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Biochemistry

Kaolin exogenous application boosts antioxidant capacity and phenolic content in berries and leaves of grapevine under summer stress

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ABSTRACT

Heat waves, high light intensities and water deficit are becoming important threats in many important viticultural areas worldwide, so the implementation of efficient and cost-effective mitigation strategies is crucial for the production of premium wines while maintaining productivity. In this context, the foliar application of kaolin, a chemically inert mineral with excellent reflective properties, is being developed and experimented as a strategy to reduce the impact of heat and drought in Douro vineyards (Northern Portugal), already revealing promising results. In the present study we investigated if an improved antioxidant capacity is part of the beneficial effects of kaolin, by studying changes in the enzymatic and nonenzymatic antioxidant system in leaves and berries (cv Touriga Nacional). Results showed that mature grape berries contained higher amounts of total phenols (40%), flavonoids (24%), anthocyanins (32%) and vitamin C (12%) than fruits from control vines, and important changes were also measured in leaves. In parallel, kaolin application improved the antioxidant capacity in berries, which was correlated with the observed increased content in secondary metabolites. Kaolin application also regulated secondary metabolism at the transcriptional level through the increase in the transcript abundance of genes encoding phenylalanine ammonia lyase and chalcone synthase.

1. Introduction

Douro Demarcated Region (DDR), recognized in 2001 by UNESCO as World Heritage, is one of the iconic wine regions in the world. This widespread area of vineyards, with a crucial socioeconomic and cultural relevance, has peculiar soil and climatic characteristics providing a suitable "terroir" for premium wine production (Likar et al., 2015). Excess of temperature, high irradiance and water scarcity are well-known environmental stress factors that severely limit grapevine productivity in Douro, which are becoming particularly frequent in the context of ongoing climate change (Fraga et al., 2014).

High irradiance, including UVB radiation, is absorbed by cellular components such as proteins and nucleic acids, resulting in biomass reduction, impaired photosynthesis, reduced protein synthesis, damage to DNA and to other chloroplast functions (Schultz, 2000). Also, leads to oxidative stress (Molassiotis et al., 2006) when excess of reactive oxygen species (ROS) are produced from the disruption of metabolic activities and through the activation of membrane localized NADPH oxidase (Majer and Hideg, 2012). It is well known that oxygen radicals are remarkably reactive and cytotoxic in all organisms, since they can react with unsaturated fatty acids and thus induce the peroxidation of essential membrane lipids or intracellular organelles (Zimmermann and Zentgraf, 2005). Peroxidation leads to cell leakage, fast dehydration and finally cell death. Damage

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to intracellular membranes may influence mitochondrial respiration and induce the degradation of pigments and a loss of the CO_2 fixation ability as well as photoinhibition (Zimmermann and Zentgraf, 2005).

The negative impacts of extreme heat, water scarcity and high irradiance in vineyards prompted the search for short mitigation strategies through the application of exogenous compounds that could maintain or even improve plant productivity under such environmental stresses. Although the literature reports encouraging results in other crops, these strategies are so far less explored in grapevine. Indeed, exogenous mannitol application to salt-stressed wheat, a plant unable to synthesize this polyol, significantly increased its salt tolerance, mainly by stimulating the activity of antioxidant enzymes (Seckin et al., 2009). Also, in maize, an exogenous application of glycinebetaine limited the adverse effects of water stress by modulating water relations (Nawaz and Ashraf, 2007), and its application in tomato (Park et al., 2006), a fleshy fruit, enhanced tolerance to chilling by protecting membranes and macromolecules directly and by inducing antioxidant enzymatic mechanisms. Abscisic acid (ABA) is an important phytohormone responsible for activating drought resistance, and has been successfully experimented as an exogenous protective compound against abiotic stress in maize (Hose et al., 2000), tomato (Aroca et al., 2008), spring wheat and poplar (Du et al., 2013).

From a set of promising compounds, we experimented the exogenous application of kaolin, which is a chemically inert mineral with excellent reflective properties that has yielded promising results. Kaolin reduced leaf surface temperatures, and improved fruit maturation and quality in apple (Wand et al., 2006), and reduced leaf surface temperature and increased CO_2 assimilation rates in olive (Nikoleta-Kleio et al., 2012). In tomato, kaolin application reduced the number of sunburned fruits, exhibited protective properties against insect attack (Cantore et al., 2009) and influenced the physiological response to salinity (Boari et al., 2014). Also, in Merlot grape (*Vitis vinifera* L.), kaolin application enhanced the total amount of berry anthocyanins (Song et al., 2012) and we recently showed that in Touriga Nacional it induced a protective effect on photosystem II structure and function (Dinis et al., 2016). The protective properties of kaolin make imperative to investigate in more detail the protective properties of kaolin in grapevine against heat waves, high irradiance and water scarcity, and if an improved antioxidant capacity is part of its beneficial effects also due to its potential useful role in water management.

In the present study the effect of kaolin exogenous application was investigated on the enzymatic and nonenzymatic antioxidant capacity of leaves and berries from grapevine (cv Touriga Nacional) through approaches that included ROS-scavenging assessment by non-enzymatic methods, such as ABTS$^{\bullet+}$ and β-carotene and hydroxyl radical-scavenging activity assays, the quantification of key secondary compounds and antioxidants, and molecular biology approaches to study the effect of kaolin on the expression of two important genes of the secondary metabolism, *VvPAL1* and *VvCHS1*, which encode phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), respectively. The observed lower ROS levels, increased hydroxyl radical scavenging and enhanced production of antioxidants compounds, including phenolics, flavonoids and anthocyanins, all key metabolites in berry, are likely important mechanisms underlying the mitigation effects of kaolin on adverse abiotic climatic stresses in grapevines.

2. Material and methods

2.1. Chemicals

All chemicals and reagents were of analytical grade and were obtained from commercial sources (Sigma–Aldrich, Merck, and Pronalab). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2. Plant material and samples preparation

Samples were obtained from Touriga Nacional (*Vitis vinifera* L.) cultivar grafted on 110 R located in a commercial vineyard "Quinta do Vallado" in the Douro Demarcated Region (Denomination of Origin Douro/Porto) located at Peso da Régua (41°09′44.5″N 07°45′58.2″W), in northern Portugal. Touriga Nacional is considered the finest Portuguese red grape variety, and in Douro wine region is particularly important in the production of Port. 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. The soil, essentially of schist origin with a loam-dominated texture, is classified as dystric-surribi aric anthrosols (Agroconsultores and Coba, 1991). Vines were managed without irrigation and grown using standard cultural practices as applied by commercial farmers. Monthly maximum temperature (T_{max}) and precipitation values (April to October) are shown in Fig. 1. Temperature values were higher in July and September, while precipitation values were lower in June and August.

Three vineyard rows, with twenty plants each, were sprayed soon after veraison (17th July 2014) with 5% (w/v) Kaolin (Surround WP; Engelhard Corp., Iselin, NJ), according to previous work done by our team (Dinis et al., 2016). A second application in the same day was done to ensure Kaolin adhesion uniformity. Other three vineyard lines, with twenty plants each, were maintained as control, i.e. without Kaolin application. All rows are located side-by-side (ensuring the same edaphoclimatic conditions) on a steep hill with an N-S orientation. The vines, with 7 years, were trained to unilateral cordon and the spurs were pruned to two nodes each with 10–12 nodes per vine.

Leaves (six fully expanded leaves of the first third of the shoot per treatment) were sampled in three different dates: 23th July (one

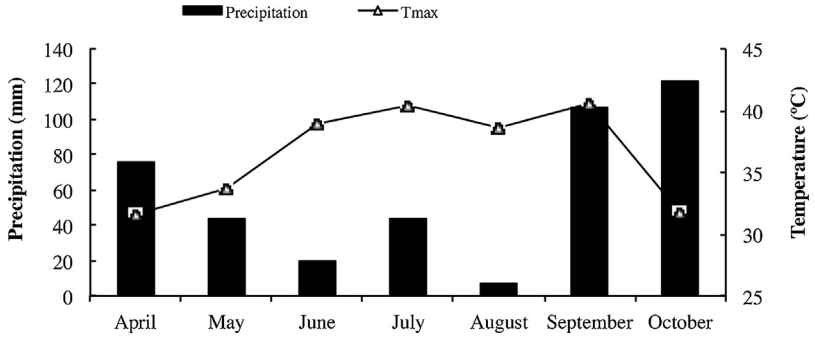

Fig. 1. Monthly values of maximum temperature (T_{max}) and precipitation at the experimental site during 2014.

week after pulverization), 21st August (one month after pulverization) and 3rd September (one month and half after pulverization). The berry samples were randomly collected ($n = 200$ per row, from different positions in the clusters and in the vine) in two different dates: 28th August and 12th September (close to harvest). Samples (leaves and berries) were kept at −80 °C and protected from light prior to further use.

For extraction of antioxidant compounds, samples were lyophilized for 48 h and converted to a fine dried powder before analysis (ground with liquid nitrogen). Sample extraction was carried out combining 0.5 g (triplicated per sample—one for each treatment row) of each sample with 5 ml of 50% (v/v) methanol under shaken for 1 h. This step was repeated three times to increase the yield of extraction. All samples were dissolved in the same solvent at a concentration of 20 mg/ml.

2.3. ROS quantification

Reactive oxygen species (ROS) were determined with 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma–Aldrich, Germany) (Kong et al., 2014). A 25 mM solution was prepared in dimethyl sulphoxide for pending use. Twenty microliter of each sample were loaded into a small well ELISA plate containing 0.2 ml of PBS buffer (pH 7.4) and 12 μM of DCFH-DA and incubated for 20 min at 25 °C.

Fluorescence was measured at 485 nm and 530 nm (excitation and emission wavelength, respectively), in a CARY 50 Bio (Eclipse, Australia) every 15 min until 60 min after the incubation. A calibration curve ($y = 0.0097x + 0.3874$; $R^2 = 0.99988$) was prepared with 2′,7′-dichlorofluorescein and the results were expressed as nM DCF mg^{-1} protein.

2.4. Determination of total antioxidant components

The concentration of total phenolics was quantified by the Folin–Ciocalteu colorimetric method (Singleton and Rossi, 1965) using a gallic acid (GAE) calibration curve ($y = 0.0459x + 0.0184$; $R^2 = 0.99921$), and the results were expressed as mg of gallic acid equivalents/g of dry weight (GAEs).

Flavonoids concentrations in the extracts were determined by a colorimetric method at 510 nm (Jia et al., 1999). A calibration curve ($y = 0.0124x + 0.0573$ $R^2 = 0.99857$) was prepared with (+)-catechin and the results were expressed as μg of (+)-catechin equivalents per g of dry weight (DW).

The concentrations of β-carotene and lycopene were determined according to previously published method (Nagata and Yamashita, 1992), and the following equations were used:

$$\beta\text{-Carotene(mg/100 ml)} = 0.216A663\text{–}1.220A645 - 0.304A505 + 0.452A453$$

$$\text{Lycopene(mg/100 ml)} = -0.0458A663 + 0.204A645 - 0.304A505 + 0.452A453$$

The results were expressed as μg/g (DW) of extract.

For the determination of the total concentration of carotenoids (Car) (Lichtenthaler, 1987), leaves were ground with liquid nitrogen and crushed in 80% acetone. The absorbance was measured at 663, 645 and 470 nm. The results were expressed as μg/g DW of extract according the followed equation: $(1000A_{470} - 1.82C_a - 85.02C_b)/198$; where C_a—chlorophyll $a = 12.25A663 - 2.79A645$ and C_b—chlorophyll $b = 21.50A645 - 5.10A663$.

Ascorbic acid concentration was measured at 515 nm (Klein and Perry, 1982). A calibration curve ($y = 2.4704x + 0.0005$; $R^2 = 0.9723$) was prepared with L-ascorbic acid and the results were expressed as μg/g DW.

Total anthocyanin concentration was estimated using the pH differential method (Meng et al., 2012). Extracts were diluted with buffers at pH 1.0 and 4.5 to attain the same dilution. Absorbance was measured at 520 and 700 nm in both pH 1.0 and 4.5 buffers. Total anthocyanin (expressed in terms of cyanidin-3-glucoside per DW) were calculated using the following formula:

$$\text{Anthocyaninconcentration} = \frac{A \times \text{DF} \times \text{MW}}{\varepsilon \times C}$$

$$A = (A_{520} - A_{700})\,\text{pH}_{1.0} - (A_{520} - A_{700})\,\text{pH}_{4.5}$$

where MW is the molecular weight of cyanidin-3-glucoside (449 g/ mol); DF is the dilution factor; ε is the molar extinction coefficient of cyanidin-3-glucoside (29,600); C is the concentration of extracted volume.

2.5. Antioxidant activity by chemical and biochemical assays

2.5.1. ABTS•+ radical-scavenging activity

The radical-scavenging activity of leaf and fruit extracts were determined by ABTS•$^{+}$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical cation decoloration assay (Re et al., 1999). The ABTS•+ radical was generated through the reaction of 2,20-azinobis-(3-ethylbenzo- thiazoline-6-sulphonic acid) diammonium salt (ABTS, 7 mM), with potassium persulphate (2.45 mM), in water. The mixture was left to stand for 12–16 h in the dark at room temperature. Absorbance of the reactant was later adjusted to 0.70 ± 0.02, at room temperature, at a wavelength of 734 nm in a UV/vis VARIAN spectrophotometer (CARY 100 Bio, Australia). The radical-scavenging activity (RSA_{ABTS}) was calculated as a percentage of ABTS• + discoloration, using the equation: % $RSA_{ABTS} = [(A_{ABTS} - A_S)/A_{ABTS}] \times 100$, where A_{ABTS} is the absorbance of the ABTS• + solution and A_S the absorbance of the solution with the sample extract (Dinis et al., 2012). The extract concentration providing 50% of radical-scavenging activity relative to the tested concentration (EC_{50}) was calculated from the graph of RSA_{ABTS} percentage against extract concentration in the range between 0.25 and 1.5 mg ml^{-1} of Trolox (hydrophilic homologous of α-tocopherol) used as standard ($y = -0.0287x + 0.5535$; $R^2 = 0.9976$).

2.5.2. DPPH radical-scavenging activity

Various concentrations of leaf and fruit extracts (0.5 ml) were mixed with methanolic solution containing DPPH radicals (60 μlmol/l) (Dinis et al., 2012). The mixture was vigorously shaken and left to stand for 5 min in the dark. The reduction of the DPPH radical was measured by monitoring continuously the absorbance at 517 nm. The radical-scavenging activity (RSA_{DPPH}) was calculated as a percentage of DPPH reduction, using the same equation as for ABTS. The extract concentration providing 50% of radical-scavenging activity relative to the tested concentration range (relative EC_{50}) was calculated from the graph of RSA_{DPPH} percentage against extract concentration in the range between 0.1–0.6 mg ml^{-1} of extract. Trolox was used as standard ($y = -0.021x + 0.3825$; $R^2 = 0.9976$).

2.5.3. Ferric reducing activity (FRAP assay)

The FRAP assay was carried out according to a modification of the original medicinal biochemical assay (Benzie and Strain, 1996). The antioxidants present in the samples reduce the Fe(III)/tripyridyltriazine (TPTZ) complex to the blue ferrous form,

Table 1
Seasonal variation of total phenol (mg g^{-1} DW), flavonoid (μg g^{-1} DW), vitamin C (μg g^{-1} DW) and anthocyanin concentrations (mg g^{-1} DW) (mean ± SD) in leaves and fruits of grapevines treated with Kaolin and untreated (n = 6).

		Month	Controlo	Kaolin	p value
Total phenols (mg g^{-1})	Leaf	July	46.8 ± 3.65a	49.1 ± 3.16a	0.266
		August	40.6 ± 2.36b	39.0 ± 3.35b	0.391
		September	40.9 ± 1.16b	38.5 ± 0.820b	0.002
		p value	0.002	<0.001	
	Fruit	August	29.6 ± 4.38	39.0 ± 3.16	0.003
		September	23.4 ± 1.64	39.0 ± 2.78	<0.001
		p value	0.010	0.928	
Flavonoids (μg g^{-1})	Leaf	July	13.6 ± 0.350b	16.3 ± 0.143a	<0.001
		August	15.1 ± 0.053a	11.1 ± 0.108c	<0.001
		September	9.40 ± 0.261c	11.9 ± 0.117b	<0.001
		p value	<0.001	<0.001	
	Fruit	August	9.82 ± 0.619	9.08 ± 0.384	0.033
		September	8.25 ± 0.343	10.9 ± 0.481	<0.001
		p value	<0.001	<0.001	
Vitamin C (μg g^{-1})	Leaf	July	74.5 ± 3.47a	65.3 ± 4.25	0.045
		August	67.8 ± 2.89b	68.8 ± 2.08	0.639
		September	68.2 ± 0.984ab	68.3 ± 2.16	0.934
		p value	0.038	0.426	
	Fruit	August	57.0 ± 0.488	55.4 ± 0.695	0.035
		September	51.6 ± 0.768	58.9 ± 0.131	<0.001
		p value	0.003	0.003	
Anthocyanins (mg g^{-1})	Leaf	July	22.8 ± 2.75a	22.8 ± 1.12b	0.953
		August	4.56 ± 1.23c	2.7 ± 1.41c	0.037
		September	9.05 ± 1.59b	37.3 ± 10.5a	<0.001
		p value	<0.001	<0.001	
	Fruit	August	488.9 ± 5.65	485.0 ± 14.2	0.542
		September	355.6 ± 15.6	521.8 ± 10.4	<0.001
		p value	<0.001	<0.001	

In each column different letters mean significant differences (p < 0.05) during the season. In each row p < 0.05 represent significant differences between treatments.

Table 2
Seasonal variation of β-carotene and lycopene concentrations (μg g^{-1} DW) (mean ± SD) and total carotenoids cconcentration (mg g^{-1} DW) in leaves and fruits of grapevines treated with kaolin and untreated (n = 6).

		Month	Control	Kaolin	p value
β-Carotene (μg g^{-1})	Leaf	July	28.1 ± 0.062c	27.7 ± 0.111c	0.004
		August	37.6 ± 0.123a	35.9 ± 0.120b	<0.001
		September	31.8 ± 0.066b	40.0 ± 0.197a	<0.001
		p value	<0.001	<0.001	
	Fruit	August	2.09 ± 0.088	2.42 ± 0.471	0.307
		September	1.19 ± 0.068	1.20 ± 0.065	0.881
		p value	<0.001	0.011	
Lycopene (μg g^{-1})	Leaf	July	19.0 ± 0.047c	18.7 ± 0.066c	0.004
		August	24.9 ± 0.068a	23.6 ± 0.627b	0.023
		September	20.9 ± 0.042b	26.1 ± 0.215a	<0.001
		p value	<0.001	0.005	
	Fruit	August	1.26 ± 0.033	1.38 ± 0.138	0.239
		September	0.712 ± 0.061	0.718 ± 0.024	0.876
		p value	<0.001	0.001	
Total carotenoids (mg g^{-1})	Leaf	July	1.11 ± 0.087a	1.02 ± 0.205	0.362
		August	0.738 ± 0.043b	0.914 ± 0.032	<0.001
		September	0.685 ± 0.132b	0.930 ± 0.133	0.010
		p value	<0.001	0.397	
	Fruit	August	0.042 ± 0.009	0.044 ± 0.005	0.671
		September	0.058 ± 0.006	0.048 ± 0.013	0.149
		p value	0.008	0.546	

In each column different letters mean significant differences (p < 0.05) during the season. In each row p < 0.05 represent significant differences between treatments.

with an increase in absorbance at 593 nm. The working FRAP reagent was freshly prepared by mixing 300 mM acetate buffer, pH 3.6, with 10 mM tripyridyltriazine (TPTZ) in 40 mM hydrochloric acid and 20 mM ferric chloride ($FeCl_3$) in water solution. A reagent blank reading was taken at 593 nm ($A_{reagentblank}$; t = 0 min). Different concentrations of extracts (0.2 ml) were added to freshly FRAP reagent, and were shaken for 30 min (Dinis et al., 2012). Afterwards, the absorbance was read and selected as final reading (A_{sample}). Sample blank reading, using extract and adequate volume of acetate buffer, was taken also ($A_{sampleblank}$). The difference between A_{sample} and $A_{sampleblank}$ was calculated as $A1_{sample}$. The FRAP value was calculated according the change in absorbance of $A1_{sample}$ against $A_{reagentblank}$. The extract concentration providing 0.5 of absorbance relative to the tested concentration range (relative EC_{50}) between 0.25 and 1.5 mg ml^{-1} of extract, was calculated from the graph. Aqueous solutions of known FeII concentrations, in range of 10–100 mM ($FeSO_4 7H_2O$) and trolox were used as standard ($y = -0.0019x + 0.157$; $R^2 = 0.9934$).

2.5.4. Inhibition of lipid peroxidation using the β-carotene linoleate mode system

The antioxidant activity of leaf and fruit grapevine extracts was evaluated by the β-carotene linoleate model system (Mi-Yae et al., 2003). A solution of β-carotene was prepared dissolving 2 mg of β-carotene in 10 ml of chloroform. Two ml of this solution were transferred into a 100 ml round-bottom flask. After removing the chloroform at 40 °C under vacuum, 40 mg of linoleic acid, 400 mg of Tween 80 emulsifier, and 100 ml of distilled water were added to the flask with vigorous shaking. This emulsion (4.8 ml) was transferred to different test tubes containing 0.2 ml of different concentrations of the extracts. The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. Absorbance readings were then recorded at 20 min intervals until the control sample had changed colour. A blank, devoid of β-carotene, was prepared for background subtraction. Lipid peroxidation (LPO) inhibition was calculated using the following equation: LPO inhibition = (β-carotene concentration after 2 h of assay/initial β-carotene concentration) × 100. The extract concentration providing 50% antioxidant activity relative to the tested concentration range (relative EC_{50}) was calculated from the graph of antioxidant activity percentage against extract concentration (0.25–4.00 mg ml^{-1}). As standard we used TBHQ ($y = 2.4965x^3 - 19.87x^2 + 53.464x + 7.7787$; $R^2 = 0.9910$).

2.5.5. Hydroxyl radical-scavenging activity

The OH-scavenging activity was obtained according to a modified biochemical assay (Chung et al., 1997). Succinctly, the reaction mixture was prepared with 10 mM $FeSO_4.7H_2O$, 10 mM EDTA, 10 mM 2-deoxyribose, 0.1 M phosphate buffer and sample in a test tube to give a total volume of 1.8 ml. Finally, 200 μl of H_2O_2 was added to the mixture, which was incubated at 37 °C for 4 h. After that, 1 ml trichloroacetic acid (2.8%) and 1 ml thiobarbituric acid (1%) were added to the test tube, which was boiled for 10 min. After cooling, its absorbance was measured at 520 nm in a Shimadzu 160-UV spectrophotometer. The OH-scavenging activity (in%) was calculated using the following equation: Inhibition (%) = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control (without sample) and A_1 is the absorbance in the presence of the sample. Tests were carried out in triplicate. The sample concentration providing 50% inhibition (EC_{50}) was obtained by plotting the inhibition percentage against extract concentrations (0.25–1.75 mg ml^{-1}). Mannitol was used as positive control ($y = -0.055x + 91.705$; $R^2 = 0.9957$)

2.5.6. RNA extraction and gene expression analysis by real time qPCR

A total of 200 mg of grape berry previously grounded on liquid nitrogen was used for total RNA extraction (Reid et al., 2006). The obtained RNAs were then purified on RNeasy Mini Spin columns (Qiagen). After treatment with DNase I (Qiagen), cDNA was synthesised from 1 μg of total RNA using Omniscript Reverse Transcription Kit of Qiagen.

Gene expression analysis of *VvPAL1* and *VvCHS1* on grape berries at the previously mentioned maturation stages from control or kaolin treated plants was performed by real-time qPCR. QuantiTect SYBR Green PCR Kit (Qiagen) was used for qPCR analysis, using 1 μl cDNA (diluted 1:10 in ultra-pure distilled water) in a final reaction volume of 20 μl per well. For reference genes, *VvACT1* (actin) and *VvGAPDH* (glyceraldehyde-3-phosphate dehydrogenase), two genes proven to be very stable and ideal for qPCR normalization purposes in grapevine (Reid et al., 2006) were chosen. Gene specific primer pairs used for *VvPAL1* transcriptional analysis were: Forward-5′-CGAACCGAATCAAGGACTG-3′ and Reverse -5′-GTTCCAGCCACTGAGACAAT-3′; and for *VvCHS1* transcriptional

Table 3
EC_{50} values (A) and *p*-values (B) obtained in the antioxidant activity assays of leaves and fruits of grapevine treated with Kaolin and untreated.

A	Leaf				Fruit					
EC_{50} (mg ml^{-1})	July		August		September		August		September	
	Control	Kaolin	Control	Kaolin	Control	Kaolin	Control	Kaolin	Control	Kaolin
ABTS (RSA)	0.483 ± 0.016	0.416 ± 0.017	0.626 ± 0.128	0.826 ± 0.040	0.812 ± 0.024	0.788 ± 0.037	0.771 ± 0.061	0.678 ± 0.013	0.681 ± 0.047	0.593 ± 0.103
DPPH (RSA)	0.176 ± 0.003	0.414 ± 0.045	0.202 ± 0.019	0.492 ± 0.049	0.445 ± 0.032	0.454 ± 0.020	0.476 ± 0.009	0.429 ± 0.007	0.590 ± 0.029	0.386 ± 0.011
FRAP	0.726 ± 0.035	0.643 ± 0.043	0.643 ± 0.083	0.671 ± 0.054	0.741 ± 0.031	0.734 ± 0.027	0.599 ± 0.059	0.549 ± 0.069	0.555 ± 0.109	0.611 ± 0.016
β-Carotene	0.423 ± 0.207	0.190 ± 0.049	1.14 ± 0.074	1.56 ± 0.321	2.50 ± 0.271	4.72 ± 0.259	2.94 ± 0.497	4.409 ± 0.817	1.99 ± 0.136	0.818 ± 0.014
Hydroxyl (RSA)	28.5 ± 2.26	38.3 ± 4.04	42.7 ± 3.30	49.1 ± 1.80	46.6 ± 6.86	88.4 ± 18.7	30.5 ± 2.95	26.2 ± 6.15	20.15 ± 1.09	14.96 ± 0.363

B	Leaf			Fruit		
p value	July	August	September	Control	Kaolin	August	September	Control	Kaolin
ABTS (RSA)	0.008bB	0.051abA	0.398aA	0.005	<0.001	0.052	0.247	0.115	0.227
DPPH (RSA)	0.001b	0.001b	0.702a	<0.001	0.141	0.002	<0.001	0.003	0.005
FRAP	0.05	0.659	0.789	0.142	0.094	0.392	0.424	0.567	0.202
β-Carotene	0.130cC	0.090bB	0.001aA	<0.001	<0.001	0.046	<0.001	0.002	0.007
Hydroxyl (RSA)	0.022bB	0.042aB	0.022aA	0.007	0.003	0.34	0.001	0.005	0.034

In each column different letters mean significant differences ($p < 0.05$). In each month *p*-values mean the significance between treatments and in each treatment mean the significance between months. Lowercase represents significant differences between months in control grapevine plants and uppercase represents significant differences between months in Kaolin treated plants.

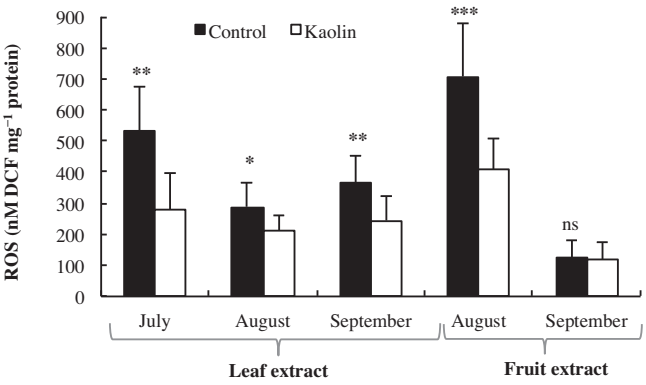

Fig. 2. ROS concentration (nM DCF mg^{-1} protein) of leaf and fruit extracts in control and Kaolin treated grapevine plants in different development stages.

analysis were: Forward-5′-CGAGCTCACCACCGAGCACCTTACCT - 3′ and Reverse-5′-CCGCTCGAGTGTTGGCTACCTGCTTCACT-3′. The specific primers for the reference genes *VvACT1* and *VvGAPDH* (Conde et al., 2015) and respectively: Forward-5′-GTGCCTGCCATGTATGTTGCCATTCAG-3′ and Reverse-5′-GCAAGGTCAAGACGAAGGATAGCATGG-3′; and Forward-5′-CACGGTCAGTGGAAGCATCATGAACTC-3′ and Reverse-5′-CCTTGTCAGTGAACACACCAGTTGACTC-3′. Melting curve analysis was performed for specific gene amplification confirmation. The efficiencies of the PCR reaction for each gene were assessed using serial dilutions of template cDNA and were taken into account for gene expression values. The expression values of *VvPAL1* and *VvCHS1* are normalized by the average of the expression of the reference genes (Pfaffl, 2001). For all experimental conditions tested, three independent experiments, one for each biological replicate, with duplicates were performed.

2.5.7. Statistical analysis

For total antioxidant compounds determinations, all the assays were repeated six times per treatment in each harvest. The results are expressed as mean values ± standard deviation (SD). For the other experiments, assays were done in triplicate. The differences between the extracts were analyzed using one-way analysis of variance, followed by Tukey Test using SPSS 20.0 software. The EC_{50} values and their correlation (regression analysis) with non-enzymatic antioxidant compounds for antioxidant activity were obtained by GraphPad Prism 6 software.

3. Results

3.1. Kaolin decreases ROS levels and stimulates changes in antioxidants components in berries and leaves

Results showed that the total amount of ROS in leaves from control plants decreased from July till August and then increased again in September. Moreover, the amount of ROS in leaves from kaolin treated plants was consistently lower in all sampling dates, particularly in July when ROS decreased by 48%. ROS levels decreased abruptly from August to September in berry tissues but the protective effect of kaolin treatment was evident only in August (42% reduction by comparing with control berries) (Fig. 2).

Table 1 shows the total phenol, flavonoid, vitamin C and anthocyanin concentrations in extracts from leaves and berries sampled from July to September from grapevines treated with kaolin and control plants. As can be seen, while the total amount of phenols did not change substantially in leaves between control and kaolin treated plants, they increased by 32% (in August) and 66% (in September) in berries of treated plants. Regarding flavonoids, the

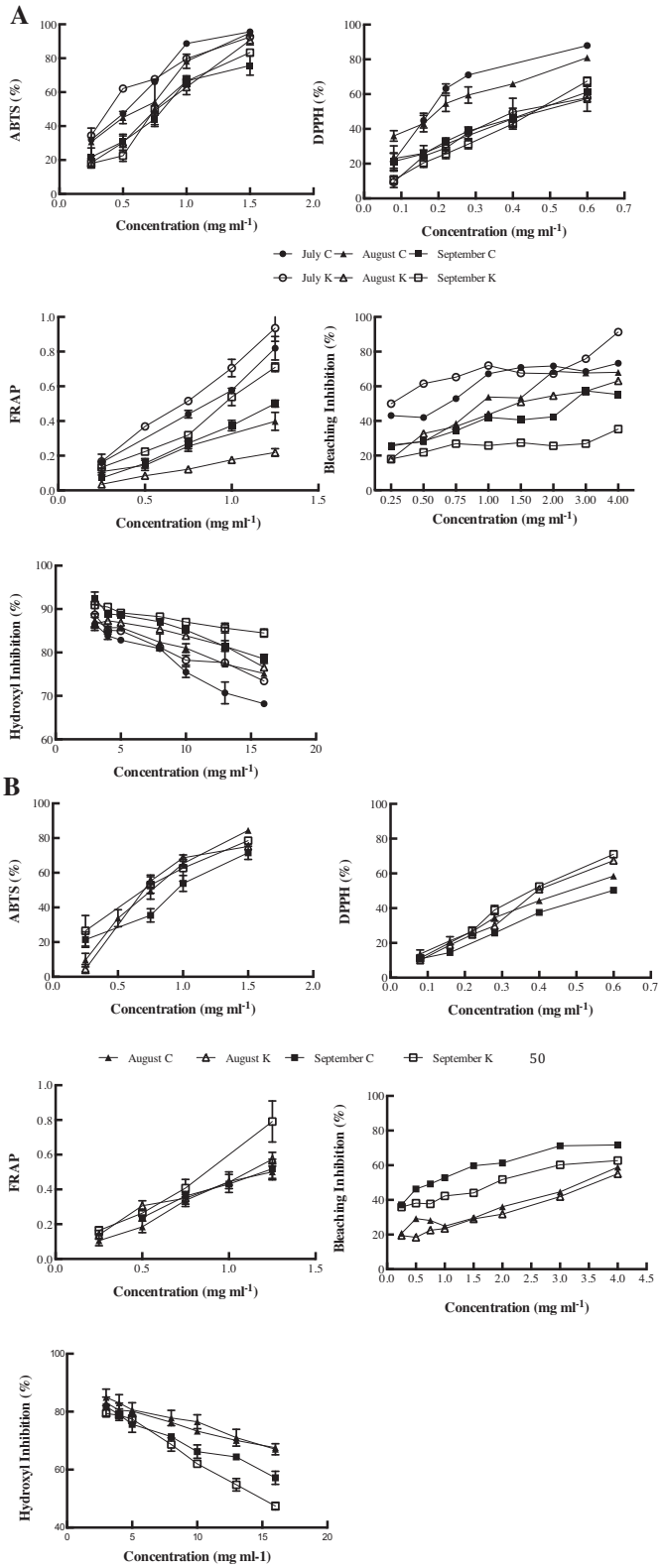

Fig. 3. Antioxidant activity of leaf (A) and fruits (B) extracts of Touriga Nacional (*Vitis vinifera* L.) with (K) and without Kaolin (C) in different growth stages. Scavenging activity on ABTS, DPPH and hydroxyl radicals (%), FRAP and bleaching inhibition (%).

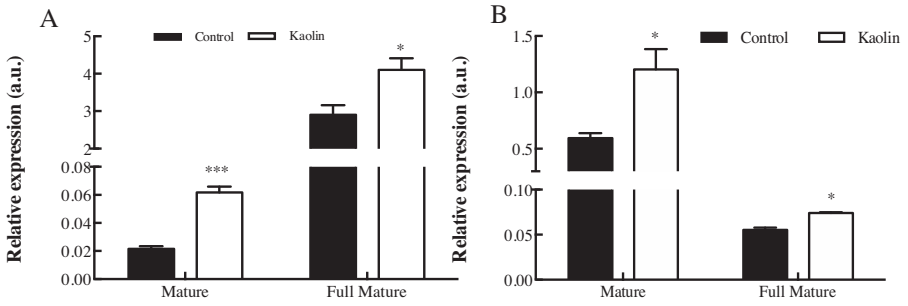

Fig. 4. The effect of Kaolin application in the transcript levels of the grapevine *VvPAL1* (A) and *VvCHS1* (B) in grape berries. Gene expression analysis, by real-time qPCR, of *VvPAL1* and *VvCHS1* on grape berry tissues collected at the mature and full mature ripening stages from vines subjected to control (without application) or Kaolin-treated grapevines. *VvPAL1* and *VvCHS1* relative expression levels were obtained after normalization with the expression of the reference genes *VvACT1* and *VvGAPDH*. Two independent qPCR runs with triplicates were performed for each tested mRNA. Values are the mean ± SE.

most relevant result was observed in berries at the mature stage (September) when an increase by 32% between control and kaolin treated vines was measured. Regarding vitamin C, an increase by 14% in mature berries between control and kaolin-treated vines was observed. Kaolin application also showed a stimulating effect on the production of anthocyaninn in leaves and fruits. In leaves from kaolin-treated plants a strong increase (14-fold) in anthocyanins content from August to September was observed, while in control plants anthocyanins concentration only duplicated in the same period. Moreover, the concentration of anthocyanins increased by 47% in mature berries between control and kaolin-treated plants.

Table 2 shows the concentrations of β-carotene, lycopene and total carotenoids in leaves and berries. The β-carotene concentration increased by 31% in leaves from July to September in kaolin-treated vines, while in control samples its concentration picked in August before decreasing till September. Consequently, in September the concentration of β-carotene was 45% higher in leaves from kaolin-treated vines than in control plants. While in leaves from control and kaolin treated plants the pattern of lycopene variation from July to September was similar to the reported above for β-carotene, in berries the concentration of β-carotene was not influenced by the treatment.

Regarding total carotenoids, while in leaves from control plants its concentration decreased by 38% from July to September, the application of kaolin seemed to prevent this abrupt decrease. Consequently, in September the total carotenoid concentration in leaves increased by 26% between control and kaolin-treated plants. In berries, the total carotenoids concentration was similar between treatments.

3.2. Kaolin application boosts the antioxidant activity of berry tissues

The antioxidant potential of leaves (Fig. 3A) and berries (Fig. 3B) was estimated from the scavenging activity of the corresponding extracts on ABTS, DPPH and hydroxyl radicals, with the FRAP assay and from the inhibition of lipid peroxidation using the β-carotene linoleate system.

As expected, the antioxidant activity of both extracts increased with the increase of the concentration of each extract. The corresponding EC_{50} values for each concentration range test (see material and methods) are presented in Table 3. While in leaves the results of the different antioxidant assays were somewhat variable, in fruits, the increase of the antioxidant activity mediated by kaolin application was very clear from the results of the ABTS, DPPH and Hydroxyl assays both in August and September, while the β-carotene assay showed that kaolin improved berry antioxidant activity only in September.

3.3. Kaolin application regulates the biosynthesis of phenolics compounds at the transcriptional level

Real-time qPCR analysis showed that the transcript levels of phenylalanine ammonia lyase (*VvPAL1*) (Fig. 4A), an enzyme that catalyses the first step in the phenylpropanoid pathway in which trans-cinnamic acid is produced, and key in grape berry secondary metabolism; and of a chalcone synthase (*VvCHS1*) (Fig. 4B), an enzyme that catalyses the first step of the flavonoid pathway, increased in the final maturation stages in berries form kaolin-treated plants.

Table 4
Correlation matrix (*r* value) established between total phenol, flavonoid, anthocyanin and Vitamin C concentrations, and scavenging effect on ABTS, DPPH, hydroxyl radicals, FRAP and β-carotene.

Assays	Leaf							
	Phenols		Flavonoids		Vitamin C		Anthocyanins	
	Control	Kaolin	Control	Kaolin	Control	Kaolin	Control	Kaolin
ABTS (RSA)	−0.826	−0.939	−0.645	−0.987	−0.722	0.562	−0.504	−0.023
DPPH (RSA)	−0.62	−0.527	−0.924	−0.658	−0.404	0.487	−0.23	−0.321
FRAP	0.073	−0.151	−0.716	0.157	0.147	0.191	0.418	0.808
β-Carotene	−0.766	−0.843	−0.84	−0.622	−0.531	0.348	−0.432	0.717
Hydroxyl (RSA)	−0.862	−0.734	−0.441	−0.504	−0.646	0.191	−0.701	0.706
Fruit								
ABTS (RSA)	0.593	0.398	0.847	−0.83	−0.622	0.42	0.655	−0.709
DPPH (RSA)	−0.947	−0.459	−0.811	−0.862	0.889	0.957	−0.978	−0.816
FRAP	0.71	0.095	0.784	0.62	−0.728	−0.629	0.838	0.679
β-Carotene	0.911	−0.484	0.857	−0.802	−0.976	0.948	0.966	−0.638
Hydroxyl (RSA)	0.957	−0.423	0.902	−0.928	−0.857	0.908	0.947	−0.878

4. Discussion

Results showed that kaolin decreased ROS levels and stimulated changes in non-enzymatic antioxidants components in berries and leaves, including phenols, flavonoids, anthocyanins and β-carotene, part of them with well-known contribution to berry and wine quality. In agreement, the results of the ABTS, DPPH and Hydroxyl assays showed a very consistent increase of the antioxidant activity in fruits mediated by kaolin. Phenols and flavonoids were the components that exhibited a better correlation with EC_{50} values for the antioxidant activity assays in leaves, excluding the FRAP assay, while lower correlations were found between antioxidant activity and concentration of anthocyanins and vitamin C (Table 4). Of note, phenols, flavonoids, anthocyanins and vitamin C contents showed a consistent positive correlation with antioxidant activity in berries (Table 4).

The observed stimulation of anthocyanin synthesis by kaolin was particularly significant in mature berries, in which an increase by 47% was observed. This stimulation may result from an increase in the partitioning of carbohydrates into the fruit, following previous observations that kaolin promotes canopy photosynthesis and protects photosystem II structure and function (Dinis et al., 2016; Glenn et al., 2010). Indeed, the relationship between sugar and anthocyanin content is well documented (Dai et al., 2014; Hunter et al., 1991; Larronde et al., 1998; Pirie and Mullins, 1977). The protective role of kaolin against heat, which is reported to inhibit the synthesis of anthocyanins and/or to stimulate anthocyanins degradation (Ramesh et al., 2010) may also explain the observed differences in the levels of anthocyanins in fruits, which is very relevant at both basic scientific and agronomical standpoints.

As previously mentioned, phenylalanine ammonia lyase (PAL) is a key enzyme in the beginning of the phenylpropanoid pathway that provides the substrate for the activity of chalcone synthase (CHS) that marks the beginning of the flavonoid biosynthesis pathway, in which, among other compounds, anthocyanins are synthesized. The increase of *VvPAL1* and *VvCHS1* expression is indeed in total agreement with the observed significant increase in total phenol and flavonoid concentration, suggesting that kaolin application positively influences secondary metabolism also at the transcriptional level.

The observed improvement in polyphenols in grapes may also result from increased water availability in grapevines, as suggested before (Iriti et al., 2011), because kaolin particle film could reduce water loss through the increase of canopy reflectance of infrared and ultraviolet radiations, thereby reducing leaf and fruit tissue temperature (Khaleghi et al., 2015). These effects could also explain why kaolin lowered the decrease of carotenoids in leaves from July to September, because heat, PAR and UV are known to activate chlorophyllases (Glenn et al., 2003). Moreover, due to the reflective properties of kaolin, leaves were probably better adapted to excessive solar radiation leading to this result.

Phenolic substances of grapes are of remarkable interest due to their antioxidant and free radical scavenging properties (Orak, 2006) that might be explored biotechnologically in biomedicine to alleviate oxidative stress associated with various human disorders, including cardiovascular and neurodegenerative diseases, atherosclerosis, cataracts and chronic inflammation (Dinis et al., 2012). Relevant nutraceutical and pharmacologic properties, including anti-allergic, anti-inflammatory and anti-carcinogenic effects, as well as diabetes prevention and vision improvement (Ghosh and Konishi, 2007), have been attributed to the complex mixture of phenols present in berry tissue (Barreira et al., 2008; Teixeira et al., 2013). In this regard, grapes and wines from Kaolin treated plants deserve further attention in what regards the above organoleptic and health related parameters.

In conclusion, the present work showed that kaolin exogenous application mitigates the effect of adverse abiotic climatic stresses in grapevines, in part by contributing to lower ROS levels, increased hydroxyl radical scavenging and enhanced production of antioxidants compounds, including phenolics, flavonoids and anthocyanins, all metabolites with great positive influence on berry quality. An increase in the transcript abundance of *VvPAL1* and *VvCHS1* genes apparently contributed to these changes in phenolic concentration. Overall, the results reinforce the promising nature of kaolin application as summer stress mitigation strategy.

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References

Agroconsultores, Coba, 1991. Carta de solos, carta de uso actual da terra e carta de aptidão da terra, do Nordeste de Portugal. In: Douro UdT-o-MeA, editor. Vila Real, Portugal.

Aroca, R., Alguacil, M.D., Vernieri, P., Ruiz-Lozano, J.M., 2008. Plant responses to drought stress and exogenous ABA application are modulated differently by mycorrhization in tomato and an ABA-deficient mutant (sitiens). Microb. Ecol. 56, 704–719.

Barreira, J.C.M., Ferreira, I.C.F.R., Oliveira, M.B.P.P., Pereira, J.A., 2008. Antioxidant activities of the extracts from chestnut flower, leaf, skins and fruit. Food Chem. 107, 1106–1113.

Benzie, I.F.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. Anal. Biochem. 239, 70–76.

Boari, F., Cucci, G., Donadio, A., Schiattone, M.I., Cantore, V., 2014. Kaolin influences tomato response to salinity: physiological aspects. Acta Agric. Scand. Secti. B: Plant Soil Sci. 64, 559–571.

Cantore, V., Pace, P., Albrizio, R., 2009. Kaolin-based particle film technology affects tomato physiology, yield and quality. Environ. Exp. Bot. 66, 279–288.

Chung, S.K., Osawa, T., Kawakishi, S., 1997. Hydroxyl radical scavenging effects of species and scavengers from brown mustrad (*Brassica nigra*). Biosci. Biotechnol. Biochem. 61, 118–123.

Conde, A., Regalado, A., Rodrigues, D., Costa, J.M., Blumwald, E., Chaves, M.M., 2015. Polyols in grape berry: transport and metabolic adjustments as a physiological strategy for water-deficit stress tolerance in grapevine. J. Exp. Bot. 66, 889–906.

Dai, Z.W., Meddar, M., Renaud, C., Merlin, I., Hilbert, G., Delrot, S., Gomès, E., 2014. Long-term in vitro culture of grape berries and its application to assess the effects of sugar supply on anthocyanin accumulation. J. Exp. Bot. 65, 4665–4677.

Dinis, L.-T., Oliveira, M.M., Almeida, J.R.C., Gomes-Laranjo, J., Peixoto, F., 2012. Antioxidant activities of chestnut nut of *Castanea sativa* Mil. (Cultivar Judia) as function of origin ecosystem. Food Chem. 132, 1–8.

Dinis, L.-T., Ferreira, H., Pinto, G., Bernardo, S., Correia, C.M., Mutinho-Pereira, J., 2016. Kaolin-based, foliar reflective film protects photosystem II structure and function in grapevine leaves exposed to heat and high solar radiation. Photosynthetica 54, 47–55 http://dx.doi.org/10.1007/s11099-015-0156-8.

Du, Y.L., Wang, Z.Y., Fan, J.W., Turner, N.C., He, J., Wang, T., 2013. Exogenous abscisic acid reduces water loss and improves antioxidant defence, desiccation tolerance and transpiration efficiency in two spring wheat cultivars subjected to a soil water deficit. Funct. Plant Biol. 40, 494–506.

Fraga, H., Malheiro, A.C., Moutinho-Pereira, J., Jones, G.V., Alves, F., Pinto, J.G., Santos, J.A., 2014. Very high resolution bioclimatic zoning of Portuguese wine regions: present and future scenarios. Reg. Environ. Change 14 (1), 295–306.

Ghosh, D., Konishi, T., 2007. Anthocyanins and anthocyanin-rich extracts: role in diabetes and eye function. Asia Pac. J. Clin. Nutr. 16, 200–208.

Glenn, D.M., Erez, A., Puterka, G.J., Gundrum, P., 2003. Particle films affect carbon assimilation and yield in 'Empire' apple. J. Am. Soc. Hortic. Sci. 128, 356–362.

Glenn, D.M., Cooley, N.M., Walker, R.R., Clingeleffer, P.R., Shellie, K.C., 2010. Impact of kaolin particle film and water deficit on wine grape water use efficiency and plant water relations. Hortscience 45, 1178–1187.
Hose, E., Steudle, E., Hartung, W., 2000. Abscisic acid and hydraulic conductivity of maize roots: a study using cell- and root-pressure probes. Planta 211, 874–882.
Hunter, J., De Villiers, O., Watts, J., 1991. The effect of partial defoliation on quality characteristics of *Vitis vinifera* L. cv Cabernet Sauvignon grapes. II. Skin color, skin sugar, and wine quality. Am. J. Enol. Vitic. 42, 13–18.
Iriti, M., Vitalini, S., Di Tommaso, G., D'Amico, S., Borgo, M., Faoro, F., 2011. New chi- tosan formulation prevents grapevine powdery mildew infection and improves polyphenol content and free radical scavenging activity of grape and wine. Aust. J. Grape Wine Res. 17, 263–269.
Jia, Z., Tang, M., Wu, J., 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 64, 555–559.
Khaleghi, E., Arzani, K., Moallemi, N., Barzegar, M., 2015. The efficacy of kaolin particle film on oil quality indices of olive trees (*Olea europaea* L.) cv 'Zard' grown under warm and semi-arid region of Iran. Food Chem. 166, 35–41.
Klein, B.P., Perry, A.K., 1982. Ascorbic acid and vitamin A activity in selected vegetables from different geographical areas of the United States. J. Food Sci. 47, 941–945.
Kong, L., Wang, F., Jisheng Si Feng, B., Zhang, B., Li, S., 2014. Increasing in ROS levels and callose deposition in peduncle vascular bundles of wheat (*Triticum aestivum* L.) grown under nitrogen deficiency. J. Plant Interact. 8, 109–116.
Kottek, M., Grieser, J., Beck, C., Rudolf, B., Rubel, F., 2006. World Map of the Köppen-Geiger climate classification updated. Meteorol. Z. (Berl.), 259–263.
Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol. 148, 350–382.
Larronde, F., Krisa, S., Decendit, A., Cheze, C., Deffieux, G., Mérillon, J., 1998. Regulation of polyphenol production in *Vitis vinifera* cell suspension cultures by sugars. Plant Cell Rep. 17, 946–950.
Likar, M., Vogel-Mikus, K., Potisek, M., Hancevic, K., Radic, T., Necemer, M., 2015. Importance of soil and vineyard management in the determination of grapevine mineral composition. Sci. Total Environ. 505, 724–731.
Majer, P., Hideg, E., 2012. Developmental stage is an important factor that determines the antioxidant responses of young and old grapevine leaves under UV irradiation in a green-house. Plant Physiol. Biochem. 50, 15–23.
Meng, J.F., Fang, Y.L., Qin, M.Y., Zhuang, X.F., Zhang, Z.W., 2012. Varietal differences among the phenolic profiles and antioxidant properties of four cultivars of spine grape (*Vitis davidii* Foex) in Chongyi County (China). Food Chem. 134, 2049–2056.
Mi-Yae, S., Tae-Hun, K., Nak-Ju, S., 2003. Antioxidants and free radical scavenging activity of Phellinus baumii (*Phellinus* of Hymenochaeta-ceae) extracts. Food Chem. 82, 593–597.
Molassiotis, A., Sotiropoulos, T., Tanou, G., Diamantidis, G., Therios, I., 2006. Boron-induced oxidative damage and antioxidant and nucleolytic responses in shoot tips culture of the apple rootstock EM 9 (*Malus domestica* Borkh). Environ. Exp. Bot. 56, 54–62.
Nagata, M., Yamashita, I., 1992. Simple method for simultaneous determination of chlorophyll and carotenoids in tomato fruit. Nippon Shokuhin Kogyo Gakkaishi 39, 925–928.
Nawaz, K., Ashraf, M., 2007. Improvement in salt tolerance of maize by exogenous application of glycinbetaine: growth and water relations. Pak. J. Bot. 39, 1647–1653.
Nikoleta-Kleio, D., Peter, A.R., Theodoros, D., Vassilios, S., 2012. Comparative effects of exogenous glycine betaine, kaolin clay particles and Ambiol on photosynthesis, leaf sclerophylly indexes and heat load of olive cv. Chondrolia Chalkidikis under drought. Sci. Hort. 137, 87–94.
Orak, H., 2006. Total antioxidant activities, phenolics, anthocyanins, polyphenoloxidase activities of selected red grape cultivars and their correlations. Sci. Hort., 235–241.
Park, E.J., Jeknic, Z., Chen, T.H., 2006. Exogenous application of glycinebetaine increases chilling tolerance in tomato plants. Plant Cell Physiol. 47, 706–714.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, 45.
Pirie, A., Mullins, M., 1977. Interrelationships of sugars, anthocyanins, total phenols and dry weight in the skin of grape berries during ripening. Am. J. Enol. Vitic. 28, 204–209.
Ramesh, C., Khanal, L.R.H., Ronald, L.P., 2010. Effect of heating on the stability of grape and blueberry pomace procyanidins and total anthocyanins. Food Res. Int. 43, 1464–1469.
Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved abts radical cation decolorization assay. Free Radi. Biol. Med. 26, 1231–1237.
Reid, K.E., Olsso, N., Schlosser, J., Peng, F., Lund, S.T., 2006. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biol. 6, 27.
Seckin, B., Sekmen, A.H., Turkan, I., 2009. An enhancing effect of exogenous mannitol on the antioxidant enzyme activities in roots of wheat under salt stress. J. Plant Growth Regul. 28, 12–20.
Singleton, V.L., Rossi, J.A.J., 1965. Colorimetric of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16, 144–158.
Song, J., Shellie, K.C., Wang, H., Qian, M.C., 2012. Influence of deficit irrigation and kaolin particle film on grape composition and volatile compounds in Merlot grape (*Vitis vinifera* L.). Food Chem. 134, 841–850.
Schultz, H.R., 2000. Climate change and viticulture: a European perspective on climatology, carbon dioxide and UV-B effects. Aust. J. Grape Wine Res. 6, 2–12.
Teixeira, A., Eiras-Dias, J., Castellarin, S.D., Gerós, H., 2013. Berry phenolics of grapevine under challenging environments. Int. J. Mol. Sci. 14, 18711–18739.
Wand, S.J.E., Theron, K.I., Ackerman, J., Marais, S.J.S., 2006. Harvest and post-harvest apple fruit quality following applications of kaolin particle film in South African orchards. Sci. Hortic. 107, 271–276.
Zimmermann, P., Zentgraf, U., 2005. The correlation between oxidative stress and leaf senescence during plant development. Cell. Mol. Biol. Lett. 10, 515–534.