

Optimising grapevine summer stress responses and hormonal balance by applying kaolin in two Portuguese Demarcated Regions

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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 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 ABA-responsive genes (*VvHVA22a* and *VvSnRK2.6*) mainly in TF, indicating different varietal responses to kaolin application under fluctuating periods of summer stress.

KEYWORDS

Abcisic acid, high light, induced resistance, NCED gene, photosynthesis, stomatal conductance, sunburn

Supplementary data can be downloaded through: <https://oenone.eu/article/view/4502>

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 (Moutinho-Pereira *et al.*, 2004; Jones *et al.*, 2005; 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 (Peleg and Blumwald, 2011; Bernardo *et al.*, 2018; Balfagón *et al.*, 2020).

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; Gomez-Cadenas *et al.*, 2015; Dinis *et al.*, 2018a). However, antagonistic reports indicate no correlation between ABA accumulation and stomatal closure in plants subjected to combined abiotic stresses (Zandalinas *et al.*, 2016; Balfagón *et al.*, 2019). 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 (Deluc *et al.*, 2009; Balint and Reynolds, 2013; Niculcea *et al.*, 2013). In grapevines, for example, Soar *et al.* (2006) 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 (Wu *et al.*, 2016; Jia *et al.*, 2017).

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 (Dinis *et al.*, 2016b; Dinis *et al.*, 2016a; Brito *et al.*, 2019a). 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 (Duchene, 2016; Ollat *et al.*, 2017), 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.

MATERIALS AND METHODS

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. 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.

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 (Winkler *et al.*, 1974; Jones *et al.*, 2010).

4. Leaf gas exchange

Leaf gas exchange was evaluated using a portable infrared gas analyser (LCpro+, 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 , $\mu\text{mol m}^{-2} \text{s}^{-1}$), stomatal conductance (g_s , $\text{mmol m}^{-2} \text{s}^{-1}$), transpiration rate (E , $\text{mmol m}^{-2} \text{s}^{-1}$), and the ratio of intercellular to atmospheric CO_2 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).

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).

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). [²H₆]-ABA (Sigma-Aldrich, USA), [²H₂]-IAA (Sigma-Aldrich, USA), and [¹³C₆]-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 μL of water : methanol (9:1), filtered through 0.22 μm PTFE filters, and directly injected into an UPLC system (Waters[™] Acquity SDS, Waters Corporation, 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 \times 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 $\mu\text{L min}^{-1}$. Results were processed using Masslynx[™] v4.1 software, and the phytohormone contents were obtained using a calibration curve prepared with commercial standards.

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 Fisher 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 Fisher 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 in Supplementary Table 1 (ST1). The amplification was performed via a reaction comprising 1 μL of cDNA, 5 μL of Maxima[™] SYBR[™] Green/ROX qPCR mix (Thermo Fisher 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. G_SVIVT00000988001), *VvNCED2* (Phytozome accession no. G_SVIVT01021507001), *VvNCED3* (Phytozome accession no. G_SVIVT01038080001), *VvHVA22a* (Phytozome accession no. G_SVIVT01012547001), and *VvSnRK2.6* (Phytozome accession no. G_SVIVT01009074001) was obtained using the Relative Expression Software Tool Solver v.2 (REST-MCS) (Pfaffl, 2001; Pfaffl, 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.

8. Statistical analysis

Statistical analyses of leaf gas exchange parameters, soluble sugars, and phytohormone content were performed using a SigmaPlot™ 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.

RESULTS

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).

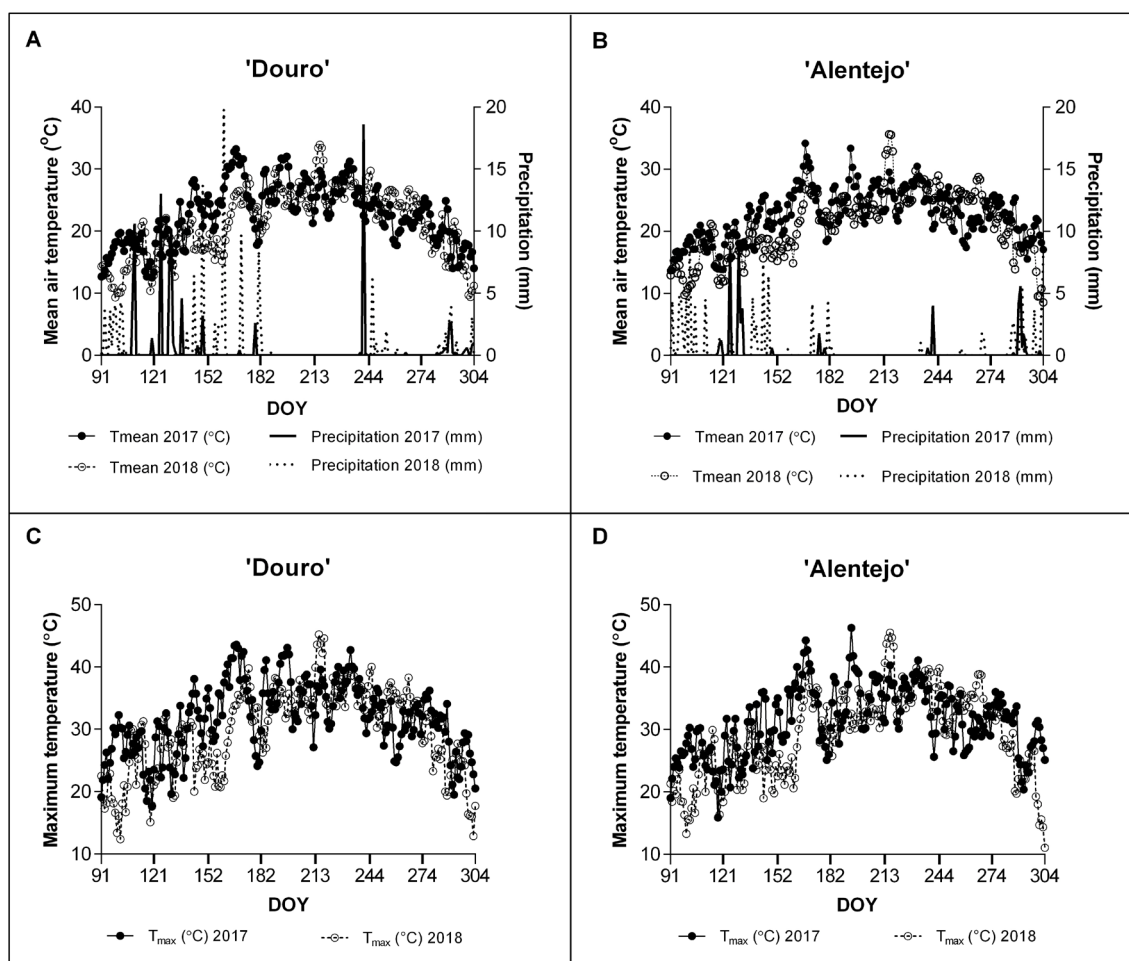


FIGURE 1. Daily mean air temperature (°C), precipitation (mm) and maximum temperature (°C) of 2017 and 2018 growing seasons in both 'Douro' and 'Alentejo'.

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).

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 (DOY 192–197), 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).

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. 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.

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.

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.

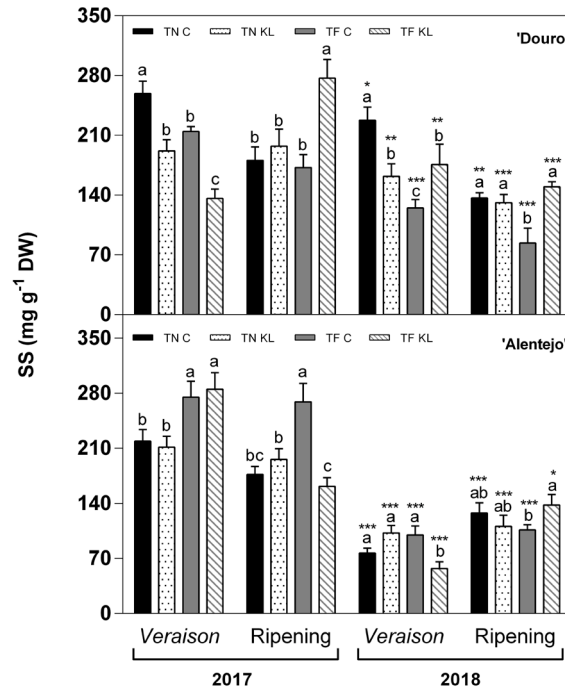


FIGURE 2. Leaf total soluble sugar (SS) content in the ‘Douro’ and ‘Alentejo’ grapevine leaves (Touriga-Nacional control - TN_C and kaolin – TN_KL; Touriga-Franca control – TF_C and kaolin – TF_KL) at 2017 and 2018 *veraison* and ripening stages.

Data are mean ± 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.

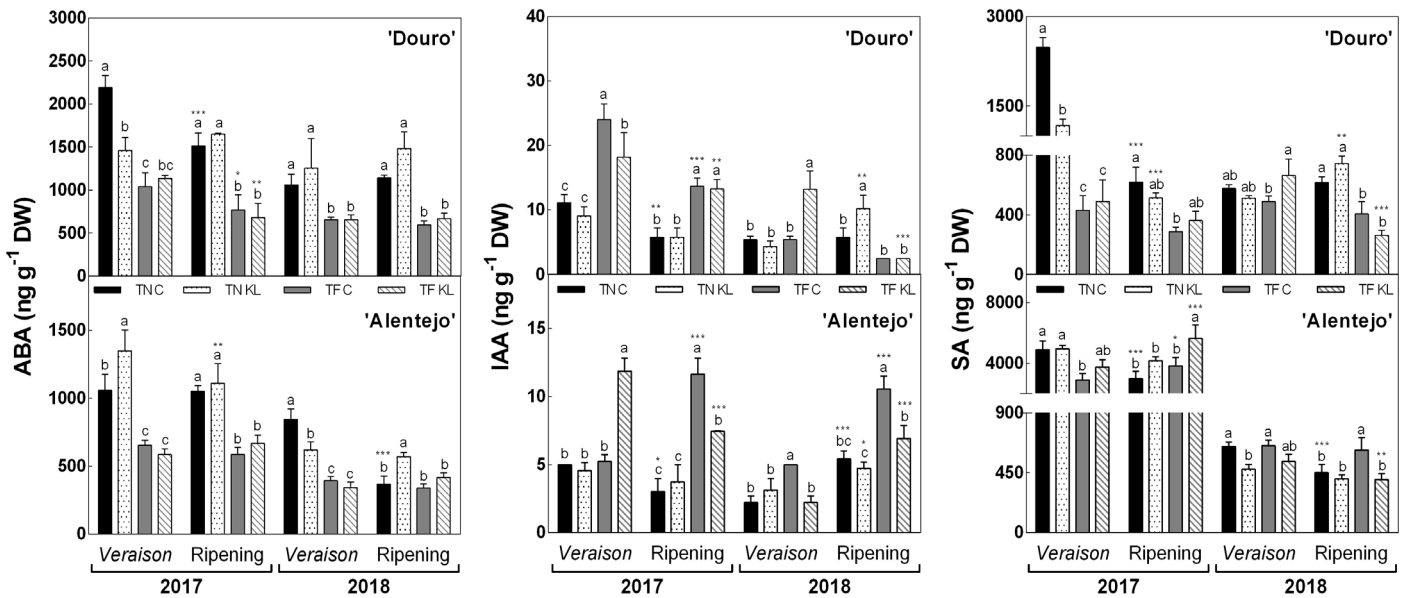


FIGURE 3. Phytohormones (abscisic acid - ABA, salicylic acid – SA, and indole-3-acetic acid - IAA) content in the ‘Douro’ and ‘Alentejo’ grapevine leaves (Touriga-Nacional control - TN_C and kaolin – TN_KL; Touriga-Franca control – TF_C and kaolin – TF_KL) throughout 2017 and 2018 summer seasons.

Data are mean ± 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.

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.

Leaf measurements	09:00 GTM+1						14:00 GTM+1								
	g_s	P_N	P_N/g_s	C_i/C_a	E	g_s	P_N	P_N/g_s	C_i/C_a	E	g_s	P_N	P_N/g_s	C_i/C_a	E
	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 ^c	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 ± 1.6 ^a	72.3 ± 6.7 ^b	0.635 ± 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 ^c	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 ^c	68.5 ± 4.9 ^{ab}	0.669 ± 0.020 ^b	1.81 ± 0.17 ^c	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 ^c	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 ^c	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 ^c	6.55 ± 0.47 ^b	50.9 ± 3.7	0.748 ± 0.015	2.46 ± 0.21					
TF_KL	257.5 ± 23.6 ^{b***}	13.5 ± 1.3 ^{ab***}	52.5 ± 3.3 ^{a***}	0.714 ± 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}					

Stomatal conductance (g_s , $\text{mmol m}^{-2} \text{s}^{-1}$), net CO_2 assimilation rate (P_N , $\mu\text{mol m}^{-2} \text{s}^{-1}$), intrinsic water use efficiency (P_N/g_s , $\mu\text{mol mol}^{-1}$), ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a), and transpiration rate (E, $\text{mmol m}^{-2} \text{s}^{-1}$). 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.

TABLE 2. Leaf gas exchange values for Touriga-Nacional with no kaolin (control - TN_C) and with kaolin (TN_KL) and for the 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.

Time:	09:00 GTM+1						14:00 GTM+1					
	Leaf measurements	g _s	P _N	P _N /g _s	C _i /C _a	E	g _s	P _N	P _N /g _s	C _i /C _a	E	
		Veraison 2017										
TN_C	172.6 ± 4.7 ^b	8.43 ± 0.41 ^c	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 ^c	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 ^c	67.1 ± 4.0 ^{ab}	0.653 ± 0.018	3.71 ± 0.17 ^a		
TF_C	180.7 ± 5.4 ^c	9.22 ± 0.70 ^c	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 ± 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 ± 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 ± 7.5 ^{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 ± 4.2 [*]	0.676 ± 0.018	3.69 ± 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 ^c	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		

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 (C_i/C_a), 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.

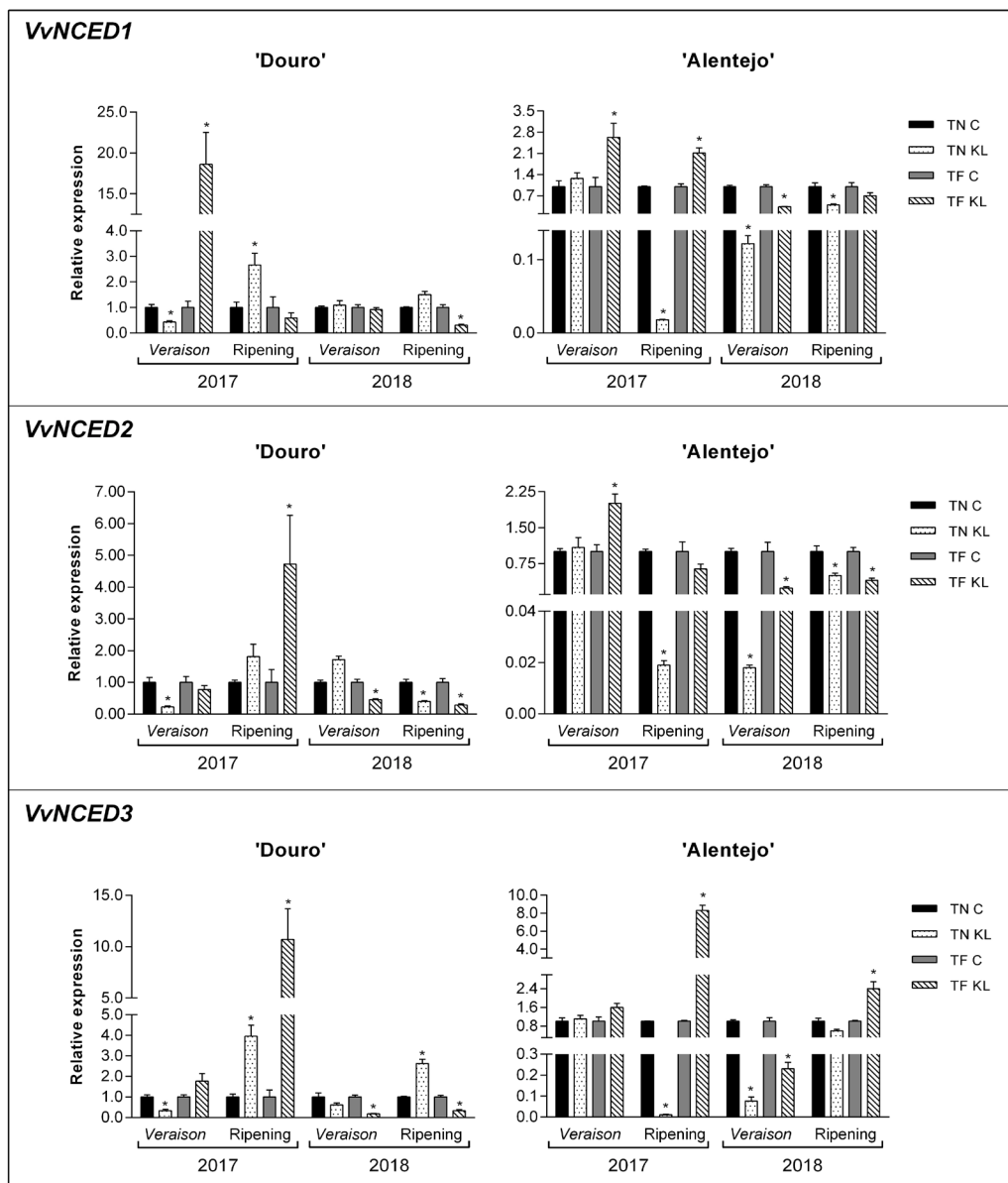


FIGURE 4. Relative expression of *VvNCED1*, *VvNCED2*, and *VvNCED3* genes of TN and TF grapevine leaves (Touriga-Nacional control - TN_C and kaolin – TN_KL; Touriga-Franca control – TF_C and kaolin – TF_KL) at ‘Douro’ and ‘Alentejo’ throughout the 2017 and 2018 summer season.

* denote significant difference between control and kaolin treated vines of each variety within the same developmental stage (*veraison* or ripening).

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’.

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.

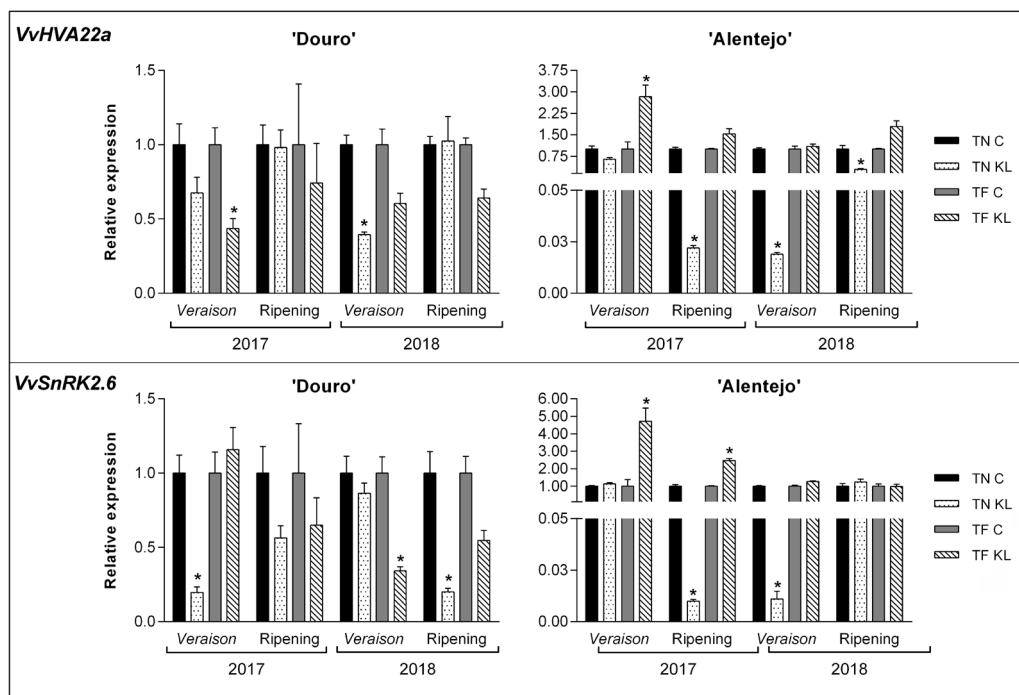


FIGURE 5. Relative expression of VvHVA22a, and VvSnRK2.6 genes of grapevine leaves (Touriga-Nacional control - TN_C and kaolin – TN_KL; Touriga-Franca control – TF_C and kaolin – TF_KL) at ‘Douro’ and ‘Alentejo’ throughout the summer season.

* denote significant difference between control and kaolin treated vines of each variety within the same developmental stage (*veraison* or *ripening*).

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 (Figure 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.*, 2019b) 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 ripening, which was previously shown to be linked to increasing photosynthetic rates, and reserve mobilisation and export (Sami *et al.*, 2016; Brito *et al.*, 2019b). Furthermore, the lower leaf SS content found in TF kaolin-treated leaves located in ‘Alentejo’ 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 (Figure 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 & Blumwald 2011). Generally, kaolin application decreased ABA, IAA, and SA accumulation in 2017 in ‘Douro’, indicating a prompt response to summer stress under adverse environmental conditions. In the equivalent period, 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, indicating that under non-limiting stress factors, kaolin plants may boost plant 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 (Zhang *et al.*, 2009; Pilati *et al.*, 2017). 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.

CONCLUSION

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 strategies under applied contexts. Furthermore, kaolin-treated leaves showed lower ABA accumulation, reducing the investment in ABA signalling associated with gene expression, which was triggered by increasing summer stress conditions.

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