UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

Understanding vine response to Mediterranean summer stress for the development of rationale adaptation strategies: the kaolin case

Tese de Doutoramento em Cadeias de Produção Agrícola - Da mesa ao campo

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JURY COMPOSITION

I hereby declare that the Doctoral thesis is in accordance with the technical and scientific standards required by the regulations applied by the University of Trás-os-Montes and Alto Douro. The doctrines presented are the exclusive responsibility of the author.

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When I find myself in times of trouble, Mother Mary comes to me Speaking words of wisdom, let it be And in my hour of darkness she is standing right in front of me Speaking words of wisdom, let it be

Let it be, let it be, let it be, let it be Whisper words of wisdom, let it be

And when the broken hearted people living in the world agree There will be an answer, let it be For though they may be parted, there is still a chance that they will see There will be an answer, let it be

Let it be, let it be, let it be, let it be There will be an answer, let it be

And when the night is cloudy there is still a light that shines on me Shinin'until tomorrow, let it be I wake up to the sound of music, Mother Mary comes to me Speaking words of wisdom, let it be...

The Beatles - Let it be

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RESUMO

As previsões climáticas até ao final do presente século apontam para alterações globais nos ecossistemas vitivinícolas e nos respetivos contextos económicos e sociais. Nesse sentido, espera-se que a influência combinada de diversos fatores ambientais em regiões com clima tipicamente mediterrânico (luz e temperatura elevadas e baixa disponibilidade hídrica) possa prejudicar os mecanismos intrínsecos de resistência da videira, diminuindo consequentemente a respetiva produtividade e qualidade das uvas. Embora a videira seja considerada uma espécie geneticamente resistente a esses stresses abióticos, tais mecanismos poderão ser insuficientes para evitar consequências nefastas no normal desenvolvimento vegetativo e reprodutivo. Assim, a adequada interação entre múltiplos fatores ambientais e vitícolas (*p.ex.* clima, casta, porta-enxerto e práticas culturais) representa um desafio efetivo e emergente para a sustentabilidade da viticultura Mediterrânea.

A aplicação foliar de caulino é uma estratégia de curto prazo amplamente conhecida e aplicada em várias culturas frutícolas porque incrementa a reflexão das radiações ultravioleta, visível e infravermelha, reduzindo a temperatura foliar e a consequente suscetibilidade ao escaldão de folhas e frutos. Contudo, no caso específico da videira, variáveis como a casta, estado hídrico, práticas culturais, fenologia e condições edafoclimáticas podem determinar a sua maior ou menor eficácia. Face ao exposto, nesta tese estudou-se o efeito do caulino (5%) nas castas Touriga-Franca (TF) e Touriga-Nacional (TN) implantadas em duas Regiões Demarcadas portuguesas (Douro e Alentejo) durante dois anos consecutivos (2017 e 2018; 2017 foi mais quente e seco que 2018 devido à ocorrência de duas ondas de calor e baixos níveis de precipitação). Várias metodologias de cariz fisiológico, bioquímico e molecular foram usadas para monitorizar as respostas das videiras durante o período estival, especialmente nas fases do pintor e próximo da vindima. Com o intuito de avaliar as funções foto / termoprotetoras do caulino, estudou-se detalhadamente as relações entre a aplicação desta argila e a acumulação de pigmentos fotossintéticos, o metabolismo de carotenóides, a regulação do ciclo das xantofilas e o possível envolvimento nos processos de quenching não fotoquímico (NPQ). Ao nível do fruto, foram igualmente avaliados vários atributos de qualidade, bem como o conteúdo hormonal e atividade anti-radicalar desde o pintor até à plena maturação.

Os resultados demonstraram que os efeitos benéficos do caulino foram maioritariamente observados em 2017. Sucintamente, foi evidente uma melhoria da eficiência intrínseca do uso da água (23% no Douro e 13% no Alentejo), das taxas de assimilação de CO₂ (P_N; 72% no Douro e 25% no Alentejo) e do teor em açúcares solúveis, assim como uma menor acumulação

de alguns reguladores de crescimento, nomeadamente de ácido abscísico (ABA) e de ácido salicílico (SA) à vindima. Relativamente aos pigmentos fotossintéticos, os efeitos do caulino variaram consoante a região: no Douro, ao longo do verão de 2017, as folhas tratadas com caulino apresentaram menor teor em clorofila e carotenoides e maior decréscimo de NPQ à vindima. Em contrapartida, no Alentejo as plantas TN tratadas com caulino apresentaram um aumento significativo do teor de clorofila enquanto na TF não se registaram alterações significativas, indicando que supostamente a aplicação de caulino nesta casta, nas condições ambientais prevalecentes, não foi tão necessária. Quanto aos carotenoides individuais, os respetivos teores aumentaram nas folhas tratadas de ambas as castas e locais, com uma concomitante regulação positiva da expressão dos genes da violaxantina de-epoxidase (VvVDE1) e da zeaxantina epoxidase (VvZEP1), indicando uma regulação otimizada do ciclo das xantofilas. Simultaneamente, o estado de de-epoxidação (DPS) e os valores de NPQ foram menores nas folhas tratadas, sugerindo uma modulação indireta das xantofilas nos processos de dissipação de energia durante o verão. Em 2017, à vindima, apenas nos bagos da TF das duas parcelas experimentais, constatou-se também que o efeito do caulino contribuiu para o aumento dos níveis de ABA e SA, assim como para uma ligeira melhoria na respetiva conservação da acidez. Embora a acumulação de antocianinas nos bagos não tenha sido consistente, no tratamento com caulino registámos maiores teores de flavonóides, orto-difenóis e taninos em 2017. Em contrapartida, em 2018, os bagos de TF e TN tratadas com caulino apresentaram menor acumulação de ABA e SA. Também se observou uma redução geral do teor de açúcares solúveis, sem comprometer os níveis de ácido málico e tartárico, bem como a produção de espécies reativas de oxigénio (ROS), durante o amadurecimento.

Resumidamente, o trabalho desenvolvido no âmbito desta tese realçou a complexidade de estudar as respostas da videira ao stresse estival e respetivas associações com características varietais, variabilidade climática e eficácia do caulino. Dado que a ação deste protetor aparentou ser mais robusta em anos mais quentes e secos, esta estratégia reúne boas indicações para ser considerada uma prática sustentável para minimizar os impactos do stresse estival em videiras cultivadas em regiões de clima tipicamente mediterrânico fortemente ameaçadas pelas alterações climáticas.

Palavras-chave: fisiologia da videira; fitohormonas; metabolitos secundários; qualidade potencial da uva; stresse estival; xantofilas

ABSTRACT

The foreseen climate change points to shifts in agricultural patterns worldwide, which may impact ecosystems directly and the economic and cultural contexts of the wine industry. Moreover, the combined effects of environmental threats (light, temperature and water availability) at different levels are expected to impair natural grapevine mechanisms, decreasing yield and the quality of grapes. Though grapevines present several mechanisms to sustain growth and development upon prevailing stress factors, it might not be enough to counterpart the increasing magnitude of summer stress impacts in Mediterranean areas. Hence, the interaction between several factors (*e.g.* climate, grapevine varietal stress responses, and management practices) represent a serious challenge for sustainable Mediterranean viticulture, promoting the development of adaptive strategies to cope with environmental stresses.

The foliar application of kaolin particle film is a well-known short-term strategy applied in many fruit crops that increases the reflection of ultraviolet, photosynthetically active and infrared radiation, reducing leaf temperature and preventing leaf and fruit sunburn damage. However, kaolin's effectiveness in improving grapevine resilience when subjected to severe environmental conditions can significantly change according to the variety, water status, management practices, phenology, and edaphoclimatic conditions. Hence, this thesis focused on the comprehensive study of kaolin foliar application (5%) to Touriga-Franca (TF) and Touriga-Nacional (TN) grapevine varieties located in two Portuguese Demarcated Regions (Douro and Alentejo) to study its effects on several defence mechanisms and related metabolites at the leaf and fruit level during two consecutive growing seasons (2017 and 2018).

This study aims to evaluate kaolin application effects on the physiological performance of vines, photochemistry processes, hormonal balance, and expression of leaf stress related genes at both *veraison* and harvest stages. Besides, to get a closer insight regarding kaolin photoprotective functions, we focused on the relations between kaolin treatment and photosynthetic pigments accumulation, carotenoids metabolism, xanthophyll cycle regulation, and its putative role on the non-photochemical quenching (NPQ) processes during 2017. Several quality traits and secondary metabolites were evaluated at the fruit level, as well as the hormonal content and antiradical activity throughout ripening.

Data show that 2017 was warmer and drier than 2018 due to the occurrence of two heatwave events and low rainfall levels, which can modulate kaolin efficiency in alleviating summer stress impacts. Indeed, kaolin effects were mainly observed in the first year of study, enhancing the intrinsic water use efficiency of vines (23 % in Douro and 13 % in Alentejo), net CO_2

assimilation rates (P_N; 72 % in Douro and 25 % in Alentejo), and soluble sugars content, while decreasing the accumulation of plant growth regulators, namely abscisic (ABA) and salicylic (SA) acids at the ripening stage. Regarding pigments accumulation and high light responses, kaolin effects differed among locations. In the Douro vineyard, TF and TN treated leaves displayed lower chlorophyll and carotenoids content throughout the 2017 summer season and reduced NPQ at ripening, suggesting a long-term response to summer stress. Reversely, TN kaolin treated vines from the Alentejo vineyard showed a significant increase of chlorophyll content compared to untreated ones, while no changes were found in TF, which might indicate a lower need for particle-film technology in this variety under the prevailing environmental conditions. Moreover, individual carotenoids were mainly higher in treated leaves, with a concomitant up-regulation of the violaxanthin de-epoxidase (*VvZEP1*) and zeaxanthin epoxidase (*VvZEP1*) gene expression in both varieties and locations, indicating optimised regulation of the xanthophyll cycle. Simultaneously, the de-epoxidation state (DPS) and NPQ values were lower in treated leaves, suggesting that there might be other components influencing NPQ levels beyond zeaxanthin, with an indirect role in long-lasting NPQ processes.

Under the severe summer conditions of 2017, kaolin application increased ABA and SA levels in TF berries at harvest in both vineyards, with slight effects on berry acidity conservation, while no significant differences were observed in TN. Conversely, TF and TN kaolin treated berries showed lower SA and ABA accumulation in 2018. Though kaolin treatment had no consistent effect on anthocyanins accumulation, flavonoids, *ortho*-diphenols, and tannins increased in the berries from kaolin treated grapevines in 2017, along with lower sugar content without compromising malic and tartaric acid levels, as well as reactive oxygen species (ROS) accumulation throughout berry ripening.

In summary, the work developed in the scope of this thesis revealed the complexity of understanding plant stress responses under field conditions and their interactions with varietal and climate features and kaolin effectiveness, which seems stronger under harsh environmental conditions. Nonetheless, kaolin application can be considered a promising practice to minimise summer stress impacts in grapevines grown in Mediterranean-type climate regions.

Keywords: berry quality potential; grapevine physiology; phytohormones; secondary metabolites; summer stress; xanthophylls

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LIST OF ABBREVIATIONS

A

Abscisic acid
Absorption energy per excited cross-section
Absorption energy per reaction centre
Ascorbic acid

B

β-car Beta-carotene

С

С	Control
Car	Carotenoids
CE	Catechin equivalents
Chl	Chlorophyll
Chla	Chlorophyll a
Chl _b	Chlorophyll b
Chl _(a+b)	Total chlorophyll
CI	Cool night index
C_i/C_a	Ratio of intercellular to atmospheric CO ₂ concentration
CO_2	Carbon dioxide
CTAB	Cetyltrimethylammonium bromide

D

DAsA	Dehydroascorbic acid
DI	Dryness index
DI ₀ /RC	Dissipation energy flux per reaction centre
DNA	Deoxyribonucleic acid
DOY	Day-Of-Year
DPS	De-epoxidation state
DW	Dry weight

E

E	Transpiration rate
ECE	Epicatechin equivalents
EL35	Eichhorn and Lorenz growth stage 35 (veraison)
EL38	Eichhorn and Lorenz growth stage 38 (harvest)
ET_0	Reference evapotranspiration

ET ₀ /RC	Electron transport per reaction centre
ΨE_0	Quantum yield of electron transport
Ψ_0	Electron transport probability

F

Fo	Basal fluorescence
F _m	Maximal fluorescence
Fs	Light-adapted steady-state fluorescence yield
F_v	Variable fluorescence
F_v/F_m	Maximum quantum efficiency of photosystem II
FW	Fresh weight

G

GAE	Gallic acid equivalents
GDD	Growing degree days
gs	Stomatal conductance

H

H_2O_2	Hydrogen peroxide
HI	Huglin heliothermal index
HPLC	High-performance liquid chromatography
HSP70	Heat shock protein 70
HVA22a	HVA22-like protein a

I

IAA	Indole-3-acetic acid
IRGA	Infra-red gas analyzer

K

K or KL	Kaolin
IX OF IXL	Raomi

L

LHCII	Light-harvesting complexes
Lut	Lutein

\mathbf{M}

MDA Malondialdehyde

XXXII

Ν

Ν	Nitrogen
NCED	9-cis-epoxycarotenoid dioxygenase
NPQ	Non-photochemical quenching
N _x	Neoxanthin

0

O_2	Molecular oxygen
O_2 .	Superoxide anion
·OH	Hydroxyl radical

P

PAR	Photosynthetically active radiation
PCA	Principal component analysis
PCR	Polymerase chain reaction
PI _{ABS}	Performance index parameter
P _N	Carbon assimilation rates
P_N/g_s	Intrinsic water use efficiency
PPFD	Photon flux density
PSI	Photosystem I
PSII	Photosystem II
PSY1	Phytoene synthase
ϕP_0	Maximum quantum yield of primary photochemistry

Q

Photochemical	quenching
	Photochemical

R

Reaction centre
Peroxyl radical
Relative humidity
Ribonucleic acid
Alkoxyl radical
Reactive oxygen species
Quantitative real-time polymerase chain reaction

S

S Developmental stage

SA	Salicylic acid
SS	Soluble sugars
SnRK2.6	Sucrose non-fermenting 1-related protein kinase 2 family member

Т

Т	Treatment
TBARS	Thiobarbituric acid reactive substances
TF	Touriga-Franca
T _{max}	Maximum air temperature
T _{mean}	Mean air temperature
TN	Touriga-Nacional
TR ₀ /RC	Trapping energy per excited reaction centre

U

UPLC	Ultra-performance liquid chromatography
UV-B	Ultraviolet B radiation

V

V VDE1 V _x V _x +A _x +Z _x	Variety <i>Violaxanthin de-epoxidase</i> Violaxanthin Sum of violaxanthin, anteraxanthin and zeaxanthin
W	
WI	Winkler index
WRKY18	WRKY transcription factor 18
X	
XC	Xanthophyll cycle
Y	
YAN	Yeast available nitrogen
	č
Z	
Z _x	Zeaxanthin

ZEP1	Zeaxanthin	epoxidase

CHAPTER 1

GENERAL INTRODUCTION
Viticultural and winemaking practices are among the most ancestral legacies, shaped by climate patterns and managed by men, with a strong cultural and socio-economic relevance worldwide (Pagnoux *et al.* 2021). Climate patterns are currently changing faster along with increasing industrial activities, which will impose a pressing challenge to all wine sectors in the upcoming times (van Leeuwen and Darriet 2016). Based on several climatic indices and modelling studies, foreseen climate change points to increases in the mean air temperature and altered rainfall patterns in terms of intensity and periodicity, with negative consequences for viticulture sustainability (Fraga *et al.* 2017, Malheiro *et al.* 2010, Santos *et al.* 2020). In Mediterranean-like climate regions, which were considered climate change hotspots, these impacts are exacerbated during the summer season, characterised by prolonged periods of high light and temperature, and water shortage (Diffenbaugh *et al.* 2007, Giorgi and Lionello 2008, Santillán *et al.* 2019). Persistent climate warming in these regions can lead to molecular, biochemical, and physiological changes and increase yield and quality losses, also caused by several pests and diseases associated with environmental factors (Caffarra *et al.* 2012, Mira de Orduña 2010, Scholasch and Rienth 2019).

Though grapevines are reasonably tolerant to heat load and drought, the increasing incidence of abiotic stress factors will certainly limit the viticultural and winery potential of vines, causing damages throughout the growing cycle (e.g. leaf scorching, sunburn, leaf senescence and abscission, shoot and root growth inhibition, fruit damage, sugars/acids and sugars/phenolics imbalances) (Moutinho-Pereira et al. 2007, Wahid et al. 2007, Zhu 2016). Grapevine abiotic stress responses are complex and differ depending on the tissue or organ involved and the stress exposure type and magnitude (Chaves et al. 2003, Cramer et al. 2011, Zsófi et al. 2009). However, the most common responses include shifts in photosynthesis and growth, protein synthesis changes, hormonal metabolism and crosstalk, gene expression regulation, and activation of cellular defence mechanisms (Carvalho et al. 2015b, Chaves et al. 2010, Degu et al. 2016, Moutinho-Pereira et al. 2004). Nevertheless, climate forecasts for Southern European viticulture are alarming, and challenged winegrowers, oenologists and researchers to develop and validate sustainable adaptation strategies to mitigate the adverse effects of summer stress (Fraga 2020, Mozell and Thach 2014). These topics are deeply reviewed in Chapter 2 – State of the art, with an overview of climate change dynamics and impacts on grapevines, particularly on Mediterranean-type climate regions, describing summer stress impacts and grapevine responses, adaptation strategies (short- and long-term), and tools available to assess grapevine performance and fruit quality towards sustainable viticulture.

Kaolin particle-film technology is a well-known short-term strategy applied under controlled and field-grown conditions to avoid sunburn, presenting cost-effective and ecofriendly features, with positive yield and quality effects on several Mediterranean crops (Brito et al. 2019). Due to its excellent reflective properties, kaolin foliar application is associated with leaf cooling effects (Abou-Khaled et al. 1970, Shellie and King 2013), physiological performance and water use efficiency improvements (Brillante et al. 2016, Frioni et al. 2019), photosynthetic pigments accumulation (Dinis et al. 2018b, Frioni et al. 2020), modulation of abscisic acid (ABA) and indole-3-acetic acid (IAA) dynamics (Dinis et al. 2018a), stimulation of the primary metabolome (Conde et al. 2018) and the enzymatic and non-enzymatic defence machinery (Bernardo et al. 2017, Dinis et al. 2016). At the fruit level, although not fully consensual, kaolin application improved general fruit quality (Coniberti et al. 2013), increased polyphenolic composition and radical scavenging activity (Dinis et al. 2016, Luzio et al. 2021), anthocyanins and tannins accumulation (Ferrari et al. 2017, Lobos et al. 2015), triggering the phenylpropanoid and flavonoid pathways (Conde et al. 2016). It was recently also observed a positive effect on berry size and acidity in white varieties, with lower soluble sugars content (Dinis et al. 2020). However, an increasing body of literature points out that kaolin effectiveness depends on several factors, such as concentration and application timing, varietal features, edaphoclimatic conditions over the season, and cultural practices applied (Boari et al. 2014, Ferrari et al. 2017, Gindaba and Wand 2005, Lobos et al. 2015, Luzio et al. 2021).

One of the main drawbacks of studying plants' responses in applied contexts is the complexity of pulling out straightforward conclusions since abiotic stresses act synergistically and plants are continuously exposed to several constraints (Carvalho *et al.* 2015a), which might modulate kaolin efficiency. On the other hand, it can also be considered a pragmatical approach to uncover kaolin efficiency in vineyards from distinct winegrowing regions. In this sense, the **overall objective** of this thesis is to assess kaolin treatment as a reliable tool against summer stress in two red varieties (Touriga-Franca and Touriga-Nacional) grown in distinct winegrowing regions (Alentejo and Douro Demarcated Regions). Besides, through the assessment of stress exposed vineyards and exogenous kaolin application, this work intends to: (i) characterise, in the Douro Superior sub-region and Alentejo region, grapevine stress resilience at a leaf and fruit level in two consecutive growing seasons (2017 and 2018); (ii) understand grapevine primary stress signals and summer stress responses combined with kaolin treatment effect on fruit quality attributes in both regions.

Under field conditions, kaolin effects on grapevines have been assessed exclusively at a local scale (Dinis et al. 2018b, Poni et al. 2019), with little research focused on adult plants grown in distinct winegrowing regions or environmental conditions. Moreover, detailed kaolin functions on several physiological, biochemical and molecular responses to summer stress and acclimation processes are also scarce. Despite modulating ABA and IAA accumulation in leaves (Dinis et al. 2018a, Frioni et al. 2020), associated with improved physiological performance, putative kaolin functions on the rate-limiting step of ABA biosynthesis triggered by 9-cis-epoxycarotenoid dioxygenase (NCED) gene expression, is still unknown. Therefore, this subject was explored in Chapter 3. To underpin a broader picture of summer stress responses and kaolin effects on the physiological performance of vines, Chapter 4 addressed the photochemistry performance by transient chlorophyll a fluorescence analysis using the JIPtest, and evaluation of stress-related metabolites and gene expression. Likewise, photosystem II (PSII) photoprotection mechanisms involving the xanthophyll cycle and chlorophyll a fluorescence dynamics in kaolin-treated leaves were deeply analysed in Chapter 5.1 and 5.2. A targeted approach to uncover kaolin impacts on balancing berry phytohormones, phenolic composition, and quality traits during ripening was also performed (Chapter 6.1 and 6.2), followed by a general discussion (Chapter 7), combining kaolin effects at the leaf and fruit levels in both varieties and winegrowing regions. Lastly, major conclusions and possible future research lines will be addressed in Chapter 8. The present thesis is organised in 8 chapters, and the general framework is outlined in Figure 1.



Figure 1. General framework of the thesis.

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CHAPTER 2

STATE OF THE ART

Briefing note

This chapter covers the current knowledge on grapevine abiotic stress assessment and available strategies to mitigate summer stress impacts in Mediterranean-like climate regions. Within those strategies, foliar protectants' application will be deeply reviewed, describing kaolin treatment effects and highlighting the need to develop, improve, and validate climate change adaptation strategies in a broader sense.

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Abstract

Foreseen climate change points to shifts in agricultural production patterns worldwide, which may impact ecosystems directly, as well as the economic and cultural contexts of the wine industry. Moreover, the combined effects of environmental threats (light, temperature and water relations) at different scales are expected to impair natural grapevine mechanisms, decreasing yield and the quality of grapes. Hence, the interaction between several factors, such as climate, *terroir* features, grapevine stress responses, site-specific spatial-temporal variability, and the management practices applied, which represents and effective challenge for sustainable Mediterranean viticulture, allowed researchers to develop adaptive strategies to cope with environmental stresses. Here we review the effects of abiotic stresses on Mediterranean-like climate viticulture and the impacts of summer stress on grapevine growth, yield, and quality potential, as well as the subsequent plant responses and the available adaptation strategies for winegrowers and researchers. Our main findings are (1) environmental stresses can trigger dynamic responses in grapevines, comprising photosynthesis, phenology, hormonal balance, berry composition, and the antioxidant machinery, (2) field research methodologies, laboratory techniques and precision viticulture are essential tools to evaluate grapevine performance and the potential quality for wine production, and (3) advances in the existing adaptation strategies are vital to maintain sustainability and regional wine identity in a changing climate. Also, these topics suggest that rational and focused management of grapevines may enlighten grapevine summer stress responses, and improve the resilience of agro-ecosystems under harsh conditions. Despite the challenge of developing different strategic responses, winegrowers should clearly define their objectives, so applied research can provide rational technical support for the decision making process towards sustainable viticulture.

Keywords: Climate change; Mediterranean climate; Growth; Yield; Berry quality potential; Resilience

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1.Introduction

In the recent past, higher temperatures and moderate water deficit have increased wine quality in most wine-growing regions, whereas yields have generally decreased. However, based on the latest climate projections worldwide, this pattern will undoubtedly change (Van Leeuwen and Darriet 2016). Scientific evidence sharply states that climate change represents a dominant challenge for viticulture in the upcoming decades (Fraga *et al.* 2013b, Giorgi and Lionello 2008, Hannah *et al.* 2013). Over the last 10 years, the number of publications regarding abiotic stresses in *Vitis vinifera* L. increased by around 90% as shown by the results on PubMed, showing the significance of climate change and abiotic constraint impacts on viticulture, as well as the scientific efforts performed towards climate change adaptation.

Driven by multiple factors, such as the emission of greenhouse gases, temperature, precipitation, and human activities, climate change is expected to directly impact ecosystems, thus leading to shifts in agricultural production patterns (Fraga et al. 2016, Hannah et al. 2013). The major perceptible effect of climate change is the increase in the growing-season mean temperatures that can be already observed (Jones et al. 2005). Several climate-based models predict temperatures to increase up to 3.7 °C until the end of the century, respecting the reference period of 1985–2005 (Fraga et al. 2013b, IPCC 2014, Jones et al. 2005, Malheiro et al. 2010). Despite the lower consensus regarding rainfall trends, it is widely accepted that the patterns will vary in terms of periodicity and intensity depending on the region (IPCC 2014). These changes can also have impacts in the Mediterranean region, which lies in a transition zone between the arid climate of North Africa and the moderate temperate and rainy climate of central Europe. Exhibiting a typical Mediterranean climate (mild and wet winters; warm and dry summers), the suitability of the Mediterranean region for grapevine production also arises from the complex topography, coastline, and vegetation that covers this region (Giorgi and Lionello 2008, Hannah et al. 2013). Even though the best wine quality rankings encompass Mediterranean-like climate countries, the impacts of climate change on viticulture and winemaking go beyond the economic and cultural dynamics of this industry. Future trends point to an impairment of numerous plant natural mechanisms, affecting grapevine growth, physiology, and berry ripening, which can cause severe losses regarding yield and the quality of vines (Fig. 1).



Fig. 1. Impacts of climate change on grapevine canopy (A) and clusters (B) in Mediterranean-like climate regions, particularly in the Douro Valley, Portugal

Due to the overall projected impacts of climate change on agriculture, assessing the magnitude of the potential risk for vines will assist the development of rationale and sustainable adaptation strategies for winegrowers (Iglesias *et al.* 2007). Also, the study of climate change effects on viticulture will lead to a better understanding of grapevine stress responses. Through the development of different grapevine stress assessment methodologies, it might be possible to increase the quality, profitability, efficiency, and sustainability of the wine industry in a changing climate. Hence, adaptation strategies should be explored to sustain grapevine yield and quality towards a sustainable viticulture (Fig. 2).



Fig. 2. Conceptual framework of climate change dynamics and its impacts on grapevine stress responses towards a sustainable viticulture

Adaptation strategies include all the set of actions and processes that can be performed in response to climate change (Akinnagbe and Irohibe 2014). A classic example of an adaptive strategy applied in viticulture is the sustainable management of vineyards, which can act as a carbon sink and improve the resilience of agro-ecosystems under harsh conditions, providing a batch of ecosystem services (Brunori *et al.* 2016). Besides, by adjusting natural and human systems, three significant purposes can be achieved: (1) reduce the risk of damage, (2) develop the capacity to manage certain damages, and (3) find opportunities with climate change (Ollat and Touzard 2014). Recent multidisciplinary research had focused not only on the impacts of climate change on the physical, biological, and molecular aspects of grapevines but also on the current adaptation strategies that can be generally applied (Mosedale *et al.* 2016, Neethling *et al.* 2016). However, we still require more information on the combined effects of environmental threats (light, temperature, and water relations) at local and regional scales, especially in Mediterranean-like climate countries, where the environmental thresholds can be reached during the summer season.

Although some research has been carried out on grapevine varietal resilience to summer stress, there is still little scientific understanding of varietal sensibility regarding interactions between environmental parameters and plant acclimation responses (Ollat and Touzard 2014). Moreover, few studies have addressed the sustainability and the validation of the adaptation

strategies nowadays available in an industrial and applied context (Duchêne 2016, Ollat and Touzard 2014). Also, there is uncertainty regarding the capacity of winegrowers to adapt to changing conditions. In this sense, the first purpose of this paper is to review current knowledge regarding summer stress impacts in Mediterranean areas, on a socio-economic and biological perspective, as well as the responses triggered in grapevines by those stimuli. Next, we will focus on short-term and long-term adaptation strategies that can be adopted by the winegrowers to cope with summer stress. Then, some field and laboratory methodologies will be considered to approach grapevine performance and fruit quality potential. Finally, we will discuss future perspectives and research topics regarding adaptation strategies applied in viticulture, which could support the decision-making process towards sustainable adaptation strategies.

2. Climate change on Mediterranean-like climate regions

According to Wardlaw (1972), stress can be defined as a factor that is potentially unfavourable to an organism, and it is nowadays unanimous that those factors can be either environmental (abiotic) or caused by other organisms (biotic). Despite the increasing scientific concern focused on abiotic and biotic stresses, other variables must be taken into consideration when extending this concept to a field crop, such as yield and quality (Cramer *et al.* 2011, Keller 2010a). The overall effects of individual or combined climate change-related variables, such as interactions between high radiation levels and high temperatures, and both soil and atmospheric water deficits, may have negative impacts on vineyards yield, specifically in most Mediterranean-like climate regions (Ferrandino and Lovisolo 2014, Fraga et al. 2013b). In fact, under the current management conditions for much of the Mediterranean region, decreases in crop yields up to 40% are predicted (Iglesias et al. 2007). Also, yield variability is predicted to increase, while a decrease in water availability is foreseen, alongside an increase in water demand (Iglesias et al. 2007). Decreasing water resources in some areas may also affect soil structure while reduced soil drainage may lead to increased salinity (Hu and Schmidhalter 2005). However, it is expected that an increase in the frequency and intensity of floods would be likely to occur in some areas presenting significant winter rainfall, leading to the loss of Mediterranean species diversity (Fraga et al. 2013a, Van Leeuwen and Darriet 2016).

2.1.Viticultural climatic indices

Several bioclimatic indices have been proposed for estimating the risk of moisture-induced diseases, which showed that areas displaying a Mediterranean-like climate would tendentiously

present low risks of contamination, particularly in southern Europe (Bois *et al.* 2017, Fraga *et al.* 2013a). While drier environments may lead to higher insect and viral outbreaks, wet periods are expected to trigger cryptogamic and bacterial diseases, which can indirectly disturb population dynamics of insect pests (Katsaruware-Chapoto *et al.* 2017). In fact, current research suggests that mildews remains the major phytosanitary threat in most wine-growing regions, even in dry climate vineyards, as a result of the irrigation practices applied, but also because of the fact that mildews can be highly damaging, thus requiring a rapid intervention (Bois *et al.* 2017). Moreover, existing pests are likely to expand, as well as invasion by new insect pests as a consequence of the foreseen increased frequency of extreme weather events, temperature, carbon dioxide levels, and changes in moisture conditions (Jaworski and Hilszczański 2013). However, an accurate quantification of the potential impact of climate change on biotic stresses represents an important challenge, since pest and host responses to environmental shifts are highly variable and complex (Katsaruware-Chapoto *et al.* 2017).

Along with these considerations, the Mediterranean region features unique characteristics (cover vegetation, coastline, and topography) that may modulate the regional climate, which is particularly decisive in viticulture, where the concept of terroir is closely related to wine quality and typicity (Giorgi and Lionello 2008, Van Leeuwen and Seguin 2006). The terroir not only includes the key elements of a delimited geographical area defined from society but also embraces the physical elements of the vineyard itself: the vine, subsoil, location, drainage, and microclimate, which altogether are essential to delineate vine-growing regions (Unwin 2012). Nonetheless, it is difficult to define the ideal terms of the natural environment (climate, soil, and geology) and their interactions with human factors, agronomic approaches, and the vine water uptake conditions, which can modulate the quality of wines (Chone *et al.* 2017, Van Leeuwen and Seguin 2006).

The integration of climatic variables, such as heliothermal conditions over the growing cycle, temperature summation, rainfall, potential soil water balance over the growing cycle, night temperature during berry ripening or seasonal weather data, with other non-climate-related indicators (e.g., potential quality of grapes at harvest and management practices) improved winegrowers' adaptive strategies, both in time and space (Malheiro *et al.* 2010, Tesic *et al.* 2002). This ongoing process in decision-making can be supported by the improvement and development of several viticultural climatic indices, describing the climate of wine-growing regions worldwide at different scales. One of the earliest indices was the heat unit concept, using a growing degree base of 10 °C (degree-days) since grapevines need a specific

heat accumulation to complete the growing cycle (Winkler and Amerine 1944). The cool night index (CI), which accounts for minimum temperatures during maturation, and the diurnal temperature range, are other thermal indices that can estimate the production of high-quality wines (Ramos *et al.* 2008, Tonietto and Carbonneau 2004). However, grapevine variety and day-temperature can also influence the effect of night temperature on the ripening process (Kliewer and Torres 1972). Based on the potential water balance of the Riou Index (Riou *et al.* 1994), Tonietto and Carbonneau (2004) developed the dryness index (DI) that considers the soil–water availability at the beginning of the growing cycle, besides the potential evapotranspiration and precipitation. Also, these authors integrated the Huglin heliothermal index (HI) in their proposed model, which adjusts the value of heliothermic index for different latitudes, and created a multicriteria climatic classification system (Geoviticulture MCC System) for the grape growing regions worldwide (Tonietto and Carbonneau 2004). This system represents a research tool for viticultural zoning, allowing the assessment of the potential suitability for grape production at different scales for economically sustained viticulture in a changing environment.

2.2.Summer stress impacts

Shifts in climate patterns leading to abiotic stresses encompass the set of environmental conditions that decrease growth and yield below optimal levels (Skirycz and Inzé 2010). The most common abiotic stresses comprise drought (water deficit), salinity, soil acidification, high temperatures, and excessive radiation exposure, being difficult to discriminate the individual impacts of each stress in an open field situation, since all these environmental factors are interrelated (Tester and Bacic 2005). Generally, the term summer stress describes the combination of various abiotic stresses, such as water deficit, high sunlight, and high temperature, which are more severe during the summer season (Cramer et al. 2011). The relationship between sunlight exposure and temperature of grape clusters is important to perceive grapevine metabolism, since many of the biochemical pathways are both light and temperature sensitive (Spayd et al. 2002). Previous studies have shown that shaded berries were often 2.4 °C above ambient temperature, whereas sun-exposed clusters were up to 12.4 °C above ambient (Millar 1972, Smart and Sinclair 1976). Similarly, Crippen and Morrison (1986) reported that sun-exposed clusters were warmer than shaded clusters during the day and cooler during the night, indicating greater net radiation loss by the sun-exposed berries at night. Moreover, it is widely known that high temperatures can cause damages throughout the growing

cycle, including scorching of leaves, sunburn, leaf senescence and abscission, shoot and root growth inhibition, fruit damage, and reduced yield (Vollenweider and Günthardt-Goerg 2005, Wahid *et al.* 2007). Another related topic is the increased incoming radiation, particularly in the UV-B range, which despite having a positive impact on skin phenolics, is also likely to affect grape aromas, and consequently the quality potential of wines (Schultz 2000, Van Leeuwen and Darriet 2016, Van Leeuwen and Destrac-Irvine 2017). Vine water status depends, not only on climatic parameters, but also on soil water retention capacity, while a period of high frequency and intensity of water stress, when transpiration exceeds the ability of the root system to supply water to the transpiring leaves, may impair photosynthesis, due to severe the water deficit (Chone et al. 2017, Van Leeuwen and Destrac-Irvine 2017). However, since grapevine features a reasonable tolerance to drought, moderate water deficit may induce changes in the source to sink relationships (competition for carbon resources), reducing both shoot vigour and berry size, and consequently increasing skin surface/mass berry ratio (Castellarin et al. 2007). Besides, mild water deficits are known to cause embolism in the xylem shoot apex, which can have positive effects on berry skin anthocyanin and tannin content in red grape varieties, due to the lower competition between vegetative growth and reproductive development for sink resources (Chone et al. 2017, Schultz and Matthews 1993). These findings also suggest that vines exposed to moderate water deficit may have richer must and wine quality (Chone et al. 2017).

3. Grapevine stress responses

Plants perceive abiotic stress signals and acquire complex and dynamic defence responses, either elastic (reversible) or plastic (irreversible), depending on the duration and intensity of the stress (acute vs chronic), as well as the organ or tissue involved (Cramer *et al.* 2011). Since grapevine features a reasonable tolerance to drought, moderate water deficit may induce changes in the source to sink relationships (competition for carbon resources), reducing both shoot vigour and berry size, and consequently increasing skin surface/mass berry ratio (Castellarin *et al.* 2007). Besides, the association between several biotic and abiotic factors and the capacity of a plant to acclimate to extreme stress conditions may determine plant resilience, despite being genotype dependent (Cramer 2010). Stress, in turn, has several biological consequences for the plants, hampering the adaptation, profitability, quality, and even the survival of many crops with high economic impact on a global scale (Fraga *et al.* 2013b). These considerations influence the complexity of the response. The most common stress responses

include shifts in photosynthesis, growth, changes in protein synthesis, hormonal metabolism, transcription, signalling networks, and stimulation of the cellular defense machinery (Moutinho-Pereira *et al.* 2004, Vierling and Kimpel 1992, Zhu 2016).

3.1. Effects on phenology, growth, and yield

Climate plays a crucial role regarding the development of vines, through optimal thermal requests, water availability over the growing cycle, and radiation intensities and extent, which may compromise plant growth, yield, and quality (Dinis *et al.* 2014, Mira de Orduña 2010). In nature, plant responses to abiotic stresses may follow a different sequence of internal events. However, the initial growth inhibition arises before inhibition of photosynthesis or respiration (Pellegrino *et al.* 2005, Zhou *et al.* 2007). Plants' ability to osmotically adjust or conduct water may modulate their growth, meaning that during stress exposure, morfo-anatomical and metabolic changes will gradually occur (Cramer *et al.* 2011). The structural dynamic of the grapevine canopy over the growing cycle is closely linked to growth and production of grapes of high quality potential (Wahid *et al.* 2007). However, increasing sunlight penetration into the canopy structure can have impacts on the ratio between older and younger leaves at berry softening stage, mainly when reaching environmental thresholds. These consequences are likely to trigger impairments in the vegetative growth and the reproductive development and functioning of plants (Wahid *et al.* 2007).

Some studies have considered the effects of environmental stresses and canopy management practices throughout the growing cycle, enlighten the dynamic between grapevine microclimate and farming practices and their effects on fruit ripening, yield, and quality potential (Dokoozlian 1996, Jackson and Lombard 1993, Mabrouk and Sinoquet 1998, Smart 1985). Phenology is considered one of the first biological indicators of stress used to quantify the magnitude of climate change impact in vines during the main grapevine phenological stages (bud break, flowering, and veraison) and at harvest (García de Cortázar-Atauri *et al.* 2017, Menzel *et al.* 2006). Several models have been applied to predict the onset of vines phenology, and to enlighten the factors that may interfere in the development of vines under different conditions (Daux *et al.* 2012, Duchêne and Schneider 2005, Jones 2013, Parker *et al.* 2011). These studies based on phenology evolution models showed that all main grapevine phenological stages would advance in the upcoming years, being more perceptible in northern vineyards, while earlier onset of grapevine phenophases often precedes changes in growth (García de Cortázar-Atauri *et al.* 2017). Previous research established that increasing mean

temperatures are negatively correlated with the number of flowers per inflorescence (Keller 2010a). Besides, the upward shift in seasonal temperatures is expected to settle the typical development pattern of grapevines towards an earlier onset of flowering, veraison, and harvest (García de Cortázar-Atauri *et al.* 2017). Earlier veraison suggests that the critical ripening stage may deviate towards the warmest period of the season, affecting yield and fruit composition, mainly sugars, organic acids, and phenolics (Ferrandino and Lovisolo 2014, Fraga *et al.* 2013b).

Generally, the number and size of grape clusters formed during grape development determine harvest yield, which is influenced by several key stages of vine phenology and seasonal conditions; however, the response of berry growth and physiology to abiotic stresses varies during the ripening process (Van Leeuwen and Destrac-Irvine 2017). In fact, although region dependent, several studies have observed a relationship between increasing summer stress and reduced grapevine yield and quality (Duchêne 2016, Petrie and Clingeleffer 2005, Pratt 1971, Watt et al. 2008). For instance, water stress induces a lower yield by restraining photosynthesis, meaning that only a limited amount of berries can achieve full ripeness (Zulini et al. 2007). In addition to carbon metabolism impairment, water stress also affects nitrogen metabolism and assimilation, through decreases in nitrate reductase activity (Bertamini et al. 2006). Moreover, Huffaker et al. (1970) suggested that a pronounced decrease of the nitrogen assimilation pathway could be associated with biochemical acclimation to drought, through the reduction of energy requirements during stress exposure, which prevents the accumulation of nitrite and ammonium. These findings suggest that it is vital to develop management tools, adapted to match specific cultivar/ rootstock/site combinations, in order to maximize grapevine quality in a changing climate.

3.2. Effects on photosynthesis

The physiological processes of grapevine initiate when the average temperature is around 10 °C; however, above 35 °C, plants start triggering acclimation mechanisms (Ferrandino and Lovisolo 2014). The most pronounced effects of summer stress on plant physiology comprise the decrease of photosynthetic rates by photoinhibition of photosystem II (PSII) and reduction in stomatal conductance (Dinis *et al.* 2016b, Moutinho-Pereira *et al.* 2007, Pinheiro and Chaves 2011). Non-photochemical quenching (NPQ) is the primary protective mechanism against photoinhibition, involving xanthophylls for the dissipation of excessive non-radiative energy (Hendrickson *et al.* 2004). Moreover, summer stress increases respiratory activity, which can overcome CO₂ fixation, leading to unbalanced growth (Millar *et al.* 2003).

Studies have revealed that high temperatures induce anatomical and structural changes in the organization of the photosynthetic membranes of chloroplasts, leading to a decrease in the photosynthetic and respiratory activities (Wahid *et al.* 2007, Zhang *et al.* 2005). For instance, Yamada *et al.* (1996) showed that chlorophyll fluorescence, the ratio of variable fluorescence to maximum fluorescence (F_v/F_m), and the basal fluorescence (F_0) are physiological parameters correlated to stress tolerance (Yamada *et al.* 1996). Similarly, other authors have reported a sustained decrease in F_v/F_m of dark-adapted grapevine leaves along with an increase in F_0 , suggesting the occurrence of photoinhibitory damage in response to high temperature and drought (Gamon and Pearcy 1989, Zulini *et al.* 2007). Moreover, studies regarding the effect of rootstock on grapevine physiological performance in a stressful environment appear to be interlinked with photochemical changes and stomatal limitations (Iacono *et al.* 1998, Toumi *et al.* 2007).

Nonetheless, the combined effect of water deficit, high temperature, and light are presumably the main constraints for photosynthesis, particularly under severe soil water deficits (Flexas *et al.* 1998). Chlorophyll degradation is also a consequence of summer stress and appears to be associated with the production of reactive oxygen species (ROS) (Camejo *et al.* 2006, Guo *et al.* 2006). Besides pigment degradation, high temperatures, light, and drought can also decrease soluble protein contents and alter the rate of rubisco regeneration (Salvucci and Crafts-Brandner 2004, Todorov *et al.* 2003). In fact, though some authors have observed a decrease in rubisco regeneration in stressed plants, little effect was observed on rubisco activity, indicating that this activity, and consequently photosynthetic efficiency, depends on the water deficit conditions and the species under study (Flexas *et al.* 1998, Galmes *et al.* 2010).

3.3. Oxidative stress and antioxidants

One of the main physiological consequences of abiotic stress lays on the inevitable leakage of electrons from different cellular compartments to oxygen (O₂), which disturbs redox homeostasis by the overproduction of ROS, ultimately leading to a state of oxidative stress (Sharma *et al.* 2012). In turn, oxidative stress can lead to shifts in enzymatic activity and the regulation of genes, which may compromise plant survival. ROS exist either as radicals, such as superoxide anion (O₂·⁻), hydroxyl (·OH), peroxyl (RCOO·), and alkoxyl (RO·) radicals, and non-radicals, all of them capable of propagating chain reactions and targeting biomolecules (DNA, lipids, pigments, and proteins) (Møller *et al.* 2007, Sharma *et al.* 2012). Although photochemical events, as well as photorespiration, are considered to represent the main sources of ROS during day light exposure, enzymes like NADPH-oxidase, xanthine oxidase, peroxidases, and amine oxidase can also contribute to ROS production (Schmidt and Schippers 2015). Furthermore, hydrogen peroxide (H₂O₂) has received particular attention as a signal molecule involved in the regulation of specific biological processes, in numerous series of environmental stresses (high light, heat, salinity, drought, and cold stress) and pathogen invasions (Bienert *et al.* 2007). In grapevines, H₂O₂ is also considered a key regulator of small heat shock proteins and many genes of the anthocyanin metabolic pathway (Grimplet *et al.* 2009, Guo *et al.* 2016).

Phenolic accumulation is also linked with several environmental disturbances (Ferrandino and Lovisolo 2014). Indeed, under high temperatures exposure, the probably increased degradation and inhibition of anthocyanins synthesis may lead to higher H₂O₂ production with forwarding induction of the antioxidant machinery (Bernardo *et al.* 2017, Conde *et al.* 2016, Mori *et al.* 2015). Flavonoids act as primary antioxidants in plant responses to a wide range of stresses, inhibiting ROS production and reducing ROS levels once they are formed (Agati *et al.* 2012). Due to the multiple disturbances to which plants are exposed, it becomes essential to expand the research on their enzymatic and non-enzymatic antioxidant defences, as well as concerning the signalling mechanisms and metabolic pathways behind plant stress responses.

3.4.Hormonal balance

Hormones are essential regulators of plant stress responses, with abscisic acid (ABA), ethylene, and auxins, representing the most preponderant for the defence mechanisms acquired by plants (Pieterse *et al.* 2012). Several studies extensively reported the oxidative effects of environmental stresses on plant responses and their interaction with hormones (Cramer *et al.* 2011, Dinis *et al.* 2018a, Spoel and Dong 2008). Changes in ABA concentrations are correlated with abiotic stress regulation, while biotic stress responses are in turn mediated by other hormones such as salicylic acid, ethylene, and jasmonic acid (Rejeb *et al.* 2014). Besides, hormonal dynamics, like auxin-ABA crosstalk, have been demonstrated to increase the sensitivity to ABA in plants (Tognetti *et al.* 2012). ABA plays a central role in stress responses, acting either rapidly, without involving transcriptional activity (e.g., control of stomata aperture) or slower, when stress signals trigger transcriptional responses, such as the regulation of growth and germination (Hubbard *et al.* 2010, Pieterse *et al.* 2012). Moreover, ABA also regulates essential physiological responses to summer stress, including photoprotection and stomatal conductance. Under water deficit, ABA plays a vital role in controlling water relations

in grapevines, by increasing its concentration and flux in the xylem vessels and influencing hydraulic conductance, aquaporin gene expression, and embolism repair (Schachtman and Goodger 2008). Furthermore, interactions between ABA signalling pathways and sugars have been reported to control sugar transport in grapevines (Cramer et al. 2011). The onset of grapevine ripening is proved to be tied to sugar accumulation, being followed by a marked increase in ABA concentration (Gambetta et al. 2010). Additionally, the synergetic effect of ABA and sucrose, concerning anthocyanin accumulation in grapevine, was observed through ABA exogenous application trials, highlighting the role of ABA during grape ripening (Pirie and Mullins 1976, Xi et al. 2012). Also, Conde et al. (2011) highlighted the role of ABA exogenous application in triggering drought resistance mechanisms, due to the increased expression of transport proteins, improved carbon metabolism, and through the expression of stress resistance-related proteins. During a stress-induced stimulus, apart from its function through signal transduction pathways on cells, ABA may also regulate some genes, and gene products that control the expression of stress adaptive-specific genes, featuring a pivotal role in plant survival under environmental fluctuations (Ferrandino and Lovisolo 2014, Sah et al. 2016). In this sense, some authors suggested that ABA may increase berry quality potential through the accumulation of secondary metabolites, since many key genes of the flavonoid biosynthetic pathways were proven to be upregulated during the ripening stage (Tardieu et al. 2010).

3.5. Effects on berry composition

Abiotic stresses, particularly high temperatures, may cause shifts in grape chemistry, which are reflected in over-ripened fruits, with low acidity, high sugar, and thus increased alcohol levels, as well as aroma and colour modifications (Mira de Orduña 2010, Mozell and Thach 2014, Pons *et al.* 2017). Within specific ranges, sun exposure of grape clusters boosts the production of secondary metabolites, which play a central role in fruit and wine quality potential (Cohen *et al.* 2008). Indeed, the temperature has been shown to play an essential role in anthocyanin synthesis since modifications in phenolic compounds are relevant, cultivar dependent, and temperature associated. Under field conditions, Sadras and Moran (2012) observed decoupling of anthocyanin and sugar contents in red wine varieties exposed to stressful environments, suggesting that a moderate water deficit before veraison could partially restore the anthocyanin concentrations, higher temperatures ($T \ge 30$ °C) can lead to a

decrease in anthocyanin synthesis, and even to its inhibition, when temperatures rise above 37 °C. As a consequence, wine quality can be affected by the reduced grape colour and increased volatilization of aroma compounds (Buttrose and Hale 1971, Coombe 1987, Spayd *et al.* 2002, Tarara *et al.* 2008). Moreover, Pons *et al.* (2017) pointed that the absence of herbaceous notes in wines may be associated with exposure to high temperatures during berry ripening. Grape berry composition is also affected by sunlight, since many of the biochemical pathways are both temperature and light sensitive. In fact, increased UV-B radiation has shown positive effects on skin phenolics accumulation, and in the development of berry aroma and aroma precursor profiles (Van Leeuwen and Destrac-Irvine 2017).

In berries, apart from total anthocyanin levels, compositional changes related to summer stress have also been associated with the increased formation of malvidin, petunidin and delphinidin derivatives in berries (Tarara *et al.* 2008). As summer temperature rises to atypical values, the anthocyanin biosynthetic genes are downregulated, reducing berry skin anthocyanin biosynthesis (Conde *et al.* 2016). For instance, Tarara *et al.* (2008) showed that high temperatures are associated with decreases in grapevine delphinidin, cyanidin, petunidin, and peonidin based anthocyanin contents, but found no influence on malvidin derivatives' concentrations.

The expected earlier onset of grapevine vegetative cycle also brings considerable consequences for grape composition, such as increased berry sugar contents and lower acidity. Increased sugar contents, leading to higher alcohol content in wine, can alter wine flavours and mouthfeel, which triggers a reduction in anthocyanin content and consequently the colour potential of red grapevine varieties (Keller 2010b). Summer stress can also have effects on the content of organic acids in grapes. Malic and tartaric acids represent the most common organic acids in grapevine fruits, featuring variable regulation over the ripening stage (Conde et al. 2007). Typically, although both acids reach their highest concentrations near veraison, it is believed that once synthesized, tartaric acid remains stable whereas malic acid is metabolized and used as an energy source during the ripening process (Rienth et al. 2016, Sweetman et al. 2014). Recent research points that water stress during the summer season, along with high light and temperature, can induce changes in aroma, increased skin phenolic content, and reduced malic acid concentrations in berries (Van Leeuwen and Destrac-Irvine 2017). Also, since berry volume increases during ripening, decreases in tartaric acid concentration are often assigned to a dilution effect (Conde et al. 2007, Dokoozlian 2000). In this sense, while tartaric acid is moderately stable to upward temperature, malic acid levels are firmly dependent on temperature and maturity (Buttrose and Hale 1971, Mira de Orduña 2010). Moreover, decreases in total grape acidity are usually linked with higher pH, though this relationship is affected by increased potassium accumulation, which is also temperature dependent, particularly during the ripening phase (Coombe 1987). Therefore, climate change has brought an impending challenge to wine industries, derived from grape composition and condition, such as the increased temperature of harvested grape delivered to the winery, higher environmental temperatures during the fermentation process, increased berry sugars and lower acidity levels (Mira de Orduña 2010).

4. Adaptation strategies

The effects of climate change, along with the future climate projections, pose severe challenges to the winemaking sector. However, winegrowers display great uncertainty regarding future climate trends, being thus essential to improve practical and scientific-based knowledge to enhance adaptive vine responses (Jones *et al.* 2005, Neethling *et al.* 2016). Therefore, adaptation strategies should be developed and optimized to sustain yield and quality. The climate projections are predicting shifts in the ripening period of grapes, meaning that winegrowers will have to adapt, by delaying the growth cycle of the vine. In various wine-growing regions, this will require a highly modified approach to viticulture, through the implementation of strategies to delay ripeness rather than techniques to improve it (Van Leeuwen and Destrac-Irvine 2017). Adaptation measures can be focused on specific threats (short-term), aiming to the optimization of grapevine development and growth, or could embrace a strategic response (long-term), letting actions to be taken before critical thresholds are reached (Fraga *et al.* 2013b, Schultz 2010).

4.1.Short term

The evolution of viticultural techniques applied worldwide allowed winegrowers to focus on additional aspects of grapevines' adaptation to a changing environment, such as the choice for grape quality potential rather than yield, which has significant implications across the soil, canopy and harvest management (Barbeau *et al.* 2014, Battaglini *et al.* 2008, Van Leeuwen and Darriet 2016). The increasing interest in understanding soil influence on vine and grape growth and development, as well as the evolution of the grape maturation concept, promoted a greater balance between technological parameters, such as the ratio between sugar and acidity levels, and the physiological variables associated with ripening, such as phenolic maturation (Neethling *et al.* 2016, Van Leeuwen and Destrac-Irvine 2017, Van Leeuwen and Seguin 2006).

Moreover, the recognition of vine vigour and grape yield as essential fractions of grapevine maturation process and quality, revitalized some existing practices such as adjustments of bud number per cane, shoot trimming, soil amendments, the introduction of cover cropping, and rational leaf removal (Martínez de Toda *et al.* 2014, Van Leeuwen and Darriet 2016).

4.1.1.Cultural practices

Once grape harvesting is occurring earlier in the season because of summer stress, shortterm adaptive measures can be undertaken towards vine phenology delay to avoid quality reduction (Keller 2010b, Van Leeuwen and Darriet 2016). For this purpose, viticulturists can use training systems with higher trunks to decrease bunch zone temperature and limit maximum temperatures on dry and stony soils. On the other hand, winegrowers can adopt the so-called goblet training system, used over centuries, characterized by shorter trunks and lower total leaf area, to promote water use efficiency (Lereboullet et al. 2013, Van Leeuwen and Darriet 2016). The main drawback of this system is converting mechanical harvest into a difficult challenge (Van Leeuwen and Darriet 2016). Besides, late pruning can delay bud break, and thus, the subsequent phenological stages. Reduced leaf area/fruit mass ratio can also delay maturity and decrease sugar/acid ratio in grapes (Parker et al. 2015). Rational hedging and selective defoliation can promote a sustainable ripening, improving the balance between skin phenolics' synthesis and UV-B radiation exposure in the bunch zone. The reduced sunlight exposure on grapes will promote a cooler microclimate, allowing grapes to retain more acidity, and a slower sugar accumulation (Lereboullet et al. 2013, Teixeira et al. 2013, Van Leeuwen and Darriet 2016).

Previous studies have shown the effect of pre-flowering leaf removal on grapevine growth, wood carbohydrates reserves, and chlorophyll fluorescence (Drenjančević *et al.* 2017, Risco *et al.* 2014). Palliotti *et al.* (2011) reported that early defoliation was effective in limiting yield per vine and berry weight, while improving berry skin mass in consecutive years, besides improving the control of vigour. Such features, alongside increased anthocyanin contents in berries, suggest that this technique may improve grape composition and wine quality potential (Palliotti *et al.* 2011). Also, regulation of vine vigour can be obtained through vine inter-row practices, for instance, by using cover cropping during wet growing seasons and, instead of removing it, applying the mulching technique, which will promote the self-reproduction of cover vegetation during dry growing seasons (Lereboullet *et al.* 2013, Møller *et al.* 2007, Van Leeuwen and Darriet 2016). Furthermore, if feasible, changes in row orientation should also be

considered, since this is one of the main factors influencing solar radiation interception (Hannah *et al.* 2013). Another alternative to avoid the effects of climate change is the use of irrigation strategies that modify vine water uptake conditions. Despite the positive effect on yield, sugar, and skin phenolics, irrigation systems represent an economic, environmental, and social cost, since water scarcity is increasing, while a balanced decrease in the water use can improve grapevine water use efficiency without changing terroir expression (Chaves *et al.* 2010, Fraga *et al.* 2013b, Lereboullet *et al.* 2013). In the driest wine-growing regions, Van Leeuwen and Seguin (2006) pointed that only deficit irrigation can bring economically acceptable yields with high-quality potential grapes. However, the ideal water status, aiming at grape quality, is highly dependent on yield. Under dry conditions, severe water stressed vines might lead to fine red wines as long as yield is low, whereas higher yields may benefit berry quality potential when water deficit is mild (Van Leeuwen and Seguin 2006).

Besides its effects on vegetative growth, several authors have studied the management of cultural practices towards the improvement of berry growth and ripening, which may also have impacts on sugar accumulation and berry quality potential (Greer and Weedon 2014, Hochberg *et al.* 2015, Matsui *et al.* 1986, Oliveira *et al.* 2014). For instance, the application of shading panels on "Semillon" grapevine variety has been suggested to delay ripening and decrease canopy temperatures, besides sugar concentration in grapes (Greer and Weedon 2013). However, other authors found no differences in berry sugar accumulation during ripening in "Shiraz" grapevines exposed to high air temperatures, suggesting that vine responses to summer stress are possibly varietal dependent (Soar *et al.* 2009).

Nutrient management represents an essential issue for winegrowers since it impacts grapevine growth, yield, berry composition, and the quality of wines (Leibar *et al.* 2017). Although research in grapevine nutrition has been conducted in several wine growing regions, little is known regarding micronutrient distribution and uptake in grapevines (Pradubsuk and Davenport 2011). Under environmental constraints, increasing evidence suggests that appropriate mineral nutrition may play a critical role in increasing both yield and stress tolerance mechanisms in agricultural crops (Cakmak 2005, White and Brown 2010). Besides, consumers and legislators are requiring sustainable production practices, aiming to decrease vineyard inputs and environmental impacts, which may lead to changes in the nutrient management of vines (Leibar *et al.* 2017). The effects of nutrient management in grapevines can be either direct, unbalancing berry composition and wine aroma, or indirect, through the influence on vegetative growth (Proffitt and Campbell-Clause 2012). Generally, grapevine

nutrient requirements are moderate; however, abiotic stresses can compromise the nutritional balance of vines, leading to a lack of acidity in wine, when the fertilization is excessive, while nutrient deficiency has been reported to increase plant oxidative processes (Delgado *et al.* 2004, Waraich *et al.* 2012). Hence, since macronutrients display different roles in vines, appropriate nutrition is essential for sustaining plant structural integrity and many key physiological processes (Moutinho-Pereira *et al.* 2001, Waraich *et al.* 2012).

Nitrogen (N) application effects in grapevines have been widely explored, pointing to increased vegetative growth, pruning weights, and lateral shoot length (Choné 2001, Keller 2010a, Keller 2010b). In grapes, several studies suggested a relationship between moderate N applications and increased berry size and fruit set (Bell and Robson 1999, Martín *et al.* 2004, Zerihun and Treeby 2002). However, Martín *et al.* (2004) found no change in "Tempranillo" grapevines yield and berry size as a response to N application under the conditions of their trial. In the same study, the authors observed that increased N doses delayed berry sugar accumulation during ripening and that an average N supply (50 g N vine–1) increased skin anthocyanin content, which significantly increased wine colour. Nonetheless, Keller *et al.* (2001) showed that an appropriate N supply might reduce symptoms of inflorescence necrosis, improve fruit set and acidity, and also decrease grape sugar. At a leaf level, Kato *et al.* (2003) reported that plants grown under high light and high N supply had greater tolerance to photo-oxidative damage and increased photosynthesis capacity, than those grown under similar high light with a low N supply, indicating that adequate N levels in plants may trigger their defence mechanisms.

4.1.2. Application of protective compounds

Exogenous application of some protective elements, such as phytohormones (e.g., ABA, gibberellic acid, jasmonic acid, salicylic acid, etc.), signalling molecules, elicitors (methyl jasmonate, yeast extracts, etc.), osmoprotectants (proline, glycine betaine, etc.), trace elements (selenium, silicon, etc.), and nutrients, have been found to be helpful in alleviating the damage of summer stress in plants (Hasanuzzaman *et al.* 2013, Wahid *et al.* 2007). In grapevines, many studies regarding the preharvest application of several treatments demonstrated benefits in managing plant stress responses and improving stress tolerance (Table 1). For instance, a number of authors demonstrated that exogenous applications of ABA, auxins, salicylic acid, gibberellin and kinetin, enhanced yield and graft union formation, increased total phenols, and

promoted changes in berry sugar, acidity, and colour (Abdel-Salam 2016a, Blazquez *et al.* 2014, Degaris *et al.* 2017, Deytieux-Belleau *et al.* 2007, Köse and Güleryüz 2006, Zhang 2011).

Table 1 – Effects of pre-harvest treatments on several Vitis spp. varietie	es.
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Treatment	Concentration	Variety	Protective effect	References
Abscisic acid (ABA)	26 mg.L ⁻¹ 300 mg.L ⁻¹	´Shiraz` ´Cabernet franc` ´Chambourcin´	Reduced transpiration; Inhibition of shoot growth; Induced endodormancy Increased grape colour	Degaris et al. (2017) Zhang (2011)
Indole-3-acetic acid (IAA)	35 mg.L ⁻¹	'Merlot`	Changes in sugar, acidity, colour and Botrytis sensibility	Deytieux-Belleau et al. (2007)
Kinetin Benzyladenine	250-500 mg.L ⁻¹	´Erenköy` ´Italia`	Improved graft union formation	Köse and Güleryüz (2006)
Gibberellin	100 mg.L ⁻¹	'Tamnara`	Delayed fruit set	Blazquez et al. (2014)
Salicylic acid Chitosan Fulvic acid	100-150 mg.L ⁻¹ 500 mg.L ⁻¹ 500 mg.L ⁻¹	"Thompson"	Improved cluster and berry weight; Increased yield, total leaf area, and chlorophyll content; Changes in acidity and total phenols levels; Delayed ripening	El-Kenawy (2017)
Methyl jasmonate	2.24 g.L ⁻¹	'Tempranillo`	Increased phenylalanine content	Garde-Cerdán et al. (2016)
Ethephon (C ₂ H ₆ ClO ₃ P)	144 mg.L ⁻¹	´Shiraz`	Stimulation of IAA biosynthesis Induced ethylene synthesis	Böttcher et al. (2013)
Kaolin (Al ₂ Si ₂ O ₅ (OH) ₄)	50 g.L ⁻¹	'Touriga Nacional` 'Sauvignon blanc`	Reduced leaf and berry skin temperature; Increased ABA and IAA levels; Improved water use efficiency, stomatal conductance and photosynthesis; Changes in antioxidant activity; Improved sucrose synthesis and photoassimilate transport	Coniberti et al. (2013) Dinis et al. (2016, 2017, 2018) Conde et al. (2018)
			capacity in leaves; Decreased DNA methylation; Stimulated anthocyanins accumulation	Bernardo et al. (2017)

Treatment	Concentration	Variety	Protective effect	References
Melatonin	100 mg.L ⁻¹	'Merlot`	Increased phenolic content and antioxidant activity	Meng et al. (2018)
Glycinebetaine	7.65 g.L ⁻¹	'Pinot noir`	Increased leaf area and specific leaf weight	Mickelbart et al. (2006)
Humic acid Citric acid Ascorbic acid	300 mg.L ⁻¹ 2 g.L ⁻¹ 2 g.L ⁻¹	'Ruby`	Higher cluster weight; Increased total phenols, anthocyanins and total chlorophyll content	Abdel-Salam (2016)
SNAP (S-nitroso-N- acetylpenicillamine)	22 mg.L ⁻¹	'Sultanina`	Increased chlorophyll content	Riquelme et al. (2017)
Polyamines Putrescine Spermidine	322 mg.L ⁻¹ 291 mg.L ⁻¹	´Olhoghi` ´Rishbaba`	Changes in phenolic content; Increased antioxidant activity and anthocyanin levels	Mirdehghan and Rahimi (2015)
Urea	1.61g.L ⁻¹	´Garnacha` ´Graciano` ´Tempranillo`	Changes in grape amino acid content	Garde-Cerdán et al. (2014) Gutiérrez-Gamboa et al. (2018)

 Table 1 (continued). Effects of pre-harvest treatments on several Vitis spp. varieties.

Moreover, Meng *et al.* (2019) reported that melatonin pre-harvest application could benefit phenolic content and antioxidant activity in the "Merlot" variety (Meng *et al.* 2019). Similarly, Böttcher *et al.* (2013) suggested that the application of an ethylene-releasing compound in grapevines might stimulate auxins biosynthesis, which may assist the development of late ripening strategies for winegrowers (Böttcher *et al.* 2013). Alternatively, other types of treatments can be applied to increase grapevine leaf and berry pigments, such as the application of humic acid or polyamines (Abdel-Salam 2016b, Mirdehghan and Rahimi 2016). Many authors have focused their research on the development of environmentally friendly practices, like kaolin application, to sustain yield and quality in a challenging climate, through the reduction of leaf and fruit berry surface temperature, thus improving the antioxidant machinery (Boari *et al.* 2015, Dinis *et al.* 2016b, Glenn 2012). In fact, foliar application of solar protectants, has already shown promising results regarding grapevine increased yield, physiological performance, and general fruit quality potential, in the context of climate change at a local scale (Bernardo *et al.* 2017, Conde *et al.* 2018, Coniberti *et al.* 2013, Dinis *et al.* 2018b).

Nitrogen composition in must also plays an important role in grape and wine quality potential, affecting yeast metabolism, fermentation kinetics, the amino acid content, and synthesis of volatile fermentative compounds, since the amino acids are precursors of volatile compounds (Arias-Gil *et al.* 2007, Bell and Henschke 2005). Despite the existence of some contrasting results about the impact of foliar nitrogen sources application on amino acid composition of must (Gutiérrez-Gamboa *et al.* 2017, Gutiérrez-Gamboa *et al.* 2018, Pérez-Álvarez *et al.* 2017), the majority of the studies report an increase in must amino acids and yeast available nitrogen (YAN) content in grapevines treated with nitrogen sources (Choné *et al.* 2006, Lacroux *et al.* 2008). However, these studies suggest that the effects of foliar application of nitrogen sources in grapevines might be varietal dependent.

Even though these short-term measures might enlighten specific plant stress-based responses and the possible mechanisms behind them, an interdisciplinary and applied approach should be adopted, in order to fully understand those processes in different wine-growing regions (Keller 2010b).

4.2.Long-term strategies

Long-term measures rely on a strategic response encompassing changes in varietal and land allocations, changes to cooler sites with lower solar exposure, or to higher altitudes, selection

of appropriate rootstocks, besides genetic enhancement approaches (Cramer et al. 2011, Fraga et al. 2016, Giorgi and Lionello 2008). Genetic variability and plasticity may maximize the adaptation potential of the existing varieties, including clonal diversity, to specific growing regions, to produce a broad range of different wines from the same varieties, or to breed new varieties better adapted to diverse wine-growing regions (Dai et al. 2011, Duchêne 2016, Van Leeuwen and Destrac-Irvine 2017). These adaptations may be also oriented towards lateripening varieties, or genotypes found among the traditional varieties in some wine-growing areas (Mozell and Thach 2014). Through the development of climatic data-based models, it would be possible to predict the grapevine pheno-phases onset in the future, which may optimize adaptation strategies and action boundaries for winegrowers over the growing cycle (Fila et al. 2014, Parker et al. 2013, Parker et al. 2011). Besides, other studies have focused on achieving high fruit to leaf ratio and late veraison dates, breeding new varieties with reduced sugar content and thus low alcohol levels. However, few studies have addressed the actual weight of the genetic variability in sugar metabolism (Duchêne 2016). Since stress has numerous impacts on different grapevine quality and yield components, recent research has been focused on the varietal characterization of phenolic profiles in order to breed or unveil new varieties whose colour would be less affected by high temperatures (Barnuud et al. 2013, Fournier-Level et al. 2009, Huang et al. 2012, Kliewer and Torres 1972).

Despite the challenge of developing different strategic responses, winegrowers should clearly define their objectives to provide rational technical support to the wine industry and to improve scientific-applied knowledge.

5. Grapevine performance and fruit quality assessment

5.1. Field research methodologies

Plant stress manifestation can often be silent, yet there are certain symptoms in different plant organs that can be accurately detected. Also, there are symptoms exhibited by plants that are common to biotic and abiotic stress exposures (Jackson 1986). Overall, field crops present a set of signals that reflect the simultaneous occurrence of various stresses, such as water deficit, nutrient deficiency, high temperature, radiation, and salinity (Ramegowda and Senthil-Kumar 2015). In agricultural crops, several parameters, such as yield and berry weight, are of the upmost relevance, with productivity representing essential data to estimate the resistance of a plant to specific environmental deviations. In viticulture, it is also common to quantify the

number of grape bunches to more accurately estimate the influence exerted by the vine training systems on yield (Keller 2010b).

Stress induces a plant response, which can cause several changes to the grapevine, thus serving as a warning for the viticulturist, and a protection signal. Tropisms and nastic movements are phenomena where the plant reacts to different stimuli, environmental or biotic, for instance, in cases of excessive light, one of the most frequent types of tropism is the paraheliotropism, in which the plant's leaves move to reduce injuriously intense light (Keller 2010a). Winding leaves can be also indicative of some biotic or abiotic stress exposure, as well as their yellowing and the appearance of necrosis. In berries, water deficit can be also detected by their deformed appearance, showing dehydration signals (Chaves *et al.* 2010).

The morphological characteristics of the plant, in each phenological stage, are also important to monitor the phytosanitary status of grapevines, allowing to obtain values of growth and development, which are useful to estimate the plant resilience potential to specific environmental deviations (Keller 2010b).

There are various non-destructive and prompt tools to accurately evaluate the physiological state of the plant in real time, which can be quantified in vivo, revealing detailed information about photosynthetic performance, nitrogen and water status of each plant, from the leaf to the whole canopy. The high level of environmental heterogeneity hinders the conduction of physiological field measurements, particularly in regions where climate varies frequently (Sebastian *et al.* 2016). Thus, stable and reliable measurements of physiological parameters are only possible when experiments are conducted between morning to mid-afternoon on a sunny day, since photosynthetic rates measured in overcast days are usually lower and inaccurate (Greer and Weedon 2012). Measurement of gas exchanges with IRGA (infra-red gas analyzer) is the most commonly used approach for research purposes to evaluate photosynthesis by individual leaf or by whole canopy. Gas exchange measurements provide direct measures of net rate photosynthetic carbon assimilation and data regarding stomatal conductance, internal CO₂ concentration, transpiration, leaf temperature, and photosynthetic photon flux density (PPFD).

During the process of light harvesting, excited chlorophylls dissipate the excessive energy in the form of heat and fluorescence, the latter being possible to determine under field conditions and in real time (Krause and Weis 1991). The use of a pulse-amplitude-modulated fluorimeter allows researchers to calculate fluorescence yield, PPFD incident on the leaf plane, leaf temperature, maximum and effective quantum efficiency of PSII, apparent relative electron transport rates, and photochemical and non-photochemical fluorescence quenching (qP and NPQ, respectively). Fluorescence parameters represent an essential tool to assess crop tolerance to an individual or combined stresses, which can be fully understood with the JIP test. The JIP test is a tool to analyze the polyphasic rise of Chl *a* fluorescence transient from basal to maximal fluorescence (F_0 and F_m , respectively), corresponding to the redox states of PSII and photosystem I (PSI), and to electron transfer effectiveness (Papageorgiou 2004). The polyphasic fluorescence rise (O, J, I, and P steps) is observed after the illumination of dark-adapted leaves, providing information on the relationship between function and structure of PSII reaction centre and core complexes (Dinis *et al.* 2016b).

In grapevines, water status represents an important factor for berry growth and quality potential that can be influenced by environmental and cultural conditions. Besides, the evaluation of crop water status is also required to monitor vine water uptake conditions, through the development of sustainable irrigation strategies and by measuring or modelling variations in soil water content, or by means of physiological indicators so the crop water demands can be supplied (Choné 2001, Gambetta 2016, Pellegrino *et al.* 2005). The pressure chamber is a plantfocused monitoring system that integrates both soil and climatic conditions for determining plant water status (Scholander *et al.* 1964). Pressure chamber measurements can provide values of predawn leaf water potential, daily leaf and stem water potential, and can be performed in open-field or in laboratorial environments (Choné 2001). The evaluation of bulk leaf water relations parameters, mainly the capability for osmotic adjustment, and the maximum bulk modulus of elasticity of cells, is an important tool which can be also monitored with this equipment through the development of pressure–volume curves (Moutinho-Pereira *et al.* 2007, Rodrigues *et al.* 1993).

Leaf chlorophyll content is also closely related to plant stress and senescence (Steele *et al.* 2008). The amount of solar radiation absorbed by the leaf results from its contents in photosynthetic pigments, with the chlorophylls representing the essential pigments for the conversion of radiative energy to stored chemical energy (Foyer *et al.* 1982). Moreover, Chl gives an indirect estimation of the nutritional status, since much of the leaf nitrogen is incorporated in Chl (Steele *et al.* 2008). For instance, the portable N-tester tool measures leaf nitrogen status, which enables fast and accurate field specific recommendations to monitor N application during the growing season (Spring and Zufferey 2000). In grapevines, inexpensive and rapid alternative solutions have been recently developed for analyzing leaf pigments by non-destructive optical methods, which are applicable in a field setting and on a larger leaf area

(Buschmann and Nagel 2007). These methods are based on numerical transformations derived from spectral reflectance or absorbance, providing reliable estimations of leaf Chl to the researcher or winegrower (Moutinho-Pereira *et al.* 2012).

5.2.Laboratory research tools

Laboratorial methods can complement the valuable information recorded in the field, besides adding essential data and outcomes. Some methods assess the general physiological state of the plant, such as pigment quantification, sugars and starch content, protein levels, and the evaluation of lipid peroxidation (Bertamini 2003, Lazo-Javalera *et al.* 2015). Stress imbalances plant homeostasis, triggering specific processes and pathways that will promote a response. Hormonal signalling, which regulates specific physiological responses (e.g., stomatal closure) reveals a broader perception of stress by the plants (Ferrandino and Lovisolo 2014). Therefore, hormones tracking and quantification can provide vital information regarding, not only hormonal signalling pathways, but also hormonal crosstalk and stress responses (Sah *et al.* 2016).

The quantification of total or individual ROS gives a perception of the plant redox state, besides, in association with ascorbate quantification data and osmolytes content, it may also provide an overview of the plant defence mechanisms. The antioxidant potential is also relevant when assessing plant stress–based responses, which can be estimated by the presence of compounds with antioxidant activity, while many of them also exhibit biological activity with relevance for health purposes (Cramer 2010, Sah *et al.* 2016). Besides, complementary analysis can be undertaken in berries to evaluate fruit quality potential, such as the determination of colourimetric parameters and biometric features. Nevertheless, a molecular approach is also crucial to understand, in a robust, assertive, and clear way, the regulation of plant stress–based responses (Cramer *et al.* 2011).

5.3. Precision viticulture tools

Precision viticulture is a strategy that integrates the advanced information technologies and field research methodology data, aiming to maximize production efficiency, quality potential, and profitability, while minimizing environmental impacts (Hall *et al.* 2002, Rey-Caramés *et al.* 2015). Modern and sustainable viticulture requires objective and regular monitoring of key parameters for rational and differentiated agronomic management of vineyards regarding spatio-temporal variability of growth, yield, and grape composition at a local scale (Ferreiro-

Armán *et al.* 2006). Through the acquisition of spectral data from several platforms (satellites, aircrafts, and remotely aerial systems), remote sensing is one of the tools used in precision viticulture to assess fine-scale temporal and spatial changes in soil moisture, canopy growth, water status, chlorophyll, and carotenoids levels, as well as grape composition and quality potential (Ferreiro-Armán *et al.* 2006, Lamb *et al.* 2008, Meggio *et al.* 2010, Zarco-Tejada *et al.* 2013). For instance, a recent study by Silva *et al.* (2018) introduced a model combining hyperspectral imaging and support vector regression to predict anthocyanin concentration, pH index and sugar content in *Touriga Franca* variety, which can be potentially used for a wider variety of grapevines in an environmentally friendly approach. Also, Acevedo-Opazo *et al.* (2008) proposed a possible site-specific approach to characterize grapevine water status variability.

Several studies showed that remote sensed hyperspectral data could be also used for grapevine varietal mapping, representing a practical tool for winegrowers to manage grapevine variability, and for inventory purposes (Ferreiro-Armán *et al.* 2006, Hall *et al.* 2002). Hence, the association of high resolution information and the development of site-specific agricultural management can produce a potential computer-based model, allowing the characterization of spatial-temporal variability at a vineyard level, with minimum impacts for the vine (Hall *et al.* 2002). Moreover, along with all the field research methodologies applied in grapevines, this technology represents a novel reliable approach to support the decision-making process.

6. Conclusion

Climate change and its impacts represent a primary concern for the winemaking sector, which boosted interdisciplinary scientific research to cope with a challenging world (Jones *et al.* 2005). Future climate trends may point to shifts in vine growing regions, where it will be difficult to maintain the high-quality standards with the traditionally cultivated varieties (Hannah *et al.* 2013, IPCC 2014, Mozell and Thach 2014). Moreover, evidence suggests that climate change will affect both grapevine physiology and biochemistry, as well as the methods usually used during the winemaking process. Therefore, focused adaptation strategies should be adopted to maintain grapevine yield and quality potential in a changing environment (Schultz 2010, Van Leeuwen and Destrac-Irvine 2017). As terroir defines each wine-growing region, understanding the interrelation between contextual factors (physical, environmental, social, and economic) and climate change, at local and regional scales, should be the first step to identify and prioritize sustainable adaptation strategies (Neethling *et al.* 2016, Ollat *et al.* 2017). To
address these issues, it is crucial to coordinate efforts on the description of standard methods, development of data management tools, besides the maintenance and enhancement of cultivar and clone collections through multidisciplinary programs, which will define our capacity to adapt to climate change (Ollat *et al.* 2017).

Despite the increasing research on grapevine environmental stresses, we still require more information regarding how plants, micro-organisms, and pathogens, will respond to an increase in CO_2 concentration, temperature and water deficit under field conditions. Hence, future research may be focused on these issues (Mozell and Thach 2014).

Besides the direct impact of high temperatures and radiation on grapevine physiology, grape berry chemistry, and wine character, the secondary effects associated with climate change have to be also considered. For instance, climate change has triggered the incidence of forest and bushfires that, along with the significant damage of green areas, will bring consequences for the viticulture and enology sectors (Mira de Orduña 2010, Overpeck *et al.* 1990).

From the field to the winery, further efforts should be made to measure the global winesector contribution to climate change so that new adaptation strategies can be developed to the wine business in a sustainable process (Schultz 2010).

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CHAPTER 3

EFFECTS ON LEAF GAS EXCHANGE, HORMONAL BALANCE AND ABA REGULATION

Briefing note

This chapter outlines several physiological, biochemical and molecular responses to summer stress and kaolin application, exploring kaolin functions on leaf gas exchange, hormonal balance and related gene expression in two Portuguese grapevine varieties (Touriga-Franca and Touriga-Nacional) located in distinct Mediterranean-like climate vineyards to optimize acclimation processes under a changing climate. This chapter answers the specific objective of the thesis (i) characterise, in the Douro Superior sub-region and Alentejo region, grapevine stress resilience at a leaf level in two consecutive growing seasons (2017 and 2018), opening the discussion regarding kaolin treatment as a reliable short-term strategy against Mediterranean summer stress conditions (overall objective).

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Abstract

In Mediterranean-like climate areas, field-grown grapevines are typically exposed to severe environmental conditions during the summer season, which can negatively impact the sustainability of viticulture. Despite the short-term mitigation strategies available nowadays to cope with climate change, little is known regarding their effectiveness in different demarcated winegrowing regions with differing climate features. Hence, we applied a kaolin suspension (5%) to Touriga-Franca (TF) and Touriga-Nacional (TN) grapevine varieties located in two Portuguese demarcated regions (Alentejo and Douro) with different mesoclimates to study its effect on the physiological performance, hormonal balance and ABA-related grapevine leaf gene expression during the 2017 and 2018 growing seasons. Data show that 2017 was warmer than 2018 due to the occurrence of two heatwaves in both locations, highlighting the protective effect of kaolin application under severe environmental conditions. In the first study year, at midday, kaolin enhanced water use efficiency (23 % in Douro and 13 % in Alentejo), carbon assimilation rates (P_N; 72 % in Douro and 25 % in Alentejo), and the soluble sugar content of grapevine leaves, while decreasing the accumulation of plant growth regulators (ABA, IAA,

and SA) during the ripening stage. The results show an up-regulation of ABA biosynthesisrelated genes (*VvNCED*) in TF treated vines from the Douro vineyard mainly in 2017, suggesting an increased stress response under severe summer conditions. Additionally, kaolin triggered the expression of ABA-responsive genes (*VvHVA22a* and *VvSnRK2.6*) mainly in TF, indicating different varietal responses to kaolin application under fluctuating periods of summer stress. The results show an up-regulation of ABA biosynthesis-related genes (*VvNCED*) in TF treated vines from the Douro vineyard mainly in 2017, suggesting an increased stress response under severe summer conditions. Additionally, kaolin triggered the expression of ABAresponsive genes (*VvHVA22a* and *VvSnRK2.6*) mainly in TF, indicating different varietal responses to kaolin application under fluctuating different varietal responses to kaolin application under fluctuating different varietal

Keywords: Abscisic acid, high light, induced resistance, NCED, photosynthesis, stomatal conductance, sunburn

1. Introduction

Viticulture is an important socioeconomic and cultural sector in many countries and regions worldwide, whose sustainability is expected to be seriously challenged by climate change in the coming years (Bernardo *et al.* 2018, Santos *et al.* 2020). Indeed, the predicted increase in periodicity of extreme weather events (e.g., heatwaves and prolonged drought), along with the simultaneous incidence of high luminosity, high temperatures and water scarcity during the summer, may impact photosynthetic productivity, hormonal regulation and cell homeostasis, thus hampering growth and crop yield (Jones *et al.* 2005, Moutinho-Pereira *et al.* 2004, Ollat *et al.* 2016). Likewise, abiotic stresses also trigger several plant defence responses and adaptation strategies, including osmotic and hydraulic adjustments, energy dissipation mechanisms, antioxidant defence systems, and hormonal regulation and crosstalk in complex signalling networks (Balfagón *et al.* 2020, Bernardo *et al.* 2018, Peleg and Blumwald 2011).

Overall, it has been well documented that abscisic acid (ABA) interacts with other hormones, such as salicylic acid (SA) and indole-3-acetic acid (IAA), controlling stomatal closure, aquaporin gene expression and embolism repair during water deficit (Cramer 2010, Dinis et al. 2018a, Gomez-Cadenas et al. 2015). However, antagonistic reports indicate no correlation between ABA accumulation and stomatal closure in plants subjected to combined abiotic stresses (Balfagón et al. 2019, Zandalinas et al. 2016). Furthermore, several studies have highlighted the existence of a varietal-dependent hormonal sensitivity to abiotic stress factors in different plant species, mainly due to their ability to control ABA metabolism under stress (Balint and Reynolds 2013, Deluc et al. 2009, Niculcea et al. 2013). In grapevines, for example, Soar et al. (2004) reported higher ABA accumulation in 'Grenache' leaves compared with 'Shiraz' under water deficit conditions, and a significant up-regulation of key genes involved in the ABA biosynthetic pathway. ABA signalling networks comprise genes involved in the biosynthesis, degradation and transport of ABA, which ultimately determine its cellular content and the genes involved in the perception and signalling cascade (Pilati et al. 2017). The conversion of neoxanthin to xanthoin is considered the rate-limiting step of ABA biosynthesis, catalysed by 9-cis-epoxy carotenoid dioxygenase (NCED). NCEDs are encoded by multigene families (e.g., NCED1, NCED2, and NCED3), being strongly modulated in response to stress (Nambara and Marion-Poll 2005). Moreover, the regulation of many ABA-responsive genes has also showed that this hormone has a key role in triggering stress adaptation responses (Jia et al. 2017, Wu et al. 2016).

Recent multidisciplinary research on climate variability and climate change short-term mitigation strategies in grapevines has shown that the application of solar protectants with reflective properties, such as kaolin particle film, can notably improve plant water relations and reduce leaf temperature, increasing its ability to cope with summer stress (Brito et al. 2019b, Dinis et al. 2016a, Dinis et al. 2016b). In addition, studies performed in field-grown grapevines have demonstrated that kaolin application can lower ABA and increase IAA accumulation in leaves, showing a strong negative correlation with stomatal conductance, and a better water status (Dinis et al. 2018a). Recently, Frioni et al. (2020) explored kaolin-induced modulation of ABA biosynthesis in potted vines under progressive water stress conditions with xanthophyll cycle pigment dynamics; their results indicated that kaolin treatment reduced the conversion of the carotenoid zeaxanthin into neoxanthin, which consequently decreased ABA levels in leaves. However, it is still not clear if the rate-limiting step of ABA biosynthesis, which is triggered by NCED gene expression, can be directly affected by kaolin application, nor the possible association with several hormonal responsive genes and crosstalk, which can trigger summer stress tolerance. Besides, we still require more knowledge on the combined effects of environmental threats at local and regional scales, especially in Mediterranean-like climate areas, where environmental thresholds can be reached during the summer (Mosedale et al. 2016). Furthermore, few studies have linked the interactions between different varietal sensitivities, environmental variables and plant acclimation responses which would validate kaolin application as a suitable and environmentally friendly practice applied in the wine industry at local scales. Since NCED genes are the cornerstones of ABA biosynthesis, this study hypothesises that kaolin treatment can regulate VvNCED gene expression, modulating ABA, IAA, and SA content with different climatic fluctuations over consecutive growing seasons, thus optimising grapevine summer stress responses. Our study therefore aims to better understand the effects of kaolin in on two red grapevine varieties, Touriga-Franca (TF) and Touriga-Nacional (TN), in two Portuguese demarcated regions (Douro and Alentejo) during the 2017 and 2018 growing seasons. For this purpose, leaf gas exchange, soluble sugar content, phytohormone accumulation, ABA biosynthesis (VvNCED1, VvNCED2, VvNCED3) and responsive (VvHVA22a, VvSnRK2.6) gene expression were assessed.

2. Material and Methods

2.1. Site and plant material

The experiments were carried out under field conditions during the 2017 and 2018 growing seasons in two different winegrowing regions: i) Douro Demarcated Region ("*Quinta do Orgal*" commercial vineyard: 41° 04' N, 7° 04' W, 169 m), in Northeast Portugal, hereafter referred to as 'Douro', and ii) Alentejo Demarcated Region ("*Herdade do Esporão*", 38° 23' N, 7° 33' W, 220 m), in the southeast part of the country, hereafter referred to as 'Alentejo'.

These regions have a warm-temperate climate with hot, dry summers (Kottek *et al.* 2006) with most rainfall occurring mainly during the winter months. An automatic weather station was set up on each trial site to record standard meteorological variables. According to the world reference base for soil resources (FAO 2015), the soil mapping of both regions is classified as luvisols, characterised by a uniform clay-enriched subsoil. The 'Douro' site has a steep slope (30° N) and E-W orientation, and is composed of 6-year-old vines grafted onto 110R rootstock and trained to a unilateral cordon. The 'Alentejo' experiment displays a slight slope (5° N) and N-S orientation, is composed of 8-year-old vines grafted onto 1103P rootstock and is also trained to a unilateral cordon. In both vineyards, spacing is 2.20 x 1.0 m between vines. In both locations, two *Vitis vinifera* L. varieties were selected - Touriga-Franca (TF) and Touriga-Nacional (TN) - due to their notable winery potential.

2.2. Treatments and monitoring

The experimental set up was adapted to the existing features of each commercial vineyard to ensure similar edaphoclimatic conditions and sun exposure among treatments and varieties. In 'Douro', 60 vines per variety were selected and divided into three blocks with 20 vines each. In 'Alentejo' we selected 120 vines per variety planted in one extended row, and with half the row as the control group, and the other half as the treated group; in each half row, the vines were also divided into three blocks with 20 plants each. All vines were managed according to the growers' commercial organic practices and deficit irrigated (30% of the reference evapotranspiration) to prevent plant death. In both experiments, the plants were divided into two experimental groups: the control or untreated group of each variety (TF_C and TN_C), and the kaolin-treated group (TF_KL and TN_KL). Treated vines were sprayed with kaolin (Surround® WP, Engelhard Corporation, Iselin, New Jersey), which was prepared in an aqueous solution at the manufacturer recommended dosage of 5% (w/v), supplemented with 0.1% (v/v) Tween 20 to improve adherence, and directly applied to leaves according to standard operating procedures adjusted for agricultural practices. In 2017 and 2018, kaolin was applied in the 'Douro' experiment on the windless mornings of DOY 177 and DOY 205 respectively,

and in 'Alentejo' trial on DOY 198 in both growing seasons. The adjacent control plants were carefully protected by a plastic film during the kaolin application. For all the physiological measurements, six healthy, fully-expanded, mature leaves in a similar position were sampled per row and treatment during two periods of the day (predawn and midday). The measurements were also undertaken during two different developmental stages: i) at *veraison*, corresponding to DOY 199 and DOY 212 in the 'Douro' and to DOY 208 and DOY 209 in 'Alentejo' in 2017 and 2018 respectively, and ii) at ripening, corresponding to DOY 234 and DOY 254 in 'Douro' and to DOY 237 and DOY 243 in 'Alentejo' in 2017 and 2018 respectively. Leaf samples were immediately frozen in liquid nitrogen, posteriorly ground to a fine powder, and then they were stored at -80°C for further analysis.

2.3. Heat accumulation – Growing degree days (GDD)

In this study, GDD was computed using the Winkler index (WI), referring to the degree day units accumulated during the growing season from April to October, with a base temperature of 10°C (Jones *et al.* 2010, Winkler *et al.* 1974).

2.4. Leaf gas exchange

Leaf gas exchange was evaluated using a portable infrared gas analyser (LC Pro+, ADC, Hoddesdon, UK), operated in the open mode. The measurements were performed on cloudless days under natural light conditions in the morning (09:00 GTM +1) and at midday (14:00 GTM +1). Net photosynthetic rate (P_N , μ mol m⁻²s⁻¹), stomatal conductance (g_s , mmol m⁻²s⁻¹), transpiration rate (E, mmol m⁻²s⁻¹), and the ratio of intercellular to atmospheric CO₂ concentration (C_i/C_a) were estimated according to von Caemmerer and Farquhar (1981). The intrinsic water use efficiency was calculated as the ratio of P_N/g_s to eliminate the possible effects of air humidity and temperature on transpiration (Iacono *et al.* 1998).

2.5. Determination of leaf total soluble sugars

Leaf soluble sugars (SS) were extracted by heating 10 mg of lyophilised tissue in 5.0 mL ethanol:water (80:20, v/v) for 1 hr at 80 °C. Quantification of SS was performed following an anthrone-sulfuric acid method adapted to microplate (Leyva *et al.* 2008). The anthrone reagent, containing 0.1 g of anthrone (0.1 %) dissolved in 100 mL of concentrated sulfuric acid (98 %), was prepared immediately before analysis and then added to the extracts. Determination of leaf SS was made in triplicate by reading the absorbance at 625 nm in a microplate multiscan reader

(SPECTROstar Nano, BMG Labtech GmbH, Germany). The colorimetric response was compared to a standard curve based on glucose, and total SS was expressed as mg/g of dry weight (DW).

2.6. Analysis of phytohormones

Abscisic acid (ABA), indole-3-acetic acid (IAA) and salicylic acid (SA) content was determined by high-performance liquid chromatography coupled to a triple quadrupole mass spectrometer (Micromass, Manchester, UK) through an orthogonal Z-spray electrospray ion source (Durgbanshi et al. 2005). Briefly, 100 mg of lyophilised leaf samples were extracted in 2.0 mL of distilled water using mill ball equipment (MillMix20, Domel, Železniki, Slovenia). ^{[2}H₆]-ABA (Sigma-Aldrich, USA), ^{[2}H₂]-IAA (Sigma-Aldrich, USA), and ^{[13}C₆]-SA (Sigma-Aldrich, USA) were used as internal standards. After centrifugation at 10.000 x g, the supernatants were recovered and the pH was adjusted to 2.8–3.2 using 30% acetic acid. Extracts were partitioned twice with diethyl ether and the supernatants were evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France) at room temperature. The dry residue was then resuspended in 500 μ l of water:methanol (9:1), filtered through 0.22 µM PTFE filters, and directly injected into an UPLC system (Waters Acquity SDS, Waters Corp., Milford, MA) interfaced with a TQD triple quadrupole (Micromass Ltd. Manchester, UK) mass spectrometer through an orthogonal Z-spray electrospray ion source. A reversed-phase C18 column (Gravity, 50×2.1 mm 1.8 µm particle size, Macherey-Nagel GmbH, Germany) was used to achieve the chromatographical separation using a methanol:water gradient, supplemented with 0.1 % acetic acid at a flow rate of 300 μ l min⁻¹. Results were processed using Masslynx v4.1 software, and the phytohormone contents were obtained using a calibration curve prepared with commercial standards.

2.7. Quantitative real-time PCR

RNA was extracted from frozen leaves according to Gambino *et al.* (2008). RNA samples were then treated with DNAse I RNase-free (Thermo Scientific, Waltham, MA, USA) to degrade the possible extracted DNA. The RNA concentration was estimated using the absorbance values at 260 nm with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), while the purity of each sample was determined calculating the 260/280 and 260/230 ratios. Finally, total RNA (1 μ g) was reverse transcribed to cDNA using Primescript RT Reagent Kit (Takara, Shiga, Japan). Quantitative real-time PCR (RT-qPCR)

was conducted with an ABI Step One detection system (Applied Biosystems, Foster City, CA, USA). Gene specific primer pairs used for each target or reference gene are listed in Suplementary material (Table ST1). The amplification was performed via a reaction comprising 1 µL of cDNA, 5 µL of Maxima SYBR Green/ROX qPCR mix (Thermo Scientific), 1 µL of primers (a mix of forward and reverse, 10 µM) and 3 µL of sterile deionised water. RT-qPCR reactions included a pre-incubation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 20 s. Actin and tubulin were used as housekeeping genes to normalise the results among samples. Relative expression of VvNCED1 (Phytozome accession no. GSVIVT00000988001), VvNCED2 (Phytozome accession GSVIVT01021507001), VvNCED3 (Phytozome no. accession no. GSVIVT01038080001), VvHVA22a (Phytozome accession no. GSVIVT01012547001), and VvSnRK2.6 (Phytozome accession no. GSVIVT01009074001) was obtained using the Relative Expression Software Tool Solver v.2 (REST-MCS) (Pfaffl 2001,2002). Each analysed gene was considered significantly up-regulated and down-regulated in the kaolin treated groups (TN KL and TF KL), when its relative expression fold change was ≥ 2.0 and ≤ 0.5 respectively.

2.8. Statistical analysis

Statistical analyses of leaf gas exchange parameters, soluble sugars, and phytohormone content were performed using a Sigma-Plot 12.3 programme (SPSS Inc.). After testing for ANOVA assumptions (homogeneity of variances with the Levene's mean test and normality with the Kolmogorov-Smirnov test), statistical differences among treatments and varieties were evaluated by two-way factorial ANOVA, followed by the post hoc Tukey's test. Afterwards, statistical differences between years (2017 *vs* 2018) within each sampling group were evaluated by one-way analysis of variance (ANOVA), followed by the post hoc Tukey's test. Different lower case letters represent significant differences between treatments and varieties (TN_C, TN_KL, TF_C, TF_KL) within each location and developmental stage. Significant differences were considered when p < 0.05. The asterisks (*** p < 0.001, ** p < 0.01 and * p < 0.05) represent significant differences between sampling years (2017 *vs* 2018) within each variety, treatment and developmental stage. Absence of letters and asterisks indicate no significant difference.

3. Results

3.1. Weather conditions

The daily mean air temperatures from April (DOY 91) to October (DOY 304) in 2017 and 2018 in the Douro trial were 22.6 °C and 21.3 °C respectively, with total precipitation of 92.2 mm in 2017 and 256.2 mm in 2018 (Figure 1A). In 'Alentejo', the daily mean air temperature registered for the equivalent period in 2017 was 22.5 °C, with a total precipitation of 47.0 mm, while in 2018, the mean air temperature recorded from April to October was 21.0 °C with 228.8 mm of total rainfall (Figure 1B). The calculated GDD indicated that 'Alentejo' had the lowest accumulated thermal units in both growing seasons (2683 °C and 2361 °C GDD in 2017 and 2018 respectively), while 'Douro' had the highest (2705 °C and 2416 °C GDD in 2017 and 2018 respectively). Based on the WI classification regions (I–V), most of the GDD calculated fitted into region V, except for the Douro site in 2017 (2705 °C GDD), which slightly exceeded the thresholds of the warmest category (Region V: 2222-2700 °C), and was thus classified as "too hot" (Jones *et al.* 2010).



(°C) of 2017 and 2018 growing seasons in both 'Douro' and 'Alentejo'.

To assess the possible occurrence of heatwaves during the experiments, we counted the number of days with maximum temperatures above 40 °C in both locations and growing seasons. In 2017, a total of 23 days with maximum temperatures above 40 °C was registered at the Douro location (Figure 1C), with two periods of at least five consecutive days each in June

(DOY 165-169) and July (DOY 193-198). Similarly, at 'Alentejo', there were two periods of five consecutive days of maximum temperature above 40 °C (Figure 1D) recorded in June (DOY 167-171) and July (DOY192-197), but with 10 days less of high temperatures throughout the season than 'Douro'. In 2018, a total of 10 and 11 days of extreme temperatures were recorded in the 'Douro' and 'Alentejo' locations respectively, with only one period of six consecutive days having a maximum temperature above 40 °C in both regions (DOY 213-218).

3.2. Leaf gas exchange parameters

From *veraison* to ripening, kaolin application boosted leaf P_N , g_s , and P_N/g_s in both varieties and locations, particularly in the midday period of the 2017 growing season (Table 1 and Table 2). Overall, g_s and P_N values were higher in 'Alentejo' (Table 2) in both seasons compared to 'Douro' (Table 1). In 2017, particularly in the midday period of the ripening stage, TN_KL and TF_KL plants showed significantly higher g_s , P_N , P_N/g_s , and lower C_i/C_a , in both locations. In 2018, these effects were only observed at the *veraison* stage, mainly in TN grapevines located in the Douro experiment (Table 1). At the 'Douro' ripening stage of 2018, TN_KL showed lower P_N and g_s , whereas TF_KL exhibited higher P_N/g_s and lower C_i/C_a . In 'Alentejo', TN_KL showed lower g_s and E at midday and increased P_N/g_s levels only at the *veraison* stage of 2018. The effect of kaolin on the physiological performance of the TF variety was mainly noticed at ripening, showing higher g_s and E values (Table 2).

3.3. Leaf soluble sugars

Between the summer of 2017 and that of 2018, we observed a general decrease in the total content of leaf soluble sugars (SS; Figure 2). At *veraison* in 'Douro', kaolin application decreased leaf total SS content by 26 % in the TN variety in 2017, and by around 29 % in the following season. In contrast, leaf SS accumulation in TF-treated vines increased by 41 % at *veraison* and by 78 % at ripening in the 2018 growing season, while no significant differences were detected in the TN variety at ripening. In 'Alentejo', TF_KL grapevines showed 40 % less leaf SS levels at the ripening stage of 2017 and decreased by around 43 % at *veraison* in 2018, contrasting with the results obtained in the ripening period of 2018 in the same variety.

Table 1. Leaf gas exchange values for Touriga-Nacional with no kaolin (control - TN_C) and with kaolin (TN_KL), and for Touriga-Franca control (TF_C) and with kaolin (TF_KL) in the morning (09:00 GTM+1) and at midday (14:00 GTM+1) in the 2017 and 2018 summer seasons in the Douro site. Stomatal conductance (g_s , mmol m⁻² s⁻¹), net CO₂ assimilation rate (P_N , µmol m⁻² s⁻¹), intrinsic water use efficiency (P_N/g_s , µmol mol⁻¹), ratio of intercellular to atmospheric CO₂ concentration (Ci/Ca), and transpiration rate (E, mmol m⁻² s⁻¹). Data are mean ± SD of six replicates. Different lower case letters represent significant differences between treatments and varieties (TN_C, TN_KL, TF_C, TF_KL) within each period of the day, developmental stage, and sampling year. *** p < 0.001, ** p < 0.01, and * p < 0.05 represent significant differences between sampling years (2017 vs 2018) within each variety, treatment, developmental stage and period of the day.

Time:	09:00 GTM+1					14:00 GTM+1	-				
Leaf measurements	gs	P _N	P _N /g _s	C _i /C _a	Ε	gs	P _N	P_N/g_s	C _i /C _a	Ε	
Veraison 2017											
TN_C	110.4±9.1 ^b	7.58±0.79 ^b	68.9 ± 7.4^{b}	0.663±0.035 ^a	2.56 ± 0.56^{b}	74.2±10.4 ^a	4.88±1.18 ab	65.6±11.7 ^b	0.671±0.051 ^a	2.49±0.42 ^a	
TN_KL	88.0±5.1 °	7.22±0.45 ^b	82.4±8.2 ^a	0.609±0.035 b	2.30±0.47 ^b	49.7±4.6 ^b	3.48±0.49 ^b	69.7±5.4 ab	0.661±0.025 ^a	1.82±0.42 ^b	
TF_C	135.8±15.6 ^a	9.50±1.22 ^a	70.0 ± 5.2^{b}	0.649±0.026 ^a	2.89±0.52 ab	89.0±19.7 ^a	5.48±1.43 ^a	61.1±4.1 ^b	0.689±0.018 ^a	2.69±0.63 a	
TF_KL	149.3±13.4 ^a	10.8±1.60 ^a	72.3±6.7 ^b	$0.635 {\pm} 0.033$ ab	3.39±0.74 ^a	84.1±10.2 ^a	6.62±0.84 ^a	79.3±11.2 ^a	0.611±0.045 ^b	2.60±0.57 ^a	
Ripening 2017											
TN_C	73.3±7.8 ^b	4.28±0.549 ^b	58.4±3.2 ^b	0.721±0.015 ^a	1.56±0.18 ^b	26.5±1.1 °	1.47±0.23 ^b	55.5±9.1 bc	0.728±0.041 ab	0.927 ± 0.080 ^b	
TN_KL	90.3±10.1 ^b	6.15±0.486 °	68.5±4.9 ab	0.669 ± 0.020 ^b	1.81±0.17 °	62.9 ± 13.7 ^{ab}	4.81±1.06 ^a	76.6±2.9 ab	0.626 ± 0.012 bc	1.99±0.28 ^a	
TF_C	92.5±19.0 ^b	5.22±1.20 bc	74.0±10.2 ^a	0.654 ± 0.035 ^b	1.74±0.26 bc	48.1±3.9 ^b	2.54 ± 0.23^{b}	52.9±4.9 °	0.733±0.021 ^a	1.65±0.08 ^a	
TF_KL	136.6±8.1 ^a	8.66±0.608 ^a	63.4±3.3 ^{ab}	0.679 ± 0.015 ^{ab}	2.32±0.12 ^a	64.0±4.4 ^a	5.21±1.20 ^a	81.1±15.4 ^a	0.607 ± 0.069 °	2.00±0.13 ^a	
Veraison 2018											
TN_C	274.3±17.4 b***	12.9±1.37 a***	46.8±3.2 ab***	0.747±0.017 ab***	4.07±0.15 a***	167.4±9.0 ^b	7.22±0.80 ^b	43.1±2.8	0.779±0.014	2.79±0.12	
TN_KL	358.6±29.2 a***	14.6±1.0 b***	40.9±2.4 b***	0.759±0.012 a***	4.51±0.12 a***	211.3±10.8 ^a	9.56±1.20 ^a	45.3±5.8	0.758±0.031	3.26±0.18	
TF_C	235.1±20.6 c***	13.0±1.2 ab***	55.3±1.8 a***	0.706±0.011 b***	3.24±0.24 ^b	128.7±3.5 °	6.55±0.47 ^b	50.9±3.7	0.748 ± 0.015	2.46±0.21	
TF_KL	$257.5\pm23.6^{bc^{***}}$	13.5±1.3 ab***	52.5±3.3 a***	$0.714 \pm 0.017 \ ^{b^{***}}$	3.27 ± 0.26^{b}	143.8±10.9 bc	7.49±1.01 ^b	51.9±3.2	0.740 ± 0.017	2.60 ± 0.09	
Ripening 2018											
TN_C	109.6±7.6 a***	5.75±0.61 ^{b*}	52.4±1.9 ^b	0.731±0.009 ^a	2.09±0.14 ab***	88.5±8.0 a***	5.32±0.60 ^{a***}	60.3±7.2 ^{ab}	0.684±0.033 ^{ab}	2.42±0.15 a***	
TN_KL	115.9±12.5 a***	7.38±0.73 ^{a*}	64.4±9.6 ab	0.669±0.042 ^b	2.35±0.13 a***	51.0±4.8 ^{b*}	3.41±0.77 ^{b*}	66.8±13.9 ab	0.665 ± 0.065 ab	1.52±0.13 b*	
TF_C	129.6±5.8 a***	7.09±1.16 ab**	54.8±8.9 ab**	0.713±0.043 ^{ab*}	2.44±0.05 a***	56.0±2.7 ^b	3.21±0.33 ^b	57.5±7.6 ^b	0.706±0.035 ^a	1.64±0.06 ^b	
TF_KL	84.2±3.8 b***	5.88±1.42 ab***	69.4±13.8 ^a	0.657 ± 0.062 ^b	1.82±0.06 b***	53.9±9.0 ^b	4.28±0.61 ab	79.7±4.5 ^a	0.605 ± 0.038 ^b	1.91±0.68 ab	

Table 2. Leaf gas exchange values for Touriga-Nacional with no kaolin (control - TN_C) and with kaolin (TN_KL), and for Touriga-Franca control (TF_C) and with kaolin (TF_KL) in the morning (09:00 GTM+1) and at midday (14:00 GTM+1) in the 2017 and 2018 summer seasons in the Alentejo site. Stomatal conductance (g_s , mmol m⁻² s⁻¹), net CO₂ assimilation rate (P_N , μ mol m⁻² s⁻¹), intrinsic water use efficiency (P_N/g_s , μ mol mol⁻¹), ratio of intercellular to atmospheric CO₂ concentration (Ci/Ca), and transpiration rate (E, mmol m⁻² s⁻¹). Data are mean ± SD of six replicates. Different lower case letters represent significant differences between treatments and varieties (TN_C, TN_KL, TF_C, TF_KL) within each period of the day, developmental stage, and sampling year. *** p < 0.001, ** p < 0.01, and * p < 0.05 represent significant differences between sampling years (2017 vs 2018) within each variety, treatment, developmental stage and period of the day

Time:	09:00 GTM+1				14:00 GTM+1					
Leaf measurements	gs	P _N	P _N /g _s	C _i /C _a	Ε	gs	P _N	P _N /g _s	C _i /C _a	Е
					Veraison 2017					
TN_C	172.6±4.7 ^b	8.43±0.41 °	48.9±3.1 ab	0.741±0.015	3.40±0.08 ^b	70.2±2.1	4.57±0.61 b	64.7±9.7 ^b	0.678±0.039 ^a	1.84±0.11 ^b
TN_KL	204.0±10.3 ^b	10.8±1.1 ^b	53.1±4.9 ^a	0.710±0.027	3.76±0.14 ab	69.9±7.7	5.22±0.69 ^b	74.5±3.6 ^a	0.641±0.019 ^{ab}	1.80±0.22 ^b
TF_C	278.5±6.1 ^a	12.4±0.4 ^b	44.6±0.9 ^b	0.744 ± 0.006	4.04±0.12 ^a	82.2±4.5	5.21±0.01 b	63.6±3.4 ^b	0.679±0.015 ^a	2.92±0.12 ^a
TF_KL	291.7±22.0 ^a	14.7±0.8 a	50.6±4.3 ab	0.708±0.021	3.77±0.26 ab	93.4±17.8	7.08±1.15 ^a	76.1±2.0 ^a	0.620±0.003 ^b	3.21±0.48 ^a
					Ripening 2017					
TN_C	264.9±12.6 ^a	11.5±1.2 ab	43.5±3.3 ^b	0.753±0.021	3.28±0.14 ^a	89.4±4.8 °	6.28±0.23 ^b	70.4±4.3 ^b	0.651±0.019	2.65±0.16 ^b
TN_KL	229.6±23.3 ^b	10.2±1.2 bc	44.5±1.3 ^b	0.754 ± 0.011	3.20±0.25 ^a	117.6±8.4 ^b	7.89±0.66 °	67.1±4.0 ab	0.653±0.018	3.71±0.17 ^a
TF_C	180.7±5.4 °	9.22±0.70 °	51.0±2.5 ab	0.736±0.014	2.56±0.06 ^b	117.8±19.7 ^b	6.68±1.23 bc	56.8±2.4 ^a	0.703±0.013	3.58±0.45 ^a
TF_KL	232.8±23.7 ^b	12.3±0.8 ^a	53.2±3.7 ^a	0.711±0.016	2.92±0.24 ab	156.7±21.2 ^a	9.89±1.52 ^a	63.2±4.9 ab	0.663±0.024	4.20±0.44 ^a
		Veraison 2018								
TN_C	344.9±19.7 c***	12.0±0.6 c***	34.7±1.9 a***	0.759±0.012	4.89±0.12 b***	249.7±13.1 a***	12.0±0.6 ab***	48.3±2.8 b***	0.674±0.013 ^{ab}	7.69±0.19 a***
TN_KL	529.3±48.0 a***	14.4±0.6 ab***	27.5±2.9 ^{b***}	$0.778 \pm 0.017^{***}$	6.15±0.23 a***	200.4±15.3 b***	11.1±0.6 b***	55.4±4.4 a***	0.648±0.022 ^b	6.38±0.34 b***
TF_C	495.8±27.1 ab***	15.4±0.8 a***	31.2±1.6 ab***	0.706±0.011	3.24±0.24 c***	250.5±30.6 a***	12.8±0.9 a***	51.3±4.9 ab***	0.654±0.023 ^{ab}	7.92±0.60 a***
TF_KL	454.0±30.5 b***	13.6±1.1 ^b	30.1±2.3 ab***	$0.714 \pm 0.017^{***}$	3.27±0.26 c***	230.4±15.6 a***	11.1±0.5 b***	48.2±2.5 b***	0.676±0.013 a***	7.68±0.25 a***
	Ripening 2018									
TN_C	100.2±6.1 b***	4.57±0.61 b***	45.5±3.7 ^b	0.757±0.019 ^a	2.29±0.09 c***	$98.1{\pm}7.5^{\text{ ab}}$	5.54 ± 0.72	56.9±10.1**	0.683 ± 0.046	3.63±0.39 ab***
TN_KL	151.1±6.6 a***	7.15±1.00 a***	47.2±4.9 ab	0.737±0.028 ^a	3.19±0.32 ^b	99.1±12.1 ab**	5.75±0.60***	$58.2 \pm 4.2^*$	0.676±0.018	$3.69{\pm}0.40$ ab
TF_C	116.5±9.3 b***	5.71±0.68 ab***	49.3±7.8 ab	0.736±0.038 ^a	2.47±0.25 °	93.4±7.1 b**	5.08±0.49**	54.6±6.6	0.698±0.031	3.33±0.23 ^b
TF_KL	118.6±10.0 b***	6.42±0.72 a***	54.1±4.3 ^a	0.692±0.022 b	4.16±0.20 a***	114.4±10.2 a***	6.21±0.76***	54.3±4.3*	0.692±0.022	4.6±0.26 ^a



Figure 2. Leaf total soluble sugar (SS) content in the 'Douro' and 'Alentejo' grapevine leaves (Touriga-Nacional control - TN_C and kaolin – TN_KL; Touriga-Franca control – TF_C and kaolin – TF_KL) at 2017 and 2018 *veraison* and ripening stages. Data are mean \pm SD of three replicates. Different lower case letters represent significant differences between treatments and varieties within each developmental stage and sampling year. *** p < 0.001, ** p < 0.01, and * p < 0.05 represent significant differences between sampling years (2017 vs 2018) within each variety, treatment, and developmental stage.

3.4. Phytohormone contents

At *veraison*, in the 2017 summer season of the 'Douro' assay, the kaolin treatment decreased ABA by 33.3 % and SA content by 52.8 % in TN, and it lowered IAA levels by 24.2 % in the TF variety, while no significant effect was observed in either variety during the ripening stage (Figure 3). In the following summer season, the kaolin coating increased leaf IAA content at *veraison* in TF (by 144 %) and in TN (by 76 %) at ripening. In 'Alentejo' at the *veraison* stage of 2017, TN_KL plants showed 27.6 % higher ABA concentrations, whereas TF_KL exhibited 128 % higher IAA content. At ripening, IAA accumulation in TF_KL decreased by around 36 % compared to the control plants. In 2018, ABA content in TN_KL leaves shifted from lower values at *veraison* compared to the control group, to increased ABA levels at the ripening stage, while no significant effects were observed for the TF variety. In addition, IAA and SA accumulation decreased in kaolin-treated plants at both developmental stages, particularly in the TF variety.



Figure 3. Phytohormones (abscisic acid - ABA, salicylic acid – SA, and indole-3-acetic acid - IAA) content in the 'Douro' and 'Alentejo' grapevine leaves (Touriga-Nacional control - TN_C and kaolin – TN_KL; Touriga-Franca control – TF_C and kaolin – TF_KL) throughout 2017 and 2018 summer seasons. Data are mean \pm SD of three replicates. Different lower case letters represent significant differences between treatments and varieties within each developmental stage and sampling year. *** p < 0.001, ** p < 0.01, and * p < 0.05 represent significant differences between sampling years (2017 vs 2018) within each variety, treatment and developmental stage.

3.5. Expression of ABA-related genes

At the 'Douro' 2017 veraison stage, VvNCED1, VvNCED2, and VvNCED3 genes were down-regulated in TN_KL compared to the control group, while in TF at both developmental stages all VvNCED genes were up-regulated in kaolin treated plants (Figure 4). In 2018, the relative expression of all VvNCED analysed genes was lower in kaolin treated plants, except for TN_KL in the ripening period. At the 'Alentejo' 2017 veraison stage, the relative expression of VvNCED genes only changed significantly in TF_KL. At ripening, TF treated plants continued to exhibit higher levels of VvNCED gene expression; in contrast, TN_KL showed an opposite pattern with a pronounced down-regulation of all VvNCED genes analysed in this study. Overall in 2018, VvNCED gene expression of kaolin treated plants was mostly down-regulated in both varieties in 'Alentejo'.



Figure 4. Relative expression of *VvNCED1*, *VvNCED2*, and *VvNCED3* genes of TN and TF grapevine leaves (Touriga-Nacional control - TN_C and kaolin – TN_KL; Touriga-Franca control – TF_C and kaolin – TF_KL) at 'Douro' and 'Alentejo' throughout the 2017 and 2018 summer seasons. * denote significant difference between control and kaolin treated vines of each variety within the same developmental stage (*veraison* or ripening).

TN_KL gene expression of *VvHVA22a* and *VvSnRK2.6* was significantly down-regulated throughout both summer seasons (2017 and 2018) at the Douro location, and, despite no significant changes being observed in the TF variety, there was also a trend for lower expression

levels (Figure 5). Similarly, *VvHVA22a*, *VvSnRK2.6* relative expression was also reduced in TN_KL in the 'Alentejo' trial in both sampling years, particularly in the ripening period of 2017, and at the *veraison* stage of 2018. Conversely, TF_KL showed an up-regulation of *VvHVA22a* and *VvSnRK2.6* gene expression, which was only perceived during the 2017 summer season.



Figure 5. Relative expression of VvHVA22a, and VvSnRK2.6 genes of grapevine leaves (Touriga-Nacional control - TN_C and kaolin - TN_KL; Touriga-Franca control - TF_C and kaolin - TF_KL) at 'Douro' and 'Alentejo' throughout the summer season. * denote significant difference between control and kaolin treated vines of each variety within the same developmental stage (*veraison* or ripening).

4. Discussion

In this study, the environmental conditions recorded over two growing seasons in two different winegrowing regions revealed that stress intensity and extent were widely present, particularly in 2017, as shown by the occurrence of at least two heatwaves in both locations (Fig. 1). In 2017, weather data indicated that the 'Douro' site had higher heat accumulation (2705 °C GDD) than 'Alentejo', which triggered different plant responses in both locations that can, in turn, modulate kaolin efficiency in mitigating summer stress impacts. Shifts in net photosynthesis, stomatal conductance, and water use efficiency are outcomes reported in

grapevines exposed to summer stress, whose efficiency has been improved by kaolin application in vineyards in the Douro region (Dinis et al. 2018b). In agreement with this, the results of the leaf gas exchange analysis (Table 1 and Table 2) showed that, in 2017, treated leaves from TN and TF had higher P_N , g_s , and water use efficiency (P_N/g_s) in both regions, which is consistent with the results obtained for other Mediterranean crops, such as olive trees (Brito et al. 2019a) and hazelnut trees (Cabo et al. 2019). Throughout the experiments, the effects of kaolin on transpiration were positively associated with increasing stomatal conductance and negatively related to P_N/g_s . However, during the midday period of the ripening stage of 2018 in the Douro region, decreased leaf P_N and g_s in TN_KL plants - without significant effects on leaf P_N/g_s and C_i/C_a parameters - may corroborate the hypothesis that kaolin efficiency is higher under more severe summer stress conditions (Brito et al. 2018). Conversely, TF_KL grapevines showed improved leaf P_N/g_s and decreased C_i/C_a in the same period, suggesting that beyond stress severity, which can modulate grapevine physiological responses (Moutinho-Pereira et al. 2004), kaolin efficiency as a short-term mitigation strategy may also depend on intrinsic varietal features. Moreover, the improved leaf gas exchange of grapevines located in the Alentejo region over the two summer seasons, indicates that the grapevines were subjected to better environmental conditions for sustainable plant growth and development. This result may partly be explained by the different row orientation in each vineyard (Hunter et al. 2020), since the E-W orientation of the Douro vineyard suggests higher midday sunlight canopy exposition compared to N-S orientation of the Alentejo vineyard. In addition, heat accumulation during the experiment also increased in 'Douro'. Nevertheless, in the warmer year of the experiment (2017), plants benefited from kaolin application, particularly during the midday period, which is in agreement with previous studies (Dinis et al. 2018a, Dinis et al. 2018b).

Beyond their role in supplying energy, carbohydrates can regulate a wide range of mechanisms, including photosynthesis, sugar transport, defence reactions, secondary metabolism, hormonal balance and berry development (Lecourieux *et al.* 2014), as reported in this study (Figure 2). Since summer stress was more prominent in 2017, and particularly in the Douro region, high leaf SS accumulation may promote carbohydrate storage and growth, maintaining cell homeostasis in kaolin treated leaves, as recently observed in some Mediterranean field crops (Brito *et al.* 2018, Dinis *et al.* 2018b). However, under non-limiting summer stress conditions, such as those recorded during the 2018 growing season, kaolin application decreased foliar carbohydrate accumulation at *veraison* for TN at 'Douro' and TF

at 'Alentejo', which was previously shown to be linked to increasing photosynthetic rates, and reserve mobilisation and export (Brito *et al.* 2019a, Sami *et al.* 2016). Furthermore, the lower leaf SS content found in TF kaolin-treated leaves located in 'Alentejo' at ripening in 2017 indicates that this variety was able to withstand even more intense periods of stress, revealing its ability to adapt to different environmental conditions. The higher SS content found in TF at the ripening stage of 2018 in both regions might also indicate that kaolin application under non-limiting summer stress conditions promotes plant growth and development, which can be varietal dependent and associated with increased expression of sugar transporters as reported by Conde *et al.* (2018).

Phytohormones are key players in modulating several plant responses and stress tolerance, through changes to their synthesis and catabolism, transport, crosstalk and signalling pathways (Gomez-Cadenas et al. 2015). Throughout the experiment, leaf ABA content was higher in 2017 compared to 2018 in both varieties (TF and TN) and treatments (control and kaolin) mainly at the Douro site, highlighting the need to explore and invest in acclimation strategies in vineyards with critical climatic up lines (Fig. 3). The modulating effect of kaolin on hormonal accumulation differed depending on the variety and sampling year, demonstrating the arduous challenge of studying stress responses under field conditions (Peleg and Blumwald 2011). At veraison, kaolin application decreased ABA, IAA, and SA accumulation in 2017 in 'Douro', indicating a prompt response to summer stress under adverse environmental conditions. At the ripening stage, IAA accumulation also decreased in treated leaves in 'Alentejo', whereas SA content increased, suggesting a possible defence signal to reduce greater damage to the photosynthetic machinery (Gururani et al. 2015). However, SA and IAA contents increased in 2018 at veraison in 'Douro', indicating that under non-limiting stress factors, kaolin plants may boost TF growth, development and abiotic stress resistance without restraining the stomatal conductance and water use efficiency of plants (Dinis et al. 2018a).

Interestingly, kaolin-treated plants in 'Alentejo' appear to have adopted a slightly different strategy, with lower IAA and SA accumulation from *veraison* to ripening, particularly in the TF variety in 2018. These results are in line with those obtained by Tombesi *et al.* (2015), who found that stomatal closure was induced by hydraulic signals and maintained by ABA in drought-stressed grapevines, showing the extent of anisohydric behaviour in distinct grapevine varieties and how ABA levels may modulate stomatal aperture upon stress recovery. Thus, the absence of differences in ABA levels in TF_KL observed in the 2018 summer season in

'Alentejo', along with higher g_s , suggests improved hydraulic-mediated mechanisms and anisohydric performance in the TF variety compared to TN.

Transcriptional analyses by RT-qPCR performed on genes involved in ABA biosynthesis and drought stress tolerance showed that kaolin treatment promoted several changes in VvNCED genes throughout grapevine development, depending on the variety, location and growing season. In 'Douro', VvNCED gene expression was up-regulated in kaolin-treated leaves during the 2017 growing season, particularly in the TF variety, but not in the following growing season; this suggests a different varietal sensitivity for ABA synthesis and regulation with kaolin treatment, which seems higher in TF under conditions of intense summer stress. Interestingly, despite the sharp VvNCED up-regulation found in treated vines, particularly in TF, ABA accumulation did not change significantly, contrasting with the results of Dinis et al. (2018a) and Frioni et al. (2020), who reported a reduction in ABA content in kaolin-treated grapevines under summer and water stress conditions. Nonetheless, the water use efficiency of kaolin-coated vines (Table 1 and Table 2) increased in both locations and growing seasons, suggesting a better water status and improved abiotic stress tolerance under harsh environmental conditions (Pilati et al. 2017, Zhang et al. 2009). In 2018, most VvNCED genes were down-regulated in treated grapevines in both locations, supporting the hypothesis that acclimated plants can limit non-essential cellular responses under moderate stress conditions (Larkindale and Vierling 2008). The decreased expression of VvNCED genes in kaolin-treated plants might also be due to changes in the upstream pathway of ABA synthesis in leaves, involving carotenoid metabolism and xanthophyll cycle activation, which play an essential role in protecting plants against water deficit as recently demonstrated by Frioni et al. (2020). Regarding the effects of kaolin in terms of triggering ABA-responsive gene expression, the results showed that VvHVA22a, and VvSnRK2.6 were down-regulated in TN in both regions and sampling years (Figure 5), possibly related to lower ABA levels (Figure 3), suggesting reduced ABA-dependent plant development (Brands 2002, Kulik et al. 2011). Furthermore, the up-regulation of VvHVA22a, and VvSnRK2.6 observed in TF KL in the 'Alentejo' region suggests that, in periods of severe summer stress, kaolin application could boost TF abiotic stress acclimation mechanisms, pointing to an improved varietal ability to cope with multiple stresses under field conditions.

5. Conclusions

In this study, the foliar application of kaolin to Touriga-Franca and Touriga-Nacional varieties over two consecutive growing seasons highlighted its role in modulating the extent to which grapevine can promote abiotic stress responses and acclimation in two different vineyards with similar mesoclimates. The results demonstrate the challenge of understanding stress-related responses and hormonal balance under field conditions. Nonetheless, even when taking into account the inter-annual variability of the environmental conditions in both locations, the foliar application of kaolin improved the water use efficiency and carbon assimilation rates of both grapevine varieties in both locations, thus preventing water restraint, and leading to sustainable plant growth and development, particularly for the TF variety. By modulating the intrinsic plant growth regulator content and signalling throughout the summer season, the kaolin treatment only induced IAA and SA accumulation in the Douro vineyard. This suggests that climate plays a primary role in triggering kaolin effectiveness, different plant stress responses and acclimation, reducing the investment in ABA signalling associated with gene expression, which was triggered by increasing summer stress conditions.

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Supplementary material

Gene	Gene unique ID		Primer (5'-3')	Size (bp)
VvNCED1	GSVIVT00000988001	Fw Rv	CTGGGCTCGTCTATTTCAAC GAGCTGACCCTCGAAGTCGT	125
VvNCED2	GSVIVT01021507001	Fw Rv	GCGGACTCCATTTTTAACGA GCTTCCAGGTTCACTTGCTC	131
VvNCED3	GSVIVT01038080001	Fw Rv	GGGCTCGTCTATTTCAACCG GCTGACCCTCGAAGTCGTAG	121
VvHVA22a	GSVIVT01012547001	Fw Rv	CTTCTTGGCTGGCCTCTATG GTACCCATTCAAGGAGCTTG	166
VvSnRK2.6	GSVIVT01009074001	Fw Rv	GGGCGTATCCCTTTGAAGAT CTGGGTTTGCCACAAAAACT	150
VvACT2	GSVIVT01026580001	Fw Rv	GCCATCCAAGCTGTTCTCTC CAGTAAGGTCACGTCCAGCA	157
VvTUB2	GSVIVT01037405001	Fw Rv	CAACTCTGACCTCCGAAAGC CTTGGAGTCCCACATTTGCT	154

Table ST1. List of primers used for real-time quantitative PCR.

CHAPTER 4

EFFECTS ON CHLOROPHYLL *a* FLUORESCENCE TRANSIENTS AND SUMMER STRESS RESILIENCE

Briefing note

This chapter outlines several physiological, biochemical and molecular responses to summer stress and kaolin application, exploring kaolin effects on chlorophyll a fluorescence transient analysis, leaf cooling and expression of heat stress and stress tolerance related genes (*VvHSP70* and *VvWRKY18*) in two Portuguese grapevine varieties (Touriga-Franca and Touriga-Nacional) located in distinct Mediterranean-like climate vineyards to optimize acclimation processes under a changing climate. This chapter answers the specific objective of the thesis (i) characterise, in the Douro Superior sub-region and Alentejo region, grapevine stress resilience at a leaf level in two consecutive growing seasons (2017 and 2018), assembling the discussion regarding kaolin treatment as a reliable short-term strategy against Mediterranean summer stress conditions (overall objective).

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Abstract

At a local scale, kaolin particle-film technology is considered a short-term adaptation strategy to mitigate the adverse effects of global warming on viticulture. This study aims to evaluate kaolin application effects on photochemistry and related defence responses of Touriga-Franca (TF) and Touriga-Nacional (TN) grapevines planted at two Portuguese winegrowing regions (Douro and Alentejo) over two summer seasons (2017 and 2018). For this purpose, chlorophyll *a* fluorescence transient analysis, leaf temperature, foliar metabolites, and the expression of genes related to heat stress (*VvHSP70*) and stress tolerance (*VvWRKY18*) were analysed. Kaolin application had an inhibitory effect on *VvHSP70* expression, reinforcing its protective role against heat stress. However, *VvWRKY18* gene expression and foliar metabolites accumulation revealed lower gene expression in TN-treated leaves and higher in TF at Alentejo, while lipid peroxidation levels decreased in both treated varieties and regions. The positive kaolin effect on the performance index parameter (PI_{ABS}) increased at ripening, mainly in TN, suggesting that stress responses can differ among varieties, depending on the initial acclimation

to kaolin treatment. Moreover, changes on chlorophyll fluorescence transient analysis were more pronounced at the Douro site in 2017, indicating higher stress severity and impacts at this site, which boosted kaolin efficiency in alleviating summer stress. Under applied contexts, kaolin application can be considered a promising practice to minimise summer stress impacts in grapevines grown in Mediterranean-like climate regions.

Keywords: Foliar spray; heat stress; high light; JIP-test; particle-film; resilience

1. Introduction

In the last decades, the Mediterranean-like climate regions were mostly classified as climate change hotspots, where the impacts of climate variability are likely to change the well-known conceptual factors (i.e., social, cultural, environmental, and economic) of the viticultural sector in the upcoming years (Giorgi 2006, Santos *et al.* 2020). Along with the foreseen occurrence of extreme weather events, the combined incidences of several environmental stresses over the summer season (e.g., high irradiance, temperature, and drought) in Mediterranean-type climate areas have also been related to impaired photochemistry and cell homeostasis, limiting both growth and crop yield (Bernardo *et al.* 2018). Simultaneously, climate variability promotes several physiological and cellular responses, enabling plants to acclimate under climatic uplines (Santos *et al.* 2020, Zandalinas *et al.* 2018).

It is well known that environmental stresses, particularly heat stress, induce gene expression and synthesis of heat-shock proteins (HSP), known by their functions as chaperones for newly synthesised proteins, protecting plant cell from damage (Carvalho *et al.* 2015). In addition, abiotic stresses can also change the expression pattern of a large family of genes (*WRKY* family) that is involved in drought, heat, and salinity tolerance mechanisms, as well as on the regulation of plant development, seed dormancy and germination, leaf senescence, and hormonal signalling (Vives-Peris *et al.* 2018). Moreover, the accumulation of osmolytes, like proline and antioxidant compounds (e.g., ascorbate), is a typical plant response to stress exposure (Akram *et al.* 2017, Masouleh *et al.* 2019). Although most research on plant stress responses has been carried out in young plants and under controlled conditions (Mittler 2006, Zandalinas *et al.* 2018), there is still little scientific understanding when transposing that knowledge to adult plants under field and commercial conditions.

Scientific outcomes regarding particle film application in grapevines reported a generally positive effect on plant photosynthetic performance, leaf cooling, leaf water status, hormonal balance, and sugar transport, decreasing lipid peroxidation and proline accumulation (Brillante *et al.* 2016, Dinis *et al.* 2018b, Frioni *et al.* 2020, Glenn *et al.* 2010, Shellie and King 2013). However, it is still unclear whether kaolin (K) particle film efficiency can be varietal dependent in its interactions with climate variability over consecutive years. In this sense, this study examines kaolin application effects on Touriga-Franca (TF) and Touriga-Nacional (TN) grapevine varieties over two consecutive growing seasons, located in distinct Portuguese wine growing-regions (Douro and Alentejo) through a multi-level approach (physiological, biochemical, and molecular). By assessing *VvHSP70* and *VvWRKY18* gene expression,

accumulation of stress-related foliar metabolites, and photochemistry responses and performance by transient chlorophyll *a* fluorescence analysis using the JIP-test, this study aims to uncover kaolin foliar coating effectiveness in adult field-grown grapevines in two successive summer seasons.

2. Material and Methods

2.1. Site description

The experiments were carried out over the 2017 and 2018 growing seasons, at two commercial vineyards located in two distinct winegrowing regions: Douro demarcated region ("*Quinta do Orgal*" commercial vineyard: 41° 04' N, 7° 04' W, 169 m), in the northeast of Portugal, hereafter designated as Douro; and Alentejo demarcated region ("*Herdade do Esporão*", 38° 23' N, 7° 33' O, 220 m), in the southeast part of the country, hereafter referred to as Alentejo. The Alentejo trial displays a slight slope (5° N) and N–S orientation, composed by 8-year-old vines grafted onto 1103P rootstock and trained to bilateral cordon, while the Douro vineyard is installed on a steep slope (30° N) with E–W orientation, composed by 6-year-old vines grafted onto 110R rootstock, and trained to unilateral cordon. Both vineyards present 2.2 × 1.0 m spacing between vines. According to the world reference base for soil resources (FAO 2015), the soil mapping of both regions is classified as Luvisols, characterised by a clay-enriched subsoil.

2.2. Plant material, treatments and sampling

In both locations, two *Vitis vinifera* L. red varieties were selected, Touriga-Franca (TF) and Touriga-Nacional (TN), due to their ability to ripen under intense heat and relevance for the potential quality and typicity of regional Portuguese red wines. The experimental setup was adapted to the existing characteristics of each vineyard to ensure similar edaphoclimatic conditions and sun exposure among treatments and varieties. At Douro, 60 vines per variety were selected, divided into three rows with 20 vines each, while at 'Alentejo', we selected 120 vines per variety planted in one extended row, and considered half row as the control group, and the other half as the treated group. In each half row, vines were distributed in three blocks with 20 plants each. Vines were managed according to the growers' commercial organic practices and deficit irrigated (30 % of the reference evapotranspiration) to prevent plant death. In both sites, plants were split into two experimental groups: the control or untreated group of each variety (TF_C and TN_C), and the kaolin treated group (TF_K and TN_K). Treated vines

were sprayed with kaolin (Surround[®] WP, Engelhard Corporation, Iselin, NJ, USA), prepared in an aqueous solution at the manufacturer recommended dosage of 5 % (w/v), supplemented with 0.1 % (v/v) Tween [®] 20 (Sigma-Aldrich, St. Louis, MO, USA, CAS number 9005-64-5) to improve adherence, which was directly applied to leaves according to standard operating procedures adjusted for agricultural practices. In 2017 and 2018, kaolin was applied at the Douro experiment in the windless mornings of 26 June (day-of-year (DOY) 177) and 24 July (DOY 205), respectively. At the Alentejo vineyard, kaolin was applied on July 17 (DOY 198) in both growing seasons. The adjacent control plants were carefully protected by a plastic film during the kaolin application. For all the physiological measurements, 18 healthy, fully expanded, and mature leaves, in a similar position, were sampled per treatment at midday in each sampling date. The measurements were also undertaken at two different developmental stages, according to the Coombe (1995) classification: EL35, corresponding to veraison (DOY 199 and DOY 212 at Douro, and DOY 208 and DOY 209 at Alentejo in 2017 and 2018, respectively), and EL38, corresponding to the ripening stage (DOY 234 and DOY 254 at Douro, and DOY 237 and DOY 243 at Alentejo in 2017 and 2018, respectively). For all biochemical and molecular assays, leaves were immediately frozen in liquid nitrogen and stored at -80 °C for further analyses.

2.3. Weather conditions and characterisation of the study areas

The regions are characterised by a warm-temperate climate with dry and hot summers (Kottek *et al.* 2006), and rainfall concentrated during the winter months. Based on the Multicriteria Climatic Classification System (MCC System), three bioclimatic indices were chosen (Tonietto and Carbonneau 2004): (i) the Huglin Heliothermal Index (HI), which includes mean and maximum temperatures and a day-length factor for a proxy for radiation; (ii) the Cool Night Index (CI), a strictly thermal index, which accounts for mean minimum temperature during maturation (September in the northern hemisphere); (iii) and the Dryness Index (DI), consisting of an adaptation of the potential soil water balance. Regarding thermal conditions, the HI indicated a very warm viticultural climate structure during the experiments (Table 1), excepting the 2018 growing season in Alentejo, which was classified as warm. Complementary to HI and thermal regime, the CI indicated that night temperature conditions in both regions were mostly considered as very dry in both regions under study. An automatic weather station was set up in each trial site, recording standard meteorological variables, such

as the minimum and maximum air temperatures, and precipitation (Figure 1). The occurrence of heatwave events was also assessed by sorting at least five consecutive days with maximum air temperature above 40 °C (Fraga *et al.* 2020). In the Douro experiment, two heatwaves were recorded in 2017 (DOY 165–169 and DOY 193–198), and one in 2018 (DOY 213–218). Similarly, two heatwave events were also recorded at the Alentejo site in 2017 (DOY 167–171 and DOY 192–197), and one in the 2018 growing season (DOY 213–218). In 2017, total precipitation at the Douro and Alentejo regions during the experiments was 65.0 and 31.6 mm, respectively, whereas in 2018, total precipitation of 173.8 and 80.4 mm was recorded in the Douro and Alentejo sites, respectively.

Table 1. Huglin Heliothermal Index (HI), Cool Night Index (CI, °C), and Dryness Index (DI, mm) values for 2017 and 2018 growing seasons in the Douro and Alentejo sites were considered according to Tonietto and Carbonneau (2004).

Region	Season	HI	Class	CI	Class	DI	Class
Douro -	2017	3283	Very warm	14.2	Temperate nights	-147.1	Very dry
	2018	3022	Very warm	16.5	Temperate nights	-47.0	Moderately dry
Alentejo -	2017	3148	Very warm	13.5	Cool nights	-207.6	Very dry
	2018	2887	Warm	16.4	Temperate nights	-119.5	Very dry



Figure 1. Daily minimum and maximum air temperature (°C), and precipitation (mm) of 2017 and 2018 growing seasons at both Douro and Alentejo sites.

2.4. Gene expression by RT-qPCR

RNA was extracted from frozen leaves (0.1 g in triplicates) previously grounded in a fine powder, following the rapid- cetyltrimethylammonium bromide (CTAB) method of Gambino et al. (Gambino *et al.* 2008) in both EL35 and EL38 stages of 2017 and 2018 growing seasons. Afterwards, RNA samples were treated with DNAse I RNase-free (Thermo Scientific, Waltham, MA, USA) to degrade the possible extracted DNA following the manufacturer instructions. The RNA concentration was estimated using the absorbance values at 260 nm with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), while the purity of each sample was determined calculating the 260/280 and 260/230 ratios. Finally, total RNA (1 µg) was reverse transcribed to cDNA using PrimeScript RT Reagent Kit (Takara, Shiga, Japan). Quantitative real-time PCR (RT-qPCR) was conducted with an ABI Step One detection system (Applied Biosystems, Foster City, CA, USA). Gene specific primer pairs used for each target or reference gene are listed on supplementary material (Table ST1).

The amplification was performed in a reaction containing 1 µL of cDNA, 5 µL of Maxima SYBR Green/ROX qPCR mix (Thermo Scientific), 1 µL of primers (a mix of forward and reverse, 10 µM), and 3 µL of sterile deionised water. RT-qPCR reactions included a preincubation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 20 s. Actin (*VvACT2*) and tubulin (*VvTUB2*) were used as housekeeping genes to normalise the results among samples. Relative expression of *VvHSP70* (Phytozome accession no. GSVIVT01008331001), and *VvWRKY18* (Phytozome accession no. GSVIVT01035885001) was achieved using the Relative Expression Software Tool Solver v.2 (REST-MCS) (Pfaffl 2001,2002). For each analysed gene, they were considered significantly upregulated and downregulated in the kaolin treated groups (TN_KL and TF_KL) when their relative expression fold change was \geq 2.0 and \leq 0.5, respectively.

2.5. Leaf temperature and chlorophyll measurements

In 2017 and 2018 growing seasons, leaf temperature was measured with an infrared thermometer (Infratrace KM800S, Welwyn Garden City, Hertfordshire, UK) with a 15° field view at stages EL35 and EL38, during the midday period. Measurements were performed on sun-exposed and fully expanded leaves at the middle of the shoots. The average temperature of 30 randomly selected leaves of each experimental groups was obtained by holding the thermometer approximately 1 m above the foliar surface. Chlorophyll concentration per area was estimated using a Chlorophyll Content Meter–CCM-300 (Opti-Sciences, Hudson, NH,

USA) at the midday period in the same 30 leaves used for the leaf temperature measurements at both developmental stages under study. Measurements were determined by the average of three readings in distinct parts of the sun-exposed leaf surface

2.6. Determination of foliar metabolites

The lipid peroxidation products were quantified according to Hodges *et al.* (1999). The extraction was performed by adding 3.0 mL of 20 % (w/v) trichloroacetic acid, with measurements of the supernatant at 440, 532, and 600 nm in a microplate multiscan reader (SPECTROstar Nano, BMG Labtech GmbH, Offenburg, Germany). After subtracting the nonspecific absorbance at 600 nm, the thiobarbituric acid reactive substances (TBARS) were calculated using the malondialdehyde (MDA) extinction coefficient of 157 mM cm^{c1}. Lipid peroxidation was expressed in mmol MDA equivalents g^{-1} dry weight (DW). Free proline content was extracted with 3 % (w/v) sulfosalicylic acid (SSA), and centrifuged at 4000 rpm for 15 min at 4 °C as described by Bates et al. (1973). In a test tube (2.0 mL), the reaction mixture containing 250 µL extract, 250 µL acid ninhydrin, and 250 µL glacial acetic acid was incubated in a boiling water bath for 1 h. Then, 500 µL of toluene was added and mixed for 20 s. The upper reddish-pink coloured phase was separated, and absorbance was read at 520 nm in a microplate reader. The colorimetric response was compared to a standard curve based on commercial proline, and results were expressed as µmol g⁻¹ of DW. Ascorbic (AsA) and dehydroascorbic (DAsA) acids were determined following the method of Okamura (1980) with slight modifications. Briefly, 10 mg of leaf sample were homogenised in 3.0 mL 6% TCA, and centrifuged for 30 min at 4000 rpm and 4 °C. Then, 100 µL of extract, 100 µL of 150mM-NaH₂PO₄ buffer (pH 7.4), and 50 µL of 10mM dithiothreitol (DTT) were added to test tubes (2.0 mL), mixed vigorously in a vortex, and incubated 15 min on ice to reduce the DAsA present in the extract. To remove excess DTT, 50 μ L of 0.5 % (w/v) N-ethylmaleimide were added. The samples were then mixed and incubated for 5 min at 25 °C. For the quantification of AsA, water was added instead of DTT, being the volume of both samples equal. To both samples, the following reagents were added consecutively: 200 μ L of 10 % (w/v) TCA, 200 μ L of 44 % (v/v) phosphoric acid, 200 μ L of 4 % (w/v) 2,2'-dipyridyl in 70 % ethanol, and 100 μ L of 3 % (w/v) FeCl₃. After mixing, the samples were incubated 1 h at 37 °C, and absorbance was recorded at 525 nm. The concentration of DAsA was estimated by subtracting the AsA concentration measured from the total ascorbate quantified. Calibration was done using a standard curve prepared with L-ascorbic acid (Sigma) in 6% TCA, and results were expressed in mg g^{-1} of dry weight (DW).

2.7. Chlorophyll a fluorescence measurements

Chlorophyll *a* fluorescence measurements were carried out in both growing seasons (2017 and 2018), at EL35 and EL38 stages, during the midday period, in six fully expanded and sun exposed leaves per treatment, using a portable chlorophyll fluorimeter *OS-30p* (Opti-Sciences Inc., Hudson, NH, USA). The leaves were dark-adapted with clips for 30 min before chlorophyll a fluorescence transient measurements. The transients were induced by 1 s illumination providing a maximum light intensity of 3000 µmol (photon) m⁻² s⁻¹. The fast fluorescence kinetics (F₀ to F_m) was recorded from 10 µs to 1 s. The fluorescence intensity at 50 µs was considered as F₀ (Strasser and Strasser 1995).

2.8. Analysis of fluorescence transients using JIP-test parameters

The relative change of the JIP test variables in the Douro and Alentejo regions, for both growing seasons, regards to the midday period of each developmental stage (EL35 and EL38), since is considered a critical period of extreme atmospheric demand conditions. The biophysical parameters derived from the OJIP transient were calculated according to the JIP test equations (Strasser *et al.* 2000, Strasser *et al.* 2004), providing structural and functional information regarding photosystem II (PSII). The following parameters were used: (1) specific energy fluxes per reaction centre (RC)–absorption (ABS/RC); electron transport (ET₀/RC); trapping (TR₀/RC), and dissipation (DI₀/RC); (2) phenomenological energy fluxes per excited crosssection (CS)–absorption (ABS/CS); (3) flux ratios or yields–maximum quantum yield of primary photochemistry (ϕ P₀), electron transport probability (Ψ ₀), and the quantum yield of electron transport (Ψ E₀); (4) performance index (PI_{ABS}) on an absorption basis, measuring the performance up to the photosystem I (PSI) end electron acceptors.

2.9. Statistical analysis

Statistical analyses of leaf temperature, chlorophyll content, foliar metabolites, and chlorophyll *a* fluorescence transients were performed using Sigma-Plot 14.0 program (SPSS Inc., San Jose, CA, USA). After testing for ANOVA assumptions (homogeneity of variances with the Levene's mean test, and normality with the Kolmogorov–Smirnov test), statistical differences among treatments and varieties were evaluated by two-way factorial ANOVA, followed by the post hoc Tukey's test. Afterwards, statistical differences between years (2017)

vs. 2018) within each sampling group were evaluated by one-way analysis of variance (ANOVA), followed by the post hoc Tukey's test. For the specific case of chlorophyll a fluorescence transient measurements, statistical differences were evaluated by one-way analysis of variance (ANOVA), followed by the post-hoc Tukey's test (p < 0.05). Different lower case letters represent significant differences between treatments and varieties (TN_C, TN_K, TF_C, TF_K) within each region, developmental stage, and growing season. The asterisks (* p < 0.05) represent significant differences between developmental stages (EL35 vs EL38) within each variety, treatment and sampling year. Absence of letters and asterisks indicate no significant difference.

3. Results

3.1. Kaolin effects on VvHSP70 and VvWRKY18 gene expression

In order to understand kaolin inducing and/or repressive effect on regulating multiple stress responses, we analysed the expression of a heat stress related gene (*VvHSP70*), and a transcription factor related to stress tolerance (*VvWRKY18*) (Figure 2). The relative expression of *VvHSP70* was significantly downregulated in TN kaolin-treated grapevines at stage EL35 in both growing seasons at Alentejo (Figure 2b), whistle in the Douro (Figure 2a) this effect was only noticed in 2017. Conversely, kaolin treatment effects on TF were mostly found in the 2018 growing season, showing approximately an 11-fold *VvHSP70* downregulation compared to the respective control group. Excepting stage EL35 of 2018 in the Douro trial, kaolin effects on *VvWRKY18* gene expression were repressive in TN variety in both regions (Figure 2c,d). In contrast, *VvWRKY18* gene expression in TF treated leaves was upregulated in Alentejo at stage EL38 in both sampling years, while at Douro an opposite trend can be observed during the 2018 growing season.

3.2. Leaf temperature and chlorophyll content

Kaolin effects on leaf cooling and chlorophyll content are shown in Table 2. Though the results do not follow a consistent change throughout the assay in both locations, in the 2017 growing season, TN_K and TF_K leaf temperature decreased 11.0 % and 4.4 %, respectively, at stage EL35 in the Douro trial. At Alentejo, significant kaolin effects on leaf cooling were only found in TF variety at stage EL38 in 2017, and at stage EL35 in the following growing season. Regarding total chlorophyll levels, similar responses can be observed in 2017 in both locations and varieties, with a significant increase in treated leaves throughout the assay,

particularly in TF. This response was only found at Douro in the following growing season, whereas TN and TF treated leaves showed 17.5 % and 58.8 % higher chlorophyll content at stage EL35, respectively.



Figure 2. Relative expression of *VvHSP70* (**a**,**b**), and *VvWRKY18* (**c**,**d**) genes of TN and TF grapevine leaves (Touriga-Nacional control-TN_C and kaolin–TN_KL; Touriga-Franca control–TF_C and kaolin–TF_KL) at Douro and Alentejo throughout 2017 and 2018 summer seasons. Values are means of three replications \pm standard error.* denote significant difference between control and kaolin treated vines of each variety within the same developmental stage (EL35 or EL38).

Table 2. Kaolin effects on leaf temperature ($^{\circ}$ C) and total content of chlorophyll (mg m⁻²) in Touriga Nacional and Touriga Franca varieties in two developmental stages (EL35 and EL 38), at the Douro and Alentejo trials, during 2017 and 2018 growing seasons.

				Dou	ro		Alentejo				
	Year	Stage	Touriga	-Nacional	Touriga	-Franca	Touriga	-Nacional	Touriga	a-Franca	
			Control	Kaolin	Control	Kaolin	Control	Kaolin	Control	Kaolin	
	2017	2017	EL35	45.33±1.78 a	40.33±0.99 c	41.78±1.30 b	39.94±0.92 c	31.20±1.29 ab	30.56±1.17 b	33.24±2.26 a	31.94±1.20 ab
Leaf Temperature 201	2017	EL38	37.31±0.82 b*	37.78±0.70 ab*	39.35±1.55 a*	38.70±0.76 ab	32.22±0.93 c	32.69±1.55 cb*	36.20±2.18 a*	34.72±1.35 ab*	
	2019	2018 EL35 EL38	31.39±1.18	31.38±0.80	31.17±1.12	30.89±1.72	30.93±0.91 b	30.65±1.29 b	35.89±1.68 a	32.78±1.36 b	
	2018		37.33±1.43 *	36.61±1.97 *	36.61±2.07 *	35.89±2.24 *	36.50±1.87 b*	35.28±1.98 b*	39.61±1.93 a*	37.06±1.91 b*	
	2017	EL35	285.3±20.2 c	316.0±17.8 c	386.0±26.9 b	453.2±21.8 a	316.0±22.7 b	431.3±10.0 a	328.0±32.2 b	460.7±11.2 a	
Total — Chlorophyll — 20	2017	EL38	353.0±24.0 b*	344.7±27.4 b	380.6±30.7 b	425.5±34.5 b	298.7±16.5 c	393.5±27.5 a*	365.5±26.3 ab	351.8±27.1 b*	
	2019	EL35	333.8±32.4 c	392.2±24.0 b	283.7±14.3 d	450.5±38.1 a	445.0±26.2	438.2±54.7	439.8±34.0	409.0±20.4	
	2018	EL38	241.3±23.1 b*	247.8±27.2 b*	297.8±8.0 a	309.0±16.4 a*	401.0±24.1 *	410.3±10.7	409.0±48.5	406.8±35.6	

3.3. Transient chlorophyll a fluorescence analysis by JIP-test

The relative change of the JIP test parameters in the Douro and Alentejo regions for both growing seasons (Figures 3 and 4), respective to the midday period of each developmental stage (EL35 and EL38), a critical period of extreme atmospheric demand conditions. The phenomenological (ABS/CS) and specific (ABS/RC, ET₀/RC, DI₀/RC and TR₀/RC) energy fluxes, quantum efficiencies (ϕP_0 , Ψ_0 and ΨE_0), and performance index (PI_{ABS}) of the Douro trial are shown in Figure 3. At the beginning of the study (Figure 3a), specific energy fluxes show that kaolin treatment resulted in a decrease of the trapping (TR_0/RC) and electron transport (ET_0/RC) fluxes in both varieties. At the same time, it increased the dissipation energy (DI₀/RC) only in TF. Similarly, the absorbed photon flux per cross-section (ABS/CS), which corresponds to the basal fluorescence, and per reaction centre (ABS/RC) were higher in TF treated grapevines, whereas PIABS increased exclusively in TN treated vines. On the other hand, the quantum yield and probability of electron transport (Ψ_0 and ΨE_0) increased with kaolin application in TN and decreased in TF. In contrast, the quantum yield of primary photochemistry (ϕP_0) was higher in TF treated grapevines, and lower in TN_K. At EL38 of 2017 growing season (Figure 3b), most of the differences triggered by kaolin application were found in TN, presenting higher quantum efficiencies (ϕP_0 , Ψ_0 , and ΨE_0), PI_{ABS}, ET₀/RC, and DI₀/RC. In TF, the positive effects of particle-film application were only noticed on PI_{ABS} and ABS/CS parameters. In 2018, no significant changes were found for TF at EL35 (Figure 3c), whereas TN_K showed lower relative values in all specific energy fluxes (ABS/RC, TR₀/RC, ET₀/RC, and DI₀/RC), and PI_{ABS}. At the last stage of the study (Figure 3d), all quantum yield parameters (ϕP_0 , Ψ_0 , and ΨE_0), as well as the absorption energy per cross-section and the performance index were increased in both treated varieties. Concerning the specific energy fluxes expressed per reaction centre, kaolin positively influenced ABS/RC and TR₀/RC parameters in TN variety, and ET_0/RC in TF, at stage EL38.



Figure 3. Radar plots of JIP parameters deduced from chlorophyll a fluorescence OJIP transients in the grapevine leaves of Touriga-Nacional control (TN_C) and kaolin (TN_C), and Touriga-Franca control (TF_C) and kaolin (TF_K) in the Douro site at stages EL35 and EL38 of 2017 (**a**,**b**) and 2018 (**c**,**d**). Data are mean \pm SD (n = 18). For each parameter, the lower value represents relative change against the maximum value, set as 100 %. Closed circles (**●**) and triangles (**▼**) at the top of each parameter represent significant differences between treatments in Touriga-Nacional and Touriga-Franca, respectively.

Regarding the Alentejo assay, different tendencies were observed on PI_{ABS} index at stage EL35 of 2017, in which kaolin had a significant positive effect only in TN (Figure 4a). Additionally, at this stage, TF_K showed lower quantum efficiencies (ϕP_0 , Ψ_0 , and ΨE_0), and lower electron transport in an active RC. Throughout the 2017 growing season, kaolin effects on TN and TF photochemistry were weakened in EL38 (Figure 4b), decreasing both the apparent antenna size of an active RC (ABS/RC) and the trapping energy by the RC in TF, without changing the electron transport flux (ET₀/RC). Similarly to the results obtained in the previous year, the PI_{ABS} index was lower in the treated plants of both varieties at stage EL35 of 2018 (Figure 4c). Still, TF_K showed increased absorption flux per cross-section (ABS/CS), and higher electron transport (ET₀/RC) and effective dissipation (DI₀/RC) per active RC at this

stage. At EL38 of 2018 (Figure 4d), PI_{ABS} index remained lower in the kaolin treated plants of both varieties, as well as the ET_0/RC and TR_0/RC specific fluxes in TF. Oppositely, ET_0/RC , DI_0/RC , and the apparent antenna size (ABS/RC), increased in TN_K.



Figure 4. Radar plots of JIP parameters deduced from chlorophyll a fluorescence OJIP transients in the grapevine leaves of Touriga-Nacional control (TN_C) and kaolin (TN_C), and Touriga-Franca control (TF_C) and kaolin (TF_K) in the Alentejo site at stages EL35 and EL38 of 2017 (**a**,**b**) and 2018 (**c**,**d**). Data are mean \pm SD (n = 18). For each parameter, the lower value represents relative change against the maximum value, set as 100 %. Closed circles (**●**) and triangles (**▼**) at the top of each parameter represent significant differences between treatments in Touriga-Nacional and Touriga-Franca, respectively.

3.4. Kaolin effects on lipid peroxidation, proline, and ascorbate accumulation

Tables 3 and 4 show different kaolin application responses regarding lipid peroxidation, proline, and ascorbate content depending on the variety. At Douro (Table 3), lipid peroxidation was significantly prevented in TN treated leaves during 2017, while in 2018, this effect was only evident at stage EL35, showing 59.6 % less TBARS levels compared to its respective

control. In contrast, TF_K showed higher lipid peroxidation levels at this stage in both growing seasons, being this effect diluted, and even inverted in 2018, at stage EL38.

Table 3. Kaolin effects on the total content of thiobarbituric acid reactive substances (TBARS, mmol MDA_{eq} g^{-1} DW), proline (µmol g^{-1} DW), ascorbate (AsA, mg g^{-1} DW), dehydroascorbate (DAsA, mg g^{-1} DW), and percentage of ascorbate reduction (%) of Touriga-Nacional and Touriga-Franca varieties in two developmental stages (EL35 and EL 38), at the Douro trial, during 2017 and 2018 growing seasons.

	G	C.	Touriga-Na	acional	Touriga-F	Touriga-Franca		
Douro trial	Season	Stage –	Control	Kaolin	Control	Kaolin		
	2017	EL35	13.14 ± 1.37 a	10.87 ± 2.17 a	$6.17\pm1.06~\mathrm{b}$	13.36 ± 0.95 a		
	2017	EL38	23.68 ± 2.03 a*	$15.20\pm0.27b^*$	$1.67 \pm 0.26 \text{ c}^*$	$2.07 \pm 1.03 \text{ c}^*$		
IBARS	2019	EL35	12.35 ± 2.52 a	$4.99 \pm 1.09 \text{ bc}$	$2.75\pm0.56\ c$	$6.96\pm1.62~b$		
	2018	EL38	$9.58 \pm 0.72 \text{ b*}$	13.18 ± 2.34 a*	$12.62 \pm 1.02 a^*$	$4.93\pm0.31\ c$		
	2017	EL35	8.20 ± 0.85	8.35 ± 0.77	7.20 ± 0.57	7.53 ± 0.40		
Derting	2017	EL38	$6.73 \pm 0.61 \text{ b*}$	$6.32 \pm 0.56 \text{ b*}$	$9.85 \pm 0.40 \text{ a}^*$	$7.09\pm0.73~b$		
Proline	2019	EL35	16.79 ± 1.64 b	10.94 ± 0.79 c	13.26 ± 0.34 c	21.16 ± 2.29 a		
	2018	EL38	16.25 ± 1.61 a	$8.76\pm0.90~b$	$10.80\pm0.37b^*$	15.34 ± 1.42 a*		
	2017	EL35	2.70 ± 0.16 a	$1.50 \pm 0.10 \text{ c}$	$0.67 \pm 0.07 \; d$	$1.84\pm0.08~b$		
	2017	EL38	$2.35 \pm 0.25 \text{ a*}$	$1.59\pm0.16~b$	$0.73\pm0.06\ c$	$1.54 \pm 0.19 \text{ b*}$		
ASA	2019	EL35	$4.50 \pm 0.26 \text{ a}$	$2.45\pm0.15~b$	$1.66 \pm 0.12 \text{ c}$	$2.52\pm0.32~b$		
	2018	EL38	$3.44 \pm 0.31 a^*$	3.23 ± 0.21 ab*	$2.87 \pm 0.27 \ a^*$	$2.49 \pm 0.19 \text{ b*}$		
	2017	EL35	$1.23\pm0.06~c$	$1.43\pm0.02~b$	$0.34 \pm 0.01 \text{ d}$	1.64 ± 0.06 a		
	2017	EL38	$1.58 \pm 0.14 a^*$	$0.20 \pm 0.02 \; d^*$	$0.56 \pm 0.05 \ c^*$	$0.89\pm0.08~b^*$		
DASA	2019	EL35	$1.90 \pm 0.30 \text{ ab}$	$1.77 \pm 0.05 \text{ ab}$	$1.61\pm0.16~\mathrm{b}$	1.92 ± 0.18 a		
	2018	EL38	$1.49 \pm 0.17 \text{ bc}^*$	$2.57 \pm 0.12 \text{ a}^*$	$1.77\pm0.11~b$	$1.21 \pm 0.04 \text{ c}^*$		
0/	2017	EL35	81.65 ± 3.72 a	31.15 ± 0.73 c	$52.53 \pm 2.42 \text{ b}$	12.88 ± 1.76 d		
%	2017	EL38	$78.73\pm1.93~b$	$89.28 \pm 3.24 \ a^*$	$33.25 \pm 1.65d*$	$59.66 \pm 8.88 \ c^*$		
ascorbate	2018	EL35	49.81 ± 2.53 a	30.68 ± 2.73 b	42.52 ± 5.95 a	$24.05\pm1.97~b$		
ascorbate	2018	EL38	53.80 ± 2.21 b	67.51 ± 4.90 a*	$27.17 \pm 4.96 \text{ c*}$	$55.52 \pm 2.40b^{*}$		

¹ Data are mean \pm SD (n = 6). Different lower case letters represent significant differences between treatments and varieties within each developmental stage (EL35 and EL38), and sampling year. The asterisks (*) indicate significant differences (p < 0.05) between developmental stages (EL35 vs. EL38) within each variety, treatment, and sampling year.

At Alentejo (Table 4), analyses from 2017 to 2018 growing seasons showed a similar pattern in kaolin coated leaves of both varieties, though lipid peroxidation levels were generally higher at the Alentejo trial in all sampling dates regardless the treatment. Regarding proline content, no significant effects were detected in 2017. In 2018, despite presenting an opposite varietal effect, similar responses to kaolin treatment were detected in both regions within each variety, particularly at stage EL38. At this stage, TN treated leaves from Douro and Alentejo showed 46.1 % and 7.3 % lower proline content, respectively, while TF_K showed an increase of 42.0 % and 75.3 %, respectively. Kaolin coating effects on AsA and DAsA were mostly observed at the Douro trial (Table 3) with an opposite trend between varieties. While TN treated leaves showed lower AsA accumulation throughout the season, TF_K showed higher AsA content, excepting at stage EL38 of the 2018 growing season. Nevertheless, kaolin

effect on ascorbate reduction was identical in both varieties and growing seasons at Douro, with significant lower percentage reduction at the stages EL35, and higher at EL38.

Table 4. Kaolin effects on the total content of thiobarbituric acid reactive substances (TBARS, mmol MDA_{eq} g^{-1} dry weight (DW)), proline (µmol g^{-1} DW), ascorbate (AsA, mg g^{-1} DW), dehydroascorbate (DAsA, mg g^{-1} DW), and percentage of ascorbate reduction (%) of Touriga-Nacional and Touriga-Franca varieties in two developmental stages (EL35 and EL 38), at the Alentejo trial, during 2017 and 2018 growing seasons.

	G	C1	Touriga-N	acional	Touriga-Franca		
Alentejo trial	Season	Stage	Control	Kaolin	Control	Kaolin	
	2017	EL35	43.11 ± 2.09 a	$28.52 \pm 3.30 \text{ b}$	$13.17 \pm 1.82 \text{ d}$	21.85 ± 1.66 c	
	2017	EL38	$18.17 \pm 0.84 \text{ a*}$	14.41 ± 1.03 ab*	$13.61 \pm 0.45 \text{ b}$	$13.48 \pm 1.29 \text{ b*}$	
IBARS	2010	EL35	30.86 ± 4.27 a	20.94 ± 2.52 b	12.41 ± 0.88 c	$6.80\pm0.42~d$	
	2018	EL38	20.30 ± 3.17 a*	$11.07 \pm 3.08 \text{ b*}$	11.36 ± 1.72 b	$10.00\pm2.48~b$	
	2017	EL35	$4.73\pm0.50~\text{b}$	$4.29\pm0.08~\text{b}$	7.47 ± 0.82 a	7.84 ± 0.52 a	
D., 1'.,	2017	EL38	$6.86 \pm 0.51 \text{ b*}$	$8.13 \pm 0.66 a^*$	7.51 ± 0.50 ab	8.00 ± 0.85 ab	
Proline	2018	EL35	$8.65\pm1.08~\mathrm{b}$	8.46 ± 0.79 b	12.04 ± 0.88 a	13.65 ± 0.86 a	
		EL38	$11.10 \pm 0.27 \text{ a*}$	$10.29 \pm 0.47 \text{ a}^*$	$9.86 \pm 0.85 a^*$	$17.28 \pm 2.23 \text{ b*}$	
	2017	EL35	4.65 ± 0.40 a	$2.45\pm0.27~b$	$1.21 \pm 0.07 \ d$	$1.47 \pm 0.15 \text{ c}$	
		EL38	$1.41 \pm 0.06 \text{ a*}$	1.39 ± 0.12 a*	$0.65 \pm 0.03 \text{ b}^*$	$0.54 \pm 0.05 \text{ b}^*$	
AsA	2018	EL35	$3.82\pm0.40\ b$	4.98 ± 0.55 a	$2.55\pm0.18~c$	$3.03\pm0.14\ c$	
		EL38	$3.64\pm0.38~\text{b}$	$4.16 \pm 0.34 a^*$	$1.51 \pm 0.12 \text{ d}^*$	$2.46 \pm 0.14 \text{ c}^*$	
	2017	EL35	2.22 ± 0.26 a	$1.10\pm0.08~b$	$0.65 \pm 0.07 \ c$	$0.69\pm0.04~c$	
DALA	2017	EL38	$0.70 \pm 0.09 \text{ a}^*$	$0.75 \pm 0.04 \ a^*$	$0.27 \pm 0.02 \text{ b*}$	$0.18 \pm 0.02 \ b^*$	
DASA	2019	EL35	$1.29\pm0.07~c$	1.46 ± 0.15 c	2.66 ± 0.21 a	$1.91\pm0.25~b$	
	2018	EL38	1.98 ± 0.18 a*	$1.32\pm0.12~\mathrm{b}$	$1.42 \pm 0.07 \text{ b*}$	$1.42 \pm 0.20 \text{ b*}$	
	2017	EL35	$58.05 \pm 4.63 \text{ b}$	$59.58 \pm 4.14 \text{ b}$	71.63 ± 6.60 a	$60.42 \pm 1.79 \text{ b}$	
% reduction	2017	EL38	$70.38 \pm 4.05 \text{ b*}$	$71.32 \pm 5.96 \text{ b*}$	$59.31 \pm 1.78 \text{ c}^*$	$92.17 \pm 8.90 \text{ a}^*$	
ascorbate	2018	EL35	70.14 ± 3.14 a	70.38 ± 3.04 a	$7.08 \pm 1.66 \ c$	$28.15\pm3.52\ b$	
	2010	EL38	$44.12 \pm 1.30 \text{ b*}$	69.14 ± 2.64 a	$29.08 \pm 6.13 \text{ c}^*$	$68.45 \pm 1.78 \text{ a}^*$	

¹ Data are mean \pm SD (n = 6). Different lower case letters represent significant differences between treatments and varieties within each developmental stage (EL35 and EL38), and sampling year. The asterisks (*) indicate significant differences (p < 0.05) between developmental stages (EL35 vs. EL38) within each variety, treatment, and sampling year.

4. Discussion

The combined hot and dry local conditions (Table 1 and Figure 1) can lead to frequent and persistent damages at physiological, biochemical, and molecular levels, highlighting the critical role of using mitigation practices in alleviating summer stress impacts (Brito *et al.* 2018, Conde *et al.* 2018).

Overall, the downregulation of *VvHSP70* gene expression in treated leaves (Figure 2), particularly at the Alentejo site, reinforces the kaolin protective role against summer stress, considering that most HSP groups are generally upregulated under stressful conditions (Kobayashi *et al.* 2010). In agreement, a similar effect was observed upon stress exposure and

recovery of 'Cabernet Sauvignon' leaves, demonstrating that most HSP genes were upregulated by heat stress, but not during recovery, supporting the hypothesis particle film technology may alleviate heat stress factors on grapevines. Likewise, VvWRKY18 gene expression was mainly downregulated in treated grapevines from TN in both regions and upregulated in TF at the Alentejo trial, indicating different varietal responses to kaolin application that could depend on other factors, such as rootstock, terroir, and stress severity (Bernardo et al. 2018, Carvalho et al. 2015, Frioni et al. 2019). It is also plausible that these differences might be related to intrinsic varietal features, such as the phenological onset of the veraison stage and leaf senescence mechanisms (Costa et al. 2019, Kobayashi et al. 2010). One of the major impacts of climate change in temperate climate regions is the earlier onset of several phenological stages associated with variations in the maximum temperature and varietal heat requirements (García de Cortázar-Atauri et al. 2017, Reis et al. 2020). Indeed, in a comparative study on grapevine phenology performed in the same varieties of this work, Costa et al. (2019) reported an earlier veraison timing for TF respecting TN in most climatic models applied, supporting the varietal differences found in VvHSP70 and VvWRKY18 gene expression in the current conditions. Nevertheless, these findings suggest that the use of particle film technology lowers the need for triggering heat stress tolerance mechanisms, and related gene expression of grapevines grown in Mediterranean-type climate vineyards.

Kaolin leaf cooling effects differed among regions (Table 2), with lower leaf temperature mostly found at stage EL35 at Douro, suggesting that regional edaphoclimatic conditions could be the paramount factor in shaping plant stress responses. Leaf cooling effect by reflective particle films application was extensively reported in previous studies in grapevines (Brillante *et al.* 2016, Dinis *et al.* 2018b, Glenn *et al.* 2010) and other crops (Abou-Khaled *et al.* 1970, Brito *et al.* 2019). However, it is also worth stating that the extent of this effect may be varietal dependent and affected by the leaf water status (Frioni *et al.* 2019). Besides, the present effect of kaolin on promoting chlorophyll accumulation under stressful conditions, and thus preventing photo-oxidative damage, supports evidence from previous research on several crops, such as grapevines (Dinis *et al.* 2016, Shellie and King 2013), wheat (Abdallah *et al.* 2019) and olive (Brito *et al.* 2018), and apple (Faghih *et al.* 2019) trees.

Regarding chlorophyll *a* fluorescence transient analysis, the specific energy flux data, combined with the quantum yield analysis, highlight different varietal responses in both regions (Figures 3 and 4), particularly in 2017 at the *veraison* stage (EL35). At this stage, specific energy fluxes (TR₀/RC and ET₀/RC), yield of primary photochemistry (ϕ P₀), and ABS/RC

decreased in TN_K, indicating an apparent antenna size reduction and lower inactivation of RC's at the beginning of the experiment, which might explain the higher performance index (PI_{ABS}) found in TN_K at both sites, as previously observed by Dinis et al. (2016). Interestingly, TF seems to have adopted a slightly different light absorption strategy, showing decreased TR₀/RC and ET₀/RC, lower Ψ_0 , and ΨE_0 , but increased ϕP_0 , ABS/RC, and DI₀/RC in kaolin treated grapevines, with no influence on the PIABS index. These results suggest a safe downregulation mechanism, which includes a decrease of the fraction of fully active RC, and increase of the heat sink centres to dissipate excess energy, as pointed on the findings of Beneragama et al. (2014). Nonetheless, this downregulation can also be related to non-quinone A (Q_A) reducing RC, known as silent RC (Strasser 1997). The positive effects of kaolin on grapevine performance (PIABS) increased at ripening (EL38), in both varieties from the Douro site (Figure 3), but not in Alentejo (Figure 4), indicating that particle film efficiency on promoting plant stress responses might be different from site-to-site, depending on stress severity and extent, as well as on the initial foliar acclimation mechanisms to kaolin treatment. In agreement, results from 2018 show no significant influence of particle film application on chlorophyll transient analysis, particularly at the Alentejo, whereas at Douro, treated grapevines continued exhibiting higher PIABS, suggesting increased kaolin effectiveness under severe environmental conditions. Since 2017 was warmer and drier than 2018, with the occurrence of two heatwave events and low rainfall levels, it seems likely that stress severity and impacts were more pronounced in the 2017 growing season, which can modulate kaolin efficiency in alleviating summer stress.

The levels of reduced and oxidised ascorbate, as well as the percentage reduction, indicated that TF and TN have different basal levels of ascorbate, and that kaolin promoted different responses to ascorbate accumulation. Overall, kaolin foliar treatment promoted the accumulation of reduced and oxidised ascorbate only at Douro (Table 3), indicating some predisposition to react under stressful conditions (Carvalho *et al.* 2015). Despite the general reducing effect on the lipid peroxidation levels observed in both treated varieties within each region, which reinforces the protective role of kaolin, the findings of the current study do not clearly support the tendency for lowering proline accumulation in kaolin treated grapevines exposed to summer stress (Bernardo *et al.* 2017, Dinis *et al.* 2018a). In fact, at Alentejo, proline levels were mainly higher in TF treated vines, indicating a lower need for kaolin application on this variety at the beginning of ripening. Even so, kaolin application under milder stress conditions, such as those recorded in 2018 that were characterised by the occurrence of only

one heatwave event and higher rainfall levels compared to 2017, could also induce positive feedback on plant stress responses by increasing the accumulation of metabolites responsible for cellular homeostasis. Moreover, it should also bear in mind that plant response to multiple factors can be unique and differ from a single stress factors (Mittler 2006, Rizhsky *et al.* 2004). Under field conditions, these observations suggest that some stress factors prevail among others, changing the accumulation of several foliar stress-related metabolites.

5. Conclusions

In summary, the assessment of kaolin particle film efficiency in climate change hotspot regions through multiple-based approaches (physiological, biochemical, and molecular) revealed regulation of heat stress responses and tolerance mechanisms, and improved summer stress responses and photochemistry modulation under stress conditions. The results indicate different varietal responses to kaolin application in each region, while highlighting the viticultural environment as the paramount factor in shaping grapevine stress responses. Moreover, this research allows studying plant stress responses and acclimation mechanisms pragmatically and reveals the complexity of studying adult plants in commercial vineyards. From a climate change perspective, comparative studies should be further explored under controlled and field conditions to elucidate the advantages of particle film application on other Mediterranean crops' production and quality.

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Supplementary material

Table	ST1.	List o	f Primers	Used fo	or Real-Time	Quantitative	PCR
Lanc	DII.	LISCO	1 I I IIIICI S	Useu It	n Rear Thire	Quantitative	I CIV.

Gene	Gene Unique ID		Primer (5'-3')	Size (bp)
VvHSP70	GSVIVT01008331001	Fw Rv	GACCTTGGGGGGTGGTACTTT GCCAACCAATCCACAACTCT	131
VvWRKY18	GSVIVT01035885001	Fw Rv	GAAGCCAAGAGAAAGCACCA GGCTCTGGGAGAAGGGTTAT	145
VvACT2	GSVIVT01026580001	Fw Rv	GCCATCCAAGCTGTTCTCTC CAGTAAGGTCACGTCCAGCA	157
VvTUB2	GSVIVT01037405001	Fw Rv	CAACTCTGACCTCCGAAAGC CTTGGAGTCCCACATTTGCT	154

CHAPTER 5

EFFECTS ON XANTHOPHYLL CYCLE DYNAMICS AND PHOTOPROTECTION

Briefing note

This chapter strengthens knowledge on grapevine photoprotection under summer stress conditions, exploring kaolin effects on photosynthetic pigments accumulation and energy dissipation processes. It is divided into two sub-chapters to detail kaolin effectiveness in managing excessive energy absorption in each studied region, characterising kaolin effects on chlorophylls, carotenoids, and xanthophyll cycle dynamics and their influence on light dissipation processes, at the Douro (Chapter 5.1) and Alentejo trials (Chapter 5.2).

This chapter tackles the specific objective of the thesis (i) characterise, in the Douro Superior sub-region and Alentejo region, grapevine stress resilience at a leaf level in two consecutive growing seasons (2017 and 2018), strengthen the discussion regarding kaolin treatment as a reliable short-term strategy against Mediterranean summer stress conditions (overall objective).

Chapter 5.1. Particle film technology modulates xanthophyll cycle and photochemical dynamics of grapevines grown in the Douro Valley

Adapted from:

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Chapter 5.2. Fine-tuning of grapevine xanthophyll-cycle and energy dissipation under Mediterranean conditions by kaolin particle-film (Alentejo)

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Chapter 5.1. Particle film technology modulates xanthophyll cycle and photochemical dynamics of grapevines grown in the Douro Valley

Abstract

Field-grown grapevines are often exposed to multiple environmental stresses, which challenges wine-growers to develop sustainable measures to sustain vine growth, yield, and quality. Under field conditions this task is demanding, due to differences in the magnitudes of stresses and associated plant responses. In this study we explored the hypothesis that kaolinparticle film application improves grapevine photoprotection through the regulation of xanthophyll cycle genes, limiting the thermal dissipation of excess energy under harsh environmental conditions. Hence, we selected two grapevine varieties, Touriga-Nacional (TN) and Touriga-Franca (TF), grown in the Douro Demarcated Region, and evaluated changes in light dissipation mechanisms, xanthophyll cycle components, and the expression of xanthophyll cycle genes during the 2017 summer season. The results showed that, from *veraison* to ripening, kaolin triggered the up-regulation of violaxanthin de-epoxidase (VvVDE1) and zeaxanthin epoxidase (VvZEP1) genes, indicating optimised regulation of the xanthophyll cycle. Kaolin treatment also decreased chlorophyll (Chl_a, Chl_b, Chl_(a+b)) and carotenoid (Car) accumulation under increasing summer stress conditions in both varieties and lowered the non-photochemical quenching (NPQ) of grapevines on ripening, suggesting a long-term response to summer stress. In addition, kaolin-treated grapevines showed increased Chla/Chlb and lower Chl(a+b)/Car ratios, displaying some features of high light adapted leaves. Overall, this study suggests that kaolin application enabled grapevines to benefit from fluctuating periods of summer stress by managing chlorophyll and carotenoid content and limiting down-regulation of both photochemistry and photoinhibition processes. Under Mediterranean field conditions, kaolin application can be considered an efficient method of minimising summer stress impact on grapevines.

Keywords: Gene expression, Non-photochemical quenching, Photochemistry, Photoprotection, Zeaxanthin

1. Introduction

In Mediterranean-like climate regions, the summer season is usually characterised by periods of combined drought, high light levels, and high temperatures, imposing a serious challenge to sustainable plant growth and development (Bernardo et al. 2018). According to the latest projections, it is expected that climate change will impair the productivity and quality of many crops with strong socio-economic relevance worldwide, especially during the summer season (Fraga et al. 2018) due to intense and prolonged stress factors. However, plants have acquired several acclimation strategies to sustain growth and development under a changing climate, such as adjustments to fluctuations in the light environment, thermal dissipation of excessive energy, osmotic regulation, and photochemical processes (Walters 2005, Zhen and van Iersel 2017).

Light acclimation strategies involve complex mechanisms to control light absorption capacity and to manage the absorbed light, protecting the photosynthetic apparatus from possible damage (Chaves et al. 2003). Thus, the balance between light absorption and utilisation in light limiting conditions, as well as the processes involved in the dissipation of excessive energy, is paramount in determining plants' acclimation efficiency and vulnerability to damage (Zhen and van Iersel 2017).

It is well known that carotenoids (e.g. xanthophyll cycle components) play a critical role in managing light-harvesting and utilisation, and on the thermal dissipation of excess energy in higher plants (Jahns and Holzwarth 2012). Under high light, non-photochemical processes require the presence of the de-epoxidised xanthophyll zeaxanthin (Zx), formed by the enzymatic de-epoxidation of violaxanthin (Vx). This process relieves excitation pressure on photosystem II (PSII), thereby allowing greater openness of PSII centres to be maintained (Ruban 2014).

Apart from their essential role in photoprotection, carotenoids also function as potent antioxidants and act as precursors of apocarotenoid hormones, such as abscisic acid and strigolactones, and other stress signalling molecules (e.g. β -cyclocitral) (Rodriguez-Concepcion et al. 2018). Phytoene is the first compound to be synthesized in the carotenoid biosynthetic pathway, catalysed by the enzyme phytoene synthase), and is described as the rate-limiting step of the pathway (Rodriguez-Concepcion et al. 2018). Besides the xanthophyll cycle components, lutein and β -carotene are other carotenoids involved in PSII photoprotection. The former exhibits a structural role in the stability of the light-harvesting complexes (LHCII), and the latter protecting the PSII reaction centres against photobleaching (Bonente et al. 2008).

Research regarding grapevine stress responses in natural environments is far from reaching a consensus (Chaumont et al. 1997, Palliotti et al. 2014). There are few studies of the molecular mechanisms underlying physiological responses in the field, where complex interactions may occur due to combined stressors, which can lead to ambiguous conclusions (Chaves et al. 2003). In spite of the photoprotective mechanisms employed by plants, increasing summer stress factors (high light levels, temperature, and drought) are already compromising viticulture in Mediterranean-like climates, challenging wine-growers and researchers to develop adaptive strategies to cope with the changing climate (Bernardo et al. 2018).

Among the multiple short-term strategies used in viticulture, the foliar application of kaolin, a white clay mineral found to reflect excess radiation and reduce the risk of sunburn, has been considered an efficient practice to mitigate the effects of environmental pressures in grapevine (Dinis et al. 2015, Dinis et al. 2017, Frioni et al. 2020). Depending on the degree, duration, and type of stressor, recent studies have also linked kaolin treatment to a general improvement in physiological performance, antioxidant defence mechanisms, hormonal crosstalk, and fruit quality (Conde et al. 2018, Dinis et al. 2018, Frioni et al. 2019).

Although several studies have elucidated kaolin application effects on field-grown grapevine stress responses (Dinis et al. 2015, Frioni et al. 2020), its putative function on PSII photoprotection mechanisms involving xanthophyll cycle regulation and chlorophyll *a* fluorescence dynamics remain unclear. Therefore, we addressed the hypothesis that kaolin application may improve grapevine photoprotection by modulating xanthophyll cycle components and gene expression (*zeaxanthin epoxidase VvZEP1* and *violaxanthin deepoxidase VvVDE1*), linked to thermal dissipation processes. For this purpose, we evaluated the effects of kaolin application on grapevine photosynthetic pigment composition, xanthophyll cycle components, chlorophyll fluorescence variables, and the expression of the xanthophyll cycle regulating genes throughout the summer season. Gene expression of *phytoene synthase* (*VvPSY1*) was also analysed to clarify grapevine summer stress acclimation strategies involving the rate limiting step of the carotenoid biosynthetic pathway.

2. Materials and Methods

2.1. Growth conditions and plant material

The study was conducted in a commercial vineyard ("Quinta do Orgal", 41° 04' N, 7° 04' W) located in one of the most important wine regions of Portugal, the Douro Demarcated Region, in the Northeast of the country. The local climate is typically Mediterranean, a warm-

temperate climate with hot dry summers (Kottek et al. 2006) and higher precipitation during the winter months. An automatic weather station recorded variables such as the rainfall and air temperature during the period of the experiment (Fig. 1). Mean air temperature from May (Day-Of-Year [DOY] 121) to September (DOY 273) was 25.1 °C, with 63.4 mm of precipitation. The number of days with a maximum air temperature above 40 °C was also counted in order to evaluate the occurrence of heatwaves during the experiment, recording a total of 23 days. Considering that a range of five consecutive days with maximum temperatures above 40 °C constitutes a heatwave (Fraga et al. 2020), two heatwave events were observed, at DOY 165 -169 and DOY 193 - 198. According to the World Reference Base for Soil Resources (FAO 2015), the soil of the region is classified as a luvisol, characterised by a clay-enriched subsoil. Two native red grapevine varieties, Touriga-Nacional (TN) and Touriga-Franca (TF), were selected due to their regular grape quality potential and moderate adaptability to warm and dry climates. The six-year-old vines were grafted on 110R, arranged in an east-west orientation at altitude 169 m with a steep slope (30° N), trained to unilateral cordon, and with vine spacing of 2.2 x 1.0 m. The experimental set up comprised 120 vines per variety, distributed across six rows with 20 vines each. Vines were managed with deficit irrigation (30% of the reference evapotranspiration) to prevent plant death and grown using the organic production practices applied by commercial farmers.



Figure 1. Daily mean air temperature (°C) and precipitation (mm) during the 2017 growing season at the vineyard experiment field in the Douro Region. Vertical grey filled bars represent the two heatwave events occurred during the experiment. DOY: Day-Of-Year; Kaolin application – DOY 177; Veraison stage – DOY 199; Ripen stage – DOY 234.

2.2. Treatments and monitoring

From the six rows selected per variety, two treatments were set up with 60 vines each: an experimental control (TN_C and TF_C) and a kaolin treated group (TN_KL and TF_KL) distributed in three consecutive rows. The kaolin treatment (Surround® WP, Engelhard Corporation, Iselin, New Jersey) was prepared in an aqueous solution following the manufacturer's recommended dosage of 5% (w/v), supplemented with 0.1% (v/v) Tween 20 to improve adherence, and applied according to standard operating procedures adjusted for agricultural practices. Kaolin was applied on the windless morning of June 26 (DOY 177) (Fig. 1). The adjacent control plants were carefully protected by a plastic film during kaolin spraying. For all the physiological and biochemical measurements, six healthy, fully expanded, and mature leaves in a similar position were collected per row and treatment at two periods of the day (pre-dawn and midday). Measurements were also carried out at two different developmental stages: veraison (DOY 199) and ripen (DOY 234), corresponding respectively to three weeks and two months after kaolin application. Leaf samples were immediately frozen in liquid nitrogen and delivered to the facilities of the Plant Physiology Laboratory at the University of Trás-os-Montes and Alto Douro. Samples were ground to a fine powder with liquid nitrogen and stored at -80 °C until analysis.

2.3. Extraction and quantification of chlorophyll and carotenoids

Photosynthetic pigments were extracted following Rodrigo et al. (2003). Briefly, fresh frozen ground leaves (approximately 0.175 g) were extracted with a solution containing 2.0 mL MeOH and 1.5 mL of Tris-HCl (50 mM, pH 7.5, containing NaCl 1 M) over a period of 30 min. Chloroform (4.0 mL) was then added to the mixture, which was stirred for 30 s and centrifuged. The aqueous phase was re-extracted with chloroform until it was colourless. The combined chloroform extracts were dried in a rotary evaporator at 35 °C and re-dissolved with acetone:petroleum ether:diethyl ether (0.7:9:1, v/v/v). An aliquot was used for determination of the chlorophyll (a+b) content, which was achieved by measuring the absorbance at 644 and 662 nm and calculated as described by Smith and Benitez (Smith and Benitez 1955). After chlorophyll measurement, the ethereal pigment solution was dried and saponified using a 10% methanolic KOH solution. The carotenoids were subsequently re-extracted with diethyl ether until the hypophase was colourless. An aliquot of the ether extract was used for quantification of total carotenoid content, and this was calculated by measuring the absorbance of the saponified extract at 450 nm (Davies 1976). The samples were dried under an N₂ stream and
kept at -20 °C until analysis. Determinations were carried out at least in triplicate, and all procedures were performed on ice under dim light to prevent photodegradation, isomerisation, and structural changes of carotenoids.

2.4. HPLC-PAD analysis of individual carotenoids

The carotenoid composition of each sample was analysed using high-performance liquid chromatography (HPLC) with a Waters Liquid Chromatography System, equipped with a 600E pump and a 2998 photodiode array detector (PAD), and Empower software (Waters, Barcelona, Spain). A C₃₀ carotenoid column (250 mm \times 4.6 mm, 5 μ m) coupled to a C₃₀ guard column (20 mm × 4.0 mm, 5 µm) (YMC, Teknochroma, Spain) was used. Samples were prepared for HPLC by dissolving the dried carotenoid extracts in CHCl₃:MeOH:acetone (3:2:1, v:v:v). A ternary gradient elution of MeOH water and methyl ter-butyl ether (MTBE) was used for carotenoid separation, as described by Rodrigo et al. (2003). The PAD detector was set to scan from 250 to 540 nm, and for each elution, a Maxplot chromatogram was obtained, which plots each carotenoid peak at its corresponding maximum absorbance wavelength. Carotenoids were identified by their retention time, absorption, and fine spectra (Britton et al. 1998, Rodrigo et al. 2004, Rodrigo et al. 2003). The carotenoid peaks were integrated at their individual maxima wavelength, and their contents were calculated using calibration curves of zeaxanthin (Extrasynthese), β -carotene (Sigma) for α - and β -carotene, β -cryptoxanthin (Extrasynthese), lutein (Sigma), anteraxanthin (CaroteNature), and violaxanthin (CaroteNature) for violaxanthin and neoxanthin isomers. Phytoene, phytofluene, and ζ-carotene were previously purified as described by Lado et al. (2015). Results are given as $\mu g.g^{-1}$ fresh weight (FW). De-epoxidation state (DPS) was expressed as a percentage, and calculated as described by Rivas et al. (2011).

2.5. Measurement of chlorophyll fluorescence

Chlorophyll *a* fluorescence emission was measured at pre-dawn (05:00–06:00; local time) and midday (14:00–15:30; local time) on fully expanded leaves in both developmental stages with a Pulse Amplitude Modulation Fluorometer (mini-PAM, Photosynthesis Yield Analyzer; Walz, Effeltrich, Germany), using two scripts.

In the first script measurements were performed on leaves that were fully exposed to sunlight (except in the pre-dawn period). For this procedure after a 35-s exposure to actinic light (1450 μ mol.m⁻².s⁻¹), light-adapted steady-state fluorescence yield (F_s) was averaged, followed by exposure to a saturating light pulse (6000 μ mol.m⁻².s⁻¹) for 0.6 s to establish F_m'.

The sample was then shaded for 5 s with a far-red light source to determine F_0 '. Several fluorescence attributes were calculated using these measurements (Bilger and Schreiber 1986, Genty et al. 1989): photochemical quenching ($qP = (F_m' - F_s)/(F_m' - F_0')$), non-photochemical quenching ($NPQ = (F_m - F_m')/F_m'$), and efficiency of electron transport as a measure of the effective quantum efficiency of PSII ($\Phi_{PSII} = \Delta F/F_m' = (F_m' - F_s)/F_m'$) (Bilger and Schreiber 1986).

In the second script, using the dark leaf clip (DLC-8), the same leaf portion used in the first script was immediately dark acclimated for 30–45 min. After this, the maximum photochemical efficiency of PSII was given by $F_v/F_m = (F_m - F_0)/F_m$, where F_0 corresponds to the minimum fluorescence level excited by the very low intensity of the measuring light to keep PSII reaction centres open, and F_m corresponds to the maximum fluorescence level elicited by a pulse of saturating light (6000 µmol.m⁻².s⁻¹) which closes all PSII reaction centres (Bilger and Schreiber 1986).

2.6. Gene expression analysis using RT-qPCR

RNA was extracted from 100 mg of frozen leaves following the rapid CTAB-based procedure of Gambino et al. (2008). Afterwards, RNA samples were treated with DNAse I, RNase-free (Thermo Scientific, Waltham, MA, USA) to degrade the possible extracted DNA. The RNA concentration was estimated using the absorbance values at 260 nm with a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA), while the purity of each sample was determined by calculating the 260/280 and 260/230 ratios. Finally, total RNA (1 µg) was reverse transcribed to cDNA using Primescript RT Reagent Kit (Takara, Shiga, Japan). Quantitative real-time PCR was conducted with an ABI Step One detection system (Applied Biosystems, Foster City, CA, USA). Gene specific primer pairs used for each target or reference gene are listed in Suppl. Table S1.

Amplification was performed in a reaction containing 1 μ L of cDNA, 5 μ L of Maxima SYBR Green/ROX qPCR Mix (Thermo Scientific), 1 μ L of primers (a mix of forward and reverse, 10 μ M) and 3 μ L of sterile water. PCR reactions included pre-incubation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 20 s. *Actin* (*VvACT2*, Phytozome accession no. GSVIVT01026580001) and *tubulin* (*VvTUB2*, Phytozome accession no. GSVIVT01037405001) were used as reference genes to normalise the results among samples. Relative expression of *VvVDE1* (Phytozome accession no. GSVIVT01026405001), *VvZEP1*

(Phytozome accession no. GSVIVT01022128001) and *VvPSY1* (Phytozome accession no. GSVIVT01035255001) was determined using *Relative Expression Software Tool Solver v.2* (REST-MCS) (Pfaffl 2001, 2002). Genes were considered to be significantly up-regulated or down-regulated in the kaolin treated groups (TN_KL and TF_KL), when their relative expression fold change was ≥ 2 and ≤ 0.5 , respectively, for each gene analysed.

2.7. Statistical analysis

Statistical analysis of individual carotenoids, xanthophyll cycle components, and chlorophyll fluorescence was performed using the Sigma-Plot 14.0 program (SPSS Inc.). After testing that analysis of variance (ANOVA) assumptions were satisfied (homogeneity of variances using Levene's test and normality using the Kolmogorov-Smirnov test), statistical differences among treatments and varieties were evaluated by two-way factorial ANOVA, followed by post hoc Tukey's test. Afterwards, statistical differences between periods of the day (pre-dawn *vs* midday) within each sampling group were evaluated by one-way ANOVA, followed by post hoc Tukey's test. Different lower case letters represent significant differences between treatments and varieties (TN_C, TN_KL, TF_C, TF_KL) within each period of the day and developmental stage. Significant differences were considered where p < 0.05. The presence of asterisks mean significant differences between periods of the day (pre-dawn *vs* midday) within each variety, treatment, and developmental stage. An absence of letters and asterisks indicate no significant difference.

3. Results

3.1. Changes in the accumulation of photosynthetic pigments

Table 1 shows chlorophyll and carotenoid content, and their respective ratios at midday at both the *veraison* and ripened stages. Kaolin application showed contrasting effects depending on the variety and developmental stage. At *veraison*, TN_KL plants showed lower Chl_a and Chl_b accumulation, while TF_KL grapevines showed a general increase in pigments levels. In contrast, this effect was reversed in TF_KL at the ripened stage, which presented lower Chl_a, Chl_b, Chl_(a+b), and Car levels, whereas treated TN vines continued to exhibit a trend of decreasing pigment content. Despite having a lower total chlorophyll content, TN_KL showed an 8% lower relative decrease in chlorophyll from *veraison* to ripeness, with a decay of 13% compared to the 21% loss of chlorophyll observed in TN_C. Regarding the chlorophyll and carotenoid ratios, kaolin treatment increased Chl_a/Chl_b in both varieties throughout the season, particularly in TN at *veraison* and in TF at the ripened stage. At the same time, $Chl_{(a+b)}/Car$ was lower in treated vines throughout the experiment.

Table 1: Leaf chlorophylls (Chl_a, Chl_b, Chl_(a+b), μ g g⁻¹ FW), total carotenoids (Car, μ g g⁻¹ FW), and related ratios (Chl_a/Chl_b, Chl_(a+b)/Car) of Touriga-Nacional (TN) and Touriga-Franca (TF) grapevines in control (TN_C, TF_C) and kaolin-treated (TN_KL, TF_KL) leaves at *veraison* and ripen stages. Data are mean ± SD of three replicates. Different lower case letters represent significant differences between treatments and varieties (TN_C, TN_KL, TF_C, TF_C, TF_KL) within each developmental stage. * represent significant differences (p<0.05) between developmental stages (*veraison vs* ripen) within each variety and treatment.

	Touriga-Nacional		Touriga-Franca	
	Control	Kaolin	Control	Kaolin
Veraison				
Chl _(a+b)	1330.9 ± 11.1	1108.5 ± 5.6	1162.3 ± 89.6	1328.7 ± 69.4
Car	247.1 ± 12.1	230.8 ± 9.3	240.8 ± 15.9	265.3 ± 10.1
Chl_a	$986.0\pm6.7~^{\rm a}$	840.1 ± 27.8 $^{\rm b}$	$897.7\pm68.8~^{ab}$	1024.1 ± 49.0 ^a
Chl_b	$344.9\pm4.4~^{a}$	$268.4\pm7.8\ ^{b}$	$264.6\pm20.8\ ^{b}$	$304.6\pm20.4~^{ab}$
Chl _a /Chl _b	$2.86\pm0.02~^a$	$3.13\pm0.01~^{b}$	$3.39\pm0.01~^{c}$	$3.37\pm0.06~^{c}$
Chl _(a+b) /Car	5.39 ± 0.22 ª	$4.80\pm0.04~^{b}$	$4.83\pm0.05~^{b}$	$5.18\pm0.23~^{ab}$
Ripen				
Chl _(a+b)	1044.5 ± 73.3 ^{ab*}	$962.1\pm84.9~^{ab}$	1181.2 ± 110.1 ^a	$935.6 \pm 60.0 \ ^{b*}$
Car	218.6 ± 21.3	197.0 ± 3.7 *	223.3 ± 14.8	208.1 ± 10.1 *
Chl_a	855.1 ± 54.3 ^{ab*}	$748.9\pm34.8~^{b}$	$947.9\pm42.0~^{\rm a}$	733.9 ± 47.0 ^{b*}
Chl_b	266.3 ± 19.0 ^{a*}	$237.8\pm15.3~^{ab}$	263.1 ± 6.1 ^a	201.7 ± 13.0 ^{b*}
Chl _a /Chl _b	$2.92\pm0.01~^{c}$	3.04 ± 0.10 $^{\circ}$	$3.49\pm0.03~^{b}$	3.64 ± 0.01 ^{a*}
Chl _(a+b) /Car	5.01 ± 0.18 ^{a*}	$4.76\pm0.17\ ^{ab}$	5.06 ± 0.17 a	4.44 ± 0.15 b*

3.2. Changes in PSII photochemistry

A distinct varietal response was noticed in the analysis of chlorophyll *a* fluorescence, especially during the midday period, due to the increased severity of summer stress during the daytime. Analysis of the F_v/F_m variation throughout the experiment indicated a progressive decay of the maximum quantum efficiency of PSII, which was more pronounced at ripen (Fig. 2A). Overall, no significant changes in quenching by the process of photosynthesis (qP) were observed (Fig. 2B) as a result of the kaolin treatment. Contrasting changes in the light-adapted parameter Φ_{PSII} due to kaolin application were detected only in the midday period at the ripened stage (Fig. 2C), with higher values in TF_KL and lower values in TN_KL compared to their respective control groups. Non-photochemical quenching (NPQ) was increased in both kaolin-treated varieties at the midday period of *veraison* (Fig. 2D) but was lower in the equivalent period of the ripened stage, particularly in TN. Interestingly, both treated and untreated TF grapevines showed lower NPQ levels than TN, mainly at midday.

3.3. Changes in individual carotenoids and xanthophyll cycle pool components

The diurnal variation in xanthophyll pigments showed that the xanthophyll cycle pool (VAZ) was similar between kaolin-treated and control leaves in both varieties (Fig. 3A) and there was a sharp decrease in Vx levels from pre-dawn to midday while Zx content increased. Kaolin-treated leaves showed 28.7% higher Zx accumulation in TF (Fig. 3B) at the midday period of the *veraison* stage, and 22.3% lower Zx at the ripened stage for TN_KL. Regarding Vx (Fig. 3C), no significant differences were found between treatments, excepting TF_KL at the midday period of the ripened stage, which showed around 13.7% lower Vx content than the respective control vines.

Overall, kaolin application enhanced leaf phytoene (Fig. 3D) accumulation throughout the experiment, especially in the TF variety at the ripened stage. Lutein (Fig. 3E) and β -carotene (Fig. 3F) accumulation increased from pre-dawn to midday in all sampling groups, but the β -carotene levels in TN_KL remained similar at the ripened stage. Kaolin treatment promoted opposite effects on lutein accumulation at *veraison* (Fig. 3E), depending on variety, its content increased in TN (43.6%), and decreased in TF (11.5%) vines. Other components of the xanthophyll cycle, namely the de-epoxidation state (DPS), also increased in TF_KL leaves and decreased in TN_KL leaves at both developmental stages during the midday period (Table 2).



Figure 2. Pre-dawn and midday chlorophyll fluorescence parameters of Touriga-Nacional (TN) and Touriga-Franca (TF) grapevines in control (TN_C, TF_C) and kaolintreated (TN_KL, TF_KL) leaves at *veraison* and ripen stages. Maximum (F_v/F_m , A) and effective (Φ_{PSII} , C) quantum efficiency, photochemical quenching (qP, B), and nonphotochemical quenching (NPQ, D). Data are mean \pm SD of six replicates. Different lower case letters represent significant differences between treatments and varieties within each developmental stage and period of the day. * represent significant differences



Figure 3. Pre-dawn and midday content of $V_x+A_x+Z_x$ (A), zeaxanthin (B), violaxanthin (C), phytoene (D), lutein (E), and β -carotene (F) of Touriga-Nacional (TN) and Touriga Franca (TF) control and kaolin-treated (TN_KL, TF_KL) leaves at *veraison* and ripen stages. Data are mean \pm SD of three replicates. Different lower case letters represent significant differences between treatments and varieties within each developmental stage and period of the day. * represent significant differences (p<0.05) from pre-dawn to midday within each variety, treatment, and developmental stage.

	Touriga	-Nacional	Touriga	-Franca
—	Control	Kaolin	Control	Kaolin
Veraison				
Xanthophylls (µg g ⁻¹)	187.3 ± 0.9	176.2 ± 9.2	190.5 ± 10.1	208.4 ± 18.5
XC Pool (%)	$25.6\pm0.3~^{b}$	$27.6\pm0.2^{\:bc}$	29.5 ± 0.4 ac	$31.6\pm0.1~^a$
DPS (%)	71.0 ± 0.2 $^{\rm a}$	$70.0\pm0.6^{\:a}$	63.3 ± 1.7 ^c	$67.2\pm0.4~^{b}$
Ripen				
Xanthophylls (µg g ⁻¹)	170.8 ± 15.6	150.4 ± 5.9	186.4 ± 31.3	153.0 ± 13.4
XC Pool (%)	$28.1\pm0.4~^{a^*}$	28.4 ± 1.4 $^{\rm a}$	$32.8\pm0.7~^{b*}$	37.2 ± 0.1 c*
DPS (%)	70.6 ± 0.4 $^{\rm a}$	61.6 ± 1.5 b*	68.6 ± 0.5 ^{a*}	75.9 ± 0.3 c*

Table 2. Xanthophylls (μ g g⁻¹ FW), xanthophyll cycle pool (XC Pool, %), and de-epoxidation state (DPS, %) of Touriga-Nacional (TN) and Touriga-Franca (TF) grapevines in control (TN_C, TF_C) and kaolin-treated (TN_KL, TF_KL) leaves at *veraison* and ripen stages.

XC Pool and DPS are expressed as a percentage, and calculated as $((Zx+Ax+Vx/total xanthophylls) \times 100)$ and $((Zx+Ax/Zx+Ax+Vx) \times 100)$, respectively. Data are mean \pm SD of three replicates. Different lower case letters represent significant differences between treatments and varieties (TN_C, TN_KL, TF_C, TF_KL) within each developmental stage. * represent significant differences (p<0.05) between developmental stages (*veraison vs* ripen) within each variety and treatment.

3.4. Changes in the expression of carotenoids biosynthetic pathway genes

Depending on developmental stage, the relative expression of genes involved changed with kaolin treatment (Fig. 4): *VvVDE1*, *VvZEP1*, and *VvPSY1* expression decreased in TN_KL plants compared to TN_C at the *veraison* stage, while *VvZEP1* was up-regulated at the ripened stage. At this stage, the kaolin effect was clearly observed in TF leaves, showing three-fold and nine-fold upregulation of *VvVDE1* and *VvZEP1*, respectively, in relation to TF_C.



Figure 4. Relative expression of VvVDE1, VvZEP1, and VvPSY1 genes of Touriga-Nacional (TN) and Touriga-Franca (TF) grapevines in control (TN_C, TF_C) and kaolin-treated (TN_KL, TF_KL) leaves at veraison and ripen stages. * denote significant differences between the control and kaolin treated vines of each variety.

4. Discussion

4.1. Kaolin particle-film application highlighted distinct varietal responses in chlorophyll accumulation and xanthophyll cycle pool regulation at version

Under summer stress conditions, the exogenous foliar application of solar protectants in vines has shown a general improvement in their physiological performance at a local scale (Dinis et al. 2018, Frioni et al. 2019). At the beginning of the experiment, the effect of kaolin on photosynthetic pigment accumulation, excess energy dissipation mechanisms, and xanthophyll cycle gene expression was greater in TN compared to TF, but showing opposite trends. While Chl_a and Chl_b content (Table 1) increased in treated TF leaves, preventing chlorophyll degradation (Dinis et al. 2015), TN_KL showed lower chlorophyll content and Chl_(a+b)/Car ratio, and increased Chl_a/Chl_b ratio, displaying some features of high light acclimated plants (Walters 2005). Furthermore, these distinct varietal responses could be related to the impact of summer stress on advancements in phenology (Hall et al. 2016).

Recently, Costa et al. (2019) predicted an earlier occurrence of *veraison* for TF compared to TN in the Douro region, highlighting the need to adjust established management practices. As the climate is the primary driver of phenology (Hall et al. 2016) and considering that the two heatwave events (DOY 165 – 169 and DOY 193 –198) (Fig. 1) occurred around the time of kaolin application (DOY 177) and soon before *veraison* was reached (DOY 199), it seems possible that *veraison* onset was advanced in TF, modulating kaolin effectiveness and plant stress responses.

Despite the opposite effects on chlorophyll content observed, both treated varieties showed higher NPQ (Fig. 2) at *veraison* than their respective control at midday, suggesting a strategy to maintain PSII reaction centres openness under high light (Ruban and Horton 1999). It has been clearly demonstrated that NPQ activation is associated with xanthophyll cycle activity, namely epoxidase-free zeaxanthin (Demmig-Adams et al. 1996, Demmig-Adams et al. 1995). Accordingly, DPS (Table 2) and Zx content (Fig. 3) increased in TF_KL at *veraison*, indicating improved photoprotective function of xanthophyll as a quencher of excitation energy by switching LHCII antenna into a dissipative state (Havaux and Tardy 1999, Horton et al. 2000). Hence, the initial rise of NPQ (Fig. 2) in kaolin-treated grapevines at *veraison* may be explained by the safe down-regulation of PSII efficiency, suggesting an optimised response to deal with severe summer stress conditions.

Beyond components of the xanthophyll cycle, the presence of other carotenoids can also indicate strategies of grapevine acclimation to combined high light and temperature and water stress (Demmig-Adams and Adams 1992). Lutein and β -carotene are other carotenoids involved in photoprotection, displaying the ability to quench singlet oxygen and chlorophyll triplets, thus improving plant acclimation to long light exposure (Jahns and Holzwarth 2012). Moreover, it has also been proposed that lutein plays an essential structural role in PSII photoprotection, converting efficient LHCII into potential dissipating centres under stressful conditions (Ramel et al. 2012). Both TF_KL and TN_KL presented lower lutein accumulation (Fig. 3) at *veraison*, particularly in the pre-dawn period, which might indicate conditions of lower stress and lower need to invest in photoprotection during the morning period (Dinis et al. 2015).

Efficient light stress responses depend on the interconversion of the xanthophyll cycle pigments Vx and Zx (Wilson and Ruban 2020) through the activation of the enzymes violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZEP) under high and non-saturated light conditions (Demmig-Adams et al. 1995). The results show that kaolin treatment

limited VvVDE1 and VvZEP1 gene expression (Fig. 4) at veraison only in the TN variety, without any evident influence on xanthophyll cycle pool dynamics. However, it should be borne in mind that VDE and ZEP gene expression, and the respective protein synthesis, may not occur at the same pace (Kim et al. 2019), particularly in field-grown grapevines that are continuously exposed to several stressors. To further investigate the effect of kaolin treatment on carotenoid synthesis during the summer season, both phytoene synthase gene expression (VvPSYI) and phytoene content were analysed. Phytoene synthase catalyses the first committed step of the carotenoid biosynthetic pathway and is one of the main reactions to control the carotenoid metabolic flux (Rodriguez-Concepcion et al. 2018). Kaolin treatment limited VvPSY1 gene expression only at veraison, highlighting the contribution of other components during the light acclimation processes, such as the DPS state, the Vx+Ax+Zx pool size, and neoxanthin synthase activity (Frioni et al. 2020). An alternative explanation could be that high levels of light in the xanthophyll cycle act directly on the VvZEP1 and VvVDE1 genes and this does not necessarily imply general stimulation of the pathway by up-regulation of VvPSY1. Nonetheless, increased levels of phytoene and a lower expression of VvPSY1 at veraison indicate that, depending on the magnitude of the stressor at the beginning of the summer season and the variety under study, kaolin treatment may improve the regulation of carotenoid metabolism under the prevailing conditions.

4.2. Kaolin particle-film promoted long-lasting high light acclimation mechanisms throughout the summer season

From *veraison* to ripening, although the underlying mechanisms behind the protective role that kaolin performs are challenging to elucidate, the results showed a general decrease of chlorophylls (Table 1) in kaolin-treated leaves at ripening, suggesting a long-term response to summer stress. In agreement with these results, Savitch et al. (2000) reported that leaves with low chlorophyll levels exhibit a typical long-term response to high light through a balanced reduction in the peripheral antenna size and a smaller absorption cross-section. Therefore, this response can be considered to be an effective protective mechanism against light stress and photoinhibition during persistent stress conditions (Chaves et al. 2003), contrasting with the results of Shellie and King (2013) and Brito et al. (2018).

The association between chlorophyll fluorescence, carotenoids, and chlorophyll data suggests the existence of dynamic and complex plant stress responses, particularly during the midday period. Since kaolin application limited chlorophyll accumulation, one would expect a

decline in the photochemical processes (Fig. 2), such as the maximum (F_v/F_m) and operating (Φ_{PSII}) efficiency of PSII, and an increase in thermal energy dissipation, measured as non-photochemical quenching (Verhoeven et al. 1997). However, no significant changes in the F_v/F_m and qP parameters were detected at midday in kaolin-treated plants at the ripened stage, while NPQ was lower, indicating that kaolin treatment increased light collection in both TF and TN and decreased the thermal dissipation of excess energy and the need to activate the xanthophyll cycle at the end of the season. Indeed, treated leaves showed lower Zx and Vx accumulation at ripen (Fig. 3), highlighting the persistence of particle film effectiveness throughout the season, despite its being a washable product (Dinis et al. 2017).

From *veraison* to ripening, *VvZEP1* and *VvVDE1* gene expression patterns in treated leaves (Fig. 4) seem to be associated with higher Zx accumulation at *veraison* in TN, and lower at ripening in both varieties, suggesting an improved tolerance to prolonged high light exposure and efficient regulation of the components of the xanthophyll cycle. Moreover, this result may also indicate that different mechanisms, apart from the xanthophyll cycle pigments, play a role during initial high light acclimation. These data agree with the findings of Frioni et al. (2020), who reported an increase in DPS state in kaolin-treated grapevines, suggesting a lower conversion of Zx into neoxanthin (ABA precursor) caused by triggering ZEP activity or by reducing neoxanthin synthase activity. In addition, the overexpression of xanthophyll cycle genes may regulate NPQ induction and relaxation in fully developed leaves, protecting plants from photodamage (Leonelli et al. 2016). Therefore, it is likely that the strong over-expression of *VvZEP1* in kaolin-treated leaves, particularly in TF, also triggered NPQ relaxation levels, optimising grapevine stress responses under the prevailing conditions. These findings support the hypothesis that kaolin application under summer stress conditions may boost the plasticity of grapevine stress responses and photoprotection mechanisms on several levels.

5. Conclusions

In this study, foliar application of kaolin improved grapevine plasticity and ability to deal with prolonged periods of summer stress, optimising their capacity to control light absorption and managing the absorbed light. Despite the challenge of understanding plant stress responses under field conditions and their interactions with varietal features, management practices, and other terroir components, this study outlines the dynamics of the photoprotective mechanisms triggered by summer stress in different grapevine varieties grown in Mediterranean-type climate regions. In addition, kaolin treatment before *veraison* relieved the impact of summer

stress on grapevines by managing pigment accumulation, modulating the expression of key genes in the xanthophyll cycle, and also through a combination of improving light-harvesting processes and decreasing the thermal dissipation of excess energy.

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Supplementary material

Table ST1.	List of primers	used for real-time	quantitative PCR.
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Gene	Gene unique ID		Primer (5'–3')	Size (bp)
VvZEP1	GSVIVT01022128001	Fw Rv	GTTCAGTGCAGCCTTCGATT GGCTTCAGCCAAAGTAGCTT	167
VvVDE1	GSVIVT01026405001	Fw Rv	CCAAACCAGCCTGGAATACT CGCCATATCCATCCCATGCA	154
VvPSY1	GSVIVT01035255001	Fw Rv	CCTGGGACTCAGAGCTTGTT CTGTTCTCCGACACCACACA	151
VvACT2	GSVIVT01026580001	Fw Rv	GCCATCCAAGCTGTTCTCTC CAGTAAGGTCACGTCCAGCA	157
VvTUB2	GSVIVT01037405001	Fw Rv	CAACTCTGACCTCCGAAAGC CTTGGAGTCCCACATTTGCT	154

Chapter 5.2. Fine-tuning of grapevine xanthophyll-cycle and energy dissipation under Mediterranean conditions by kaolin particle-film (Alentejo)

Abstract

Kaolin-particle film has been considered a low-cost technology to mitigate the adverse effects of high light and temperature, and drought in several crops. However, the underlying excess energy absorption and dissipation mechanisms, and related components associated with kaolin photoprotective effects in grapevines are poorly explored. This study aims to understand the interactions between kaolin treatment and photosynthetic pigments accumulation, carotenoids metabolism, xanthophyll cycle regulation, and its putative role on the nonphotochemical quenching (NPQ) processes in Touriga-Franca (TF) and Touriga-Nacional (TN) varieties due to their winery potential. Over the summer season, kaolin enhanced TN chlorophyll accumulation up to 114 % at EL35 (veraison) and 123 % at EL38 (full mature), highlighting its protective role on chlorophyll degradation, while no changes were found in TF, which might indicate a lower need for particle-film technology in this variety under the current environmental conditions. Individual carotenoids were mainly higher in treated leaves, as well as the xanthophyll cycle pigments zeaxanthin (Z_x) and violaxanthin (V_x) . Simultaneously, the de-epoxidation state (DPS) and NPQ values were lower in treated leaves (1.92 - 2.36) compared to control (3.19 - 3.24), suggesting that there might be other components influencing NPQ levels beyond Z_x, with an indirect role in long-lasting NPQ processes. In addition, in leaves of TF kaolin-treated violaxanthin de-epoxidase (VvVDE1) and zeaxanthin epoxidase (VvZEP1) gene expression were respectively 3-fold and 4-fold upregulated at stage EL35, while VvZEP1 gene expression decreased at stage EL38 in TN kaolin-treated, indicating an optimised regulation of the xanthophyll cycle. These findings suggest that kaolin treatment promoted a fine-tuning of the carotenoids metabolism under sustained summer stress factors, by managing xanthophyll cycle activity, carotenoids and chlorophyll accumulation. Under applied contexts, kaolin application can be considered an efficient practice to minimise summer stress impacts in grapevines grown in Mediterranean-like climate regions.

Keywords: acclimation; chlorophyll; de-epoxidation state; non-photochemical quenching; violaxanthin; zeaxanthin

1. Introduction

Mediterranean crops are frequently subjected to several environmental constraints during the summer season, impairing plant growth and development, with negative impacts on yield and fruit quality potential. According to the latest climate projections, particularly for grape production, the prevalence of summer stress factors (e.g. high light, high temperature and prolonged drought) will certainly increase on Mediterranean-type climate regions in the upcoming decades, imposing a serious challenge for the wine industry (Fraga *et al.* 2013).

Within the numerous short-term climate change adaptation strategies applied in viticulture, the use of kaolin particle-film technology has been studied over the last decade as a potential tool to mitigate high light stress and elevated temperature conditions, as well as under water shortage (Bernardo *et al.* 2018, Shellie and Glenn 2008, Shellie and King 2013). Indeed, most of the research regarding kaolin application reports general positive effects on leaf cooling, water use efficiency, enhanced chlorophyll and carotenoids accumulation, improving the general photochemical and excess energy dissipation processes of grapevines (Brillante *et al.* 2016, Correia *et al.* 2015, Dinis *et al.* 2015, Dinis *et al.* 2017, Frioni *et al.* 2019). However, the underlying excess energy absorption and dissipation mechanisms and related components associated with kaolin photoprotective effects in adult grapevines remain unclear.

It is known that light acclimation under field conditions demands flexible photoprotective responses, through the dissipation of excess radiative energy as heat, also known as non-photochemical quenching (NPQ), and the enduring repair of PSII, which requires a rapid reorganisation of the thylakoid membrane (Kress and Jahns 2017). Excessive energy dissipation involves the activation of the xanthophyll cycle, through the de-epoxidation of violaxanthin (V_x) to zeaxanthin (Z_x) by the enzyme violaxanthin de-epoxidase (VDE), which were related to NPQ activation (Demmig *et al.* 1987). Nevertheless, it is still a matter of debate whether the photoprotective role of xanthophylls is based on a direct (e.g. quencher of excitation energy) or indirect (e.g. modulator of energy dissipation) function, as well as its antioxidant potential in the lipid phase of membranes (Horton *et al.* 2000, Kress and Jahns 2017).

Beyond its role in photoprotection, the accumulation of xanthophyll cycle components can also be a sign of acclimation to a combined high light and water stress (Demmig-Adams and Adams 1992). Likewise, other carotenoids can boost photoprotection, such as lutein and β carotene, through their capacity to avoid the accumulation of reactive oxygen species, improving plants acclimation to long light exposures (Jahns and Holzwarth 2012). Moreover, it has also been proposed that lutein plays an essential structural role in PSII photoprotection, converting efficient light harvesting complexes (LHCII) into potential dissipating centres under stressful conditions (Ramel *et al.* 2012).

Recently, Frioni *et al.* (2020) suggested that kaolin treatment under progressive water deficit in potted grapevines limited Z_x 's conversion into neoxanthin (N_x), an abscisic acid precursor, maintaining a functional xanthophyll cycle running and avoiding excess energy. However, to the best of our knowledge, the interactions between kaolin treatment and photosynthetic pigments accumulation, carotenoids metabolism, and xanthophyll cycle regulation are poorly understood, as well as the dynamic link with NPQ activation in field grown-grapevines. Hence, this study aims to enlighten kaolin photoprotective functions regarding NPQ activation under summer stress conditions, and its putative role in regulating xanthophyll cycle dynamic in Mediterranean-type climate regions. For that purpose, we assessed the chlorophyll and individual carotenoid contents of Touriga-Franca (TF) and Touriga-Nacional (TN) grapevine varieties, analysed the fluorescence of chlorophyll *a*, and determined the expression of key genes of the xanthophyll cycle (*VvVDE1* and *VvZEP1*) and the first committed step of the carotenoid biosynthetic pathway (*VvPSY1*) throughout the summer season.

2. Materials and Methods

2.1. Growth conditions and plant material

The field trial was conducted in an ampelographic field (9.7 ha) with 189 grapevine varieties distributed in side-by-side rows in the Alentejo Demarcated Region, in the Southeast part of Portugal (38° 23' N, 7° 33' W). In the 2017 growing season, the six-year-old vines of Touriga-Franca (TF) and Touriga-Nacional (TN) were selected due to their good winery potential and cultural expression in most Portuguese winegrowing regions, displaying an N-S orientation with a slight slope (5° N) and 220 m of elevation. Vines were grafted on 1103P, trained to bilateral cordon, with a 2.2 m by 1.0 m vines by row spacing. The local climate is typically Mediterranean-like, with a warm-temperate climate with dry and hot summers (Kottek *et al.* 2006), and higher precipitation during the winter months. An automatic weather station recorded weather variables such as the rainfall, mean air temperature (T_{mean}), and maximum air temperature (T_{max}) during the experiment (Fig. 1). The mean air temperature registered from April (DOY 91) to October (DOY 304) was 22.5 °C, with total precipitation of 47 mm. Considering the range of five consecutive days with temperatures above 40°C, two heatwave events were recorded during the experiment (DOY 167-171 and DOY 192-197).



Figure 1. Daily mean (T_{mean}) and maximum (T_{max}) air temperature (°C) and precipitation (mm) at the vineyard site. Vertical grey filled bars represent the heatwave events occurred during the experiment.

According to the world reference base for soil resources 2014 (FAO 2015), the soil mapping of the region is classified as luvisol, characterised by a clay-enrichment subsoil. At the vineyard site, soil composition is derived from granodiorite, with a very similar texture that varies between sandy loam and sandy loam-clay, well-drained, and with some water holding capacity without limitation due to the appearance of the original rock up to 1.50 m deep. Vineyard management reflected local commercial organic production practices, with deficit irrigation (30% of the reference evapotranspiration) to prevent plants from death.

2.2. Treatments and monitoring

The experimental set up was adapted to the commercial vineyard's existing features to ensure similar edaphoclimatic conditions and solar exposure among treatments and varieties. In each extended row, 120 vines per variety were selected, considering half row as the control group, and the other half as the treated group. Within each group, vines were also distributed in three blocks with 20 plants each. Two treatments were then established: an experimental control (TF_C and TN_C), and a kaolin treated group (TF_KL and TN_KL). Kaolin suspension (Surround® WP, Engelhard Corporation, Iselin, New Jersey) was prepared in an aqueous solution following manufacturer's recommended dosage of 5 % (w/v), supplemented with 0.1 % (v/v) Tween 20 to improve adherence, and applied according to standard operating procedures adjusted for agricultural practices. Kaolin was applied in the windless morning of

July 17 (DOY 198). For all the physiological and biochemical measurements, six healthy, fully expanded and mature leaves in a similar position were collected per treatment and variety at two periods of the day (pre-dawn and midday). The measurements were also carried out at two different developmental stages according to the Coombe (1995) classification: EL35 (DOY 208), corresponding to *veraison*, and EL38 (DOY 237), corresponding to the harvest date. Leaf samples were immediately frozen in liquid nitrogen and delivered to the plant physiology laboratory of the University of Trás-os-Montes and Alto Douro. Samples were grounded to a fine powder with liquid nitrogen and stored at -80 °C until analysis.

2.3. Chlorophyll and carotenoid extraction and quantification

Photosynthetic pigments were extracted essentially as described in Rodrigo et al. (2003). Briefly, fresh frozen ground leaves (approximately 0.175 g) were extracted with a solution containing 2.0 mL MeOH and 1.5 mL of Tris-HCl (50 mM, pH 7.5 containing NaCl 1 M) during 30 min. Chloroform (4.0 mL) was then added to the mixture, vortex during 30 s and centrifuged at 3500 g at 4 °C. The aqueous phase was re-extracted with chloroform until it was colourless. The combined chloroform extracts were dried on a rotary evaporator at 35 °C and re-dissolved with acetone:petroleum ether:diethyl ether (0.7:9:1, v/v/v). An aliquot was used for determination of the chlorophyll (a+b) content by measuring the absorbance at 644 and 662 nm and calculated as described by Smith and Benitez (1955). After chlorophyll measurements, the ethereal pigment solution was dried and saponified using a 10 % methanolic KOH solution. The carotenoids were subsequently re-extracted with diethyl ether until the hypophase was colourless. An aliquot of the ether extract was used for quantification of total carotenoids content, and this was calculated by measuring the absorbance of the saponified extract at 450 nm (Davies 1976). The samples were dried under an N₂ stream and kept at -20 °C until analysis. Determinations were carried out at least in triplicates, and all procedures were performed on ice under dim light to prevent photodegradation, isomerisations and structural changes of carotenoids.

2.4. Analysis of individual carotenoids

Carotenoid composition of each sample was analysed by HPLC with a Waters liquid chromatography system equipped with a 600E pump and a 2998 photodiode array detector (PAD), and Empower software (Waters, Barcelona, Spain). A C₃₀ carotenoid column (250 mm \times 4.6 mm, 5 µm) coupled to a C₃₀ guard column (20 mm \times 4.0 mm, 5 µm) (YMC, Teknochroma,

Spain) was used. Samples were prepared for HPLC by dissolving the dried carotenoid extracts in CHCl₃:MeOH:acetone (3:2:1, v:v:v). A ternary gradient elution of MeOH water and methyl *ter*-butyl ether (MTBE) was used for carotenoid separation as described by Rodrigo *et al.* (2003). The PDA detector was set to scan from 250 to 540 nm, and for each elution, a Maxplot chromatogram was obtained, which plots each carotenoid peak at its corresponding maximum absorbance wavelength. Carotenoids were identified by their retention time, absorption and fine spectra (Britton *et al.* 1998, Rodrigo *et al.* 2004, Rodrigo *et al.* 2003). The carotenoid peaks were integrated at their individual maxima wavelength, and their contents were calculated using calibration curves of zeaxanthin (Extrasynthese), β -carotene (Sigma) for α - and β -carotene, β cryptoxanthin (Extrasynthese), lutein (Sigma), anteraxanthin (CaroteNature), and violaxanthin (CaroteNature) for violaxanthin and neoxanthin isomers. Phytoene was previously purified as described (Lado *et al.* 2015). Results are given as $\mu g g^{-1}$ fresh weight (FW). The de-epoxidation state (DPS) of the xanthophyll cycle was calculated as DPS = $(Z_x + 0.5A_x)/(V_x + Z_x + A_x)$, where V_x , A_x , and Z_x are violaxanthin, antheraxanthin and zeaxanthin, respectively.

2.5. Chlorophyll fluorescence measurement

Within each experiment, chlorophyll *a* fluorescence emission was measured at pre-dawn (05:00 – 06:00; local time) and midday (14:00 – 15:30; local time) on six fully expanded leaves in both EL35 and EL38 developmental stages with a Pulse Amplitude Modulation Fluorometer (mini-PAM, Photosynthesis Yield Analyzer; Walz, Effeltrich, Germany), using two scripts: In the first script, measurements were done on well sun exposed leaves (except on pre-dawn period). In this procedure, after a 35-s exposure to actinic light (1,450 µmol m⁻² s⁻¹), light-adapted steady-state fluorescence yield (F_s) was averaged, followed by exposure to a saturating pulse light (6,000 µmol m⁻² s⁻¹) for 0.6 s to establish F_m'. The sample was then shaded for 5 s with a far-red light source to determine F₀'. From these measurements, several fluorescence attributes were calculated (Bilger and Schreiber 1986, Genty *et al.* 1989): photochemical quenching (qP = (F_m' – F_s)/(F_m' – F₀')), non-photochemical quenching (NPQ = (F_m - F_m')/F_m'), and efficiency of electron transport as a measure of the effective quantum efficiency of PSII ($\Phi_{PSII} = \Delta F/F_m' = (F_m' - F_s)/(F_m')$.

In a second script, using the dark leaf clip (DLC-8), the same leaf portion used in the first script was immediately dark acclimated for 30-45 min. After this, the maximum photochemical efficiency of PSII was given by $F_v/F_m = (F_m - F_0)/F_m$, where F_0 corresponds to the minimum fluorescence level excited by the very low intensity of measuring light to keep PSII reaction centres open, and F_m corresponds to the maximum fluorescence level elicited by a pulse of

saturating light (6,000 μ mol m⁻² s⁻¹) which closes all PSII reaction centres (Bilger and Schreiber 1986).

2.6. Gene expression analysis using RT-qPCR

RNA was extracted from frozen leaves following a rapid CTAB-based procedure (Gambino *et al.* 2008). Afterwards, RNA samples were treated with DNAse I RNase-free (Thermo Scientific, Waltham, MA, USA) to degrade the possible extracted DNA. The RNA concentration was estimated using the absorbance values at 260 nm with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), while the purity of each sample was determined calculating the 260/280 and 260/230 ratios. Finally, total RNA (1 μ g) was reverse transcribed to cDNA using Primescript RT Reagent Kit (Takara, Shiga, Japan). Quantitative real-time PCR was conducted with an ABI Step One detection system (Applied Biosystems, Foster City, CA, USA). Gene specific primer pairs used for each target or reference gene are listed on supplementary table ST1.

The amplification was done in a reaction contained 1 µL of cDNA, 5 µL of Maxima SYBR Green/ROX qPCR mix (Thermo Scientific), 1 µL of primers (a mix of forward and reverse, 10 µM) and 3 µL of sterile water. PCR reactions included a pre-incubation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 20 s. *Actin (VvACT2*, phytozome accession no. GSVIVT01026580001) and *Tubulin (VvTUB2*, phytozome accession no. GSVIVT01037405001) were used as reference genes to normalise the results among samples. Relative expression of *VvVDE1* (Phytozome accession no. GSVIVT01022128001), and *VvPSY1* (Phytozome accession no. GSVIVT01035255001) was achieved using the *Relative Expression Software Tool Solver v.2* (REST-MCS) (Pfaffl 2001,2002). For each gene analysed, they were considered significantly upregulated and down-regulated in the kaolin treated groups (TN_KL and TF_KL), when their relative expression fold change were ≥ 2 and ≤ 0.5 , respectively.

2.7. Statistical analysis

Statistical analyses of individual carotenoids, xanthophyll cycle components, and chlorophyll fluorescence were performed with Sigma-Plot 14.0 program (SPSS Inc.). After testing for ANOVA assumptions (homogeneity of variances with the Levene's mean test, and normality with the Kolmogorov-Smirnov test), statistical differences among treatments and

varieties were evaluated by two-way factorial ANOVA, followed by the post hoc Tukey's test. Afterwards, statistical differences between periods of the day (pre-dawn *vs* midday) within each sampling group were evaluated by one-way analysis of variance (ANOVA), followed by the post hoc Tukey's test. For total chlorophylls, carotenoids, xanthophylls content, DPS, and related ratios, statistical differences between developmental stages (EL35 *vs* EL38) within each sampling group were evaluated by one-way ANOVA, followed by the post hoc Tukey's test. Different lower case letters represent significant differences (p < 0.05) between treatments (TF_C, TF_K, TN_C, TN_K) within each developmental stage, and * (p < 0.05) represent significant differences between developmental stages. (EL35 *vs* EL38) within each treatment and variety. Absence of letters and asterisks indicate no significant difference.

3. Results and Discussion

Kaolin particle-film technology has been widely studied in several Mediterranean crops as a tool for sustainable management, improving the photosynthetic performance and water use efficiency of vines, as well as boosting leaf photosynthetic pigments accumulation, particularly under high light, high temperature, and drought conditions (Bernardo et al. 2017, Brillante et al. 2016, Glenn 2012). According to our results, kaolin treatment enhanced Chl_(a+b), Chl_a, and Chl_b contents of TN throughout the season (Table 1), highlighting its protective role on chlorophyll degradation (Dinis et al. 2016). However, in TF variety this response was only observed at stage EL38, whose control group showed already 92.7 % and 42.8 % higher chlorophyll and carotenoids levels, respectively, at stage EL35 compared to TN_C, indicating different varietal responses to summer stress, and possibly a lower need for kaolin application in TF. Moreover, Chl_(a+b)/Car ratio (Table 2) of TN_K leaves increased up to 33.3 % at stage EL35 and 45.9 % at stage EL38 compared to TN_C, as previously observed in kaolin-treated 'Malbec' leaves subjected to reduced irrigation levels (Shellie and King 2013), suggesting that kaolin leaf cooling effect lead to pigment concentration changes. This response was also reported by Nanos (2015) and Brito et al. (2018) in kaolin-sprayed olive trees grown in Mediterranean-type climate areas, indicating that kaolin application could promote some lowlight adapted features. By reducing Chl_a/Chl_b and increasing Chl_(a+b)/Car ratios, treated leaves might better capture and utilise low-energy and far-red radiation, and increase total lightharvesting capacity, which was also reflected in a higher quantum yield efficiency ($\Phi PSII$) (Palliotti et al. 2000, Ruban 2014).

	Touriga-Franca		Touriga-Nacional		
	Control	Kaolin	Control	Kaolin	
EL35					
Chl _(a+b)	$1418.9 \pm 59.0 \text{ b}$	1472.6 ± 283.1 b	736.9 ± 88.7 a	$1578.7 \pm 80.0 \text{ b}$	
Car	$260.2 \pm 18.4 \text{ b}$	$280.3 \pm 23.0 \text{ b}$	182.2 ± 15.4 a	$261.7 \pm 6.0 \text{ b}$	
Chl_a	$1093.4 \pm 45.1 \text{ b}$	$1120.4 \pm 212.2 \text{ b}$	649.2 ± 65.3 a	1193.7 ± 58.6 b	
Chl_b	$325.5 \pm 14.0 \text{ b}$	$352.2 \pm 70.9 \text{ b}$	213.1 ± 23.4 a	$385.0 \pm 21.3 \text{ b}$	
Xanthophylls	$214.2 \pm 17.8 \text{ b}$	229.1 ± 15.7 b	116.3 ± 11.4 a	$205.3 \pm 14.7 \text{ b}$	
DPS	$0.786 \pm 0.009 \text{ c}$	$0.730 \pm 0.010 \text{ b}$	$0.746 \pm 0.022 \text{ b}$	0.635 ± 0.007 a	
EL38					
Chl _(a+b)	$968.4 \pm 16.0 \text{ b*}$	1315.0 ± 13.9 c	754.0 ± 55.8 a	1682.7 ± 132.2 d	
Car	227.3 ± 9.7 b*	262.6 ± 7.7 c	$164.2 \pm 9.1 \text{ a}$	241.6 ± 12.7 c	
Chl_a	734.8 ± 22.0 ac*	$1004.8 \pm 11.6 \text{ bc}$	564.9 ± 43.1 a	1255.2 ± 105.0 b	
Chl_b	$233.6 \pm 6.0 \text{ ac}^*$	$310.3 \pm 2.3 \text{ c}$	207.0 ± 12.7 a	$427.5 \pm 27.2 \text{ b}$	
Xanthophylls	161.6 ± 18.4 b*	$193.3 \pm 11.0 \text{ b*}$	107.9 ± 10.2 a	142.6 ± 10.2 ab*	
DPS	$0.647 \pm 0.022 \text{ b*}$	$0.600 \pm 0.016 \text{ ab*}$	$0.446 \pm 0.028 a^*$	0.573 ± 0.033 a*	

Table 1. Midday leaf chlorophylls (Chl_a, Chl_b, Chl_(a+b), μ g g⁻¹ FW), total carotenoids (Car, μ g g⁻¹ FW), total xanthophylls (μ g g⁻¹ FW), and de-epoxidation state (DPS) of Touriga-Franca and Touriga-Nacional control and kaolin-treated grapevines at EL35 and EL38 developmental stages.

Table 2. Midday chlorophyll a/b ratio (Chl_a/Chl_b), total chlorophyll/ total carotenoids ratio (Chl_(a+b)/Car), xanthophyll cycle pool per unit of Chl (VAZ/Chl_(a+b)), lutein per Chl ratio (Lut/Chl_(a+b)), and β -carotene per Chl ratio (β -car/ Chl_(a+b)) in Touriga-Franca and Touriga-Nacional control and kaolin-treated grapevines at EL35 and EL38 developmental stages. Data are mean \pm SD of three replicates. Different lower case letters represent significant differences between treatments and varieties within each developmental stage. * represent significant differences (p<0.05) between developmental stages (EL35 *vs* EL38) within each variety and treatment.

	Touriga-Franca		Tour	iga-Nacional
	Control	Kaolin	Control	Kaolin
EL35				
Chl _a /Chl _b	$3.36\pm0.01\ b$	3.24 ± 0.12 ab	3.08 ± 0.07 a	3.10 ± 0.02 ab
Chl _(a+b) /Car	5.26 ± 0.31 ab	5.17 ± 0.66 a	4.65 ± 0.20 a	$6.20\pm0.22~b$
VAZ/Chl _(a+b)	$0.053 \pm 0.003 \; b$	$0.051 \pm 0.006 \; b$	$0.046\pm0.002~b$	0.035 ± 0.001 a
Lut/Chl _(a+b)	$0.085 \pm 0.010 \text{ ab}$	$0.106 \pm 0.011 \text{ b}$	$0.093 \pm 0.003 \text{ b}$	0.080 ± 0.003 a
β -car/Chl _(a+b)	$0.031 \pm 0.002 \; b$	$0.037 \pm 0.003 \text{ c}$	0.025 ± 0.002 a	0.022 ± 0.002 a
EL38				
Chl _a /Chl _b	$3.15 \pm 0.18 \text{ ab*}$	$3.24\pm0.01\ b$	3.00 ± 0.05 ab	$2.93\pm0.06~b$
Chl _(a+b) /Car	4.52 ± 0.16 a	5.12 ± 0.25 a	4.97 ± 0.21 a	$7.25\pm0.22~b$
VAZ/Chl _(a+b)	$0.055 \pm 0.004 \text{ c}$	$0.044 \pm 0.001 \text{ b}$	$0.038 \pm 0.005 \text{ b*}$	$0.023 \pm 0.001 a^*$
Lut/Chl _(a+b)	$0.103 \pm 0.007 \text{ b*}$	$0.088 \pm 0.001 \text{ b}$	$0.096 \pm 0.009 \text{ b}^*$	$0.057 \pm 0.004 \ a^*$
β -car/Chl _(a+b)	$0.024 \pm 0.002 \text{ b*}$	$0.019 \pm 0.002 \ b*$	$0.014 \pm 0.001 \text{ a*}$	$0.009 \pm 0.001 a^*$

In the current study, no changes were detectable on the maximum photochemical quantum efficiency of PSII (F_v/F_m) (Fig. 2A), whereas Φ PSII increased up to 33.0 % in TF_K and up to 31.4 % in TN_K at stage EL38 on midday (Fig. 2C), compared to their control groups, along with a slight increase in qP values (20.1 %) found exclusively in TN (Fig. 2B). In accordance with the present results, previous studies have reported higher Φ PSII and similar qP in kaolin-coated leaves (Brito *et al.* 2019, Dinis *et al.* 2018), indicating a higher efficiency of the light

absorbed by PSII antenna, which is likely due to lower thermal dissipation of excessive energy. In fact, our results show that kaolin treatment decreased NPQ (Fig. 2D) mainly at stage EL38, whose values ranged between 1.92 - 2.36 in treated leaves, and between 3.19 - 3.24 in the control ones, indicating better conditions for plant growth and development under Mediterranean summer conditions (Bernardo *et al.* 2018). Interestingly, at EL35 stage, both qP (Fig. 2B) and NPQ (Fig. 2D) decreased in TF_K, indicating that there might be other components influencing photochemical processes and NPQ relaxation levels, such as stomatal conductance, photosynthetic rates, and chlorophyll and carotenoids accumulation, among others (Demmig-Adams *et al.* 2014). Therefore, this unlikely result denotes that light acclimation features putatively promoted by kaolin coating should be carefully explored, as they seem to depend on several factors, such as stress magnitude, species and varieties under study, as well as kaolin application timing and concentration (Ferrari *et al.* 2017, Glenn 2012).



Figure 2. Maximum (F_v/F_m , A) and effective (Φ_{PSII} , C) quantum efficiency, photochemical quenching (qP, B), and non-photochemical quenching (NPQ, D) of Touriga-Franca (TF) and Touriga-Nacional (TN) control (TF_C, TN_C) and kaolin-treated (TF_K, TN_K) leaves at EL35 and EL38 stages at pre-dawn and midday periods. Data are mean \pm SD of six replicates. Different lower case letters represent significant differences between treatments and varieties within each developmental stage and period of the day. * represent significant differences (p<0.05) between the period of the day (pre-dawn vs midday) within the same variety, treatment and developmental stage.

In line with the findings of Dinis *et al.* (2017), total carotenoids content (Table 1) increased in both kaolin-treated varieties throughout the experiment, as well as total xanthophyll content. Likewise, the xanthophyll cycle pigment concentrations (Fig. 3), V_x and Z_x , and the $V_xA_xZ_x$ pool increased mainly in TN_K, which was previously reported on both C₃ and C₄ plants acclimated to high light, in parallel with an increased NPQ capacity (Brugnoli *et al.* 1994, Brugnoli *et al.* 1998).



Figure 3. Pre-dawn and midday content of $V_x+A_x+Z_x$, zeaxanthin, and violaxanthin, phytoene (D), lutein (E), and β -carotene (F) of Touriga-Franca (TF) and Touriga-Nacional (TN) control (TF_C, TN_C) and kaolin-treated (TF_K, TN_K) leaves at EL35 and EL38 stages. Data are mean \pm SD of three replicates. Different lower case letters represent significant differences between treatments and varieties within each developmental stage and period of the day. * represent significant differences (p<0.05) from pre-dawn to midday within each variety, treatment, and developmental stage.

It has been clearly demonstrated that NPQ activation is associated with xanthophyll cycle activity, namely the epoxidase-free zeaxanthin (Demmig-Adams 1990, Demmig-Adams and Adams 1996, Demmig *et al.* 1987). In agreement, the simple correlations between NPQ levels and xanthophylls (Table 3) revealed a strong positive correlation with the levels of Z_x (r = 0.58), neoxanthin (N_x) (r = 0.53) and de-epoxidation state (DPS) (r = 0.75), and a strong negative correlation with V_x content (r = - 0.70).

Table 3. Simple correlation between the levels of non-photochemical quenching (NPQ) and individual carotenoids, de-epoxidation state (DPS), and Vx+Ax+Zx pool.

	NPQ	
	Pearson r	p-value
β-carotene	0.46	0.008**
Lutein	0.46	0.009**
Neoxanthin	0.53	0.002**
Phytoene	0.17	0.346
Violaxanthin	-0.70	< 0.001***
Zeaxanthin	0.58	< 0.001***
Vx+Ax+Zx	0.25	0.172
DPS	0.75	< 0.001***

In spite of the greater Z_x accumulation, and the significant correlation with NPQ parameter, one might expect increased NPQ values in kaolin-treated leaves. This outcome contributes to the long-lasting debate regarding xanthophyll photoprotective direct function as a quencher of excitation energy or indirect function as a modulator of energy dissipation, as well as its antioxidant role in the lipid phase of the membrane (Havaux and Niyogi 1999, Horton et al. 2000, Ruban and Horton 1999). Recently, Kress and Jahns (2017) also found that the extent of NPQ was independent of Z_x accumulation with increasing light intensity, suggesting an indirect role of Z_x in long-lasting NPQ processes, which could explain the discrepancy between NPQ and Z_x parameters observed under the current study. Hence, the proposed indirect function of xanthophylls might be related to an allosteric regulation of NPQ processes, or a wide photoprotective function by modulation of the membrane fluidity, or by acting as an antioxidant (Horton et al. 2000, Papadatos et al. 2017). Treated leaves showed higher V_x and Z_x accumulation (Fig. 3), and lower NPQ levels without compromising photochemical processes (Fig. 2), suggesting that kaolin treatment boosted the plasticity of grapevine stress responses and energy cost-saving, by managing xanthophylls accumulation and limiting both downregulation of photochemistry and photoinhibition (Chaumont et al. 1997).

Meanwhile, the de-epoxidation state (DPS) was significantly lower in kaolin sprayed vines at EL35 stage, and no significant differences were found at EL38 stage. This result partly contrasts with the recent findings of Frioni *et al.* (2020), who reported higher DPS in 'Sangiovese' kaolin-treated grapevines under water shortage, along with constant levels of neoxanthin (N_x) and increased $V_xA_xZ_x$ pool, proposing that kaolin treatment reduced the conversion of Z_x into N_x. Beyond the increased $V_xA_xZ_x$ pool, the present results show a concomitant N_x accumulation in kaolin-treated vines (Fig. 4), particularly at stage EL35.



Figure 4. Pre-dawn and midday content of phytoene, lutein, β -carotene, and neoxanthin of Touriga Franca (TF) and Touriga-Nacional (TN) control (TF_C, TN_C) and kaolin-treated (TF_K, TN_K) leaves at EL35 and EL38 stages. Data are mean \pm SD of three replicates. Different lower case letters represent significant differences between treatments and varieties within each developmental stage and period of the day. * represent significant differences (p<0.05) from pre-dawn to midday within each variety, treatment, and developmental stage.

Besides, lutein and β -carotene contents (Fig. 4) also increased in treated vines, with a greater effect on TF at pre-dawn, and in TN at both pre-dawn and midday periods, suggesting different varietal acclimation strategies and stress recovery timings (Carvalho *et al.* 2015). Moreover, lutein and β -carotene showed an equal positive correlation to NPQ (Table 3), highlighting the photoprotective function of other carotenoids apart from the xanthophyll cycle major players (Z_x and V_x), as previously described by Niyogi *et al.* (1997). Nonetheless, on a Chl_(a+b) basis, both V_xA_xZ_x and lutein accumulation decreased in kaolin-treated grapevines (Table 2), particularly in TN variety. Considering that the two heatwave events (DOY 167 – 171 and DOY 192 - 197) (Fig. 1) occurred soon before reaching EL35 stage (DOY 208), the individual carotenoids data might indicate that kaolin treatment promoted a fine-tuning of the carotenoids metabolism under sustained summer stress factors in field-grown grapevines, which can also be associated to better physiological performance (Demmig-Adams and Adams 1992).

To understand the putative role of kaolin on regulating xanthophyll cycle and carotenoids biosynthesis in field-grown grapevines under summer stress, the relative gene expression of phytoene synthase (*VvPSY1*), violaxanthin de-epoxidase (*VvVDE1*), and zeaxanthin epoxidase (*VvZEP1*) were investigated (Fig. 5).



Figure 5. Relative gene expression of *VvVDE1*, *VvZEP1*, and *VvPSY1* of Touriga-Franca (TF) and Touriga-Nacional (TN) grapevines in control (TF_C, TN_C) and kaolin-treated (TF_K, TN_K) leaves at EL35 and EL38 stages. * denote significant differences between the control and kaolin treated group of each variety.

The relative VvPSYI gene expression was similar among treatments and varieties, indicating that kaolin treatment had no effect on the first rate limiting-step of the carotenoid biosynthetic pathway (Rodriguez-Concepcion *et al.* 2018). At stage EL35, VvVDE1 and VvZEP1 gene expression of TF_K were respectively around 3-fold, and 4-fold upregulated compared to its control group, explaining the Z_x and V_x accumulation in treated leaves, although no significant changes were observed in TN gene expression at this stage.

This outcome complements one evidence proposed by Frioni *et al.* (2020), which suggested that kaolin promotes the activity of the $V_xA_xZ_x$ cycle under stressful conditions. At the end of the season, only *VvZEP1* gene expression was significantly lower in TF treated vines, which could reflect an effort to retain Z_x in the lipid phase, regulating the stability and fluidity of the membrane (Kress and Jahns 2017). Since field-grown grapevines are permanently subjected to several abiotic and biotic stress factors, involving complex and dynamic interactions, it is difficult to establish a direct association between ZEP gene expression and Z_x accumulation, which do not occur at the same pace (Kim *et al.* 2019). Therefore, future research on the multiple functions of particle film technology application in Mediterranean grapevine production, related with other management practices, should be explored in both controlled and applied scenarios, to provide an accurate frame of plant stress responses, as well as the potential benefits of using short-term climate change adaptation strategies.

4. Conclusions

The present study indicates a varietal-dependent summer stress response that can influence kaolin efficiency on the light absorption and dissipation processes throughout the season. By fine-tuning xanthophylls and $V_xA_xZ_x$ cycle metabolism under Mediterranean summer stress conditions, kaolin prevented the thermal dissipation of excess energy, which may optimise the plasticity of grapevine stress responses and energy cost- saving. In summary, pre-*veraison* kaolin application improved the down-regulation of photochemistry, which consequently enhanced field-grown grapevines physiology under adverse environmental conditions.

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Supplementary material

Table ST1. List of primers used for real-time quantitative PCR.

Gene	Gene unique ID		Primer (5'–3')	Size (bp)
VvZEP1	GSVIVT01022128001	Fw Rv	GTTCAGTGCAGCCTTCGATT GGCTTCAGCCAAAGTAGCTT	167
VvVDE1	GSVIVT01026405001	Fw Rv	CCAAACCAGCCTGGAATACT CGCCATATCCATCCCATGCA	154
VvPSY1	GSVIVT01035255001	Fw Rv	CCTGGGACTCAGAGCTTGTT CTGTTCTCCGACACCACACA	151
VvACT2	GSVIVT01026580001	Fw Rv	GCCATCCAAGCTGTTCTCTC CAGTAAGGTCACGTCCAGCA	157
VvTUB2	GSVIVT01037405001	Fw Rv	CAACTCTGACCTCCGAAAGC CTTGGAGTCCCACATTTGCT	154
CHAPTER 6

EFFECTS ON BERRY PHYTOHORMONES, PHENOLIC COMPOSITION AND QUALITY TRAITS

Briefing note

This chapter strengthens knowledge on the balance of berry phytohormones, phenolic composition, and berry quality traits throughout ripening under summer stress conditions. It is divided into two sub-chapters to underpin kaolin effectiveness in managing berry ripening timing, oxidative damage, secondary metabolites accumulation, berry acidity, and antioxidant activity at the Douro (Chapter 6.1) and Alentejo vineyards (Chapter 6.2).

This chapter tackles the specific objective of the thesis (ii) understand grapevine primary stress signals and summer stress responses combined with kaolin treatment effect on fruit quality attributes in both regions, strengthen the discussion regarding kaolin treatment advantages for the wine sector in Mediterranean summer stress conditions (overall objective).

Chapter 6.1. Uncovering the effects of kaolin on balancing berry phytohormones and quality attributes of *Vitis vinifera* grown in warm-temperate climate regions (Douro)

Adapted from:

Bernardo, S., Dinis, L-T., Machado, N., Barros, A., Pitarch-Bielsa, M., Malheiro, A.C., Gómez-Cadenas, A., Moutinho-Pereira, J. (2021). Uncovering the effects of kaolin on balancing berry phytohormones and quality attributes of *Vitis vinifera* grown in warm-temperate climate regions. Journal of the Science of Food and Agriculture. 102 (2): 782-793 DOI: 10.1002/jsfa.11413

Chapter 6.2. Kaolin impacts on hormonal balance, polyphenolic composition and oenological parameters in red grapevine berries during ripening (Alentejo)

Adapted from:

Bernardo, S., Dinis, L-T., Machado, N., Barros, A., Pitarch-Bielsa, M., Malheiro, A.C., Gómez-Cadenas, A., Moutinho-Pereira, J. (2021). Kaolin impacts on hormonal balance, polyphenolic composition and oenological parameters in red grapevine berries during ripening. Journal of Berry Research. 11 (3): 465-479

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Chapter 6.1. Uncovering the effects of kaolin on balancing berry phytohormones and quality attributes of *Vitis vinifera* grown in warm-temperate climate regions (Douro)

Abstract

The application of kaolin particle-film is considered a short-term strategy against several environmental stresses in areas with a Mediterranean-like climate. However, it is known that temperature fluctuations and water availability over the season can jeopardise kaolin efficiency in many Mediterranean crops. Hence, this study aims to evaluate the effects of kaolin foliar application on berry phytohormones, antioxidant defence and oenological parameters at veraison and harvest stages of Touriga-Franca (TF) and Touriga-Nacional (TN) grapevines in two growing seasons (2017 and 2018). The 2017 growing season was considered the driest (-147.1 DI) and the warmest (2705°C GDD) of the study. In 2017, TF kaolin-treated berries showed lower salicylic acid (SA) (-26.6% compared with unsprayed vines) and abscisic acid (ABA) (-10.5%) accumulation at veraison, while SA increased up to 28.8% at harvest. In a less hot season, TN and TF kaolin-treated grapevines showed a 2-fold and 3-fold increase in the ABA and indole-3-acetic-acid (IAA) content at *veraison*, respectively, and lower ABA levels (83.8%) compared with unsprayed vines at harvest. Treated berries showed decreased sugar content without compromising malic and tartaric acid levels, as well as reactive oxygen species (ROS) accumulation throughout berry ripening. The results suggest kaolin exerts a delaying effect in triggering ripening-related processes under severe summer stress conditions. Treated berries responded with improved antioxidant defence and phytohormone balance, showing significant interactions between kaolin treatment, variety and developmental stage in both assessed years.

Keywords: Abscisic acid; Antioxidants; Berry ripening; Indole-3-acetic acid; Redox homeostasis; Salicylic acid

1. Introduction

In grapevines, ripening onset and balance are extensively modulated by temperature and precipitation levels, which affect the yield and the quality potential of berries, particularly in regions with a Mediterranean-type climate that were recently described as climate change hotspots (Conde *et al.* 2007, Kuhn *et al.* 2013, Leolini *et al.* 2020, Reis *et al.* 2020). In those regions, the summer season is characterised by prolonged periods of high light, high temperature and water scarcity challenging winegrowers to sustain grapevine production in warmer and drier conditions (Reis *et al.* 2020). Driven by increased air temperature and changes in rainfall patterns, anticipated harvest dates have been reported in southern European winegrowing regions, along with higher berry sugar contents, lower acidity and sub-optimal phenolic maturation (Bernardo *et al.* 2018, Reis *et al.* 2020).

Besides yield and quality losses, environmental stresses also promote the leakage of electrons from cellular compartments, increasing reactive oxygen species (ROS) production and disturbing redox homeostasis (Carvalho *et al.* 2015). However, plants display several defence strategies under limiting environmental conditions to trigger stress resilience, such as the synthesis of radical scavenging compounds (Hasanuzzaman *et al.* 2020). In additional, hormonal changes followed by secondary metabolite production can coordinate the berry ripening process, adding value to the wine industry due to their potential role in sustaining redox homeostasis (Kuhn *et al.* 2013, Soto *et al.* 2015).

Phytohormones play a central role in several plant physiological processes during grapevine development and fruit ripening, regulating metabolic changes caused by interactions with biotic and abiotic stress factors, such as temperature, light, UV-B radiation and water availability (Fortes *et al.* 2015). Grape ripening onset is usually characterised by abscisic acid (ABA) accumulation; however, it is currently considered a complex process involving crosstalk with other plant growth regulators, instead of being governed by the production of a single phytohormone (Kuhn *et al.* 2013, Pérez-Llorca *et al.* 2019). Several studies have reported ABA accumulation under stressful conditions at *veraison* coordinated with the activation of complex hormonal signalling, which can ultimately impact anthocyanin content, berry size, colour development, sugar accumulation and ripening timing (Niculcea *et al.* 2013, Parada *et al.* 2017).

The foliar application of kaolin particle film is a well-known short-term strategy applied in many fruit crops that increases the reflection of ultraviolet, photosynthetically active (PAR) and infrared radiation, reducing leaf temperature and preventing leaf and fruit sunburn damage (Glenn and Puterka 2010). However, the effectiveness of kaolin in improving leaf resilience

when subjected to severe environmental conditions, mainly through gas exchange assessment (e.g. stomatal conductance, carbon assimilation rates and water use efficiency), can significantly change according to the variety, water status and stress severity (Attia et al. 2014, Dinis et al. 2018b, Frioni et al. 2019, Shellie and King 2013). For example, Brillante et al. (2016) reported reduced photosynthetic rates and stomatal conductance in kaolin-treated Cabernet Sauvignon cv. vines in dry years. By contrast, other authors have found a concomitant increase in gas exchange parameters for kaolin-sprayed leaves, essentially when the environment shows persistent limiting factors (Attia et al. 2014, Dinis et al. 2018b, Frioni et al. 2019). Under these conditions, kaolin application in the Douro region showed positive effects on vines' physiological performance and sucrose concentration and transport in leaves while reducing susceptibility to photoinhibition (Conde et al. 2018, Dinis et al. 2016b, Dinis et al. 2018b, Luzio et al. 2021). Furthermore, the effects of kaolin on hormonal dynamics suggest modulation of indole-3-acetic acid (IAA), salicylic acid (SA) and ABA levels in grapevine leaves in areas with a Mediterranean-type climate (Bernardo et al. 2021, Dinis et al. 2018a). This response was subsequently related to bottlenecks on the carotenoid biosynthetic pathway that leads to ABA biosynthesis in water-stressed Sangiovese cv. leaves (Frioni et al. 2020, Tombesi *et al.* 2015).

At the fruit level, berry analysis of kaolin-sprayed vines indicates quite beneficial effects on cluster cooling, uniform colour, berry size, total acidity, monomeric anthocyanin and total phenolic content, stimulating the phenylpropanoid and flavonoid pathways in berries exposed to multiple environmental stresses (Brillante *et al.* 2016, Conde *et al.* 2016, Dinis *et al.* 2020, Dinis *et al.* 2016a, Ferrari *et al.* 2017, Luzio *et al.* 2021, Shellie 2015). Nonetheless, it is still unclear whether kaolin application might modulate the hormonal levels of berries throughout ripening, neither its function on balancing grape ripeness or quality over consecutive growing seasons in adult vines growing in a Mediterranean-type climate. Hence, this study explores the effects of kaolin application on several berry quality traits, phytochemicals, antioxidant activity and hormonal screening of two field-grown Portuguese grapevine varieties, Touriga-Franca (TF) and Touriga-Nacional (TN), located in the Douro Demarcated Region over two consecutive growing seasons.

2. Materials and Methods

2.1. Site description and weather conditions

The experiments were performed in the 2017 and 2018 growing seasons, at a commercial vineyard located in the Douro Demarcated Region ('Quinta do Orgal'): 41°04'N, 7°04'W, 169 m above sea level), in Northeast Portugal. The vineyard is installed on a steep slope (30°N) with an east-west orientation, composed by 6-year-old vines grafted onto 110R rootstock, growing using the unilateral Cordon training system with vertical shoot positioning and distances between and along rows of 2.2 m and 1.0 m, respectively. The vines were winter pruned with about 8 buds left per vine. The vines were managed according to the growers' commercial organic practices (use of organic fertilisers, non-synthetic pesticides and spontaneous cover cropping) and deficit irrigated (30% of the reference evapotranspiration), using a drip irrigation system consisting of 2.2 l h⁻¹ self-compensating drippers spaced 1.0 m apart. According to the world reference base for soil resources (FAO 2015), the soil mapping in this region is classified as luvisols, characterised by a clay-enriched subsoil. The region is characterised by a warmtemperate climate with dry and hot summers and rainfall periods mostly concentrated in the winter months (Kottek et al. 2006). An automatic weather station was installed in the vineyard to record standard meteorological variables, such as air temperature and precipitation (Fig. 1). To characterise the weather conditions of the field trial in the 2017 and 2018 growing seasons, two bioclimatic indexes were computed: one related to heat accumulation over the growing seasons (growing degree days – GDD),(Jones et al. 2010) and another related to the level of potential soil water availability (dryness index - DI) (Tonietto and Carbonneau 2004) (Table 1). According to the classifications of Tonietto and Carbonneau (2004) and Jones et al. (2010) the 2017 growing season was considered the driest (-147.1 DI) and the warmest (2705°C GDD) of the study.

Table 1. Dryness index (DI, mm) and growing degree days (GDD, °C) values for the 2017 and 2018 growing seasons in the vineyard site. The DI and GDD classes were considered according to Tonietto and Carbonneau (2004) and Jones et al. (2010), respectively.

Season	DI	Class	Class limits	GDD	Class	Class limits
2017	- 147.1	Very dry	≤ -100	2705	Too hot	>2700
2018	- 47.0	Moderately dry	$\leq 50 > -100$	2416	Very warm	2222 - 2700



Figure 1. Daily mean air temperature (°C) and precipitation (mm) of the 2017 and 2018 growing seasons from May to September (DOY 121-273). Vertical grey bars (dark grey -2017; light grey -2018) indicate the heatwaves events recorded during the experiment.

2.2. Plant material, treatments and sampling

Two Vitis vinifera L. red varieties were selected, TF and TN, due to their ability to ripen under intense heat and importance for the typicity of regional Portuguese red wines. A total of 60 vines per variety were randomly selected and divided into three side-by-side rows with 20 vines each. In both growing seasons, plants were divided into two experimental groups: the control or untreated group of each variety (TF C and TN C), and the kaolin-treated group (TF_K and TN_K). Kaolin treatment (Surround® WP, Engelhard Corporation, Iselin, New Jersey) was prepared in an aqueous solution at the manufacturer's recommended dosage of 5.0% (w/v), supplemented with 0.1% (v/v) Tween 20 to improve adherence. It was sprayed on the whole canopy with a machine operated air blast sprayer at a rate of 300 l ha⁻¹. The adjacent control plants were carefully protected by a plastic film during the application of kaolin. In 2017 and 2018, kaolin was applied in the windless mornings of 26 June (Day-Of-Year – DOY 177) and 17 July (DOY 198), respectively. In 2017, the vineyard environment featured a relative humidity (RH) of 50.1%, maximum (T_{max}) and mean (T_{mean}) air temperature of 34.7°C and 24.7°C, respectively, with reference evapotranspiration (ET₀) of 6.1 mm and no precipitation records. In the following growing season, the vineyard environment showed an RH of 45.3%, T_{max} of 35.7°C, and T_{mean} of 26.5°C, with matching records of ET₀ and precipitation to the previous growing season.

Berry sampling was undertaken at both *veraison* and harvest stages. In 2017, the *veraison* stage occurred at DOY 199 and harvest at DOY 234, whereas in 2018 these respective periods corresponded to DOY 212 and DOY 254 (Fig. 1).

For all biochemical assays, a total of 300 berries were collected within each treatment, variety and developmental stage; frozen in liquid nitrogen; and stored at -80°C until the lyophilisation procedure. The fruits were then divided into three groups of 100 berries each, lyophilised (SCANVAC 55-4 Pro, LaboGene, Lynge, Denmark) for 120 h, ground to a fine powder and stored at room temperature.

2.3. Phytohormones

Abscisic acid (ABA), indole-3-acetic acid (IAA), and salicylic acid (SA) content were determined by high-performance liquid chromatography coupled to a triple quadrupole mass spectrometer (Micromass, Manchester, UK) through an orthogonal Z-spray electrospray ion source (Durgbanshi et al. 2005). Briefly, 100 mg lyophilised fruit samples were extracted in 2.0 ml distilled water using a mill ball apparatus (MillMix20, Domel, Železniki, Slovenija). After centrifugation at 10,000 g, supernatants were recovered, and pH was adjusted to 2.8-3.2 with 30% acetic acid. Extracts were partitioned twice with diethyl ether, and the supernatants were evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France) at room temperature. The dry residue was then resuspended in 500 µl water:methanol (9:1), filtered through 0.22 µM polytetrafluoroethylene (PTFE) filters and injected directly into an ultra-performance liquid chromatography (UPLC) system (Waters Acquity SDS, Waters Corp., Milford, MA) interfaced to a TQD triple quadrupole (Micromass Ltd. Manchester, UK) mass spectrometer through an orthogonal Z-spray electrospray ion source. A reversed-phase C18 column (Gravity, 50 × 2.1 mm, 1.8-µm particle size, Macherey-Nagel GmbH, Germany) at 40°C was used to achieve the chromatographical separation using a methanol:water gradient, supplemented with 0.1% acetic acid at a flow rate of 300 μ l min⁻¹. The aqueous phase was maintained at 90% during the first 2 min, down until 10% up to 6 min, and increasing to 90% up to 7 min, maintaining this proportion until the end of the run at 8 min. Regarding mass spectrometry parameters, the triple quadrupole was operated in multiple reaction monitoring (MRM) mode, using nitrogen as a drying and nebuliser gas, with a cone gas flow of 250 l h⁻¹ and a desolvation flow of 1200 l h⁻¹, and argon as collision gas, setting the cone voltage and collision energies according to Durgbanshi et al. (2005) with a few modifications. $[^{2}H_{6}]$ -ABA, $[^{2}H_{2}]$ -IAA and $[^{13}C_{6}]$ -SA were used as internal standards. The results were processed using the Masslynx v4.1 software, and the phytohormone contents were determined by the interpolation of the response obtained from the phytohormone and internal standard areas by a calibration curve prepared with commercial standards for ABA (y = 7.0377x - 0.0161; $R^2 = 1$), IAA (y = 25.624x - 0.0601; $R^2 = 0.9994$) and SA (y = 4.4612x - 0.0597; $R^2 = 0.9999$).

2.4. Berry quality traits

Soluble sugars (SS) were extracted by heating 10 mg lyophilised fruit tissue in 5.0 ml ethanol:water (80:20, v/v) for 1 h at 80°C. SS was quantified following an anthrone-sulfuric acid method adapted to 96-well microplates (Leyva *et al.* 2008). The anthrone reagent, containing 0.1 g anthrone (0.1%) dissolved in 100 ml concentrated sulfuric acid (98%), was prepared right before analysis and then added to the extracts. SS was determined in triplicate by reading the absorbance at 625 nm in a microplate multiscan reader (SPECTROstar Nano, BMG Labtech GmbH, Offenburg, Germany). The colourimetric response was compared to a standard curve based on glucose, and total SS is expressed as milligrams per gram dry weight (DW).

The physicochemical parameters of grapes were assessed in 100 fresh berries with triplicates, collected per variety, treatment and developmental stage. Total acidity and pH were analysed following the OIV international methods of wine and must analysis (OIV 2003). The tartaric and malic acid levels were measured enzymatically, using an automated clinical chemistry analyser (Miura One, TDI, Spain) (Franquès *et al.* 2018).

2.5. Phenolic composition and radical scavenging activity

Phenolic compounds were extracted as previously described by Mendes Lemos *et al.* (2020) Total phenols, *ortho*-diphenols and flavonoids were measured in triplicate, following the methods adapted by Gouvinhas *et al.* (2018). Briefly, total phenols were determined by mixing 20 µl fruit extract, 100 µl Folin–Ciocalteu reagent and 80 µl sodium carbonate (7.5%) in a microplate well, and incubated at 45°C for 30 min. Absorbance was then recorded at 750 nm. For the evaluation of *ortho*-diphenols, a colourimetric reaction was recorded at 375 nm by previous incubation of 40 µl sodium molybdate (50 g l⁻¹) with 160 µl fruit extract at room temperature for 15 min. The colourimetric response of both total phenols and *ortho*-diphenols measurements was compared to a standard curve based on gallic acid, and the results are expressed as milligrams gallic acid equivalents (GAE) per gram DW. The flavonoid content was determined in a reaction mixture containing 24 μ l fruit extract, 28 μ l sodium nitrite (50 g L⁻¹), 28 μ l aluminium chloride (100 g L⁻¹) and 120 μ l sodium hydroxide (1.0 M). The absorbance was immediately recorded at 510 nm, and the results are expressed as milligrams catechin equivalents (CE) per gram DW.

Analysis of total monomeric anthocyanins was performed using a pH differential method (Lee *et al.* 2005). At two distinct pH values (pH 1.0 potassium chloride [0.025 M] buffer, and pH 4.5 sodium acetate [0.4 M] buffer), measurements were recorded at 700 nm and 520 nm in a microplate reader (SPECTROstar Nano). The total anthocyanin content is expressed as equivalents of malvidin-3-*O*-glucoside, based on its molar extinction coefficient (28,000 l cm⁻¹ mol⁻¹).

The tannin content was measured by the methyl cellulose precipitable assay (Dambergs *et al.* 2012) with slight modifications. The method was performed in 1.5 ml test tubes, by adding 100 μ l fruit extract, 300 μ l methyl cellulose (0.04%) and 200 μ l a saturated ammonium sulphate solution, adjusting the final volume to 1 ml with distilled water. In control samples, distilled water was added instead of methyl cellulose, and all samples were measured at 280 nm in UV-adapted microplates. The tannin concentration was obtained by subtracting the absorbance of control samples and using epicatechin (EC) as standard. The results are expressed as milligrams epicatechin equivalents (ECE) per gram DW.

The free radical scavenging activity based on the 2,2-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid (ABTS) radical decolourisation was determined as described by Gouvinhas *et al.* (2018). Briefly, 188 μ l ABTS working solution was mixed with 12 μ l methanolic fruit extract, incubated for 30 min and then the absorbance was recorded at 734 nm in a microplate reader.

Hydroxyl radical (OH[•]) scavenging activity was determined using the deoxyribose assay with slight adaptations (Chung *et al.* 2014, Luqman and Kumar 2012). Different concentrations of fruit extracts (50–400 μ g ml⁻¹) were added to a solution of 2-deoxyribose (3.0 mM) dissolved in phosphate buffer (10 mM, pH 7.4), along with 0.1 mM FeCl₃, 0.1 mM EDTA and 0.1 mM ascorbic acid in a final volume of 1.2 ml. In parallel, a duplicate for each sample was prepared without the presence of EDTA, and a blank was prepared with phosphate buffer instead of sample extract. After 1 h incubation at 37°C, 1.0 ml trichloroacetic acid (TCA) (2.8%) and 1.0 ml TBA (1%) were added, heated at 100°C for 15 min and the absorbance was read at 532 nm. Mannitol was used as a positive control, and the results are expressed in IC₅₀ values.

2.6. Analysis of stress-related metabolites

The lipid peroxidation products were quantified according to Hodges *et al.* (1999). The extraction was performed by adding 3.0 ml 20% (w/v) TCA, with measurements of the supernatant at 440, 532 and 600 nm in a microplate multiscan reader (SPECTROstar Nano). After subtracting the non-specific absorbance at 600 nm, the thiobarbituric acid reactive substances (TBARS) were calculated using the malondialdehyde (MDA) extinction coefficient of 157 mM cm⁻¹. Lipid peroxidation is expressed in mmol MDA equivalents per gram DW.

The free proline content was extracted with 3% (w/v) sulphosalicylic acid (SSA) and centrifuged at 9,000 g for 15 min at 4°C as described by Bates *et al.* (1973). In a 2.0 ml test tube, the reaction mixture containing 250 μ l extract, 250 μ l acid ninhydrin and 250 μ l glacial acetic acid was incubated in a boiling water bath for 1 h. Then, 500 μ l toluene was added and mixed for 20 s. The upper reddish-pink-coloured phase was separated, and absorbance was read at 520 nm in a microplate reader. The colourimetric response was compared to a standard curve based on commercially available proline, and the results are expressed as μ mol proline per gram DW.

The hydrogen peroxide (H₂O₂) content was determined following the method of Junglee *et al.* (2014). Under dim light, 50 mg dried fruit was homogenised with 250 μ l TCA (0.1%), 500 μ l potassium iodide (KI) and 250 μ l phosphate buffer (10 mM, pH 7.4); it was then centrifuged for 15 min at 1500 g and 4°C. For the removal of tissue colour background, a duplicate for each sample was prepared by using distilled water instead of KI. The supernatant (200 μ l) was then transferred to a 96-well microplate, incubated for 20 min protected from light and absorbance was read at 350 nm. The results were calculated based on a standard curve of H₂O₂ and are expressed as μ mol H₂O₂ per gram DW.

ROS were assessed according to Kong *et al.* (2013) with a 2',7'-dichlorofluorescein diacetate (DCFH-DA) solution (25 mM), prepared in dimethyl sulphoxide immediately before use. Briefly, fruit extracts (20 μ l) were loaded into a small-well microplate containing 0.2 ml phosphate-buffered saline (pH 7.4) and 12 μ M DCFH-DA and incubated for 20 min at 25°C. Fluorescence was measured at 485 nm and 530 nm in a CARY 50 Bio (Eclipse, Australia) every 15 min until 60 min after the incubation. A calibration curve was obtained using 2',7'-dichlorofluorescein, and the results are expressed as nmol DCF per milligram protein.

2.7. Statistical analysis

Statistical analyses of phytohormones, berry quality traits and stress-related metabolites were performed using Sigma-Plot 14.0 (SPSS Inc., San Jose, CA, USA). After testing for analysis of variance (ANOVA) assumptions (homogeneity of variances with Levene's mean test and normality with the Kolmogorov-Smirnov test), statistical differences among treatments and varieties within each developmental stage were evaluated by two-way factorial ANOVA, followed by the post hoc Tukey's test. Subsequently, statistical differences between developmental stages (veraison vs harvest) within each sampling group were evaluated by oneway ANOVA, followed by the post hoc Tukey's test. For the specific case of OH' scavenging activity, IC₅₀ values were obtained using GraphPad Prism 6. Principal component analysis (PCA) was performed to examine summer stress effects throughout berry ripening of control and treated plants in both growing seasons, using SigmaPlot 14.0. To detect the effect of the growing season, variety, treatment and their interaction, a multivariate analysis of variance (MANOVA) was performed using Pillai's trace statistic test in the SPSS 22.0 software. Different lowercase letters represent significant differences (p < 0.05) between treatments (TF_C, TF_K, TN_C, TN_K) within each developmental stage, and *** p < 0.001, ** p < 0.01 and * p < 0.05 represent significant differences between developmental stages (veraison vs harvest) within each treatment and variety. The absence of letters and asterisks indicate no significant difference.

3. Results

3.1. Weather conditions

The daily mean air temperature from May (DOY 121) to September (DOY 273) was 24.5°C and 23.5°C in 2017 and 2018, respectively, with precipitation values of 63.6 mm in 2017 (total of 185.4 mm from DOY 1) and 173.8 mm in 2018 (total of 466.4 mm from DOY 1) (Fig. 1). To determine the possible occurrence of heatwaves during the experiments, we assessed the number of days with a maximum temperatures above 40°C represented by vertical grey bars (Fig. 1). In 2017, there was a total of 23 days with maximum temperatures above 40°C at the vineyard site, with two periods of at least five consecutive days in June (DOY 165–169) and July (DOY 193–198) that occurred just before *veraison*. In 2018, there was a total of 10 days of extreme temperatures, with six consecutive days with a maximum temperature above 40°C (DOY 213–218) that occurred at *veraison*.

3.2. Phytohormones

Figure 2 shows the contents of ABA, IAA and SA phytohormones throughout berry ripening. In 2017, kaolin treatment showed no significant effects on IAA accumulation in either variety or stage. Conversely, in the following growing season, kaolin treatment boosted the IAA content of TN_K (+197.4% on average compared with unsprayed vines) and TF_K (+134.4% on average compared with control vines) at *veraison*, with no effects at the end of the experiment. Overall, ABA levels decreased throughout the experiment in both growing seasons and varieties. Besides, kaolin exerted a general inhibitory effect in both varieties. At *veraison*, ABA accumulation decreased in TF_K (10.5%) and TN-K (24.5%) in 2017 and 2018, respectively, whereas at harvest, this effect was exclusively observed in TN (45.6%) in 2018. In general, TF displayed higher SA levels compared with TN at most of the sampling dates. The effects of kaolin on SA accumulation were mainly detected in TF, with antagonist effects depending on the growing season. In 2017, TF_K showed 26.6% lower SA than TF_C at *veraison*, whereas at harvest, SA levels increased about 28.8%. In the following growing season, TF_K berries showed an opposite trend.



Figure 2. Phytohormones (abscisic acid - ABA, salicylic acid – SA, and indole-3-acetic acid - IAA) content in Touriga-Franca and Touriga-Nacional grapevine berries (Touriga-Franca control – TF_C and kaolin – TF_K; Touriga-Nacional control - TN_C and kaolin – TN_K) throughout 2017 and 2018 summer seasons. Data are mean \pm SD (n=6). Different lower case letters represent significant differences between treatments and varieties within each developmental stage and sampling year. Asterisks represent significant differences (*** p < 0.001; ** p < 0.01; * p < 0.05) between *veraison* and harvest within each variety, treatment, and sampling year.

3.3. Fruit quality traits

The analysis of several oenological variables revealed that kaolin-treated fruit showed lower total SS than their respective control groups in both growing seasons, particularly at the *veraison* stage of 2017 (Table 2). Within this stage, the total sugar content was relatively similar among treatments and varieties in 2017, while in 2018, it was almost 50% lower in TN compared with TF. In 2018, the overall sugar content was also lower at harvest compared with 2017. By contrast, total acidity and tartaric and malic acid levels were higher in 2018 compared with 2017 in both varieties, mainly at *veraison*. Overall, kaolin application promoted tartaric acid accumulation and increased total acidity in both growing seasons, with significant effects in TN.

Table 2. Total content of soluble sugars and oenological attributes of Touriga-Franca and Touriga-Nacional control and kaolin-treated berries in two stages (*veraison* and harvest) during the 2017 and 2018 growing seasons. Data are mean \pm SD (n = 6). Different lower case letters represent significant differences between treatments and varieties within each stage (*veraison* and harvest) and sampling year. *** p < 0.001, ** p < 0.01, *p < 0.05 represent significant differences between stages (*veraison* and harvest) within the same treatment and sampling year. The absence of letters indicates no significant differences between treatments and varieties.

			Touriga-Franca		Touriga-Nacional	
	Season	Stage	Control	Kaolin	Control	Kaolin
Soluble sugars (mg g ⁻¹ DW)	2017	Veraison	697.78 ± 33.52 a	579.97 ± 15.36 b	639.11 ± 17.50 a	$546.96 \pm 31.83 \text{ b}$
		Harvest	$785.11 \pm 67.56 \text{ bc}^{***}$	713.71 ± 54.55 c***	$876.20 \pm 29.58 \ a^{***}$	$804.42 \pm 29.76 \text{ ab}^{***}$
	2018	Veraison	354.36 ± 27.00 a	367.94 ± 9.97 a	$161.36\pm18.48~\text{b}$	$140.72 \pm 14.22 \ b$
		Harvest	$509.83 \pm 18.34 \ ab^{***}$	$494.75 \pm 18.90 \text{ bc}^{***}$	$544.63 \pm 7.09 \; a^{***}$	$474.17 \pm 22.29 \text{ c}^{***}$
рН	2017	Veraison	$3.16\pm0.03\ a$	$3.17\pm0.03~a$	$3.01\pm0.03~b$	$2.97\pm0.03\ b$
		Harvest	$3.91 \pm 0.04 \; a^{***}$	$3.86 \pm 0.02 \ a^{***}$	$3.76 \pm 0.08 \ b^{***}$	$3.73 \pm 0.04 \ b^{***}$
	8	Veraison	$2.65\pm0.17\ a$	$2.73\pm0.04\ a$	$2.49\pm0.01\ b$	$2.43\pm0.06\ b$
	20	Harvest	$3.52 \pm 0.04 \ a^{***}$	$3.49 \pm 0.05 \ ab^{***}$	$3.35 \pm 0.02 \ b^{***}$	$3.38 \pm 0.03 \ b^{***}$
Total	2017	Veraison	$6.63\pm0.09\ b$	$6.31\pm0.32~b$	10.52 ± 0.47 a	$11.15\pm0.59~a$
acidity (g L ⁻¹ Tartaric acid)		Harvest	$3.80 \pm 0.16 \ b^{***}$	$3.75 \pm 0.08 \ b^{***}$	$4.35 \pm 0.15 \ ab^{***}$	$4.53 \pm 0.10 \; a^{***}$
	2018	Veraison	$14.68 \pm 1.51 \text{ c}$	$13.25\pm1.21\ c$	$29.35\pm0.58\ b$	$33.78 \pm 1.28 \ a$
		Harvest	$4.19 \pm 0.09 ***$	4.15 ± 0.21 ***	$4.70 \pm 0.06 ***$	5.03 ± 0.44 ***
Tartaric acid (g L ⁻¹)	2017	Veraison	$4.63\pm0.13\ b$	$4.77\pm0.08\ b$	$6.83\pm0.06\ a$	$6.93\pm0.35~a$
		Harvest	$2.48 \pm 0.20 \ b^{***}$	$2.31 \pm 0.02 \ b^{***}$	$2.88 \pm 0.13 \; a^{***}$	$3.00 \pm 0.18 \ a^{***}$
	2018	Veraison	$7.56\pm0.72\ c$	$6.70\pm0.53~c$	$15.23\pm0.50\ b$	$17.53\pm0.76\ a$
		Harvest	2.63 ± 0.24 ***	$2.61 \pm 0.06 ***$	3.05 ± 0.26 ***	3.01 ± 0.02 ***
Malic acid (g L ⁻¹)	2017	Veraison	$1.49\pm0.12\ b$	$1.22\pm0.08~b$	$3.05\pm0.13\ a$	$3.16\pm0.14\ a$
		Harvest	1.09 ± 0.09 *	1.12 ± 0.08	$1.12 \pm 0.06 ***$	$1.10 \pm 0.09 ***$
	18	Veraison	$6.31\pm0.64\ c$	$5.50\pm0.20\ c$	$12.67\pm0.21\ b$	$14.57\pm0.55\ a$
	20	Harvest	$1.06 \pm 0.06 ***$	1.04 ± 0.08 ***	0.86 ± 0.02 ***	$0.93 \pm 0.09 ***$

3.4. Stress-related metabolites

Kaolin-treated fruit showed lower total ROS accumulation in both varieties, with more pronounced effects in 2017. By contrast, fruit of TN kaolin-treated vines showed higher H₂O₂ levels than control berries at harvest in 2017 (Fig. 3). Except for TF_K at *veraison*, there were no significant changes in lipid peroxidation levels (TBARS) in 2017. In the 2018 growing season, the effects of kaolin on reducing TBARS accumulation were only noticed in TN at *veraison*. Conversely, lipid peroxidation levels increased in TN fruit from treated vines (+30.9% on average compared with control vines) at harvest. Kaolin application decreased proline accumulation in both varieties and growing seasons, with greater effects on TF at harvest.



Figure 3. Total content of reactive oxygen species (ROS), thiobarbituric acid reactive substances (TBARS), hydrogen peroxide (H₂O₂), and proline of the samples: control (C) and kaolin (K) treated berries of both Touriga-Franca (TF) and Touriga-Nacional (TN), in two developmental stages (EL35 and EL 38), during 2017 and 2018 growing seasons. Data are mean \pm SD (n=6). Different lower case letters represent significant differences between treatments and varieties within each developmental stage and sampling year. Asterisks represent significant differences (*** p < 0.001; ** p < 0.05) between *veraison* and harvest within each variety, treatment, and sampling year.

3.5. Phenolic compounds and radical scavenging activity

Overall, kaolin treatment promoted a slight increase in the total phenol content in both varieties and growing seasons (Table 3). Interestingly, the flavonoid content increased around 76% in TF_K compared with TF_C from veraison to harvest in 2018, results that were in contrast to the 13% reduction in flavonoid accumulation observed in their control group. Besides, kaolin treatment promoted ortho-diphenol accumulation in 2017 in TF (70.8%) and TN (39.2%) fruit compared with fruit from their respective control vines, particularly at harvest. Similarly, TF_K showed higher total anthocyanin accumulation than its control group at this stage, whereas no major effects were detected in TN. Throughout the experiment, the tannin content was higher in 2017 in both treatments and varieties compared with 2018. The results show a trend for tannin accumulation in TF_K in 2017, mainly at veraison. In the same period of the following growing season, the kaolin effects were reversed in TF but not in TN. Antiradical activity showed that the kaolin effects were only observed by the ABTS method. Kaolin promoted different antioxidant responses in both varieties by increasing the anti-radical activity in TN berries and decreasing the TF response, mainly at veraison. In addition, there was higher antioxidant activity in 2018 compared with 2017 in both varieties, treatments and developmental stages.

Table 3. Total content of phenols, flavonoids, ortho-diphenols, anthocyanins, tannins and antiradical activity based on ABTS and hydroxyl radical scavenging activity (OH[•]) in control and kaolin-treated berries of Touriga-Franca and Touriga-Nacional in two developmental stages (*veraison* and harvest) during the 2017 and 2018 growing seasons. Data are mean \pm SD (n=6). Different lower case letters represent significant differences between treatments and varieties within each developmental stage (*veraison* and harvest), and sampling year. *** p < 0.001, ** p < 0.01, *p < 0.05 represent significant differences between developmental stages (*veraison* and harvest) within the same treatment and sampling year. Absence of letters indicates no significant differences between treatments and varieties.

			Touriga-Franca		Touriga-Nacional	
	Season	Stage	Control	Kaolin	Control	Kaolin
Total phenols (mg GAE g ⁻¹ DW)	2017	Veraison	$28.35\pm0.94~b$	30.57 ± 1.52 ab	30.89 ± 1.57 ab	31.37 ± 2.59 a
		Harvest	16.12 ± 1.71 b***	20.52 ± 1.42 a***	$15.20 \pm 0.84 \ b^{***}$	$15.57 \pm 0.79 \text{ b***}$
	2018	Veraison	$59.12 \pm 5.60 \text{ c}$	60.60 ± 4.64 c	$80.18\pm2.88~b$	91.10 ± 6.98 a
		Harvest	29.03 ± 2.61 ***	35.90 ± 3.49 ***	27.58 ± 3.30 ***	27.76 ± 4.70 ***
Flavonoids	2017	Veraison	24.25 ± 1.38 a	21.48 ± 1.90 a	37.71 ± 1.94 b	$36.46\pm2.66~b$
$(mg CE g^{-1})$		Harvest	$7.95 \pm 0.71 \text{ b}^{***}$	$10.82 \pm 0.86 \text{ b}^{***}$	$9.31 \pm 1.24 \text{ b}^{***}$	$15.16 \pm 0.48 \ a^{***}$
	18	Veraison	$74.66 \pm 4.76 \text{ b}$	53.16 ± 5.38 c	129.71 ± 8.15 a	120.53 ± 6.46 a
	20	Harvest	$64.69 \pm 3.39 \text{ b}^{***}$	$93.30 \pm 3.05 \ a^{***}$	$57.61 \pm 6.69 \text{ bc}^{***}$	$46.25 \pm 3.30 \text{ c}^{***}$
Ortho-	2017	Veraison	21.90 ± 3.10 bc	20.22 ± 1.74 c	23.93 ± 1.22 ab	25.34 ± 1.75 a
diphenols (mg GAE g ⁻¹		Harvest	$10.00 \pm 0.87 \text{ bc}^{***}$	$17.08 \pm 0.65 a^{***}$	$7.58 \pm 0.84 \ c^{***}$	$10.55 \pm 0.44 \ b^{***}$
DW)	8	Veraison	48.12 ± 2.85 c	42.57 ± 4.32 c	$69.99\pm2.90~b$	84.06 ± 4.69 a
	20	Harvest	$19.01 \pm 1.45 \text{ b}^{***}$	25.04 ± 2.50 a***	$19.30 \pm 1.41 \text{ b}^{***}$	22.27 ± 2.27 ab***
Anthocyanins	18 2017	Veraison	6.74 ± 0.31 a	6.48 ± 0.38 a	$4.50\pm0.38~\text{b}$	3.42 ± 0.23 c
(mg MVE g ⁻ DW)		Harvest	$3.95 \pm 0.37 \ c^{***}$	$5.82 \pm 0.27 \ a^*$	$5.14\pm0.26\ ab$	$4.51 \pm 0.70 \ bc^{***}$
,		Veraison	$5.46\pm0.26~\text{b}$	$7.63\pm0.46\ a$	$5.98\pm0.58\ b$	$6.23\pm0.11\ b$
	20	Harvest	$7.51 \pm 0.79 \ b^{***}$	$8.29\pm0.48\ b$	$10.29 \pm 0.34 \ b^{***}$	$9.42 \pm 0.33 \ b^{***}$
	2017	Veraison	$21.70\pm2.73~b$	33.58 ± 3.84 a	$25.78\pm1.18\ b$	$22.61\pm1.60~\text{b}$
Tannins		Harvest	10.43 ± 0.73 a***	10.62 ± 1.95 a***	$5.01 \pm 0.73 \text{ b***}$	$7.53 \pm 0.62 \text{ ab}^{***}$
(IIIg ECE g DW)	2018	Veraison	$4.28\pm0.40\ c$	$3.76\pm0.25\ d$	$8.47\pm0.30\ b$	$9.67 \pm 0.35 \text{ a}$
,		Harvest	$2.12 \pm 0.33 a^{***}$	$2.04 \pm 0.22 \ a^{***}$	$1.34 \pm 0.07 \text{ b***}$	$1.04 \pm 0.10 \ b^{***}$
ABTS [•] (μmol TE g ⁻¹ DW)	2017	Veraison	$194.25 \pm 9.65 \text{ c}$	$183.82 \pm 9.05 \text{ c}$	$274.84 \pm 17.34 \ b$	295.19 ± 9.56 a
		Harvest	$70.46 \pm 8.04 \ a^{***}$	$47.11 \pm 4.40 \ b^{***}$	$41.19 \pm 3.85 \text{ ab}^{***}$	$52.50 \pm 3.56 \text{ b}^{***}$
	18	Veraison	$587.47 \pm 44.80 \text{ c}$	$474.47 \pm 21.27 \ d$	$775.28 \pm 62.19 \ b$	883.56 ± 29.25 a
	20	Harvest	332.32 ± 15.69 ***	321.86 ± 19.64 ***	309.87 ± 15.18 ***	$277.43 \pm 21.65 ***$
OH.	18 2017	Veraison	58.06 ± 2.59	56.82 ± 6.29	56.63 ± 4.89	64.89 ± 8.15
$(IC_{50} \mu g mL^{-1})$		Harvest	105.01 ± 7.58 ***	99.15 ± 5.15 ***	102.32 ± 7.62 ***	97.22 ± 8.67 ***
		Veraison	31.88 ± 2.15 ab	38.43 ± 3.42 a	30.52 ± 0.29 ab	$26.13\pm1.61\ b$
	20	Harvest	85.63 ± 6.86 ***	79.01 ± 3.29 ***	88.68 ± 6.39 ***	78.77 ± 6.24 ***

3.6. Multivariate analysis

To understand the effects of summer stress on the grapevine antioxidant defence system and hormonal balance throughout the growing seasons, as well as the effects of kaolin on two grapevine varieties, we performed a PCA (Fig. 4) and a MANOVA (Table 4) for each growing season. The PCA data corresponded to both varieties, treatments and developmental stages. In 2017 (Fig. 4a-b), the PCA showed that 77.2% of the total variability was explained by PC1 and PC2. PC1 revealed that ABTS, total phenols, flavonoids, ortho-diphenols, tannins, ABA and ROS were positively correlated, which can be attributed to the general level of antioxidant defence (Fig. 4a). PC2 showed that both anthocyanins and SA were negatively correlated with total SS accounting mainly for components related to berry ripening. The second plot (Fig. 4b) showed the position of each sampling group (TF_C, TF_K, TN_C, TN_K) and developmental stage (veraison and harvest). PC1 showed a clear opposition between developmental stages, with grapes from *veraison* showing higher levels of components of antioxidant defence. PC2 separated the grapevine varieties with parameters associated with ripening-delaying factors, such as higher SA levels and lower SS levels in TF, particularly in kaolin-treated berries at harvest. In 2018 (Fig. 4c-d), the PCA explained 81.7% of the total variability, revealing that phenolic compounds, ABA and ABTS were positively correlated in PC1 and that ROS and SS were negatively correlated with IAA and SA. Similarly to 2017, PC1 showed an opposition between developmental stages, and PC2 separated mainly the varieties under study, with no perceptible effects of kaolin treatment in either variety or developmental stage. To assess differences in constitutive phytohormone levels (ABA, IAA, SA), antioxidant defence (total phenols, flavonoids, ortho-diphenols, ABTS) and quality attributes (tannins, anthocyanins, SS), several MANOVAs were performed for each growing season with variety, treatment, developmental stage and their interactions as factors. These analyses showed significant differences due to variety, treatment and developmental stage on hormone levels, antioxidant defence and quality traits in both 2017 and 2018 growing seasons (Table 4). In 2017, no significant interaction by treatment and variety was observed in the quality attributes evaluated or in the variety, treatment and developmental stage interaction, whereas in 2018, all factors and interactions were significant (p < 0.001) in all the parameters evaluated.



Figure 4. Principal component analysis (PCA) plot scores for berry ripening traits and antioxidant defence components of Touriga-Franca (TF) and Touriga-Nacional (TN) control (C) and kaolin-treated (K) berries in two developmental stages (*veraison* and harvest). A PCA has been undertaken for each growing season (2017 – a,b; 2018 - c,d).

	2017	2018
Phytohormones		
V	2270.52***	1011.31***
Т	69.75***	2225.84***
S	3683.41***	1897.77***
$V \times T$	166.04***	430.26***
$V \times S$	691.13***	1143.21***
$T \times S$	298.94***	874.70***
$V \times T \times S$	58.87***	619.43***
Antioxidant defence		
V	822.29***	2312.00***
Т	53.25***	32.33***
S	1577.71***	570.47***
$V \times T$	21.91***	49.10***
$V \times S$	950.56***	3140.34***
$T \times S$	12.76***	27.07***
$V \times T \times S$	10.46***	46.78***
Quality attributes		
V	1578.88***	2498.67***
Т	35.09***	59.61***
S	2843.57***	2875.85***
$V \times T$	1.847	49.06***
$V \times S$	1471.15***	2550.59***
$T \times S$	23.49***	40.22***
$V \times T \times S$	2.82	49.07***

Table 4. Results (F-values) of MANOVA Pillai's trace test for the effects of the variety (V), treatment (T), developmental stage (S), and their interactions on phytohormones (ABA, IAA, SA), antioxidant defence parameters (total phenols, flavonoids, ortho-diphenols, ABTS), and quality traits (SS, anthocyanins, tannins) in the 2017 and 2018 growing seasons.*** indicates p < 0.001. Absence of asterisks indicate non-significant effect.

4. Discussion

The foreseen occurrence of extreme weather events (e.g. heatwaves) in areas with a Mediterranean-type climate during the summer season can impose serious effects on grapevine phenology and berry ripening, compromising yield and the sustainability of the wine sector (Reis *et al.* 2020). The bioclimatic index data indicate that the 2017 growing season was the warmer $(2705^{\circ}C \text{ GDD})$ and the drier (-147.1 DI) of the two seasons in this study due to the record of more than 10 days with temperatures above 40°C, along with the occurrence of two heatwaves just before *veraison* (DOY 165–169 and DOY 193–198), and lower precipitation levels (- 110.2 mm) compared with 2018. Therefore, the differences regarding stress severity found between growing seasons might influence the effectiveness of kaolin in balancing berry ripening and quality potential in red grapevine varieties grown in Mediterranean-like climate regions. Nonetheless, kaolin application effects in grapevines can also vary according to the variety under study (Luzio *et al.* 2021, Singh *et al.* 2020) because the accumulation of several stress-related metabolites and the hormonal content differed among samples.

One of the expected impacts of climate variability in temperate climate regions is the earlier onset of several phenological stages (Leolini *et al.* 2020). Indeed, Costa *et al.* (2019) predicted an earlier phenophase timing for TF compared with TN, a phenomenon that could explain the varietal differences found in this study regarding berry hormonal content, particularly in 2017, when stress severity was more pronounced. Hormonal content and accumulation throughout berry ripening depend on many environmental and biotic factors (Coelho *et al.* 2019); ABA, IAA and SA play antagonistic roles on ripening-related processes (Kuhn *et al.* 2013). While ABA is considered a ripening promoter, IAA and SA are known to delay this process (Deytieux-Belleau *et al.* 2007, Lo'ay 2019). Because summer stress was more prominent in 2017, it seems plausible that under severe conditions, kaolin might have a ripening-delaying effect due to lower SA and ABA accumulation at *veraison* found in TF_K, and the high SA content at harvest observed in both varieties. In a less hot season, the kaolin delaying effect on ripening-related processes was also observed through a sharp increase in IAA content at *veraison* and lower ABA levels at harvest, mainly in TN (Fig. 2).

Managing grapevine ripening with environmental fluctuations under field conditions can be a challenging task. However, particle film technology has shown beneficial effects on several fruit quality traits, preserving berry acidity, promoting a balanced synthesis of secondary metabolites and sugar transport throughout ripening (Conde et al. 2018, Conde et al. 2016). In agreement, the results revealed that kaolin application decreased fruit SS content under summer stress conditions (Table 2) and slightly preserved berry acidity, mainly tartaric acid, with no effects on pH at the last stage of maturation, as recently described by Singh et al. (2020). These attributes are highly appreciated by the wine industry due to their potential to sustain wine balancing and quality (Moran et al. 2019). Moreover, several studies have shown a yield increase in several crops after kaolin application, which was correlated to increasing growing season temperatures, highlighting the efficiency of kaolin in mitigating environmental stresses (Brito et al. 2018, Cantore et al. 2009, Glenn 2009, Glenn 2012). In grapevines, this effect might vary at least according to the variety and edaphoclimatic conditions (Brillante et al. 2016, Dinis et al. 2018b). Under the stressful conditions of the Douro region, Dinis et al. (2018b) observed a yield increase of around 15% in kaolin-sprayed vines, while Brillante et al. (2016) found no effects in this parameter in Cabernet Sauvignon grown in South Italy under drought conditions. Lobos et al. (2015) also reported no significant changes in fruit composition and gas exchange parameters in Cabernet Sauvignon vines upon kaolin treatment. Nevertheless, the incidence and severity of fruit dehydration in vines grown in Mediterranean-type climate

regions were significantly lower in kaolin-treated compared with unsprayed plants (Lobos *et al.* 2015). Recently, Garrido *et al.* (2019) found that kaolin could also modulate the photosynthetic activity of both exocarp and seed integuments of Alvarinho berries due to higher PAR reflection at the inner zones of the canopy in non-irrigated vines, suggesting that canopy structure, incident radiation and irrigation settings can also affect kaolin effectiveness.

Overall, kaolin application limited the total ROS content in both varieties and growing seasons, data that are in agreement with previous findings for TN (Dinis *et al.* 2016a). Besides, H_2O_2 levels were positively influenced by kaolin treatment in TN, indicating greater oxidative stress signalling, which can improve the regulation of several biological processes in grapevines, such as the synthesis of heat shock proteins and anthocyanins (Carvalho *et al.* 2015, Niculcea *et al.* 2013). Earlier studies on the effects of kaolin in TN berries have related lower H_2O_2 and TBARS content with decreased proline accumulation (Bernardo *et al.* 2017). However, the present study does not associate such trends in both varieties, suggesting that H_2O_2 , TBARS and proline accumulation might be varietal and climate dependent, with other factors influencing proline content over grape ripening, such as sugar accumulation (Hasanuzzaman *et al.* 2020, Kavi Kishor and Sreenivasulu 2014).

Overall, kaolin treatment showed no major effects on oxidative stress parameters while improving the non-enzymatic antioxidant defence compared with control vines (Table 3). Indeed, kaolin enhanced the total phenol, anthocyanin, tannin and ortho-diphenol content, as shown in previous works (Bernardo et al. 2017, Dinis et al. 2016a). The latter displays an important role in radical stability, as recently reported in other Portuguese grapevine varieties (Dinis et al. 2020, Mendes Lemos et al. 2020) and olive fruit (Brito et al. 2018). Radicalscavenging activity in fruit extracts was also higher in treated plants, mainly in the 2017 growing season, indicating a close interplay between climate variability and kaolin effectiveness, which seems stronger under severe environmental conditions (Brito et al. 2018). Nevertheless, the interactions between kaolin application, variety and developmental stage were significant for the antioxidant defence parameters in both growing seasons (Table 4), suggesting long-lasting kaolin effectiveness, as shown by Cabo et al. (2019) in hazelnut trees. The response of grapevine antioxidant defence system and phytohormone levels in two successive growing seasons showed significant differences between varieties, growing seasons and developmental stages (Fig. 4 and Table 4). In the veraison stage of 2017 (Fig. 4 a-b), berries showed higher components of the antioxidant defence and secondary metabolite accumulation, such as phenolic compounds, ROS and anti-radical activity, which were lower at harvest, particularly in TN. These data, along with the changes found in SA and SS content in TF_K, reinforce the hypothesis that kaolin application promotes changes in the berry ripening timing, depending on the variety and the magnitude of environmental stress. In addition, the MANOVA analysis (Table 4) indicates non-significant interactions in 2017 by treatment, variety and developmental stage regarding berry quality attributes. The absence of an interactive effect among multiple factors could be ascribed to the different ripening timings of each variety, as suggested by the PCA analysis (Fig. 4). In a less hot season (Fig. 4 c-d), TN showed higher levels of antioxidant defence and ripening related components, such as sugars and anthocyanins, compared with TF at *veraison*, indicating a varietal sensitivity for ripening onset, which is likely advanced in TN under the current settings. Besides, the effects of kaolin on delaying TF ripening were only distinguished in 2017, suggesting that stress severity triggers the effectiveness of kaolin. This possibility should be taken into consideration when planning particle-film application and dosage.

5. Conclusions

The present study suggests that kaolin application in wine regions with a Mediterraneantype climate could be a low-cost strategy (approximately 50 euros/ha) to modulate berry ripening under adverse environmental conditions, beyond its described effects on improving the vines' physiological performance. Despite the complexity of studying adult plants in commercial vineyards, our results demonstrate that climate conditions are the primary trigger for ripening onset, along with simultaneous changes in the hormonal content, accumulation of secondary metabolites, sugars and ROS. Kaolin application promoted different varietal responses in both growing seasons, delaying ripening timing of TF under harsh summer conditions, and probably of TN in a less hot season, indicating higher stress resilience and acclimation in TF during the experiment. Besides, kaolin treatment enhanced berry quality traits and anti-radical activity in both varieties under study. From a climate change perspective, comparative studies should be performed under controlled and field conditions and followed to the wine industry to elucidate the advantages of particle film application on increasing the performance of the winemaking process and potential berry quality.

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Chapter 6.2. Kaolin impacts on hormonal balance, polyphenolic composition and oenological parameters in red grapevine berries during ripening

Abstract

Kaolin particle-film application is a well-known strategy to avoid fruit damage. However, its putative role in balancing berry ripening under a changing climate remains poorly explored. We assessed kaolin treatment effect on several ripening berry components, hormonal balance and oenological parameters of the field-grown Touriga-Franca (TF) and Touriga-Nacional (TN) grapevine varieties at veraison (EL35) and ripening (EL38) during two growing seasons (2017 and 2018). Under the adverse summer conditions (two heatwave events) of 2017, kaolin application increased 211.2 % and 51.4 % the salicylic acid (SA) and abscisic acid (ABA) levels in TF berries at EL38, while no significant differences were observed in TN. Conversely, TF, and TN kaolin treated berries showed lower SA and ABA accumulation in 2018, respectively. Tartaric acid content increased about 17.2 %, and 24.2 % in TF and TN treated berries at stage EL35 in the 2017 growing season. Though kaolin treatment had no consistent effect on anthocyanins accumulation, flavonoids, ortho-diphenols and tannins increased in kaolin treated grapevines in 2017. This study highlights the key role of climate in triggering ripening related processes and fruit quality potential. Nevertheless, kaolin treated grapevines displayed an improved response to oxidative stress signals by increasing secondary metabolites accumulation in warm vintages. Kaolin application promoted different varietal responses, with a possible ripening delaying effect in TF, reinforcing its efficiency in alleviating severe summer stress impacts.

Keywords: Abscisic acid; Antioxidants; Foliar spray; Heat stress; Indole-3-acetic acid; ripening; Salicylic acid; Kaolin

1. Introduction

Sustainable grape production and quality are the central cornerstones of current viticulture, which can be seriously affected by climate change in the coming decades (Fraga *et al.* 2016). Indeed, the expected increase in the mean air temperature and changes in the rainfall patterns, particularly in Mediterranean-like climate regions, will harmfully affect grapevine sustainability, imposing a pressing challenge to the winemaking sector (Santos *et al.* 2020).

One of the main effects of climate variability is the earlier onset of several phenological stages (Jones and Davis 2000), resultant from a foreseeing general advancement of 6 to 25 days for distinct grapevine varieties (Jones 2007). Likewise, in a recent modelling study performed on 37 varieties with different heat requirements, Reis *et al.* (2020) classified 'Avesso', 'Castelão', and 'Chardonnay' as early grapevine varieties for budburst; 'Touriga-Nacional' (TN), 'Touriga-Franca' (TF), and 'Gouveio' for flowering; and 'Alfrocheiro', 'Jean', 'Tinta Barroca', and TF for the onset of *veraison* due to their lower heat requirements. Consequently, earlier harvest dates are also predicted in several Mediterranean-type climate areas, where summers are characterised by prolonged periods of high light, temperature and water scarcity (Santos *et al.* 2020). As a result, the sugar and organic acid metabolisms and secondary metabolites accumulation can be desynchronised during ripening (Reis *et al.* 2020, Rienth *et al.* 2016).

Grape ripening onset is also coordinated by abscisic acid (ABA) accumulation and crosstalk with other plant growth regulators, such as salicylic acid (SA) and indole-3-acetic acid (IAA), known by their potential role on plant growth and defence signalling (Kuhn *et al.* 2013, Pérez-Llorca *et al.* 2019). Moreover, phytohormones play a pivotal role over grapevine development and fruit ripening, regulating metabolic changes caused by complex interactions with abiotic stress factors, such as temperature, light, UV-B radiation, and water availability (Fortes *et al.* 2015). Several studies have reported a synchronised ABA accumulation and activation of complex hormonal signalling under stressful conditions at *veraison*, influencing anthocyanin content, berry size, colour development, sugar levels, and ripening timing (Niculcea *et al.* 2013, Parada *et al.* 2017).

Though grapevines present various antioxidant defence mechanisms, the implementation of short-term adaptation strategies seems imperative to sustain acclimation under severe environmental conditions (Bernardo *et al.* 2018, Hasanuzzaman *et al.* 2020). Within those strategies, the foliar application of kaolin (Al₂Si₂O₅(OH)₄), a chemically inert white clay mineral, is a well-known short-term strategy applied in many fruit crops that increases the

reflection of ultraviolet, photosynthetically active (PAR) and infrared radiation. Kaolin-based particle film application has already shown beneficial effects on leaf cooling, gas exchange and water use efficiency, boosting the primary metabolome of leaves, the phenylpropanoid and flavonoid pathways in berries (Conde *et al.* 2018, Dinis *et al.* 2016) and the acidity of some white varieties (Dinis *et al.* 2020). Additionally, a possible role in modulating IAA and ABA dynamics in grapevine and olive leaves under severe environmental conditions in Mediterranean-like climate areas was also proposed (Brito *et al.* 2019, Dinis *et al.* 2018). Nonetheless, at a fruit level, it is still unclear whether kaolin application might modulate the hormonal balance of berries throughout ripening, neither its effects on balancing grape ripening under a changing climate. Thus, this study addresses the hypothesis that kaolin treatment can lead to several changes in berry quality attributes, phenolic compounds, antioxidant activity, and hormonal content of two field-grown red grapevine varieties during two successive growing seasons (2017 and 2018).

2. Materials and Methods

2.1. Site description and weather conditions

The experiment was outlined in an ampelographic vineyard (9.7 ha) composed of 189 grapevine varieties distributed in side-by-side rows in the Alentejo Demarcated Region, in the Southeast part of Portugal (38° 23' N, 7° 33' W). Two native varieties, Touriga-Franca (TF) and Touriga-Nacional (TN), grafted on 1103P rootstock, trained to the bilateral cordon, and with a vine spacing of 2.2 m x 1.0 m were chosen due to their finest winery potential and moderate suitability to warm and dry climates (Fraga et al. 2016). The six-year-old vines displayed an N-S orientation with a slight slope (5° N) and 220 m of elevation. The chosen varieties are installed in nearby rows within the parcel. The local climate is typically Mediterranean-like, with a warm-temperate climate with dry and hot summers (Kottek et al. 2006), and higher precipitation during the winter months. An automatic weather station recorded weather variables such as the rainfall, and maximum air temperature (T_{max}) during the experiment (Fig. 1). The occurrence of heatwave events was also assessed considering a range of five consecutive days with maximum air temperatures above 40°C (Fraga et al. 2020). According to the world reference base for soil resources 2014 (FAO 2015), the soil mapping of the region is classified as luvisols, characterised by a clay-enrichment subsoil. At the vineyard site, soil composition is derived from granodiorite, with a very similar texture that varies between sandy loam and sandy loam-clay, well-drained, and with some water holding capacity

without limitation due to the appearance of the original rock up to 1.50 m deep. Vineyard management reflected local commercial organic production practices, with deficit irrigation (30% of the reference evapotranspiration) to prevent plants from death.



Figure 1. Daily maximum (T_{max}) air temperature (°C) and precipitation (mm) at the vineyard site. Vertical filled bars represent the heatwave events occurred during the experiment (light grey – 2017; dark grey – 2018). Kaolin application – DOY 198; Stage EL35 (2017 - DOY 208; 2018 – DOY 209); Stage EL38 (2017 – DOY 237; 2018 – DOY 243)

2.2. Plant material, treatments and sampling

The experimental set up was adapted to the commercial vineyard's existing features to ensure similar edaphoclimatic conditions and solar exposure among treatments and varieties. In each extended row, 120 vines per variety were selected, considering half row as the control variant, and the other half as the treated variant. Within each variant, vines were distributed in three blocks with 20 vines each. Two treatments were then established: an experimental control (TF_C and TN_C), and a kaolin treated variant (TF_KL and TN_KL). Kaolin suspension (Surround® WP, Engelhard Corporation, Iselin, New Jersey) was prepared in an aqueous solution following manufacturer's recommended dosage of 5 % (w/v), supplemented with 0.1 % (v/v) Tween 20 to improve adherence, and applied on the whole canopy with a machine

operated air blast sprayer at a rate of 300 L/ha. Kaolin suspension was applied on the windless morning of July 17 (DOY 198) in both growing seasons (2017 and 2018), whereas control vines did not receive any treatment. Berry sampling was carried out at two developmental stages according to the Coombe (1995) classification: EL35, corresponding to *veraison*, and EL38, corresponding to the full maturation stage (harvest date). The EL35 stage was achieved on the 27th (DOY 208) and 28th (DOY 209) of July in 2017 and 2018, respectively, whereas EL38 corresponded to the 25th (DOY 237) and 31st (DOY 243) of August, respectively.

For all biochemical assays, a total of 300 berries were collected from the western and eastern sides of the vines within each treatment, variety, and developmental stage. Berries were then frozen in liquid nitrogen and stored at -80 °C until the lyophilisation procedure. Fruits were then divided into three groups of 100 berries each, lyophilised (SCANVAC 55-4 Pro, LaboGene, Lynge, Denmark) for 120 h, ground to a fine powder with liquid nitrogen by using and a mortar and pestle, and stored at room temperature.

2.3. Phytohormones

ABA, IAA, and SA content were determined by high-performance liquid chromatography coupled to a triple quadrupole mass spectrometer (Micromass, Manchester, UK) through an orthogonal Z-spray electrospray ion source (Durgbanshi et al. 2005). Briefly, 100 mg of lyophilised fruit samples were extracted in 2.0 mL of distilled water using a mill ball equipment (MillMix20, Domel, Železniki, Slovenija). [²H₆]-ABA, [²H₂]-IAA, and [¹³C₆]-SA were used as internal standards. After centrifugation at 10.000 x g, supernatants were recovered, and pH was adjusted to 2.8-3.2 with 30% acetic acid. Extracts were partitioned twice with diethyl ether, and the supernatants were evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France) at room temperature. The dry residue was then resuspended in 500 µl of water:methanol (9:1) and filtered, through 0.22 µM PTFE filters, and directly injected into an UPLC system (Waters Acquity SDS, Waters Corp., Milford, MA) interfaced to a TQD triple quadrupole (Micromass Ltd. Manchester,UK) mass spectrometer through an orthogonal Z-spray electrospray ion source. A reversed-phase C18 column (Gravity, 50×2.1 mm 1.8-µm particle size, Macherey–Nagel GmbH, Germany) was used to achieve the chromatographical separation using a methanol-water gradient, supplemented with 0.1% acetic acid at a flow rate of 300 µl/min. Results were processed using the Masslynx v4.1 software, and the phytohormone contents were achieved by a calibration curve prepared with commercial standards.

2.4. Berry quality traits

Soluble sugars (SS) were extracted by heating 10 mg of lyophilised fruit tissue in 5.0 mL ethanol-water (80:20, v/v), during 1 h, at 80 °C. Quantification of SS was performed following an anthrone-sulfuric acid method adapted to 96-well microplates (Leyva *et al.* 2008). The anthrone reagent, containing 0.1 g of anthrone (0.1%) dissolved in 100 mL of concentrated sulfuric acid (98%), was prepared right before analysis and then added to the extracts. Determination of SS was taken in triplicate by reading the absorbance at 625 nm in a microplate multiscan reader (SPECTROstar Nano, BMG Labtech GmbH, Germany). The colourimetric response was compared to a standard curve based on glucose, and total SS was expressed as mg/g of dry weight (DW).

The physicochemical parameters of grapes were assessed in 100 fresh berries with triplicates, collected per variety, treatment, and developmental stage. Total acidity and pH were analysed following the OIV international methods of wine and must analysis (OIV 2003). The tartaric and malic acid were measured enzymatically, using an automated clinical chemistry analyser (Miura One, TDI, Spain) (Franquès *et al.* 2018).

2.5. Phenolic composition and radical scavenging activity

Phenolic compounds were extracted as previously described by Mendes Lemos *et al.* (2020). Total phenols, *ortho*-diphenols, and flavonoids were measured in triplicates, following the methods adapted by Gouvinhas *et al.* (2018). Briefly, total phenols were determined by mixing 20 μ L of fruit extract, 100 μ L of Folin-Ciocalteu reagent, and 80 μ L of sodium carbonate (7.5 %) in a microplate well, and incubated at 45 °C, during 30 min. Absorbance was then recorded at 750 nm. For the evaluation of *ortho*-diphenols, a colourimetric reaction was recorded at 375 nm by previous incubation of 40 μ L of sodium molybdate (50 g/L) with 160 μ L of fruit extract at room temperature, during 15 min. The colorimetric response of both total phenols and *ortho*-diphenols measurements was compared to a standard curve based on gallic acid, and expressed as milligrams (mg) of gallic acid equivalents (GAE) per gram of dry weight (DW). Flavonoids content were determined in a reaction mixture containing 24 μ L of fruit extract, 28 μ L of sodium nitrite (50 g/L), 28 μ L of aluminium chloride (100 g/L), and 120 μ L of sodium hydroxide (1.0 M). The absorbance was immediately recorded at 510 nm, and results were expressed as mg of catechin equivalents (CE) per gram of dry weight (mg CE/g DW).

Analysis of total monomeric anthocyanins was performed using a pH differential method (Lee *et al.* 2005). At two distinct pH values (pH 1.0 potassium chloride ([0.025 M] buffer, and

pH 4.5 sodium acetate [0.4 M] buffer), measurements were recorded at 700 nm and 520 nm in a microplate reader (SPECTROstar Nano, BMG Labtech GmbH, Germany). Total anthocyanins content was expressed as equivalents of malvidin-3-*O*-glucoside (MVE), based on its molar extinction coefficient (28 000 L/cm/mol).

Tannin content was measured by the methyl cellulose precipitable assay (Dambergs *et al.* 2012) with slight modifications. The method was performed in 1.5 mL test tubes, by adding 100 μ L of fruit extract, 300 μ L of methyl cellulose (0.04 %), and 200 μ L of a saturated ammonium sulphate solution, adjusting the final volume up to 1.0 mL with distilled water. In control samples, distilled water was added instead of methyl cellulose, and all samples were measured at 280 nm in UV-adapted microplates. Tannins concentration was obtained by subtracting the absorbance of control samples and using epicatechin (ECE) as standard. The results were expressed as mg ECE/g DW.

The free radical scavenging activity based on the 2,2-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid (ABTS) radical decolourisation was determined as described by Gouvinhas *et al.* (2018). Briefly, 188 μ L of ABTS working solution was mixed with 12 μ L of methanolic fruit extract, incubated for 30 min, and then absorbance was recorded at 734 nm in a microplate reader. Trolox ® was used as standard, and the results expressed as equivalents to trolox (TE) concentration per DW of sample (μ mol TE/g DW).

2.6. Statistical analysis

Statistical analyses of phytohormones, berry quality traits, and secondary metabolites were performed using Sigma-Plot 14.0 program (SPSS Inc.). After testing for ANOVA assumptions (homogeneity of variances with the Levene's mean test, and normality with the Kolmogorov-Smirnov test), statistical differences among treatments and varieties within each developmental stage were evaluated by two-way factorial ANOVA, followed by the post hoc Tukey's test. Afterwards, statistical differences between developmental stages (EL35 *vs* EL38) within each sampling variant were evaluated by one-way ANOVA, followed by the post hoc Tukey's test. The relationship between the concentration of phenolic compounds and the antioxidant activity was analysed by the Pearson correlation test using Sigma-Plot 14.0. Principal component analysis (PCA) was performed to examine summer stress effects throughout berry ripening of control and treated plants using STATISTICA 8.0 program. Different lower case letters represent significant differences (p < 0.05) between treatments (TF_C, TF_K, TN_C, TN_K) within each developmental stage, and the asterisks (*p < 0.05) represent significant differences

between developmental stages (EL35 *vs* EL38) within each treatment, variety and growing season. Absence of letters and asterisks indicate no significant difference.

3. Results and Discussion

3.1. Environmental features

Mediterranean-like climate areas are currently classified as climate change hotspots, particularly in Southern European regions, which may impair grapevine production and grape quality potential (Fraga et al. 2018). In this study, climate variability (Fig. 1) assessed by the variables maximum air temperature and precipitation from April (DOY 91) to October (DOY 304), revealed in 2017 the occurrence of two heatwave events (DOY 167-171 and DOY 192-197) soon before reaching stage EL35 (DOY 208). In the following growing season (2018), only one heatwave was also recorded (DOY 213-218) soon after stage EL35 (DOY 209). As climate is the primary driver of phenology, the recent trends and future projections of harvest dates in early, mid, and late-ripening varieties were associated with variations in the maximum air temperature along the season (García de Cortázar-Atauri et al. 2017, Pagay and Collins 2017). While early ripening varieties were more associated with the variations in maximum air temperatures during March to July, mid- and late-ripening varieties appeared to be rather affected by maximum air temperatures during the ripening period (Koufos et al. 2020), which can influence berry composition and quality. Besides, total precipitation over the experiment was almost 5-fold higher in 2018 (228.8 mm) than the equivalent period of 2017 (47.0 mm), which may also influence grapevine summer stress responses and kaolin efficiency in preserving berry quality attributes.

3.2. Berry hormonal balance

Hormonal content and accumulation throughout berry ripening lean on many environmental and biotic factors, with antagonist roles on ripening-related processes displayed by IAA, SA, and ABA (Kuhn *et al.* 2013). While ABA is considered a ripening promoter, SA and IAA are known to delay this process (Deytieux-Belleau *et al.* 2007, Lo'ay 2019). Our results showed that in 2017, kaolin effects were mainly observed in TF berries at stage EL38 (Fig. 2), with 211.2 %, 51.4 % and 76.8 % higher SA, ABA and IAA content, respectively, suggesting an improved stress response and hormonal modulation under adverse environmental conditions (Parada *et al.* 2017), such as those recorded during the first year of the study (low rainfall with several days with maximum temperatures above 40 °C).


Figure 2. Phytohormones (abscisic acid - ABA, salicylic acid – SA, and indole-3-acetic acid - IAA) content in Touriga-Franca and Touriga-Nacional control (TF_C and TN_C) and kaolin berries (TF_K and TN_K) throughout the 2017 and 2018 summer seasons. Data are mean \pm SD (n=3). Different lower case letters represent significant differences between treatments and varieties within each developmental stage and sampling year. * represent significant differences (p<0.05) between the period of the day (pre-dawn vs midday) within the same variety, treatment and developmental stage.

In the 2018 growing season, the tendency was different, particularly regarding SA levels, with a sharp increase in all treatments, varieties and developmental stages. In this year, TF kaolin treated berries showed lower SA accumulation consistently throughout ripening, while TN_K berries presented higher SA content and lower ABA accumulation at stage EL38, suggesting that kaolin could delay TF berry ripening compared to TN. The regulation of SA signalling and crosstalk is associated with thermotolerance and biotic stress responses during

plant growth and development (Klessig et al. 2018), which were also investigated through controlled SA application in several crops exposed to heat and water stress conditions (Brito et al. 2019, Oraei et al. 2019, Wang et al. 2010). However, Khan et al. (2015) reported that SA application seems beneficial for plants even under optimal conditions, highlighting the challenge of characterising hormonal responses and dynamics in field-grown plants (Abreu and Munné-Bosch 2008). At stage EL35, TF and TN treated vines presented fruits, respectively, with 26.7 % and 36.7 % less IAA content than their correspondent controls, reinforcing kaolin modulating effect on berry ripening, which seems associated to stress intensity and length (Frioni et al. 2019a). On the other hand, independently of the varietal effect, data also suggests that grapevines displayed better fruit growth and maturation conditions under mild environmental conditions (2018), which can also influence ripening timing and kaolin effectiveness (Ferrari et al. 2017). At the leaf level, previous studies have shown the influence of kaolin on grapevine ABA and IAA dynamics associated with improved water use efficiency and general physiological performance under stressful conditions (Dinis et al. 2018, Frioni et al. 2020). In fact, one of the primary impacts of climate variability in temperate climate regions is the earlier onset of several phenological stages (Leolini *et al.* 2020). Indeed, in a comparative study on grapevine phenology performed in the same varieties of this work, Costa et al. (2019) predicted an earlier phenophase timing for 'Touriga-Franca' respecting 'Touriga-Nacional', which is in accordance with the general differences found in this study regarding varietal hormonal content. Since summer stress was greater in 2017, it seems plausible that under severe conditions, kaolin might promote a ripening delaying effect in TF under the current conditions to sustain a balanced ripening.

3.3. Fruit quality traits

Shellie (2015) reported a decline in particle-film effectiveness in 'Merlot' berries with the simultaneous onset of cooler air temperatures and decreasing radiation intensity. Indeed, in the 2017 growing season, kaolin treatment decreased about 19.1 % TF fruit soluble sugars content (Table 1) at stage EL35, and up to 30.2 % at stage EL38, suggesting an improved kaolin efficiency on preserving berry oenological potential under severe summer stress conditions such as those recorded in 2017. In contrast, no major effects were observed in TN in all sampling dates, indicating distinct varietal responses to kaolin application. Similarly to our findings, Shellie and Glenn (2008) and Shellie and King (2013) have reported a varietal-dependent effect on fruit soluble solids accumulation by kaolin treatment. In accordance with

several studies (Brillante *et al.* 2016, Frioni *et al.* 2019b, Lobos *et al.* 2015), pH was consistently unaffected in red grapevine varieties.

Table 1. Total soluble sugars content, and oenological parameters of berries from Touriga-Franca and Touriga-Nacional control and kaolin-treated grapevines in two developmental stages (EL35 and EL 38) during 2017 and 2018 growing seasons. Data are mean \pm SD (n=3). Different lower case letters represent significant differences between treatments and varieties within each developmental stage (EL35 and EL38), and sampling year. The asterisks (*p < 0.05) represent significant differences between developmental stages (EL35 vs EL38) within the same treatment, variety and sampling year. Absence of letters indicate no significant differences between treatments and varieties.

Fruit			Touriga-F	Touriga-Franca		Touriga-Nacional	
parameters		Date	Control	Kaolin	Control	Kaolin	
Soluble sugars (mg/g DW)	17	EL35	633.03 ± 26.01 a	$512.21 \pm 20.31 \ b$	614.00 ± 14.78 a	622.80 ± 23.12 a	
	20	EL38	$932.52 \pm 20.90 \; a^*$	$650.55 \pm 31.92 \text{ bc}*$	$608.30 \pm 18.12 \; c$	$671.93 \pm 40.06 \ b^*$	
	18	EL35	$210.53 \pm 14.99 \; a$	$256.23 \pm 14.39 \; a$	$125.00 \pm 12.85 \ b$	$85.12\pm7.93~\text{c}$	
	20	EL38	547.70 ± 34.34 *	540.57 ± 51.15 *	528.45 ± 50.78 *	582.57 ± 23.37 *	
рН	2017	EL35	3.22 ± 0.07	3.10 ± 0.10	3.08 ± 0.04	3.06 ± 0.06	
		EL38	3.89 ± 0.25 *	3.81 ± 0.10 *	3.73 ± 0.01 *	$3.69 \pm 0.09 *$	
	18	EL35	2.57 ± 0.02	2.58 ± 0.02	2.56 ± 0.04	2.52 ± 0.01	
	20	EL38	$3.52 \pm 0.06 \ a^*$	$3.50 \pm 0.03 \ a^*$	$3.24\pm0.03\ b^*$	$3.29\pm0.04\ b^*$	
Total acidity (g/L Tartaric acid)	17	EL35	$6.48\pm0.53~b$	$8.46\pm0.80\ a$	$8.95\pm0.60~a$	$8.88\pm0.19\;a$	
	50	EL38	3.90 ± 0.08 *	3.95 ± 0.23 *	4.38 ± 0.21 *	4.50 ± 0.26 *	
	2018	EL35	$23.83\pm2.14~b$	$25.45 \pm 1.73 \text{ b}$	32.03 ± 0.38 a	33.18 ± 0.17 a	
		EL38	4.38 ± 0.16 *	4.50 ± 0.08 *	5.63 ± 0.14 *	5.34 ± 0.30 *	
Tartaric acid (g/L)	2017	EL35	$4.29\pm0.20\ b$	$5.03\pm0.32~a$	$4.13\pm0.32\ b$	$5.13\pm0.32~a$	
		EL38	$2.97 \pm 0.25 \ a^*$	$2.84\pm0.14\ a^*$	$2.08\pm0.06\ b^*$	$2.84\pm0.20\ a^*$	
	2018	EL35	$12.13 \pm 1.01 \text{ b}$	$13.20\pm1.01~b$	16.90 ± 0.36 a	17.27 ± 0.31 a	
		EL38	2.22 ± 0.12 *	2.42 ± 0.03 *	2.36 ± 0.13 *	2.59 ± 0.16 *	
Malic acid (g/L)	2017	EL35	$1.43\pm0.12\;c$	$1.39\pm0.12\ c$	3.16 ± 0.13 a	$2.74\pm0.10\ b$	
		EL38	$1.20\pm0.11\ ab^*$	$0.90\pm0.09~c^{\ast}$	$1.39 \pm 0.10 \ a^*$	$1.08\pm0.11~bc^*$	
	18	EL35	$10.30\pm0.90\ b$	$11.00\pm0.72~b$	$13.83\pm0.15~a$	$14.33 \pm 0.06 \text{ a}$	
	201	EL38	1.52 ± 0.11 *	1.59 ± 0.04 *	2.08 ± 0.18 *	1.80 ± 0.10 *	

Regarding berry acidity, kaolin effects were also noticed in the 2017 growing season. While no consistent changes were detected on total acidity, tartaric acid accumulation increased about 17.2 % and 24.2 % in TF and TN treated berries at stage EL35, respectively, as recently reported in 'Pinot noir' berries (Frioni *et al.* 2019b). In contrast, malic acid content at stage EL38 (2017) decreased in both treated varieties, though in TN this effect was already observed at stage EL35. In the following year, with milder environmental conditions, no significant differences were observed between treatments and varieties for all oenological parameters analysed. Although most studies found no difference on organic acids concentration, it has been recently observed small preservation of malic and tartaric acids at harvest in white 'Cerceal' kaolin berries during

warm vintages (Dinis *et al.* 2020). As the kaolin-particle film has a significant effect on leaf and cluster cooling (Garrido *et al.* 2019, Glenn 2012), thus avoiding sunburn, treated vines may display favourable fruit growth conditions (Dinis *et al.* 2020). Hence, the present malic acid changes in 2017 might be due to a dilution factor. Recently, Bigard *et al.* (2019) indicated a malic acid breakdown or dilution promoted in late or slow-ripening berries grown in a semi-arid climate. In this sense, it is reasonable to suggest that in the present conditions, a pre-*veraison* kaolin treatment could delay berry ripening under sustained summer stress, though being varietal dependent (Costa *et al.* 2019, Dai *et al.* 2011).

3.4. Polyphenolic compounds and antioxidant activity

Phenolic compounds play an essential role in grape quality potential due to their significant contribution to wine colour and aroma, with antioxidant functions, that can be affected by environmental factors and management practices (Sharma et al. 2019). In accordance with Singh et al. (2020), while no consistent response was found for total phenols and anthocyanins accumulation throughout the experiment (Table 2), flavonoids, ortho-diphenols, and tannin levels were mainly affected by kaolin treatment in 2017 at stage EL38. This effect corroborates the recent findings of Luzio et al. (2021). At this stage, flavonoids and ortho-diphenols content decreased up to 32.0 %, and 16.7 % in TF kaolin berries, respectively, and tannins increased by about 60.3 % related to control berries, while in TN this effect was reduced. However, in the following growing season, flavonoids, anthocyanins and tannins increased about 149.3 %, 20.7 %, and 72.0 % at the same stage (EL38), respectively, while no effects were detected on orthodiphenols and total phenols content. Conversely to our results found in 2017, most of the studies regarding kaolin effect on berry phenolics report an increment of total phenols and flavonoids in several grapevine varieties at ripening (Frioni et al. 2019b, Lobos et al. 2015). However, since kaolin treatment can have a positive impact on berry size at veraison, with 14.3 % higher volume in grapevines grown under similar edaphoclimatic conditions (Dinis et al. 2020), this result might be theoretically due to a dilution factor since berry size indirectly affects the final concentration of phenolics (Holt et al. 2008). On the other hand, seasonal changes in tannins and anthocyanins content appear to be more related to biosynthesis and/or transport rather than berry size itself (Holt *et al.* 2008), being influenced by several climate and varietal features, as well as the degree of ripeness, particularly during very hot summers (Rodríguez Montealegre et al. 2006). Indeed, tannins accumulation increased in kaolin berries at stage EL38, which was also recently observed in 'Meili' treated berries (Wang et al. 2020). In contrast, no significant

response was found in the same varieties of this study grown in another Portuguese winegrowing region (Singh et al. 2020), with distinct mesoclimate (Fraga et al. 2017). This somewhat inconsistent response highlights that climate is the paramount factor in shaping ripening related processes and fruit quality potential. Though kaolin treatment had no evident effect on anthocyanins accumulation throughout the seasons, flavonoids, ortho-diphenols, and tannins increased in treated grapevines in 2017, indicating an optimised response to oxidative stress signals and a balanced ripening (Kuhn et al. 2013, Mendes Lemos et al. 2020). Likewise, the radical scavenging activity by the ABTS method was higher during the 2017 summer season in treated vines, ranging between 160.51 - 202.08 and 162.04 - 228.89 µmol TE/g DW in the TF and TN control variants, respectively, and between 230.35 - 247.31 and 203.00 - 226.08 umol TE/g DW in the treated ones. As expected, antioxidant activity was significantly correlated (Table 3) with the variables total phenols (r = 0.952), flavonoids (r = 0.771), and ortho-diphenols (r = 0.958), showing that phenolics have a strong association with the antioxidant capacity of berries. This effect was previously reported in 'Touriga-Nacional' grapevines (Dinis et al. 2016), and recently described in olives from rainfed trees (Brito et al. 2018), indicating kaolin treatment as a suitable strategy to mitigate the adverse summer stress conditions of some Mediterranean-like climate areas.

Table 2. Kaolin effects on phenols, flavonoids, ortho-diphenols, anthocyanins, and tannins content, and antiradical activity based on ABTS in the berries of Touriga-Franca and Touriga-Nacional at two developmental stages (EL35 and EL 38) during the 2017 and 2018 growing seasons. Data are mean \pm SD (n=6). Different lower case letters represent significant differences between treatments and varieties within each developmental stage (EL35 and EL38), and sampling year. The asterisks (*p < 0.05) represent significant differences between developmental stages (EL35 vs EL38) within the same treatment and sampling year. Absence of letters indicate no significant differences between treatments and varieties.

Fruit analysis			Touriga-Fra	anca	Touriga-Nacional		
		Date	Control	Kaolin	Control	Kaolin	
Total phenols (mg GAE/g DW)	2018 2017	EL35	$31.67 \pm 1.65 \text{ ab}$	33.49 ± 1.74 a	$33.03\pm2.82~a$	$29.07\pm1.13~b$	
		EL38	$23.29 \pm 2.06 \text{ a*}$	$22.86\pm1.38~ab^*$	$20.22 \pm 1.57 \text{ bc*}$	$18.71 \pm 1.38 \text{ c}^*$	
		EL35	$67.90\pm3.60\ b$	$72.27\pm3.89~b$	$80.83 \pm 3.69 \text{ a}$	83.33 ± 5.13 a	
		EL38	$43.74 \pm 3.60 \ a^*$	$35.66 \pm 2.10 \text{ b}*$	$32.86 \pm 1.81 \ b^*$	$29.20 \pm 2.01 \text{ b*}$	
	17	EL35	$32.44\pm2.00\ b$	$44.19 \pm 3.05 \text{ a}$	$18.60 \pm 1.82 \ c$	$15.09\pm0.96\ d$	
Flavonoids	20	EL38	$25.12 \pm 1.16 \ a^*$	$17.08 \pm 1.73 \text{ bc}^*$	$20.46\pm1.13\ b$	$15.60\pm0.90\ c$	
(mg CE/g DW)	18	EL35	63.95 ± 0.43 a	$45.02 \pm 2.37 \text{ c}$	$60.48\pm5.78~a$	$52.72\pm1.79~b$	
	20	EL38	$11.97 \pm 1.12 \ b^*$	$10.11 \pm 1.37 \text{ b*}$	$16.99 \pm 1.59 \ b^*$	$42.35 \pm 6.25 \ a^*$	
<i>Ortho</i> -diphenols (mg GAE/g DW)	17	EL35	34.68 ± 1.81 a	37.34 ± 2.65 a	$31.07\pm3.08\ b$	$28.01\pm2.43\ b$	
	20	EL38	$25.25 \pm 1.95 \ a^*$	$21.04 \pm 1.25 \ b^*$	$19.38 \pm 1.33 \ b^*$	$18.41 \pm 1.37 \ b^*$	
	2018	EL35	$69.85 \pm 2.33 \text{ c}$	$64.09\pm2.07~d$	$77.84 \pm 3.24 \ b$	$90.72 \pm 4.17 \text{ a}$	
		EL38	$35.06 \pm 1.09 \ a^*$	$30.24 \pm 2.84 \text{ ab*}$	$27.45 \pm 0.95 \ b^*$	$31.55 \pm 2.12 \ ab^*$	
	17	EL35	$7.01\pm0.60\ a$	$4.63\pm0.55\ bc$	$3.82\pm0.25\;c$	$5.30\pm0.38\ b$	
Anthocyanins	20	EL38	5.19 ± 0.77 *	4.64 ± 0.54	4.78 ± 0.39 *	4.21 ± 0.33 *	
(mg MVE/g DW)	18	EL35	$5.18\pm0.26\ b$	$6.02\pm0.58~a$	$5.47\pm0.46\ ab$	$5.30\pm0.41\ ab$	
	20	EL38	$9.11 \pm 0.24 \ a^*$	$7.01\pm0.57\ b^*$	$6.05 \pm 0.35 \ c^*$	$7.30\pm0.22\ b^*$	
	17	EL35	33.53 ± 3.25 a	33.37 ± 2.67 a	$34.72\pm3.02\ a$	$27.44\pm1.76\ b$	
Tannins	20	EL38	$16.09 \pm 0.60 \ b^*$	$25.80 \pm 2.67 \ a^*$	$12.77 \pm 1.04 \ b^*$	$12.04 \pm 1.18 \text{ b*}$	
(mg ECE/g DW)	18	EL35	$8.22\pm0.31\ d$	$9.06\pm0.42\ c$	$11.37\pm0.50\ b$	$13.21 \pm 0.40 \text{ a}$	
	20	EL38	$1.36\pm0.17\ b*$	$2.79\pm0.34\ a*$	$1.25 \pm 0.12 \ b^*$	$2.15\pm0.32\ a*$	
	17	EL35	$160.51 \pm 7.75 \text{ b}$	230.35 ± 15.50 a	$162.04 \pm 13.82 \text{ b}$	203.00 ± 16.20 a	
ABTS'	20	EL38	$202.08 \pm 16.38 \ b^*$	247.31 ± 12.91 a	$228.89 \pm 23.26 \ ab^*$	$226.08\pm13.23\ ab$	
$(\mu mol TE/g DW)$	18	EL35	$676.60 \pm 47.30 \; c$	$655.78 \pm 6.38 \ c$	$781.73 \pm 39.09 \ b$	940.21 ± 75.81 a	
	20	EL38	285.97 ± 28.49 *	234.06 ± 21.70 *	293.30 ± 10.78 *	247.25 ± 25.91 *	

Table 3. Pearson correlation matrix between the antioxidant capacity and polyphenol content. Correlations are significant at p < 0.001 level (***).

	Total phenols	Flavonoids	<i>Ortho-</i> diphenols	Anthocyanins	Tannins	ABTS
Total phenols	1					
Flavonoids	0.764***	1				
Ortho-diphenols	0.980***	0.834***	1			
Anthocyanins	0.104	-0.107	0.026	1		
Tannins	-0.258	-0.047	-0.172	-0.525	1	
ABTS	0.952***	0.771***	0.958***	-0.037	-0.318	1

3.5. Principal components analysis

In order to understand the influence of kaolin treatment on grapevine summer stress responses and ripening during two successive growing seasons, we performed a principal components analysis (PCA) for each developmental stage (Fig. 3). The results showed that PC1 and PC2 explained 85.1 % of the total variability at stage EL35, and 69.5 % at stage EL38. The response of grapevine antioxidant defence system and ripening components to summer stress and kaolin treatment showed apparent differences between growing seasons. At stage EL35, berries from the 2017 growing season displayed higher components related to berry ripening, such as tannins, soluble sugars and ABA content which were positively correlated. Besides, at this stage, TF control berries showed the highest score in both PC1 and PC2, possibly indicating a higher ripening rate. These data, along with the changes found in SA and soluble sugars content in TF_K, reinforces the hypothesis that kaolin treatment could have a ripening delaying effect in TF under adverse summer stress conditions. Conversely, no major difference was observed between treatments in 2018, highlighting the significance of climate variability on kaolin efficiency and varietal acclimation. At stage EL38, the results also showed a clear difference between sampling years, with high levels of phenolics, antioxidant activity and total acidity in the 2018 growing season, indicating better conditions for balanced ripening, likely due to less severe environmental conditions. Regarding kaolin effects at this stage, TF control berries presented the highest level of total phenols, anthocyanins, tartaric acid, and soluble sugars in both sampling years, while showing lower acidity related components and antioxidant activity in the 2017 growing season. In this sense, kaolin effects on modulating berry ripening seem to depend on the variety and stress severity, since under mild environmental conditions its effectiveness can be questionable.



Figure 3. Principal component analysis (PCA) plots for berry ripening traits and antioxidant defence components of Touriga-Franca (TF) and Touriga-Nacional (TN) control (C) and kaolin (K) treated berries in two developmental stages (EL35 and EL38). A PCA has been undertaken for each developmental stage.

4. Conclusions

The present study suggests that kaolin application in Mediterranean climate type vineyards could represent a promising strategy to modulate grape berry ripening under severe environmental conditions. Despite the complexity of studying adult plants in commercial vineyards, our results demonstrate that climate is the primary trigger in shaping ripening related processes and kaolin effectiveness, along with changes on the hormonal content, accumulation of secondary metabolites, soluble sugars, and acidity. Kaolin application promoted different varietal responses, with a possible ripening delaying effect in Touriga-Franca variety under harsh summer conditions in the Alentejo region. Nonetheless, future research on foliar protectants under controlled and open-field scenarios should be extended towards the wine industry to elucidate the advantages of particle film application on improving berry quality and the winemaking performance.

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CHAPTER 7

GENERAL DISCUSSION

General discussion

In Mediterranean-type climate regions, the summer season is usually characterised by periods of combined drought, high temperatures and light levels, imposing a serious challenge to sustainable plant growth, yield, and fruit quality potential (Bernardo *et al.* 2018, Santos *et al.* 2020). Indeed, the foreseen increase of extreme weather events (*e.g.* heat waves and prolonged drought) during the summer season can jeopardise vines beyond yield and quality decays, such as sunburn of leaves and clusters, impairment of photosynthetic productivity, hormonal regulation, and cell homeostasis (Fraga *et al.* 2020, Ollat *et al.* 2017).

Though grapevines present several mechanisms to sustain growth and development upon prevailing stress factors, like adjustments to fluctuations in the light environment, osmotic adjustment, excess energy dissipation mechanisms, hormonal regulation, and antioxidant defences, it might not be enough to counterpart the impacts of the increasing magnitude of summer stress in Mediterranean areas (Bernardo *et al.* 2018, Walters 2004, Zhen and van Iersel 2017). Therefore, researchers and winegrowers have studied and applied several long and short-term strategies to overcome this issue, the former considered as structural strategies (*e.g.* rootstock and varietal selection, row orientation, etc.), and the latter as conjunctural strategies, such as the application of stress alleviating compounds (reviewed in **Chapter 2**) (Bernardo *et al.* 2018).

The foliar application of kaolin particle film is a well-known short-term strategy applied in many fruit crops that increases the reflection of ultraviolet, photosynthetically active (PAR) and infrared radiation, reducing leaf temperature and preventing leaf and fruit sunburn damage (Glenn and Puterka 2010). However, kaolin's effectiveness in improving grapevine resilience when subjected to severe environmental conditions, mainly through gas exchange assessment (*e.g.* stomatal conductance, carbon assimilation rates and P_N/g_s), can significantly change according to the variety, water status, management practices, plant age, and edaphoclimatic conditions (Brillante *et al.* 2016, Dinis *et al.* 2018b, Frioni *et al.* 2019, Shellie and King 2013). The weather conditions' characterisation was performed by analysing several bioclimatic indexes to underpin the climate variability influence on the grapevine summer stress responses and kaolin effectiveness during the experiment (2017 and 2018). Weather data showed that 2017 was the warmest and driest year of study in both locations (Douro and Alentejo), which influenced kaolin effectiveness in promoting summer stress acclimation, as reported in other crops (Brito *et al.* 2019, Cabo *et al.* 2019), suggesting that kaolin effectiveness is greater under adverse environmental conditions. In agreement, in the Douro site, two heatwave events were

recorded in 2017 (DOY 165-169 and DOY 193-198), and one in 2018 (DOY 213-218), as well as in the Alentejo site (2017: DOY 167-171 and DOY 192-197; 2018: DOY 213-218), which could explain most of the differences found between treatments (control *vs* kaolin) and growing seasons within each region.

Under field conditions, research on kaolin efficiency in promoting stress resilience of different grapevine varieties is still unclear (Brillante *et al.* 2016, Dinis *et al.* 2018b, Ferrari *et al.* 2017, Frioni *et al.* 2019, Shellie and King 2013), pushing forward this project's core to explore kaolin effectiveness in different regions and distinct varieties (Touriga-Nacional - TN and Touriga-Franca – TF).

In the **Douro site**, the photoprotective effect of kaolin was mainly observed in 2017, particularly at *veraison*, by decreasing leaf temperature and increasing photosynthetic rates, stomatal conductance and P_N/g_s of TN and TF grapevines (for more details see Chapter 3). These responses were previously reported in several crops and associated with lower ABA accumulation in leaves, reduced chlorophyll degradation and NPQ, and improved photochemistry, suggesting better conditions for plant growth and development under adverse environmental conditions (Dinis et al. 2018a, Dinis et al. 2016b, Frioni et al. 2020). In this study, kaolin effects on hormonal balance throughout the summer season of 2017 revealed that ABA and SA accumulation decreased in TN. Beyond their antagonistic regulation, a positive crosstalk between SA and ABA pathways was also reported, suggesting a synergistic interaction to control stomatal closure and drought tolerance (Bharath et al. 2021, Lim et al. 2015, Tombesi et al. 2015). Besides, the down-regulation of key genes involved in ABA synthesis (VvNCEDs) observed in this study indicates a lower need for ABA synthesis that could modulate stomatal conductance and improve vines' physiological performance as described by (Dinis et al. 2018a, Frioni et al. 2020). Under high light, decreased SA levels in plants usually indicate slightly impairment of PSII operating efficiency, enhanced thermal energy dissipation and limited photosynthetic electron transport efficiency, suggesting that appropriate levels of SA and other signalling molecules are required for proper light acclimation responses (Janda et al. 2014). Additionally, the down-regulation of VvNCED_s gene expression found in TN kaolin treated leaves might also suggest changes in the upstream pathway of ABA synthesis in this organ, involving carotenoids metabolism and xanthophyll cycle activation, which play an essential role against high light and water stress, as recently demonstrated by Frioni et al. (2020). Although the sharp VvNCED_s up-regulation found in TF kaolin treated vines in 2017, ABA accumulation did not change significantly, whereas P_N/g_s increased, suggesting an improved resilience to summer stress conditions.

The analysis of chlorophyll a fluorescence transients of kaolin treated leaves at *veraison* showed i) decreased maximum quantum yield of primary photochemistry (ΦP_0), ii) reduced absorption (ABS/RC), electron transport (ET_0/RC), and trapping (TR_0/RC) energy per reaction centre, but iii) higher performance index (PI_{ABS}), indicating an apparent antenna size reduction and lower inactivation of reaction centres at the beginning of the experiments (for more details see Chapter 4). These findings corroborate the ones of Dinis et al. (2016b), (2018b), who reported a positive kaolin effect on PIABS, linked to changes in the antenna conformation and oscillation in the light interception, increased leaf reflectance, and consequently lower leaf temperature and transpiration. Light acclimation processes comprise the balance between light absorption, utilisation, and excess energy dissipation, determining plants' acclimation efficiency and vulnerability to damage (Chaves et al. 2003, Zhen and van Iersel 2017). In order to get a detailed insight into kaolin functions on the light acclimation processes, this project focused on the accumulation of the photosynthetic pigments, xanthophyll cycle modulation, and light utilisation and dissipation processes upon summer stress conditions (high light, temperature, and water shortage) and kaolin treatment during the 2017 growing season. At the Douro site, TN treated leaves presented a lower Chl_(a+b)/Car ratio at veraison, while Chl_a/Chl_b increased, displaying some features of high light acclimated plants (Schumann et al. 2017). Besides, chlorophyll (Chla and Chlb) content decreased in TN and TF treated leaves from veraison to ripening, suggesting a long-term response to summer stress. Moreover, while NPQ increased at *veraison* in both treated varieties, it decreased at ripening compared to untreated vines. This quite puzzling finding suggests a trade-off between reductions in photosynthetic efficiency and reductions in high light stress induced damage that could relate with switches in the LHCII functionality from the light-harvesting to the photoprotective state (Liguori et al. 2015, Wu et al. 2020). Hence, the initial rise of NPQ observed in both kaolin treated varieties at *veraison* may be explained by a safe down-regulation of PSII efficiency and a greater effort to maintain PSII reaction centres openness under high light (Ruban 2014).

It is known that xanthophylls play an important role in light stress responses, depending on the interconversion of V_x and Z_x through the activation of the enzymes VDE and ZEP under high and non-saturated light conditions (Demmig-Adams *et al.* 1995). Curiously, kaolin coating limited xanthophyll cycle genes expression in TN at *veraison* without evident influence on the xanthophyll cycle pool dynamic, highlighting that *VvVDE1* and *VvZEP1* expressions and their respective protein synthesis may not occur at the same pace (Kim *et al.* 2019), particularly in field-grown grapevines that are continuously exposed to several stressors (for more details see **Chapter 5.1**). Under high light, NPQ processes require the presence of Z_x to relieve excitation pressure on PSII (Ruban and Horton 1999). In accordance with Frioni *et al.* (2020), Z_x levels increased in kaolin treated vines at *veraison*, indicating an improved photoprotective function of xanthophyll as a quencher of excess energy by switching LHCII antenna into a dissipative state, without restraining the photosynthetic performance of vines, through a fine-tuning of light acclimation responses. At the ripening stage, *VvZEP1* was up-regulated in both treated varieties, along with lower Z_x and NPQ levels, suggesting increased light collection and improved tolerance to prolonged high light exposure by an efficient regulation of the xanthophyll cycle (Bernardo *et al.* 2021).

In the 2018 growing season, kaolin effects on leaf gas exchange were diluted throughout the season. TN treated grapevines showed at ripening lower carbon assimilation rates, and stomatal conductance without effects on P_N/g_s , while TF_K exhibited improved P_N/g_s and lowered C_i/C_a. These results corroborate the hypothesis that kaolin effectiveness is lower under mild summer stress conditions that may depend on intrinsic varietal features (Brito et al. 2018a, Dinis et al. 2018b, Shellie and King 2013). The higher soluble sugars found in TF at ripening also indicate that this variety can benefit from kaolin application in less hot years. In addition, SA and IAA contents were positively affected in TF treated vines at veraison, whereas ABA content did not change significantly, indicating improved development and resistance to abiotic stresses without limiting the P_N/g_s and stomatal conductance of plants. The leaf cooling effect triggered by kaolin treatment was weaker in 2018 than in 2017, however, treated grapevines displayed higher energy dissipation (DI₀/RC), absorption (ABS/CS), quantum yield of electron transport (ΨE_0), chlorophyll content, and PI_{ABS} particularly at ripening, suggesting that plant stress responses could also depend on the initial foliar acclimation to kaolin treatment (Dinis et al. 2016b, Lobos et al. 2015). Nevertheless, the down-regulation of VvHSP70 and VvWRKY18 gene expression observed in treated grapevines, mainly in TF, reinforces the protective role of kaolin against summer stress since most heat shock protein groups are up-regulated under stressful conditions and a lower need to activate stress tolerance mechanisms (Kobayashi et al. 2010).

At the **Alentejo site**, the association between chlorophyll fluorescence, carotenoids, gas exchange, and chlorophyll data indicates the existence of complex and dynamic stress responses, particularly during the midday period. In 2017, from *veraison* to ripening, kaolin

boosted the accumulation of chlorophylls in both varieties, highlighting its protective role in chlorophyll degradation (for more details see Chapter 5.2). Besides, kaolin treatment showed a positive effect on the Chl_(a+b)/Car ratio exclusively in TN variety, as previously observed in kaolin-treated grapevines and olive leaves subjected to reduced irrigation levels (Brito et al. 2018a, Shellie and King 2013), promoting in a higher quantum yield efficiency (Φ_{PSII}) (Ruban 2014). Likewise, both kaolin treated varieties showed higher Φ_{PSII} at ripening than their control groups in 2017, and higher q_P values in TN_K, which is likely due to lower thermal dissipation of excessive energy (for more details see Chapter 4). Indeed, NPQ decreased in TN and TF kaolin treated grapevines at ripening, indicating better plant growth and development conditions under Mediterranean summer stress conditions. Interestingly, at veraison, both qP and NPQ decreased in TF kaolin treated vines, suggesting that photochemical processes and NPQ relaxation levels can also be influenced by other factors, such as stomatal conductance, photosynthetic rates, and photosynthetic pigments accumulation (Demmig-Adams et al. 2014). Therefore, this unlikely result denotes that light acclimation features putatively promoted by kaolin coating should be carefully explored, as they seem to depend at least on the stress magnitude, species and varieties under study, as well as kaolin application timing and concentration (Ferrari et al. 2017). Regarding the modulating effect of kaolin on hormonal accumulation, there were no significant differences observed in TN variety in 2017, whereas TF kaolin treated plants showed lowered IAA content and increased SA at ripening, suggesting a possible defence signal to reduce greater damage to the photosynthetic machinery (Gururani et al. 2015) (for more details see Chapter 3). In agreement, the analysis of fluorescence transients revealed lower absorption (ABS/RC) and trapping (TR_0/RC) energy per RC in TF sprayed vines than control ones at ripening. Besides, kaolin treatment increased carbon assimilation rates, stomatal conductance and P_N/g_s in both varieties in growing seasons, indicating that kaolin protective functions can also be noticed in less hot years at Alentejo. This finding contrasts with the ones obtained in the Douro vineyard, possibly due to the different row orientation of the studied vineyards, among other factors, since the E-W orientation of the Douro vineyard suggests higher midday sunlight canopy exposition compared to the N-S orientation of the Alentejo vineyard.

In line with the findings of Frioni *et al.* (2020), V_x , Z_x , and $V_xA_xZ_x$ pool increased in kaolin treated vines in 2017, mainly in TN, which was previously reported in high light acclimated plants, in parallel with an increase in NPQ capacity (Brugnoli *et al.* 1994, 1998). In spite of the greater Z_x accumulation, one would expect increased NPQ values in treated leaves, which was

not observed in this location. This outcome contributes to the long-lasting debate regarding xanthophyll photoprotective direct function as a quencher of excitation energy or indirect function as a modulator of energy dissipation (Havaux and Niyogi 1999, Horton *et al.* 2000, Ruban and Horton 1999). Moreover, Kress and Jahns (2017) also found that the extent of NPQ was independent of Z_x accumulation with increasing light intensity, suggesting an indirect role of Z_x in long-lasting NPQ processes, which might explain the discrepancy found between NPQ and Zx content at the Alentejo site. Kaolin treated leaves at this site showed higher V_x and Z_x accumulation and lower NPQ levels without compromising photochemical processes, suggesting that kaolin treatment boosted the plasticity of grapevine summer stress responses by managing the xanthophyll cycle accumulation and limiting the down-regulation of photochemistry. Additionally, the relative *VvPSY1* gene expression was similar among treatments and varieties, indicating that kaolin treatment had no effect on the first rate limiting-step of the carotenoid biosynthetic pathway (Rodriguez-Concepcion *et al.* 2018).

Overall, *VvWRKY18* was mainly down-regulated in treated leaves from TN, and upregulated in TF at ripening, indicating different varietal responses to kaolin treatment that could depend on intrinsic varietal features (*e.g.* phenology and leaf senescence) and stress severity and extent (Bernardo *et al.* 2018, Carvalho *et al.* 2015a). Nevertheless, the use of particle film technology in Mediterranean-type climate vineyards lowers the need for triggering heat stress tolerance mechanisms through a reduction in *VvHSP70* gene expression found in both sampling years. Besides, *VvNCEDs* gene expression was generally lower in both varieties at the Alentejo site, particularly in the 2018 growing season, supporting the hypothesis that acclimated plants can limit non-essential cellular responses under moderate stress conditions (Larkindale and Vierling 2008).

Managing grapevine berry ripening with environmental fluctuations under field conditions can be a challenging task. Still, particle film technology has shown beneficial effects on several fruit quality traits, improving the synthesis of secondary metabolites and sugar accumulation throughout ripening (Conde *et al.* 2016, Coniberti *et al.* 2013, Dinis *et al.* 2016a). **In the Douro site**, the results revealed that the berries from kaolin sprayed vines displayed lower soluble sugars content, particularly in 2017, slight preservation of berry acidity, mainly tartaric acid, with no significant effects on pH, as recently pointed by Luzio *et al.* (2021) (for more details see **Chapter 6.1**). Overall, kaolin application limited total ROS accumulation in both varieties and growing seasons, in accordance with other studies performed by our group (Dinis *et al.* 2016a, 2018b). Interestingly, H_2O_2 levels were positively influenced by kaolin coating in TN, suggesting greater oxidative stress signalling, which can improve the regulation of several biological processes in grapevines (*e.g.* synthesis of heat shock proteins and anthocyanins) (Carvalho *et al.* 2015b). Earlier studies on kaolin effects in TN berries have related lower H₂O₂ and lipid peroxidation levels with decreased proline accumulation (Bernardo *et al.* 2017). However, this study does not associate such trends in both varieties, suggesting the presence of other factors that could influence proline content over ripening, such as sugar accumulation (Kavi Kishor and Sreenivasulu 2014). On the other hand, kaolin treatment triggered the non-enzymatic antioxidant defence of berries, namely total phenols, anthocyanins, tannins, and *ortho*-diphenols content, as shown in previous works (Dinis *et al.* 2016a, Luzio *et al.* 2021). Besides, radical scavenging activity of fruit extracts was also higher in treated plants, mainly in 2017, indicating a close interplay between climate variability and kaolin effectiveness, being stronger under severe environmental conditions.

One of the expected impacts of climate variability in temperate climate regions is the earlier onset of several phenological stages (Leolini *et al.* 2020). Indeed, Costa *et al.* (2019) predicted an earlier phenophase timing for TF than TN, which could partly explain the varietal differences between varieties and kaolin effects on the berry hormonal content, mainly in 2017. Hormonal content and accumulation throughout berry ripening lean on many environmental and biotic factors, with antagonistic roles in ripening-related processes (Coelho *et al.* 2019, Kuhn *et al.* 2013). While ABA is considered a ripening promoter, IAA and SA are known to delay this process (Deytieux-Belleau *et al.* 2007, Lo'ay 2019). Since berries from TF treated vines showed lower ABA and SA content at *veraison* and higher SA at harvest in 2017, it seems plausible that kaolin application could have a ripening delaying effect in TF in warmer years, which was not so noticed in TN, despite presenting lower ABA content at harvest in the following growing season.

The assessment of berry hormonal balance at the **Alentejo** vineyard showed an increase in ABA, SA, and IAA content in TF treated vines at ripening in 2017, suggesting a prompt stress response and hormonal modulation under adverse environmental conditions (Peleg and Blumwald 2011). This stress modulating effect was also noticed in the following growing season, particularly in berries from TN kaolin treated vines, by increasing SA content and decreasing ABA accumulation at ripening (for more details see **Chapter 6.2**). However, this response did not lead to significant changes in most fruit quality traits in 2018. On the contrary, under the quite stressful conditions of 2017, kaolin treatment decreased soluble sugars content in TF, while tartaric acid increased in both varieties only at *veraison*, suggesting an improved

kaolin efficiency on preserving berry oenological potential under severe summer stress conditions (Dinis *et al.* 2020).

Phenolic compounds play an essential role in grape quality potential due to their significant contribution to wine colour and aroma, with antioxidant functions affected by environmental factors and management practices (Sharma *et al.* 2019). In 2017, while no consistent response was found for total phenols and anthocyanins accumulation throughout ripening, flavonoids, *ortho*-diphenols, and tannins levels were mainly increased in kaolin treated vines at ripening. In the following growing season, flavonoids, anthocyanins, and tannins increased in the berries of TF treated vines, highlighting that climate is the paramount factor in shaping ripening related processes and fruit quality potential (Carvalho *et al.* 2015a, 2015b). Nonetheless, the phenolic compounds decay from *veraison* to ripening occurred on a lesser extent in both treated varieties, indicating an optimised response to oxidative stress signals (Kuhn *et al.* 2013). Indeed, the radical scavenging activity was higher during the 2017 summer season in the berries of treated grapevines, which was also recently described in rainfed olive orchards (Brito *et al.* 2018b), indicating kaolin treatment as a suitable strategy to mitigate the adverse summer stress conditions of some Mediterranean-like climate areas.

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CHAPTER 8

FINAL REMARKS AND FUTURE PERSPECTIVES

The kaolin foliar application to Touriga-Franca and Touriga-Nacional varieties over two consecutive growing seasons in distinct locations (Douro and Alentejo) highlighted its role in modulating the extent to which grapevine can promote abiotic stress responses and acclimation in two different vineyards with similar mesoclimatic Mediterranean characteristics. The main outcomes of this thesis project are summarised below:

- Weather conditions play a primary role in triggering kaolin effectiveness, plant stress responses, and acclimation strategies in applied contexts. As so, this project allowed studying plant stress responses and acclimation mechanisms pragmatically and reveals the complexity of studying adult plants in commercial vineyards;
- 2. The kaolin foliar application improved the intrinsic water use efficiency and net carbon assimilation of both grapevine varieties in both locations, thus preventing water restraint and leading to improved plant growth and development;
- 3. Kaolin foliar application improved grapevine ability to deal with prolonged periods of summer stress, optimising their capacity to control light absorption respective use efficiency. Besides, kaolin treatment before *veraison* relieved the impact of summer stress on grapevines by managing pigment accumulation, modulating the expression of key genes of the xanthophyll cycle, and also through a combination of improving light-harvesting processes and decreasing the thermal dissipation of excess energy;
- Kaolin treated leaves showed lower ABA accumulation, reducing the investment in ABA signalling associated with gene expression, which was endorsed by increasing summer stress conditions;
- 5. Kaolin application in Mediterranean climate-type wine regions could be a suitable strategy to modulate berry ripening under adverse environmental conditions beyond its described effects in improving the vines' physiological performance. Kaolin foliar spraying enhanced berry quality traits, secondary metabolites, antiradical activity, and modulated hormonal content in both varieties and vineyards, with a possible ripening delaying effect under severe summer stress conditions.

Considering the aforementioned outcomes, future research on eco-friendly foliar protectants under controlled and open-field scenarios should be extended to the wine industry to elucidate the advantages of particle-film application on improving berry quality and winemaking performance. Besides, merging several management practices throughout the growing cycle, such as canopy and soil management practices, could also provide an accurate frame of plant stress responses and resilience and the potential benefits of using short-term climate change adaptation strategies.

Despite the increasing research on grapevine environmental stresses, we still require more information regarding how plants, micro-organisms, and pathogens will respond to an increase in CO_2 concentration, temperature and water shortage. Therefore, it is crucial to coordinate efforts through multidisciplinary programs and knowledge transfer to develop and maintain cultivars and clone collections and management techniques or strategies, which will define our capacity to adapt to climate change.

In summary, as *terroir* defines each wine-growing region, understanding the interrelation between contextual factors (physical, environmental, social, and economic) and climate change at local and regional scales should the first approach to identify and prioritise sustainable adaptations strategies.