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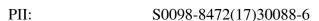
Kaolin particle film application lowers oxidative damage and DNA methylation on grapevine (*Vitis vinifera* L.)

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Highlights

1. Kaolin protects grapevine plant against oxidative damage.

2. Kaolin causes the global DNA demethylation.

3. Promising nature of kaolin application as summer stress mitigation strategy.

Abstract

The exogenous kaolin application in grapevine has shown a great potential as summer stress mitigation strategy because it positively impacts fruit quality as a result of many molecular and biochemical changes. In the present study we wanted to address the hypothesis that the observed improved antioxidant capacity could also result from a more efficient enzymatic antioxidant system response. For that purpose, the enzymatic activity of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APx), glutathione reductase (GR) and glutathione peroxidase (GPx) were measured in kaolin treated plants, and correlated to some biomarkers of oxidative status, including hydrogen peroxide (H_2O_2), total thiobarbituric acid reactive substances (TBARS) and proline. Also, to assess if kaolin particle film could mediate epigenetic modifications, the levels of DNA methylation (5-methylcytosine) were assessed in leaf tissues through a stereological analysis. Results showed that the activity of the ascorbate-glutathione cycle key enzymes was boosted in kaolin treated plants, which translated into a less oxidative damage in leaf and berry tissues (lower H_2O_2 levels and lipid peroxidation). Results also showed that the methylation of DNA, which seems to be stimulated by the harsh environmental conditions, was decreased in leaves from kaolin treated plants.

1. Introduction

Drought, elevated air temperature, and high evaporative demand are increasingly frequent during summer in grape growing areas like the Mediterranean basin, limiting grapevine productivity and berry quality. The foliar exogenous application of kaolin, a radiation-reflecting inert mineral, has proven effective in mitigating the negative impacts of these abiotic stresses in grapevine and other fruit crops (Conde et al., 2016; Glenn et al., 2010; Shellie, 2015; Song et al., 2012). Under the current climate changing scenario, the mean temperature is projected to increase up to 4°C by the end of this century (IPCC, 2007), being important to evaluate potentially different impacts of this abiotic factor, and others, on grapevine productivity in Douro Region (Ferreira et al., 2012; Moutinho-Pereira et al., 2004).

Considering the above concerns, previous studies indicated that heat, water deficit and high photosynthetic photon flux density (PPFD) and UV-B levels are well known to disturb cellular redox homeostasis (Krasensky and Jonak, 2012; Mittler, 2006). In fact, under adverse environmental conditions, unrestrained accumulation of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) were recruited in different cellular components (Ahmad et al., 2010; Mittler, 2002) and may lead to oxidative stress when excess of ROS are produced. In a recent work, grapevines treated with kaolin have been correlated with a reduction of ROS in berries and leaves, reinforcing the mitigation of adverse abiotic climatic stresses (Dinis et al., 2016b). The imbalance of ROS alters membrane fluidity and permeability, leading to the denaturation and inactivation of some enzymes, degradation of proteins, bleaching of pigments and disruption of DNA strands, which culminates in programmed cell death (Apel and Hirt, 2004; Vacca et al., 2004). In order to counteract ROS and alleviate oxidative damage, plants have several

enzymatic and non-enzymatic systems, the later including lipophilic membrane associated antioxidants and hydrophilic reductants (Ozden et al., 2009). Antioxidants are an integrative participant in the ROS scavenging pathways, such as the water-water cycle in the chloroplast and the ascorbate-glutathione cycle in the chloroplast, mitochondria, peroxisomes, apoplast and the cytosol (Slooten et al., 1995). Among the non-enzymatic antioxidants, proline works like an electron sink mechanism and it has also been suggested that it functions as a cellular osmotic regulator between the cytosol and vacuole. Moreover, proline can detoxify ROS and promote membrane protection and stabilization of antioxidant enzymes (Ozden et al., 2009; Sharma and Dietz, 2006).

Beyond the improvement of antioxidant activity in leaves and berries in this species (Dinis et al., 2016), environmental stresses induce also genetic (Conde et al., 2016) and epigenetic changes that trigger DNA methylation (Correia et al., 2016; Meijon et al., 2009; Schellenbaum et al., 2008; This et al., 2007). DNA methylation is a well characterized indicator of epigenetic responses to biotic and abiotic factors (Feil and Fraga, 2012; Madlung and Comai, 2004; Mirbahai and Chipman, 2014). Altered DNA methylation is associated with changes in gene expression and signal transduction, being a well characterized indicator of epigenetic responses (Bräutigam et al., 2013; Kinoshita and Seki, 2014; Valledor et al., 2007). However, there is relatively few information concerning alterations in DNA methylation following exposure of plants to environmental stress with a chemically inert mineral with excellent reflective properties, such as kaolin.

In this conceptual framework, although it has been reported that environmental factors influence grapevine productivity, how epigenetic mechanisms such as DNA methylation and antioxidant enzymatic mechanisms may interfere with vine tissues is poorly studied. Thus, the purpose of the present study was to fulfill the identified gap of

knowledge by research how environmental stresses may disrupt biochemical and epigenetic levels in leaves and/or berries after a kaolin treatment. For this purpose, enzymatic activities alterations (superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APx), glutathione reductase (GR) and glutathione peroxidase (GPx) were quantified. The oxidative damage through the quantification of hydrogen peroxide (H_2O_2) and total thiobarbituric acid reactive substances (TBARS) was also evaluated, likewise, a signaling molecule (proline) synthetized as a response to abiotic stress. On the other hand, in attempting to explore the effects of kaolin on DNA methylation dynamics in grapevine leaves, global 5-methylcytosine (5-mdC) was also assessed by immunohistochemistry and quantified through a stereological analysis.

Keywords: Antioxidative enzymes, hydrogen peroxide, lipid peroxidation, epigenetic marks

2. Material and methods

2.1. Experimental design

Samples were obtained from *Vitis vinifera* L. (Touriga Nacional cv.), grafted on 110 R rootstock, located in a commercial vineyard "Quinta do Vallado" in the Douro valley located at Peso da Régua (41º09'44.5"N 07º45'58.2"W), in northern Portugal. The climate is typically Mediterranean-like, with a warm-temperate climate and dry and hot summers (Kottek et al., 2006), with higher precipitation during the winter months and very low during the summer. According to the World reference base for soil resources 2014 (FAO, 2015) the soil mapping of the region is classified as dystric technosols taking in account their acidity and deep modification by human actions. Vines were managed without irrigation and grown using standard cultural practices as applied by

commercial farmers. Two exposure conditions were set up: an experimental control and another pulverised (17th July 2014) with 5 % (w/v) kaolin (Surround WP; Engelhard Corp., Iselin, NJ), both in three vineyard rows (n = 20 per row and treatment). To ensure the same edaphoclimatic conditions, all rows are located side-by-side on a steep hill with an N-S orientation. The 7-year old vines, were trained to unilateral cordon and the spurs were pruned to two nodes each with 10-12 nodes per vine. A schematic representation of the experiment procedure is presented in Fig. 1.

For the biochemical procedure, mature leaves that were well exposed to solar radiation (n = 6/ per row and treatment) were sampled in three different dates after pulverization: one week (23rd July), one month (21st August) and one month and half (3rd September). Berry samples were randomly collected from different positions in the clusters and in the vine (n = 200 per row and treatment) in two different dates: 28th August and 12th September (close to harvest) (Fig. 1). Samples (leaves and fruits) were frozen in liquid nitrogen and stored at −80 °C, posteriorly lyophilized for 48 h and converted to a fine dried powder in two sub-samples per row (n = 6/per treatment) before analysis.

For the immunohistochemistry procedure, leaves were collected at 21st August (n = 3/per row and treatment) and fixed according to Meijón (Meijon et al., 2009) with some modifications. The tissues were fixed in Phosphate Buffered Saline (PBS) with 4% (w/v) paraformaldehyde containing mercaptoethanol and later stored in PBS containing 0.1% (w/v) paraformaldehyde at 4 ºC until further use.

2.2. Quantification of TBARS and H_2O_2 concentrations

The oxidative damage to lipids in grapevine leaf and fruit extracts was quantified as previously described (Dinis et al., 2014). Briefly, the lyophilized samples were frozen in

liquid nitrogen and ground in 20% (w/v) trichloroacetic acid with mortar and pestle. The absorbance of the supernatant was measured at 532 nm, with subtraction of the value measured as non-specific absorption at 600 nm. TBARS concentration was expressed in terms of mg g^{-1} dry weight (DW), using an extinction coefficient of 155 M cm^{-1} (Costa et al., 2002).

Hydrogen peroxide (H_2O_2) concentration was measured following the method of Ozden et al. (2009). Leaf and fruit lyophilized extracts were ground in 1% (w/v) trichloroacetic acid (Rosati et al., 2006). The supernatant was added to 0.5 ml of 10 mM K-phosphate buffer (pH 7.0) and 1.0 ml of 1 M KI. The absorbance of the reaction mixture was measured at 350 nm and the H_2O_2 concentration was calculated from a standard calibration curve (up to 1000 µmol ml^{-1}) and expressed as µmol g^{-1} DW.

2.3. Proline concentration

Proline quantification was determined according to Bates et al. (1973). The lyophilized extracts of leaf and fruit samples were homogenized in 3% (w/v) sulphosalicylic acid (SSA) and the mixture was centrifuged for 10 min at 10,000 × g after filtration through a Whatman filter paper. Thereafter, 1 ml of each supernatant was mixed with 1 ml of glacial acetic acid and 1 ml of freshly prepared acid–ninhydrin solution. The mixtures were incubated for 1 hour in a water bath at 100 ºC and then cooled in an ice bath to 0 ºC. Afterwards, 4 ml of toluene were added to each previous mixture and vortexed for 15 s. The toluene phase was then carefully collected and absorbance was measured at 520 nm in a spectrophotometer using toluene as a blank. The proline concentration was calculated using a standard curve.

2.4. Extraction of antioxidant enzymes and determination of enzymatic activities

Antioxidant enzymes were extracted according to Papadakis et al. (2001). The lyophilized samples were homogenized with an Ultra Turrax RZR1 (Heidolph) at 20,000 rpm/4 ºC. The extraction buffer contained 0.2 M of Tris-HCl (pH 8.0), 5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10% (w/v) glycerol, 0.25% (w/v) Triton X-100, and 2% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). Each extract was centrifuged for 30 min at 40,000 × g, and the supernatants aliquoted and frozen at -80 °C. Protein determination was performed according to Bradford (1976). Prior to the ascorbate peroxidase (APx) enzymatic assay, 1 mM of ascorbate was added to the protein extract. All enzymatic assays were performed at saturating substrate concentrations to ensure the determination of the maximal velocities.

SOD (EC 1.15.1.1) activity determination was performed by the WST (2–(4–iodophenyl– 3–(4–nitrophenyl)–5–(2,4–disulphophenyl)–2H–tetrazolium, monosodium salt) reduction method, with the SOD assay kit WST (19160–1KT–F, SIGMA). The ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) was followed spectrophotometrically at 440 nm.

CAT (CAT; EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 at 240 nm (Chance and Maehly, 1955) (extinction coefficient 0.0436 mM^{-1} cm^{-1}) for 2 min. Ascorbate peroxidase (EC 1.11.1.11) activity was assayed by monitoring the decrease in absorbance at 290 nm for 2 min (extinction coefficient 2.8 mM^{-1} cm^{-1}) (Rao et al., 1995).

GR (GR; EC 1.8.1.7) activity was measured according the method described by Foyer and Halliwell (Foyer and Halliwell, 1976). The reaction mixture (final volume 2 mL) contained 0.1 ml of protein extract, 1 mM EDTA, 1 mM glutathione, 0.05 mM

NADPH and 100 mM buffer adjusted to pH 7.5 with HC1. The decrease in absorbance at 340 nm due to the oxidation of NADPH to NADP+ was observed and determined.

GPx (GPx; EC 1.11.1.9) activity assay was performed according to Flohe and Gunzler with some modifications (Flohe and Gunzler, 1984). For that, the consecutive glutathione reductase reaction was measured and monitored by the oxidation of NADPH at 340 nm for 2 min. The reaction mixture contained phosphate buffer (pH 7), 10 mM GSH, GSH reductase (0.24 U), 12 mM TBHP, 10 mM NADPH and sample in a final volume of 2 ml.

2.5. Immunohistochemistry

After fixation (referred in 2.1), samples were introduced in a cryostat medium (Tissue-Tek, Killik; Sakura Finetek USA, Inc., Torrance, CA, USA) and were frozen at -23 ºC to posteriorly monitor global 5-methylcytosine (5-mdC) distribution. Leaf sections of 50 μm were cut with a sliding cryotome CM1510S (2002 Leica Microsystems, Wetzlar, Germany), collected on slides and conserved at -20 ºC until the analysis (Correia et al., 2013). Sections were immersed in ascending and descending 25, 50, 75 and 100% ethanol series (5 minutes each one) and finally for 5 minutes in PBS buffer. Before incubating overnight with the primary antibody (anti-5-mdC mouse antibody; Eurogentec, Cat. no. BI-MECY-0100) diluted 1/50 in 1% bovine serum albumin (BSA, Sigma), slides were pre-treated with 10% BSA for 10 min to reduce non-specific binding. For negative controls the primary antibody was omitted and replaced by 1% of BSA. After washing (2 times, 10 minutes each) in PBS buffer with tween (Phosphate Buffered Saline + 0.1 % (v/v) Tween-20-phosphate-buffered saline, pH 7.2), sections were incubated for 1 h in darkness with Alexa Fluor 488-labelled anti-mouse polyclonal antibody (Molecular Probes, Cat. no. A-11001) diluted 1/25 as the secondary antibody

anti-mouse. Afterwards, slides were rinsed in PBS buffer with tween (2 times, 5 minutes each). Finally, the slides were counterstained with DAPI (4',6- diamidino-2-phenylindole; Fluka) during 20 minutes. Sections were washed in MilliQ water (2 times, 5 minutes each), and coverslipped using the mounting media Mowiol (Sigma–Aldrich Co., St. Louis, MO, USA). Fluorescence was visualized using a confocal microscope (Leica TCS-SP2-AOBS) connected to a workstation and the images were processed with Fiji Software.

2.6. Global DNA methylation quantification by stereological analysis

The volume density (V_V) is defined as the percentage of the total volume, of a well defined reference space, occupied by any given component within it. To determine the V_V (%) of the cells presenting methylation (5-mdC) stained within the leaf (the present reference space), a classical stereological technique was performed based on point counting (Freere and Weibel, 1968; Parkhurst, 1982) using the following formula:

V_V (structure, reference) = [P(structure) × 100] ÷ [k × P(FAO)]

where P(structure) is the number of test points within each structural component, P(FAO) is the total number of test points lying over the reference space (leaf), and k is the ratio between the number of points on the grid used for the structure of interest and for the reference space.

Counting was done using the images captured with the confocal microscope. The stereological test grids used had two sets of points, which were previously established depending on the frequency and size of the targeted compartment, and were super-imposed on the live image of the monitor. A 1:4 grid was used, with a total of 48 coarse points targeting the reference space, and 192 points targeting all immunohistochemistry staining of global methylation antibody.

Point counting was made in the systematically sampled fields working with the 40×
Olympus UIS-2 objective lens (Olympus Co., Ltd., Tokyo, Japan). The first field of
vision was randomly selected and the following fields were systematically sampled by
stepwise movements of the stage in the x- and y-directions (220 μm × 170 μm),
covering the complete section area of the leaf in order to count all
immunohistochemically stained cells.

2.7. Statistics

Statistical analyses were performed with Sigma-Plot 12.0 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
months and treatments were evaluated by two-way factorial ANOVA, followed by the
post hoc Tukey's test. Significant differences were considered for $p<0.05$.
Measurements were carried out in samples collected in three different dates after
pulverization: at 23th July (one week), 21st August (one month) and 3rd September (one
month and half) for leaves and 28th (veraison) and 12th September (maturation) for
fruits. Values are presented in Figure 2 and 3 as mean ± standard deviation (SD),
n=6/per row for leaves and n=200/per row for fruits. Statistical analysis was performed
using a two-way factorial ANOVA. Different lower case letters represent significant
differences between the season (July, August and September) within the same treatment
($p < 0.05$) and *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ represent significant differences
between treatments (control *versus* kaolin) within the same month. Absence of
superscript indicates no significant difference between treatments.

3. Results and discussion

3.1. Kaolin application protects leaves and fruits against oxidative damage

The effects of kaolin application on TBARS and H_2O_2 concentrations in grapevine leaf and fruit extracts are shown in Table 1. The leaves shown lower lipid peroxidation levels than grape berries, and this difference was more pronounced in the control. In August and September TBARS levels in leaf tissues from kaolin treated plants were lower than in control leaves, and the difference was even higher between berries from control and kaolin treated plants. The addition of kaolin appeared to activate the antioxidant systems to reduce the peroxidation extent compared to the control. It is known that lipid peroxidation can originate cellular damage by reacting with other lipids, proteins and nucleic acids (Almeras et al., 2003) and that kaolin application may prevent membrane injury in some plants (Beis and Patakas, 2012; Mobin and Khan, 2007). In all treatments, the H_2O_2 concentration in the leaves was higher than that in the fruits during all the experimental period. In august, the addition of kaolin only significantly reduced the berry H_2O_2 concentration; however, in September, it can remarkably reduce the leaf and berry H_2O_2 concentration. Depending on the intensity and duration of the stress, H_2O_2 display two distinct roles in plants, acting as a signal molecule involved in the acclimation process at low concentrations or, at high concentrations, it can lead to programmed cell death due to its involvement on the Fenton-Haber-Weiss reactions (Gill and Tuteja, 2010). The reaction's product is the highly reactive hydroxyl radical, which in turn reacts with all biological molecules.

Proline concentration was much higher in fruits than in leaves and kaolin significantly reduced the proline concentration being more evident in September (38% reduction) (Figure 2). Stressful conditions results in an overproduction of proline, that also performs as a radical scavenger against oxidative damage and as a signaling molecule, maintaining a sustainable environment for plants growth under certain

boundaries (Hayat et al., 2012; Kisgor K. et al., 2014; Rizhsky et al., 2004). In grapevines treated with kaolin, especially in berries, the reduced levels of TBARS and H_2O_2 correlated with lower amount proline was in agreement with previous reports (Carvalho et al., 2015; Lin and Kao, 1998; Upadhyaya et al., 2007). In fact, these studies showed that the increase of proline concentration with higher H_2O_2 concentrations and lipid peroxidation, suggesting a crosstalk between proline and H_2O_2 (Carvalho et al., 2015; Lin and Kao, 1998; Upadhyaya et al., 2007). It has been reported that plants exposed to various abiotic stresses show an increase in TBARS concentration due to ROS production (Dinis et al., 2016), indicating that kaolin application may prevent membrane injury (Beis and Patakas, 2012; Mobin and Khan, 2007).

3.2. Kaolin exogenous application boosts enzymatic defences

The assessment of CAT, SOD, APx, GR and GPx activities in leaf and berry extracts are shown in Fig. 3 (A, B, C, D and E, respectively). Kaolin treated plants exhibited higher CAT and SOD activity (Fig. 3A and 3B) than control ones throughout the experimental period. At the final ripening stage (September), kaolin treated leaves had an increased activity of 42% and 22%, for CAT and SOD respectively. From July to September, CAT activity (Fig. 3A) increased 56% in control samples and 71% for kaolin ones, demonstrating the stimulating effects of kaolin exogenous application on the enzymatic antioxidant defences. SOD activity (Fig. 3B) in leaves presented a similar tendency, being the enzyme which activity was more accentuated in leaves from kaolin treated plants, increasing 57% from the beginning of the study (July) up to the end of the experimental period. The fruits, from both treatment, presents a slight decrease in CAT activity from August until the harvest date, still, kaolin plants presenting 62%

more activity than control ones. On the contrary, SOD activity in berries revealed an opposite trend, exhibiting an increasing pattern from the first until the last sampling date. However, when compared to control grapevines, kaolin treated plants showed 24% more activity at harvest. The balance between ROS formation and consumption is tightly controlled by non-enzymatic and enzymatic antioxidants, as well as by developmental stages since the fruit ripening process also generates a certain amount of oxidative stress (Masia et al., 1998). Previous studies done in other species reported that the natural reaction of ROS production in stress conditions, triggers the antioxidant machinery in which SOD and CAT are more effective than other antioxidant enzymes in influencing the patterns of fruit ripening (Singh et al., 2012). Despite the fact that of grapevines were continuously exposed to summer stress, kaolin application showed protective effects, allowing greater conditions for plants to counteract the oxidative damage and enhanced the enzyme-mediated antioxidative systems in plants.

Generally, APx activity was higher on kaolin treated grapevines (Fig. 3C). Significant differences were found in leaves after kaolin application, exhibiting an increase of 92% in APx activity compared to control in the first month of the experiment. Overall the assay, kaolin berries also feature higher APx activity than control, being significant at the harvest date. In August, fruits obtained from kaolin treated plants had 55% more activity comparatively to control ones. The results showed a decrease in APx activity for kaolin treated fruits from August until the harvest date (27%), while the ones from control did not registered major alterations in enzymatic activity. The APx is a crucial enzyme of the antioxidant machinery, displaying higher affinity for H_2O_2 detoxification than CAT, though was found to be more sensitive in its response to oxidative stress (Blokhina et al., 2003; Payton et al., 2001). Some studies regarding plant defence systems against abiotic stresses suggest that APx activation

resulted from an increase in H_2O_2 concentration (Ozden et al., 2009). Thus, there is an opposite correlation between APx activity and H_2O_2 concentration, which seems similar to the effects observed in the grapevines treated with kaolin particle film. Beyond the possible crosstalk between APx activity and H_2O_2 levels, Balla *et al.* (2009) demonstrated that the enhanced activity of APx results in greater tolerance to heat stress, showing the efficiency and importance of the glutathione – ascorbate cycle (Balla et al., 2009; Lee et al., 2007; Pang and Wang, 2010). Since the improvement of stress tolerance is often related to enhancement of antioxidant enzymes activity in plants, our study points that kaolin boosts grapevine performance and growth, as well as protection against oxidative damage (Ma et al., 2008). Data collected could also indicate that kaolin treated grapevines are better prepared to develop tolerance mechanisms against summer stress.

In the ascorbate – glutathione cycle, GPx uses H_2O_2 to form the oxidized glutathione (GSSH), which is then cycled back to the reduced form (GSH) by GR (Suzuki et al., 2012). GR and GPx activities for both leaf and berry extracts are shown in Fig. 3D and 3E, respectively. In leaf tissues, the results indicate the higher activity of GR than GPx, suggesting the positive performance of the ascorbate – glutathione cycle, since glutathione also participate as an electron donor for dehydroascorbate reductase (DHAR) activity, which contributes in the regeneration of ascorbate pool (Foyer and Noctor, 2011). Despite of having lower H_2O_2 concentrations, kaolin leaf extracts exhibited higher GR and GPx activities, mainly in September, with an increase in 32% of GR activity compared to control. GR also plays a fundamental role in the antioxidative system by regenerating the glutathione pool in the ascorbate - glutathione cycle, indicating that the accumulation of GSH causes an increase in GR activity, which trigger stress tolerance in plants (Asada, 2006; Foyer and Noctor, 2005). As in our

study, it has also been reported that during heat stress, GR activity increased in some plant species (Contour-Ansel et al., 2006; Hernandez et al., 2001). From August to September, the results showed a decrease in 46% of GPx activity in kaolin treated leaf extracts. However, as described above, data relating to APx activity suggest an efficient response to summer stress. Treated plants with kaolin exhibited higher levels of GR and GPx activities when compared to control ones, suggesting that control plants are not able to respond in the same terms to stress factors as kaolin treated grapevines. After kaolin pulverization (July), the differences observed between control and kaolin for GPx and GR activities of leaf extracts were slightly significant, specifically for GR. Nevertheless, fruit extracts demonstrated an opposite trend, i.e. higher GPx activity instead of GR. In berry extracts, though GR activity did not reached significant values during overall the experimental period, significant differences can be observed in GPx activity, mainly at the harvest date, with 82% less activity in extracts from kaolin treated berry comparatively to control. The increased H_2O_2 levels, the reduced APx activity in control fruit extracts, as well as the ascorbate levels (data shown in Dinis et al. 2016a), point to an imbalanced role of GR activity in the control grapevines of our study. Despite the higher GR activity in control fruit extracts, our study suggests that one cannot endure the necessary ascorbate pool to greatly sustain the ascorbate – glutathione cycle performance. Previous studies reported that heat stress stimulate the enzymatic antioxidant machinery (CAT, SOD, APx and GR), showing a coupled relation between reduced oxidative damage and elevated levels of enzymatic and non enzymatic antioxidants. So, it reveals that the up regulation of the antioxidant defence systems could influence stress tolerance in plants (Hasanuzzaman et al., 2013). The levels of H_2O_2, APx and ascorbate indicate that kaolin extracts are more available to greatly respond in stress conditions, supporting the role of the antioxidant defence

system in conferring summer stress tolerance (Dinis et al., 2016). The fact that lower levels of H_2O_2 were observed, in parallel with the increase of antioxidant enzyme activities strongly suggests that this increase is not due to greater stressful conditions and cellular impairment, but instead that it indeed was stimulated somehow by kaolin and played a probable role in the increased tolerance of kaolin treated plants to the deleterious effects of summer stress. A model for the biochemical pathways involved is proposed in Figure 4 based in our results, supported by the recent knowledge (white arrows). Under summer stress kaolin exogenous application boosts the antioxidant defense systems in fruit and leaf extracts (green arrow) compared to grapevines without this particle film (blue arrow). Leaf and fruit extracts converge on the activation of the same antioxidant defense, except for GPx and GR pathways which are inversely proportional in berry ones (Fig. 4).

3.3. Changes of the global level of DNA methylation under Kaolin application

Positive labelling of DNA methylation estimated by global 5-methylcytosine (5-mdC) in grapevine leaves were detected one month after kaolin pulverization (Fig. 5). 5-mdC signal is mainly detected in palisade cells of mesophyll both in control and kaolin treatment. The presence of these immunopositive cells, identified by their green fluorescing staining, clearly indicate occurrence of DNA methylation and the relative volumes of these cells were also quantified. Overall, the global relative volumes of DNA methylation were distinct among control and kaolin, being significantly decreased from 30.8% to 11.7% in leaves (p = 0.024) after exposure to kaolin treatment (Fig. 5 A, C and D, F to kaolin and control leaves, respectively). These results indicate that kaolin seems to induce the demethylathion of DNA when compared to the methylation status of the control group.

DNA methylation is a well characterized indicator of epigenetic responses of biotic and abiotic factors (Feil and Fraga, 2012; Madlung and Comai, 2004; Mirbahai and Chipman, 2014). Abiotic stresses, such as unfavorable temperature, are known to induce negative effects on growth and development of plants modulating a host of genetically programmed responses (Conde et al., 2016; Madlung and Comai, 2004). The role of epigenetic mechanisms, such as DNA methylation in many genes and histone acetylation were clearly crucial in acclimation and survival of plants to high temperatures, such as *Quercus suber* L. (cork oak) (Correia et al., 2013). For instance, it was reported that higher temperature resulted in several occasions and models results hypermethylation, whereas lower temperature resulted in the demethylation of DNA extracted from *Antirrhinum majus* (snapdragon) (Hashida et al., 2003). DNA methylation and demethylation in response to abiotic stresses is however variable, depending on the genes it affects, on the abiotic stress ocurred (as well as its intensity), and on the cells, organs and plants subjected to the stress. Our results also suggested that high temperature and solar radiation with low water availability are associated to the hypermethylation of DNA as we can see in control grapevines leaves (Fig. 5F), which are subjected to these extreme conditions (Dinis et al., 2016b). Oppositely, as kaolin improves leaf reflectance in the infrared and ultraviolet region, this reduces heat and light stress in plants (Dinis et al., 2016b) and seems to induce the global demethylation of DNA. This demethylation indicates a potential reduction of stress effects in the exposed group. Additional studies to investigate a possible correlation between enzymatic and molecular results are necessary to determine if demethylation is somehow associated/deactivation of biochemical pathways in grapevines involved in the synthesis of important compounds of an organoleptic standpoint, as phenylpropanoid and flavonoid pathways previously reported by Conde et al 2016, or

even involved in stress-responsive mechanisms. Importantly, environmental conditions induce epigenetic changes that can be imprinted in genes, so DNA methylation could generate novel and heritable phenotypic variations in grapevine, such as heat tolerance, which can be useful under the current climate changing scenario.

4. Conclusions

Kaolin exogenous application boosts the antioxidant defense systems in grapevines exposed to summer stress, by increasing enzymatic activities in fruits, which leads to a decrease in ROS production and oxidative damage. Interestingly, the present study suggested also that kaolin might cause the global DNA demethylation and consequently the regulation of transcriptional changes on genes associated to the DNA methylation/demethylation. Overall, the results reinforce the promising nature of kaolin application as summer stress mitigation strategy and opens new research questions in the molecular field that deserves to be explored.

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Figure 1. Experimental design overview and timeline. Grapevines (*Vitis vinifera*) were exposed to a pre-veraison kaolin (5%) application up to maturation harvest.

Figure 2. Proline concentration in leaf and fruit extracts in response to kaolin exogenous application in grapevine.

Figure 3. Enzymatic activities in leaf and fruit extracts in response to kaolin exogenous application in grapevine: **A** – Catalase (CAT); **B** – superoxide dismutase (SOD); **C** – ascorbate peroxidase (APx); **D** – glutathione reductase (Gr) and **E** – glutathione peroxidase (GPx).

Figure 4. Schematic representation of grapevine (*Vitis vinifera*) leaf and berry antioxidant machinery based in biochemical results (control - blue arrow; kaolin - green arrow), supported by the current knowledge (white arrows). APx – ascorbate peroxidase; AsA – ascorbic acid; CAT – catalase; DHA – dehydroascorbic acid; DHAR – dehydroascorbate reductase; GPx – glutathione peroxidase; GR – glutathione reductase; GSH – glutathione; GSSG – glutathione disulfide; H_2O_2 – hydrogen peroxide; MDHAR – monodehydroascorbate reductase; MDHA – monodehydroascorbate; $O_2^{\bullet-}$ – superoxide radical; $OH^{\bullet}$ – hidroxil radical; and SOD – superoxide dismutase.

Figure 5. Immunodetection of DNA methylation in section of *Vitis vinifera* leaves from control and kaolin treatments using confocal microscope (x20). Differential interference contrast (A and D), DAPI with blue signal (B and E), and DNA methylation with green signal (C and F). **A**, **B** and **C** are from leaves untreated (control); **C**, **D** and **E** are from

kaolin treated leaves. Abbreviations: ME = mesophyl cells and VV = vascular vessel.

Three biological replicates of each treatment and a negative control were performed.

Table 1 – Thiobarbituric acid reaction substances (TBARS) and hydrogen peroxide (H_2O_2) concentration of grapevines leaves and fruits in response to kaolin exogenous application. Measurements were carried out in samples collected at 23th July (one week after pulverization), 21st August (one month after pulverization) and 3rd September (one month and half after pulverization). Each point is the mean of six replicates with standard deviation. Values were normalized by dry weight (DW).

Statistical analysis was performed using a two-way factorial ANOVA. Different lower case letters represent significant differences during the season (different months), in the same treatment (column), and the * represent the significance of different treatments (control/kaolin) within the same month.

		Month	Control	Kaolin
TBARS ($\mu g\ g-1$)	Leaf	**July**	1.86±0.294b	3.08±0.275a***
		August	2.36±0.092a	1.98±0.067b**
		September	2.31±0.155a	1.36±0.312c***
	p value	Treatment	*0.018*	
		Month	*0.003*	
		Treatment x Month	*0.004*	
	Fruit	**August**	9.37±1.172	4.76±0.776**
		September	8.66±0.620	1.05±0.522***
	p value	Treatment	*<0.001*	
		Month	*0.066*	
		Treatment x Month	*0.861*	
H$_2$O$_2$ ($\mu mol\ g-1$)	Leaf	**July**	5.63±0.177a	5.72±0.807a
		August	4.88±0.112b	5.66±0.400a**
		September	5.20±0.632ab	3.66±1.20b**
	p value	Treatment	*0.355*	
		Month	*0.001*	
		Treatment x Month	*0.001*	
	Fruit	**August**	2.40±0.486	1.66±0.294**
		September	1.79±0.310	0.838±0.229***
	p value	Treatment	*<0.001*	
		Month	*<0.001*	
		Treatment x Month	*0.489*	

Control group

Kaolin group

Vitis vinifera leaf

DAPI

DNA methylation