and their plasticity in response to soil water availability seem crucial parameters to estimate rootstock performance in any drought prone area (e.g. Tsegay et al., 2014; Ollat et al., 2017; Tandonnet et al., 2018). Despite the extensive knowledge gained in these studies, our knowledge on rootstock architecture in the field remains limited due to the difficulty in accessing the root system of the vine, which restricts phenotyping throughput (Soar and Loveys, 2007; De Herralde et al., 2010; Tandonnet et al., 2010; Dumont et al., 2016; Archer and Saayman, 2018; Tandonnet et al., 2018; Ollat et al., 2019). Knowledge about early root development and root morphology of grapevines grown in the field and particularly their relationship with vine performance in established vineyards, root growth plasticity and root growth dynamics seems particularly limited (Ollat et al., 2017). This prevents a better understanding of root deployment in the field, an important step to address issues with practical relevance such as grower uncertainties regarding the optimal rootstock choice to support the survival of young vines.

A range of traits are commonly used in the literature to describe grapevine root architecture and growth (Table 1), among them static root traits (i.e. measurable at a single point of time) and dynamic root traits (i.e. related to spatio-temporal changes, De Dorlodot et al., 2007).

Many of these root traits play a context-dependent role in the drought tolerance of grapevines. For instance, in intermittent drought scenarios prevailing in most central European winegrowing regions, the ability to take up water in topsoil when summer precipitation becomes available (either by maintaining or reinitiating growth of fine roots and high total root length density) is likely to contribute to drought tolerance (Cuneo et al., 2021), but such traits would be of limited value in storage-driven hydrology typical of vineyards in Mediterranean climates, in which rooting depth seems to play a crucial role (Tron et al., 2015).

Soil water depth is another major parameter of the drought stress scenario and determines the strategy to ensure plant performance. In soils with available deep water, a strategy to outgrow the water deficit might be best suited for plant survival and productivity (e.g. high root length density at depth). In rather shallow soils or soils where no deep water is available, a reduction of metabolic investment into root development may be beneficial for vine survival, since a trade-off exists between the carbon costs of root systems and the benefit of increased water uptake under drought, limiting the necessity of investing into large root system under specific growing conditions (Tardieu et al., 2017). It is, however, unclear whether such parsimonious strategies will benefit vine survival and productivity in case there is cover crop competition (see Figure 1).

Management practices such as planting density, cover cropping strategy or canopy management, and soil parameters such as penetration resistance also exert a strong role in shaping root system architecture and its development (Richards, 1983; Reimers et al., 1994; Smart et al., 2006; Celette et al., 2008; Hunter et al., 2016). Describing the complex interplay of rootstock genotypes and their interactions with various environment and management factors would require an enormous amount of field phenotyping studies - an impossible task considering the difficulties in accessing the root network.

To get a better understanding of the complex interactions shaping root growth, a combination of phenotyping methods with increased throughput or increased spatio-temporal resolution and advanced modeling is suggested in the following paragraphs.

4 Phenotyping techniques to capture root system development

Root phenotyping studies in the field and, under significant limitations, in the greenhouse, are needed to capture root architecture traits under conditions as close as possible to practical viticulture. In the following section, we briefly discuss methods to capture root architectural traits and discuss their advantages and drawbacks.

Rhizoboxes and rhizotrons (hereinafter referred to as rhizoboxes) are specialized growth chamber systems. Usually, simple designs comprising a frame, at least one transparent pane and an opaque cover are used to monitor the temporal below-ground development of young grapevine plants, cuttings or seedlings grown in a soil-like medium in a non-destructive way and to characterize a range of static and dynamic root architecture traits in a limited space, yet basically in 2 dimensions (Dumont et al., 2016; Baldi et al., 2018; Krzyzaniak et al., 2021; Yee et al., 2021). The benefit of this simple and cost-effective approach is the investigation of root growth under controlled conditions with low space requirement and high throughput. However, there are limitations in the use of rhizoboxes in perennial plants like grapevines. In particular, the design of rhizoboxes restricts root growth (e.g. maximum rooting depth, 2D), thus determining the

TABLE 1 Overview of root architecture traits commonly used in grapevine research.

Trait	Unit	References
rooting angle	°	Smart et al., 2006; De Herralde et al., 2010; Fort et al., 2017; Cochetel et al., 2019; Schmitz et al., 2021
rooting depth	cm; m	Morlat and Jacquet, 2003; Smart et al., 2006; De Herralde et al., 2010; Tsegay et al., 2014; Hunter et al., 2016; Kocsis et al., 2016; Fort et al., 2017; Cochetel et al., 2019
total root length	mm; cm; m	Bassoi et al., 2003; De Herralde et al., 2010; Alsina et al., 2011; Dumont et al., 2016; Kocsis et al., 2016; Ollat et al., 2017; Peccoux et al., 2018; Yıldırım et al., 2018; Peiró et al., 2020; Schmitz et al., 2021; Burgess, 2022
root length density	mm/cm^3; cm/cm^3; m/m^3	Bassoi et al., 2003; Soar and Loveys, 2007; Tsegay et al., 2014; Peccoux et al., 2018; Burgess, 2022
root length area	cm/cm^2	Peccoux et al., 2018
root density	$no./m^2$	Morlat and Jacquet, 2003; Hunter et al., 2016; Ferlito et al., 2020
root diameter	mm; cm	Bauerle et al., 2008; De Herralde et al., 2010; Tsegay et al., 2014; Barrios-Masias et al., 2015; Dumont et al., 2016; Kocsis et al., 2016; Peccoux et al., 2018; Peiró et al., 2020
total number of roots	no.	Morlat and Jacquet, 2003; Bauerle et al., 2008; Comas et al., 2010; Hunter et al., 2016; Kocsis et al., 2016; Tandonnet et al., 2018; Cochetel et al., 2019; Schmitz et al., 2021
root biomass	g	Bassoi et al., 2003; Morlat and Jacquet, 2003; De Herralde et al., 2006; Soar and Loveys, 2007; De Herralde et al., 2010; Tandonnet et al., 2010; Gambetta et al., 2012; Dumont et al., 2016; Hunter et al., 2016; Fort et al., 2017; Tandonnet et al., 2018; Yıldırım et al., 2018; Ferlito et al., 2020; Bartlett et al., 2021
root volume	m^3	Soar and Loveys, 2007; Jones, 2012; Gambetta et al., 2020; Peiró et al., 2020; Schmitz et al., 2021
root surface area	cm^2; m^2	Bassoi et al., 2003; Gambetta et al., 2012; Dumont et al., 2016; Yıldırım et al., 2018; Peiró et al., 2020; Bartlett et al., 2021; Cuneo et al., 2021
specific root length	m/g	Pérez-Harguindeguy et al., 2013; Zhu et al., 2021
rooting index	no. of roots < 2 mm/no. of roots > 2 mm	Swanepoel and Southey, 1989; Ferlito et al., 2020
ramification/number of lateral roots/ branching frequency	no.; no./volume; no./branching point	De Herralde et al., 2010; Cochetel et al., 2019; Schmitz et al., 2021
root growth plasticity	mm/cm^2 per season	Bauerle et al., 2008
root growth/elongation rate	mm/d; cm/d; mm/h	Dumont et al., 2016; Mahmud et al., 2018; Cuneo et al., 2021
root production pattern	no./period; mm/ period; cm/ period	Comas et al., 2005; Bauerle et al., 2008; Comas et al., 2010; Fort et al., 2017

boundaries of the experiment spatially and temporally (Poorter et al., 2012).

To phenotype root systems of field grown grapevines with minimal soil disturbance, soil coring and minirhizotrons provide complementary methods for characterizing spatio-temporal differences in root growth traits (e.g. Soar and Loveys, 2007; Linsenmeier et al., 2010). These techniques are particularly suited for studies where comparisons of multiple genotypes or locations are of interest, or where there is a requirement to follow the development of root systems with repeated observations at a limited spatial resolution (Soar and Loveys, 2007; Bauerle et al., 2008). The information obtained from soil coring should be complemented with soil moisture monitoring in assessing the functional implications of measured root distribution and can assist in parametrizing water balance models where depth of water uptake and relative share of soil water with cover crops can be used as inputs (Hofmann et al., 2014; Zhu et al., 2021). For minirhizotrons, the ability to make more frequent observations down to a scale of individual roots provides further functional insight by allowing detailed assessments of root elongation rates, root lifespan, and seasonal fine root growth dynamics (e.g. Comas et al., 2010; Savi et al., 2018). Aspects of both methods are labor intensive, but technological developments in image collection and analysis, as well as opportunities to apply molecular techniques in the study of soil and roots collected from cores is greatly increasing the value of information that can be obtained (Haling et al., 2011; Lobet et al., 2013). For example, molecular techniques could allow to reliably discriminate cover crop from grapevine roots and hence obtain information about the spatial distribution of the roots of multiple species in a cover cropped vineyard. In addition, inverse estimation methods have been used to derive root architecture traits such as maximum length, elongation rate, insertion angles, and

numbers of zero-order roots from soil coring (Morandage et al., 2021), a strategy also applicable to minirhizotron data (Schnepf et al., 2018a).

Root architecture traits of field grown grapevines can be acquired at very high spatial resolution by excavating entire root systems followed by 3D-digitization (e.g. using a low magnetic field digitizer such as Fastrak, Polhemus, Colchester, VT, USA). 3D-digitization has successfully been applied to phenotype aboveground grapevine growth (Schmidt et al., 2019) and to digitize root architecture of different tree species (Danjon et al., 2005; Danjon et al., 2013; Danquechin Dorval et al., 2016), but to the best of our knowledge has not been applied to root systems of grapevines. Manual excavation is laborious, whereas removing the soil with high-pressure air is efficient without harming fine and coarse roots (Danjon et al., 2005). Manual uprooting can be done for smaller plants, but for larger plants, mechanical uprooting using a mechanical shovel is generally much more rapid. In this case, the number of roots lost during uprooting is large in the peripheral part of the root system. Once excavated, the root system can be measured *in situ* or brought to the laboratory, provided that the roots are rigid enough to maintain the overall 3D structure of the root system. *In situ* measurement is specifically suitable for young plants to reduce the loss of roots and to accurately measure root system geometry. After the excavation or uprooting the root system can be digitized. According to the downstream data distribution method, the digitized root geometry data are available in different formats: simple lists, structured lists (compare Schmidt et al., 2019) or multiscale tree graph (MTG) format file (Godin and Caraglio, 1998), where the root system is defined as a set of root axes subdivided into segments. Although both parts of this method, excavation and digitization, are very time-consuming and come at the expense of throughput, the high-resolution data output along with a functional annotation makes such data sets ideally suited for the integration into root growth models.

Of the methods described here, only rhizoboxes allow for a throughput in the scale needed to run genetic studies on root traits and/or screen larger breeding collections. Connecting field-based observations taken from older vines to seedling and/or cutting-based root measurements (e.g. on adventitious roots), for example *via* genetic correlation analyses, would provide highly valuable information for breeding regarding potential proxy-traits that genetic improvement programs could target at much higher throughput.

5 Modeling root growth

Root phenotyping data may be used to inform or parametrize models that might advance our understanding of the interaction of root architecture and drought stress response in specific environments, both for young and mature grapevines. This can be achieved by integrating traits of individual rootstocks into existing models, by the extension of existing models and by the parameterization of new root growth models. Root growth models describe growth of roots over time – often in relation to drivers such as water availability or nutrients. In this respect, such models are often part of classical crop models (e.g. APSIM, Keating et al., 2003). They typically consider root growth processes in relation to soil depth, focusing on one dimension only. Models for root architecture explicitly consider positioning of root segments in the soil, either in 2D or in 3D (e.g. Leitner et al., 2010; Postma et al., 2017; Barczi et al., 2018; Schnepf et al., 2018a; Schnepf et al., 2018b; Morandage et al., 2021). A root architectural model might belong to the class of functional-structural model (FSPM), if it integrates interactions with physiological processes. Functional-structural models may be static or dynamic over time. A dynamic FSPM includes both the growth and development (appearance) of new organs (Buck-Sorlin, 2013).

Among existing plant growth models that include root architectural traits, SurEau (Cochard et al., 2021) and APSIM grapevine (Zhu et al., 2021) are examples for a mechanistic plant model and a crop model, respectively, that allow the integration of a variety of rootstock traits. SurEau was developed to test the effect of drought stress on woody species hydraulics in the soil-plant-atmosphere continuum, and has been applied to study the effects of drought stress on hydraulic failure of several grapevine scion genotypes (Dayer et al., 2020; Dayer et al., 2022). Soil in SurEau is divided into several layers, each with its own root distribution. It however seems to have limitations in the application to vineyard situations, as it does not consider the row structure of the vineyard, or effects of cover cropping. APSIM is a modeling framework that has recently been parametrized for grapevine (Zhu et al., 2021). It can integrate several root traits (e.g. rooting depth; biomass accumulation; root length density; fine root distribution in specific vineyard zones like the inter-row space) in specific soil layers. One limitation of APSIM grapevine is that the variety of drought stress functions available in the parent framework have not been integrated into APSIM grapevine yet.

Further progress can be achieved by extending or modifying existing models. Modifying models such as SurEau to represent vineyard situations more accurately (e.g. by introducing row structure or a cover crop module), and possibly integrate a larger number of root traits will greatly expand our possibility to analyze drought damage to grapevines as a function of rootstock genotype. Similar output, but with a stronger focus on vineyard water balance on a large scale, might be obtained by expanding existing water balance models such as the one published by Hofmann et al. (2022) with root architecture traits. If an interface between physiological models (e.g. SurEau) and vineyard water balance models can be achieved, it may become possible to simulate vine hydraulic failure risk on a regional scale as a function of scion and rootstock, provided that scion/rootstock interactions are known.

The application of such models, however, would have the drawback that they do not yet integrate dynamic root traits nor root system architectural traits *sensu stricto*. Frameworks such as APSIM contain features that may allow for a dynamic simulation of root growth. Additional knowledge can be gained from the application of dynamic FSPMs, which are able to represent the development of plant architecture in time and space. Generic root FSPMs such as CRootBox (Schnepf et al., 2018a; Schnepf et al.,

2018b; Zhou et al., 2020; Morandage et al., 2021), OpenSimRoot (Postma et al., 2017) or DigR (Barczi et al., 2018) may be used to predict the architecture of mature vines from young vine phenotyping data and are thus ideally suited to transfer results from limited greenhouse studies (e.g. in rhizoboxes) to the field. Also, FSPMs have already successfully been applied to model intercropping systems (Bourke et al., 2021 and references therein) and will hence be ideally suited to simulate root development of grapevines and cover crops, as well as their mutual interaction. For the parametrization of FSPMs, data obtained by 3D-digitisation are optimally suited (Schmidt et al., 2019; Schmidt et al., 2022). The application of FSPMs would additionally require a rather detailed representation of the spatial heterogeneity of soil water content due to its influence on the direction of root growth (De Dorlodot et al., 2007). The environments for *in silico* studies with grapevine FSPMs can be generated by water balance models with high spatial resolution. Ideally, such models could integrate a variety of drought tolerance related traits of below- and aboveground parts, but such models will be extremely complex and computationally expensive. To simplify the complex interplay of soil water balance and root architecture, Tron et al. (2015) linked a 1D-water balance model to a 3D-dynamic root growth model (Leitner et al., 2010) by downscaling 3D root data to a 1D sink term.

The high requirements on phenotyping and soil data may explain that FSPMs have not yet been used to simulate grapevine root growth so far. However, first above-ground FSPMs for grapevine already exist (e.g. Zhu et al., 2018; Schmidt et al., 2019) and are successfully applied to predict growth and plant water status under varying environments, demonstrating the potential of FSPMs as a powerful tool for grapevine rootstock research in the future.

6 Rootstock architectural models to guide predictive breeding

Given the critical role of rootstocks for grapevine performance under abiotic and biotic stresses, rootstock breeding is gaining an increasing attention as a strategy to tackle the impacts of climate change. Extending rootstock breeding pipelines to incorporate physiological modelling could enable a more informed definition of future breeding targets with the goal to deliver performance improvement under forecasted climatic fluctuations. Developing high-throughput phenotyping tools that enable population screenings for key root traits used as model parameters will be critical in order to integrate both physiological and genetic modelling in future rootstock genetic improvement programs. Modern breeding tools such as genomic selection that uses dense genomic marker maps (Meuwissen et al., 2001), or phenomic selection that uses non-destructive high-throughput phenotyping data (e.g. from hyperspectral imaging, Rincent et al., 2018) to predict the genotypic value of individuals for traits of interest are particularly promising. With the broad range of modelling approaches available, genomic and phenomic selection could be directly coupled with spatio-temporal modelling of physiological processes in order to better capture impacts of G x E interaction on crop performance (e.g. Cooper et al., 2014; Technow et al., 2015). Such transdisciplinary approaches would enable a more targeted exploration of the highly complex multi-dimensional G x E x M space with the potential to identify workable breeding paths that deliver novel solutions for performance improvement under climate change (Cooper et al., 2021).

7 Conclusion

The choice of drought tolerant grapevine rootstocks presents a viable means of adapting viticulture to relevant drought scenarios prevailing in winegrowing regions. The current state of knowledge allows us to simulate future drought stress scenarios of vineyards with a high spatial resolution and to predict scion transpiration and mortality risk as a function of water uptake by the roots. The knowledge of rootstock traits, however, still impedes predicting the role of rootstock genotypes in grapevine drought tolerance under given growing conditions (i.e. drought scenario, soil properties, management decisions). This gap of knowledge has so far hindered the knowledge transfer into practical viticulture and rootstock breeding, potentially explaining why the majority of the existing variety of rootstocks are only scarcely used in practice.

Although our knowledge on the importance of individual or sets of traits relevant for the drought tolerance of a grapevine rootstock (or conferred by it) is far from comprehensive, a large body of evidence points towards the high importance of root architectural traits, such as rooting depth, root length density or specific root length. Data on the spatio-temporal development of root architecture are still extremely scarce, considering the large variability of root growth brought about by differences in soil structure, and hence the need for a relatively large database to provide robust information. To increase available knowledge on root structure and development, as well as to characterize root growth modification by grapevine x cover crop interactions, rhizoboxes, minirhizotrons, soil coring and excavation/digitization are methods that have the potential to increase throughput or spatial/temporal resolution. Data on the spatio-temporal pattern of grapevine root development can be used as model inputs to evaluate the effects of root architectural traits on resource acquisition during root development in a given drought stress scenario. FSPMs seem ideally suited for this task. An integration of additional drought related traits as well as aboveground plant growth and function into crop or plant models may in the future provide for a comprehensive understanding of drought related traits for rootstock and overall grapevine performance and survival under water deficit. While such knowledge would represent a milestone in grapevine drought stress physiology, it would still need to be integrated into a highly interdisciplinary network of experts involving agronomists, soil scientists, climatologists, modelers, plant physiologists and plant geneticists that provides decision support for the sustainable climate change adaptation of viticulture.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

LF and MF designed the overall concept of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the German Federal Environmental Foundation (Deutsche Bundesstiftung Umwelt, DBU). We acknowledge the support by the Open Access Publishing Fund of Hochschule Geisenheim University.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 27 March 2023
DOI 10.3389/fpls.2023.1145274

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

RECEIVED 15 January 2023
ACCEPTED 01 March 2023
PUBLISHED 27 March 2023

CITATION
Müller K, Keller M, Stoll M and Friedel M (2023) Wind speed, sun exposure and water status alter sunburn susceptibility of grape berries.
Front. Plant Sci. 14:1145274.
doi: 10.3389/fpls.2023.1145274

Wind speed, sun exposure and water status alter sunburn susceptibility of grape berries

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In the context of climate change, yield and quality losses from sunburn necrosis are challenging grape growers around the world. In a previous review, we identified the role of wind speed, duration of heat exposure, drought stress and adaptation as major knowledge gaps that prevent a better predictability of sunburn events. In this paper we present results of targeted experiments aiming to close these knowledge gaps. The effects of drought stress and adaptation on sunburn susceptibility were investigated in a combined drought stress/defoliation experiment. Riesling grapevines growing in an arid climate were fully irrigated or drought stressed, and clusters were exposed to sunlight by fruit-zone leaf removal (defoliation) at two developmental stages. Sunburn symptoms were induced using infrared heaters while fruit surface temperature was measured using thermal imaging enabling the establishment of threshold temperatures. The influence of the duration of heat exposure of berries was examined by heating grape clusters to a stable temperature and monitoring the evolution of sunburn symptoms over time. To examine the effects of wind speed on the appearance of sunburn necrosis symptoms, fruit surface temperatures and sunburn severity were measured along an artificially induced wind speed gradient in two cultivars using thermal imaging and visual inspection. Longer durations of heat exposure required lower fruit surface temperatures to induce damage, while the differences in temperature after 60 min and 90 min of exposure were marginal (47.82 ± 0.25 °C and 47.06 ± 0.26 °C). Clusters of vines grown under water deficit were less susceptible to sunburn compared to those of well-irrigated plants following defoliation. The lethal temperature of clusters exposed to sunlight for seven days did not differ from those exposed to sunlight for 28 days, indicating that a full adaptation ocurred within this period. Higher wind speeds led to lower cluster temperatures and reduced sunburn severity. First evidence of a drought priming induced heat tolerance of grapevine berries was found, while adaptation had a more pronounced effect on the susceptibility to sunburn compared to water stress.

KEYWORDS

***Vitis vinifera*, sunburn, drought-stress, heat stress, fruit surface temperature, wind speed**

Abbreviations: SN, Sunburn necrosis; ND, not defoliated cluster zone; ED, early defoliated cluster zone (pea-size, EL-31); LD, late defoliated cluster zone (lag-phase, EL-33); FI, fully irrigated; DS, drought stressed.

Introduction

In the context of climate change, grapevines have to cope with higher temperatures and lower soil water availability during the vegetation period (Schultz, 2019; Santos et al., 2020). Heat load during key phenophases is projected to rise not only due to rising temperatures, but also due to an acceleration of phenological development (Yang et al., 2022). In addition, more intense, more frequent and longer lasting heatwaves during key phenophases are expected to occur (Meehl and Tebaldi, 2004). This can result in substantial yield losses due to sunburn necrosis (SN) or in a loss of crop value due to sunburn browning (Gambetta et al., 2021). Water scarcity is arguably the most critical threat to agricultural productivity and in many important winegrowing regions as climate change intensifies. Drought might exacerbate adverse effects of heat stress by increasing canopy temperatures through reduced transpiration (Schrader et al., 2003). Higher yield losses were observed following a heatwave in vineyards that concurrently suffered from drought stress in Australia (Webb et al., 2010).

The berry temperature plays a key role in the development of SN symptoms. Berry temperature is mostly a function of the degree of exposure to direct sunlight, as the absorption of radiation may cause berry temperatures to rise well above ambient (Smart, 1985; Gambetta et al., 2021). The ability to adapt to heat stress and to acquire thermotolerance during phases of sublethal heat exposure has been shown for various fruit species, including grapevine (Morales-Quintana et al., 2020; Gambetta et al., 2021). Sublethal stress was shown to enhance the transcription of several heat shock factors and heat shock proteins that are important for acquired thermotolerance (Morales-Quintana et al., 2020). Berries growing under elevated temperatures accumulated heat shock factors that are associated with the formation of molecules that mediate heat stress in cells (Pillet et al., 2012).

Sun-exposed berries may adapt to high light and temperature exposure by forming a thicker epidermis which increases the amount of photoprotective pigments like chlorophylls, carotenoids or phenolic compounds that are related to stress mitigation. The formation of a more plate-like and thicker epicuticular wax layer under these conditions may additionally reflect radiation more efficiently (Gambetta et al., 2021). To increase the accumulation of quality-related compounds, improve spray coverage of clusters for disease control, and accelerate the drying of clusters after rain events, partial or total defoliation of the cluster zone has become a popular practice in viticulture (Smart, 1985; Reynolds and Vanden Heuvel, 2009).

When performing partial defoliation during a period of relatively mild temperatures, grape berries may experience a sublethal stress through elevated temperatures that do not lead to injuries (Morales-Quintana et al., 2020). The same stimulus, if performed during a period of excessive temperatures causes lethal damage of the cells and hence leads to SN or to sunburn browning (Lopes et al., 2019).

Due to an alteration in gas exchange and growth rate, water deficit increases canopy porosity and sun exposure, and hence the temperature of the fruit (Keller et al., 2016). Even though water stress may be a limiting factor for natural shading of the clusters by the canopy, the acquisition of thermotolerance might be enhanced *via* cross-priming reactions induced by drought stress (Morales-Quintana et al., 2020). Drought stress promotes the accumulation of some secondary metabolites that prevent oxidative damage, including carotenoids in white cultivars or anthocyanins in red cultivars (Gambetta et al., 2020). An increase in flavonols observed in berries of water stressed vines is more likely induced due to a higher light exposure (Torres et al., 2021).

Apart from absorbed radiation and ambient temperature, wind speed is a key determinant of berry temperature (Smart and Sinclair, 1976). By reducing the boundary layer resistance and thus increasing surface heat loss *via* forced convection, higher wind speed may have the potential to prevent heat damage. In a field trial, the wind speeds within a defoliated canopy were consistently higher compared to a non-defoliated canopy (Thomas et al., 1988), which could attenuate the effects of higher sunlight exposure to some degree.

The aim of this study was to address knowledge gaps around heat damage formation in grapes to make sunburn events more predictable and thus manageable. We hypothesized that berries are able to enhance their resilience to SN damage under water stress conditions and light exposure treatments by leaf removal and that altered wind speeds are able to reduce SN severity. To this end, we examined possible interactions of drought stress, the timing of leaf removal, and the developmental stage on the susceptibility of grape berries to sunburn by implementing a method to investigate differences in sunburn susceptibility as described in Müller et al. (2022) for *Vitis vinifera* L. cv. Riesling, one of the major white grape cultivars worldwide. The influence of the duration of heat exposure on the occurrence of SN symptoms and the influence of wind speed on berry temperature and SN development were tested in two additional experiments.

Materials and methods

Irrigation and defoliation experiment

The experiment was conducted in 2022 in a vineyard planted in 2010 at the Irrigated Agriculture Research and Extension Center (46.29°N; 119.73°W; 345 m a.s.l.) near Prosser, Washington, USA. In this arid climate zone in the Yakima Valley the mean annual precipitation is ~200 mm with average annual temperature of 12°C, average growing season (April-October) temperature of 16.5°C, and average seasonal growing degree days (GDD > 10°C) of 1400°Cd. The rows planted with *V. vinifera* cv. Riesling (clone FPS 09, own-rooted) were north-south orientated. Vines were trained to vertical shoot positioning (VSP) and spaced 1.82 m apart with row spacing of 2.74 m. The soil is a Warden silt loam with a pH of 8.0 and 26% (v/v) soil moisture at field capacity and 8% at the permanent wilting point.

The field experiment included two irrigation treatments (main plots) with four rows each and three defoliation treatments (sub-plots, early defoliation (ED, EL-27, 10 days after fruit set), late

defoliation (LD, EL-32, 25 days after fruit set) and a non-defoliated control (ND)), in a split-plot design with five replicate blocks. Only the fruit zone on the east side of the canopy was defoliated to avoid excessive heat damage on the clusters. Two to three leaves were removed manually from the base of each shoot so that the clusters were exposed to sunlight during the morning hours. (sub-plots). Each replicate block consisted of 6 vines, whereas the two outer vines served as a buffer to the neighboring blocks. A full irrigation treatment (FI, no water stress) and a drought stress treatment (DS) was established using drip irrigation. Vines were irrigated weekly to a target soil moisture of 16% and a vine water status, measured as midday leaf water potential, ranging from -0.8 to -1.0 MPa. DS vines were not irrigated from 13 June to 08 August (EL-27 to EL-35). Before budbreak and after harvest, soil water was replenished to field capacity to avoid water stress before bloom and during winter.

Monitoring for SN was done prior to harvest (23 September) with 25 clusters per block being evaluated on both sides of the canopy. SN was monitored on a free scale from 0-100 as a percentage of damage of the whole cluster.

Temperature (°C) and light intensity (lx) sensors (HOBO Pendant Data Logger, Onset Computer Corporation, Bourne, MA, USA) were placed next to representative clusters on the east and west side of the canopy. The sensors were set up in the DS treatment to evaluate differences in the defoliation treatments. Data was collected in 30 min intervals starting from day of year (DOY) 192, approximately 14 days after fruit set and 4 days after ED was applied. The dataloggers on the west side of the canopy and those in non-defoliated treatments were placed underneath a layer of leaves in the fruit zone, and their correct positioning was periodically monitored. Temperatures were calculated as mean temperatures per hour with two measurements per hour. During the heating experiments (described below), some of the sensors of the non-defoliated controls were exposed due to partial defoliation; their measurements were excluded from the data set from that timepoint forward. Temperature (°C) and solar radiation (W m^{-2}) values at 2 m height were measured at hourly intervals by a weather station located at the north-east corner of the vineyard.

Sunburn was induced by heat treatment using the method described by Müller et al. (2022). Briefly, grape clusters were heated to a target temperature using infrared lamps and temperature sensors inserted in the center of the clusters. The power of the lamps was regulated by a control unit, which continuously read the actual temperature given by the sensors and altered the power of the lamps using a control algorithm. Six lamps were controlled individually by the control unit consisting of a single-board computer connected to a power stage with phase control modulator and a touchscreen for manual operation.

Sunburn was induced by heating at two developmental stages: berries at pea-size (EL-31, 19 and 20 July) and lag phase (EL-33, 13 and 14 August) for each of the three defoliation treatments. The first sunburn induction occurred 7 days after ED, and the second sunburn induction occurred 7 days after LD. Immediately before the sunburn induction, the fruit zones of treated vines and non-treated control vines were defoliated to ensure that sunlight reached the clusters during the heat treatment. Six clusters per replicate were heated to a sensor target temperature of 48°C for 30 min. The distance of single berries to the center of the lamp, their position in the cluster and thus their distance to the sensor alters their recorded surface temperature. This yields a range of berry temperatures along the cluster which is necessary for the calculation of a lethal temperature. Midday leaf water potential (Ψ_L) was measured between 13:00 and 14:00 PST around the days of the experiments for each vine of the experiment. One mid-sized leaf per vine, approximately 5-6 nodes from the top of the shoot was selected and enclosed in a plastic bag before excision. The measurements were performed with a pressure chamber (model 615D, PMS Instrument Company, Albany, OR, USA).

The fruit surface temperature (FST) of single berries was measured using infrared thermography. A thermal image of the cluster was taken using an infrared camera (model: H2640, Nippon Avionics Co., Ltd., Yokohama, Japan). Thermograms were analyzed with the InfReC Analyzer NS9500 LT software (Version 5.0C, Nippon Avionics Co., Ltd., Yokohama, Japan). Mean FST of an individual berry was calculated as the mean temperature of all pixels of a manually selected circular area following the shape of the berries on the thermogram.

Heat duration experiment

The effect of the duration of heat applied to grape berries on their susceptibility to sunburn was examined at an early developmental stage (pea size, EL-31). Six clusters on two vines each were heated to a target temperature of either 46°C or 48°C without direct sunlight on the clusters. Thermal and digital images of each cluster were taken before the start of the heating and then after 15, 30, 60 and 90 min. To take thermal images at the same timepoints, heating of each cluster started with a delay of 60 sec from the start of the heating of the previous cluster. For each cluster, the mean FST of all visible berries was calculated so that each berry was repeatedly measured for each timepoint. Each berry was classified as either symptomatic or non-symptomatic for SN by visually examining the digital images from each timepoint. The surface temperatures were calculated as mean temperatures from the beginning of the experiment to each point of measurement by adding the previous measured temperatures and dividing the sum by the time that had passed. Berries that were annotated as symptomatic were excluded from the dataset for the subsequent timepoints.

Wind experiment

The experiment was conducted on 19 July 2022 in a vineyard planted in 2015 at Hochschule Geisenheim University (49.99°N; 7.95°E, 110 m a.s.l.). The region has a mean annual precipitation of ~540 mm with average annual temperature of 11°C, average growing season (April-October) temperature of 15.7°C (1991-2020). Rows were alternately planted with *V. vinifera* cv. Riesling (clone 365 grafted to SO4 rootstock) and Calardis Blanc ((Bacchus x Seyval Blanc) x Seyve Villard 39-639 grafted to SO4 rootstock) and oriented north-south. From a breeder´s perspective, Calardis blanc compared to Riesling can be considered as resilient to sunburn, even

in very dry and hot years (JKI, 2023). Vines were cane-pruned to a single cane and trained to a VSP system and spaced 1.2 m apart with a row spacing of 2 m. The soil is a silty loam with an available field capacity (2 m) of 300 mm or 15% (v/v).

At the time of the experiment, both cultivars were at phenological stage EL-32. Before the experiment, 13 and 12 shaded clusters per cultivar were selected on a 3 m stretch of canopy and marked. The selected clusters were all located at a comparable height above ground (1.0 – 1.2 m). After solar noon (14:00 CET), all clusters were exposed to direct sunlight by leaf removal on the western side of the canopy. Although all leaves in the cluster zone were removed, clusters located on the eastern side of the canopy were not sun-exposed throughout the experiment, and thus a portion of clusters remained in canopy shade. Immediately after leaf removal, cluster temperatures were measured by infrared thermography. After the thermograms were taken, a wind gradient was applied along the cluster zone using column fans (DO1100E, Duracraft, Southborough, MA, USA). Wind speed along the 3 m stretch of canopy was measured by a hand-held anemometer (Agrotop, Obertraubling, Germany). The distance of all marked clusters from the fans was recorded. Thermograms were taken at 0, 10, 30, 60, 120 and 180 min and wind was maintained until the cluster zone was shaded by the adjacent rows (about 18:00 CET). Visible SN symptoms were documented on a scale from 0-100% the day after (9:00 CET) for all marked clusters and for all clusters in the 3 m canopy stretch, divided into 30 cm sections.

Data analysis

R software (v. 4.0.3, R Core Team, 2020) was used for statistical analysis. Depending on the experiment, a binary logistic regression model was calculated with SN symptoms as the response variable and either 'irrigation treatment', 'defoliation treatment' and 'developmental stage', or 'surface temperature' and 'heating time' as predictors. A Wald chi-squared test ($p < 0.05$) was used to test the significance of the predictors. Using the *MASS* package, the lethal temperature for 50% of the berries (LT_{50}) was calculated for each heating time (Venables et al., 2002). A linear model was calculated to predict LT_{50} based on heating time. Sunburn severity was tested for normal distribution using an Anderson-Darling test. The test was statistically significant ($A = 1.268$, $p = < 0.001$) indicating no compliance with the normality assumption. A linear mixed model ANOVA was performed prior to a pairwise comparison using Z-tests, corrected with Holm's sequential Bonferroni procedure ($p < 0.001$).

Results

Heat duration experiment

Figure 1A shows the predicted probability of SN symptoms to appear after berries were exposed to high temperatures. The FST was measured at five timepoints throughout the heat exposure. A binary logistic regression model was used to calculate the contribution of the two predictors *Surface temperature* (Wald = 163.62, $p < 0.001$) and *Time* (Wald = 123.66, $p < 0.001$). For each 1°C increase in FST, the probability for berries to show SN symptoms was 1.007 times higher. Likewise, with an increase of 1 min of heat exposure, berries were 3.34 times more likely to show SN symptoms. Both effects are visualized by different slopes of the prediction curves and by the shift of each prediction curve on the abscissa. Symptomatic and non-symptomatic berries were determined for each timepoint and are visualized in Figure 1B as percentages of symptomatic berries.

With longer durations of heat exposure, lower FST were required for berries to show symptoms of SN. LT_{50}s for the timepoints 15, 30, 60 and 90 min were 53.79 ± 1.10°C, 49.94 ± 0.41°C, 47.82 ± 0.25°C and 47.06 ± 0.26°C, respectively. The calculated LT_{50} predicted the duration of heat exposure required to induce damage at that temperature ($R^2 = 0.94$, $F(1,2) = 46.48$, $p = 0.021$) as visualized by the regression fit (solid line) in Figure 1C.

Irrigation and defoliation experiment

In the irrigation and defoliation experiment conducted in Washington, the hourly mean temperatures, measured by dataloggers at cluster level, reached a maximum of about 50°C on the west side of the canopy (Figure 2). The highest temperatures were found on DOY 208 to 212, when the weather station registered about 40°C daily maximum temperatures. The ED, east-side cluster zone had a 1.5 ± 0.3°C cooler daily mean temperature compared to the ND cluster zones. The highest temperatures were found on the ND west-side of the canopy between 16:00 to 19:00 PST with 46.1°C mean temperature (Suppl. Data 1).

Midday leaf water potential was measured on two developmental stages prior to the heating (i.e., sunburn induction) experiments. In the FI block, the mean Ψ_L was -0.82 ± 0.07 MPa at EL-31 and -0.8 ± 0.09 MPa at EL-33. In the DS block, the mean Ψ_L was -1.28 ± 0.05 MPa at EL-31 and -1.34 ± 0.69 MPa at EL-33.

The LT_{50} in three defoliation treatments and in two irrigation plots was calculated for two developmental stages as shown in Figure 3. At EL-31, the LD treatments were not evaluated since they were not defoliated at that time, thus were congruent to the ND treatment. LT_{50s} were higher in ED and LD compared to ND for FI vines and likewise higher for DS at both developmental stages. While ED treatments at EL-31 had similar LT_{50} between FI and DS, the LT_{50} was 2°C higher at EL-33 with LD compared to ND following a similar trend. ED and LD were at a similar level at EL-33 for FI (-0.5°C) and DS (+0.1°C). The LT_{50} of the ND clusters in the DS plots was 1.2°C lower at EL-33 compared to ND clusters in the FI plots.

The highest SN severities as shown in Figure 4 were found in the defoliated cluster zone (east) of the ED and LD treatment in the FI plot, with no significant difference between both treatments ($p = 0.09$). On this canopy side, LD of the FI plot was significantly higher compared to LD of the DS plot ($p < 0.001$), while ED was not different between the irrigation plots ($p > 0.05$). Pairwise comparison across all DS defoliation treatments showed no significant differences. No significant differences in SN severity were found between the east side of ND treatments of both irrigation plots. Sunburn severity on the west side of the FI

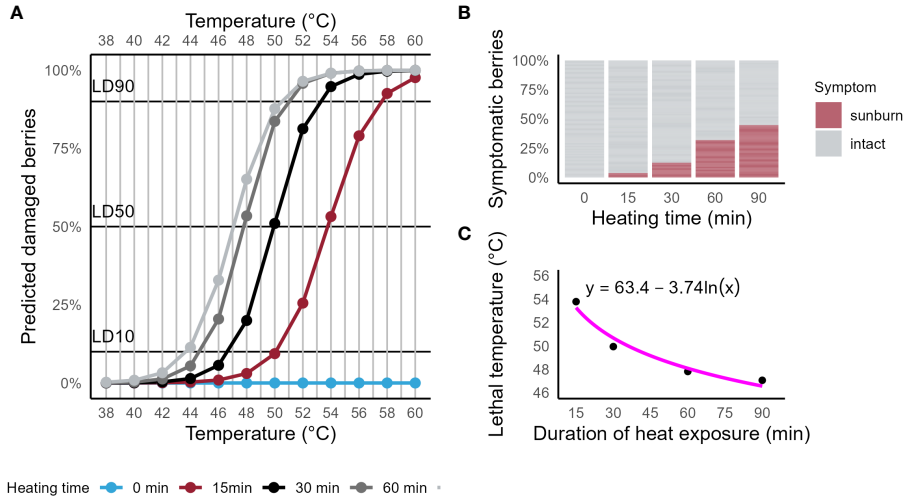

FIGURE 1
(A) Prediction plot for the occurrence of sunburn symptoms on Riesling berries exposed to high temperatures with periodic measurements of berry surface temperature. Binary logistic regression model showed significance for predictors *Surface temperature* (Wald = 123.66, $p < 0.001$) and *Time* (Wald = 163.62, $p < 0.001$). **(B)** Bar plot of the cumulative percentage of berries observed with symptoms of sunburn necrosis for four timepoints (0, 15, 30, 60 and 90 min). **(C)** Scatter plot of calculated lethal temperatures (LT_{50}, probability for symptomatic berries = 50%) for four durations of high heat exposure of grape berries (15, 30, 60 and 90 min). A regression fit is represented by the solid line ($R^2 = 0.94$, $F(1,2) = 46.48$, $p = 0.021$).

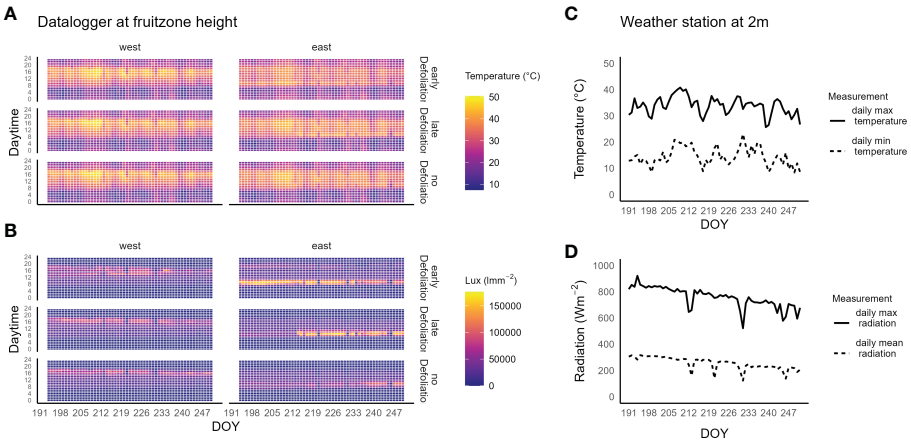

FIGURE 2
(A) Heatmap of the hourly mean temperature of Riesling grapes in a vineyard in southeastern Washington, USA. **(B)** Heatmap of the hourly mean light intensity. Measurements were taken on drought stressed vines at fruit-zone height on the east and west side of the canopy for three defoliation treatments (early defoliation, 10 days after fruit set, late defoliation, 25 days after fruit set and no defoliation). **(C)** Daily maximum (solid line) and daily minimum (dashed line) temperature measured at 2 m height at the weather station located next to the vineyard. **(D)** daily maximum (solid line) and mean (dashed line) global radiation.

treatment was relatively low for all defoliation treatments with no significant differences between them or to the ND treatments at the east side of each irrigation plot ($p > 0.05$)

Wind experiment

In the wind experiment, Calardis Blanc and Riesling showed a significantly different sunburn damage ($p < 0.001$, $n = 108$), with 0.8% and 8.9% severity, respectively, in the control (no wind) despite showing comparable berry temperatures. The wind speed gradients for both cultivars were comparable as shown in Figure 5, and measured wind speed ranged from 0.1 m s^{-1} to 3.25 m s^{-1} at 260 cm and 40 cm distance from the fan. Integrating the results across all clusters, it was shown that after 30 min, berry temperatures were stable along the wind gradient until the end of the experiment. Cluster temperature was significantly correlated to wind speed after stable temperatures were reached ($p < 0.001$, $R^2 = 0.39$, $n = 140$ bunch sections). The mean temperature of the clusters at 160-190 cm from the fan was about 3°C warmer than that of

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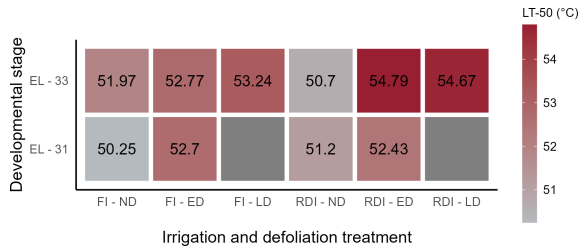

FIGURE 3
Heatmap of the predicted Lethal Temperature 50 (LT_{50}, Temperature for 30 min of heat exposure at which the probability of SN symptoms to occur is 50%). The LT_{50} was calculated for two developmental stages (pea-size (EL-31) and lag-phase (EL-33)), two irrigation treatments (FI, fully irrigated; DS, drought-stressed) and three defoliation treatments (ND, not defoliated, ED; early defoliated; LD, late defoliated).

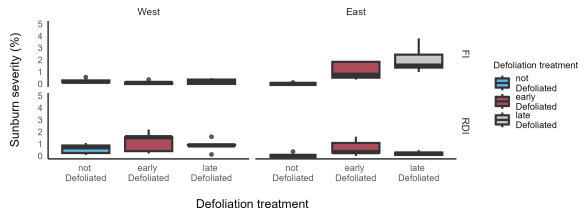

FIGURE 4
Boxplots of sunburn severity prior to harvest (23 September) in two irrigation treatments (FI, fully irrigated; DS, drought-stressed) and three defoliation treatments (ND, not defoliated; ED, early defoliated; LD, late defoliated) on both east and west side of the canopy in a vineyard in southeastern Washington.

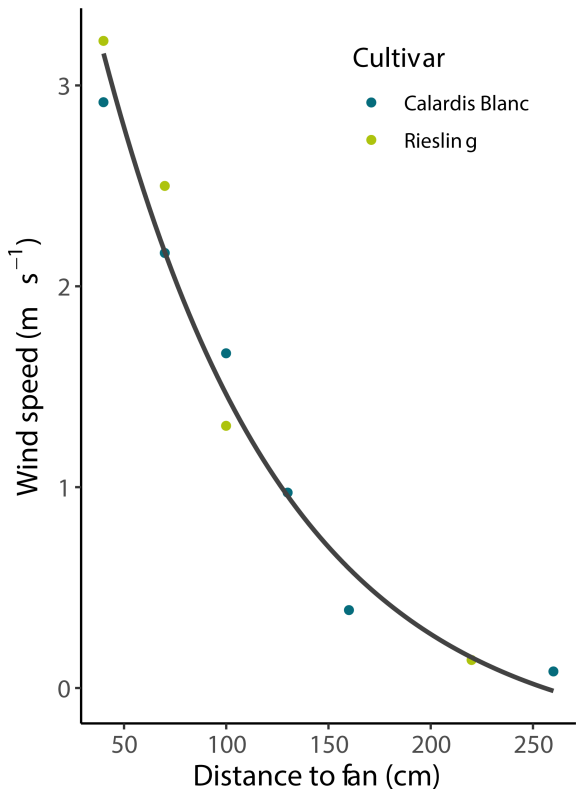

FIGURE 5
Wind speed as affected by the distance from the column fan. Line represents a second order polynomial fit that was used to predict wind speed at the location of individual clusters by measuring their distance to the column fan.

clusters at 20-40 cm distance (43.5 and 40.5°C, respectively). As visualized in Figure 6, the SN damage observed in the marked, previously shaded clusters correlated significantly with the wind speed predicted for the individual clusters in both cultivars (n = 13, $p < 0.001$ for Riesling and n = 12, $p < 0.01$ for Calardis Blanc, respectively). Sunburn damage in Riesling, however, occurred even at high wind speeds of 2.25-3.9 m s^{-1} in all previously shaded clusters, although the damage was lower than at low wind speeds. No cooling effect could be detected at a distance > 190 cm (wind speed ~0.3 m s^{-1}) in either cultivar and damage severity was relatively uniform.

Discussion

This study demonstrated that the duration of short-term exposure to high temperatures modulates the LT_{50} and provided evidence for priming and cross-priming effects that increase heat tolerance of grapevine berries after exposure to drought. It further demonstrated that wind decreases sunburn incidence and severity by reducing the temperature of sun-exposed clusters.

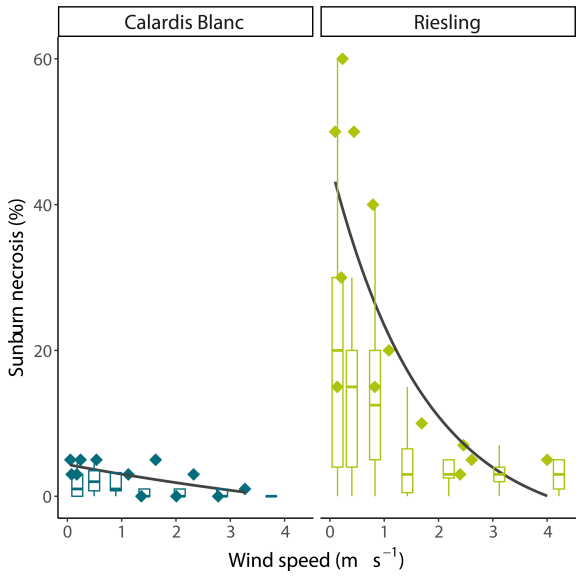

FIGURE 6
Effect of wind speed on sunburn necrosis severity of Calardis Blanc and Riesling grape clusters in a vineyard at Geisenheim. Box plots represent all clusters in the canopy, symbols represent marked, formerly shaded clusters. Solid lines represent logarithmic fits of necrosis severity of marked clusters vs. wind speed.

Heat duration experiment

The probability of a berry to develop symptoms of sunburn necrosis depends on both intensity and duration of the heat that it receives. Berry surface temperatures of 50 ± 0.2°C led to a 10% probability for damage after 15 min, 50% probability after 30 min and close to 90% probability after 90 min of heat exposure. Other authors found symptoms of sunburn necrosis on apple peel after 10 min of heat exposure to 52.2°C with sunlight excluded and symptoms of sunburn browning after 1 h with temperatures of about 48°C with sunlight during the heating time (Schrader et al., 2001). Whilst the present study added to our understanding of the effects of duration and intensity of heat on the development of SN symptoms for grape berries, we did not observe any sunburn browning. Schrader et al. (2001) found symptoms of SN when apples were sunlit while being heated to a certain threshold, thus direct sun exposure is likely to be an indispensable requirement for SN on grape berries too. The strong negative logarithmic correlation of FST and exposure time to heat intensity showed that a threshold temperature range might exist that leads to SN. When the exposure time exceeded 60 min, temperatures of about 46-47°C were sufficient to induce sunburn for berries previously grown in shade in an early stage of development (EL-31). Our observations during the wind experiment in Geisenheim support this idea. The time of exposure of clusters to direct sunlight *in vivo* depends on numerous biotic and abiotic factors (Smart, 1985; Gambetta et al., 2021) but as seen from Figure 1 can last up to three hours for a defoliated fruit zone.

Irrigation and defoliation experiment

Exposure of grape berries by leaf removal enhanced their resilience to heat damage. Leaf removal after bloom time is a common practice to prevent fungal diseases (Mundy et al., 2022), and our study demonstrated that it lowers the berries' susceptibility to sunburn after an acclimation period of about 7 days. The same effect was shown for both early (pea size) and late (lag phase) defoliation. These results help in the decision making of growers for timing of leaf removal, since a relatively short period of acclimation can be matched with weather forecast data to perform leaf removal in a time of relatively low temperatures or low solar radiation. In the context of climate change, water stress does not necessarily lead to a higher susceptibility of grape berries to SN (Santos et al., 2020). In apple fruit, Makeredza et al. (2013) found low soil water contents linked to higher fruit surface temperatures and consequently to higher sunburn incidences at harvest. In contrast to apples, grape clusters are commonly found in the lower part of the canopy of most training systems and are often protected by one or several layers of leaves (Smart, 1985). Due to a very low number of stomata, which become dysfunctional by veraison, the bulk of grape berry transpiration occurs *via* the cuticle. Berry transpiration rate is thus mainly driven by the atmospheric vapor pressure deficit (VPD), is dependent on berry size and changes throughout development (Zhang and Keller, 2015). By removing leaves from the cluster zone, the evaporative demand was increased throughout various training systems in a study by English et al. (1990). The leaves may therefore not only provide natural shading but could also help to cool the berries by transpiration and consequently lower the VPD in the cluster zone.

Berries on water-stressed grapevines were less or similarly susceptible to SN compared to fully irrigated vines, giving first evidence of a drought priming induced heat tolerance in grapevine. This phenomenon has been observed in several other species, mainly in field crops (Zhang and Huang, 2020). Drought induced priming was more evident in the final SN assessment, where exposed bunches (eastern side) from the FI treatment showed higher damage than those in the drought stressed treatment. The LT_{50} of drought stressed vines in the heating experiment was higher only 3 out of 5 comparisons than that of FI vines only in, yielding rather inconclusive results.

Although daily maximum temperatures exceeded 40°C on several days in late July, sunburn severity in the 2022 growing season was generally low in our field trial and rarely exceeded 2% total damage. Our findings show that clusters under FI were more or similarly susceptible to SN when defoliation was applied on the east side of the canopy. No defoliation was conducted on the west side of the canopy. Nevertheless, the damage on the west side of the DS treatment was comparable to that on the defoliated east side of the FI treatment.

Depending on the trellis system and the overall growth, a more porous canopy structure of vines under DS may lead to a higher percentage of sunlight-exposed berries (Keller et al., 2016). Additionally, drought stress reduces the leaf area growth of a canopy and can alter leaf angles (Briglia et al., 2020). Compared with FI vines, the vines in the DS treatment had lower vigor due to water stress (-1.2 MPa), which limited the development of foliage that might provide shade to the fruit and exposed berries on the west side of the canopy to high temperatures in the afternoon (Figure 2). Consequently, exposed berries might accumulate more light-dependent metabolites that protect the berries (Gambetta et al., 2020) but in turn are more likely to suffer sunburn injury than shaded berries, since higher surface temperatures are more likely to be reached in sun-exposed berries. This is especially true for the west side of a north-south orientated vineyard, where foliage manipulation should be carried out with particular care to minimize heat damage on clusters.

Wind experiment

Wind reduces sunburn incidence and severity. In order to highlight the importance of wind in the surrounding of the bunches, we have deliberately chosen two extremes between a sunburn-sensitive grape variety and a less susceptible variety. The results of this case study showed two opposing reactions which was observed in two distinct grape cultivars (Riesling and Calardis Blanc), although sunburn damage was negligible in the latter, confirming field observations at the breeding station (JKI, 2023). These results clearly show that extreme berry temperatures (in this case roughly > 48°C for Riesling) cause sunburn, especially when clusters are suddenly exposed to heat. Wind velocities applied in our

study closely resemble meteorological conditions found in the area, with the closest weather station (~300 m from the experimental site) showing mean wind velocities of 2 m s^{-1} for the afternoon hours of July 2022 (14:00 – 18:00 CET) at 2 m above ground. Consequently, wind is an important factor in predicting sunburn formation and should be carefully considered when modelling sunburn formation. From a practical point of view, under similar ambient temperatures wind-exposed vineyards will have a reduced risk of sunburn formation due to lower peak berry temperatures. This makes such vineyards better suited for the application of canopy management techniques such as leaf removal, which might also have implications for berry quality and disease control. Mean average cluster temperatures in this experiment did not exceed 43.5°C, only about 5.5°C above ambient temperature, perhaps because the presence of shaded clusters had a buffering effect on the mean cluster temperatures. Maximum berry temperatures reached more than 53°C, which were limited to a few compact clusters with surfaces perpendicular to the sun zenith. These observations highlight the variability of cluster temperatures in grapevine canopies even after leaf removal. They also explain why sunburn damage in temperate climates is often localized to the most exposed parts of the most exposed clusters in a canopy, while damage is more widespread in warm climates.

Conclusion

Our study provides new insights into the main abiotic factors that alter grape susceptibility to sunburn. Cooling effects through wind can be considered as a crucial parameter to lessen sunburn damage. Higher wind velocities were shown to reduce heat damage on susceptible Riesling grapes by reducing the fruit surface temperature through forced convection. The duration of berries being exposed to heat had a pronounced effect on the probability of sunburn development, with longer exposures decreasing the temperature required to induce damage. Berries grown under water stress conditions were less or equally susceptible to sunburn than berries on fully irrigated vines at two pre-veraison developmental stages. Defoliation and subsequent adaptation of berries through sunlight exposure within about 7 days had a more pronounced effect on the susceptibility to sunburn compared to water stress. These findings support growers in decision making concerning row orientation, water management, and timing of leaf removal.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

KM, MK, MS and MF conceived and planned this study with equal contributions, KM, MK and MF conducted the experiments and carried out the measurements. KM and MF drafted the original manuscript and finalized the manuscript. MK and MS reviewed and edited the manuscript. All authors approved the manuscript for publication.

Funding

Research in Washington was partly funded by the Washington State Grape and Wine Research Program and the Chateau Ste. Michelle Distinguished Professorship in Viticulture. Financial support was provided by FDW (Forschungsring des Deutschen Weinbaus) and by GFHG (Gesellschaft zur Förderung der Hochschule Geisenheim). We acknowledge support by the Open Access Publishing Fund of Geisenheim University.

Acknowledgments

We thank Lynn Mills, Charles Obiero, Steven Jachym, Melissa Manzo Parra, Sami Sanders and Lukas Fichtl for skilled technical assistance.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1145274/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 25 April 2023
DOI 10.3389/fpls.2023.1155888

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

RECEIVED 31 January 2023
ACCEPTED 03 April 2023
PUBLISHED 25 April 2023

CITATION
Buesa I, Yeves A, Guerra D, Sanz F, Chirivella C and Intrigliolo DS (2023) Testing field adaptation strategies for delaying grape ripening and improving wine composition in a cv. Macabeo Mediterranean vineyard.
Front. Plant Sci. 14:1155888.
doi: 10.3389/fpls.2023.1155888

Testing field adaptation strategies for delaying grape ripening and improving wine composition in a cv. Macabeo Mediterranean vineyard

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Under semiarid and warm climates, field practices for climate change adaptation have to be defined in order to modulate grape composition according to the desired wine styles. Under this context, the present study investigated several viticulture practices in cv. Macabeo for Cava production. The experiment was carried out over 3 years in a commercial vineyard located in the province of Valencia (eastern Spain). The techniques tested were (i) vine shading, (ii) double pruning (bud forcing), and (iii) the combined application of soil organic mulching and shading, all of them tested against a control. Double pruning significantly modified phenology and grape composition, improving the wine alcohol-to-acidity ratio and reducing the pH. Similar results were also achieved by shading. However, the shading strategy did not significantly affect yield, unlike double pruning, which reduced vine yield even in the year following its application. Shading alone or in combination with mulching significantly improved the vine water status, suggesting that these techniques can also be used to alleviate water stress. Particularly, we found that the effect of soil organic mulching and canopy shading on stem water potential was additive. Indeed, all the techniques tested were useful for improving wine composition for cava production, but double pruning is only recommended for premium Cava production.

KEYWORDS

climate change, double-pruning, phenology, shading nets, vine performance, water stress

1 Introduction

Climate change projections for the 21st century indicate that Mediterranean-like areas are especially vulnerable to the potential effects of temperature, shifts in rainfall patterns, and the frequency of extreme events (IPCC et al., 2014). Grapevine yield and quality depend on complex interactions between temperature, soil water availability, plant material, and field practices (van Leeuwen et al., 2019). For instance, a study conducted for 50 years across many European regions and cultivars showed that phenological timing has advanced, between 6 and 18 days depending on the cultivar (Jones et al., 2005; van Leeuwen et al., 2019). Also, a great spatial and temporal variability in temperature and phenology within a region has been reported (Neethling et al., 2019; de Rességuier et al., 2020). Overall, the combined effect of advanced phenology and increased temperatures and reference evapotranspiration (ET_o) has resulted in warmer and drier conditions during the grape-ripening phase (Previtali et al., 2022). On the one hand, under warmer conditions, grapes have an increased sugar concentration, which results in a higher alcohol content in wines, and decreased organic acid content, while aromas and aroma precursors are dramatically changed (Duchêne and Schneider, 2005; Neethling et al., 2012; van Leeuwen and Destrac-Irvine, 2017). Moreover, increased soil water deficit and higher ET_o are expected to reduce vine vigor and yield. In this context, adaptation strategies that minimize these effects on vine performance and on berry composition and, consequently, on wine quality must be achieved.

Possible adaptation strategies to climate change could include earlier harvesting, although this is not feasible as grapes would not have the correct phenolic maturity (Sadras and Moran, 2012), the relocation of vineyards to cooler locations, in altitude or latitude (Jones et al., 2005; Ollat et al., 2016), and changing the current genetic material used (grapevine varieties, clones, and rootstocks) (Schultz, 2000; Medrano et al., 2015; van Leeuwen and Destrac-Irvine, 2017). In addition, other strategies include changes in field management techniques such as grapevine architecture, light interception modulation, the adjustment of the source-to-sink balance, canopy management, soil management, irrigation, and shifting vine phenology (Intrigliolo and Castel, 2011; Ollat et al., 2016; Martínez-Moreno et al., 2019; Buesa et al., 2020a; Buesa et al., 2020b; Buesa et al., 2021c; Naulleau et al., 2021; Previtali et al., 2022). Regardless of the strategy chosen, the success of the adaptation techniques to climate change strongly depends on the interaction of ecological and socioeconomic factors assessed locally (Lereboullet et al., 2013).

The present work aimed at evaluating shading, double pruning, and shading + mulching as adaptation strategies in a 'Macabeo' vineyard. This cultivar can be used to make still and sparkling wines. The latter, if prepared with traditional methods, can be called Cava. The Macabeo cultivar (syn. 'Viura') ripens early as compared to other white cultivars authorized by the Cava designation of origin. It is usually harvested early in order to maintain a good balance between alcohol and acidity as low levels of acidity would lead to a lack of brightness and aromas (Sweetman et al., 2009). Moreover, total acidity (TA) is directly related to the microbiological stability of the wine and, therefore, to the aging capacity of sparkling wines, carbonic maceration, or malolactic fermentation (Poni et al., 2018). However, under the projected climate change scenario, field strategies must be adopted for this cultivar to be used for premium sparkling wine production (Jones et al., 2014). Knowledge about the relationship between microclimatic conditions (mainly temperature and light environment around the clusters) and grape composition is scarce.

The first adaptation strategy evaluated was the modulation of light intercepted through the use of shading nets due to their effects on canopy microclimate and thus in determining grape composition (Greer et al., 2010; Caravia et al., 2016). The reduction in the amount of solar radiation intercepted by the vineyard can reduce grapevine photosynthetic capacity and canopy transpiration, slowing down the ripening process and alleviating vine water stress (Zufferey et al., 2000; Palliotti et al., 2014; Basile et al., 2015; Martínez-Lüscher et al., 2020). Thus, both water and heat stress can be alleviated by regulating the sunlight intercepted by vineyards (Intrieri et al., 1998; Williams and Ayars, 2005). Moreover, the reductions in light interception during grape ripening greatly affect leaf and cluster microclimate conditions, affecting vine physiology and grape ripening (Medrano et al., 2012; Manja and Aoun, 2019). This could mitigate the excessive exposure to sunlight of the cluster and overheating, reducing subsequent grape acidity catabolism and aromatic composition alterations, as well as berry sunburn and shriveling (Keller, 2010; Caravia et al., 2016; Pons et al., 2017; Gambetta et al., 2021). The exposure of the cluster to high solar radiation could increase the polyphenol content in the berry, which is considered a drawback for white wine production as it affects the final color of the wine (van Leeuwen and Destrac-Irvine, 2017).

The second adaptation strategy evaluated was a double pruning technique (i.e. bud forcing) to delay vine phenology for several months and thus grape ripening timing as well (Gu et al., 2012; Martínez-Moreno et al., 2019; Gutiérrez-Gamboa et al., 2021). It consists of green cane pruning and removing all the non-perennial parts (lateral branches, leaves, and clusters), to force the growth of the primary buds formed for the subsequent season. This technique can greatly reduce or not affect yield at all, as compared to unforced vines, depending on the timing and number of parts retained. However, interestingly, grapes from double-pruned vines show higher acidities, a lower pH, and much higher phenolic content as compared to the berries from the unforced vines (Cabral et al., 2023). Overall, a further postponement of the grape-ripening process seems to be a quite promising tool for addressing the detrimental effects of high temperatures and ET_o on fruit and wine quality in warm and semiarid regions. Nevertheless, large aroma differences in wine typicity are expected under such changes in the weather conditions during ripening (Jones et al., 2014; Vilanova et al., 2019; Buesa et al., 2021a).

Furthermore, the combination of adaptation strategies may have additive effects, which might provide better solutions for adapting to climate change (Naulleau et al., 2021). In this sense, the combination of shading nets and organic mulch application to the soil may improve the vine water status due to the reduction in evaporation from soil mulching (Buesa et al., 2021b). Even under drip irrigation, soil evaporation can be, in fact, an important

component of the vineyard water balance, accounting for up to 30% of the entire vineyard evapotranspiration when canopies were managed with vertical shoot positioning (López-Urrea et al., 2020).

The study hypothesis is that the adaptation strategies proposed can improve grape and wine composition while preserving yield, through both the amelioration of vineyard microclimate, and the improvement of the vine water status.

2 Materials and methods

2.1 Vineyard site

The trial was carried out from 2017 to 2019 in a commercial 'Macabeo' vineyard (*Vitis vinifera* L.) located in Requena, Valencia, Spain (39°30′02′′N, 1°13′48′′W; elevation 740 m a.s.l.). The vineyard was planted in 2002 with 161-49C rootstock at a spacing of 2.5 × 1.5 m (2,666 vines ha^{-1}). This rootstock (*V. riparia* × *V. berlandieri*) is well adapted to calcareous clay soils; it confers medium vigor to the scion, provides a steady yield, and promotes early ripening (Chomé Fuster et al., 2003). Vines were winter-pruned to a 16-bud count per vine on a bilateral cordon de Royat and trained to a vertical trellis system oriented in the south–west north–east direction.

The farm's soil was a Typic Calciorthid according to the Soil Taxonomy (Soil Survey Staff, 1999), with a clay loam-to-light clay texture according to the U.S. Department of Agriculture (USDA), highly calcareous (37%) and with low fertility (<1% in organic matter). Soil depth was greater than 2 m with 200 mm m^{-1} of available water capacity. The climate in this area is classified as semiarid hot-summer Mediterranean (Rodríguez-Ballesteros, 2016); the heliothermal index of Huglin (Huglin, 1978) was 2,291°C, corresponding to a temperate-warm viticultural climate, with cool nights and moderately dry according to the classification system for grape-growing regions proposed by Tonietto and Carbonneau (2004). At the experimental site, the annual average values (for the 2002–2016 period) of the reference evapotranspiration (ET_o) and rainfall were 1,114 and 402 mm, respectively. Sustained deficit irrigation was applied from early June to the end of September by the vineyard manager according to standard practices carried out in the area of study by the irrigation association, which normally delivers 60–100 mm per season of irrigation water (Ramírez-Cuesta et al., 2023). Drip irrigation was applied using a single pipe per vine row with two 4 L h^{-1} compensated emitters per plant.

2.2 Experimental design

The experiment consisted of three treatments: (i) control, winter-pruned; (ii) shading, the application of shading nets 1 m over the canopy to reduce photosynthetic active radiation on the vines by 50% once the phenological stage of pea size was reached (Supplemental Figure 1A); and (iii) double pruning and winter pruning plus severe green pruning 20 days after bloom (Supplemental Figure 1B). In 2019, in the double pruning treatments, the vines were pruned as the control in order to assess the carry-over effect of the application of the technique in the previous seasons on vine performance.

The experimental design was a complete block layout with four replicates. Each block comprised 10 rows of 7 vines. The shading treatment was located in the middle of each replicate due to the installation of the metal structure. Each experimental unit (EU), a combination of block × treatment, consisted of 10 experimental vines plus the surrounding perimeter vines acting as borders.

Moreover, in 2018 and 2019, the combination of field adaptation strategies was assessed only on the effects on the water status of the grapevine. This was done by including a subtreatment consisting of an organic mulch covering the soil in the drip zone under shaded conditions (shading + mulching). This subexperiment was carried out on four replicates of four vines within the borders of the shading treatment. Mulching consisted of the application of mechanically chopped vine prunings in the vine rows. This organic mulch was 1 m wide on the soil and 3–5 cm thick.

2.3 Field measurements and laboratory determinations

Weather data were recorded at an automated meteorological station located within the farm perimeter. Reference evapotranspiration (ET_o) was calculated with the Penman–Monteith equation (Allen et al., 1998). Time periods were measured as days of the year (DOYs). Midday Ψ_{stem} was determined throughout the season with a pressure chamber (Model 600, PMS Instrument Company, USA) on bag-covered leaves from two representative vines per EU at noon (measurements were carried out between 11:30 and 12:30 solar time). The leaves used for these measurements were located on the west side of the row and enclosed in hermetic plastic bags covered with aluminum foil for at least 1 h prior to the measurements (Choné et al., 2001).

In addition, the additive effect of water deficit duration and intensity was accounted for by the water stress integral (S_Ψ) computed as the sum of Ψ_{stem} measured every day during a given period (Myers, 1988). It was calculated from the Ψ_{stem} values over the veraison to harvest periods, subtracting those with the least negative value registered in a fully irrigated vineyard by Buesa et al. (2017) (−0.24 MPa) and considering the number of days in between measurements.

Harvest was carried out in each treatment aiming a level of 18°–19° Brix in the must, which is the standard for the traditional method of making sparkling wines (Jones et al., 2014). Therefore, harvest was performed at different dates within the year depending on the treatment. Grape yield, the number of clusters per vine, average cluster mass, and shoot fruitfulness (the number of clusters per shoot) were determined at harvest on each experimental vine. Shoots per vine were counted at the end of the season, and pruning fresh mass was weighted on each experimental vine. In addition, the yield-to-pruning ratio, known as the Ravaz index, was calculated to estimate the vine balance.

2.4 Grape and wine composition

Berry ripening evolution was assessed approximately every 10 days, starting from shortly before veraison to harvest. Berry fresh mass was determined on two random samples of 100 berries per EU. Berry samples were crushed and hand-pressed through a metal screen filter and used to evaluate technologically defined maturity. Must total soluble solids (TSSs) were determined by refractometry (PR-101, Series Palette, Atago Co, LTD, Japan); pH and titratable acidity (TA) were measured with an automatic titrator (Metrohm, Herisau, Switzerland). Must was titrated with a 0.1 N solution of NaOH to an end point of pH 7, and results were expressed in tartaric acid equivalents. Malic acid and tartaric acid were determined by colorimetric methods using an automated sequential analyzer (Easychem Plus, Systea, Anagni, Italy). All analytical determinations in musts were performed in duplicate. In addition, the TSS-to-TA and tartaric-to-malic acid ratios were calculated.

Wines were separately made from the grapes of each EU at the experimental winery. Thus, in the 2017 season, 12 vinifications were performed. However, in 2018 and 2019, the double pruning treatment was not vinified as in 2018. The grapes from this treatment did not reach commercial TSS content, while in 2019, only the carry-over effect of this technique on vine performance was evaluated. Grapes were mechanically crushed, destemmed, pressed, and fermented at a temperature of approximately 22 °C in 20 L stainless-steel containers. In all the musts, $K_2S_2O_5$ was added at a ratio of 10 g/100 L of must. Afterward, these were then inoculated with 20 g of commercial *Saccharomyces cerevisiae* yeast that was previously hydrated at 37°C for 30 min (FR Excellence, Lamothe-Abiet). All the wines were stored at approximately 20°C for 3–4 months before analytical determinations. Fourier-transform infrared spectroscopy (BACHUS II, TDI, Barcelona, Spain) was used for determining alcohol content, TA, pH, citric and lactic acids, and glycerol content. All analytical determinations in wine were done in duplicate.

2.5 Statistical analysis

A two-way analysis of variance (ANOVA) was used to test the effect of the treatment and year and the treatment per year interaction (T*year) on vine performance variables. As significant interactions between T*year were observed in most variables, one-way ANOVAs were used to assess the effect of the treatment within each year. Similarly, the effect of the treatment on grape and wine composition variables were assessed by a one-way ANOVA. In the case that the ANOVA detected significant effects ($P < 0.05$), a mean separation was performed with the Duncan multiple range test. The ANOVAs and *post hoc* tests were carried out using the Statgraphics Centurion XVI package (version 16.0.07) (Statgraphics Technologies, The Plains, VA, USA). Additionally, regressions were calculated using SigmaPlot (version 11.0) (Systat Software, San Jose, CA, USA).

3 Results

3.1 Climate and water relations

The results presented correspond to two dry years, 2017 and 2019, and a very wet one, 2018 (Figure 1). The values of rainfall were 322, 515, and 354 mm for 2017, 2018, and 2019, respectively, while the ET_o was 1,251, 1,144, and 1,218 mm, respectively. Maximum, minimum, and average temperatures showed a fairly similar pattern during the growing season (from April to October). On the other hand, from April to October, rainfall was 126, 314, and 250 mm in 2017, 2018, and 2019, respectively.

The seasonal evolution of Ψ_{stem} showed that the vine water status was clearly affected by the different adaptation strategies (Figure 2). Both shading and double pruning treatments significantly reduced vine water stress as compared to that of the control in most of the measurement dates. The fast effect of the installation of the shading net on the Ψ_{stem} as compared to the control was remarkable (see arrows in Figure 2). The average Ψ_{stem} reduction in the shading treatments as compared to that of the control was 0.15, 0.23, and 0.17 MPa for 2017, 2018, and 2019, respectively. The reductions in Ψ_{stem} of the double pruning treatment as compared to the control were even greater (Figure 2).

Moreover, the addition of mulching in the shading treatment significantly decreased the values of the S_Ψ during veraison to harvest in the 2018 and 2019 seasons as compared to the shading application alone (Figure 3). The reduction in S_Ψ of the shading + mulching treatment as compared to the control was 22%, while the one between the shading treatment and the control was only 16%.

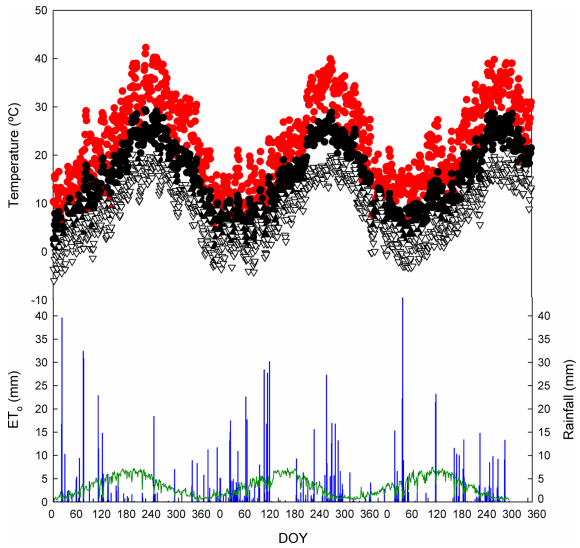

FIGURE 1
Seasonal patterns of the daily maximum air temperature (•), mean temperature (•), and minimum temperature (▽) in Requena, Valencia, Spain. Rainfall is represented with blue bars and reference evapotranspiration (ET_o) with a green line. DOY, day of the year.

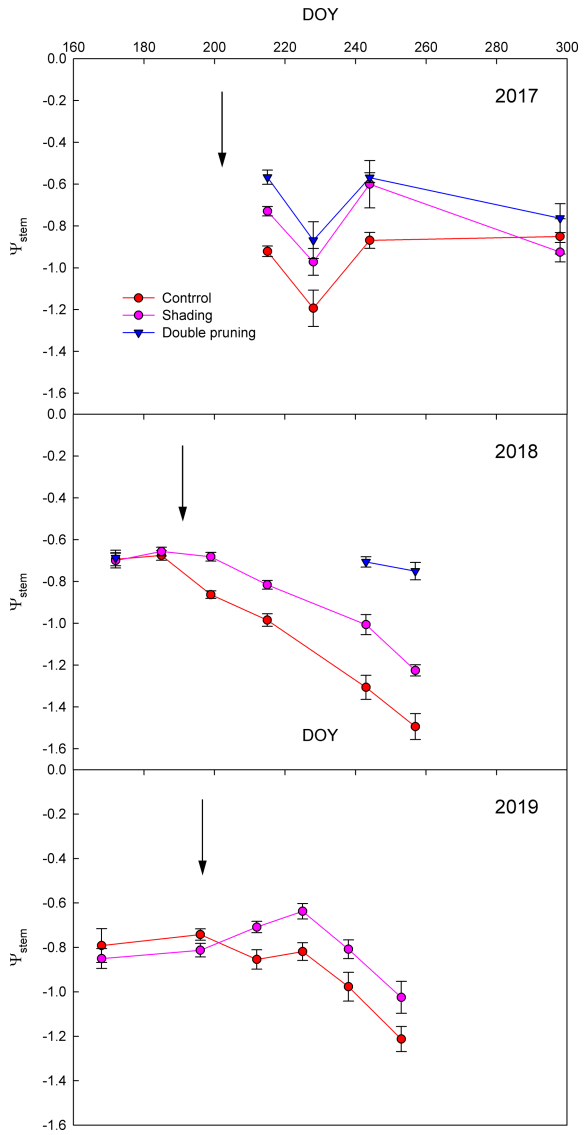

FIGURE 2
Effect of shading (•), double pruning (▼), and control (•) treatments on the seasonal evolution of the midday stem water potential (Ψ_{stem}) of Macabeo grapevines in **(a)** 2017, **(b)** 2018, and **(c)** 2019 seasons. Data are the averages and standard errors of 16 leaves per treatment and date. The installation of nets (↓) is indicated.

3.2 Agronomic and physiological response

Differences in the number of shoots per vine among treatments were not consistent across seasons, with a significant T*year interaction observed (Table 1). The shading treatment, applied shortly before veraison, did not cause phenological differences with respect to the control. The double pruning treatment was effective in provoking the regrowth of the vine as no differences in the number of shoots per vine as compared to the control were found in any season. The double pruning treatment indeed showed a proper development of the entire growing cycle, although it delayed the phenology of the crop by several months.

Vegetative vigor, measured through the pruning mass per vine, showed significant differences between treatments (Table 1). Control and shading treatments did not differ between them in this parameter. However, the pruning mass was significantly lower in the double pruning than in the other two treatments. Differences with the control were of 65% and 71% in 2017 and 2018, respectively. In 2019, when no second pruning was performed, the differences in pruning mass were only 22%, confirming the carry-over effects of the previous seasons of double pruning.

Regarding grape yield, differences between treatments were even higher than in vigor (Table 1). Significant differences between shading and control were found only in 2018, where the adaptation strategy reduced yield by 27%. The reductions in the double pruning treatment compared to both the control and shading were, in the two first experimental seasons, of 84% and 87%, respectively. The carry-over effect reduced by 47% in 2019. Differences in yield were mainly due to a reduction in the number of clusters per vine, as well as in cluster mass. Shading significantly reduced this parameter as compared to control, but only in 2018 (Table 1). Nevertheless, the ratio between the number of clusters and the number of shoots, known as shoot fruitfulness, did not significantly change in response to shading as compared to control, with it being 1.05 and 1.09 on average, respectively. The cluster mass in the shading treatment was similar to that of the control in 2017 and 2019 but significantly reduced in 2018. In this season, there was a general Botrytis affection in the clusters, and the shading treatment was more affected than the control. Regarding berry mass, there were no differences between shading and control treatments.

The double pruning technique reduced the number of clusters per vine in 2017 and 2018 as compared to that of the control. Thus, the fruitfulness in the double pruning treatment was significantly reduced by 62% on average, as compared to that of the control. Cluster mass was also significantly lower in the double pruning treatment than in the control, with reductions of 79% in the first two seasons and of 19% in 2019 (Table 1). Berry mass was, on average, 30% lower in the double pruning treatment as compared to the control (Table 1).

The Ravaz index was not affected by shading as compared to control, in 2017 and 2019, but it was significantly lower in 2018 due to the reduction in yield, whereas this index was significantly reduced by the double pruning in every season as compared to both control and shading treatments (Table 1).

3.3 Grape and wine composition

The control and shading treatments were harvested at the end of August or September, depending on the season (Table 2). The shading treatment was harvested between 4 and 8 days after the control, whereas the double pruning treatment was picked at the end of October or even in November. This means that harvest was delayed 43 and 62 days more than the shading treatment in 2017 and 2018, respectively. This harvest delay was related to the delays observed in grape veraison, with the control and shading veraison occurring in late July and early September, while the double pruning occurred during the first week of October.

Differences in the TSS content at harvest were not consistent among treatments and seasons (Table 2, Figure 4). Despite the

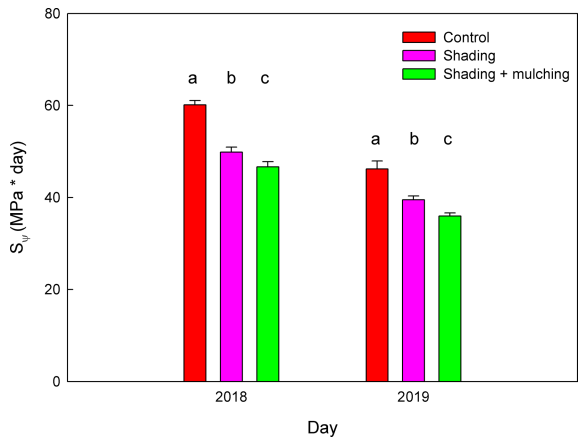

FIGURE 3
Values of the water stress integral (S_Ψ) during the period from veraison to harvest during the 2018 and 2019 seasons for Macabeo grapevines subjected to different management strategies. Data are the average and standard errors of eight measurements per date and season (n = 24). Different letters mean a significant difference among treatments within each season (Duncan test; $p < 0.05$).

harvest criteria being the same for all treatments, aiming a level of 18°–19° Brix in the must, in 2017, the TSSs were significantly higher in the double pruning treatment than in the other two. However, in 2018, the TSS content at harvest was higher in the control than in the other treatments, despite the latter being harvested later in the season (Table 2, Figure 4). Regardless the level of grape ripeness reached by each treatment at harvest, the grape ripening kinetics showed that the control fruits had higher TSSs than the other treatments at each measurement dates (Figure 4). On the contrary, the level of TA during the ripening process tended to be lower in the control than in other treatments for the same date. In the 2017 harvest, TA was significantly higher in the double pruning treatment than in the control and shading ones (Table 2). In 2018 and 2019, however, the control treatment showed significantly lower TA. Must pH showed quite similar differences among treatments as the ones observed in TA, although not fully consistently (Table 2). This can be partially explained by the differences found in the tartaric-to-malic acid ratios between treatments. On the one hand, grapes from the control treatment tended to show lower levels of both tartaric and malic acid at harvest (Table 2), and, on the other hand, double pruning produced grapes with the highest malic acid levels. Thus, the tartaric-to-malic acid ratios were significantly lower in the double pruning treatment, followed by the shading one, and with the highest ratios found in the control. Similar effects were also found in the TSS-to-TA ratios (Table 2).

Regardless of the level of ripening at harvest, the relationship between TSSs and TA across the grape- ripening period in the 2017–2019 seasons was similar in the control and shading treatments (Figure 5). Double pruning, however, did show an overall effect of an increased TA for similar TSS levels than the control and shading treatments. In the tartaric-to-malic acid ratio, there was a clear effect of the treatments across seasons (Figure 5). This ratio was higher for the control than for the shading and for the shading than for the double pruning treatment.

Regarding wine composition, the differences among treatments in alcohol content (Table 3) were similar to those found in grape TSSs (Table 2). In any case, the TA and pH of the wines were significantly higher in the shading treatment as compared to the control, but the wines from the shading treatment had lower a TA than the double pruning one. Thus, the alcohol-to-acidity ratio was lower in the wines made from shaded and double-pruned vines as compared to that of the control. Similar effects as TA were found in 2017 in volatile acidity (VA), without significant differences between control and shading wines in 2018 and 2019 (Table 3). The citric acid content in wines was significantly higher in the wines from the double pruning treatment than in the ones from the control, which, in turn, had higher values than the ones from the shading treatment in every season. The malic acid content tended to be lower in the control than in the other treatments, while the opposite was observed in the lactic acid content (Table 3). Finally, the glycerol content was higher in wines from double-pruned vines than from that from the control or shading treatments. In 2017 and 2018, the glycerol content was significantly higher in control wines than in the shading treatment ones, whereas the opposite was observed in 2019 wines.

4 Discussion

In many Mediterranean-like climate conditions, the main agronomic and oenological challenges faced by wine producers are the low acidity and high pH of the grape must, rather than yield losses (Sadras et al., 2017). The present results have shown that through the use of field strategies, it is possible to modify the composition of grapes and significantly influence the quality of the base wines for the subsequent production of sparkling wines. Nevertheless, our results also showed an interactive effect between these practices and the season. In this sense, the effectiveness of these strategies was revealed since they allowed the microclimate of the cluster to be modified during the ripening phase, and the TSS-to-TA ratio to be reduced in grapes, and thus, the alcohol-acidity ratio in base wines for sparkling wine productions. Moreover, changes were observed in the content of different acids in the grapes and wines. Overall, the effect of the adaptation strategies evaluated was different in both the vine performance and grape ripening. Therefore, the effectiveness of these practices, as well as the combined use of shading + mulching, is individually discussed below, focusing on the effects of each strategy on the vine water status, vine performance, and grape and wine composition.

4.1 Shading effects

Grapevine shading reinforced the general idea that maximizing light interception particularly under high solar radiation conditions might not always optimize crop productivity (Corelli-Grappadelli and Lakso, 2007). Despite leaf or whole canopy gas exchange not determined in our experiment, vine water status was clearly improved by shading as compared to control, which perhaps helped to counteract the expected effect of lower light availability

TABLE 1 Average values of vegetative growth and yield components over the 3 years of the experiment in 'Macabeo' grapevines subjected to different treatments.

Parameter	Year	Treatments												Significance of effects		
		Control				Shading				Double pruning				Treat	Year	T*year
Shoots vine^{-1}	2017	16.1	±	0.4	ab	15.7	±	0.4	a	17.0	±	0.4	b	0.152	<0.0001	<0.0001
	2018	16.9	±	0.4	b	15.6	±	0.4	a	16.8	±	0.4	b			
	2019	18.4	±	0.7	a	22.2	±	0.7	b	19.5	±	0.6	a			
Pruning mass (Kg vine^{-1})	2017	0.80	±	0.06	b	0.89	±	0.06	b	0.28	±	0.06	a	<0.0001	0.008	<0.0001
	2018	0.96	±	0.06	b	1.10	±	0.06	b	0.32	±	0.06	a			
	2019	0.87	±	0.05	b	0.85	±	0.06	b	0.68	±	0.06	a			
Clusters vine^{-1}	2017	14.6	±	0.7	b	14.5	±	0.6	b	10.7	±	0.7	a	<0.0001	<0.0001	0.0001
	2018	21.3	±	0.8	c	18.9	±	0.7	b	11.6	±	0.7	a			
	2019	20.1	±	1.0	b	22.2	±	0.9	b	13.1	±	0.8	a			
Shoot fruitfulness (clusters shoot^{-1})	2017	0.91	±	0.04	b	0.91	±	0.04	b	0.62	±	0.04	a	<0.0001	<0.0001	0.061
	2018	1.06	±	0.05	b	1.16	±	0.04	b	0.81	±	0.04	a			
	2019	1.10	±	0.05	b	1.00	±	0.04	b	0.63	±	0.04	a			
Cluster mass (g)	2017	426	±	16	b	445	±	15	b	91	±	15	a	<0.0001	0.0005	<0.0001
	2018	438	±	14	c	336	±	13	b	70	±	13	a			
	2019	375	±	19	b	304	±	16	a	299	±	18	a			
Berry mass	2017	2.3	±	0.1	b	2.3	±	0.1	b	1.6	±	0.1	a	–	–	–
	2018	1.7	±	0.0	b	1.8	±	0.0	b	1.2	±	0.0	a			
	2019	1.9	±	0.1	a	1.8	±	0.1	a	–	–	–	–			
Yield (kg vine^{-1})	2017	6.2	±	0.3	b	6.4	±	0.3	b	1.0	±	0.3	a	<0.0001	<0.0001	<0.0001
	2018	9.3	±	0.3	c	6.3	±	0.3	b	0.8	±	0.3	a			
	2019	7.5	±	0.4	b	6.6	±	0.4	b	3.9	±	0.3	a			
Ravaz index	2017	8.6	±	0.7	b	8.2	±	0.7	b	4.2	±	0.7	a	<0.0001	0.07	0.0001
	2018	10.1	±	0.5	c	6.3	±	0.5	b	2.7	±	0.5	a			
	2019	9.0	±	0.5	b	7.9	±	0.5	ab	6.5	±	0.5	a			

Data are average and standard errors of four replicates per treatment and season (n = 4). Different letters mean a significant difference among treatments within each season (Duncan test; $p < 0.05$).

on the leaf photosynthesis rate and transpiration (Prieto et al., 2012; Prieto et al., 2020). Indeed, the photosynthetic response to light decreases under progressive water stress (Escalona et al., 2003). A field trial in which source-to-sink manipulation and irrigation strategies were investigated revealed that the vine water status was a more determinant factor influencing vine performance (Mirás-Avalos et al., 2017). In addition, a meta-analysis confirmed those results, highlighting that the effect of the water status was even clearer for white wines than for red ones (Mirás-Avalos and Intrigliolo, 2017). In our research, shading improved the vine water status by 0.2 MPa on average, as previously reported in other cultivars (Martínez-Lüscher et al., 2020). This effect did not allow vines to reach mildly severe water stress as recorded in control vines in the 2018 and 2019 experimental seasons. Indeed, the Ψ_{stem} values reached in the control treatment during the grape- ripening stage would indicate that water stress provoked severe damages to leaf function, thus impairing net photosynthesis up to thresholds at which the grapes would not ripen properly (Romero et al., 2010). In this regard, a recent study using photovoltaic panels that shaded 70% of the total incident solar radiation reported that the vine water status was also improved by 0.15–0.2 MPa, even if the vines were fully watered and able to maintain near-optimum water status conditions (Ferrara et al., 2023).

In our trial, in the last season after three consecutive years of 50% photosynthetically active radiation (PAR) exclusion, the shading treatment did not affect yield or pruning mass and, consequently, the Ravaz index, in comparison with the control vines. Only in 2018 did the rainy and cool conditions favor the development of fungal infections, which became more severe in the shading treatment. This is probably why there was a reduction in yield in the shading treatment as compared with the control in 2018. Nonetheless, it is worth noting that in this season, the control

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TABLE 2 Harvest date and average values of berry composition at harvest over the 3 years of the experiment in Macabeo grapevines subjected to different treatments.

Parameter	Year	Treatments											
		Control				Shading				Double pruning			
Harvest date (DOY)	2017	233				237				299			
	2018	261				269				312			
	2019	252				252				–			
TSS (°Brix)	2017	19.6	±	0.4	a	18.4	±	0.4	a	21.3	±	0.4	b
	2018	18.4	±	0.3	b	15.6	±	0.3	a	14.9	±	0.3	a
	2019	17.9	±	0.4	a	16.7	±	0.4	a	–	–	–	–
pH	2017	3.28	±	0.02	b	3.27	±	0.02	b	3.15	±	0.02	a
	2018	3.18	±	0.02	b	3.29	±	0.02	c	3.12	±	0.02	a
	2019	3.39	±	0.02	b	3.25	±	0.02	a	–	–	–	–
TA (g/L)	2017	5.8	±	0.2	a	6.2	±	0.2	a	9.9	±	0.2	b
	2018	5.8	±	0.3	a	7.8	±	0.3	b	7.2	±	0.3	b
	2019	5.9	±	0.2	a	7.4	±	0.2	b	–	–	–	–
Tartaric acid (g/L)	2017	5.8	±	0.1	a	5.8	±	0.1	a	7.2	±	0.1	b
	2018	7.6	±	0.1	a	9.1	±	0.1	c	8.6	±	0.1	b
	2019	6.1	±	0.2	a	6.4	±	0.2	a	–	–	–	–
Malic acid (g/L)	2017	2.7	±	0.2	a	2.9	±	0.2	a	5.9	±	0.2	b
	2018	1.4	±	0.2	a	3.3	±	0.2	b	7.5	±	0.2	c
	2019	1.7	±	0.1	a	3.2	±	0.1	b	–	–	–	–
TSS-to-TA	2017	3.4	±	0.1	c	3.0	±	0.1	b	2.2	±	0.1	a
	2018	3.2	±	0.1	b	2.0	±	0.1	a	2.1	±	0.1	a
	2019	3.1	±	0.1	b	2.3	±	0.1	a	–	–	–	–
Tartaric/malic ratio	2017	3.9	±	0.3	c	2.3	±	0.3	b	1.2	±	0.4	a
	2018	5.8	±	0.4	c	2.8	±	0.4	b	1.1	±	0.4	a
	2019	3.7	±	0.2	b	2.0	±	0.2	a	–	–	–	–

Data are average and standard errors of four replicates per treatment and season (n = 4). Different letters mean a significant difference among treatments within each season (Duncan test; $p < 0.05$).

reached 24.8 tons/ha, which is more than double the amount allowed by the Cava Appellation of Origin for sparkling wine production. Therefore, the overall unaffected vine performance in response to such reduction in PAR suggests that improved light capture counterbalanced the irradiance reduction (González et al., 2019). Shoot fruitfulness was not affected by shading as compared to the control, which was intended by the installation of the nets after the formation of inflorescences (Lavee and May, 1997).

On the other hand, as intended, clear effects of shading on grape composition were recorded. The changes in microclimatic conditions in the shaded vines as compared to that of the control, were effective in delaying grape ripening and, consequently, harvest date. Not only the changes in the microclimate of the canopy and the cluster but also an additional ripening period (i.e., 1 week), under less hot weather conditions, allowed for a reduction of the TSS-to-TA ratio. This is in agreement with previous findings in a similar shading treatment applied to Cabernet-Sauvignon (Lu et al., 2021), in Pinot noir and Chardonnay (Ghiglieno et al., 2020), and in Riesling (Friedel et al., 2016). Nevertheless, this ratio was dependent on the ripeness level at harvest, which showed differences in TSSs among treatments. Thus, the relationship between TSSs and TA during ripening was not remarkably modified by the shading treatment as compared to the control. This suggests that although the shading treatment most likely reduced berry respiration and thus acid catabolism (Lu et al., 2021), it also reduced the grape's accumulation of photoassimilates by photosynthesis to a similar extent (Hernández-Montes et al., 2022). In contrast, the relationship between tartaric and malic acids was significantly affected by shading. This was to be expected as a higher temperature and light intensity in the cluster zone generally results in an increase in the metabolic activity of the berries (Spayd et al., 2002; Sweetman et al., 2014). Nevertheless, the

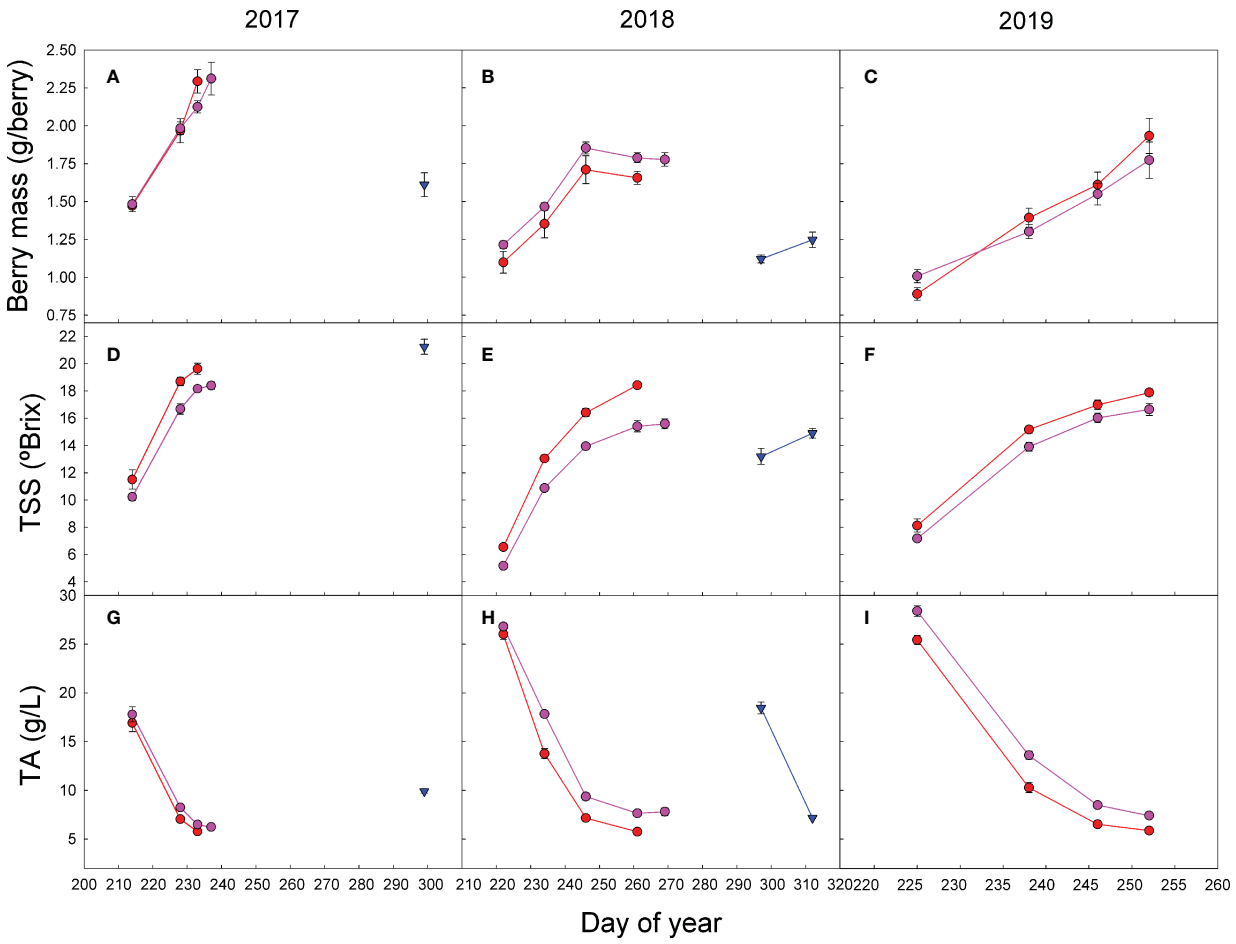

FIGURE 4
Effect of shading (•), double pruning (▼), and control (•) treatments on the seasonal evolution of **(A–C)** fresh berry mass; **(D–F)** total soluble solids (TSSs); and **(G–I)** the total acidity (TA) of 'Macabeo' grapes in **(A, D, G)** 2017, **(B, E, H)** 2018, and **(C, F, I)** 2019 seasons. Data are the average and SE of four values per treatment and date.

kinetics of malic acid, tartaric acid, pH, and potassium accumulation in grapes are not fully understood. A recent study points to potassium as a possible candidate to clarify the relationship between these parameters (Poni et al., 2018). Furthermore, the effect of temperature on tartaric acid and potassium, which, together with malic acid, determines the pH of must and wine remains to be explored.

The shading strategy, in our study, was effective in reducing wine pH as compared to that of the control in every season. The malic and lactic acid results are not fully conclusive as spontaneous malolactic fermentations seem to have taken place in some of the wines. However, the higher concentration of citric acid in the wines from the shading treatment confirms its effects of changes in acid synthesis and degradation in the grape as compared to the control.

4.2 Double pruning

Forcing vine regrowth during summer drastically shifted vine phenology and delayed grape ripening as compared to the other treatments. In addition, yield was reduced both in the season of application and in the subsequent one. Double pruning strongly affected vegetative growth and correct bunch formation. In fact, differences in yield components suggest that the main yield component affected by the double pruning technique was the number of berries per bunch. Nevertheless, the fruitfulness also decreased as compared to that of the control. This is in agreement with previous experiments that assessed this technique (Martínez de Toda et al., 2019; Martínez-Moreno et al., 2019; Cabral et al., 2023), and it may be attributable to a possible reduction in the nutritional reserves of the vines (Lebon et al., 2008; Smith and Holzapfel, 2009). Moreover, it should be noted that, in 2018, the double pruning vines suffered from powdery mildew during flowering, which can affect the fruit set percentage. Thus, the large shift in phenology provoked by this technique might increase the risk of suffering from fungal diseases. Moreover, given the large delay in the phenology caused by double pruning, the periods of maximum evapotranspirative demand occurred in different phenological stages than in the control or shaded vines. This may increase water requirements in forced vines as compared to the control, as Oliver-Manera et al. (2023) recently observed. Furthermore, they suggested that double-pruned vines were extremely sensitive to even mild water stress.

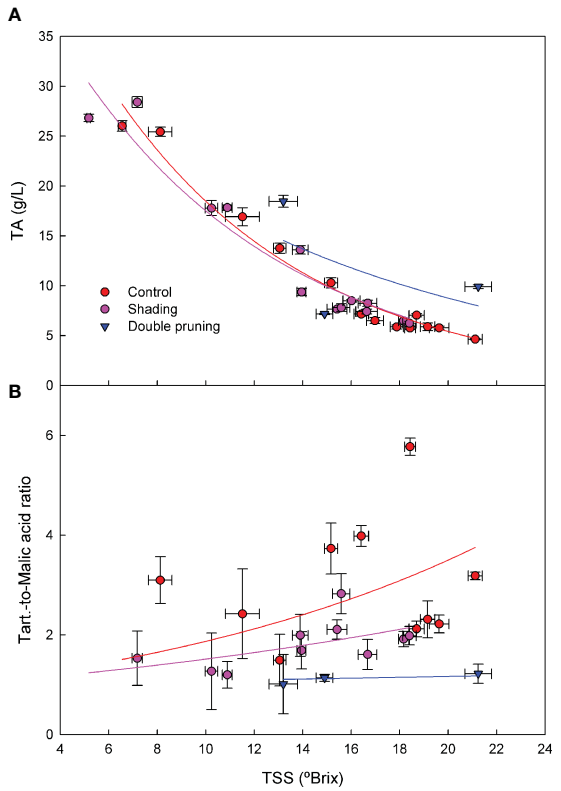

FIGURE 5
Effect of shading (•), double pruning, (▼) and control (•) treatments on the relationship between **(a)** TSSs and TA and **(b)** TSSs and tartaric-to-malic acid ratio for 'Macabeo' grapes in the 2017–2019 seasons. Data are the average of four replicates per treatment and date. TA, titratable acidity; TSSs, total soluble solids.

Regarding grape composition, our results clearly indicated that the TSS-to-TA ratio can be improved and, consequently, the alcohol-to-TA ratio. The later grape ripening confirmed that the temperature and humidity conditions in September and October were more favorable for a balanced grape composition. Thus, double pruning did increase the TA in relation to TSSs, and the tartaric-to-malic acid ratio in relation to TSSs, as other authors reported in other cultivars (Gu et al., 2012; Martínez de Toda et al., 2019; Martínez-Moreno et al., 2019; Oliver-Manera et al., 2023). These increases can be beneficial for wine stability as higher titratable acidity and a low tartaric-to-malic acid ratio decrease must pH. Glycerol content was also higher in the wines made from double-pruned grapes, which might be related to the enhancement of the smoothness sensation of the wine as it increases its density and viscosity (Silvestroni et al., 2018).

4.3 Combination of field strategies for climate change adaptation

Our research demonstrated the possibility of combining field strategies to add their effects. The Shading, together with the application of mulch, further reduced the water stress integral (S_Ψ) from veraison to harvest during the 2018 and 2019 seasons as compared with Shading alone. Thus, both techniques, combined, had an additive effect in improving the vine water status. Shading likely reduced the evapotranspirative demand of the vines, while mulching reduced soil evaporation, as previously established (López-Urrea et al., 2020). This is particularly relevant today as the common agriculture policies economically support the application of organic mulching in vineyards in order to protect them from soil erosion and to improve the vineyard water balance, as demonstrated in the present study. Organic mulching could also be applied on the entire vineyard floor without a soil tillage operation (Buesa et al., 2021b). Moreover, other field strategies such as the application of sprinkler cooling within the cluster zone during ripening can also help reduce the impact of high temperatures (Caravia et al., 2017) beyond the improved water status of the techniques tested here, which may help to face heat waves. It is worth noting the positive effect that these techniques may have on soil water balance as the reduction in vine water stress is due to the reduction in evapotranspirative demand (Ramírez-Cuesta et al., 2023). Moreover, mulching could also affect root respiration and vine water status, due to not only soil water increases but also soil temperature modification (Hernández-Montes et al., 2017; Costa et al., 2018). Recently, Hernández-Montes et al. (2022) indeed highlighted the importance of respiratory processes on the carbon balance during vine phenology. For all of the above reasons, field adaptation strategies can be employed to face the detrimental effects of climate change in wine grapes. The two alternative practices tested differed both in terms of the infrastructure required, and the expected changes in vine phenology and development, and grape ripening. The high cost of installation of Shading nets may, however, preclude their final utilization, although the potential of this technique to minimize the risks of hail and wind damage and the impact of heat waves is also worth noting (Basile et al., 2014; Manja and Aoun, 2019; Martínez-Lüscher et al., 2020). The main limitation for the Double pruning strategy is the yield reduction and its carry-over effects, as it has been reported in other previous trials conducted with other wine grape varieties (Martínez de Toda et al., 2019; Cabral et al., 2023). This makes this technique profitable only for premium wine production of very high commercial value. However, the carry-over effects of this technique (Martínez-Moreno et al., 2019), together with its sensitivity to water deficit (Oliver-Manera et al., 2023), question its feasibility. On the other hand, the addition of mulching seems to be an easy and cheap technique to implement at the plot level, and with a proven potential to alleviate the impact of vine water stress (Buesa et al., 2021b). Moreover, the evaluated field strategies and their combination can be applied in the short-term. Nevertheless, the genetic material as a potential strategy should also be considered as a long-term adaptation (Medrano et al., 2015; Tortosa et al., 2016; van Leeuwen and Destrac-Irvine, 2017; Buesa et al., 2021c).

5 Conclusion

In the short term, grapevine adaptation to climate change can be achieved by using field strategies such as shading of the vines,

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TABLE 3 Average values of wine composition at harvest over the 3 years of the experiment in 'Macabeo' grapevines subjected to different treatments.

Parameter	Year	Treatments											
		Control				Shading				Double pruning			
Alcohol (%)	2017	10.5	±	0.2	b	9.6	±	0.2	a	13.2	±	0.2	c
	2018	10.7	±	0.1	b	8.5	±	0.1	a	–		–	–
	2019	10.8	±	0.2	b	8.7	±	0.2	a	–		–	–
TA	2017	6.48	±	0.10	a	6.88	±	0.10	b	8.69	±	0.10	c
	2018	6.68	±	0.06	a	8.60	±	0.09	b	–		–	–
	2019	6.46	±	0.09	a	8.12	±	0.09	b	–		–	–
VA	2017	0.38	±	0.01	a	0.44	±	0.01	b	0.59	±	0.01	c
	2018	0.31	±	0.01	a	0.29	±	0.01	a	–		–	–
	2019	0.31	±	0.01	a	0.29	±	0.01	a	–		–	–
pH	2017	3.08	±	0.02	b	2.98	±	0.02	a	3.09	±	0.02	b
	2018	2.93	±	0.02	b	2.64	±	0.02	a	–		–	–
	2019	2.99	±	0.03	b	2.68	±	0.03	a	–		–	–
Citric acid (g/L)	2017	0.31	±	0.01	b	0.27	±	0.01	a	0.41	±	0.01	c
	2018	0.32	±	0.01	b	0.25	±	0.01	a	–		–	–
	2019	0.37	±	0.01	b	0.32	±	0.01	a	–		–	–
Malic acid (g/L)	2017	1.52	±	0.10	a	1.70	±	0.10	a	2.58	±	0.10	b
	2018	1.11	±	0.04	a	2.03	±	0.06	b	–		–	–
	2019	1.32	±	0.05	a	2.06	±	0.05	b	–		–	–
Lactic acid (g/L)	2017	0.61	±	0.02	b	0.59	±	0.02	b	0.48	±	0.02	a
	2018	0.65	±	0.02	b	0.45	±	0.03	a	–		–	–
	2019	0.66	±	0.02	b	0.40	±	0.02	a	–		–	–
Glycerol	2017	6.7	±	0.1	b	6.2	±	0.1	a	8.4	±	0.1	c
	2018	5.5	±	0.0	b	5.0	±	0.1	a	–		–	–
	2019	3.8	±	0.1	a	4.1	±	0.1	b	–		–	–
Alcohol-to-TA ratio (%/g/L)	2017	1.60	±	0.05	b	1.40	±	0.03	a	1.53	±	0.04	ab
	2018	1.68	±	0.04	b	1.10	±	0.03	a	–		–	–
	2019	1.62	±	0.02	b	1.00	±	0.03	a	–		–	–

Data are average and standard errors of four replicates per treatment and season (n = 4). Different letters mean a significant difference among treatments within each season (Duncan test; $p < 0.05$).

double pruning, or shading + mulching, which can be all used to improve the composition of the wine base for making cava. The effectiveness of shading will depend to a large extent on the climatic conditions during the grape-ripening period, and more attention should be paid to the sanitary status of the grapes. In rainy and cool years, the application of the shading technique would not be recommended as it would be much more effective in hotter and drier vintages. On the other hand, double pruning can only be recommended for the production of premium Cava with a high commercial value, due to its drastic yield reduction and carry-over effects. All the investigated techniques are effective in improving the vine water status, and, in addition, the effect of the combination of shading and mulching was additive. This suggests the convenience of applying field practices in combination in light of future and more critical climate change scenarios and highlights the importance of further studies on the use of combined field adaptation strategies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

IB and DI contributed to the conception and design of the study. IB, AY, DG, and FS acquired the data. IB, AY, and DI performed the data analysis and interpretation. IB prepared the first-draft. IB and DI reviewed and edited the manuscript. IB and DI supervised the work. IB and DI acquired the funding. All authors contributed to the article and approved the submitted version.

Funding

This study was funded by the BestCava and BestCava2.0 research grants from the Fundación Ciudad de Requena and the financial support of Spanish Ministry of Economy and Competitiveness with FEDER cofunding, grant numbers AGL2017-83738-C3-3-R and PID2021-123305OB-C31. IB acknowledges the postdoctoral financial support received from the "Juan de la Cierva" Spanish Postdoctoral Program (FJC2019-042122-I).

Acknowledgments

Thanks are due to the Lucio Gil Fagoaga Foundation for the maintenance of the experimental plot.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1155888/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Photographs of (A) the Shading and Control treatments and (B) the Double pruning treatment at the time of the second pruning.

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frontiers | Frontiers in Plant Science

TYPE Review
PUBLISHED 09 May 2023
DOI 10.3389/fpls.2023.1145137

OPEN ACCESS

EDITED BY
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RECEIVED 15 January 2023
ACCEPTED 20 March 2023
PUBLISHED 09 May 2023

CITATION
Costa JM, Egipto R, Aguiar FC, Marques P, Nogales A and Madeira M (2023) The role of soil temperature in mediterranean vineyards in a climate change context. *Front. Plant Sci.* 14:1145137. doi: 10.3389/fpls.2023.1145137

The role of soil temperature in mediterranean vineyards in a climate change context

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The wine sector faces important challenges related to sustainability issues and the impact of climate change. More frequent extreme climate conditions (high temperatures coupled with severe drought periods) have become a matter of concern for the wine sector of typically dry and warm regions, such as the Mediterranean European countries. Soil is a natural resource crucial to sustaining the equilibrium of ecosystems, economic growth and people's prosperity worldwide. In viticulture, soils have a great influence on crop performance (growth, yield and berry composition) and wine quality, as the soil is a central component of the *terroir*. Soil temperature (ST) affects multiple physical, chemical and biological processes occurring in the soil as well as in plants growing on it. Moreover, the impact of ST is stronger in row crops such as grapevine, since it favors soil exposition to radiation and favors evapotranspiration. The role of ST on crop performance remains poorly described, especially under more extreme climatic conditions. Therefore, a better understanding of the impact of ST in vineyards (vine plants, weeds, microbiota) can help to better manage and predict vineyards' performance, plant-soil relations and soil microbiome under more extreme climate conditions. In addition, soil and plant thermal data can be integrated into Decision Support Systems (DSS) to support vineyard management. In this paper, the role of ST in Mediterranean vineyards is reviewed namely in terms of its effect on vines' ecophysiological and agronomical performance and its relation with soil properties and soil management strategies. The potential use of imaging approaches, e.g. thermography, is discussed as an alternative or complementary tool to assess ST and vertical canopy temperature profiles/gradients in vineyards. Soil management strategies to mitigate the negative impact of climate change, optimize ST variation and crop thermal microclimate (leaf and berry) are proposed and discussed, with emphasis on Mediterranean systems.

KEYWORDS

radiation, row-crops, sustainable soil management, thermal data, water, soil temperature sensing, cover crops

1 Introduction

1.1 European viticulture and climate change

Agriculture is a nature-based, climate-dependent sector and is strongly affected by climate change. A recent report from the European Environment Agency indicates that the overall impacts of climate change can decrease significantly the EU's agricultural sector production (up to 16% loss in income by 2050), with large regional variations (EEA, 2019). Even in regions not experiencing a decrease in rainfall, air temperature rise will result in higher evapotranspiration (Seneviratne et al., 2010; Abad et al., 2021). For this reason, the agricultural sector must build up the capacity to adapt to increasing dry and warm conditions induced by climate change. Soil characteristics and soil management have a major role in this adaptation (EEA, 2019), but a better understanding is needed for Mediterranean viticultural systems.

The EU protects high-quality wines by linking them to legally defined geographic areas, specific sustainable production practices, traditional varieties and soil characteristics (Candiago et al., 2022; Onofre, 2022). The contribution of Mediterranean viticulture (e.g. Spain, Italy, France, Portugal and Greece) to the global wine industry is large, accounting for more than 50% of the world production and about 55% of world exports (OIV, 2022). However, Mediterranean viticulture is highly vulnerable to climate change (Costa et al., 2016; Fraga et al., 2016; Santos et al., 2020; Xyrafis et al., 2022), especially to the combination of longer warmer and drier periods. The same occurs for other Mediterranean perennial crops, such as olive groves and almond orchards (Andrade et al., 2014; Garcia-Tejero et al., 2018; Fraga et al., 2021).

Higher air temperature promotes earlier bud break, flowering, maturation and harvest, which can be negative for berry composition (e.g. higher sugar concentration and decreasing acidity) and can result in unbalanced wines (Bonada et al., 2015; Droulia and Charalampopoulos, 2022). Drier conditions exacerbate the effects of heat stress because dry soils cannot provide latent heat cooling by evapotranspiration, resulting in higher and more stressful temperatures at the plant level (Seneviratne et al., 2010; Stéfanon et al., 2014; Guion et al., 2022). This not only affects vine's phenology but also yields and vines longevity and, ultimately the overall sustainability of the sector (economical, environmental and social) (Santos et al., 2020; Costa et al., 2022; Droulia and Charalampopoulos, 2022). In addition, these climatic scenarios may limit the expansion of the cultivated area in some regions of Mediterranean countries and may force the relocation of vineyards at higher altitudes (Jones, 2012).

1.2 Soil, climate change and surface energy balance

Soils are critical to sustain the equilibrium of ecosystems, economic growth and people's prosperity worldwide (Brady and Weil, 2017). Soils provide multiple ecosystem services and socio-economic activities and in viticulture, they are an important component of the *terroir*, since they are one of the major factors influencing berry traits, wine characteristics and styles (White, 2015; Van Leeuwen et al., 2018; Sremac et al., 2021). Soils have a relevant function in the adaptation of the agricultural sector to adverse climatic conditions and more sustainable soil management is needed to ensure food security but also to improve adaptation to climate crises (EEA, 2019; Cataldo et al., 2021). Soil characteristics govern vegetation growth and influence heat, water and carbon fluxes between soil and the atmosphere (Evett, 2000; Lorenz et al., 2010; Liu et al., 2022).

Soil-atmosphere temperature relations are particularly important in the context of climate change (Hirschi et al., 2011). They involve partitioning of the surface energy into sensible (H) and latent heat (LE) fluxes (Figure 1), which depend on soil moisture content (Wang and Yang, 2018). Under dry conditions, the available net radiation (Rn) energy is converted into H fluxes, which increases air temperature. The relationship (coupling) between ST and soil moisture regimes explains the use of both variables in natural resource management, to better quantify and predict climate change impacts (Houle et al., 2012; Bradford et al., 2019). The energy balance equation for soil is commonly expressed as: $Rn = LE + H + G$, in which Rn is the net flux density of radiation (W/m^2), and G is the soil heat flux,

Soil characteristics and soil management influence the energy balance at the soil's surface and on the plant's energy balance due to the reflection of shortwave irradiation that becomes a source of longwave radiation for plants (Nobel, 2005) (Figure 1). Furthermore, ST influences physical, chemical and biological processes taking place in the soil and regulates energy and matter exchange with the atmosphere (Baver, 1965; Hillel, 2004; Brady and Weil, 2017). Soil temperature influences evaporation, aeration and the type and rates of chemical reactions occurring in soils (Hillel, 2004).

Predictions for air temperature increase due to climate change are well described in the literature (IPCC, 2021). However, less information is available for ST. In a recent study, Schultz (2022) reports a progressive increase of ST for Northern European countries (e.g. Germany) in the last decades. Nonetheless, this trend observed for ST is expected to be more marked in Southern Europe. The Mediterranean region has a warm season transitional climate, in which evapotranspiration is limited by low soil moisture rather than by solar radiation (Feng et al., 2022). In this context, low soil moisture will amplify heat anomalies and extremes in the region (Seneviratne et al., 2010; Vogel et al., 2021; Feng et al., 2022) with a potential major impact on growth and yield of both crop and weed species.

In Mediterranean areas, ST and soil moisture regimes are classified as xeric, as precipitation concentrates in the winter and summers are dry, and the mean annual ST can range between 15 and 22°C (Soil Survey Staff, 2014). Soil temperature is one of the major drivers of grapevine physiology, growth and productivity. Soil temperature affects physical and biological processes at soil's surface (e.g. weed and crop phenology, growth, respiration, etc.) (Bullied et al., 2003; Howell et al., 2020). At deeper soil layers ST influences root metabolism and growth, soil respiration, water and nutrient uptake, microbial diversity and activity, organic matter (OM) dynamics, soil bio-chemistry) (Akter et al., 2015; Onwuka and Mang, 2018; Mehdizadeh et al., 2020a; Mehdizadeh et al., 2020b; Shah et al., 2022).

Mean temperatures of air and soil, and in particular their extremes, influence weeds and crop physiology (seeds, fruits,

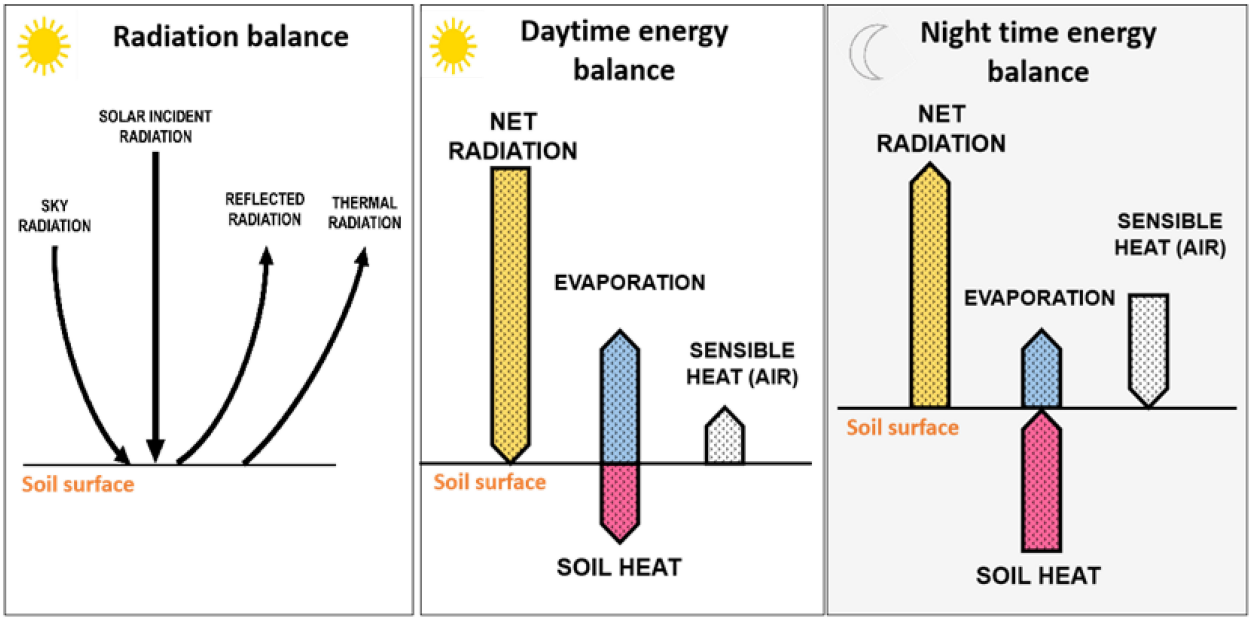


FIGURE 1
Schematic representation of the radiation balance, the daytime energy balance, and the night time energy balance. Net radiation at soil surface represents the sum "solar radiation plus sky radiation" minus the sum of "reflected radiation plus thermal radiation". Most of the radiation that reaches earth's surface in the daytime is used for evapotranspiration or reflected and emitted to the atmosphere. Evaporation translates the latent heat. The arrows indicate the direction of the exchange and arrow lengths tentatively indicate the magnitude of the different fluxes (Adapted from Evett, 2000; Hillel, 2004 and Brady and Weil, 2017).

leaves, and roots) (Bullied et al., 2003; Chaves et al., 2016; Field et al., 2020; Gambetta et al., 2021; Sremac et al., 2021). In vineyards, the proximity of leaves and clusters to soil surface enhances the warming effect of ST and soil sensible heat fluxes on berries, clusters and leaves. This is observed for the worldwide used Vertical Shoot Positioning (VSP) system in which the cluster zone and basal leaves often get warmer than the upper part of the canopy due to soil sensible fluxes, under warm and dry conditions (Costa et al., 2019).

A deeper understanding of vineyard soils, including their properties, functions, ecological roles, and management is required to increase the resilience of Mediterranean viticulture systems to more extreme climate conditions. There is a need to integrate the components of the *terroir* related to ST and the solutions for adaptation to climate change. This must be done at local level and should consider the trade-offs between adaptation strategies (Naulleau et al., 2021). In the following section, some of the major determinants of ST are presented.

2 Determinants of soil temperature in vineyards

Soil properties (e.g. color, texture, structure, moisture content) together with dominant atmospheric conditions (e.g. air temperature, solar radiation and wind) (Baver, 1965; Evett, 2000) influence soil heat and water fluxes and ultimately ST variables (thermal conductivity, thermal regime, maximum and minimum temperatures) (Brady and Weil, 2017). On the other hand, anthropological conditions, including agricultural soil management strategies (Table 1), can modify heat and water fluxes between soil, plant and atmosphere influencing ST variation (Neilsen et al., 1986; Rességuier et al., 2023).

The impact of climatic conditions on surface energy balance and consequently on ST is expressed by daily and seasonal variations in surface ST. In summer months (June–July in the northern hemisphere) maximum incident global radiation is closely related to maximum ST at midday (Figure 2) (Costa et al., 2019; Sremac et al., 2021). The stronger seasonal warming response of soils in summer as compared to autumn period mainly relates to decreased soil moisture content at the top layers of soil during summer (Schultz, 2022). Together with the effect of climate conditions, various soil properties and soil management (e.g. irrigation, mulching) can influence ST to a different extent (Table 1).

2.1 Topography and soil temperature

Soil temperature is related to the amount of incident radiation (Figure 1). Topographic components (e.g. slope and exposition to sunlight) influence ST and soil moisture regimes (Baver, 1965; Griffiths et al., 2009; Brady and Weil, 2017). Slopes with a southern aspect have higher levels of insolation, and consequently, higher heat accumulation and are usually considered ideal (Yau et al., 2014). Usually, the temperature of corrugated fields is higher than that of flattened ones due to different degrees of incident and reflected radiation (Brady and Weil, 2017). Radke (1982) in turn, reported that inclined ridge surfaces absorbed about 10% more solar radiation than flat surfaces contributing to higher ST.

TABLE 1 Non-exhaustive list of major determinants influencing soil temperature (ST), and general individual effect on the increase (↑), decrease (↓), reliable with other factors, such as climate (↓↑).

FACTORS	GENERAL EFFECT ON ST	SOURCE
Topographic		
Slope	flat↑ large sloping ↓	Baver (1965)
Exposition	North↓ South↑	Brady and Weil (2017)
Soil properties		
Color and albedo	dark ↑ light ↓	Meinhold et al. (2010)
Soil texture	silty ↑ sandy ↓	Sremac et al. (2021)
Organic matter content	OM and darker color↓ poor/lighter color↑	Brady and Weil (2017)
Soil structure	stable large round aggregates↓ unstable or platy, prismatic, blocky aggregates↑	Sremac et al. (2021)
Soil moisture	dry ↑ wet ↓	Brady and Weil (2017); Wang and Yang (2018); Krapez et al. (2012)
Soil and canopy management		
Tillage	↑	Radke (1982); Pradel and Pieri, (2008)
Plant density	high ↓ low ↑	White (2015)
Canopy size/shedding	large ↓ small ↑	White (2015)
Irrigation	↓	Costa et al. (2019); Costa et al. (2020); Krapez et al. (2012)
Row orientation	↑ ↓	Hunter et al. (2021); White (2015); Pisciotta et al. (2021)
Soil cover[1)]	↓	Baver (1965); Lazcano et al. (2020); Akter et al. (2015); Brady and Weil (2017)
Soil and canopy cover[2)]	↓	Marigliano et al. (2022); Tadayon and Hossein (2022); Pradel and Pieri, (2008)

[1)]mulching, natural vegetation, cover crops; [2)]nets and other covering structures.
Extremes of the scale, when pertinent, are given as indicators of the general effect on ST.

2.2 Soil properties and soil temperature

2.2.1 Soil albedo and color

The surface albedo represents the reflectivity of the Earth's surface for incident solar radiation (Evett, 2000). The amount and type of reflected radiation depend on the characteristics of the surface and of the vegetation cover or mulch beneath crops, and soil properties (Meinhold et al., 2010). Soil vegetation controls the amount of sunlight that hits the ground surface, and bare soils cool down and warm up faster than soils covered with vegetation (Akter et al., 2015; Brady and Weil, 2017). Regarding soil color, dark-colored soils can warm more than light-colored soils, since they absorb more radiation (Baver, 1965). Nevertheless, large amounts of OM in dark soils can increase their water retention, which can offset the increased heat absorption due to the dark color (Brady and Weil, 2017). Soil's albedo can be manipulated by different management strategies such as soil conservation tillage (Brady and Weil, 2017) or the use of certain products such as biochar (Verheijen et al., 2013), or the use of other organic (Burg et al., 2022) and inorganic mulches (Marshall et al., 1996; Aragüés et al., 2014).

2.2.2 Soil texture and structure

Soil texture refers to the proportion of sand, silt and clay sized particles that make up the mineral fraction of the soil, while soil structure refers to the organization of soil particles and the tendency of individual soil particles to combine into aggregates (Marshall et al., 1996; Hillel, 2004). The degree of aggregation influences water and air transport in the soil, solutes movement and soil's biological activity (Brady and Weil, 2017). The texture influences soil thermal behaviour and soil surface temperature. Sandy soils tend to warm up faster than clay soils due to their lower heat capacity, lower thermal conductivity, and lower evaporative cooling (Hillel, 2004). On the other hand, the amplitude of the daily ST variation decreases in the order sand > loam > clay. Soil moisture at the surface and in the subsurface moderates the daily range of ST (Krapez et al., 2012) (Figure 3). In orchards, sandy soils usually have a higher night time cooling rate than clay soils, due to a faster energy loss and lower minimum air temperature (Sremac et al., 2021).

Soil structure also influences ST. Soil structure controls pore spaces due to different arrangements of soil particles and soils with a more spherical structure warm up faster due to higher aeration and reduced waterlogging conditions (Brady and Weil, 2017). Soil structure is negatively affected by compaction which increases soil density and thermal conductivity which also enables faster changes in ST and affects root growth and morphology (Brady and Weil, 2017; Gürsoy, 2021).

2.2.3 Soil water content

Soil water and heat fluxes are coupled (Wang and Yang, 2018) and their study is highly relevant for climate research (e.g. climate models) (Lanyon et al., 2004; Seneviratne et al., 2010) and

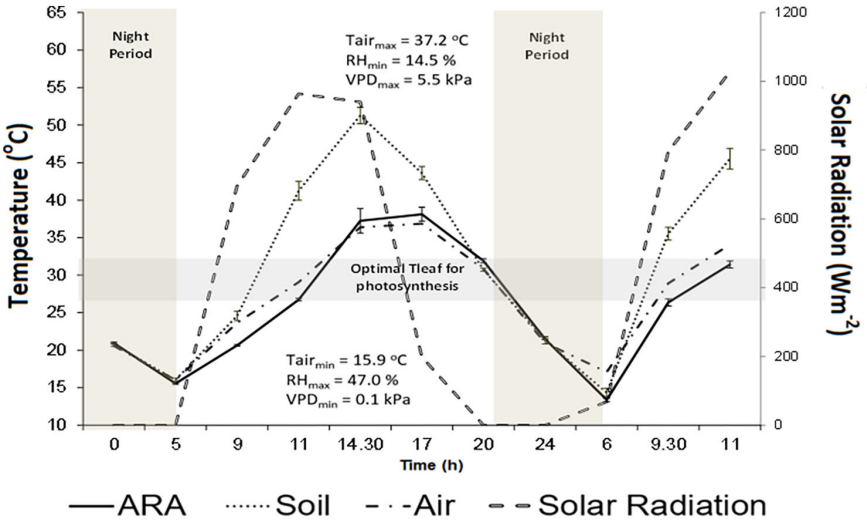
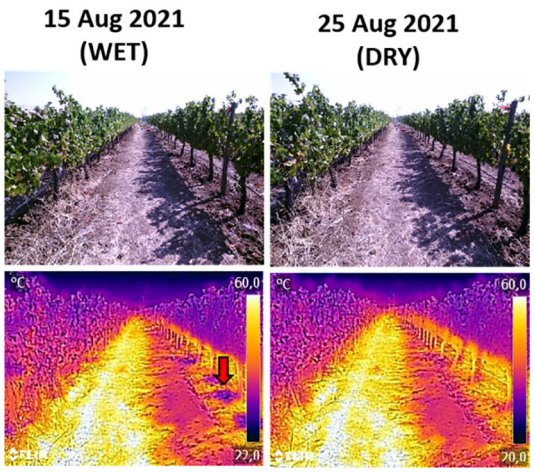


FIGURE 2
Diurnal variation of solar radiation (Wm^{-2}) (– –), air temperature (T_{air}), soil surface temperature (·····, Soil) and vine's canopy temperature (‾ ARA) for the the *Vitis vinifera* cv Aragonez (syn. Tempranillo) (ARA), subjected to deficit irrigation, in a vineyard located in Alentejo (Southern Portugal). Data were collected along 8–9 July 2015 under the following climatic conditions ($T_{air\ min/max}$ = 37.2 °C/15.9 °C; RH $_{min/max}$ = 14.5%/47.0%; Wind speed $_{min/max}$ = 0.6 m s^{-1}/4.7 m s^{-1}). Soil surface temperature was assessed by thermal imaging (Adapted from Costa et al., 2019).

agronomical and remote sensing research (Krapez et al., 2012; Kustas et al., 2022). The specific heat of water is higher than that of soil, and consequently, soils with high moisture have higher specific heat than dry soils, resulting in lower ST (Baver, 1965). Therefore, higher soil water content makes the variation (increase/decrease) in ST occur more slowly than in dry soils (Brady and Weil, 2017). Lower moisture content results in a higher conversion of solar radiation into sensible heat (measurable as temperature) (Figure 1), in opposite to high soil moisture conditions, in which the incident solar energy is used to evaporate water (Heilman et al., 1994). Soil water evaporation reduces ST, and the temperature difference between soil and the atmosphere is proportional to the evaporation rate (Zeng et al., 2021) and as a consequence soil surface temperature inversely correlates with soil water content (Krapez et al., 2012). As a result, the typical wet-dry irrigation cycles occurring in irrigated crops often result in spikes in ST (See Figure 3).

Severe precipitation events and flooding can greatly affect soil characteristics, leading in general to soil erosion, compactation and nutrient runoff, with detrimental effects on crops (root growth, yield) and soil fauna, and influencing soil temperature (Mancuso and Shabala, 2010; Ruperti et al., 2019).



	Daylight time	
	Avg (°C)	Accum (°C.day)
15 Aug 2021 (WET)		
ΔT (top canopy - air)	0.8	11.3
ΔT (canopy cluster zone - air)	1.3	17.6
ΔT (air below canopy - air)	0.6	8.9
ΔT (soil - air)	- 3.0	- 41.2
25 Aug 2021 (DRY)		
ΔT (top canopy - air)	2.8	36.7
ΔT (canopy cluster zone - air)	3.2	42.0
ΔT (air below canopy - air)	1.4	18.5
ΔT (soil - air)	3.0	39.3

FIGURE 3
RGB and thermal images taken with a medium cost thermal camera (Flir C5, 160 x 120 pixels, 8-14µm, Emissivity = 0,96) from the inter row and rows with *Vitis vinifera* cv Tempranillo, taken at 16:00 hours, on 15 and 25 August 2021, showing the marked effect of shadow and sunlit soil sides as well as effect of irrigation on soil temperature (arrow) as part of a typical wet-drying cycle in irrigated vineyards. (Adapted from Egipto et al., 2022).

2.2.4 Soil organic carbon content

Soil organic carbon (OC) content depends on the balance between carbon inputs and outputs. Carbon inputs relate to plant productivity, while carbon outputs relate to microbial decomposition of OM. Soil OM decomposition is controlled by ST during wet periods and by the combined effect of soil water and ST during dry periods (Yuste et al., 2007). On the other hand, high ST promotes OM mineralization rates and drives several physical, chemical and biological changes, which accelerates microbial decomposition of soil OM and, decreases soil fertility (Guoju et al., 2020). Soil microbial respiration uses soil OC and releases CO_2, and higher ST promotes soil respiration and higher CO_2 release to the atmosphere (Karhu et al., 2014).

Soil management practices (tillage, the use of cover crops, mulching) combined with changes in soil water content due to precipitation or irrigation, influence C dynamics in soils and soil biodiversity (Haddaway et al., 2017; Crystal-Ornelas et al., 2021). Soil tillage promotes CO_2 release and disrupts protected OM in soil aggregates, increasing its availability for microorganisms (Haddaway et al., 2017). Precipitation and irrigation modify CO_2 fluxes in soil, and the wet-dry cycles due to precipitation or irrigation events result in marked fluctuations in soil CO_2 efflux and in dynamic responses in soil C pools (Yu et al., 2022).

3 The impact of soil temperature in vineyards

3.1 Grapevine responses

Vitis vinifera is a crop species well adapted to dry and warm conditions. However, more variable and extreme climatic conditions (heat and drought) pose risks to the wine sector. Temperature is a primary environmental factor influencing grapevine development, growth and physiological processes occurring in roots, shoots/leaves and berries, including growth and phenology, respiration and photosynthesis, flowering and fruit set, yield and berry composition (Chaves et al., 2016; Pagay and Collins, 2017; Field et al., 2020; Bernardo et al., 2021; Gambetta et al., 2021; Keller et al., 2022). Warmer conditions lead to earlier bud break and earlier harvests, resulting in wines with lower organic acids, higher pH levels, higher ethanol levels and altered sensory characteristics (Van Leeuwen and Destrac-Irvine, 2017; Venios et al., 2020). Heat stress due to air temperatures above 35°C decreases the synthesis of secondary metabolites, reduces photosynthesis rates and vegetative growth (Moutinho-Pereira et al., 2004; Chaves et al., 2010) and may affect plant water transport (Galat Giorgi et al., 2020). Excessively high temperatures (air and soil) in wine-growing regions can reduce berry color due to inhibition of anthocyanin biosynthesis or their degradation and promote the synthesis of reactive oxygen species (ROS) (Carvalho et al., 2014; Venios et al., 2020).Higher average air temperatures in the growing season can negatively impact yield and quality (Bonada et al., 2015) and brief episodes of extreme temperatures are detrimental when occurring at specific phenological stages, e.g. flowering and fruit set (Pagay and Collins, 2017; Gambetta et al., 2021; Keller et al., 2022).

High air temperatures and drought stress can influence leaf morphology and structure resulting in larger but thinner leaves, with smaller cells, and higher stomatal density (Pierce et al., 2022). High diurnal air temperatures and low night air temperatures ensure a low pH in berries which is highly relevant for wine production in warm areas (e.g. Mediterranean), which are increasingly experiencing an increase in night time temperatures (Venios et al., 2020).

The response of grapevine to heat and drought stress depends on several factors that include the atmospheric climatic conditions, the genotype (variety/rootstock), soil characteristics and soil and crop management (Lopes et al., 2011; Bota et al., 2016; Simonneau et al., 2017). Leaf gas exchange traits (e.g. photosynthesis, transpiration or stomatal conductance) respond fast to abiotic stresses, namely to drought and high temperatures (Chaves et al., 2010; Simonneau et al., 2017). Optimal leaf temperatures for photosynthesis range between 25 and 30° C (Greer and Weedon, 2012), but stomatal response to the environment depends on the genotype and their strategy (isohydric or anisohydric) to cope with water stress while optimizing thermal regulation (Chaves et al., 2010; Chaves et al., 2016; Simonneau et al., 2017).

Current studies on crop response to high-temperature stress are mainly focused on the effects of air temperatures on the aerial part of plants/crops (shoots, leaves) and its immediate environment, while the potential adverse effects of high ST are less examined (Dong et al., 2016; Costa et al., 2019). This applies to the effects of day time and night time temperatures under scenarios of warmer nocturnal air temperatures that tend to increase root-zone ST (Dong et al., 2016).

Metabolic processes such as respiration, photosynthesis and transpiration are sensitive to short-term temperature fluctuations and air and ST influence carbohydrate relations in grapevine (Chaves et al., 2016; Venios et al., 2020; Gambetta et al., 2021). Carbohydrate reserves are most abundant in grapevine roots, and ST regulates their mobilization to shoot and trunk (Rogiers et al., 2011). Soil warming up to 24°C promote shoot growth by increasing the use of starch reserves, while soil cooling (13°C) result in starch accumulation in both roots and stem and shift the overall biomass partitioning to the root system (Field et al., 2020). High night air temperature reduces carbohydrates exportation from leaves, but promotes respiration resulting in lower leaf carbohydrate contents (Tombesi et al., 2019). Moreover, higher air temperatures coupled with higher surface ST during early evening may promote excessive carbon loss due to maintenance of higher respiration (Escalona et al., 2012). Therefore, carbon losses and modified carbohydrate dynamics due to increased respiration must be better quantified for vines growing under dry and warm conditions (Medrano et al., 2016).

In grapevine, pot-based experiments showed that the highest biomass production and shoot growth rates were achieved under warmer treatment regimens (24°C compared to 13°C) (Field et al., 2020). However, above certain critical temperatures, growth is often hindered due to lower net assimilation rates, and in more extreme cases leaf overheating and death (Chaves et al., 2016). Supra-optimal ST may also affect the root system. Root survival can be negatively affected by ST above 35°C, which can lead to root death

(Huang et al., 2005). Fluctuations in air temperature and ST modulate grapevine's morphology. ST can affect root characteristics (size, architecture, and function) (Luo et al., 2020; Gavelienė et al., 2022). Exposure of plant roots to temperatures above their optimum often decreases primary root length and lateral root density, reducing the volume of soil explored by roots and consequently, reducing water and nutrient uptake (Koevoets et al., 2016). Meanwhile, the root architecture may dynamically adapt to spatial and temporal temperature changes by acclimation of root structure and geometry (Nagel et al., 2009; Fonseca de Lima et al., 2021; Fichtl et al., 2023). Nevertheless, it is difficult to quantify the effect of root temperature in real conditions, as the distribution of roots changes according to soil characteristics and conditions of the soil surface (Pradel and Pieri, 2008).

Temperature influences grapevine hormonal relations at root and shoot level (Walker and Winter, 2006; Chaves et al., 2016; Field et al., 2020; Bernardo et al., 2021). Soil temperature was found to regulate hormone content such as cytokinins (CKs) in grapevine xylem sap (Field et al., 2020) and also of abscisic acid (ABA) (Bernardo et al., 2021).The effects of ST on vine performance still need to be better quantified and the interaction between air and ST and soil moisture on vines must be evaluated for their physiology and agronomical performance under extreme dry and high-temperature conditions

3.2 Vineyard weeds and spontaneous vegetation

Soils of vineyards in the Mediterranean region are often subjected to intensive labor to reduce or eliminate competition by light, water, and nutrients, between vines and the weedy flora. Therefore, vineyard landscapes depend on a great investment in tillage, mowing, or herbicide application (Winter et al., 2018). Intensive soil management practices result in increasing ST with feedback loops on soil seed bank, altered seed dormancy, seed longevity and germination patterns, along with general plant composition changes towards resilient species to heat and water stress (Kathiresan and Gualbert, 2016). ST and soil moisture are key determinants for seed dormancy breaking and a trigger for germination, along with exposure to flashes of light on non-deep buried seeds caused by soil disturbance (Sauer and Struik, 1964). Higher ST due to bare soils and warmer climate conditions can promote synchronized mass seed germination of certain species, resulting in homogeneous and well-adapted weedy plant communities, which can be more damaging if agrochemicals (fertilizers and pesticides) are used, stimulating growth and weed resistance to herbicides. However, the mechanisms and traits of weed species can differ, and for some summer annual species, dormancy breaking occurs under low ST conditions, whereas optimal germination is trigged by higher ST (Forcella, 1998). Soil growing degree days (GDD) has been used with success for weeds, estimated from ST to predict emergence rates of weed seedlings. Following germination, higher air temperature and ST usually favoured rapid development of weed seedlings and plant growth, and GDD based on air temperatures can be used to estimate post-emergence seedling height growth (Forcella, 1998).

The proportion of bare soil was tested as a predictor of the taxonomic diversity of plant communities and vine yield, and results pointed to slightly lower berry productivity for higher plant diversity, corresponding to lower bare soil area, and lower ST (Guerra et al., 2022). Nevertheless, soil management decisions made by winegrowers involve cost-effectiveness of weed control that needs to be addressed locally and over the long term. These factors include, for instance, the weed resistance to herbicides, the role of weeds as a refuge for pests and diseases, or as resources for pollinators and pest predators, amongst other goods and services that spontaneous flora can provide to the well-being and society (Paiola et al., 2020). Higher ST promotes the dominance of exotic weedy species from tropical climates, such as *Conyza* spp. or native Mediterranean species that have clear positive photoblastic germination mechanisms, such as *Dittrichia viscosa* (Parolin et al., 2014). Evolutionary mechanisms of exotic annuals or seed-dispersed perennials out of the native range are likely to take place as an environmental adaptation, which increases the risk of unbalanced agroecosystems (Clements and Ditommaso, 2011; Garcia et al., 2018).

Effects of extreme ST on plants ecophysiology were studied for a few species and mostly on crops or grasslands but in general, extreme high ST affected photosynthesis by reducing carboxylation efficiency, with differences between C3 and C4 plants (Nóia Júnior et al., 2018). A reduction in leaf stomatal conductance, relative water content and increased concentration in intercellular CO_2 occurred and C4 plants are likely to be more affected than C3, given the differences in photosynthetic pathways. In turn, extreme low ST on C4 plants resulted in higher leaf stomatal resistance and reduced photosynthetic rates.

Weeds and spontaneous vegetation present diverse seasonal dynamics that, together with the vine's phenology, produce a dynamic ecosystem across time and space, on the rhizosphere and above ground. Relations with ST and vineyard management must be addressed by looking at the seasonality of the complex of crop-spontaneous vegetation and weedy flora, and the constraints and objectives of wine producers (Garcia et al., 2018).

3.3 Soil organisms

The biological component of the soil is a vital part of agricultural ecosystems, including vineyards, and is composed of a diverse set of macro- and micro-organisms like insects, myriapods, worms, nematodes, bacteria, archaea, fungi, actinomycetes, protozoa, algae (Pritchard, 2011; Sassenrath et al., 2018). They compose the soil food web and can be divided into four groups according to their body size and functional roles: micro-organisms and macro-, meso- and micro-fauna (Giffard et al., 2022). Those organisms have important ecological functions and lead crucial processes in the soil that determine soil and plant health, such as OM decomposition, nutrient cycling, soil structure improvement, including soil aeration and increased water and nutrient retention, as well as pest, disease and weed control (Giffard et al., 2022). Climate change and increased ST are likely to affect their diversity and

community dynamics, which may have a strong influence on the overall food soil web and on the ecosystem services they provide, which may ultimately affect grapevine performance.

3.3.1 Soil microbiota

In the particular case of soil microbiota, a rich and diverse community of soil microorganisms can ensure productive soils, because they largely influence nutrient cycling and soil fertility, promote pathogen suppression, enhance CO_2 sequestration and increase soil OM mineralization rates. Due to those crucial roles for agro-ecosystem functioning, microbial biodiversity is considered as an important determinant of the *terroir* (Gobbi et al., 2022).

In vineyards, soils are the main reservoir of microorganisms for the grapevine phyllosphere and endosphere, since every growing season, aboveground plant organs, including leaves and berries, obtain their microbes mainly from the soil (Compant et al., 2011; Zarraonaindia et al., 2015). Nowadays, it is well accepted that balanced grapevine–associated microbial communities are essential not only for plant growth and biotic and abiotic stress tolerance (Pinto et al., 2014), but affect the organoleptic properties of the must (Zarraonaindia et al., 2015). It can also influence the fermentation dynamics at the winery and ultimately wine quality (Belda et al., 2017). Hence, any changes in soil microbial community composition and structure may influence plant performance and berry composition, given their direct and indirect influence on grapevine growth, health, stress tolerance and berry development (Di Giacinto et al., 2020).

Temperature is one of the most important determinants of microbial growth and metabolic rates. However, the assessment of the overall soil microbial community response as a function of temperature is still challenging (Jansson and Hofmockel, 2020) and an acclimatization of microbial communities to soil warming cannot be excluded (Pritchard, 2011; Snyder and Callaham, 2019). The increase of ST may have two contrasting consequences on soil organisms and microbiota. Since there is a linear relationship between temperature and respiration, it could be expected that in response to temperature rises, soil microbial respiration will also increase, releasing CO_2 to the atmosphere (Bond-Lamberty et al., 2018), which contributes to the greenhouse effect, and further temperature increase. On the other hand, at higher temperatures, the metabolism of OM decomposers (mainly fungi) is expected to be more active (Schindlbacher et al., 2011) and, therefore, microbial OM mineralization rates could be faster. Under such circumstances, nutrient release is accelerated (e.g. nitrogen), which can be translated into faster plant growth, contributing to carbon sequestration. This issue is even more complex, with several other interacting factors in the local soil environment (e.g. high temperatures also affect the nitrification process or soil oxygen concentration) that ultimately affect microbial growth and metabolism (Bai et al., 2013; Hu et al., 2016; Jansson and Hofmockel, 2020).

Some studies demonstrate that as temperature increases, population shifts, and variations in microbial community structure and changes in functional genes occur (Zhang et al., 2005; Schindlbacher et al., 2011; Melillo et al., 2017; Romero-Olivares et al., 2017). In the case of vineyards, in a recent study across the world, Gobbi et al. (2022) found a positive correlation between temperature and fungal alpha diversity, but not between prokaryotic alpha diversity and temperature, indicating that fungal communities might be more sensitive to temperature than soil bacteria and archaea. Větrovský et al. (2019) also found that among the different environmental factors (climatic, soil and vegetation parameters), temperature and precipitation were the key factors regulating fungal diversity and community composition in soils. Nevertheless, to our knowledge, no studies have been conducted yet in vineyards to assess the direct effect of an increase in ST in key microbial soil processes as well as in the community composition/structure of soil micro-organisms. In particular, due to the major roles of fungal communities in vineyard soils as OM decomposers, as plant mutualists (and therefore promoting plant tolerance to stress factors) and also as pathogens, more knowledge is needed on the specific ways in which ST affects these communities. This will allow to develop better strategies to manage vineyard soils to buffer the negative effects of climate change on particular soil microbial functional groups.

3.3.2 Soil fauna

Soil macro, meso and micro-fauna have important roles in the soil. They are involved in OM decomposition, attract microbial communities that mineralize nutrients, and contribute to improve soil structure by creating aggregates and soil pores and mixing the soil (Culliney, 2013). For instance, macroinvertebrates like earthworms, ants and thermites can move large portions of soil, creating new microhabitats for other soil organisms, and can assimilate plant materials, integrating them into the soil as OM (Snyder and Callaham, 2019). On the other hand, isopods and myriapods promote OM decomposition by feeding on carbon-based compounds and by excreting enzymes and feces into the soil, thereby enhancing the proliferation of microbial decomposer communities, that release nutrients into the soil (Hendrix, 2000; Zagatto et al., 2021).

The study of how soil warming affects soil fauna is challenging, since depending on the methodological approach (air, soil or air and soil warming), the outcome can be substantially different (Snyder and Callaham, 2019). Therefore, artificial/experimental temperature manipulation may not lead to a real response of soil fauna under natural conditions (Snyder and Callaham, 2019), which makes drawing conclusions somewhat challenging. Moreover, ST and moisture are directly linked, and therefore, differentiating the independent effects of each factor on soil fauna is often difficult. In addition, distinct taxonomical or functional groups may react differently to ST increases and to the indirect effects that this entails in the soil ecosystem.

In a model described by Snyder and Callaham (2019), the increase in ST leads first to changes in animal behavior, such as up- or downward movements in the soil profile. It can also lead to physiological changes that have consequences on their fitness and reproduction, with a subsequent effect on soil animal taxa abundance, community structure and diversity. This can

ultimately lead to changes in their functions, including nutrient cycling, OM decomposition and soil respiration. In their review, Snyder and Callaham (2019) also present a conceptual model generalizing how soil warming may affect the soil environment and summarize the direct and indirect interactions that may occur between vegetation, microorganisms, soil macro-, meso- and microfauna and OM under a global warming context.

Although some studies already describe the diversity and ecosystem functions of soil fauna in vineyards (Ghiglieno et al., 2020; Gonçalves et al., 2021; Andrés et al., 2022; Giffard et al., 2022), much less information is available on the effects of soil warming on soil fauna (Snyder and Callaham, 2019). It is known that agricultural soils, and thus vineyards, tend to have low soil invertebrate diversity, which are often characterized by species that are well-adapted to environmental disturbances (Callaham et al., 2006). However, the management regime can strongly influence their populations, with a generally beneficial effect observed in arthropod abundance and diversity in organic vineyards (Caprio et al., 2015; Gagnarli et al., 2015; Ghiglieno et al., 2019; Ghiglieno et al., 2020; Bosco et al., 2022). According to Blankinship et al. (2011), soil fauna may be more sensitive to soil moisture content, and therefore to changes in precipitation, than to mild increases in temperature.

In a study conducted by Ghiglieno et al. (2020) in Italian vineyards, Collembola and Acari were the most frequent taxa observed in vineyard soils. While taxa of the first group showed a variety of responses related to ST, Acari, as well as Thysanoptera, Diplopoda and Hymenoptera were more related to lower ST. Contrastingly, Diptera, Isopoda, Hemiptera, as well as Coleoptera and Diptera larvae taxa abundance, was related to higher ST. This may lead to the hypothesis that the latter taxa may be more negatively affected by soil warming in the context of climate change than the former group, which could be benefited, since they appear to be thermophilic (Eisenbeis and Wichard, 1987; Reddy and Venkataiah, 1990; Zhu et al., 2018). This would be the case of ants, as some studies showed that they increase their activity at higher temperatures (Dunn et al., 2009; Snyder and Callaham, 2019).

More knowledge is needed on how soil fauna, in particular invertebrates, will react to soil warming and the associated changes in the above-ground vegetation and soil moisture. Understanding how those animal communities will respond to increased ST may help to decide on the most appropriate vineyard soil management strategies that can buffer ST changes and foster the proliferation of taxa that benefit both soil and crops.

4 Soil temperature measurements

Ecological patterns and processes are often more related to below-canopy soil temperature rather than to well-ventilated air temperatures (Lembrechts et al., 2022. Moreover, near-surface, rather than air temperature can work as better predictors of ecosystem functions and processes such as OM decomposition, soil respiration and other components of the global carbon balance (Lembrechts et al., 2022). Therefore, ST measurements are highly relevant and needed to achieve good reference data for specific ecological conditions as well as to use ST as a major variable to support modelling of ecosystem processes. However, due to the complexity and large labour costs of ST measurements, *in situ* observations of ST are less commonly described in literature than those of precipitation and air temperature (Li et al., 2020).

Soil surface temperature measurements are usually carried out with thermocouples and radiometers, but these devices have limitations concerning logistics, access, and technician costs (Frodella et al., 2020). In turn, measuring ST at different depths can be done with different types of thermometers and sensors installed at various depths (Abdel-Ghany et al., 2022), which do requires knowledge and significant manpower.

Consequently, there is a general consensus about the need to achieve soil spatial information (e.g. temperature, moisture) faster and with fewer human resources. The use of remote sensing technologies can offer an alternative or a complementary solution for localized and punctual measurements as it allows to retrieve a larger set of spatial data to study vegetation or soil properties at different resolution scales (temporal and spatial) (Jones and Vaughan, 2010; Wulf et al., 2014). In the last decades, it has increased the interest in developing methodologies for remotely measuring soil surface and vegetation temperature and to assess soil moisture conditions by using spaceborne, airborne or ground-based sensors (Jones and Vaughan, 2010; Krapez et al., 2012; Frodella et al., 2020; Xu et al., 2020; Diago et al., 2022).

Thermal imaging emerged as a highly flexible and non-contact measurement technique that enables small to large scale, surface temperature sensing and it can be used as an alternative or as a complementary tool for conventional soil surface temperature and moisture monitoring technologies, in a wide variety of geo-environmental and agricultural applications (Jones and Vaughan, 2010; Frodella et al., 2020; Zeng et al., 2021; Diago et al., 2022). Ground-based thermal imaging sensors, such as thermal cameras, experienced a fast technological development (e.g. focal plane array uncooled microbolometer sensors) that increased detectors' accuracy, spatial resolution, and decreased costs (Jones and Vaughan, 2010).

Thermal imaging was successfully used in viticulture to monitor canopy and ST variation at different time scales and different irrigation conditions (Jones et al., 2002; Gutiérrez et al., 2018; Costa et al., 2019; Gago et al., 2020; Diago et al., 2022; Kustas et al., 2022) (Figure 3). Thermography has been used to monitor the effects of soil, irrigation and different soil covering materials on ST in vineyards (Frodella et al., 2020). Other studies using thermography helped to characterize the mechanisms behind soil desiccation cracking (Zeng et al., 2021), or to study heat transfer processes in vineyards (Kustas et al., 2022). Thermography has been also used as an alternative method to monitor and detect soil microbial activity (Schwarz et al., 2021).

Satellite remote sensing has been developed for thermal applications, but data calibration and validation remain complex and costly (Frodella et al., 2020). Indeed, there are still limitations concerning image resolution because satellite measurements still have limited spatial and temporal resolution (Basurto-Lozada et al., 2020), when considering their practical aplication to field crops.

Atypical vineyard canopy architecture and row disposition, characterizes by a large amount of bare soil/cover crop separating rows of trellised vines, which demands higher imaging spatial resolution (of one meter or less) for high robustness (Basurto-Lozada et al., 2020). Nevertheless, a combination of ground-based *in-situ* measurements with aerial and satellite imaging may be a solution to monitor ST more effectively (Xu et al., 2020). The same applies to methodological approaches based on the fusion of information retrieved from thermal and multispectral sensors to generate estimates of ST by using computational intelligence models (Basurto-Lozada et al., 2020).

Other techniques such as soil resistivity measurements can be used as a proxy for ST: soils with high resistivity have generally coarse-textured and are warm in contrast to low resistivity soils that are richer in clay and are cooler (Van Leeuwen et al., 2020). Soil electrical conductivity (or its reciprocal soil electric resistivity) reflects a combination of soil mineralogy, salts, moisture and texture, which makes it a robust parameter to characterize soil properties. The advantage of this proximal sensing methodology gives high-resolution maps of the soil resistivity, which can be further related to ST. Furthermore, regression equations have been developed to predict and map moisture content, topsoil thickness, and clay content (Samouëlian et al., 2005).

The development of digital soil science, that is the study of soil using the tools of the digital convergence (Wadoux and McBratney, 2021), also opens new possibilities for imaging studies applied to ST and their effects on plants and soil. In addition, the existing cooperative works and data sources on ST (Lembrechts et al., 2022) can open new opportunities to use ST data in agriculture.

5 Strategies to manage soil temperature in vineyards

Sustainable water and soil management are the core of several sustainability programs in the wine sector (Costa et al., 2022) and other perennial woody crops, such as olive groves and almond orchards (Andrade et al., 2014; Garcia-Tejero et al., 2018; Fraga et al., 2021). More sustainable practices related to soil management can help to alleviate the harmful effects of more extreme drought and heat events due to climate change. This is an increasingly important issue for Mediterranean viticulture and must combine effective soil and canopy management strategies (Figure 4), together with more efficient use of irrigation water and better-adapted varieties/rootstock combinations (Andrade et al., 2014; Costa et al., 2016; Cataldo et al., 2021; Naulleau et al., 2021).

Irrigation is probably the most important and effective short-term adaptation strategy to face the impacts of climate change in Mediterranean vineyards, attending to its high effectiveness in moderating thermal microclimatic extremes at both soil, plant and atmosphere levels (Andrade et al., 2014; Costa et al., 2016) (see Figures 3, 5). Watering and higher soil moisture promote transpiration and the related evaporative cooling in plants, and also favor soil water evaporation (Figures 1, 3). As a result, irrigation in

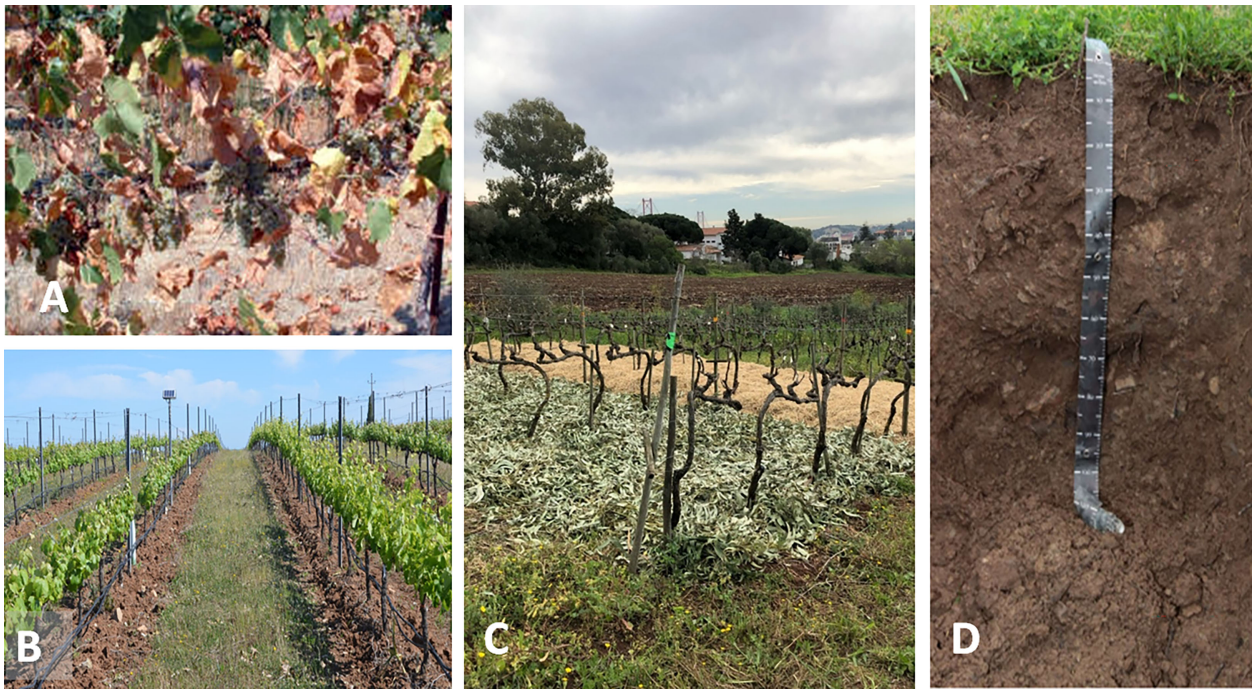


FIGURE 4
(A) The impact of dry and extreme heat in the basal leaves of a Mediterranean vineyard (South Portugal) and sustainable management practices in Mediterranean vineyards, **(B)** Soil grass cover in the inter row combined with row tillage in a vineyard in Alentejo's wine region (South Portugal), **(C)** Mulching with different organic materials (rice straw and *Eucalyptus* foliage) and **(D)** Soil profile characterization as a tool to support best practices in soil management (ISA campus U. Lisboa).

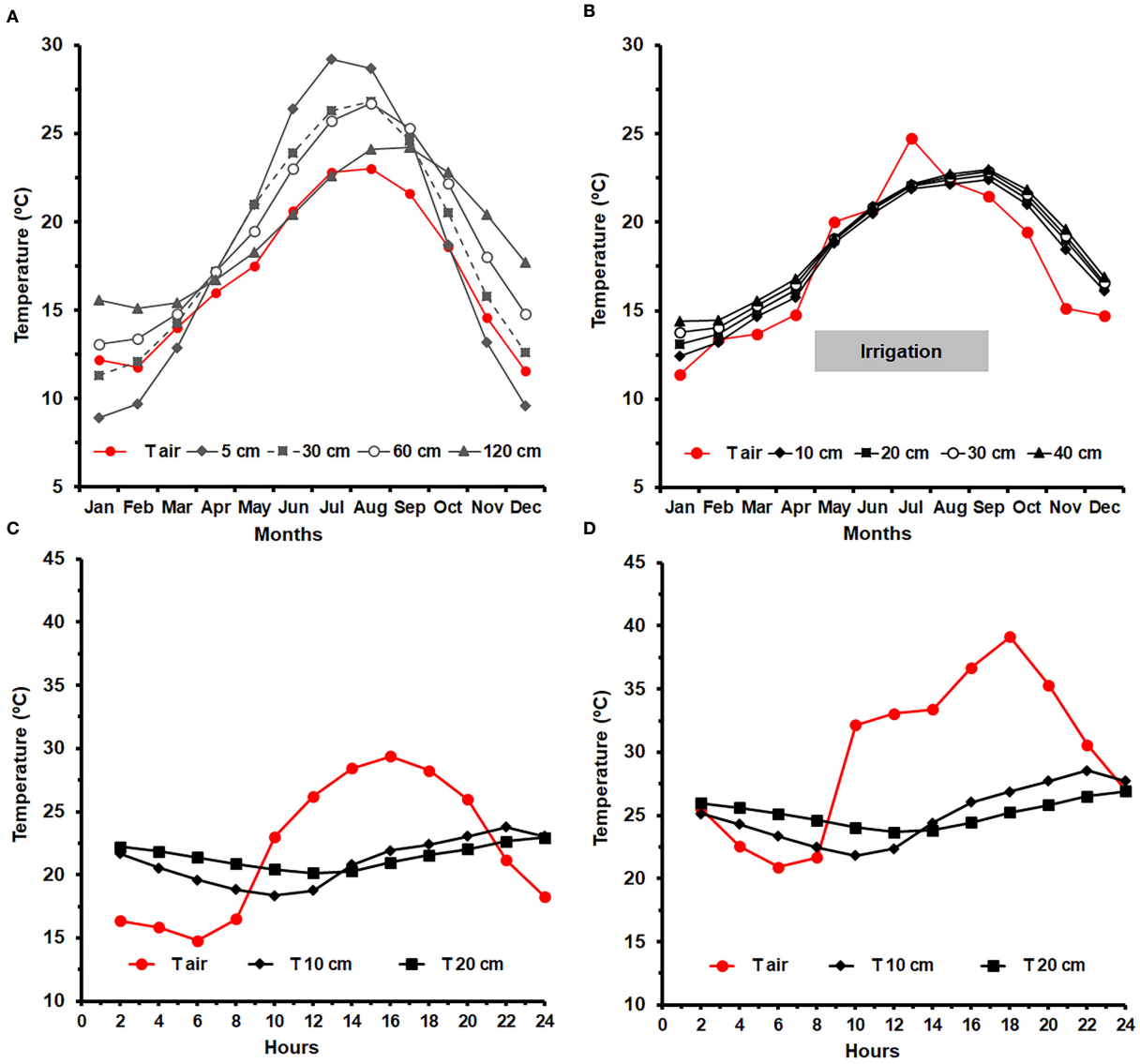


FIGURE 5
(A) Annual variation of soil monthly mean temperature (ST) at 5, 10, 30, 60 and 120 cm depth, for a 30-yr period (1931-1960), measured at the campus of the Instituto Superior de Agronomia (ISA), Lisbon (38°42'27.5"N; 9°10'56.3"W), under rainfed conditions (Botelho da Costa, 1995). **(B)** Monthly variation ST during the year of 2022, at 10, 20, 30 and 40 cm, in an irrigated vineyard, at ISA (data provided by Hidrosoph, Oeiras, Portugal). The red line indicates variation in the monthly mean air temperature, and the peak in July relates to a heat wave event; Daily ST variation during 1st July 2022 **(C)** and 9th July 2022 **(D)**, respectively before and at the end of a heat wave, measured every two hours at 10 and 20 cm depth, in the irrigated vineyard; the red line indicates the air temperature.

vineyards expanded fast in southern Europe (Costa et al., 2016; Fraga et al., 2018; Gambetta et al., 2021), but water resources are increasingly scarce and demand more precise irrigation management in Mediterranean vineyards (Mirás-Avalos and Araujo, 2021).

A detailed soil characterization (soil profile, soil properties, fertility) (Figure 4) in new vineyards and in the already installed ones is crucial to support more efficient irrigation and fertilization programs. Soil characterization is also essential for an effective distribution of soil water sensors across the vineyard. In addition, thermal measurements of soil, air and plants (punctual and image-based) coupled with computer–based information systems can support Decision Support Systems (DSS) (Figure 6) for more efficient vineyard management (Costa et al., 2020; Naulleau et al., 2021). DSS systems using these multi-parameter thermal data besides supporting precise irrigation strategies are potential indicators of water and heat stress in vineyards that can help to predict and mitigate climate risks.

In addition to irrigation, ST (and plant temperature) can be regulated in vineyards under warmer and dryer conditions, by promoting the use of spontaneous soil cover vegetation, selected cover crops, or mulching. Soil cover protects against soil erosion, increases infiltration and water retention, reduces evaporation, and in the case of living mulches or maintenance of adequate spontaneous vegetation, they act as a source of nutrients and OM, and can improve physical, chemical and biological conditions

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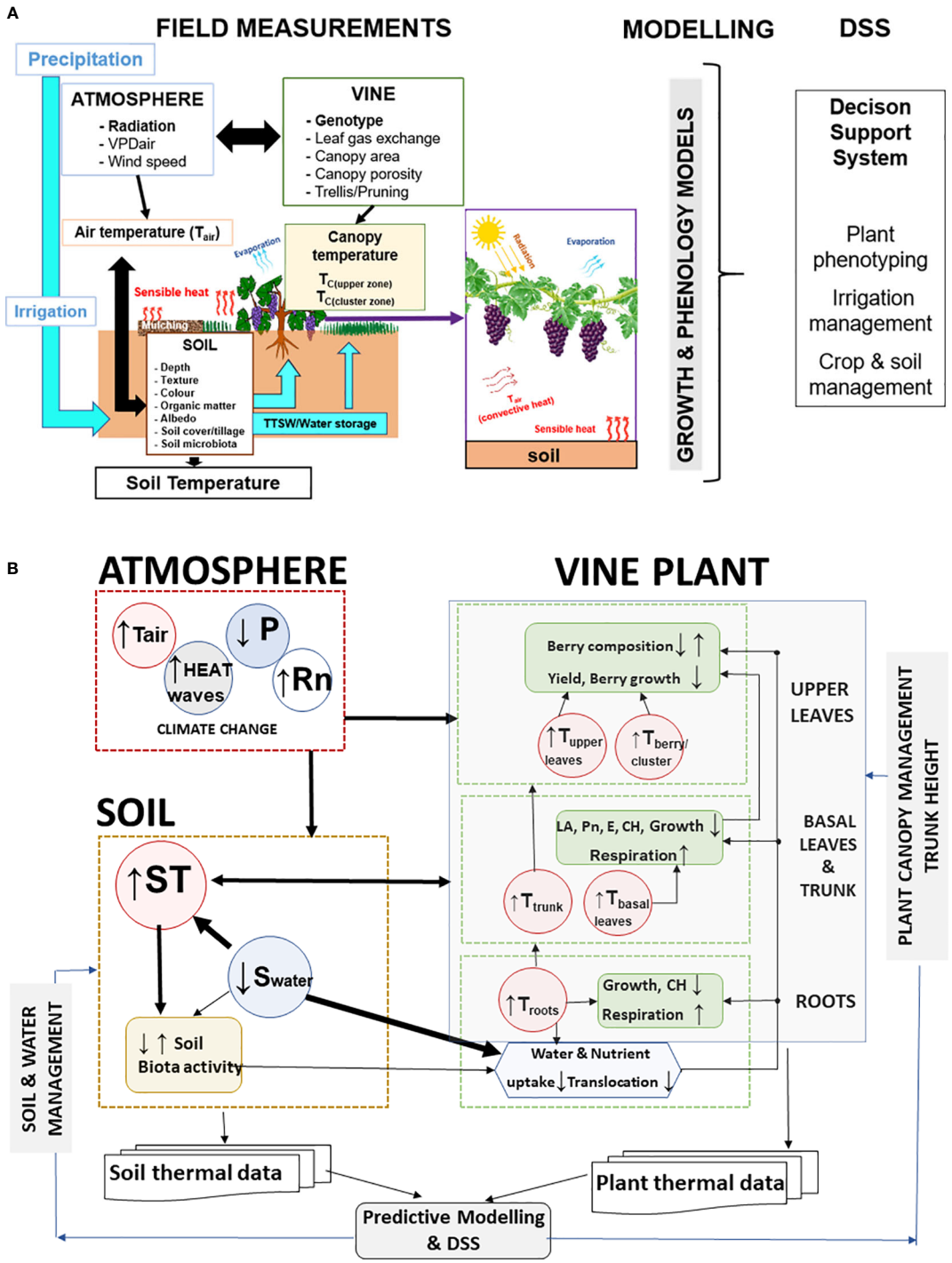

FIGURE 6
(A) Diagram illustrating the potential interactions between soil, canopy and berries in terms of heat exchange and temperature regulation and the use of air temperature (T_{air}), soil temperature (TS), canopy temperature (T_C), and cluster or berry temperature as parameters to feed models to support decision on plant phenotyping, and vineyard management (irrigation, canopy and soil). TTSW – total transpirable soil water; VPDair - Air Vapour-Pressure Deficit (Adapted from Costa et al., 2020). **(B)** Conceptual framework showing the interaction between soil temperature (ST) and soil water (Swater) on vines temperature (roots, trunk, basal and upper leaves, berry/cluster) in a context of climate change (higher radiation -Rn, air temperature -Tair, less precipitation - P). Atmospheric conditions and ST influence vine plants T and morpho-physiological and biophysical processes (Leaf area - LA, Net photosynthesis - Pn, Transpiration - E, Carbohydrates - CH, Soil temperature - ST) depends on atmospheric climate conditions (Rn, Tair, P). Higher Rn and Tair and lower P, promote soil warming and increase evapotranspiration. Soil and plant management influence the thermal regulation of both soil and plants and can be optimized by thermal data (atmosphere, plant and soil) and Decision Support Systems - DSS.

(Marshall et al., 1996; Lazcano et al., 2020). Specifically, natural or spontaneous vegetation cover can also stimulate deeper vine root distribution and promote the use of resources in deeper soil layers (Pradel and Pieri, 2000). The replacement of mineral fertilizers and herbicides with cover crops or vegetation will take years to have a proper impact on soil nutrients and microbial activity, apart from the need to monitor and maintain soil cover (Döring et al., 2019).

Mulching can help to control pests and weeds and maintain yield levels under adverse climatic conditions. Fraga and Santos (2018) investigated the effects of mulching in a typical Mediterranean climate region in Southern Portugal, under future climate change scenarios. Although ST was not directly addressed, their results suggest that mulch can mitigate the adverse effects of hotter and drier weather and extreme events, expressed by an estimated increase of yield by 10 to 25% as compared to bare soil vineyards. The use of soil covering material (mulches) influences maximum summer ST, minimum winter ST, as well as the daily ST fluctuations. In apple orchards, for example, plastic mulching promoted more extreme maximum and minimum ST, but the effect on weed control and water losses was positive and resulted in no major negative effects on plants' performance (Neilsen et al., 1986).

A more sustainable soil management involving no-tillage or improved tillage strategies is key to minimize soil erosion, decrease soil compaction and avoid the formation of impermeable layers which influence soil thermal and water regimes, nutrient cycles and crop performance. Tillage strategies must be based on a good spatial characterization of the soil profile and properties, avoiding the numerous drawbacks of its use and at larger spatial scales, as it hampers surface water run-off, increases greenhouse gas emissions, difficult the groundwater recharge and promote biodiversity losses (Gürsoy, 2021) (Figure 4).

Adaptation to increasing ST may encompass larger rooting depth and involve the use of rootstocks with a wider root-zone temperature optimum to enhance the future performance of woody perennial crops (Koevoets et al., 2016; Darriaut et al., 2022). Therefore, selecting new rootstocks to specific environments should be a challenge to face cultivation problems associated with global climate change.

Other strategies to minimize soil and canopy insulation, control ST and protect crops from light stress and high temperatures may be envisaged. However, they are costly and/or may have negative environmental impacts (e.g. visual pollution; recycling issues). This is the case of the use of shading nets in VSP trellis systems as a strategy to mitigate the negative impact of heat waves and sun exposure of berries. Indeed, partial shading (less than 60% of solar radiation) at the cluster zone reduced by about 4 °C the cluster temperature as compared to sunlit clusters (Marigliano et al., 2022). Shadowing in combination with water availability can avoid berry dehydration during the last phases of ripening with positive effects on anthocyanins and flavonols, as compared to sun exposed clusters (Martínez-Lüscher et al., 2020; Marigliano et al., 2022). Nowadays, the installation of photovoltaic panels over crops ("agrivoltaic" farming) is being advertised as a win-win solution for climate change adaptation of vineyards and to produce energy (Frauhnofer, 2022). Nevertheless, vine's morpho-physiological responses must be taken into account and the respective cost-benefits analysis must be carried out, along with the quantification of the damaging effects on the landscape (e.g. loss of the aesthetics of the natural rural landscapes). Furthermore, row orientation has a dramatic effect on the vine's exposition to sunlight and consequently on ST and canopy temperature (White, 2015; Hunter et al., 2020; Pisciotta et al., 2021). In addition, row spacing, and trellis design influence ST by varying the percentage of shading of solar radiation on the soil. For example, the soil layers of east – west oriented rows reach their highest temperature in the afternoon, and ST generally increases in the two top layers and decreases in the lower layers from mid-morning to late afternoon (Hunter et al., 2020). A combination of higher planting density with shading can also be considered, which favors both natural and artificial shading in vineyards and minimizes the impacts of extreme radiation and heat conditions on both crops and soil cover crops and benefit the activity of the relevant soil micro-organisms (microbiota).

6 Conclusions

More sustainable agricultural, hydrological, and environmental management in the context of climate change demands a better understanding of soil resources variability, at increasingly higher resolutions (Wulf et al., 2014). Though soil temperature maps are already available for many regions of the world (Lembrechts et al., 2022), high resolution data on ST that can be representative of microhabitat conditions for below-ground organisms is still needed, and especially for deeper soil layers.

The effects of spatiotemporal variation of temperature on ecological processes and functioning of agroecosystems has been investigated but the predictive capacity remains low, and more studies focused on the interaction of soil-organisms-crop productivity and quality are still required (Pipan et al., 2021). This knowledge at fine scales would help to better understand the roles of soil and soil management on climate change adaptation and will help to cope with current and future challenges of climate change by supporting predictive modelling and decision-making applied to perennial crops systems, such as grapevine or other typical Mediterranean crops

Long-term field measurements using sensors of both ST and soil moisture are being developed and tested in vineyards and other perennial crops (Garcia-Tejero et al., 2018; Costa et al., 2019; Wild et al., 2019). Thermal sensors have become less expensive and offer larger robustness and energy autonomy though limitations such the low contact of the logger in certain soil types (e.g. drying clay soils) were reported (Wild et al., 2019).

Climate warming may have diverse effects on ST according to the diverse types of heat stress (heat shocks, heat waves, or increasing warming conditions), leading to diverse physiological and molecular responses at leaf and fruit levels, and on root morphology as well as on reproductive traits. There is evidence that the phenological stage of crops influences crops vulnerability to increase temperature, either by pulses or in a continuous trend (Jagadish et al., 2021). This applies to grapevines. Future research should encompass a better understanding of the mechanism(s) by

which ST affects leaf and berry traits across different grapevine varieties, clones and/or rootstocks.

Soil remains sidelined in viticulture research, suggesting a lack of attention to non-new but highly relevant issues such as the detailed spatial distribution and characterization of soil types before designing and planting new vineyards. As consequence there is an urgent need to improve monitoring and better evaluate the roles of soil properties and ST in Mediterranean vineyards, which are increasingly exposed to more adverse climatic conditions and increasing irrigation limitations (Costa et al., 2016). A better understanding of the roles of soil properties on soil microbiota, weed and vine morpho-physiology, and on heat and water fluxes, is crucial to achieve a more efficient soil and crop management, and to ensure a more sustainable Mediterranean viticulture under extreme stress conditions. This achievement will certainly contribute to the Sustainable Development Goals, namely in terms of soil and biodiversity protection and more sustainable water management. Ultimately, we must consider the feasibility and economic implications of the proposed management strategies that vary with the wine regions and the fact that improved soil and canopy management solutions will be only achieved with multidisciplinary knowledge and more trained professionals.

Author contributions

JMC: idea, writing, editing, reviewing and funding. MM, FA, AN and RE writing, editing, and reviewing. JMC, FA, PM: illustrations and editing. All authors contributed to the article and approved the submitted version.

Funding

JMC and AN were funded by national funds through FCT – Fundação para a Ciência e a Tecnologia, I.P., under the project UIDB/04129/2020 of LEAF-Linking Landscape, Environment, Agriculture and Food, Research Unit. MM and FCA acknowledge the support by the Centro de Estudos Florestais, a research unit funded by Fundação para a Ciência e a Tecnologia I.P. (FCT) Portugal, through the project UIDB/00239/2020 and FCA is supported by national funds *via* FCT under the contracts Norma Transitória - DL57/2016/CP1382/CT0028.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Plant Science

TYPE Review
PUBLISHED 11 July 2023
DOI 10.3389/fpls.2023.1227628

OPEN ACCESS

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RECEIVED 23 May 2023
ACCEPTED 15 June 2023
PUBLISHED 11 July 2023

CITATION
Poni S, Frioni T and Gatti M (2023) Summer pruning in Mediterranean vineyards: is climate change affecting its perception, modalities, and effects?
Front. Plant Sci. 14:1227628.
doi: 10.3389/fpls.2023.1227628

Summer pruning in Mediterranean vineyards: is climate change affecting its perception, modalities, and effects?

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Summer pruning encompasses a series of operations typically performed on the grapevine during the growing season. This review provides an update on the research conducted over the last 20 years on the modalities and strategies of main summer pruning operations, which include shoot positioning and thinning, shoot trimming, leaf removal, and cluster thinning, with a special focus on their adaptation to climate change occurring in Mediterranean areas. Three main novelties emerged from the survey. First, due to a common need to shelter clusters against overheating and sunburn-related damages, shoot thinning and leaf removal are practices that are now being applied in a much more cautious and conservative manner. Second, the meaning of summer pruning is evolving because operations are being used as precious tools to direct ripening toward a desired direction rather than being received passively. Third, some operations, such as leaf removal, have disclosed very high plasticity, which means that, depending on the timing and modalities of the intervention, yield can be either increased or decreased and ripening anticipated or postponed. In an era where economic and environmental sustainability have to find a good compromise, cluster thinning is increasingly being depicted as an extraordinary operation that should be left to occasional occurrences of overcropping. Moreover, summer pruning is a tool through which growers can, to an extent, exploit the potentialities offered by climate change. For instance, the crop-forcing technique, under the different configurations of single and double cropping within the same season, has been trialed promisingly in several regions and cultivars. The principle of forcing is to unlock the dormant bud during the first year by removing at least the young organs present on the shoot within a time window between the end of the flowering and pea-size stages. In particular, when it is applied in a double-cropping mode, the preliminary results related to Pinot noir, Grenache, Tempranillo, and Maturana tinta indicate that two harvests separated by 30–50 days can be obtained, with the latter having superior quality in terms of a lower level of pH and higher levels of acidity, anthocyanins, and phenolics.

KEYWORDS

vegetative growth, yield, grape composition, mechanization, source-to-sink balance

1 Introduction

Summer or green pruning encompasses any manual or mechanical operation performed in vineyards when even a minimal sign of vegetative growth is perceivable in the canopy. Therefore, in the strictest sense, even a very delayed winter pruning (Poni et al., 2022), performed, for instance, when buds on the canopy have already developed a few unfolded leaves, can be categorized as summer (green) pruning. The four main categories of summer pruning can be distinguished: shoot thinning and positioning, shoot trimming, leaf removal, and cluster thinning. All these share a common feature, i.e., to trigger a dynamic (and sometimes difficult to describe or quantify) seasonal change of the canopy leaf-area-to-yield (LA/Y) ratio, with possible impacts on the speed of ripening and based on the degree a given task changes the cluster microclimate. This feature renders the idea that any strategy for summer pruning operation unavoidably interacts with the ongoing and predicted climate change effects.

Among the examples that could be given to underline the aforementioned interaction, two are particularly effective. First, warming effects bound to climate change translate into a higher number of hot days as well as a higher frequency and/or severity of meteorological drought (Fraga et al., 2012; Schultze and Sabbatini, 2019; Van Leeuwen et al., 2019), leading to reduced canopy vigor. This explains why damages due to organ overheating, sunburn, and desiccation are nowadays considered increasing threats in vineyards. It is quite well established that several summer pruning operations do directly (i.e., leaf removal) or indirectly (i.e., a shoot trimming shifting the lateral regrowth on the apical part of the shoot and, consequently, limiting the cluster leaf cover from the basally located laterals) alter the cluster microclimate; therefore, we must question whether the technique of summer pruning needs to be adapted or changed. Second, it is similarly very stimulating that summer pruning could potentially be leveraged to address increasing complaints about environments with excessively fast sugar accumulation that becomes increasingly decoupled from phenolic and flavor ripeness.

If one perception is that the modalities of summer pruning need revisiting, perhaps a deeper change is at hand. Thanks to the vast knowledge that is presently available regarding the physiological background and expected effects of summer pruning operations, they can be turned into precious tools that the grower might utilize to orientate growth and ripening toward a desired direction, rather than perceive them as preemptive and unavoidable "things to do."

The purpose of this review was to summarize the most recent findings about the most common summer pruning operations and determine whether there is any room to re-think their interventional modalities under the pressure of past, ongoing, and predicted effects of climate change having Mediterranean areas as the main focus.

2 Shoot positioning and thinning

In the context of vineyards, shoot positioning has recently acquired a broader meaning, depending upon the evolution of training systems. In fact, at present, traditional shoot positioning, which pertains to vertical shoot-positioning (VSP) hedgerow trellises (Figures 1A–D), has a few alternatives linked to a given training system. For instance, in the case of Geneva Double Curtain (GDC) system, positioning refers to as the action of moving the shoots growing inward to an outward position (Figure 1E). This is because the two "divided" curtains will have to maintain a spatial separation during the season while radiation is allowed to reach the inner part of the two curtains. Then, depending on the choice of trellis (e.g., goblet or single high-wire cordon, with both featuring no foliage wires for shoot attachment), shoot positioning might not be required (Figure 1F). In fact, in the case of a sprawling canopy, the main aim is to obtain a naturally and mostly erect canopy that, once assisted by moderate shoot trimming, does not need any other adjustment. The research conducted on Shiraz and Grenache trained for both VSP and sprawl types (Louarn et al., 2008) has shown that non-positioned shoot systems offer the possibility of combining a high level of light interception, proper sun exposure and ventilation around clusters, and reduced labor-intensive practices for vineyards under conditions of moderate vigor. (Zahavi et al., 2001) performed the same comparison on Chardonnay and Cabernet Sauvignon, which are grown in the Golan region of Israel. This led to the conclusion that the high level of irradiance assured by the sprawl canopies was effective at reducing powdery mildew infection in moderate- and high-level diseases.

From a strictly physiological perspective, it has also been shown that when sprawl-trained Chardonnay canopies were enclosed in plastic chambers to monitor daily and seasonal whole-canopy gas exchanges, and the same canopies were squeezed between catch wires to simulate a VSP pattern, the whole-canopy net CO_2 exchange rate diminished by 26% (Intrieri et al., 1997). This demonstrates that the foliage packing typical of a VSP-constricted canopy impairs the efficiency of light utilization. Based on these positive outcomes that were once linked to the challenges imposed by the climate, it is apparent that the no-positioning option offered by the sprawl canopy type is a direction that should be pursued. This is not only useful in saving time during a notoriously labor-intensive operation but the dim light regime broken by sun flecks—which an open, semi-erect canopy can provide to the subtending clusters—is very likely a winning option when overheating and sunburn have to be mitigated (Mabrouk and Sinoquet, 1998; Downey et al., 2006; Tarara et al., 2008; Poni et al., 2018).

Shoot positioning is applied in a VSP trellis with the primary goals of avoiding shoots growing towards the alleyway, allowing better light exposure of basal nodes to be retained in winter for next year's cropping (i.e., the case of short pruning) (Figure 1B), and, above all, building an ordered and uniform vertical canopy that can be ideally shoot-trimmed to the required number of main leaves. However, it is detrimental when a canopy with large gaps and/or several shoots invading the alleyways originates due to poor or absent shoot positioning, which is sometimes coupled with inexperienced wire distancing along the canopy wall (e.g., a situation where the first couple of catch wires are too distant from the main support wire, so most growing shoots deviate too soon from verticality and escape from the wire capture track). With mechanical trimming, these will be shortened to just a few leaves above the clusters, and their ripening potential will be compromised.

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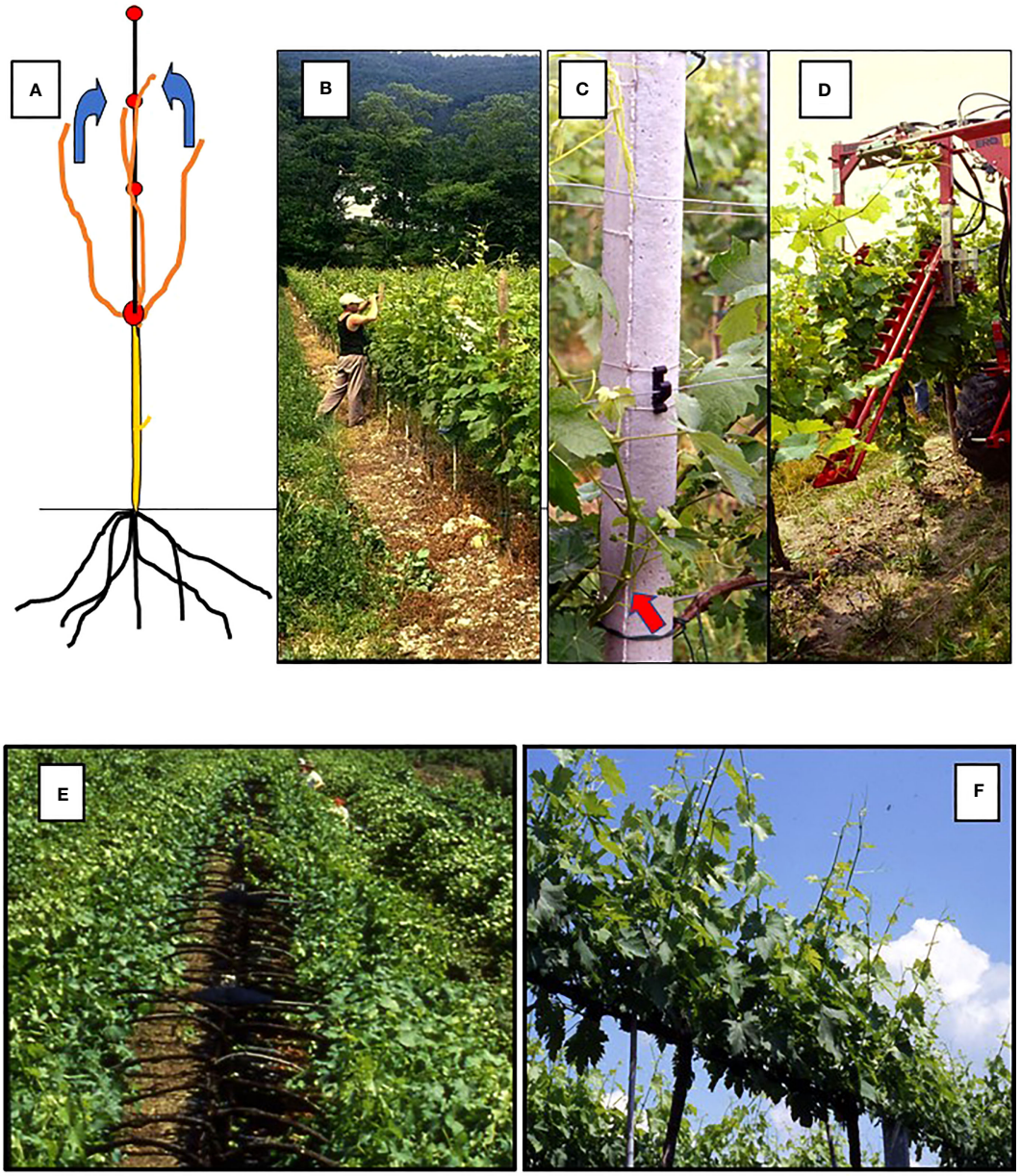

FIGURE 1
Diagram of a vertical shoot positioning (VSP) training system **(A)** and of hand **(B)**, plastic-hook aided **(C)** and mechanical **(D)** shoot positioning, The red arrow in panel B indicates the basal shoot nodes from which next season spurs will be obtained. Bottom panels: in **(E)** manual shoot positioning under way in a Geneva Double Curtain (GDC) training system: it is apparent how the operation is crucial for maintaining the two parallel canopies physically separated; in **(F)** a sprawl canopy type (single high wire cordon) which does not need any shoot positioning.

In certain viticultural regions, and especially in Tuscany, Italy, traditional shoot positioning followed by trimming in a VSP trellis is replaced with horizontally wrapping the growing shoots along the top wire and leaving them untrimmed. Faralli et al. (2022) in their two-year-long study on Cabernet Franc/SO4 grown in northern Italy, provided a direct comparison between the two solutions. They found that the wrapping treatment produced the highest polyphenol and anthocyanin contents as well as the highest must acidity. Two main reasons were provided to explain this effect: (i) higher shading is cast on clusters from more leaves concentrated at the top of the canopy and (ii) most of the laterals are still concentrated in the basal nodes as the wrapped shoots are mostly untrimmed and the apical dominance is left undisturbed, which contributes to the casting of additional leaf cover onto the clusters. When shoot wrap was applied on Cabernet Franc grown in the cool Finger Lakes Region (NY, US) no effects on grape quality were seen (Logan et al., 2021). However, in a climate quite conducive to cluster rot, shoot wrap also achieved the desirable features of reduced fruit zone lateral lengths by up to 50% and cluster compactness by up to 2.4 fewer berries per centimeter rachis.

Shoot thinning is usually applied in viticulture in medium-to-high-vigor areas as a tool to prevent excessive canopy density at the cluster level targeting a final shoot density of around 10–15 shoots/m (Smart, 1985; Reynolds et al., 2005) (Figure 2). This situation occurs

more often in cane-pruned systems when several double shoots are burst at each node, as well as in spurred systems when the development of primary shoots occurs with the concurrent growth of other shoots from secondary or base buds, thereby creating leaf clumping around the spur itself. Although the effects of shoot thinning have been somewhat neglected by the scientific community compared to those of other summer pruning operations, here is a summary of results obtained thus far: (i) as a result of an operation performed early in the season (the shoot length is, on average, around 15–20 cm), the growth compensation due to the remaining shoots usually allows for full recovery in terms of the final leaf area (Reynolds et al., 1994; Bernizzoni et al., 2011) or the total pruning weight per vine (Naor et al., 2002); (ii) a shoot thinning performed on Barbera vines, which reduced density from 30 to 15 shoots per meter, led to full canopy photosynthesis recovery at only 17 days after the thinning was performed (Bernizzoni et al., 2011); and (iii) in the large majority of cases, total soluble solids (TSS) and total anthocyanins increased with decreasing shoot density (Reynolds, 1989; Mota et al., 2010; Bernizzoni et al., 2011; Silvestroni et al., 2016) as result of reduced Ravaz index (i.e., yield-to-pruning-weight ratio) or increased LA/Y ratio (m^2/kg).

As in the case of shoot positioning, it is important to question whether the objectives and effects of a shoot thinning operation are sensitive to climate change. A study conducted on Cabernet Sauvignon grown in Oakville (CA), focused on the effects of shoot thinning and leaf removal on flavonoid content, confirming the enhancing effect of shoot thinning on sugars, phenolic substances, hue, and proanthocyanins polymerization (Torres et al., 2021). However, it was also surprisingly found that shoot thinning decreased wine's antioxidant capacity due to diminished catechin and quercetin content.

At present, when shoot thinning has to be performed to balance an otherwise excessively dense canopy, a limiting factor is that despite the deserving yet occasional attempts of mechanization (Kurtural and Fidelibus, 2021), the operation is manually performed and requires approximately 20–40 hours/hectare. This is mostly due to the difficulty in accessing the organs to be thinned and the high degree of selectivity required. However, today, robotics associated with the approaches of machine learning and artificial intelligence is seriously tackling the automation of this kind of operation, along with winter pruning (Guadagna et al., 2023; Teng et al., 2023). A similar approach is being taken for shoot thinning, with some preliminary research aiming to assess cordon shapes by using deep learning networks (Majeed et al., 2021). The preliminary results are quite encouraging. A sixth-degree polynomial model could fit approximately 80% of cordon trajectories with an R^2 = 0.98. Thus, this model might allow for the detection of cordons even if it is too heavily occluded by shoots to precisely position and orient thinning end-effectors for automated shoot thinning.

Considering the above-cited results together, it can be concluded that, when performed in areas allowing for good vegetative vigor, decreased shoot density leads to early/enhanced ripening, primarily because of an LA/Y ratio that might increase in quantity (especially when shoot thinning also regards some fruitful shoots, thereby lowering the yield level) and quality (especially when the growth compensation of the remaining shoots prolongs the formation of new leaves, which might then reach maturity at the right time to boost ripening) compared to non-thinned vines. Overall, when shoot thinning is used in warm environments where an excessively fast sugar accumulation often decoupled from adequate phenolic maturity is almost the rule, its use should be more cautiously regarded.

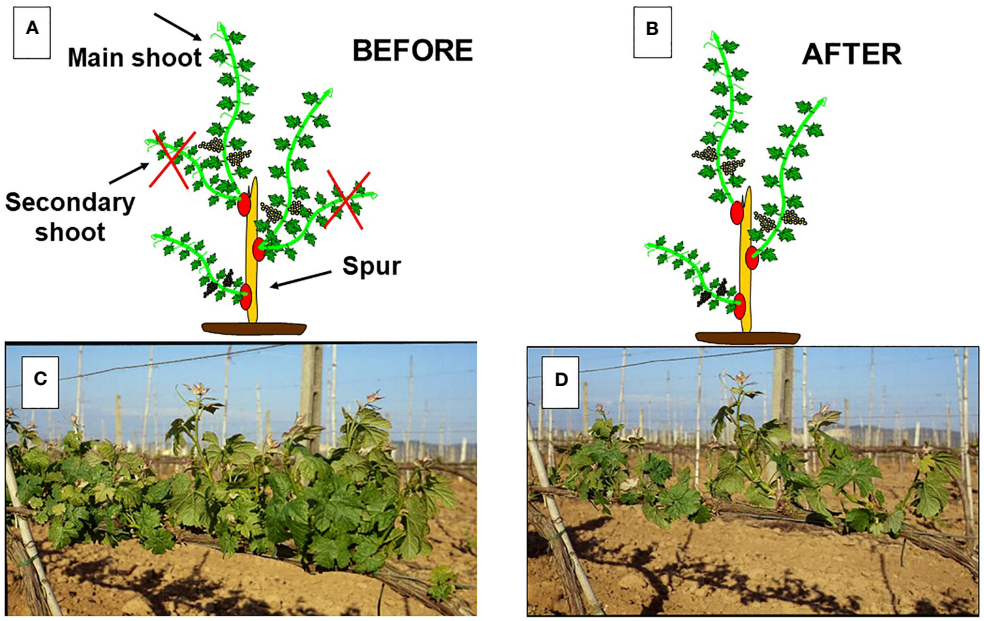

FIGURE 2
In **(A)**: representation of a spur with three count nodes which has originated a total of five shoots. In **(B)**: final set-up after manual shoot thinning which has removed the secondary shoots. In **(C)**: pre-thinning canopy density; in **(D)**: post thinning canopy density.

3 Shoot trimming

Among summer pruning operations, shoot trimming, which involves removing the shoot apex and some of the subtending young leaves (Figure 3A), is likely the best example of a practice that has been traditionally regarded as either neutral or mild in terms of affecting the yield and grape composition but is driven by the need of maintaining a more regular canopy size so as to not hinder the vineyard traffic and other vineyard operations (e.g., spraying or soil management). With more attention put into the physiological effects triggered by shoot trimming, this operation has been re-evaluated in terms of its ability to alter ripening dynamic, which might end up being either promoted or delayed depending on the timing and severity of the trimming, as well as the environmental conditions and the crop load after the trimming (Keller et al., 1999; Poni and Giachino, 2000; Mota et al., 2010; Dokoozlian, 2012; King et al., 2012; Poni et al., 2014b).

Today, in the context of climate change, the significance of shoot trimming has grown because it appears to be the right tool to obtain two concurrent advantages: on one side, reducing canopy size through shoot trimming would also result in lower canopy water use, whereas on the other side, if shoot trimming causes a permanent reduction in the LA/Y ratio, the ripening process could, consequently, be delayed.

With regard to former desirable effect, (Williams et al., 2022) confirmed the reliability of estimating seasonal grapevine crop coefficients (K_c) from the shaded areas beneath grapevine canopies that, in turn, are a function of several factors, among which trellis geometry and canopy size changes induced by summer pruning are prioritized. However, although it has been demonstrated that adopting a split canopy such as the Lyra system leads to a mid-season K_c of 0.96 vs. 0.49 measured in a VSP trellis at the same between-row spacing (2.74 m) (Williams et al., 2022), the same proportionality is more dubious when the water use or water status of a tall, untrimmed canopy is compared to that of a canopy shortened by trimming. Over a three-year period, (Abad et al., 2019), conducted a study comparing the vine water status—assessed as stem water potential (Ψ_{ST})—of untrimmed and severely trimmed vines at berry pea size in different locations of northern Spain. Their findings revealed occasional and inconsistent differences in Ψ_{ST} between the two treatments, which reached significance ($\Delta\Psi_{ST} \geq$ -0.2 MPa) only under year x site combinations marked by high evaporative demand. Most notably, in a two-year-long study conducted on Merlot under well-watered conditions in Northern Italy (Herrera et al., 2015), no seasonal effects on Ψ_{ST} were reported for severe shoot trimming made at veraison by leaving only six primary leaves compared to light trimming (i.e., 12 main leaves retained).

Interestingly, when short (~90 cm) and tall (~130 cm) canopies were compared in a semi-arid Tempranillo vineyard along with three irrigation strategies, the tall canopy maintained more negative Ψ_{ST} in both study areas even under full irrigation (Mirás-Avalos et al., 2017). However, it should be noted that, in this specific study, the standard canopy management was represented by the 90-cm-tall canopy, whereas the 130-cm-tall canopy was a purposely extended canopy aimed at creating more limiting conditions.

A typical problem that occurs any time the effect of the management of canopy water use needs to be assessed pertains to determining the time and spatial scales at which the measurements should be taken. Shortening a canopy by severe shoot trimming would indeed cause a perceivable instantaneous response, after which, the progressive compensation in water use would become a primary function of the extent and duration of the lateral regrowth. Additionally, leaving the assessment of whole-canopy transpiration to extrapolation from single-leaf readings has been proven to be quite unsafe Poni et al. (2009a); Medrano et al. (2015). In such a context, Poni et al. (2021) reported a notable use case where the reaction to severe trimming (i.e., six main leaves left) performed at the end of the flowering (end of May) or pea-size (mid-June) stages was followed in terms of season's whole-canopy transpiration (T_c) by using an enclosure system (Poni et al., 2014a). Due to the vigorous vegetative regrowth, the earlier trimming already offset the initial T_c drop (-23.6% vs. the pre-trimming rates) at the end of July, whereas the later trimming registered an instantaneous T_c drop by 44% against the pre-trimming rates while also achieving full T_c compensation around the same time.

Overall, the picture emerging from the several studies that attempted to assess shoot trimming as a tool to contain canopy transpiration is quite uncertain. Indeed, two different scenarios can be envisioned. On one hand, in case the technique is applied under non-limiting water conditions, either due to sufficient precipitation or the availability of irrigation, T_C is temporarily curtailed and then, progressively replenished by vegetative compensation. On the other hand, if severe shoot trimming is applied under conditions that are not conducive to any significant leaf area compensation, the T_C reduction has a more permanent character and is usually reflected in the better leaf water status of the retained leaves.

Within the aforementioned framework, the decision to proceed with severe trimming is driven by the probability of achieving a significant ripening delay with a desirable reduction of the decoupling between sugar and phenolic ripening. In other words, the wish is to slow down the pace of sugar accumulation without affecting flavonoid accumulation. This presents two distinct cases. The first scenario is when severe trimming is performed under conditions that favor partial or full replenishment of the removed leaf area (i.e., early intervention, low crop level, and irrigation availability), thereby originating a dynamic LA/Y evolution after the cut. Here, the existing literature is quite consistent in demonstrating that both sugar and color accumulation are more or less equally delayed not just because of a limiting LA/Y ratio but also due to the direct late-season competition exerted by the lateral shoots (Keller et al., 1999; Poni and Giachino, 2000; Poni et al., 2014b; Santesteban et al., 2017), This practice is quite solid if the goal is to postpone ripening to a cooler period, although the risk is that full ripening is never achieved in climates where the growing season is not that long or when medium-to-late-ripening cultivars are grown.

The second scenario that a fairly late severe shoot trimming might disclose is that, depending on specific conditions (i.e., semi-arid climate, dry farming, and high crop level), the abrupt decrease in the LA/Y ratio is revealed to be almost permanent as negligible vegetative regrowth occurs after the cut. Under such circumstances, two main hypotheses can be drawn. If the post-trimming LA/Y ratio is at a level (i.e., > 1 m^2/kg) that is considered to be still not too

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FIGURE 3
In **(A)** representation of a standard shoot trimming followed by the regrowth of some laterals. In **(B)** a cutter bar machine operating hedging and topping cuts in a vertically shoot positioning (VSP) trained vine row; in **(C)** the same machine at work on a sprawl, single high wire cordon. In the latter, cutter bars can operate even at a very close distance from the cordon as no foliage wires are present.

detrimental for ripening, then scant differences in the maturation patterns of untrimmed and trimmed vines are to be expected. Conversely, if severe trimming results in a strong and permanent source limitation (e.g., LA/Y ≤ 0.5 m^2/kg), then a significant ripening delay should occur. According to (Bobeica et al., 2015; Silva et al., 2017; Zhu et al., 2019), in these circumstances, berries might use a higher proportion of fixed carbon for sugar accumulation under carbon limitation than under carbon sufficiency. Thus, under carbon limitation, the grape berry can manage the metabolic fate of carbon in such a way that sugar accumulation is maintained at the expense of secondary metabolites.

The studies conducted by (Herrera et al., 2015) and (Lu et al., 2023) fall in the former category (i.e., a non-limiting LA/Y ratio even after trimming) and share a mild limitation in berry sugar

accumulation, along with reporting no change (Herrera et al., 2015) or minor change (Lu et al., 2023) in the total anthocyanin content. Conversely, (Filippetti et al., 2015; Caccavello et al., 2017; Valentini et al., 2019; Bubola et al., 2022) fall in the other category (i.e., a limiting LA/Y ratio after trimming). Surprisingly, while delayed sugar ripening was registered in all these cases, the flavonoid content, and particularly the total anthocyanin accumulation, was less delayed if not affected at all. This leads to the tempting conclusion that, regardless of the source-to-sink ratio after severe shoot trimming, the vine metabolism tends to delay sugar accumulation more than color accumulation. Despite this being a quite desirable outcome, its physiological bases are unclear. Indeed, two factors can play a role in this. First, if severe shoot trimming is performed late in the season and not followed by any significant canopy regrowth, the obvious instantaneous variation in the amount of the LA/Y ratio is unlikely to be exhaustive. The issue is that the quality of the source progressively deteriorates due to basal leaf aging (Poni et al., 2009b), and this phenomenon might have a stronger impact on sugar accumulation. Second, the total anthocyanin accumulation and degradation are also functions of the local microclimate at the cluster level (Mabrouk and Sinoquet, 1998; Haselgrove et al., 2000). As regards red cultivars, it has been found that berry temperatures exceeding 35°C might inhibit the synthesis of color while also enhancing its degradation (Mori et al., 2007), with some varieties such as Pinot noir, Barbera and Sangiovese being especially sensitive to this (Poni et al., 2018). The cluster microclimate is not directly impacted by late severe shoot trimming, which might justify the lower sensitivity of flavonoid synthesis to the altered LA/Y ratio.

In more general terms, the potential of late and severe shoot trimming to delay ripening has been validated from the aforementioned work. However, the efficacy and transferability of a new practice depend upon several factors, among which practical feasibility and the degree of mechanization are significant. Most of the experiments conducted have imposed manual severe shoot trimming, which leaves six-to-eight main leaves on the main shoot. In a VSP trellis, its mechanical execution is quite impractical regardless of when this operation will be performed, as the commonly used cutter bar machines can perform topping and hedging along an unhindered path only (Figure 3B). Therefore, the most severe affordable mechanical cut is the one that is performed when most of the shoots outgrow the top foliage wire. However, this prevents the performance of a truly severe cut as in a canopy wall usually extending for at least 1.2–1.4 m above the main wire, the minimum number of leaves left cannot be lower than 14–16. The alternative is double: to wait until the main shoots have started to bend, so that, after trimming, the number of source leaves removed will be higher. However, waiting until that late for the first trimming can lead to heavy interrow hindrances and difficulties in machinery transit. Second option could be using an over-row rotating disks machine able to negotiate rigid obstacles during the row passage. Indeed, this machine type is slower, more expensive and less flexible than a cutter bar machine. The problem is fully solved when a sprawl canopy is adopted. The free space around the support wire allows any pruning machine to get close to the cordon and perform a short cut (Figure 3C) (Poni et al., 2014b).

4 Leaf removal

Among all summer pruning operations, leaf removal is the one that has attracted the most attention from the scientific community as well as the one whose modalities and scope have been most significantly affected by global warming. This change has also led to leaf removal getting a different meaning assigned compared to the past. Traditionally, leaf removal has been associated with the plucking of leaves around clusters, which is performed anywhere between fruit set and veraison, to improve light exposure as well as ventilation and facilitate sprays penetration (Koblet et al., 1994; Zoecklein et al., 1998; Kok, 2016; Bubola et al., 2017; Reynolds, 2022). Such goals are still prominent in cool and wet growing regions where the impact of climate change is less and where fruit and wine quality still benefit from lower canopy density and better air circulation at the cluster level (Smith et al., 1988; Zhuang et al., 2014; Frioni et al., 2017). In warmer areas, the current interpretation of leaf removal incorporates two main changes. There is a shared perception that if global warming poses increasing concerns about overheating, sunburn, and desiccation events, leaf removal has to be either applied more judiciously or simply rendered unnecessary. Moreover, leaf removal has been demonstrated to be an extraordinarily flexible operation, as adjusting the timing and severity of the intervention is possible to modulate the effects, thereby piloting them toward a desired direction (i.e., enhancing or delaying ripening) (Figure 4).

As regards the actual need for leaf removal, if worries related to multiple summer stresses are increasing (Palliotti and Poni, 2015), then maintaining some leaf cover around the clusters is at least advisable. Recent work on the relationship between the timing of leaf removal and the susceptibility to sunburn (Diago et al., 2012; Ferrari et al., 2017; Gambetta et al., 2019; Gambetta et al., 2021; Rustioni et al., 2023) concluded that early leaf removal (i.e., at fruit set rather than at veraison) will reduce the incidence of sunburn. Moreover, extensive analytics performed by (Gambetta et al., 2021) for photo-protectant compounds (i.e., flavonoids, carotenoids, and chlorophylls) demonstrated that all molecules are especially receptive to the light stimulus during the green phase of berry growth. In fact, their concentration significantly increased after fruit-set leaf removal, whereas a much weaker response was found for late-season leaf removal. From veraison onward, berries lose most of their potential to synthetize photo-protectant compounds; therefore, their acclimation potential is reduced, and their sensitivity to berry sunburn is increased. Furthermore, in cultivars such as Pinot noir, Gamay, Merlot, Chasselas, and Doral (Verdenal et al., 2019), early leaf removal almost doubled the berry skin thickness, whereas in a trial on Barbera (Poni and Bernizzoni, 2010), the same treatment achieved higher relative skin growth than the non-defoliated vines despite having larger berries.

In a paradox, the need of maintaining some leaf cover around clusters during summer has shifted attention to mechanized leaf removal. As a matter of fact, the "imperfect" work of a de-leafing machine—in which some leaves are stripped, others are partially broken, and the rest remain untouched—naturally meets the need of avoiding cluster overexposure, as some leaf cover is always preserved. The evolution of leaf removal has generated two

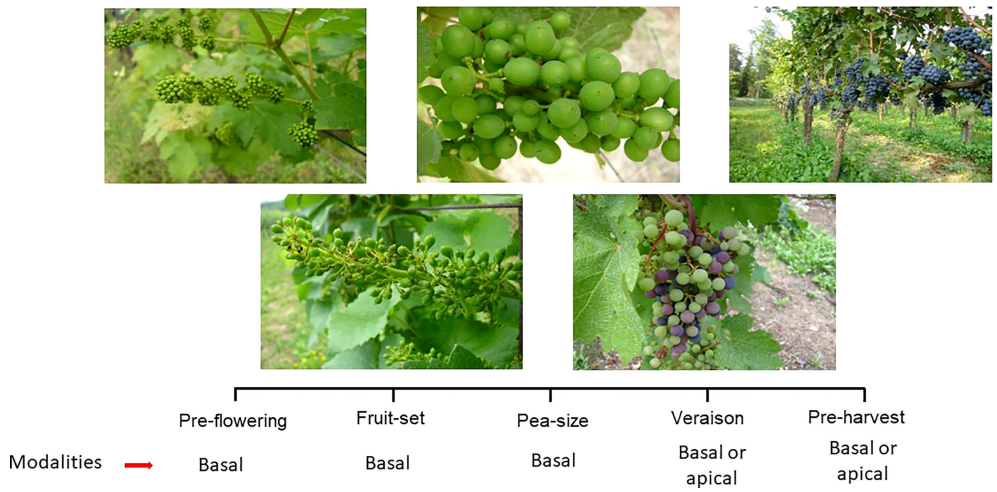

FIGURE 4
Phenological stages at which leaf removal can be performed on the grapevine. Depending of growing stage and purpose, leaf removal can target different canopy portions (i.e. basal vs apical).

variants of the technique, which have drawn the attention of several research groups.

The most tested technique is early (pre-flowering) leaf removal (ELR), the objectives of which are quite different from those of traditional leaf removal (Poni et al., 2006) (Figure 5). Based on the strong physiological principle according to which any source limitation caused around the flowering stage will negatively impact fruit set (Coombe, 1962), the technique is considered best suited to cases of high-yielding vineyards with heavy and compact clusters that are quite susceptible to rot and often incapable to reach full ripening. A meta-analysis study was conducted by (VanderWeide et al., 2021), which, after initially identifying 175 publications on the topic of "early leaf removal," thinned them down to 59 after eight data-curation steps. It returned a clear and consistent picture: ELR systematically lowered cluster rot disease through reduced compactness of clusters, which was mostly due to lower fruit set. Moreover, ELR promoted a significant increase in fruit total soluble solids, which was related to the increase in the LA/Y ratio. Additionally, the total anthocyanin content also tended to increase in ELR, albeit with more variability among the observed responses. Interestingly, the ELR effects were mildly affected by the climate, which supports the hypothesis that the technique has a solid physiological foundation, whereas, cultivar (Verdenal et al., 2019; Mucalo et al., 2021) and rootstock were found to have a larger influence on the success of ELR.

The large-scale adoption of ELR in several viticulture areas around the world and especially where cluster rot diseases are a concern is also due to at least two more factors. First, it has been shown that the operation can be fully mechanized (Intrieri et al., 2008; Tardaguila et al., 2010; VanderWeide et al., 2018) using, at the proper timing (i.e., still no flowers open on the cluster), a mechanical leaf-blower machine that allows for handling larger surfaces (Figure 5B). Second, in case no hand labor or machines are available to perform the operation, a viable alternative is using antitranspirants that can coat the leaves and reproduce a source-limiting effect (Intrieri et al., 2013; Mphande et al., 2023). This last solution vastly broadens applicability of this practice as the source limitation is achieved without a physical alteration of the cluster microclimate, which might be quite helpful when the application site features high temperature and radiation loads.

An agreeable side effect of ELR is that the induced yield limitation is proportional to the number of source leaves removed. Some authors (Gatti et al., 2012; Verdenal et al., 2019; VanderWeide et al., 2020; Chalfant and Dami, 2021) have suggested that yield regulation can be effectively achieved through ELR while avoiding collateral negative effects, which might be associated with a cluster-thinning approach where the retained clusters grow more, leading to higher compactness and larger berries. As regards Chambourcin grapes, (Chalfant and Dami, 2021) also reported that performing basal leaf removal at pre-bloom or bloom reduced bud cold injury during the dormant season.

Recently, (O'Brien et al., 2021) demonstrated an original approach to leaf removal where this practice was tested in a sprawl canopy type as a tool to delay ripening in Cabernet Sauvignon without achieving consistent results. However, the source-sink balance of the de-leafed treatments was always above the 1.5 m^2/kg threshold, and this might explain why the imposed defoliations were rather ineffective.

The second variant that bursts from traditional leaf removal can be regarded as an up-side-down technique compared to ELR. It is applied late in the season (pre-veraison until a sugar concentration in berries of approximately 10–12°Brix) and targets the apical canopy portions (Figure 6) while the cluster microclimate is unchanged (Poni et al., 2013; Buesa et al., 2019; De Bei et al., 2019; Gatti et al., 2019). Overall, the principle that the technique exploits to delay ripening is the same that is pursued in the case of severe trimming, i.e., provoking a calibrated source limitation by removing a portion of functional foliage. However, when compared to severe trimming, one main technical difference exists: apical canopy portion leaf removal aims at opening a window of about half

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FIGURE 5
In **(A)** a row section of a vertical shoot positioning (VSP) trellis where manual early (pre-flowering) leaf removal has been completed. The first six basal leaves have been taken out from all shoots under the main purpose to decrease fruit set, control yield and obtain looser clusters less susceptible to rot. In **(B)** the same operation performed at the same stage with a Pellenc^{TM} leaf plucker.

a meter length in a fruitless canopy area. This has two apparent advantages: (i) the operation is fully mechanizable, although two passages per row are needed to optimize leaf removal, and (ii) the task encounters the favor of the driver who knows that defoliation has to be carried out in a fruitless portion, thereby eliminating any fear of possible cluster damage.

The whole-canopy physiology changes brought about by late apical leaf removal have been investigated by (Poni et al., 2013) who studied potted Sangiovese vines to ascertain that both pre- and post-veraison apical leaf removal were effective at significantly delay sugaring and retain higher acidity without affecting phenolic maturity. This happened despite the final source-sink balance of the defoliated treatments was not limiting (LA/Y ratio of approximately 10–11 cm^2/g and a carbon-to-yield ratio of 9.8–10.4 mg CO_2/g berry fresh weight), with the latter not differing from the control's values (11 mg CO_2/g berry fresh weight). Moreover, a two-year-long field study on Sangiovese mirrored the same results (Palliotti et al., 2013), with vines defoliated at a TSS level of approximately 12°Brix showing, at harvest, 1.2°Brix less than the undefoliated control and an unchanged phenolic maturation. When

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FIGURE 6
In **(A)**, a leaf plucker machine starts working on the apical canopy portion of a vertically shoot positioning (VSP) trained vine row with the purpose of removing a significant portion of source leaves to induce a ripening delay. In **(B)** the results of the work performed in cv Ortrugo.

tested on Semillon and Shiraz in Australia, the technique was found to be ineffective in the first year of application, whereas in the second year, it achieved a delay in ripening of 10 days in Semillon and 20 days in Shiraz, which suggests that the source limitation can be buffered from reserves storage during the first year (De Bei et al., 2019). As shown in (Palliotti et al., 2013) and (Poni et al., 2013), it is remarkable that a sugar ripening delay was obtained even in the absence of a limiting source-to-sink balance. The hypothesis explaining this outcome is that although the final or seasonal LA/Y ratio might not differ between the two treatments, when the leaf removal is performed (around veraison) targeting the youngest apical leaves, the abrupt source decrease is likely strong enough to temporarily limit the sugar trend, which at that time is either at the inflection point or going through the steepest sugar accumulation rate.

5 Cluster thinning

By definition, cluster thinning is configured as an extraordinary operation of canopy management that intervenes either when an overcropping status exists or when the sought wine target embraces

overripe flavors. If so, a climate change scenario that inherently speeds up ripening should lead to a more conservative application of cluster thinning.

However, it is quite difficult to trace the evolution in the popularity of cluster thinning since a broad meta-analysis study has not yet been conducted. Indeed, in premium areas for red wine, the habit to use cluster thinning as the primary crop load controlling tool (rather than winter pruning) is still widespread. However, despite several publications available on the matter, a primary question related to cluster thinning remains unanswered: is cluster thinning always followed by an increase in grape quality that might offset yield loss and added costs due to the required human labor?

The following physiological principle (Figure 7) should drive the cluster thinning operation: if the anomalous values in the LA/Y ratio (e.g., lower than 1 m^2/kg) or the Ravaz index (e.g., higher than 8–10 kg/kg) warn about a likely overcropping status, then cluster thinning should lead to the expected results of significantly improving grape and wine quality while helping to remain within yield limits imposed by law. However, when cluster thinning is performed in vines with adequate crop load, the advantages of these technique significantly diminish, resulting in reduced economic return due to the additional cost required to implement the technique (Berkey et al., 2011; Preszler et al., 2013; Schamel and Schubert, 2016).

Summary of main results of 20 papers on cluster thinning are shown in Table 1 which also reports, along with the effects on yield and grape quality, the evaluation of the LA/Y ratio or the Ravaz index (when available) in the different treatments. An overall analysis of the group of 20 papers led to the following conclusions:

i. The aforementioned hypothesis holds true in several cases (Keller et al., 2005; Intrigliolo and Castel, 2011; Sun et al., 2012; Zhuang et al., 2014; Mawdsley et al., 2019; Martínez-Lüscher and Kurtural, 2021; Copp and Levin, 2022), although significant deviations were also found (Gatti et al., 2012; Sivilotti et al., 2020; Aru et al., 2022 a, b).
ii. In most cases cluster thinning resulted in a significant final yield reduction, suggesting that yield compensation mechanisms from the retained clusters have been overall minor. Exceptions were data from Intrigliolo and Castel (2011) and Mucalo et al. (2022).
iii. In the few studies where the same CT treatments were repeated for three years on the same vines, the adjustment to CT increased over time to offset between-treatment differences in the second or third year (Mawdsley et al., 2019; Martínez-Lüscher and Kurtural, 2021; Netzer et al., 2022).
iv. In almost all the studies where CT induced significant yield limitation, the total soluble solids at harvest were the most responsive variable (Reynolds, 1989; Dami et al., 2006; Gil-Muñoz et al., 2009; Kok, 2011; Reščič et al., 2015), whereas the total anthocyanin content was often less affected (Guidoni et al., 2002; Santesteban et al., 2011; Sun et al., 2012; Copp and Levin, 2022).
v. It was apparent that the effects induced by cluster thinning were sensitive to cultivar and specific environmental conditions of the site, rendering the forecasting of its effects hard to predict and endangering the repeatability of the effects.

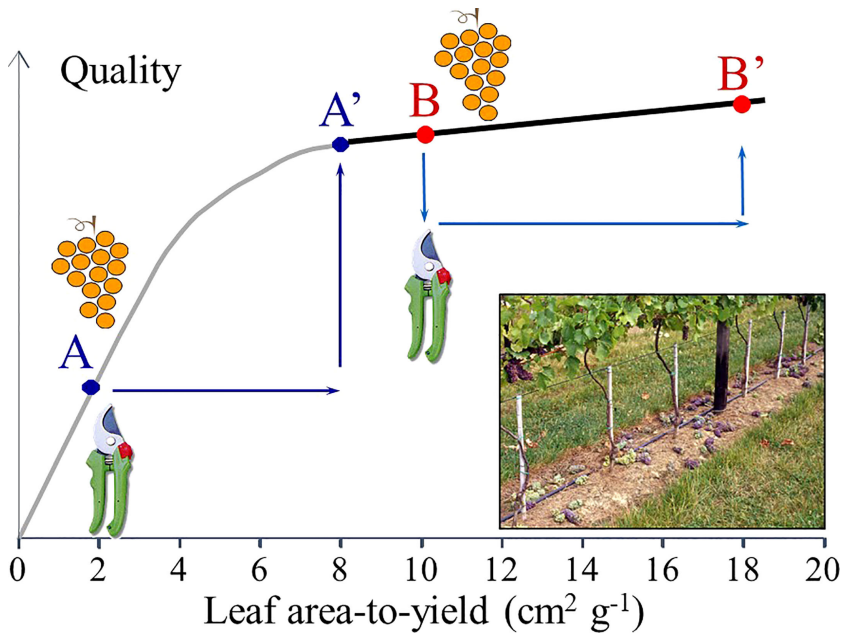

FIGURE 7
Representation of a physiological hypothesis for prediction of cluster thinning effects. If grape "quality" (defined in general terms as the desired grape composition needed for a given wine style) is plotted vs the leaf area-to-yield ratio, two different cases are envisaged. The gray line represents the improvement in fruit quality from A to A' when cluster thinning is done in over cropped vines. The black line represents the negligible effect of cluster thinning in fruit quality from B to B' when vine source-sink is properly balanced.

TABLE 1 Synoptic information from 20 research papers on the impact of cluster thinning strategies on yield, fruit quality and source to sink balance of grapevine.

Country/ cultivar	Trial years	Timing/ intensity of CT	Effects on yield	Effects on grape quality	LA/Y ratio or Ravaz index	Notes	Reference
USA/Cabernet Sauvignon	3	Fruit set/33-66% fruit removal	–	+TSS	++ LA/Y	3rd Year, TSS and LA/F ns	(Martínez-Lüscher and Kurtural, 2021)
Italy/Refosco dal peduncolo rosso	2	Pre-veraison/50% fruit removal	–	+TSS, + color	++ LA/Y	LA/Y in C = 1.90m^2/kg; LA/Y in CT = 3.34m^2/kg	(Sivilotti et al., 2020)
USA/Pinot noir	3	4,8,12 weeks after bloom	-(Y1); ns (Y2-3)	+ or ns TSS;	Ravaz, ns	Ravaz varying between 1-3 kg/kg	(Mawdsley et al., 2019)
Australia/Semillon - Shiraz	2	Veraison/50% fruit removal	–	++TSS	N/A		(Wang et al., 2019)
USA/Pinot noir	3	Peppercorn/1 cluster/shoot center	-or –	+ or ns TSS; ns color	-or ns Ravaz		(Copp and Levin, 2022)
Denmark/Solaris	1	Veraison/66% fruit removal	–	++TSS	ns, LA/Y	LA/Y in C = 13 m^2/g; LA/Y in CT = 10 cm^2/g	(Aru et al., 2022a; Aru et al., 2022b)
Israel/Malbec	3	Pre-veraison/50% fruit removal	-(Y1); -ns (Y2-3)	+ (Y2) or ns (Y1 and 3)	N/A	C and CT wines perceived as similar	(Netzer et al., 2022)
Croatia/Maraština	1	Veraison/35% fruit removal	ns	ns	N/A	CT wines had better aroma than C wine	(Mucalo et al., 2022)
USA/Chambourcin	5	10(L), 20(M), 30(H) clusters/ vine	M vs H ns - in L in 3Y out of 5	+TSS in L in 3Y out of 5	N/A		(Dami et al., 2006)
Spain/Tempranillo	2	11(L), 20(M), 27(H) clusters/ vine	Occasional lower yield in L	Occasional higher TSS in L	LA/Y varying from 0.7 to 3.1 m^2/kg	Increased response to thinning for LA/ Y < 1.5 m^2/kg	(Intrigliolo and Castel, 2011)
Turkey/Sauvignon blanc	1	4,6,8,10,12 weeks after bloom/ 1 cluster per shoot	-35% vs C	+TSS	N/A		(Kok, 2011)
Slovenia/Blauer Portugieser	3	Pea size/25 and 45% fruit removal	-or ns in25% – in 45%	ns TSS in 25% +TSS in 45%	N/A	25% fruit removal quite ineffective	(Reščič et al., 2015)
USA/Cabernet Franc	2	Fruit set, 3 weeks before veraison and veraison/40(L) and 80(H) clusters/vine	-38% in the L treatment	ns TSS + color in L for second year only	Lower Ravaz in L in both years		(Zhuang et al., 2014)
Italy/Sangiovese	3	Flowering and lag phase; 50% fruit removal	-45% vs C	++ TSS ++ color	LA/Y = 1.03 m^2/kg in C; LA/Y = 1.65 m^2/kg in CT		(Gatti et al., 2012)
USA/Coron	2	Pea size/removal of distal clusters in CT	– in CT	+ TSS ns color	Ravaz varying from 2.3 and 7.1 kg/kg	Very vigorous vines	(Sun et al., 2012)
USA/Cabernet Sauvignon, Riesling, Chenin blanc	5	Pea size and pre-veraison/30% to 39% fruit removal	-17% to 36% in CT	ns TSS ns color	LA/Y = 1.7-1.9m^2/kg in C; LA/Y = 1.6-3.7 m^2/kg in CT		(Keller et al., 2005)
Spain/Tempranillo	4	Veraison/one cluster/shoot in CT	- in 10 out of 12 vineyards	+ TSS in 6 out 12 vineyards; + color in 4 out of 12 vineyards	N/A		(Santesteban et al., 2011)
\Italy/Nebbiolo	3	Pea size/50% fruit removal	-21% in CT	+TSS and color in 2 out of 3 years	N/A		(Guidoni et al., 2002)

(Continued)

TABLE 1 Continued

Country/ cultivar	Trial years	Timing/ intensity of CT	Effects on yield	Effects on grape quality	LA/Y ratio or Ravaz index	Notes	Reference
Spain/Tempranillo and Shiraz	3	Pre-veraison/one cluster/shoot in CT	-40% in CT	+TSS +color	N/A		(Gil-Muñoz et al., 2018)
Canada/Riesling	3	Fruit set/1 and 2 clusters/ shoot vs C	-30% in one cluster/ shoot vs C	++TSS	N/A	No differences in final wines	(Reynolds, 1989)

Y, year; C, control; CT, cluster thinning; L, low; M, medium; H, high; LA/Y, leaf area-to-yield ratio in m^2/kg or cm^2/g; Ravaz index as yield-to-pruning weight ratio (kg/kg); TSS, total soluble solids; color = total anthocyanins unless otherwise stated. One single – or + sign means significant vs C at $p < 0.05$. Two – or + signs mean significant vs C at $p < 0.01$. ns = non-significant.

Global warming is causing excessively fast ripening, especially in semi-arid areas with increasing decoupling between sugar accumulation and phenolic ripening (Nicholas, 2015). Among the several solutions available to slow and postpone ripening to a cooler period (Palliotti et al., 2014), the reuse of unripen, thinned clusters was also evaluated (Kontoudakis et al., 2011) with the specific aim of reducing alcohol content and pH. The idea was to use a fraction of the thinned clusters at veraison to produce a very acidic must (TSS at 5° Brix, total acids at 17.8 g/L, and pH = 2.78) in Merlot, Cabernet Sauvignon, and Bobal. At the end of fermentation, the wine was treated with activated carbon and bentonite to remove phenolics and aggressive green flavors and obtain an odorless as well as colorless product. To adjust the composition of wines derived from the normal harvests, an aliquot of the green must was added to replace an equivalent amount of standard must for each cultivar. Within batches of 8 kg of grapes and a must yield of 6.4 L, the replaced must fraction was 0.85, 1.50, and 2.0 L for Cabernet Sauvignon, Merlot, and Bobal, respectively. The results met the expectations, as the final alcohol content in the blended wines was reduced by 0.9% (Cabernet Sauvignon), 1.7% (Merlot), and 3.0% (Bobal) compared to traditional wines. Moreover, due to a significant reduction in wine pH, blended wines also registered higher total anthocyanin concentrations.

6 Summer pruning and interactions with late frost and hail events

A very consistent trait accompanying the climate change era is, indeed, the increase in both frequency and severity of so-called "extreme" weather events (AghaKouchak et al., 2020). Climate becomes extreme when damaging events turn unusually violent or anomalous in terms of their frequency and duration. In Europe, it has been estimated that the frequency of extreme events registered over the last three decades causing significant economic losses has increased by about 60% (Sánchez et al., 2004; Beniston et al., 2007). While we have covered the relationship between specific summer pruning operations and drought and/or sunburn in the single chapters of this review, late frost and hail occurrences require a specific treatment.

Within a global warming scenario, two effects in particular can contribute to render late frost a fearsome event: i) earlier phenology is one of the most characteristic and consistent changes induced by higher heat summations. In the grapevine, a more advanced bud burst can overwhelm any benefit derived from global warming, leading to a reduction (rather than a widening) of the "frost free period" (Leolini et al., 2018); ii) in the event of a potentially damaging temperature, the vegetative development of the vine can be definitely more advanced, thus inherently increasing damages to tissues (Fuller and Telli, 1999).

A technique named "late winter pruning" (LWP) has been tested mostly over the last two decades as a prevention or mitigation tool against late frost damages in vineyards (Poni et al., 2022).

This LWP is based on an extraordinarily simple physiological principle that can be related to grapevine acrotony. In the grapevine, regardless of the length and position of the productive unit (i.e., a spur or a cane of variable length), the distal nodes are the first to commence growth in spring, after which the proximal buds gradually follow. Based on this principle and to prevent or minimize late frost damage, the goal of LWP is to postpone final winter pruning until shoot growth has not commenced on the apical nodes. Advantages are: i) time elapsing between date of budburst and actual late winter pruning date is the first mechanism lowering chances to incur into frost damages; ii) lag time needed to the basal nodes to burst and reach a growth stage susceptible to frost (e.g swollen bud onward) is an additional warrant against frost damage. Details of the LWP protocols suited for either spur pruned and cane pruned vines are described in Poni et al. (2022) and also shown in Figure 8.

In the above- cited review paper, summary of the results from 21 trials which applied LWP under an array of locations and cultivars leads to the following main conclusions: i) bud burst was delayed by 5 to 56 days according to local condition and experimental layout; ii) yield was either unchanged or variably reduced vs a standard winter pruning treatment. A common trait of several works is that when the final pruning or hand finishing takes place beyond the stage of 2-3 unfolded leaves, yield of the current season can be seriously lowered, and iii) in a significant number of cases the bud burst delay caused by LWP carries on until harvest with notable improvement especially in terms of total anthocyanins and phenolics; iv) an inherent hurdle of this kind of study is that, even if all the treatments are correctly planned, it is virtually impossible to predict the actual occurrence of a significant frost damage. However, such a situation occurred on 29 April 2019 during a two-year trial on Lemberger, when a freezing event

occurred when the phenological stage of the control averaged between woolly buds and green leaf tips visible, respectively (Persico et al., 2023). In 2019, late-pruned (1 May) vines had 61% greater yield than control vines, reflecting differences in shoot freeze damage between the two treatments. Moreover, final grape quality was not affected.

A close connection between summer pruning and frost damage also occurs when it has to be decided if and how to intervene to facilitate vine recovery and yield capacity restoration. While actions to be taken need surely to take into account timing and severity of frost damage, here we would like to concentrate on different behaviors to be maintained according to the adopted pruning system.

While in a spur-pruned cordon care should be taken that the post-frost new shoots grow upward along the foliage wires, thereby safeguarding their integrity, in cane-pruned systems, some additional concerns might arise. In a Guyot trellis, after a very severe frost damage, secondary shoots are usually developed at each node position along a horizontal or arched cane. If the specific site allows vigorous regrowth, such shoots can be maintained to build a new efficient leaf area. Conversely, in low-vigor sites or locations suffering, for instance, from summer drought, allowing all the shoots to develop increases the risk that a relatively low growth potential is partitioned among an excessive number of shoots. The final undesirable outcome is that no good renewal canes are borne on the head of the vine. If this is the case, anticipating the removal of the damaged canes can result a winning choice as the residual vine regrowth potential will be concentrated on the few buds that will develop from the vine head.

A trickier situation occurs when the mortality of the primary shoots after the frost event is lower; for instance, < 50% of the total shoots. Once again, different pruning systems will require different approaches. In spur-pruned cordons, chances of achieving a decent crop level as well as canes robust enough to warrant good spur selection for the next cropping are quite high, even in cases where no post-frost operations are executed. Conversely, for long-cane pruning lack of intervention would likely be inappropriate. For instance, laterals already developing from the basal nodes of the partially injured shoots will soon achieve apical dominance and will become the primary vegetative sinks. In a cane-pruned system, those laterals might represent a wasteful process as they usually stem from canopy positions unsuited to provide a renewal cane. Moreover, the energy the grapevine invests into that growth will be detrimental to the development of a long cane in a suitable position (i.e., on the head of the vine and possibly below the supporting wire).

For hail events, forecast models predict a progressive decrease of relative humidity and an increase of convective instability, mostly due to atmospheric overheating; in turn, this might lead to an increase in the frequency of hail events as well as the possibility of larger hail grains (Raupach et al., 2021). As in the case of late frost damages, it would be useful to address advisable post-hail summer pruning interventions. For severe hail damage (i.e., canopy defoliation higher than 50%) occurring at a still early stage (roughly by fruit set), prompt intervention is mandatory to safeguard normal cropping level for the next year. Severe damage is likely to compromise the integrity of the canes that will have been

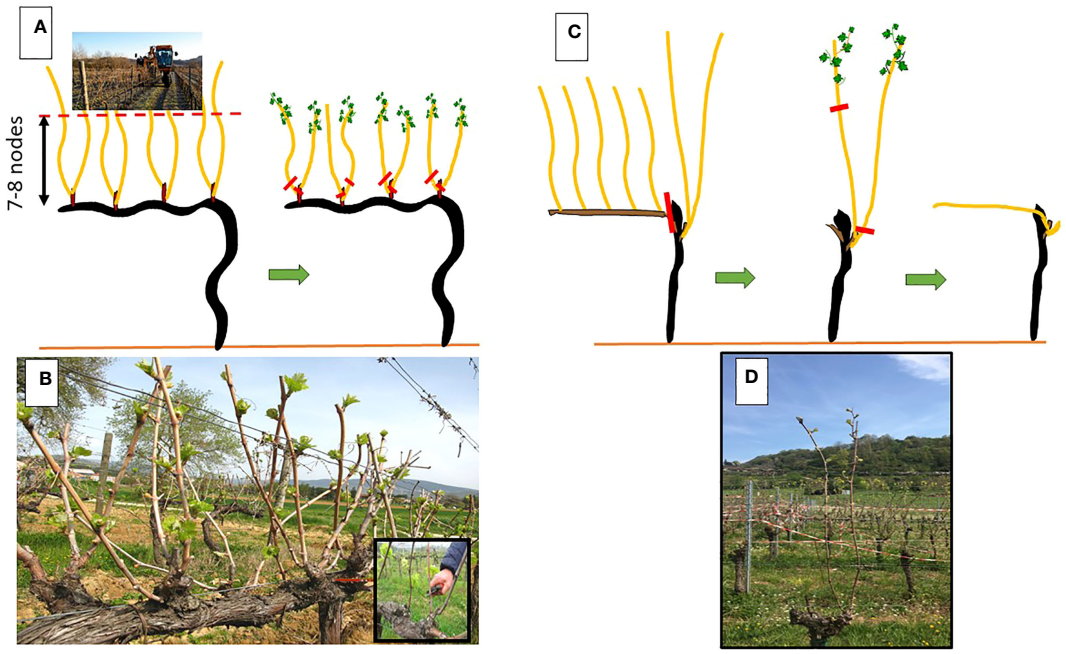

FIGURE 8
Representation of the Late Winter Pruning (LWP) protocol applied on spur-pruned **(A, B)** and cane pruned **(C, D)** vines. In spur-pruned vines, a mechanical pre-pruning **(A)** is performed during dormancy to shorten the canes **(B)** before final spur pruning is made (inset of panel **B**). In cane pruned vines, cane selection of unshortened canes is made in winter **(C, D)** and then followed by final shortening and positioning of the cane along main wire. Red lines indicate pruning cuts. In both pruning systems, recommendation is not to wait beyond the stage of 2-3 unfolded leaves to perform the late cuts.

used for the replenishment of fruiting spurs or new canes at winter pruning; therefore, it is crucial to concentrate on the post-hail vine regrowth of those growing points that are deemed useful for winter pruning.

However, the scenario changes again in relation to spur-pruned or cane-pruned systems. In spur-pruned cordons, the main goal is to form new canes with basal portions that are thick and mature enough for the successful selection of future spurs. Conversely, in cane-pruned systems, the new cane must be long enough to fill the space available along the support wire. Probability that the new canes stimulated by the post-hail intervention will complete a regular bud induction and differentiation process depends on the speed of growth resumption as well as the environmental conditions (namely light and temperature) accompanying the growth of the new shoots/canes. If, besides a standard sanitation spray aimed at disinfecting wounds and facilitating the healing process, a "no intervention" option is chosen, the worst possibility is that most of the regrowth vine potential will be concentrated on fostering the regrowth of erratically distributed laterals, which mostly happens at canopy positions unsuited to winter pruning needs.

Consequences of hail damage are especially fatal when it occurs late in the season, e.g., at a time when even the growth of laterals has almost ceased. Under such circumstances, in addition to the damage to the clusters usually being more severe, it becomes very difficult, if not impossible, to promote the growth of new wood for the forthcoming winter pruning. However, in a spur-pruned system, late-season hail damage followed by prompt and aggressive reform pruning with the aid of any possible forcing tool (e.g., late-season irrigation, foliar fertilization, removal of a competitive cover crop, etc.) might still allow the formation of some new wood, which might give rise to a few renewal spurs. But this is very unlikely to happen in a cane-pruned system, and, quite often, a late hail event causes the cropping function to be compromised for two consecutive years, rendering business sustainability a very serious concern.

7 Crop forcing in *Vitis vinifera*: a summer pruning consequence?

An increasing number of studies have reported on the shifts in timing and length of the growing season, based on phenological, satellite, and climatological studies (Bradshaw and Holzapfel, 2006; Christiansen et al., 2011; Cook and Vizy, 2012). The evidence points to a lengthening of the growing season of ca. 10–20 days in the last few decades, where an earlier onset of the start is most prominent (Linderholm, 2006). Variations in the timing and length of the growing season may not only have far-reaching consequences for plant and animal ecosystems, but persistent increases in the length of the growing season may lead to long-term increases in carbon storage and changes in vegetation cover, which may affect the climate system.

As a deciduous fruit tree, the grapevine is, of course, quite receptive to such changes (Jones and Davis, 2000; Mesterházy et al., 2018), and a lengthening of the growing season is stimulating new agronomic approaches, one of which, unsurprisingly, is based on various combinations of summer pruning. The terminology of "crop forcing" is quite effective in describing an attempt to shift cropping and maturation at a later stage compared to calendar ripening dates. The physiological principle behind the technique is quite simple: in a *Vitis vinifera* L. cultivar grown in a temperate or continental climate where buds typically undergo winter dormancy, the cropping cycle and ripening can be consistently postponed if the dormant bud is forced to grow during its first year of induction and differentiation (Figure 9). This happens if its para-dormancy status is broken making the dormant bud acting as a prompt bud. The pioneering study that paved the way for this technique was by (Gu et al., 2012) who, in the hot environment of Fresno, California, trimmed the primary shoots to six nodes at different dates while also removing any main leaf, laterals, and primary clusters. When the trimming was performed by pea-size (at the latest), the results were astonishingly good. The forced (F) crop was only 13% less than the primary crop achieved on the unforced control (C) vines (6.45 kg/vine vs. 7.35 kg/vine). The forcing shifted the harvest by over two months (from mid-August to mid-October and even further) and the fruits from the forced crop had similar TSS, smaller berries, lower pH levels, higher acidity, and enhanced total anthocyanin and phenolic concentrations compared to C vines.

Since then, other studies have followed. However, these are difficult to compare as a "crop forcing" technique might follow different strategies, which depend upon the type of organs that are retained on the vine at the time when the forcing is imposed. In their study, Gu et al. (2012) left no organs on the trimmed shoots of the forced vines. The same approach was followed in more recent studies (Martínez et al., 2019; Martínez-Moreno et al., 2019; de Toda, 2021; Martinez De Toda, 2021; Cabral et al., 2023; Lavado et al., 2023a; Lavado et al., 2023b) conducted on Touriga Nacional, Tempranillo, and Maturana tinta, which delivered a surprisingly consistent outcome: in all the studies, the forced crop (replacing in full the removed primary crop under the current circumstances) had similar TSS to the unforced control while displaying distinctly higher acidity (especially malic), lower pH, and greatly enhanced total anthocyanin and phenolic concentrations. The weak point shared by all the studies is that the grape yield borne on the forced shoot was severely curtailed compared to the primary crop harvested on the unforced vines, as the yield reduction was 70–85% in Tempranillo and Maturana tinta (Martínez et al., 2019), 57% in Tempranillo (Lavado et al 2023a), 88–90% in Touriga Nacional (Cabral et al., 2023). Evidence suggests that such a negative impact on yield carried by the forced primary shoots is largely due to the severe source limitation, which parallels the unlocking and development of the primary bud whose differentiation might result incomplete originating clusters with fewer and smaller berries. Moreover, depending on the timing at which the forcing is performed, the dormant bud might not have yet completed the floral induction process, thereby resulting in several vegetative shoots. Such yield constraint associated with the high amount of labor required for organ removal renders this practice economically unsustainable.

A new light has been shed on the forcing technique by authors who followed a different approach. As already discussed by (Gu et al., 2012), crop forcing application can differentiate depending on

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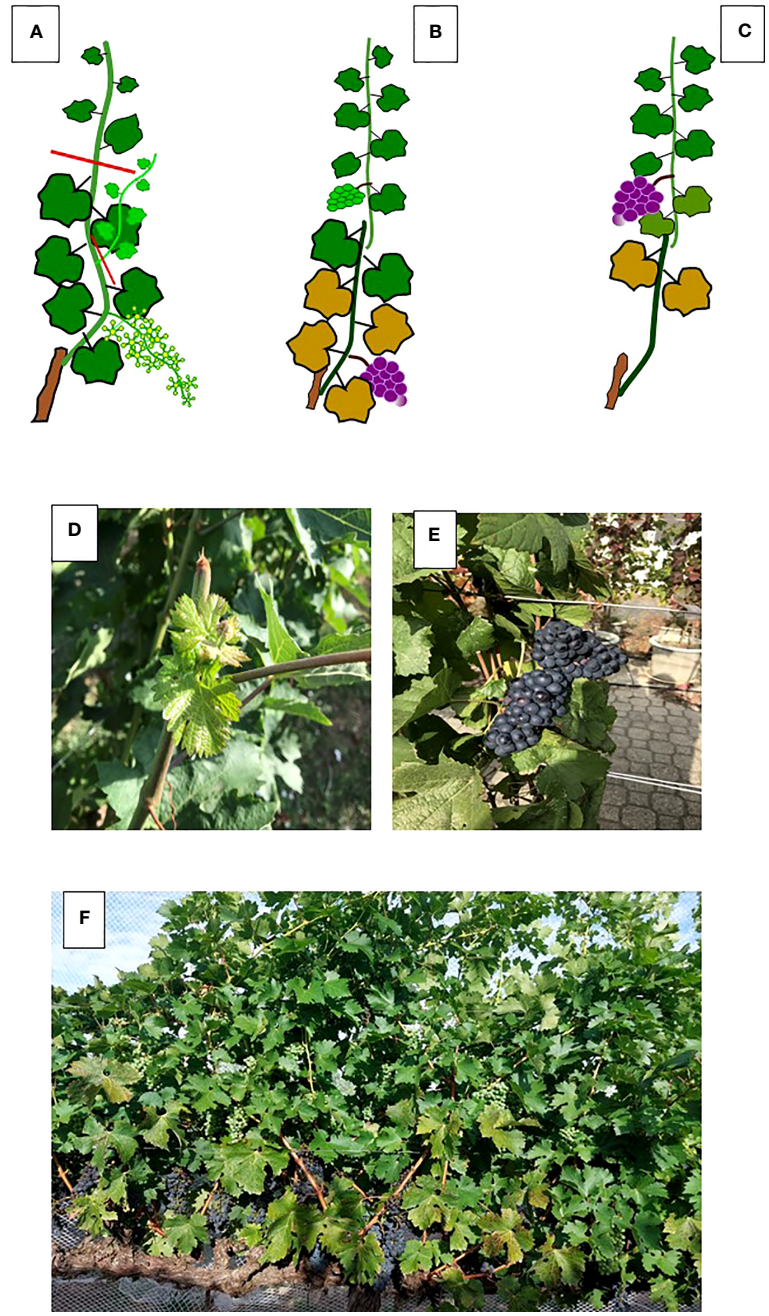

FIGURE 9
Representaion **(A–C)** of the crop forcing technique leading to two harvests in the same season. In **(A)** main shoot is trimmed at six leaves and all laterals removed. This will unlock the dormant buds. In **(B)**, the primary crop is ripen, whereas the forced crop is at about lag phase of berry growth; in **(C)** the forced crop is ripen while leaf shedding has already started from the basal part of the canopy. In **(D)** a detail of the forced shoot originated by the dormant bud and in **(E)** two forced Pinot noir clusters close to maturity. In **(F)** a Cabernet Saubvignon canopy where, in the lower portion, the primary clusters can be seen whereas, in the upper portion, the still green forced clusters are also visible.

the types of organs that are retained or removed from the vine. Therefore, (Poni et al., 2020; Martinez De Toda, 2021; Poni et al., 2021) decided to retain primary leaves and clusters while removing laterals after severe trimming of the primary shoots. The purpose behind this was to head toward "double cropping" with an early and late harvest to be completed on the same vines (Figure 9). The same forcing type was preliminarily tested by (Gu et al., 2012), who reported a 74% yield decrease for the forced shoots compared to the primary crop setting at 6.85 kg/vine. More recently, "double cropping" was evaluated on a two-year basis in Grenache, Tempranillo, and Maturana tinta by (Martinez De Toda, 2021) and in Pinot noir (Poni et al., 2021) following a very similar experimental protocol, where the main shoots were trimmed to six-to-seven nodes between the end of the flowering and the pea size stage, and all the laterals were progressively removed. In the former experiment, across cultivars, the time distance between primary and

forced harvests went from 32 to 52 days (the latest harvest was on November 4 in Tempranillo) and TSS was lower in any forced treatment, whereas the acids and the total anthocyanin concentration were greatly enhanced. Overall, the forced crop per vine averaged over the three cultivars was 1.24 kg, which is 42% of the primary crop. The results obtained on Pinot noir were similar, if not better. The crop obtained in the forced primary shoots was about 40–50% of that borne on the regular primary shoots, and, interestingly, the second crop's grape quality scored higher TSS, total anthocyanin and phenolic concentrations, and total acidity. While ripening in the standard crop was reached within the second week of August (which is a rather usual pattern for an early ripening variety grown in an environment assuring 1800–1900 GDD), the forced crop was harvested on October 7 and 8, respectively.

An enticing feature of this daring technique is not only its ability to pursue two harvests within the same season but also its potential to furnish batches of grapes of such different compositions as to allow different market segments to be profitably targeted. In terms of acceptance from growers, the "double cropping" version has an inherent advantage: it is difficult to convince any grower to remove the entire primary crop under the "hope" of obtaining a forced one. At present, more work is required from an operational standpoint to ease the quite laborious shoot trimming and lateral removal operations. Technically, shoot trimming already is an easy and fully mechanized summer pruning practice in vineyards; however, under the protocol envisaged by bud forcing, the trimming should take place above six-to-eight nodes on the main shoots, which means that the machine will not act without the hindrance of posts, stakes, and wires. A solution to this problem is provided by replacing a traditional cutter bar trimmer (which performs topping on vertically positioned shoots when they have outgrown the top foliage wires) with an over-the-row rotating disk machine. The principle of functioning for such machines allows for the navigation of posts and stakes, and once the distance of the bottom pair of disks from the height of the cordon or cane is regulated to correspond to an average cut at six-to-eight nodes, the machine action could also be revealed to be effective at removing or stripping all the young laterals.

In summary, the crop forcing technique applied in a double cropping mode is very interesting. However, to complete a simultaneous double reproductive cycle and active vegetation having to be sustained in mid-summer, vines need high vigor and high resources in terms of nutrients and water. As a matter of fact, Martinez De Toda, (2021) heavily irrigated vines with 4.5 L per day, whereas Poni et al. (2021) used potted grapevines. While longer term studies are needed, initial recommendation would be to start trialing this practice in irrigated vineyards and/or environments having no major summer drought occurrences.

8 Conclusions

The modalities and scope of vineyard summer pruning are evolving along with progressive effects bound to global warming. Although generalization is a difficult task, the following main changes seem to stand out:

1) An almost globally felt requirement is that more leaf cover around the clusters is needed today compared to the past. Such a need would render operations such as shoot thinning and leaf removal less stringent, as these exert more of a direct impact on fruit microclimate than others. Moreover, the same need should give more impulse to mechanical leaf removal compared to hand leaf removal.
2) Summer pruning represents a burden in terms of workload. Practices, such as shoot trimming, trunk de-suckering, and leaf removal, are easily mechanizable, whereas others, such as shoot and cluster thinning, are still bound to laborious hand work, which has an impact on vineyard profitability.
3) The most prominent advancement has been a change in mentality, which has configured a given summer pruning as not just a "necessary evil" but rather as a tool to pilot ripening into a desired direction. Early basal leaf removal, apical to the cluster leaf removal, and, more recently, the crop forcing that eventually leads to two harvests in the same season, are very good working examples of this.
4) Summer pruning will likely be impacted by precision viticulture and robotics, and first attempts to automize highly selective operations such as shoot and cluster thinning are underway.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Funding

Universitá Cattolica del Sacro Cuore contributed to the funding of this research project and its publication (Linea D3.1, year 2023).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor [PS] declared a past collaboration with the author(s) [SP and TF].

Publisher's note

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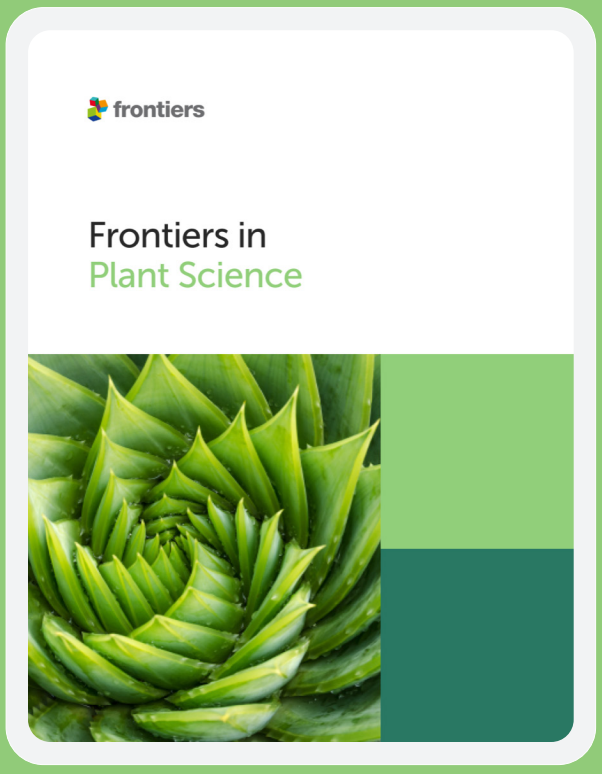

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