SUPPLEMENTARY MATERIAL

Ecophysiological and phytochemical response to ozone of wine grape cultivars of *Vitis vinifera* L.

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Abstract

Vitis vinifera sensitivity to tropospheric ozone (O_3) has been evidenced in several studies. In this work, physiological and metabolic effects of O_3 on two wine cultivars of *V. vinifera* (i.e. Maturano and San Giuseppe) have been studied. Moreover, chlorogenic acid (CGA) production, in consideration of its importance in the biosynthetic pathway of polyphenols and as antioxidant, has been investigated. Maturano cultivar resulted more sensitive to O_3 , as evidenced by the gas exchange reduction at the early stage of treatment, and by the increase of C_i/C_a and the decoupling of net photosynthesis and the stomatal conductance at the end of the treatment. Unexpectedly, O_3 did not activate stilbene production. Ozone induced an early CGA decrease, significantly more consistent in cv. Maturano, and an increase after 8 days, more consistent in cv. S. Giuseppe. These results suggest that CGA could be considered a biochemical marker of O_3 induced stress in *V. vinifera*.

Keywords: *Vitis vinifera*; tropospheric ozone; HPTLC; chlorogenic acid; stomatal conductance; JIP test.

Experimental

Plant material, plant growth conditions and ozone fumigation

The experiments were carried out inside two closed “walk-in” chambers (2.5 m x 3.9 m x 3.0 m h), one used as control and one for O_3 fumigation (Salvatori et al. 2013). Air temperature was maintained at 24 °C and 20 °C, during day and night, respectively, and relative humidity was 65%. In each chamber, a PAR of approximately 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at sample leaf height was provided for 12 h per day by 6 metal halide lamps (1000 W, Philips HPI-T). Microclimatic conditions were monitored at 6-min interval, and did not differ significantly between the chambers. Two Italian cultivars of *V. vinifera* L. (San Giuseppe and Maturano, red and white grape, respectively), autochthonous of the Latium region (Comino and River Liri valleys), were used as models. They were kindly supplied by the Italian National Council for Research (CNR) and the Agricultural Research Council (CREA-ENC, Velletri, Rome Italy) that assured identity and cloned by *in vitro* micropropagation (Damiano et al. 2007) and acclimatisation. Two years old plants were transferred into 18 L pots (P & b) and watered daily at field capacity with a sub-irrigation system, in which water was delivered from below the pots, absorbed upwards, and the excess collected for recycling. After 4 weeks of acclimation, 4 plants per cultivar were exposed to 250 ppb of O_3 for 4 h per day (9:00 and 13:00) for a period of 10 days of fumigation (DOF). O_3 was generated by flowing pure

oxygen on a UV light source (Helios Italquartz, Milan, Italy), and then added to the chamber air inlet by a Teflon tube. Four plants per cultivar without exposition to O₃ were also used as control. The O₃ concentration at plant height was continuously monitored with a photometric O₃ detector (Model 205, 2B Technologies, Boulder, CO, USA). The accumulated O₃ exposure over a threshold of 40 ppb, i.e. the AOT40 index, was calculated as the sum of the differences between the hourly mean O₃ concentration in ppb and 40 ppb for each hour of fumigation.

Ecophysiological measurements and assessment of visible O₃ injury

Gas exchanges and chlorophyll fluorescence were measured on fully expanded leaves at day 0 (before fumigation), and after the O₃ fumigation at DOF 1 to 10. Measurements were made between 13:30 and 15:00 (CET). Net photosynthesis (Pn, $\mu\text{molCO}_2 \text{ m}^{-2}\text{s}^{-1}$), leaf transpiration (E, $\text{mmolH}_2\text{O m}^{-2}\text{s}^{-1}$), stomatal conductance (g_s, $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) and sub-stomatal CO₂ concentration (C_i, ppm) were measured with a CIRAS-2 infrared gas analyser (PP Systems, Hitchin, UK), which also recorded environmental parameters such as irradiance (PAR, $\mu\text{mol m}^{-2} \text{ s}^{-1}$), relative humidity (RH, %), ambient and leaf temperature (T_a, T_l, °C). The instantaneous ratio between sub-stomatal and external CO₂ concentration (C_i/C_a) was also calculated.

Chlorophyll “a” fluorescence was recorded with a direct fluorometer (Handy PEA, Plant Efficiency Analyser, Hansatech Instruments Ltd, UK) after 0, 3 and 10 DOF, during the same time and on the same leaves of the gas exchange measurements, after a dark adaptation of at least 40 min. The fluorescence transient were induced by a red light (peak at 650 nm) of 3000 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ through an array of six light-emitting diodes. The values of F₀ (approximated as florescence value at 10 μs) and F_m were recorded. F₀ is fluorescence measured when all RCs of PSII are considered open, i.e. all the primary acceptors, quinone QA, are fully oxidized. F_m is the maximal fluorescence yield in the dark, when the excitation intensity is high enough to close all RCs of PSII, i.e. all the QA is fully reduced. Values of the maximum quantum yield for primary photochemistry (F_v/F_m, i.e. the maximal efficiency that an exciton is trapped by a RC of PSII) was calculated as (F_m – F₀)/F_m. The Biolyzer software (Bioenergetics Lab., Geneva, CH) was used to calculate the JIP-test parameters detailed in Table 1 – Supplementary material, following Strasser and collaborators (2010).

In both chambers, plants were inspected daily, assessing the extent of ozone-like foliar symptoms. Following the Horsfall-Barrett method (1945) the total number of developed leaves was counted for each plant, and those with injured area were recorded. The percentage of injured leaves per plant was then calculated.

STATISTICAL ANALYSIS

All data were subjected to one-way ANOVA (Analysis of Variance) and significant differences between means of control and fumigated plants of each cultivar were determined by the post-hoc Newman–Keuls test at the significance level of 0.05. The Statistica 7 software package (StatSoft, Inc. – Tulsa, OK – USA) was used for all statistical analyses.

Preparation of leaf extracts

Samples of fully expanded leaves (3 grams fresh weight for each sample), both from untreated and treated plants at day 0 (before fumigation), and after O₃ fumigation at 24, 48, 192, 216 e 240 h have been randomly collected. The leaves were weighed, frozen in liquid nitrogen and grounded to a fine powder. All the samples were extracted with ethanol/ water (7/3 v:v), in three successive 24 h steps; the final ratio was 1 g fresh weight/10 mL of extractive mixture. The hydroalcoholic extracts were dried under vacuum (about 30°C), redissolved in the extractive mixture, and then filtered through a 0.22 µm PTFE membrane (Whatman, New Jersey, USA) and analyzed by HPLC and HPTLC.

HPLC analysis

HPLC analysis was focused on identification and quantification of resveratrol in all the extracts obtained from leaves of the two cultivars. Extracts were dissolved in 10 ml of EtOH/H₂O (7:3, v/v). Solutions were cleaned through syringe filters of 0.45 µm diameter pores and filtered solutions analyzed by HPLC. The apparatus consisted of a high pressure pump (Waters 1525) with a dual detector (Dual λ Detector Waters 2487), an inverted phase C₁₈ column 4.6x150 mm with pores 5 µm (Waters). The mobile phase was a gradient solution (A) H₂O-HCOOH (95:5, v/v) and (B) acetonitrile, optimized for qualitative analysis of stilbenes and delivered at a rate of 1 ml/min. Detection was set at 280 and 330 nm, on the basis of UV absorbance of stilbenes. Injection volumes, both for standards and samples, were 20 µl and analyses were performed for 35 min.

HPTLC analysis

The HPTLC system (CAMAG, Muttenz, Switzerland) consisted of Linomat 5 sample applicator using 100 µL syringes and connected to a nitrogen tank; Chamber ADC 2 containing twin trough chamber 20 x 10 cm; Camag TLC Plate Heater III; Camag TLC Visualizer; Camag TLC Scanner 3 linked to winCATS software. Glass plates 20 cm x 10 cm (Merck, Darmstadt, Germany) with glass-backed layers silica gel 60 (2 µm thickness). Before use, plates were prewashed with methanol and dried for 3 min at 100 °C. The analysed samples were directly applied to the plate with nitrogen flow. The operating conditions were: syringe delivery speed, 10 s µL⁻¹ (100 nL s⁻¹); injection

volume, 4 μL ; band width, 7 mm; distance from bottom, 15 mm. CGA was dissolved in MeOH at four different concentrations (0.25 mM, 0.5 mM, 1 mM and 2 mM).

The HPTLC plates were developed in the automatic and reproducibly developing chamber ADC 2, saturated with the same mobile phase, AcOEt/ CH_2Cl_2 / CH_3COOH / HCOOH / H_2O 100:25:10:10:11, for 20 min at room temperature. The developing solvents (i.e. type of solvents and ratios) were carefully optimized before the analyses. The length of the chromatogram run was 70 mm. The developed layers were allowed to dry on Camag TLC Plate Heater III for 5 min and then derivatised with selected solutions, including Natural Product Reagent (NPR) (1 g diphenylborinic acid aminoethylester in 200 mL of ethyl acetate) and anhyaldehyde. All treated plates were allowed to dry on Camag TLC Plate Heater III for 5 min and then inspected under a UV light at 254 or 366 nm or under white light upper and lower (WRT), respectively, at a Camag TLC visualizer, before and after derivatization. CAMAG DigiStore2 digital system with winCATS software 1.4.3 was used for the documentation of derivatized plates.

Sample solutions were applied on the same HPTLC plate and the chromatogram evaluated for additional band. Similarly, band stability was checked by keeping the resolved peaks and inspecting at intervals of 12, 24 and 49 h. Sample solutions were found to be stable at 4 $^{\circ}\text{C}$ for at least 1 month and for at least 3 days on the HPTLC plates. Repeatability was determined by running a minimum of three analyses. RF values for main selected compounds varied less than 0.02%. The effects of small changes in the mobile phase composition, mobile phase volume, duration of saturation were minute and reduced by the direct comparison. On the contrary, the results were critically dependent on prewashing of HPTLC plates with methanol.

For the densitometric analysis the scanner was set at 350 nm, after that a multi-wavelength scanning between 190 and 800 nm in the absorption mode had been preliminarily tried. A minimum background compensation was performed on the x -axis during the scanning. The sources of radiation were deuterium and tungsten lamps. The slit dimension was kept at 6.00 x 0.45 mm and the scanning speed used was 100 mm s^{-1} . Data in Figure 3 were reported as an average of three replicates.

Technical fluorescence parameters	
F_t	Fluorescence emission from a dark-adapted leaf at the time t
F_0	Fluorescence intensity at 50 μs
$F_{100\mu s}; F_{300\mu s}$	Fluorescence intensity at 100 μs and at 300 μs
F_J	Fluorescence intensity at the J step (at 2 ms)
F_I	Fluorescence intensity at the I step (at 30 ms)
F_m	Maximal fluorescence intensity
M_0	Slope of the curve at the origin of the relative variable fluorescence rise dV/dt_0 . It is a measure of the rate of the primary photochemistry. $M_0 = 4(F_{300\mu s} - F_0)/(F_m - F_0)$
V_J	Relative variable fluorescence at 2 ms. $V_J = (F_J - F_0)/(F_m - F_0)$
V_I	Relative variable fluorescence at 30 ms. $V_I = (F_I - F_0)/(F_m - F_0)$
ΔV_{IP}	Amplitude of the IP-phase. It indicates the electron transport around the PSI to reduce the final acceptors of the electron transport chain, i.e., ferredoxin and NADP. $\Delta V_{IP} = 1 - V_I = (F_m - F_{30 ms})/(F_m - F_0)$
Quantum efficiency or flux ratios	
$\phi_{P_0} = TR_0/ABS = F_v/F_m$	Trapping probability or maximum quantum yield of primary photochemistry. Expresses the probability that an absorbed photon will be trapped by the PSII RC and will reduce one Q_A . $\phi_{P_0} = (F_m - F_0)/F_m$
$\phi_{E_0} = ET_0/ABS$	Electron transport efficiency. Expresses the probability that an absorbed photon will enter in the electron transport chain. $\phi_{E_0} = [1 - (F_0 - F_m)] \Psi_0$
$\Psi_{E_0} = ET_0/TR_0$	Expresses the probability that a photon trapped by the PSII RC enters the electron transport chain. $\Psi_{E_0} = 1 - V_J$
$\phi_{R_0} = RE_0/ABS$	Quantum yield of electron transport from Q_A^- to the PSI end electron acceptors. $\phi_{R_0} = [1 - (F_0/F_m)](1 - V_I) = 1 - (F_t/F_m)$
$\delta_{R_0} = RE_0/ET_0$	Probability that an electron is transported from reduced PQ to the electron acceptor side of PSI. $\delta_{R_0} = (1 - V_I)/(1 - V_J) = (F_m - F_t)/(F_m - F_J)$
Specific fluxes or specific activities (expressed in arbitrary units)	
ABS/RC	Effective antenna size of an active reaction center (RC). Expresses the total number of photons absorbed by Chl molecules of all RC divided by the total number of active RCs. $ABS/RC = M_0(1/V_J)(1/\phi_{P_0})$
ET_0/RC	Electron transport in an active RC. $ET_0/RC = M_0(1/V_J) \Psi_{E_0}$
TR_0/RC	Maximal trapping rate of PSII. Describes the maximal rate by which an excitation is trapped by the RC. $TR_0/RC = M_0(1/V_J)$
RE/RC	Electron flux reducing end electron acceptors at the PSI acceptor side, per RC. $RE/RC = M_0(1/V_J)(1 - V_I)$
DI_0/RC	Effective dissipation in an active RC. $DI_0/RC = (ABS/RC) - (TR_0/RC)$
Density of RCs	
RC/CS_0	It is a measure for Q_A -reducing RCs per excited leaf cross section; $RC/CS_0 = \phi_{P_0}(V_J/M_0)F_0$
Performance Indices	
PI_{ABS}	Performance Index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors. $PI_{ABS} = (RC/ABS)[\phi_{P_0}/(1 - \phi_{P_0})][\Psi_{E_0}/(1 - \Psi_{E_0})]$
PI_{tot}	Performance Index (potential) for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors. $PI_{tot} = PI_{ABS}[\delta_{R_0}/(1 - \delta_{R_0})]$

Table 1. Description of the JIP-test parameters used in the text