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A B S T R A C T

Photosynthetic performances and energy dissipation mechanisms were evaluated on the anisohydric cv. Sangiovese and on the isohydric cv. Montepulciano (*Vitis vinifera* L.) under conditions of multiple summer stresses. Potted vines of both cultivars were maintained at 90% and 40% of maximum water availability from fruit-set to veraison. One week before veraison, at predawn and midday, main gas- exchange and chlorophyll fluorescence parameters, chlorophyll content, xanthophyll pool and cycle and catalase activity were evaluated. Under water deficit and elevated irradiance and temperature, contrary to cv. Montepulciano and despite a significant leaf water potential decrease, Sangiovese's leaves kept their stomata more open and continued to assimilate CO_2 while also showing higher water use efficiency. Under these environmental conditions, in comparison with the isohydric cv. Montepulciano, the protective mechanisms of energy dissipation exerted by the anisohydric cv. Sangiovese were: (i) higher stomatal conductance and thermoregulation linked to higher transpiration rate; (ii) greater ability at dissipating more efficiently the excess energy via the xanthophylls cycle activity (thermal dissipation) due to higherVAZ pool and greater increase of de-epoxidation activity.

1. Introduction

Under field conditions, plants are rarely affected by a single abiotic stress but rather from multiple environmental limiting factors which might include excessive light and temperature, too low relative humidity, water shortage, etc. (Mittler, 2006). Usually, when combined, these abiotic stresses have synergistic effects, causing a significant decrease in CO_2 fixation capability due to stomata closing and/or to impairment of photochemical reactions that limit growth, yield and fruit composition up to threatening plant's survival. This situation is very frequent in semi-arid environments, typical of countries of the Mediterranean basin where grapes are grown since long time, or in other overseas countries where grape growing is more recent (i.e., Australia, Chili and some areas of Argentina).

It is known that environmental stresses lead to a chain of morpho-structural, physiological, biochemical and molecular changes which influence plant growth and yield (Chaves, 2002; Wang et al., 2003). According to the species and to the timing and intensity of abiotic stresses, different mechanisms have been evolved, at both molecular and cellular level, to increase tolerance just after the onset of stress conditions. Under these conditions, the gene expression profiles result significantly altered, especially in specific functional categories such as "antioxidative response", "signaling" and "protein metabolism" and it improves thereby the

Abbreviations: P_n, net photosynthesis; g_s, stomatal conductance; WUE_i, intrinsic water use efficiency; Chl, chlorophyll; ETR, electron transport rate; NPQ, non photochemical quenching; P_r, photorespiration; F_v/F_m, maximum photochemical efficiency; J_c, electron flow to carboxylation; Φ_{PSII}, actual maximum quantum yield of PSII; CAT, catalase; H_2O_2, hydrogen peroxide; W_l, leaf water potential; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; WW, well-watered; WS, water-stressed.

abiotic stress defense pathways (Carvalho et al., 2011). Drought and high temperatures cause oxidative stress and consequently may determine the denaturation of structural and functional proteins. However, usually these abiotic stresses activate a different categories of genes coding for heat-shock proteins, chaperones, osmoprotectants, free radical scavengers, acquaporins and ion transporters as well as proteins which protect the membranes (Wang et al., 2003). Recently, Rocheta et al. (2013) has reported that under acute heat stress the cv. Aragonez expresses a huge number of genes with high energetic costs, whereas under water deficit it reduces the gene expression to a minimum and increases only those genes necessary to survive.

There is a fairly shared consensus that the *Vitis vinifera* L., due to the more than 10,000 varieties cultivated worldwide (Alleweldt and Dettweiler, 1992), displays large variation in terms of tolerance to drought which is primarily related to stomatal behavior (Escalona et al.,1999; Chaves and Oliveira, 2004) as between-genotype variation in photosynthetic rate seems to be minor under both well watered and water deficit conditions (Chaves et al., 1987; Schultz, 1996; Bota et al., 2001).

As it has been previously reported, the two red grape cultivars most commonly cultivated in Italy, i.e., Sangiovese and Montepulciano currently grown on more than 100,000 ha acreage, show intraspecific differences in morpho-physiological characteristics and productive performances under non limiting water conditions (Palliotti et al., 2014). Unlike Montepulciano, which under drought is classified as a isohydric genotype, the near-anisohydric Sangiovese retains higher whole vine carbon fixation, thereby being considered a genotype more adapted to drought (Palliotti et al., 2008, 2009, 2014; Tombesi et al., 2014).

Under drought conditions, stomatal regulation versus water loss determines the level of carbon gain and therefore plant growth, productivity and survival. Recently, it has emerged that the mechanisms that allow the various varieties to adapt to summer stresses are mediated by stomatal sensitivity and are genetically controlled (Medrano et al., 2003; Beis and Patakas, 2012; Hochberg et al., 2013; Palliotti et al., 2014). According to stomata kinetics under drought, *V. vinifera* embraces varieties classified as isohydric, which are capable of maintaining a fairly constant midday leaf water potential regardless of soil water availability by means of early stomatal closure, and anisohydric, which maximize photosynthetic gain by maintaining stomata open despite a large leaf water potential drop (Tardieu and Simmoneau, 1998). In truth, a whole range of responses between completely isohydric and anisohydric behaviors can be detected in different plant species, and the two strategies can even occur within different cultivars of the same species or as a function of different water stress intensity, duration and time of application as well as interactions with excessive irradiance and temperature (Poni et al., 2007; Chaves et al., 2010). This is likely to depend on the fact that some responses were defined using one- or two-year old vines, often without clusters, and/or with water stress artificially applied after the end of growth. After the end of growth, responses to drought usually are limited to only short-term adjustment responses mainly at leaf level. Indeed, adjustment processes at plant level, such as plant architecture and hydraulic, occurs only when the stresses are experienced during the shoot and root growth (Chaves et al., 2002; Schultz, 2003; Choat et al., 2010; Palliotti et al., 2014).

This study investigates physiological changes and some energy dissipation mechanisms showed by primary leaves of outdoor grown vines of cvs. Sangiovese and Montepulciano during the hottest hours of summer days under concurrent light, heat and water stresses. Catalase activity and one of the major marker of oxidative stress, i.e. the leaf bulk of H_2O_2 concentration, as well as total chlorophyll content and xanthophyll-cycle pigments were analyzed at midday and compared with values measured at predawn.

2. Materials and methods

2.1. Experimental conditions and trial layout

This study was conducted in 2011 on eight-year-old potted (60 L) vines of *V. vinifera* L. cv. Sangiovese (clone VCR30) and cv. Montepulciano (clone R7) grafted onto 1103 Paulsen rootstock and grown in an outdoor area close to the Faculty of Agriculture of the University of Perugia (Region of Umbria, central Italy, 42°58′N, 12°24′E, elevation 405 m a.s.l.). All the pots were filled with loam soil with a field capacity of 30.2% [(vol water/vol soil)× 100] and a wilting point of 16.7%. At the end of February, each vine was pruned to retain 4 spurs with 2 buds each. All shoots were oriented up-right using suitable stakes. Ten vines per cultivar were used and maintained at about 90% of maximum water availability (WW, well-watered vines) and 10 vines received, from fruit-set to veraison, 40% of maximum water availability (WS, water-stressed vines). During water limitation, all stressed pots were covered with a plastic film to avoid interference due to rainfall and soil water evaporation. The water supply per pot was determined by monitoring the soil water content with a Diviner 2000® capacitance probe (Sentek Environ. Tech., Australia) using access tubes.. In each pot, in June, July and August the water was supplied every day at 20:00 h.

2.2. Leaf water potential, gas-exchange and chlorophyll fluorescence measurements

In 2011, one week before veraison, gas exchange readings of single leaves from both Sangiovese and Montepulciano vines were taken at midday (between 13:00 and 14:00 h) using a portable, open system, LCA-3 infrared gas analyzer (ADC Bio Scientific Ltd., Herts, UK). The system was equipped with a broad leaf chamber with a 6.25 cm^2 window and all readings were taken at ambient relative humidity with an air flow adjusted to 350 mL min^{-1}. For each cultivar, twenty average-size leaves, chosen between nodes 14 and 16 of primary shoots, were sampled under saturating light (PAR > 1400 μmolphotons m^{-2} s^{-1}). Net photosynthetic rate (P_n) and stomatal conductance (g_s) were calculated from inlet and outlet CO_2 and H_2O concentrations. Intrinsic water use efficiency (WUE_i) was then calculated as Pn/g_s ratio. Just after the gas-exchange measurements, the leaf temperature was evaluated on the same leaves (20 per each treatment and variety) with an infrared thermometer (Mod. TM909L9, Assi-control, Italy) and the leaf dark respiration (R_d) was measured in the dark (PAR level of 0 μmolphotons m^{-2} s^{-1}) obtained by covering the broad leaf chamber with a black sheet. The time needed to reach the steady state was between 2 and 6 min according to leaf temperatures, in accordance with Zufferey et al. (2000) during this time the leaf temperatures equilibrated with ambient temperature. Moreover, between 13:00 and 14:00 h, leaf water potential (W_l) was measured on ten leaves for each cultivar and treatment using a portable pressure chamber (model 1000, PMS Instruments Co., USA). In order to highlight possible instability of the photochemical apparatus, on the same leaves sampled for gas exchange, modulated chlorophyll fluorescence measurements were carried out around midday (between 13:00 and 14:00 h) using a field-portable pulse modulated fluorometer (FMS-2, Hansatech Instruments, Norfolk, UK). Leaves were dark adapted for 30 min to obtain open PSII centers using the instrument leaf-clips to ensure maximal photochemical efficiency. The fiber optic and its adaptor were fixed to a ring located over the leaf-clip at approximately 1 cm from the sample

and the different light pulses were applied following the standard routines programmed within the instrument. Signal recordings and calculations were performed using the data analyzer and control software provided with the FMS-2. The fluorescence origin (F_0) was measured by applying a week modulated light pulse (<1 μmol m^{-2} s^{-1}) to avoid appreciable variable fluorescence. Maximum fluorescence (F_m) was induced by a 0.8 s saturating light pulse at 9000 μmol m^{-2} s^{-1}. The maximum efficiency of PSII photochemistry was calculated as F_v/F_m, where F_v (variable fluorescence) was calculated asthe difference between F_m and F_o. Steady-state fluorescence yield (F_s) was determined by averaging the fluorescence signal under the ambient light; a saturating pulse at 8000 μmol photons m^{-2} s^{-1} was imposed to determine the maximum fluorescence in the light-adapted state (F_m'). The PSII actual efficiency (Φ_{PSII}) was computed as $(F_m'-F_s)/(F_m')$ (Genty et al., 1989). The non-photochemical quenching was calculated as NPQ = $(F_m/F_m')-1$ (Bilger and Björkman, 1990). The apparent electron transport rate (ETR) through PSII was computed as $\Phi_{PSII} \times PAR \times 0.5 \times 0.84$ (Björkman and Demmig-Adams, 1987), where PAR is the photosynthetic active radiation, 0.5 implies an equal distribution of excitation between PSI and PSII and 0.84 assumes that leaf absorbance was the same as in other grapevine cultivars (Schultz, 1996).

Photorespiration (P_r) and electron transport used for carboxylation (J_c) were estimated according to Valentini et al. (1995) using the following equations:

$$P_r = \frac{1}{12}[ETR - 4(P_n + R_d)]$$

$$J_c = \frac{1}{3}[ETR - 8(P_n + R_d)].$$

The relative significance of P_r as a photoprotective mechanisms was also evaluated by estimating the ETR/P_n ratio.

2.3. Chlorophyll and xanthophyll determination

These analysis were made on the same leaves used for gas-exchange measurements at pre-dawn (5:00 h) and midday (13:00 h). Three samples per treatment, each consisting of 8 pieces of different leaves taken between nodes 14 and 16 of primary shoots, were cut and immediately wrapped in aluminum foil and dipped into liquid N_2. The samples were then stored at 80 °C until pigment analyses. The pigments extraction was carried out fol- lowing the method of Lashbrooke et al. (2010). All the operations were done under subdued light to avoid degradation or isomer- ization of carotenoids. Twoμg of *trans*-ß-apo-8^r-carotenal were used as internal standard (IS). The separation by RP-HPLC-DAD was carried out on an Agilent1260HPLC system (Agilent Technolo- gies, Palo Alto, California, USA) equipped with a DAD system. A YMC30 column (3 μm, 150 4.6 mmid), protected by a guard col- umn(5 μm, 10 4 mmid) (both from YMC Europe, Schermbeck, Germany) was used and the G1315C Agilent ChemStation software was used for data processing. Mobile phase solvents consisting of 3% H_2O in methanol containing 0.2% (w/v) ammonium acetate 2 M (solvent A) and 100% methyl-*t*-butyl ether (MTBE) (solvent B) were employed; both solvents contained 0.005% (w/v) triethylamine (TEA). The optimal conditions of separation were found to be at a flow rate of 0.8 mL min^{-1} with the column temperature maintained at 20 °C and an injection volume of 20 μL. Elution was done according to the following program: isocratic at 15% B for 4 min followed by a linear increase from 15 to 20% B in 2 min, an isocratic at 20% B for 9 min, a linear increase to 50% B in 3 min, an isocratic at 50% B for 2 min, a linear increase to 70% B in 5 min and isocratic at 70% B for 1 min. The column was equilibrated for 5 min at the starting conditions before each injection. The elution of the various carotenoid and chlorophyll pigments was acquired by scanning from 280 to700 nm. Pigments were identified by comparison of their retention times and spectral properties with those of authentic standards. The authentic standards *trans*-ß-carotene, chlorophyll *a* and *b*, *trans*-ß-apo-8^r-carotenal and lutein were purchased from Sigma–Aldrich. CaroteNature GmbH (Lupsingen, Switzerland) supplied lutein epoxide, zeaxanthin, neoxanthin, violaxanthin and antheraxanthin. Standards were prepared as indicated in Lashbrooke et al. (2010) with an initial stock solution of each standard at 1 mg mL^{-1} in chloroform containing 0.1% BHT. Standard curves for the quantification of carotenoids and chlorophylls were obtained by plotting the analytes to IS peak areas ratios versus analytes to IS nominal concentrations ratios, at the wavelength chosen for each compound. The curves were created by triplicate injections of 20 μLof serial dilutions of the stocks solutions (10, 50, 100, 500, 1000, 5000 ng mL^{-1}) containing 2 μg of IS (as for samples) corresponding to a concentration of 2000 ng mL^{-1}.

2.4. Catalase activity, H_2O_2 content and total protein determination

On the same leaves used for Chl and xanthophyll analysis, catalase (CAT) enzyme was extracted and measured following the method reported in Ozden et al. (2009), slightly modified. Frozen leaf samples were grounded under liquid N_2 and 0.5 g of powder were weighted and suspended in 2 mL of a 50 mM K-phosphate buffer at pH 7.0 containing 2 mM of EDTA and 1% of PVP. After 10 min of centrifugation at 10,000×*g* and 4 °C, the supernatant representing the crude extract was collected and destined to the successive enzymatic assay. Specific CAT activity was evaluated at 240 nm, with a Lambda 3B UV–vis spectrophotometer (PerkinElmer Instruments Ltd., Seer Green, Beaconsfield, U.K.), by recording the H_2O_2 disappearance over 10 min of reaction at 25 °C. The mixture of the assay was composed by 2.88 mL of 50 mM K-phosphate buffer at pH 7.0, 70 μL of 20 mM H_2O_2, and 50 μL of crude extract which was added the last to start the reaction. The blank was represented by the same mixture where the crude enzyme was replaced by the buffer. The activity was calculated as μmol of H_2O_2 consumed by the enzyme during 1 min of linearity per g of fresh tissue and/or per mg of total proteins. Hydrogen peroxide (H_2O_2) concentration was measured slightly adapting the method described by Loreto and Velikova (2001). 0.15 g of frozen leaf tissue, grounded under liquid N_2, was suspended in 0.5 mL of 1% tricloracetic acid (TCA). After stirring, the suspension was centrifuged at 10,000 × *g* for 10 min and 4 °C, then the supernatant collected and destined to the spectrophotometric assay. H_2O_2 concentration was appreciated by a Lambda 3B UV–vis spectrophotometer (PerkinElmer Instruments Ltd., Seer Green, Beaconsfield, U.K.) by reading, at 390 nm, a mixture containing: 0.75 mL of 50 mM K-phosphate buffer at pH 7.0, 1.5 mL of 1 M of KI solution, and 0.75 mL of supernatant. The blank was prepared by replacing the buffer to the supernatant. The concentration of H_2O_2 was calculated from a standard curve plotted in the range from 100 to 1000 μmol/ml and expressed as μmol/g of fresh weight and/or per mg of proteins. Total proteins were extracted and measured following the Bradford's method (1976). Their amount was destined to be included in the calculation of CAT enzyme activity and H_2O_2 concentration.

2.5. Statistical analysis

Differences between means within each cultivar, treatment and hour of sampling were assessed by two-way ANOVA (genotype vs. water availability) using Student–Newman–Keuls test ($p < 0.05$). To test differences in photosynthetic pigments, CAT activity and H_2O_2 content between the two hours of sampling (05:00 and 13:00 h), a one-way ANOVA followed by *t*-Student test ($p < 0.05$) was applied.

All statistical analysis were performed using SigmaStat software (SPSS Science, USA).

3. Results

3.1. Leaf water potential, gasexchange and chlorophyll fluorescence

Regardless of the cultivar, during the hottest hours of the day, the median leaves of WW-vines showed a similar W_l (about −0.6 MPa), as well as g_s and WUE_i values, whereas Montepulciano exhibited a significant higher P_n than Sangiovese (+12%) (Fig. 1). Over the same time interval, characterized by an average air temperature of 37.8 °C and an incident PAR of about 1900 μmol photons $m^{-2} s^{-1}$, the median leaves of WS-Sangiovese vines had a significant decrease of W_l (about −0.8 MPa compared to homologous leaves of WW-vines) reaching −1.42 MPa, whereas under the same conditions the W_l reduction in WS-Montepulciano vines was significantly lower and equal only to −0.4 MPa as compared to WW-vines (Fig. 1). Concurrently, in comparison to WW-vines, the WS-Montepulciano vines displayed a significant lower P_n and g_s compared to WS-Sangiovese vines. Contrary to Montepulciano, in Sangiovese vines such drought conditions significantly increased the WUE_i calculated during the hottest hours of the day (Fig. 1). Under the same drought conditions, F_v/F_m values did not change between cultivars, whereas dark respiration (R_d) increased by about 20% in Sangiovese and 28% in Montepulciano, respectively, as compared to non-stressed vines (Table 1).

Regardless of cultivar, photorespiration (P_r), electron flow to carboxylation (J_c), actual photochemical efficiency of PSII (Φ_{PSII}) and electron transport rate (ETR) showed significantly lower values in leaves of stressed-vines, whereas an opposite behavior was observed for non photochemical quenching (NPQ) (Table 1). Nevertheless, P_r expressed as percentage of P_n increased under drought at a faster rate in Montepulciano than in Sangiovese, namely 73% and 52%, respectively.

The ETR/P_n ratios, which can be used to evaluate photorespiration as a photoprotective mechanism, significantly increased under multiple summer stresses (Table 1). However, in WS-Montepulciano leaves this parameter showed an higher increase in comparison to homologous leaves of WS-Sangiovese vines, precisely +75% against +18% only, respectively.

3.2. Chlorophyll, xanthophyll, catalase activity and H_2O_2 content

The Chl content changed according to unit of measurement; indeed when expressed on a per leaf area basis, drought conditions significantly reduced the total Chl content irrespective of cultivar and sampling time (Table 2); conversely, the Chl content was not affected in both cultivars when given on a g of leaf fresh weight basis. Finally, when expressed on a dry weight basis, the Chl content was significantly increased in Sangiovese under drought (+12% and +18% at 5:00 and 13:00 h, respectively) and remained unchanged in Montepulciano. In both cultivars, there was no variation in the total Chl content according to the sampling time. Apart from treatment and time of measurement, Sangiovese leaves had lower Chl content than Montepulciano, around −20% (Table 2).

Contrary to Montepulciano, in Sangiovese leaves the drought conditions increased the content of lutein (+25% in the leaves sampled at 5:00 h and +22% in those sampled at 13.00 h, compared to WW-vines), whereas the lutein 5,6-epoxide was never detected (Table 2).

Regardless of time of sampling and treatment, an higher VAZ pool was found in Sangiovese leaves compared to Montepulciano ones (Fig. 2). The characterization of xanthophylls composition during the day showed that in the leaves of WS-Sangiovese vines, V significantly decreased from pre-dawn to midday (about −80%) compared to Montepulciano leaves (−55%) (data not shown).

In both cultivars, under drought conditions the VAZ pool was enhanced and it did not change according to time of sampling (Fig. 2). Sangiovese leaves had an higher de-epoxidation state which, at midday, reached a value of about 0.8 against less than 0.6 found in Montepulciano.

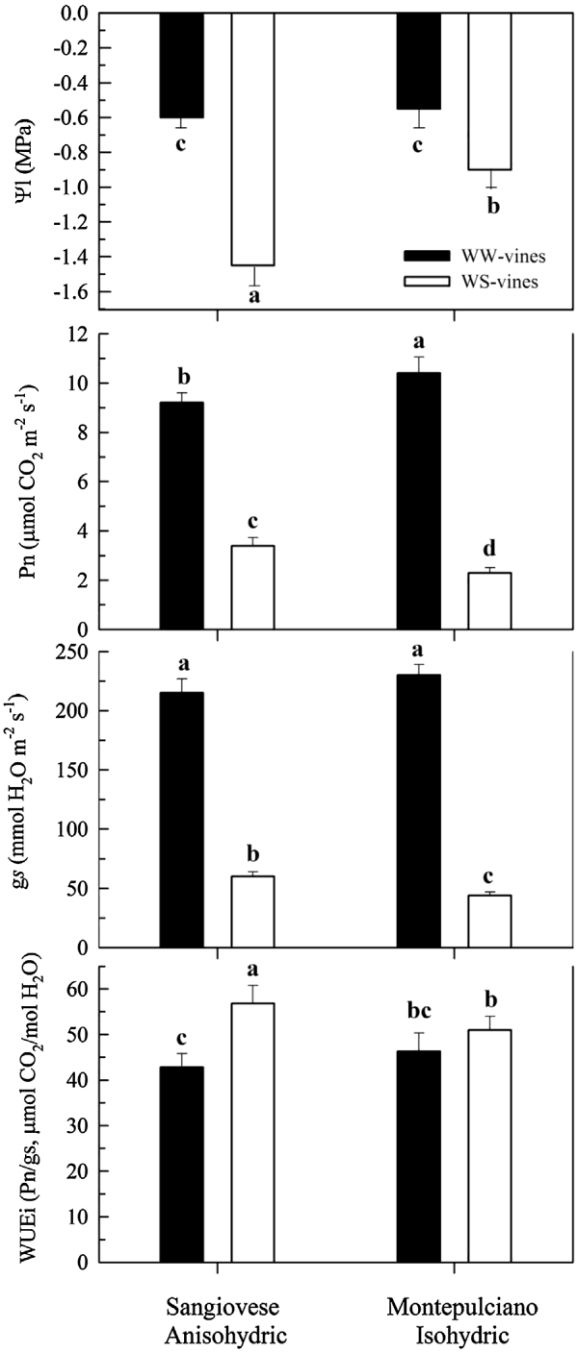

Fig. 1. Leaf water potential (W_l), net photosynthesis (P_n), stomatal conductance (g_s) and intrinsic water use efficiency (WUE_i) measured in leaves of 60-L potted well-watered (WW-vines) and water-stressed (WS-vines) Sangiovese and Montepulciano vines. Data were taken 1 week before veraison, after 7 weeks of water deficit, during 12–13:00 h interval under heat stress + light stress + water stress (air $T°$ of ~37–38 °C, incident PAR of ~1900 μmol photons $m^{-2} s^{-1}$ and soil moisture set at 40% of maximum water availability). Data represent the average value of 20 leaves ± S.E. (for W_l the leaves used were 10). Different letters indicates significant differences between treatments ($p < 0.05$) based on Student–Newman–Keuls test.

Table 1
Changes in leaf temperature, maximal photochemical efficiency (F_v/F_m, arbitrary units), dark respiration (R_d, μmol CO_2 m^{-2} s^{-1}), non photochemical quenching (NPQ, relative units), photorespiration (P_r, μmol CO_2 m^{-2} s^{-1}), electron flow to carboxylation (J_c, μmol CO_2 m^{-2} s^{-1}), actual photochemical efficiency of PSII (Φ_{PSII}), electron transport rate (ETR, μmol e^- m^{-2} s^{-1}) and ETR/P_n ratio (μmol e^- μmol^{-1} CO_2) in mature leaves of 60-L potted well-watered (WW) and water-stressed (WS) Sangiovese and Montepulciano vines. Data were taken 1 week before veraison, after 7 weeks of water deficit, at midday (12–13:00 h interval) under heat stress + light stress + water stress (air $T°$ of ~37–38 °C, incident PAR of ~1900 μmol photons m^{-2} s^{-1} and soil moisture set at 40% of maximum water availability).

	cv. Sangiovese		cv. Montepulciano	
	WW-vines	WS-vines	WW-vines	WS-vines
Leaf temperature (°C)	39.1 ± 1.2 b	42.6 ± 0.9 a	39.0 ± 0.9 b	42.5 ± 1.0 a
F_v/F_m	0.801 ± 0.01 a	0.790 ± 0.02 a	0.816 ± 0.01 a	0.802 ± 0.01 a
R_d	1.01 ± 0.08c	1.21 ± 0.05 b	1.22 ± 0.04 b	1.55 ± 0.09 a
NPQ	0.97 ± 0.08 c	1.39 ± 0.10 a	0.94 ± 0.05 c	1.20 ± 0.09 b
P_r[x]	3.2 ± 0.5 a	1.8 ± 0.2 b	3.3 ± 0.7 a	1.7 ± 0.3 b
P_r (% of P_n)	34 ± 4 c	52 ± 5 b	32 ± 5 c	73 ± 7 a
J_c[y]	54 ± 6 a	26 ± 5 b	60 ± 8 a	22 ± 6 b
Φ_{PSII}	0.51 ± 0.06 a	0.37 ± 0.04 b	0.55 ± 0.04 a	0.31 ± 0.04 b
ETR	82 ± 12 a	41 ± 8 b	88 ± 14 a	37 ± 6 b
ETR/P_n	9.1 ± 0.4 c	10.8 ± 0.4 b	7.3 ± 0.5 d	12.8 ± 0.6 a

Means followed by different letters are significantly different at $p < 0.05$ according to Student–Newman–Keuls test.

[x] $P_r = 1/12\ [ETR - 4\ (P_n + R_d)]$ (Valentini et al., 1995).

[y] $J_c = 1/3\ [ETR + 8\ (P_n + R_d)]$ (Valentini et al., 1995).

Table 2
Changes in total chlorophyll, lutein and lutein 5,6-epoxide in mature leaves of 60-L potted well-watered (WW) and water-stressed (WS) Sangiovese and Montepulciano vines. Data were taken 1 week before veraison, after 7 weeks of water deficit, at predawn (5:00 h) and at midday (12–13:00 h interval) under heat stress + light stress + water stress (air $T°$ of ~37–38 °C, incident PAR of ~1900 μmol photons m^{-2} s^{-1} and soil moisture set at 40% of maximum water availability).

	Hours of the day	cv. Sangiovese		cv. Montepulciano	
		WW-vines	WS-vines	WW-vines	WS-vines
Total chlorophyll (μg cm^{-2})	5.00	35.4 ± 4 c	28.6 ± 3 d	58.0 ± 6 a	48.1 ± 5 b
	13.00	33.9 ± 6 c	27.1 ± 5 d	60.2 ± 4 a	49.0n± 6 b
		ns	ns	ns	ns
Total chlorophyll (μg g^{-1} FW)	5.00	1598 ± 55 b	1702 ± 62 b	2401 ± 81 a	2226 ± 68 a
	13.00	1681 ± 45 b	1792 ± 46 b	2358 ± 65 a	2220 ± 64 a
		ns	ns	ns	ns
Total chlorophyll (μg g^{-1} DW)	5.00	4990 ± 135 b	5528 ± 76 b	7232 ± 88 a	7022 ± 64 a
	13.00	5285 ± 102 c	6243 ± 122 c	7102 ± 67 a	7003 ± 74 a
		ns	ns	ns	ns
Lutein (μg g^{-1} DW)	5.00	376 ± 17 b	470 ± 51 a	518 ± 57 a	534 ± 58 a
	13.00	399 ± 38 b	484 ± 42 a	552 ± 39 a	535 ± 34 a
		ns	ns	ns	ns
Lutein 5,6-epoxide (μg g^{-1} DW)	5.00	Non detected			
	13.00	Non detected			

Within each hour of measurement, means followed by different letters are significantly different at $p < 0.05$ according to Student–Newman–Keuls test.
ns, no significant difference (*t*-Student test, $p < 0.05$).

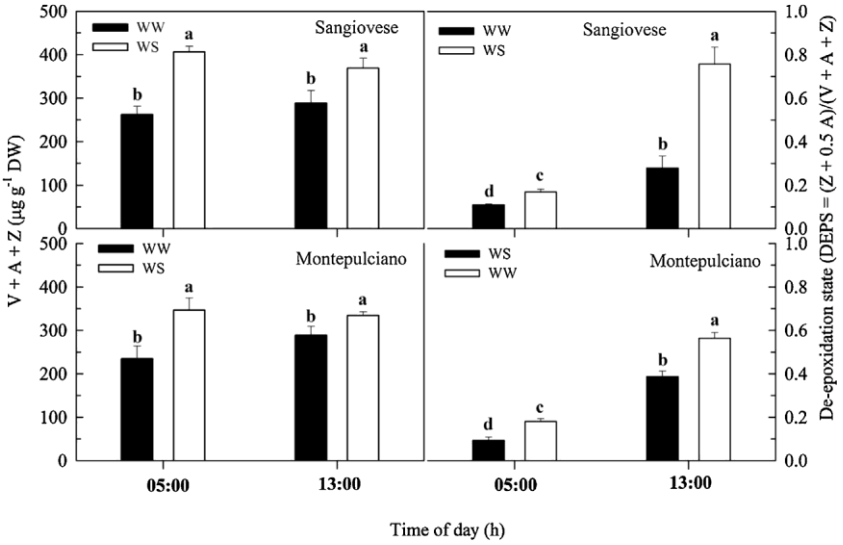

Fig. 2. Xanthophyll cycle pool size [(violaxanthin (V) + zeaxanthin (Z) + antheraxanthin (A)] and the de-epoxidation state of xanthophylls cycle pigments collected at predawn (05:00 h) and midday (13:00 h) in leaves of 60-L potted well-watered (WW) and water-stressed (WS) Sangiovese and Montepulciano vines. Data were taken 1 week before veraison, after 7 weeks of water deficit; at midday, data were collected under heat stress + light stress + water stress (air $T°$ of ~37–38 °C, incident PAR of ~1900 μmol photons m^{-2} s^{-1} and soil moisture set at 40% of maximum water availability). Data represent the average value of 3 analyses ± S.E. Different letters indicates significant differences between treatments ($p < 0.05$) based on Student–Newman–Keuls test.

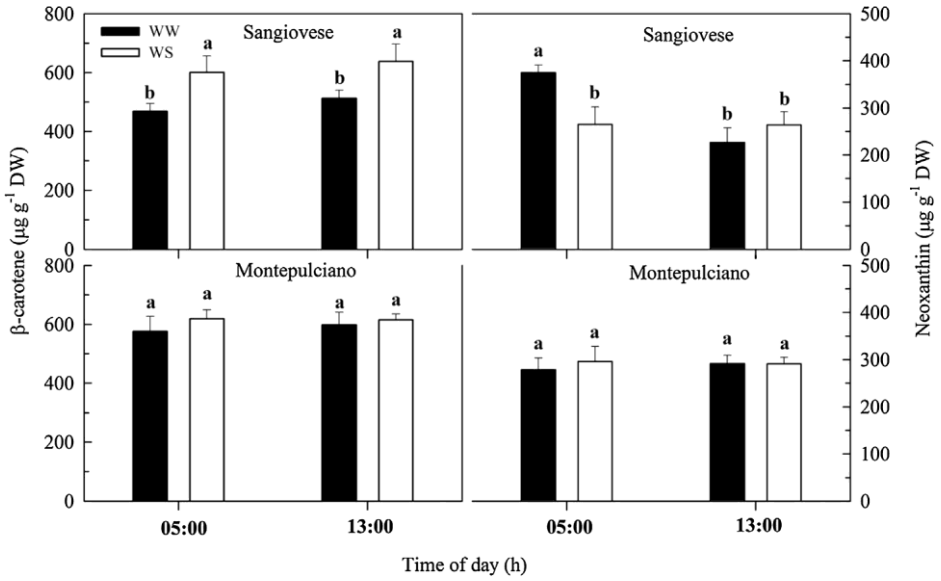

Fig. 3. ß-Carotene and neoxanthin content collected at pre-dawn (05:00 h) and midday (13:00 h) in leaves of 60-L potted well-watered (WW) and water-stressed (WS) Sangiovese and Montepulciano vines. Data were taken 1 week before veraison, after 7 weeks of water deficit; at midday, data were collected under heat stress + light stress + water stress (air T° of ~37–38 °C, incident PAR of ~1900 μmol photons $m^{-2} s^{-1}$ and soil moisture set at 40% of maximum water availability). Data represent the average value of 3 analyses ± S.E. Different letters indicates significant differences between treatments ($p < 0.05$) based on Student–Newman–Keuls test.

Contrary to Montepulciano, in Sangiovese leaves the ß-carotene and the neoxanthin content did not change during the hottest hour of the day, whereas at pre-dawn a significant increase of ß-carotene and a decrease in the neoxanthin content were found in WS-Sangiovese leaves compared to WW-Sangiovese ones (Fig. 3).

Regardless of sampling time and applied treatments, Sangiovese leaves had more total protein, H_2O_2 content and catalase (CAT) activity in comparison with homologous leaves of Montepulciano (Table 3). Contrary to values of leaves sampled at predawn (5:00 h), at midday the CAT activity increased in both cultivars. However, WS-Montepulciano leaves had a greater increase of CAT activity in comparison with WS-Sangiovese, namely +195% against an increase of +95% when compared with the respective WW-vines. Contrary to Sangiovese, in WS-Montepulciano vines the H_2O_2 and total protein contents significantly increased at midday (namely 46% and 19%, respectively).

4. Discussion

Under multiple summer stresses, i.e. air temperature of37–~ 38 °C, incident PAR of~ 1900 μmol photons $m^{-2} s^{-1}$ and soil moisture set at 40% of maximum water availability, during the hottest hours of the day (12–13:00 h interval), Sangiovese showed a clear anisohydric behavior. In fact, despite the expected more negative midday W_l (−1.45 vs. −0.90 MPa of Montepulciano), Sangiovese retained a higher relative P_n (+48%) due to more open stomata, evidenced by an higher g_s (+37%). This behavior led to increased intrinsic WUE_i by about 13% as compared with Montepulciano.

In both grapevine cultivars the VAZ pool and cycle enhanced under drought; same results were reported for different grapevine varieties by Medrano et al. (2002) and Chaumont et al. (1995). However, the greater VAZ pool and de-epoxidation activity found at midday in WS-Sangiovese leaves certainly plays a positive role in tolerance acquisition through increased photostability of PSII as suggested in previous studies (Düring, 1988; Adams and Demmig-Adams, 1992). In fact, from pre-dawn to midday, in WS-Sangiovese leaves about 80% of V was de-epoxidated in Z + A (corresponding to 275 μg g^{-1} DW) versus 55% only measured in WS-Montepulciano leaves (corresponding to 163 μg g^{-1} DW). This behavior suggests, for the WS-Sangiovese, an elevated energy utilization through the VAZ cycle and, therefore, an high thermal dissipation of excessive excitation energy in the chloroplasts (Demmig-Adams and Adams, 1996). Similar results were found in Manto Negro, a drought-resistant Spanish grapevine cultivar, which showed 90% of the xanthophylls in the de-epoxidation state against only 70% found in cv. Tempranillo (Medrano et al., 2002). Yet, leaves of field-grown cvs. Pinot Noir and Fernao Pires had a maximum de-epoxidation state of about 85% and 75%, respectively, whereas in non-acclimated cuttings of Pinot Noir the maximum de-epoxidation state was only 60% (Chaumont et al., 1995, 1997).

As found also by other authors (Flexas et al., 1999, 2002; Medrano et al., 2002, 2003; Guan and Gu, 2009), the significant increase of NPQ found on WS-Sangiovese leaves compared to WS-Montepulciano leaves indicates an elevated thermal dissipation of excessive excitation energy in the chloroplasts. On the other hand, NPQ covariate with the de-epoxidation state of the xanthophyll cycle similarly to what previously reported in grapevines (Chaumont et al., 1997; Flexas et al., 2000). The largest part of NPQ at the stress level tested in this experiment was mostly due to energy-dependent quenching (qE) where as photoinibitory quenching (qI) was limited (in our experiment F_v/F_m ratio did not vary between WS and WW plants) and state-transition quenching had a limited incidence on the photoprotection processes (Niyogi, 1999). qE is regulated by pH reduction in the thylakoid lumen and the acidification of lumen pH is considered to be the trigger of violaxanthin de-epoxidation via the activation of violaxanthine de-epoxidase (VDE) (Gilmore, 1997; Müller et al., 2001). We hypothesize that this mechanism can explain the correlation between NPQ and xanthophyll de-epoxidation observed in our experiment and previously reported on *Vitis* in the literature. However, it is noteworthy that in WW conditions, xantophyll de-epoxidation in Montepulciano is higher than in Sangiovese thought NPQ was similar. Such difference, that apparently is in contrast with the coordination of these two factors can suggest the important role of the genotype that in *Vitis* account for large variability in the mechanism used to withstand to drought stress. Furthermore it should be considered that the magnitude of the contribution of each mechanism largely depend on the severity of drought stress that can induce different adaptation depending on the cultivar.

Table 3
Changes in catalase (CAT) activity, hydrogen peroxide (H_2O_2) and total protein content in mature leaves of 60-L potted well-watered (WW) and water-stressed (WS) Sangiovese and Montepulciano vines. Data were taken 1 week before veraison, after 7 weeks of water deficit, at predawn (5:00 h) and at midday (12–13:00 h interval) under heat stress + light stress + water stress (air $T°$ of ~37–38 °C, incident PAR of ~1900 μmol photons m^{-2} s^{-1} and soil moisture set at 40% of maximum water availability).

	Hours of the day	cv. Sangiovese		cv. Montepulciano	
		WW-vines	WS-vines	WW-vines	WS-vines
CAT activity (μmol H_2O_2 min^{-1} mg^{-1} protein)	5.00	3.8 ± 0.2 a	3.2 ± 0.3 b	2.2 ± 0.1 d	2.6 ± 0.2 c
	13.00	4.1 ± 0.2 c ns	8.0 ± 0.5 a *	2.1 ± 0.3 d ns	6.2 ± 0.6 b *
H_2O_2 (mmol mg^{-1} protein)	5.00	21.0 ± 1.2 a	19.4 ± 2.3 a	16.8 ± 2.0 b	15.4 ± 0.9 b
	13.00	20.2 ± 1.7 c ns	24.9 ± 0.8 a *	12.2 ± 1.0 d ns	22.5 ± 2.4 b *
Total protein (μg g^{-1} FW)	5.00	13.2 ± 1.8 a	12.1 ± 1.9 a	7.1 ± 1.0 b	7.9 ± 0.8 b
	13.00	11.2 ± 0.6 a ns	12.2 ± 0.9 a ns	7.0 ± 0.5 c ns	9.4 ± 0.7 b ns

Within each hour of measurement, means followed by different letters are significantly different at $p < 0.05$ according to Student–Newman–Keuls test.
* Indicate significant differences at $p < 0.01$ (comparisons between the two hours of sampling were performed by *t*-Student test); ns, no significant differences.

The increase of VAZ-cycle pigments, mainly Z from pre-dawn to midday, found in WS-Sangiovese leaves may reduce and/or avoid ROS production by enhancing the capacity of thermal dissipation of excess radiant energy in the chloroplasts and preserve thylakoid membranes from oxidation (Havaux, 1998; Logan et al., 2000). In this regard, our data point out that under drought and during the hottest hours of summer days in WS-Sangiovese leaves there was no variation of H_2O_2 content, whereas under the same conditions in the WS-Montepulciano leaves the H_2O_2 content doubled. These behavior implies that in the cv. Sangiovese the VAZ-cycle pigments seems to play a significant role in photoprotection of PSII helping maintenance of good physiological activity under very critical environmental conditions.

Although ROS were considered to be detrimental to cells, it is now widely recognized that redox regulation involving ROS is a key factor modulating cellular activities (Mittler, 2006). As a matter of fact, the accumulation of H_2O_2 below the toxic threshold induce the expression of various defense-related genes, like HSP, glutathione S transferase and phenylalanine ammonia lyase (Neill et al., 1999; Volkov et al., 2006). One of the possible sources of H_2O_2 production in plants is the high P_r which usually occurs in the peroxisomes. In *V. vinifera* several studies have ascertained that the rate of P_r is cultivar-dependent (Düring, 1988; Beis and Patakas, 2012; Hochberg et al., 2013). Indeed, contrary to cv. Sabatiano, a significant increase of P_r was found in cv. Mavrodafni under severe drought and such response was paralleled by an increase in H_2O_2 production and CAT activity (Beis and Patakas, 2012). The cv. Cabernet Sauvignon subjected to drought stress exhibited a higher P_r rate than cv. Shiraz (Hochberg et al., 2013). Düring (1988) reported that P_r greatly increased under stress conditions from about 35% of P_n in cvs. Riesling and Trollinger to about 52% of P_n in cv. Phoenix. Yet, the ratio of P_r to P_n on cvs. Chasselas and Riesling strongly increased with temperature and, at a leaf temperature higher than 35 °C, P_r accounted for more than 50% of P_n irrespective of leaf age (Zufferey et al., 2000). In our study, P_r expressed as percentage of P_n increased under drought at a faster rate in Montepulciano than in Sangiovese (i.e. 73% against 52%, respectively) suggesting a primary role in photoprotection of PSII, and a concomitant and significant increase of H_2O_2 production and CAT activity (+84% and +195%, respectively) was assessed in comparison with leaves from WW-vines.

It is well-established that the P_r protects C3 plants under excess of irradiance (Kozaghi and Takeba, 1996); in a drought-tolerant mutant of barley with reduced activity of photorespiratory enzymes the P_r strongly increased under drought conditions (Wingler et al., 1999). In apple leaves it has been reported that photoinhibition could be effectively prevented by ROS produced from photorespiration and Mehler reaction (Jia et al., 2003). However, in Jingxiu and Beta grapevine cultivars the heat tolerance increased after heat pretreatment and this behavior was associated with less energy partitioned in non-regulated energy dissipation (i.e. through the alternative pathways to NPQ), less lipid peroxidation, and higher antioxidant enzymes activities than in control vines under heat stress (Wang et al., 2009). Nevertheless, the relationship to photosynthetic energy partitioning remains partly unknown as well as heat acclimation and/or adaptation effects in improve heat tolerance.

An additional, slower reversible mechanism of photoprotection is represented by the lutein epoxide (Lx) cycle which joins the VAZ cycle, especially in shade plants (Garcia-Plazaola et al., 2007). The Lx cycle occurs in the α-carotene branch of carotenoid biosynthetic pathway and works similarly to the V-pathway of the β-branch. In general, Lx is synthesized from L by an epoxidation reaction catalyzed by an epoxidase (presumable ZE) by reaction analogous to the epoxidation of Z to form A and V. Garcia-Plazaola et al. (2007) reported that the Lx cycle has a quite irregular taxonomical distribution having been found in leaves of 116 out of 188 species and in the 58% of 50 families (angiosperms and gymnosperms). Moreover, Lx photoconversion in L has been found only in 56 species. Our analysis showed the lack of this additional reverse mechanism of photoprotection since no lutein 5,6-epoxide was found in the leaves of both grapevine varieties, treatment examined and hour of sampling.

Regardless of cultivar, the significantly lower values of J_c, Φ_{PSII} and ETR found in leaves of stressed-vines are a common responses in grapevine and were reported for different varieties by several authors (Medrano et al., 2003; Guan et al., 2004; Guan and Gu, 2009; Beis and Patakas, 2012).

The ETR/P_n ratio, which can be used to evaluate photorespiration as a photoprotective mechanism, significantly increased under multiple summer stresses. Similar results were found in other grapevine genotypes subjected to drought (Flexas et al., 2002; Medrano et al., 2003; Beis and Patakas, 2012). However, the response was more marked in WS-Montepulciano leaves confirming the great role of photorespiration in this genotype; in more details the significantly high values of ETR/P_n may suggest that the unbalance between electrons generated photosynthetically and depleted through P_n calls for an alternative sink for electrons, such as the Mehler reaction. Thus, a higher risk of oxidative damage in WS-Montepulciano leaves is expected since electrons in excess could react with O_2 generating ROS.

In *V. vinifera* the P_n increases with irradiance up to the saturation point which is primarily a function of the light regime leaves underwent during their development (Düring, 1988; Palliotti et al., 2000; Zufferey et al., 2000). In grapevine the light saturation point is usually reached at about 700–900 μmol photons m^{-2} s^{-1}, but with increasing temperatures the light saturation point can increase

until 1200–1300 μmol photons m^{-2} s^{-1}. thus in several areas of grapevine cultivation worldwide, where under clear skies irradiance typically exceeds 1900–2000 μmol photons m^{-2} s^{-1}, the light is surely in excess. Under this situation the mechanisms triggered by the plants to avoid and/or limit irreversible photoinhibition in the leaf tissues can be divided in preventive and protective. A reduction of light absorbance by means of an adjustment of Chl concentration and/or antenna size has been reported as an effective preventive mechanism against light stress and photoinhibition (Anderson, 1986; Galmés et al., 2007). In grapevine, several authors have found a significant reduction of Chl content under drought stress conditions (Maroco et al., 2002; Palliotti et al., 2008, 2014; Martim et al., 2009; Beis and Patakas, 2012), whereas others have not ascertained any significant effect (Chaumont et al., 1997; Palliotti et al., 2001). These discrepancies could be attributable to different severity, duration and type of stress applied as well as the cultivar examined and, lastly, to how the Chl content is expressed. Indeed, only when expressed on a per leaf area basis, drought conditions significantly reduced the total Chl content irrespective of cultivar. This reduction in Chl content has been ascribed to loss of chloroplast membranes, excessive swelling, distortion of lamellae vesciculation and appearance of lipids droplets (Kaiser et al., 1981), the latter to be considered as a typical symptom of oxidative stress.

5. Conclusions

Under open-field conditions and simultaneous high light and temperature and water deficit the physiological measurements carried out during the hottest hours of summer day allow to assert that the cv. Sangiovese exhibits an anisohydric behavior, since it is capable to maximize photosynthetic gain, by maintaining stomata open despite a high drop of leaf water potential, as well as increase the WUE. Vice versa, the cv. Montepulciano confirms its isohydric behavior since it closes the stomata and maintains a fairly constant midday leaf water potential, whereas P_n and g_s result significantly penalized. Under such environmental conditions, the protective mechanisms of energy dissipation showed by the anisohydric cv. Sangiovese (the more drought resistant genotype) are: (i) higher stomatal conductance and thermoregulation linked to higher transpiration rate; (ii) greater ability at dissipating more efficiently the excess energy via the xanthophylls cycle (thermal dissipation) due to higher VAZ pool and greater increase of de-epoxidation activity. The isohydric cv. Montepulciano (more susceptible to drought), that under well-watered conditions had larger VAZ de-epoxidation activity, could base its photoprotection primarily on enabling photorespiration, even if more studies are needed. However, these highly-energy-expensive mechanisms evidenced in the more drought resistant cv. Sangiovese are coordinated with other mechanisms, mainly of preventive nature, aiming at dissipating excess of energy and avoiding photoinhibition and/or photodamage without energy expenditure, such as: (i) lower content of total Chl in comparison to the drought susceptible cv. Montepulciano when expressed on a leaf area basis and, in turn, lower leaf absorbance and greater leaf transmittance which reduce and/or avoid excessive light and heat absorption (Palliotti et al., 2008, 2009); (ii) changes in leaf lamina inclination assuming a more vertical orientation during the hottest hours of the days to reduce the amount of intercepted light, hence heat load and transpiration rate (Merli et al., 2015; Poni et al., 2007; Palliotti et al., 2008, 2009); (iii) voluntary chronic photoinhibition and subsequent fall of the major part of the basal older leaves which become chlorotic and necrotic (Palliotti et al., 2009, 2014); (iv) lower xylem vulnerability to cavitation and reduced hydraulic conductance (Tombesi et al., 2014).

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