Uncovering the effects of kaolin on balancing berry phytohormones and quality attributes of *Vitis vinifera* grown in warm-temperate climate regions

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Abstract

BACKGROUND: 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 jeopardize 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 dryness index) and the warmest (2705 °C growing degree days) of the study.

RESULTS: In 2017, TF kaolin-treated berries showed lower salicylic acid (−26.6% compared with unsprayed vines) and abscisic acid (ABA) (−10.5%) accumulation at veraison, whereas salicylic acid increased up to 28.8% at harvest. In a less hot season, TN and TF kaolin-treated grapevines showed a twofold in ABA content and a threefold increase in the indole-3-acetic acid content at veraison and lower ABA levels (83.8%) compared with unsprayed vines at harvest. Treated berries showed a decreased sugar content, without compromising malic and tartaric acid levels, and reactive oxygen species accumulation throughout berry ripening.

CONCLUSION: 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.

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Keywords: abscisic acid; antioxidants; berry ripening; indole-3-acetic acid; redox homeostasis; salicylic acid

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.1-4 In those regions, the summer season is characterized by prolonged periods of high light, high temperature, and water scarcity, challenging winegrowers to sustain grapevine production in warmer and drier conditions.5,6 Driven by increased air temperature and changes in rainfall patterns, earlier harvest dates have been reported in southern European wine-growing regions, along with higher berry sugar contents, lower acidity, and suboptimal phenolic maturation.7,8

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.7 However, plants display several defence strategies under limiting environmental conditions to trigger stress resilience, such as the synthesis of radical-scavenging compounds.8 In addition, hormonal changes followed by secondary metabolite production can coordinate the berry ripening process,
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, ultraviolet (UV) B radiation, and water availability.\(^{10}\) Grape ripening onset is usually characterized 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.\(^{6,11}\) 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.\(^{12,13}\)

The foliar application of kaolin particle film is a well-known short-term strategy applied in many fruit crops that increases the reflection of UV, photosynthetically active, and infrared radiation, reducing leaf temperature and preventing leaf and fruit sunburn damage.\(^{14}\) 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.\(^{15-19}\) For example, Brillante et al.\(^{20}\) reported reduced photosynthetic rates and stomatal conductance in kaolin-treated cv. Cabernet Sauvignon 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.\(^{15-17}\) Under these conditions, kaolin application in the Douro region showed positive effects on vine physiological performance and sucrose concentration and transport in leaves while reducing susceptibility to photoinhibition.\(^{16,21-23}\) 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.\(^{24,25}\) This response was subsequently related to bottlenecks on the carotenoid biosynthetic pathway that leads to ABA biosynthesis in water-stressed cv. Sangiovese leaves.\(^{26,27}\)

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.\(^{26,28-33}\) 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.

**MATERIAL AND METHODS**

**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 of 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 eight buds left per vine. The vines were managed according to the growers’ commercial organic practices (use of organic fertilizers, 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,\(^ {34}\) the soil mapping in this region is classified as Luvisols, characterized by a clay-enriched subsoil. The region is characterized by a warm-temperate climate with dry and hot summers and rainfall periods mostly concentrated in the winter months.\(^ {35}\) An automatic weather station was installed in the vineyard to record standard meteorological variables, such as air temperature and precipitation (Fig. 1). To characterize 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),\(^{36}\) and another related to the level of potential soil water availability (dryness index, DI)\(^{37}\) (Table 1). According to the classifications of Tonietto and Carbonneau\(^{37}\) and Jones et al.\(^{36}\) the 2017 growing season was considered the driest (−147.1 DI) and the warmest (2705 °C GDD) of the study.

**Plant material, treatments, and sampling**

Two *Vitis vinifera* L. red varieties were selected, TF and TN, owing 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
The tartaric and malic acid levels were measured at room temperature. The dry residue was then resuspended in a centrifuge concentrator (Speed Vac; Jouan, Saint Herblain, France) at 40 °C, with matching records of ET0 and precipitation to the vineyard environment showed an RH of 45.3%, Tmax of 35.7 °C, and Tmean 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 lyophilization procedure. The fruits were then divided into three groups of 100 berries each, lyophilized (SCANVAC 55-4 Pro; LaboGene, Lynge, Denmark) for 120 h, ground to a 100 mm particle size; Macherey-Nagel GmbH, Düren, Germany) at 40 °C until the lyophilization process at 45 °C for 30 min. Absorbance was then recorded at 750 nm. For the evaluation of ortho- and para-diphenols, and flavonoids were measured in triplicate, collected per variety, treatment, and developmental stage. Total acidity and pH were analyzed following the OIV international methods of wine and must analysis. The tartaric and malic acid levels were measured enzymatically, using an automated clinical chemistry analyser (Miura One, TDI, Barcelona, Spain).

Phenolic composition and radical scavenging activity
Phenolic compounds were extracted as previously described by Mendes Lemos et al. Total phenols, ortho-diphenols, and flavonoids were measured in triplicate, following the methods adapted by Gouvinhas et al. 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 incubation at 45 °C for 30 min. Absorbance was then recorded at 750 nm. For the evaluation of ortho-diphenols, a colorimetric 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 colorimetric response of both total phenols and ortho-diphenols measurements was compared with a standard curve based on gallic acid, and the results are expressed as milligrams gallic acid equivalents per gram 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 mode, using nitrogen as a drying and nebulizer 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. with a few modifications. [1H3]-ABA, [1H7]-IAA, and [12C6]-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; R2 = 1), IAA (y = 25.624x−0.0601; R2 = 0.9994), and SA (y = 4.4612x−0.0597; R2 = 0.9999).

Berry quality traits
Soluble sugars (SS) were extracted by heating 10 mg lyophilized 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. 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 colorimetric response was compared with a standard curve based on glucose, and total SS is expressed as milligrams per gram dry weight (DW).

The physical-chemical parameters of grapes were assessed in 100 fresh berries with triplicates, collected per variety, treatment, and developmental stage. Total acidity and pH were analyzed following the OIV international methods of wine and must analysis. The tartaric and malic acid levels were measured enzymatically, using an automated clinical chemistry analyser (Miura One, TDI, Barcelona, Spain).

Table 1. Dryness index (DI, mm) and growing degree days (GDD, °C) values for the 2017 and 2018 growing seasons in the vineyard site

<table>
<thead>
<tr>
<th>Season</th>
<th>DI</th>
<th>Class</th>
<th>Class limits</th>
<th>GDD</th>
<th>Class</th>
<th>Class limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>2017</td>
<td>−147.1</td>
<td>Very dry</td>
<td>≤−100</td>
<td>2705</td>
<td>Too hot</td>
<td>&gt;2700</td>
</tr>
<tr>
<td>2018</td>
<td>−47.0</td>
<td>Moderately dry</td>
<td>≤50 to &gt;−100</td>
<td>2416</td>
<td>Very warm</td>
<td>2222–2700</td>
</tr>
</tbody>
</table>

The DI and GDD classes were considered according to Tonietto and Carbonneau and Jones et al. respectively.
The flavonoid content was determined in a reaction mixture containing 24 μL fruit extract, 28 μL sodium nitrite (50 g L⁻¹), 28 μL aluminium chloride (100 g L⁻¹), and 120 μL sodium hydroxide (1.0 mol L⁻¹). The absorbance was immediately recorded at 510 nm, and the results are expressed as milligrams catechin equivalents per gram DW.

Analysis of total monomeric anthocyanins was performed using a pH differential method. At two distinct pH values (pH 1.0 and 4.0) to two distinct pH values (pH 1.0 and 4.0) to two distinct pH values (pH 1.0 and 4.0) to two distinct pH values (pH 1.0 and 4.0) to two distinct pH values (pH 1.0 and 4.0), the extraction was performed by adding 3.0 mL of sample extract. After 1 h incubation at 37 °C, 1.0 mL trichloroacetic acid (TCA; 2.8%) and 1.0 mL thiobarbituric acid (1%) were added to a duplicate for each sample was prepared without the presence of EDTA, and a blank was prepared with phosphate buffer instead of TCA. The supernatant (200 μL) was then transferred to a 96-well microplate, incubated for 20 min protected from light, and the absorbance then read at 350 nm. The results were calculated based on a standard curve of H₂O₂ and are expressed as micromoles H₂O₂ per gram DW.

ROS were assessed according to Kong et al. with a 2',7'-dichlorofluorescein diacetate (DCFH-DA) solution (25 mM L⁻¹), prepared in dimethyl sulfoxide immediately before use. Briefly, fruit extracts (20 μL) were loaded into a small-well microplate containing 0.2 mL phosphate-buffered saline (pH 7.4) and 12 μmol L⁻¹ DCFH-DA and incubated for 20 min at 25 °C. Fluorescence was measured at 485 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 nanomoles dichlorofluorescein per milligram protein.

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 test. Subsequently, statistical differences between developmental stages (veraison vs harvest) within each sampling group were evaluated by one-way ANOVA, followed by the post hoc Tukey test. For the specific case of OH• scavenging activity, IC₅₀ values were obtained using Prism 6 (GraphPad Software, San Diego, CA, USA). 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 Statistics 22.0 software (IBM Corp., Armonk, NY, USA). 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.

RESULTS

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 temperature 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.

**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. In addition, 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 this effect was exclusively observed in TN (45.6%) in 2018 at harvest. In general, TF displayed higher SA levels than TN did 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 the SA levels at harvest increased about 28.8%. In the following growing season, TF_K berries showed an opposite trend.

**Fruit quality traits**

The analysis of several oenological variables revealed that kaolin-treated fruit showed lower total SS than their respective control groups did 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, whereas it was almost 50% lower in TN in 2018 compared with TF. In 2018, the overall sugar content was also lower at harvest than in 2017. By contrast, total acidity and tartaric and malic acid levels were higher in 2018 than in 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.

**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 did 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.

**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. In addition, 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 than in 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. Anti-radical activity showed that the kaolin effects were only observed by the ABTS method. Kaolin promoted different antioxidant responses in both varieties by increasing the antiradical activity in TN berries and decreasing the TF response, mainly at veraison. In addition, there was higher antioxidant activity in 2018 than in 2017 in the two varieties, treatments, and developmental stages.

**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 the two grapevine varieties, we performed a PCA (Fig. 4) and a MANOVA (Table 4) for each growing season. The PCA data refer to the two varieties, treatments, and developmental stages. In 2017 (Fig. 4(a), (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. 4(a)). 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. 4(b)) 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. 4(c), (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. Similar 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.

**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**

<table>
<thead>
<tr>
<th>Season</th>
<th>Stage</th>
<th>Soluble sugars (mg g⁻¹ dry weight)</th>
<th>Total acidity (g L⁻¹ tartaric acid)</th>
<th>Tartaric acid (g L⁻¹)</th>
<th>Malic acid (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Kaolin</td>
<td>Control</td>
<td>Kaolin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2017</td>
<td>Veraison</td>
<td>697.78 ± 33.52 a</td>
<td>579.97 ± 15.36 b</td>
<td>639.11 ± 17.50 a</td>
<td>546.96 ± 31.83 b</td>
</tr>
<tr>
<td>Harvest</td>
<td></td>
<td>785.11 ± 67.56 bc***</td>
<td>713.71 ± 54.55 c***</td>
<td>876.20 ± 25.98 a***</td>
<td>804.42 ± 29.76 ab***</td>
</tr>
<tr>
<td>2018</td>
<td>Veraison</td>
<td>354.36 ± 27.00 a</td>
<td>367.94 ± 9.97 a</td>
<td>161.36 ± 18.48 b</td>
<td>140.72 ± 14.22 b</td>
</tr>
<tr>
<td>Harvest</td>
<td></td>
<td>509.83 ± 18.34 ab***</td>
<td>494.75 ± 18.90 bc***</td>
<td>544.63 ± 7.09 a***</td>
<td>474.17 ± 22.29 c***</td>
</tr>
<tr>
<td>2017</td>
<td>Veraison</td>
<td>3.16 ± 0.03 a</td>
<td>3.17 ± 0.03 a</td>
<td>3.01 ± 0.03 b</td>
<td>2.97 ± 0.03 b</td>
</tr>
<tr>
<td>Harvest</td>
<td></td>
<td>3.91 ± 0.04 a***</td>
<td>3.86 ± 0.02 a***</td>
<td>3.76 ± 0.08 b**</td>
<td>3.73 ± 0.04 b***</td>
</tr>
<tr>
<td>2018</td>
<td>Veraison</td>
<td>2.65 ± 0.17 a</td>
<td>2.73 ± 0.04 a</td>
<td>2.49 ± 0.01 b</td>
<td>2.43 ± 0.06 b</td>
</tr>
<tr>
<td>Harvest</td>
<td></td>
<td>3.52 ± 0.04 a***</td>
<td>3.49 ± 0.05 ab***</td>
<td>3.35 ± 0.02 b**</td>
<td>3.38 ± 0.03 b**</td>
</tr>
<tr>
<td>2017</td>
<td>Veraison</td>
<td>6.63 ± 0.09 b</td>
<td>6.31 ± 0.32 b</td>
<td>10.52 ± 0.47 a</td>
<td>11.15 ± 0.59 a</td>
</tr>
<tr>
<td>Harvest</td>
<td></td>
<td>3.80 ± 0.16 b***</td>
<td>3.75 ± 0.08 b***</td>
<td>4.35 ± 0.15 ab***</td>
<td>4.53 ± 0.10 a***</td>
</tr>
<tr>
<td>2018</td>
<td>Veraison</td>
<td>14.68 ± 1.51 c</td>
<td>13.25 ± 1.21 c</td>
<td>29.35 ± 0.58 b</td>
<td>33.78 ± 1.28 a</td>
</tr>
<tr>
<td>Harvest</td>
<td></td>
<td>4.19 ± 0.09 ***</td>
<td>4.15 ± 0.21 ***</td>
<td>4.70 ± 0.06 ***</td>
<td>5.03 ± 0.44 ***</td>
</tr>
<tr>
<td>2017</td>
<td>Veraison</td>
<td>4.63 ± 0.13 b</td>
<td>4.77 ± 0.08 b</td>
<td>6.83 ± 0.06 a</td>
<td>6.93 ± 0.35 a</td>
</tr>
<tr>
<td>Harvest</td>
<td></td>
<td>2.48 ± 0.20 b***</td>
<td>2.31 ± 0.02 b***</td>
<td>2.88 ± 0.13 a***</td>
<td>3.00 ± 0.18 a***</td>
</tr>
<tr>
<td>2018</td>
<td>Veraison</td>
<td>7.56 ± 0.72 c</td>
<td>6.70 ± 0.53 c</td>
<td>15.23 ± 0.50 b</td>
<td>17.53 ± 0.76 a</td>
</tr>
<tr>
<td>Harvest</td>
<td></td>
<td>2.63 ± 0.24 ***</td>
<td>2.61 ± 0.06 ***</td>
<td>3.05 ± 0.26 b**</td>
<td>3.01 ± 0.02 b**</td>
</tr>
<tr>
<td>2017</td>
<td>Veraison</td>
<td>1.49 ± 0.12 b</td>
<td>1.22 ± 0.08 b</td>
<td>3.05 ± 0.13 a</td>
<td>3.16 ± 0.14 a</td>
</tr>
<tr>
<td>Harvest</td>
<td></td>
<td>1.09 ± 0.09 *</td>
<td>1.12 ± 0.08</td>
<td>1.12 ± 0.06 ***</td>
<td>1.10 ± 0.09 ***</td>
</tr>
<tr>
<td>2018</td>
<td>Veraison</td>
<td>6.31 ± 0.64 c</td>
<td>5.50 ± 0.20 c</td>
<td>12.67 ± 0.21 b</td>
<td>14.57 ± 0.55 a</td>
</tr>
<tr>
<td>Harvest</td>
<td></td>
<td>1.06 ± 0.06 ***</td>
<td>1.04 ± 0.08 ***</td>
<td>0.86 ± 0.02 ***</td>
<td>0.93 ± 0.09 ***</td>
</tr>
</tbody>
</table>

Data are mean plus/minus standard deviation (n = 6). Different lower-case letters represent significant differences between treatments and varieties within each stage (veraison and harvest) and sampling year. **P < 0.01, *P < 0.05** and *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.
The bioclimatic index data indicate that the 2017 growing season was the warmer (2705 °C GDD) and the drier (40 °C) than in 2018. Therefore, the differences regarding stress severity found between growing seasons might influence the effectiveness of kaolin in balancing berry ripening and potential quality 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, because the accumulation of several stress-related metabolites and the hormonal content differed among varieties.

The predicted 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 pheno-ology and berry ripening, compromising yield and the sustainability of the wine sector. The bioclimatic index data indicate that the 2017 growing season was the warmer (2705 °C GDD) and the drier (40 °C) than in 2018. Therefore, the differences regarding stress severity found between growing seasons might influence the effectiveness of kaolin in balancing berry ripening and potential quality 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, because the accumulation of several stress-related metabolites and the hormonal content differed among varieties.

### DISCUSSION

The predicted 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. The bioclimatic index data indicate that the 2017 growing season was the warmer (2705 °C GDD) and the drier (40 °C) than in 2018. Therefore, the differences regarding stress severity found between growing seasons might influence the effectiveness of kaolin in balancing berry ripening and potential quality 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, because the accumulation of several stress-related metabolites and the hormonal content differed among varieties.

One of the expected impacts of climate variability in temperate climate regions is the earlier onset of several phenological stages. Indeed, Costa et al. 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. ABA, IAA, and SA play antagonistic roles on ripening-related processes. Whereas ABA is considered a ripening promoter, IAA and SA are known to delay this process. 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
In agreement, the results revealed that kaolin application limited the total ROS content in both varieties and growing seasons, data that are in agreement with previous findings for TN.28 Besides, H$_2$O$_2$ 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.7,12 Earlier studies on the effects of kaolin in TN berries have related lower H$_2$O$_2$ and TBARS content with decreased proline accumulation.64 However, the present study does not associate such trends in both varieties, suggesting that H$_2$O$_2$, TBARS and proline accumulation might be varietal and climate dependent, with other factors influencing proline content over grape ripening, such as sugar accumulation.8,65

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 contents, as shown in previous studies.29,64 The latter displays an important role in radical stability, as recently reported in other Portuguese grapevine varieties30,42 and olive fruit.58 Radical-scavenging 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. 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. 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, 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 antiradical 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 (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, than in TF at veraison, indicating a varietal sensitivity for ripening onset, which is likely advanced in TN under the current settings. In addition, 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.

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: (a,b) 2017; (c,d) 2018. ABA: abscisic acid; ABTS: 2,2-azino-bis(3-ethylbenzothiazoline)-6 sulfonic acid; IAA: indole-3-acetic acid; ROS: reactive oxygen species; SA: salicylic acid; T.Phenols: total phenols.
Table 4. Results (F-values) of multivariate analysis of variance using Pillai’s trace test for the effects of the variety (V), treatment (T), developmental stage (S), and their interactions on phytohormones (abscisic acid, indole-3-acetic acid, salicylic acid), antioxidant defence parameters (total phenols, flavonoids, ortho-diphenols, 2,2-azino-bis(3-ethylbenzothiazoline)-6 sulfonic acid), and quality traits (soluble sugars, anthocyanins, tannins) in the 2017 and 2018 growing seasons.

<table>
<thead>
<tr>
<th></th>
<th>2017</th>
<th>2018</th>
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<tr>
<td><strong>Phytohormones</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>2270.52*</td>
<td>1011.31*</td>
</tr>
<tr>
<td>T</td>
<td>69.75*</td>
<td>2225.84*</td>
</tr>
<tr>
<td>S</td>
<td>3683.41*</td>
<td>1897.77*</td>
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<tr>
<td>V x T</td>
<td>166.04*</td>
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<td>691.13*</td>
<td>1143.21*</td>
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<tr>
<td>T x S</td>
<td>298.94*</td>
<td>874.70*</td>
</tr>
<tr>
<td>V x T x S</td>
<td>58.87*</td>
<td>619.43*</td>
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<tr>
<td><strong>Antioxidant defence</strong></td>
<td></td>
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</tr>
<tr>
<td>V</td>
<td>822.29*</td>
<td>2312.00*</td>
</tr>
<tr>
<td>T</td>
<td>53.25*</td>
<td>32.33*</td>
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<tr>
<td>S</td>
<td>1577.71*</td>
<td>570.47*</td>
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<td>21.91*</td>
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<tr>
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<td>950.56*</td>
<td>3140.34*</td>
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<tr>
<td>T x S</td>
<td>12.76*</td>
<td>27.07*</td>
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<tr>
<td>V x T x S</td>
<td>10.46*</td>
<td>46.78*</td>
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<tr>
<td><strong>Quality attributes</strong></td>
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<tr>
<td>V</td>
<td>1578.88*</td>
<td>2498.67*</td>
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<tr>
<td>T</td>
<td>35.09*</td>
<td>59.61*</td>
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<tr>
<td>S</td>
<td>2843.57*</td>
<td>2875.85*</td>
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<td>V x T</td>
<td>1.847</td>
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<td>V x S</td>
<td>1471.15*</td>
<td>2550.59*</td>
</tr>
<tr>
<td>T x S</td>
<td>23.49*</td>
<td>40.22*</td>
</tr>
<tr>
<td>V x T x S</td>
<td>2.82</td>
<td>49.07*</td>
</tr>
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* P < 0.001.

CONCLUSION

The present study suggests that kaolin application in wine regions with a Mediterranean-type climate could be a low-cost strategy (approximately €50 per hectare) to modulate berry ripening under adverse environmental conditions, beyond its described effects on improving vine 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 and 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. In addition, kaolin treatment enhanced berry quality traits and antiradical activity in both varieties under study. From a climate change perspective, comparative studies should be performed under controlled and field conditions and followed in 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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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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