Improvement of grapevine physiology and yield under summer stress by kaolin-foliar application: water relations, photosynthesis and oxidative damage

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Abstract

Knowledge about short-term climate change adaptation strategies for Mediterranean vineyards is needed in order to improve grapevine physiology and yield-quality attributes. We investigated effects of kaolin-particle film suspension on water relations, photosynthesis and oxidative stress of field-grown grapevines in the Douro region (northern Portugal) in 2012 and 2013. Kaolin suspension decreased leaf temperature by 18% and increased leaf water potential (up to 40.7% in 2013). Maximum photochemical quantum efficiency of PSII was higher and the minimal chlorophyll fluorescence was lower in the plants sprayed by kaolin. Two months after application, net photosynthesis and stomatal conductance at midday increased by 58.7 and 28.4%, respectively, in treated plants. In the same period, kaolin treatment increased photochemical reflectance, photosynthetic pigments, soluble proteins, soluble sugars, and starch concentrations, while decreased total phenols and thiobarbituric acid-reactive substances. Kaolin application can be an operational tool to alleviate summer stresses, which ameliorates grapevine physiology and consequently leads to a higher yield.

Additional key words: biochemistry; Douro region; oxidative stress; *Vitis vinifera*.

Introduction

Europe presents the largest vineyard area in the world (about 38%), that is located under Mediterranean climate and is characterized by long growing seasons, mild to warm temperatures, and dry summers (Fraga *et al.* 2016). In the last years, much attention has been focused on climate change and its impact on viticulture (Jones *et al.* 2005, Hannah *et al.* 2013, Fraga *et al.* 2014a). For these Mediterranean-like climatic regions, future projections point to an increased warming and drying, which is expected to result in lower water availability (IPCC 2013).

This combined effect, including longer periods of high air temperatures followed by high evaporative demands and drought, could result in damaging effects during the grapevine growing season, not only on a yield but also on grape and wine quality (Chaves *et al.* 2007, Fraga *et al.* 2014b). Wine-making regions, which are already on the limits of *V. vinifera* L. climatic suitability, could face further threats in order to preserve high quality wine production (Jones *et al.* 2005, Fraga *et al.* 2013). Furthermore, traditionally cultivated grapevine varieties, which are used in the production of renowned high quality wines, may experience additional stresses, which may challenge wine typicity (Ferrandino and Lovisolo 2014). It is known that the occurrence of these stresses may promote dramatic decrease in plant carbon assimilation as the result of severe decay of net photosynthesis, as well as a partial loss of canopy leaf area (Chaves *et al.* 2007, dos Santos *et al.* 2007, Moutinho-Pereira *et al.* 2004). The high temperature conditions also affect numerous physiological processes, including modifications in photosynthesis efficiency (Moutinho-Pereira *et al.* 2007), transport and accumulation of assimilates (Sampol *et al.* 2003), respiration and mineral nutrition. The photosynthetic limitation decreases sugar synthesis, leading to reduction not only of the yield, but also of the fruit quality (dos Santos *et al.* 2007). Additionally, an increase in total polyphenols is induced under such stressful conditions (Moutinho-Pereira *et al.* 2007, Wen *et al.* 2008).

As irrigation becomes economically unsustainable in the Douro region, special attention must be focused on minimizing these adverse effects. Foliar sprays with inert reflective materials, such as kaolinite clay [$Al_2Si_2O_5(OH)_4$], have been used in greenhouses and orchard systems in order to increase light reflectance and to reduce canopy temperature (Shellie and King 2013) and water stress. The kaolin particles has been provided to reflect potentially damaging ultraviolet and infrared radiation, as well as to allow the transmission of photosynthetically active radiation (Glenn and Puterka 2005), to reduce leaf temperature (Shellie and King 2013) and sunburns, and to increase leaf carbon assimilation (Glenn *et al.* 2001). In the same experiment during three years, we found that kaolin interferes with some parameters that are related to the PSII photochemical performance (Dinis *et al.* 2016a) and fruit quality (Dinis *et al.* 2016b). In this work, with the purpose of complementing information, we presented their effects on spectral leaf reflectance, leaf temperature, water status, carbon metabolism, oxidative stress indicators, and yield. In this context, the aim of this study was to i) evaluate the efficiency of foliar-sprayed kaolin in canopy cooling and in mitigation of damages associated with excessive sun leaf exposure, ii) and to determine how this exposure influences water relations, photosynthesis, and yield in grapevines under a typical Mediterranean-like climate.

Materials and methods

Plant material, growth conditions and treatments: The trial was carried out in 2012 and 2013 in "Quinta do Vallado", a commercial vineyard of Douro Demarcated Region (Denominations of Origin Douro and Porto) located at Peso da Régua (41°09'44.5"N 07°45'58.2"W), northern Portugal. The climate is typically Mediterranean, with high precipitation during the winter months and dry and hot in the summer (Kottek *et al.* 2006). An automatic weather station recorded rainfall, mean air temperature, and solar radiation during the trial period. According to the World Reference Base for Soil Resources 2014 (FAO 2015), the soil mapping of the region is classified as dystric technosols taking into account their acidity and deep modification by human actions. The rainfed plants were managed under commercial viticultural practices. In order to study the potential of kaolin application, the Portuguese Touriga nacional variety (*Vitis vinifera* L.), grafted onto 110 R rootstock, was selected. In a steep hill with an N-S row orientation, 6-year-old vines were trained to unilateral cordon and the spurs were pruned to two nodes each with 10–12 nodes per vine. Three vineyard rows, with twenty plants each, were sprayed soon after veraison (11 July in 2012 and 17 July in 2013) with 5% (w/v) kaolin (*Surround WP, Engelhard Corp.*, Iselin, New Jersey). A second application on the same days was performed to ensure kaolin adhesion uniformity. Other three vineyard rows,

side-by-side (for ensuring similar edaphoclimatic conditions between treatments), with twenty plants each, were used as control, *i.e.* without kaolin application. The control plants were carefully protected by a plastic film during the kaolin application. In 2012, an additional kaolin application was done (30 July), to compensate for the partial kaolin removal due to a high precipitation event.

The supplementary data (Fig. 1S, *supplement available online*) shows monthly total values of rainfall (Rf, April to October), solar radiation, and air temperature from May to October in 2012 and 2013. The Rf values were higher in 2012 than that in 2013, except of those in October. Relating to temperature, values were significantly higher in 2013, except those in May. Light incident was also higher in 2013, except in May and June. Thus, 2013 was warmer and drier than 2012.

Reflectance: By using the same leaf samples to measure the gas exchange and chlorophyll (Chl) *a* fluorescence, the spectral reflectance was determined following the procedures described in Moutinho-Pereira *et al.* (2012). Briefly, ten scans per sample were integrated (integration time of 10 ms) and a total of three measurements were acquired within the marked homogeneous area of each leaf. Then the average of these measurements was calculated to establish a single representative reflectance

spectrum per leaf and from this, several reflectance indices were computed for measuring the changes between leaves with and without the kaolin treatment. Chl reflectance index [$RI_{red\ edge}$] was calculated as $[(R_{750-800})/(R_{695-740})] - 1$ (index with broad spectral band and positively correlated with Chl content) (Gitelson and Merzlyak 2004).

Leaf temperature was measured with an infrared thermometer (*Infratrace KM800S*, England) with a 15° field view, throughout veraison (late July) and ripening stage (August and early September). Measurements were performed under clear sunny days and on sun-exposed and fully expanded leaves at the middle of the shoots (usually between 8th and 11st nodes on the shoot axes). The average temperature of two randomly selected leaves (in eight plants) in each plot was obtained by holding the thermometer at about 1 m above the foliar surface.

Water relations: The volumetric water content of the soil was monitored by time-domain reflectometry using a *MiniTrase* (*SoilMoisture*, USA) at approximately 20–30 d intervals over the growing seasons. For this purpose, in each treatment, six replicates of 0.6 m heavy-duty wave guides were inserted in the vine row, at 0.30 m apart from the trunk. At a given date, the fraction of available soil water was computed as the difference between the soil water content on the day of measurements and the minimum soil water content, and that was divided by the total available water in the defined soil profile (0–0.6 m).

Leaf water potential (Ψ) was determined according to Dinis *et al.* (2014). Briefly, Ψ was determined with a pressure chamber (*Model 1000*, *PMS Instrument Company*, Albany, USA) at pre-veraison (July), veraison (August), and the maturation (early September) stage. Measurements were performed on eight fully expanded leaves at predawn (6:00 h, Ψ_{pd}) and midday (13:00–14:00 h, Ψ_{md}).

Leaf gas exchange was measured with an infrared gas analyser (*LC Pro+*, *ADC Bioscientific Ltd.*, UK), operating in the open mode. Measurements were carried out in the morning (09:00–10:30 h) and midday (14:00–15:30 h) period on 31 July and on 4 September (maturation) in 2012, and on 11 September in 2013. In 2013, only one day of measurements (September) was performed due to infrared gas analyser failure in previous months. Net CO_2 assimilation rate (P_N), stomatal conductance (g_s), transpiration rate (E), and internal CO_2 concentration/ambient CO_2 ratio (C_i/C_a) were estimated according to von Caemmerer and Farquhar (1981). To eliminate the possible effects of air humidity and temperature on transpiration, the P_N/g_s ratio, rather than the P_N/E ratio, was calculated in order to evaluate the intrinsic water-use efficiency (WUE_i) (Iacono *et al.* 1998).

Chl *a* fluorescence was obtained *in situ* at the same periods of gas-exchange measurements by using a pulse-amplitude-modulated fluorimeter (*FMS 2, Hansatech Instruments*, Norfolk, England). Maximum quantum efficiency of PSII was calculated as $F_v/F_m = (F_m - F_0)/F_m$, by measuring the fluorescence signal from 30-min dark-adapted leaves, when all reaction centres are open, using a low intensity pulsed measuring light source (F_0), and during a pulse saturating light [0.7 s pulse of 15,000 µmol(photon) m^{-2} s^{-1} of white light], when all reactions centres are closed (F_m). Following F_v/F_m estimation, after a 20-s exposure to actinic light [1,500 µmol(photon) m^{-2} s^{-1}], light-adapted steady-state fluorescence yield (F_s) was averaged over 2.5 s, followed by exposure to saturating light [15,000 µmol(photon) m^{-2} s^{-1}] for 0.7 s to establish F_m'. The sample was then shaded for 5 s with a far-red light source to determine F_0'. From these measurements, several fluorescence attributes were calculated (Bilger 1986, Genty *et al.* 1989) as the photochemical quenching [$q_P = (F_m' - F_s)/(F_m' - F_0')$], nonphotochemical quenching ($q_N = 1 - (F_v'/F_v)$), and efficiency of electron transport evaluated as Φ_{PSII} [$\Phi_{PSII} = \Delta F/F_m' = (F_m' - F_s)/F_m'$]. The photosynthetic electron transport rate (Marinari 2007) was estimated as ETR = $(\Delta F/F_m') \times PPFD \times 0.5 \times 0.84$, where PPFD is the photosynthetic photon flux density incident on the leaf, 0.5 is the factor that assumes equal distribution of energy between the two photosystems, and the leaf absorbance used was 0.84 because is the most common value for C_3 plants (Bilger 1986).

Foliar pigments, metabolites and oxidative stress indicators: The assays were analysed in the same leaves used for gas exchange and Chl *a* fluorescence measurements at two different stages (July and September in 2012 and 2013). They were treated according to Dinis *et al.* (2014). Leaf sections were ground in 80% acetone for Chl and carotenoid (Car) determination. Chl *a*, Chl *b*, total Chl (*a+b*), the Chl *a/b* ratio (Šesták *et al.* 1971), and total Car (Lichtenthaler 1987) were determined in *UV/Vis VARIAN* spectrophotometer (*CARY 100 Bio*, Australia). The total phenols (TP) in leaf extracts were determined according to the Folin-Ciocalteu's procedure (Singleton and Rossi 1965) on the same extract used for pigment analysis.

Soluble sugars (SS) were extracted by heating leaf discs in 80% ethanol and quantified according to the method of Irigoyen *et al.* (1992). The absorbance at 625 nm was determined in *UV/Vis VARIAN* spectrophotometer (*CARY 100 Bio*, Australia). Starch (St) was extracted with 30% perchloric acid (Osaki *et al.* 1991) and its concentration was determined by the anthrone method. Glucose was used as standard for both soluble sugars and starch. The total soluble proteins (SP) were extracted using an extraction buffer containing phosphate of pH 7.5 with ethylenediaminetetraacetic acid (EDTA) and quantified according to the method of Bradford (1976). The lipid peroxidation products in grape leaves were estimated as discribed by Heath and Packer (1968), with measurements of the supernatant at 532 and 600 nm in *UV/Vis VARIAN* spectrophotometer (*CARY 100 Bio*, Australia). The extration were done with 3 ml of 20% (w/v) trichloroacetic

acid (Rosati *et al.* 2006) and total thiobarbituric acid-reactive substances (TBARS) was quantified using an extinction coefficient of 157 M^{-1} cm^{-1} (Costa *et al.* 2002).

Yield: Total fruit mass was determined in each plant using a hand held balance ($n = 60$ plants per treatment).

Statistics: Statistical analyses were performed with *Sigma-Plot 12.0* program (*SPSS Inc.*). After testing for analysis of variance (*ANOVA*) assumptions (homogeneity of variances with the *Levene*'s mean test, and normality with the *Kolmogorov-Smirnov*'s test), statistical differences between years and treatments were evaluated by two-way factorial *ANOVA*, followed by the *post hoc Tukey*'s test. In the specific case of gas-exchange parameters data were submitted to one-way factorial *ANOVA*. Significant differences were considered for $p<0.05$.

Results

Reflectance: The kaolin application resulted in significant increases in the blue and green light (400–630 nm) reflectance, and in the near infrared (750–1000 nm) wavelength regions compared with the control (Fig. 2S, *supplement available online*). Relatively to $RI_{red\ edge}$, no significant differences were observed in July 2012, whereas two months after kaolin application this index was significantly higher in the treated plants than that in control ones (Fig. 1). In 2013, the $RI_{red\ edge}$ values were significantly higher than those obtained in 2012.

Leaf temperature values were higher in the control plants, while no significant differences were observed in both day periods in August and September 2012 (Fig. 2). Major differences between treatments were found in July in the morning (18.2 and 17.7% in 2012 and 2013, respectively), in July in midday (11.4 and 12.1% in 2012 and 2013, respectively), and in September 2013 (13.3 %). The major differences between years were observed in the midday period.

Water relations: The Ψ_{pd} of the kaolin-sprayed plants was higher in July, mainly in 2013 (40.7%), whereas the Ψ_{md} of these plants were higher in July and also in September (6.55 and 17.9%, respectively) (Table 1). Meanwhile, Ψ_{pd} and Ψ_{md} values were also dependent on the year, with higher Ψ_{pd} in 2013 (July and September). These differences were not associated with available soil water because this fraction at the 0–0.6 m soil profile showed a similar decreasing pattern in the kaolin-treated and control plots during both years. Further, no significant differences were found between treatments, though that fraction was generally lower in 2012 due to a drier winter.

Gas-exchange parameters showed that the treated plants exhibited significantly higher values of P_N (31.8% higher) in July 2012, only in the midday period (Table 2). Regarding to September, independently of the year, the kaolin-treated plants presented significantly higher values of g_s and P_N in both periods. However, in 2013, significant differences were also obtained in C_i/C_a, P_N/g_s, and E in both morning and midday periods. The kaolin-treated plants showed higher P_N/g_s and E, contrary to lower values of C_i/C_a. In most cases, the treatment effect was highly significant and accounted for most of the total variation than that of the year effect.

Chl *a* fluorescence: Relating to F_0, in the morning period, all values were lower in the treated plants, except in July 2013 (Table 3). Only obtained differences were found in the F_m values during the midday period, when the treated plants showed the higher values. The maximum quantum efficiency of PSII, estimated by the F_v/F_m ratio, was generally higher in the kaolin-treated plants compared with the control plants. Concerning of Φ_{PSII}, there was no significant difference between treatments, but in July 2012 during the midday period. In both periods and years, the leaves with kaolin had higher Φ_{PSII} values than that of the leaves without the clay particles. The q_P values seemed to be affected by the year (different conditions) rather
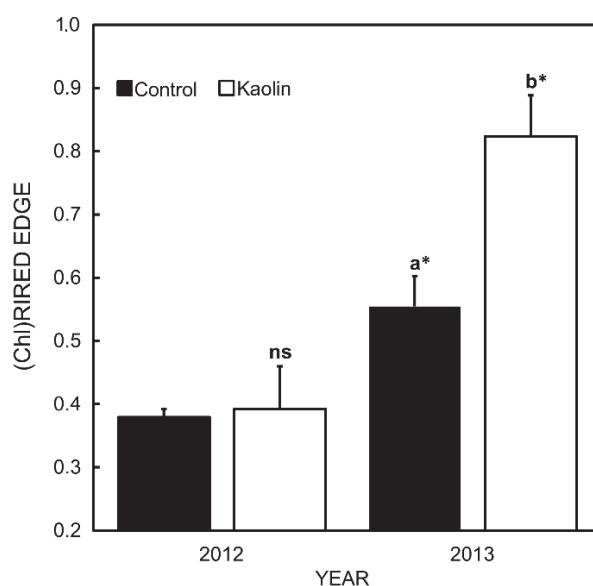
Fig. 1. Chlorophyll reflectance index (ChlRI$_{red\ edge}$) of grapevine leaves in September of 2012 and 2013. Values are presented as mean ± standard deviation (SD), $n = 8$ per treatment. Statistical analysis was performed using a two-way factorial *ANOVA*. Different *lowercase letters* represent significant differences between the treatment (control *vs.* kaolin), in the same year. * – significant differences between years within the same treatment ($p<0.05$).
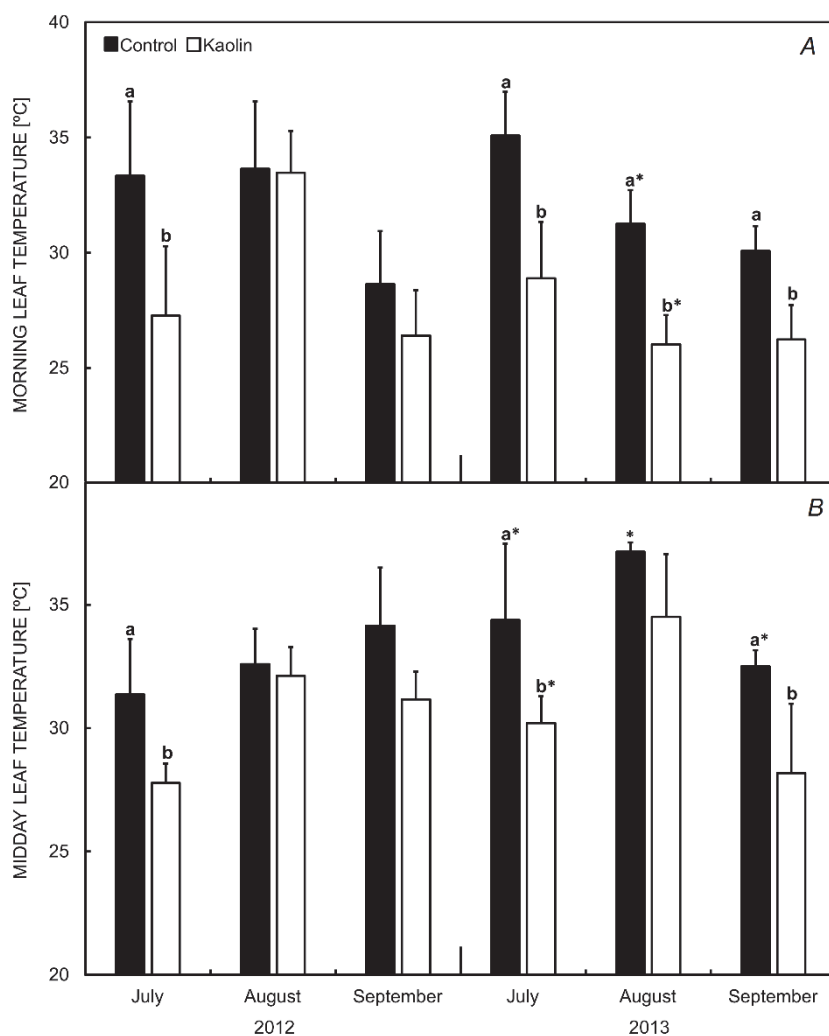
Fig. 2. Leaf temperature of kaolin-sprayed leaves and control ones measured in the morning and midday periods in July, August and September 2012 and 2013. Values are presented as mean ± standard deviation (SD), $n = 8$ per treatment. Statistical analysis was performed using a two-way factorial *ANOVA*. Different *lowercase letters* represent significant differences between the treatment (control *vs.* kaolin), in the same year, and * represents significant differences between years within the same treatment ($p<0.05$).

Table 1. Predawn (Ψ_{pd}) and midday (Ψ_{md}) leaf water potential of control and kaolin-sprayed grapevine leaves in 2012 and 2013. Values are presented as mean, $n = 8$ per treatment. Statistical analysis was performed using a two-way factorial *ANOVA*. Different *lowercase letters* represent significant differences between the treatment (control *vs.* kaolin), in the same year, and * represents significant differences between years within the same treatment ($p<0.05$).

	Ψ_{pd} [MPa]	Ψ_{md} [MPa]	Ψ_{pd} [MPa]	Ψ_{md} [MPa]	Ψ_{pd} [MPa]	Ψ_{md} [MPa]
	July		August		September	
2012						
Control	−0.445	−1.16	−0.545	−1.37	−0.850	−1.48
Kaolin	−0.413	−1.05	−0.546	−1.39	−0.867	−1.37
2013						
Control	−0.408[a]	−1.23[a*]	−0.550	−1.41	−0.538[*]	−1.45[a]
Kaolin	−0.242[b*]	−1.15[b]	−0.454	−1.43	−0.467[*]	−1.19[b*]
Two-way *ANOVA* (*p*-values)						
Treatment	0.003	0.047	0.180	0.353	0.610	<0.001
Year	0.002	0.197	0.220	0.109	<0.001	<0.001
Treatment × year	0.087	0.197	0.173	0.901	0.412	0.005

Table 2. Kaolin application effects on stomatal condutance (g_s, mmol m^{-2} s^{-1}), net CO_2 assimilation (P_N, μmol m^{-2} s^{-1}), intrinsic water-use efficiency (P_N/g_s, μmol mol^{-1}), intercellular CO_2 concentration to ambient CO_2 concentration ratio (C_i/C_a), and transpiration rate (E, mmol m^{-2} s^{-1}) in July (one week after kaolin application) and September (two months after kaolin application) 2012 and 2013. Values are presented as mean, $n = 8$ per treatment. Statistical analysis was performed using a two-way factorial *ANOVA*. Different *lowercase letters* represent significant differences between the treatment (control *vs.* kaolin), in the same year, and * represents significant differences between years within the same treatment ($p<0.05$).

	g_s	P_N	P_N/g_s	C_i/C_a	E	g_s	P_N	P_N/g_s	C_i/C_a	E
July 2012	Morning					Midday				
Control	199.7	7.43	57.8	0.688	3.79	99.8	5.92	60.8	0.659	3.16
Kaolin	165.5	10.8	65.8	0.640	4.39	123.0	7.80	64.8	0.631	3.81
Significance	ns	ns	ns	ns	ns	ns	*	ns	ns	ns
September 2012	Morning					Midday				
Control	47.5^a	4.19^a	80.2	0.493	2.20	30.2^a	2.65^a	85.1	0.555	1.49^a
Kaolin	93.6^b	7.85^b	88.5	0.543	2.73	45.9^b	3.98^b	87.1	0.544	2.02^b
2013										
Control	67.8^a	3.96^a	57.4^{a*}	0.675^{a*}	3.46^{a*}	35.9^a	1.87^a	46.6^{a*}	0.755^{a*}	1.12^a
Kaolin	132^{b*}	9.14^b	71.7^{b*}	0.588^b	5.42^{b*}	69.7^{b*}	6.58^{b*}	96.4^b	0.515^b	2.27^b
Two-way *ANOVA* (*p*-values)										
Treatment	<0.001	<0.001	0.039	0.533	0.010	<0.001	<0.001	0.027	0.016	<0.001
Year	0.035	0.552	0.001	0.001	<0.001	0.003	0.119	0.193	0.088	0.738
Treatment × year	0.494	0.398	0.565	0.028	0.117	0.05	0.007	0.039	0.025	0.086

than by the treatment, mainly in July. The q_N values were 22.5 and 58.0% higher in the kaolin-treated plants in 2012 and 2013, respectively, compared with those of the control during the midday period in September. Relating to ETR, the plants treated with kaolin had significantly higher values than the control ones. The F_v'/F_m' values in the plants treated with kaolin showed significant differences in September and the sprayed plants exhibited the higher values.

Foliar metabolic assays and oxidative stress indicators: No significant differences were found in photosynthetic pigment concentrations in July (Table 4). Conversely, kaolin particles caused a significant effect on the leaf pigment contents two months later (Table 4). In September, the Chl and Car concentrations were significantly higher in the kaolin-treated leaves. However, neither Chl *a/b* nor Chl/Car ratio were affected by the particle pulverization. In July, all parameters seemed to be influenced by the year. However, in September, only the Chl/Car ratio was affected.

Discussion

Vines sprayed with kaolin showed lower leaf temperature due to clay particles impact on leaf reflectance (Fig. 2S). According to Rosati *et al.* (2006), this effect reduces potentially damage caused by visible and ultraviolet radiations, and consequently reduces heat stress and sunburn damage. Our results are in agreement with those obtained for many tree species including apple, pomegranate, pecan, walnut, almond, grapefruit, pear, and citrus, where kaolin application reduced leaf and/or

Concerning foliar metabolites, only SS concentration was not significantly affected in July 2012 (Table 5). In this period, all other parameters were lower in the kaolin-treated leaves. In the treated plants, almost all biochemical variables were affected by the year during this month. The SS and St concentrations were significantly enhanced in the treated leaves in September (Table 5). The SS contents increased approximately 19.5 and 13.1% in 2012 and 2013, respectively, and the St concentration increased by approximately 46.5 and 6.3% in 2012 and 2013, respectively. The TP content, in this period, only showed significant differences between 2012 and 2013 and the SP content was higher in the kaolin-sprayed leaves. In this period, the amount of TP was lower.

Yield: The kaolin-treated plants showed higher values, with increases of 15.0 and 16.2% in 2012 and 2013, respectively (Fig. 3). In 2013, both treated and untreated plants boosted their production for more than the double, with significant differences.

canopy temperature by 1–6 °C (Glenn *et al.* 2003, Rosati *et al.* 2006, Shellie and King 2013). Together, the kaolin effects caused, in this specific study on field-grown grapevines, a significant increment of g_s and P_N, when compared with the untreated plants, mainly two months after the application. The lower values of P_N and g_s in the control plants at the morning and midday periods suggested that the decline in P_N could be related to stomatal limitations which are closely associated with

Table 3. Grapevine leaves minimal fluorescence (F_0), maximum fluorescence (F_m), maximum (F_v/F_m) and actual (Φ_{PSII}) quantum efficiencies of photosystem II, photochemical (q_P) and nonphotochemical (q_N) fluorescence quenching, electron transport rate (ETR, μmol e·m^{-2} s^{-1}) and capture efficiency of excitation energy by open PSII reaction centres (F'_v/F'_m) determined in the morning and midday periods in July (one week after Kaolin application) and September (two months after Kaolin application) of 2012 and 2013. Values are presented as mean, $n = 8$ per treatment. Statistical analysis was performed using a two-way factorial *ANOVA*. Different *lowercase letters* represent significant differences between years within the same treatment (control *vs.* kaolin), in the same year. * – significant differences between the treatment ($p<0.05$).

| | Morning | | | | | | | | Midday | | | | | | | |
	F_0	F_m	F_v/F_m	Φ_{PSII}	q_P	q_N	ETR	F'_v/F'_m	F_0	F_m	F_v/F_m	Φ_{PSII}	q_P	q_N	ETR	F'_v/F'_m
July																
2012																
Control	232.3a	960.5	0.757a	0.229a	0.668	0.973	144.4a	0.346a	168.8	870.4	0.689	0.284	0.311	0.620	178.3	0.494
Kaolin	189.2b	999.3	0.810b	0.272b	0.614	0.847	171.6b	0.428b	141.7	738.0	0.716	0.311	0.284	0.599	178.8	0.474
2013																
Control	213.6*	1009.0	0.830*	0.178*	0.473*	0.620*	124.4	0.401*	291.0*	768.5a	0.661*	0.290	0.685*	0.779	182.8	0.454
Kaolin	201.4*	990.2	0.841	0.203*	0.546*	0.533*	128.0*	0.404	244.5*	966.6b*	0.747	0.352	0.707*	0.975*	221.7	0.497
Two-way *ANOVA* (*p*-values)																
Treatment	0.628	0.732	0.009	0.010	0.769	0.142	0.084	0.011	0.059	0.464	0.205	0.056	0.959	0.300	0.405	0.761
Year	0.919	0.503	<0.001	<0.001	0.001	<0.001	0.001	0.336	0.002	0.167	0.975	0.541	<0.001	0.004	0.317	0.812
Treatment × Year	0.388	0.329	0.072	0.465	0.062	0.787	0.179	0.018	0.764	0.001	0.508	0.248	0.627	0.201	0.415	0.401
September																
2012																
Control	180.0a	983.3	0.808a	0.268	0.614	0.907	161.3	0.414	299.3	566.0a	0.651a	0.150a	0.406	1.26	103.8a	0.409a
Kaolin	155.1b	878.5	0.830b	0.278	0.618	0.876	194.8	0.498	218.0	834.6b	0.722b	0.264b	0.504	1.17	154.8b	0.487b
2013																
Control	185.6a	966.0	0.786a	0.295	0.577	0.938	185.5	0.475	318.0	608.5	0.745a*	0.181	0.494	1.13a	99.3a	0.353a
Kaolin	175.9b*	1010.0*	0.851b*	0.387*	0.658*	0.893	243.8*	0.594	228.1*	755.5	0.847b	0.230	0.551	0.758b*	144.9b	0.423b*
Two-way *ANOVA* (*p*-values)																
Treatment	0.009	0.446	<0.001	0.035	0.279	0.133	0.012	0.001	0.002	0.007	0.029	<0.001	0.112	0.024	0.001	0.002
Year	0.043	0.160	0.097	0.006	0.972	0.338	0.040	0.006	0.560	0.790	0.008	0.933	0.159	0.010	0.574	0.009
Treatment × Year	0.232	0.072	0.087	0.035	0.324	0.781	0.465	0.504	0.862	0.380	0.680	0.105	0.658	0.155	0.835	0.844

Table 4. Leaf photosynthetic pigments in control and kaolin-sprayed grapevine in July (kaolin application) and September 2012 and 2013. Values are presented as mean ($n = 6$ per treatment). Statistical analysis was performed using a two-way factorial *ANOVA*. Different *lowercase letters* represent significant differences between the treatment (control *vs*. kaolin), in the same year, and * represents significant differences between years within the same treatment ($p<0.05$). Chl – chlorophyll; Car – carotenoids.

	Chl *a/b*	Chl ($a+b$) [mg g^{-1}(DM)]	Car [mg g^{-1}(DM)]	Chl/Car
July 2012				
Control	2.68	5.08	1.11	4.59
Kaolin	2.55	4.63	1.02	4.60
2013				
Control	3.13*	3.60*	0.936*	3.84*
Kaolin	3.03*	3.45*	0.914	3.77*
Two-way *ANOVA* (*p*-values)				
Treatment	0.049	0.138	0.275	0.827
Year	<0.001	<0.001	0.009	<0.001
Treatment × year	0.870	0.436	0.512	0.773
September 2012				
Control	2.36	3.37[a]	0.774a	4.35
Kaolin	2.37	4.65b	0.894b	4.95
2013				
Control	2.70	2.93a	0.761[a]	3.82
Kaolin	2.73	3.81[b]	0.910[b]	3.93*
Two-way *ANOVA* (*p*-values)				
Treatment	0.897	0.011	0.049	0.242
Year	0.039	0.114	0.977	0.016
Treatment × year	0.948	0.606	0.822	0.416

lower leaf water potential (Table 1). The regulation of stomatal aperture limits the CO_2 entry into leaves and consequently leads to a decrease of P_N as a result of the reduced CO_2 availability (Centritto *et al.* 2005). In addition, the E was also lower in the control plants, which is in close association with the variation in g_s, as reported by Poni *et al*. (2009). Moreover, as the stress progressed, the nonstomatal component of photosynthesis was also affected, as demonstrated by the decrease of P_N and g_s which was associated with the increase of C_i/C_a ratio and the decrease of P_N/g_s during the midday period in 2013 (Table 2). The higher C_i/C_a and lower P_N/g_s in the control plants occurred due to nonstomatal limitations, either through CO_2 diffusion, carboxylation efficiency (Medrano *et al.* 2002), and/or photochemical perturbations. This last aspect was confirmed by Chl *a* fluorescence analysis. In general, total electron flow through PSII was more inhibited in the control plants, as indicated by reductions in the F_v/F_m, Φ_{PSII}, F_v'/F_m', and ETR (Table 3), mainly in the September 2013. The sharp increase in F_0 associated with a decrease in F_v/F_m, as observed in the control plants, means that the photoprotective capacity of the leaves was exceeded and photoinhibitory damage in the PSII had occurred (Valladares and Pearcy 1997). This process was also observed in plant response to high temperature (Gamon and Pearcy 1989) and water stress (Epron *et al.* 1992) (Table 3).

Contrary to the control plants, a noticeably lower decrease was found in g_s, P_N, and PSII photochemistry from morning to midday period in the kaolin-treated plants (Jifon and Syvertsen 2003). These results together, with the increase in q_N (for control plants), mainly at midday period in September, suggested a limited regulation for electron transport that was enough to match the decreased demand for electrons through $NADP^+$ consumption (DaMatta *et al.* 2002), which means a reduction in the opening of reaction centres (reduced quinones). The high value of q_N means a high dissipation of the excess energy by heat, avoiding the photosystem damages by oxidation (Baker 2008).

The $RI_{red\ edge}$, evaluated through leaf reflectance, is used for Chl prediction, and follows similar trends to those indicated in a study with apple (Merzlyak *et al.* 2003). As mentioned above, two months after the kaolin treatment, the $RI_{red\ edge}$ was higher in the treated leaves (Fig. 1) and during the same period, kaolin-treated plants had also higher Chl ($a+b$) contents (Table 4). The lower Chl ($a+b$) concentration in the control plants revealed a sign of oxidative stress due to Chl degradation and/or reduced Chl synthesis (Smirnoff 1993). Also, the kaolin-treated leaves showed the higher Car content, which has an important role in preventing Chl degradation. Thus, the relationship between Chl and Car may be used as a potential indicator of photooxidative damage (Moutinho-Pereira *et al.* 2007).

Table 5. Grapevine leaves concentration of soluble sugars (SS), starch (St), total phenols (TP), soluble proteins (SP), and total thiobarbituric acid-reactive substances concentration (TBARS) quantified in September 2012 and 2013 after application of kaolin in July. Values are presented as mean (n = 6 per treatment). Statistical analysis was performed using a two-way factorial *ANOVA*. Different *lowercase letters* represent significant differences between the treatment (control *vs*. kaolin), in the same year, and * represents significant differences between years within the same treatment ($p<0.05$).

|	SS [mg g^{-1}(DM)]	St [mg g^{-1}(DM)]	TP [mg g^{-1}(DM)]	SP [mg g^{-1}(DM)]	TBARS [µmol g^{-1}(DM)]
July 2012					
Control	110.7	19.7[a]	316.0[a]	79.2[a]	10.8[a]
Kaolin	117.8	10.3[b]	236.8[b]	64.8[b]	8.55[b]
2013					
Control	183.5*	20.9	308.2	69.5*	6.10*
Kaolin	197.9*	19.0*	301.9*	71.2*	5.98
Two-way *ANOVA* (*p*-values)					
Treatment	0.228	0.046	0.012	0.031	0.536
Year	<0.001	0.010	0.078	<0.001	0.043
Treatment × year	0.679	0.004	0.029	<0.001	0.491
September 2012					
Control	145.4	42.3[a]	268.0	103.0	4.96
Kaolin	180.7	79.0[b]	285.2	146.3	4.73
2013					
Control	197.5[a]*	16.4*	364.7[a]*	128.0[a]*	5.93[a]
Kaolin	227.3[b]*	17.5*	292.8[b]*	204.8[b]*	5.13[b]
Two-way *ANOVA* (*p*-values)					
Treatment	0.020	<0.001	0.119	0.049	0.041
Year	0.001	<0.001	<0.001	<0.001	0.283
Treatment × year	0.832	<0.001	0.049	0.461	0.648
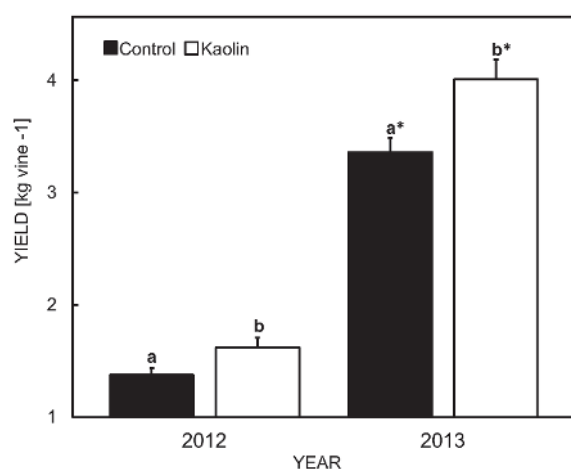
Fig. 3. Yield of kaolin-sprayed vines and control ones. Values are presented as mean ± standard deviation (SD), n = 60 plants per treatment. Statistical analysis was performed using a two-way factorial *ANOVA*. Different *lowercase letters* represent significant differences between the treatment (control *vs*. kaolin), in the same year, and * represents significant differences between years within the same treatment ($p<0.05$).

Another indicator of oxidative stress in the control leaves was observed in September 2013 due to the increase in lipid peroxidation (Table 5). It was extensively described that reactive oxygen species cause lipid peroxiETRdation leading to membrane damage (Shalata and Tal 1998). Our

results suggested that kaolin application reduced the oxidative damage of cell membranes which was indicated by the decrease in lipid peroxidation. In this context, the control plants needed to develop some defense mechanisms against oxidative stress, such as the increase in TP concentration (Bacelar *et al.* 2006). This increase in total polyphenols could be a consequence of phenylalanine ammonia lyase activated under high temperature (Fig. 2) (Wen *et al.* 2008). According to the growth/differentiation balance theory (Koricheva *et al.* 1998), the pathways which compete for phenylalanine as a precursor, can lead either to phenylpropanoid or to protein synthesis. Under stress conditions, carbon is diverted to phenylpropanoid synthesis, which might explain the increase of phenolic compound contents in the control plants. However, under adequate water availability and light conditions, the growth and development are priority and the bulk of phenylalanine is used for protein synthesis (Koricheva and Larsson 1998), which seems to be occurring in the kaolin-treated leaves in September (Table 5). Changes in soluble proteins are important indicators to understand the impact of stress on cell proteolysis and protein synthesis (Santos and Caldeira 1999). The lower values of SP in the control plants (Table 5) might be a consequence of the hydrolysis of some proteins in response to an increased request for amino acids induced by higher leaf temperature under water stress (Yadav *et al.* 1999). One of the most important responses of plants to water stress is an overproduction of

diverse types of compatible solutes (Ashraf and Harris 2004). It has been already demonstrated by our group that kaolin-treated plants showed lower contents of proline (Dinis *et al.* 2016), which reinforces the idea that this particle film is a protecting agent for grapevines growing under water stress, strong light, and high temperature conditions.

The kaolin-treated plants had higher P_N, sink capacity, and consequently the yield per vine, which led to a higher SS concentration than that of the control plants (Table 5). Thus, with lower photosynthetic limitations in the kaolin-treated plants, SS values increased, leading to a temporary increase in the St concentration (Table 5). Overall, taken together these results can explain the higher yield per plant in grapevines treated with kaolin. Meanwhile, more rain in April/May 2012, coinciding with the flowering and fruit-set stage, explained the decrease in the production per vine, relatively to the 2013 season.

In summary, this study showed that kaolin foliar spray reduced leaf temperature and increased leaf water potential, g_s, and P_N (improving overall plant physiological performance). The increase of photosynthesis, soluble proteins, soluble sugars, and starch, and the reduction of solar damage, make the application of *Surround®* (kaolin) a promising short-term climate change mitigation strategy. Its effectiveness in reducing summer stress on grapevines and improving their yield quality reinforced experimental data published earlier (Dinis *et al.* 2016), but also added new information. Kaolin can be efficiently used to alleviate the negative impacts of summer stress in the Mediterranean-like climates, especially taking into account the climate change projections for these regions. Moreover, kaolin is a natural product, which can be used in organic production.

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